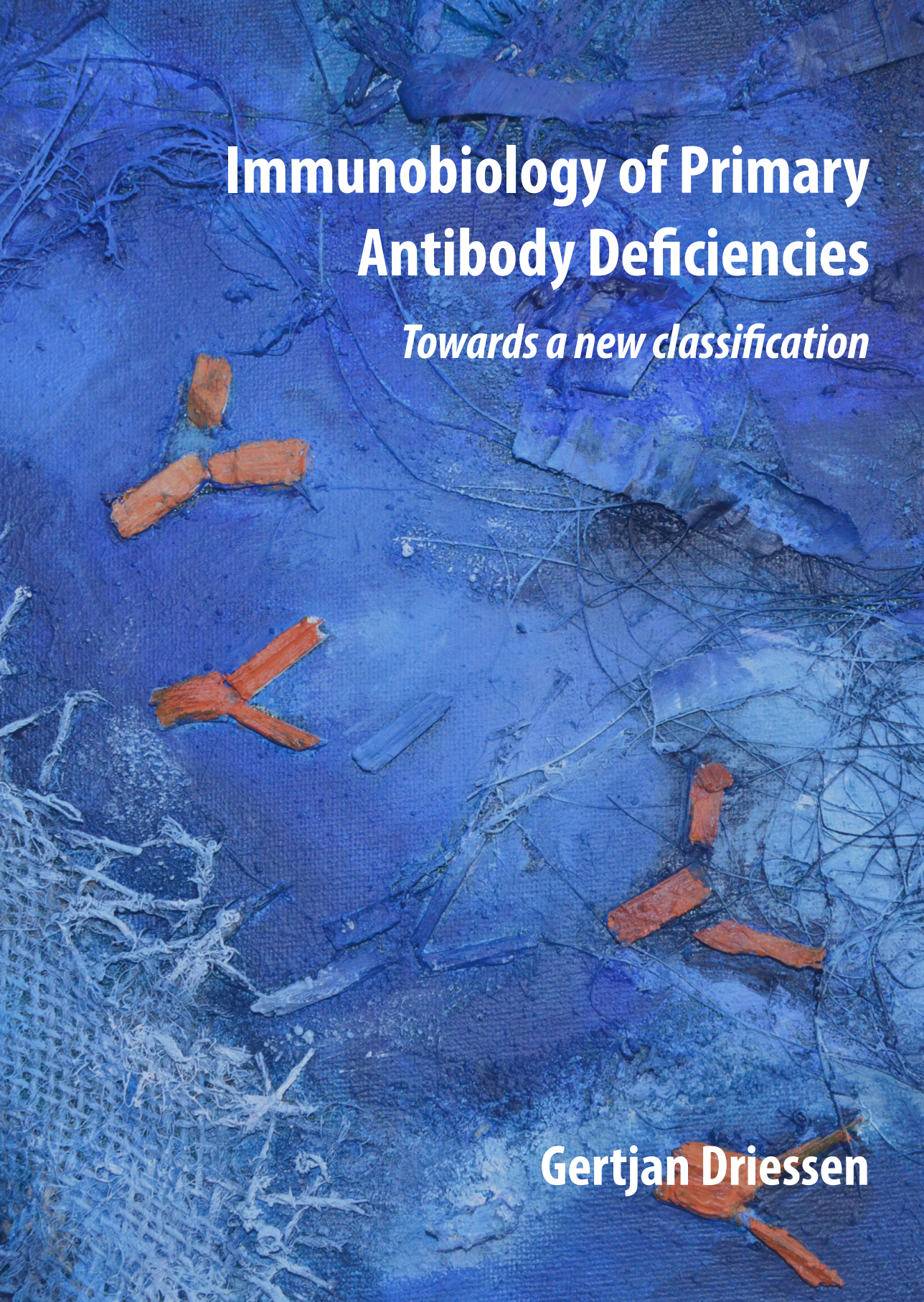
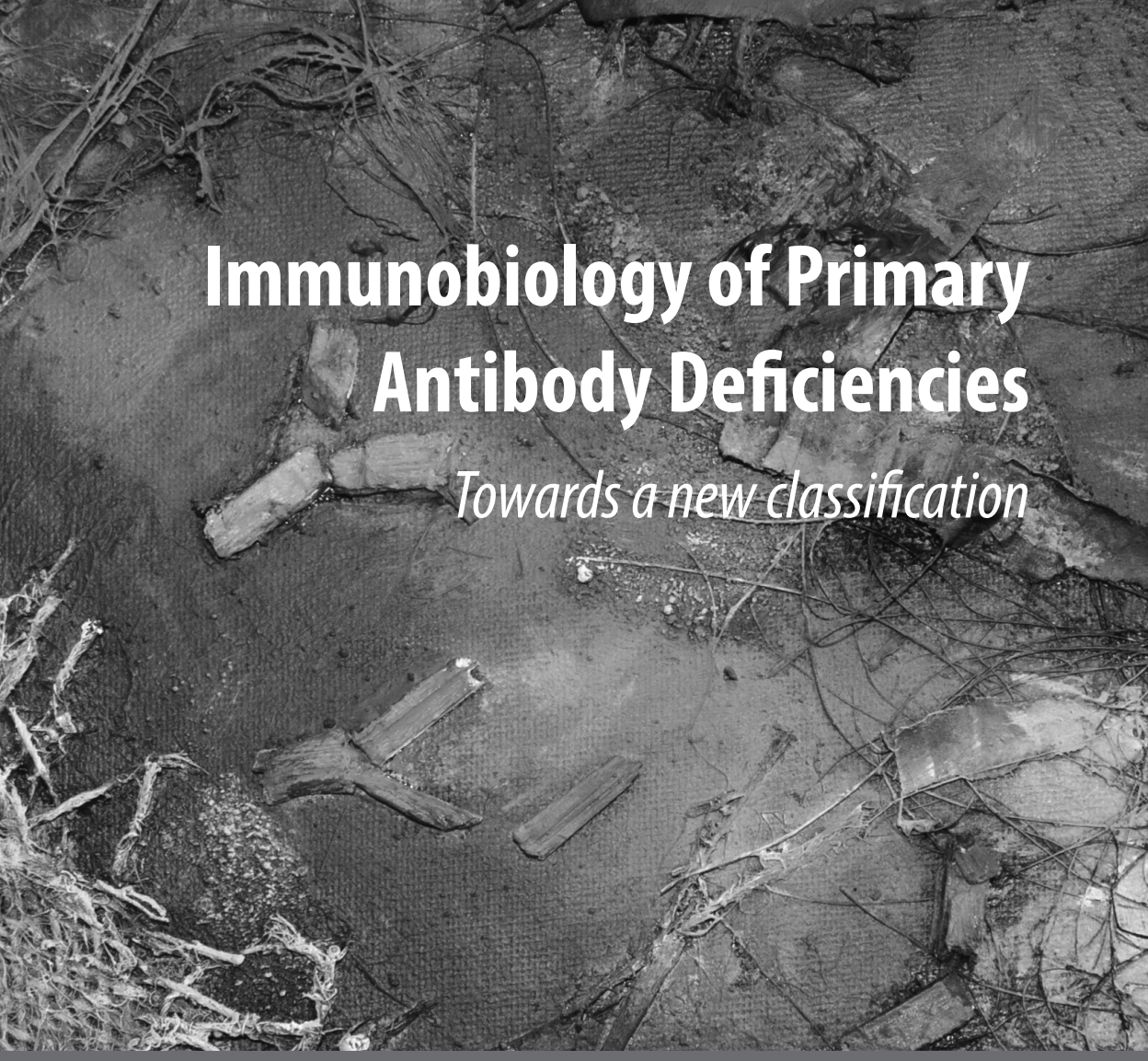

Immunobiology of Primary Antibody Deficiencies
Towards a new classification
Gertjan Driessen



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Towards a new classification

Gertjan Driessen

Molecular Medicine
Postgraduate School

The research for this thesis was performed within the framework of the Erasmus Postgraduate School Molecular Medicine.

The studies described in the thesis were performed at the Department of Immunology and Pediatrics, Erasmus MC, University Medical Center Rotterdam, Rotterdam, the Netherlands and collaborating institutions.

The printing of this thesis was supported by:
Erasmus MC and Department of Immunology

ISBN: 978-94-91811-03-6

Illustrations: Sandra de Bruin-Versteeg
Cover: Petronette van Jaarsveld-de Bakker
Lay-out: Caroline Linker
Printing: Haveka B.V., Alblasserdam, the Netherlands

Immunobiology of Primary Antibody Deficiencies

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Immunobiologie van primaire antistofdeficiënties

op weg naar een nieuwe classificatie

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus

Prof.dr. H.G. Schmidt

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 16 oktober 2013 om 13.30 uur

door

Gerardus Johannes Andreas Driessen

geboren te Son en Breugel

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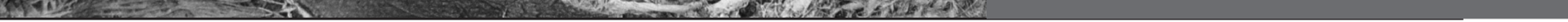
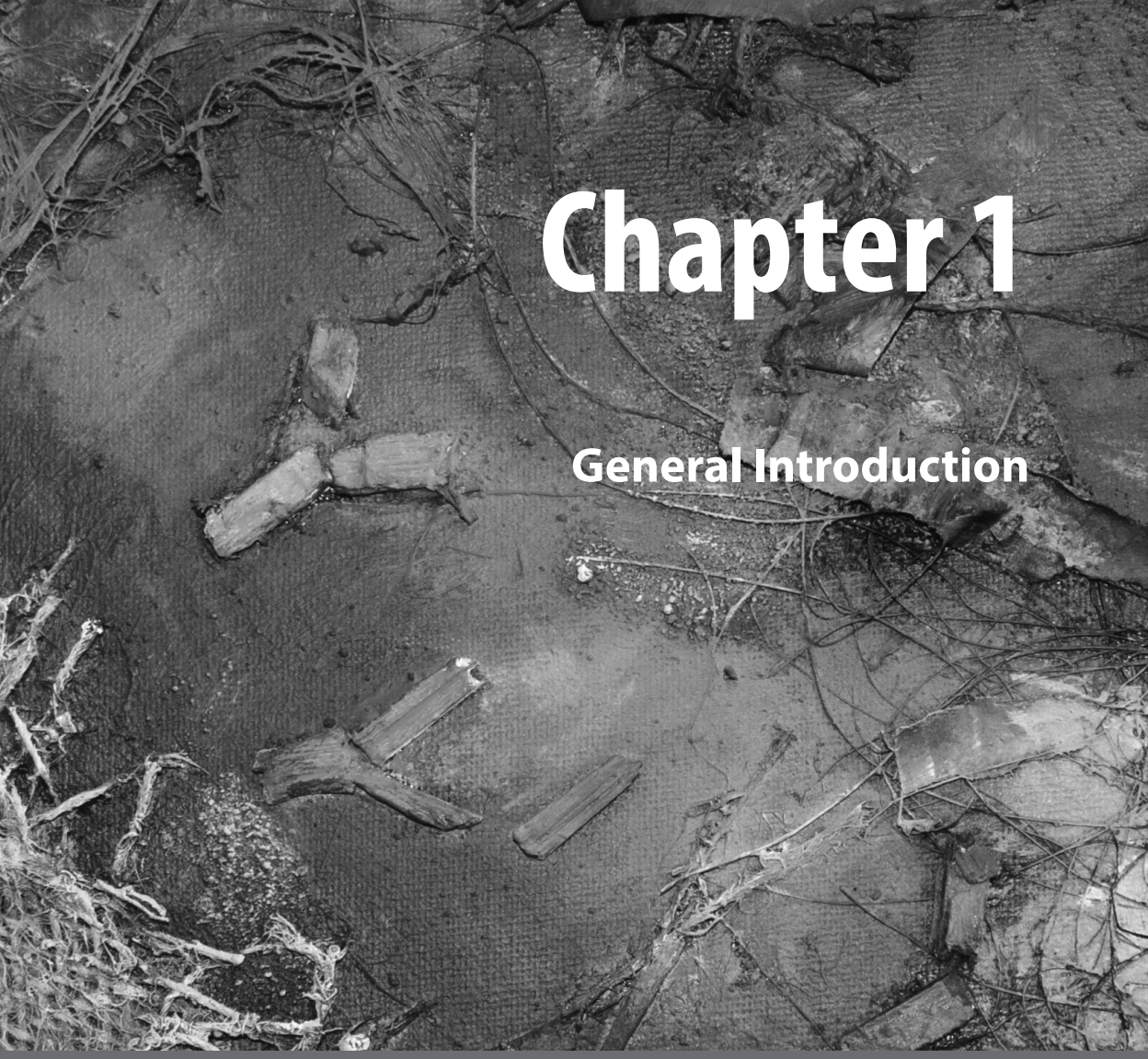
CONTENTS

CHAPTER 1	General Introduction	**9**
CHAPTER 2	Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways	**31**
CHAPTER 3	Immunobiology and classification of antibody deficiencies of unknown etiology	**63**
3.1	B-cell replication history and somatic hypermutation status identify distinct pathophysiological backgrounds in Common Variable Immunodeficiency	**65**
3.2	Common Variable Immunodeficiency and Idiopathic Primary Hypogammaglobulinemia: two different conditions within the same disease spectrum	**91**
CHAPTER 4	Immunobiology of antibody deficiency in patients with known genetic or chromosomal defects	**109**
4.1	Antibody deficiency in Ataxia Telangiectasia is caused by disturbed B and T cell homeostasis and reduced immune repertoire diversity	**111**

4.2	Autosomal dominant germline mutations in *PTEN* impair class switch recombination and somatic hypermutation and are associated with CVID like hypogammaglobulinemia.	143
4.3	Defective B-cell memory in patients with Down syndrome	163
CHAPTER 5	General Discussion and future perspectives	187
ADDENDUM		213
	Abbreviations	215
	Summary	217
	Samenvatting	221
	Dankwoord	227
	Curriculum Vitae	233
	PhD Portfolio	235
	Publications	239





Chapter 1

General Introduction

Parts of this chapter were published in:

Driessen G.J., van der Burg M. Primary antibody deficiencies. European Journal of Pediatrics 2011;170:703-702

Van der Burg M, van Zelm M.C., Driessen, G.J., van Dongen J.J.M. New frontiers of primary antibody deficiencies. Cellular and Molecular Life Sciences 2012; 69: 59-73

GENERAL INTRODUCTION

Primary antibody deficiencies (PADs) are the most common primary immunodeficiencies[1]. The hallmark of PADs is a defect in the production of normal amounts of antigen specific antibodies. These antibodies or immunoglobulins are indispensible for the adaptive immune response against a wide variety of pathogens. A defect in antibody production results in recurrent and/or severe infections. PADs represent a heterogeneous spectrum of conditions, ranging from often asymptomatic selective IgA and IgG subclass deficiencies to the severe congenital agammaglobulinemia's, in which antibody production of all immunoglobulin isotypes is severely decreased. Apart from recurrent infections there is a wide range of other clinical complications associated with primary antibody deficiency[2-4], affecting quality of life and life expectancy. Primary antibody deficiencies are the result of primary or secondary defects in B-cell development.

This Chapter will discuss the principles of adaptive immunity including normal B-cell development, followed by an introduction of the known immunogenetic and clinical characteristics of primary antibody deficiency. Next, we give an overview of potential pathophysiological mechanism in idiopathic primary immunodeficiency. Finally, the aims of the thesis are explained.

PRINCIPLES OF ADAPTIVE IMMUNITY

The cells of the adaptive immune response (B- and T-lymphocytes) are powerful players in the immune system. Each lymphocyte creates a unique antigen receptor for recognition of pathogens during precursor differentiation in bone marrow or thymus, respectively. Together, this results in a large repertoire of antigen receptors with the potential to specifically recognize many different pathogens. On top of this broad repertoire, the lymphocytes that actually recognize antigen are selected and are capable of undergoing enormous clonal proliferation, thereby generating huge numbers of daughter cells with the potential to recognize the same pathogen. This clonal expansion generates effector cells for a strong response and long term memory in the form of memory B- and T-cells and immunoglobulin (Ig)-producing plasma cells. The host requires a highly dynamic immune system, which maintains a tight balance between the production of a large repertoire of cells with unique receptors and a strong immune response of groups of cells with a highly-specific and thereby a more limited (selected) repertoire.

NORMAL B-CELL DEVELOPMENT

Generation of naive mature B-cells by stepwise differentiation in bone marrow

Precursor B-cells are generated from hematopoietic stem cells in the bone marrow, where they undergo stepwise differentiation independent from antigen (Figure 1). The main objective is to create a unique B-cell antigen receptor (BCR), which is composed of two Ig heavy chains (IgH) and Ig light chains (Igκ or Igλ). Ig genes are subjected to a genomic rearrangement process, called V(D)J recombination, to form functional proteins. During V(D)J recombination in the *IGH* locus, one Variable, one Diversity and one Joining gene segment are randomly combined to form a functional exon. Similar rearrangements are initiated between one V and one J gene segment in the *IGK* and *IGL* loci. The rearrangement process is accompanied by deletion and random insertion of nucleotides at the ends of V, D and J gene segments resulting in unique junctions. The combination of V, (D) and J gene segments and the processing of junctional regions contribute enormously to the BCR diversity between precursor-B cells. Ig gene rearrangements are initiated in pro-B cells at the *IGH* locus with D to J rearrangements, followed by V to DJ rearrangements in the pre-B-I cell stage[5]. Upon formation of a functional *IGH* gene rearrangement, an Igμ chain is expressed together with surrogate light chain proteins VpreB and λ14.1 as a pre-BCR (Figure 2). The cells are now identified as large pre-B-II cells in which proliferation is induced by expression of the pre-BCR, which signals via the CD79 complex and a network of downstream kinases and linker proteins (Figure 2)[6]. This clonal expansion phase is followed by G1 arrest, during which the surrogate light chain is down-regulated and the rearrangement process is continued at the Ig light chain loci (*IGK* followed by *IGL* in small pre-B-II cells). At the immature B-cell stage, the complete BCR is tested for functionality without high affinity for auto-antigens, upon which the cell can migrate to the periphery as transitional B-cell (Figure 1). The continuous production of B-cells in bone marrow ensures a high BCR diversity of the naive B-cell pool.

Antigen-dependent B-cell Maturation in Secondary Lymphoid Organs

Transitional B-cells are immature in their migration capacity and response to antigen, but develop rapidly into naive mature B-cells, which form the bulk of B-lymphocytes in peripheral blood. Naive mature B-cells are thought to be short-lived unless they are activated upon antigen encounter with their specific BCR. Upon binding to its cognate antigen, the BCR induces downstream signaling using the same pathways as the pre-BCR, to initiate a Ca^{2+} flux and target gene transcription (Figure 2). The CD19-complex, consisting of CD19, CD21, CD81 and CD225, is necessary for sufficiently strong signaling of the BCR (Figure 3)[7-9]. Specifically, signaling molecules are recruited upon phosporylation of multiple tyrosine residues in the intracellular tail of CD19.

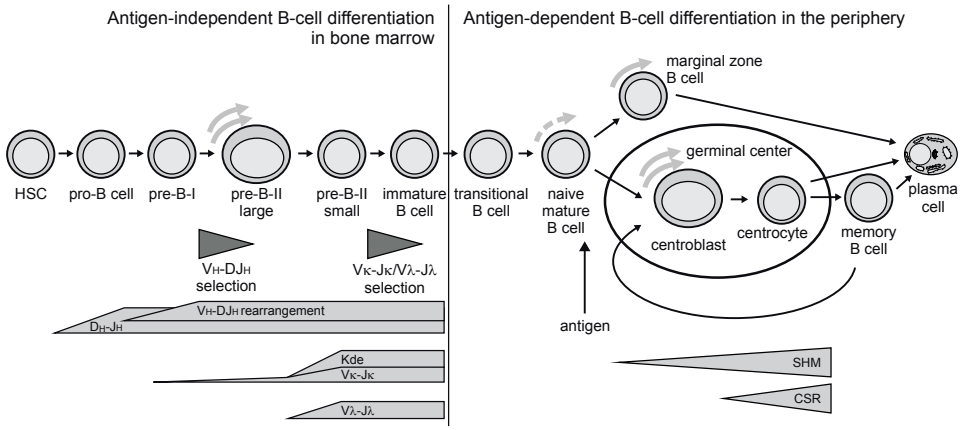


Figure 1. Molecular processes during the stepwise development of B-cells from hematopoietic stem cells (HSC) to memory B-cells and plasma cells. The Ig gene rearrangements and the selection of their functionality in the bone marrow compartment, followed by antigen-induced proliferation and selection processes in the periphery represent a highly dynamic cascade of events, which requires a tight balance between the antigen independent and antigen-dependent B-cell differentiation stages.

The B-cell antigen response depends greatly on the strength of the BCR-antigen interaction and the presence of co-stimulatory signals. Cognate CD4+ TH-cell help results in the strongest humoral response in lymph nodes and other secondary lymphoid organs. Upon CD40L-CD40 interaction (Figure 4), the activated B-cells undergo extensive proliferation and form highly organized structures: germinal centers. In germinal centers, a dark and a light zone can be identified. The dark zone mainly consists of proliferating B-cells (centroblasts) in a network of follicular dendritic cells. These cells from stromal origin present complete, unprocessed antigen via Fc and complement receptors to stimulate proliferation and survival of antigen-specific B-cells. The proliferating centroblasts induce somatic hypermutation (SHM) in their Ig genes, which changes their affinity for antigen. The centroblasts become resting centrocytes and can undergo Ig class switch recombination (CSR) and selection based on high affinity for antigen in the light zone of the germinal center (Figure 1).

The CD40-CD40L interaction induces translocation of NF-κB to the nucleus, where NF-κB activates transcription of target genes, including the gene that encodes activation-induced cytidine deaminase (AID) (Figure 4). AID deaminates cytidine residues in Ig genes, which are processed by error-prone DNA-repair proteins such as UNG and PMS2, that are finally responsible for CSR and SHM[10]. Ultimately, B-cells with high affinity BCRs

exit the germinal center and differentiate into antibody-producing plasma cells or long-lived memory cells. Whereas plasma cells generate the antibodies to neutralize antigens, memory-B-cells are long-lived resting cells that can take part in additional germinal center reactions upon new encounter with the same antigen (Figure 1).

B-cell responses can also occur independently of T-cell help in the marginal zone of the spleen or in the lamina propria in the gut[11-12]. These B-cells can be sufficiently activated by the repetitive nature of antigens recognized on blood borne pathogens[13]. Alternatively, these B-cells recognize antigens on pathogens, which also stimulate other receptors of the B-cell, such as Toll-like receptors[14]. Marginal zone like B-cells can be found recirculating in peripheral blood (also called "natural effector B-cells"), have a memory phenotype, and carry SHM[15].

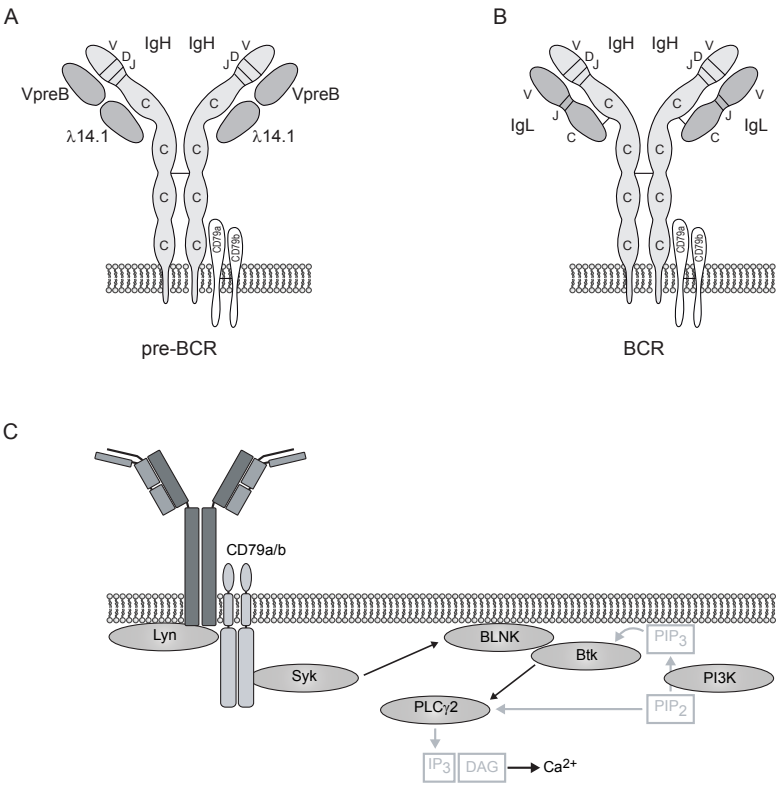


Figure 2. Pre-BCR and BCR signaling complexes. A. pre-BCR complex. **B.** BCR-complex. **C.** Schematic representation of important kinases and linker proteins involved in downstream signaling from the pre-BCR.

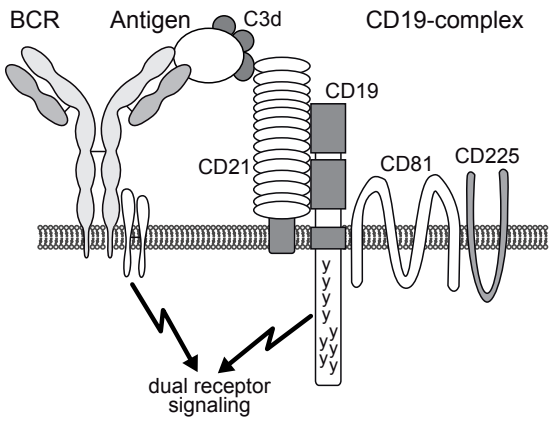


Figure 3. Schematic representation of "dual receptor signaling" upon binding of antigen to the BCR and the CD19-complexes. In this signaling process, the CD19 complex functions to decrease the threshold for BCR signaling upon antigen-binding.

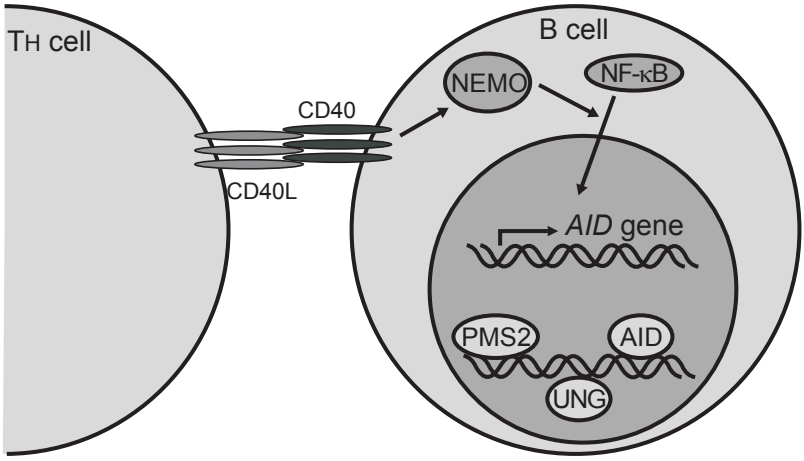


Figure 4. Induction of CSR and SHM by TH-cell – B-cell interaction in germinal centers. Upon CD40-CD40L interaction, NEMO supports translocation of NF-κB to the nucleus, where it activated *AID* gene transcription. AID introduces single strand DNA lesions in Ig genes, which can result in CSR or SHM when repaired by error-prone mechanisms involving UNG and PMS2.

IMMUNOBIOLOGY AND CLINICAL CHARACTERISTICS OF PRIMARY ANTIBODY DEFICIENCY

Defects in all critical stages of B-cell development have the potential to cause PAD (Figure 5A). Over the past 20 years, 22 genetic defects have been identified as underlying PAD (Figure 5B). The genetic basis of most cases of agammaglobulinemia and Ig CSR deficiency has been unravelled. In contrast, for CVID and partial antibody deficiency, gene defects have only been identified over the past 10 years, and in the majority of patients, a genetic defect has not (yet) been identified. Here, we give a clinical description and an historical overview of the identification of genetic defects in PAD.

CONGENITAL AGAMMAGLOBULINEMIA'S

The first report of a congenital agammaglobulinemia dates from 1952[16], when Bruton described a boy with recurrent infections and a deficiency of gammaglobulins. Many years later it appeared that boys with X-linked agammaglobulinemia (XLA) suffer from a defect in the gene for Bruton's tyrosine kinase or *BTK*[17], which is crucial for (pre)B-cell receptor signalling. Btk deficiency causes an early block in B-cell development in the bone marrow, resulting in the (near) absence of B-lymphocytes in peripheral blood and peripheral lymphoid tissues. As a result, antibody production of all immunoglobulin isotypes, including the response to vaccinations, is severely impaired. XLA accounts for 85% of all cases of congenital agammaglobulinemia.

Following the identification of BTK mutations, other components of the preBCR signaling complex became candidate genes for autosomal recessive (AR) agammaglobulinemia. The preBCR signaling complex is composed of two identical Igμ chains and the surrogate light chain proteins VpreB and λ14.1 together with the anchoring molecules CD79α and CD79β. In 1996, the first genetic defects were indeed identified in AR agammaglobulinemia in the Igμ heavy chain (IGHM)[18]. In the following years, two other genetic defects affecting preBCR expression were described, i.e., in λ14.1 and CD79α[19-20]. In addition, a mutation was described in BLNK, which is a signaling molecule downstream of the preBCR. It was not until 2007 that the first mutation in CD79β was described[21]. In 2012, a mutation in the P85α regulatory subunit of PI3K, which is also part of the (pre-)BCR signalling complex, was identified by whole exome sequencing of a patient with agammaglobulinemia and absent B-cells.[22] Apart from hypogammaglobulinemia this patient suffered from colitis.

Over half the XLA patients present before one year of age and more than 90% are diagnosed at the age of five years[23]. Fewer than 10% of the patients have symptoms in the

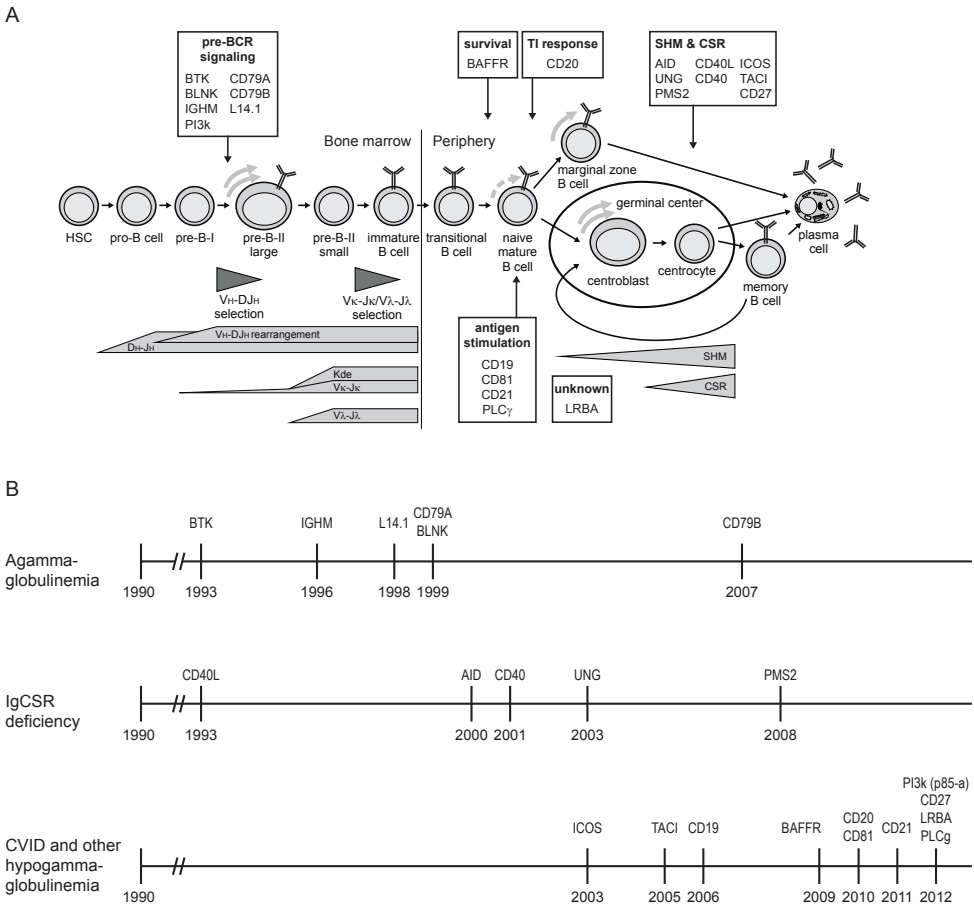


Figure 5. A. B cell differentiation. Molecular processes during the stepwise differentiation of B cells from hematopoietic stem cells (HSC) to memory B cells and plasma cells. The Ig gene rearrangements and the selection of their functionality in the bone marrow compartment are followed by antigen-induced proliferation and selection processes in the periphery. The identified PAD gene defects and the impaired differentiation steps are indicated in boxes. **B.** Identification of genetic defects in agammaglobulinemia, IgCSR deficiencies, and CVID from 1990 to 2012.

first three months, because of protection by placentally transferred maternal antibodies. Recurrent ENT and airway infections are the most frequent presenting symptoms, but children may also present with severe bacterial infections in other organ systems[23]. The clinical problems of patients with autosomal recessive forms of agammaglobulinemia are

comparable to XLA, but the clinical phenotype tends to be more severe, because of a more absolute block in early B-cell development[24].

Apart from a severe antibody deficiency, 11% of children with XLA suffer from concomitant neutropenia, which can be misdiagnosed as congenital neutropenia. Patients with congenital agammaglobulinemia's have low levels of all immunoglobulin isotypes[25]. Subsequent lymphocyte subset analysis will reveal that B-cells in the peripheral blood are severely decreased. In case B-cells are present, other PADs have to be considered, especially transient hypogammaglobulinemia of infancy and class switch recombination deficiencies (discussed below).

CLASS SWITCH RECOMBINATION DEFICIENCIES

IgCSR deficiencies were previously called hyper IgM syndromes because the patients are generally characterized by increased levels of serum IgM in combination with reduced levels of IgG and IgA. However, patients with a IgCSR defect can also have a normal serum IgM level. Therefore, the term IgCSR deficiency has been introduced in the WHO classification[26]. The disease causing mechanism is either a disturbed co-stimulation of B-cells and T-cells in the germinal centre, affecting the initiation of CSR, or a deregulation of the class switch process itself. In 1993, the first genetic defect in patients with an IgCSR deficiency was identified in the X-linked CD40L gene (Figure 5B)[27] Eight years later, in 2001, a mutation in the receptor of CD40L, CD40, was identified[28]. CD40-CD40L interaction plays an important role in T-cell-dependent B cell proliferation and differentiation and in the induction of CSR and somatic hyper mutations (SHM) (see below). As such, it is the prototype of a co-stimulation defect[27,29-31]. CD40L deficiency not only causes a PAD, but also results in a T-cell deficiency, because CD40 triggering plays a central role in T-cell-mediated activation of monocytes and dendritic cells. Therefore CD40L deficiency is nowadays primarily classified as a T-cell disorder[26]. Because of the T-cell deficiency in CD40L patients, an important difference with other PADs is the occurrence of opportunistic infections. Apart from a bacterial pneumonia, which occurs in 80% of the patients, 41% suffer from pneumocystis jiroveci pneumonia[31].

In 2000, homozygosity mapping in eight consanguineous families with patients with a hyper IgM syndrome pointed to a genomic region harboring the AID gene[32]. From mouse studies it was known that AID expression is strictly restricted to B cells and induces CSR and SHM, therefore this gene was sequenced in these families and was found to be mutated. This made AID the first candidate gene for autosomal recessive B-cell-intrinsic

IgCSR deficiencies. Apart from recurrent infections patients with AID deficiency often suffer from lymphoid hyperplasia, inflammatory bowel disease and auto-immunity[33-34].

In 2003, the group of Durandy described UNG as a second candidate gene[34]. As the phenotype of AID deficiency resembled the phenotype of Ung-deficient mice[35], the possibility of UNG deficiency in these patients was explored, and mutations were indeed identified. UNG deficiency is characterized by impairment of CSR and a partial disturbance of the SHM pattern.

In 2008, the same group described—in patients with an IgCSR defect without a disturbed SHM process—mutations in the PMS2 gene[36], which is a component of the mismatch repair system known to play a role in CSR[37].

Apart from the B-cell intrinsic CSR defects, abnormalities of CSR have been reported in syndromic forms of primary immunodeficiency. X-linked anhidrotic ectodermal dysplasia with immunodeficiency secondary to mutations in *NEMO* affect CD40 mediated B-cell differentiation[38]. Furthermore, Ataxia Telangiectasia has been associated with a clinical phenotype of CSR deficiency[39]. Ataxia Telangiectasia results from mutations in the *ATM* gene. The ATM protein is implicated in Non-homologous End Joining, a DNA repair process that is important for V(D)J recombination as well as CSR[40].

There is still a group of patients with a defined IgCSR defect in combination with a normal SHM frequency in whom the genetic defect has not yet been unravelled (reviewed by Kracker et al.[41]).

COMMON VARIABLE IMMUNODEFICIENCY AND OTHER HYPOGAMMAGLOBULINEMIA'S

Common Variable Immunodeficiency (CVID) is the most prevalent idiopathic primary antibody deficiency characterized by hypogammaglobulinemia. The estimated prevalence is 1:25000. CVID is defined by serum IgG levels below 2 SD of normal controls in the presence of decreased IgA and/or IgM levels, recurrent infections, impaired response to immunization, exclusion of defined causes of hypogammaglobulinemia, and an age above two years (ESID-PAGID-criteria "probable CVID", www.esid.org). A considerable group of patients suffer from idiopathic primary hypogammaglobulinemia, but do not fulfil all the diagnostic criteria. These patients are usually diagnosed as "possible" CVID or as having a "CVID-related" or "CVID-like" primary antibody deficiency. Most CVID patients present in young adulthood, but symptoms start in childhood in more than half of the cases[3]. Consequently, a diagnostic delay of more than five years is the rule[2,42]. Sometimes CVID is preceded by IgA deficiency, IgG subclass deficiency or a specific anti-polysaccharide

antibody deficiency. The presenting symptoms in CVID are diverse, but recurrent recurrent ENT and airway infections are present in more than 90% of the patients. Non-infectious clinical complications are present in one fifth of the patients. These complications include auto-immune disease, most often auto-immune cytopenia´s, granulomatous inflammation of tissues such as the lungs and gastrointestinal tract, chronic diarrhoea secondary to unexplained enteropathy and haematological malignancies, which are an important case of death. CVID patients who suffer from at least one non-infectious complication have a much higher mortality compared to patients who only suffer from infectious complications[43-45]. Less than 10% of the CVID patients have a positive family history[2] and a genetic defect has only been identified in less than 5% of the patients who have been reported to the ESID primary immunodeficiency database with the clinical phenotype of CVID[1].

The first genetic defect in patients with a CVID clinical phenotype was identified by Grimbacher et al. and concerned a homozygous deletion of exons 2 and 3 of the "inducible costimulator" or ICOS gene (Figure 5A)[46]. Initially, the same mutation had been reported in nine patients from four families, indicating a founder effect in these families[46-48]. In 2009, a second ICOS mutation was identified in two Japanese siblings[49]. ICOS is expressed on activated T cells and interacts with ICOSL on B cells and dendritic cells[50]. ICOS-ICOSL is important for T-B-co-activation, CD40-mediated CSR, secretion of cytokines, and development of a Th2 immune response[49,51].

In 2005, mutations in TACI (transmembrane activator and CAML interactor) were identified in patients with CVID and IgA deficiency by two independent groups[52-53]. TACI belongs together with BAFF-R and BCMA to the TNF receptor superfamily, and interacts with the ligands BAFF and APRIL is crucial for development and maintenance of humoral immune response[53]. Heterozygous TACI mutations result in increased disease susceptibility, but are not likely to be disease causing, because these heterozygous mutations are also found in healthy individuals[54]. In 2009, a homozygous BAFF-R mutation was reported in two siblings with reduced serum IgM and IgG levels but with normal IgA concentrations[55]. The deficiency was identified by screening the CVID cohort for individuals with potential defects in genes regulating B cell survival and homeostasis. Only one of these patients had recurrent infections, which indicates that a BAFF-R deficiency does not always result in a clinically manifest immunodeficiency[55].

Another category of CVID concerns deficiencies of the CD19 complex. This complex, consisting of CD19, CD21, CD81, and CD225, reduces the threshold for antigen-dependent stimulation via the B cell receptor. In 2006, the first genetic defect in the CD19 gene was described, which illustrated that a defect in the CD19 complex gives rise to antibody deficiencies[56]. In the following years, a total of seven different mutations were described in nine patients[57]. In 2010, a mutation in the CD81 gene was identified[58]. CD81 is essential for CD19 expression, but for the other two complex members it is not known whether they

are as essential for CD19 expression as CD81. In 2012, the first human CD21 deficiency has been reported [59].

In 2012 three other mutations have been associated with CVID-like hypogammaglobulinemia[60-62]. Apart from hypogammaglobulinemia and infections, patients had more extensive clinical presentation. Patients with CD27 deficiency suffered from persistent symptomatic EBV viremia[62]. Lack of CD27 expression was associated with impaired T cell-dependent B-cell responses and T-cell dysfunction. Defects in PLCγ2 were characterized by cold urticaria and variable manifestations such as atopy, granulomatous rash and autoimmune thyroiditis.[60] PLCγ2-expressing cells, including B-cells, had diminished cellular signaling at 37 degrees °C, but enhanced signaling at sub-physiologic temperatures. Patients with mutations in LRBA (lipopolysaccharide responsive beige-like anchor protein) are prone to auto-immunity, especially auto-immune cytopenia's.[61] LRBA mutations resulted in disturbed B cell development, defective in vitro B cell activation, plasmablast formation, immunoglobulin secretion and low proliferative responses.

The identified genetic defects in CVID affect different steps or processes of B cell differentiation (Figure 5), which supports the assumption that the immunopathological causes of CVID are heterogeneous. Pathophysiological mechanism of patients with known mutations can serve as a disease model for the idiopathic cases. The heterogeneity of the immunological and clinical features of CVID hampers the discovery of underlying disease causing mechanisms, clinically relevant prognostic factors and genetic defects.

PARTIAL ANTIBODY DEFICIENCIES

Selective IgA, IgG_2 subclass and Specific anti-polysaccharide Antibody Deficiency.

These three PADs tend to appear in combination. As single conditions they are often asymptomatic, but a combination more often leads to a clinically significant immunodeficiency characterized by recurrent respiratory infections. The partial antibody deficiencies can be considered as part of a spectrum of idiopathic primary antibodies deficiencies which include CVID and CVID related PAD.

Selective IgA-deficiency (sIgAD) is defined as a decrease of serum IgA <2SD of age matched controls. The prevalence of sIgAD in Europe varies between 1:163 and 1:875 [63-64]. The incidence is much lower in Asian populations [65]. Although the cause of sIgAD is unknown, mutations in *TACI* increase disease susceptibility, similar to CVID[66]. Furthermore CVID and sIgA deficiency cluster in families. Furthermore sIgAD is associated with a higher prevalence of allergy/atopy and a range of auto-immune diseases, including auto-immune cytopenia's[67-69].

The four IgG-subclasses are defined by the structure of their constant regions. Of the IgG subclass deficiencies, at least IgG_2 deficiency is clinically relevant. A decrease in IgG_1 cannot be considered as an IgG subclass deficiency, because a decrease of IgG_1 is usually associated with hypogammaglobulinemia. Antibodies against encapsulated bacteria are mainly of the IgG_2 subclass, and IgG_2 deficiency increased the susceptibility to these bacteria. Symptomatic children with IgG_2 subclass deficiency should be tested for a concomitant specific anti-polysaccharide antibody deficiency (SPAD) if they are older than two years[69]. Children under the age of 10 may recover spontaneously[70]. IgG_2 deficiency has been reported in patients with DNA repair disorders such as Ataxia Telangiectasia[71], but in most patients the cause in unknown.

Specific antipolysaccharide antibody deficiency.

Although the pathophysiology is unknown in most patients, a deficiency of CD20 results in an impaired T-cell-independent (TI) antibody response[72]. The antibody response to polysaccharide antigens is impaired in healthy children under the age of two to three years, which contributes to their susceptibility to infections with encapsulated bacteria. However, some infants are able to produce normal responses to certain pneumococcal serotypes[73-74]. After the age of two to three years children should be able to mount a sufficient response to pneumococcal polysaccharides. An insufficient response after this age defines the presence of a SPAD.

AIMS OF THE THESIS

Many genetic defects have been identified that cause primary antibody deficiency (PAD), but in the majority of patients with PAD the underlying pathophysiological mechanism and causative genetic defects are still unknown. Most of these patients suffer from CVID or closely related heterogeneous disorders. In several other categories of patients with defined genetic defects or chromosomal abnormalities associated with antibody deficiency, the underlying pathophysiological mechanisms have not been fully explored. Examples are the DNA repair disorder Ataxia Talangiectasia and Down syndrome.

To address and understand the heterogeneity in CVID, several classification systems have been developed based on immunophenotyping of B cell subsets. These classification systems aimed for correlating immunophenotypes to clinical complications in subgroups of CVID patients[75-78]. In several studies decreased proportions of switched memory B-cells in the peripheral blood have been associated with auto-immunity, granuloma's and respiratory infections[78-81]. The response to vaccination was used as an alternative approach for the classification CVID patients. Non-responders to meningococcal polysaccharide

vaccination tended to suffer from respiratory infections and bronchiectasis[82]. Furthermore, analyses of T-cell subsets classified CVID patients in subgroups with different profiles of clinical complications[83-85]. Finally, others divide patients in subgroups based on KREC and TREC analysis of full blood or peripheral blood mononuclear cells[86-87]. So far, the only CVID classification that has demonstrated to predict mortality in CVID is based on clinical phenotypes; Patients with non-infectious disease related complications, such as auto-immune cytopenia's, granulomatous inflammation and/or enteropathy have impaired long term survival compared to patients without these complications[43-45].

Although most of these studies demonstrated a relationship between immunological markers and existing clinical complications, there are several important issues that have not been addressed in the current classifications:

1. Identification of relevant immunological prognostic factors of morbidity and mortality.
2. Association with pathophysiological mechanisms
3. Use of age-matched reference values of well defined B-cell subsets in children.

The aim of the studies described in this thesis was to increase insight into the pathophysiological mechanisms that underlie primary antibody deficiency in children and adults. To achieve this we used two different strategies. First, we performed a detailed analysis of peripheral B-cell development by flow cytometric and molecular approaches in patients with idiopathic hypogammaglobulinemia in order to create homogenous subgroups with a similar pathophysiology. Furthermore, we explored the clinical complications of these subgroups.

Secondly, we aimed to unravel the disease-causing mechanism in patients with a known genetic or chromosomal defects associated with abnormal B-cell development, because immunological studies in these patients will not only have the potential to understand the pathophysiological mechanisms in these particular conditions, but can also be used to shed light on the pathophysiology of idiopathic antibody deficiencies such as CVID.

OUTLINE OF THE THESIS

In Chapter 2 we characterized circulating memory B-cell subsets to determine their origin and maturation pathways through analysis of their molecular characteristics, in order to use these data to explore memory B-cell differentiation in patients with primary antibody deficiency.

Chapter 3.1 links the composition of the peripheral B-cell compartment in children and adults to *in vivo* B-cell replication and somatic hypermutation status, thereby identifying pathophysiological mechanisms in immunologically homogenous CVID subgroups with specific B-cell patterns.

Chapter 3.2 further explores the approach of Chapter 2 and 3 in patients with a poorly defined hypogammaglobulinemia, which we named Idiopathic Primary Hypogammaglobulinemia (IPH). We aimed to clarify whether IPH is a clinically relevant antibody deficiency and to determine pathophysiological aspects of IPH compared to CVID. This study was facilitated by the generation of age matched normal reference values for peripheral B-cell subsets.

In Chapter 4 we describe peripheral B-cell development in patients with known genetic or chromosomal abnormalities to generate insight into pathophysiological mechanisms of PAD.

In Chapter 4.1 the consequences of *ATM* mutations for peripheral B-cell development and immunological disease severity was studied. We analyzed the peripheral B-cell and T-cell development in 15 AT patients with different degrees of the severity of their immunodeficiency by flow cytometry, *in vivo* B-cell replication history by KREC analysis, SHM and CSR to IgA and IgG subclasses and B-cell repertoire with molecular techniques.

Chapter 4.2 aimed at identifying the mechanisms of disease in patients with heterozygous germline mutations in *PTEN*. Conditional knockout of *PTEN* in mice B-cells (*bPTEN*$^{-/-}$) is associated with abnormalities in B-cell development and antibody production. Mutations in *PTEN* have not been associated with primary antibody deficiency in humans. By using a comparable approach as described in Chapter 4.1 we identified a novel disease causing mechanism of primary antibody deficiency in humans.

Chapter 4.3 aimed at identifying abnormalities in B-cell development in patients with Down syndrome, who have a very variable immunodeficiency. We showed that defects in B-cell development patients in Down syndrome resemble a subgroup of CVID patients.

The implications of the studies are discussed in the General Discussions (Chapter 5) which also gives directions for future research.

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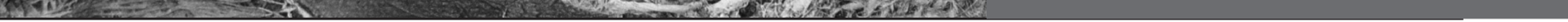

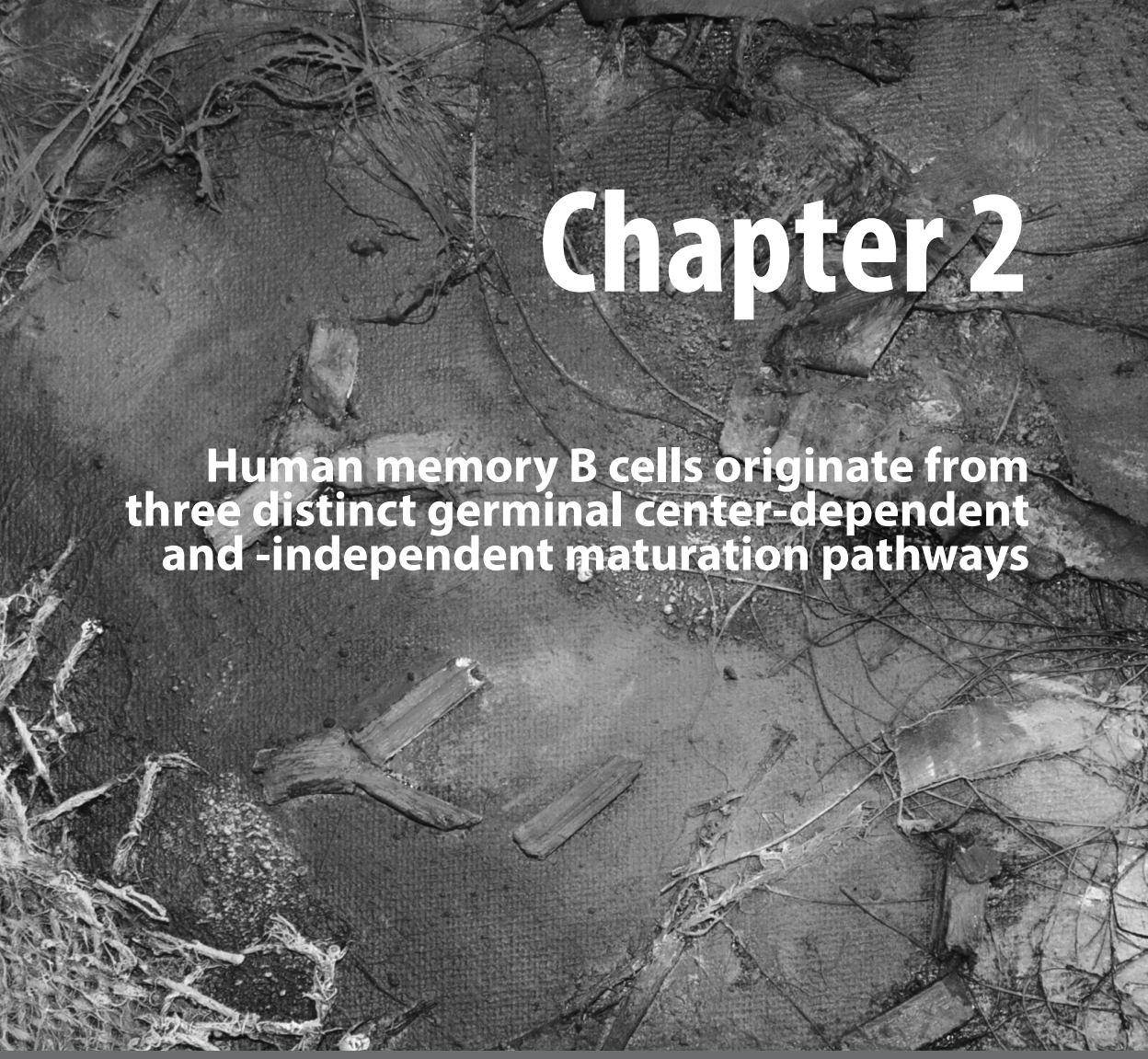

Chapter 2

Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways

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Blood. 2011 August 25; 118(8): 2150–2158

ABSTRACT

Multiple distinct memory B-cell subsets have been identified in humans, but it remains unclear how their phenotypic diversity corresponds to the type of responses from which they originate. Especially, the contribution of germinal center-independent responses in humans remains controversial. We defined 6 memory B-cell subsets based on their antigen-experienced phenotype and differential expression of CD27 and IgH isotypes. Molecular characterization of their replication history, Ig somatic hypermutation, and class-switch profiles demonstrated their origin from 3 different pathways. $CD27^{-}IgG^{+}$ and $CD27^{+}IgM^{+}$ B cells are derived from primary germinal center reactions, and $CD27^{+}IgA^{+}$ and $CD27^{+}IgG^{+}$ B cells are from consecutive germinal center responses (pathway 1). In contrast, natural effector and $CD27^{-}IgA^{+}$ memory B cells have limited proliferation and are also present in CD40L-deficient patients, reflecting a germinal center-independent origin. Natural effector cells at least in part originate from systemic responses in the splenic marginal zone (pathway 2). $CD27^{-}IgA^{+}$ cells share low replication history and dominant Igλ and IgA2 use with gut lamina propria IgA^{+} B cells, suggesting their common origin from local germinal center-independent responses (pathway 3). Our findings shed light on human germinal center-dependent and -independent B-cell memory formation and provide new opportunities to study these processes in immunologic diseases.

INTRODUCTION

Antigen-specific memory formation after a primary infection contributes greatly to human health. Immunologic memory lies in long-lived T and B cells derived from the initial immune response. Precursor B cells develop from hematopoietic stem cells in the bone marrow and create a unique receptor by V(D)J recombination in their immunoglobulin (Ig) loci.[1–3] After antigen recognition, mature B cells proliferate and can further optimize antigen-binding by the introduction of point mutations in the V(D)J exons of their Ig heavy and light chains (somatic hypermutations; SHMs) and the subsequent selection for high-affinity mutants.[4] Furthermore, the antibody effector functions can be modified by changing the isotype of the *IGH* constant region from μ to α, δ, ϵ, or γ (Ig class-switch recombination; CSR).[5] Both processes are mediated by activation-induced cytidine deaminase (AID), which preferentially targets specific DNA motifs.[6,7]

In addition to antigen recognition via the B-cell antigen receptor (BCR), B cells need a second signal to become activated.[8] Activated T cells can provide such a signal via CD40L that interacts with CD40 on B cells. T cell–dependent B-cell responses are characterized by germinal center (GC) formation, extensive B-cell proliferation, affinity maturation, and Ig CSR.[9] Thus, high-affinity memory B cells and Ig-producing plasma cells are formed. In addition, B cells can respond to T cell–independent (TI) antigens that either activate via the BCR and another (innate) receptor (TI-1) or via extensive cross-linking of the BCR because of the repetitive nature of the antigen (TI-2).[10] TI responses are directed against blood-borne pathogens in the splenic marginal zone and in mucosal tissues (reviewed in Cerutti *et al*[11] and Weill *et al*[12]).

A substantial fraction of B cells in blood of human subjects has experienced antigen and shows hallmarks of memory B cells: SHMs of rearranged Ig genes and fast recall responses to antigen.[13] Initially, human memory B cells were identified based on the expression of CD27.[14,15] IgA and IgG class-switched $CD27^+$ B cells are derived from T cell–dependent responses in the GC and contain high loads of SHMs in their Ig genes.[16–18] $CD27^+IgM^+$ B cells contain less SHMs but show molecular footprints of (early) GC generation.[19] Interestingly, in contrast to $CD27^+IgM^+IgD^-$ "IgM-only" cells, $CD27^+IgM^+IgD^+$ "natural effector" B cells are present in patients with CD40 or CD40L deficiency, indicating that at least part of this subset can be generated independently of T-cell help.[17,20,21] Furthermore, natural effector B cells resemble splenic marginal zone B cells and have a limited replication history compared with GC B cells (both centroblasts and centrocytes) and $CD27^+IgD^-$ memory B cells.[17,18]

More recently, $CD27^-$ IgG and IgA class-switched B cells have been identified. $CD27^-IgG^+$ B cells contain fewer SHMs in their Ig genes and have increased IgG3 use compared with their $CD27^+$ counterparts.[22,23] Thus, 6 B-cell subsets have been described to contain genetic hallmarks of B-cell memory. This raises the question whether all these subsets

show functional characteristics of memory B cells[25] and whether the phenotypic diversity reflects functional diversity or an origin from different maturation pathways.

We performed detailed analyses on 6 phenotypically distinct memory B-cell subsets, which all seem to display an activated phenotype and molecular signs of antigen recognition. The comparative analyses of replication history, SHM, and CSR profiles of these subsets enabled us to trace their origins to 3 different germinal center-dependent and -independent maturation pathways.

METHODS

Flow cytometric immunophenotyping and purification of B-cell subsets from human peripheral blood, tonsils, and colon

Peripheral blood, tonsil, and colon samples were obtained with informed consent following the Declaration of Helsinki and according to the guidelines of the Medical Ethics Committee of Erasmus MC and the Institutional Review Board of Weill Medical College of Cornell University.

Immunophenotyping and cell sorting details are provided in supplemental Methods.

Hematoxylin and eosin staining

Up to 30 000 cells from each sorted population were applied to poly-l-lysine–coated slides and stained with Diff-Quik staining set (Medion Diagnostics). Pictures were acquired on an Axioskop microscope using a Plan-NEOFLUAR 63/1.25 oil objective, MRc5 digital camera, and Axio Vision Release 4.8.1 software (Carl Zeiss).

CD40L-deficient patients

All 5 CD40L-deficient patients lacked expression of CD40L protein on activated T cells as shown after 5-hour stimulation with phorbol 12-myristate 13-acetate (Sigma-Aldrich) and calcium ionophore (Sigma-Aldrich). Mutations were detected by exon sequencing of the *CD40L* gene. Details of the patients are shown in supplemental Table 3.

Sequence analysis of complete IGH gene rearrangements and Ig switch regions

DNA was isolated from each sorted subset with the GenElute Mammalian Total DNA Miniprep kit, and RNA was isolated from Ig class-switched B-cell subsets using the GeneElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich). Complete *IGH* gene rearrangements and hybrid switch regions were amplified and analyzed as described in supplemental Methods.

Replication history analysis using the KREC assay

The replication history of sorted B-cell subsets was determined with the Igκ-deleting recombination excision circles (KREC) assay as described previously.[18] In brief, the amounts of coding and signal joints of the *IGK*-deleting rearrangement were measured by real-time quantitative-PCR in DNA from sorted B-cell populations on an ABI Prism 7000 sequence detection system (Applied Biosystems). Signal joints, but not coding joints are diluted 2-fold with every cell division.[18] To measure the number of cell divisions undergone by each population, we calculated the ratio between the number of coding joints and signal joints. The previously established control cell line U698 DB01 (InVivoScribe) contains 1 coding and 1 signal joint per genome and was used to correct for minor differences in efficiency of both real-time quantitative-PCR assays.

IgκREHMA

The frequency of mutated *IGK* alleles was determined with the Igκ restriction enzyme hot-spot mutation assay (IgκREHMA) as described previously.[18,26] In brief, PCR was performed on genomic DNA using a hexachlorofluorescein phosphoramidite (HEX)–coupled *IGKV3-20* intron forward primer and two 5-carboxyfluorescein–coupled *IGKJ* reverse primers recognizing all 5 *IGKJ* genes. The PCR products were digested by the KpnI and Fnu4HI restriction enzymes and run on an ABI Prism 3130 XL genetic analyzer. Fnu4HI recognizes 2 adjacent sites in the unmutated gene product in the hot-spot region of IGKV-complementarity-determining region (CDR) 1. Unmutated gene products can therefore be visualized as 244- or 247-bp HEX-coupled fragments. KpnI cuts the gene product in FR2 downstream of the Fnu4HI sites, resulting in a 262-bp HEX-coupled mutated fragment. The unmutated B cell line CLL-1 was used as a positive control for complete digestion with Fnu4HI. The digests hardly contained undigested gene products of 481 bp, indicating complete digestion by KpnI.

Statistical analyses

Statistical analyses were performed with the Mann-Whitney U test, or χ^2 test as indicated in details in figure legends. P values < .05 were considered statistically significant.

RESULTS

Phenotypic characterization of memory B-cell subsets in healthy individuals

To study the diversity in the human B-cell compartment, we defined and purified 2 naive and 6 memory B-cell subsets (Figure 1A). Within the $CD19^+$ B-cell compartment, we defined $CD38^{hi}CD24^{hi}$ transitional B cells. $CD38^{dim}CD24^{dim}$ B cells were subdivided based

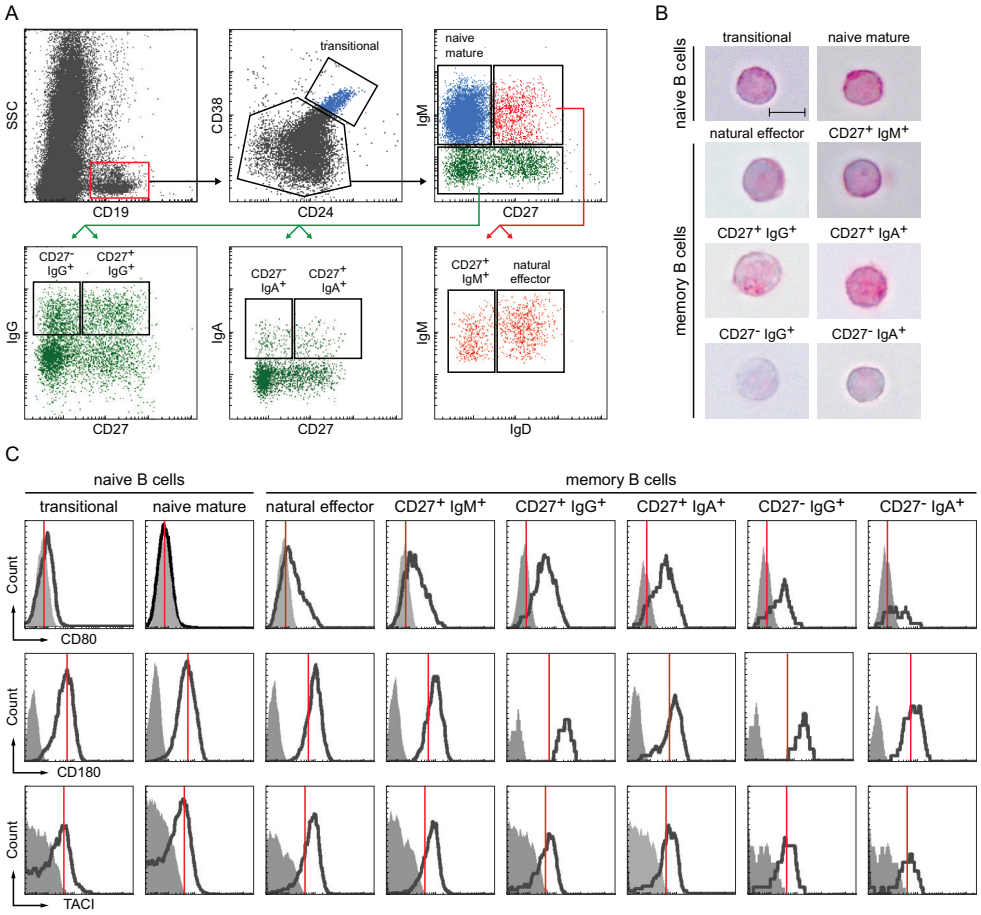

Figure 1. Isolation and phenotypic characterization of peripheral blood memory B-cell subsets. (A) Gating strategy to identify 2 naive and 6 memory B-cell subsets based on expression of CD24, CD38, CD27, and IgH isotypes. (B) H&E staining of sorted subsets revealed a typical lymphocytic morphology with large nucleus (purple) and little cytoplasm (pink; ×63, original magnification; bars represent 5 μm). (C) All 6 memory B cell subsets showed up-regulation of CD80, CD180 and TACI as compared with naive B cells. Expression levels are shown in black and isotype controls as filled, gray histograms. Red lines indicate mode expression levels for each molecule on naive mature B cells.

on the expression of IgM and CD27. Naive mature B cells were defined as $CD27^{-}IgM^{+}$. $CD27^{+}IgM^{+}$ B cells were separated into IgD^{+} "natural effector" B cells and IgD^{-} "IgM-only" B cells. Finally, IgM-negative B cells were separated into 4 class-switched B cell populations based on the expression of IgA, IgG, and CD27.

All 8 purified subsets had a typical lymphocytic morphology with a large nucleus and little cytoplasm as observed after hematoxylin and eosin staining (Figure 1B). Furthermore,

all 6 memory B-cell subsets showed an immunophenotype that was characteristic for activated cells; with increased expression of the B7 family member CD80, TLR-related CD180, and TNF receptor superfamily member TACI compared with naive B-cell subsets (Figure 1C).[25,27] In addition, all B-cell subsets highly expressed BAFFR, and all memory B-cell subsets showed bimodal expression of inhibitory collagen receptor CD305 and were dimly positive for CD95 (data not shown).[25,28] Thus, all 6 subsets we studied had the phenotype that was reported to be important for fast and powerful memory responses.

Ig repertoire selection in memory B-cell subsets

To study whether the memory B-cell subsets showed molecular signs of antibody selection, we sequenced *IGH* gene rearrangements from sorted fractions of healthy adult donors and compared these with naive B-cell subsets from adult blood as well as with GC B cells from childhood tonsils. We analyzed gene use for the most frequent *IGHV* subgroups: *IGHV3* and *IGHV4*.[29,30] All subsets showed diverse usage of *IGHV3* subgroup genes with *IGHV3-23*, *IGHV3-21*, and *IGHV3-30* predominating (Figure 2A). Naive mature B cells showed dominant use of the *IGHV4-34* and *IGHV4-59* genes (Figure 2B), probably resulting from increased recombination frequency because of highly efficient recombination signal sequences.[31,32] Importantly, *IGHV4-34* was hardly used in memory B-cell subsets, indicating selection against this inherently autoreactive gene.[33,34]

Of the 3 CDRs, the VDJ-junction encoded CDR3 region is the most dominant in establishment of antigen binding specificity. Long IGH-CDR3s are associated with auto- and polyreactivity.[35] We observed diverse IGH-CDR3 sizes in transitional and naive mature B cells, with a median of 17 amino acids (Figure 2C). The median size was slightly reduced to 16 in both centroblasts and centrocytes. All memory B-cell subsets had significantly ($P < .05$) shorter IGH-CDR3s (median of 14-15 amino acids) compared with naive mature B cells. Thus, all 6 memory B-cell subsets showed comparable signs of Ig repertoire selection.

Distinct degrees of replication history and SHMs in memory B-cell subsets

Typical hallmarks of memory B cells are extensive antigen-induced proliferation and SHMs. We showed previously that GC B cells in tonsils from young children have undergone ~8 cell cycles, by calculating the ratio between genomic coding joints and signal joints on KREC of the *IGK*-deleting rearrangement.[18] This replication history was similar in childhood $CD27^+IgD^-$ B cells but clearly higher in adulthood $CD27^+IgD^-$ cells, probably because of consecutive GC reactions. Proliferation of GC B cells was accompanied by SHMs in their Ig loci and further enrichment of mutated *IGKV3-20* alleles in memory B cells, both in children and adults.[18] We quantified the replication history, the frequency of mutated nucleotides in rearranged *IGHV* genes, and the frequency of mutated *IGKV3-20* alleles in 2 naive and

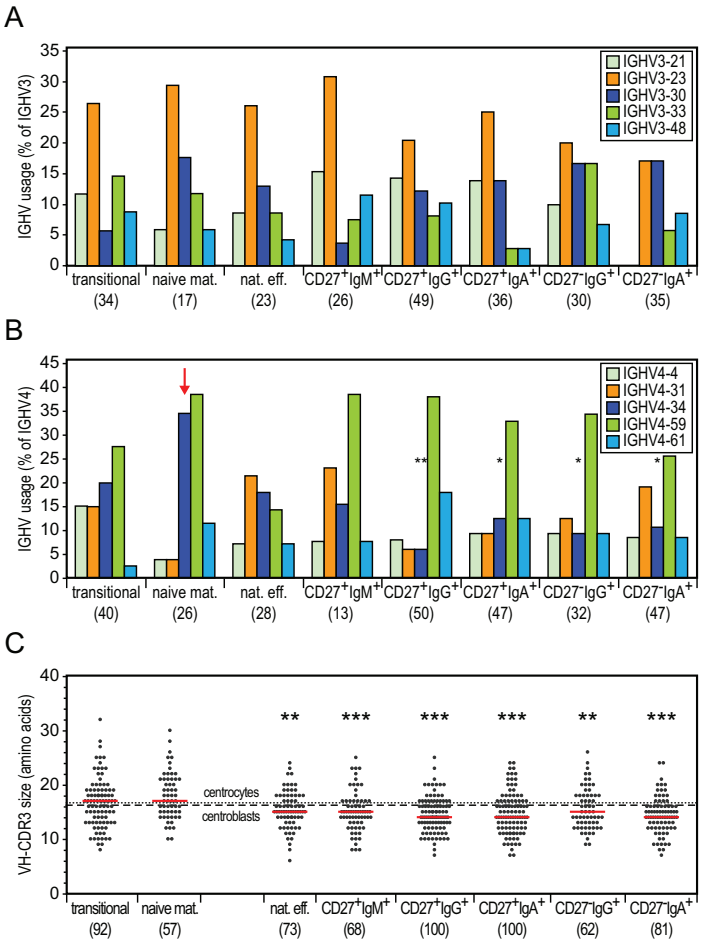

Figure 2. Selection against the *IGHV4-34* gene and long IGH-CDR3s in all 6 memory B-cell subsets. (A) Frequencies of the most commonly used *IGHV3* genes in cloned *IGH* gene rearrangements. Differences between each memory B-cell subset compared with naive mature B cells were statistically analyzed with the χ^2 test. (B) Frequencies of the most commonly used *IGHV4* genes in cloned *IGH* gene rearrangements. An arrow indicates *IGHV4-34* gene use in naive mature B cells. Differences between each memory B-cell subset compared with naive mature B cells were statistically analyzed with the χ^2 test. $^{*}P < .05$, $^{**}P < .01$. (C) IGH-CDR3 size distributions. All individual sizes are indicated for each subset as gray dots, with red lines representing the median values. The dashed and dotted lines represent median values for centroblasts (n = 67) and centrocytes (n = 55), respectively. Differences between each memory B-cell subset compared with naive mature B cells were statistically analyzed with the Mann-Whitney test. $^{**}P < .01$, $^{***}P < .001$.

all 6 memory B-cell subsets. As shown before, transitional B cells did not undergo proliferation since their release from bone marrow, whereas naive mature B cells underwent ~2 cell cycles in absence of SHMs (Figure 3).[18] Conventional adult CD27^{+}IgG^{+} and CD27^{+}IgA^{+}

B cells underwent the highest number of cell divisions (~10) with high levels of SHMs. Both proliferation and SHM levels were clearly higher than in GC B cells from childhood tonsils. This might suggest additional proliferation and mutation in consecutive GC reactions.

IgM-only and CD27$^-$IgG$^+$ B cells underwent ~9 cell divisions and had similar SHM levels in rearranged *IGHV* genes as GC B cells but increased frequencies of mutated *IGKV3-20* alleles. The characteristics of both subsets suggest an origin from primary GC responses followed by selection for mutated *IGKV3-20*.

Finally, natural effector and CD27$^-$IgA$^+$ B-cell subsets showed less proliferation compared with GC B cells (Figure 3A). Natural effector B cells showed only 7 cell cycles, whereas the *IGHV* mutation loads were similar to GC B cells, and these cells were enriched for mutated *IGKV3-20* alleles. These proliferation and SHM levels were clearly higher than those observed for natural effector cells in childhood tonsils.[18] Still, these results indicate that a substantial fraction of this population had been generated independently from a GC. Finally, we observed only 4 cell divisions for CD27$^-$IgA$^+$ B cells. Interestingly, the *IGHV* gene mutation loads were increased as compared with GC B cells, although the frequency of mutated *IGKV3-20* alleles was similar. These results indicate a GC-independent origin of CD27$^-$IgA$^+$ B cells but with high AID activity generating high SHM levels and IgA class switching. Still, these cells lacked selection for mutated *IGKV3-20* alleles.

Targeting and selection of SHMs in rearranged IGHV genes

We analyzed type and targeting of SHMs in the memory B-cell subsets to obtain insight into the activity of AID, POLη, and UNG. Neither the SHM targeting nor the nucleotide substitution spectra and transition/transversion ratios were significantly different between the memory B-cell subsets and centrocytes (supplemental Table 1 and supplemental Figure 1). Furthermore, the targeting of specific nucleotides in motifs was largely similar between subsets (supplemental Table 2). Thus, we conclude that the differences in mutation frequencies did not result from altered AID, UNG, and Polη activities; rather, they probably reflect the duration of exposure to these enzymes.

Generally, a high ratio of replacement versus silent mutations (R/S) in IGHV-CDRs is regarded as a molecular sign of affinity maturation. Nevertheless, a clear cut-off value, which would reflect antigenic selection, remains difficult or even impossible to define.[36] We found accumulation of replacement mutations in CDR1 and CDR2 of rearranged *IGHV* genes in all analyzed subsets (supplemental Figure 2). IGHV-CDR R/S ratios were similar between all memory B-cell subsets, ranging between 3.3 and 4.0, except for CD27$^-$IgG$^+$ B cells that had a slightly lower IGHV-CDR R/S ratio of 2.3 (supplemental Table 1).

Alignment of rearranged *IGHV* genes revealed the existence of recurrent amino acid changes (ie, the same amino acid replacement at the same position) in all except the natural effector, transitional, and naive mature B-cell subsets. In centrocytes, we identified

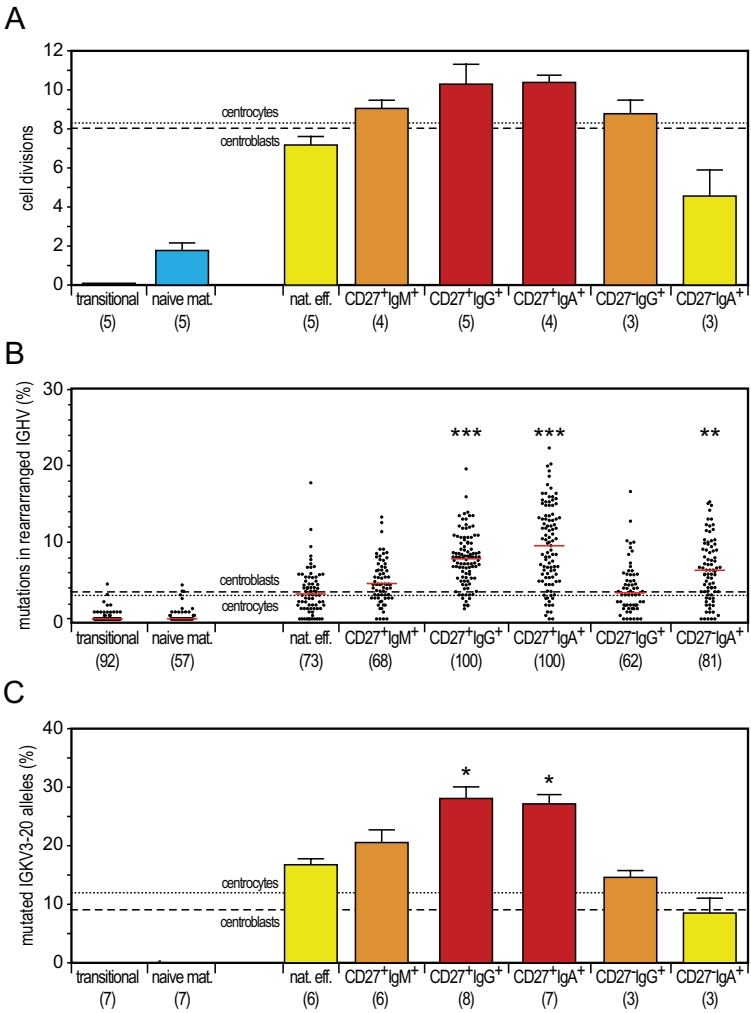

Figure 3. Discrimination of GC-dependent and -independent B-cell maturation pathways based on quantitative analysis of the replication history and SHM levels. (A) Replication history of 2 naive and 6 memory B-cell subsets as measured with the KREC assay.[18] Three different levels of extensive proliferation in memory B-cell subsets in contrast to naive B cells (blue) could be identified: lower than GC (yellow bars), similar to GC (orange bars) and increased compared with GC (red bars). Bars represent mean values with SEM. In the whole figure, dashed and dotted lines represent values for centroblasts and centrocytes, respectively. Differences between each memory B-cell subset compared with centrocytes were statistically analyzed with the Mann-Whitney test. (B) Frequency of mutated nucleotides in rearranged *IGHV* genes. All individual data points are shown as gray dots, with red lines indicating the median value. Differences between each memory B-cell subset compared with centrocytes were statistically analyzed with the Mann-Whitney test. $^{**}P < .01$, $^{***}P < .001$. (C) Frequency of mutated *IGKV3-20* genes as measured with the IgκREHMA assay. Bars represent mean values with SEM. Differences between each memory B-cell subset compared with centrocytes were statistically analyzed with the Mann-Whitney test. $^{*}P < .05$.

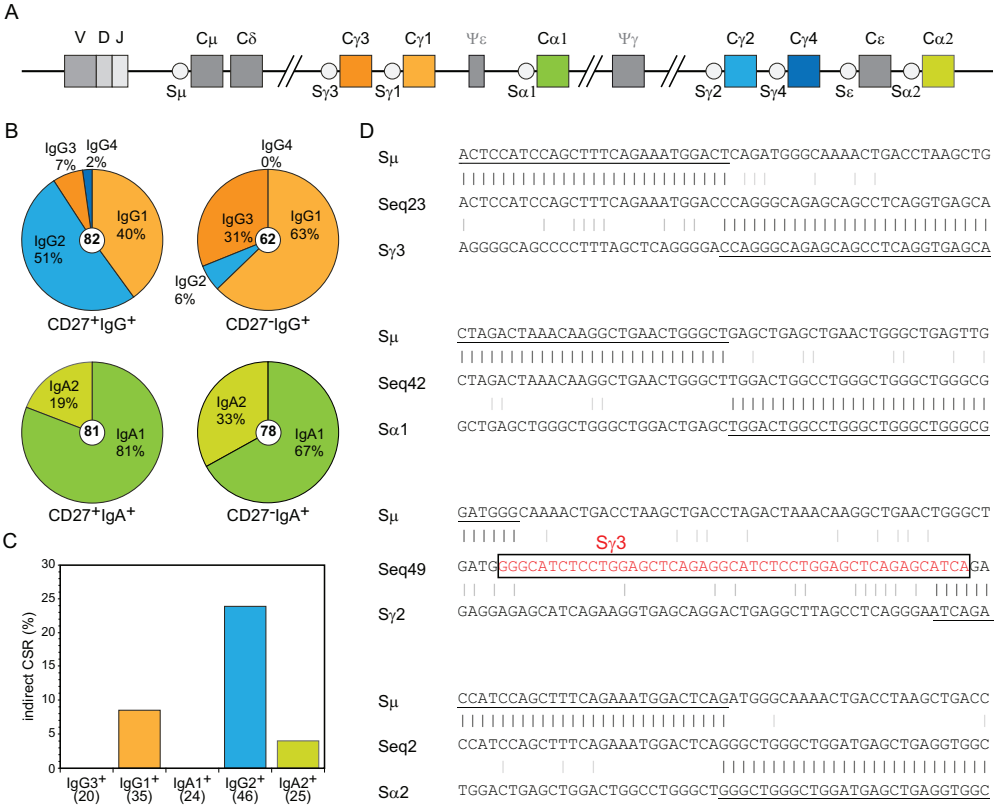

Figure 4. Molecular analysis of Ig class switching in IgA⁺ and IgG⁺ memory B-cell subsets. (A) Schematic representation of the constant region of the human *IGH* locus. (B) Distribution of IgA and IgG receptor subclass use in *IGH* rearrangements of class-switched memory B-cell subsets. Total number of analyzed sequences is indicated in the center of each plot. Differences in the distribution were statistically analyzed with the χ^2 test and were found significant for both $CD27^+IgG^+$ vs $CD27^-IgG^+$ ($P < .0001$) and $CD27^+IgA^+$ vs $CD27^-IgA^+$ ($P < .05$) B-cell subsets. (C) Frequency of Sμ-Sα and Sμ-Sγ rearrangements bearing remnants of indirect class switching. Number of analyzed sequences is given in brackets. (D) Examples of direct and sequential class switching; a piece of Sγ3 sequence in the Sμ-Sγ2 junction is indicated boxed in red font.

a cluster of 5 sequences with identical VDJ gene use and closely similar if not identical IGH-CDR3s (always of identical length), pointing to their common ancestry. In addition to recurrent mutations, the sequences exhibited a different number and distribution of SHMs, indicating that the process of antigen-driven clonal expansion also was accompanied by intraclonal diversification.

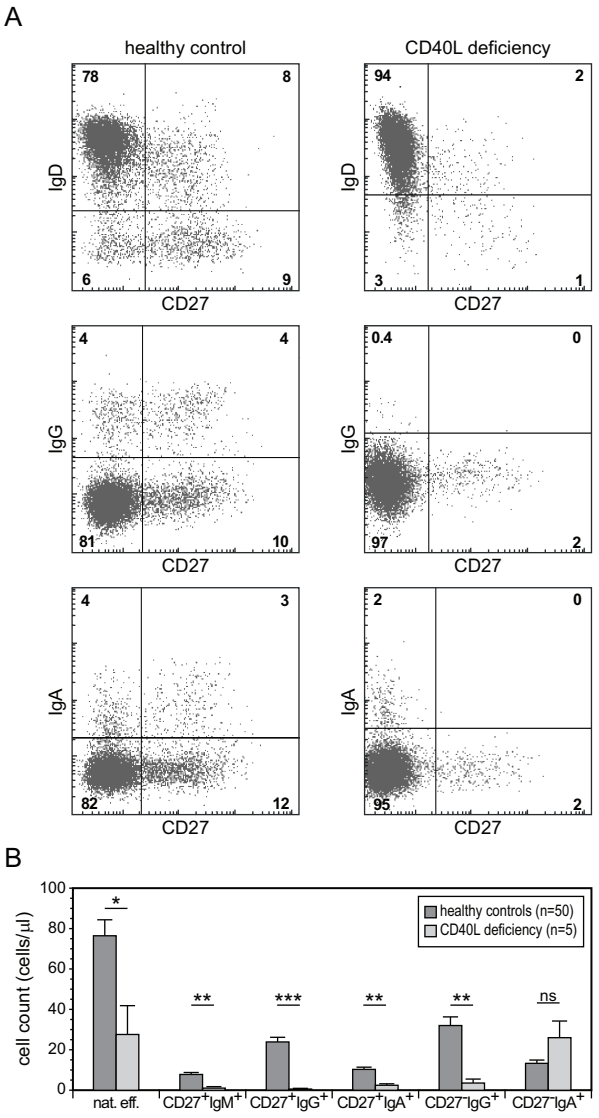

Figure 5. GC-independent generation of natural effector and CD27⁻IgA⁺ memory B cells. (A) Memory B-cell subset distribution was analyzed in 5 CD40L-deficient patients (age, 1-13 years) and 50 healthy controls (age, 1-5 years). Representative FACS plots of B-cell subsets. (B) Absolute cell numbers of 6 memory B-cell subsets. Bars represent mean values with SEM. Statistical significance was calculated with the Mann-Whitney test. * P < .05, **P < .01, ***P < .001.

IgG and IgA subclass distribution in class-switched memory B-cell subsets

In addition to differential CD27 expression, both IgG^+ and both IgA^+ memory B-cell subsets varied in their replication history and SHM levels (Figure 3). This suggests different origins and functions for the $CD27^+$ and $CD27^-$ B-cell subsets. Because the constant region of an antibody molecule is important for its function and the human *IGH* locus contains 4 *IGHG* and 2 *IGHA* constant genes (Figure 4A), we studied the Ig subclass use in sequenced *IGH* transcripts. We found a dominant use of *IGHG2* (51%) and *IGHG1* (40%) and low *IGHG3* and *IGHG4* in $CD27^+IgG^+$ cells (Figure 4B). In contrast, $CD27^-IgG^+$ cells showed a dominant use of *IGHG1* (63%) and *IGHG3* (31%) with little *IGHG2* and no *IGHG4*.[22,37] Thus, the $CD27^-IgG^+$ cells showed a dominant use of *IGHM*-proximal *IGHG3* and *IGHG1* regions (94%), whereas this was reduced to only 47% in $CD27^+IgG^+$ cells ($P < .0001$). Ig CSR to distal constant genes can occur indirectly via an *IGHM*-proximal gene. Analysis of hybrid switch regions (Sμ-Sγ2) in genomic DNA of sorted populations indeed revealed that 24% of junctions had remnants of Sγ3, Sγ1, or Sα1, whereas only 9% of Sμ-Sγ1 junctions had Sγ3 remnants (Figure 4C-D). Furthermore, the *IGHV* genes in *IGHG2* and *IGHG4* transcripts contained higher SHM loads than *IGHG1* and *IGHG3* (supplemental Figure 3A). The (indirect) switching to downstream *IGHG* genes accompanied by increased SHM frequencies suggests more prolonged AID activity in $CD27^+IgG^+$ cells, potentially reflecting multiple GC reactions.

The IgA^+ memory B-cell subsets also showed differential subclass use: $CD27^-IgA^+$ memory B cells contained significantly more *IGHA2* transcripts (33%) than $CD27^+IgA^+$ memory B cells (19%; Figure 4B; $P < .05$). Even though *IGHA2* is the most downstream constant gene in the human *IGH* locus (Figure 4A), only 4% of hybrid Sμ-Sα2 regions contained remnants of more proximal S regions (Figure 4C-D), suggesting that most of the switching toward *IGHA2* occurred directly from Sμ. No evidence for indirect class switching was found in Sμ-Sα hybrid switch regions. Furthermore, there was no difference in the mutation frequencies between *IGHA1* and *IGHA2* transcripts (supplemental Figure 3B). These results imply that switching toward *IGHA2* occurs mainly directly from Sμ and the molecular differences between $CD27^+IgA^+$ and $CD27^-IgA^-$ memory B cells most likely reflects their generation via separate response pathways, rather than consecutive GC reactions as observed for $CD27^-IgG^+$ versus $CD27^+IgG^+$ memory B cells.

T cell–independent generation of B-cell memory in CD40L-deficient patients

Replication history analyses indicated a GC-independent origin of natural effector and $CD27^-IgA^+$ B cells. To demonstrate that these subsets can be generated in the absence of the T-cell help, we analyzed their presence in 5 CD40L-deficient patients (supplemental Table 3). We found a clear population of natural effector B cells in CD40L-deficient patients, confirming previous observations that at least part of the blood natural effector B-cell population can be generated independently from T-cell help.[17,20,21,38] Still, this subset was ~3

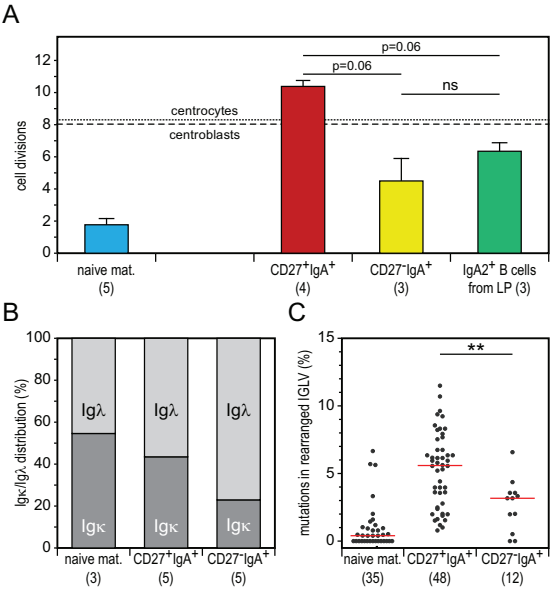

Figure 6. CD27⁻IgA⁺ memory B cells resemble colon lamina propria IgA⁺ B cells. (A) Replication history in naive mature, IgA⁺ memory B-cell subsets and CD19⁺IgA2⁺ B cells isolated from human colon *lamina propria* as measured with the KREC assay. Bars represent mean values with SEM. Statistical significance was calculated with the Mann-Whitney test. (B) Igκ and Igλ isotype distribution of naive mature and IgA⁺ memory B cell subsets as determined with flow cytometric analysis. (C) Frequency of mutated nucleotides in rearranged *IGLV* gene segments. All individual data points are showed as gray dots, with red lines indicating the median value. Statistical significance was calculated with the Mann-Whitney test. $^{**}P < .01$.

times reduced in number compared with age-matched healthy controls (Figure 5), highlighting the fact that in healthy controls a major part of this subset has a germinal center origin. More importantly, blood of CD40L-deficient patients also contained CD27⁻IgA⁺ memory B cells, and their numbers were similar compared with healthy controls (Figure 5). Thus, in addition to natural effector cells, T cell–independent humoral responses in human can generate IgA class-switched memory B cells.

CD27⁻IgA⁺ memory B cells resemble colon lamina propria IgA⁺ B cells

T cell–independent responses have been demonstrated to generate IgA-producing B cells in the *lamina propria* of human gut.[39,40] Furthermore, these IgA⁺ B cells showed predominant use of *IGHA2*.[41] These similarities with blood CD27⁻IgA⁺ memory B cells encouraged us to study whether these cells had been generated in similar responses. First, we analyzed the replication history of IgA2⁺ B cells isolated from colon *lamina propria*. Similar to CD27⁻IgA⁺ B cells, these cells had proliferated less than GC B cells in childhood tonsils and significantly less than GC-derived CD27⁺IgA⁺ memory B cells in adult blood (Figure 6A). In addition, because it was suggested previously that a broad Igλ repertoire may be beneficial for responses in the human gastrointestinal tract,[42] we analyzed the κ/λ light chain isotype ratios of blood B-cell subsets by flow cytometry. We found a high frequency of Igλ⁺ cells (80%) within the CD27⁻IgA⁺ B-cell subset compared with both CD27⁺IgA⁺ cells (55%) and naive mature B cells (45%; Figure 6B). Sequence analysis of *IGLV-IGLJ* rearrangements

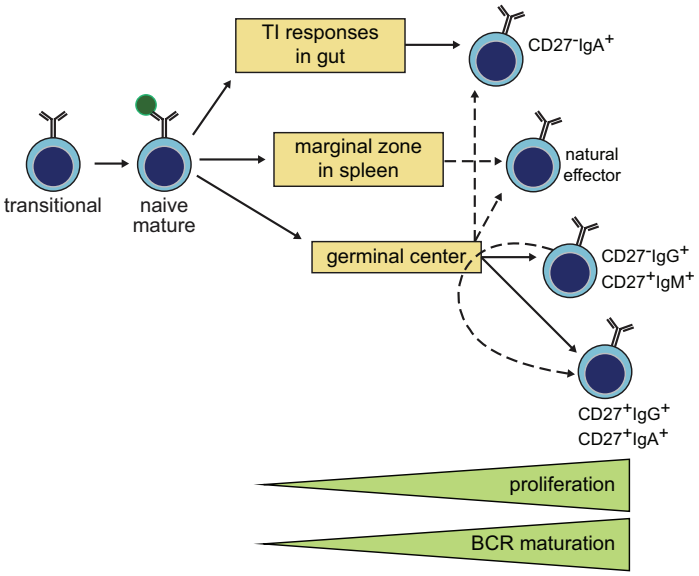

Figure 7. Model of human memory B-cell generation from GC-dependent and -independent pathways. Six purified memory B-cell subsets showed differential levels of proliferation and BCR maturation. Ig class-switching profiles and immunophenotyping of blood of CD40L-deficient patients supported delineation of these 6 subsets from T cell-dependent and -independent maturation pathways.$CD27^-IgA^+$ and natural effector B cells can be derived independently from T-cell help, probably locally in the gastrointestinal tract and from systemic responses in splenic marginal zone, respectively. The molecular profiles of $CD27^-IgG^+$ and $CD27^+IgM^+$ memory B cells resembled those of primary GC cells, whereas $CD27^+IgG^+$ and $CD27^+IgA^+$ memory B cells has increased proliferation and SHM levels suggestive of further maturation in consecutive GC response.

revealed fewer mutations in $CD27^-IgA^+$ than in $CD27^+IgA^+$ memory B cells, despite similar *IGLV* and *IGLJ* gene use and IGL-CDR3 size and composition (Figure 6C; supplemental Table 4). The molecular similarities between $CD27^-IgA^+$ B cells and gut lamina propria IgA-producing B cells suggest a common origin of these cells from local responses in the gastrointestinal tract.

Model of memory B-cell generation from 3 distinct pathways

Here, we demonstrate by molecular analysis of Ig genes that 6 distinct memory B-cell subsets can be identified based on their IgH isotype and expression of CD27. To recapitulate our findings, we propose a modified scheme of memory B-cell generation (Figure 7): $CD27^-IgA^+$ B cells and natural effector B cells (at least in part) are derived from local and systemic GC-independent responses, respectively; $CD27^-IgG^+$ and $CD27^+IgM^+$ B cells are derived from primary GC responses and $CD27^+IgG^+$ and $CD27^+IgA^+$ B cells (at least in part) from secondary GC responses.

DISCUSSION

In this study, we set out to relate distinct memory B-cell subsets to the diverse humoral response types that have been documented in the literature. We defined 6 memory B-cell subsets with phenotypic and molecular signs of antigen encounter. Detailed comparative analysis of their Ig genes, comparison with tissue-derived B-cell subsets, and analysis of memory B-cell subsets in CD40L-deficient patients allowed us to distinguish 3 unique maturation pathways: GC-independent local and systemic responses and GC-dependent responses. Furthermore, we delineated primary and consecutive phases of GC responses.

The $CD27^+IgA^+$ and $CD27^+IgG^+$ subsets are generally regarded as true B-cell memory.[25] Whereas this qualification is somewhat controversial for $CD27^+IgM^+$ subsets and $CD27^-$ class-switched subsets, our results strongly support these to be true memory B cells based on the (1) high expression of activation and costimulatory molecules; (2) selection against inherently autoreactive V_H domain characteristics; (3) extensive replication history compared with naive B cells; and (4) SHM profiles of Ig heavy and light variable genes with high R/S ratios in IGH-CDRs. Despite these common features of B-cell memory, we found clear quantitative differences in proliferation, SHM, and CSR processes among these subsets. We conclude that these differences reflect different origins and maturation pathways before becoming memory B cells. Consequently, these differences justify dividing the memory B-cell compartment into subsets.

Of the 6 memory B-cell subsets, the $CD27^+IgG^+$ and $CD27^+IgA^+$ B cells had the highest degrees of proliferation and SHMs. Interestingly, these levels were higher than those of GC B cells from childhood tonsils. We previously observed increased proliferation and SHMs in $CD27^+IgD^-$ cells from adults compared with children and concluded that in adults at least part of these cells had undergone additional immune responses on secondary or tertiary antigen encounter.[18] Our current results showed similar additional proliferation and SHMs for both $CD27^+IgA^+$ and $CD27^+IgG^+$ B cells. Furthermore, the increased proliferation was accompanied by increased use of distally located *IGHG2* and *IGHG4* genes with signs of indirect CSR. Thus, these results support the concept that at least part of the $CD27^+IgA^+$ and $CD27^+IgG^+$ B-cell subsets in healthy adults has undergone multiple immune responses.

Interestingly, the *IGHV* gene mutation frequency was clearly higher in $CD27^+IgA^+$ compared with $CD27^+IgG^+$ B cells. Because the targeting of mutations was similar, AID and UNG activities seemed unaffected. Rather, $CD27^+IgA^+$ B cells might have experienced prolonged AID and UNG activities. Because IgA class switching mostly occurs in mucosa-associated lymphoid tissues, this difference might reflect the location of the immune response. Still, despite these higher mutation loads, we found no differences in replacement mutation patterns in *IGHV* genes or the frequency of mutated *IGK* alleles, suggesting similar selection mechanisms for both $CD27^+IgA^+$ and $CD27^+IgG^+$ B cells.

We conclude that IgM-only and $CD27^-IgG^+$ B cells are derived from primary GC responses. This was based on their highly similar replication history and *IGHV* gene mutation loads compared with GC B cells from childhood tonsils and is further supported by the dominant use (> 90%) of the *IGHM*-proximal *IGHG1* and *IGHG3* genes in $CD27^-IgG^+$ B cells. In contrast to *IGHV* gene mutation loads, the frequencies of mutated *IGKV* alleles were increased in both subsets compared with GC B cells. We previously found this increased frequency in tonsillar $CD27^+IgD^-$ memory B cells.[18] Because this occurred in the absence of additional proliferation, it probably reflects positive selection for the mutated hot-spot in memory B cells rather than additional mutations.

IgM responses are initiated early in primary infection. Dogan et al[43] described that following primary immunization of mice, IgM^+ memory B cells were formed that on secondary challenge could class switch toward $IgG1^+$ cells. Furthermore, clonally related IgM^+ and IgG^+ B cells were found in human GCs and peripheral blood.[19,44] Thus, compared with $CD27^+IgA^+$ and $CD27^+IgG^+$ memory B cells, $CD27^+IgM^+$ memory B cells are early GC emigrants that did not undergo class switching.[45] Still, 2 issues have hampered proper studies on IgM^+ memory B cells in recent years. First, the $CD27^+IgM^+IgD^-$ and $CD27^+IgM^+IgD^+$ subsets have not always been separated, despite evidence that only the $CD27^+IgM^+IgD^+$ subset contains cells that have been generated independently from GCs (Figure 5B).[17,21] Second, often the $CD27^+IgD^-$ population is not further subdivided. As a consequence, this is a mixed population of Ig class-switched and IgM^+ memory B cells. Our results demonstrate that this has no major implications, because these subsets all seem GC dependent. However, it should be noted that the $CD27^+IgD^-$ subsets contain a substantial fraction of IgM^+ memory B cells, particularly in young children; therefore, it should be avoided to address these as "Ig class-switched memory."

The low SHM loads in $CD27^-IgG^+$ B cells compared with $CD27^+IgG^+$ B cells have lead to speculations on the origin of these cells: from T cell-independent responses or first wave from a GC reaction.[22,23] We found that the replication history and SHM levels of $CD27^-IgG^+$ B cells highly resemble GC B cells. Furthermore, $CD27^-IgG^+$ B cells were hardly detectable in CD40L-deficient patients and they have dominant use of IgM-proximal IgG3 and IgG1 subclasses. Thus, we conclude that similarly to $CD27^+IgM^+$ cells, $CD27^-IgG^+$ cells are derived from primary GC-dependent responses.

Several studies have shown an expansion of both $CD27^+IgM^+IgD^-$ and $CD27^-IgG^+$ memory B cells in autoimmune diseases.[17,22,23] Interestingly, $CD27^-IgG^+$ B cells dominantly use IgG1 and IgG3, which are potent activators of the complement system and inducers of antibody-dependent cell-mediated cytotoxicity.[46] Thus, our observations of the different IgG subclass use in $CD27^+IgG^+$ versus $CD27^-IgG^+$ B cells suggest a potential role of $CD27^-IgG^+$ cells in autoimmunity. Still, additional studies need to address whether many

$CD27^-IgG^+$ B cells carry an autoreactive BCR or whether other mechanisms result in deregulation of $CD27^-IgG^+$ B cells in patients with an autoimmune disease.

In contrast to the other memory B-cell subsets, natural effector and $CD27^-IgA^+$ B cells showed limited proliferation compared with GC B cells and were present in CD40L-deficient patients. Thus, we concluded that these cells can be generated independent from T-cell help. It is debated whether $CD27^+IgM^+IgD^+$ natural effector B cells in healthy adults are generated from germinal center responses or independently of T-cell help in the splenic marginal zone.[17,19–21] We describe reduced replication history and SHM levels in natural effector B cells compared with IgM-only memory B cells. Because IgM-only memory B cells highly resemble germinal center B cells on the molecular level, we conclude that in healthy adults part of the natural effector B cells can be generated outside of a GC. Thus, the natural effector B-cell subset in healthy individuals is probably a mixed population of GC-derived and splenic marginal zone-derived memory B cells. Interestingly, a recent study described the presence of $CD27^+CD43^+CD20^+$ B1 cells in umbilical cord blood and in adult peripheral blood.[47] It is possible that the T cell–independent characteristics ascribed to $CD27^+IgM^+IgD^+$ natural effector B cells are specific for the $CD43^+$ fraction. This should be further investigated.

The $CD27^-IgA^+$ memory B-cell subset was the smallest population we studied and, to our knowledge, we showed for the first time that these cells can be derived independent from T-cell help. TI IgA responses have been observed both in human and mouse, both systemically in the splenic marginal zone and locally in the gastrointestinal system.[40,48–49] Potential mediators of CD40-independent IgA CSR are BAFF and APRIL.[39] Because blood $CD27^-IgA^+$ B cells and gut *lamina propria* IgA-producing B cells were highly similar in their limited replication history, and dominant IgA2 and Igλ light chain isotypes, we conclude that these cells have been generated in similar responses. Although the anatomic location of TI CSR toward IgA in human gut remains controversial,[40,50] on the basis of our findings, we can state that $CD27^-IgA^+$ memory B cells resemble IgA^+ cells from the gut *lamina propria* and seem to be a blood counterpart of this IgA-producing population. Even though analysis of the memory B-cell compartment showed that $CD27^-IgA^+$ B cells seem completely TI, we cannot exclude that in physiologic conditions a minor fraction of $CD27^-IgA^+$ B cells is generated in a primary immune response analogous to $CD27^-IgG^+$ B cells.

The human memory B-cell compartment is more complex than originally thought and actually consists of diverse subsets that have originated from functionally distinct responses. Interestingly, differential expression of CD27 was the key to identification of the diverse subsets. The function of CD27 on B cells remains unclear. CD27-CD70 interactions can trigger plasma cell differentiation and provide negative feedback signals. Thus, $CD27^+$ and $CD27^-$ memory B cells might function differently. Still, the similar up-regulation of many other costimulatory molecules on these cells might compensate for the lack of

CD27. Alternatively, the differential CD27 expression might reflect the different types of responses in which the cells have been generated and thus represents a useful marker to discriminate between these subsets.

Different levels of memory B-cell responses seem to reflect the phylogenetic evolution of the immune system from local TI responses, via systemic TI responses to most advanced T cell–dependent responses in the GC. These different origins suggest unique physiologic functions in protection against pathogens.

In this study, we dissected the human memory B-cell compartment into 6 distinct subsets. Molecular analysis of these memory B cells in healthy controls and comparison with memory B cells from CD40L-deficient patients and colon *lamina propria* B cells enabled us to delineate their origin from 3 different maturation pathways: local and systemic TI responses and primary or secondary GC responses. Because these B-cell subsets are present in blood, our study provides new opportunities to analyze these processes in patients with (auto)inflammatory conditions, B-cell immunodeficiencies, and nodal and extranodal B-cell malignancies.

ACKNOWLEDGEMENTS

The authors are indebted to D. van den Heuvel, E. F. E. de Haas, and S. J. W. Bartol for technical support; to S. de Bruin-Versteeg for assistance with preparing the figures; and to Dr N. S. Longo for assistance with JOINSOLVER (http://joinsolver.niams.nih.gov) analysis.

This work was supported by a grant from the Erasmus University Rotterdam (EUR-Fellowship to M.C.v.Z.).

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SUPPLEMENTS

Flow cytometric immunophenotyping and purification of B cell subsets from human peripheral blood, tonsil and colon

Blood B cells were isolated from buffy coat post-ficoll mononuclear cells by magnetic separation with CD19 beads (Miltenyi Biotech). From these, two naive and 6 memory B-cell subsets were purified on a FACSAria cell sorter (BD Biosciences). Antibodies used to discriminate and characterize B-cell populations were: CD24-FITC (gran-B-ly-1; Sanquin), IgM-FITC, IgD-PE, IgG-PE, IgA-PE (all goat polyclonal from SBA), Igλ-FITC (rabbit polyclonal; Dako), Igκ-PB (A8B5; Exbio), BAFF-R (11C1), CD19-PerCP-Cy5.5 (SJ25C1), CD27-APC (L128), CD38-APC-H7 (HB7), LAIR-1-PE (DX26), IgD-biotin (IA6-2) (all from BD Biosciences), CD80-FITC (MAB104), CD95-FITC (UB2; both from Beckman Coulter), CD180 (MHR73-11; eBioscience) and TACI-bio (G112; PeproTech). Biotinylated antibodies were detected with Streptavidin PE-Cy7 (eBioscience) and unlabeled CD180 was detected with goat anti-mouse IgG-PE. Mouse IgG1-FITC and IgG1-PE (BD Biosciences) were used as isotype controls.

Tonsillar B-cell subsets from three children and $IgA2^+$ lamina propria B cells from three colonic samples were isolated as described before.[1–3] Centroblasts ($CD19^+CD38^+IgD^-CD77^+$) and centrocytes ($CD19^+CD38^+IgD^-CD77^-$) were labeled with $CD19^-$PE-Cy7 (SJ25C1),

CD38⁻APC (HB7), CD77⁻FITC (5B5; all from BD Biosciences) and IgD-PE (goat polyclonal from SBA), and CD19⁺IgA2⁺ cells with CD19⁻PerCP-Cy5.5 and IgA2-PE (IS11-21E11; Miltenyi Biotec). All subsets were sorted on the FACSAria cell sorter.

All fractions were obtained with a purity of >95% as determined by post-sort analysis.

Sequence analysis of complete IGH gene rearrangements and Ig switch regions

Complete *IGH* gene rearrangements were amplified from the genomic DNA of IgM⁺ B-cell subsets using 6 IGHV-FR1 forward primers and one JH consensus reverse primer.4 After reverse transcription using random hexamers, *IGA* and *IGG* transcripts were amplified using the same six IGHV-FR1 forward primers in combination with an *IGHA* (5'GTGGCATGTCACGGACTTG 3') or an *IGHG* (5'CACGCTGCTGAGGGAGTAG 3') consensus reverse primer. All PCR products were cloned into pGEM-T easy vector (Promega, Madison, WI) and prepared for sequencing on the ABI Prism 3130 XL fluorescent sequencer (Applied Biosystems). Obtained sequences were analyzed with IMGT database (http://imgt.cines.fr/) to assign the *IGHV*, *IGHD* and *IGHJ* gene, and to identify somatic mutations.[5] From each unique clone, the mutation frequency was determined within the *IGHV* gene, as was the length and composition of the IGH-CDR3. Where applicable, the IgA and IgG receptor subclasses were determined using the *IGH* reference sequence (NG_001019).

Targeting and selection of SHM in framework regions (FR) 1, 2 and 3 and CDR1 and 2 in rearranged *IGHV* genes were analyzed with the JoinSolver program.[6–7] The following parameters were examined: (1) targeting of SHM to RGYW/WRCY, WA/TW motifs and (2) to individual nucleotides within these motifs; (3) frequencies of transition and transversion mutations; (4) replacement/silent mutation ratios in FR and CDR; (5) nucleotide substitution frequencies and patterns in rearranged *IGHV*.

Hybrid Sμ-Sγ and Sμ-Sα regions were amplified in a nested approach as described previously.[8–9] PCR products were prepared for sequencing on the ABI Prism 3130 XL and obtained sequences were aligned with the IGH reference sequence (NG_001019).

Table S1. Targeting and selection of individual mutations in rearranged IGHV

	nat.eff. (53)	$CD27^+IgM^+$ (59)	$CD27^+IgG^+$ (100)	$CD27^+IgA^+$ (100)	$CD27^-IgG^+$ (62)	$CD27^-IgA^+$ (81)	centroblasts (67)	centrocytes (55)
RGYW (%)	142.3/500 (28.5)	184.4/650 (28.4)	501.3/1760 (28.5)	591.1/2104 (28.1)	159.8/618 (25.8)	321.5/1176 (27.3)	162.9/634 (25.7)	165.8/576 (28.8)
WRCY (%)	77.8/500 (15.6)	111.4/650 (17.1)	273.0/1760 (15.5)	318.9/2104 (15.2)	89.8/618 (14.5)	178.5/1176 (15.2)	97.4/634 (15.4)	73.8/576 (12.8)
WA (%)	72.1/500 (14.4)	89.8/650 (13.8)	261.8/1760 (14.9)	299.0/2104 (14.2)	84.6/618 (13.7)	161.6/1176 (13.7)	94.1/634 (14.8)	90.4/576 (15.7)
TW (%)	25.9/500 (5.2)	36.4/650 (5.6)	131.9/1760 (7.5)	142.0/2104 (6.7)	47.9/618 (7.8)	81.4/1176 (6.9)	46.6/634 (7.4)	39/576 (6.8)
Transitions (%)	273/500 (54.6)	359/650 (55.2)	890/1760 (50.6)	1100/2170 (52.3)	336/618 (54.4)	610/1176 (51.9)	344/634 (54.3)	296/576 (51.4)
Transversions (%)	227/500 (45.4)	291/650 (44.8)	870/1760 (49.4)	1004/2170 (47.7)	282/618 (45.6)	566/1176 (48.1)	290/634 (45.7)	280/576 (48.6)
FR R/S (ratio)	202/112 (1.8)	239/156 (1.5)	654/402 (1.6)	770/568 (1.4)	242/149 (1.6)	463/286 (1.6)	258/163 (1.6)	240/130 (1.8)
CDR R/S (ratio)	143/40 (3.6)	181/50 (3.6)	504/155 (3.3)	588/159 (3.7)	151/65 (2.3) **	341/86 (4.0)	163/50 (3.3)	168/38 (4.4)

FR indicates framework region; CDR, complementarity determining region; R/S is the ratio between replacement (R) and silent mutations (S); The number of analyzed sequences is indicated in the brackets next to the population name.

All analyses were performed with the JoinSolver program and the differences between each analyzed population as compared with centrocytes were statistically analyzed with the X^2 test. Significant differences are depicted in bold. **, $p<0.01$

Table S2. Targeting of individual nucleotides in hypermutable motifs

	nat.eff. (53)		$CD27^+IgM^+$ (59)		$CD27^+IgG^+$ (100)		$CD27^+IgA^+$(100)		$CD27^-IgG^+$ (62)		$CD27^-IgA^+$ (81)		centroblasts (67)		centrocytes (55)	
G																
inside RGYW	93.5/466.5 (20.0)	**6.4**	127/514 (24.7)	**6.9**	311/956 (32.5)	**5.9**	333/931 (35.8)	**5.3**	107/596.5 (17.9)	**5.3**	194/748.5 (25.9)	**5.4**	90/613.5 (14.7)	**4.8**	103.5/506.5 (20.4)	**5.3**
All other G	91.5/2937.5 (3.1)		114/3198 (3.6)		287/5178 (5.5)		344/5069 (6.8)		110/3257.5 (3.4)		201/4207.5 (4.8)		108/3523.5 (3.1)		110.5/2891.5 (3.8)	
C																
inside WRCY	47.5/383 (12.4)	**5.0**	68/419 (16.2)	**4.6**	168/749 (22.4)	**4.5**	209/745 (28.1)	**4.6**	63/480.5 (13.1)	**4.1**	107/624.5 (17.1)	**4.1**	55.4/486.9 (11.4)	**3.6**	39.5/418.5 (9.4)	**3.4**
all other C	70.5/2821 (2.5)		107/3007 (3.6)		251/5047 (5.0)		305/4995 (6.1)		101/3167.5 (3.2)		180/4331.5 (4.2)		105.6/3367.1(3.1)		76.5/2748.5 (2.8)	
A																
inside WA	70.5/750.5 (9.4)	**3.3**	93.5/840.5 (11.1)	**3.6**	299/1536.5 (19.5)	**3.8**	334/1479 (22.6)	**3.0**	85.5/940.5 (9.1)	**3.5**	189/1197 (15.8)	**4.1**	107/1045 (10.2)	**3.6**	104.5/864 (12.1)	**3.8**
all other A	65.5/2262.5 (2.9)		76.5/2482.5 (3.1)		199/3849.5 (5.2)		281/3708 (7.6)		63.5/2436.5 (2.6)		120/3098 (3.9)		74/2572 (2.9)		67.5/2090 (3.2)	
T																
inside TW	32.5/609.5 (5.5)	**3.7**	45.5/701.5 (6.5)	**7.3**	154/1146.5 (13.4)	**5.5**	174.5/1091(16.0)	**4.8**	58/746.5 (7.8)	**6.0**	106/960 (11.4)	**1.9**	61/779 (7.8)	**5.9**	48.5/623.5 (7.8)	**6.2**
all other T	28.5/1953.5 (1.5)		18.5/2080.5 (0.9)		91/3729.5 (2.4)		123.5/3689 (3.3)		30/2333.5 (1.3)		179/3059 (5.9)		33/2481 (1.3)		25.5/2044.5 (1.2)	
A																
inside RGYW	24/194.5 (12.3)	**3.2**	50.5/242 (20.9)	**5.4**	122/523.5 (23.3)	**3.0**	146/464.5 (31.4)	**3.2**	32/299 (10.7)	**2.8**	76/362 (21.0)	**3.5**	35.5/300 (11.8)	**2.7**	38/252 (15.1)	**3.0**
all other A	112/2818.5 (4.0)		119.5/3081 (3.9)		376/4862.5 (7.7)		469/4722.5 (9.9)		117/3078 (3.8)		233/3933 (5.9)		145.5/3317 (4.4)		134/2702 (5.0)	
A																
inside WRCY	19/311.5 (6.1)	**1.4**	13.5/303 (4.5)	**0.9**	95/672.5 (14.1)	**1.7**	85/705.5 (12.0)	**1.0**	20/420 (4.8)	**1.1**	49/574 (8.5)	**1.2**	29.5/302 (9.6)	**2.1**	26/384.8 (6.8)	**1.2**
all other A	117/2701.5 (4.3)		156.5/3020 (5.3)		403/4713.5 (8.5)		530/4481.5(11.8)		129/2957 (4.4)		260/3721 (7.0)		151.5/3315 (4.6)		146/2569.2 (5.7)	

The frequency of underlined (commonly mutated) nucleotides inside and outside the motifs was calculated with the JoinSolver software. The ratio between the frequency of mutations inside and outside the motifs is depicted in bold and used as a measurement of targeting. Number of analyzed sequences is indicated in brackets next to the name of each population.

Table S3. Baseline characteristics and laboratory findings in five patients with CD40L deficiency

CD40L-deficient patient	Age [years]	Sex	Mutation - cDNA	Mutation - protein	CD40L protein expression	$CD3^+$ [cells/µl]	$CD19^+$ [cells/µl]	$CD56^+CD16^+$ [cells/µl]	Serum IgG [g/l]	Serum IgA [g/l]	Serum IgM [g/l]
1	13	M	c.761C>T	p.Thr254Met	absent	958	151[b]	56[b]	1.15[b]	<0.20[b]	1.24
2	1	M	c.761C>T	p.Thr254Met	absent	8720[a]	1310	390	0.8[b]	<0.01[b]	<0.3[b]
3	2	M	c.474delG	p.Gly-158fsX4	absent	2950	1002	50[b]	0.8[b]	0.47	1.54
4	1	M	c.154A>T	p.Lys52X	absent	6013	796	367	1.52[b]	0.06[b]	1.78
5	1	M	c.521A>C	p.Gln174Pro	absent	5600	200[c]	590	0.92[b]	<0.06[b]	1.21

Indicated age is the age of B-cell subset analysis; Ig serum levels were measured at diagnosis;
[a] Cell count or serum Ig level >95 percentile for the age category;
[b] Cell count or serum Ig level <5 percentile for the age category.

Table S4. Characteristics of IGLV–IGLJ junctions from naive mature and IgA+ class-switched memory B cells

	Number of sequences	IGL-CDR3 [aa]	length [nt]	del (IGLV)	P (IGLV)	N (IGLV-IGLJ)	P (IGLJ)	del (IGLJ)
naive mature	33	10.73	32.55	4.73	0.03	3.94	0.21	1.27
$CD27^+IgA^+$	49	10.62	31.88	4.48	0.02	3.44	0.02	1.67
$CD27^-IgA^+$	13	10.25	30.75	3.75	0	2	0.25	1.5

The data shown are the mean of 2 (naive mature) or 3 donors;
The following abbreviations are used: Del, deletion; P, palindromic nucleotides; N, non-template nucleotides;
No statistically significant differences were found with the Mann-Whitney test.

Natural effector (53) $CD27^{-}IgG^{+}$ (82)

To	A	C	G	T	
A	-	36	67	33	136
C	15	-	29	74	118
G	100	68	-	17	185
T	14	32	15	-	61
					500

To	A	C	G	T	
A	-	7.2	13.4	6.6	27.2
C	3.0	-	5.8	14.8	23.6
G	20.0	13.6	-	3.4	37.0
T	2.8	6.4	3.0	-	12.2
					100.0

To	A	C	G	T	
A	-	43	72	34	149
C	24	-	44	96	164
G	119	77	-	21	217
T	20	49	19	-	88
					618

To	A	C	G	T	
A	-	7.0	11.7	5.5	24.1
C	3.9	-	7.1	15.5	26.5
G	19.3	12.5	-	3.4	35.1
T	3.2	7.9	3.1	-	14.2
					100.0

$CD27^{+}IgM^{+}$ (59) $CD27^{-}IgA^{+}$ (81)

To	A	C	G	T	
A	-	42	93	35	170
C	20	-	45	110	175
G	123	100	-	18	241
T	19	33	12	-	64
					650

To	A	C	G	T	
A	-	6.5	14.3	5.4	26.2
C	3.1	-	6.9	16.9	26.9
G	18.9	15.4	-	2.8	37.1
T	2.9	5.1	1.8	-	9.8
					100.0

To	A	C	G	T	
A	-	78	154	77	309
C	34	-	91	162	287
G	186	159	-	50	395
T	38	**108***	39	-	185
					1176

To	A	C	G	T	
A	-	6.6	13.1	6.5	26.3
C	2.9	-	7.7	13.8	24.4
G	15.8	13.5	-	4.3	33.6
T	3.2	9.2	3.3	-	15.7
					100.0

$CD27^{+}IgG^{+}$ (100) centroblasts (67)

To	A	C	G	T	
A	-	122	236	140	498
C	51	-	119	249	419
G	272	242	-	84	598
T	56	133	56	-	245
					1760

To	A	C	G	T	
A	-	6.9	13.4	8.0	28.3
C	2.9	-	6.8	14.1	23.8
G	15.5	13.8	-	4.8	34.0
T	3.2	7.6	3.2	-	13.9
					100.0

To	A	C	G	T	
A	-	42	100	39	181
C	12	-	51	98	161
G	**85***	81	-	32	198
T	12	**61***	21	-	94
					634

To	A	C	G	T	
A	-	6.6	15.8	6.2	28.5
C	1.9	-	8.0	15.5	25.4
G	13.4	12.8	-	5.0	31.2
T	1.9	9.6	3.3	-	14.8
					100.0

$CD27^{+}IgA^{+}$ (100) centrocytes (55)

To	A	C	G	T	
A	-	163	298	154	615
C	68	-	122	324	514
G	314	275	-	88	677
T	77	164	57	-	298
					2104

To	A	C	G	T	
A	-	7.7	14.2	7.3	29.2
C	3.2	-	5.8	15.4	24.4
G	14.9	13.,1	-	4.2	32.2
T	3.7	7.8	2.7	-	14.2
					100.0

To	A	C	G	T	
A	-	45	82	45	172
C	12	-	32	72	116
G	105	84	-	25	214
T	19	37	18	-	74
					576

To	A	C	G	T	
A	-	7.8	14.2	7.8	29.9
C	2.1	-	5.6	12.5	20.1
G	18.2	14.6	-	4,3	37.2
T	3.3	6.4	3.1	-	12.8
					100.0

Figure S1. Substitution of individual nucleotides in rearranged IGHV. Individual nucleotide substitutions for all analyzed subsets are given both absolute number (left panels) and percentage (right panels). The number of analyzed sequences is indicated in brackets next to the population name. All analyses were performed with the JoinSolver program and the statistical significance was calculated between each analyzed population and centrocytes. Statistically significant differences were calculated with the X^2 test and are depicted in bold. *, $p<0.01$

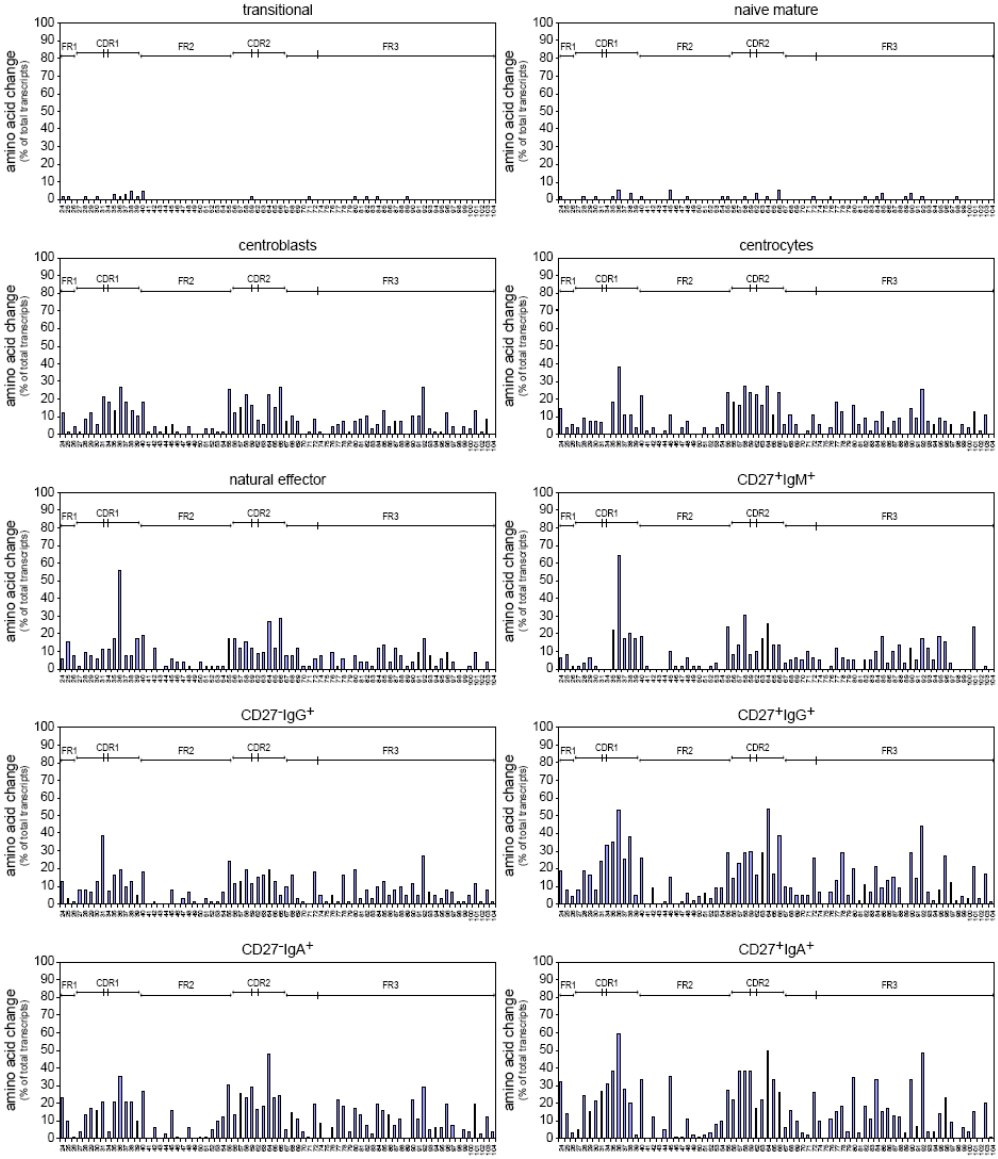

Figure S2. Distribution of replacement mutations substitutions in rearranged IGHV genes from blood and GC B-cell subsets. Each bar represents the frequency replacement mutations at each amino acid position starting from 24 (first codon following primer sequence) to 104 (last codon of the FR3 region). FR denotes framework region and CDR denotes complementarity determining region.

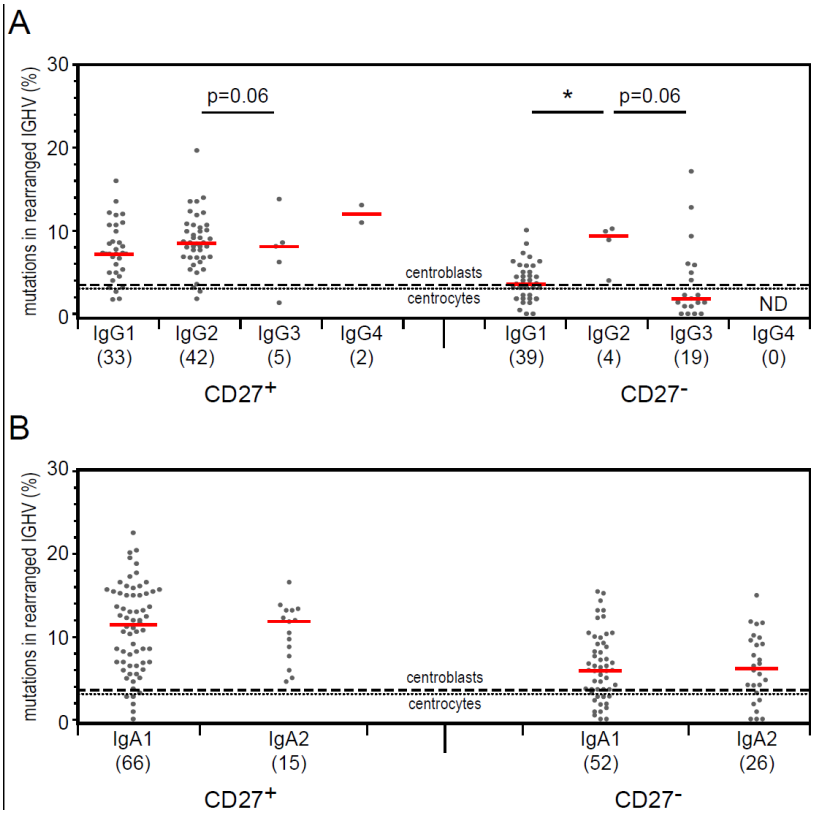

Figure S3. IGHV genes mutation frequencies of distinct Ig subclass transcripts in IgG⁺ (A) and IgA⁺ (B) memory B cells. All individual data points are shown as grey dots with red lines indicating the median value. The dashed line and dotted line represent median frequency of mutations for centroblasts and centrocytes, respectively. The number of sequences analyzed is indicated in brackets for each subset. Number of sequences for centroblasts, 67; and centrocytes, 55; ND denotes not detected; Statistical significance was calculated with the Mann-Whitney test. *, $p<0.05$

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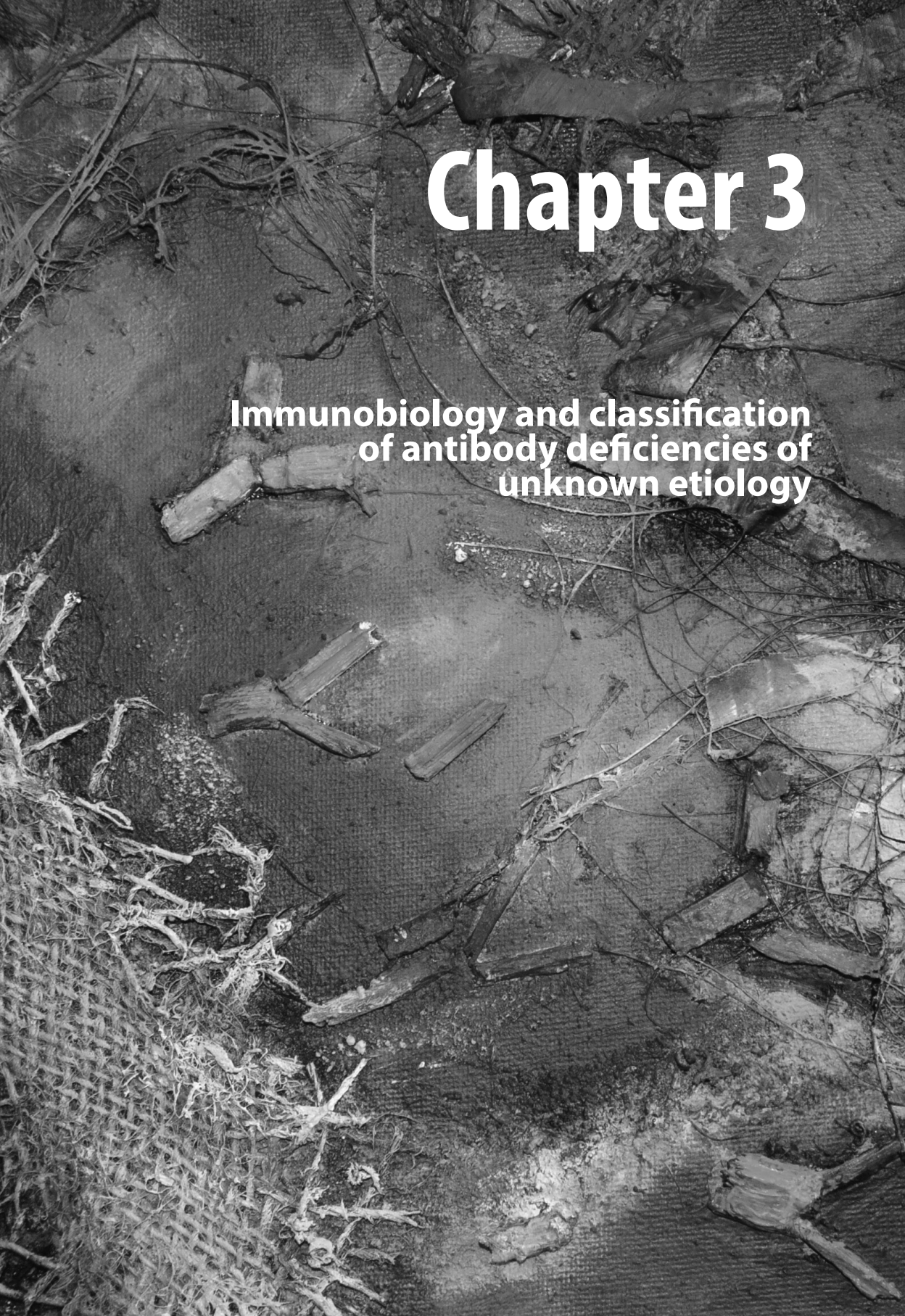
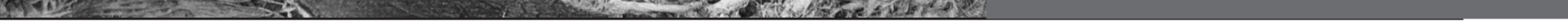
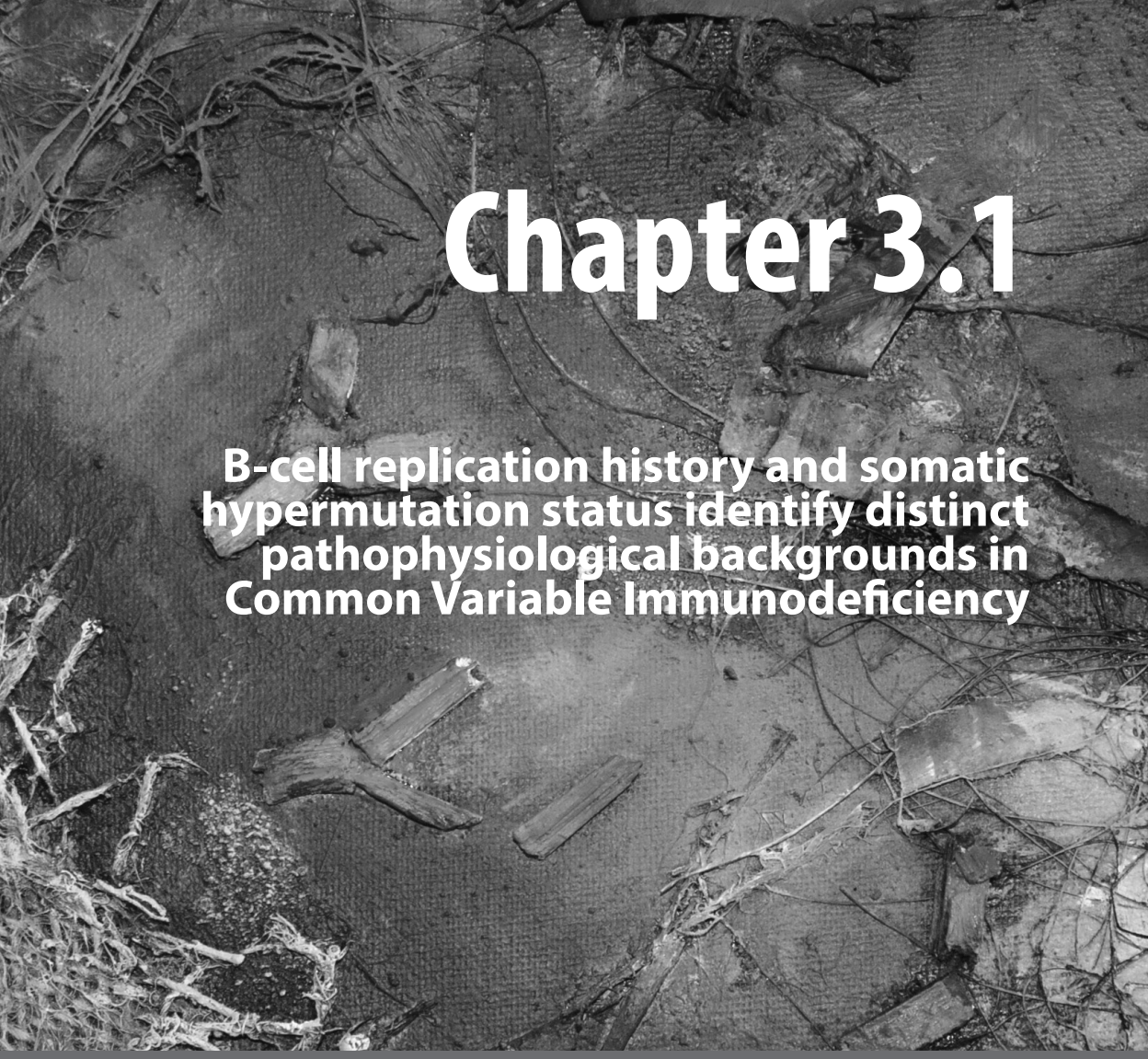


Chapter 3

Immunobiology and classification of antibody deficiencies of unknown etiology





Chapter 3.1

B-cell replication history and somatic hypermutation status identify distinct pathophysiological backgrounds in Common Variable Immunodeficiency

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Blood 2011; 118(26): 6814-6823

ABSTRACT

Common Variable Immunodeficiency Disorders (CVID) is the most prevalent form of primary idiopathic hypogammaglobulinemia. Identification of genetic defects in CVID is hampered by clinical and immunological heterogeneity. By flow cytometric immunophenotyping and cell sorting of peripheral B-cell subsets of 37 CVID patients, we studied the B-cell compartment at the B-cell subset level using the KREC assay to determine the replication history and the IgκREHMA assay to assess the somatic hypermutation (SHM) status. Via this approach five B-cell patterns were identified, which delineated groups with unique replication and SHM characteristics. Each B-cell pattern reflected an immunologically homogenous patient group for which we proposed a different pathophysiology: 1) a B-cell production defect (n=8, 18%); 2) an early peripheral B-cell maturation or survival defect (n=4, 11%); 3) a B-cell activation and proliferation defect (n=12, 32%); 4) a germinal center defect (n=7, 19%) and 5) a post-germinal center defect (n=6, 16%). In conclusion, the here presented study provides for the first time insight into the underlying pathophysiological background in five immunologically homogenous groups of CVID patients. Moreover, this study forms the basis for larger cohort studies with the here defined homogenous patient groups and will facilitate the identification of underlying genetic defects in CVID.

INTRODUCTION

Common Variable Immunodeficiency Disorders (CVID) is the most prevalent form of primary idiopathic hypogammaglobulinemia, frequently leading to clinical complications.[1-3] CVID is defined by serum IgG levels below 2 SD of normal controls in the presence of decreased IgA and/or IgM levels, recurrent infections, impaired response to immunization, exclusion of other defined causes of hypogammaglobulinemia, and an age above two years (ESID-PAGID-criteria www.esid.org). CVID patients suffer from sinopulmonary infections, which eventually result in bronchiectasis in more than 30% of cases. In addition, they may develop complications, such as auto-immune disease, granulomatous disease and malignancies.[2-8]

Over the last years, deficiencies of ICOS,[9-10] TACI,[11-12] CD19,[13-14] BAFF-R,[15] CD20,[16] and CD81[17] have been identified in patients with CVID or CVID-like conditions. However, less than 10% of the CVID patients have a positive family history[2] and a genetic defect has only been identified in less than 10% of the patients who have been reported to the ESID primary immunodeficiency database.[1,18] The immunological and clinical heterogeneity of CVID hampers the discovery of underlying disease causing mechanisms, genetic defects, and clinically relevant prognostic factors in the majority of patients.

CVID patients fail to produce sufficient amounts of antigen-specific antibodies, which can be caused by defects in any critical stage of B-cell differentiation and maturation.[7,19] B-cells are continuously produced in the bone marrow followed by migration to peripheral lymphoid organs where they mediate antigen-specific responses. Multiple B-cell subsets circulate in peripheral blood. Transitional B-cells are early bone marrow emigrants and constitute only a small part of the peripheral B-cell pool. In healthy controls, transitional B-cells do not proliferate, but differentiate into naive mature B-cells, which do undergo homeostatic proliferation of 1 to 2 cell cycles, thereby expanding the naive B-cell pool.[20] Activation of the B-cell receptor (BCR) complex by antigen stimulates further B-cell differentiation and maturation. B-cells can be activated with T-cell help in a germinal center (GC) in lymphoid tissue or independently of T-cell help, e.g. in the marginal zone of the spleen. Activated B-cells generate Activation Induced Cytidine Deaminase (AID) dependent somatic hypermutations (SHM) in the variable region of the immunoglobulin (Ig) heavy and Ig light chains. Subsequent class switch recombination (CSR) changes the IgH constant region to form Ig isotypes with different effector functions. Finally, memory B-cells and plasma cells are formed, responsible for long lasting immunological memory and the production of large amounts of Ig molecules. T-cell independent B-cell responses in the splenic marginal zone are thought to generate a substantial fraction of circulating natural effector B-cells.[20-22]

Recently, CD21^{low}CD38^{low} B-cells have been described as a distinct sub-population. Whereas their origin and specific function are disputed, they contain mostly autoreactive unresponsive clones and might represent anergic or innate-like B-cells.[23-24] CD21^{low}CD38^{low} B-cells are very infrequent in healthy individuals, but expansions have been found in several autoimmune diseases and in a subgroup of CVID patients.[25-26]

In the past decade, the "Freiburg" and "Paris" CVID classifications have been developed based on the composition of the peripheral B-cell compartment.[27-28] The main aim of these classifications was to predict clinical complications. In the recent EUROclass consensus classification of CVID, a relative decrease of switched memory B-cells was associated with splenomegaly, granulomatous disease and auto-immunity.[5] The other reported associations in this study were an increased proportion of transitional B-cells with lymphadenopathy and a decreased proportion of CD21^{low}CD38^{low} B-cells with splenomegaly. Additionally, decreased proportions of marginal zone like B-cells[29] and an abnormal T-cell phenotype[30] have been found to associate with clinical complications. Because abnormalities in different immune pathways may account for the immune defects in CVID, a classification independent of immune parameters has been proposed by Chapel et al, grouping patients into clinically homogenous categories with a different prognosis.[31] Despite multiple attempts in classifying CVID patients, understanding the heterogeneity in terms of immunological and genetic defects as well as clinical prognosis still imposes a major challenge.

The aim of this study was to identify immunologically homogenous subgroups of CVID patients based on B-cell subset abnormalities. Using a combined flow cytometric and molecular approach, we provide a link between the composition of the peripheral B-cell compartment and in vivo B-cell replication and somatic hypermutation status. This resulted in a model that describes five different pathophysiological backgrounds in immunologically homogenous CVID subgroups. Defining these immunologically homogenous groups of CVID patients will facilitate the identification of prognostic factors and the underlying genetic defects.

MATERIALS AND METHODS

Patients

Peripheral blood samples and clinical data were collected of 37 patients with Common Variable Immunodeficiency. In addition we collected blood from 86 healthy age matched. The research was approved by the Medical Ethics Committee of the Erasmus MC and all patients and controls provided written informed consent.

Flow cytometric analysis

Six-color flow cytometric immunophenotyping of peripheral blood was performed on a LSRII (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 (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 (SouthernBiotechnologies), 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; Beckman Coulter). The absolute sizes of the peripheral B-cell subsets (transitional B-cells, naive mature B-cells, marginal zone like B-cells and memory B-cells) were determined by flow cytometric immunophenotyping and compared to age matched healthy controls. The gating strategy is depicted in Figure 1. Considering the gating of transitional B-cells, the lower border of the transitional B-cell gate, which is separating transitional B-cells from the naive mature B-cell population, was set in a standardized way to ensure a homogenous way of analysis. A decrease or increase of a B-cell subset was defined as a value below the 5^{th} or above the 95^{th} percentile of 86 healthy age matched controls. Analysis of the precursor B-cell compartment was performed as described previously.[32]

High speed cell sorting of B-cell subsets from peripheral blood

Four B-cell subsets were purified from blood samples of all 37 patients and 20 healthy controls using FACS DiVa cell sorter (BD Biosciences) after staining of post-Ficoll mononuclear cells with CD24-FITC (1B5), IgD-PE, ITK diagnostics, CD19-PerCP-Cy5.5 (SJ25C1), CD27-APC (L128) and CD38 PE-Cy7 (HB7). CD3 APC-Cy7 (SK7) was used as exclusion marker (55). The following CD19-positive populations were sorted: transitional B-cells ($CD27^{-}CD24^{high}CD38^{high}$), naïve mature B-cells ($CD27^{-}$ $CD24^{dim}CD38^{dim}$), marginal zone like ($CD27^{+}IgD^{+}$) and memory B-cells ($CD27^{+}IgD^{-}$). DNA was extracted from the sorted cell fractions using a direct lysis method.[33-34]

KREC assay to determine the replication history of B-cells

The replication history of B-cells was determined using the KREC assay (In Vivo Scribe, San Diego, CA), which is based on a quantification of coding joints and signal joints of an Ig kappa-deleting rearrangement (intron RSS-Kde) by real time quantitative PCR (RQ-PCR).(20) The ΔC_T between the signal joint and the coding joint exactly represents the number of cell divisions a B cell has undergone. The RQ-PCR mixture of 25µl contained TaqMan Universal MasterMix (Applied Biosystems), 900nM of each primer, 100nM

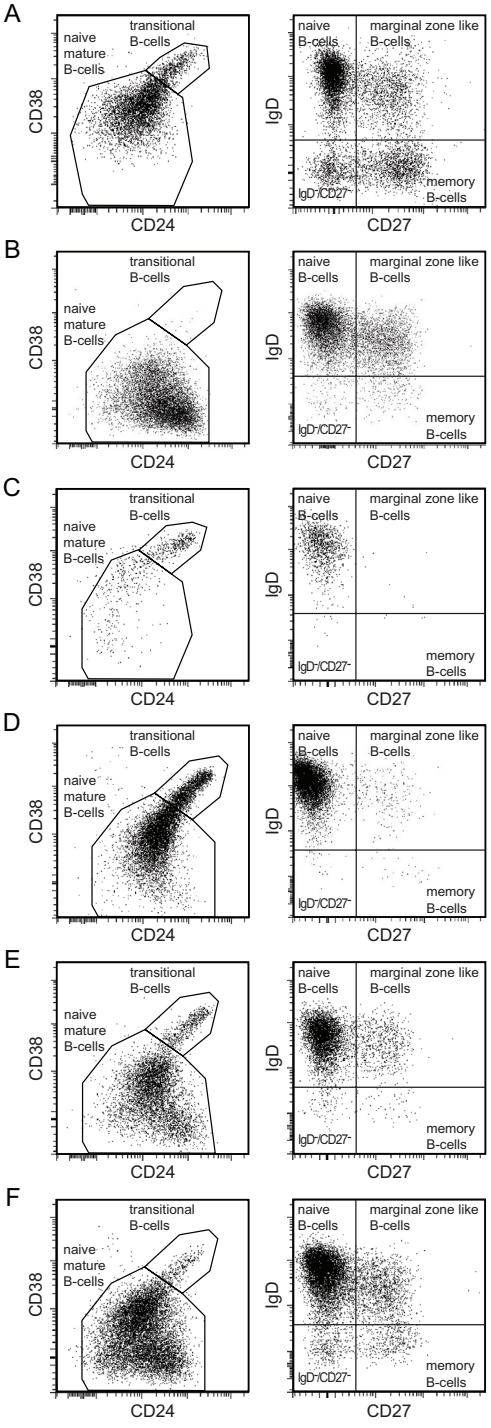

Figure 1. Flow cytometric analysis of blood B-cell subsets in normal controls and CVID patients. All B-cell subsets are determined within the CD19+ lymphogate. Naive B-cell subsets (transitional B-cells and naive mature B-cells) are defined within the CD27-IgD+ lymphogate based on expression of CD24 and CD38. **A**. normal control **B-F**. B-cell patterns observed in CVID patients; **B**. B-cell pattern 1; low transitional and memory B-cells. **C**. B-cell pattern 2; low naive mature, marginal zone like and memory B-cells. **D**. B-cell pattern 3; low marginal zone like and memory B-cells. **E**. B-cell pattern 4; low memory B-cells. **F.** B-cell pattern 5; normal marginal zone like and memory B-cells. Naive mature B-cells cells in CVID patients (B-F) were more often $CD38^{low}$ compared to controls (A) and represent, at least for a large part, the $CD21^{low}CD38^{low}$ B-cell population within the naive B-cell compartment.

FAM-TAMRA-labeled probe, 25ng of DNA, 0.4 ng BSA and was run on the ABIPRISM 7700 detection system (Applied Biosystems).[20]

SHM analysis using a Vκ3-20-specific restriction enzyme hot-spot mutation assay (IgκREHMA) on genomic DNA

To investigate the occurrence of SHM in the B-cell subsets, the IgκREHMA assay for genomic DNA was used.[20,35] In short, a PCR reaction was performed with a HEX-coupled Vκ3-20 intron forward primer and two FAM-coupled Jκ reverse primers recognizing all five Jκ gene segments. The PCR products (500bp) were digested with KpnI and Fnu4HI and run on a capillary sequencer ABI3130 (Applied Biosystems). Unmutated gene products can be visualized as 244 or 247-bp HEX-coupled fragments and mutated gene products as 262-bp HEX-coupled fragments.[20]

STATISTICS

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. Whenever multiple groups with continuous outcomes were compared the non-parametric Kruskal-Wallis rank sum test was used, followed by pair wise Mann Whitney tests if the former indicated significant differences. Correlation coefficients given are Spearman's. For categorical variables the chi-square was used or Fisher's exact test if required. Statistical significance was set at two sided $P<0.05$.

RESULTS

Patients

Thirty-seven CVID patients (19 males) were included in this study. The patient's age range was 6-76 years, with 22 adults and 15 children. All patients fulfilled the ESID-PAGID-criteria for CVID. All patients received immunoglobulin replacement therapy. Excluded from the study were males with decreased peripheral B-cells and mutations in the *BTK* gene, males with CD40L deficiency, patients with other genetic defects known to cause hypogammaglobulinemia, such as UNG and AID deficiency, patients with a secondary hypogammaglobulinemia and patients under immunosuppressive therapy.

The mean age of onset of symptoms was 15.6 years (range 0.3-64) and the mean age of diagnosis was 24.3 years (2.5-71.0), resulting in a mean diagnostic delay of 8.7 years. The mean age of inclusion in the study was 31.0 years (6.0-76.0).

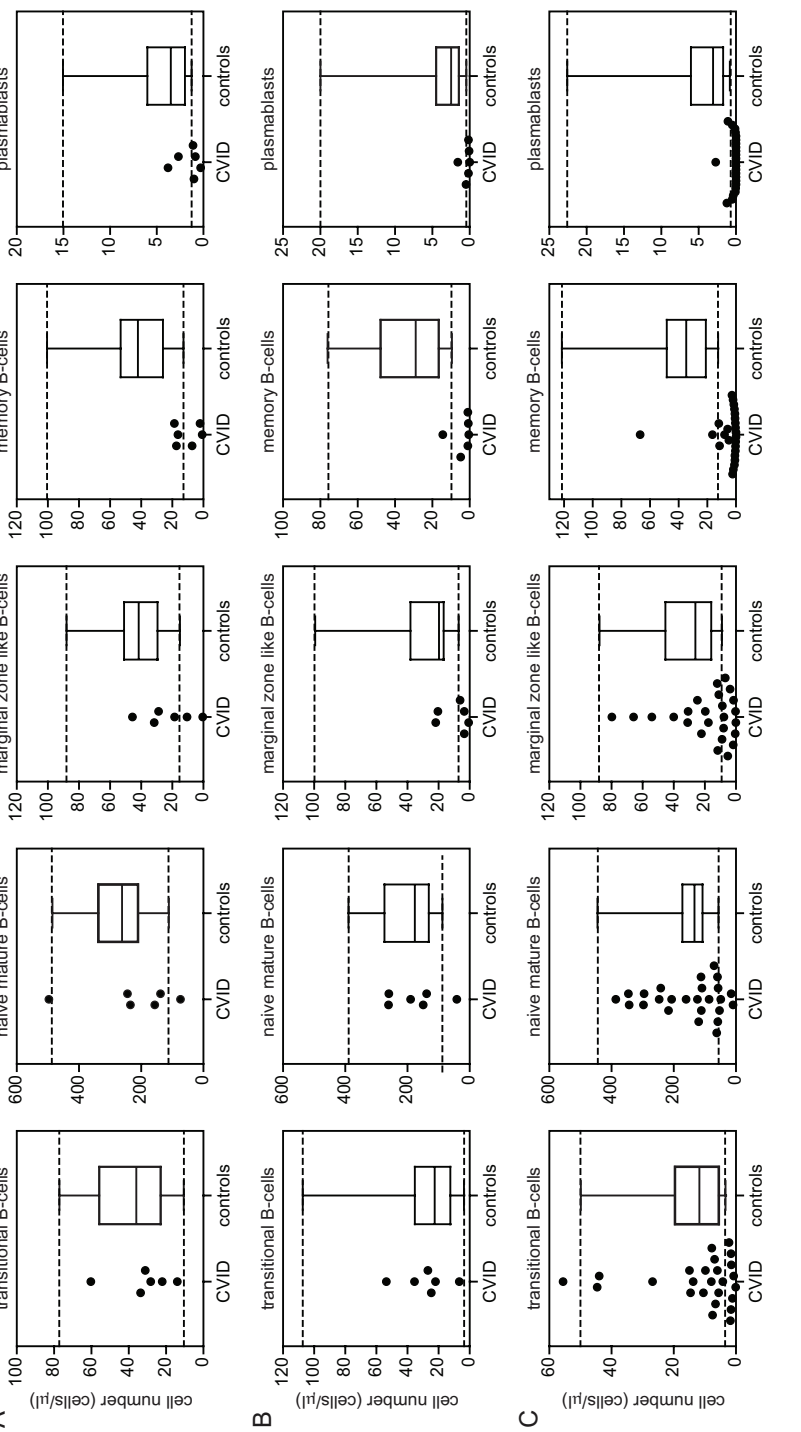

Figure 2. Absolute numbers of B-cells per B-cell subset of 37 CVID patients and 86 healthy age-matched controls. Patients and controls are divided into three age groups: 5-10 years (n=30) **A**, 10-16 years (n=28) **B** and >16 years (n=28) **C.** The three age groups contained 6, 6 and 25 CVID patients, respectively. Boxes depict median values, 25 and 75 percentiles; whiskers, extended by interrupted lines, depict 5th and 95th percentiles age matched normal controls in the three age groups.

Composition of the peripheral B-cell compartment in CVID

Flow cytometric analysis of blood B-cell subsets was performed in 37 CVID patients and 86 healthy age matched controls. The following peripheral CD19+ B-cell subsets were defined: Transitional B-cells as $CD27^{-}IgM^{+}IgD^{+}CD24^{high}CD38^{high}$ B-cells and naive mature B-cells as $CD27^{-}IgM^{+}IgD^{+}CD24^{dim}CD38^{dim}$. In addition to these two naive B-cell subsets, two $CD38^{dim}$CD27+ B-cell subsets were identified, i.e. CD27+IgD+IgM+ marginal zone like B-cells and CD27+IgD- memory B-cells (Figure 1A). Finally, plasmablasts were defined as $CD24\text{-}CD38^{hi}$. The B-cell subset sizes were calculated as cells per microliter blood, because in contrast to relative sizes, the absolute size of a specific B-cell subset is not influenced by increase or decrease of the other B-cell subsets.

The CVID cohort was divided into three age groups (5-10 year, 10-16 years and >16 years) at the time of inclusion in the study, in order to compare the B-cell subset counts of the individual patients with the 5^{th} and 95^{th} percentiles of age matched controls (Figure 2A-C, Table 1). A peripheral B-cell subset size was considered reduced when the value was below the 5^{th} percentile. Twenty-two percent of CVID patients had reduced numbers of transitional B-cells, 14% had reduced numbers of naive mature B-cells and 48% and 84% had reduction in marginal zone like B-cells or memory B-cells, respectively. Most patients (81%) also showed a reduction of plasmablasts compared to age matched controls.

Subsequently, we divided the CVID patients into groups with a specific composition of the peripheral B-cell compartment (B-cell patterns) based on absolute reductions of transitional, naive mature, marginal zone like or memory B-cells. We identified five main B-cell patterns (Figure 1 B-F) consisting of at least three patients, a prerequisite for statistical analysis. Eight patients (22%) showed decreased numbers of transitional B-cells in combination with a reduction of memory B-cells (B-cell pattern 1). Of the patients with normal transitional B-cells, four patients (11%) showed a reduction of naive mature, marginal zone like and memory B-cells (B-cell pattern 2); 12 patients (32%) showed a reduction

Table 1. Age related normal values of B-cell subset absolute counts

B-cell subset *age*	transitional	naive mature	marginal zone like	memory	plasmablast
5-10 yrs n=30	11-77	111-486	15-88	13-100	1-15
10-16 yrs n=28	4-108	87-390	7-90	10-76	0.5-20
>16 yrs n=28	3-50	57-447	9-88	13-122	1-23

Depicted values are 5^{th} and 95^{th} percentiles of normal controls in cells/μL

n= number of normal controls per age group

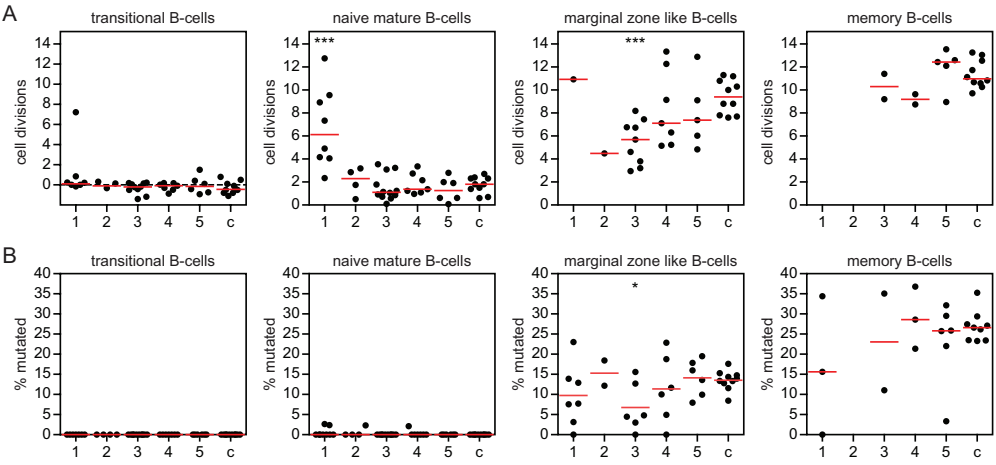

Figure 3. B-cell replication history and somatic hypermutation levels in five different B-cell patterns compared to controls. **A**. The *in vivo* replication history of B-cell subsets as determined by KREC assay in sorted peripheral B-cell subsets of patients and controls (depicted as c, n=10) is given in number of cell divisions. In patients with B-cell pattern 1, proliferation of marginal zone like and memory B-cells was above the detection limit of the KREC assay in seven patients. **B**. The somatic hypermutation frequency given in percentage mutated hotspot in a rearranged Vk3-20 gene segment was determined by the IgκREHMA assay and compared to 10 healthy controls (depicted as c). The five B-cell patterns are: 1) low transitional and memory B-cells, 2) low naive mature, marginal zone like and memory B-cells, 3) low marginal zone like and memory B-cells, 4) low memory B-cells and 5) normal marginal zone like and memory B-cells. Individual data points are displayed and bars indicate medians. Groups are compared to controls with the Mann Whitney test. Significant values compared to normal controls are indicated. *** P=<0,0005 ** P<0,005 * P<0.05.

of both marginal zone like and memory B-cells (B-cell pattern 3) and seven (19%) an isolated reduction of switched memory B-cells (B-cell pattern 4). Six patients (16%) did not have a reduction in marginal zone like or memory B-cells (B-cell pattern 5). Remarkably, none of the CVID patients showed an isolated reduction of marginal zone like B-cells. Thus, by using absolute numbers of B-cell subsets, five main B-cell patterns could be identified.

Comparison of B-cell patterns to the EUROclass CVID classification

B-cell patterns that have been described previously in CVID classification systems are based on relative B-cell subset sizes and include reductions of marginal zone like and memory B-cells and an expansion of transitional B-cells (Supplemental Table 1 for comparison to the EUROclass CVID classification). B-cell pattern 1, i.e. with reduced numbers of transitional B-cells, has not been described so far as separate B-cell phenotype, which also

applies for B-cell pattern 2 with a reduction of naive B-cells, marginal zone like and memory B-cells. Furthermore we showed that six patients who are classified as smB+ (switched memory B-cells >2% of lymphocytes) in EUROclass actually have decreased age matched memory B-cell counts. Using absolute cell counts, we also noted that only one patient showed a minimal increase of transitional B-cells (Figure 2C), whereas 15 patients could be classified as Tr^{hi} according to EUROclass. Therefore, a relative expansion of transitional B-cells in CVID is the result of a reduction of the other B-cell subsets. In conclusion, the here defined B-cell patterns only show a limited overlap with the EUROclass CVID classification.

Abnormalities in B-cell proliferation and somatic hypermutation

To study whether reductions of peripheral B-cell subsets in the five B-cell patterns were associated with aberrant B-cell proliferation, the *in vivo* B-cell replication history was determined of sorted B-cell subsets of patients and controls by calculating the ratio between genomic coding joints and corresponding signal joints on kappa-deleting recombination excision circles (KRECs) of the *IGK*-deleting rearrangement.[20] In addition, somatic hypermutation (SHM) levels were determined by measuring the frequency of a mutated hotspot in rearranged Vκ3-20 gene segments with a restriction enzyme hotspot mutation assay (IgκREHMA).[20,35]

Transitional B-cells are recent bone marrow emigrants that have not undergone proliferation in healthy individuals.[20] In virtually all CVID patients the replication history of transitional B-cells was normal, including patients with reduced transitional B-cells (Figure 3A). Only in a single patient with low transitional B-cells, the transitional B-cells had undergone seven cell divisions. These findings indicate that the absolute number of transitional B-cells in CVID patients is not influenced by deregulated proliferation, but rather reflects reduced bone marrow output and/or increased cell death. In two patients with low transitional B-cells, we examined the precursor B-cell compartment in bone marrow. These patients had a reduced proportion of immature B-cells, supporting the hypothesis of a decreased bone marrow output of B-cells (Figure 4).

Naive mature B-cells of controls had undergone a median of 1.8 (range 0.7-2.7) cell divisions in the absence of SHM (Figure 3A and 3B), which is known as antigen-independent homeostatic proliferation[20]. Naive mature B-cells of CVID patients with reduced absolute numbers of transitional B-cells (B-cell pattern 1) showed significantly increased proliferation, which did not result in increased naive B-cells numbers. These naive mature B-cells were not clonal or oligoclonal based on a normal Igκ/Igλ ratio and a polyclonal pattern on IgH-CDR3 spectratyping (data not shown). Based on absence of SHM, antigenic stimulation was also excluded as cause of increased proliferation (Figure 3B). Increased proliferation was not observed in any of the other CVID patients. In summary, patients with

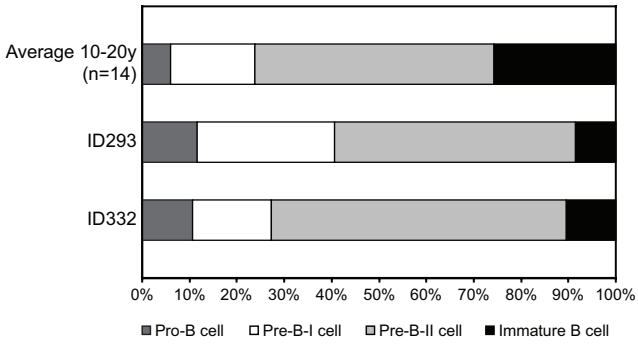

Figure 4. Composition of the bone marrow precursor B-cell compartment of two patients with low transitional and memory B-cells (B-cell pattern 1). Using flow cytometric immunophenotyping, four major precursor B-cell subsets can be identified (pro-B, pre-B-I, pre-B-II and immature B). In healthy donors the immature B-cell fraction composes 25% of the total precursor B-cell compartment. The proportion of immature B-cells in CVID patients with pattern 1, was decreased compared to controls.

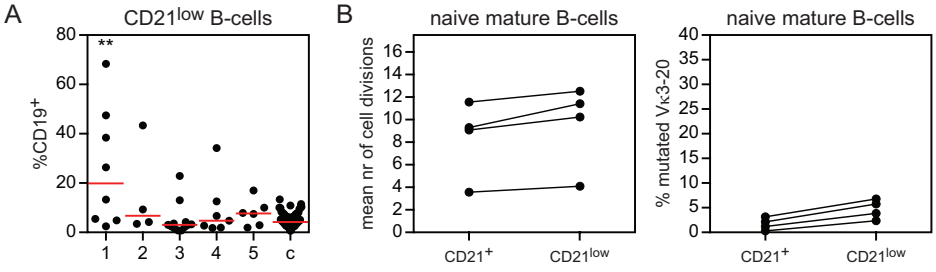

Figure 5. Frequency and proliferation history of CD21^{low} B-cells in CVID patients. A. CD21^{low} B-cells are depicted as proportions of CD19+ B-cells and compared to normal controls (c) according to their B-cell subset pattern. Individual data points are displayed and bars indicate medians. B-cell patterns were compared to controls with the Mann Whitney test. **B.** Number of cell divisions and frequency of somatic hypermutations of sorted CD21^{low} CD27-IgM+IgD+ naive B-cells compared to sorted CD21+ CD27-IgM+IgD+ naive B-cells in patients with >20% CD21^{low} B-cells and a naive B-cell replication history of > 4 cell divisions.

a combined decrease of transitional and memory B-cells showed increased proliferation of naive mature B-cells, which did not result in an increased subset size.

Patients with decreased naive mature B-cells, marginal zone B-cells and memory B-cells (B-cell pattern 2) showed a normal replication of transitional and naive mature B-cells. The few naive mature B-cells did not show an increase of their homeostatic proliferation to compensate for low naive mature B-cell numbers. Since the majority of B-cells did not survive beyond the transitional B-cell stage, we propose that these patients suffer from an early defect in peripheral B-cell maturation or survival. The replication history and SHM status of the marginal zone and memory B-cell subsets could not be determined in most of these patients because of very low cell numbers. Thus, B-cell pattern 2 seems to be the result of an early defect in peripheral B-cell maturation or survival.

Marginal zone like B-cells of patients with a combined reduction of marginal zone like and memory B-cells (B-cell pattern 3) showed a significantly decreased number of cell divisions. In marginal zone like B-cells of controls, the median number of cell divisions was 9.4 (7.6-11.3). This proliferation is antigen-driven, reflected by the presence of SHM (median 14%; 8-18%) (Figure 3B). Decreased proliferation in B-cell pattern 3 was accompanied by reduced SHM levels (Figure 3B), which is indicative for impaired response to antigen. Therefore we propose that the reduction in marginal zone B-cells is caused by reduced (antigen driven) proliferation.

In healthy controls, the memory B-cells showed the highest number of cell divisions (median 11.0; 9.7-13.3) and SHM levels (median 27%, 23-35%) (Figure 3A and 3B). Due to limited memory B-cell numbers, the KREC assay could only be performed in 16 of 37 patients. In most CVID patients, replication of memory B-cells showed at least 9 cell divisions, which was in the normal range (Figure 3A). Apparently, memory B-cell subset reductions cannot be solely explained in terms of a B-cell proliferation defect.

In patients with a normal marginal zone like and memory B-cells, no significant abnormalities in B-cell replication and SHM could be detected (B-cell pattern 5). Thus, patients with normal absolute numbers of peripheral B-cell subsets did not show aberrancies in B-cell proliferation and SHM. The seemingly normal B-cell subsets suggest that the immunodeficiency is likely the result of impaired antibody production by plasma cells rather than a B-cell differentiation defect.

CD21^{low}CD38^{low} B-cells and B-cell proliferation

A subgroup of CVID patients show increased frequencies of CD21^{low}CD38^{low} B-cells.[5,25] Therefore, we studied the frequency of these aberrant cells that occupy the B-cell compartment in our patient groups. The proportions of CD21^{low}CD38^{low} B-cells were significantly increased in patients with low transitional B-cells and memory B-cells (B-cell pattern 1) (Figure 5A). Since the naive mature B-cells of these patients showed increased

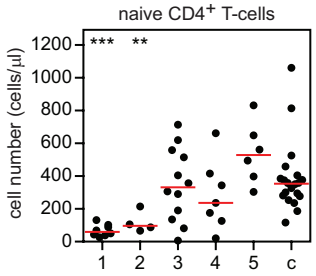

Figure 6. CD4+ naive T-cells in five different B-cell patterns compared to controls. Absolute counts in cells/µL of CD3+CD4+CD27+RA+RO- naive T-cells in patients with five different B-cell patterns compared to controls. The five B-cell patterns are: 1) low transitional and memory B-cells, 2) low naive mature, marginal zone like and memory B-cells, 3) low marginal zone like and memory B-cells, 4) low memory B-cells and 5) normal marginal zone like and memory B-cells. Individual data points are displayed and bars indicate medians. Groups are compared to controls with the Mann Whitney test. Significant values compared to normal controls are indicated. *** P=<0,0005 ** P<0,005 * P<0.05.

Table 2. Clinical complications in CVID patients

Clinical complication	**B-cell pattern**					**Total** n=37
	1 n=8	**2** n=4	**3** n=12	**4** n=7	**5** n=6	
Recurrent RTI and/or ENT infections	8 (100%)	4 (100%)	12 (100%)	7 (100%)	6 (100%)	37 (100%)
Recurrent severe pneumonia	4 (50%)	2 (50%)	4 (33%)	0 (0%)	0 (0%)	10 (27%)
Bronchiectasis	3 (38%)	2 (50%)	4 (33%)	1 (14%)	0 (0%)	10 (27%)
Auto-immune disease	4 (50%)	2 (50%)	1 (8%)	1 (14%)	1 (17%)	9 (24%)
Granulomatous inflammation	1 (13%)	1 (25%)	2 (17%)	0 (0%)	0 (0%)	4 (11%)
Splenomegaly	6 (75%)	1 (25%)	3 (25%)	0 (0%)	0 (0%)	10 (27%)
Recurrent herpes zoster	3 (38%)	1 (25%)	3 (25%)	1 (14%)	0 (0%)	8 (22%)
Recurrent lymphadenopathy	3 (38%)	2 (50%)	1 (8%)	2 (29%)	1 (17%)	12 (32%)

RTI; respiratory tract infection, ENT; ear nose throat, n=number of patients. B-cell patterns are discussed in the text. Recurrent severe pneumonia; >1 episode of: infiltrate on the chest X-ray, hospitalization and i.v. antibiotics

proliferation and most CD21^{low}CD38^{low} B-cells have a naive mature B-cell phenotype, we questioned whether the frequency of these cells was related to the number of cell divisions of naive mature B-cells. We sorted CD21^{+} and CD21^{low}CD38^{low} CD27-IgM+IgD+ naive B-cells from four patients with increased B-cell proliferation (>4 cell divisions) and >20% CD21^{low}CD38^{low} B-cells within the total B-cell compartment. In these four patients, CD21^{+} and CD21^{low}CD38^{low} naive mature B-cells showed similar increased levels of proliferation (Figure 5B). Since both fractions did not show a significant increase of mutated *IGK* alleles (Figure 5B), it is unlikely that the hyperproliferation was antigen driven. Thus, CD21^{low}CD38^{low} B-cells were significantly increased in patients with low transitional and memory B-cells, but did not show more proliferation as compared with their CD21+ counterparts and lacked clear signs of antigenic stimulation.

Naive CD4+ T-cells

The composition of the B-cell compartment has been associated with abnormalities in naive CD4+ T-cells numbers[30] and low naive CD4+ T-cells are associated with clinical complications.[36] Therefore we determined the number of naive CD4+ T-cells in CVID patients with the different B-cell subset patterns. B-cell patterns 1 and 2 were associated with a decrease of naive CD4+ T-cells compared to healthy controls (Figure 6). A decrease of naive CD4+ T-cells in addition to the severe disturbance of peripheral B-cell development suggests that the immunological defect in these groups is not limited to the B-cell lineage.

Clinical complications

Having defined the B-cell replication and somatic hypermutation characteristics of our CVID patients, we aimed to relate these with the clinical complications (Table 2). The age of inclusion in the study did not differ significantly between patients with different B-cell patterns. However, the mean age of initial symptoms and diagnosis was higher in patients with B-cell pattern 1 (mean 30 years, range 4.0-59.0 and 38.0 years, range 19.0-63.0 respectively) as compared to patients with B-cell pattern 2 (11, 4.0-19.0 and 17.8, 12.0-28.0), B-cell pattern 3 (9.8, 0.3-64.0 and 16.0, 4.0-71.0 years) and B-cell pattern 5 (3.9, 0.5-9.0 and 11, 2.5-29.0). The differences in age of onset of symptoms and diagnosis of patients with B-cell pattern-1 supports the hypothesis of a different pathophysiologal background.

The occurrence of bronchiectasis was not associated with age of onset of symptoms, diagnostic delay, IgG level at diagnosis (as reported previously by others[31]) or a specific B-cell pattern. However, patients with bronchiectasis experienced more episodes of severe pneumonia, defined as an infiltrate on the chest X-ray, hospitalization, and the need for intravenous antibiotics (Supplemental Figure 1A). The occurrence of splenomegaly (n=10; defined by ultrasound or by clinical examination) was associated with decreased numbers of transitional B-cells and increased proliferation of naive mature B-cells (Supplemental

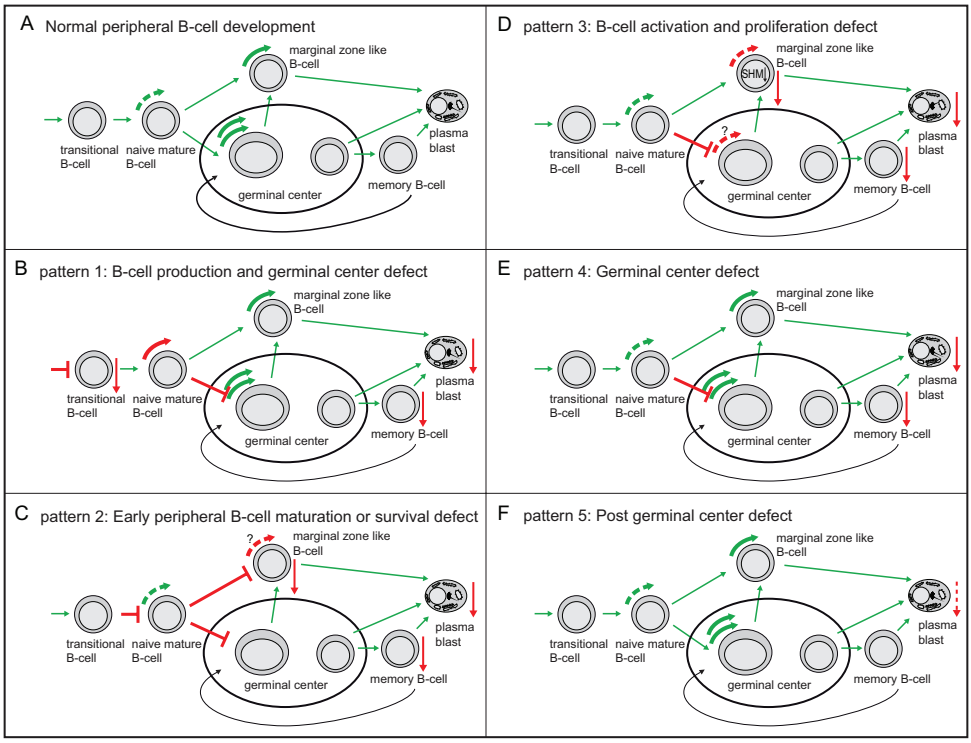

Figure 7. Model of the pathophysiological background of five B-cell patterns in CVID patients based on proliferation history and somatic hypermutation levels. A: Normal peripheral B-cell development. Green curved arrows depict normal B-cell proliferation. **B-F**: abnormal peripheral B-cell development in the five B-cell patterns. Left upper side: proposed pathophysiology and corresponding B-cell pattern. Red straight arrows depict decreased subset size (arrow pointing downwards). T-shaped red bars depict a proposed block in B-cell development. **B**: Red curved arrow depicts increased proliferation of naive B-cells. **C.** Red interrupted curved arrows depict decreased proliferation of marginal zone like B-cells.

Figure 1B). Splenomegaly significantly clustered in patients with B-cell pattern 1 (P=0.007). Lymphadenopathy, autoimmunity and granulomatous disease were not significantly correlated with one of the five B-cell patterns. However, autoimmunity was associated with an increased proportion of $CD21^{low}CD38^{low}$ B-cells (Supplemental Figure 1C). Thus, only splenomegaly was associated with a specific B-cell pattern, although we cannot draw firm conclusions about the association between B-cell patterns and clinical complications because of the limited number of patients.

DISCUSSION

CVID represents a heterogeneous group of disease entities which are expected to result from various underlying immunopathological mechanisms. The level of immunological heterogeneity has been mainly described in terms of abnormalities in the relative size of B-cell subsets in CVID patients[5,27-28] and studies unravelling the immunological causes are limited. Using a combined flow cytometric and molecular approach, we identified in our CVID cohort five unique B-cell patterns based on reductions in absolute numbers of specific B-cell subsets and linked these five B-cell patterns to abnormalities in B-cell replication and somatic hypermutation. These results provide new insight into understanding of the different pathophysiological backgrounds of CVID.

In this study, we used healthy age-matched controls and absolute B-cell subset numbers to define reductions in the various B-cell subsets. This has the advantage over relative frequencies, because the absolute size of a specific B-cell subset is not influenced by increase or decrease of the other B-cell subsets. In this way, we could demonstrate that a relative increase of transitional B-cells in CVID patients as has been reported in the EUROclass classification[5] is the result of reductions in the other B-cell subsets, rather than of an expansion of transitional B-cells.

CVID patients with low numbers of transitional B-cells and memory B-cells (n=8) showed increased proliferation of naive mature B-cells, without an increase of the naive mature B-cell subset. Furthermore, two of these patients had a reduced frequency of immature B-cells in bone marrow, which might reflect a reduced production of B-cells in the bone marrow (Figure 7B). Therefore, the increased number of cell divisions likely compensates for decreased bone marrow output or for increased cell death of immature or naive mature B-cells. In addition, these patients had decreased memory B-cells, which is indicative for a germinal center defect (Figure 7B). A partial defect in precursor B-cell development at the pre-B-I to pre-B-II stage has recently reported by Ochtrop et al. in 9 of 25 CVID patients and was associated with low transitional B-cells.[37] This subgroup of CVID patients probably shows overlap with B-cell pattern 1 and supports the hypothesis that these patient have a different pathophysiology. We identified a similar immunophenotype with increased B-cell proliferation of naive mature B-cells in patients with the Nijmegen Breakage Syndrome (NBS).[38] NBS patients have a DNA repair defect, which leads to a quantitative V(D)J recombination defect and consequently a defect of precursor B-lymphopoiesis, which is compensated by increased proliferation of naive mature B-cells.[38] In addition, NBS patients have a germinal center defect defined by defective somatic hyper mutation and class switch recombination.[39] The observed B-cell pattern in CVID patients with decreased transitional and memory B-cells might be compatible with a DNA repair defect. Several studies show that increased radiosensitivity of lymphocytes and aberrancies in DNA repair

genes can be found in part of the CVID patients.[40-41] Therefore, we are currently investigating DNA-repair defects in CVID patients with this B-cell pattern. Apart from DNA repair defects, other defects that affect precursor B-cell development are potentially involved in the pathophysiology of B-cell pattern 1.

Naive CD4+ T-cells were also reduced in patients with low transitional and memory B-cells. Although this finding is compatible with a defect in DNA repair,[42] the decrease of naive CD4+ T-cells and increased incidence of splenomegaly also show similarities to the new CVID subset with Late Onset Combined Immunodeficiency (LOCID), as proposed by Malphettes et al.[43], although our patients did not suffer from opportunistic infections. In line with our observation in naive mature B-cells, the reduction of naive CD4+ T-cells in CVID patients has been associated with decreased thymic output[44] and increased proliferation and apoptosis of naive T-cells.[30] Apparently, the compensatory hyperproliferation seems not to be limited to the B-cell lineage. Serana et al.[44] reported that a subgroup of CVID patients show decreased thymic output as measured with TRECs in combination with an increased proliferation of total B-cells, as measured with the KREC assay. The observed increase of B-cell proliferation in a subgroup of CVID patients supports our observation of increased naive mature B-cell proliferation in patients with B-cell pattern 1. However, B-cell pattern 1 does not fully correspond to the findings of Serana et al., who report a normal proportion of memory B-cells in patients with increased B-cell proliferation. Since we studied B-cell replication at the B-cell subset level, our analysis gives more accurate information of the impact of B-cell subset proliferation on the composition of the peripheral B-cell compartment. Increased naive mature B-cell proliferation was associated with increased $CD21^{low}$ B-cells. Rakhmanov *et al*. reported a more extensive proliferative history of $CD21^{low}$ B cells in CVID patients compared to naive B cells of controls.[23] We showed that in patients with increased naive mature B-cell proliferation both the $CD21^{low}$ naive B-cells and the CD21+ naive B-cells hyperproliferated, so the aberrant proliferation was present in all naive mature B-cells irrespective of CD21 expression. Since $CD21^{low}$ B-cells contain mostly autoreactive unresponsive clones[24], we hypothesize that downregulation of CD21 expression on hyperproliferating naive mature B-cells could be a mechanism to silence them.

Patients with reduced numbers of naive mature, marginal zone like and memory B-cells (B-cell pattern 2) suffer from an early block in peripheral B-cell development affecting B-cell maturation and survival after the transitional B-cell stage (Figure 7C). As a result, also marginal zone like B-cells and memory B-cells are severely decreased. Subsequent analysis of the CD27+ B-cell subsets for replication history and SHM was therefore not possible. Also in patients with BAFF-R deficiency, B-cell development is arrested at the transitional B-cell stage[15]. However, naive CD4+ T-cells were also severely decreased in association with B-cell pattern 2, so in these patients a combined B- and T-cell defect is more likely.

The existence of a combined decrease of marginal zone like and memory B-cells has been described previously in CVID patients, but the pathophysiology remains unclear.[5,28] We showed that a decrease of marginal zone like B-cells in 12 CVID patients was associated with decreased proliferation. Furthermore, the frequency of somatic hypermutations was decreased, which is indicative for an impaired response to antigen. Therefore, these data suggest that impaired activation and subsequently impaired proliferation is implicated in the pathophysiology of this group of CVID patients (Figure 7D). The number of memory B-cells was also severely reduced, but the replication history of the few generated memory B-cells could not be reliably established because of the extremely low cell numbers. In line with our hypothesis of decreased B-cell activation and subsequent proliferation, a combined decrease of marginal zone and memory B-cells has been observed in patients with CD19, CD81 deficiency.[13,17] Thus far, only in vitro B-cell proliferation defects have been reported in CVID patients with defective B-cell TLR9 signaling.[45-46] Mutations in the TLR9 gene were absent in these patients, suggesting that decreased TLR9 signaling was a secondary phenomenon. Information about the immunophenotype of these patients is scarce, but also points towards a combined decrease of marginal zone and memory B-cells.[46] Based on our own data and the observations in CD19 and CD81 deficient patients we hypothesize that a combined decrease of marginal zone like and memory B-cells could best be explained by impaired response to antigen, although more detailed studies are necessary to define the defect more precisely.

An isolated reduction of memory B-cells was associated with a proliferation of this subset in the lower normal range. Since most CVID patients with decreased memory B-cells showed at least 9 cell divisions, this number apparently is a prerequisite for memory B-cell development. An isolated reduction of memory B-cells as we identified in seven CVID patients is compatible with defects that predominantly affect the generation of switched memory B-cells in the GC (Figure 7E)[9,47-48]. Yet unidentified co-stimulation or CSR defects could underlie this B-cell phenotype.

CVID patients with normal memory B-cells and marginal zone like B-cells (n=6) represent a group without B-cell proliferation and somatic hyper mutation abnormalities and show less clinical complications compared to patients with low memory B-cells. We hypothesize that this B-cell pattern is compatible with a predominantly post germinal center defect, most likely a terminal plasma cell maturation or homing defect (Figure 7F). Taubenheim et al[49] showed that B-cell could only reach the initial stage of plasma cell differentiation in lymph nodes of three reported CVID patients. Analysis of terminal plasma cell development in lymphoid tissues has the potential to unravel the pathophysiology of this B-cell pattern.

In conclusion, our combined flow cytometric and molecular approach resulted in the identification of five main B-cell patterns in CVID, delineating five immunological

homogenous patient groups for which different pathophysiological backgrounds are proposed.

Detailed studies in the here defined homogenous groups of patients are needed to further unravel the defects at a molecular level. Furthermore, this approach might well be applicable to "CVID-like" disorders. Recently, progress has been made by uncovering multiple novel susceptibility loci for CVID using genome-wide analysis of single nucleotide polymorphisms and copy number variations.[50] Integration of (high throughput) genomic analysis, detailed flow cytometric immunophenotyping, functional molecular assays and clinical data collection in a large cohort of CVID patients is important for the identification of the clinical correlates, prognostic factors and underlying genetic defects in CVID patients with different B-cell patterns.

ACKNOWLEDGMENTS

The authors are indebted to Mr. E.F.E. de Haas, Mrs. S. Postumus-van Sluijs and Mr B. Van Turnhout for technical assistance; to Mrs. S. De Bruin-Versteeg for technical support and assistance with preparing the figures; To Mrs. P van Jaarsveld-Bakker and Mrs. M.W. van der Ent van for assistance with collecting blood samples and clinical data.

AUTHORSHIP CONTRIBUTIONS

GJD, MvH, NGH, AW, EdV, BB and IP performed the research. WH assisted in statistical analysis of the data. MvdB and MCvZ designed the research. GJD, MCvZ, JJMvD and MvdB wrote the paper.

All Authors declare no conflict of interest.

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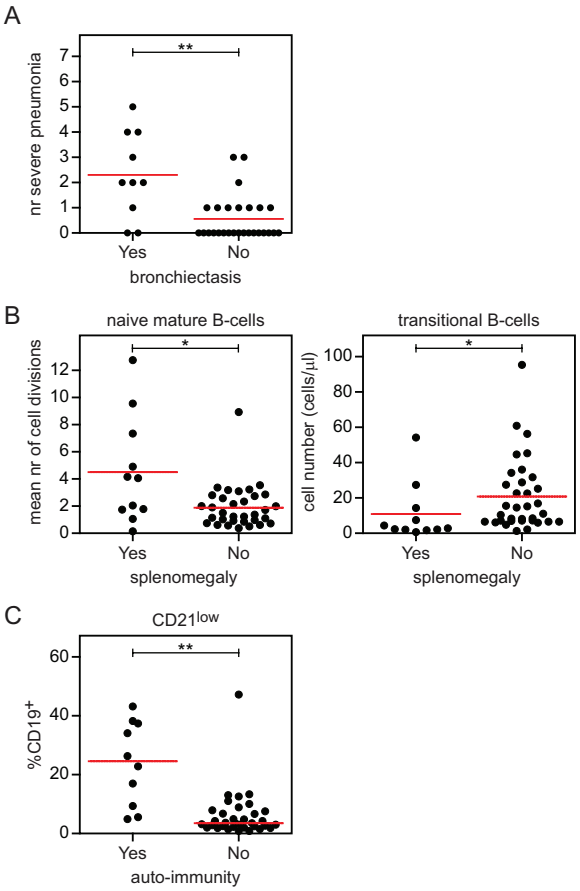
SUPPLEMENTS

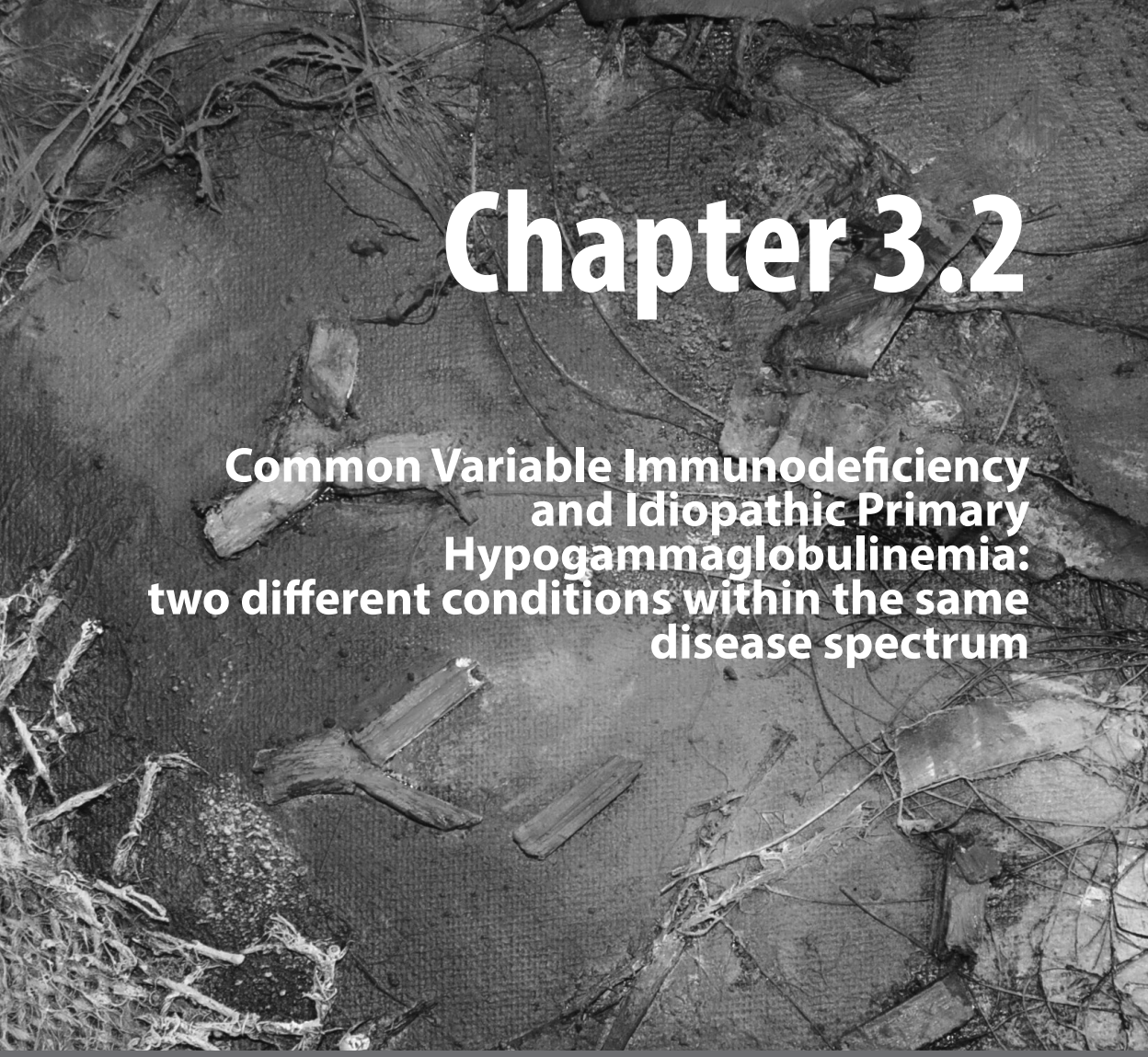


Supplemental Figure 1. Clinical complications and associated B-cell subset abnormalities in patients with CVID. A. Patients with bronchiectasis had significantly higher number of severe pneumonia, which was defined as an infiltrate on the chest X-ray, hospitalization and the need for intravenous antibiotics. B. Patients with splenomegaly had a significantly lower number of transitional B-cells and a significantly higher number of cell divisions of the naive mature B-cells. C. Auto-immunity was correlated with an increased proportion of CD21low B-cells. Individual data points are displayed and bars indicate medians. Groups were compared with the Mann Whitney test. Significant values compared to normal controls are indicated. *** P=<0,0005 ** P<0,005 * P<0.05.

Supplemental Table 1. B-cell patterns compared to the EUROclass[5] CVID classification.

		EUROclass			
B-cell pattern		smB-	smB+	$CD21^{lo}$	Tr^{hi}
1	n=8	7	1	5	0
2	n=4	3	1	1	4
3	n=12	11	1	2	6
4	n=7	4	3	2	3
5	n=6	0	6	1	2

Depicted values are number of patients, all patients were B+; CD 19+ B-cells >1% of lymphocytes. smB-: switched memory B-cells <2% of B-cells, smB+ Switched memory B-cells >2% of B-cells, $CD21^{lo}$: CD21low B-cells >10% of B-cells, Tr^{hi}: Transitional B-cells >9% of B-cells



Chapter 3.2

Common Variable Immunodeficiency and Idiopathic Primary Hypogammaglobulinemia: two different conditions within the same disease spectrum

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Haematologica. 2013 Jun 10. [Epub ahead of print] doi:10.3324/haematol.2013.085076

ABSTRACT

Patients with hypogammaglobulinemia who do not fulfill all the classical diagnostic criteria for Common Variable Immunodeficiency (reduction of two immunoglobulin isotypes and a reduced response to vaccination) constitute a diagnostic and therapeutic dilemma, because information concerning the clinical and immunological characteristics of these patients with Idiopathic Primary Hypogammaglobulinemia is not available. In 44 Common Variable Immunodeficiency and 21 Idiopathic Primary Hypogammaglobulinemia patients we determined the clinical phenotypes and performed flow cytometric immunophenotyping to assess the pathophysiological B-cell patterns and memory B-cell subset counts. Age-matched B-cell subset reference values were generated of 130 healthy donors. Severe pneumonia and bronchiectasis occurred at similar frequencies in Idiopathic Primary Hypogammaglobulinemia and Common Variable Immunodeficiency. Although IgG levels were only moderately reduced compared to Common Variable Immunodeficiency, 12/21 Idiopathic Primary Hypogammaglobulinemia patients required immunoglobulin replacement. Non-infectious disease related clinical phenotypes (auto-immune cytopenia, polyclonal lymphocytic proliferation and persistent unexplained enteropathy) were exclusively observed in Common Variable Immunodeficiency and were associated with early peripheral B-cell maturation defects or B-cell survival defects. T-cell dependent memory B-cell formation was more severely affected in Common Variable Immunodeficiency. Furthermore, 14/21 Idiopathic Primary Hypogammaglobulinemia patients showed normal peripheral B-cell subset counts, suggestive for a plasma cell defect. In conclusion, Idiopathic Primary Hypogammaglobulinemia patients who do not fulfill all diagnostic criteria of Common Variable Immunodeficiency have moderately decreased immunoglobulin levels and often a normal peripheral B-cell subset distribution, but still suffer from serious infectious complications.

INTRODUCTION

Common Variable Immunodeficiency (CVID) is the most prevalent form of symptomatic primary antibody deficiency[1]. It is defined by 1) a marked decrease in serum IgG and IgA or IgM of at least 2 SD below the mean for age 2) absent isohemagglutinins and/or poor response to vaccines 3) onset of immune deficiency at greater than 2 years of age, and 4) other defined causes of hypogammaglobulinemia have been excluded (1). Patients with CVID suffer from recurrent infections and non-infectious complications (autoimmune cytopenia, polyclonal lymphocytic proliferation and persistent unexplained enteropathy), of which the latter are associated with increased mortality[2-3]. By definition, CVID excludes a group of patients with Idiopathic Primary Hypogammaglobulinemia, who suffer from hypogammaglobulinemia, but do not fulfill CVID diagnostic criteria with respect to a reduction of two immunoglobulin isotypes and/or a reduced response to vaccination. According to the CVID diagnostic classification of the European Society for Immunodeficiency diseases (ESID; www.ESID.org), some of these patients with Idiopathic Primary Hypogammaglobulinemia (further referred to as IPH) can be classified as "possible" CVID, and in the ESID database of primary immunodeficiencies they are classified as "other hypogammaglobulinemia's". Remarkably, according to the Primary Immunodeficiency Classification of the International Union of Immunological Societies (IUIS) these patients cannot be sufficiently classified within any of the subcategories of "Predominantly Antibody Deficiency"[4]. In comparison, the ICD10 (International Classification of Diseases, 10th version; www.WHO.int/classifications/icd/en/) classifies IPH as "hypogammaglobulinaemia not otherwise specified" with the same ICD10 code as CVID. Patients with IPH are regularly encountered in clinical practice, but information concerning the prevalence and the clinical and immunological characteristics is not available. It is important to obtain insight in the frequency and severity of the clinical complications of IPH, to clarify whether IPH is a clinically relevant antibody deficiency and to develop appropriate treatment strategies. In addition, analysis of immunological parameters will enable the comparison of pathophysiological aspects of IPH and CVID.

Therefore, we aimed to determine the position of IPH in the spectrum of idiopathic antibody deficiencies through clinical and immunological comparison with CVID. First, we annotated the patients with the clinical phenotypes as established by Chapel *et al*.[2,5]. In addition, we performed flow cytometric immunophenotyping in order to analyze T-cell dependent and independent memory B-cell subset counts[6] and blood B-cell patterns, which are associated with differences in pathophysiological background[7].

METHODS

Patients

Peripheral blood samples and clinical data were collected of 44 CVID patients and 21 IPH patients. IPH was diagnosed if patients had a reduction of IgG at least 2 SD below the mean for age, an onset of the immunodeficiency at greater than 2 years of age, exclusion of defined causes of hypogammaglobulinemia and if they did not fulfill the CVID diagnostic criteria with respect to a reduction of two immunoglobulin isotypes and/or a reduced response to vaccination. The group of CVID patients includes the 37 patients that have been reported in our original description of the B-cell patterns[7]. In addition we collected blood from 130 healthy age matched controls and 26 cord blood samples. This study was approved by the Medical Ethics Committee of the Erasmus MC.

Clinical phenotyping

Clinical data was collected from all IPH and CVID patients to annotate their clinical phenotypes as previously described by Chapel *et al*.[2,5]. These phenotypes are: 1) no disease related complications (infections only); 2) auto-immune cytopenia's; 3) polyclonal lymphoproliferation (granuloma/LIP/persistent unexplained lympadenopathy); and 4) unexplained persistent enteropathy. In addition, data was collected concerning the frequency and severity of infections and modes of treatment. Pneumococcal polysaccharide vaccination responses were interpreted according to Borgers et al.[8] as an adequate response to half of the measured pneumococcal serotypes.

Flow cytometric analysis and assignment of B-cell patterns

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) were compared to age matched healthy controls. A decrease or increase of a B-cell subset was defined as a value below the 5th or above the 95th percentile. B-cell patterns were determined as described previously[7] and are summarized together with their pathophysiological background in Table 1.

Table 1. B-cell patterns associated with different pathophysiological backgrounds in CVID (ref. 7.)

B-cell pattern	Corresponding B-cell immunophenotype	Pathophysiological background
1	reduction of transitional B-cells and CD27+IgD- memory B-cells	defect in B-cell production and germinal center
2	normal transitional B-cells and a reduction of naive mature, CD27+IgD+IgM+ and CD27+IgD- memory B-cells	defect in early B-cell maturation or survival
3	reduction of CD27+IgD+IgM+ and CD27+IgD- memory B-cells.	defect in B-cell activation and proliferation
4	isolated reduction CD27+IgD- memory B-cells	defect in Germinal Center function
5	normal CD27+IgD+IgM+ and CD27+IgD- memory B-cells	post Germinal Center defect

Reduction: < 5th percentile of age matched controls, normal: >5th percentile of age matched controls

STATISTICS

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. Whenever multiple groups with continuous outcomes were compared the non-parametric Kruskal-Wallis rank sum test was used, followed by pair-wise Mann Whitney tests if the former indicated significant differences. For categorical variables the chi-square was used or Fisher's exact test if required. Statistical significance was set at two sided $P<0.05$.

RESULTS

Patient characteristics

In this study, 44 CVID patients were included, with a mean age of 32 years (range 6-77 years) and 21 patients with IPH with a mean age 28 years (range 7-74). The age and sex distribution was comparable between both groups. In the CVID group, 2/44 patients were from consanguineous families and in 10/44 patients the family history was positive

Table 2. Clinical characteristics of patients with Idiopathic Primary Hypogammaglobulinemia

Patient nr	Age in yrs	Sex	IgA#	IgM#	IgG#	IgG1 #&	IgG2 #&	IgG3 #&	IgG4 #&	Response to pneumococcal polysaccharide immunization**	Bronchiectasis on HRCT thorax	History of severe pneumonia	Other pulmonary diagnosis	Other serious or frequent infections$	Immunoglobulin replacement	Prophylactic antibiotics
1	11	M	**0,3**	0,5	**5,0**	**3,65**	1,36	0,15	0,1	normal	no	no		no	no	no
2	9	M	**0,2**	0,6	**4,3**	**3,66**	**0,64**	0,23	0,15	normal	no	no		ENT	yes	no
3	18	M	**0,5**	0,6	**4,1**	**3,27**	**0,5**	0,7	0,09	normal	no	yes		ENT	yes	no
4	47	F	**0,2**	**0,3**	**5,7**	ND	ND	ND	ND	normal	no	no		no	no	yes
5	47	M	1,2	**0,3**	**5,1**	**3,29**	1,63	0,17	0,2	normal	no	no		no	no	no
6	10	F	0,3	**0,3**	**4,9**	3,94	**0,82**	0,19	0,17	normal	no	no	asthma	ENT	yes	no
7	7	F	0,4	**0,3**	**4,7**	3,8	0,63	0,15	0,08	normal	no	no		no	yes	no
8	22	F	0,7	0,4	**5,0**	4,09	1,29	0,41	0,09	normal	no	yes		meningitis	no	yes
9	74	F	0,9	0,5	**4,4**	ND	ND	ND	ND	normal	no	yes	COPD	no	yes	no
10	14	M	1,5	0,8	**4,7**	**2,11**	1,31	0,46	0,05	normal	yes	no	asthma	molluscae	yes	no
11	9	F	0,6	0,6	**5,2**	**3,7**	1,39	0,28	0.33	normal	yes	yes		no	yes	no
12	60	M	0,9	1,1	**4,9**	4,36	1,57	0,22	0,68	normal	yes	yes		ENT	no	yes
13	16	F	1,7	1,2	**6,1**	4,15	2,06	0,18	0,35	normal	no	yes	asthma	no	no	no
14	9	F	0,4	1,0	**4,5**	**3,57**	**0,78**	0,33	0,05	normal	no	no	asthma	ENT	yes	no
15	44	F	1,2	0,4	**5,5**	**3,42**	1,38	0,21	0,45	unknown	no	no		skin	no	no
16	7	M	0,7	0,9	**4,3**	**2,8**	0,95	**0,11**	0,06	unknown	no	no		skin	no	no
17	18	M	0,7	1,6	**5,6**	4,2	1,39	0,55	0,05	unknown	yes	yes	asthma	no	yes	yes
18	32	F	1,6	2,1	**6,6**	**3,29**	0,95	0,39	0,06	unknown	yes	yes		no	yes	yes
19	10	F	1,0	0,5	**4,0**	4,76	1,67	0,29	0,3	abnormal	no	yes		no	yes	no
20	59	F	1,6	0,8	**6,1**	**3,76**	3,16	0,32	0,31	abnormal	no	yes		no	yes	no
21	57	M	1,3	0,4	**6,0**	ND	ND	ND	ND	abnormal	no	no		no	no	yes

bold values are decreased levels (in g/L) compared to age specific normal values (Supplemental Table 1).

&IgG subclasses were sometimes performed at a different time points than IgG, IgA and IgM levels.

**response to pneumococcal polysaccharides was interpreted according to Borgers et al. (8).

$ all patients experienced recurrent upper respiratory tract infections.

ENT; frequent and serious Ear Nose and Throat infections (all these patients got multiple ENT operations)

Asthma; asthmatic bronchitis, COPD; chronic obstructive pulmonary disease

ND; not determined

for hypogammaglobulinemia and/or IgA deficiency, compared to 1/21 and 6/21 in IPH, respectively.

Details regarding immunoglobulin levels, response to immunization and infectious clinical complications of IPH patients are provided in Table 2. All IPH patients displayed reduced serum IgG levels, but did not fulfill the diagnostic criteria for CVID due to: adequate response to immunization (7 patients), normal levels of IgA and IgM (3 patients) or both (7 patients) (Table 2). Four patients had normal levels of both IgA and IgM (Table 2; patient 15-18), but vaccination data were not available. The median IgG levels at diagnosis were lower in patients with CVID (3.0 g/L, range 0-5.7) compared to IPH (4.9 g/L, range 3.6-6.6) (Figure 1A). Considering the IgG subclass levels, most patients had IgG1 levels just below the normal range, with IgG2, IgG3 and IgG4 levels in the (lower) normal range (Table 1). In six patients IgG subclasses were in the (lower) normal range in the presence of a decreased total IgG. In addition, IgM and IgA levels were lower in CVID, which was an expected finding because of the clinical definition of CVID and IPH.

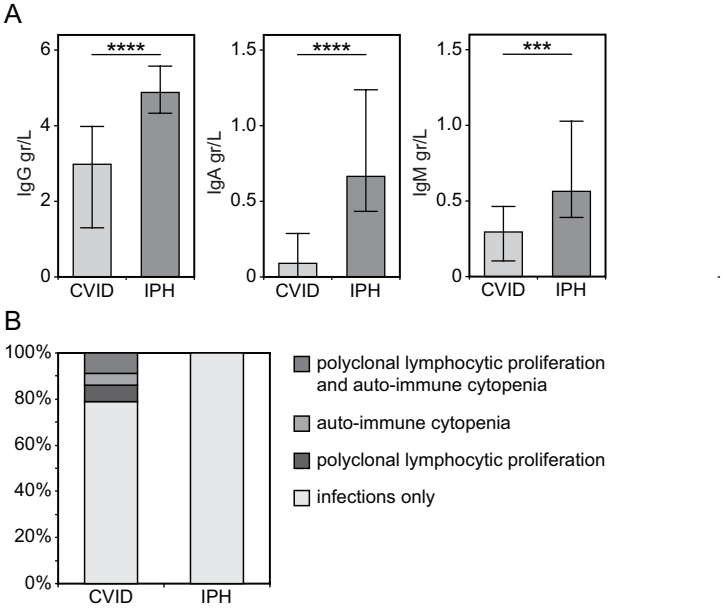

Figure 1. Immunoglobulin levels and clinical phenotypes in CVID and IPH. A. Immunoglobulin levels at diagnosis in CVID and IPH. Groups are compared with the Mann Whitney test. Significant values are indicated. **** P<0.0001, *** P=<0.0005 **B**. Clinical phenotypes according to Chapel et al. (ref 5 and 12).

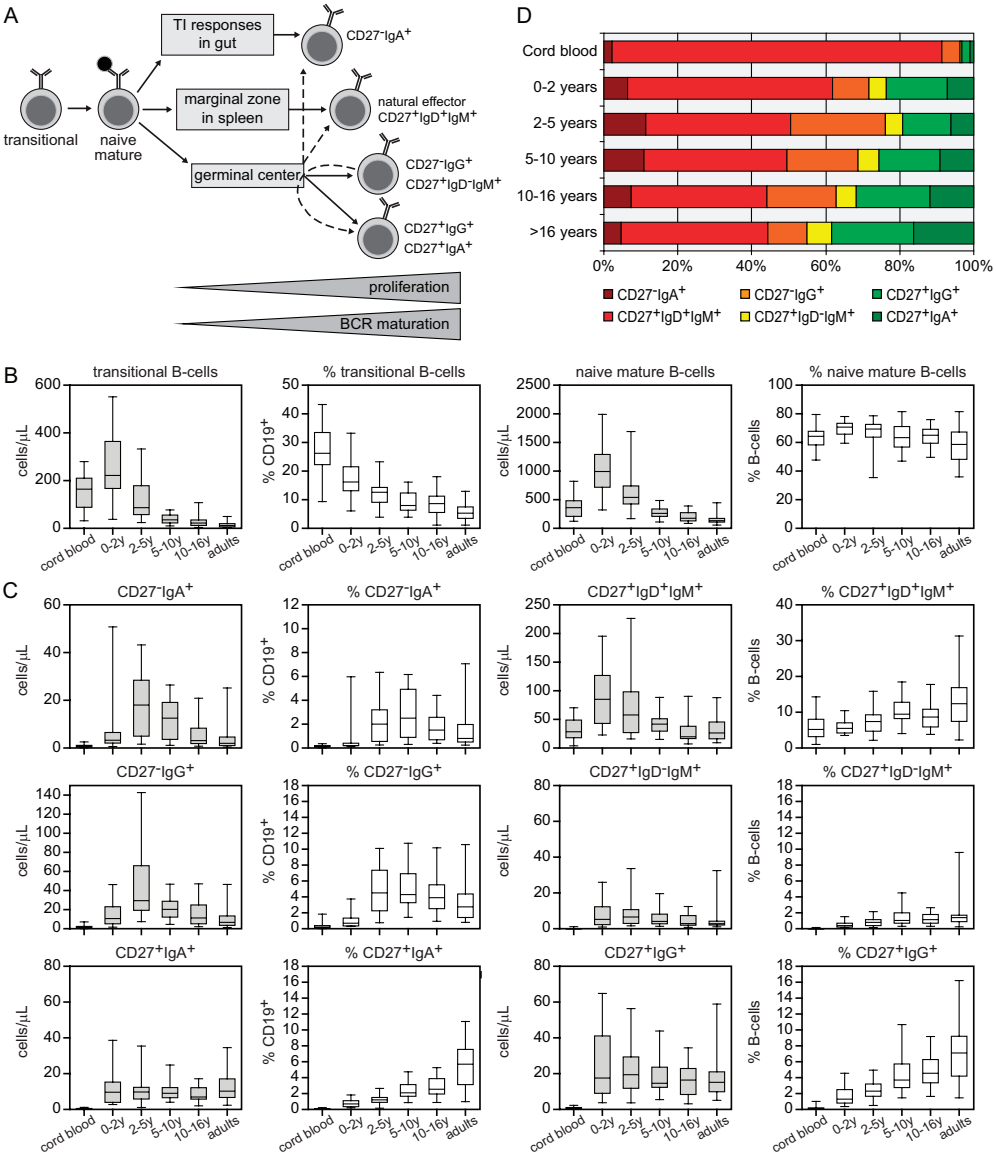

Figure 2. Naive and memory B-cell subsets in normal controls. A. Overview and definition of peripheral B-cell subsets **B**. Normal values of transitional and naive B-cells. **C**. Normal values of memory B-cell subsets. Grey bars represent absolute counts and open bars proportions of CD19+ cells. Bars indicate median with 25 and 75 percentiles. Whiskers represent 5th and 95th percentiles. **D**. Relative distribution of memory B-cell subsets per age category.

Clinical phenotypes in CVID and IPH

All patients with CVID and IPH suffered from recurrent upper respiratory tract infections. For IPH, the infectious complications are summarized in Table 2. Many IPH patients suffer from severe pneumonia (one ore more episodes with hospital admission) and bronchiectasis. These complications were present in a similar frequency compared to CVID patients (50% versus 48% and 24% versus 27%, respectively).

Specific non-infectious disease-related clinical phenotypes (auto-immune cytopenia, polyclonal lymphocytic proliferation and persistent unexplained enteropathy) are associated with an increased mortality in patients with CVID[2-3]. In our cohort, 79% of the CVID patients suffered from infections only, with the remaining 21% experiencing one ore more disease-related non-infectious clinical complications (Figure 1B). In contrast, all IPH patients suffered only from infections. Therefore, non-infectious disease related complications were exclusively observed in CVID (P=0.02).

Treatment

All CVID patients were treated with immunoglobulin replacement therapy. Of the IPH patients, 12/21 (57%) received immunoglobulin replacement (sometimes in combination with prophylactic antibiotics). Of the remaining nine patients, three were treated with prophylactic antibiotics and six received antibiotics during infectious episodes as the only mode of treatment.

B-cell subsets in healthy controls

In order to detect abnormalities in peripheral B-cell subset distribution in patients, we generated age related normal values of all B-cell subsets in a cohort of 130 healthy controls (Figure 2A-2D, Table 3). Absolute numbers of transitional B-cells and naive mature B-cells directly increase after birth and decrease after the age of 2 years. The frequency of transitional B-cells decreases over time, while the frequency of naive B-cells remains stable (Figure 2B).

The human memory B cell compartment consists of six subsets[6] CD27+IgD+IgM+ natural effector B cells are the only memory subset that constitutes a considerable part of the B-cell compartment in cord blood (Figure 2C). Absolute counts of natural effectors increase after birth to show a decline from 2-5 years. In contrast to absolute counts, the proportion of natural effector B cells gradually increases over time and form the largest proportion of memory B cells within the memory B cell compartment. CD27- switched (IgA and IgG) memory B cells are present at birth in very small amounts and reach maximum values at 2-5 years, after which they decline to values just above those in cord blood (Figure 2C-D). CD27+IgM+IgD- (IgM-only) memory B cells and CD27+ switched (IgA+ and IgG+) memory B cells are hardly present in cord blood, but increase rapidly after birth to absolute counts

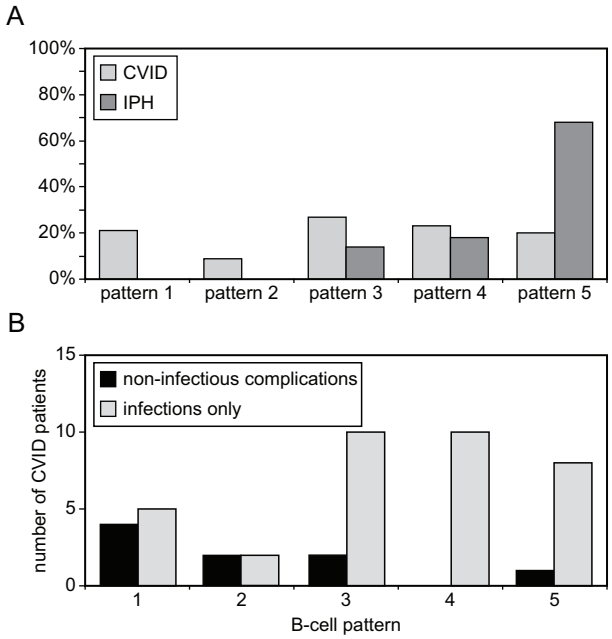

Figure 3. Pathophysiological B-cell patterns in CVID and IPH and relation to clinical phenotypes. A. Comparison of B-cell patterns between CVID (n=44) and IPH (n=21). B-cell pattern 1 and 2 are exclusively observed in CVID (P=0.02). **B.** B-cell patterns and clinical phenotypes in CVID (n=44). Non-infectious complications (auto-immunity and polyclonal lymphocytic proliferation) were more often observed in B-cell pattern 1-2 compared to B-cell pattern 3-5 (P=0.003).

Table 3. Reference values of peripheral B-cell subset absolute counts

	Cord blood n=26	0-2 years n=21	2-5 years n=23	5-10 years n=30	10-16 years n=28	>16 years n=28
Transitional	164 (32-278)	222 (38-551)	87 (24-333)	36 (11-77)	22 (4-108)	12 (3-50)
Naive mature	362 (124-821)	992 (322-1991)	540 (170-1691)	262 (111-486)	176 (87-390)	134 (57-447)
CD27-IgA+	0.7 (0-2.5)	3 (0.6-51)	18 (1.6-43)	13 (1.1-26)	3 (0.8-21)	2 (0.4-25)
CD27+IgD+IgM+	29 (4-70)	85 (23-195)	58 (16-226)	42 (15-88)	20 (7-90)	26 (9-88)
CD27-IgG+	0.8 (0-7)	11 (2-46)	29 (7-143)	20 (5-47)	11 (2-47)	7 (1-46)
CD27+IgD-IgM+	0 (0-1)	5 (1-26)	7 (2-34)	4 (1-20)	3 (1-12)	3 (1-33)
CD27+IgA+	0.1 (0-1)	10 (3-39)	10 (1-35)	9 (4-25)	7 (2-17)	10 (2-35)
CD27+IgG+	0.5 (0-2)	18 (4-65)	19 (45-56)	15 (6-44)	16 (3-34)	15 (5-59)

Median (5^{th} and 95^{th} percentile) in cells/uL

that remain stable over all age groups until adulthood. The relative proportions of these CD27+ memory B-cell subsets show an impressive increase over time (Figure 2D), which mainly reflects the declining absolute number of transitional and naive mature B cells.

Peripheral B-cell subset patterns are different in CVID and IPH

CVID patients can display one of five distinct B-cell patterns[7]. The patterns are based on the composition of the peripheral B-cell compartment and the replication history and somatic hyper mutation frequency of the individual B-cell subsets and are indicative for the pathophysiological background of the antibody deficiency[7]. The B-cell patterns can be easily determined by flow cytometry. Our earlier detailed molecular assays on sorted peripheral B-cell subsets were used to characterize the patterns, but are not used to define the pattern in individual patients.

The five B-cell patterns with their corresponding pathophysiological background are summarized in Table 1. The distribution of B-cell patterns was different between CVID and IPH (Figure 3A, P=0.003). B-cell pattern 1 and 2 that reflect B-cell production or early peripheral B-cell maturation/survival defects, were exclusively present in CVID (9/44 and 4/44 respectively) and not in IPH. B-cell pattern 3 and 4, which are associated with B-cell activation, proliferation and germinal center defects, were more common in CVID (12/44 and 10/44, respectively) as compared to IPH (3/21 and 4/21 respectively). In contrast, most IPH patients showed a normal peripheral B-cell subset distributions (B-cell pattern 5, 14/21), which is higher in frequency than CVID (9/44). The differences in B cell patterns between CVID and IPH patients were reminiscent of the differences in clinical phenotypes: CVID patients with non-infectious complications predominantly displayed B-cell patterns 1 and 2 (Figure 3, P=0.003).

Memory B-cell subsets are more severely affected in CVID than in IPH patients

Data of memory B-cells subsets of CVID and IPH patients were compared to age-matched controls and the proportion of patients with normal (>5th percentile of age matched normal controls) and reduced (<5th percentile) memory B-cell subset size was determined cut of values are summarized in Table 3). The results are depicted in Figure 4. The T-cell independent CD27-IgA+ memory B-cell subset was normal in the majority of CVID and IPH patients. In CVID, four memory B-cell subsets were more frequently reduced compared to IPH. T-cell dependent class switched CD27+IgA+ and CD27+IgG+ memory B-cells showed the most significant difference between the groups. In line with these findings, 19/21 (90%) IPH patients could be classified as smB+ (CD27+IgD- B-cells >2% of B-cells) according to the EUROclass CVID classification[9] and only 2/21 (10%) as smB- (CD27+IgD- B-cells </= 2% of B-cells), whereas in CVID 25/44 (57%) patients were smB- and

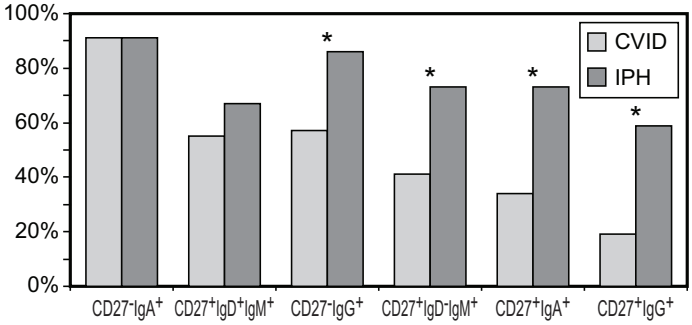

Figure 4. Memory B-cell subsets in CVID and IPH. The proportion of patients with normal memory B-cell subset size (>5th percentile of age matched normal controls) is depicted. Data of CVID and IPH were compared with Fischer's exact test, * P<0.05.

19/44 smB+ (43%). In conclusion, memory B-cell formation was more severely affected in CVID compared to IPH, primarily affecting GC dependent memory B-cell subsets.

DISCUSSION

This study describes the clinical and immunological characteristics of a group of patients with idiopathic primary hypogammaglobulinemia, who do not fulfill the criteria for CVID with respect to a reduction of two immunoglobulin isotypes and/or an impaired response to vaccination. These IPH patients have not yet been well described in the literature and cannot be sufficiently classified within the current PID diagnostic classification system[4]. It has been demonstrated previously that some "CVID" patients have the ability to respond to vaccination[10-11], so these patients might show similarities to some of the IPH patients described in this study. Our data raise the question whether an impaired response to vaccination should be used for the diagnosis of CVID, especially since solid criteria for the interpretation of vaccination responses are lacking.

Important differences were observed between IPH and CVID in clinical phenotypes, pathophysiological B-cell pattern and memory B-cell subset sizes, but also similarities with respect to infectious complications. IPH patients have less severe hypogammaglobulinemia compared to CVID, but most patients with IPH still suffered from recurrent or serious infections. The occurrence of severe pneumonia and bronchiectasis was not significantly different from patients with CVID and more than half of the patients with IPH required immunoglobulin replacement. These data support the idea that IPH is a clinically relevant

antibody deficiency. In contrast to CVID, several IPH patients were in a good clinical condition without immunoglobulin replacement or antibiotic prophylaxis. Apparently, the specific antibody production in these patients was sufficient to prevent the occurrence of severe or frequent infections. Long term follow up studies are necessary to examine which IPH patients require immunoglobulin replacement and/or antibiotic prophylaxis and to monitor whether IPH can over time develop into a full CVID phenotype.

Over the past years Chapel *et al.* described the different clinical phenotypes of CVID patients[2,12] and convincingly showed that non-infectious clinical complications (auto-immune cytopenia, polyclonal lymphocytic proliferation and persistent unexplained enteropathy) are associated with increased mortality compared to patients with infections only[2,12]. We showed that non-infectious clinical complications were present in CVID but not in IPH, suggesting that the latter condition might have a better prognosis. Due to the relatively small cohort of IPH patients, we cannot exclude some of them can present with or develop non-infectious clinical complications.

In line with the clinical differences, analysis of the earlier presented B-cell patterns[7] revealed that defective B-cell production and early peripheral B-cell maturation or survival defect (B-cell pattern 1 and 2, respectively) were exclusively seen in CVID. In addition, we showed that these two B-cell patterns are associated with non-infectious disease related clinical complications. Early defects in B-cell development apparently tend to result in the full CVID phenotype and more often give rise to immune deregulation. Further analysis of B-cell patterns showed that more than half of the CVID patients displays B-cell pattern 1-3, indicative of defects in peripheral B-cell development before the GC stage. In contrast, the majority of IPH patients did not show abnormalities in peripheral B-cell distribution, suggesting a defect in post-GC (terminal) B-cell or a plasma cell defect such as a differentiation, a survival and/or homing defect. Thus, the identification of B-cell patterns is useful to detect differences in pathophysiological background and has the potential to become clinically relevant in the follow up of CVID and IPH, because of the association with non-infectious clinical complications.

We analyzed memory B-cell subsets in CVID and IPH and compared these to 130 age matched controls. We showed that absolute numbers of memory B-cell subsets in healthy individuals reach adult levels within 2 years of age and do not substantially increase afterwards. The observed relative increase in memory B-cells mainly reflects a reduction of transitional and naive B-cells over time. Our data is in line with previously published B-cell subset reference values[13-17]. Memory B-cells were reduced in the majority of CVID patients and mainly involved GC dependent maturation pathways. IPH patients showed less frequently abnormalities in memory B-cell subsets, in line with our hypothesis that most of these patients suffer from plasma cell survival or homing defects.

CVID and IPH are two partly overlapping conditions. IPH is similar to CVID with respect to infectious complications, but is not the same with respect to non-infectious clinical complications, immunoglobulin levels, distribution of B-cell patterns and memory B-cell counts. Functional immunological studies should focus on plasma cell differentiation and homing. Clinical follow up studies of larger numbers of IPH patients should be performed to assess the prognosis, facilitate the development of optimal treatment strategies and determine the place of IPH in current PID classification systems.

AUTHORSHIP AND DISCLOSURES

The authors' contributions are: GJD, MvdB designed the research; VD, PMvH, NGH, MvR, AW, AvR, EdV contributed clinical data and provided material necessary for performing experiments; JJMvD and MvZ provided conceptual advice; BHB, SP, IP performed and interpreted the experiments; HAG and GJD analyzed the data; GJD and MvdB wrote the manuscript; and VD, PMH, NGH, AW, AvR, EdV, MvZ and JJMvD commented on the manuscript. MvdB and GJD received an unrestricted research grant from Baxter.

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SUPPLEMENT

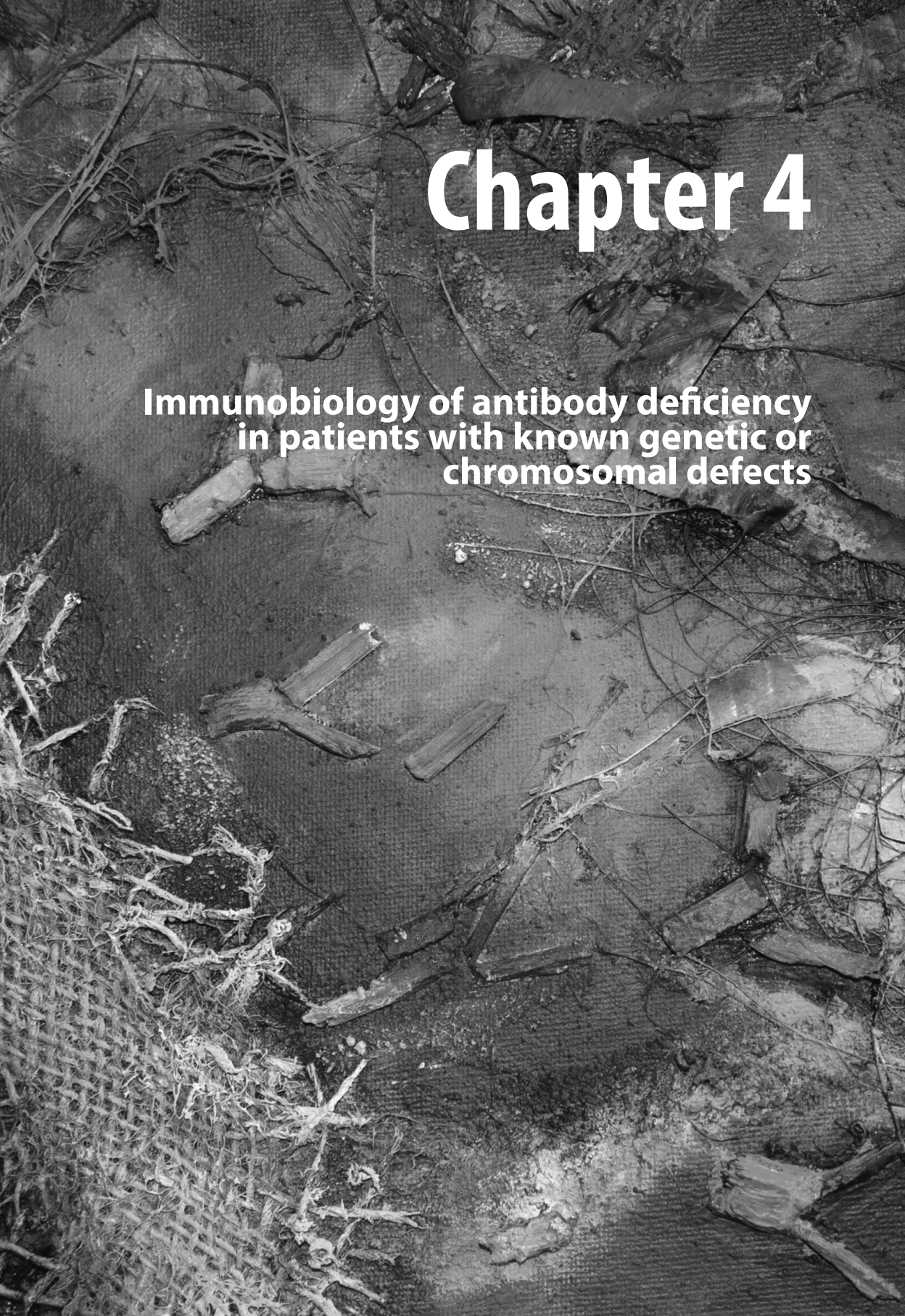
Supplemental Table 1. Age related normal values of immunoglobulin levels*

Age group	IgM (g/L)	IgG (g/L)	IgA (g/L)
newborn	0,06–0,3	6,1–15,4	0,01–0,04
3-6 months	0,3–1,0	2,5–5,6	0,05–0,5
6 months-1 year	0,3–1,0	2,5–6,7	0,08–0,7
1-2 year	0,4–1,7	3,3–11,6	0,1–1,0
2–6 year	0,5–1,8	4,0–11,0	0,1–1,6
7–12 year	0,5–2,0	6,0–12,3	0,3–2,0
Adults	0,4–2,3	7,0–16,0	0,7–4,0

	IgG1	IgG2	IgG3	IgG4
0-1 month	2,4-10,6	0,8-4,1	0,14-0,55	0,04-0,6
1-6 month	1,8-7,0	0,4-2,1	0,14-0,70	<0,03-0,4
6 month-2 years	2,2-8,2	0,4-2,4	0,15-1,0	<0,03-0,6
2-7 years	3,5-10,0	0,6-3,5	0,14-1,3	<0,03-1,2
7-18 years	3,8-10,0	0,9-5,0	0,15-1,5	<0,03-2,1

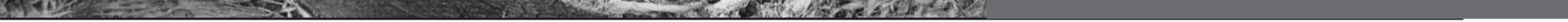
*adapted from Vries E de, Kuijpers TW, Tol MJD van, et al. Ned Tijdschr Geneeskd 2000;144:2197-203

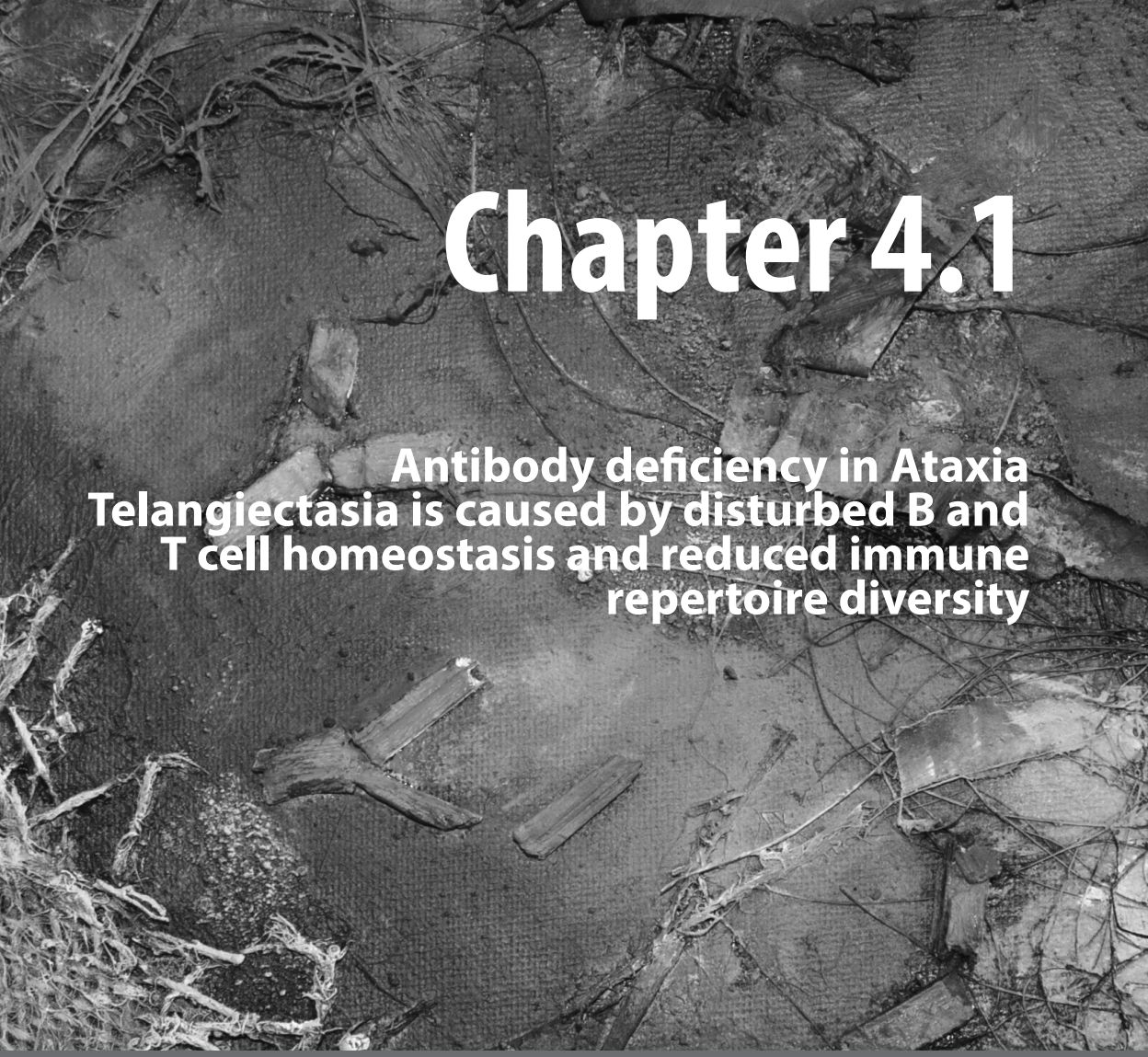

3.2



Chapter 4

Immunobiology of antibody deficiency in patients with known genetic or chromosomal defects





Chapter 4.1

Antibody deficiency in Ataxia Telangiectasia is caused by disturbed B and T cell homeostasis and reduced immune repertoire diversity

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J Allergy Clin Immunol. 2013;131(5):1367-75.e9

ABSTRACT

Background. Ataxia Telangiectasia (AT) is a multisystem DNA-repair disorder caused by mutations in the *ATM* gene. AT patients have reduced B- and T-cell numbers and a highly variable immunodeficiency. ATM is important for V(D)J recombination and immunoglobulin class switch recombination (CSR), however, little is known about the mechanisms resulting in antibody deficiency severity. *Objective.* To examine the immunological mechanisms responsible for antibody deficiency heterogeneity in AT. *Methods.* In this study, we included patients with classical AT plus early onset hypogammaglobulinemia (n=3); classical AT (n=8); and variant AT (late onset; n=4). We studied peripheral B- and T-cell subsets, B-cell subset replication history, somatic hypermutation frequencies, CSR patterns, B-cell repertoire and ATM kinase activity. *Results.* Classical AT patients lacked ATM kinase activity, while variant AT patients showed residual function. Most patients had disturbed naive B-cell and T-cell homeostasis as evidenced by low cell numbers, increased proliferation, a large proportion $CD21^{low}CD38^{low}$ anergic B cells and decreased antigen receptor repertoire diversity. Impaired formation of T-cell dependent memory B-cells was predominantly found in AT plus hypogammaglobulinemia. These patients had extremely low naive CD4+ T-cell counts, which were more severely reduced compared to classical AT patients without hypogammaglobulinemia. Finally, AT deficiency resulted in defective CSR to distal constant regions that might reflect impaired ability of B-cells to undergo multiple germinal center reactions.

Conclusion. The severity of the antibody deficiency in AT correlates with disturbances in B and T-cell homeostasis resulting in reduced immune repertoire diversity, which consequently affects the chance of successful antigen-dependent cognate B-T interaction.

INTRODUCTION

Ataxia Telangiectasia (AT) is an autosomal recessive multisystem disorder resulting from mutations in the *ATM* gene (Ataxia Telangiectasia Mutated). AT is characterized by cerebellar ataxia, oculocutanous teleangiectasias, radiosensitivity, chromosomal instability, a propensity for the developing (mainly hematological) malignancies, growth retardation and endocrine abnormalities.[1] Furthermore, AT has been recognized as a primary immunodeficiency.[2]

ATM is critically important for processes in lymphocyte development that rely on DSB repair,[3, 4] such as V(D)J recombination[5] and Class Switch Recombination (CSR)[6, 7] of immunoglobulin (Ig) genes. Similar to patients with the Nijmegen Breakage Syndrome (NBS),[8] a closely related DNA repair disorder, AT patients have low circulating B- and T-cell numbers. Considering the role of ATM in V(D)J recombination, this could be due to reduced numbers of precursor cells that are able to successfully rearrange their antigen receptor genes[8]. CSR depends on repair of DSBs at recombining Ig switch (S) regions.[9] ATM deficiency affects DSB recognition and/or repair during CSR and as a consequence alternative pathways of error-free joining are used.[6, 7, 10, 11]

Although the effects of *ATM* mutations on the V(D)J recombination and CSR processes have been studied in detail, little is known about the consequences of *ATM* mutations on the degree of immunodeficiency. Clinically, the immunodeficiency in AT is highly variable with a predominant antibody deficiency. Patients with early onset disease are referred to as classical AT.[12,13] A subset of patients with classical AT has a severe early onset hypogammaglobulinemia, reminiscent of a CSR deficiency.[14] Variant AT patients have a later onset and a less severe antibody deficiency.[12, 15]

To understand the immunological mechanisms responsible for AT disease heterogeneity, we analyzed the blood B- and T-cell compartments of 15 AT patients with different degrees of antibody deficiency severity extensively with flow cytometric and molecular analysis[16,17] Reminiscent of NBS, naive B and T cells showed extensive replication histories and a restricted antigen receptor repertoire, and disease severity was clearly correlated with numbers of circulating naive T cells.

MATERIALS AND METHODS

Patients

Peripheral blood samples and clinical data were collected from 15 patients with Ataxia Telangiectasia and 45 healthy age-matched controls. These studies were approved by the

Medical Ethics Committees of the Radboud University Nijmegen Medical Center and the Erasmus MC Rotterdam.

Flow cytometric analysis and high speed cell sorting of blood B cell subsets

Six-color flow cytometric immunophenotyping of peripheral blood was performed on a FACS LSRII (BD Biosciences) and data were analyzed using FACS Diva software (BD Biosciences) as described previously[16]. Memory B-cell subsets were characterized as described previously[16]. Naive mature B-cells were sorted from post-Ficoll mononuclear cells on a FACS AriaII (BD Biosciences) followed by DNA extraction with a direct lysis method.[18]

KREC and TREC assays to determine the replication history of B- and T-cells

The replication history of sorted B cell subsets was determined with the Kappa-deleting Recombination Excision Circles (KREC) assay as described previously.[17] The proliferation of T cells was measured by the γδTREC as previously described.[19]

Sequence analysis of complete IGH gene rearrangements

RNA was isolated from mononuclear cells using the GeneElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich). After reverse transcription using random hexamers, *IGA* and *IGG* transcripts were amplified as described previously.[20] Obtained sequences were analyzed with IMGT database (http://imgt.cines.fr/) and JoinSolver program (http://joinsolver.niaid.nih.gov).

ATM kinase activity

ATM kinase activity was measured as described previously.[21]

Repertoire analysis

VH-JH rearrangements were amplified from 200ng sorted naive B cells in a multiplex PCR using the Biomed-2 VH1-6 FR1 and JH consensus primers[22]. The primers were adapted for 454 sequencing by addition of an adaptor, the 'TCAG' key and multiplex identifier (MID). PCR products were purified by gel extraction (Qiagen, Valencia, CA) and Agencourt AMPure XP beads (Beckman Coulter). Subsequently, the concentration of the PCR product was measured using the Quant-it Picogreen dsDNA assay (Invitrogen, Carlsbad, CA). For every individual, 3 independent PCRs were performed and sequenced on the 454 GS junior instrument according the manufacturer's recommendations, using the GS junior Titanium emPCR kit (Lib-A), sequencing kit and PicoTiterPlate kit (454 Life Sciences, Roche, Brandford, CT). The sequences of >250bp were separated per individual based on the MID tag and trimmed based on quality score 0.05 in CLC genomic workbench software. The reads were exported in Fasta format and uploaded to IMGT High V-Quest[23]. From this

output, the number of unique junctions (as defined by *IGHV*, *IGHD* and *IGHJ* gene usage and CDR3 region) per PCR reaction was determined.

STATISTICS

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. Whenever multiple groups with continuous outcomes were compared, the non-parametric Kruskal-Wallis rank sum test was used, followed by pair wise Mann Whitney tests if the former indicated significant differences. For categorical variables the χ^2 or Fisher's exact tests were used. Correlation coefficients given are Spearman's. Statistical significance was set at two sided $P<0.05$.

RESULTS

Patients

Patient characteristics are summarized in Table 1. Genotype phenotype correlations of the patients (among others) have been reported elsewhere[15]. AT patients were divided in three groups: classical AT plus hypogammaglobulinemia (n=3), classical AT (n=8) and variant AT (n=4), i.e. patients with late onset. None of the classical AT patients showed ATM kinase activity, whereas patients with variant AT showed residual activity.

Patients with classical AT plus hypogammaglobulinemia were diagnosed with severe hypogammaglobulinemia (IgG levels <1 gr/L, Table 2) before the age of one year, before the diagnosis of AT was made. Patient AT1 and AT3 presented with recurrent infections. Patient AT2 was screened for hypogammaglobulinemia prior to the development of infections, because she was sibling of patient AT3. They were treated with immunoglobulin replacement therapy. In contrast, patients with classical AT had total IgG levels > 5 gr/L and normal IgG_1 levels. Total IgG levels were slightly decreased for age in only three cases and one of them received immunoglobulin replacement. Most classical AT patients suffered from an IgG_2 and/or IgA deficiency. None of them showed progression of the antibody deficiency over time.

Of the variant AT patients, only one showed a mild IgG_2 deficiency. Variant AT patients showed pneumoccal polysaccharide antibody levels (without booster vaccination) above protective level (0.35 µg/ml).

Table 1. Characteristics of patients with Ataxia Telangiectasia

Patient	Sex	Age*	*ATM* mutation		ATM activity	Age of onset ataxia	Tele-angiect.	Weel-chair bound Age	Infections	IVIG
			allele 1	allele 2						
Classical hypogamma										
AT1	F	13	c.2554 C>T	c.2554C>T	no	1.5	yes	10	URTI pneumonia	yes
AT2	F	8	c.5188 C>T	c.5188 C>T	no	1	yes	7	no	yes
AT3	F	13	c.5188 C>T	c.5188 C>T	no	1.5	yes	10	URTI	yes
Classical										
AT4	M	39	c.1563_ 1564delGA	unidentified	no	1	yes	8	no	no
AT5	M	8	c.6082 C>T	c.6082 C>	no	1.5	yes	9	no	no
AT6	M	13	c.484 C>T	c.1898+2 T>G	no	1	yes	10	URTI	no
AT7	M	10	c.7875_7876delTGinsGC	c.7875_7876delTGinsGC	no	1	yes	9	no	no
AT8	M	17	c.3741-1G>C	c.5197 G>C	no	1	yes	11	no	no
AT9	M	10	c.309 C>G	c.1369 C>T	no	1.5	yes	13	no	no
AT10	M	15	c.790_790delT	c.1563_1564delAG	no	1.5	yes	10	URTI	yes
AT11	F	17	c.3576 G>A	c.3576 G>A	no	5	yes	9	no	no
Variant										
AT12	F	51	c.2922-1G>A	c.8147 T>C	yes	39	minimal	no	no	no
AT13	F	37	c.331+5 G>A	c.331+5 G>A	yes	15	minimal	20	no	no
AT14	M	34	c.331+5 G>A	c.331+5 G>A	yes	15	no	21	no	no
AT15	F	35	c.5932 G>T	c.8147 T>C	yes	29	no	no	no	no

ND = not determined, URTI = recurrent upper respiratory tract infections, IVIG = intravenous immunoglobulin replacement

*Age at evaluation, ages are given in years

Low transitional B-cells and increased proliferation of naive mature B-cells in AT

To investigate the difference in severity of antibody deficiency in AT patients, detailed analysis of the lymphocyte subsets was performed. Absolute B-cell numbers were reduced in all classical AT patients with hypogammaglobulinemia patients, in 5/8 classical AT patients and in only 1/4 variant AT patients (Table 2).[24] 7/11 patients with classical AT (with or without hypogammaglobulinemia) showed a reduction of total T-cell numbers as well. NK-cell numbers were in the normal range for all patients.

ATM deficiency results in impaired DSB repair during V(D)J recombination,[25] potentially affecting B-cell production in the bone marrow explaining the reduced peripheral B-cell numbers. To study this, we quantified early emigrants from bone marrow, i.e. circulating transitional B-cells.[26] All AT patients showed reduced numbers of transitional B cells as compared to healthy controls, irrespective of residual ATM kinase activity (Figure 1A). Thus, bone marrow output or homeostasis of new emigrant B cells seemed affected in AT.

Naive mature B cells were reduced in 13/15 (87%) of AT patients (Figure 1A). To study whether the naive mature B-cells showed increased (compensatory) proliferation, the *in vivo* B-cell replication history was determined in sorted naive mature B-cells of 6 patients with classical and 3 with variant AT. Naive mature B-cell proliferation of classical AT patients was increased with a median of 5.1 cell divisions as compared to 1.8 in controls (Figure 1B). Subsequently, we studied $CD21^{low}CD38^{low}$ B-cells, which is a distinct B-cell population containing mostly autoreactive unresponsive clones that might represent anergic or innate-like B-cells.[27] In AT, the proportion of $CD21^{low}CD38^{low}$ B-cells was increased in all three AT groups (Figure 1C), most prominently in AT with hypogammaglobulinemia.

Reduced antigen receptor repertoire of naive mature B-cells in AT

Based on the reduced B-cell egress from bone marrow and increased proliferation of naive B-cells, a restricted B-cell repertoire was assumed. To address this issue, DNA was isolated from sorted naive B-cells and antigen receptor repertoire diversity was assessed by next generation sequencing of *IGH* gene rearrangements. Given that each newly generated B cell has a unique *IGH* gene rearrangement, amplification of identical sequences in independent PCR reactions (i.e. coincidences) would indicate restriction of the Ig repertoire.[28] Indeed, AT patients showed increased numbers of coinciding *IGH* sequences in independent PCR reactions of sorted naive B-cells as compared to controls (Table 3, Supplemental Table 2). Thus, on top of their reduced numbers, naive B-cells in AT showed a reduced antigen receptor repertoire diversity.

Decreased memory B-cells in AT

Six memory B-cell subsets were studied that are thought to derive from GC-dependent and –independent pathways[16] (Figure 2A). Patients with classical AT plus

Table 2. Lymphocyte subsets, immunoglobulin levels and specific antibodies

patient	Age at Evalu-ation	B-cells (cells/µL)	T-cells (cells/µL)	NK-cells (cells/ µL)	IgG (g/l)	IgA (g/l)	IgM (g/l)	IgG1 (g/l)	IgG2 (g/l)	IgG3 (g/l)	IgG4 (g/l)	Pneumo type 3 (µg/ml)	Pneumo type 4 (µg/ml)	Pneumo type 9 (µg/ml)
Classical hypogamma														
AT1	13	**70**	1,500	410	**0.3**	**< 0.07**	**0.17**	ND	ND	ND	ND	ND	ND	ND
AT2	8	**30**	**390**	370	**0.5**	**< 0.07**	2.2	ND	ND	ND	ND	ND	ND	ND
AT3	13	**10**	**650**	360	**0.18**	**< 0.07**	0.42	ND	ND	ND	ND	ND	ND	ND
Classical														
AT4	39	140	1,000	290	**6.13**	**< 0.07**	7.92	5.23	**< 0.17**	**<0.03**	<0.07	0.58	0.40	0.31
AT5	8	**140**	**630**	330	6.84	**< 0.07**	0.6	5.09	**0.69**	0.26	< 0.05	0.06	0.01	<0.01
AT6	13	**70**	**290**	210	**6.27**	0.6	1.44	5.11	**0.22**	0.32	< 0.05	<0.01	<0.01	<0.01
AT7	10	**160**	2,340	450	6.68	0.69	1.95	5.15	**0.26**	0.59	< 0.05	0.13	0.05	0.32
AT8	17	160	**640**	450	8.3	**< 0.07**	0.46	6.38	**0.21**	0.16	0.07	<0.01	<0.01	<0.01
AT9	10	**90**	**350**	540	11.7	**< 0.07**	1.26	12.5	**0.39**	0.21	0.06	ND	ND	ND
AT10	15	**90**	**760**	150	**5,78**	**< 0.07**	1.23	5,34	**<0,17**	0,19	ND	ND	ND	ND
AT11	17	180	1,001	280	10,1	1,14	1	7,05	1,46	0,36	<0,05	0.71	0.01	0.01
Variant														
AT12	51	110	820	530	13.2	1.76	2.58	8.54	2.74	0.66	0.27	1.97	1.6	2.05
AT13	37	150	940	210	8.71	2.63	2.3	7.31	**0.51**	0.28	< 0.05	1.54	0.59	0.41
AT14	34	110	920	180	9.76	2.07	2.91	6.84	2.32	0.24	0.58	ND	ND	ND
AT15	35	**70**	860	300	9.71	1.62	1.53	6.05	2.72	0.47	0.29	3.06	1.26	1.44

ND= not determined. Values of lymphocyte subsets and immunoglobulin levels marked in bold are below the age related normal value. For normal values of lymphocyte subsets see Comans-Bitter *et al.*[24] For normal values of immunoglobulin levels; Supplemental Table 1. Pneumo type = specific antibody level against pneumococcal serotype.

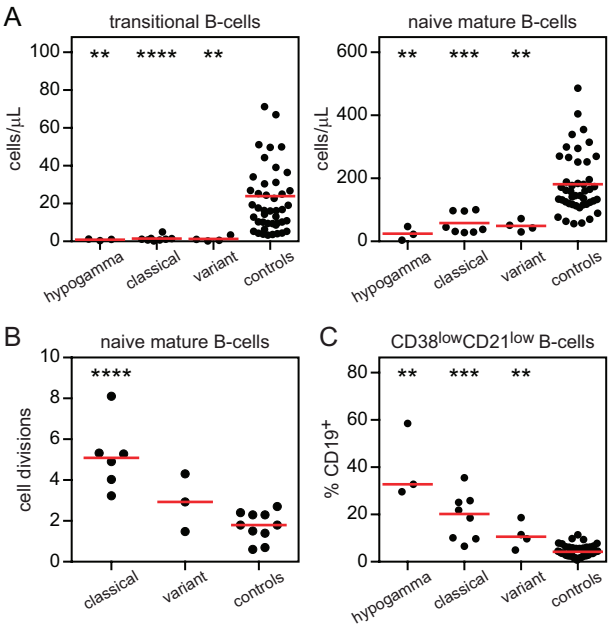

Figure 1. Naive and CD21^{low}CD38^{low} defects in Ataxia Telangiectasia. A. Absolute numbers of blood transitional B-cells (CD19+CD27^{-}CD24^{high}CD38^{high}) and naive mature B-cells (CD19+CD27^{-}CD24^{dim}CD38^{dim}) in three categories of AT patients. **B.** Naive B cell replication history as measured with the KREC assay**. C.** Proportions of CD21^{low}CD38^{low} B-cells. Data are compared to normal controls using the Mann-Whitney test. Individual data points are displayed and bars indicate medians. Significant values are indicated: ****, P<0.0001; ***, P=<0.0005; **, P<0.005; *, P<0.05.

hypogammaglobulinemia showed the most severe reduction of B-cell memory: all subsets were decreased, except for the T-cell independent CD27-IgA+ memory B-cells (Figure 2B). Patients with classical AT only displayed reduced CD27+IgA+ memory B-cells, whereas patients with variant AT only showed reduced CD27+IgG+ memory B-cells despite normal serum IgG levels. The relative distribution of the six memory B-cells (Figure 2C) shows that CD27+ class switched memory B-cells were most severely reduced in AT plus hypogammaglobulinemia.

Low naive CD4+ T-cell counts are associated with hypogammaglobulinemia and low memory B-cell numbers

To study whether T-cell abnormalities contributed to the reduced numbers of (germinal center derived) memory B-cells, we assessed blood T-cell subsets in our patients with flow

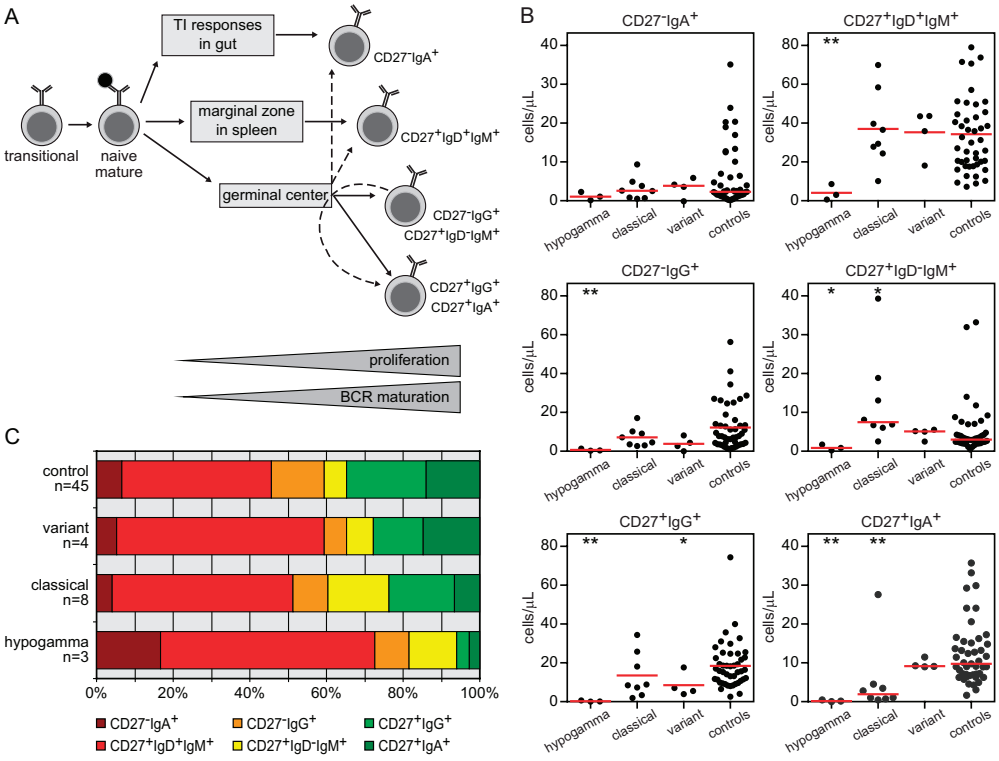

Figure 2. Memory B-cell subset distribution in AT. A. Memory B-cell subsets according to Berkowska et al Blood 2011[16] **B**. Absolute numbers of memory B-cell subsets in three categories of AT patients. **C**. Relative distributions of memory B-cell subsets. Data are compared to normal controls using the Mann-Whitney test. Significant values are indicated: ***, P<0.0005; **, P<0.005; *, P<0.05.

Table 3. B-cell repertoire analysis of *IGH* sequences of naive B-cells

	Total sequences	Coincidences		
		none	2	3
Control 1 (17 yr)	25,280	25212	34	0
Control 2 (10 yr)	22,429	22421	4	0
Control 3 (25 yr)	32,537	32537	0	0
AT15	22,043	21628	191	11
AT7	16,027	15779	124	0
AT11	12,463	12409	27	ND

ND = not determined. Each AT patients is different from each control by χ^2 test (all P<0.0001), except for AT11 compared to control 1 (P=0.06).

cytometic immunophenotyping. Total CD3+, CD4+ and CD8+ numbers were decreased as compared to controls, but did not differ significantly between the three groups of AT patients (Figure 3A and 3B). However, patients with classical AT plus hypogammaglobulinemia showed fewer naive CD4+ T-cells than patients with classical AT and variant AT (Figure 3A). Naive CD8+ cells were reduced in all three AT groups as compared to controls. Memory and effector CD4+ and CD8+ T-cells were not significantly differenct from controls (Figure 3A and 3B). The reduction of naive T-cell subsets was also apparent from the relative distribution of the subsets within the CD4+ and CD8+ compartments (Figure 3C). TRECs were decreased in patients with classical AT, indicative of decreased thymic output and/or increased peripheral T-cell proliferation (Figure 3D).

Because naive CD4+ T-cell numbers differed between the three groups of AT patients, we calculated the correlations between naive CD4+ T-cell counts, memory B-cell subset counts and immunoglobulin levels. Naive CD4+ T-cell counts strongly correlated with CD27+IgA+ memory B-cells subset counts ($r=0.93$, $P=0.001$), and with IgG_2 levels ($r=0.73$ $P=0.01$). These results indicate that low naive CD4+ T-cell counts are associated with poor memory B-cell formation and a more severe antibody deficiency.

Somatic hypermutation and Ig class switch recombination in ATM deficiency

The frequencies of SHM in *IGHG* and *IGHA* transcripts were not different from controls (Supplemental Figure 1A), and replacement mutations in rearranged *IGHV* genes were predominantly targeted to CDR regions (Supplemental Figure 1B). Thus, affinity maturation appeared to be normal in the (reduced number of) antigen-experienced B-cells.

To study Ig CSR in more detail, we determined the *IGHG* and *IGHA* subclass usage in amplified *IGH* transcripts (Figure 4A and 4B). In classical AT and AT plus hypogammaglobulinemia, the usage of downstream *IGHG* regions (*IGHG2* and *IGHG4*) was severely reduced as compared to controls (Figure 4B; $P<0.0001$ and $P=0.02$ respectively), suggesting that CSR to the more downstream *IGHG2* and *IGHG4* gene segments was impaired. Although variant AT also seemed to display reduced *IGHG2* and *IGHG4* used as compared to controls, the difference did not reach significance ($P=0.053$). Analysis of *IGHA* transcripts revealed that patients with classical AT and patients with variant AT showed significantly reduced usage of *IGHA2* transcripts ($P<0.0001$ and $P=0.006$, respectively). Only 12 *IGHG* transcripts and no *IGHA* transcripts could be amplified from patients with AT plus hypogammaglobulinemia, which prevented a meaningful analysis. In these patients, CSR to all Ig subclasses is likely decreased, because of the severely decreased number of class-switched memory B-cells and the profound hypogammaglobulinemia.

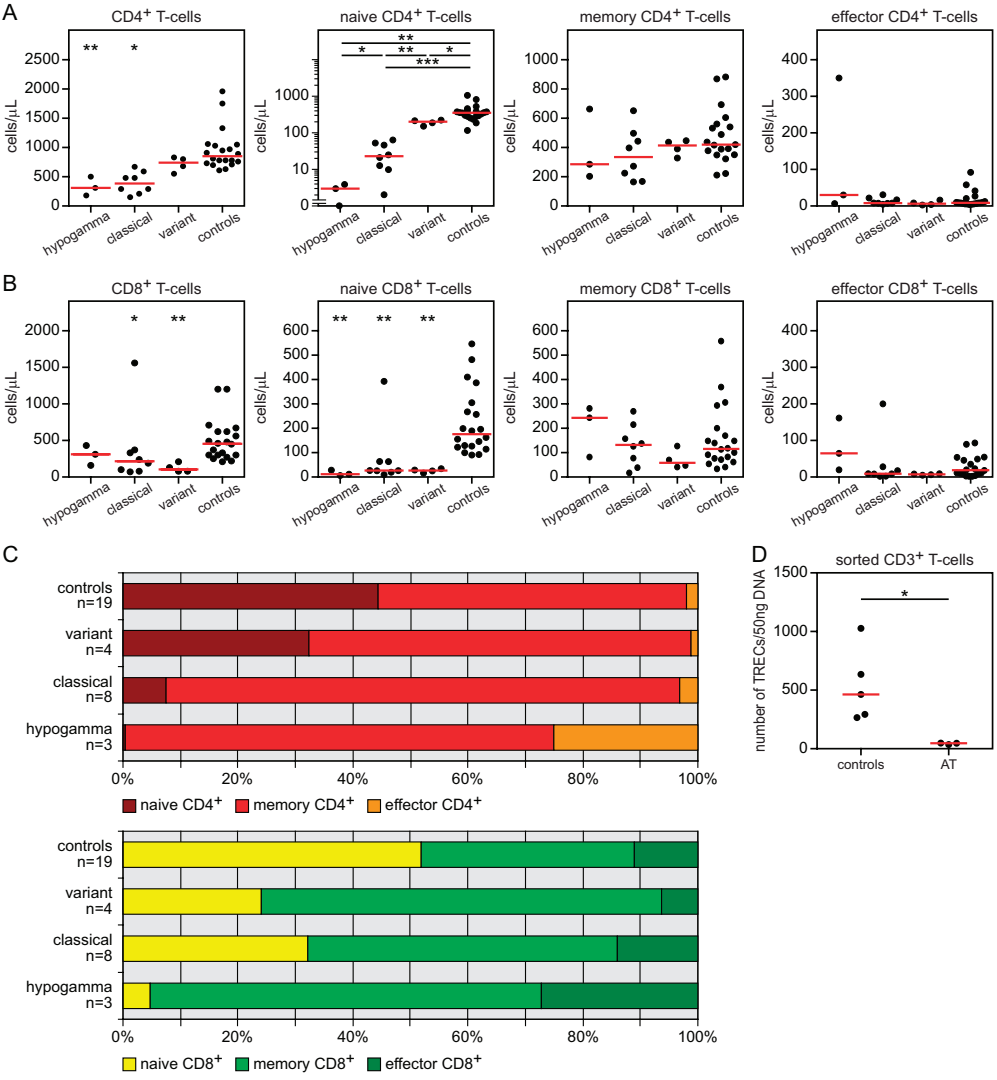

Figure 3. T-cell subset distribution in AT. Absolute numbers of CD4+ (**A**) and CD8+ T-cell subsets in three categories of AT patients **B**. **C**. Relative distributions of T-cell subsets **D**.TRECs in sorted T-cells. Naïve T-cells (CD45RA+CD27+); memory T-cells (CD45RA-CD27+) and effector T-cells (CD45RA+/-CD27-). Significant values are indicated: ***, P<0.0005; **, P<0.005; *, P<0.05.

DISCUSSION

In this study, we demonstrated that the antibody deficiency in AT is caused by disturbed naive B- and T-cell homeostasis leading to reduced immune repertoire formation and reduced memory B-cell formation. While these defects are present in all patients, three clinical subgroups can be defined, of which the disease severity correlated with circulating memory B cells and naive T cells.

Reduction of transitional and naive mature B-cell counts is the hallmark of abnormal naive B-cell homeostasis and was observed in all AT patients. This finding shows strong resemblance with reduced levels of naive mature B-cells in NBS patients.[29] We previously showed that in NBS the production of precursor B-cells in bone marrow is impaired due to loss of juxtaposition of RAG-induced immunoglobulin DNA ends, thereby obstruction DSB repair during V(D)J recombination.[29] In AT deficient mice, the DSB repair phase during V(D)J recombination is also impaired,[5] which is in concordance with the observed low transitional B-cell counts in AT patients. Despite increased proliferation, the number of naive B-cells is low, similar as found in NBS patients[29]. Increased proliferation could be a mechanism to compensate for decreased bone marrow output. Alternatively, it could be the result of lack of cell cycle control by ATM during V(D)J recombination. Irrespective of the mechanism of increased naive B-cell proliferation, it will result in a peripheral B-cell compartment with a restricted B-cell repertoire. We were able to confirm the decrease of naive B-cell repertoire by deep sequencing of *IGH* gene rearrangements. We recently described increased naive B-cell proliferation in a subgroup of CVID patients with a B-cell pattern similar to AT patients (low transitional and memory B-cells), which could therefore point to a DNA repair disorder.[29] An increase of $CD21^{low}CD38^{low}$ anergic B-cells was present in CVID patients with increased naive B-cell proliferation[29, 30] as well as in AT patients, indicating that increased proliferation of the naive B-cell compartment is associated with B-cell anergy.

Similar to the reduced naive B-cells, naive CD4+ T-cells and TREC levels were also reduced in AT. This was shown to result from reduced thymic output and a concomitant reduction of TRECs, increased proliferation and a consequently restricted TCR repertoire.[31] Recently, these findings were attributed to premature aging of the immune system.[32, 33] Both naive CD4+ and naive CD8+ T-cells were most severely decreased in patients with classical AT plus hypogammaglobulinemia, followed by classical AT and variant AT.

Despite reduced naive T-cell numbers, AT patients mainly suffer from an antibody deficiency and not from opportunistic infections related to T-cell deficiencies. We found normal numbers of circulating memory and effector CD4+ and CD8+ T-cells in all three AT categories. This indicates that peripheral T cells of AT patients have a normal terminal

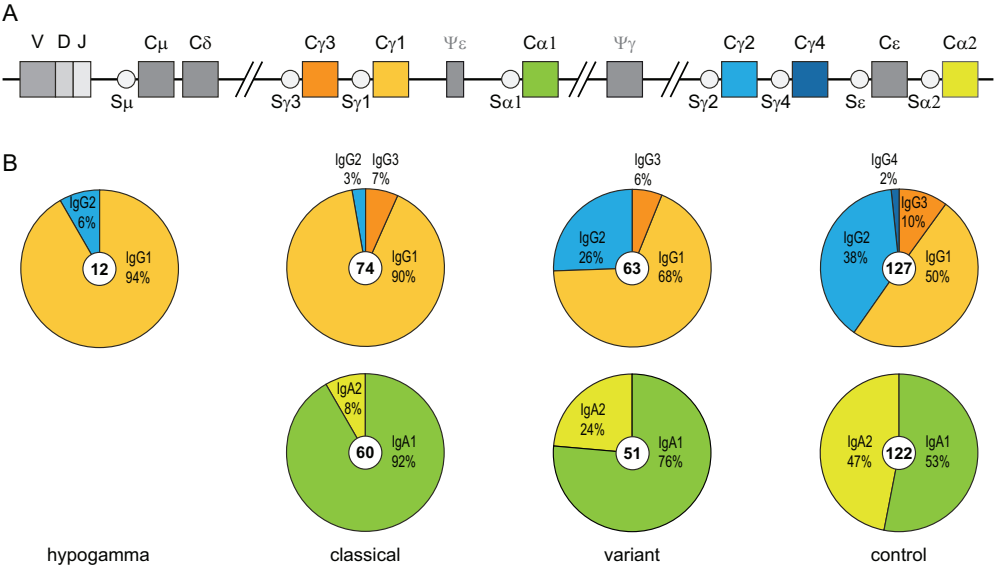

Figure 4. IgA and IgG class switching in *IGH* transcripts of AT patients. A. Schematic representation of the constant regions in the *IGH* locus. **B.** Frequencies of *IGHG2* and *4* and *IGHA2* transcripts in classical AT plus hypogammaglobulinemia, classical AT and variant AT were compared to controls (for details see text). In the center of each plot the number of analyzed transcripts is depicted.

differentiation upon antigenic stimulation without giving apparent clinical signs of a T-cell deficiency despite the low number of naive T-cells.

It is unknown why only part of the patients with classical AT have severe hypogammaglobulinemia, because they all lack ATM kinase activity. In this study, we showed that the absolute number of naive CD4+ T-cells and naive B-cells was significantly lower in AT plus hypogammaglobulinemia, implying a more severe V(D)J recombination defect. The currently used methods to measure ATM kinase activity assays might not be sensitive enough to detect low levels of residual ATM kinase activity or, alternatively, might not measure all ATM protein functions important for B and T-cell development, i.e. V(D)J recombination and CSR.

Memory B-cell formation was impaired in all AT patients. Five out of six memory B-cell subsets were decreased in classical AT plus hypogammaglobulinemia, whereas in classical AT and variant AT only T-cell dependent germinal center reactions were affected. These data suggest that naive CD4+ T-cells seem to play an important role in the severity of the antibody deficiency in AT, most probably by affecting T-cell dependent germinal center reactions. We hypothesize that due to the limited number and limited repertoire of both

(functional) naive mature B-cells and naive CD4+ T-cells the chance of an antigen-dependent cognate B-T interaction, which is required for initiation of a germinal center reaction, is decreased in AT. At this moment, we cannot rule out that also other intrinsic B-cell or T-cell factors contribute directly to memory B-cell formation.

It has been shown that ATM deficiency affects DSB recognition and/or repair during CSR.[6, 7] Sm-Sg junctions in AT patients have severely reduced mutations or insertions, indicating that the predominantly used error prone NHEJ pathway in CSR is impaired in AT patients.[11] However, the effect of the CSR deficiency on the subclass distribution of *IGH* constant genes has not been explored so far. In this study, we showed that the proportion of distal *IGHG2*, *IGHG4* and *IGHA2* constant regions was reduced. These findings are in line with studies in ATM-deficient mice that suggest a defect in joining of distant switch regions.[6] Frequently Ig CSR to distal constant genes occurs indirectly via an *IGH*-proximal gene. Berkowska et al.[16] showed that 24% of hybrid switch regions (Sμ-Sγ2) in genomic DNA of sorted populations of normal controls contained remnants of Sγ3, Sγ1, or Sα1, whereas only 9% of Sμ-Sγ1 junctions had Sγ3 remnants. In addition, *IGHG2* and *IGHG4* switch regions contain higher SHM loads, potentially reflecting multiple GC reactions. Based on these data, defective switch to distant constant regions in AT could be explained by an impaired ability of B-cells to undergo multiple successful GC responses, could point towards a role for ATM in the use of distal switch regions or both.

Patients with variant AT did not have a clinically apparent antibody deficiency, which is in line with earlier observations[15]. However, they still showed signs of a CSR deficiency at the molecular level, reminiscent of a sub-clinical antibody deficiency. We hypothesize that the subclinical antibody deficiency in variant AT might become clinically apparent with progressive ageing of the immune system.

In conclusion, AT patients have disturbed naive B-cell and T-cell homeostasis most likely due reduced B and T-cell production linked to disturbed V(D)J recombination and consequently have a limited B-cell and T-cell receptor repertoire. Therefore, the chance of successful initiation of a germinal center reaction is reduced leading to reduction of especially T-cell dependent memory B-cell populations. Consequently, AT patients suffer from an antibody deficiency with variable severity depending on the presence of residual ATM kinase activity and naive T-cell counts.

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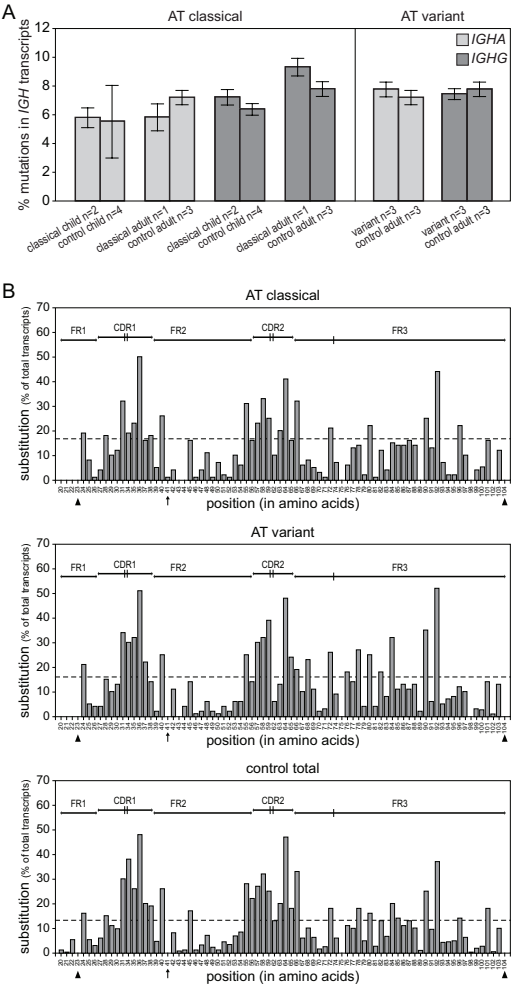
SUPPLEMENTS



Supplemental figure 1. Frequency of somatic hypermutations in IGHA and IGHG transcripts A. SHM in IGHGA and IGHG transcripts in classical and variant AT; children and adult compared to age matched controls. Data are compared with the Mann Whitney test. Significant values are indicated *** P=<0,0005 ** P<0,005 * P<0.05. B. Distribution of replacement mutation substitutions in rearranged IGHV genes in classical AT, variant AT and controls. CDR: complementarity determining region, FR: framework region.

Supplemental Table 1. Normal value of immunoglobulin levels

Age	IgA (g/L)	IgM (g/L)	IgG (g/L)	IgG1 (g/L)	IgG2 (g/L)	IgG3 (g/L)	IgG4 (g/L)
7-12 year	0.3-2.0	0.5-2.0	6.0-12.3	3.8-10.0	0.9-5.0	0.15-1.5	<0.03-2.1
>12 year	0.70-4.0	0.4-2.3	7.0-16.0	3.8-10.0	0.9-5.0	0.15-1.5	<0.03-2.1

Based on de Vries E, Kuijpers TW, Tol MJD van et al. Ned Tijdschr Geneeskd 200;144:2197-203

Supplemental Table 2. Sequences of coincidences

VH	DH	JH	CDR3 (aa)	JUNCTION
controle 1				
IGHV4-30-2*01	IGHD3-3*01	IGHJ6*02	17	tgtgccagagtagtatcggatttttggagcggcgagttgtacggtatggacgtctgg
IGHV1-69*06	IGHD1-26*01	IGHJ6*02	17	tgtgcccggggagtgggagcccatgggtcatactactactacggtatggacgtctgg
IGHV4-30-2*01	IGHD3-3*01	IGHJ6*02	15	tgtgccggattttggagtggttcctactactactacggtatggacgtctgg
IGHV3-23*01	IGHD6-13*01	IGHJ4*02	14	tgtgcgaaagattcccatagcagcagcttcacttattttgactactgg
IGHV3-30*03	IGHD2-15*01	IGHJ6*02	17	tgtgcgaaagattttagtgggcggaccacgcgctactactacggtatggacgtctgg
IGHV1-2*04	IGHD3-3*01	IGHJ6*02	22	tgtgcgaaagcggcccgccccaaacacaacgtattacgctttttggagtggcctcacggtatg-gacgtctgg
IGHV3-30*03	IGHD4-11*01	IGHJ4*02	12	tgtgcgaaaggcgactacggaaataggtactttgagtattgg
IGHV3-23*01	IGHD3-22*01	IGHJ3*02	14	tgtgcgaaagggtgtagtggttatttccttgatggttttgatatctgg
IGHV3-30-3*01	IGHD2-8*01	IGHJ6*02	24	tgtgcgagaccccacatcaaggatattgtactaatggtgtatgccaggcacccattggcgtacg-gtatggacgtctgg
IGHV4-59*08	IGHD6-19*01	IGHJ4*02	18	tgtgcgagactagtttttcgtggcagtggctggtataggggggcgctactttgactactgg
IGHV1-69*06	IGHD2-2*01	IGHJ6*02	20	tgtgcgagagaactggtagtaccagctgctccggcttactactactacggtatggacgtctgg
IGHV1-18*01	IGHD6-13*01	IGHJ4*02	12	tgtgcgagagaagagatagcagcagctgaagctgactactgg
IGHV4-34*01	IGHD5-18*01	IGHJ6*02	22	tgtgcgagagaagtggactacagctatggtaagacggggccccgttactactactacggtatg-gacgtctgg
IGHV1-18*01	IGHD6-6*01	IGHJ6*02	15	tgtgcgagagacccgtgggtagcagctcgccactacggtatggacgtctgg
IGHV1-18*01	IGHD3-16*01	IGHJ6*02	16	tgtgcgagagacgcaattactttcggttactactactacggtatggacgtctgg
IGHV1-18*01		IGHJ6*02	7	tgtgcgagagacggtatggacgtctgg
IGHV1-2*04	IGHD3-3*01	IGHJ6*02	24	tgtgcgagagagagaatgaattcgaggggaatttttggagtggttatgggctctgtaggctacg-gtatggacgtctgg
IGHV4-39*07	IGHD3-16*01	IGHJ6*02	13	tgtgcgagagagggattaggttatcactacggtatggacgtctgg
IGHV1-2*04	IGHD3-22*01	IGHJ4*02	11	tgtgcgagagagtccccaggctattactatgatagctgg
IGHV4-39*07	IGHD5-12*01	IGHJ4*02	11	tgtgcgagagatcatgatatagtggctacgaaccactgg
IGHV3-7*03	IGHD3-10*01	IGHJ4*02	11	tgtgcgagagattggcggttcggggagttgaattactgg
IGHV4-59*01	IGHD3-22*01	IGHJ5*02	18	tgtgcgagagatttgaggtattactatgatagtaccaccgggtggtggttcgacccctgg
IGHV3-11*03	IGHD2-15*01	IGHJ6*02	16	tgtgcgagagcctatgtcggcatggtggctgcgacctacggtatggacgtctgg
IGHV4-34*01	IGHD1-26*01	IGHJ4*02	12	tgtgcgagaggccggagatactatgatatgattgactactgg
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IGHV4-59*08	IGHD1-14*01	IGHJ4*02	11	tgtgcgagaggggcggacccctggtactttgactactgg
IGHV4-39*07	IGHD6-6*01	IGHJ6*02	15	tgtgcgagaggttcagctcgtccttgggtctactacggtatggacgtctgg
IGHV4-59*01	IGHD5-24*01	IGHJ4*02	16	tgtgcgagatggacacctcgacggatggctacaattacgggacttgactactgg
IGHV4-39*01	IGHD4-17*01	IGHJ5*02	11	tgtgcgagccctacggagtacaactggttcgacccctgg
IGHV4-34*01	IGHD6-13*01	IGHJ6*02	16	tgtgcgagccttgggtttagcagcccctactactactacggtatggacgtctgg
IGHV3-7*03	IGHD3-16*01	IGHJ6*02	15	tgtgcgaggcttcgacccgatgactactactactacggtatggacgtctgg
IGHV4-39*07	IGHD6-6*01	IGHJ6*02	13	tgtgcgagggtcccccaagaatactactacggtatggacgtctgg
IGHV4-39*07	IGHD6-19*01	IGHJ4*02	13	tgtgcgctagcagtggctggcccgaacttggcctttgactactgg
IGHV3-23*01	IGHD5-24*01	IGHJ4*02	11	tgtgcgtctgggggagatggctacaatcttgactactgg

VH	DH	JH	CDR3 (aa)	JUNCTION
Controle 2				
IGHV3-23*01	IGHD4-17*01	IGHJ5*01	11	tgtgcgaaagatcctcacgactacggtgacggctactgg
IGHV4-34*01	IGHD2-2*01	IGHJ1*01	18	tgtgcgagagcggccgtggtatattgtagtagtaccaactgcggatacttccagcactgg
IGHV4-59*01	IGHD3-10*01	IGHJ5*02	14	tgtgcgagagtaaatagggttcggggattttactggttcgacccctgg
IGHV4-34*03	IGHD2-21*02	IGHJ6*03	15	tgtgcgagagtcactgctcgttactactactactactacatggacgtctgg
AT15				
IGHV3-21*01	IGHD3-9*01	IGHJ4*02	10	tgcgcgagttccgataattacttccttgactcctgg
IGHV3-15*01	IGHD3-22*01	IGHJ5*02	19	tgtaccacggtaccgtattactatgatagtttccgaacctgggacaactggttcgacccctgg
IGHV3-64*01	IGHD3-22*01	IGHJ3*02	22	tgtacgagagacttaaagctccaaagcgatagtagtgggagctactcccagtcacatggttttgatatctgg
IGHV3-7*01	IGHD6-13*01	IGHJ4*02	11	tgtacgagagagggtataacagcaggggcggactactgg
IGHV3-7*01	IGHD5-18*01	IGHJ4*02	16	tgtacgagggacgtggatacagctccccaggtttgtccctactttgactactgg
IGHV1-46*01		IGHJ3*02	8	tgtactagaggaaatgcttttgatatctgg
IGHV3-73*01	IGHD3-16*02	IGHJ5*02	17	tgtactagcagatactacattacgtttgggggagttatctaccggttcgacccctgg
IGHV3-9*01	IGHD3-10*01	IGHJ4*02	13	tgtgcaaaagatttctccacctcgttcggggagttaagagattgg
IGHV3-9*01	IGHD3-9*01	IGHJ4*02	13	tgtgcaaaagccaatcttggctgggggggattactttgactactgg
IGHV3-9*01	IGHD4-23*01	IGHJ4*02	14	tgtgcaaaagccctgggggggtaactacataggccccttggactactgg
IGHV6-1*01	IGHD6-13*01	IGHJ3*02	20	tgtgcaagagagaacctcccgggtatagcagcagctggtacccccaatgatgcttttgatatctgg
IGHV6-1*01	IGHD6-13*01	IGHJ4*02	14	tgtgcaagagagcccgatagcagcagcgggactctctttgactactgg
IGHV6-1*01	IGHD5-24*01	IGHJ4*02	11	tgtgcaagagagggatggctacaaagctttgactactgg
IGHV6-1*01	IGHD6-19*01	IGHJ6*02	16	tgtgcaagagagtcaggtagcagtggcttttctccctacggtatggacgtctgg
IGHV6-1*01	IGHD5-12*01	IGHJ4*02	9	tgtgcaagagatcgatcctactttgactactgg
IGHV3-74*01	IGHD2-15*01	IGHJ4*02	13	tgtgcaagagatcggacctggggtagctcctactttgactactgg
IGHV6-1*01	IGHD6-6*01	IGHJ6*03	18	tgtgcaagagatcgggagtatagcagctcgacttactactactactacatggacgtctgg
IGHV6-1*01	IGHD6-13*01	IGHJ4*02	13	tgtgcaagagatctatcagcagcagctgtcaggtttgactactgg
IGHV6-1*01	IGHD1-1*01	IGHJ3*02	16	tgtgcaagagatcttatggactggaacgacgttgagagtgcttttgatatctgg
IGHV6-1*01	IGHD1-14*01	IGHJ4*02	15	tgtgcaagagattactccccggaaccacgaaggtattactttgactactgg
IGHV3-74*01	IGHD3-3*01	IGHJ3*02	10	tgtgcaagagatttttctgatgcttttgatatctgg
IGHV6-1*01	IGHD3-16*01	IGHJ6*02	14	tgtgcaagagctcggtcgggggtctactactacggtatggacgtctgg
IGHV3-74*01	IGHD3-10*01	IGHJ4*02	20	tgtgcaagaggagactatggttcggggagttattataacgatgcctccggaactttgactactgg
IGHV3-74*01	IGHD6-13*01	IGHJ4*02	9	tgtgcaagagggagcagctggattacctactgg
IGHV3-74*01	IGHD6-19*01	IGHJ4*02	14	tgtgcaagagggggggtatagcagtggctggtaccactttgactactgg
IGHV6-1*01	IGHD1-26*01	IGHJ5*02	13	tgtgcaagagggggtgggagctactacggctggttcgacccctgg
IGHV6-1*01	IGHD1-1*01	IGHJ6*02	8	tgtgcaagaggtaccggtatggacgtctgg
IGHV3-13*01	IGHD3-22*01	IGHJ6*02	19	tgtgcaagaggtagtagtggttattaccctaattactactactactacggtatggacgtctgg
IGHV6-1*01	IGHD1-1*01	IGHJ3*02	17	tgtgcaagagtaaatgggtacaactggaactacccgccaggggctttgatatctgg
IGHV3-74*01	IGHD3-22*01	IGHJ3*02	17	tgtgcaagagtcggtagtgggtattactatgatactaagggtgcttttgatatctgg

VH	DH	JH	CDR3 (aa)	JUNCTION
AT15				
IGHV6-1*01	IGHD2-15*01	IGHJ4*02	14	tgtgcaagattggtggcaggggaccttcgtactactttgactactgg
IGHV3-74*01		IGHJ6*02	10	tgtgcaagcgactacgcctacggtatggacgtctgg
IGHV3-74*01	IGHD4-17*01	IGHJ2*01	15	tgtgcaaggggagggactacggtgactgggctgtggtacttcgatctctgg
IGHV6-1*01	IGHD3-3*01	IGHJ4*02	11	tgtgcaagtgggtcggacccccggtcattatactattgg
IGHV3-33*01	IGHD5-24*01	IGHJ4*02	9	tgtgcaattctacaaggattctttgactattgg
IGHV4-59*01	IGHD4-11*01	IGHJ1*01	11	tgtgcacgatacacgaacgctgaatacttccagcactgg
IGHV3-21*01	IGHD6-13*01	IGHJ5*02	15	tgtgccaggaagcagcagctggtaaaaagaggagactggttcgacccctgg
IGHV4-34*01	IGHD5-18*01	IGHJ4*02	19	tgtgcccgaagcgggaaggggctatggttaggggcctcgggtcggtcctactttgaccactgg
IGHV3-66*02	IGHD3-3*01	IGHJ6*03	12	tgtgcccgatattactactactactacatggacgtctgg
IGHV3-23*01	IGHD5-18*01	IGHJ3*02	15	tgtgcgaaagaagatacagctatggttacaaatgatgcttttgatatctgg
IGHV3-23*01	IGHD1-1*01	IGHJ4*02	12	tgtgcgaaagacccccaagaggagtactactttgactactgg
IGHV3-23*01	IGHD6-19*01	IGHJ6*02	16	tgtgcgaaagacggcagtggccattactactactactacggtatggacgtctgg
IGHV3-53*01	IGHD5-18*01	IGHJ3*02	11	tgtgcgaaagacgggatacatgatgcttttgatatctgg
IGHV3-30*03		IGHJ4*02	7	tgtgcgaaagacgtctttgactactgg
IGHV3-23*01	IGHD3-10*01	IGHJ1*01	14	tgtgcgaaagagggtgaggggtccgaccttgaatacttccagcactgg
IGHV3-23*01	IGHD3-22*01	IGHJ4*02	14	tgtgcgaaagataaacaggactatgatagtagtccaattgactactgg
IGHV3-23*01	IGHD1-26*01	IGHJ3*01	10	tgtgcgaaagataacagtgggagctacgggcactgg
IGHV3-23*01	IGHD3-10*01	IGHJ5*02	18	tgtgcgaaagatccgtccgccttattactatggttcggggagggggtggttcgacccctgg
IGHV3-30*03	IGHD1-7*01	IGHJ6*02	17	tgtgcgaaagatcgcgggtataactggaactacgacgggtacggtatggacgtctgg
IGHV3-30*03	IGHD3-10*01	IGHJ6*02	19	tgtgcgaaagatcggagttcggggagttattatagccttacgaggtacggtatggacgtctgg
IGHV3-30*03	IGHD1-20*01	IGHJ6*02	15	tgtgcgaaagatcgggcgctgtataactggaactacggtatggacgtctgg
IGHV3-23*01	IGHD6-25*01	IGHJ6*02	15	tgtgcgaaagatctcctgcggcggcactactactacggtatggacgtctgg
IGHV3-23*01	IGHD3-3*01	IGHJ6*03	21	tgtgcgaaagatgcgcggtcctacgatttttggagtggttatcactactactactacatggacgtctgg
IGHV3-23*01	IGHD2-15*01	IGHJ4*02	13	tgtgcgaaagattgggagcagcttttgtactactttgactactgg
IGHV3-30*03	IGHD6-19*01	IGHJ4*02	14	tgtgcgaaagattgggggatgggcagtggtactactttgactactgg
IGHV3-23*01	IGHD6-13*01	IGHJ4*02	17	tgtgcgaaagatttgacactccttagcagcagacccctttactactttgacttctgg
IGHV3-23*01	IGHD2-2*01	IGHJ4*02	10	tgtgcgaaagcaggcccccagtactttgactactgg
IGHV3-30*03	IGHD3-22*01	IGHJ1*01	17	tgtgcgaaagcgggctactatgatagtagtggttattggaggtacttccagcactgg
IGHV3-23*01	IGHD3-10*01	IGHJ4*02	19	tgtgcgaaaggtcggcccctaggggtcctatggttcgggagtcaagactactttgactactgg
IGHV3-23*01	IGHD5-18*01	IGHJ4*02	15	tgtgcgaaagtcaccttggttggatacaactatggttgttttgactactgg
IGHV3-23*01	IGHD3-22*01	IGHJ3*02	21	tgtgcgaaatacaggcaatggaattactatgatagtagtgcttattacacggatgcttttgatatctgg
IGHV3-23*01	IGHD6-6*01	IGHJ4*02	14	tgtgcgaaatccctaactgcagctcgtccgaactactttgactactgg
IGHV3-23*01	IGHD1-26*01	IGHJ6*03	21	tgtgcgaacggccaagacgggggggcagtgggagctactattctctactactactacatggacgtctgg
IGHV3-66*01	IGHD6-13*01	IGHJ4*02	10	tgtgcgaaggggatatagcagcagctgcgcggtgg
IGHV3-23*01	IGHD6-19*01	IGHJ4*02	16	tgtgcgaatctatcacccgggtatagcagtggctgggaagactttgactactgg

VH	DH	JH	CDR3 (aa)	JUNCTION
AT15				
IGHV4-59*08	IGHD3-10*01	IGHJ6*03	17	tgtgcgactaggggaggggggagccccctactactactactacatggacgtctgg
IGHV3-23*01	IGHD2-8*01	IGHJ4*02	19	tgtgcgagaaacggacgttgggatattgtactaatggtgtatgctctccctttgggctactgg
IGHV3-66*01	IGHD6-13*01	IGHJ6*02	17	tgtgcgagagatcggatagcagcagctggtaccgactactacggtatggacgtctgg
IGHV3-21*01	IGHD1-14*01	IGHJ6*02	16	tgtgcgagagatcgggaccgccattactactactactacggtatggacgtctgg
IGHV3-7*01	IGHD3-16*02	IGHJ4*02	20	tgtgcgagagatctcgatgattacgtttgggggagttatcgttacccaagccccttttgactactgg
IGHV3-48*01	IGHD5-12*01	IGHJ4*02	13	tgtgcgagagatctcggatatagtggctacgattacggctactgg
IGHV1-18*01	IGHD3-22*01	IGHJ5*01	17	tgtgcgagagatctctatgatagtagtggtcgtatatatacccccgaccgtctactgg
IGHV4-59*01	IGHD3-22*01	IGHJ3*02	13	tgtgcgagagatgccctagtggttggaaatgcttttgatatctgg
IGHV3-48*01	IGHD2-21*01	IGHJ6*02	19	tgtgcgagagatggaggcgaggggaccgaggtagactactactactacggtatggacgtctgg
IGHV3-21*01	IGHD2-15*01	IGHJ4*02	20	tgtgcgagagatgggtggttttgtagtggtggtccctgccaccgtctatactactttgactactgg
IGHV3-21*01	IGHD3-9*01	IGHJ4*02	16	tgtgcgagagattacgatattctgactgctaattcatactactttgactactgg
IGHV1-18*01	IGHD3-22*01	IGHJ1*01	14	tgtgcgagagattactactatgatagtagtggttatccccattactgg
IGHV4-59*01	IGHD5-12*01	IGHJ6*03	18	tgtgcgagagattcaaggggctccggttattactactactactacatggacgtctgg
IGHV3-48*01	IGHD3-10*01	IGHJ4*02	8	tgtgcgagagattcgcggggtgactactgg
IGHV3-21*01	IGHD3-22*01	IGHJ3*02	22	tgtgcgagagattcggcttattactatgatagtagtggttatcaaagagggttcgatgcttttgatatctgg
IGHV4-59*01	IGHD6-19*01	IGHJ4*02	16	tgtgcgagagattggagcagtggctggaccccgcggtactactttgactactgg
IGHV4-34*01	IGHD4-17*01	IGHJ4*02	14	tgtgcgagagcaaacccagactacggtgatgaaatgcttgactactgg
IGHV3-21*01	IGHD6-19*01	IGHJ4*02	15	tgtgcgagagcagagagtggctggtaccagaagtactactttgactactgg
IGHV4-4*07	IGHD5-18*01	IGHJ3*02	19	tgtgcgagagcattcgcggatacaactatggttttatcgggccttttatgcttttgatatctgg
IGHV1-3*01	IGHD2-21*01	IGHJ4*02	14	tgtgcgagagctctgggggtgaactcaccgtactactttgactactgg
IGHV3-11*01	IGHD5-18*01	IGHJ4*02	12	tgtgcgagagctgtaagtggatacagctatgttaactactgg
IGHV4-59*01	IGHD3-10*01	IGHJ5*02	19	tgtgcgagaggaagtagggttacctattactatggttgggggtgtggccagttcgacccctgg
IGHV1-2*04	IGHD6-6*01	IGHJ6*03	15	tgtgcgagaggacagcagctcgcctactactactactacatggacgtctgg
IGHV4-34*01	IGHD4-17*01	IGHJ5*02	16	tgtgcgagaggacgaaccgcctctacggtgaagcgactgtggttcgacccctgg
IGHV3-23*01	IGHD6-19*01	IGHJ5*02	11	tgtgcgagaggaggatggtacaactggttcgacccctgg
IGHV3-7*01	IGHD6-6*01	IGHJ6*02	16	tgtgcgagaggaggcccaacccgttactactactactacggtatggacgtctgg
IGHV4-34*01	IGHD3-10*01	IGHJ5*02	21	tgtgcgagaggcaaagtagggtcacgtattactatggttcggggagttatttcctccttcgacccctgg
IGHV4-34*01	IGHD5-18*01	IGHJ4*02	11	tgtgcgagaggcacagatacaactacgggagtctactgg
IGHV4-34*01	IGHD2-2*01	IGHJ4*02	20	tgtgcgagaggcaggaccggatattgtagtagtaccagctgctatgttaccccatttgactactgg
IGHV4-34*01	IGHD1-26*01	IGHJ4*02	11	tgtgcgagaggcccccagtgggagcgaattgactactgg
IGHV4-34*01	IGHD3-3*01	IGHJ4*02	21	tgtgcgagaggcccgccgcctaagtattacgatttttggagtggttattattcctactttgactactgg
IGHV4-34*01	IGHD6-13*01	IGHJ4*02	11	tgtgcgagaggccgaagtaagctggaacttgactactgg
IGHV4-34*01	IGHD6-13*01	IGHJ5*02	15	tgtgcgagaggccgatatagcagcaggggagggaactggttcgacccctgg
IGHV4-34*01	IGHD4-17*01	IGHJ4*02	13	tgtgcgagaggccgtcacggtgactacgccgggtttgactactgg
IGHV4-34*01	IGHD4-11*01	IGHJ4*02	16	tgtgcgagaggcgtcatctacagtaacgatcgcaggtactactttgactactgg
IGHV1-8*01	IGHD3-9*01	IGHJ6*02	19	tgtgcgagaggctccgtattacgatattttgaccgctactactactacggtatggacgtctgg

VH	DH	JH	CDR3 (aa)	JUNCTION
AT15				
IGHV1-8*01	IGHD3-9*01	IGHJ6*02	19	tgtgcgagaggctccgtattacgatattttgaccgctactactactacggtatggacgtctgg
IGHV1-69*04	IGHD1-26*01	IGHJ4*02	8	tgtgcgagagggaactactttgactactgg
IGHV3-11*01	IGHD4-17*01	IGHJ6*02	19	tgtgcgagagggaccattgactacggagaatattactactactactacggtatggacgtctgg
IGHV3-66*02	IGHD3-16*02	IGHJ4*02	9	tgtgcgagaggggaattaccgtggactactgg
IGHV3-21*01	IGHD3-22*01	IGHJ4*02	9	tgtgcgagaggggttaactactttgactactgg
IGHV4-59*01	IGHD3-16*02	IGHJ3*02	9	tgtgcgagagggtcttttgcttttgatatctgg
IGHV3-7*01	IGHD2-15*01	IGHJ4*02	14	tgtgcgagagggtggtctggtggctccttgtactactttgactactgg
IGHV3-21*01	IGHD3-22*01	IGHJ4*02	16	tgtgcgagaggtaccctccctacgtattactatgatagtagtgtttactactgg
IGHV4-34*01	IGHD1-26*01	IGHJ4*02	11	tgtgcgagaggtagtgggagtcaaacttttgactactgg
IGHV3-20*01	IGHD3-9*01	IGHJ5*02	17	tgtgcgagaggtccaggcgatattttgactggttattacaactggttcgacccctgg
IGHV4-34*01	IGHD3-10*01	IGHJ5*02	19	tgtgcgagaggtgactatggttcggggagttatatacctcccgcgcggtggttcgacccctgg
IGHV3-21*01	IGHD2-21*01	IGHJ4*02	13	tgtgcgagaggtggggagggcgatggctactactttgactactgg
IGHV4-34*01	IGHD6-13*01	IGHJ4*02	20	tgtgcgagagtaatgtatagcagcagctggtacgccgtagccctcccgggctactttgactact-gg
IGHV4-34*01	IGHD5-18*01	IGHJ4*02	16	tgtgcgagagtgaggcgtggatacagctatggtggggtctactttgactactgg
IGHV4-34*01	IGHD5-24*01	IGHJ4*02	13	tgtgcgagagtggggatggctacaattactggtttggactactgg
IGHV3-23*01	IGHD4-17*01	IGHJ4*02	13	tgtgcgagagttcactacggtgacttcccgtactttgactactgg
IGHV3-48*03	IGHD3-22*01	IGHJ4*02	18	tgtgcgagagttcccccgagcgattattactatgatagtagtggttattctgactactgg
IGHV4-34*01	IGHD5-18*01	IGHJ4*02	17	tgtgcgagagttgggagaacgtacatacaactatggtcccgacctttgactactgg
IGHV3-48*01	IGHD6-19*01	IGHJ6*02	16	tgtgcgagagtttcgggctggtacggtcactactactacggtatggacgtctgg
IGHV4-b*01	IGHD1-26*01	IGHJ5*02	15	tgtgcgagatcggcgatagtgggagctagggtattctggttcgacccctgg
IGHV3-21*01	IGHD3-3*01	IGHJ4*02	23	tgtgcgagatctcaacgtattacgatttttggagtggttattatacggccaggtcagcggggct-ttgactactgg
IGHV5-51*01	IGHD6-13*01	IGHJ1*01	14	tgtgcgagatgtatagcagcagctggtacaggttacttccagcactgg
IGHV4-61*02	IGHD1-26*01	IGHJ3*02	13	tgtgcgagattggcgttgcgtgggaactatgcttttgatacctgg
IGHV4-59*01	IGHD4-17*01	IGHJ4*02	11	tgtgcgagatttcatgactacggtgacttcgccgactgg
IGHV3-21*01	IGHD3-10*01	IGHJ3*02	10	tgtgcgagcctttcgcgtgatgcttttgatatctgg
IGHV4-59*01	IGHD5-18*01	IGHJ6*03	19	tgtgcgagcggtggatacagctatggttacgactactactactactacatggacgtctgg
IGHV3-23*01	IGHD3-22*01	IGHJ4*02	14	tgtgcgagcgttgatagtagtggttattacttctactttgactactgg
IGHV3-21*01	IGHD3-16*01	IGHJ6*02	13	tgtgcgagctatggggaagactactactacggtatggacgtctgg
IGHV4-59*01	IGHD5-24*01	IGHJ4*02	13	tgtgcgaggagagagatggctatgattggatactttgactactgg
IGHV4-59*01	IGHD2-15*01	IGHJ5*02	17	tgtgcgaggagtcattgtagtgggagtagctgctacctcgactggttcgacccctgg
IGHV3-64*01	IGHD1-26*01	IGHJ3*02	15	tgtgcgaggcccctcagggtgggagctactcatgatgcttttgatatctgg
IGHV4-34*01	IGHD2-2*01	IGHJ6*03	14	tgtgcgagggtccccagttactactactactacatggacgtctgg
IGHV4-59*01	IGHD1-26*01	IGHJ6*02	11	tgtgcgagggtcggatactactacggtatggacgtctgg
IGHV4-34*01	IGHD3-10*01	IGHJ6*03	18	tgtgcgagggttcggggagttattattgggcgctactactactactacatggacgtctgg
IGHV4-59*01	IGHD4-17*01	IGHJ4*02	12	tgtgcgaggtcctactacggtgactactactttgactactgg
IGHV4-34*01	IGHD3-10*01	IGHJ4*02	11	tgtgcgaggttctcggacagctactactttgactactgg

VH	DH	JH	CDR3 (aa)	JUNCTION
AT15				
IGHV4-34*01	IGHD3-10*01	IGHJ4*02	11	tgtgcgaggttctcggacagctactactttgactactgg
IGHV1-2*02	IGHD6-19*01	IGHJ4*02	14	tgtgcgagtaccggagggccgcgggatagcagtggcgctcacgggtgg
IGHV4-39*01	IGHD1-1*01	IGHJ6*02	13	tgtgcgagtactggagactactactactacggtatggacgtctgg
IGHV3-33*01	IGHD4-17*01	IGHJ6*03	12	tgtgcgagttacggtgactactactactacatggacgtctgg
IGHV3-7*01	IGHD2-21*01	IGHJ4*02	14	tgtgcgagttccacccggggattcctcccagactactttgactactgg
IGHV1-8*01		IGHJ6*02	10	tgtgcgatttactactactacggtatggacgtctgg
IGHV3-30*03	IGHD3-9*01	IGHJ6*03	19	tgtgcgcgagaggcgggattacgatattttgactggttatactactactacatggacgtctgg
IGHV4-4*07	IGHD1-1*01	IGHJ5*02	14	tgtgcgcgaggggcaactggatcaccatacaattggttcgacccctgg
IGHV1-18*01	IGHD6-19*01	IGHJ4*02	15	tgtgcgcggggatttatccctgaccagtggctggtacccctttgactactgg
IGHV4-59*01	IGHD2-15*01	IGHJ3*02	12	tgtgcgcggtggctgccaacggatgatgcttttgatatctgg
IGHV1-58*02	IGHD3-3*01	IGHJ5*02	15	tgtgcggcaggggcccaagattacgatttttggagtggttattcactttgg
IGHV1-2*02	IGHD5-18*01	IGHJ3*02	20	tgtgcgggtagacaccacagctatggttacaattgggttgcaataaatgatgcttttgatatct-gg
IGHV5-51*01	IGHD1-26*01	IGHJ4*02	11	tgtgcggtgagtaggagctactactactttgactactgg
IGHV1-46*01	IGHD5-12*01	IGHJ4*02	12	tgtgctagagggcttgacacggtggctacgattagttactgg
IGHV6-1*01	IGHD6-13*01	IGHJ4*02	11	tgtgcaagagaaggcagcagctggtacgtagactattgg
IGHV6-1*01	IGHD3-10*01	IGHJ3*02	9	tgtgcaagaggtgcgggtgcttttgatatctgg
IGHV1-8*01	IGHD1-26*01	IGHJ6*03	15	tgtgcaagagtaaatggtgggagctactactactactacatggacgtctgg
IGHV3-30*03	IGHD6-13*01	IGHJ4*02	15	tgtgcgaaacatggtgagggagagcagcagctggcgtactttgactactgg
IGHV3-23*01	IGHD3-22*01	IGHJ4*02	14	tgtgcgaaagatggttactatgatagtagtgcttattttgtctactgg
IGHV3-23*01	IGHD3-9*01	IGHJ4*02	14	tgtgcgaagcaactggattacgatattttgactggttgtgactactgg
IGHV4-34*01	IGHD6-6*01	IGHJ6*02	16	tgtgcgaccctagcagctcgtccgccctactattattatggtatggacatctgg
IGHV4-34*01	IGHD3-3*02	IGHJ5*02	16	tgtgcgagacaattttggagtgcttatcccgaatacaactggttcgacccctgg
IGHV4-59*01	IGHD5-24*01	IGHJ4*02	12	tgtgcgagacgaactggagagatggcgagctttgactactgg
IGHV5-51*01	IGHD6-13*01	IGHJ4*02	11	tgtgcgagagagagcgatagcagcagctggttctactgg
IGHV3-11*01	IGHD4-17*01	IGHJ5*02	11	tgtgcgagagagtcccgcatgactacggtgacgcggtgg
IGHV3-21*01	IGHD4-17*01	IGHJ4*02	10	tgtgcgagagataagactacgcactttgactactgg
IGHV3-7*01	IGHD3-10*01	IGHJ4*02	9	tgtgcgagagatcgcggatgttttgactactgg
IGHV3-21*01	IGHD6-25*01	IGHJ4*02	14	tgtgcgagagatcggttgggggggtatagcagtggcgacggactactgg
IGHV3-21*01	IGHD5-24*01	IGHJ5*02	18	tgtgcgagagcgggagggatggctacaataatttgggagtacaactggttcgacccctgg
IGHV1-8*01	IGHD5-24*01	IGHJ4*02	17	tgtgcgagaggccccaacctgagatggctacaattatacggctactttgactactgg
IGHV4-34*01	IGHD2-2*01	IGHJ5*02	16	tgtgcgagaggcttgagccttaccagagactcctctaactggttcgacccctgg
IGHV3-7*01 I	GHD2-21*02	IGHJ4*02	16	tgtgcgaggattacgagtgcatattgtggtggtgactgccctcttgactactgg
IGHV3-30*03	IGHD6-13*01	IGHJ4*02	16	tgtgcgaggggacagaggggcagcagctggcccgattatctaattgactactgg
IGHV1-2*02	IGHD3-22*01	IGHJ4*02	13	tgtgcgagggttgatagtagtggttattactactttctctactgg
AT7				
IGHV3-15*07	IGHD5-18*01	IGHJ4*02	14	tgtaccacagaggcacgcaacgtggatacagctatggttgactactgg

VH	DH	JH	CDR3 (aa)	JUNCTION
AT7				
IGHV3-15*07	IGHD1-26*01	IGHJ3*02	14	tgtaccaccttagtgggagctactactgcgtatgcttttgatatctgg
IGHV3-49*04	IGHD6-19*01	IGHJ4*02	13	tgtactagagagtatgggcagtggctggtccccttttgactactgg
IGHV3-49*04	IGHD3-22*01	IGHJ3*02	17	tgtactagagcgcgttactatgatagtagtggttcccctgatgcttttgatatctgg
IGHV3-9*01	IGHD3-10*01	IGHJ3*02	9	tgtgcaaaagcccggggcgcttttgatatctgg
IGHV1-24*01	IGHD1-1*01	IGHJ4*02	7	tgtgcaacacacctctttgactactgg
IGHV1-24*01	IGHD6-25*01	IGHJ4*02	15	tgtgcaacagagtttagtctcgggtacccacaatactactttgactactgg
IGHV1-24*01	IGHD1-26*01	IGHJ4*02	16	tgtgcaacagtctatagtgggagctactacggcgggtactactttgactactgg
IGHV3-74*01	IGHD3-10*01	IGHJ3*02	9	tgtgcaagagaggttcgggcttttgatatctgg
IGHV6-1*01	IGHD6-6*01	IGHJ4*02	14	tgtgcaagagatcctagtataacaactcgtctcgactttgactactgg
IGHV3-74*01	IGHD1-14*01	IGHJ4*02	8	tgtgcaagagatcggaactttgactactgg
IGHV6-1*01	IGHD1-7*01	IGHJ4*02	14	tgtgcaagagatcgtctcaactggaactacggtacatttgactactgg
IGHV6-1*01	IGHD5-18*01	IGHJ4*02	15	tgtgcaagagatgagggaggaatacagctatggtccggttttgactactgg
IGHV3-13*01	IGHD1-26*01	IGHJ4*02	15	tgtgcaagagccaagggccgaaggagtgggagctacgttcttgactactgg
IGHV3-74*01	IGHD3-22*01	IGHJ4*02	13	tgtgcaagaggggatagtagtggttattccccctttgactactgg
IGHV6-1*01	IGHD3-10*01	IGHJ3*02	19	tgtgcaagagtttacactatggttcggggagttactatagattcccatgcttttgatatctgg
IGHV2-5*10	IGHD4-11*01	IGHJ6*02	14	tgtgcacacagaccccagtactactactactacggtatggacgtctgg
IGHV4-30-2*01	IGHD3-22*01	IGHJ3*02	14	tgtgccagaacccatagtagtggttattacggtgcttttgatatctgg
IGHV3-23*01	IGHD5-12*01	IGHJ4*02	15	tgtgcgaaaatccatagtggctacgattccccgtactactttgactactgg
IGHV3-23*01	IGHD6-13*01	IGHJ4*02	19	tgtgcgaaacaaccaaccgggtatagcagcagctggtacggtcaaggatactttgac-tactgg
IGHV3-23*01	IGHD2-21*02	IGHJ4*02	18	tgtgcgaaaccaagagcatattgtggtggtgactgctatcctccttactttgactactgg
IGHV3-23*01	IGHD6-25*01	IGHJ2*01	19	tgtgcgaaactgtgggcgcggtatagcagctcctacccattaaactggtacttcgatctct-gg
IGHV3-23*01	IGHD7-27*01	IGHJ4*02	17	tgtgcgaaagaagactctcacaaactggggatggtgggggactactttgactactgg
IGHV3-23*01	IGHD6-19*01	IGHJ4*02	16	tgtgcgaaagaccatagcagtggctggtcaggccggttctactttgactactgg
IGHV3-23*01	IGHD6-19*01	IGHJ4*02	15	tgtgcgaaagacccaacgggtatagcagtggctggttcttttgactactgg
IGHV3-23*01	IGHD3-22*01	IGHJ4*02	16	tgtgcgaaagatccctatgatagtagtggttattacaccacttttgactactgg
IGHV3-23*01	IGHD2-21*02	IGHJ4*02	14	tgtgcgaaagatcgccgggggggcggtgactactatctttgactactgg
IGHV3-23*01	IGHD6-19*01	IGHJ4*02	18	tgtgcgaaagatcgggggggtagtatagcagtggctggtaataggggttttgactactgg
IGHV3-23*01	IGHD4-23*01	IGHJ4*02	16	tgtgcgaaagatcgtaggctttacggtggtaacccctactactttgactactgg
IGHV3-23*01	IGHD3-22*01	IGHJ1*01	17	tgtgcgaaagatcgtggaacgtattactatgatagtagtggttattacctacactgg
IGHV3-23*01	IGHD6-6*01	IGHJ6*02	17	tgtgcgaaagatctcagctcgtccggcccttactactactacggtatggacgtctgg
IGHV3-23*01		IGHJ3*02	8	tgtgcgaaagatgatgcttttgatatctgg
IGHV3-15*07	IGHD1-26*01	IGHJ3*02	14	tgtaccaccttagtgggagctactactgcgtatgcttttgatatctgg
IGHV3-49*04	IGHD6-19*01	IGHJ4*02	13	tgtactagagagtatgggcagtggctggtccccttttgactactgg
IGHV3-49*04	IGHD3-22*01	IGHJ3*02	17	tgtactagagcgcgttactatgatagtagtggttcccctgatgcttttgatatctgg
IGHV3-9*01	IGHD3-10*01	IGHJ3*02	9	tgtgcaaaagcccggggcgcttttgatatctgg
IGHV1-24*01	IGHD1-1*01	IGHJ4*02	7	tgtgcaacacacctctttgactactgg

VH	DH	JH	CDR3 (aa)	JUNCTION
AT7				
IGHV1-24*01	IGHD6-25*01	IGHJ4*02	15	tgtgcaacagagtttagtctcgggtacccacaatactactttgactactgg
IGHV1-24*01	IGHD1-26*01	IGHJ4*02	16	tgtgcaacagtctatagtgggagctactacggcgggtactactttgactactgg
IGHV3-74*01	IGHD3-10*01	IGHJ3*02	9	tgtgcaagagaggttcgggcttttgatatctgg
IGHV6-1*01	IGHD6-6*01	IGHJ4*02	14	tgtgcaagagatcctagtataacaactcgtctcgactttgactactgg
IGHV3-74*01	IGHD1-14*01	IGHJ4*02	8	tgtgcaagagatcggaactttgactactgg
IGHV6-1*01	IGHD1-7*01	IGHJ4*02	14	tgtgcaagagatcgtctcaactggaactacggtacatttgactactgg
IGHV6-1*01	IGHD5-18*01	IGHJ4*02	15	tgtgcaagagatgagggaggaatacagctatggtccggttttgactactgg
IGHV3-13*01	IGHD1-26*01	IGHJ4*02	15	tgtgcaagagccaagggccgaaggagtgggagctacgttcttgactactgg
IGHV3-74*01	IGHD3-22*01	IGHJ4*02	13	tgtgcaagaggggatagtagtggttattccccctttgactactgg
IGHV6-1*01	IGHD3-10*01	IGHJ3*02	19	tgtgcaagagtttacactatggttcggggagttactatagattcccatgcttttgatatctgg
IGHV2-5*10	IGHD4-11*01	IGHJ6*02	14	tgtgcacacagaccccagtactactactactacggtatggacgtctgg
IGHV4-30-2*01	IGHD3-22*01	IGHJ3*02	14	tgtgccagaacccatagtagtggttattacggtgcttttgatatctgg
IGHV3-23*01	IGHD5-12*01	IGHJ4*02	15	tgtgcgaaaatccatagtggctacgattccccgtactactttgactactgg
IGHV3-23*01	IGHD6-13*01	IGHJ4*02	19	tgtgcgaaacaaccaaccgggtatagcagcagctggtacggtcaaggatactttgac-tactgg
IGHV3-23*01	IGHD2-21*02	IGHJ4*02	18	tgtgcgaaaccaagagcatattgtggtggtgactgctatcctccttactttgactactgg
IGHV3-23*01	IGHD6-25*01	IGHJ2*01	19	tgtgcgaaactgtgggcgcggtatagcagctcctacccattaaactggtacttcgatctct-gg
IGHV3-23*01	IGHD7-27*01	IGHJ4*02	17	tgtgcgaaagaagactctcacaaactggggatggtgggggactactttgactactgg
IGHV3-23*01	IGHD6-19*01	IGHJ4*02	16	tgtgcgaaagaccatagcagtggctggtcaggccggttctactttgactactgg
IGHV3-23*01	IGHD6-19*01	IGHJ4*02	15	tgtgcgaaagacccaacgggtatagcagtggctggttcttttgactactgg
IGHV3-23*01	IGHD3-22*01	IGHJ4*02	16	tgtgcgaaagatccctatgatagtagtggttattacaccacttttgactactgg
IGHV3-23*01	IGHD2-21*02	IGHJ4*02	14	tgtgcgaaagatcgccgggggggcggtgactactatctttgactactgg
IGHV3-23*01	IGHD6-19*01	IGHJ4*02	18	tgtgcgaaagatcgggggggtagtatagcagtggctggtaatagggtttttgactact-gg
IGHV3-23*01	IGHD4-23*01	IGHJ4*02	16	tgtgcgaaagatcgtaggctttacggtggtaacccctactactttgactactgg
IGHV3-23*01	IGHD3-22*01	IGHJ1*01	17	tgtgcgaaagatcgtggaacgtattactatgatagtagtggttattacctacactgg
IGHV3-23*01	IGHD6-6*01	IGHJ6*02	17	tgtgcgaaagatctcagctcgtccggcccttactactactacggtatggacgtctgg
IGHV3-23*01		IGHJ3*02	8	tgtgcgaaagatgatgcttttgatatctgg
IGHV3-23*01	IGHD3-3*01	IGHJ3*02	16	tgtgcgaaagatggaggggcccgccctctaccccatgatgcttttgatatctgg
IGHV3-30*03	IGHD1-26*01	IGHJ4*02	16	tgtgcgaaagatggtcgggtgggagccgaacctaaaaactactttgactactgg
IGHV3-23*01	IGHD6-19*01	IGHJ4*02	15	tgtgcgaaagattgggcaggagcagtggctggtaactactttgactactgg
IGHV3-23*01	IGHD3-3*01	IGHJ4*02	10	tgtgcgaaagattgggggtggacctttgactactgg
IGHV3-23*01	IGHD3-9*01	IGHJ1*01	12	tgtgcgaaagccgtgtccgtttctgaatacttccagcactgg
IGHV3-23*01	IGHD1-26*01	IGHJ4*02	15	tgtgcgaaagcggcggtgtgggagctccaaacgccgtactttgactactgg
IGHV3-23*01	IGHD3-22*01	IGHJ6*02	15	tgtgcgaaagctaaggtagcagtcaactactactacggtatggacctctgg
IGHV3-23*01	IGHD3-22*01	IGHJ4*02	17	tgtgcgaaagggaaggggaattactatgatagtagtggttattactttgactactgg
IGHV3-30*03	IGHD3-22*01	IGHJ1*01	18	tgtgcgaaaggttattactatgatagtagtggttattaccctgaatacttccagcactgg

VH	DH	JH	CDR3 (aa)	JUNCTION
AT7				
IGHV3-23*01	IGHD3-22*01	IGHJ4*02	13	tgtgcgaaagtagggcgatactatgatagtagctttgactactgg
IGHV3-53*01	IGHD4-17*01	IGHJ4*02	15	tgtgcgaaagtggggcctacggtgactacgatgtactactttgactactgg
IGHV3-23*01	IGHD3-16*01	IGHJ2*01	11	tgtgcgaaggcaggggggaaactggtacttcgatctctgg
IGHV3-30*01	IGHD3-10*01	IGHJ6*02	22	tgtgcgaccgagcccctattactatggttcggggagttacctagcgggtactactacggtatggacgtctgg
IGHV4-39*01	IGHD4-23*01	IGHJ4*02	16	tgtgcgagaacacactacggtggtaactccgccctttactactttgactactgg
IGHV3-7*01	IGHD3-22*01	IGHJ4*02	19	tgtgcgagaagaagagaggaggggtattactatgatagtagtggttattactttgactactgg
IGHV3-30*01	IGHD5-12*01	IGHJ4*02	18	tgtgcgagacaattacgtggatatagtggctacgatgtacagggaaattttgactactgg
IGHV5-a*01	IGHD6-19*01	IGHJ3*02	10	tgtgcgagacaccccaggggtgcttttgatatctgg
IGHV4-39*01	IGHD6-13*01	IGHJ6*02	17	tgtgcgagacataatgctatagcagcagctggttactactacggtatggacgtctgg
IGHV4-39*01	IGHD6-19*01	IGHJ3*02	15	tgtgcgagacatggaactagcagtggctggttagatgcttttgatatctgg
IGHV4-39*01	IGHD6-25*01	IGHJ4*02	16	tgtgcgagacatggggatcgtccaatagcagcagccacgaactttgactactgg
IGHV4-39*01	IGHD6-25*01	IGHJ4*02	13	tgtgcgagacatgtccatatagcagctggtacggttcactactgg
IGHV4-39*01	IGHD6-13*01	IGHJ4*02	15	tgtgcgagacatgtgctacagcagctgggaggttactactttgactactgg
IGHV4-39*01	IGHD1-26*01	IGHJ4*02	12	tgtgcgagaccgtatagtgggagctacactcttgactactgg
IGHV3-20*01	IGHD3-9*01	IGHJ3*02	18	tgtgcgagacgaggttacgatattttgactggttattcccctggtgcttttgatatctgg
IGHV4-39*01	IGHD2-15*01	IGHJ6*02	14	tgtgcgagacgtcgtgggagctactactactacggtatggacgtctgg
IGHV5-51*01	IGHD2-2*01	IGHJ5*02	20	tgtgcgagacgttcaagcggatattgtagtagtaccagctgcagagacaactggtcgacccctgg
IGHV3-33*01	IGHD6-13*01	IGHJ4*02	11	tgtgcgagagaaggggacaggccttactttgactactgg
IGHV4-b*01	IGHD6-6*01	IGHJ4*02	14	tgtgcgagagacttccatgaggttgggagctactactttgactactgg
IGHV3-48*03	IGHD6-13*01	IGHJ3*02	20	tgtgcgagagaggaatactaccggtcctttgggggagcagcagctggccgagcttttgatatctgg
IGHV3-33*01	IGHD5-18*01	IGHJ4*02	13	tgtgcgagagaggtggatacagctatggtttactttgactactgg
IGHV1-46*01	IGHD5-18*01	IGHJ1*01	16	tgtgcgagagaggtttgggatacagctatggccccgggatacttccagtactgg
IGHV3-7*01	IGHD2-15*01	IGHJ6*02	19	tgtgcgagagataaagtggtggtagctgctacggactactactactacggtatggacgtctgg
IGHV3-7*03	IGHD6-19*01	IGHJ4*02	16	tgtgcgagagataaggatagcagtggctggtacggccactactttgactactgg
IGHV3-48*03	IGHD1-26*01	IGHJ3*02	18	tgtgcgagagataatcggggtgggagctactacaagtgttgtgatgcttttgatatctgg
IGHV3-48*03		IGHJ4*02	6	tgtgcgagagatattgactactgg
IGHV4-59*01	IGHD3-10*02	IGHJ5*02	15	tgtgcgagagatcacctcaactggggccggggaaactggttcgacccctgg
IGHV3-48*03	IGHD3-10*01	IGHJ2*01	18	tgtgcgagagatccaccccagcccttttggttcggggaagtactggtacttcgatctctgg
IGHV4-61*01	IGHD2-2*01	IGHJ3*02	10	tgtgcgagagatcgccaggatgcttttgatatctgg
IGHV3-33*01	IGHD6-6*01	IGHJ3*02	10	tgtgcgagagatcgccctgatgcttttgatatctgg
IGHV3-7*03	IGHD4-23*01	IGHJ1*01	15	tgtgcgagagatctgactacggtggtgcgcgctgaatacttccagcactgg
IGHV3-21*01	IGHD5-12*01	IGHJ4*02	16	tgtgcgagagatgggtatagtggctacgattacaagaactactttgactactgg
IGHV1-18*01	IGHD6-6*01	IGHJ4*02	17	tgtgcgagagattccggtagcagctcgtccttaggtcgtcgctcctttgactactgg
IGHV4-59*01	IGHD3-10*01	IGHJ4*02	14	tgtgcgagagattcggcagctatggttaggggattctttgactactgg
IGHV1-46*01	IGHD7-27*01	IGHJ3*02	11	tgtgcgagagattctggggatgatgcttttgatatctgg

VH	DH	JH	CDR3 (aa)	JUNCTION
AT7				
IGHV1-3*01	IGHD6-19*01	IGHJ4*02	17	tgtgcgagagattttgaggagatagcagtggctggtaccggctactttgactactgg
IGHV4-59*01	IGHD2-15*01	IGHJ4*02	11	tgtgcgagagcccggaagctgtactactttgactactgg
IGHV3-7*01	IGHD6-19*01	IGHJ4*02	16	tgtgcgagagccctgatagcagtggctggtaaggggtactactttgactactgg
IGHV3-21*01	IGHD3-22*01	IGHJ4*02	19	tgtgcgagagccttaaacgaccttcctaattactatgatagtagtggttattactactgctgg
IGHV3-21*01	IGHD2-8*01	IGHJ4*02	18	tgtgcgagaggccagggatattgtactaatggtgtatgctattactactttgactactgg
IGHV4-34*01	IGHD3-3*01	IGHJ6*02	17	tgtgcgagaggcctgcgcgatttttggagttactactactacggtatggacgtctgg
IGHV1-69*01	IGHD5-24*01	IGHJ5*02	12	tgtgcgagaggcgtagagatctggaactggttcgacccctgg
IGHV3-7*03	IGHD1-26*01	IGHJ4*02	12	tgtgcgagaggctgggagcgggcgggtatgattgactactgg
IGHV3-33*01	IGHD1-26*01	IGHJ4*02	10	tgtgcgagaggctggtgggagccacccggctactgg
IGHV3-48*03	IGHD3-9*01	IGHJ4*02	18	tgtgcgagagggacgtattacgatattttgactgccaataaggggtactttgactactgg
IGHV4-34*01	IGHD6-19*01	IGHJ3*02	15	tgtgcgagagggatagcagtggctggtcagagtgatgcttttgatatctgg
IGHV1-2*04	IGHD6-6*01	IGHJ4*02	12	tgtgcgagagggcatagcagctcgtcggactttgactactgg
IGHV1-18*01	IGHD1-1*01	IGHJ4*02	9	tgtgcgagagggctggaaatctttgactactgg
IGHV1-2*04	IGHD3-22*01	IGHJ4*02	17	tgtgcgagaggggattactatgatagtagtggttatccgtactactttgactactgg
IGHV4-59*01	IGHD6-25*01	IGHJ4*02	11	tgtgcgagaggggcagcagcaacaaactttgactactgg
IGHV3-7*01	IGHD6-6*01	IGHJ4*02	14	tgtgcgagaggggctcgtatagcagctcggtactactttgactactgg
IGHV3-30*01	IGHD3-10*01	IGHJ4*02	10	tgtgcgagaggggattatactactttgactactgg
IGHV3-33*01	IGHD3-16*01	IGHJ5*02	9	tgtgcgagaggggggagcgggttcgacccctgg
IGHV4-34*01		IGHJ2*01	9	tgtgcgagagggtactggtacttcgatctctgg
IGHV4-34*01	IGHD1-26*01	IGHJ4*02	14	tgtgcgagagggttgggagctaccgtctcgtactactttgactactgg
IGHV1-69*01	IGHD6-13*01	IGHJ6*02	23	tgtgcgagaggtgactcgtatagcagcagctgggcagctgggaggtactactactac-tacggtatggacgtctgg
IGHV1-69*06	IGHD4-17*01	IGHJ2*01	10	tgtgcgagagtaggctacggtgactacagtagctgg
IGHV3-30*01	IGHD5-18*01	IGHJ4*02	13	tgtgcgagagtatacagttatggctatcgtgactttgactactgg
IGHV3-53*01	IGHD5-18*01	IGHJ4*02	14	tgtgcgagagtatcacagctatggcagacaggatactttgactactgg
IGHV1-69*06	IGHD6-13*01	IGHJ4*02	17	tgtgcgagagtccgaagccccgggtatagcagcagccggtacgggcttgactactgg
IGHV3-30-3*01	IGHD6-19*01	IGHJ3*02	17	tgtgcgagagtcgaggggggatagcagtggctggtacggggatgcttttgatatctgg
IGHV1-69*01	IGHD2-15*01	IGHJ6*02	20	tgtgcgagagtctcggatattgtagtggtggtagctgcagactactactacggtatg-gacgtctgg
IGHV4-34*01	IGHD5-12*01	IGHJ6*02	16	tgtgcgagagtgcaggctgggatcctctactactacggtatggacgtctgg
IGHV4-61*01	IGHD6-13*01	IGHJ4*02	16	tgtgcgagagtggggagcagcagctggtatccctcctactactttgactactgg
IGHV1-2*04	IGHD6-19*01	IGHJ3*02	15	tgtgcgagagtggttagcagtggctggtacgagggtgcttttgatatctgg
IGHV3-30*01	IGHD2-15*01	IGHJ3*02	14	tgtgcgagagttaaggccagctgctactcatgtgcttttgatatctgg
IGHV1-69*01	IGHD5-18*01	IGHJ4*02	17	tgtgcgagagttccccgcctacgtggatacagctatggttacgactttgactactgg
IGHV4-34*01	IGHD6-6*01	IGHJ4*02	17	tgtgcgagagttggtatagcagctcgtccgggccgggaagcacatcttgactactgg
IGHV4-34*01	IGHD3-9*01	IGHJ5*02	21	tgtgcgagatcgggagccggggtgatacgatattttgactggttacctcggaactggt-tcgacccctgg
IGHV3-30-3*01	IGHD6-6*01	IGHJ4*02	12	tgtgcgagattcaggtatagcagctcgtccgtgggctactgg

VH	DH	JH	CDR3 (aa)	JUNCTION
AT7				
IGHV3-11*03	IGHD1-7*01	IGHJ3*02	11	tgtgcgagatttcgaactcgtggtgcttttgatatctgg
IGHV4-59*01	IGHD6-19*01	IGHJ3*02	13	tgtgcgagctaccagtggctggcacctggtgcttttgatatctgg
IGHV3-30*01		IGHJ3*02	8	tgtgcgaggaatgatgcttttgatatctgg
IGHV3-33*01	IGHD3-22*01	IGHJ4*02	18	tgtgcgaggctgtattactatgatagtagtggttattacaacgggggctttgactactgg
IGHV4-39*01		IGHJ6*02	12	tgtgcgagggccccttactactactacggtatggacgtctgg
IGHV1-3*01	IGHD2-2*01	IGHJ6*02	20	tgtgcgaggggaattgtagtagtaccagctgctatgtactactactactacggtatg-gacgtctgg
IGHV1-69*01	IGHD1-26*01	IGHJ4*02	13	tgtgcgaggtccttgacgatagtgggagcctactttgactactgg
IGHV3-53*01	IGHD4-23*01	IGHJ3*02	9	tgtgcgagtacctctgatgcttttgatatctgg
IGHV4-34*01	IGHD1-26*01	IGHJ3*02	16	tgtgcgagtatccagtgggagctactgaatcgaccttctgcttttgatatctgg
IGHV3-64*05	IGHD3-10*01	IGHJ4*02	15	tgtgtgaaagatctatttactatggttcggggagttggccttgactactgg
IGHV3-64*05	IGHD3-3*01	IGHJ4*02	13	tgtgtgaaagcttacgatttttggagtggttattatgactactgg
IGHV3-30*01		IGHJ4*02	6	tgtgcgagggactttgactactgg
AT11				
IGHV6-1*01	IGHD3-3*01	IGHJ5*02	10	tgtgcaagagagaggggttactggttcgacccctgg
IGHV3-23*01	IGHD5-18*01	IGHJ4*02	15	tgtgcgaaagataaactacggatacagctatgggcctcgattgactactgg
IGHV4-34*01	IGHD1-1*01	IGHJ1*01	9	tgtgcgagaaacgactttcctttccagcactgg
IGHV4-39*07	IGHD6-13*01	IGHJ2*01	12	tgtgcgagaatgggcagcttctactggtacttcgatctctgg
IGHV4-59*08	IGHD5-12*01	IGHJ4*02	12	tgtgcgagacacgtagtggctacggccccttatagttactgg
IGHV4-59*08	IGHD3-10*01	IGHJ5*02	10	tgtgcgagacgtcggggtcgctggttcgacccctgg
IGHV4-59*08	IGHD6-13*01	IGHJ4*02	17	tgtgcgagacttccgtatagcagcagctggtacgggtcctactactttgactactgg
IGHV4-34*01	IGHD5-24*01	IGHJ1*01	5	tgtgcgagagaccaattctgg
IGHV3-21*01	IGHD2-21*01	IGHJ5*02	13	tgtgcgagagagggcttcgacggtgctgctgagttcgtcacctgg
IGHV4-34*01	IGHD2-15*01	IGHJ5*02	21	tgtgcgagagcgggtcgtgattattgtagtggtggtagctgcaactcacacaggtggt-tcgacccctgg
IGHV4-31*03	IGHD1-26*01	IGHJ4*02	15	tgtgcgagagcttggggaaagtgggagctacccagcgcctttgactactgg
IGHV4-34*01	IGHD1-26*01	IGHJ4*02	17	tgtgcgagaggacggggcggattgcaagtgggagctactatatactttgactactgg
IGHV3-21*01	IGHD2-21*02	IGHJ4*02	6	tgtgcgagaggactgccctactgg
IGHV4-34*01	IGHD4-17*01	IGHJ4*02	20	tgtgcgagaggagagccgccggcgaatccccggccaggtacggtgacctgttgctttgactact-gg
IGHV4-34*01	IGHD5-12*01	IGHJ4*02	11	tgtgcgagaggcagggccacccactactttgactactgg
IGHV4-34*01	IGHD6-13*01	IGHJ4*02	11	tgtgcgagaggcagggcgtcggagccttttgactactgg
IGHV4-34*01	IGHD2-2*01	IGHJ4*02	9	tgtgcgagaggccaggactactttgactactgg
IGHV4-34*01	IGHD4-11*01	IGHJ4*02	12	tgtgcgagaggcccggacaataaccactcttttgactactgg
IGHV4-34*01	IGHD6-13*01	IGHJ6*02	17	tgtgcgagaggcgtatattggtcggggtatagcagcttttacggtatggacgtctgg
IGHV3-30*04	IGHD1-26*01	IGHJ4*02	20	tgtgcgagaggtttgcggggaaatatagtgggagctacaaaggggcttgactactttgactact-gg

VH	DH	JH	CDR3 (aa)	JUNCTION
AT11				
IGHV4-31*03	IGHD2-2*01	IGHJ3*02	21	tgtgcgagagtggtgaatctaggatattgtagtagtaccagctgctatgcgggggcttttgatatctgg
IGHV3-7*01	IGHD3-22*01	IGHJ3*02	18	tgtgcgaggggtgattaccatgatagtagtggttattgggtcgatgcgtttgatatctgg
IGHV4-31*03	IGHD2-2*01	IGHJ3*02	21	tgtgcgagagtggtgaatctaggatattgtagtagtaccagctgctatgcgggggcttttgatatctgg
IGHV3-7*01	IGHD3-22*01	IGHJ3*02	18	tgtgcgaggggtgattaccatgatagtagtggttattgggtcgatgcgtttgatatctgg
IGHV4-39*01	IGHD1-26*01	IGHJ4*02	12	tgtgcgaggtatagtgggagctacggctactttgactactgg
IGHV4-34*01	IGHD3-22*01	IGHJ4*02	11	tgtgcgcgaggcgcccgtagtagtggttatcacttctgg
IGHV4-34*01	IGHD3-10*01	IGHJ4*02	15	tgtgcggcaggtgcccttctcggtttagggagcctccttttttgactactgg
IGHV4-59*01	IGHD2-15*01	IGHJ3*02	10	tgtgcggtggtagctgccggggctttgatatctgg
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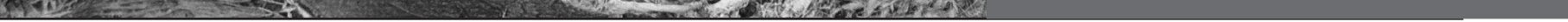

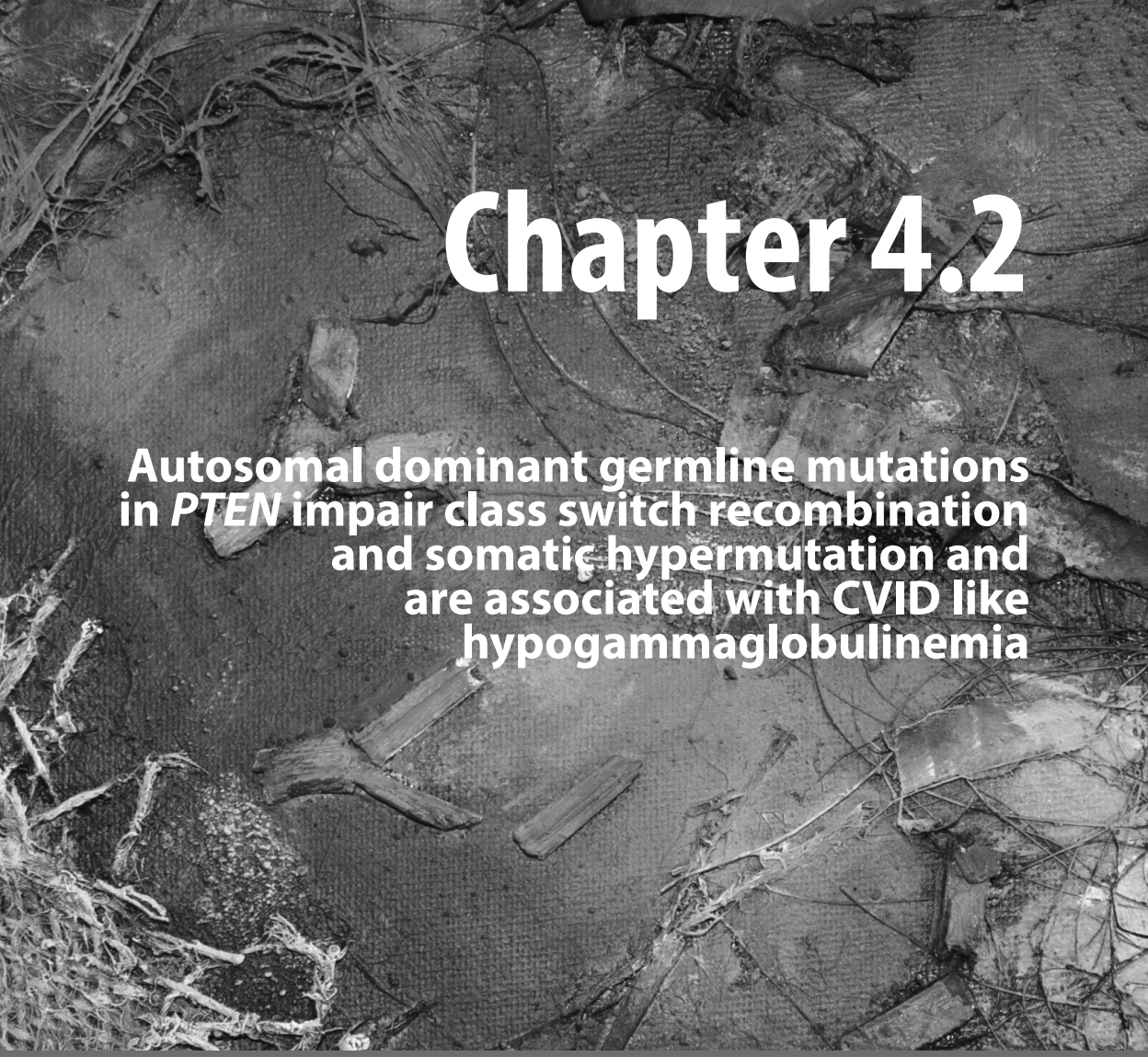

Chapter 4.2

Autosomal dominant germline mutations in *PTEN* impair class switch recombination and somatic hypermutation and are associated with CVID like hypogammaglobulinemia

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Manuscript in preparation

ABSTRACT

Background. Autosomal dominant germline mutations in *PTEN* are associated with PTEN Hamartoma Tumor Syndrome (PHTS). Immunodeficiency has not been reported to be part of the clinical spectrum of PHTS, although mice data indicate that *PTEN* mutations affect Class Witch Recombination (CSR) by Akt mediated inhibition of Activation Induced Cytidine Deaminase. *Aim*. To examine the immunological mechanisms responsible for the antibody deficiency in patients with PHTS. *Methods*. We studied three patients with heterozygous germline *PTEN* mutations who suffered from PHTS and hypogammaglobulinemia and six patients with PHTS without antibody deficiency. We explored peripheral naive and memory B-cell subsets, B-cell subset replication history, somatic hypermutation (SHM) frequencies and CSR patterns in *IGH* transcripts and B-cell activation by calcium flux. *Results*. The clinical phenotype of the PHTS with hypogammaglobulimia patients fulfilled Common Variable Immunodeficiency diagnostic criteria. CSR and SHM were impaired in PHTS, irrespective of the presence of hypogammaglobulinemia, as exemplified by reduced proportions of class switched memory B-cells and at the molecular level by impaired CSR to IgG_2, IgG_4 and IgA_2, as well a reduction of SHM frequencies in *IGH* transcripts. PHTS patients without antibody deficiency could compensate their CSR deficiency by generating increased absolute counts of transitional and naive B-cells, normal counts of class switched memory B-cells and increased plasmablasts. *Conclusions*. Autosomal dominant germline mutations in *PTEN* cause CSR and SHM deficiency and are associated with CVID like hypogammaglobulinemia. Comparison to mice data suggest that the probable pathophysiological mechanism is PI3K/Akt mediated inhibition of AID, due to loss of negative regulation of PI3K by PTEN. Deregulated Akt signaling should also be considered as a potential causative mechanism in CVID, especially since it is associated with auto-immunity, lymphoproliferation and the propensity to develop malignancies.

INTRODUCTION

PTEN (phosphate and tensin homologue deleted on chromosome 10) is a tumor suppressor gene located on chromosome 10q23. Autosomal dominant germline mutations in *PTEN* are associated with three partly overlapping clinical syndromes: Cowden syndrome[1-2], Bannayan-Riley-Ruvalcaba[3-4] syndrome and Proteus syndrome[5-6]. Together, these conditions are referred to as PTEN Hamartoma Tumor Syndromes (PHTS). Important clinical manifestations of PHTS are hamartoma's in multiple organs, increased susceptibility to malignant tumors (breast, thyroid, endometrium), macrocephaly[7], autism and developmental delay. Immunodeficiency has not been reported to be part of the clinical spectrum of PHTS. However, conditional knockout of *PTEN* in B-cells is associated with defective B-cell development[8-9] in mice.

Normal B-cell development starts in the bone marrow, where a diverse repertoire of B-cells is generated by V(D)J recombination of the immunoglobulin (Ig) genes. After migration to the periphery, transitional B-cells differentiate into naive mature B-cells, which can be activated by antigen through the B-cell receptor complex. This takes place with T-cell help in a germinal center (GC) in lymphoid tissue or independently of T-cell help, e.g. in the marginal zone of the spleen. Cognate interaction between B and T-cells generate Activation Induced Cytidine Deaminase (AID) dependent somatic hypermutations (SHM) in the variable region of the Ig heavy and Ig light chains, in order to increase the affinity of the BCR. Subsequent AID dependent class switch recombination (CSR) changes the IgH μ constant region to form Ig isotypes with different effector functions (γ, α or ε), resulting the development of IgG, IgA or IgE producing plasma cells and class-switched memory B-cells. B-cell responses in the splenic marginal zone are thought to generate a substantial fraction of circulating natural effector B-cells.

PTEN affects B-cell development by inhibition of phosphatidyl inositol 3-kinase (PI3K)/Akt signaling, which regulates survival, proliferation, SHM, CSR and plasma cell differentiation, as reviewed by Omori et al.[10] and Werner et al.[11] (Figure 1). Type 1 PI3K consists of a regulatory subunit (P85, P55 or P50) and a catalytic subunit (p110α, p110β or p110δ). Of three PI3Ks, PI3Kδ is highly expressed in lymphocytes[12] and converts phosphatidylinositol 4,5, biphosphate (PIP2) into phosphatidyl inositol 3,4,5 triphosphate (PIP3). PIP3 activates the downstream effectors PDK1, Akt and Btk[13]. This pathway is negatively regulated by PTEN, which is thought to be constitutively active, and by SHIP, which is activated by co-ligation of the BCR and FcγRIIb[14]. Conditional deletion of *PTEN* in B cells in mice results in increased Akt activity, which is responsible for an increase of naive mature B cells, B1 B cells[15] and marginal zone B cells[9, 15]. PTEN-deficient B cells are hyperproliferative[15] and exhibit a lower threshold for activation through their BCR. Furthermore, bPTEN$^{-/-}$ mice produce more IgM antibody secreting cells[8]. In contrast, CSR is suppressed by Akt mediated

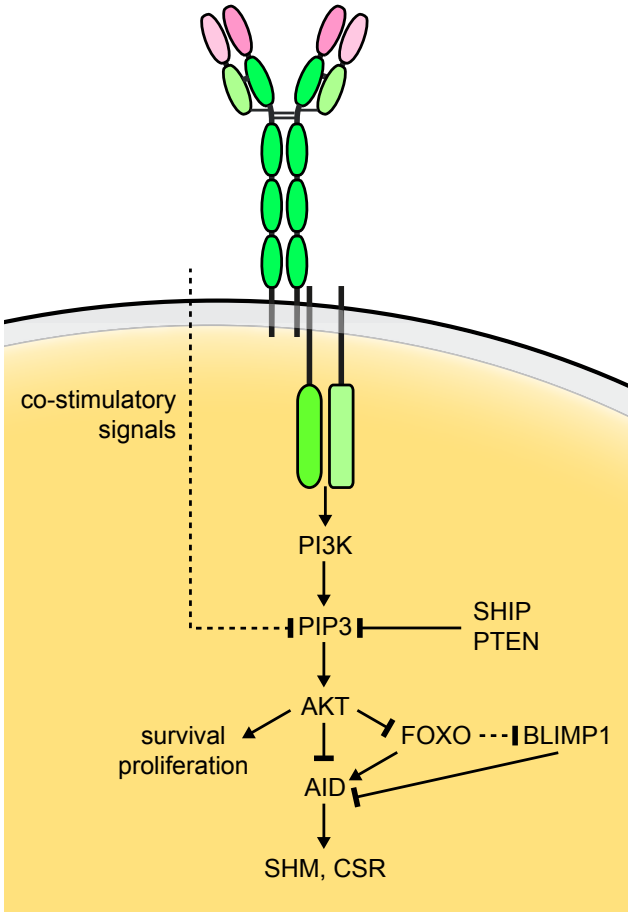

Figure 1. Model of the role of PI3K/Akt signaling on class switch recombination and somatic hypermutation. Figure modified from Omori et al (ref 10) and Werner et al. (ref 11). Arrows indicate stimulatory signals, bars indicate inhibitory signals. B-cell receptor signaling results in activation of PI3K and conversion of PIP2 to PIP3, which subsequently activates the Akt pathway. PTEN antagonizes this pathway by conversion of PIP3 in PIP2.

inhibition of AID[9], so the specific IgG and IgA antibody responses are impaired. Inhibition of PI3K by a specific inhibitor (IC87114) restores CSR activity and inhibits the formation of IgM antibodies[8]. Heterozygous germline *PTEN* mutations in mice result in lymphocyte resistance to apoptosis and auto-immunity[16], rather than CSR deficiency.

In humans, mutations in *PTEN* have not been associated with class switch recombination deficiency. Hypogammaglobulinemia has once been reported in a patient with Proteus syndrome[17], but mutation analysis for *PTEN* was not available for this patient. Cowden

disease has occasionally been associated with variable abnormities in B-, T-cell and NK-cell cells, of which a decrease of T-cells was the most frequent observation[18]. These studies mostly involved single cases and in the majority of reports mutation analysis of the *PTEN* gene has not been reported. Hypogammaglobulinemia has been described in two children with macrocephaly of unknown origion. These children could potentially sufferfrom PHTS. We identified three patients with heterozygous *PTEN* mutations who suffered from hypogammaglobulinemia, including one of the children with macrocephaly in the above mentioned study[19]. We performed a detailed study of the peripheral B-cell compartment of these patients and of patients with PHTS who did not suffer from antibody deficiency. We demonstrate that patients with *PTEN* mutations have CSR and SHM deficiency, most likely because of Akt mediated inhibition of AID.

METHODS

Patients

We included 9 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 normal controls. Furthermore, we screened 42 CVID patients for the presence of mutations in *PTEN*. The research was approved by the Medical Ethical Committee of the ErasmusMC.

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) were compared to controls.

PCR amplification

To investigate the status of SHM and CSR, PCR amplification was applied on cDNA and PCR-products using the V_H3 and V_H4 forward primers and the V_H-Cγ and V_H-Cα reverse

primers. The mastermix included 2.5 µl 10x gold PCR buffer (Perkin Elmer, 4311818), 0.1 µl Ampli Taq Gold (Perkin Elmer, N808-0249), 0.25 µl dNTP's (20 mM, Pharmacia Biotech, 27-2050-01), 1.5 µl $MgCl_2$ (25 mM, Perkin Elmer, N808-0249), 10 pmol primers, 0.5 µl BSA and 16.65 µl autoclaved Milli-Q water for each reaction. Depending on DNA quality, 1-2 µl DNA was added. PCR products were visualized by electrophoresis on a 1% agarose gel. The expected size of the PCR product comprised between 400 and 500bp.

Cloning, sequencing and IGH sequence analysis

V_H3-Cα, V_H4-Cα, V_H3-Cγ, and V_H4-Cγ fragments were amplified using PCR. PCR-products were ligated into a pGEM T-Easy vector (Promega, Leiden, Netherlands) and transformed into competent *E. coli* bacteria (strain DH5α, Invitrogen, Breda, Netherlands) using the protocol provided by the manufacturer. Transformed *E. coli* bacteria were cultured on agar plates containing 50µg ampicillin (Sigma, Aldhrich), bromo-chloro-indolyl-galactopyranoside (X-gal, 0.002%, Bioline, Taunton, MA) and isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.2mM, Fermentas, Burlington, ON). The presence of product in positive clones was confirmed by PCR using the PUC and SP6 primer pairs. Selected PCR products were sequenced using Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an ABI Prism 3130 XL fluorescence sequencer (Generic Analyzer, 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 was determined for V_H gene segment of each transcript. Additionally, the replacement/silence (R/S) ratio of these mutations for the framework and for the CDRs was determined.

Calcium flux upon BCR stimulation

Peripheral Blood Mononuclear Cells (PBMC's) were incubated with 6 µg/ml Indo-1 (Molecular Probes, Invitrogen) and used to assess the Ca^{2+} fluxes upon BCR stimulation. Free intracellular Ca^{2+} concentrations were determined in CD20-positive B lymphocytes by flow cytometry using a FACSVantage station (BD Biosciences) before and after stimulation with 20 µg/ml goat anti-human IgM-F(ab')$_2$ (Jackson ImmunoResearch Laboratories Inc.). Subsequently, 2 µg/ml ionomycin (Molecular Probes) was added after each response for intracellular loading of Indo-1.

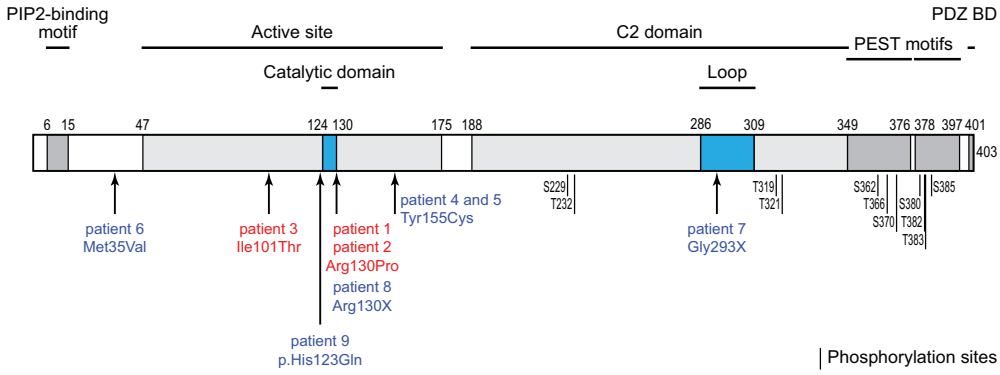

Figure 2. A structural overview of the PTEN protein with type of mutations. The overview includes distinct domains, phosphorylation sites and the locations as well as the type of mutations within the patient cohort. Red fonts indicate patients with hypogammaglobulinemia, blue fonts patients without hypogammaglobulinemia.

RESULTS

Patient characteristics

We included 9 patients bearing heterozygous germline mutations in the *PTEN* gene. An overview of the mutations in the *PTEN* gene is given in Figure 2. Three patients suffered from hypogammaglobulinemia and six did not have an antibody deficiency. Mutations of most PHTS patients localized near the catalytic domain in the active side of the PTEN protein, irrespective of the presence of hypogammaglobulinemia.

The patient characteristics are summarized in Table 1. We screened 42 patients with Common Variable Immunodeficiency Disorders (CVID) for abnormalities in the *PTEN* gene, but no mutations could be detected (data not shown).

Patients with PTEN mutations and hypogammaglobulinemia

Three patients suffered from hypogammaglobulinemia consistent with the CVID diagnostic criteria; a reduction of two immunoglobulin isotypes and a deceased response to immunization.

Patient 1 is a 40 year old woman who suffered from recurrent 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

Table 1. Characteristics of PHTS patients

A. Sex and age distribution, ***PTEN*** mutation analysis and clinical manifestation

Patient	Sex	Age	*PTEN* Mutation	Clinical manifestations
1	F	43	exon 5: c.389G>C (p. Arg130Pro)	PHTS, CVID
2	F	12	exon 5: c.389G>C (p. Arg130Pro)	PHTS, CVID
3	M	6	exon 5: c.302T>C (p. Ile101Thr)	PHTS, CVID
4	M	6	exon 5: c.464A>G (p.Tyr155Cys)	PHTS
5	M	37	exon 5: c.464A>G (p.Tyr155Cys)	PHTS
6	M	8	exon 2: c.103A>G (p.Met35Val)	PHTS
7	M	7	exon 8: c.877G>T (p.Gly293X)	PHTS
8	F	18	exon 5: c.388C>T (p.Arg130X)	PHTS
9	F	32	exon 5: c.369C>G (p.His123Gln)	PHTS

M; Male, F; Female, PHTS; PTEN Hamartoma Tumor Syndrome, CVID; Common Variable Immunodeficiency.

B. Lymphocyte subsets and antibody levels

Patient ID	B-cells*	T-cells*	CD4*	CD8*	CD4/ CD8 ratio	NK cells*	IgG g/L	IgA g/L	IgM g/L	Specific antibodies
1	0.16	1.37	0.58	0.71	0.82	0.06	4.7	<0.07	0.3	low
2	0.38	1,18	0,53	0,51	1,03	0.26	5.5	0.16	0.65	low
3	0.41	1,77	0,93	0,68	1,38	0.36	1.46	0.07	0.69	low
4	1.01	3,20	1,69	0,95	1,78	1,29	9.0	0.80	0.9	normal
5	0.18	1,01	0,68	0,28	2,41	0,43	14.0	2.60	1.8	normal
6	0.76	2,25	1,25	0,85	1,48	0,16	10.0	0.70	0.5	normal
7	0.91	1,50	0,97	0,42	2,32	0,33	7.0	0.50	1.7	normal
8	0.20	1.28	0.78	0.23	1.83	0.11	12.4	0.82	1.68	normal
9	0.23	1,07	0,70	0,31	2,27	0,12				

* x 10^9/L

in bronchiectasis. 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 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, IgG2 deficiency and specific polysaccharide antibody deficiency. Within two years of follow up, she developed hypogammaglobulinemia at the age of 12 years. She is not receiving immunoglobulin replacement therapy, because of a stable clinical condition.

Patient 3 is a 6 year old boy, who has previously been published as a case of macrocephaly and hypogammaglobulinemia[19] (case 2). This child of non-consanguinous 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. At the age of 21 month a diagnosis of hypogammaglobulinemia was made and immunoglobulin replacement was initiated. At the age of 6 years, a de novo heterozygous *PTEN* mutation was detected.

Patients 4-9. For comparison, we studied six patients with a confirmed clinical and genetic diagnosis of PHTS, but without signs of infections and/or antibody deficiency.

Immunoglobulin levels and lymphocyte subsets

Patients with PHTS with hypogammaglobulinemia showed a decrease of IgG, IgA and specific antibody production (Table 1). Since no other cause for the hypogammaglobulinemia could be identified and patients were older than 2 years of age, the hypogammaglobulinemia fulfilled the CVID diagnostic criteria. Five patients with PHTS had normal levels of immunoglobulins and specific antibodies to vaccination antigens. In one patient immunoglobulin levels were not available, but she never suffered from infections.

Lymphocyte subset levels were in the normal range (Table 1). The CD4/CD8 ratio in PHTS with hypogammaglobulinemia was lower compared to controls and compared to patients with PHTS without antibody deficiency syndrome (data not shown).

Peripheral B-cell homeostasis is disturbed in PHTS

Peripheral B-cell subset distribution was studied by analyzing the relative and absolute B-cell subset size of two naive (Figure 3) and six memory B-cell subsets and plasmablasts

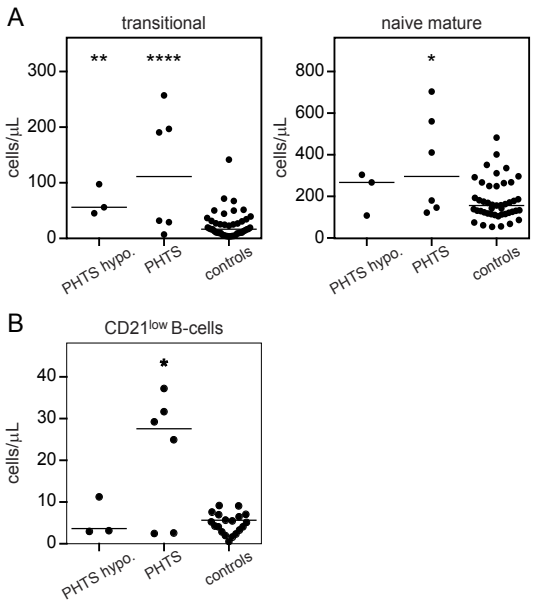

Figure 3. Naive B-cell subsets and CD21^{low} B-cells in PHTS patients. A. Transitional B-cells (CD27-CD38hi CD24hi) and naive mature B-cells (CD27-IgD+CD38lowCD24low) absolute counts **B.** Frequency of CD21^{low} B-cells. Data are compared to normal controls using the Mann-Whitney test. Individual data points are displayed and bars indicate medians. Significant values are indicated: ****, P<0.0001; ***, P=<0.0005; **, P<0.005; *, P<0.05. PHTS hypo; PHTS with hypogammaglobulinemia.

(Figure 4). Two groups of patients with *PTEN* mutations were separately analyzed: patients with hypogammaglobulinemia (PHTS hypogamma) and PHTS without antibody deficiency (PHTS without ADS). Data were compared to 45 normal controls.

Naive B-cell subsets

Absolute counts of transitional B-cells were increased in both groups, indicative of an increased bone marrow output or survival of transitional B-cells. Naive mature B-cells were increased in PHTS without ADS but not in PHTS hypogamma (Figure 3A). The proportion of anergic CD21^{low} B-cells was increased in patients with PHTS without ADS.

In a single patient with PHTS hypogamma (Patient 1), we sorted peripheral B-cell subsets to perform the KREC assay, in order to establish the proliferative history. Transitional B-cells had not proliferated whereas naive mature B-cells showed a slightly increased

proliferation of 3.4 cell divisions (normal 0.7-2.7). Natural effector B-cells showed normal proliferative history of 8.2 cell divisions (normal 7.6-11.3), whereas in memory B-cells proliferations was slightly decreased with 9.3 cell divisions (9.7-13.3).

Decrease of T-cell dependent memory B-cell subsets

Data of six memory B-cells subsets were compared to controls. In PHTS without ADS, the absolute count of T-cell independent CD27-IgA+ memory B-cells was increased (Figure 4A). Analysis of the relative proportions of memory B-cell subsets (Figure 4B) revealed

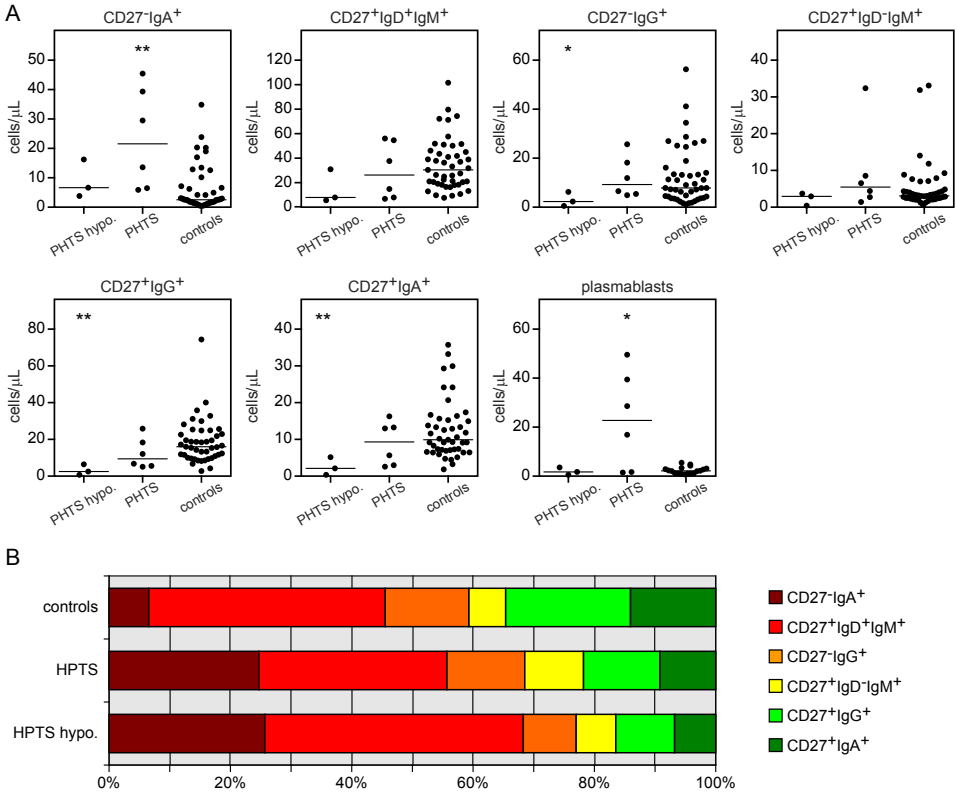

Figure 4. Memory B-cell subset analysis in PHTS patients. A. Absolute counts of six memory B-cell subsets **B**. relative distribution of memory B-cell subset. Data are compared to normal controls using the Mann-Whitney test. Individual data points are displayed and bars indicate medians. Significant values are indicated: ****, P<0.0001; ***, P=<0.0005; **, P<0.005; *, P<0.05. PHTS hypo; PHTS with hypogammaglobulinemia.

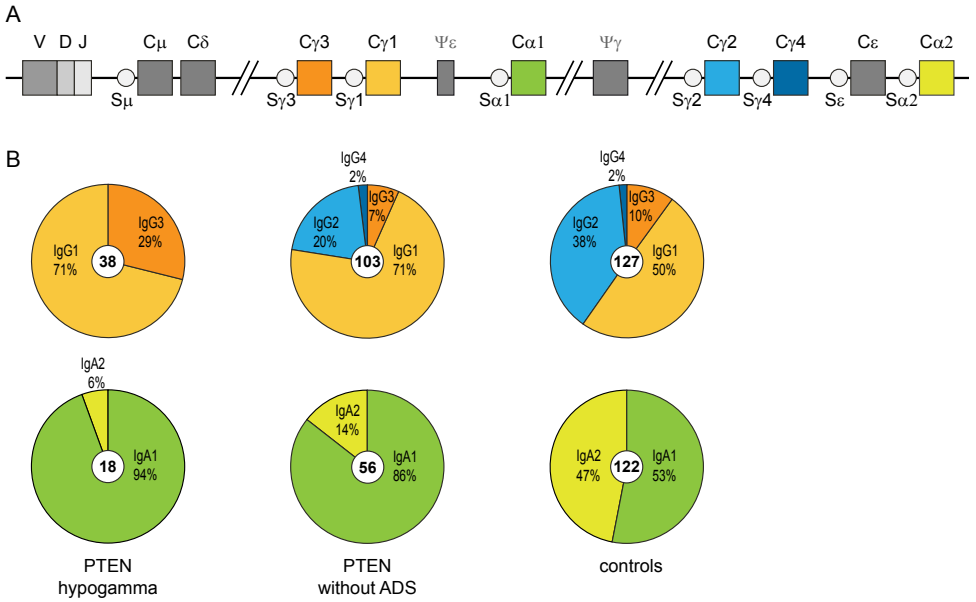

Figure 5. IgA and IgG class switching in *IGH* transcripts of PHTS patients. A. Schematic representation of the constant regions in the *IGH* locus. **B.** Frequencies of *IGHG2* and *4* and *IGHA2* transcripts in PHTS with hypogammaglobulinemia and PHTS without ADS were compared to controls (for details see text). In the center of each plot the number of analyzed transcripts is depicted.

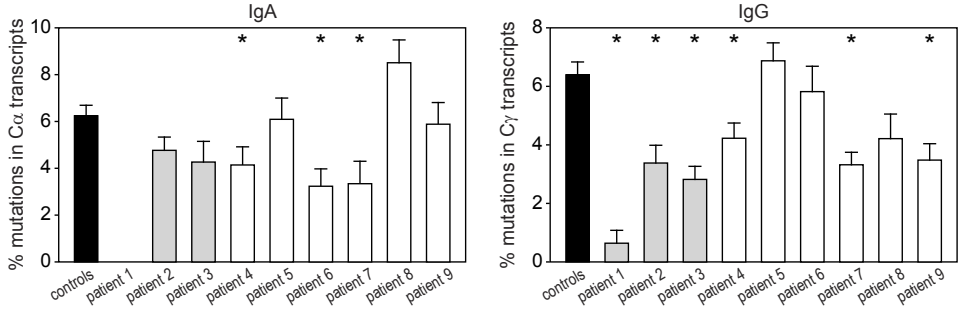

Figure 6. Somatic hypermutation analysis of *IGHG* and *IGHA* transcripts. Grey bars represents the PHTS patients with hypogammaglobulinemia and white bars PHTS patients without ADS. Data are compared to normal controls using the Mann-Whitney test. Significant values are indicated: * P<0.05.

that both in PHTS hypogamma and PHTS without ADS, CD27-IgA+ memory B-cells were increased. In addition, plasmablasts were increased in PHTS without ADS. Absolute counts of CD27-IgG+, CD27+IgG+ and CD27+IgA+ class switched memory B-cells were decreased in PHTS hypogamma patients (Figure 4A). The relative proportions of CD27+IgG+ and CD27+IgA+ B-cells were decreased in PHTS hypogamma as well as PHTS without ADS, suggestive of a germinal center problem in both groups.

In summary, transitional B-cells were increased in PHTS without ADS and PHTS hypogamma patients. PHTS hypogamma patients have low counts of class switched memory B-cells indicative of a CSR problem. The relative proportions of class switched memory subsets were decreased in all PHTS patients, indicating that a sub-clinical class switch recombination deficiency was also present in PHTS without ADS. In contrast to PHTS-hypogamma patients, PHTS patients without ADS were able to generate normal absolute numbers of memory B-cell subset counts and showed an increase of T-cell independent CD27-IgA+ memory B-cells and plasmablasts.

Impaired CSR to IGH-distal IgA and IgG subclasses

We studied CSR at the molecular level by analyzing the IgG and IgA subclass distribution of *IGH* transcripts (Figure 5). In patients with PHTS with hypogamma CSR to IgG2 and IgG4 was decreased compared to controls ($P<0.0001$), which was also the case in PHTS without ADS ($P=0.03$). Similar results were obtained for CSR to IgA2, which was severely reduced PHTS with hypogamma ($P=0.005$) as well as PHTS without hypogamma ($P=0.007$). So irrespective of hypogammaglobulinemia, mutations in *PTEN* cause abnormalities in IgG subclass distribution.

Impaired SHM

The somatic hypermutation frequency was determined by mutational analysis of the V_H3-Cα, V_H4-Cα, V_H3-Cγ, and V_H4-Cγ transcripts (Figure 6). Mutational analyses showed decreased SHM in IgG transcripts of all three PHTS hypogamma patients and in three of six patients with PHTS. For IgA, the decrease in SHM did not reach significance for the PHTS hypogamma group, probably because of the limited number of transcripts that could be analyzed. SHM in IgA transcipts was decreased in three of six patients with PHTS without ADS. So in both groups, abnormalities in somatic hypermutation frequency were present.

Mutations in PTEN does not influence the calcium-flux

Generation of PIP2 activates PLC-γ and BTK upon BCR activation which results in increased levels of intracellular Ca^{2+}(Ca^{2+} flux). However, mutations in the PTEN gene potentially impair the activation of PLC-γ and BTK by a diminished formation of PIP2 and thereby reduce level of intracellular Ca^{2+} upon BCR stimulation[20-21]. So we performed Ca^{2+}

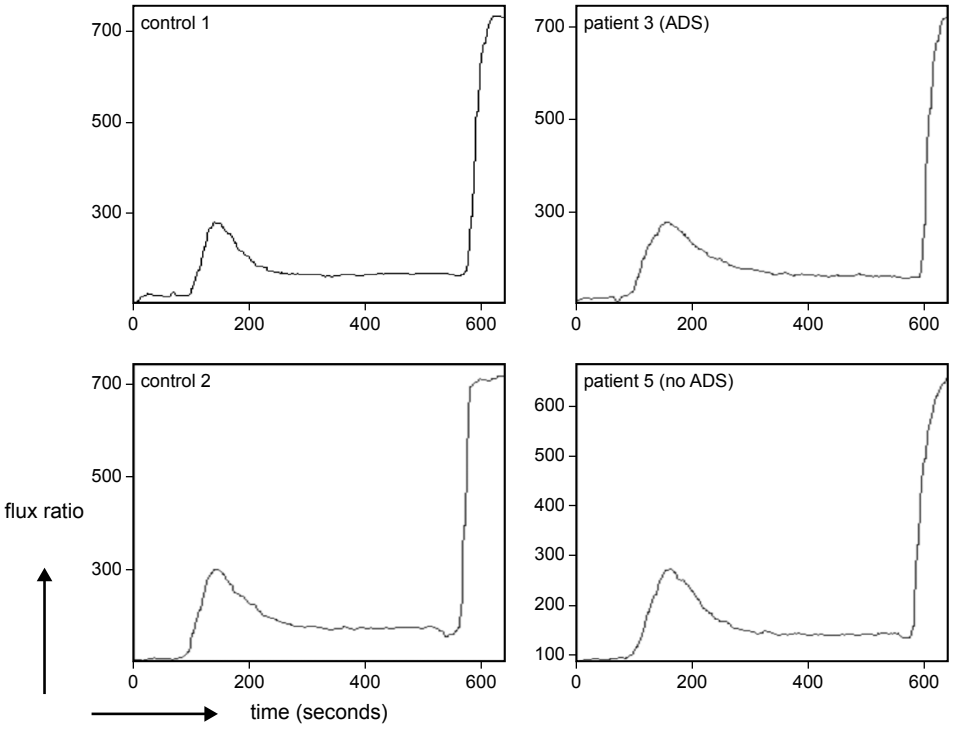

Figure 7. Calcium flux analysis of B-cells in PHTS. PBMC's were incubated with Indo-1 and used to assess the Ca^{2+} fluxes upon BCR stimulation. Free intracellular Ca^{2+} concentrations were determined in CD20-positive B lymphocytes by flow cytometry. Flux ratio's are displayed.

flux experiments to explore this hypothesis (Figure 7). We were not able to detect a defect in Ca^{2+} fluxes in PHTS compared to controls, with respect to the first flux ratio peak and the clearance of intracellular Ca^{2+}. PHTS with hypogammaglobulinemia and without hypogammaglobulinemia showed a similar Ca^{2+} pattern, which is indicative for an active and functional PLC-γ and BTK pathway.

DISCUSSION

Autosomal dominant germline mutations in *PTEN* are associated with PTEN Hamartoma Tumor Syndromes (PHTS). So far, immunodeficiency has not been reported to be part of PHTS, although mice data indicate that mutations in *PTEN* affect B-cell development[8-9, 16]. We identified three patients with PHTS secondary to autosomal dominant germline mutations in *PTEN*, who suffer from hypogammaglobulinemia compatible with the diagnostic criteria of Common Variable Immunodeficiency Disorders. In addition, we studied six PHTS patients without antibody deficiency or infections. PHTS patients appeared to have abnormalities in B-cell development indicative of class switch recombination deficiency and somatic hypermutation deficiency, irrespective of the presence of hypogammaglobulinemia. In PHTS patients with hypogammaglobulinemia these abnormalities were more severe. Similar to observations in mice, Akt mediated inhibition of AID is the most probable cause of the CSR and SHM deficiency and provides an exciting new pathophysiological mechanism for primary antibody deficiency in humans, resulting in a heterogeneous clinical phenotype.

Conditional knock out of PTEN in mice B-cells (bPTEN-/-) has been shown to affect class switch recombination and induce the production of IgM+ antibody secreting cells[8-9]. The disease causing mechanism of these abnormalities is loss of negative feedback of PI3K/Akt activity by decreased PTEN function[8-9]. Increased Akt signaling inhibits AID directly and indirectly by inhibition of FOXO transcription factors[8]. AID regulation might in addition take place at the post-transcriptional level[8]. In turn, increased Akt activity induces the expression of BLIMP1, which favors ASC differentiation[10] (summarized in Figure 1). Our observations in humans with heterozygous germline mutations in *PTEN* fit with observations in bPTEN-/- mice. Evidence for CSR and SHM deficiency in PHTS patients are decreased proportions of class switched memory B-cells, and at the molecular level, impaired CSR to the downstream IgG2 and IgG4 and IgA2 constant regions. Furthermore, impaired somatic hypermutation of *IGH* transcripts, important for affinity maturation of the antibody response, was present irrespective of a clinically apparent antibody deficiency.

If CSR and SHM are impaired in all patients with PHTS, why does only a minority develop hypogammaglobulinemia? We propose that inter-individual differences in any of the multiple factors that regulate PI3K/Akt signaling differentially affect the final outcome of B-cell development. PI3K/Akt signaling is important for precursor B-cell development in the bone marrow, not only by regulating survival and proliferation of B-cells, but also by influencing V(D)J recombination, receptor editing and selection of precursor B-cells (reviewed by Werner et al[11]). The importance of PI3K function at this stage is exemplified by the observation that a missense mutation of the p85α regulatory subunit of PI3K resulted

in an arrest of B-cell development in the bone-marrow and agammaglobulinemia[22]. Another group observed variations in the lymphocyte specific p110δ subunit of PI3K in children with agammaglobulinemia of unknown ethiology[23]. In contrast, bPTEN-/- mice have an expansion of naive B-cells indicative of increased bone marrow output, B-cell proliferation and/or survival[9]. In the periphery, Akt activity is induced by BCR signaling and is negatively regulated by PTEN, SHIP and by inhibitory co-stimulatory signals[10-11]. Taking these mechanisms into account, we observed that patients with PHTS without ADS not only have an increase of transitional B-cells, compatible with increased bone marrow output and/or survival of B-cells, but also have higher naive B-cell counts. These naive B-cells have the potential to respond to antigen and develop in terminally differentiated memory B-cell or ASC. This might explain why PHTS patients without ADS have an impaired proportion of class switched memory B-cells, in the presence of normal absolute counts. In contrast, PHTS hypogammagobulinemia patients have reduced absolute counts of class switched memory B-cells. Furthermore, PHTS patients without ADS have increased numbers of circulating plasmablasts. This preferential differentiation of B-cells in ASC, which has also been observed in bPTEN-/- mice, might prevent the development of hypogammaglobulinemia in the presence of a partial defect of CSR. Apart from differences in PI3K/Akt activity at the B-cell level, differences in T-cell function might be implicated. PI3K signaling regulates the CD4/CD8 differentiation ratio[24], by augmenting the generation of CD4+ T-cells in mice. Furthermore, impaired PTEN function reduces the requirement for CD28 co-stimulation[25]. The level of PI3K/Akt signaling in B-cells as well as T-cells will therefore influence final outcome of terminal B-cell differentiation.

Patients with PHTS with hypogammaglobulinemia have a clinical phenotype fulfilling CVID diagnostic criteria. We checked whether mutations in *PTEN* were present in a cohort of CVID patients, but no mutations could be detected. However, for several reasons we consider PI3K/Akt signaling an attractive disease causing mechanism to explore in CVID patients. 1) heterozygous germline mutations in *PTEN* in mice have been associated with auto-immunity[16, 26], which is a common phenomenon in CVID 2) mice with germline mutations in PTEN suffer from lymphoproliferation[16], which is regularly encountered in CVID 3) an increased risk to develop malignancies is present in PHTS as well as CVID 4) patients with PHTS have been reported to suffer from intestinal nodular interstitial hyperplasia[27], which is commonly encountered in CVID and 5) PHTS is a very heterogeneous condition, which is also the case in CVID. The exploration of increased PI3K/Akt signaling is not only interesting as a potential disease causing mechanism, but might have therapeutic implications[28], since PI3K inhibitors are currently under investigation in clinical trials. In addition, our data show the importance of a thorough clinical evaluation of antibody deficient patients, including the evaluation of dysmorphic features, head circumference and neurodevelopmental status. Another implication of our data is that dysfunctional PI3K/

Akt signaling provides a model for an affinity maturation deficiency in the presence of normal antibody levels, which has so far not been identified as a clinical entity. Finally, the occurrence of recurrent infections in PHTS patients warrants the evaluation for antibody deficiency, since it appears to be part of the clinical spectrum of this syndrome.

In conclusion, autosomal dominant germline mutations in *PTEN* cause CSR and SHM deficiency and are associated with CVID like hypogammaglobulinemia. Increased PI3K/Akt signaling is an attractive disease causing mechanism to explore in CVID, because it is associated with many phenomena observed in this disease, such as lymphoproliferation, auto-immunity, the propensity to develop malignancies, nodular interstitial hyperplasia and clinical heterogeneity. Our data show the importance of a thorough clinical evaluation of antibody deficient patients, including the evaluation of dysmorphic features, head circumference and neurodevelopmental status. In addition, antibody deficiency should be considered in known PHTS patients with recurrent infections.

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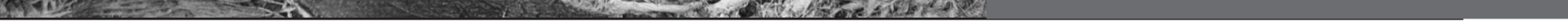

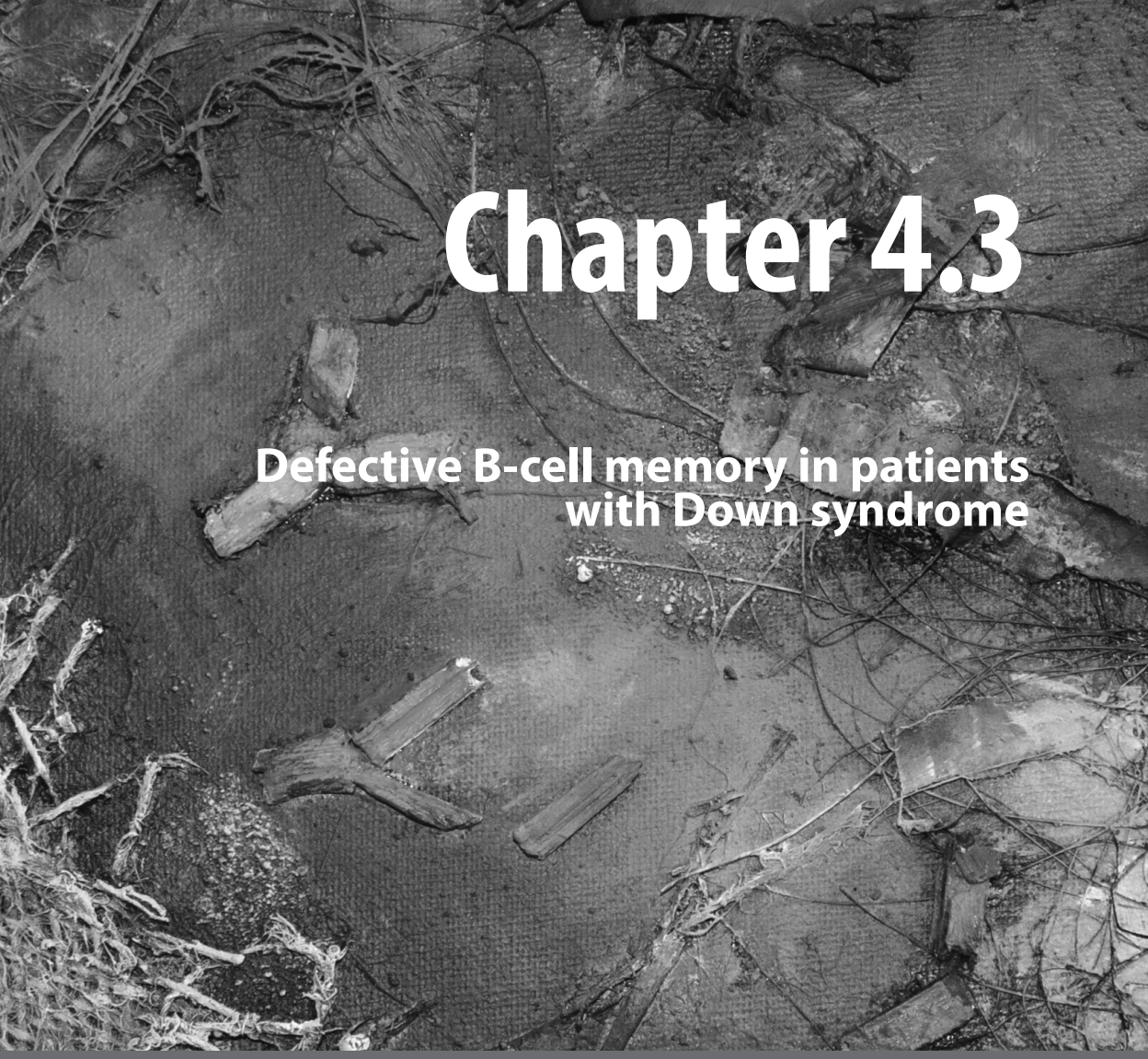

Chapter 4.3

Defective B-cell memory in patients with Down syndrome

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Submitted

ABSTRACT

Background: Patients with Down syndrome carry immunological defects as evidence by the increased risks for autoimmune diseases, hematological malignancies and respiratory infections. Moreover, the low numbers of circulating B-cells suggest impaired humoral immunity. *Objective:* To study how the immune deficiency in Down syndrome results from immunological defects in the B-cell compartment. *Methods:* We studied peripheral B-cell subsets, B-cell subset replication history, somatic hypermutation status, class switch recombination and selection processes in 17 children with Down syndrome. *Results:* Transitional B-cells were normal, but naive mature and memory B-cell numbers were reduced despite slightly increased serum BAFF levels. $CD27^+IgD^+IgM^+$ "natural effector" B-cells showed reduced proliferation and somatic hypermutation levels, while these were normal in $CD27^+IgD^-$ memory B-cells. Furthermore, IgM^+ and IgA^+, but not IgG^+, memory B-cells showed impaired molecular signs for antigen selection. The B-cell pattern was highly similar to that of common variable immunodeficiency patients with a defect in B-cell activation and proliferation. Still, Down syndrome patients had normal serum Ig levels and circulating plasma cell numbers. *Conclusion:* Despite the reduction in memory B-cell numbers, systemic B-cell immunity seems sufficient. However, local IgA and IgM responses are important for mucosal immunity. The observed molecular defects selective defects in circulating IgA and IgM B-cell memory could reflect impaired local responses, which underlie the increased susceptibility to respiratory infections of patients with Down syndrome.

INTRODUCTION

Down syndrome is the most common genetic cause of developmental delay in humans and is associated with numerous health issues.[1] Hypotonia, congenital heart disease and gastro-intestinal malformations are variably present in newborns. In older children and adults, Down syndrome is associated with recurrent respiratory tract infections, hematological malignancies and autoimmune disease, such as celiac disease, hypothyroidism and type 1 diabetes mellitus.[1-3] These clinical features suggest an immune deficiency and indeed, immunological studies in the past have shown many abnormalities.[4] Individuals with Down syndrome have decreased B-cells with lower absolute numbers of $CD21^{high}$, $CD23^{+}$ and $CD27^{+}$ B-cells.[5] Furthermore, the serum immunoglobulin (Ig) levels are affected in Down syndrome with increased IgG1, and decreased IgM, IgG2 and IgG4 levels as compared to age-matched controls.[5] Finally, Down syndrome patients show variable poor Ig responses to vaccines.[4]

Upon antigen stimulation, naive mature B-cells differentiate into memory B and plasma cells. Activated B-cells induce somatic hypermutations (SHMs) in the variable regions of their Ig heavy and light chains. The mutated Ig molecules are subsequently selected for antigen-affinity. In addition, the B-cells can induce class-switch recombination to change the IgH isotype region from IgM into IgG, IgA or IgE. Based on their IgH isotype and expression of CD27, six memory B-cell subsets can be identified in blood that have been derived from three distinct pathways (Figure 1A).[6]

Some of the clinical and immunological features found in Down syndrome resemble common variable immunodeficiency (CVID). CVID is a primary immunodeficiency, characterized by sinopulmonary infections and idiopathic hypogammaglobulinemia.[7] CVID has a heterogeneous pathophysiology, which can be visualized by flowcytometric analysis of the blood B-cell compartment.[8] An abnormal pattern of this compartment can be indicative of: a defect in B-cell production (pattern 1), early peripheral B-cell maturation/survival (pattern 2), B-cell activation and proliferation (pattern 3) or germinal center response (pattern 4). A normal B-cell subset distribution in CVID patients is indicative of a defect restricted to the plasma cell compartment. In addition to memory B-cell defects, a subset of CVID patients carry increased $CD21^{low}CD38^{-}$ B cells. These $CD21^{low}$ B cells are mostly naive and express highly autoreactive antibodies.[9] Moreover, they show decreased responses to antigen stimulation and are more prone to die by apoptosis.[9] It is therefore believed that this autoreactive B-cell population is controlled by anergy.

To study how the immune deficiency in children with Down syndrome results from immunological defects in the B-cell compartment, we performed detailed cellular and molecular analysis of their B-lymphocytes. The results were compared with age-matched healthy controls and the previously described CVID subgroups.[8]

METHODS

Patients

In this study, blood samples and clinical data were collected from 17 children with Down syndrome after written informed consent was obtained from their parents. In addition, we collected blood from 43 healthy age-matched controls and buffy coats from 10 healthy adult blood bank donors. This study was performed according to the Declaration of Helsinki and the guidelines of the Medical Ethics Committees employed by the Jeroen Bosch Hospital and the Erasmus MC.

Flow cytometric analysis of peripheral blood lymphocytes and B-cell subsets

Absolute counts of blood $CD4^+$ and $CD8^+$ T-cells, as well as $CD16/56^+$ NK-cells and $CD19^+$ B-cells were obtained with a diagnostic lyse-no-wash protocol. Furthermore, 8-color flow cytometric immunophenotyping was performed as described before to detect transitional, naive mature, $CD21^{low}$, 6 memory B-cell subsets and plasma cells on a 3-laser FACS LSRII (BD BioSciences; Figure 1B and 1C).[6] Detailed analysis of B-cell subsets was performed with CD25-FITC (2A3), CD80-FITC (L307.4), CD95-FITC (DX2, all from BD Biosciences), CD86-PE (HA5.2B7; Beckman-Coulter) and TACI-biotin (goat polyclonal from PeproTech).

Transitional, naive mature, natural effector and $CD27^+IgD^-$ memory B-cells were high-speed cell sorted from post-Ficoll mononuclear cells on a FACSAria I (BD BioSciences) as described before.[10] DNA was isolated from each sorted subset with the GenElute Mammalian Total DNA Miniprep Kit (Sigma-Aldrich) for replication history and SHM analysis. All fractions were obtained with a purity of >95% as determined by post-sort analysis.

Quantification of BAFF serum levels

BAFF serum levels were measured by ELISA and analyzed in duplicate according to the manufacturer's instructions (R&D Systems).

Molecular analysis of replication history and Ig gene rearrangements

DNA was isolated from each sorted subset with the GenElute Mammalian Total DNA Miniprep Kit (Sigma-Aldrich). The replication history of sorted B-cell subsets was determined with the Kappa-deleting Recombination Excision Circles (KREC) assay as described previously.[10] The frequency of mutated *IGK* alleles was determined with the Igκ restriction enzyme hot-spot mutation assay (IgκREHMA) as described previously.[10,11]

Total cDNA was prepared from mRNA isolated from thawed mononuclear cells as described previously.[6] After reverse transcription using random hexamers, *IGA* and *IGG* transcripts were amplified using family-specific forward primers in the leader sequence of *IGHV3* and *IGHV4* in combination with a Cα (5'-GTGGCATGTCACGGACTTG-3') or a Cγ

(5'-CACGCTGCTGAGGGAGTAG-3') consensus reverse primer.[12] In addition, rearrangements were amplified from DNA of sorted natural effector B cells using the same *IGHV3* and *IGHV4* leader primers and a consensus *IGHJ* primer.[13] All PCR products were cloned into pGEM-T easy vector (Promega) and prepared for sequencing on an ABIPRISM 3130XL. Obtained sequences were analyzed with IMGT database (http://imgt.cines.fr/) and JoinSolver program (http://joinsolver.niaid.nih.gov).[14] IgA and IgG receptor subclasses were determined using the *IGH* reference sequence (NG_001019). Additionally, we analyzed mutation patterns of Ig sequences using Bayesian estimation of Antigen-driven SELectIoN (BASELINe; http://selection.med.yale.edu/baseline/).[15, 16]

Statistics

Statistical analyses were performed using the Mann-Whitney test (SPSS version 18.0), or $\chi 2$ test as indicated in details in Figure legends. A P-value <0.05 was considered statistically significant.

RESULTS

Clinical and basic immunological characterization

Seventeen patients (6 male), aged 7-17 years, with karyotype confirmed diagnosis of Down syndrome were included. Basic clinical and immunological information of these patients is shown in Table 1. Inhaled β2-adrenergic receptor agonists and/or corticoids were used by 6 patients to treat viral induced wheezing; the included patients did not have proven asthma or allergies. The patients had not experienced any serious respiratory tract infections that required admission to a pediatric intensive care unit. Ear, nose and throat (ENT)-problems were common, as expected,[17] as was the prevalence of autoimmune disease.

Similar to previous observations,[5,18] our patients had low numbers of circulating T and NK cells (Table 1), but these were still within the normal range of age-matched healthy controls. B-cell numbers were more severely affected, and were in 10/17 patients below the 5th percentile of the normal range (Table 1). The distribution of Ig serum levels was altered as previously described in Down syndrome.[4,5,19] Thus, the clinical and basic immunological parameters of the patients in our study population were in line with previous studies.[2,4,5,17]

Composition of the blood B-cell compartment in children with Down syndrome

To study the nature of reduced total B-cell numbers, we performed detailed flowcytometric analysis of the peripheral blood B-cell compartment in 13 of the patients with Down syndrome and compared these with 43 age-matched healthy controls (Figure 1).

Table 1. Clinical and basic immunological characteristics of patients with Down syndrome

Patient	Gender	Age (yrs)	Recurrent respiratory infections	Prophylactic antibiotics	History of inhaled medication[1]	Tympa-nostomy tubes	Adeno-tonsillectomy	Hypo-thyroid disease[2]	Lymphocyte subsets (cells/μL)			Immunoglobulin levels (g/L)						
									T cells	B cells	NK cells	IgG	IgG1	IgG2	IgG3	IgG4	IgA	IgM
P1	M	7	Yes	Yes	No	Yes	No	No	1,830	296	340	11.0	8.3	0.93	0.45	0.19	0.84	0.76
P2	M	7	Yes, until age 6 yrs	No	No	No	No	No	1,160	290	150	11.5	9.4	1.25	0.81	0.23	1.23	0.99
P3	F	7	Yes	No	Yes	Yes	No	Yes	1,100	270	120	9.9	8.6	0.67	0.45	0.03	0.76	0.63
P4	M	7	No	No	No	No	No	No	ND	310	ND	11.8	8.7	1.09	1.53	0.12	1.54	1.03
P5	F	8	No	No	Yes	No	No	No	ND	153	ND	11.4	8.5	0.87	1.21	0.03	1.99	0.48
P6	F	8	Yes	Yes	Yes	No	No	No	ND	219	ND	14.2	9.6	2.68	1.50	0.14	1.71	0.48
P7	M	9	Yes, until age 6 yrs	No	Yes	No	No	No	800	87	100	14.8	10.8	1.71	0.89	0.03	1.26	1.36
P8	F	10	Yes, until age 6 yrs	No	Yes	No	No	No	ND	126	ND	11.3	8.1	1.56	1.02	0.23	1.28	0.41
P9	F	10	Yes, until age 8 yrs	No	No	Yes	No	No	980	140	200	10.2	8.1	0.74	0.74	0.12	1.64	0.43
P10	F	11	Yes, until age 8 yrs	No	No	Yes	No	Yes	950	110	110	9.7	7.1	0.63	0.72	0.08	1.84	0.57
P11	F	13	No	No	No	No	Yes	No3	1,330	406	210	12.3	8.3	2.08	1.31	0.28	0.97	0.53
P12	M	14	No	No	No	No	No	No	ND	120	ND	10.5	7.3	2.17	1.43	0.11	2.39	0.63
P13	M	15	Yes, until age 10 yrs	No	Yes	Yes	Yes	No	560	50	80	11.7	8.1	2.02	1.24	0.30	2.43	0.45
P14	F	15	No	No	No	No	No	No3	ND	68	ND	12.5	7.6	3.19	0.52	0.42	1.69	0.58
P15	F	17	No	No	No	Yes	No	Yes	ND	167	ND	12.9	9.0	2.30	0.93	0.37	1.76	0.99
P16	F	17	No	No	No	No	No	No3	1,520	60	100	18.1	14.7	1.79	0.66	<0.01	1.46	0.47
P17	F	17	No	No	No	Yes	No	Yes	1,110	68	80	16.0	10.7	2.79	1.23	0.44	1.84	0.22

None of the children showed signs of celiac disease, diabetes mellitus, hematological malignancies, asthma or allergy. History of use of inhaled β2-adrenergic receptor agonists and/or corticoids.[2] Hypothyroid disease for which thyroid hormone replacement therapy was started and anti-TPO values were increased (>25U/L).[3] Patients with normal thyroid function but increased anti-TPO values (>25U/L). Values of lymphocyte subsets and immunoglobulin levels below and above age related normal values are marked in bold and italic font, respectively. For normal values of lymphocyte subsets see Comans-Bitter et al.[18] For normal values of immunoglobulin levels see Chapter 3.2.

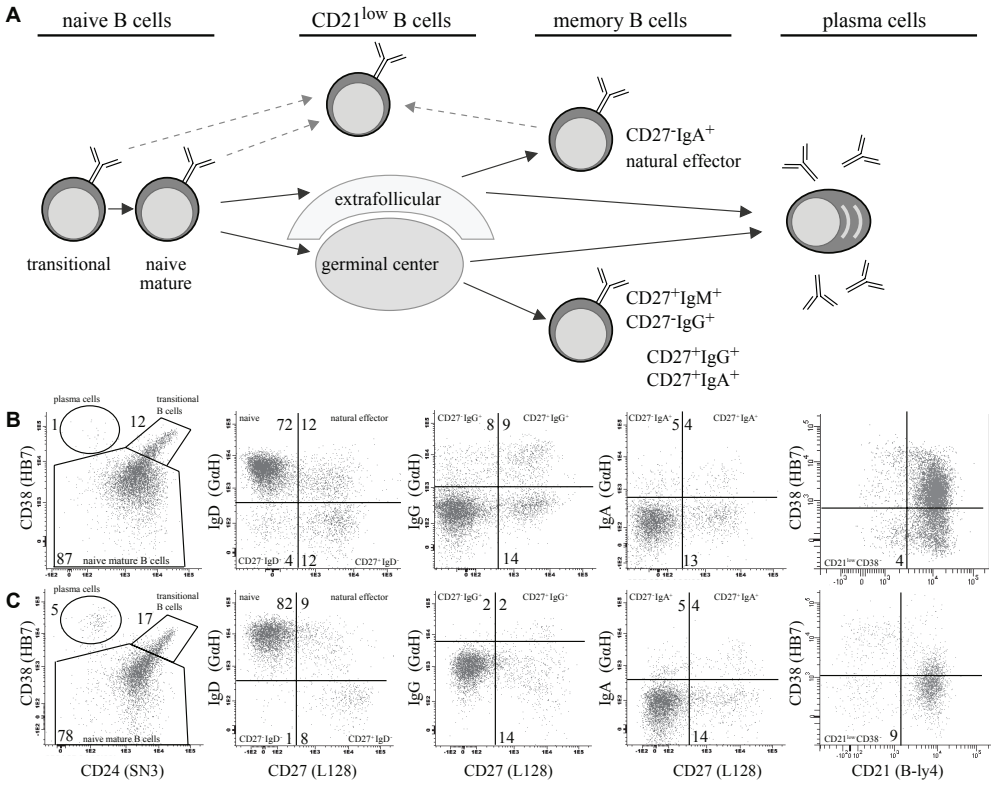

Figure 1. Definition and gating strategy of B-cell subpopulations. A. Differentiation scheme of naive and memory B cells. Naive B-cells are $CD27^-IgM^+IgD^+$. Within this population, early bone marrow emigrants, transitional B-cells, express high levels of CD38 and CD24 and naive mature B-cells are $CD38^{dim}CD24^{dim}$. Six memory subsets are derived from 3 pathways: primary T cell-dependent germinal center reactions ($CD27^-IgG^+$ and $CD27^+IgM^+$), secondary T cell-dependent germinal center reactions ($CD27^+IgG^+$ and $CD27^+IgA^+$) and T cell-independent antigen responses in the splenic marginal zone and gastrointestinal tract ($CD27^+IgM^+IgD^+$ 'natural effector' and $CD27^-IgA^+$). A distinct $CD21^{low}CD38^-$ B-cell population can be identified that can contain cells with unmutated Ig genes derived from naive B cells and cells with mutated Ig genes with a potential memory B-cell origin. B and C. Flow cytometric gating strategy in a representative control (**B**) and a patient with Down syndrome (**C**) to dissect transitional, naive mature and plasma cells within $CD19^+$ B cells and for naive and memory B-cell subsets within $CD19^+CD38^{dim}$ B cells.

The children with Down syndrome had normal numbers of $CD27^-IgM^+IgD^+CD24^{high}CD38^{high}$ transitional B cells, whereas $CD27^-IgM^+IgD^+CD24^{dim}CD38^{dim}$ naive mature B-cells were significantly decreased (Figure 2A). Of the 6 memory B-cell subsets, $CD27^+IgD^-IgM^+$ 'IgM-only' B-cells were normally present, but both natural effector and $CD27^+IgG^+$, $CD27^+IgA^+$,

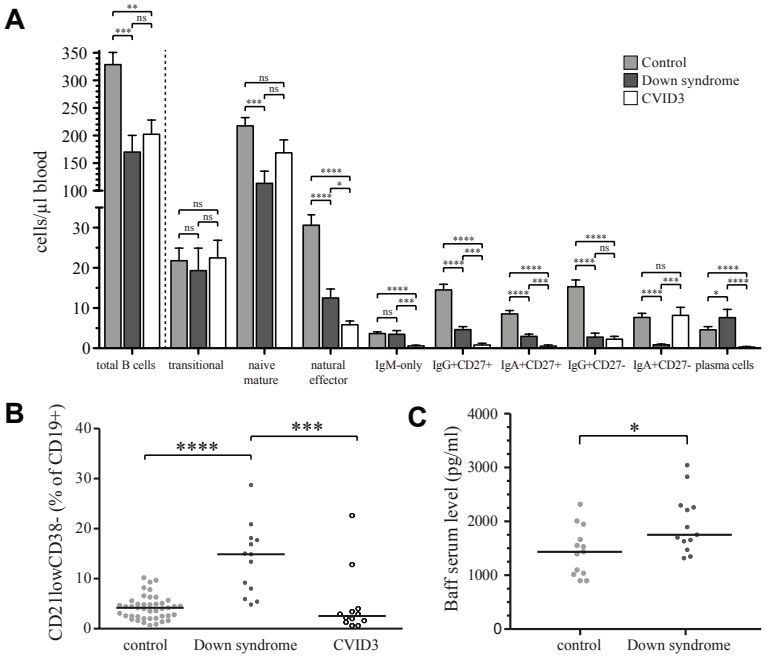

Figure 2. Naive and memory B-cell subsets are affected in patients with Down syndrome. **A**, Absolute numbers of B-cell subsets in 43 healthy controls (light grey bars), 13 patients with Down syndrome (dark grey bars) and 12 CVID patients with a potential B-cell activation and proliferation defect (CVID pattern 3; white bars). **B**, Frequencies of $CD21^{low}CD38^{-}$ B-cells in healthy controls, patients with Down syndrome and CVID3 patients. **C**, BAFF serum levels in 13 healthy controls and 13 patients with Down syndrome. Panels **A-C** include Down syndrome patients P1, P4-P8, P11-P17. Differences between controls and patient groups were statistically analyzed with the Mann-Whitney test: ns, not significant; *, $P<.05$; **, $P<.01$; ***, $P<.001$; ****, $P<.0001$.

$CD27^{-}IgG^{+}$ and $CD27^{-}IgA^{+}$ class-switched memory B-cell numbers were significantly lower in Down syndrome patients than in healthy controls. In contrast, circulating plasma cell numbers were increased in Down syndrome patients. Together with normal to high levels of serum IgG and IgA (Table 1), this indicates that Ig responses can take place in patients with Down syndrome, but these patients seem defective in generation and/or maintenance of T-cell dependent and T-cell independent memory B cells.

Of the 13 Down syndrome patients, 10 had natural effector and/or $CD27^{+}IgD^{-}$ memory B-cell numbers that were below the 5th percentile of the normal range of age-matched controls (See Table 7). Six of these patients also showed reduced naive mature B-cell numbers. Based on the reduced numbers of one or more B-cell subsets, the 10 patients could be assigned to one of the previously identified B-cell patterns in CVID patients: 4

patients displayed pattern 2 (defect in early B-cell maturation or survival; 2 patients with pattern 3 (defect in B-cell activation and proliferation); and 4 pattern 4 (defect in germinal center function).[8] The average composition of the blood B-cell compartment of the whole group of 17 Down syndrome patients showed the highest resemblance with CVID pattern 3 (CVID3; Figure 2A). Natural effector B-cells were more severely reduced in CVID3, while $CD27^+IgG^+$, $CD27^+IgA^+$ and $CD27^-IgG^+$ memory B-cells were equally affected. In contrast to the patients with Down syndrome, IgM-only B-cells were reduced in CVID3, as were circulating plasma cell numbers, while $CD27^-IgA^+$ B-cells seemed unaffected. Thus, both the patients with Down syndrome and patients with CVID3 seem impaired in generation and/or maintenance of B-cell memory. However, the observed distribution of subsets was different between the two groups: patients with Down syndrome seem capable of generating normal numbers of T-cell dependent IgM memory, while CVID3 patients seem normally capable in generating $CD27^-IgA^+$ memory from T-cell independent responses in intestinal mucosa.

$CD21^{low}CD38^{low}$ anergic B-cells

A subgroup of CVID patients with autoimmunity carries increased frequencies of $CD21^{low}CD38^{low}$ B-cells.[20] These cells with functional signs of anergy,[9] are especially increased in CVID patients with a defect in B-cell production (pattern 1). Interestingly, these $CD21^{low}$ cells were also increased in patients with Down syndrome (Figure 2B). This clearly contrasted the CVID3 group, which showed mostly normal frequencies of $CD21^{low}$ B-cells.[8] Thus, patients with Down syndrome seem to carry defects in B-cell activation, similar to CVID3, but their B-cell phenotype is unique, especially with regards to circulating plasma cells and $CD21^{low}$ B-cells.

Serum BAFF levels are not rate-limiting for blood B-cell survival in Down syndrome

The reduced numbers of naive mature and memory B-cells despite normal numbers of transitional B-cells are suggestive of a peripheral B-cell survival defect. Since soluble BAFF is a critical survival factor for mature B-cells,[21] we quantified BAFF serum levels in the Down syndrome patients. Interestingly, the BAFF serum levels were slightly increased in Down syndrome, rather than declined, as compared with age-matched controls (Figure 2C). This indicates that serum BAFF levels are not limiting and might even be increased as a result of reduced usage by the low numbers of mature B-cells.

Distinct phenotypic alterations in IgM^+, IgA^+ and IgG^+ memory B-cells

To study defects in B-cell memory, we first analyzed expression of typical memory markers on B-cell subsets of the patients. Naive mature B-cells from Down syndrome patients seemed normal with low expression of CD80, CD95 and TACI (Figure 3). In contrast to

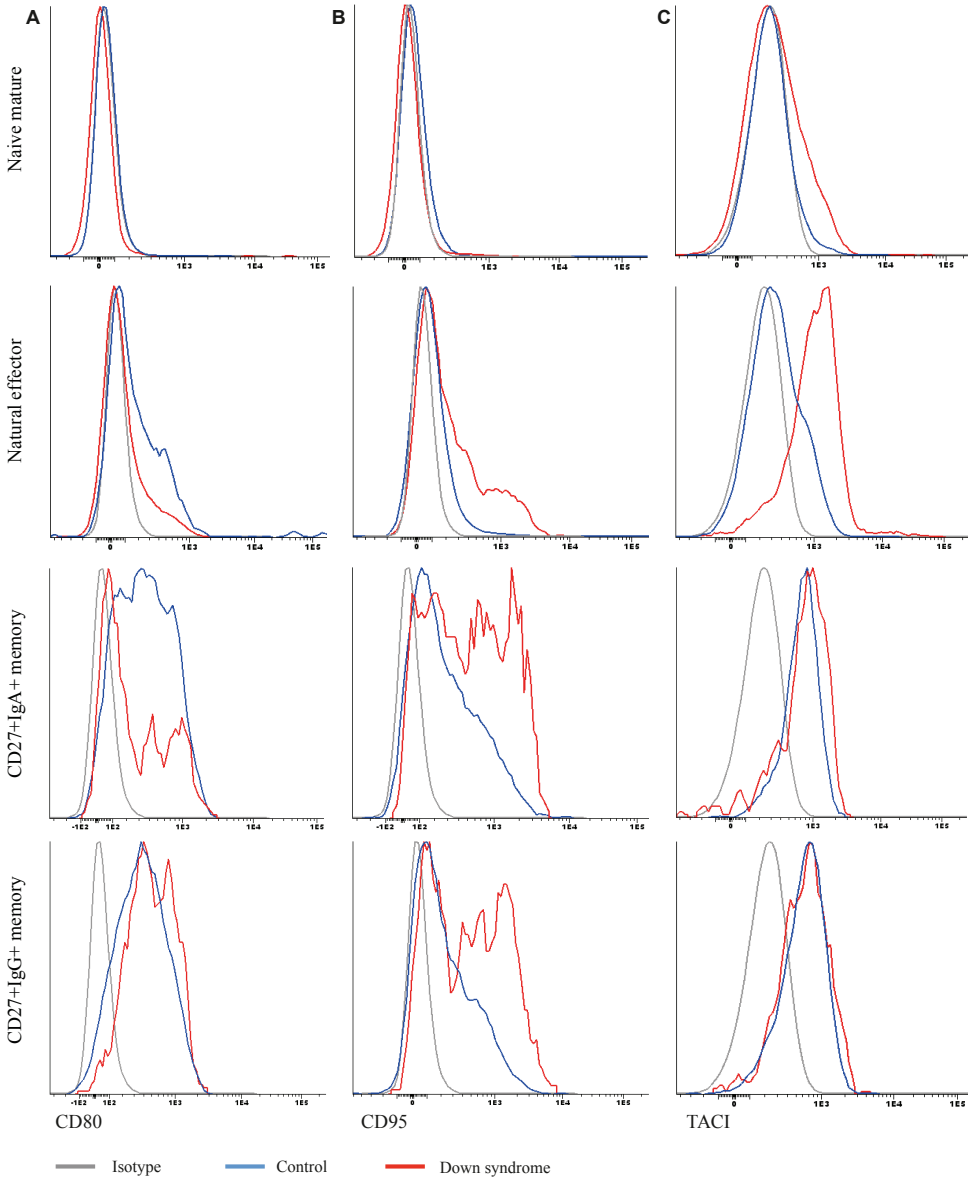

Figure 3. Abnormal phenotypes of memory B-cell subsets of Down syndrome patients. Expression levels of CD80 (A), CD95 (B), and TACI (C) on naive mature, natural effector, $CD27^+IgA^+$ and $CD27^+IgG^+$ memory B cells.

Table 2. Targeting and selection of individual mutations in rearranged IGHV

	Natural Effector				IgA Memory				IgG Memory			
	Control (n=120)		Down (n=98)		Control (n=112)		Down (n=115)		Control (n=129)		Down (n=145)	
Mutated rearrangements (%)	100/120	(83.3)	76/98	(77.6)	111/112	(99.1)	113/115	(98.3)	126 /129	(97.7)	139/145	(95.9)
Transitions (%)	504/909	(55.4)	281/552	(50.9)	997/1850	(53.9)	921/1792	(51.4)	1241/2438	(50.9)	1191/2296	(51.9)
Transversions (%)	405/909	(44.6)	271/552	(49.1)	853/1850	(46.1)	871/1792	(48.6)	1197/2438	(49.1)	1105/2296	(48.1)
Transitions at C•G (%)	298/543	(54.9)	164/311	(52.7)	563/1076	(52.3)	553/1083	(51.1)	743/1432	(51.9)	728/1401	(52.0)
Targeting of C•G (%)	543/909	(59.7)	311/552	(56.3)	1076/1850	(57.7)	1083/1792	(60.4)	1432/2438	(58.7)	1401/2296	(61.0)
RGYW (%)	244.1/909	(26.9)	106.5/552	(19.3)**	483.3/1850	(26.1)	486.4/1792	(27.1)	613.7/2438	(25.2)	606/2296	(26.4)
WRCY (%)	132/909	(14.5)	79.7/552	(14.4)	264.6/1850	(14.3)	250.6/1792	(14.0)	351.7/2438	(14.4)	321.3/2296	(14.0)
WA (%)	131.7/909	(14.5)	74.8/552	(13.5)	252.3/1850	(13.6)	207.3/1792	(11.6)	303.7/2438	(12.5)	303.8/2296	(13.2)
TW (%)	45.2/909	(5.0)	39.1/552	(7.1)	151.9/1850	(8.2)	143.7/1792	(8.0)	159.0/2438	(6.5)	137.9/2296	(6.0)
FR (R/S)	379/212	(1.8)	229/135	(1.7)	719/460	(1.6)	677/494	(1.4)	1065/618	(1.7)	903/600	(1.5)
CDR (R/S)	259/59	(4.4)	145/41	(3.5)	535/134	(4.0)	485/136	(3.6)	596/159	(3.7)	642/151	(4.3)

FR indicates framework region; CDR, complementarity determining region; R/S is the ratio between replacement (R) and silent mutations (S); the number of analyzed sequences is indicated in brackets next to the population name. All analyses were performed with the JOINSOLVER™ program and the differences between controls and patients were analyzed with the χ^2 test. Significant differences ($p<0.01$) are indicated with **.

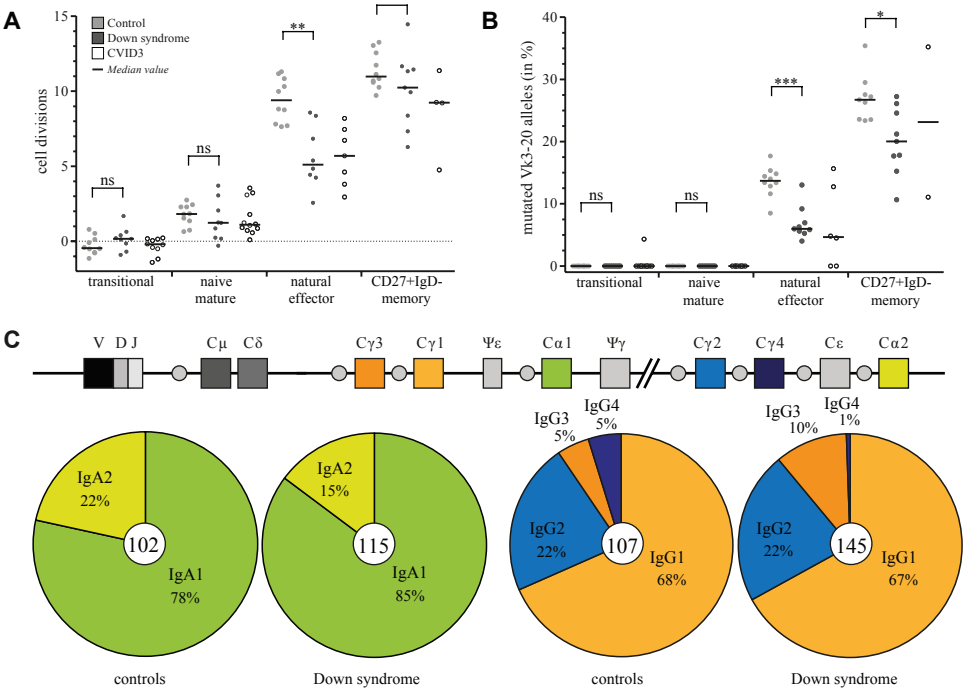

Figure 4. Replication history, somatic hypermutation and Ig subclass usage. A. The *in vivo* replication history of transitional, naive mature, natural effector and CD27$^+$IgD$^-$ B-cell subsets as determined with the KREC assay in patients and controls. **B**. The frequency of rearranged IGKV3-20 alleles with a mutation in a CDR1 hotspot determined with the IgκREHMA assay. Panels A and B include Down syndrome patients P1, P4-6, P8, P11, P12, P14, P15. Each dot represents a subset from a single patient, with healthy controls shown in light grey, patients with Down syndrome in dark grey and CVID3 patients in black outlined circles. The black lines indicate the median value. Differences between controls and Down syndrome patients were statistically analyzed for each subset with the Mann-Whitney test: ns, not significant; *, $P<.05$; **, $P<.01$; ***, $P<.001$; ****, $P<.0001$. **C**, Distribution of IgA and IgG subclass usage in *IGH* transcripts. The total number of analyzed sequences is indicated in the center of each plot. No significant differences were seen in the relative usage of IGHM-proximal IgG1, IgG3 and IgA1 versus IGHM-distal IgG2, IgG4 and IgA2 constant regions. Sequences were obtained from Down syndrome patients P1, P9-11.

CD27$^+$IgG$^+$ memory B-cells, natural effector and CD27$^+$IgA$^+$ memory B-cells showed impaired upregulation of CD80. All three memory subsets show increased upregulation of CD95. While TACI was higher on natural effector B-cells of Down syndrome patients than of controls, it was normally upregulated on IgG and IgA memory B-cells. These phenotypic profiles demonstrate that IgM memory is mostly affected in Down syndrome patients with IgA and IgG to a lesser extent.

Molecular analysis of antigen-driven B-cell maturation in Down syndrome and CVID3

To study the nature of the impaired memory B-cell compartment in patients with Down syndrome, we performed molecular analysis of their replication history, somatic hypermutations and Ig class-switch profiles. Quantification of the replication history with the KREC assay and SHM with IgκREHMA demonstrated neither had occurred in transitional B-cells of Down syndrome patients, CVID3 and controls (Figure 4A and 4B), confirming their status of recent bone marrow emigrants.[10] Naive mature B-cells of Down syndrome and CVID3 patients showed homeostatic proliferation of 1-2 cell divisions in absence of SHM, which was also not significantly different from the healthy controls. Thus, the reduced numbers of naive mature B-cells in patients with Down syndrome do not seem to result from defects in homeostatic proliferation.

Natural effector and $CD27^{+}IgD^{-}$ memory B cells of Down syndrome and CVID3 patients showed proliferation in conjunction with SHM. Still, these levels were significantly lower than healthy controls. Proliferation of $CD27^{+}IgD^{-}$ B cells of Down syndrome and CVID3 patients was similar to controls, but these cells showed reduced frequencies of mutated IGKV3-20 alleles in patients with Down syndrome. IgA and IgG subclass analysis of rearranged *IGH* transcripts of Down syndrome patients revealed no difference in usage as compared to healthy controls. The normal use of *IGHM*-downstream IgG2, IgG4 and IgA2 indicates that Ig class switching to downstream constant regions was not impaired (Figure 4C). Thus, antigen-dependent B-cell maturation is clearly impaired in patients with Down syndrome, especially with regards to natural effector B cells.

Targeting of somatic hypermutations

We further analyzed molecular signs of antigen maturation though sequencing of *IGHV* genes (Figure 5A). In line with the IgκREHMA analysis, this yielded decreased SHM frequencies in natural effector B cells. In contrast to the IgκREHMA assay, SHM frequencies were normal in IgA and IgG memory B cells (Figure 5A), and no differences were seen in SHM frequencies for the various IgA and IgG subclasses (See Figure 6).

To study whether the SHM processes were induced normally in memory B cells of Down patients, we analyzed targeting of mutations to sequence motifs.[22] Natural effector B cells of Down patients had significantly decreased targeting of the RGYW DNA motifs (R = purine, Y = pyrimidine, and W = A or T) that are direct targets of Activation-Induced Cytidine Deaminase (AID) (Table 2). IgA and IgG transcripts showed normal RGYW targeting. Furthermore, natural effector and Ig-class switched memory B cells showed normal transition/transversion ratios, as well as normal WA/TW targeting. Thus, memory B cells of Down syndrome patients showed normal repair of AID-induced lesions, and the only defect appears to be reduced AID activity in natural effector B cells.

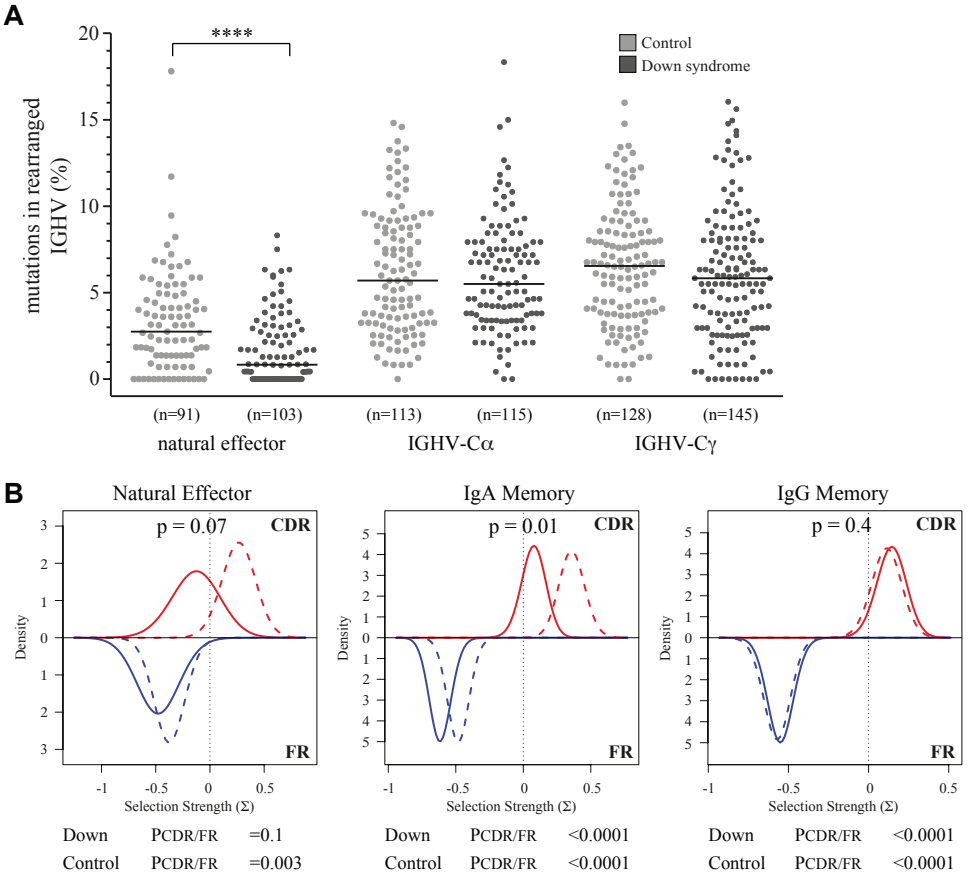

Figure 5. SHM frequency and selection in natural effector, IgA and IgG memory B cells. A. Frequencies of mutated nucleotides in rearranged *IGHV* genes from sorted natural effector B-cells and Ig subclass transcripts of switched IgA$^+$ and IgG$^+$ memory B-cell subsets. Sequences were obtained from Down syndrome patients P1 P8, P11 and P12 for natural effector B-cells and P1, P9-11 for IgA and IgG transcripts. The numbers of analyzed sequences are indicated for each subset. **B.** Selection for replacement mutations in CDR (red lines) and FR regions (blue lines) in natural effector, IgA and IgG memory B-cells of Down syndrome patients as determined with the BASELINe tool.[16] The selection strengths for sequences of healthy controls are shown in dotted lines in each plot. A selection strength >0 is indicative of positive selection. BASELINe analysis was performed on the same sequences as in panel A.

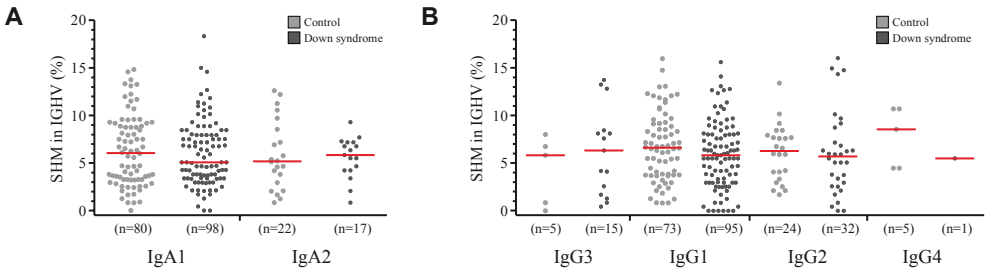

Figure 6. IGHV mutation frequencies of distinct IgA and IgG subclass transcripts. The frequency of mutated nucleotides in IGHV genes are shown for the two IgA subclasses (A) and IgG subclasses (B) are shown. Each dot represents a transcript from a healthy control (light grey) or a patient with Down syndrome (dark grey), with red lines indicating the median value for each category. The analyzed numbers of transcripts are shown in brackets. Sequences were obtained from Down syndrome patients P1, P9-11.

Molecular analysis of Ig selection processes

In healthy individuals, the use of inherently autoreactive IGHV4-34 genes and long complementarity determining regions in IGH (IGH-CDR3) are counter-selected in memory B cells and therefore less frequent than in naive B cells.[6,23-26] Natural effector B-cells of controls and Down syndrome patients showed smaller IGH-CDR3 than naive mature B cells of healthy controls (See Figure 7A). However, we did not observe decreased use of IGHV4-34 in natural effector B cells of either controls or Down syndrome patients (See Figure 7B). IGH-CDR3 sizes and IGHV4-34 use were decreased in IgA and IgG transcripts of both controls and patients as compared with naive mature B-cells (See Figure 7B). Still, the median IGH-CDR3 size of IgA transcripts in Down syndrome patients was significantly larger than in controls. Furthermore, the use of IGHV4-34 was slightly, but not significantly increased in IgA and IgG transcripts of patients with Down syndrome as compared with controls. Thus, despite minor differences with healthy controls, natural effector and Ig class switched memory B cells of patients with Down syndrome showed normal molecular signs of Ig repertoire selection.

In healthy individuals, replacement mutations are favored in CDR, whereas these are negatively selected in FR.[15,16] Initial analysis revealed increased replacement/silent mutation (R/S) ratios in regions as compared to FR in both controls and Down syndrome patients (Table 2). Furthermore, Down syndrome patients appeared to have higher replacement mutation frequencies of amino acids in CDR than in FR, similar to healthy controls (See Figure 8). However, the increased R/S ratios in CDR vs FR do not necessarily reflect selection processes, because the codon usage in CDR differs from FR in their nature to be more susceptible to replacement mutations.[27,28] To study whether the increased R/S ratios in

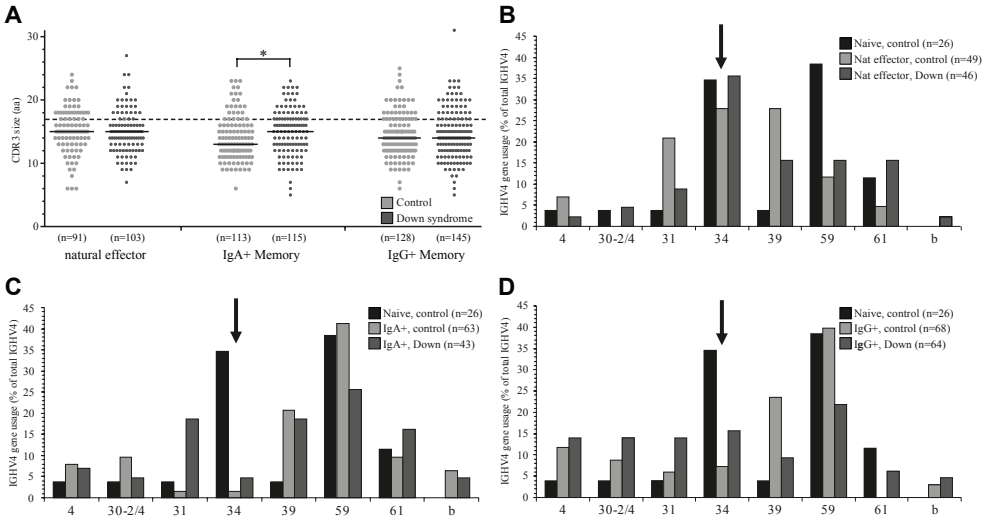

Figure 7. Distribution of replacement mutations in rearranged IGHV genes in memory B-cell subsets. Distribution of replacement mutations in rearranged IGHV genes are determined for natural effector B-cells (A), IgA+ memory B-cells (B), and IgG+ memory B-cells (C) from controls (left) and patients with Down syndrome (right). Each bar represents the frequency of replacement mutations at each amino acid position starting from 20 (first codon following primer sequence) to 104 (last codon of the FR3 region). FR denotes framework region and CDR denotes complementarity determining region. Sequences were obtained from Down syndrome patients P1 P8, P11 and P12 for natural effector B-cells and P1, P9-11 for IgA and IgG transcripts.

CDR of Down syndrome patients really reflected normal selection, we analyzed the *IGH* sequences with the BASELINe program that determines whether the mutation patterns differed from what can be expected from random targeting.[15] Similar to previous observations, we found positive selection for CDR and negative selection for FR in natural effector, IgA and IgG memory B-cells in healthy controls (Figure 5B; dotted lines). The differences between selection in CDR and FR were highly significant. In contrast, natural effector B cells of Down syndrome patients did not show positive selection of replacement mutations in CDR. While IgA and IgG transcripts of Down syndrome patients showed significant selection for replacement mutations in CDR, the selection strength of IgA was significantly lower than in healthy controls. Thus, in addition to the phenotypic profiles, natural effector and IgA memory B-cells showed defects in molecular maturation, whereas IgG memory B cells appeared normal.

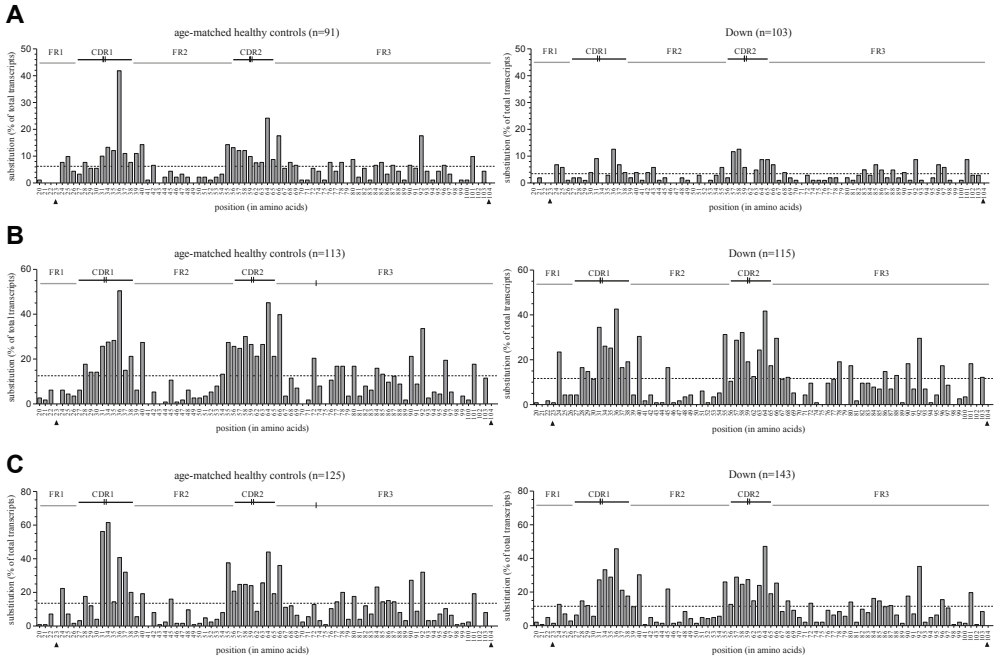

Figure 8. Selection against IGHV4-34 usage and long IGH-CDR3 in natural effector, IgA and IgG memory B cells. (A) IGH-CDR3 size distributions. All individual sizes of healthy controls (light grey) and patients with Down syndrome (dark grey) are indicated as dots with black lines representing the median values. The dashed line represents median value for centroblasts (n=67). Differences between healthy controls and patients with Down syndrome were statistically analyzed with the student's t-test. *p<0.05.

(B-D) IGHV4 gene usage in natural effector (B), IgA (C) and IgG (D) memory B cells of Down syndrome patients. IGHV4 gene usage is displayed as frequency within the total IGHV4 subgroup and in each plot the patients are depicted together with the corresponding subset and with naive mature B-cells from healthy controls. The arrows indicate highlight IGHV4-34 gene usage.

Sequences were obtained from Down syndrome patients P1, P8, P11 and P12 for natural effector B-cells and P1, P9-11 for IgA and IgG transcripts.

DISCUSSION

Through cellular and molecular analysis of the blood B-cell compartment, we showed that B-cell maturation is impaired in patients with Down syndrome. $CD27^+$ memory B cells were reduced in number and displayed impaired proliferation and antibody maturation. The B-cell pattern was reminiscent of a subgroup of CVID patients with a potential defect in

B-cell activation (CVID pattern 3).[8] Still, in contrast to CVID, patients with Down syndrome had normal numbers of circulating plasma cells and Ig serum levels. Thus, in addition to their anatomical and physiological abnormalities of the respiratory tract, Down syndrome patients carry B-cell memory defects that might contribute to the increased frequency of respiratory tract infections.

Our detailed analysis of the blood B-cell compartment in patients with Down syndrome revealed a decrease in naive mature B cells. This decrease can be the result of reduced output from bone marrow, reduced homeostatic proliferation or impaired survival. Reduced B-cell output from bone marrow has been shown to lead to reduced B-cell numbers in patients with Nijmegen Breakage Syndrome or Ataxia Telangiectasia.[29,30] These patients with multisystem DNA-repair disorders display a humoral immunodeficiency due to impaired DNA repair during V(D)J recombination in bone marrow.[29,31] Their decreased bone marrow output is reflected by reduced transitional B-cells and increased homeostatic proliferation of naive mature B-cells. This B-cell pattern was also observed in a subset of CVID patients: pattern 1.[8] However, none of our Down syndrome patients showed this B-cell pattern. It is therefore less likely that their reduced B-cell compartment is the result of impaired bone marrow output.

In addition to production, homeostatic proliferation of naive mature B-cells seemed normal with ~2 cell cycles in absence of SHM. This leaves impaired survival as the most likely cause of the reduced naive B-cell compartment. This is supported by previous studies that showed increased apoptosis of B-cells in patients with Down syndrome.[32,33] However, we found that the critical cytokine for naive B-cell survival, BAFF, was normally present in serum of patients. Therefore, the increased apoptosis is likely caused by other processes. A potential candidate is macrophage migration–inhibitory factor (MIF). This B-cell survival molecule is produced by bone marrow-resident dendritic cells.[34] Circulating dendritic cells were found to be decreased in patients with Down syndrome.[35] Defects in dendritic cells could be associated with impaired production of MIF and underlie the reduced naïve B-cell numbers in Down syndrome patients despite normal bone marrow output and homeostatic proliferation.

On top of decreased naive mature B-cell numbers, circulating $CD27^+$ memory B cells were also reduced in patients with Down syndrome. Both natural effector and IgD^- memory B-cell subsets showed increased expression levels of the FAS receptor (CD95), which is also known as the death receptor that induced apoptosis.[36] Increased CD95 expression levels can tip the balance between BCR-induced survival and CD95-induced cell death, thereby negatively affecting memory B-cell numbers.[37,38]

In addition to CD95 expression levels, natural effector B cells showed impaired proliferation and SHM levels, as well as defective selection for replacement mutations in CDR. These defects indicate that on top of a potential survival defect, Down syndrome patients

are defective in generation of IgM$^+$ B-cell memory. Since IgM responses are important for clearing blood borne pathogens,[39] this defect could underlie the increased susceptibility of patients with Down syndrome to blood borne infections.[40] Recently, MyD88-TIRAP-IRAK4-dependent Toll-like receptor signaling was found critical for generation of homeostasis of natural effector B-cells.[41] Children with genetic defects in these signaling molecules suffer from invasive bacterial infections.[42] However, the natural effector B cells in these immunodeficient children carry normal SHM frequencies.[41] Thus, patients with Down syndrome might have a TLR signaling defect that could contribute to the observed deficiency in natural effector B cells in patients with Down syndrome, but it is unlikely that this is the single cause.

In addition natural effector, IgA and IgG memory B cells were reduced in patients with Down syndrome. Despite normal SHM levels, IgA transcripts showed impaired molecular selection. Considering the role of IgA and IgM in the airways and intestinal tract, these defects might impair local mucosal immunity. Despite reduced numbers, IgG B-cell memory seemed quite normal in phenotype and molecular maturation in the patients that we studied. These features might at least partly explain why patients in this age range (7-17 years) are less susceptible to recurrent respiratory tract infections than very young children of <5 years old.[5,43]

We identified an increased population of CD21^{low} B cells in patients with Down syndrome. These cells were also found increased in multiple immune disorders, including autoimmune diseases such as rheumatoid arthritis, Crohn's disease, and systemic lupus erythematosus, as well as CVID patients with autoimmune phenomena.[9,44] The increase of CD21^{low} B-cells could be related to the increased risk for autoimmune disease in patients with Down syndrome. IGHV4-34 and long IGH-CDR3 are associated with autoreactivity of Ig.[24, 25] We did not observe any increase of these autoimmune susceptibility features in memory B-cells of our patients, since memory B-cells showed reduced IGH-CDR3 sizes and hardly used IGHV4-34 genes. The normal counter-selection against these features therefore indicates that not all autoimmunity selection checkpoints are affected in Down syndrome patients. Although the increased proportions of CD21^{low} cells are in line with findings in other autoimmune diseases, the exact mechanism by which this leads to autoimmunity remains to be determined.

Most of the Down syndrome patients we studied showed abnormalities in their blood B-cell compartment that fitted with one of the previously published CVID patterns.[8] Despite the defects in memory B cells, circulating plasma cells and serum Ig levels were normal in Down syndrome patients. Because defects in Ig levels are the hallmark of CVID, Down syndrome patients do not meet the CVID criteria as defined by the European Society for Immunodeficiencies (ESID) and Pan American Group for Immunodeficiency (PAGID; criteria available on www.esid.org). Still, the reduced memory B cell numbers in Down syndrome

patients were suggestive of a defect in B-cell activation and proliferation (CVID pattern 3). Together with the impaired molecular maturation of IgM and IgA memory, these defects in B-cell memory might have implications for subsequent encounters with the same pathogen and hence the susceptibility to recurrent infections. Thus, our study indicates that analysis of immune-competence in Down syndrome patients should included analysis of (mucosal) IgA and IgM responses.

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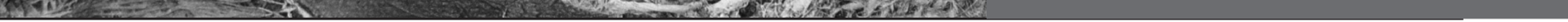
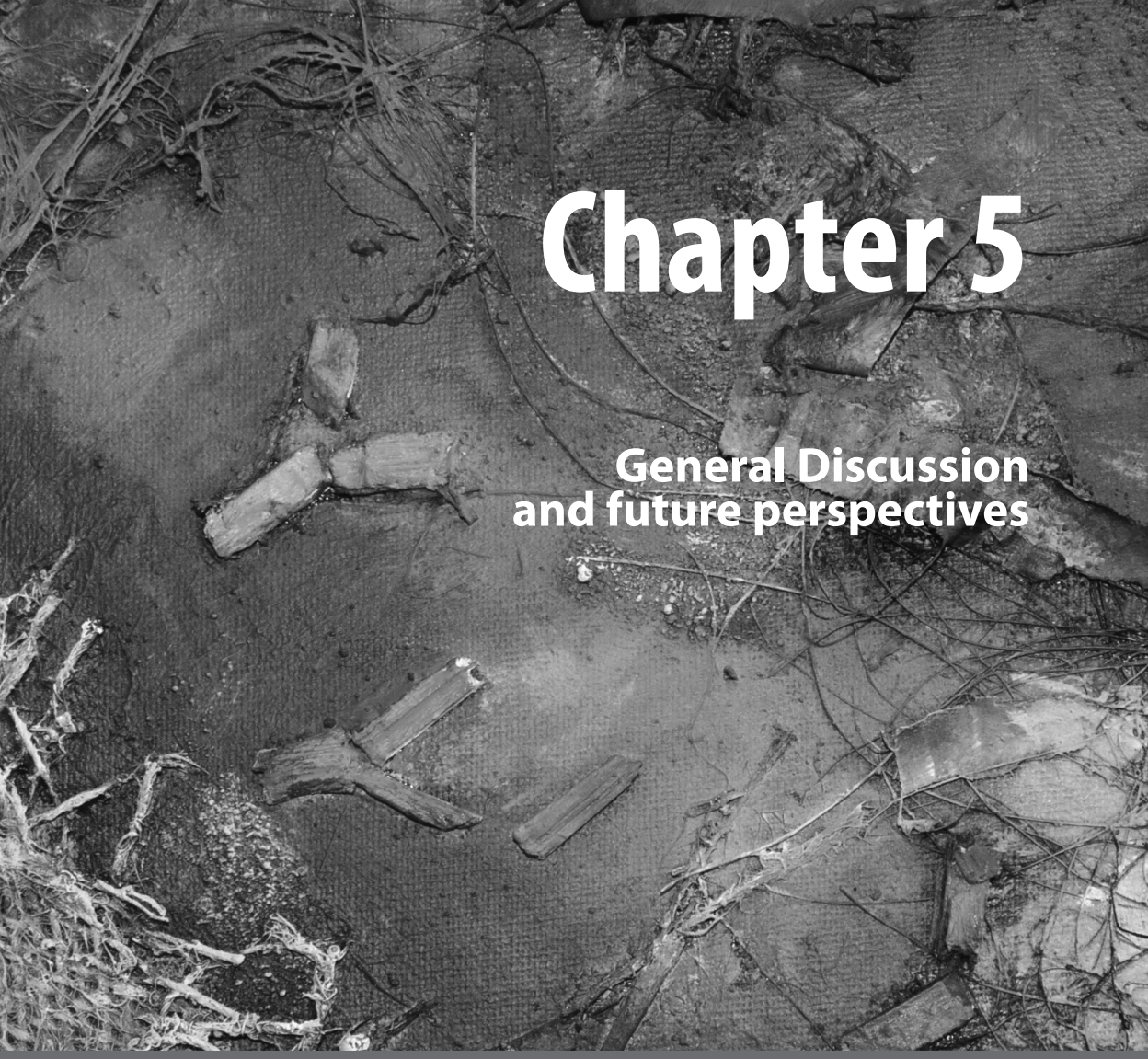




Chapter 5

General Discussion and future perspectives

Review in preparation

GENERAL DISCUSSION

Primary antibody deficiencies are characterized by a defect in the production of antigen specific antibodies and are the most prevalent primary immunodeficiencies[1], resulting in a wide range of infectious and non-infectious clinical complications and a decreased life expectancy[2-5] (see Chapter 1 for an overview). Most primary antibody deficiencies are idiopathic, which implies that the underlying genetic defect and disease causing mechanism are yet unknown. The aim of this thesis was to explore the immunobiology of primary antibody deficiencies, with a focus on Common Variable Immunodeficiency Disorders (CVID), CVID-like disorders, and a selected number of conditions associated with genetic or chromosomal defects, such as Ataxia Telangiectasia, PTEN Hamartoma Tumor Syndrome, and Down Syndrome. Knowledge of normal B-cell development is crucial to understand aberrancies[5]. Therefore, a detailed characterization of peripheral B-cell maturation was performed, including the characterization of six memory B-cell subsets[6].

DISTINCT MATURATION PATHWAYS OF MEMORY B-CELLS.

Memory B-cells and plasma cells are the end stages of B-cell development[7]. In order to understand aberrant B-cell development in patients with antibody deficiencies, it is important to characterize normal differentiation and maturation pathways. Chapter 2 delineates memory B-cell development into six memory B-cell subsets, deriving from different T-cell dependent and independent maturation pathways[6]. Recently, Berkowska *et al.* characterized two additional CD27+IgE+ and CD27-IgE+ memory B-cell subset (Berkowska *et al.*, submitted), which will not be discussed here.

CD27+IgD+IgM+ natural effector or marginal zone like B-cells have been shown to predominately develop in a T-cell independent way in the marginal zone of the spleen, but part of the CD27+IgD+IgM+ memory B-cells likely originate from the germinal center (GC), since these cells are decreased in patients with CD40L deficiency (Chapter 2) and in part carry signs of GC passage[8]. Ongoing studies aim to identify phenotypic markers to distinguish the T-cell dependent (GC) fraction from the T-cell independent (non-GC) fraction of this memory subset. The replication history and SHM status of CD27-IgG+ and CD27+IgD-IgM+ memory B-cells are compatible with primary GC responses, whereas CD27+IgG+ and CD27+IgA+ B-cells show the highest number of cell divisions and somatic mutations, reminiscent of multiple (consecutive) GC passages. Molecular characterization showed that CD27-IgA+ memory B-cells predominantly originate from T-cell independent primary immune responses in the mucosa, as exemplified by their limited replication history, low

Table 1. Definitions of CVID classifications and associations with clinical complications.

Classification	TRECs^	KRECs^	Total B-cells	Transitional B-cells	Naïve mature B-cells	CD27+ IgM+IgD+	CD27+IgM-IgD-	CD21^{low} B-cells	AI	SPL	GR	LymP	OI
Freiburg[9]# n=30													
Ia							<0,4% of lymphocytes	>20% of B-cells	+	+	-	-	-
Ib							<0,4% lymphocytes	<20% of B-cells	-	-	-	-	-
II							>0,4% lymphocytes		-	-	-	-	-
Paris[10]# n=57													
MB0						<11% CD27+B-cells	<11% CD27+B-cells		-	+	+	+	-
MB1						Normal§	</= 8% of B-cells		-	+	-	-	-
MB2						Normal in most	Normal§		-	-	-	-	-
Euroclass[11]# n=303													
B-			B-cells <1% lymf						-	-	-	-	-
smB-							<2% of B-cells		-	+	+	-	-
smB+							>2% of B-cells		-	-	-	-	-
smB-Trhi				>/=9% of B-cells			<2% of B-cells		-	-	-	+	-
smB-/CD21lo							<2% of B-cells	>10% of B-cells	-	+	+	-	-
B-cell pattern* n=37 Rotterdam[14]													
Pattern 1				**<5th perc.**	>5th perc.	</> 5th perc.	**<5th perc.**		+($)	+	+($)	-	-
Pattern 2				>5th perc.	**<5thperc.**	**<5th perc.**	**<5th perc.**		+($,&)	-	+($,&)	+&	-
Pattern 3				>5th perc.	>5th perc.	**<5th perc.**	**<5th perc.**		-	-	-	-	-
Pattern 4				>5th perc.	</>5th perc.	>5th perc.	**<5th perc.**		-	-	-	-	-
Pattern 5				</>5th perc.	</>5th perc.	>5th perc.	>5th perc.		-	-	-	-	-
KREC/TREC[13] n=40													
A	detect	detect							-	-	-	-	-
B	detect	undetect							-	-	-	-	-
C	undetect	detect							-	-	-	-	+
D	undetect	undetect							+	-	-	-	+

AI auto-immune cytopenia, SPL splenomegaly, GR granulomatous inflammation, LymP lymphoproliferation, BR bronchiectasis. OI Opportunistic infections. ^in whole blood. #based on B-cell subset relative proportions, not age matched. & according to Piatosa et al[23], who used a similar approach using relative proportions instead of absolute counts. *based on age matched B-cell subset absolute counts. + significant association with clinical complication. $ in a combined analysis of CVID1 and 2. detect = detectable, undetect = undetectable, perc. = percentile of age matched normal controls. § normal = >-2SD of controls.

somatic hypermutation (SHM) levels and use of IgA2 and Igλ. These memory B-cells numbers are mostly not affected in patients with CVID (Chapter 3.2).

In Chapter 3.2 we describe the dynamics of memory B-cell subsets counts in different age groups. An important finding is that the absolute numbers of class switched CD27+IgG+ and CD27+IgA+ memory B-cells reach already normal values in the first year of life, whereas the relative proportions of these CD27+ memory B-cell subsets show an increase throughout childhood, which mainly reflects the declining absolute number of transitional and naive mature B cells. Analysis of the memory B-cell compartment can therefore most reliably be done by evaluating absolute cell numbers rather than relative proportions.

B-CELL CLASSIFICATIONS FOR CVID

Over the last decade several CVID B-cell classifications have been proposed, which divide cohorts of at least 25 CVID patients in subgroups with similar immunophenotypic B-cell characteristics. These classification (in part) correlate with clinical complications (summarized in Table 1)[9-13]. This thesis introduces a novel strategy of classifying CVID patients in subgroups, based on flow cytometric peripheral B-cell maturation pathways and related functional molecular analysis[14]. Our novel B-cell maturation-based classification uses a different approach compared to the currently existing CVID classifications[14], because it encompasses a more complete analysis peripheral B-cell maturation pathways to get better insight in B-cell defects in CVID. Therefore, the subgroups in our classification reflect distinct pathophysiological backgrounds of the aberrant peripheral B-cell maturation.

The hallmark of the earlier CVID classifications is analysis of (a few) peripheral B-cell subsets, with an emphasis on the proportion of CD27+ switched memory B-cells in peripheral blood, which is used as the most important parameter in the Freiburg[9], Paris[10] and the EUROclass consensus classification[11]. Apart from the proportion of CD27+ switched memory B-cells, the Freiburg classification includes the proportion of CD21^{low} anergic[15] memory B-cells[9]. In addition, the Paris classification includes the proportion of CD27+IgM+IgD+ marginal zone like or natural effector memory B-cells[10]. Finally, the EUROclass consensus classification includes both CD21^{low} B-cells and an increased proportion of transitional B-cells[11] as additional parameters.

In contrast to the earlier CVID classifications, we introduced a CVID B-cell classification based on the comparison of complete peripheral B-cell maturation pathways, including the precise definition of blood B-cell subsets compared to age-matched reference values of absolute B-cell subset counts and molecular characteristics of individual B-cell subsets.

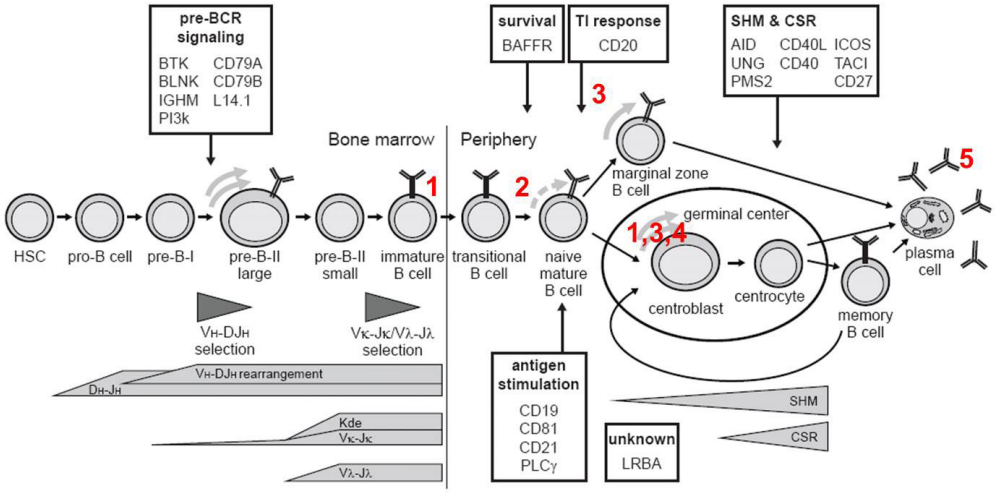


Figure 1. Overview of peripheral B-cell development, reported genetic defects and B-cell differentiation blocs per B-cell pattern. The proposed blocs in B-cell differentiation for the B-cell patterns 1-5 are depicted in red. Genetic defects are reported in the text boxes.

The classification includes four peripheral B-cell subsets: transitional B-cells, naive mature B-cells, CD27+IgM+IgD+ marginal zone like memory B-cells and CD27+IgD- memory B-cells. This approach revealed 5 major B-cell maturation patterns (for details see Chapter 3.1 and Figure 1), including a newly recognized aberrant maturation pattern characterized by a reduction of transitional B-cells as well as CD27+IgD- memory B-cells (B-cell pattern 1). In addition, we systematically performed molecular analysis of B-cell proliferation and SHM at the B-cell subset level, demonstrating that the newly identified B-cell maturation patterns represent rather homogenous CVID subgroups with distinct pathophysiological backgrounds.

The immunological homogeneity of the subgroups is this new classification is exemplified by the characteristic increase of naive B-cell proliferation in B-cell maturation pattern 1, the impaired B-cell proliferation and SHM of marginal zone like B-cells in B-cell maturation pattern 3 and the absence of proliferation and SHM abnormalities in patients with a normal peripheral B-cell distribution (B-cell maturation pattern 5).

Furthermore, we showed that the five B-cell maturation patterns in CVID patients are compatible with those of patients with genetic defects known to cause hypogammaglobulinemia. We show in chapter 4.1 that B-cell maturation pattern 1 resembles the B-cell

phenotype of patients with AT and NBS[16] and therefore most likely results from a B-cell production defect in combination with a CSR defect. Because increased radiosensitivity has been reported in CVID[17-18], DNA repair disorders might be a causative factor in some of these patients. Another example is the immunophenotype of patients with B-cell maturation pattern 2, which resembles BAFF-R deficiency[19] and as such is compatible with an early B-cell maturation or survival defect. We showed that B-cell pattern 3 is compatible with the B-cell subset distribution in CD19 deficient patients[20] and might therefore result from B-cell activation defects. Finally, we propose that B-cell pattern 4 and 5 are compatible with GC defects and defects in terminal plasma cell differentiation and/or survival, respectively. So the B-cell maturation patterns presented in this thesis describe the pathophysiological background of CVID and point to different defects in peripheral B-cell maturation. Table 2 summarizes the available functional immunological data associated with other CVID classifications. These data show that CVID patients belonging to group Ia of the Freiburg classification (decreased CD27+IgD- B-cells and increased $CD21^{low}$ B-cells) have an impaired BCR mediated Ca^{2+} flux, indicative of B-cell anergy[21]. In addition, CD76 and CD80 expression was impaired in the presence of low switched memory B-cells, pointing towards a GC defect[22]. Comparison of the Freiburg classification to our B-cell pattern classification shows that group Ia shows some overlap with B-cell pattern 1. Comparison with the EUROclass consensus classification reveals that most patients with B-cell maturation pattern 1 are smB-$CD21^{lo}$ (switched memory B-cells < 2% of B-cells and $CD21^{low}$ B-cells >10% of B-cells). However, in contrast to Freiburg Ia and EUROclass smB-$CD21^{lo}$ subgroups, our approach provides more consistent functional evidence for the homogeneity of the defined CVID subgroup. The same applies for comparison of our B-cell maturation patterns with the Piqueas or Paris classification. Functional data supporting the Paris classification is scarcely available. A small number of patients classified as MB1 (low CD27+IgD- memory B-cells and a normal proportion of CD27+IgD+IgM+ B-cells) have decreased SHM in CD27+IgD+IgM+ memory B-cells.[10] Our B-cell maturation pattern classification more sharply defines B-cell subset abnormalities than the Paris classification. We showed that impaired SHM as well as impaired B-cell proliferation in marginal zone like or natural effector B-cells are characteristics of B-cell pattern 3, in which marginal zone B-cells as well as CD27+IgD- memory B-cells are decreased. Because GC defects, such as CD40L deficiency, can also result in low CD27+IgD+IgM+ memory B-cells, our B-cell maturation pattern classification can probably not sharply divide patients with B-cell activation and proliferation defects from patients with primary GC defects, since both defects can result in low marginal zone like B-cells and CD27+IgD- memory B-cells and hence B-cell maturation pattern 3. Patients with B-cell maturation pattern 5 showed a normal maturation of CD27+IgD+IgM+ and CD27+IgD- memory B-cells and no abnormalities in B-cell proliferation and SHM, indicative of a terminal plasma cell differentiation or maturation

Table 2. Functional data supporting immunological CVID classifications based on B-cell immunophenotyping.

	B-cell stimulation assays	B-cell subset proliferation by KREC	B-cell proliferation in PBMCs by KREC	SHM B-cell subsets	Suggested B-cell defect
Freiburg[9]					
Ia	Decreased Ca flux in B-cells, decreased upregulation of CD70 and CD86 on B-cells				GC
Ib	Decreased upregulation of CD70 and CD86 on B-cells				GC
II					Terminal plasmacel differentiation, survival and/ or homing, increased metabolism of Ig
Paris[10]					
MB0			normal^		GC, antigen driven selection of B-clymphocytes
MB1			increased^	decreased in CD27+IgD+ (4 patients)	GC
MB2			increased^		"Immunoglobulin production" problem
Euroclass[11]					
B-					Precursor B-cell problem
smB-					GC
smB-Trhi					GC
smB+/smB-CD21lo					
Rotterdam[14]					
Pattern 1		increased in naive			B-cell production and GC defect
Pattern 2		normal in naive			Early B-cell maturation or survival defect
Pattern 3		decreased in CD27+IgD+		decreased in CD27+IgD+	B-cell activation and proliferation (GC)
Pattern 4		normal			GC
Pattern 5		normal		normal	Plasma cell differentiation and homing (post GC)

^data of Serana et al 2011, GC = Germinal Center

defect. We showed that these patients were all EUROclass smB+ (switched memory B-cells >2% of B-cells), but the other half of the smB+ patients in our cohort still showed absolute reductions in CD27+IgD- memory B-cells. Functional data exploring the pathophysiology of the subgroups of the EUROclass classification is not available.

In February 2013, Piatosa *et al.* published a paper in which they classified children with CVID according to age-matched peripheral B-cell subset size[23-24]. The subgroups they propose are strikingly similar to our B-cell maturation pattern classification (Table 1), with the only difference that they used age-matched relative proportions of B-cell subsets, rather than absolute counts. No functional data are provided to support the proposed classification. However, similar to our observations, evaluation of clinical complications revealed that, patients with an early B-cell maturation block were at significantly greater risk of granuloma formation and auto immune cytopenia's. Furthermore they reported an increased incidence of enteropathy and lymphoproliferation in this group. The observed abnormalities in B-cell subsets were stable over time.

A limitation of each CVID classification is the impossibility to sufficiently capture the whole range of heterogeneity in a single classification system without creating too many subgroups. So within defined "homogenous" subgroups there will always be some degree of heterogeneity. Furthermore, it should be noted that heterogeneity is also found in patients with identical immunological or genetic defects (as demonstrated in Chapter 4), because of the variable severity of the defect or other unknown factors, leading to hypomorphic clinical presentations. Since variability in CVID classification has been reported while evaluating one patient at different time points[25], results should be interpreted with care. Finally, several functional defects in B-cell development have been reported that could not directly be linked to a specific immunophenotype. Such defects include B-cell calcium signalling[26] and TLR9 signalling[27-29].

In conclusion, our B-cell maturation-based CVID classification is unique in using absolute counts of four age matched B-cell subset as starting point, in combination with molecular characteristics of individual B-cell subsets. Although no CVID classification can tackle the extensive immunological and clinical variability observed, the homogeneity of the B-cell maturation-based CVID subgroups is superior to the other B-cell CVID classifications, particularly because it is based on full B-cell maturation patterns instead of a few B-cell subsets and because it is supported by functional data and compatible with observations in patients with known genetic defects. Our B-cell maturation-based CVID classification might therefore be a valuable tool for further research in the pathophysiology of CVID and related disorders.

Table 3. Immunophenotyping and functional characterisics of T-cells in relation tot CVID classifications

	CD4 counts	Fas on CD4	Naïve CD4 T-cells	early thymic emigrants	HLA-DR on CD4 and CD8	T-cell repertoire	TRECs in PBMCs	T-cell replication by Ki67	spontaneous apoptosis of lymphocytes	IFN-γ production
Freiburg[9]										
Ia			decreased in some							
Ib										
II										
Paris[10]										
MB0	decreased	increased	decreased		increased		increased			
MB1		increased	decreased		increased					
MB2	decreased						decrease			
Rotterdam[14]										
Pattern 1	normal		decreased				decreased#			
Pattern 2	normal		decreased				decreased#			
Pattern 3	normal		normal				normal#			
Pattern 4	normal		normal				normal#			
Pattern 5	normal		normal				normal#			
KREC/TREC[13]										
A										
B										
C			decreased							
D			decreased							
Giovanetti[12]										
I	Decreased?	increased	<15% of CD4 T-cell	strong decrease	increased	restricted	decreased	increased	stongly increased	increased
II	?	increased	16-29% of CD4 T-cells	decreased					increased	increased
III	?	increased	>30% of CD4 T-cells	decreased					increased	normal

#unpublished results

T-CELLS, TRECs AND KRECs TO CLASSIFY CVID

T-cells

Giovanetti *et al.* advocate that T-cells play a key role in the pathogenesis of CVID[12] and that T-cell immunophenotyping should be used to classify CVID. The T-cell classification of Giovanetti *et al.*[12] combines flow cytometry with functional immunological T-cell analysis and shows that the CVID subgroup with a reduction of naive CD-4 T-cells have a reduced thymic output, an oligoclonal T-cell repertoire, increased T-cell activation markers, increased interferon-gamma production, and an increased occurrence of spontaneous lymphocyte apoptosis (summarized in Table 3).

The reduction of naive CD4 T-cells has been observed by other authors and is associated with the auto-immune cytopenia and polyclonal lymphocytic proliferation clinical phenotypes[30]. Comparing the classification of Giovannetti *et al.* to our B-cell patterns, it appears that B-cell pattern 1 and 2 are strongly associated with decreased naive CD4+ T-cells and as such overlap with the subgroup I of Giovannetti *et al.* (Table 3). Since low transitional B-cells and an increased proliferation of naive mature B-cells are the hallmark of B-cell pattern 1, it seems that B-cells and T-cells are equally affected. The oligoclonal T-cell repertoire with decreased thymic output and low transitional B-cells is identical to our observations in patients with Ataxia Telangiectasia, in which we also demonstrated a restricted B-cell repertoire by next generation sequencing of immunoglobulin variable regions (Chapter 4.1).

We agree with Giovannetti *et al.* that T-cells are important to classify CVID. It is important to realize that abnormalities in T-cell development almost invariably coexist with B-cell abnormalities. Combining B- and T-cell parameters in one classification system will most probably result in an improvement of the classification of CVID patients in homogenous subgroups in the future.

KREC and TREC detection in CVID; the importance of analysis at the B-cell subset level.

Recently, Kamae *et al.* introduced a new CVID classification, based on TREC (T-cell receptor excision circle) and KREC (Kappa-deleting recombination excision circle) content of whole blood[13]. Their approach is based on a screening strategy originally developed for the neonatal detection of SCID in Guthrie cards[31]. Another study exploring the use of KRECs and TRECs in CVID was published by Serana *et al.*[32]. Both studies measure KRECs in whole blood or PBMCs, and as such lack the accuracy of our approach of detecting KRECs as measure of B-cell replication at the B-cell subset level (Chapter 3.1).

Kamae *et al*. describe four groups based on the absence or presence of TRECs, KRECs or both in 40 CVID patients. A group of six patients with a combined reduction of TRECs and

KRECs displayed characteristics of a combined immunodeficiency, with a high incidence of opportunistic infections and low naive CD4 T-cells. These findings are compatible with Giovannetti group I[12] and our aberrant B-cell maturation patterns 1 and 2. We performed a similar TREC and KREC analysis in a cohort of CVID patients. Preliminary unpublished data show that group D of Kamae *et al.* almost fully overlap with our B-cell maturation pattern 1. Our observation that B-cell maturation patttern 1 and group D overlap is further supported by the observation of Kamae *et al.* that NBS and AT patients fall into group D and by our own observations described in Chapter 4.1 that AT patients display B-cell maturation pattern 1 as their predominant B-cell phenotype. As explained above, the classification of Kamae *et al.* is limited by the fact that the KRECs and TRECs are measured in whole blood and do not relate to any B-cell phenotype, in contrast to our observations that abnormalities in KRECs and TRECs at the B-cell subset level are associated with specific B-cell patterns. In addition, functional data to support the homogeneity of the classification by Kamae *et al.* is lacking. However, an advantage of their approach is that it is relatively easy to perform and inexpensive. The delineation of group D as a combined immunodeficiency is rather convincing, but the other groups appear to be more heterogeneous. We are currently comparing the TREC and KREC classification according to Kamae *et al.* and our B-cell maturation pattern classification; this will clarify the relationship between both classifications.

The second paper using TRECs and KRECs in CVID was published by Serana *et al*, who performed KREC and TREC analysis on total PBMCs[32]. Also these authors did not analyze B-cell replication at the B-cell subset level. Surprisingly, they report a much higher number of cell divisions in total PBMCs: an average number of cell divisions of 4.4 in total PBMCs compared to ~ 2 cell divisions in our experiments, which is similar to that of naive B-cells (unpublished results). This difference could be caused by a technical artefact, because they did not use a control cell line carrying one coding and one signal joint in their KREC assay, as previously proposed by van Zelm *et al*[33]. Since the vast majority of the KREC signal joints are present in naive B-cells, analysis of B-cell replication in total PBMCs merely reflects the proliferation of the naive compartment. Extensive B-cell proliferation in the small memory B-cell compartment will not be identified by measuring proliferation history at PBMC level. For example, a complete loss of the signal joint in the total memory B-cell compartment with a relative size of up to 20% of total blood B-cells (so an "unlimited" number of cell divisions as determined by KREC analysis), will not even add half a cell division to the total proliferative history at PBMC level, since the high signal joint content of the 80% predominantly naive B-cells will almost completely obscure the hyperproliferation of the 20% memory B-cells. As a rule, the replication history at PBMC level will predominantly reflect the number of cell divisions in the least proliferated B-cell subset[33]. Therefore, the assumption by Serana *et al.* that "in an unsorted pool of circulating B lymphocytes, one of the two important determinants of the average number of cell divisions should be the number

of memory B-cells" demonstrates the full misunderstanding of the authors on how the KREC assay should be interpreted. The fact that they observed low naive CD4 T-cells in the population with highest (predominantely naive!) number of B-cell divisions and low TRECs, should have been interpreted as decreased B- and T-cell production, probably reflecting the situation present in aberrant B-cell maturation pattern 1. This illustrates that TREC-KREC studies on total PBMC is only relevant for evaluation of aberrancies in production or proliferation of the naive T-cell and B-cell compartment, not for evaluation of aberrancies in more mature lymphoid subsets; in such case targeted subset analysis is a prerequisite.

BENEFITS AND LIMITATIONS OF CVID CLASSIFICATIONS

In the previous section, we compared our "B-cell maturation pattern" CVID classification to other currently existing immunological CVID classifications. Next we will discuss the benefits and limitations of CVID classifications in general. The potential benefit of a CVID classification is that:

- The classification has implications for prognosis, because it predicts clinical complications and/or mortality risk. Such classifications potentially guide follow-up schedules for different patient subgroups and might facilitate therapeutic clinical trials.
- The classification has implications for the treatment strategy; patients in different subgroups should receive different treatment modalities in order to improve their outcome.
- The classification facilitates research, because it creates (homogenous) groups of patients with a suspected different pathophysiology, which is important to detect disease causing mechanisms and/or genetic defects. To a lesser extend, such classifications might also facilitate (diagnostic or therapeutic) clinical trials.

Considering the prediction of the prognosis of CVID, the Chapel classification[3-4] shows that patients with non-infectious clinical complications have a worse outcome in terms of survival, compared to patients with infections only. This classification is based on the clinical complications itself and it is not designed to predict them. The definitions of all currently existing immunological CVID classifications and their association with clinical complications are summarized in Table 1. Although the currently existing immunological CVID classifications correlate immunological parameters to existing clinical complications, there are no follow up studies to demonstrate that these classifications predict future clinical complications or survival. Our B-cell maturation pattern classification was also designed as an attempt to correlate aberrant B-cell maturation patterns with clinical parameters.

Indeed, as shown in Chapter 3, B-cell maturation pattern 1 and 2 are associated with the occurrence of non-infectious clinical complications. Immunological classifications have the potential to become clinically relevant in predicting outcome, if they are evaluated in prospective follow-up studies. In this respect, it is important to pay special attention to the group of CVID patients with low (naive) CD4 T-cells in addition to hypogammaglobulinemia and reduced CD27+ memory B-cells, because these patients probably represent a separate disease entity with a worse outcome. Interestingly these patients have also been identified as "late onset combined immunodeficiency" or LOCID[34]

None of the currently existing CVID classifications has implications for treatment strategies, such as adaptations in dosage of immunoglobulin replacement, use of antibiotic treatment, and/or prophylaxis and use of immune suppressive of modulatory drug. In fact, there is an urgent need for clinical trials exploring the benefits of tailored treatment strategies for subgroups of CVID patients. Especially the treatment of granulomatous complications and the use of immunosuppressive drug in immunodeficient patients are challenging issues.

An important benefit of classifying CVID patients into homogenous subgroups is to facilitate research on disease causing mechanism and the targeted search and identification of genetic defects. The currently existing immunological classifications, particularly our B-cell (and T-cell) maturation-based CVID classification, serve this aim. For an optimal division of CVID patients in homogenous subgroups, an immunological classification should have the following characteristics: 1) It is supported by functional immunological characteristics to show that the identified subgroups are homogenous; 2) It is compatible with aberrant B-cell/T-cell maturation patterns of patients with known genetic defects; 3) It is applicable to all age categories, because it is based on age related reference values; 4) It is associated with clinical complications (and preferable *predicts* clinical complications); 5) It is reproducible at different follow-up time points; 6) It is easy and relatively cheap to perform; 7) It can be fully standardized, so that different laboratories can obtain fully comparable results.

Our B-cell maturation pattern CVID classification has several potential advantages over currently existing CVID classifications, because, apart from the above point 5 (which is currently under investigation), our classification fulfils all of the above mentioned characteristics. Since we believe that it is important to have a broadly applicable classification that is available for all immunodeficiency centers, we are now designing new ≥8-color flow cytometric immunostainings according to the technical guidelines and novel flow cytometric concepts of the EuroFlow Consortium[35-36]

Future perspectives of immunological CVID classifications

Future studies should concentrate on the determination of both B-cell and T-cell maturation pathways, supported by functional assays such as KREC/TREC analysis, immune repertoire analysis, etc., whenever needed for confirmation (see Chapter 4.2). Such information should be combined with long term clinical follow-up of CVID patients. Current classifications systems could be prospectively evaluated, comparability can be determined, and the best prognostic markers can be identified. The introduction of innovative strategies to improve the generation of homogenous subgroups is essential to further improve clinically relevant CVID classifications.

An improved B-cell pattern CVID classification could use multicolour flow cytometry according to standardized EuroFlow protocols. The EuroFlow approach has been demonstrated to be a powerful tool for the diagnosis of leukemia[35-37]. Currently, the EuroFlow PID consortium (www.euroflow.org) is extending this approach to develop new standardized flow cytometric. This includes the full standardization of the instrument settings, the immunostaining protocols, the antibody panels, the choice of fluorochromes, and the novel software tools for data analysis (www.infinicyte.com). This is all being performed in the PID Workpackage of the EuroFlow Consortium with participation of 8 different European PID centers.

Principle component analysis of the immunophenotyping data of leukocyte subsets, as has been performed in a limited number of CVID patients by Kalina et al[38], will contribute to the improved classification of CVID and related disorders. In addition, inclusion of additional parameters, such as TLR signalling, Ca flux assays or Akt signalling (Chapter 4.2), could provide new insights in the pathophysiology of idiopathic antibody deficiency.

Further analysis of homogenous subgroups could then identify underlying functional and/or genetic defects. "Hypothesis free" large scale high throughput strategies for genetic analysis in families and large cohorts of CVID patients is successful in identifying new candidate genes[39] or genetic defects[40-42] associated with immunodeficiency, but the interpretation of these data is challenging. Orange *et al.*[39] performed GWAS (Genome Wide Association Study) in a large cohort of CVID patients and found associations with the MHC region, the metalloproteases *ADAM28* and *ADAM7*, *ADAMDEC1* and *STC1*. Furthermore, this study showed that copy number variations in multiple genes were significantly associated with CVID. So the observed heterogeneity at clinical level was mirrored in a heterogeneous picture at the genetic level, albeit that it still has to be proven whether these genetic variations are directly related to the pathophysiology of CVID. Nevertheless, these data suggest that CVID is either a polygenetic condition resulting from multiple genetic and environmental disease susceptibility factors or a collection of rare monogenetic diseases. Future strategies using whole exome or genome sequencing might be successful when

used in multiplex families or in large cohorts of well defined homogenous subgroups of CVID patients.

Patients will only benefit from new insights in the pathophysiology of CVID if tailored treatment strategies can be developed for infectious and non-infectious[43] disease related complications of CVID subgroups. Especially the benefits of immunosuppressants[43], immunomodulary drugs and biological such as ritximab[43-44] need further exploration in the context of CVID subgroups.

DIAGNOSTIC CRITERIA FOR CVID AND OTHER IDIOPATHIC ANTIBODY DEFICIENCIES: TIME FOR A CHANGE?

In Chapter 3.2, we describe a group of patients with hypogammaglobulinemia not fulfilling the current CVID diagnostic criteria with respect to a decrease of two immunoglobulin isotypes and/or an impaired response to vaccinations. These patients with idiopathic primary hypogammaglobulinemia or IPH cannot be sufficiently classified according to the IUIS classification of primary immunodeficiencies[45]. According to the ESID criteria, some of these patients are considered "possible CVID" and the ICD10 (International Classification of Diseases version 10) classifies them as "hypogammaglobulinemia not otherwise specified" under the same code as CVID. So, the current diagnostic criteria for CVID exclude a group of hypogammaglobulinemic patients, which we demonstrated to suffer from a clinically relevant antibody deficiency.

We showed that CVID and IPH are partly overlapping conditions within one disease spectrum of B-cell deficiencies. CVID patients more often suffer from early defects in B-cell maturation, whereas IPH patients mostly show a normal peripheral B-cell distribution, suggesting a defect in terminal plasma cell differentiation, which should be the focus of further investigations into the pathophysiology of this group. Both groups suffer from respiratory tract infections, but CVID patients more often suffer from (severe) non-infectious complications.

We propose to revise the IUIS categories for predominantly antibody deficiencies in order to facilitate the inclusion of IPH. A descriptive classification could be added as a "reduction of IgG with normal levels of IgA and IgM and normal numbers of B-cells". Part of the IPH patients can be classified in the same IUIS category as CVID (a decrease of 2 immunoglobulin isotypes, with normal or reduced levels of B-cells). However, in contrast to CVID, these patients can have a normal response to vaccination. Of the CVID diagnostic criteria, an impaired response to vaccination is most controversial, because it has not been defined which antigens and which specific cut-of serum levels for the interpretation of the specific

antibody responses should be used. Orange *et al.* published a consensus document on vaccination in primary antibody deficiencies, which could be a starting point to define exact criteria for the response to immunization in CVID[46]. Alternatively, if no consensus about the interpretation can be achieved, vaccination responses should be dropped as criterion for the diagnosis of CVID.

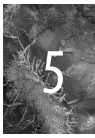
Concerning the T-cell phenotypes in CVID, it is appropriate to consider patients with decreased (naive) CD4 T-cells, compatible with Kamae group D[13], B-cell maturation pattern 1 and 2 and/or late onset combined immunodeficiency[34], as combined immunodeficiencies rather than primary antibody deficiencies, because of the occurrence of opportunistic infections, a surplus of non-infectious complications and an increased mortality.

IMMUNOBIOLOGY OF ANTIBODY DEFICIENCY IN KNOWN GENETIC OR CHROMOSOMAL DEFECTS.

Chapter 4 of this thesis explores peripheral B-cell differentiation and maturation in three conditions with a known genetic or chromosomal defect. Apart from studying disease causing mechanisms, the pathophysiologies of the B-cell defects were analyzed in relation to the clinical severity of the involved antibody deficiencies. In addition, the findings of Chapter 4 can be used to facilitate the interpretation of the findings of Chapter 3.1 and 3.2.

Restriction of immune repertoire in Ataxia Telangiectasa

In Chapter 4.1 we explored the pathophysiology of the antibody deficiency in Ataxia Telangiectasia (AT). We showed that the severity of the antibody deficiency in AT is associated with abnormalities in T-cell and B-cell homeostasis. In AT, thymic and bone marrow output is decreased because of impaired DNA repair during V(D)J recombination,[47] similar to observations in the related DNA repair disorder NBS[16]. In addition, peripheral proliferation of naive T-cells and B-cells was increased, as demonstrated by KREC and TREC analysis. We demonstrated a decreased diversity of the naive mature B-cells antigen receptor repertoire using next generation sequencing of VH-JH rearrangements. Both decreased bone marrow output and increased proliferation contribute to loss of immune receptor repertoire diversity, which is the hallmark of the immunodeficiency in AT. Next generation sequencing of VH-JH rearrangements can be used for high throughput analysis of naive as well as antigen experienced repertoire[48-49]. However, the interpretation of the results is challenging[50]. In addition, next generation sequencing permits analysis of SHM frequencies and CDR3 lengths, which provides additional information on immunoglobulin



variable region maturation. As such, next generation sequencing of immune repertoire will become increasingly relevant in the evaluation the pathophysiology of primary immunodeficiency and auto-immune disease. In combination with a proteomic approach, immune repertoire analysis can even facilitate the ex vivo generation of high affinity antibodies directed against pathogens[51-52]. Currently we are exploring the immune repertoire in CVID. Since AT resembles CVID with B-cell maturation pattern 1 (as mentioned earlier), we expect the naive immune receptor repertoire to be decreased in these patients.

We showed that Class Switch Recombination (CSR) deficiency is affected in classical AT patients with and without hypogammaglobulinemia, who all lack ATM-kinase activity. In a search for an explanation for this observation, we showed that low levels of naive CD4 T-cells correlated with the severity of the antibody deficiency. We speculated that impaired T-cell help in the GC contributes to the severity of the class switch recombination deficiency, but the low levels of naive CD4 T-cells might also reflect the level of reduced thymic and bone marrow output. Since all classical AT patients lack ATM-kinase activity, the exact reason for the variability in the level of naive CD4 T-cells remains unsolved. Further studies should clarify this issue and most likely need to focus on the V(D)J recombination process itself.

Germline mutations in PTEN are associated with CSR and SHM deficiency

In Chapter 4.2 we for the first time describe antibody deficiency in patients with germline mutations in *PTEN*. The *PTEN* (phosphate and tensin homologue deleted on chromosome 10) gene is a tumor suppressor gene located on chromosome 10q23. Autosomal dominant germline mutations in *PTEN* are associated with three partly overlapping clinical syndromes: Cowden syndrome[53], Bannayan-Riley-Ruvalcaba syndrome[54] and Proteus syndrome[55]. The majority of patients with *PTEN* hamartoma tumor syndrome (PHTS) do not suffer from antibody deficiency, indicating that the clinical phenotype is very heterogeneous and that other factors than the *PTEN* mutations might contribute to the immunodeficiency.

The importance of the studies in Chapter 4.2 is that they describe a new pathophysiological mechanism of antibody deficiency is humans. Similar to studies in mice[56-59], our data suggest that impaired CSR is caused by PI3K/Akt mediated inhibition of Activation Induced Cytidine Deaminase (AID). We now have preliminary data showing that increased PI3K/Akt activity is the result of loss of inhibition because of impaired PTEN activity in PHTS patients. In addition, we generated data to show that also in humans AID expression is regulated by Akt. Our data also indicate that immunodeficiency should always be considered in patients with syndromic conditions that have not been formerly associated with PID, if the clinical picture is characterized by recurrent infections or immune dysregulation. Inversely, patients with a diagnosed immunodeficiency might suffer from an

unrecognized syndromic condition, implying that a thorough clinical evaluation of antibody deficient patients is mandatory, including the evaluation for dysmorphic features and neurodevelopmental status.

Finally, as described in Chapter 4.2, we propose that increased PI3K/Akt signaling as observed in patients with *PTEN* mutations, is an attractive disease causing mechanism to explore in CVID patients. The identification of deregulated Akt activity might have therapeutic implications, since PI3K inhibitors are currently under investigation in clinical trials for the treatment of different cancers[60-61]. These agents can potentially also treat the immunodeficiency[56], but carry the risk of increasing the severity of auto-immune disease, in case restoration of CSR facilitates the production of high affinity auto-antibodies.

In conclusion, the discovery of deregulated PI3K/Akt activity as a potential disease causing mechanism in antibody deficiency emphasizes the association between syndromic features and antibody deficiency and opens new possibilities for therapeutic interventions.

B-cell development in Down syndrome

The increased susceptibility to (respiratory) infections in patients with Down syndrome (DS) is caused by anatomical abnormalities of the respiratory tract as well as immunodeficiency involving innate and adaptive immunity[62-63]. Although antibody deficiency is part of the variable immunodeficiency in DS patients, the peripheral B-cell compartment has not been thoroughly evaluated in these patients. Chapter 4.3 explores B-cell development in DS patients by flow cytometry and molecular techniques. DS patients did not suffer from hypogammaglobulinemia. Still, their B-cell maturation patterns were characterized by reduction of naive mature B-cells, CD27+IgD+IgM memory B-cells and CD27+IgD- memory B-cells. Similar to a subgroup of CVID patients[14], CD27+IgD+IgM memory B-cells showed impaired proliferation and SHM. The heterogeneity of the B-cell maturation patterns (patterns 2, 3 and 4) probably reflects a complex, polygenetic nature of the abnormalities in the B-cell system, but also shows that B-cell memory is invariably affected. Remarkably, plasma cell counts and immunoglobulin levels were increased or in the higher normal range. Similar abnormalities were present in patient with *PTEN* mutations (Chapter 4.2), who did not suffer from a clinically overt antibody deficiency. It is tempting to speculate that abnormalities in PI3K/Akt signaling could contribute to the immunodeficiency in DS patients. One study in a DS mouse model (TS65Dn mouse model) reported increased Akt phosphorylation in the hippocampus[64]. Regarding lymphocyte development, studies in the same mouse model revealed T-lymphocyte proliferation and apoptosis defects.[65] In addition to the use of human lymphocytes, the TS65Dn mouse model could be used to further explore the causes of the B-cell abnormalities in DS. Revealing the exact pathophysiology of the immunodeficiency in DS could have clinical implications in the future.

Table 4. Summary of most important findings of the thesis and directions for future research

Chapter	Most important findings	Directions for future research
2	- Characterization of six T-cell dependent and independent memory B-cell subsets. CD27-IgA+ memory B-cells are derived from T-cell independent responses in the gut. CD27+IgM+IgD+ natural effector memory B-cell originate from T-cell independent as well as T-cell dependent pathways.	- Identification of markers tot distinguish T-cel dependent and independent fraction of natural effector or marginal zone like B-cells. - Characterization of CD27- class switched memory B-cells. - Characterization of CD27+IgE+ memory B-cells.
3.1	- Classification of CVID patients in subgroups using 5 B-cell patterns associated with a distinct pathophysiolocal background as demonstrated by B-cell proliferation and SHM analysis at B-cell subset level.	- Further functional and characterization of subgroups with different B-cell patterns, including DNA repair defects in B-cell pattern 1. - Explore the reproducibility of the B-cell pattern classification. - Further exploration of clinical correlates of the B-cell pattern classification and comparision with other CVID classifications in large cohorts of patients. - B-cell repertoire analysis in CVID subgroups.
3.2	- Clinical and immunological characterization of patients with idiopathic primary hypogammaglobulinemia not fulfilling CVID diagnostic criteria with respect to a decrease of two immunoglobulinn isotyes and/or a decreased response to immunization; non infectious complications are rare in IPH. Most patients have a normal distribution of peripheral B-cell subsets. - Generation if age related reference values for memory B-cell subsets.	- Long term clinical follow up of IPH patients to document clinical complications. - Development of appropriate treatment strategies. - Exploration of defect in terminal plasma cell differentiation and or homing as causative defect.
4.1	- Detailed analysis of defects in peripheral B-cell development in classical and variant Ataxia Telangiectasia, demonstrate defects B-cell proliferation, memory B-cell differentiation and B-cell repertoire generation. These defects resemble observations in NBS and CVID patients with B-cell pattern 1.	- Further analysis of V(D)J recombination defects in patients with classical AT with and without hypogammaglobulinemia.
4.2	- Heterozygous germline mutations in *PTEN* affect B-cell differentiation and can result in hypogammaglobulinemia. - The likely mechanism is Akt mediated inhibition of Activation induced Cytidine Deminase.	- Providing definite proof of Akt mediated inhibition of AID as the causative mechanism in PHTS. - Exploration of Akt signalling defect as causative factor in CVID. - Investigating the therapeutic value of Akt inhibitors.
4.3	- Patients with Down syndrome show defects in B-cell development consisting of impaired proliferation and SHM of natural effector or marginal zone like B-cells and reductions of CD27+ memory B-cells.	- Trying to link the immunological defect to clinical phenotypes. - Exploration of the pathophysiological mechanism for decreased proliferation, somatic hypermutation and other memory B-cell abnormalities in DS.
5	- General discussion.	- Redefinition of clinical and immunological CVID classifications by long term follow-up of large cohorts of CVID and IPH patients, consensus meetings and innovative flow cytometry using EUROflow protocols, repertoire analysis and other functional assays. - Whole exome sequencing in multiplex families and large cohorts of well defined CVID patients to identify new genetic defects. - Development of tailored treatment strategies for patients with non-infectious clinical complications.

FINAL REMARKS

We developed a new CVID classification based on a more complete immunophenotypic and molecular analysis of peripheral maturation pathways, in order to divide patients in homogenous subgroups with the aim to facilitate research into the pathophysiology of the antibody deficiency, the identification of prognostic markers and the development of tailored treatment strategies. We succeeded to characterize five B-cell maturation patterns, which, in contrast to earlier classifications, represent subgroups with a distinct pathophysiology. The classification could also be applied to idiopathic primary hypogammaglobulinemia not fulfilling all CVID diagnostic criteria, which we showed to represent a clinically relevant, but different, antibody deficiency within the same disease spectrum. The approach of a combined immunophenotypic and functional molecular characterization was extended to study patients with known genetic defects. In Ataxia Telangiectasia it revealed defects in B-cell proliferation, memory B-cell differentiation, and B-cell repertoire as assessed by next generation sequencing of *IGH*. The defects in AT were remarkably similar to observations in CVID patients with B-cell maturation pattern 1, which demonstrates that analysis of patients with known genetic defects facilitates the study of idiopathic primary antibody deficiency. This was also exemplified by an analysis of patients with hypogammaglobulinemia caused by heterozygous germline mutations in the *PTEN:* The proposed pathophysiology of Akt mediated inhibition of activation induced cytidine deminase might be implicated in the pathophysiology of CVID. Finally, we extended the analysis to patients with DS, who suffer from B-cell memory defects in the presence of heterogeneous abnormalities of B-cell maturation pathways.

Because of its complexity and heterogeneity, primary antibody deficiencies will be subject of intensive research for the years to come. Much can be expected from large multicenter studies, integrating clinical, immunophenotypic and functional (molecular and genetic) data. This integrated approach will be fruitful in idiopathic antibody deficiency, but also in patients with known genetic defect. The directions for future research based on the most important findings of this thesis are summarized in Table 4.

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Addendum

Abbreviations

Summary

Samenvatting

Dankwoord

Curriculum Vitae

PhD Portfolio

Publications

LIST OF ABBREVIATIONS

ADS	Antibody Deficiency Syndrome
AID	Activation-induced Cytidine Deaminase
AT	Ataxia Telangiectasia
ATM	Ataxia Telangiectasia Mutated
BAFF	B-cell Activation Factor
BAFFR	BAFF receptor
BCR	B-cell antigen receptor
BTK	Bruton's tyrosine kinase
CDR	complementarity-determining region
CSR	Ig Class Switch Recombination
CVID	Common Variable Immunodeficiency Disorders
DS	Down Syndrome
DSB	Double Strand Break
ENT	Ear Nose and Throat
ESID	European Society for Immunodeficiencies
FcγR	Fc gamma receptor
FR	Framework
GC	Germinal Center
GWAS	Genome wide association study
HLA	Human Leucocyte Antigen
HSC	Hematopoietic Stem Cells
ICD10	International Classification of Diseases version 10
Ig	Immunoglobulin
IgH	Ig heavy chain
IGH	Ig heavy chain gene
IgκREHMA	Igκ restriction enzyme hot-spot mutation assay
IPH	Idiopathic Primary Hypogammaglobulinemia
IUIS	International Union of Immunological Societies
IVIG	Intravenous immunoglobulin
KREC	Igκ-deleting recombination excision circles
NBS	Nijmegen Breakage Syndrome
PAD	Primary Antibody Deficiency
PAGID	Pan American Group for Immunodeficiency
PBMCs	Peripheral blood mononuclear cells
PHTS	PTEN Hamartoma Tumor Syndrome
PID	Primary immunodeficiency

PIP2	Phosphatidylinositol 4,5 biphosphate
PIP3	Phosphatidyl inositol 3,4,5 triphosphate
PI3K	Phosphatidyl inositol 3-kinase
PTEN	Phosphate and tensin homologue deleted on chromosome 10
SHIP	Src homology domain 2 containing inositol phosphatase
SHM	Somatic hypermutation
SPAD	Specific anti-polysaccharide antibody deficiency
TACI	Transmembrane activator and CAML interactor
TCR	T-cell receptor
TD	T-cell dependent
TI	T-cell independent
TRECs	T-cell receptor excision circles
XLA	X-linked agammaglobulinemia

SUMMARY

Primary antibody deficiencies are characterized by a defect in the production of antigen specific antibodies and are the most prevalent primary immunodeficiencies, resulting in a wide range of infectious and non-infectious clinical complications and a decreased life expectancy. Many genetic defects have been identified that cause primary antibody deficiency (PAD), but in the majority of patients with PAD the underlying pathophysiological mechanism and causative genetic defects are still unknown. Most of these patients suffer from Common Variable Immunodeficiency Disorders (CVID). In several other categories of patients with defined genetic defects or chromosomal abnormalities associated with antibody deficiency, the underlying pathophysiological mechanisms have not been fully explored. The aim of this thesis was to study the immunobiology of PAD, with a focus on CVID and a selected number of conditions associated genetic or chromosomal defects; Ataxia Telangiectasia, PTEN Hamartoma Tumor Syndrome and Down Syndrome.

In Chapter 2 we defined six circulating memory B-cell subsets, based on the expression of CD27 and IgH isotypes, and determined their origin and maturation pathways through analysis of molecular characteristics. This analysis showed that CD27+IgD+IgM+ memory B-cells, also called natural effector B-cells or marginal zone like B-cells, at least in part originate in a T-cell independent way in the marginal zone of the spleen, because these cells were present in patients with CD40L deficiency. CD27-IgG+ and CD27+IgD-IgM+ memory B-cells are generated during primary germinal center (GC) responses, whereas CD27+IgG+ and CD27+IgA+ B-cells show the highest number of cell divisions and somatic mutations, reminiscent of consecutive GC passages. The CD27-IgA+ memory B-cells predominantly originate from T-cell independent primary immune responses.

Chapter 3 addresses the immunological and clinical classification of patients with CVID and related forms of idiopathic primary antibody deficiency. In the Chapter 3.1, patients with CVID where subjected to a detailed analysis of their peripheral B-cell maturation pathways, in order to divide them in homogenous subgroups with a similar pathophysiological background. By flow cytometric immunophenotyping and cell sorting of peripheral B-cell subsets of 37 CVID patients, we studied the B-cell maturation at the B-cell subset level using the KREC assay to determine the replication history and the IgκREHMA assay to assess the somatic hypermutation (SHM) status. Via this approach five B-cell maturation patterns were identified, which delineated groups with unique replication and SHM characteristics. Each B-cell maturation pattern reflected an immunologically homogenous patient group for which we proposed a different pathophysiology. B-cell maturation pattern 1, characterized by low transitional and CD27+IgD- memory B-cells, is compatible with a B-cell production defect; B-cell maturation pattern 2, characterized by low naive, marginal zone like and CD27+IgD- memory B-cells, is compatible with an early peripheral

B-cell maturation or survival defect; B-cell maturation pattern 3, characterized by low marginal zone like B-cells and CD27+IgD- memory B-cells, is compatible with a B-cell activation and proliferation defect, but can also be the result of a germinal center defect; B-cell maturation pattern 4, characterized by low CD27+IgD- memory B-cells, is compatible with germinal center defects and B-cell pattern 5, characterized by the absence of major abnormalities in peripheral B-cell subsets, is compatible with a post-germinal center or terminal plasma cell differentiation or homing defect. Thus, this approach provided insight into the underlying pathophysiological background in five immunologically homogenous groups of CVID patients.

Chapter 3.2 further explores the approach of Chapter 2 and 3 in patients with hypogammaglobulinemia, which cannot be appropriately classified with current PID classification systems. We clinically and immunologically characterized a group of patients with idiopathic primary hypogammaglobulinemia (IPH), not fulfilling all CVID diagnostic criteria, which has not been done so far. IPH was defined as a reduction of IgG of at least 2 SD below the mean for age, an onset of the immunodeficiency at greater than 2 years of age and exclusion of defined causes of hypogammaglobulinemia in patients who did not fulfill the CVID diagnostic criteria with respect to a reduction of two immunoglobulin isotypes and/or a reduced response to vaccination. We aimed to clarify whether IPH is a clinically relevant antibody deficiency and to determine pathophysiological aspects of IPH compared to CVID. We determined the clinical phenotypes and performed flow cytometric immunophenotyping to assess the pathophysiological B-cell patterns and memory B-cell subset counts. Age-matched B-cell subset reference values were generated of 130 healthy donors. Severe pneumonia and bronchiectasis occurred at similar frequencies in IPH and CVID. Non-infectious disease related clinical phenotypes (auto-immune cytopenia, polyclonal lymphocytic proliferation and persistent unexplained enteropathy) were exclusively observed in CVID and were associated with B-cell maturation pattern 1 and 2, compatible with the presence of early peripheral B-cell maturation defects or B-cell survival defects. T-cell dependent memory B-cell formation was more severely affected in CVID. Furthermore, more than half of the IPH patients showed normal peripheral B-cell subset counts, suggestive for a plasma cell defect. CVID and IPH are two partly overlapping conditions. IPH is similar to CVID with respect to infectious complications, but is not the same with respect to non-infectious clinical complications, immunoglobulin levels, distribution of B-cell maturation patterns and memory B-cell counts. Clinical follow up studies of larger numbers of IPH patients will reveal the prognosis, facilitate the development of optimal treatment strategies and determine the place of IPH in current PID classification systems. Chapter 3.2 also describes the dynamics of normal memory B-cell subsets counts in different age groups.

In Chapter 4 we describe peripheral B-cell development in patients with known genetic or chromosomal abnormalities to generate insight into pathophysiological mechanisms of PAD.

In Chapter 4.1 the consequences of *ATM* mutations for peripheral B-cell development and immunological disease severity were studied. Ataxia Telangiectasia (AT) is a multisystem DNA-repair disorder caused by mutations in the *ATM* gene. AT patients have reduced B- and T-cell numbers and a highly variable immunodeficiency. ATM is important for V(D)J recombination and immunoglobulin class switch recombination (CSR). We analyzed the peripheral B-cell and T-cell development in 15 AT patients with different degrees of the severity of their immunodeficiency by flow cytometry, *in vivo* B-cell replication history by KREC analysis, SHM and CSR to IgA and IgG subclasses and B-cell repertoire with molecular techniques. This study included patients with classical AT plus early onset hypogammaglobulinemia, classical AT, and variant AT (late onset). Classical AT patients lacked ATM kinase activity, while variant AT patients showed residual function. Most patients had disturbed naive B-cell and T-cell homeostasis and a decreased B-cell antigen receptor repertoire diversity as determined by deep sequencing of *IGH* gene rearrangements. Impaired formation of T-cell dependent memory B-cells was predominantly found in AT plus hypogammaglobulinemia. These patients had extremely low naive CD4+ T-cell counts, which were more severely reduced compared to classical AT patients without hypogammaglobulinemia. Finally, ATM deficiency resulted in defective CSR to distal constant regions that might reflect impaired ability of B-cells to undergo multiple germinal center reactions. Chapter 4.1 showed that the severity of the antibody deficiency in AT correlates with disturbances in B and T-cell homeostasis resulting in reduced immune repertoire diversity, which consequently affects the chance of successful antigen-dependent cognate B-T interaction.

Autosomal dominant germline mutations in *PTEN* are associated with PTEN Hamartoma Tumor Syndrome (PHTS). Immunodeficiency has not been reported to be part of the clinical spectrum of PHTS, although mice data indicate that *PTEN* mutations affect CSR by Akt mediated inhibition of Activation Induced Cytidine Deaminase (AID). Chapter 4.2 aimed at identifying the immunological mechanisms responsible for the antibody deficiency in patients with heterozygous germline mutations in *PTEN*. By using a comparable approach as described in chapter 4.1, we identified a novel disease causing mechanism of primary antibody deficiency. We studied three patients with heterozygous germline *PTEN* mutations who suffered from PHTS and hypogammaglobulinemia and six patients with PHTS without antibody deficiency. The clinical phenotype of the PHTS with hypogammaglobulimia patients fulfilled CVID diagnostic criteria. CSR and SHM were impaired in PHTS, irrespective of the presence of hypogammaglobulinemia. PHTS patients without antibody deficiency could compensate their CSR deficiency by generating increased absolute counts of transitional and naive B-cells, normal counts of class switched memory B-cells and increased

plasmablasts. We propose that the probable pathophysiological mechanism is PI3K/Akt mediated inhibition of AID, due to loss of negative regulation of PI3K by PTEN. Because auto-immunity, lymphoproliferation and the propensity to develop malignancies are associated with deregulated Akt signaling as well as the CVID clinical phenotype, deregulated Akt signaling should be considered as a potential causative mechanism in CVID.

Chapter 4.3 aimed at identifying abnormalities in B-cell development in patients with Down syndrome (DS), who have an increased risk of respiratory infections, autoimmune diseases and hematological malignancies, similar to CVID. We studied the peripheral B-cell compartment in 13 DS patients, using a similar approach as in patients with AT (Chapter 4.1) and PHTS (Chapter 4.2). B-cell defects were present in CD27+IgA-, CD27+IgD- class switched and $CD27^{+}IgD^{+}IgM^{+}$ natural effector B-cells. Remarkably, $CD27^{+}IgD^{+}IgM^{+}$ natural effector B-cells showed reduced proliferation and SHM frequencies, similar to a CVID-subset, while these were normal in $CD27^{+}IgD^{-}$ memory B-cells. We hypothesize that this defect may contribute to the increased susceptibility to infections in DS.

The studies described in this thesis shed light on the immunobiology of idiopathic antibody deficiencies and antibody deficiencies with known genetic defects. They show that the approach of combining immunophenotyping with molecular immunological studies and clinical data collection has additional value in exploring pathophysiological mechanisms and classifying patients with diverse causes of their antibody deficiency. Innovative techniques, such as the EuroFlow approach of immunophenotyping as well as next generation sequencing of immune repertoire will further enhance the insight in these conditions. For patients to benefit from new insights in the immunobiology and classification, tailored treatment and follow-up strategies have to be developed for infectious and non-infectious disease related complications of homogenous subgroups. In patients with AT, PHTS and Down syndrome, this thesis showed that similar genetic or chromosomal defects might give rise to differences in clinical and immunological severity of the immunodeficiency. Insight in the immunobiology of these patients gives directions for research into the pathophysiology of idiopathic antibody deficiencies. Because of its complexity and heterogeneity, primary antibody deficiencies will be subject of intensive research for the years to come. Much can be expected from large multicenter studies, integrating clinical, immunophenotypic and functional (molecular and genetic) data. This integrated approach will be fruitful in idiopathic antibody deficiency, but also in patients with known genetic defects.

SAMENVATTING

Primaire antistof deficiënties (PADs) zijn de meest voorkomende primaire immuundeficiënties. Bij PADs is er sprake van een kwantitatief en/of kwalitatief defect in de productie van antigeenspecifieke antistoffen. PADs zijn geassocieerd met diverse klinische complicaties, zoals recidiverende of ernstige (luchtweg) infecties, maar ook niet-infectieuze complicaties zoals auto-immuun aandoeningen en granulomateuze ontstekingen. In sommige patiënten met PADs kunnen erfelijke defecten aangetoond worden die verantwoordelijk zijn voor het veroorzaken van de ziekte, maar in de meeste patiënten is het erfelijke defect en het precieze pathofysiologische mechanisme niet bekend. Veel patiënten met een PAD lijden aan Common Variable Immunodeficiency Disorders (CVID) of verwante idiopathische PADs. Het gaat hierbij om een heterogene groep PADs wat betreft de frequentie en ernst van klinische complicaties. Soms is een PAD een onderdeel van een erfelijk syndroom, waarvan het exacte gendefect of chromosomale afwijking wel bekend is. Bij deze patiënten is echter de manier waarop het erfelijk defect resulteert in een PAD vaak niet opgehelderd.

Het doel van de studies in dit proefschrift was om de immunobiologie van PADs te bestuderen om meer inzicht te krijgen in de pathofysiologie in relatie tot klinische complicaties. In het eerste deel van het onderzoek lag de nadruk op het bestuderen van CVID. Vervolgens hebben we op een vergelijkbare manier gekeken naar een aantal aandoeningen met bekende genetische en chromosomale defecten, zoals Ataxia Telangiectasia (AT), PTEN Hamartoma Tumor Syndroom (PHTS) en het Syndroom van Down. Door middel van gedetailleerde immunofenotypering van B-cellen in het perifere bloed in combinatie met moleculaire analyse van de rijping van B-cellen, hebben we deze patiënten groepen in kaart gebracht.

Kennis van de normale ontwikkeling van B-cellen is nodig om afwijkingen op het spoor te komen. In Hoofdstuk 2 definiëren we zes subsets van *memory* B-cellen, uitgaande van de expressie van CD27 en IgH isotypes en laten zien dat deze subsets afstammen van verschillende routes van B-cel uitrijping. We laten hier zien dat CD27+IgD+IgM+ *memory* B-cellen, ook wel *natural effector* B-cellen genoemd, voor een deel afstammen van T-cel onafhankelijke uitrijpingsroutes in de marginale zone van de milt, omdat deze cellen ook aanwezig zijn in patiënten met een CD40 ligand deficiëntie, die niet in staat zijn tot effectieve B-T cel interactie. Verder laten we hier zien dat de CD27-IgG+ en CD27+IgD-IgM+ *memory* B-cellen voornamelijk afstammen van primaire T-cel afhankelijke reponsen in kiemcentra in lymfoid weefsel. Op basis van B-cel proliferatie en somatische hypermutatie (SHM) frequentie zijn CD27+IgG+ en CD27+IgA+ *memory* B-cellen meerdere keren het kiemcentrum gepasseerd. Tenslotte blijken CD27-IgA+ memory B-cellen voornamelijk te ontstaan uit T-cel onfhankelijke B-cel responsen in de darm.

Hoofdstuk 3 beschrijft de immunologische en klinische classificatie van CVID en verwante PADs. In Hoofdstuk 3.1 hebben we de perifere B-cel uitrijping van patiënten met CVID gedetailleerd onderzocht. Hierdoor werd het mogelijk om deze heterogene patiëntengroep in te delen in relatief homogene subgroepen met dezelfde pathofysiologische achtergrond. Met flow cytometrische immunofenotypering en door het sorteren van perifere B-cel subsets hebben we de proliferatie status (met de *KREC assay*) en de somatische hypermutatie status (met de *IgκREHMA assay*) vastgesteld. Op deze manier was het mogelijk om vijf unieke B-cel patronen te onderscheiden. Voor elke specifiek B-cel patroon hebben we aannemelijk gemaakt dat er sprake was van een verschillend defect in B-cel uitrijping. Bij B-cel patroon 1, gekenmerkt door lage transitionele B-cellen en lage CD27+IgD- *memory* B-cellen, is er sprake van een gecombineerd B-cel productie en kiemcentrum defect. B-cel patroon 2, waarbij naief mature B-cellen en zowel CD27+IgD+ als CD27+IgD- *memory* B-cellen verlaagd waren, is compatibel met een vroeg uitrijpingsdefect van de B-cellen. Voor B-cel patroon 3, met een verlaagde CD27+IgD+ en CD27+IgD- *memory* B-cel subset, hebben we laten zien dat een B-cel activatie en/of proliferatie het potentiële defect is. Bij B-cel uitrijpingspatroon 4 waren alleen de CD27+IgD- *memory* B-cellen verlaagd, hetgeen wijst op een kiemcentrum defect, terwijl bij patroon 5 er geen afwijkingen in de perifere B-cel uitrijping aanwezig waren, wat suggereert dat het defect gelokaliseerd is in de terminale B-cel uitrijping tot antistof producerende plasmacellen. Het unieke van deze benadering is dus dat B-cel patronen aanwijzingen geven voor de onderliggende oorzaak van de antistof deficiëntie in CVID.

In Hoofdstuk 3.2 worden de resultaten uit Hoofdstuk 2 en Hoofdstuk 3.1 gebruikt om een groep PAD patiënten in kaart te brengen die lijdt aan een hypogammaglobulinemie van onbekende origine, maar die niet voldoet aan de criteria van CVID. Patiënten kunnen niet goed ingedeeld worden volgens de huidige klinische classificatie systemen. Deze patiëntgroep met idiopathische primaire hypogammaglobulinemie (IPH) is niet eerder gekarakteriseerd. We hebben IPH gedefinieerd als een hypogammaglobulinemie, waarbij het IgG meer dan 2 SD verlaagd is ten opzichte van leeftijd gerelateerde controles, de hypogammaglobulinemie is ontstaan na de leeftijd van 2 jaar en andere oorzaken van hypogammaglobulinemie zijn uitgesloten. Deze patiënten voldeden niet aan de CVID criteria wat betreft van een verlaging van 2 van de 3 immuunglobuline isotypes en/of een verminderde respons op vaccinatie. We hebben een cohort van 21 IPH patiënten klinisch beschreven en hebben door middel van immunofenotypering de B-cel patronen vastgesteld volgens de methodiek van Hoofdstuk 3.1. Ook hebben we IPH zowel klinisch als immunologisch vergeleken met CVID patiënten, om te kijken of er sprake was van een relevante primaire antistofdeficiëntie. Vervolgens hebben we leeftijd gerelateerde normaalwaarden gegenereerd van de *memory* B-cel subsets in 130 gezonde kinderen en deze gebruikt voor een *memory* B-cel analyse in IPH en CVID patiënten. Ernstige pneumonieën

en bronchiectasieën kwamen bij IPH en CVID in gelijke mate voor. De niet-infectiologische complicaties zoals auto-immuun cytopenieën, polyclonale lymfocytaire proliferatie en persisterende onverklaarde enteropathie werden alleen gezien in CVID en waren geassocieerd met de aanwezigheid van B-cel patroon 1 en 2. T-cel afhankelijk *memory* B-cel ontwikkeling was ernstiger aangedaan in CVID patiënten. Meer dan de helft van de IPH patiënten toonden geen afwijkingen in perifere B-cel ontwikkeling, wat past bij een defect in terminale plasmacel uitrijping, overleving of *homing*. Dit hoofdstuk heeft aannemelijk gemaakt dat IPH en CVID op elkaar lijken wat betreft infectiologische complicaties, maar verschillen wat betreft het voorkomen van niet-infectiologische complicaties, immunoglobuline spiegels, verdeling van B-cel uitrijpingspatronen en *memory* B-cel ontwikkeling. Onderzoek in een groter cohort IPH patiënten is noodzakelijk om de prognose vast te stellen en om adequate behandelstrategieën te ontwikkelen voor deze groep patiënten. Hoofdstuk 3.2 geeft verder inzicht in de dynamiek van *normale* memory B-cel subset aantallen gedurende de kinderleeftijd.

In Hoofdstuk 4 wordt de perifere B-cel ontwikkeling van een aantal klinische syndromen met bekende genetische origine onderzocht, waarbij een PAD onderdeel uitmaakt van het ziektebeeld. In Hoofdstuk 4.1 worden de consequenties van mutaties in het *ATM* gen voor de perifere B-cel ontwikkeling bestudeerd bij patiënten met de DNA reparatie stoornis Ataxia Telangiectasia (AT), waarbij gekeken wordt naar de ernst van de immunologische afwijkingen. AT patiënten hebben verlaagde aantallen B- en T-cellen en een variabele immuundeficiëntie.. De perifere B- en T-cel ontwikkeling van AT patiënten met een verschillende ernst van de immuundeficiëntie werd in kaart gebracht door middel van immunofenotypering, in vivo B-cel replicatie met behulp van de KREC assay en CSR naar IgG en IgA subklassen en het B-cel repertoire werden bestudeerd met moleculaire technieken. De volgende patiënten werden in geïncludeerd; klassieke AT, waarbij er sprake is van een hypogammaglobulinemie op zeer jonge leeftijd, klassieke AT zonder ernstige hypogammagloblinemie en variant AT (late klinische uiting). Bij de meeste patiënten was de perifere B-cel en T-cel homeostase verstoord, waarbij ook de diversiteit van het B-cel repertoire verminderd was (gemeten d.m.v. *deep sequencing* van *IGH* gen herschikkingen). Defecten in T-cel afhankelijke B-cel *memory* waren voornamelijk aanwezig in AT patiënten met hypogammaglobulinemie. Deze patiënten hadden ook zeer lage naive CD4 positieve T-cellen, welke naar verhouding ernstiger verlaagd waren dan in patiënten zonder hypogammaglobulinemie. Verder was er een defect aanwezig in CSR naar de distale constante regio's, mogelijk als gevolg van een verminderde capaciteit van B-cellen om meerder kiemcelreacties te ondergaan. De antistof deficiëntie in AT correleert dus met afwijkingen in B- en T-cel ontwikkeling, met als gevolg een verminderde immuunrepertoire diversiteit. Dit kan tot gevolg hebben dat de kans op een succesvolle interactie tussen B- en T-cellen, die nodig is voor de T-cel afhankelijke B-cel ontwikkeling, verminderd

is. Autosomaal dominante *germline* mutaties in *PTEN* zijn geassocieerd met het PTEN hamartoma tumor syndroom (PHTS). Voor zover bekend maakt immuundeficiëntie geen deel uit van het klinische spectrum van dit syndroom. Wel zijn er studies in muizenmodellen verricht die laten zien dat PTEN een belangrijke rol speelt bij de B-cel ontwikkeling. In muizen beïnvloedt PTEN CSR door middel van Akt gemedieerde inhibitie van *Activation Induced Cytidine Deaminase* (AID). Door middel van een soortgelijke benadering die gebruikt is in Hoofdstuk 4.1, hebben we bij PHTS patiënten aangetoond dat bovengenoemd in muizenstudies beschreven mechanisme waarschijnlijk ook bij mensen verantwoordelijk is voor het ontstaan van antistof deficiëntie in sommige patiënten met PHTS. De studie beschrijft 3 patiënten met heterozygote *germline* mutaties in *PTEN* met kenmerken van PHTS, maar daarnaast een hypogammaglobulinemie die voldoet aan de CVID criteria. Als controle onderzochten we 6 patiënten met PHTS die geen antistof deficiëntie hadden. Opvallend is dat alle patiënten met PHTS afwijkingen in CSR en SHM hadden, onafhankelijk van de aanwezigheid van hypogammaglobulinemie. Mogelijk kunnen patiënten met PHTS zonder antistofdeficiëntie deze defecten compenseren, doordat bij deze patiënten verhoogde aantallen transitionele B-cellen en naïef mature B-cellen aanwezig zijn. Dit hoofdstuk maakt aannemelijk dat Akt gemedieerde inhibitie van AID secundair aan de mutaties in *PTEN* waarschijnlijk de oorzaak is van de antistofdeficiëntie in een deel van de patiënten met PHTS. Omdat lymfoproliferatie, auto-immuniteit en een verhoogde kans op het ontwikkelen van maligniteiten zowel bij PHTS als CVID voorkomen, is dit pathofysiologische mechanisme ook belangrijk om te onderzoeken bij CVID patiënten.

In Hoofdstuk 4.3 werd onderzocht of er bij het syndroom van Down, waarvan bekend is dat er enige mate van antistof deficiëntie aanwezig kan zijn, afwijkingen zijn in de B-cel ontwikkeling. Patiënten met het syndroom van Down hebben een verhoogd risico op respiratoire infecties, auto-immuun ziekten en hematologische maligniteiten, net als CVID patiënten. We bestudeerden het perifere B-cel compartiment in 13 patiënten met het syndroom van Down volgens een soortelijke benadering als in Hoofdstuk 4.1 en 4.2. Defecten in de *memory* B-cel ontwikkeling waren aanwezig in de volgende *memory* B-cel subsets; CD27+IgA-, CD27+IgD- *class switched memory* B-cellen en CD27+IgD+IgM+ *natural effector* B-cellen. Opvallend is dat deze laatste memory B-cel subset een verlaagde proliferatie historie en verlaagde SHM liet zien, identiek aan observaties bij een subgroep CVID patiënten beschreven in Hoofdstuk 3.1.Mogelijk dragen deze *memory* B-cel afwijkingen bij aan de verhoogde gevoeligheid voor infecties in het syndroom van Down.

De studies in dit proefschrift werpen licht op de immunobiologie van idiopathische PADs en antistofdeficiënties met een bekend erfelijk defect. Ze laten zien dat het combineren van immunofenotypering met moleculair-immunologische studies en analyse van klinische data toegevoegde waarde heeft om de pathofysiologische mechanismen van een divers palet patiënten met PAD op te helderen. Innovatieve technieken, zoals

de EuroFlow benadering voor immunofenotypering en het gebruik van *next generation sequencing* technieken om immuun repertoire in kaart te brengen, zullen ons inzicht in deze mechanismen in de toekomst verder kunnen vergroten. Voordat patiënten kunnen profiteren van deze nieuwe inzichten, is het belangrijk dat er nieuwe behandel en follow-up strategieën op maat worden ontwikkeld voor de verschillende subgroepen van PAD patiënten. Wat betreft patiënten met AT, PHTS en het syndroom van Down laat dit proefschrift zien dat identieke genetische defecten aanleiding kunnen geven tot verschillen in de immunologische en klinische ernst van het ziektebeeld. Verder kunnen de verkregen inzichten in deze patiënten groepen gebruikt worden om onderzoek te doen naar patiënten met idiopathische PADs. Door hun complexiteit en heterogeniteit zullen PADs onderwerp van onderzoek blijven, nu en in de toekomst. Er valt veel te verwachten van grote multicenter studies die klinisch, immunofenotypisch en functioneel (moleculair en genetisch) immunologisch onderzoek weten te combineren. Een dergelijke geïntegreerde benadering is veelbelovend voor zowel patiënten met idiopathische PADs als patiënten met bekende genetische defecten.

DANKWOORD, WORD OF THANKS

Op een of andere manier is nu toch het moment gekomen dat dit proefschrift zijn voltooiing nadert. Onderzoek doen is teamwork, zeker als je klinisch werk met onderzoek wil combineren. Dit proefschrift is dan ook het resultaat van een samenwerking met veel verschillende mensen, in het laboratorium, de kliniek, maar ook daarbuiten, waar ik met heel veel plezier aan terugdenk. Hieronder wil ik iedereen bedanken, zonder wiens inspanningen dit proefschrift niet in deze vorm tot stand zou zijn gekomen.

Allereerst wil ik alle patiënten en de ouders/verzorgers van patiënten hartelijk bedanken voor deelname aan de onderzoeken beschreven in dit proefschrift. Hopelijk kunnen de resultaten in de toekomst een bijdrage leveren aan het verbeteren van de behandeling en begeleiding van mensen met zeldzame afweerstoornissen.

Mijn dank gaat uit naar mijn promotoren, co-promotoren en andere leden van de promotiecommissie.

Prof. dr. J.J.M.van Dongen, beste Jacques, ik wil je bedanken voor het feit dat je me vanaf 2007 de mogelijkheid hebt gegeven onderzoek te doen binnen de unit moleculaire immunologie. Hier ben ik in aanraking gekomen met onderzoek op hoog niveau (dat volgens jou altijd nog een stap hoger kan). Ik ben je erkentelijk voor je inspirerende bijdragen aan brainstromsessies, persoonlijke gesprekken over de verschillende projecten, waardevolle bijdragen aan de artikelen en je visie dat goed onderzoek alleen maar kan gedijen in een open samenwerking met onderzoekspartners.

Prof. dr. P.M. van Hagen, beste Martin, dank voor de goede samenwerking de afgelopen jaren en voor het vertrouwen dat je me hebt gegeven om als kinderarts onderzoek te doen binnen het cohort volwassenen met CVID. Met veel energie heb je gestalte gegeven aan het het ErasmusMC Afweercentrum, iets waar de afdelingen kindergeneeskunde, immunologie en interne geneeskunde nu en in de toekomst de vruchten van zullen blijven plukken. Binnen deze samenwerking waardeer ik je brede kennis van de klinische immunologie, die zich soms ook naar de kindergeneeskunde laat vertalen.

Prof. dr. A.J. van der Heijden, beste Bert, bij mijn aanstelling als staflid hadden we een gentlemen agreement dat ik binnen een jaar of vier zou promoveren. Het zijn er een paar meer geworden. Dank voor je geduld en voor het feit dat je de rol van secretaris in de kleine commissie wil vervullen. Ik waardeer en bewonder de wijze waarop je in roerige tijden de belangen van de afdeling kindergeneeskunde boven je persoonlijke belangen kan zetten.

Prof. dr. H. Chapel, dear Helen, thank you very much for your willingness to join the thesis committee and for the efforts to judge the manuscript. I greatly appreciate your opinion as a leading expert in the field of PID.

Prof. dr. E.A.M. Sanders, beste Lieke, dank voor het feit dat je als autoriteit op het gebied

van immuundeficientie en vaccinaties in de kleine commissie plaats wilde nemen.

Prof. dr. T.W. Kuijpers, beste Taco, bedankt voor de bereidheid plaats te nemen in de promotiecommissie, als immunoloog met brede expertise in het vak.

Dr. M. van der Burg, beste Mirjam, in het najaar 2006 mocht ik mijn lab stage immunologie starten bij jou als werkgroepleider van de Primaire Immuundeficientie (PID) groep. Vanaf het begin voelde ik me welkom. Dankzij jou heb ik de mogelijkheid gekregen om me binnen het lab verder te verdiepen in CVID en andere immuundeficienties. Vele uren hebben we ideeën uitgewerkt, "gekauwd" op de soms taaie datasets, om die uiteindelijk te vertalen naar mooie artikelen. Dank voor je coaching en de stimulerende manier waarop je in staat bent om de mensen om je heen te motiveren om het beste uit zichzelf te halen. Dank ook voor de creatieve manier waarop je onderzoeksvragen kan vertalen in experimenten, waardoor dit proefschrift is geworden wat het nu is. Ik hoop dat we de komende jaren het synergisme tussen lab en kliniek kunnen continueren!

Dr. N.G. Hartwig, beste Nico, je hebt me opgeleid tot pediatrisch infectioloog/immunoloog. Ik ben je dankbaar dat je me in de laatste fase van het fellowship hebt aangemoedigd onderzoek te doen naar immuundeficienties. Cruciaal was het feit dat je me als staflid hebt aangenomen en me daarna de ruimte hebt gegeven om me verder te verdiepen in PIDs, een onderwerp waar je zelf veel kennis over in huis hebt. Recent heb je de bakens verzet en bent opleider geworden in het St Franciscus Gasthuis, Rotterdam, wat recht doet aan je bijzondere talent als docent. Veel dank voor de jaren meer dan plezierige samenwerking binnen onze vakgroep en voor je bijdragen aan de verschillende artikelen in het proefschrift.

Dan de leden van de PID groep (in de volksmond: "PIDjes"), door de jaren heen: Sandra (de Bruin), Barbara, Ingrid, Bob, Sandra (Posthumus), Hanna, Erik en Marjolein. Ik ben jullie heel veel dank verschuldigd. Allereerst voor het teamgevoel, waar vind je dat nog op die manier? En verder voor het geduld waarmee jullie me hebben geintroduceerd in het lab (in het begin vooral Ingrid en Barbara). Maar ook voor de belangrijke bijdragen aan alle experimenten die beschreven zijn in dit proefschift. Beste Sandra (de Bruin), na je bijdragen aan de flow cytemetrie van de CVID studie heb je me, nadat je de PID groep had verlaten, bijzonder goed geholpen met het maken van de figuren, dank daarvoor. Ingrid, jouw interesse en bijdragen liggen vooral op het gebied van de moleculaire studies, bedankt voor al het werk dat je hebt verzet en voor de gezelligheid, vooral ook tijdens ons bezoek aan het lab in Freiburg (ook al hadden vrijwel alle cellen die we van te voren hadden opgestuurd het loodje gelegd). Je bent altijd zo behulpzaam en lekker nuchter. Sandra (Posthumus), bedankt voor onder andere de grote bijdrage aan de normaalwaardenstudie bij kinderen, de CVID en de AT studie. Je bent een bescheiden harde werker en ook nog eens kei aardig (zoals ze dat in Brabant zeggen). Bob (die andere Brabander), dank voor je bijdrage aan de verschillende studies en de lekkere BBQ in Dongen. Goed dat je na het aflopen van je

contract binnen de unit een goede plek hebt gevonden. Barbara, je bent vanaf het begin betrokken geweest bij alle studies van dit proefschrift. Er is je niet snel iets teveel, je bent snel, efficient en er valt ook nog eens met je te lachen. Dank voor alle ondersteuning en het feit dat je mijn paranimf wil zijn. Erik, als de nieuwste aanwinst van de PID groep hoop ik de komende jaren nog veel met je samen te werken. Mede AIO Hanna, door jouw bijdrage aan de repertoire analyse in de AT studie is deze echt op een hoger plan gekomen en ook jouw studie geworden. Dank daarvoor! Verder heb je recent veel werk verricht om de laatste losse eindjes van de PTEN studie aan elkaar te knopen (al kunnen deze kersverse data helaas niet meer mee in dit boekje), waardoor je ook aan deze studie een belangrijke bijdrage hebt geleverd. Veel succes met het afronden van jouw proefschrift dit jaar! Marjolein, je bent dit jaar begonnen met nieuwe studies over CVID, vooral het EUROflow PID project. Heel veel succes hiermee.

De afgelopen jaren is er veel samengewerkt met de B-cel differentiatie groep (BCD). Beste Menno, je hebt als werkgroepleider BCD bijgedragen bijna alle studies in dit proefschrift. Ik waardeer je directe, opbouwend kritische manier van communiceren, je bent altijd goed voor een waardevolle visie of mening en een grondige beoordeling van een manuscript. Dank hiervoor. Beste Benjamin en Edwin (inmiddels werkzaam in het LUMC), hartelijk dank voor al het werk dat jullie in de memory B-cel studie de Down studie en CVID studie hebben gestoken. Edwin, de B-cell subset cell sorts gingen als een speer, helaas hebben de vele Ca-fluxen niet opgeleverd wat we ervan verwachtten, maar het was wel leuk om hier samen aan te werken. Benjamin, dank voor de grote bijdrage aan de Down studie. Dear Magda, you finished your PhD with an excellent thesis! Thanks a lot for the enormous amount of work you performed as first author of the memory B-cell study. I wish you a lot of success as postdoc at Sanguin laboratories! Hopelijk komt het stuk in een mooi tijdschrift! Diana en Christina: dank voor jullie bijdrage aan hoofdstuk 2 en Jorn, Halima en Magda R: dank voor de gezelligheid.

Binnen de unit moleculaire immunologie heb ik me de afgelopen jaren prima thuis gevoeld tussen nog veel meer aardige, kundige en behulpzame collega's. Mijn dank voor de plezierige samenwerking in het lab, tijdens brainstormsessies, meetings, bij de koffieautomaat of anderszins gaat uit naar de LLD en MID groep, maar ook naar de unit medische immunologie, de frontservice en het secretariaat.

In de vakgroep pediatrische infectieziekten, immunologie en reumatologie is het goed toeven. Beste Annemarie, dank voor het faciliteren van de normaalwaardestudie en voor de plezierige samenwerking binnen ons team. Je gaat het zeker maken als nieuw subhoofd van onze vakgroep. Beste Pieter, je bent een energieke nieuwe aanwinst van onze groep, dank voor je inspanningen binnen "de club", waardoor ik wat meer tijd had voor de laatste loodjes. Beste Sylvia, dankzij jou heb ik veel opgestoken over auto-immuunziekten, wat goed van pas komt bij de zorg voor kinderen met PID. Beste Conne, je staat niet graag

op de voorgrond, maar toch ga ik je hier bedanken voor alle ondersteuning de afgelopen jaren! Het opvangen van vele telefoontjes (nee, hij is nu op de immuno...), onmisbare hulp bij patiëntenzorg, begeleiding van studenten, en vele, vele dingen meer. Petronette, dank je voor je bijdrage als research verpleegkundige, maar ook als consulent voor de kinderen met immuundeficiënties. Je hebt ook veel gedaan aan de vermoeidheidsstudie, die niet meer in het boekje is gekomen. Daarnaast wil ik je bedanken voor de prachtige omslag die je hebt gemaakt, je hebt veel creatieve genen! Linda, naast je werk voor de kinderen met HIV heb je ook altijd klaar gestaan om voor de kinderen met primaire immuundeficiënties een bijdrage te leveren, wat ik enorm waardeer. Eline en Emiel, dank voor de hulp bij het verzamelen van materiaal voor het normaalwaarden onderzoek en Eline, veel succes ook als vervangster van Petronette! Renate, Marianne en Annet en Heleen, ook jullie hebben jullie steentje bijgedragen met de assistentie op de poli. Beste Annette, dank voor de samenwerking met betrekking tot de kinderen met auto-immuun problemen. Mijn dank gaat ook uit naar de medewerkers van de dagverpleging in het Sophia, voor de behandeling en begeleiding van kinderen met PID.

Beste Virgil, Paul en Jan, ik wil jullie bedanken voor de jarenlange prettige samenwerking binnen het afweercentrum en de inclusie van patiënten voor de verschillende onderzoeken. Virgil, dank ook voor je actieve bijdrage aan de IPH studie en het zetten van belangrijke stappen voor vervolgonderzoek. Ik hoop de komende jaren nog veel samen te ondernemen op het gebied van PIDs. Marianne, als nurse practitioner ben je een spil in de patiëntenzorg, maar ook in het onderzoek. Ik wil je bedanken voor de vele tijd die je in de CVID studie hebt gestoken.

De afgelopen jaren hebben diverse studenten tijdens onderzoeksstages een bijdrage geleverd aan de verschillende studies die beschreven zijn in dit proefschrift. Marleen, je was de eerste die geholpen heeft de klinische data van de CVID patiënten in kaart te brengen. Margreet, je hebt met veel energie en initiatief gewerkt aan de CVID studie, maar hebt daarnaast ook een bijdrage geleverd aan de AT en PTEN studie. Anne, uit jouw koker kwam een mooie onderzoeksstage over IPH patiënten (al hadden we ze toen nog niet zo genoemd…). Arthur, je hebt je vastgebeten in de Akt fosflow en SHM en CSR analyse van de PTEN patiënten, wat geen eenvoudige opgave was. Sandra, you also contributed to the IPH study, which resulted in a nice trip to Chicago to the CIS meeting. Ik wil jullie allemaal hartelijk bedanken voor jullie inspanningen. Thanks a lot!

Beste co-auteurs, jullie hebben een belangrijke bijdrage geleverd door het includeren van patiënten, verzamelen van klinische data, het verrichten van laboratorium analyses, het redigeren van manuscripten, maar soms ook als initiatiefnemer voor het opstarten van een studie. Heel veel dank voor de bijdragen. Dear co-authors, thank you very much for your contributions to the studies in this thesis. In willekeurige volgorde: Dr. A. A. Warris, beste Adilia, je hebt meegewerkt aan de CVID, AT en IPH studie, veel succes met je carrièreswitch

naar Schotland. Dr. M. van Deuren, Marcel, je was samen met Corry Weemaes, een belangrijke de initiator van de AT studie. Het was erg leuk om met je samen te werken, je zit altijd vol met originele ideeën. Corry, ik heb nog college van je gehad en nu had ik het voorrecht van je kennis van DNA repair stoornissen te profiteren. Dr. M.M. Verhagen, beste Mijke, de AT studie borduurde voort op jouw AT studies. Prof dr. Ásgeir Haraldsson, Dr. M. van der Flier, Prof. dr. Malcolm A. Taylor, Dr. N. Wulffraat, you contributed to the to the AT study. Dr. E. de Vries, beste Esther, je hebt bijgedragen aan de CVID en IPH studie en nam het initiatief om de B-cel differentiatie van Down patiënten in kaart te brengen en dit te linken aan de CVID data. Drs. R. Verstegen, beste Ruud, dank voor de plezierige samenwerking in het kader van de Down studie. Dr. W Hop, beste Wim, we hebben geprofiteerd van je statistische expertise. Dr. H. G.Yntema en W. Nillesen, beste Helger en Willie, jullie hebben een belangrijke bijdrage geleverd aan de genetisch analyse van de PHTS patiënten. Dr. E.A.J. Peeters, beste Els, je hebt bijdragen aan de PTEN studie. Prof. dr. N. Kutukculer and dr. O. Çogulu, you were the first to describe patients with macrocephaly and immunodeficiency, one of whom appeared to have a *PTEN* mutation. Thanks for the fruitful collaboration in this project.

De afdeling verloskunde en vrouwenziekten van het ErasmusmMC, in het bijzonder Hans Duvekot, medisch coördinator en Titia Winter en Joke Rhee-Binkhorst, research verpleegkundigen, wil ik bedanken voor het verzamelen van de navelstrengbloedmonsters voor de normaalwaardenstudie.

Dr. M. Rizzi and prof. dr. H. Eibel, dear Marta and Hermann, thank you very much for your hospitality and the collaboration on the PTEN project during my stay in you lab in the Center for Chronic Immunodeficiencies in Freiburg, Germany.

Caroline, je hebt prachtig werk afgeleverd bij het lay-outen van het boekje in een kort tijdsbestek (ook bij het bewerken van de supplemental tables van het AT artikel :-)), hiervoor wil ik je heel hartelijk bedanken.

Beste René, onze vriendschap gaat terug tot vroeg in de collegebanken. Je hebt veel eerder met succes je promotie kunnen afronden dan ik dat deed. Dank je voor het feit dat je mijn paranimf wil zijn.

Berend, Juliëtte en Britta, door jullie besef ik iedere dag dat er belangrijkere dingen zijn dan immuundeficiënties, B-cellen en promoveren. Ik heb heel wat geleerd van jullie feedback: "papa hoort echt niet wat er tegen hem gezegd wordt als hij achter de computer zit" of "pa, heb je dan echt iets nieuws gevonden, dat meen je niet…"

Lieve Hélène, je bent al meer dan 25 jaar het allerbeste dat me is overkomen! Je hebt me heel wat uurtjes moeten missen de afgelopen jaren, waardoor er veel op je schouders terecht kwam. En dan nog de verhuizing, waardoor jouw sabbatical in een verhuisverlof veranderde… We gaan weer meer tijd nemen (= ik ga je meer tijd geven) om van het leven te genieten!

OVER DE AUTEUR

Gertjan Driessen werd op 19 januari 1967 geboren te Son en Breugel. Na het behalen van zijn VWO diploma aan het Pius X college te Almelo ging hij in 1985 geneeskunde studeren aan de Katholieke Universiteit Nijmegen. Na het behalen van het artsexamen was hij van 1993-1994 werkzaamheden als arts-assistent interne geneeskunde in opleiding in het Canisius Wilhelmina Ziekenhuis te Nijmegen (opleider dr. R.W.de Koning).

In de loop van 1994 besloot hij om zijn werkzaamheden voort te zetten als tropenarts in opleiding. In het kader hiervan werkte hij in de periode 1994-1996 als arts-assistent in het ziekenhuis Gelderse vallei, locatie Bennekom (stage chirurgie, opleider drs. H.H.J. Wegdam) en locatie Ede (stage gynaecologie/verloskunde, opleider drs. P. van der Weg). Deze opleiding werd afgesloten met de nationale tropencursus voor artsen in het Koninklijk Instituut voor de Tropen, Amsterdam. Enkele maanden later, in januari 1997, werd hij voor 3 jaar als algemeen tropenarts uitgezonden naar het Holy Family Hospital te Techiman, Ghana. Tijdens deze intensieve periode heeft hij zich onder andere ingezet voor het verbeteren van de kindergeneeskundige zorg, vooral de behandeling van malaria en andere infectieziekten. In dit kader heeft hij i.s.m.de afdeling parasitologie van het st. Radboud Universitair Medisch Centrum Nijmegen (dr. J.P. Verhave) diverse studenten begeleid bij onderzoek naar de werkzaamheid van antimalaria middelen bij kinderen.

Na terugkeer in Nederland in 2000 begon hij met de opleiding kindergeneeskunde in het Medisch Centrum Rijnmond Zuid (opleider dr. E.J.A. Gerritsen). Gedurende die periode werd zijn interesse gewekt voor het vakgebied immunologie. De opleiding werd voltooid in de periode 2001-2004 in het ErasmusMC, Sophia kinderziekenhuis (opleider prof. dr. A.J. van der Heijden). Aansluitend werd hij aangenomen als fellow pediatrische infectieziekten/immunologie (opleiders prof. dr. R. de Groot en dr. N.G. Hartwig).

Gedurende de onderzoeksstage immunologie in het kader van dit fellowship, werd een aanvang gemaakt met onderzoek naar antistofdeficiënties (o.l.v. dr. M. van der Burg en prof. dr. J.J.M. van Dongen), waarna dit onderzoek als promotietraject werd voortgezet. Na zijn registratie als kinderarts infectioloog/immunoloog in 2007 heeft hij zijn werkzaamheden gecontinueerd als staflid infectieziekten/immunologie in het Sophia kinderziekenhuis (hoofd dr. N.G. Hartwig en vanaf 2012 dr. A.M.C. van Rossum), met een speciale interesse voor de diagnostiek en behandeling van primaire immuundeficiënties.

Vanaf november 2013 zal hij zich als opleider voor het fellowship pediatrische infectieziekten/immunologie binnen het Afweercentrum van het ErasmusMC inzetten om patiëntenzorg en onderwijs te combineren met translationeel onderzoek.

Gertjan is getrouwd met Hélène Driessen-Hulshof en heeft 3 kinderen, Berend (1998), Juliëtte (1999) en Britta (2003).

PHD PORTFOLIO

Name PhD student:	Gertjan Driessen
ErasmusMC departments:	Pediatrics and Immunology
Research school:	Molecular Medicine
PhD period:	2007-2013
Promotors:	Prof. dr. J.J.M. van Dongen
	Prof. dr. P.M. van Hagen
Co-promotors:	Dr. M. van der Burg
	Dr. N.G. Hartwig

1. PHD TRAINING

Courses

2007	Infection and immunity in children, University of Oxford, UK.
2007	ESID summer school Primary Immunodeficiencies, Malaga, Spain.
2010	Online course Good Clinical Practice.
2011	BROK master class, ErasmusMC, Rotterdam.

Oral scientific presentations

2007	Grand round ErasmusMC Sophia, Rotterdam.
2007	Research day Pediatrics, ErasmusMC Sophia, Rotterdam.
2008	Meeting of the Dutch Working Group for Primary Immunodeficiencies (WID), Utrecht.
2009	European Congress of Immunology, Berlin, Germany.
2009	Immunology Expert Meeting, Noordwijk.
2010	Scientific meeting of the Pediatric Infectious Disease and Immunology group of the Dutch Pediatric Society (NVK).
2010	Euro PAD net meeting, Oxford, UK.
2010	Congress of the European Society for Immunodeficiencies (ESID), Isanbul, Turkey.

2010	Scientific Meeting Dutch Working Group for Primary Immunodeficiencies (WID), Utrecht.
2010	Symposium 25 years Clinical Immunology, ErasmusMC, Rotterdam.
2011	Immunology expert meeting, Noordwijk.
2011	Congress of the European Society for Pediatric Infectious Disease (ESPID), The Hague.
2012	Meeting of the Dutch Working group for HIV in children (PHON), Utrecht.
2012	Grand round, ErasmusMC Sophia, Rotterdam.
2013	Meeting of the Dutch Society for laboratory personnel (NVLM), The Hague.

Other Seminars and workshops

2007 onwards	Meetings of the Dutch Working group for Primary Immunodeficiency, Utrecht.
2007 onwards	Meetings of the Pediatric Infectious Disease and Immunology group of the Dutch Pediatric Society, Utrecht (biannual).
2011	Chest CT in Antibody Deficiency Syndrome Group, Oxford, UK.

Poster presentations

2012	Clinical Immunological Society Meeting, Chicago, US.
2012	Congress of the European Society for Immunodeficiencies (ESID), Florence, Italy (7 posters).

2. TEACHING

2005-2010	Medical Instructor for the Advanced Pediatric Life Support course (biannual), Riel.
2004 onwards	Lecturer course tropical medicine for medical students, Erasmus Medical Center, Rotterdam (biannual).
2006 onwards	Lecturer postgraduate training for pediatricians, ErasmusMC Sophia, Rotterdam (yearly).
2007 onwards	Teacher at the faculty of medicine, ErasmusMC, multiple contributions (yearly).

2008 and 2010	Lecturer at the course for pediatric infectious diseases of the Dutch Pediatric Society.
2008 onwards	Organization and/or lecturer at the annual seminars in Tropical Pediatrics, Zeist.
2009	Lecturer at the Royal Institute for the Tropics, Amsterdam.
2011	Teaching editor of the European Journal of Pediatrics; editor of five reviews on PID.
2011 and 2013	Organization and lecturer at the pediatric immunology course for general pediatricians of the of the Dutch Pediatric Society.
2011 and 2013	Organization and lecturer at the Dutch infection and immunity day.

INTERNATIONAL PUBLICATIONS

1. **Driessen GJ**, van Kerkhoven S, Schouwenberg BJJW, Bonsu G, Verhave JP. Sulphadoxine/Pyrimethamine: an appropriate first line alternative for the treatment of uncomplicated falciparum malaria in Ghanaian children under five years of age. Trop Med Int Health 2002;7(7):577-583
2. **Driessen GJ**, Gerritsen EJA, Fischer A, Fast A, Hop WC, Veys P, Porta F, Cant A, Stward CG, Vossen JM, Uckan D, Friedrich W. Long-term outcome of haematopoietic stem cell transplantation in autosomal recessive osteopetrosis: an EBMT Report. Bone Marrow Transplantation 2003;32(7):657-663.
3. Verweel G, Burger DM, Sheehan N, Bergshoeff AS, Warris A, van der Knaap LC, **Driessen GJ**, de Groot R, Hartwig NG. Plasma concentrations of the HIV-protease inhibitor lopinavir are suboptimal in children aged two years and below. Antiviral Therapy 2007;12(4):453-458
4. **Driessen GJ**, Pereira R , Brabin BJ , Hartwig NG. Imported malaria in children: a national surveillance in the Netherlands and a review of European studies. Eur J Public Health 2008;8:184-188.
5. de Steenwinkel JEM, **Driessen GJ**, Kamphorst-Roemer MH, Zeegers AGM, Ott A, van Westreenen M. Tuberculosis mimicking ileocecal intussusception in a 5-Month-old girl. Pediatrics 2008;121(5):1434-1437
6. van Bilsen K, **Driessen GJ**, de Paus RA, van de Vosse E, van Lom K, van Zelm MC, Lam KH, Hartwig NG, Baarsma GS, van de Burg M, van Hagen PM. Low level IGF-1 and common variable immune deficiency: an unusual combination Neth J Med. 2008 ;66(9):368-372
7. van der Flier M, Verweel G, van der Knaap LC, van Jaarsveld P, **Driessen GJ**, van der Lee M, Hartwig NG, Burger DM Pharmacokinetics of lopinavir in HIV type-1-infected children taking the new tablet formulation once daily. Antivir Ther. 2008;13(8):1087-1090.
8. van Zwol AL, Lequin M, Aart-Tesselaar C, van der Eijk AA, **Driessen GJ**, de Hoog M, Govaert P. Fatal neonatal parechovirus encephalitis. BMJ Case Rep. 2009;2009: bcr05.2009.1883.
9. Poodt AE, **Driessen GJ**, de Klein A, van Dongen JJ, van der Burg M, de Vries E. TACI mutations and disease susceptibility in patients with Common Variable Immunodeficiency. Clin Exp Immunol. 2009;156(1):35-39
10. Rakhmanov M, Keller B, Gutenberger S, Foerster C, Hoenig M, **Driessen GJ**, van der Burg M, van Dongen JJM, Wiech E, Visentini M, Quinti I, Prasse A, Voelxen N, Salzer U, Goldacker S, Fisch P, Eibel H, Schwarz K, Peter HH, Warnatz K. 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):13451-13456.

11. Zubakov D, Liu F, Zelm MC van, Vermeulen J, Oostra BA, Duijn CM van, **Driessen GJ**, Dongen JJM van, Kayser MH, Langerak AW. Estimating human age from T-cell DNA rearrangements. Current Biology 2010;20(22): R970-R971.
12. Burg M van der, Zelm MC van, **Driessen GJ,** Dongen JJM. Dissection of B-Cell development to unravel defects in patients with a primary antibody deficiency. Advances in Experimental Medicine and Biology 2011;697:183-196.
13. Chaim LYT, Verhagen MMM, Haraldsson A, Wulffraat NM, **Driessen GJ**, Netea MG, Weemaes CMR, Seyger MMB, Deuren M van. Cuteneous granulomas in ataxia telangiectasia and other primary immunodeficiencies: reflection of inappropriate immune regulation? Dermatology 2011;223:13-19.
14. Vries, E. de, **Driessen GJ**. Primary immunodeficiency in children: a diagnostic challenge. European Journal of Pediatrics 2011;170:169-177.
15. **Driessen GJ,** Burg M van der. Primary antibody deficiencies. European Journal of Pediatrics 2011;170:703-702.
16. Berkowska MA, **Driessen GJ**, Bikos V, Grosserichter-Wagener C, Stamatopoulos K, Cerutti A, He B, Biermann K., Lange JF, Burg M van der, Dongen JJM van, Zelm MC van. Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways. Blood 2011;118(8): 2150-2158.
17. Pike-Overzet K, Rodijk M., Ng YY, Baert MRM, Lagresle-Peyrou C, Schambach A, Zhang F, Hoeben RC, Hacein-Bey-Abina S, Lankester AC, Bredius RGM, **Driessen GJ**, Thrasher AJ, Baum C, Cavazzana-Calvo M, Dongen JJM. Van, Staal FJT. Correction of murine Rag1 deficiency by self-inactivating lentiviral vector-mediated gene transfer. Leukemia 2011;25(9):1471-1483.
18. Schatorje EJH, Gemen EFA, **Driessen GJ**, Leuvenink J, Hout RWNM van, Burg M van der, Vries E de. Age-matched Reference Values for B-lymphocyte Subpopulations and CVID Classifications in Children. Scandinavian Journal of Immunol. 2011;74(5): 502-510.
19. **Driessen GJ**, Zelm MC van, Hagen PM van, Hartwig NG, Trip MD, Warris A, Vries E de, Barendregt BH, Pico I, Hop WCJ, Dongen JJM van, Burg M. van der. B-cell replication history and somatic hypermutation status identify distinct pathophysiologic backgrounds in common variable immunodeficiency. Blood 2011;118(26): 6814-6823.
20. van Rijn SF, **Driessen GJ**, Overbosch D, van Genderen PJ. Travel-related morbidity in children: a prospective observational study. J Travel Med. 2012;19(3):144-149
21. Schatorjé EJ, Gemen EF, **Driessen GJ**, Leuvenink J, van Hout RW, Vries E. de Paediatric reference values for the peripheral T cell compartment Scand J Immunol. 2012;75(4):436-444.

22. de Vries E; **European Society for Immunodeficiencies (ESID) members**: Patient-centred screening for primary immunodeficiency, a multi-stage diagnostic protocol designed for non-immunologists: 2011 update.Clin Exp Immunol. 2012;67(1):108-119
23. Burg M. van der, Zelm MC van, **Driessen GJ**, Dongen JJM van. New frontiers of primary antibody deficiencies. Cellular and Molecular Life Sciences 2012;69:59-73.
24. **Driessen GJ**, IJspeert H, Weemaes HMR, Haraldsson A, Trip M, Warris A, van der Flier M, Wulffraat N, Verhagen MMM, Taylor MA, van Zelm MC, van Dongen JJM, van Deuren M, van der Burg M. Antibody deficiency in Ataxia Telangiectasia is caused by disturbed B and T cell homeostasis and reduced immune repertoire diversity. J Allergy Clin Immunol. 2013;131(5):1367-1375e9
25. **Driessen GJ**, Dalm VASH, van Hagen PM, Grashoff HA, Hartwig NG, van Rossum AMC, Warris A, de Vries E, Barendregt BH, Pico I, Posthumus S, van Zelm MC, van Dongen JJM, van der Burg M. Common variable immunodeficiency and idiopathic primary hypogammaglobulinemia: two different conditions within the same disease spectrum. Haematologica 2013 Jun 10. Epub ahead of print.
26. Janssen WJ, Bloem AC, Vellekoop P, **Driessen GJ**, Boes M, van Montfrans JM. Measurement of pneumococcal polysaccharide vaccine responses for immunodeficiency diagnostics: combined IgG responses compared to serotype specific IgG responses. J Clin Immunol. 2013 Jul 24. Epub ahead of print.

NATIONAL PUBLICATIONS

1. van der Meulen M, van Hellemond JJ, van Genderen PJJ, **Driessen GJ**. Evaluatie van kinderen met koorts na terugkeer uit de tropen. Tijdschrift voor Infectieziekten. 2011;8:178-186
2. van Montfrans JM, **Driessen GJ**. Common Variable Immuundeficiency de "need to knows" voor de algemeen kinderarts. Praktische Pediatrie 2013 *in press*.
3. **Driessen GJ**, van Montfrans JM. Immunoglobulinetherapie. Praktische Pediatrie 2013 *in press*.

BOOK CHAPTERS

1. **Driessen GJ**, Waard-van der Spek FJ, Oranje AP. Erythema multiforme en toxische epidermale necrolyse in : Handboek Kinderdermatologie Waard-van der Spek FJ, Oranje AP (eds), Elsevier 2005

2. Hazelzet JA, **Driessen GJ**, Abboud P, Wheeler DS, Shanley TP, Wong HR. Chapter 100 Sepsis in: Pediatric Critical Care Medicine: Basic Science and Clinical Evidence, Eds. Wheeler D, Wong HR, Shanley T. Springer 2007.
3. Kager PA **Driessen GJ**. Hoofdstuk 19 Import infectieziekten in: Werkboek Infectieziekten. Furth AM, Wolfs TFW, Hartwig NG (eds). VU uitgeverij 2008.
4. Burg M van der, Zelm MC van, **Driessen GJ**, Barendregt, BH, Knijnenburg I, Turnhout BAC van, Posthumus -van Sluijs SJ, Dongen JJM. van. Ontwikkelingen in de diagnostiek van primaire immunodeficienties van het lymfatische systeem. In van Dongen JJM, Dik WA (eds), Nieuwe ontwikkelingen in de Medische Immunologie 2010 Rotterdam: Erasmus MC.
5. de Vries E, Terheggen-Lagro S, **Driessen GJ**. Hoofdstuk 30 afweerstoornissen. In Werkboek kinderlongziekten. van Gent R, Merkus P, Pijnenburg M, Rottier B (eds). VU uitgeverij 2012
6. **Driessen GJ**. Common Variable Immunodeficiency. In Infectieziekten en afweerstoornissen bij kinderen. van Furth AM, Ang CW, Bredius RGM, van der Kuip M, Warris A, Wolfs TFW, Kneepkens CMF (eds). Prelum uitgeverij 2013
7. **Driessen GJ**, de Vries E., Bredius R, van Montfrans JM. Hoofdstuk 4 Primaire antistof deficiënties. In Werkboek kinderimmunologie. VU uitgeverij 2013 *in press*
8. de Vries E, **Driessen GJ** en van Montfrans JM. Hoofdstuk 21 Antimicrobiele therapie en profylaxe. In Werkboek kinderimmunologie, VU uitgeverij 2013. *in press*
9. van Montfrans JM, de Vries E, **Driessen GJ**. Hoofdstuk 22 Gammaglobuline. In Werkboek kinderimmunologie, VU uitgeverij 2013 *in press*


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