Clinical and immunological diversity of recombination defects

Hanna IJspeert

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Molecular Medicine
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Clinical and Immunological Diversity of Recombination Defects

Klinische en immunologische diversiteit van recombinatie defecten

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Chapter 1

General Introduction

GENERAL INTRODUCTION

The immune system is capable to detect a large variability of pathogens and distinguish them from the body's own healthy tissue. The immune system consists of the innate- and adaptive immune system. The adaptive immune system consists of B-and T-lymphocytes, which can recognize pathogens with an antigen-specific receptor. B-lymphocytes can adapt their responses during an infection to improve recognition of the pathogen and they generate long-term immunological memory. These antigen-specific receptors are called B-cell receptor (BR) and T-cell receptor (TR), respectively. Since the number of possible antigens is innumerable, an enormous diversity of the antigen-specific receptors is required. To achieve this, the BR and TR contain a variable domain that is unique for each individual T or B cell. This variable domain is generated by recombination of the antigen receptor genes in a process which is called V(D)J recombination. This process requires lymphoid specific proteins to generate DNA double strand breaks (DSB), but is also dependent on a common DNA repair pathway to repair the DSB. Defects in V(D)J recombination hamper the production of the antigen-specific receptors, which results in a complete or partial block in B- and T-cell differentiation, leading to a combined B- and T-cell deficiency.

In this **General Introduction** the B and T-cell development will be described, with special focus on the generation of the antigen-specific receptor repertoire. The role of DNA repair during the V(D)J recombination process and during further maturation of the B cells will be highlighted. Subsequently, clinical and immunological aspects of V(D)J recombination and DNA repair defects resulting in immunodeficiency will be addressed. Finally, the aims of this thesis will be outlined.

IMMUNOGLOBULIN AND T-CELL RECEPTOR MOLECULES

B-cell receptor

The BR, also called immunoglobulin (IG) is composed of two identical heavy chains (IGH) and two identical light chains, either IGκ or IGλ (Figure 1A). The heavy and light chains all consist of a variable and a constant domain. The variable domain of the IG is encoded by a combination of one the V, D and J genes (heavy chain) (Figure 1B), or by a combination of the available V, and J genes (IGK or IGL). The IGH locus contains 9 constant genes (Cμ, Cδ, Cγ3, Cγ1, Cα1, Cγ2, Cγ4, Cε and Cα2), which have different effector functions. Naive B cells express Cμ and Cδ, but upon encountering an antigen the effector function can be changed, by replacing the constant gene during a process called class switch recombination (CSR).

T-cell receptor

A TR molecule consists of two chains, either a TRα and a TRβ chain (TRαβ) or a TRγ and a TRδ chain (TRγδ) (Figure 1C). The vast majority of mature T lymphocytes (85-95%) expresses TRαβ; a minority expresses TRγδ (5-15%).[1] The variable domain of the TR is encoded by a combination of one of the many variable (V), diversity (D) and joining (J) genes (TRB en TRD loci), or by a combination of the available V and J genes (TRA and TRG loci).[1, 2] The constant domains of the TR chains are encoded by constant (C) genes.

B-CELL DEVELOPMENT

Precursor B cells derive from multipotent hematopoietic progenitors and develop in the bone marrow into immature B cells (Figure 2). This differentiation occurs in a stepwise manner and the main objective is to create a unique IG molecule. At the immature B-cell stage, the IG molecule is tested for functionality without high affinity for auto-antigens, after which they go to the periphery and become transitional B cells. The transitional B cells are immature in their migration capacity and response to antigen, but develop rapidly into naive mature B cells. After B cells have encountered antigen, they can further mature

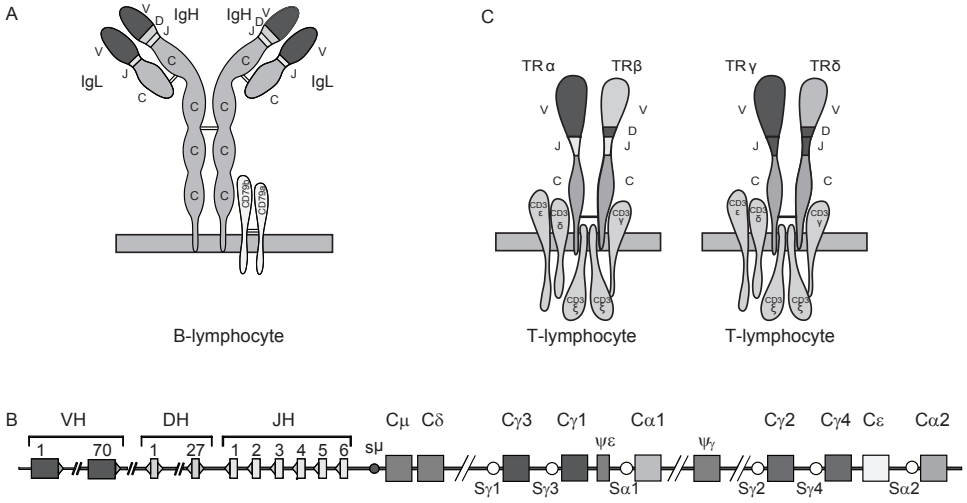

Figure 1. Composition and generation of T- and B-cell receptors. A) The B-cell receptor (BR) consist of two heavy (IGH) and two light chains (IGL). B) Schematic overview of IGH locus including the constant genes. C) The T-cell receptor (TR) consist of either a TRα and TRβ chain or a TRγ and TRδ

in a T-cell dependent or T-cell independent manner and become an antibody secreting plasma cell or a memory B cell.

IG rearrangements

During precursor B-cell differentiation, V(D)J recombination starts with the incomplete DH-JH rearrangements on both IGH loci (Figure 3),[3, 4] which occur at the pro-B cell stage (Figure 2).[5-7] Subsequently, in the pre-B-I cell stage only one allele continues with the V to DJ rearrangement, and the second allele only rearranges when the first is not productive (e.g. out of frame or with a stop codon). Once a VDJ exon is formed, RNA transcripts are produced and the exon is spliced to the Cμ exon. If the resulting Igμ heavy chain is capable of pairing with the surrogate light chain proteins λ14.1 and VpreB, this pre-B-cell receptor complex (pre-BcR) is expressed on the plasma membrane of the cell at the large pre-B-II cell stage. Expression of a functional pre-BcR is an important checkpoint in precursor B-cell differentiation.[8, 9] Expression of the pre-BcR receptor induces several processes, which include allelic exclusion and induction of proliferation. As soon as the pre-BcR complex is down regulated, proliferation will be limited and rearrangement of the IG light chains is initiated, and cells will progress to the small pre-B-II cell stage. First, a Vκ will rearrange to a Jκ, and if this junction is not productive, subsequent Vκ-Jκ rearrangements can take place until a functional Vκ-Jκ junction is formed. If not, further rearrangement to the kappa-deleting element (Kde) takes place, which removes the Cκ from the genome.[10] If the rearrangement of the first IGK allele is not functional, rearrangement of the second IGK allele takes place.

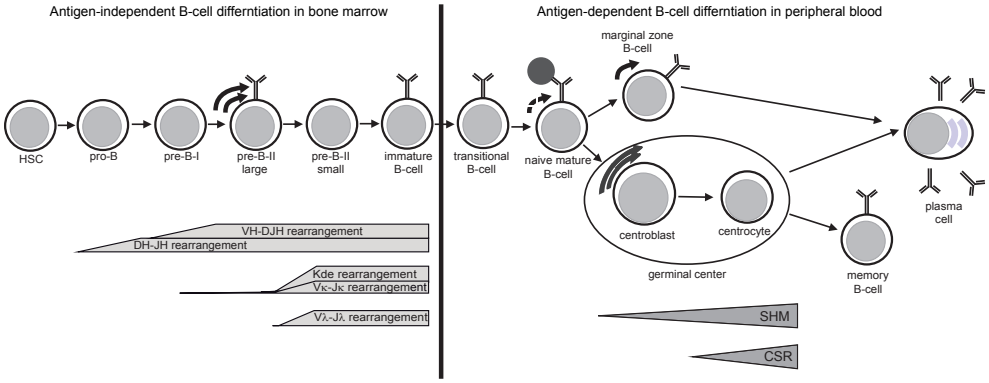

Figure 2. B cell development. Schematic overview of the stepwise development of B cells from hematopoietic stem cells (HSCT) to memory B cells and plasma cells.

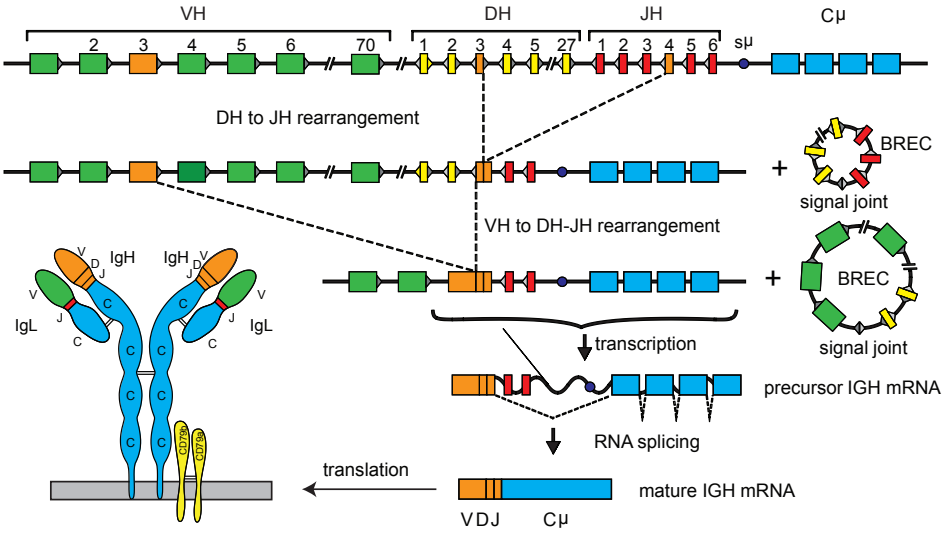

Figure 3. V(D)J recombination of the IGH locus. Rearrangements at the IGH locus start with a DH-JH rearrangement, followed by a V to DJ rearrangement. The excised DNA forms a signal joint, resulting in a B-cell receptor excision circle (BREC). Upon functional rearrangements, the RNA is spliced and can be translated into a B-cell receptor protein.

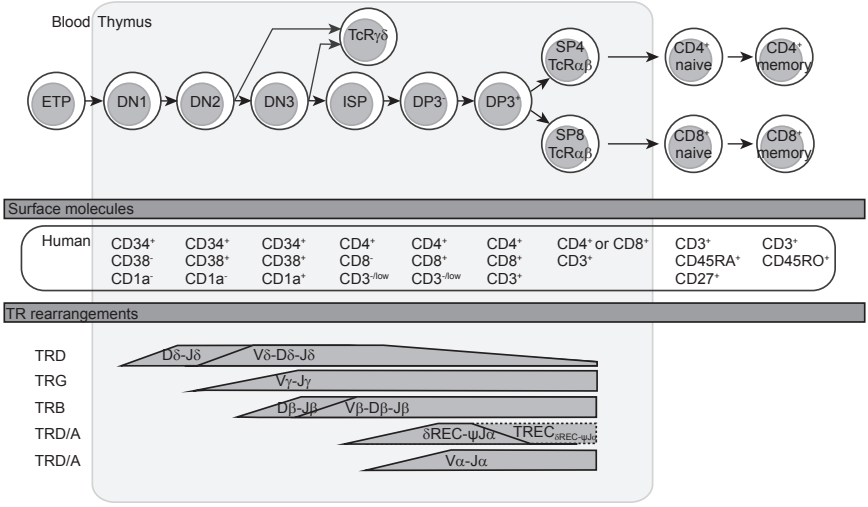

Figure 4. T cell development. Schematic overview of consecutive T-cell developmental stages. Progenitor cells that enter the thumus develop into mature T cells via consecutive developmental stages, followed by further maturation and differentiation in the peripheral blood upon encounter of antigens. The different developmental stages, can be recognized by surface markers and by TR rearrangements. ETP: early thymic progenitor, DN: double negative, ISP: immature single positive, DP: double positive, SP: single positive, TR: T-cell receptor Adapted from thesis Van der Weerd, Dik et al, van Dongen et al.[1, 16]

If both IGK alleles are non-functional, rearrangement of the IGL allele takes place.[11] Once a functional BR is formed that does not display high affinity for autoantigens, the immature B cell will further mature and migrate to the periphery as a transitional B cell (Figure 2).

B-cell maturation in the periphery

After the assembly of a functional BR and screening for poly- and auto-reactivity, B cells migrate to the periphery.[12] Upon antigen recognition and T-cell activation, reactive B cells home to germinal centers of peripheral lymphoid organs to undergo two additional maturation steps: somatic hyper mutation (SHM) and CSR.[13] Germ-line encoded variable regions often make low affinity antibodies.[13] During the process of SHM, point mutations are induced and accumulate in the rearranged V(D)J gene regions with the potential of creating a higher affinity. Due to selection, B cells with high affinity for antigen binding regions dominate the late antibody response. In addition to the process of SHM, CSR is induced (Figure 2), which results in replacement of the constant region, thereby changing the effector function of the BR.

T-CELL DEVELOPMENT

T-cell development in thymus

T cells derive from bone marrow multipotent hematopoietic progenitors that seed in the thymus.[14] T cells undergo several immunogenotypic and immunophenotypic changes, with the final aim to express a functional TR on the membrane. Four main T-cell developmental stages can be defined based on the expression of CD4 and CD8 co-receptors (Figure 4). T-cell precursors entering the thymus lack CD4 and CD8 surface expression and are therefore called double negative (DN). The DN cells can be further subdivided into DN1, DN2 and DN3 based on the expression of CD34, CD38 and CD1a (Figure 4). These DN cells subsequently develop into immature single positive (ISP) cells expressing CD4, followed by further maturation into double positive (DP) cells expressing both CD4 and CD8. During the DP stages, T cells are positively selected for recognition of self-peptides/MHC complexes and negative selected for too strong binding to the self-antigen/MHC complexes.[15] Finally, the T cells further mature into single positive (SP) mature T cells expressing either CD4 or CD8.

TR rearrangements

During T-cell development V(D)J recombination starts at the DN stage and occurs in a sequential manner starting with the TRD, followed by TRG, TRB and finally the TRA locus (Figure 4).[1, 16, 17] The TRD rearrangement is a multistep process in which Dδ to Dδ, DDδ to Jδ and Vδ to DDJδ are joined, followed by the rearrangement of a Vγ to a Jγ in the TRG

locus. If the TRD and TRG rearrangements are productive, a TRγδ receptor can be produced and cells can further develop into the TRγδ lineage.[1, 16] However, T cells with non-functional TRG/TRD rearrangements can continue with rearranging the TRB locus, followed by complex rearrangements in the TRD/A locus.[18-21] If the TRB and TRA rearrangements are productive, T cells will further develop into the TRαβ lineage.

V(D)J RECOMBINATION

Both the TR and IG loci consist of multiple V, D and J genes, which can be recombined during a process called V(D)J recombination (Figure 3), this process ensures the enormous diversity of the antigen receptors. The V(D)J recombination process consist of three phases: the initiation phase, the processing phase and the ligation phase (Figure 5).

The initiation phase

All V, D and J genes are flanked by specific recombination signal sequences (RSSs). These RSSs consist of a palindromic heptamer sequence adjacent to the coding sequence and a nonamer sequence that are separated by a less conserved spacer region of 12 or 23 base pairs.[22, 23] In principle, only RSS with different spacer lengths can join efficiently, known as the 12/23 rule.[22] The RSS flank the 3'V genes, both sides of the D genes and the 5'side of the J genes. There is a consensus sequence (CACAGTG) for the heptamer and for the nonamer (ACAAAAACC). The consensus RSS appears to be the optimal for V(D)J recombination, but only five VH genes (VH3-9, VH3-43, VH4-34, VH4-39 and VH4-59) are flanked by this consensus RSS.[24] The RSSs flanking the other genes deviate considerably from the consensus. V(D)J recombination is initiated by the lymphoid specific recombination activating gene 1 (RAG1) and 2 (RAG2) proteins (Figure 5), which introduce single strand nicks between the coding segment and the flanking RSS.[25, 26] This results in a coding end with a hairpin structure, and a blunt end at the side of the RSS (signal end). The resulting signal ends of both RSS are ligated into a signal joint.

Hairpin opening and the processing/ligation phase

The RAG proteins are lymphoid specific and are only expressed at the developmental stages where V(D)J recombination takes place,[6, 16] but the processing and repair of the DNA DSB is done by the common nonhomologous end-joining (NHEJ) pathway of DNA repair. The DNA DSBs are recognized by the DNA-dependent protein kinase (DNA-PK) complex, which is composed of the DNA-PK catalytic subunit (DNA-PKcs) and the KU70/KU80 heterodimer.[27, 28] After initial loading of the Ku70/Ku80 heterodimer onto DNA ends, DNA-PKcs is recruited to form a stable protein-DNA complex that ensures protection from

exonuclease activities and stimulates juxtaposition of DNA ends.[29] Subsequently, the Artemis protein is phosphorylated by DNA-PKcs, and opens the hairpin-sealed coding ends, which are formed by the RAG proteins.[30, 31]. To create even more variability in the receptors, the DNA ends are further processed, resulting in junctional diversity (Figure 6).

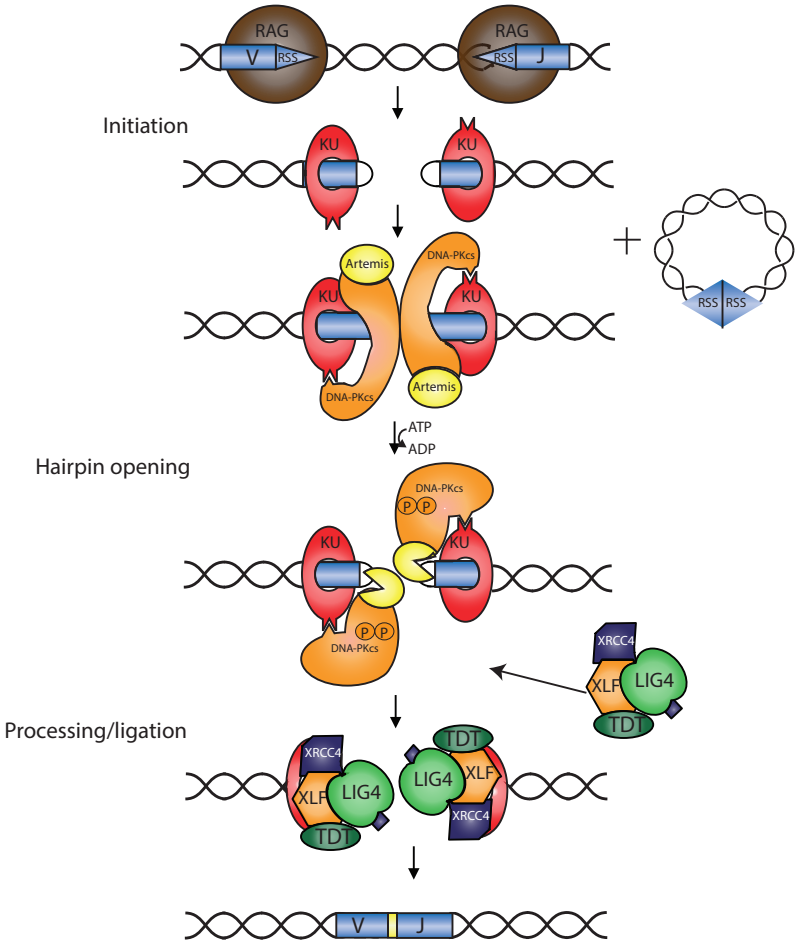

Figure 5. V to J recombination in detail. VJ recombination starts with the induction of DNA DSB between the V and J genes and the recombination signal sequences (RSSs), which result in a coding end and a signal joint. The DSB of the coding ends are recognized by the KU70/KU80 complex (indicated as KU). Subsequently, DNA-PKcs binds to the C-terminus of Ku80 and forms a complex with KU. After DNA-PKcs becomes phosphorylated, it undergoes a conformational change. DNA-PKcs activates Artemis, which opens the hairpins. Thereafter, the DNA is processed by removal of nucleotides and insertion of random N-nucleotides by TdT. Finally, the DSB are ligated by LIG4 in complex with XLF and XRCC4.

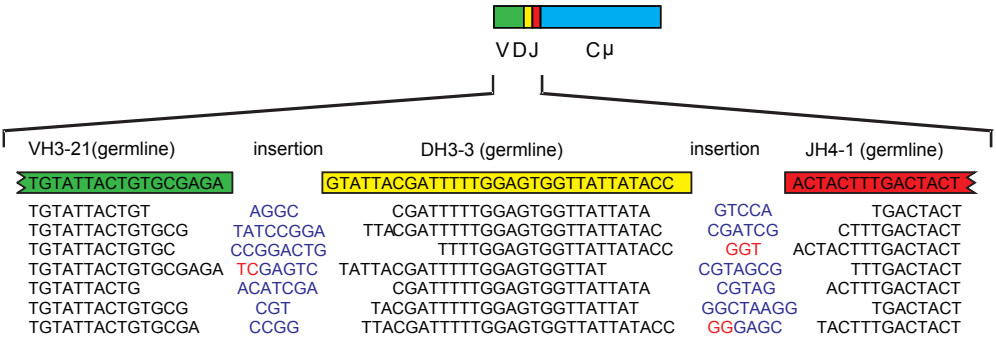

Figure 6. Junctional diversity. Examples of VDJ junctions showing junctional diversity by nucleotides that are removed, non-templated nucleotides that are added (blue) and palindromic nucleotides (red).

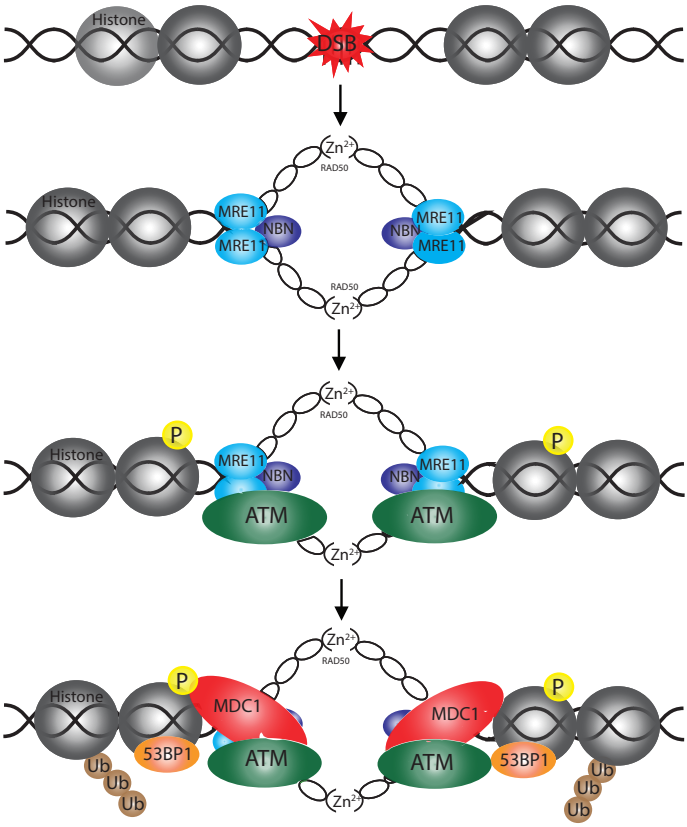

Figure 7. DNA damage response. DSB are recognized by the MRN complex (MRE11, NBN and RAD50) and keep the DNA ends in close proximity. ATM is recruited and phosphorylates histone H2AX, which mediates binding of MDC1 and initiates ubiquitination of histones proximal to the break. Finally, 53BP1 is recruited which suppresses DNA end resection and skews repair towards non-homologous end joining. Adapted from Blundred and Stewart.[147]

In case of asymmetric opening of the hairpins, palindromic (P) nucleotides are present. Nucleotides can be lost due to exonuclease activity and non-templated (N) nucleotides can be randomly added by terminal deoxynucleotidyl transferase (TdT).[31-33]

The final step of V(D)J recombination is the ligation step. The coding ends are ligated by the DNA ligase IV (LIG4)/XRCC4 complex in conjunction with Cernunnos/XRCC4-like factor (XLF) (Figure 5).[23, 34-37] LIG4 interacts with XRCC4 via its C-terminal region [38] and forms a 1:2 complex [39]. The interaction with XRCC4 is important for LIG4, since it stabilizes LIG4, and protects it from degradation [40].

DNA damage response proteins

After induction of DNA double strand breaks by RAG1 and RAG2, the MRN complex consisting of MRE11, RAD50 and NBN is activated.[41] This complex binds to the DNA ends and tethers them together to ensure that the ends remain in close proximity (Figure 7).[42, 43] In addition, the MRN complex recruits the ataxia-telangiectasia-mutated (ATM) protein to the breaks. Activation of ATM involves an autophosphorylation step that converts inactive dimers into active monomers.[44] After activation, ATM phosphorylates hundreds of targets resulting in the initiation of cell cycle arrest, activation of DNA repair pathways and initiation of apoptosis in cells that fail to repair DNA breaks.[45-48] Among these ATM targets is histone H2A variant H2AX, which gets phosphorylated and forms γH2AX.[49]

Subsequently, γH2AX mediated binding of MDC1,[50] which stabilizes the MRN complex,[51, 52] and initiates the ubiquitination of histones proximal to the break, resulting in relaxation of the chromatin surrounding the break site, making it more accessible for repair proteins, such as 53BP1.[53] The precise role of 53BP1 is not known, but 53BP1 is involved in suppressing DNA end resection, which skews the repair towards NHEJ.[54, 55]

MOLECULAR PROCESSES OF SHM AND CSR

SHM

During the first step of both SHM and CSR, activation-induced cytidine deaminase (AID) converts a cytidine (C) to a uracil (U) by deamination (Figure 8A). In the recognition of a C, AID has shown a preference for RGYW and WRCY motifs (R=Purine, Y=pyrimidine, W = A or T).[56, 57] This conversion generates a mismatch between the newly formed U and the guanine (G) on the complementary DNA strand. There are several ways this mismatch can be repaired, all likely to result in mutations (Figure 8A). During replication, the U can be recognized as a thymine (T), resulting in a C to T transition mutation (Figure 8B). Alternatively, the U can be recognized and removed by uracil-N-glycosylase (UNG), creating an abasic site recognizable for members of the base excision repair (BER) machinery.[58] Subsequently,

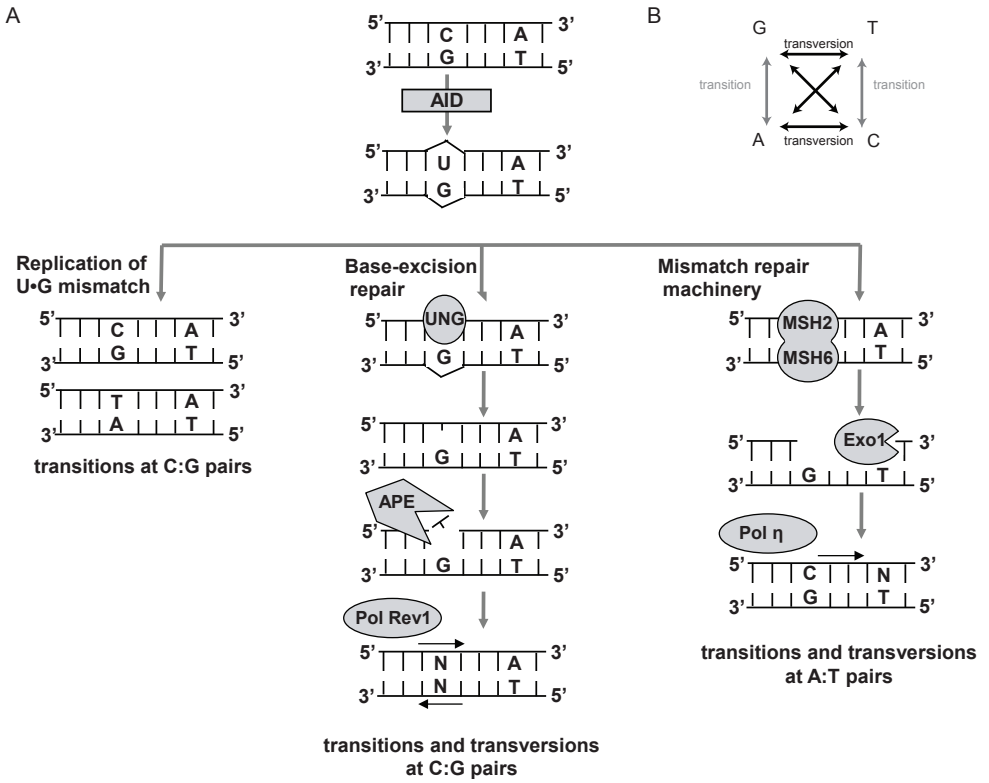

Figure 8. Somatic hypermutations. A) Somatic hypermutations are induced by deamination of a C to a U by AID. The resulting mismatch can be repaired in three different ways. Replication of the U-G mismatch, removal of the U by UNG and repair via base-excision repair (BER), or recognition of the mismatch and repair via the mismatch repair machinery. B) Transition and transversion mutations.

apyrimidimic endonuclease (APE) nicks the phosphate backbone at the abasic site, creating a single stranded (ss)DNA break.[59] Error-prone polymerases then insert a random nucleotide at the abasic site allowing both transition and transversion mutations to take place. This only results in mutations at G:C bases. Mutations at A:T bases can occur when MSH2-MSH6 heterodimer recognizes the mismatch [60-63] and recruits additional members of the mismatch repair (MMR) machinery, like exonuclease 1(Exo1).[64] Exo1 excises base pairs and leaves a ssDNA gap.[55] This gap can then be filled by polymerase η, which inserts random nucleotides at A:T pairs with preferential targeting of WA and TW motifs.[65]

CSR

When two ssDNA breaks are in close proximity, a DSB can occur. Due to the high density of AID motifs in the switch regions, multiple DSBs occur during CSR (Figure 9). When a

break occurs in the switch region of both Cμ and another constant region (Cγ, Cα or Cε), the two switch regions can be ligated to perform successful class-switching. Ligation can be established by the NHEJ pathway or the alternative end joining (A-EJ) pathway. The NHEJ pathway is the predominant pathway for DSB repair in healthy cells,[66] but when key factors in the NHEJ pathway are missing, A-EJ pathway becomes dominant.[67] Key factors in the A-EJ pathway are several members of the MMR machinery, including Exo1 and MSH2.[67] Switch regions rearranged by A-EJ show long stretches of microhomology and are more error prone.[68, 69] The intervening DNA, between the switch regions, is excised, making CSR irreversible, but switching to more downstream regions remains possible upon second activation. Contrary to SHM, CSR is not random and does not affect the affinity of the Ig molecule. Instead, cytokines regulate the accessibility of particular switch regions [70, 71]. By CSR the effector function of the antibody alters. IgM antibodies are expressed without SHM and therefore tend to have low affinity for the antigen. However, these IgM molecules form polymers which increases their avidity, but make it harder to diffuse out of the blood vessels. The antibodies of the other classes-IgG, IgA, IgE-are smaller, and diffuse easily out of the blood into the tissues. IgG is the principal class in blood and can act as neutralizing antibody, opsonisation (IgG1 and IgG3) and activation of the complement system (IgG3 and IgG1).[72] IgA is less susceptible for bacterial proteases and is mainly expressed in mucosal tissue acting as neutralizing antibodies.[73] Finally, the main function of IgE is sensitization of mast cells.[74]

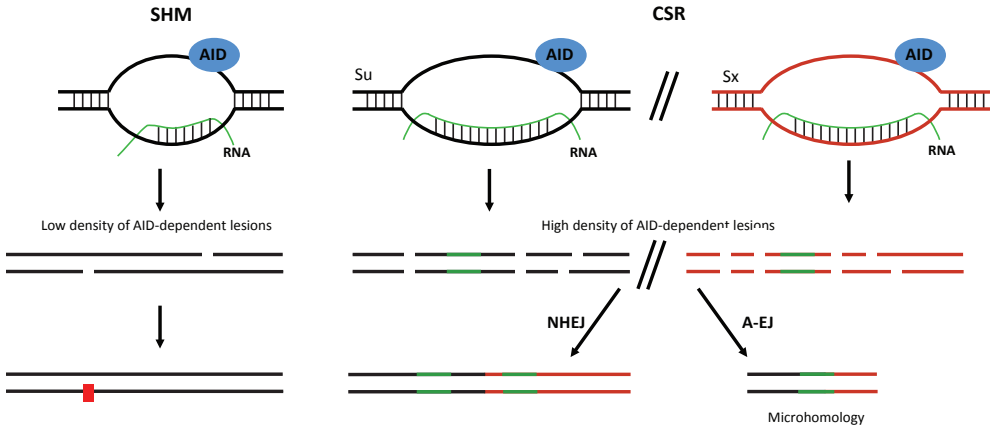

Figure 9. Class switch recombination. During SHM, AID induces DNA lesion at a low density, resulting in somatic mutations. However, during CSR, AID induces high density lesions resulting DSBs which can be repaired via NHEJ or A-EJ.

IMMUNE RECEPTOR REPERTOIRE

The immune receptor repertoire is defined as the total set of different B or T-cell receptors, and can be divided in naïve and antigen-selected repertoire. The naïve repertoire is formed upon V(D)J recombination and is not selected by antigens, in contrast to the antigen-selected repertoire.

Diversity of naïve T- and B-cell receptors

The total diversity of the unique TRs and BRs is the sum of the combinational and junctional diversity of the individual chain, but also the combination of the different chains (TRα and TRβ, TRγ and TRδ, or heavy and light chains) that are combined. The many different functional Vβ (n=44-47), Dβ (n=2) and Jβ (n=13) genes in the TRB locus and Vα (n=45) and Jα (n=53) genes in the TRA locus determine the potential V(D)J combinational diversity of the TRαβ receptors, which is estimated to be >$2x10^6$ different combinations. The total diversity of the TRαβ receptors is further increased by junctional diversity, which is estimated to result in a total repertoire of >10^{12}. The number of functional V, D and J genes is lower for the TRγδ receptors, resulting in a limited combinational diversity of >5000. However, this is compensated by the extensive junctional diversity present in these junctions, resulting in a total estimated repertoire of >10^{12} (Table 1). The IGH locus consist of 38-46 functional VH, 25 DH and 6 JH genes resulting in a combinational diversity of >$5.7x10^3$. In combination with the 200 possible Igκ and 124 Ig λ rearrangements, this

Table 1. Combinational and junctional diversity of the T- and B-cell receptors.

	TRαβ		TRγδ		IG		
	TRα	**TRβ**	**TRγ**	**TRδ**	**IGH**	**IGκ**	**IGλ**
number of genes							
V genes	45	44-47	6	6	38-46	43	38
D genes		2		3*	25		
J genes	53	13	5	4	6	5	4
Combinational diversity	>$2X10^6$		>5000		>$1.8X10^6$		
Junctional diversity	+	++	++	++++	++	±	±
Total diversity	>10^{12}		>10^{12}		>10^{12}		

*Multiple D genes can be used

results in a combinational diversity of >1.8 x10^6. Similarly to the TR, junctional diversity increases the total estimated diversity of the IG to >10^{12}.

Antigen-selected repertoire

Both the T- and B-cell receptor repertoires are changed after antigen exposure. A striking example in the TRγδ repertoire is the presence of Vγ9/Vδ2. In neonatal cord blood and infancy, the Vγ9/Vδ2 T-cells only represent 5-15% of all TRγδ+ T cells, however in older children and adults 80-90% of the TRγδ+ T cells in blood express this receptor with a specific selection epitope in the Vδ2-Jδ1 junctional region.[75, 76] In B cells, the presence of somatic hypermutations change the antigen-selected repertoire, ensuring higher affinity for the antigen.

SEVERE COMBINED IMMUNODEFICIENCY

Severe combined immunodeficiency (SCID) is an inherited primary immunodeficiency, characterized by absence or dysfunction of T lymphocytes. In these patients both the humoral and cellular immunity is defective, and are therefore highly susceptible to bacterial, viral, fungal and opportunistic infections. Most of these patients suffer within months after birth from severe opportunistic infections, chronic diarrhea, and failure to thrive. Antimicrobial prophylaxis and immunoglobulin substitution are mandatory in clinical management but curative treatment can only be obtained by allogeneic stem cell transplantation (SCT) and, in an experimental setting, gene therapy.[77-79] According to the classification system of the International Union of Immunological Societies (IUIS) SCID can be classified as T-B+SCID or T-B-SCID, based on the presence or absence of B cells.[80]

T-B+SCID is caused by mutations in cytokine-mediated signaling, and the majority (65%) of the patients have mutations in the *IL2RG* gene encoding the common γ chain (Figure 10) The *IL2RG* is located on the X chromosome, and is the only SCID gene with an X-linked inheritance. Almost half of the SCID patients we diagnosed in Rotterdam originate from Turkey. The frequency of consanguinity in Turkey is relatively high, and therefore we see a slightly lower frequency of patients with *IL2RG* mutations. A separate category of T-B+SCID patients have mutations in one of the four CD3 genes (*CD3G, CD3D, CD3E* and *CD3Z*).[81, 82] The absence of one of the CD3 chains inhibits formation of the CD3 complex and consequently expression and signaling via (pre)TR.

T-B-SCID can be caused by mutation in V(D)J recombination genes (Figure 10), or by mutations in adenylate kinase 2 (AK2) or adenosine deaminase (ADA). The latter two gene defects can also be associated with NK cell deficiency. *AK2* mutations result in defective maturation of lymphoid and myeloid cells, and absence of ADA activity results in elevation

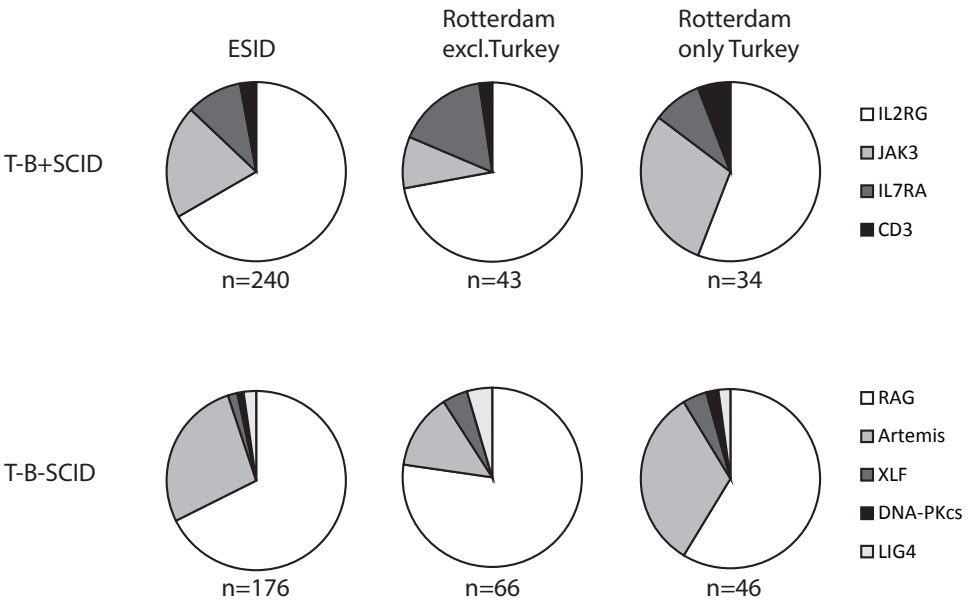

Figure 10. Distribution of SCID genes. Distribution of SCID genes in T-B+SCID and T-B-SCID according to the European society of immunodeficiency (ESID) database (January 2014), and patients diagnosed in Rotterdam excluding the patients originated from Turkey or only the patients originating from Turkey.

Table 2. Characteristics of V(D)J recombination genes.

	HUGO name	size (aa)	exons (coding)	clinical phenotype	IR sensitive	neurological abnormalities
V(D)J recombination genes defects						
RAG1	*RAG1*	1043	2 (1)	SCID, OS, leaky SCID	-	
RAG2	*RAG2*	527	2 (1)	SCID, OS, leaky SCID	-	
Artemis	*DCLRE1C*	692	14 (14)	SCID, OS, leaky SCID	+	
DNA-PKcs	*PRKDC*	4128	87 (87)	SCID	+	
LIG4	*LIG4*	911	2 (1)	SCID, OS, LIG4 syndrome	++	microcephaly
XLF	*NHEJ1*	299	8 (7)	CID	++	microcephaly
XRCC4	*XRCC4*	336	8 (7)	Primoridal dwarphism		
V(D)J recombination genes not (yet) associated with immunodeficiency in humans						
KU70	*XRCC6*	608	12 (12)			
KU80	*XRCC5*	732	23 (21)			
TdT	*DNTT*	509	11 (11)			

of lymphotoxic metabolites. V(D)J recombination defects result in a block in T and B cell development and therefore no T and B cells are present in the peripheral blood. So far, genetic defects have been identified in the *RAG1*, *RAG2*, *Artemis*, *LIG4*, *XLF, DNA-PKcs, and XRCC4* genes (Figure 10 and Table 2).[34, 35, 83-87] Most of the T-B-SCID patients have mutations in the RAG1 or RAG2 genes (70%) or in the Artemis gene (20%). The distribution of the gene defects we identified in Rotterdam were similar as in the database of the European society of immunodeficiencies (ESID). However, *Artemis* mutations were found 1.5 fold more frequently in the Turkish patients (Figure 10). In the next paragraph the V(D)J recombination defects will be discussed in more detail.

V(D)J RECOMBINATION DEFECTS

The immunological phenotype and clinical presentation of V(D)J recombination defects can be different, depending on the type of genetic defect, i.e. null mutations or hypomorphic mutations with residual V(D)J recombination activity. Importantly, RAG is only expressed in lymphocytes during V(D)J recombination, so a RAG deficiency is limited to the lymphoid system. However, the NHEJ proteins are important for the repair of DNA DSB in every cell. So, patients with defects in any of these proteins not only have a V(D)J recombination defect, but can have other clinical manifestations, such as microcephaly or neurological abnormalities. They are sensitive for ionizing radiation, and are often referred to as radiosensitive (RS) SCID patients.[88]

RAG deficiency

In approximately 70% of the T-B-SCID patients, mutations are found in the *RAG1* and *RAG2* genes.[83] Especially for the *RAG* genes, many different mutations have been described that give rise to residual activity of the mutated RAG protein.[89, 90] In the last decade it has become more apparent that different *RAG* mutations may result in a broad spectrum of clinical phenotypes,[91, 92] including:

- Classical SCID
- RAG deficiency (RAGD) with skin inflammation and αβ T-cell expansion (classical Omenn syndrome (OS))
- RAGD with skin inflammation but without T-cell expansion (incomplete OS)
- RAGD with materno-fetal transfusion
- RAGD with γδ T-cell expansion
- atypical SCID
- RAGD with granulomas
- RAGD with CD4 cytopenia and thymus hypoplasia

This broad spectrum of clinical phenotypes impedes timely recognition of RAGD and may thus delay treatment (SCT). All these different presentations have now been summarized in a new classification that defines three main categories: SCID, OS and leaky SCID.[93]

Artemis deficiency

In 2001, Moshous *et al.* identified the *Artemis* gene as being defective in a group of patients with RS-SCID.[83] Similar to RAG deficiency, hypomorphic *Artemis* mutations were described to give rise to a spectrum of clinical phenotypes including; atypical SCID,[94, 95] OS,[96] atypical SCID with chronic inflammatory bowel disease,[97] atypical SCID with granulomas (**Chapter 3.3**),[98] and atypical SCID with hyper IgM syndrome and large granular lymphocytic leukemia.[99] The defect in V(D)J recombination and NHEJ result in a block in precursor B-cell development in bone marrow and sensitivity for ionizing radiation.[95] The role of Artemis in V(D)J recombination is opening of the hairpins that are formed at the coding ends. In case of Artemis deficiency, the hairpins are opened inefficiently and therefore increased numbers of P-nucleotides are present in the Ig rearrangements.[100] Another striking feature of Artemis deficiency is the increased use of microhomology in the switch junctions.[101] Importantly, several patients with an atypical clinical presentation have developed hematological malignancies, this stresses the importance to treat these patient with SCT, but with consideration of the radiosensitivity of these patients for conditioning.[94, 99]

DNA-PKcs deficiency

For many years spontaneous *DNA-PKcs* mutations have only been reported in animals: horses of Arabian breed,[102] the classical SCID mouse,[103, 104] and Jack Russell terriers.[105] The phenotype of these animals was reminiscent of the phenotype of classical SCID in humans. Finally, in 2009, we presented the first human patient with a hypomorphic *DNA-PKcs* mutation (**Chapter 2**).[85] This patient presented with the classical SCID phenotype: she had a complete block in precursor B-cell development, and was sensitive to ionizing radiation. The patient had two homozygous *DNA-PKcs* mutations, from which the missense mutation (p.L3062R), was proven to be the disease causing mutation. Similar to Artemis deficient patients, this patient had increased numbers of P-nucleotides in the IG junctions, which suggested that the mutant inhibits Artemis activation. Woodbine *et al.* recently identified a second DNA-PKcs deficient SCID patient with dysmorphology, severe growth failure, microcephaly, seizures, and profound globally impaired neurological function.[106] The patient had compound heterozygous for two new *DNA-PKcs* mutations. One mutation resulting in loss of an exon, and appeared to be inactivating, the other mutation was a hypomorphic mutation. This patient illustrates that besides V(D)J recombination and NHEJ, DNA-PKcs is also important for neurological development.

LIG4 deficiency

To date, 28 LIG4 deficient patients have been described [86, 107-117] All patients were ionizing radiation (IR) sensitive, but clinically they can be divided into six distinct disease categories: 1) leukemia,[111] 2) LIG4 syndrome,[107, 110, 112, 113] 3) Dubowitz syndrome,[116] 4) Omenn syndrome,[114] 5) RS-SCID,[86, 108, 109] and 6) LIG4-deficiency with dwarfism.[115, 117] The leukemia patient had no overt immunodeficiency [111]. Patients with LIG4 syndrome present with microcephaly, developmental delay, mild immunodeficiency, and can develop malignancy. One patient presented with the Dubowitz syndrome, which is characterized by microcephaly, short stature, abnormal face and mild to severe mental retardation.[116] Patients with RS-SCID closely resemble patients with LIG4 syndrome but the immunodeficiency is more severe and they are diagnosed at younger age. All LIG4-deficient patients described so far have at least one allele with a hypomorphic mutation in combination with a second hypomorphic or null mutation on the other allele. Lig4 knockout mice are embryonic lethal [118-120], which suggests that LIG4 is essential for life. In this thesis, we describe a patient with a new clinical phenotype of LIG4 deficiency, which was characterized by microcephalic primordial dwarfism (**Chapter 3.4**).[115] This case broadens the clinical spectrum of LIG4 deficiencies.

XLF deficiency

In 2006, a novel NHEJ factor was described by two independent groups. Ahnesorg *et al.* identified the XLF by using a yeast-two-hybrid screen,[34] and showed that this gene was mutated in the previously reported patient with a novel SCID.[121] Buck *et al.* reported five patients with combined immunodeficiency and identified the novel NHEJ factor by functional complementation assays and called it Cernunnos. XLF is involved in ligation of DNA DSB and directly interacts with the LIG4/XRCC4 complex.[34, 122, 123] Several XLF deficient patients have been described in literature.[34, 35, 121, 124] They all have microcephaly, growth retardation, increased sensitivity for ionizing radiation, mild to severe immunodeficiency and some patients have autoimmunity.

XRCC4 deficiency

Recently, Shaheen *et al*. described the first patient with XRCC4-deficiency.[125] This patient presented with primordial dwarfism, bird-like face, small philtrum, and speech-delay. Unfortunately, no immunological information is known about this patient. The *XRCC4* mutation identified in this patient concerns a missense mutation (p.Trp43Arg) in a conserved amino acid. This mutation is likely to be hypomporphic, since XRCC4 is considered to be essential. However, the activity of this mutant has not been tested yet. Most likely, more *XRCC4* mutations will be identified in patients with primordial dwarfism.

OTHER IMMUNODEFICIENCIES CAUSED BY DEFECTS IN DNA REPAIR GENES

DNA repair pathways play an important role in lymphocyte development and differentiation. Not only defects in NHEJ factors result in immunodeficiency, but also defects in other DNA damage response proteins, and proteins involved in repair of breaks during SHM and CSR result in immunodeficiency (Table 3).

Nijmegen Breakage Syndrome

Mutations in *NBN,* the gene encoding for NBN result in Nijmegen breakage syndrome (NBS).[126] These patients have a characteristic facial appearance, microcephaly, growth retardation, immunodeficiency, increased sensitivity to ionizing radiation, and a strong predisposition to (lymphoid) malignancies.[126-128] More than 90% of the patients have a homozygous 5-nucleotide deletion (c.657del5) which causes a premature stop at codon 219.[129] NBN forms a complex with MRE11 and RAD50, and is involved in sensing DNA DSBs, keeping two DNA ends together and activation of ATM. The immunodeficiency in NBS patients is characterized by reduced absolute numbers of T and B cells, suggesting a defect in V(D)J recombination. Analysis of IG rearrangements did not show differences in length or composition of these junctions,[43, 130, 131] but NBS patients have increased loss of juxtaposition of the DNA ends and this reduces the chance for successful rearrangements.[43]

Ataxia Telangiectasia

Ataxia telangiectasia (AT) is a multisystem disorder caused by mutations in the *ATM* gene.[132] AT is characterized by cerebellar ataxia, oculocutaneous telangiectasias, radiosensitivity, chromosomal instability, a propensity for development of (mainly hematologic) malignancies, growth retardation, endocrine abnormalities and immunodeficiency.[133, 134] ATM is a protein kinase and member of the phosphoinositidyl 3-kinase related kinase (PIKK) family. As described above, ATM has many functions and is not only involved in cell cycle regulation, but also in DSB repair during V(D)J recombination and CSR. Clinically, the immunodeficiency in patients with AT is highly variable, with a predominant antibody deficiency. Patients with early-onset disease are referred to as having classical AT.[135, 136] A subset of these classical AT patients have a severe early onset hypogammaglobulinema reminiscent of a CSR deficiency.[137] Patients with variant AT, due to a *ATM* mutation leading to residual enzyme activity, have later onset and less severe antibody deficiency.[135, 138]

Bloom's syndrome

Bloom's syndrome protein (BLM) is a member of the RECQL gene family of helicases [139], together with four additional members: RECQL1, WRN, RECQL4 and RECQL5 [140]. Helicases unwind double stranded (ds) DNA, making the strands accessible for replication and repair proteins. RECQ helicases are known to be involved in DNA repair and several other cellular processes [141] Mutation in *BLM*, result in Bloom's syndrome (BS), which is characterized by short stature, photosensitivity, facial abnormalities, mental retardation, malignancies, immunodeficiency, and chromosomal instability. The role of BLM in the immune system is not well described, but most BS patients have an antibody deficiency.

Other DNA repair proteins associated with immunodeficiency

In addition to *ATM* and *NBN*, mutations in *MRE11*,[142] *RAD50*,[143] *RNF168*,[144], *LIG1*,[145] and the MMR genes are also associated with immunodeficiency (Table 3).[146] These genes are mainly involved in CSR, resulting in antibody deficiencies in these patients. For most of these diseases, the immunological phenotype is a minor aspect of their clinical phenotype, because these patients can present with neurological disorders, dysmophic features and commonly develop (lymphoid) malignancies.

Table 3. DNA repair defects associated with immunodeficiency.

Gene	HUGO name	size (aa)	exons (coding)	clinical phenotype	neurological abnormalities
ATM	*ATM*	3056	63 (62)	AT	ataxia, telangiectasia
NBN	*NBN*	754	16 (16)	NBS	microcephaly
BLM	*BLM*	1417	22 (21)	BS	
MRE11	*MRE11A*	708	20 (19)	AT-related disorder	microcephaly
RAD50	*RAD50*	1312	25 (25)	NBS-related disorder	microcephaly
RNF168	*RNF168*	571	6 (6)	RIDDLE syndrome	microcephaly
LIG1	*LIG1*	919	28 (27)		
MSH2	*MSH2*	934	16 (16)	CMMR-D	
MSH5	*MSH5*	834	25 (24)	CMMR-D	
MSH6	*MSH6*	1360	10 (10)	CMMR-D	
MLH1	*MLH1*	756	19 (19)	CMMR-D	
PMS2	*PMS2*	862	15 (15)	CMMR-D	

Ataxia Telangiectasia (AT), Nijmegen breakage syndrome (NBS), Bloom's syndrome (BS), radiosensitivity, immunodeficiency, dysmorphic features and learning difficulties (RIDDLE syndrome), constitutional mismatch-repair deficiency (CMMR-D).

OUTLINE OF THE THESIS

V(D)J recombination is necessary to create a diverse repertoire of antigens receptors, which is essential for the defense against the many different pathogens. Defects in V(D)J recombination result in T-B-SCID. Several genetic defects have been found to cause T-B-SCID. At this moment, there is a delay in making a molecular diagnosis because detailed insight in the clinical heterogeneity is lacking, and not all candidate genes (i.e. genes that can be mutated in T-B-SCID) are known. Due to the delay in the diagnostic process, the procedure of adequate treatment is often initiated (too) late leading to more serious life-threatening infections and irreversible organ damage.

The aim of this thesis was to unravel the clinical and immunogenetic heterogeneity of SCID. In **Part 2**, we describe a new genetic defect in the DNA-PKcs gene in a patient with a classical SCID phenotype (**Chapter 2**). **Part 3** describes detailed insight in the clinical heterogeneity of recombination defects. It becomes progressively clear that V(D)J recombination defects not only result in the 'classical SCID' phenotype, but can result in a spectrum of clinical phenotypes. We discuss new clinical phenotypes for RAG deficiency (**Chapter 3.1**), Artemis deficiency (**Chapter 3.3**) and LIG4 deficiency (**Chapter 3.4**), but we also show that even similar *RAG* mutations can result in a spectrum of clinical phenotypes (**Chapter 3.2**). Finally, in **Part 4** we discuss mechanisms of repertoire development and CSR. We used a new method, based on next generation sequencing, to determine the composition and diversity of the IGH repertoire in more depth. In **Chapter 4.1** we showed that XLF is essential to create junctional diversity by TdT and in **Chapter 4.2** we showed for the first time that patients with AT have a reduced repertoire diversity. Finally, the ***General Discussion*** deals with the implications of the studies described in this thesis.

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PART 2

New nonhomologous end-joining defect

Chapter 2

A DNA-PKcs mutation in a radiosensitive T-B- SCID patient inhibits Artemis activation and nonhomologous end-joining

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ABSTRACT

Radiosensitive T-B- severe combined immunodeficiency (RS-SCID) is caused by defects in the nonhomologous end-joining (NHEJ) DNA repair pathway, which results in failure of functional V(D)J recombination. Here we have identified the first human RS-SCID patient to our knowledge with a *DNA-PKcs* missense mutation (L3062R). The causative mutation did not affect the kinase activity or DNA end-binding capacity of DNA-PKcs itself; rather, the presence of long P-nucleotide stretches in the immunoglobulin coding joints indicated that it caused insufficient Artemis activation, something that is dependent on Artemis interaction with autophosphorylated DNA-PKcs. Moreover, overall end-joining activity was hampered, suggesting that Artemis-independent DNA-PKcs functions were also inhibited. This study demonstrates that the presence of DNA-PKcs kinase activity is not sufficient to rule out a defect in this gene during diagnosis and treatment of RS-SCID patients. Further, the data suggest that residual DNA-PKcs activity is indispensable in humans.

INTRODUCTION

Severe combined immunodeficiency (SCID) is an inherited primary immunodeficiency. SCID patients present in the first year of life with severe opportunistic infections, chronic diarrhea and failure to thrive. The total group of SCID patients can be divided in two main categories: T^-B^+ SCID with a T-cell signaling defect (70%) and T^-B^- SCID with a defect in V(D)J recombination (30%). V(D)J recombination assembles variable (V), diversity (D) and joining (J) gene segments of the immunoglobulin (Ig) and T-cell receptor (TR) genes during B- and T-cell differentiation in order to generate a broad repertoire of antigen specific receptors. V(D)J recombination starts with introduction of DNA breaks at the border of the gene segments and the flanking recombination signal sequences (RSSs) by the RAG1 and RAG2 proteins.[1] The resulting blunt signal ends are ligated directly, forming a signal joint. The hairpin sealed coding ends require further processing before coding joint formation. Recognition and repair of the DNA ends occur via the general nonhomologous end joining (NHEJ) pathway of DNA double strand break (DSB) repair.[2, 3]

DSBs induce ATM kinase activity, which phosphorylates histone H2AX,[4] followed by binding of 53BP1, MDC1 and a complex of MRE11, RAD50 and NBS1 (MRN complex).[5, 6] These proteins form a microenvironment that holds together the DNA ends over a relatively large distance, but still allow some degree of freedom for movement of the DNA ends and access of NHEJ proteins.[7] DNA ends are first recognized by the NHEJ factor DNA-dependent protein kinase (DNA-PK), which is composed of the DNA-PK catalytic subunit (DNA-PKcs) and the Ku70/Ku80 heterodimer.[8] After initial loading of the Ku70/Ku80 heterodimer onto DNA ends, DNA-PKcs is recruited to form a DNA end synapsis, ensuring protection from exonuclease activities and juxtaposition of DNA ends.[9] The presence of Ku70/Ku80 and DNA-PKcs at DNA ends is not rigid, but constitutes a dynamic equilibrium of DNA-bound and DNA-free protein.[10, 11] *Trans* autophosphorylation of the ABCDE cluster of DNA-PKcs (seven phosphorylation sites between residues 2609 and 2647) causes a conformational change that facilitates Artemis nuclease activity.[12-14]

During V(D)J recombination, Artemis nuclease activity is required for opening of the hairpin sealed coding ends.[15] During processing of coding ends, nucleotides can be lost due to exonuclease activity and non-templated (N) nucleotides can be added by terminal deoxynucleotidyl transferase (TdT).[16, 17] This contributes tremendously to the diversity of the antigen receptor repertoire. *Trans* autophosphorylation of the PQR cluster (six phosphorylation sites between residues 2023 and 2056) functions to limit further end processing and to specifically

promote end joining. Activation (T3950) loop autophosphorylation is also required for efficient end joining.[18] Finally, the DNA ends are ligated by the XRCC4-Ligase IV complex, which is promoted by XLF (Cernunnos)[19, 20] The requirement of XLF can differ between various species and/or various cell types.[21]

In ~70% of T^-B^- SCID patients, mutations are found in the *RAG1* and *RAG2* genes.[22, 23] The majority of the remaining patients show hypersensitivity to ionizing radiation, due to mutations in *Artemis* or *LIG4;*[24-29] mutations in the *XLF/Cernunnos* gene have been found in radiosensitive patients with growth retardation, microcephaly, and immunodeficiency due to profound T and B-cell lymphocytopenia.[19, 20] We here present a new type of RS-SCID with a defect in DNA-PKcs.

RESULTS

Flow cytometric analysis of peripheral blood and bone marrow pre-SCT and post-SCT

A girl (patient ID177) from consanguineous parents of Turkish origin (first degree relatives) was clinically diagnosed with SCID when she was 5 months old. B and T cells were virtually absent from peripheral blood, whereas NK cells were within the normal range (supplementary Table 1). Informed consent for patient material was obtained according to the guidelines of the medical ethics committees of Hacettepe University Children's Hospital and Erasmus MC. The precursor B-cell compartment in bone marrow showed a complete block in B-cell differentiation before the pre-B-II cell stage comparable to Artemis-deficient SCID[25] and RAG-deficient SCID,[30] which suggests that the patient has a defect in V(D)J recombination (Figure 1). Patient ID177 received hematopoietic stem cell transplantation (SCT) from her HLA identical cousin, which resulted in full reconstitution of the precursor B-cell compartment and production of all lymphocyte subsets after three and six months.

Supplementary Table 1. Absolute numbers of B, T and NK cells in peripheral blood (x 10^9/l) of the patient at diagnosis, three and six months post-SCT

	diagnosis	3 months post -SCT	6 months post -SCT	Age matched controls
B-cells	<0.01	0.4	0.6	0.6-2.7
T-cells	0.1	2.4	2.2	1.6-6.7
NK cells	0.4	0.4	0.1	0.2-1.2

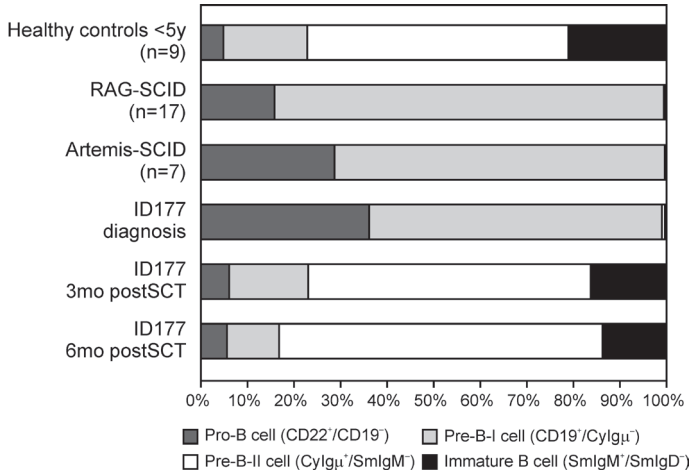

Figure 1. Flow cytometric analysis of the precursor B-cell compartment
At diagnosis ID177 had a complete block in precursor B-cell differentiation before the pre-B-II cell stage comparable to RAG- and Artemis-deficient SCID patients.[25, 30] Already three months post-SCT the precursor B-cell compartment showed a normal composition.

DNA repair defect in patient fibroblasts

Mutations in *RAG1* and *RAG2* were excluded by sequencing analysis. Subsequently, clonogenic survival assays showed that ID177 fibroblasts were X-ray sensitive (Figure 2A), indicating that patient ID177 has RS-SCID due to a defect in NHEJ. However, known candidate genes for RS-SCID (*Artemis*, *LIG4, XLF-Cernunnos*) were not mutated. Therefore, we analyzed the DSB repair defect in more detail by counting foci of the DSB marker γ-H2AX at various time points after irradiation. The kinetics of induction and disappearance of γ-H2AX foci at early time points (<6 hours) did not differ significantly between patient, control (VH10) and Artemis-deficient cells (Figure 2B). However, γ-H2AX foci disappeared with delayed kinetics in Artemis-deficient and patient fibroblasts and 15% of residual foci were still present after 72 hours, suggesting that a comparable level of unrepairable DSBs existed in patient ID177 and in Artemis-deficient cells.

Composition of D_H-J_H coding joints from bone marrow mononuclear cells

Additional information on the repair defect was obtained by analyzing incomplete *IGH* gene rearrangements (i.e. D_H-J_H) derived from bone marrow mononuclear cells. These junctions showed an average total number of 3.0 palindromic (P-) nucleotides, which is significantly higher than the observed 0.2 P-nucleotides in healthy controls (P=0.025), although not as high as in

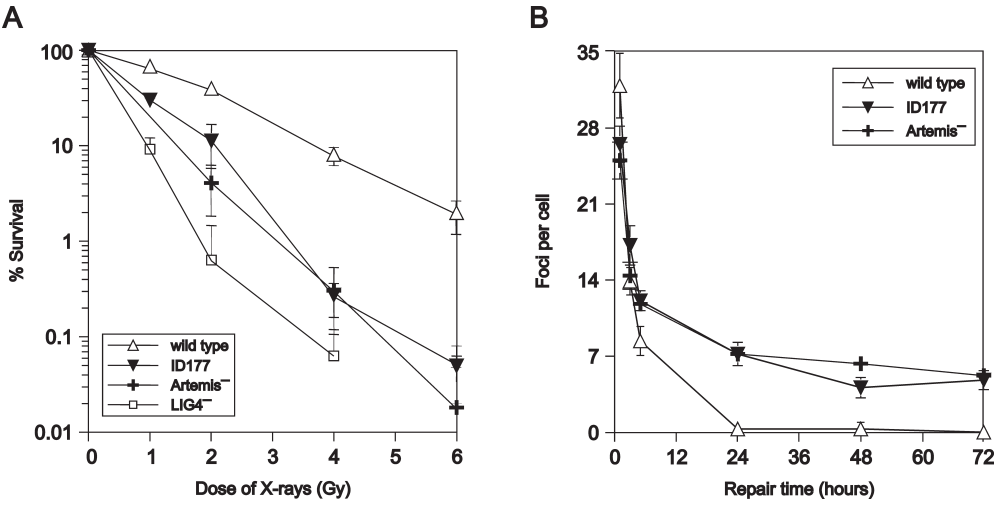

Figure 2. ID177 harbors a DSB repair defect.
Clonogenic survival assay using wild type (FN1) fibroblasts, patient fibroblasts (ID177), and fibroblasts from a Artemis-deficient patient (Artemis-1[25]) and a LIG4-deficient patient (SC2[29]). Fibroblasts were irradiated with increasing X-ray doses. ID177 fibroblasts were radiosensitive. Error bars represent the S.D. from three independent experiments.
Numbers of γ-H2AX foci per nucleus were determined at indicated time points after irradiation (average number of foci per nucleus in 40 cells) in wild type (VH10) fibroblasts, ID177 and Artemis-deficient fibroblasts (Artemis-6[31]). Error bars represent the S.D. from two to four independent experiments.

Table 1. Comparison of the junction characteristics of patient ID177 and junctions from healthy controls [29], Artemis-deficient [31] and LIG-4 deficient [29] patients.[a]

Patient (number of clones)	D_H(del)	P-nucleotides[b]	N-nucleotides[b]	P	(del) J_H	total P	total del
ID177 (23)	-2.3	1.0	4.2	2.0	-4.5	3.0	6.8
Control (15)	-4.2	0.1	7.9	0.1	-6.0	0.2	10.2
Artemis (53)	-1.9	3.0	4.0	3.8	-1.1	6.7	3.3
LIG4 (13)	-12.2	0.2	2.8	0.0	-16.0	0.2	28.2

[a]Numbers represent average numbers of nucleotides per junction
[b]P, palindromic nucleotides; N, non-templated nucleotides.

Artemis-deficient patients (6.7 nucleotides)[31] (Table 1 and supplementary Table 2). Artemis-deficient patients have a defect in DNA hairpin opening of the coding end,[9] which results in exceptionally high numbers of P-nucleotides, with high frequency of microhomology in the P-nucleotide tracts.[31, 32] The shorter P-tract tracts and the absence of P-tract microhomologies in ID177 junctions suggest that hairpin-opening defect is somewhat different from Artemis-deficiencies.

Supplementary Table 2. Analysis of DH-JH coding joints of the patient

DH gene segment	3′ deletion	P nucleotides[a]	N nucleotides[b]	P nucleotides	5′ deletion	JH gene segment
DH1-26	0				-15/-18	JH4/JH5
DH2-15	-3		CG		0	JH6
DH3-9	-4		TGA		0	JH4
DH3-22	-10		A	A	0	JH3
DH3-22	-1		GGG		-10	JH4
DH3-22	0		CCT		0	JH3
DH4-17	0		AC		-9	JH4
DH4-4	-11/-14		TC	GT	0	JH4
DH4-17	-7/-10		CC	AAGTAGT	0	JH4
DH5-5	0	GTAACCA		AAGTAGT	0	JH4
DH5-5	0	G	CCCGTTACGCCC	T	0	JH6
DH5-24	-1		GAAAAGG		-17	JH6
DH5-24	0	GTAATTG	ACC	CAAAGTAGT	0	JH4
DH5-12	-2		GGGCCCTATATCCAGATCGG		0	JH4
DH6-13	0	GTACCA		TAGTAAT	0	JH6
DH6-25	0		CGATATTGG		-5	JH6
DH6-13	-4		ACC		-5	JH6
DH6-19	0		TGGTAGGG		-7	JH4
DH6-19	-1		AAGTGTCC		0	JH1
DH6-25	-5		C	CAAAGTAGT	0	JH4
DH6-13	0	G			-2	JH6
DH6-13	0	G			-2	JH6
DH6-25	-2		CACCGCC	GT	0	JH4
Average	-2.2/-2.5	1.0		2.0	-4.7/-5.0	

[a] P nucleotides, palindromic nucleotides
[b] N nucleotides, non-templated nucleotides

Analysis of NHEJ components Ku70, Ku80, XRCC4 and DNA-PKcs

Since Artemis activity depends on DNA-PKcs autophosphorylation, we analyzed the *DNA-PKcs* locus. Before sequencing this large gene, short tandem repeat (STR) analysis was performed in the patient and twelve family members using four polymorphic markers surrounding the *DNA-PKcs* gene (Figure 3A). Patient ID177 was homozygous for all four markers and the allele defined by those four markers was present heterozygously in both parents, in the two related grandmothers (sisters) and in one uncle. No family members were homozygous for this genotype. Therefore, we sequenced the 12.3 kb *DNA-PKcs* cDNA and found two homozygous mutations (Figure 3B): a three nucleotide deletion (c.6338_6340delGAG) resulting in the deletion of a Glycine (p.delG2113) and a missense mutation, resulting in

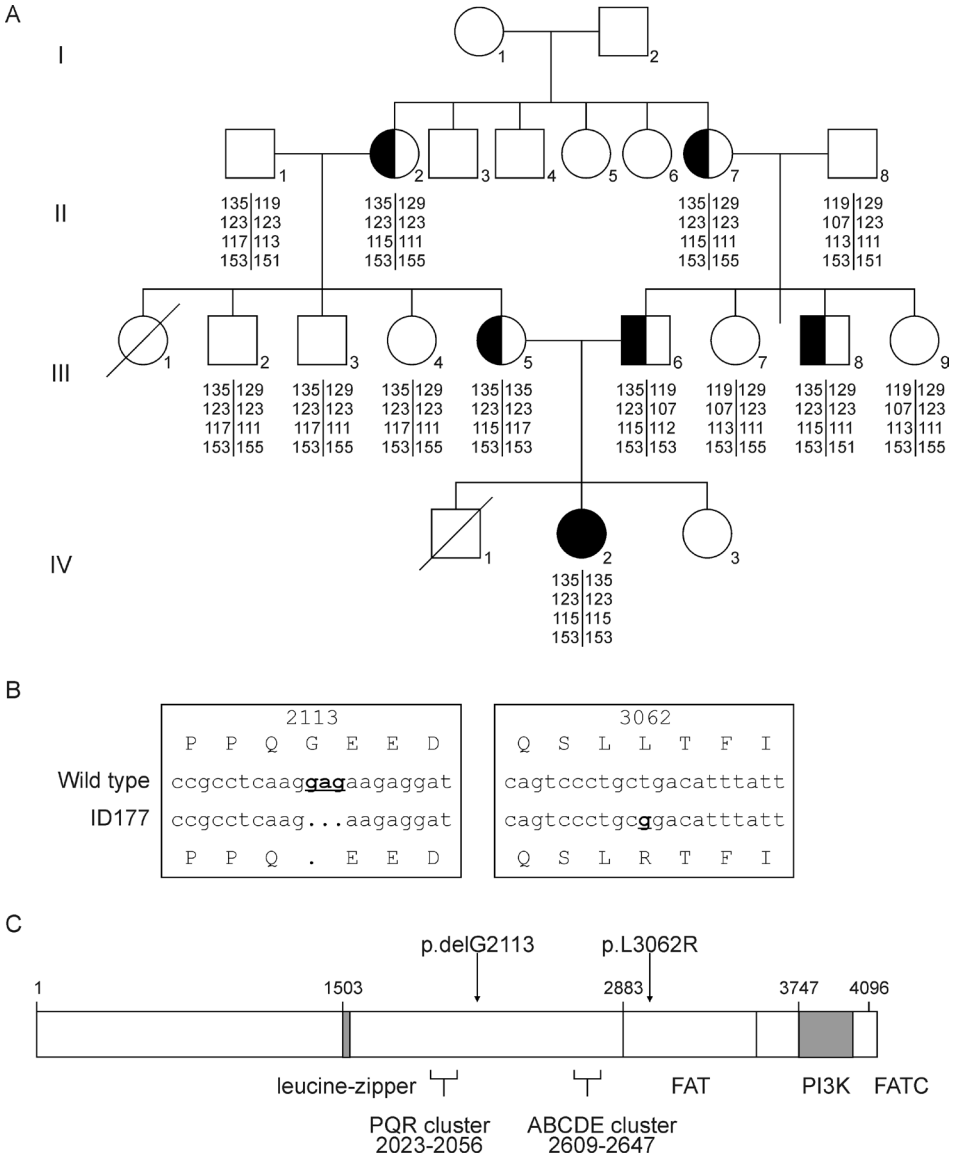

Figure 3. DNA-PKcs mutation analysis.
Family tree of patient ID177 and twelve family members. STR analysis was performed using the polymorphic markers D8S1460, D8S359, D8S531 and AF075268 surrounding the *DNA-PKcs* gene. Patient ID177 was homozygous for all four markers. This allele, defined by the four markers, was present heterozygously in both parents (III-5 and 6), the two grandmothers who are sisters (II-2 and 7) and one uncle (III-8). None of the family members were homozygous for this genotype.
Detection of two homozygous mutations in the *DNA-PKcs* gene in patient ID177; a deletion of three nucleotides (c.6338_6340delGAG) resulting in deletion of Glycine 2113 and a missense mutation (c.9185T>G) resulting in replacement of Leucine.by Arginine at position 3062.
Schematic representation of the DNA-PKcs protein and the two identified mutations delG2113 and L3062R.

the replacement of one amino acid (c.9185T>G; p.L3062R) in the DNA-PKcs FAT domain (Figure 3C). The mutations were confirmed to be present heterozygously in the parents, the two grand mothers and the uncle and were not present in the other family members. To exclude that these mutations are recurrent polymorphisms, 100 Turkish controls were analyzed and the mutations were not detected. Mutations in the other NHEJ components (*Ku70*, *Ku80* and *XRCC4*) were excluded by sequencing.

Normal DNA-PKcs expression and activity

First, we studied the effect of the *DNA-PKcs* mutations on kinase activity and protein expression. In vitro DNA-PK kinase activity appeared to be similar in extracts from patient and control cell cultures (Figure 4A). The observed phosphorylation could specifically be inhibited by the DNA-PK inhibitor NU7441 (Figure 4B) in a final concentration of 0.5mM.[33] NU7441 is specific for DNA-PK kinase inhibition (IC_{50} 0.014mM) and inhibits ATM only at high concentrations (IC_{50} >100mM).[33] ATM inhibitor KU55933 inhibited the phosphorylation activity with 50% (Figure 3B), which can be attributed to less specificity of this inhibitor.[34] Ku55933 has an IC_{50} of 0.013mM for ATM, but an IC_{50} of 2.5 mM for inhibition of DNA-PK. These data show that ID177 nuclear extracts possesses wild type levels of DNA-PK activity.

Subsequently, we studied autophosphorylation using a phophospecific antibody for one of the auto-phosphorylation sites (S2056) (Figure 4C). The S2056 site is specifically known to be an exclusive DNA-PK target site in vivo.[35] DNA-PKcs expression was detected in all fractions, while phosphorylated DNA-PKcs was only found in irradiated control and ID177 fibroblasts. These experiments showed that mutated DNA-PKcs was expressed and retained kinase and autophosphorylation capacity.

Confirmation of the disease causing DNA-PKcs mutation

As we did not observe a defect in DNA-PKcs activity we sought a different way to prove that the *DNA-PKcs* mutations have a disease-causing effect. Therefore, DNA-PKcs-deficient V3 cells expressing the mutated DNA-PKcs protein were studied in a clonogenic survival assay. Indeed, DNA-PKcs carrying both mutations did not complement the radiosensitivity of V3 cells, whereas wild type DNA-PKcs did, proving that one or both DNA-PKcs mutations in ID177 are disease-causing (Figure 5A).

Alignment of the DNA-PKcs protein sequences of several species showed that Leucine 3062 is a highly conserved amino acid in a predicted α-helix (H), whereas Glycine 2113 is located in a region with little conservation (Figure 5B). Moreover,

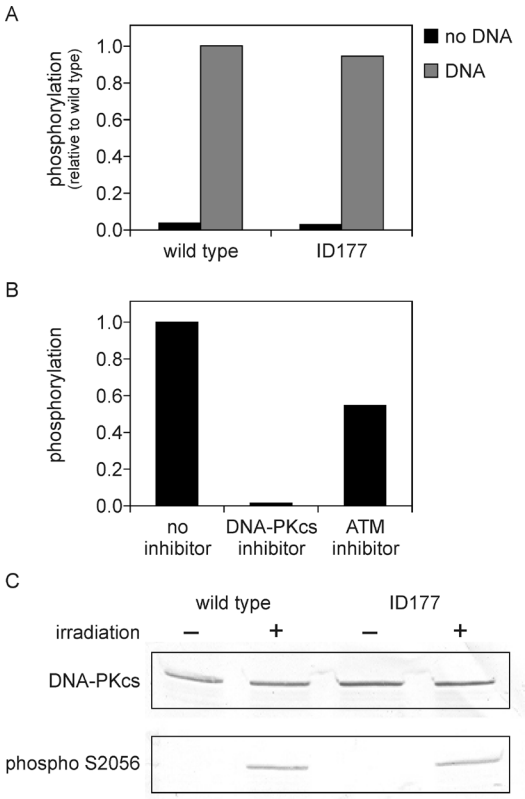

Figure 4. Measurement of DNA-PKcs activity.
DNA-PKcs kinase activity of cellular extracts of wild type (MRC5) and ID177 fibroblasts was measured by quantification of phosphorylation of a p53 peptide in the presence and absence of DNA.
DNA-PKcs kinase activity in ID177 cellular extracts was measured by quantification of phosphorylation of a p53 peptide in the presence DNA without inhibitor or with DNA-PKcs specific inhibitor NU7441,[33] or ATM-specific inhibitor KU55933[34] in a final concentration of 0.5mM. Phosphorylation is expressed relative to ID177 without inhibitor.
Western blot analysis of DNA-PKcs and phosphorylated DNA-PKcs in cellular extracts from untreated (-) and irradiated (2Gy) fibroblasts (+) of ID177 and wild type (C5RO) with the DNA-PKcs antibodies 2208 and 2129 (1:1) and the phosphospecific DNA-PKcs antibody S2056.

Canis familiaris lacks this amino acid as well. Therefore, we hypothesized that L3062R is most likely the disease-causing mutation.

We recently developed a V(D)J recombination assay, which allows detection of hairpin opening proficiency by simple analysis of junctions using restriction enzyme digestion (Figure 5C).[31] This assay is based on cotransfection of a V(D)J recombination substrate containing two RSS in inversed orientation and RAG1 and RAG2. Depending on the way of hairpin opening and coding joint formation,

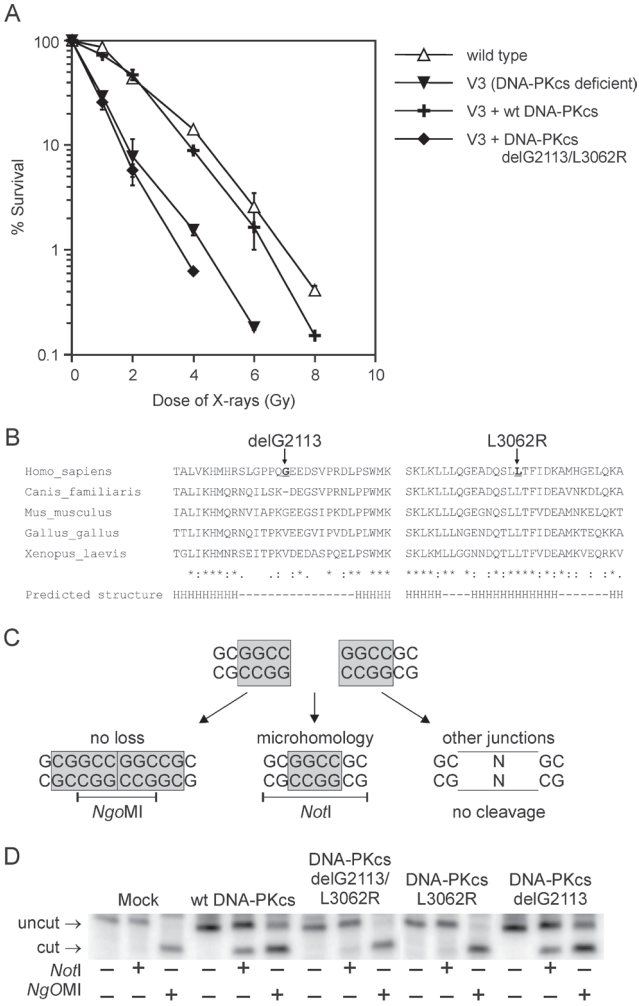

Figure 5. Confirmation of disease causing effect of DNA-PKcs mutation

Clonogenic survival assay of DNA-PKcs-deficient V3 cells [57] complemented with wild type DNA-PKcs or mutated DNA-PKcs (delG2113/L3062R) compared to wild type (AA8) cells. In contrast to wild type DNA-PKcs, mutated DNA-PKcs was not able to complement the radiosensitivity of V3 cells. Error bars represent the S.D. from three independent experiments.

Sequence alignment DNA-PKcs protein sequences of different species. G2113 is located in a region without a predicted secondary structure (indicated as (–)); L3062 is located in a predicted helix (H). Predictprotein analysis software was used.

Coding end structure of recombination substrate (pDVG93) used in the V(D)J recombination assay [31]. Depending on the way of hairpin opening and coding joint formation, three different types of coding joints can be generated and discriminated by restriction enzyme digestion. Coding joints with long stretches of homologous P-nucleotides without nucleotide loss gives an *Ngo*MI restriction site, a four-base pair microhomology of the coding ends creates a *Not*I restriction site. Junctions that have been processed differently will not contain either of the two restriction sites.

PCR and restriction enzyme digestion of coding joints recovered from DNA-PKcs-deficient V3 cells and V3 cells expressing wild type DNA-PKcs or mutated DNA-PKcs after cotransfection of pDVG93 and RAG1 and RAG2 expression constructs. Wild type DNA-PKcs and DNA-PKcs delG2113 were able to restore the shifted junction pattern as observed in DNA-PKcs-deficient V3 cells.

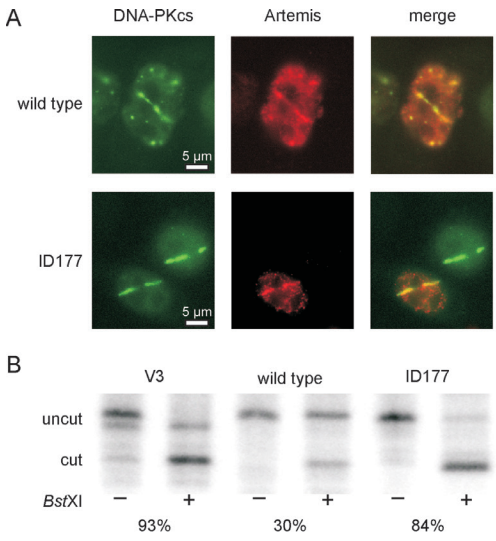

Figure 6. Residual function of DNA-PKcs defect
Immunostaining of micro-irradiated wild type and delG2113/L3062R YFP-DNA-PKcs-expressing V3 cells after cotransfection with a wild type myc-Artemis expression construct (in red). Cells were microirradiated using a multiphoton laser system. One of the two cells of V3 expressing delG2113/L3062R YFP-DNA-PKcs was not transfected with Artemis. This figure shows representative examples of the experiment.
DNA end-joining assay in DNA-PKcs-deficient V3 cells and V3 cells expressing wild type or ID177 mutant DNA-PKcs. Linearized pDVG94 can be rejoined via direct or microhomology-directed end-joining. Joining via microhomology results in the generation of a *Bst*XI restriction site.[36] Junctions were PCR amplified and PCR products were digested with *Bst*XI, as indicated.

three different types of coding joints can be generated and discriminated from each other by restriction enzyme digestion. Coding joints without nucleotide loss are *Ngo*MI sensitive, whereas a four-base pair microhomology of the coding ends creates a *Not*I restriction site. Junctions that have been processed differently will not contain either of the two restriction sites. In a wild type situation, the three different types are used in equal frequencies. Similar to Artemis-deficient cells, DNA-PKcs-deficient V3 cells showed a complete shift towards *Ngo*MI sensitive junctions (Figure 5D). Expression of wild type DNA-PKcs in V3 cells restored normal distribution of junctions. The normal junction pattern could not be restored in V3 cells by the DNA-PKcs expression construct containing both mutations or containing only the L3062R mutation. However, the DNA-PKcs delG2113 was able to correct the shifted junction pattern, indicating that the DNA-PKcs L3062R missense mutation is the disease-causing mutation.

Accumulation of mutated DNA-PKcs at DSB sites

To further pinpoint the effect of the *DNA-PKcs* mutation, we studied the ability of mutated DNA-PKcs to recruit Artemis to laser-induced DSB sites in living cells.[11] Lines of DNA damage were introduced in V3 cells expressing YFP-tagged wild type DNA-PKcs or mutated DNA-PKcs. In this assay a myc-tagged Artemis expression construct was co-transfected. Mutated DNA-PKcs protein was still able to accumulate at DSB sites and to recruit Artemis to these sites (Figure 6A). Colocalization of mutated DNA-PKcs and Artemis suggests that the point mutation did not disrupt interaction with Artemis.

Impaired end joining activity of linear substrates

Finally, the relative level of microhomology use was tested in a plasmid recircularization assay, which is based on transfection of linear DNA. This is a sensitive method to detect subtle NHEJ defects.[36] The mutated DNA-PKcs expression construct was not able to correct the increased microhomology use in DNA-PKcs-deficient V3 cells, showing that the overall NHEJ activity was affected by this mutation (Figure 6B).

DISCUSSION

We identified the first human RS-SCID patient with a mutation in the *DNA-PKcs* gene. Clinically the DNA-PKcs-deficient patient presented as a classical SCID with symptoms similar to patients with RAG or Artemis mutations, i.e. with a severe immunodeficiency due to lack of B and T cells, but without signs of microcephaly or mental retardation as found in XLF-deficient patients[20] or in patients with the LIG4 syndrome.[37] For several years, mutations in this gene have been expected to exist in (SCID) patients, because spontaneous mutations in the *DNA-PKcs* gene had already been reported in three different species: in horses of Arabian breed,[38] in mice[39, 40] and in Jack Russell Terriers,[41] which indicated that mutations in this gene are not lethal. This notion was further supported by a recent study in which a human adenocarcinoma DNA-PKcs null cell line was generated.[42] This study clearly demonstrated that under cell culture conditions, DNA-PKcs is not essential for human cells.

The disease-causing mutation in the SCID patient described here concerned a hypomorphic L3062R missense mutation in the DNA-PKcs FAT domain. This mutation did not influence *in vitro* kinase activity nor hampered DNA-PKcs autophosphorylation. Moreover, mutated DNA-PKcs protein was still able to

accumulate at DSB sites and to recruit Artemis to these sites in vivo, suggesting that Artemis interaction was not disrupted. However, subtle changes in the interaction might influence correct positioning of the nuclease domain relative to the DNA end. It is interesting to note, that a cryo-electron microscopy based 3-dimensional model of the DNA-PK structure places the FAT domain (which contains the L3062R mutation) on a protrusion that faces the DNA end.[43] Therefore, this domain could serve as the Artemis nuclease interaction site. The consequence of the L3062R mutation was insufficient Artemis activation leading to disturbed coding joint formation and concomitantly to strongly reduced numbers of B and T cells in peripheral blood. The coding joints contained long stretches of P-nucleotides, indicating that Artemis nuclease activity was strongly inhibited.

However, the effect of the L3062R mutation was not limited to the Artemis activation deficit. Although we did not observe a significant difference in DSB repair kinetics in DNA-PKcs-deficient (ID177) and Artemis-deficient cells, joining of transfected linear DNA in ID177 was shifted towards microhomology use. Such a shift in joining products has been observed before in V3 cells that completely lack DNA-PKcs activity,[36] but not in Artemis-deficient cells.[25] As *DNA-PKcs* null mutations have not been found in patients, we assume that residual DNA-PK activity is indispensable in humans. It is possible that DNA-PKcs-dependent NHEJ is partially functional in ID177. However, the essential function in humans could also be unrelated to NHEJ, possibly a DNA-PK-dependent phosphorylation event.

The L3062R mutation differs substantially from the spontaneous mutations described in SCID horses, mice and dogs, which all concerned *DNA-PKcs* mutations that resulted in truncated proteins. In SCID foals, a 5bp deletion resulted in a frame shift and premature stop codon that prevented the translation of the 967 C-terminal amino acids, which resulted in a nonstable protein.[44, 45] C.B-17 BALB/c SCID mice have a nonsense mutation in a highly conserved region with a stop codon at a position corresponding to amino acid 4045 thereby deleting the 83 C-terminal amino acids.[46] Jack Russell Terriers also have a nonsense mutation that deletes the 517 C-terminal amino acids.[41, 47] The human DNA-PKcs null cell line lacked the kinase catalytic domain (encoded by exons 81, 82 and 83) and did not express detectable levels of DNA-PKcs protein.[42] In the three mentioned species as well as in the human cell line, the DNA-PKcs defects resulted in absence of kinase activity.[41, 42, 44, 48] This is in strong contrast to the human L3062R mutation, which retained kinase activity. It is tempting to speculate that DNA-PKcs kinase activity is essential for human development. The human carcinoma cell line was viable, although these cells had a severe proliferation and genomic stability deficit, suggesting that DNA-PK activity is more important in humans than in most other

mammals. It is possible, that DNA-PK has an essential function, e.g. some kinase signaling event that is carried out by ATM in other mammals. However, we cannot exclude that the presence of DNA-PKcs activity is specific for the (hypomorhpic) L3062R mutation and that other *DNA-PKcs* mutations lacking this kinase activity can still be found in SCID patients. This study unambiguously shows that the presence of DNA-PKcs protein and DNA-PKcs activity is not sufficient to rule out a defect in this gene. This might explain the delay in identification of the first DNA-PKcs deficient patient and probably of *DNA-PKcs*mutations in other RS-SCID patients.

The question remains whether mutations in the other NHEJ genes (*Ku70, Ku80* and *XRCC4*) can also be expected in patients. Ku70 and Ku80 knock out mice are viable and have a radiosensitive SCID phenotype,[49-52] but no spontaneous animal models have been reported. In contrast, human cell lines with inactivated Ku70 or Ku80 are not viable, which suggests that Ku70 and Ku80 are essential for human cell viability.[53, 54] XRCC4-deficient mice are embryonic lethal due to apoptosis of post-mitotic neurons[55] and if this can be extrapolated to humans, functional null mutations in *XRCC4* are also unlikely. However, hypomorphic mutations in *Ku70*, *Ku80* or *XRCC4* can never be excluded and therefore testing for defects in these genes remains valid in a case of radiosensitive SCID or in case of patients with immunodeficiency, growth retardation and/or developmental defects. As shown by this study, the analysis of coding joints from the bone marrow precursor B-cells is helpful for identification of the type of V(D)J recombination or NHEJ defect and can therefore guide the molecular testing of candidate genes.

In conclusion, *DNA-PKcs* is a new candidate gene for human radiosensitive SCID. This study illustrates that the presence of DNA-PKcs kinase activity is not sufficient to rule out a defect in this gene and that coding joint analysis is a powerful tool in the diagnostic process. In addition, this study suggests that residual DNA-PKcs activity is indispensable in humans.

MATERIALS AND METHODS

Case report

A Turkish girl (patient ID177) presented at the age of 5 months with recurrent oral candidiasis and lower respiratory tract infections since the third month of life with progressive respiratory distress leading to hypoxia. At the age of four month she had a large oral aphtous lesion. She is the third child of consanguineous parents (first degree relatives). A male sibling was lost due to a congenital heart

defect at the age of 3 years. She has a healthy 3 year-old sister. Vaccination with Bacille Calmette-Guerin (BCG) went uneventful. She had minimal tonsillary tissue. She was transplanted from her HLA-identical cousin (a healthy male) without pre-transplant conditioning regimen.

Cell lines and tissue culture

Primary fibroblasts were cultured from a skin biopsy of patient ID177 and were used in all assays. Wild type human fibroblasts FN1, VH10, C5RO or MCR5 were used as positive controls. In addition, fibroblasts derived from Artemis-deficient patients (Artemis-1,[25] Artemis-6 and Artemis-7[31]) and a LIG4-deficient patient (SC2[29]) were used in several assays. Other cell cultures used in this study were the hamster cell lines AA8[56] (wild type), DNA-PKcs-deficient V3 cells[40, 57] and V3 cells that were stably transfected with the YFP-DNA-PKcs expression construct.[11] All cell lines were cultured in a 1:1 mixture of Ham's F10 medium and DMEM (BioWhittaker), supplemented with 10% FCS, penicillin (100 U/ml), and streptomycin (100 µg/ml).

Flow cytometric analysis of peripheral blood and bone marrow

At diagnosis and three and six months after stem cell transplantation (SCT), flow cytometric analysis of peripheral blood was performed aiming at the analysis of the peripheral lymphocyte subset populations and at assessing the composition of the precursor B-cell compartment as previously described.[29]

Clonogenic survival assay

Clonogenic survival assays with primary skin fibroblasts or hamster cells were done as described previously.[25, 29] In short, primary skin fibroblasts in exponential growth or hamster cells were trypsinized, and 500 to 2,000 cells (5,000 to 80,000 cells for the highest doses) were seeded into 10 cm plastic dishes (2 dishes per dose, 3 for nonirradiated control) and irradiated at room temperature at a dose of approximately 2.7 Gy/min (200kV, 4.0 mA). After 12 to 14 days, the cells were rinsed with 0.9% NaCl and stained with 0.25% methylene blue for survival assessment. Three independent survival experiments were performed. In complementation experiments only cell lines with homogeneous human YFP-DNA-PKcs expression were analyzed.

Analysis of γH2AX foci after ionizing radiation

X-ray-induced H2AX foci were analyzed in confluent serum starved primary human fibroblasts cells after irradiation at a dose of 1 Gy by staining with monoclonal anti-phospho-H2AX (Ser139) (Upstate, Billerica, MA, USA) (10 ng/ml)

and subsequently with Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes). Cell nuclei were counter stained with 4,6-diamidine-2-phenylindole (DAPI, Sigma). Foci were examined by fluorescence microscopy and 40 cells per time point were analyzed.[29]

Sequence analysis of genes involved in V(D)J recombination and NHEJ

Sequence analysis of genes involved in V(D)J recombination and NHEJ was performed by PCR analysis (for *RAG1* (NCBI M29474), *RAG2* (NCBI M94633), *Artemis/DCLRE1C* (NCBI M94633), *XLF/NHEJ1* (NCBI AJ972687), and *LIG4* (NCBI X83441)) or RT-PCR analysis (for *KU70/XRCC6* (NCBI NM_001469), *KU80/XRCC5* (NCBI NM_021141), *XRCC4* (NCBI NM_003401) and *DNA-PKcs/PRKDC* (NCBI NM_006904)) of the coding regions with the TaqGold™ amplification system followed by direct sequencing. Primer sequences are available upon request.

Analysis of DH-JH junctions from bone marrow

DNA was isolated from bone marrow mononuclear cells (MNCs) and DH-JH coding joints were amplified by PCR with family specific DH primers and a consensus JH primer[58, 59] as previously described[31] followed by cloning of the PCR products into pGEM T-easy (Promega). Individual clones were sequenced and the composition of the junctional region was determined using the information from the international ImMunoGeneTics (IMGT) information system (http://imgt.cines.fr/).

Western blot analysis for DNA-PKcs

Cellular extracts were prepared for Western blot analysis of fibroblasts of patient ID177 and control fibroblasts (C5RO) 30 min after irradiation with 2 Gy. Non-irradiated cells were used as control. DNA-PKcs protein expression was detected with a mixture of the two rabbit polyclonal antibodies produced against amino acids 3486-3677 and 356-570, respectively (antibodies 2208 and 2129)[29] to detect full length DNA-PKcs and the phosphospecific antibody against P-S2056-DNA-PKcs.[11]

DNA-PK kinase activity assay

DNA-PK activity was determined using the SignaTECT DNA-Dependent Protein Kinase Assay system (Promega). Cell extracts were prepared from fibroblasts and assayed for DNA-dependent kinase activity according to supplier's protocol. DNA-PKcs specific kinase inhibitor NU7441[33] and ATM inhibitor KU-55933[34] were obtained from Kudos Pharmaceuticals (Cambridge, UK) kindly provided by Dr.

Graeme C.M. Smith and used in a final concentration of 0.5 mM. The inhibitors were dissolved in DMSO and as control (no inhibitor) DMSO was added to the same concentration.

Short tandem repeat (STR) marker analysis

Four STR markers in the *DNA-PKcs* gene region were selected (D8S1460, D8S359, D8S531 and AF075268). PCR analysis with one FAM-labeled primer was performed on DNA samples of the patient and 12 family members. The number of STRs was determined using fragment analysis on the ABI3100.

V(D)J recombination assay

V3 cells with or without DNA-PKcs expression were transfected with a recombination substrate (pDVG93) containing two RSS in inversed orientation and RAG1 and RAG2 expression constructs. Where indicated, also a FLAG-tagged DNA-PKcs expression construct was included. All expression constructs were of human origin. After 48 h, the fibroblasts were harvested and extrachromosomal DNA was isolated to amplify the coding joints. PCR products were digested with *Not*I or *Ngo*MI. After electrophoresis, the gel was dried and PCR products were visualized by phosphorimaging.[31]

DNA-PKcs expression constructs

Mutated full-length cDNA constructs (delG2113/L3062R, delG2113 and L3062R) with FLAG or EYFP tags were generated in pBluescript II with CMV promoter and stably transfected in V3 cells.[11]

End joining assay

EcoRV/Eco47III linearized pDVG94 (with homologous ends (ATCAGC sequence)) was transfected into V3 cells and V3 cells expressing either the WT or the mutant human DNA-PKcs construct as described previously.[36]. Newly formed junctions were PCR amplified, and the relative microhomology use was determined by *Bst*XI digestion.

DNA damage experiments

DSBs were introduced in a subnuclear volume using a pulsed 800 nm (multiphoton) laser system as described.[10] Cells were transfected with human Myc-tagged Artemis (pDVG190)[31] and proteins were detected using the EYFP-signal on DNA-PKcs and anti-Myc tag antisera (Santa Cruz Biotechnology). Immunofluorescence was carried out as described.[10]

Statistical Analysis

Differences in numbers of P-nucleotides were analyzed by using the non-parametric Mann-Whitney test (one-tailed; $P > 0.05$) in the GraphPad Prism program (GraphPad Software, San Diego, CA, USA).

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PART 3

Clinical spectrum of immunodeficiency with residual V(D)J recombination activity

Chapter 3.1

Idiopathic CD4+ T lymphopenia without autoimmunity or granulomatous disease in the slipstream of *RAG* mutations

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ABSTRACT

A girl presented during childhood with a single course of extensive chickenpox and moderate albeit recurrent pneumonia in the presence of idiopathic $CD4^+$ T lymphocytopenia (ICL). Her clinical condition remained stable over the past 10 years without infections, any granulomatous disease, or autoimmunity. Immunophenotyping demonstrated strongly reduced naive T and B cells with intact proliferative capacity. Antibody reactivity on *in vivo* immunizations was normal. T-cell receptor-Vβ repertoire was polyclonal with a very low content of T-cell receptor excision circles (TRECs). Kappa-deleting recombination excision circles (KRECs) were also abnormal in the B cells. Both reflect extensive in vivo proliferation. Patient-derived $CD34^+$ hematopoietic stem cells could not repopulate *RAG2*$^{-/-IL2R}\gamma c^{-/-}$ mice, indicating the lymphoid origin of the defect. We identified 2 novel missense mutations in *RAG1* (p.Arg474Cys and p.Leu506Phe) resulting in reduced RAG activity. This report gives the first genetic clue for ICL and extends the clinical spectrum of *RAG* mutations from severe immune defects to an almost normal condition.

INTRODUCTION

Selective depletion of T lymphocytes is common in both primary and secondary immunodeficiencies. Idiopathic $CD4^+$ T lymphocytopenia (ICL) is defined by an unexplained persistent $CD4^+$ T lymphocyte count of < 300 cells/μL or < 20% of the total T-cell count.[1]

Since the discovery of human retroviruses, sporadic ICL patients were recognized with a $CD4^+$ lymphocytopenia not infected by HIV or HTLV-1.[2-7] Smith et al reviewed 230179 cases from the CDC AIDS Reporting System and described 47 ICL patients.[2,3] Of these cases, only 3 (6%) were asymptomatic. Screening of healthy blood donors confirmed a low prevalence of ICL of 0.2%-0.6%.[4,5]

The disease may have a transient nature over the years but mostly persists.[3] The immunologic parameters of ICL consist of a prolonged decrease in $CD4^+$ T cell numbers, sometimes with a concomitant decrease in $CD8^+$ T cells and B cells as well. Immunoglobulin levels are normal,[2–5] which helps to distinguish ICL from Common Variable Immunodeficiency (CVID).[1,6]

Although function declines with age, thymic output is well maintained into late adulthood.[7-9] Thymic size correlates with numbers of $CD4^+CD45RA^+$ naive T cells. At very young age T lymphocytopenia is often caused by congenital defects resulting in severe combined immunodeficiency syndromes (SCID).[10]

Null mutations in *RAG1* or *RAG2* account for 70% of SCID cases with the classic T^-B^-SCID phenotype.[10] Hypomorphic mutations with residual RAG activity occur in typical Omenn syndrome,[10] or rare cases with lympocytopenia, hypogammaglobulinemia, granulomas in the skin, mucosa, internal organs and viral complications, including EBV-related lymphomas.[11, 12] In the present paper, we describe hypomorphic *RAG1* mutations that corresponds with mild $CD4^+$ T lymphocytopenia, normal *in vitro* lymphocyte function and *in vivo* vaccination responses.

MATERIALS AND METHODS

Subjects and blood samples

Heparinized venous blood was collected from healthy (age-matched) donors, patient and family members. The study was approved by the institutional medical ethical committee and informed consent for the research purpose described was obtained from the parents of the child and age-matched controls in accordance with standards of the 1964 declaration of Helsinki.

Lymphocyte phenotyping

Absolute numbers of T cells, B cells, and NK cells were determined with Multitest 6-color (FACSCantoII; BD-Biosciences). For T- and B-cell subset analysis directly conjugated monoclonal antibodies (MoAbs) were obtained from BD except for CD45RA-RD1 (Beckman-Coulter) and CD27-FITC (Sanquin).[13, 14]

Lymphocyte activation, determination of T-cell receptor CDR3 spectratype, TRECs, and Lymphocyte proliferation was performed as described.[13] $CD45RA^+$ naive T cells were purified (> 95%) to determine CDR3 patterns in $CD4^+$ and $CD8^+$ T cells or to isolate DNA for Sj–T-cell receptor excision circles (TRECs) analysis.[14, 15]

KREC assay and Vκ3-20–specific IgκREHMA

Four B-cell subsets were purified from PBMCs using FACS-DiVa cell-sorter.[16, 17] Somatic hypermutation in these subsets was assessed by the Vκ3-20–specific Igκ-restriction enzyme hot-spot mutation assay (REHMA).[16]

RAG gene analysis and in vitro V(D)J recombination assay

The *RAG1* and *RAG2* genes were amplified by PCR and sequenced.[18] The level of recombination activity of the RAG1 mutant proteins were compared with the wt RAG1.

Case

The patient was the first child of healthy, nonconsanguineous Dutch parents, born in May 1992. At the age of 5, she contracted chickenpox with numerous large hemorrhaghic skin lesions and VZV-associated pneumonia. Skin lesions healed slowly with cicatriciation. Until the age of approximately 8 years she has suffered from recurrent episodes of fever and (viral) pneumonia for which she regularly received antibiotics. High-resolution CT scanning of neck, thorax, and abdomen around that age showed mild bronchiectasis without enlargement of perihilar lymph nodes or granulomatous lesions in lungs, liver, or spleen; a thymus was not detectable (supplemental Figure 1). Dysmorphic features were not observed by clinical geneticists. Chromosomal abnormalities (including 22q11 hemizygosity) were excluded. She remained disease-free for the past 10 years using oral cotrimoxazole as prophylaxis.

RESULTS AND DISCUSSION

Lymphocyte subpopulations and humoral immune responses

Immunophenotyping of the patient's PBMCs showed low numbers of $CD4^+$ T cells and to a lesser extent, $CD8^+$ T cells. The $CD4^+$ lymphocytopenia was persistent over time with

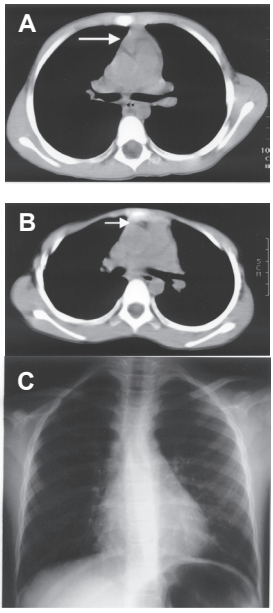

Figure S1. Thymus as demonstrated by HRCT scanning of an age-matched control suspected of pulmonary pathology (A) compared with our patient (B). The normal localization of the thymus is indicated by white arrows. The earliest pulmonary X-ray available at 4 years of age (C) already showed the absence of a thymic shadow in the patient.

Table S1. Ig spectrum and serologic reactivity in the patient

Age (years)	**4**	**6**	**12**
IgG (g/L)	5.6	6.8	7.1
IgA (g/L)	0.2	0.7	0.4
IgM (g/L)	0.6	0.8	0.7
IgE (kU/L)1	5	4	9
EBV-VCA	–	IgG +	IgG +
EBV-EBNA	nt	–	–
CMV	–	–	–
VZV	IgG +	IgG +	IgG +
Measles	IgG +	not tested	not tested
Tetanus Toxoid	0.74 U/mL	not tested	1.9 U/mL
Polio	1 : 2048	1 : 1024	1 : 20482

1 phadiatop and food allergen screenings negative
2 post-DTP booster at 10 years of age

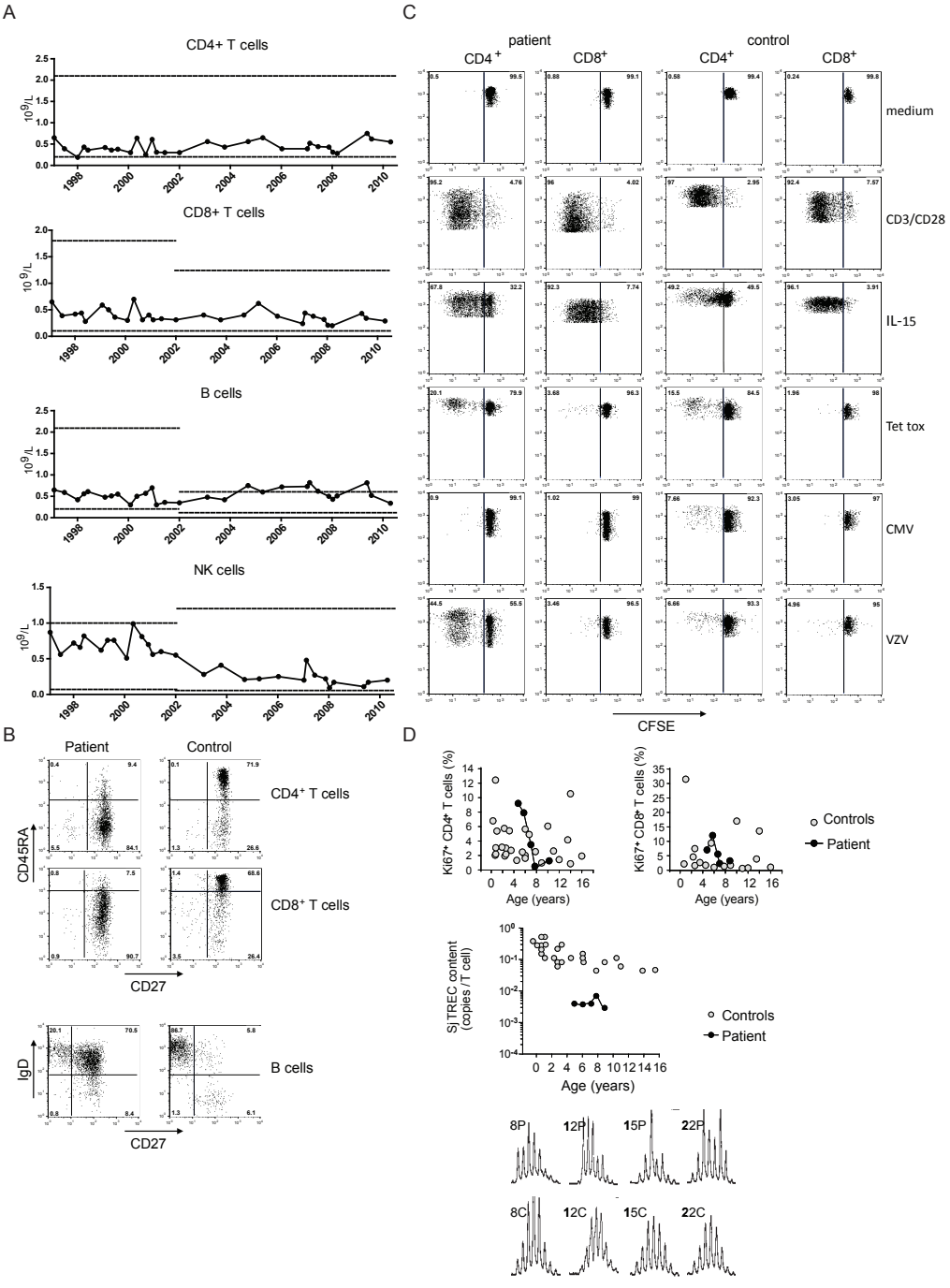
A
CD4+ T cells
CD8+ T cells
B cells
NK cells
10^9/L
1998
2000
2002
2004
2006
2008
2010
B
Patient
Control
CD4+ T cells
CD8+ T cells
CD45RA
CD27
IgD
B cells
C
patient
control
CD4+
CD8+
medium
CD3/CD28
IL-15
Tet tox
CMV
VZV
CFSE
D
Ki67+ CD4+ T cells (%)
Ki67+ CD8+ T cells (%)
Age (years)
Controls
Patient
SjTREC content (copies / T cell)
8P
12P
15P
22P
8C
12C
15C
22C

Figure 1. Absolute numbers of CD4+ and CD8+ T cells, CD19+ B cells and CD3+CD16/56+ NK cells over time. (A) Numbers of the patient's lymphocytes are indicated in closed circles;the age-matched control levels for different age categories (ie, 4-10 years; 10-18 years) are indicated in a range of 2SD (dotted lines). (B) Immunophenotyping of CD4+ and CD8+ T cells and CD19+CD20+ B cells, according to CD45RA/CD27 and sIgD/CD27, respectively, as compared with a healthy, age-matched control. Proliferative capacity of the patient's T cells. T-cell proliferation of the patient and a healthy control is shown by CFSE dilution after 6 days of culture. Polyclonal proliferation was induced by a combination of CD3/CD28 or IL-15, whereas antigen-specific proliferation was assessed by stimulation with tetanus toxoid (Tet Tox), cytomegalovirus (CMV) or varicella zoster virus (VZV). (C) As expected in the presence of negative serology, CMV antigen did not activate her T cells. Increased proliferation of the peripheral CD4_ T-cell compartment as demonstrated by nuclear Ki67 staining in naive (CD45RA_CD27_) CD4_ and CD8_ T cells, compared with healthy age-matched naive control T cells, as described.13 TRECs in patient's T cells for the early, so-called signal joint (Sj) over a period of 5 years.(D) For T-cell repertoire analysis CDR3 spectratyping in the patient's T cells was analyzed, being representative for 2 separate experiments in triplicate, more than 2 years apart.

some fluctuations (Figure 1A). The percentage of $CD4^+$ and $CD8^+$ T cells with a $CD45RO^+$ memory phenotype was strongly increased as compared with the naive T cells (Figure 1B).

Although B-cell numbers were normal, the distribution of the B-cell subsets was severely disturbed (Figure 1B). The frequency of class-switched $CD27^+$ memory B cells was low (3.4%-9.2%) whereas the frequency of nonswitched $sIgD^+CD27^+$ memory B cells showed a high and stable percentage between 58%-72% (controls between 5-15 years: 18.4 ± 7.2%, n = 40). Humoral immunity was intact (supplemental Table 1).

In vitro functionality of T cells, thymic function, and TCR repertoire

T-cell proliferation to general stimuli (ie, CD3/CD28, cytokines), and specific antigens was intact (Figure 1C; data not shown). Cytokine release, CTL or spontaneous NK-cell killing of target cells were normal (data not shown).

In the absence of a detectable thymus, peripheral *in vivo* T-cell proliferation was expected to be increased to sustain normal T-cell numbers. This was indeed demonstrated in 2 ways. First, lymphocyte expression of the nuclear proliferation marker Ki67 was not different from age-matched control samples (Figure 1D; data not shown). Second, the content of TRECs was reduced compared with controls (Figure 1D), indicative for increased T-cell proliferation. However, despite extensive peripheral T-cell expansion to sustain the number of T cells, no restriction in the TCR repertoire was demonstrated (Figure 1D).

Cellular and molecular properties of the circulating B-cell compartment

B-cell proliferation was found to be normal on BcR-dependent and BcR-independent stimulation (supplemental Figure 2A). The number of cell divisions of the transitional, naive-mature, and nonswitched B cells was strongly increased as compared with healthy controls (supplemental Figure 2B).[16,17] Naive B cells were not somatically mutated and the mutation frequency in the expanded fraction of nonswitched natural effector B cells was

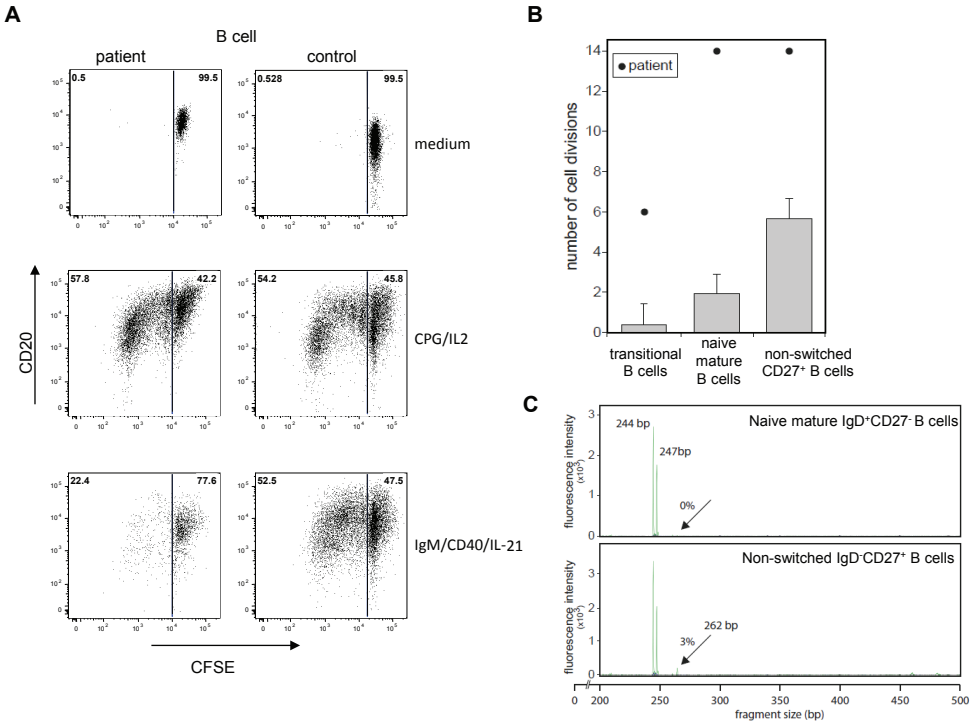

Figure S2. B-cell proliferation and B-cell repertoire analysis in the patient's B-cell subsets. Proliferation of CD19+ B cells of the patient and a control is shown by CFSE dilution after 6 days of culture with the indicated stimuli. Plots show CFSE dilution versus CD20 expression. (A). Increased proliferation of transitional, naïve mature, and non-switched natural effector B cells, as determined as the ΔCT of the intron-RSS-Kde coding joint and the signal joint PCR (B). The percentage of mutated Vκ3-20-Jκ alleles determined the IgκREHMA assay in naïve mature B cells (upper panel) and non-switched natural effector B cells (lower panel). The 244 and 247 bp fragments are unmutated and the 262 bp fragments are mutated (C).

only 4.7% (healthy controls 15%; n = 5; supplemental Figure 2C). These results indicate that the number of total peripheral B cells is normal because of extensive (antigen-independent) proliferation of the transitional, naive and nonswitched B cells.

Lymphoid origin of the defect and genetic immunodeficiency screening

T-cell maturation data suggested an intrinsic T-cell developmental defect with thymus hypoplasia from early age onward. Using a humanized *RAG2*$^{-/-}$*IL2Rγc*$^{-/-}$ mouse model,[19] normal human T-cell reconstitution on intrahepatic injection of 4×10^5 patient-derived CD34$^+$CD38$^-$ HSCs from the bone marrow of the patient failed, in contrast to control HSCs. In the blood, no patient T cells could be detected at 6 and 9 weeks, nor in the thymus

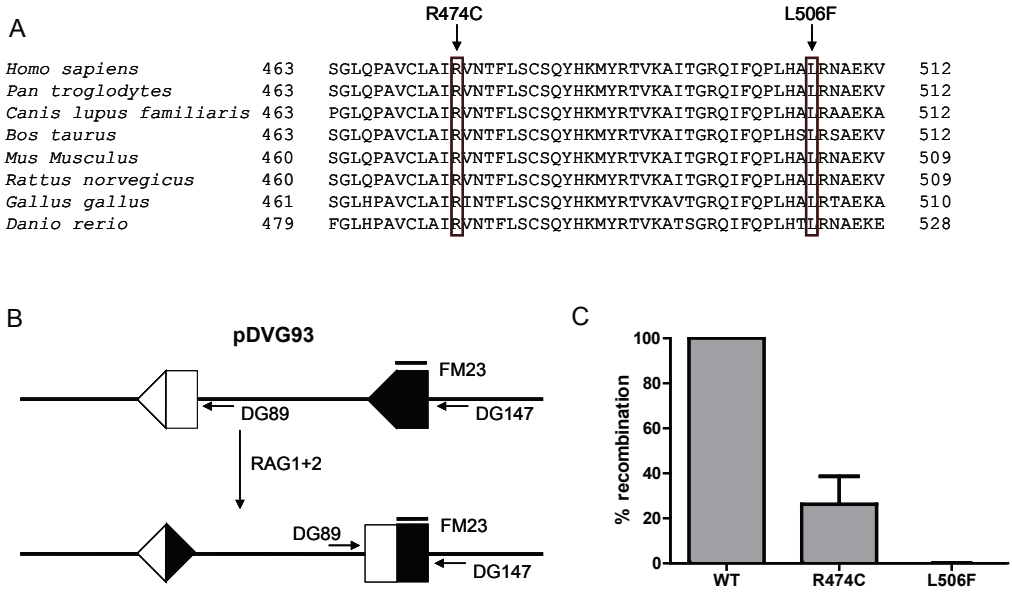

Figure 2. RAG1 gene mutations and residual recombination activity. Sequence alignment of RAG1 protein of different species. Both the arginine (R) at amino acid position 474 and the leucine (L) at 506 are conserved residues. (A) The parents were heterozygous for each of the mutation. (B) Transfection of pDVG93, RAG1 and RAG2 in 3T3 fibroblasts results in an inversion rearrangement of pDVG93, which can be detected by the primers DG89 and DG147. (C) Only the R474C mutation results in residual recombination activity, as assessed by the in vitro recombination assay using pDVG93.

at 10 weeks (supplemental Table 2), confirming the lymphoid background of the thymus hypoplasia.

RAG defect

Sequence analysis demonstrated 2 novel heterozygous missense mutations in the *RAG1* gene (c.1420C > T and c.1516C > T), affecting the evolutionary conserved amino acids Arg474 and Leu506 (Figure 2A). The RAG1 p.Arg474Cys recombinant protein had 25% recombinase activity compared with wild-type RAG1, whereas the p.Leu506Phe mutant did not have any residual activity (Figures 2B-C).

Hypomorphic mutations may result in typical Omenn patients with T cells showing an oligoclonal TCR repertoire, and poor development of precursor B cells,[18, 20] which may contribute to hyperinflammation/autoimmunity because of low-affinity autoantibody production.[21, 22] These features were absent in our patient.

Table S2. Differentiation capacity into lymphocytes of bone marrow derived HSCs, from patient and normal donor in the RAG2–/–IL2Rγc–/–mouse model

BLOOD (A)		**6w**		**9w**	
Mouse	**Donor**	**%CD45+**	**nCD45+**	**%CD45+**	**nCD45+**
1	patient	0	0	0.2	11
2	patient	0	0	0.1	3
3	control	34.5	19	3.3	114
4	control	28.6	8	5.1	230
5	control	47.1	8	12.4	565
6	control	57.1	24	20.2	303
THYMUS (B)		**10w**			
Mouse	Donor	%CD45+		nCD45+	
1	patient	0		0	
2	patient	0		0	
3	control	74.9		351094	
4	control	88.4		9530625	
5	control	83.6		16328125	
6	control	87.9		274688	

Two-day-old newborn sub-lethally irradiated (3.5Gy) *RAG2–/–IL2Rγc–/–*mice were injected via the intra-hepatic route with HSCs from bone marrow[19]. Mice 1 and 2 were injected intra-hepatic with 4x105 HSCs from the bone marrow of the patient. Mice 3-6 were injected with an equal number of HSCs from the bone marrow of a normal donor. Blood (A) was taken at 6 and 9 weeks after grafting, and all mice were euthanized 10 weeks after grafting for the analysis of the thymus (B). The percentage of human CD3+CD45+ cells was based on the cells in the life gate.

Table S3. Pre-activation state of the patient's T and B lymphocytes

	Unstimulated		**PHA-activated[1]**	
CD4+ (% pos)	**Patient**	**Controls**	**Patient**	**Controls**
Control Ab (γ1)	1 ± 1.1	1 ± 0.8	2 ± 2.1	1 ± 1.2
CD95	96 ± 11.9*	26 ± 8.1	98 ± 12.3*	45 ± 16.7
CD70	11 ± 2.8*	2 ± 1.3	57 ± 16.6	36 ± 14.4
CD27	99 ± 8.5	96 ± 4.2	72 ± 14.9	89 ± 12.1
Ox40	46 ± 11.0*	6 ± 2.3	80 ± 8.7*	46 ± 16.3

[1]PBMC in medium or activated for 4 days with PHA (1 µg/ml) and IL-2 (20 U/mL). Significant differences between patient and controls are indicated by an asterisk ($p<0.05$ [n=4], tested over 5 years). MoAbs were purchased from BD.

Epicrise

V(D)J recombination activity of the RAG1 mutant proteins was very low. Even this residual RAG1 activity provides a low level of T- and B-cell production for effective host immunity. The functional immune system in terms of the mild clinical course in the absence of a detectable thymus could be explained by cytokine-driven proliferation, as reflected by the maintenance of a polyclonal repertoire.[23] The persistent lymphocyte activation could be compatible with cytokine-driven homeostasis (Figure 2A; supplemental Table 3).

The thymus hypoplasia associated with an immune defect similar to ICL and few clinical symptoms was identified to be caused by novel heterozygous *RAG1* mutations with very low RAG activity. Instead of SCID, Omenn syndrome or extensive granulomatous disease, our case study adds to the complexity of hypomorphic mutations in the *RAG* genes.

AUTHORSHIP

Contribution: T.W.K. performed the diagnosis and treatment, designed research, and wrote the paper; H.I. performed research, cloning and expression of RAG variants, and analysis of data; E.M.M.v.L. contributed to the design of research and data analysis; M.H.J. and M.D.H. performed research and data analysis; K.C.W. performed vital mouse studies; R.A.W.v.L. contributed to the design of research and wrote the manuscript; and M.v.d.B. contributed to the design of study and wrote the manuscript.

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Chapter 3.2

Similar recombination-activating gene *(RAG)* mutations result in similar immunobiological effects but in different clinical phenotypes

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ABSTRACT

Background V(D)J recombination takes place during lymphocyte development in order to generate a large repertoire of T- and B-cell receptors. Mutations in the recombination activating genes 1 (*RAG1*) and *RAG2* result in loss or reduction of V(D)J recombination. It is known that different mutations in the *RAG* genes vary in residual recombinase activity and give rise to a broad spectrum of clinical phenotypes.

Objective To study the immunological mechanisms causing the clinical spectrum of RAG deficiency (RAGD).

Methods We included 22 patients with similar *RAG1* mutations (c.519delT or c.368_369delAA), resulting in N-terminal truncated RAG1 protein with residual recombination activity, but presenting with different clinical phenotypes. We studied precursor B-cell development, immunoglobulin (IG) and T-cell receptor repertoire formation, receptor editing, and B- and T-cell numbers.

Results Clinically, patients were divided into three main categories: T-B-severe combined immunodeficiency (SCID), Omenn syndrome and combined immunodeficiency (CID). All patients showed a block in the precursor B-cell development, low B- and T-cell numbers, normal immunoglobulin gene usage, limited B- and T-cell repertoires and slightly impaired receptor editing.

Conclusion This study demonstrates that similar RAG mutations can result in similar immunobiological effects, but different clinical phenotypes, indicating that the level of residual recombinase activity is not the only determinant for clinical outcome. We postulate a model in which the type and moment of antigenic pressure impact on the clinical phenotype of these patients.

CLINICAL IMPLICATIONS

RAG deficiency can result in a broad spectrum of clinical presentations, but the level of residual RAG activity is not always predictive for the clinical outcome.

INTRODUCTION

Defects in V(D)J recombination result in a block in the B and T cell differentiation, because formation of immunoglobulin (IG) and T cell receptors (TR) is perturbed.[1] This results in a combined immunodeficiency of B and T cells. V(D)J recombination is initiated by the recombination activating gene (RAG) 1 and RAG2 proteins by creating double strand breaks in the IG and TR loci. Subsequently, these breaks are processed and repaired by proteins involved in non-homologous end joining. So far, genetic defects have been identified in the *RAG1*, *RAG2*, *Artemis*, *Ligase IV (LIG4)*, *XLF (Cernunnos)* and *DNA-PKcs* genes.[2-8] The immunological phenotype and clinical presentation of these mutations are different, depending on the type of genetic defect, i.e. null mutations or hypomorphic mutations with residual V(D)J recombination activity. Especially for the *RAG* genes, many different mutations have been described that give rise to residual activity of the mutated RAG protein.[9] Different *RAG* mutations may result in a broad spectrum of clinical phenotypes, including SCID, RAG deficiency (RAGD) with skin inflammation and αβ T-cell expansion (classical Omenn syndrome (OS)), RAGD with skin inflammation but without T-cell expansion (incomplete OS), RAGD with materno-fetal transfusion, RAGD with γδ T-cell expansion, late-onset SCID, RAGD with granulomas, and RAGD with CD4 cytopenia and thymus hypoplasia.[9, 10] This broad spectrum of clinical phenotypes impedes timely recognition of RAGD and may thus delay treatment (hematopoietic stem cell transplantation).

In this study we selected 22 RAGD patients with similar N-terminal truncating *RAG1* mutations, to study the effect of a similar mutation on the clinical phenotype. These patients could be divided into three main different clinical phenotypes, i.e. SCID, OS, and combined immunodeficiency (CID), which includes the other phenotypes. We studied whether key immunologic parameters (e.g. precursor B-cell development, B- and T-cell numbers, B- and T-cell repertoire) might explain the differences in clinical phenotypes.

METHODS

Cell samples and flow cytometric immunophenotyping

Peripheral blood (PB), bone marrow (BM) and clinical data were obtained according to the guidelines of the Medical Ethics Committee of the Erasmus MC Rotterdam. Flow cytometric analysis was performed as previously described.[8, 11, 12]

RAG analysis and in vitro V(D)J recombination assay

The *RAG1* and *RAG2* genes were amplified by PCR and sequenced as previously described.[13] The level of recombination activity of the RAG1 expression constructs was determined by using the recombination plasmid pDVG93 as described before.[10, 13] A TaqMan-based RQ-PCR was used to measure *RAG1* and *RAG2* transcription levels in bone marrow mononuclear cells as was described before.[14]

TRB analysis

T cell receptor beta (TRB) gene rearrangements were studied as described before.[15]

Sequence analysis of Vκ and Jκ genes

Vκ-Cκ junctions were amplified in a multiplex PCR using primers specific for Vκ1-5 families (VκI: 5'-GTAGGAGACAGAGTCACCATCACT-3', VκII: 5'-TG- GAGAGCCGGCCTCCATCTC-3', VκIII:5'-GGGAAAGAGCCACCCTCTCCTG-3', VκIV:5'-GGCGAGAGGGCCACCATCAAC-3') and a Cκ primer (5'-ACTTTGGCCTCTCTGGA- TA-3'). PCR products were cloned in the pGEM-Teasy vector (Promega, Madison, WI) and prepared for sequencing on the ABI Prism 3130 XL fluorescent sequencer (Applied Biosystems). Obtained sequences were analyzed with the IMGT database (http://imgt.cines.fr/) to assign the Vκ and Jκ genes.[16, 17] The productive and unique sequences were used to determine the frequency of the Vκ and Jκ genes.

Repertoire analysis using next generation sequencing

The VH-JH junctions were amplified from post-ficoll peripheral blood (PB) mononuclear cells in a multiplex PCR using the VH1-6 FR1 and JH consensus BIOMED-2 primers.[15] The primers were adapted for 454 sequencing by adding the forward A or reverse B adaptor, the 'TCAG' key and multiplex identifier (MID) adaptor. PCR products were purified by gel extraction (Qiagen, Valencia, CA) and Agencourt AMPure XP beads (Beckman Coulter, Fullerton, CA, USA). Subsequently, the PCR concentration was measured using the Quant-it Picogreen dsDNA assay (Invitrogen, Carlsbad, CA). The purified PCR products were sequenced on the 454 GS junior instrument according the manufacturer's recommendations, using the GS junior Titanium emPCR kit (Lib-A), sequencing kit and PicoTiterPlate kit (454 Life Sciences, Roche, Brandford, CT). Using the CLC genomic workbench software

the samples were separated based on their MID sequence, trimmed and reads with a quality score below 0.05 and below 250bp were discarded. The reads were uploaded to IMGT HighV-Quest.[18] Subsequently these output files were uploaded to the custom Galaxy platform [19-21] Further processing was done in the 'R' programing language[22] to generate the tabular and graphical outputs. The CDR3 amino acid patterns were visualized using WebLogo (http://weblogo.berkeley.edu/).[23, 24]

Statistics

Differences in absolute numbers of lymphocytes subsets were analyzed by the two-tailed T-test for independent samples ($P<0.05$ was considered significant) in the GraphPad Prism program (GraphPad Software, Inc.).

RESULTS

Residual RAG1 activity in patients with N-terminal truncating RAG1 mutations

Over the past ten years, we identified one of the two mutations resulting in N-terminal truncating *RAG1* mutations in 22 patients (Table I and II). These c.519delT (hereafter abbreviated as delT) and c.368_369delAA (hereafter abbreviated as delAA) mutations have been described before in several patients.[13, 25-29] They were found to be hypomorphic,[13, 27] because translation can be reinitiated from the alternative start site methionine 202 (M202) or M183, resulting in an N-terminal truncated RAG1 protein,[13] with the same (comparable) residual RAG1 activity (<5% compared to wild type) (Figure 1A).[13] Sixteen patients were homozygous for the delAA or the delT mutation, and six patients were compound heterozygous (Table I). Three *RAG1* mutations found on the second allele were also analyzed in the *in vitro* recombination assay, showing no residual RAG1 activity (Figure 1A). In addition, we determined the presence of polymorphisms in the *RAG1* gene because these might influence the recombination activity of RAG1. The only polymorphism found was the p.Arg249His, which was shown not to affect recombination activity.[2]

N-terminal truncating RAG1 mutations result in a spectrum of clinical phenotypes

Although all patients had similar *RAG1* mutations resulting in the same N-terminal truncation of the RAG1 protein, the clinical phenotypes varied substantially. The patients could be divided into three main clinical phenotypes: "classical" T-B-SCID (N=4), OS (N=9), and CID (N=9) (Table I and II). The "classical" SCID patients were defined as low B- and T-cell numbers and age at diagnosis before the first year of life. The OS patients all suffered from generalized and pronounced erythroderma. The patients with CID were diagnosed after the first year of life and had >14% γδ T cells or had normal levels of T cells (P17 and P22).

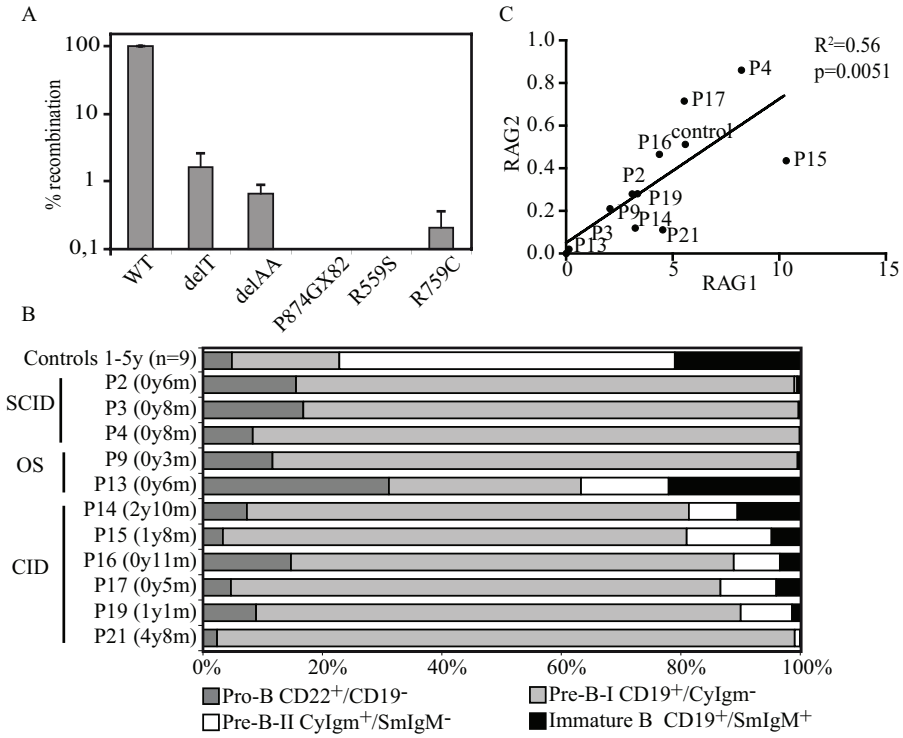

Figure 1. RAG expression and precursor B-cell compartment. Recombination activity of the c.519delT (delT), c.delA368/A369 (delAA), p.P874GX82, p.R559S and p.R759C *RAG1* mutations was compared with wild type *RAG1*. Only the delT and the delAA *RAG1* mutations result in low levels of residual recombination activity (A). Composition of the precursor B-cell compartment in controls (N=9), three "classical" SCID phenotype, two OS patients, and six CID patients (B). Relative *RAG1* expression levels correlated to *RAG2* expression in all the analyzed RAG patients as determined by using RQ-PCR (C).

Despite the same N-terminal truncation of RAG1 in the 22 patients, the range of clinical phenotypes strongly suggests that other factors than residual RAG1 activity contributes to the clinical phenotype.

All clinical phenotypes show a block in precursor B-cell development

RAGD results in a block in the precursor B-cell differentiation in BM at the B-cell stages where V(D)J recombination of the IG genes takes place.[11] To investigate precursor B-cell differentiation, the relative distribution of pro-B, pre-B-I, pre-B-II and immature B cells was assessed in BM from 11/22 patients. In healthy children, pro-B and pre-B-I cells constitute 20-25% of the precursor B cells (Figure 1B). All "classical" SCID and OS patient, except for

P13) showed a complete block before the pre-B-II cell stage (Figure 1B), while most of the CID patients had a leaky block with >10% pre-B-II and immature B cells (Figure 1B). To exclude that differences in *RAG1* transcription levels caused these difference in precursor-B cell composition, *RAG1* and *RAG2* transcription levels were determined in the bone marrow mononuclear cells (BMMNC). It is known that *RAG1* and *RAG2* transcription levels are correlated,.[30] but the levels of *RAG1* and *RAG2* in BMMNC depend on the number of cells expressing *RAG* (pre-B-I and pre-B-II). In all 11 studied patients the *RAG1* transcription level was correlated to *RAG2* (Figure 1C), indicating that the differences in severity of the precursor-B cell block were not caused by differences in expression of *RAG1*. The B-cell numbers in PB were undetectable or very low in most patients, except for P15 who had normal levels (Table II). Correlating the percentage of pre-B-II and immature cells in BM with the number of peripheral B cells showed that only patients with more than 10% pre-B-II and immature B cells in BM (P13, P14, P15, P16, and P17) had detectable B cells in PB. Collectively these data indicate that most patients with CID have a milder block in the precursor B-cell composition, and that only patients with a leaky block have detectable levels of B cells in the PB.

Immunoglobulin heavy chain combinatorial repertoire

In those patients that had detectable peripheral B cells we studied the *IGH* V(D)J recombination repertoire. *IGH* gene rearrangements were amplified from mononuclear cells derived from PB and/or BM, and subsequently sequenced using next generation sequencing in healthy controls (PB and BM), and in three CID patients (P15, P16 and P18). The frequency of unique sequences in *IGH* genes was significantly lower in RAGD patients than in controls (Table III), which is a reflection of the low numbers of B-cells present in PB. Despite the low recombination activity, the *IGHV*, *IGHD* and *IGHJ* genes usage was not restricted (Figure 2A and B and Supplemental Figure 1). 48 out of the 57 *IGHV* genes used in controls were identified in the RAGD patients, as were the 25 *IGHD* genes and all 6 *IGHJ* genes. The *IGHV*, *IGHD* and *IGHJ* usages were similar to controls, although some genes were used with different frequencies (Figure 2 and Figure E1). Most strikingly, the JH6 usage was lower, while JH4 usage was higher compared to controls. The RAGD patients had a significantly lower frequency (5.9-6.2% vs 20.9-24.3% in controls) of unproductive rearrangements (Table III), as was reported before.[31] Unproductive rearrangements were defined as out-of-frame rearrangements or rearrangements with a stop codon. So, even though the RAGD patients had reduced V(D)J recombination leading to a limited TR and IG repertoire, the *IGH* gene usage was similar to controls without preferential use of the proximal or distal genes.

Table I. Clinical data of RAG deficient patients

	onset infections (m)	age at diagnosis (m)	infections	respiratory tract infections	autoimmunity	Hepato-megaly	Spleno-megaly	Lymphadeno-pathy
SCID								
P1	3							
P2	6	6	BCG	no	ITP	no	no	no
P3	8	8		pneumonia and upper airway infections				
P4	6	8	BCG					mild
OS								
P5	0	0.5			erythroderma	yes	yes	yes
P6[a]	0	0.5		recurrent pneumonia	erythroderma	yes	no	yes
P7[a]	0	0.5	CMV	no	erythroderma	yes	yes	yes
P8	0	3.5	CMV, candida, MRSE	severe pneumonia	erythroderma			
P9	0	4			erythroderma	yes	no	yes
P10	1	1			erythroderma			yes
P11	1.5	2			erythroderma			yes
P12	1	8	BCG	recurrent pneumonia	erythroderma	yes	yes	yes
P13	3	6	candida, M. bovis, cornonavirus, rhinovirus	recurrent upper and lower airway infections	erythroderma. AIHA. ITP	yes	no	no
CID								
P14[b]	9	30	CMV, candida	recurrent bronchopneumonia		yes	yes	no
P15[b]	9	18	CMV	recurrent bronchopneumonia		yes	yes	no
P16[c]	1	11	CMV	chronic rhinitis		no	no	no
P17	4	6	CMV, BCG	pneumonia		yes	yes	yes
P18	18	60	CMV, BCG, rhinovirus	yes	AIHA. ITP	no	no	no
P19	3	13	candida	chronic rhinitis and bronchitis	AIHA	no	no	no
P20	24	48			AIHA			
P21	13	60	candida, aspergillosis	recurrent pneumonias, bronchitis	AIHA	no	no	no
P22[c]	0	17		recurrent pneumonias, bronchitis		no	no	no

a, b or c indicates relatives.

Table II. Immunological data of RAG deficient patients

	delT	delAA	other	CD3+ T abs (x10E9/l)	CD4+ T abs (x10E9/l)	CD8+ abs	CD45RA (%)	γδT (%)	CD19+ abs (x10E9/l)	NK abs (x10E9/l)
SCID										
P1		hom		0.06 (1.4-8.0)	0.04 (0.9-5.5)	0.01 (0.4-2.3)		21,8	0.03 (0.6-3.1)	0.08 (0.1-1.4)
P2		het	p.P874GfsX82	0.1 (2.4-6.9)	0.06 (1.4-5.1)	0.01 (0.6-2.2)	32.1	24.4	0.01 (0.7-2.5)	0.4 (0.1-1.0)
P3	hom			0.3 (1.6-6.7)	0.06 (1.0-4.6)	0.3 (0.4-2.1)			0 (0.6-2.7)	0.5 (0.2-1.2)
P4		het	p.R559S	0.3 (1.6-6.7)	0.2 (1.0-4.6)	0.04 (0.4-2.1)	7.7	10	0 (0.6-2.7)	0.1 (0.2-1.2)
OS										
P5	hom			20.1 (2.3-7.0)	7.56 (1.7-5.3)	12.75 (0.4-1.7)		4	0 (0.6-1.9)	2.59 (0.2-1.4)
P6 [a]		hom		3.7 (2.3-6.5)	3.1 (1.5-5.0)	0.4 (0.5-1.6)	7.2	5.2	0.03 (0.6-3.0)	0.8 (0.1-1.3)
P7 [a]		hom		36 (2.3-6.5)	10.7 (1.5-5.0)	24.9 (0.5-1.6)	3.1	1	0.03 (0.6-3.0)	0.4 (0.1-1.3)
P8		hom		3.93 (2.3-6.5)	1.45 (1.5-5.0)	2.19 (0.5-1.6)	21.4	24.4	0.02 (0.6-3.0)	0.88 (0.1-1.3)
P9	hom			1.84 (2.3-6.5)	1.48 (1.5-5.0)	0.3 (0.5-1.6)	4.6	3	0.004 (0.6-3.0)	1.64 (0.1-1.3)
P10		het	p.R737H	3.3 (2.3-7.0)	0.32 (1.7-5.3)	2.97 (0.4-1.7)	0.3	0.1	0 (0.6-1.9)	0.34 (0.2-1.4)
P11		het	p.R559S	4.33 (1.6-6.7)	4 (1.0-4.6)	0.27 (0.4-2.1)			0.01 (0.6-2.7)	0.93 (0.2-1.2)
P12		hom		2.21 (2.3-6.5)	1.34 (1.5-5.0)	0.61 (0.5-1.6)			0.07 (0.6-3.0)	0.56 (0.1-1.3)
P13		hom		0.6 (2.4-6.9)	0.6 (1.4-5.1)	0.01 (0.6-2.2)	4.8	2.9	0.07 (0.7-2.5)	0.2 (0.1-1.0)
CID										
P14 [b]		hom		0.3 (0.9-4.5)	0.1 (0.5-2.4)	0.07 (0.3-1.6)	35	49.5	0.4 (0.2-2.1)	0.8 (0.1-1.0)
P15 [b]		hom		0.5 (1.4-8.0)	0.1 (0.9-5.5)	0.2 (0.4-2.3)	38.9	64.1	0.4 (0.6-3.1)	2.9 (0.1-1.4)
P16 [c]		hom		0.16 (1.6-6.7)	0.07 (1.0-4.6)	0.02 (0.4-2.1)	26.9	46.3	0.09 (0.6-2.7)	0.32 (0.2-1.2)
P17		hom		2.7 (1.6-6.7)	0.2 (1.0-4.6)	1.5 (0.4-2.1)	90.7	90.2	0.06 (0.6-2.7)	0.7 (0.2-1.2)
P18	het		p.R759C	0.53 (0.9-4.5)	0.07 (0.5-2.4)	0.12 (0.3-1.6)		57.2	0.12 (0.2-2.1)	1.32 (0.1-1.0)
P19		hom		0.10 (1.6-6.7)	0.01 (1.0-4.6)	0.10 (0.4-2.1)		97.5	0.04 (0.6-2.7)	0.23 (0.2-1.2)
P20		hom		0.77 (0.9-4.5)	0.25 (0.5-2.4)	0.24 (0.3-1.6)		41.7	0.02* (0.2-2.1)	0.18 (0.1-1.0)
P21		het	p.A444V	0.12 (0.9-4.5)	0.10 (0.5-2.4)	0.06 (0.3-1.6)		14.4	0.001 (0.2-2.1)	0.25 (0.1-1.0)
P22 [c]		hom		1.97 (1.6-6.7)	0.42 (1.0-4.6)	1.50 (0.4-2.1)	42		0.29 (0.6-2.7)	0.72 (0.2-1.2)

The numbers in brackets indicates normal values, * indicated under rituximab treatment and a, b or c indicates relatives.

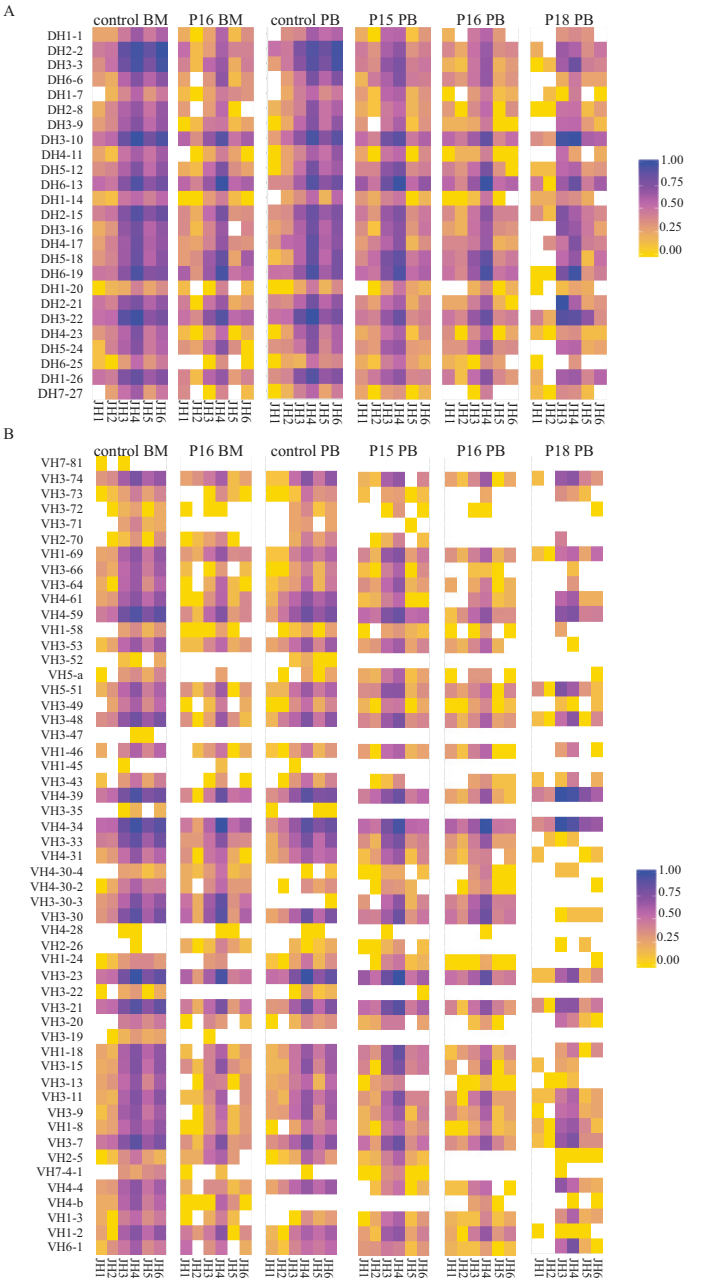

Figure 2. Immunoglobulin heavy chain gene usage. Heatmap of the different combinations of DH-JH (A) and VH-JH (B) as determined in the unique junctions (defined by the unique combination of VH, DH, JH and nucleotide sequences of CDR3).

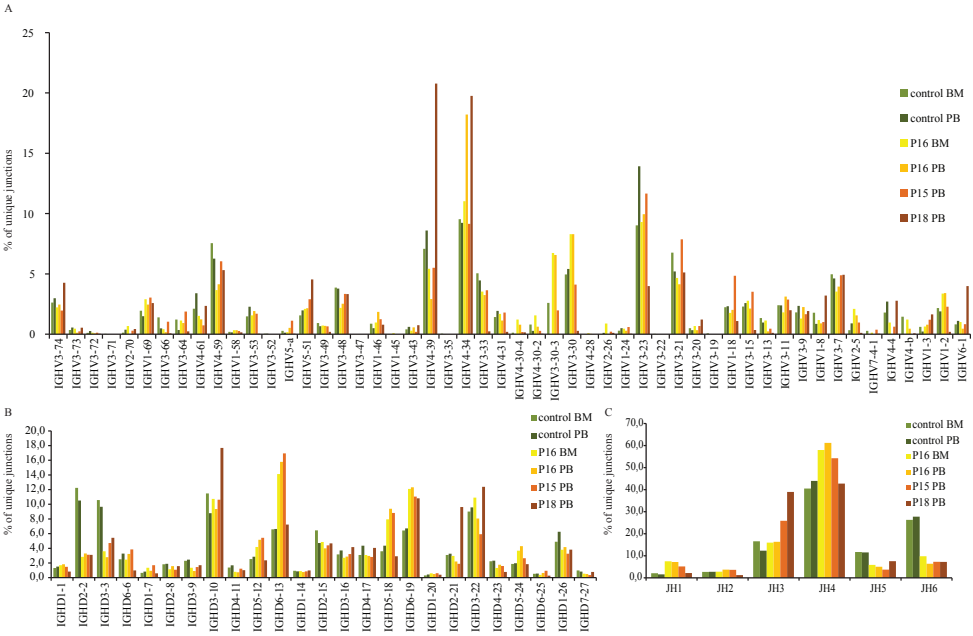

Figure E1. Immunoglobulin heavy chain gene segment usage. Frequency of IGHV (A), IGHD (B) and IGHJ (C) genes usage in control BM and PB and in P16 BM and PB, P15 PB and P18 PB.

Table III. Number of IGH sequences

	all sequences	unique sequences	unproductive	productive
control BM	35472	18241 (51.4)	8633 (24.3)	26839 (75.7)
P16 BM	12195	3325 (27.3)	1629 (13.3)	10566 (86.7)
control PB	19294	9185 (61.2)	4030 (20.9)	15003 (77.8)
P15 PB	16826	7706 (45.8)	1047 (6.2)	15779 (93.8)
P16 PB	14572	3763 (25.8)	896 (6.1)	13676 (93.9)
P18 PB	25100	3730 (14.9)	1488 (5.9)	23612 (94.1)

The number in brackets indicates percentages. Unproductive refers to out-of-frame rearrangements or rearrangements containing a stop codon in the CDR3 region.

Selection of B cells is slightly impaired

OS is characterized by autoimmune-like clinical features including severe erythrodermia, hepato-splenomegaly and lymphadenopathy.[32, 33] The immune dysregulation in OS patients may be caused by the severe abnormalities of thymic architecture and impaired expression of autoimmune regulator (AIRE) and tissue-specific antigens (TSA).[34, 35] In addition, hypomorphic Rag mouse models have shown a disturbance in B-cell tolerance.[36, 37] Besides the OS patients, also one "classical" SCID patient, and four patients with CID suffered from autoimmunity and all displayed idiopathic thrombocytopenic purpura (ITP) and/or autoimmune hemolytic anemia (AIHA) (Table I). Unfortunately, the thymic architecture and AIRE and TSA expression could not be studied in our patients, but we were able to evaluate three parameters in the *IGH* sequences that are associated with autoimmunity. These are characterized by long complementary determining regions 3 (CDR3s), and the frequency of *IGHV4-34* which is known to encode intrinsically self-reactive cold agglutinin antibodies that recognize carbohydrate antigens on erythrocytes.[38, 39] The distribution of the CDR3 length of the unique junctions in BM and PB was similar to controls (Figure 3A), except for patient 18 who seemed to have increased numbers of junctions with a CDR3 of 16 and 22 amino acids (aa). These junctions with a CDR3 length of 22 aa displayed high similarity (Figure 3B). No sequence similarity was found when all 16aa CDR3s were compared (Figure 3B), but 18.3% of these junctions used *IGHV6-1* and all these junctions had a highly similar CDR3 sequence (Figure 3B), which suggests that they might recognize a common antigenic determinant. The frequency of long CDR3s (≥15 aa) was significantly lower in patient 15 and 16 ($P<0.0001$), but not in patient 18 (Figure 3C). The frequency of *IGHV4-34* usage was significantly higher in patient 16 ($p<0.0001$) and patient 18 ($p<0.0001$) (Figure 3D). From the three patients we analyzed, patient 18 had autoimmunity, which was reflected by the high frequency *IGHV4-34* usage.

Besides selection against long CDR3s, B-cell tolerance is also generated by receptor editing of self-reactive B cells. These self-reactive B cells are induced to express the RAG proteins and edit their receptor light chains via available upstream *Vκ* and downstream *Jκ* genes, to change the affinity of their receptors. Therefore, the *Vκ-Jκ* junctions were amplified from five OS patients and four patients with CID. The *IGKV* gene usage was not significantly different from controls (Figure 3E), but less *IGKJ5* genes were used in the RAGD patients (Figure 3F) So, receptor editing seems partly affected, as deduced from the very low *IGKJ5* usage.

Difference between clinical phenotypes in absolute number of T cells but not in T cell repertoire

The hallmark of classical OS is an expansion of autologous T cells with a HLA DR+CD45 RO+ phenotypes and an oligoclonal αβ-T-cell repertoire.[40] Consistent with this, most of

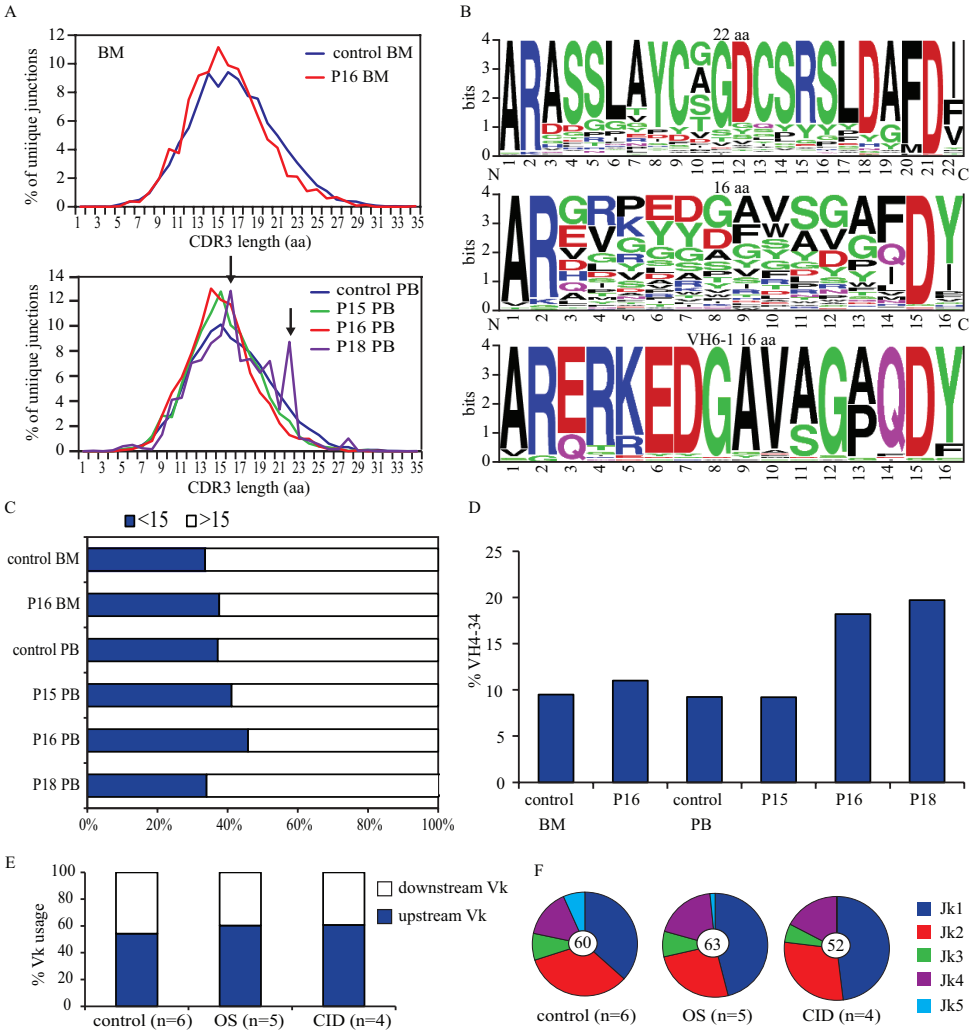

Figure 3. Functional characteristics of IGH junctions. Functional characteristics of the IGH junctions were determined in three RAGD patients in PB or BM. Distribution of CDR3 length frequencies in BM and PB was similar in control and RAGD patients (A), however P18 had increased numbers of junctions with a CDR3 length of 16 and 23 amino acids (aa). Sequence logo showed no similarity of the 16aa CDR3s of P18, but high similarity of CDR3s of 16 aa using the IGHV6-1 gene and of the 22aa CDR3s (B). The frequency of long CDR3s (≥15aa) was decreased in P15 and P16 (C). The IGHV4-34 usage was increased in P16 and P18 (D). The percentage of IGKV and IGKJ genes was determined in six controls, five OS patients, and four patient with CID. The IGKV usage was normal (E), but hardly and IGKJ5 gene was used (F).

the OS patients had normal or elevated CD3+ T-cell numbers, in addition two CID patients had normal numbers (P17 and P22), while all other patients had low absolute numbers of CD3+ T cells (Table II). Remarkably many patients had high percentage (>14%) of γδ T-cells, including 2 "classical" SCID, 1 OS and 8 CID patients (Table II). In addition, we determined the T-cell proliferation by determining the δREC-φJα T-cell receptor excision circles (TREC)[41] content per 50 ng DNA in three "classical" SCID patients, seven OS patients, and five CID patients. In 11 patients, TRECs were not detectable and in the other four patients (P2, P5, P7 and P18) the number of TRECs/50 ng DNA was lower than 1, compared to 134±75 TREC/50ng DNA in control (n=7 age 8m-11yr) (data not shown), meaning that the T cells that were present in these patients showed extensive proliferation. Furthermore, the T-cell repertoire was determined by testing the *TRB* gene rearrangements in two "classical" SCID patients, three OS patients, and two CID patients. In all patients, the TRB repertoire was clearly restricted (Figure 4). Taken together, the T cells that were present in the RAGD patients showed extensive proliferation and had a restricted TR repertoire.

DISCUSSION

Many different *RAG1* mutations have been reported to the *RAG* mutation database.[42] While most are null mutations, several have been described to result in residual recombinase activity.[11, 13, 25, 27, 43] Previously, it was hypothesized that null mutations in *RAG1* would result in "classical" T-B-SCID and partial reduction of RAG activity would result in OS or an intermediate late-onset SCID or OS phenotype.[28] Over the last few years the spectrum of reported clinical phenotypes of RAGD has broadened and now also includes RAGD with γδ T-cell expansion, RAGD with skin inflammation but without T-cell expansion (incomplete OS), RAGD with granulomas, RAGD with maternofetal transfusion, and RAGD with CD4 cytopenia and thymus hypoplasia.[10, 40] A few case reports have shown that the same *RAG* mutation can result in a different clinical phenotype.[25, 29, 44, 45] This study is the first to report an in depth immunobiological evaluation of 22 RAGD patients with similar *RAG1* mutations resulting in the same N-terminal truncation of the RAG1 protein. These similar mutations result in three different clinical phenotypes, which indicates that a specific mutation does not predict the clinical phenotype of a patient.

Since all patients had similar mutations, the residual RAG1 protein activity was expected to be comparable between all patients. The N-terminally truncated RAG1 protein is produced through translation starting from an alternative start site (M183 or M202), hence the amount of protein is dependent on how efficient these start sites are used. Since the *RAG1* transcription level correlated with those of *RAG2* we assume that all patients had similar expression of the mutant RAG1 protein (Figure 1C). We cannot exclude that epigenetics

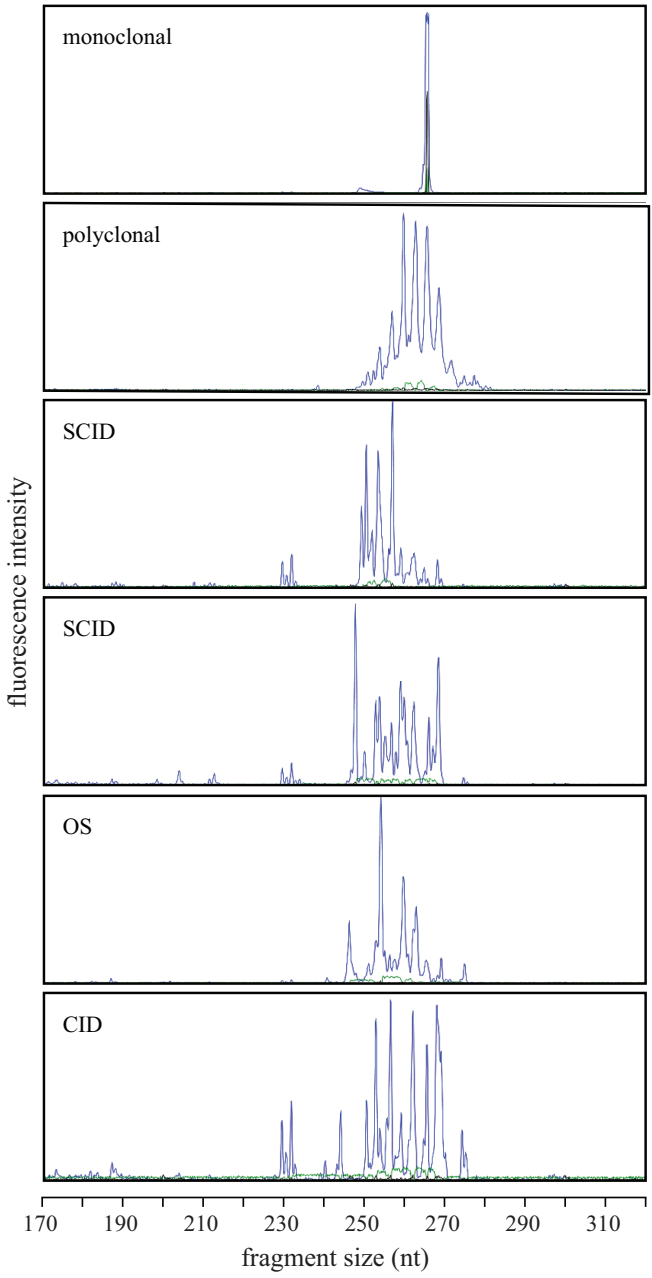

Figure 4. TRB repertoire. TR spectratyping profiles of *TRB* gene rearrangements using the BIOMED-2 TRB tube B. The upper two panels show the monoclonal and the polyclonal control. The patient panels SCID, OS, and CID show a restricted TRB repertoire.

and modifier genes accounted for small differences in RAG1 protein expression. Although a previous attempt to identify such modifier genes in humans was not successful,[46] studies in mouse models could shed more light on the contribution of epigenetics and modifier genes.

In our cohort, V(D)J recombination was not completely abolished but was strongly reduced, due to the low residual activity of the RAG1 protein. Reduced V(D)J recombination was characterized by normal *IGHV*, *IGHD* and *IGHJ* gene usage, without preferential use of proximal or distal genes. However, as was shown before,[31] the frequency of unproductive sequences was significantly lower than in healthy controls, indicating that the B cells in the RAGD patients failed to correct unproductive rearrangements by recombination of the second *IGH* allele.

As a consequence of the reduced V(D)J recombination fewer B and T cells with a functional receptor can be produced. To compensate for the low circulating B and T cells, the proliferation of the lymphocytes is increased. This idea is corroborated by the low numbers of TRECs in the RAGD patients. The increased proliferation of T cells might result in normal or elevated T-cells counts, especially in the OS patients, however the corresponding TR repertoire in all the RAGD patients remains restricted.

Most RAGD patients showed clinical signs of immune dysregulation, such as erythroderma, lymphadenopathy, hepato-splenomegaly, idiopathic thrombocytopenic purpura and autoimmune hemolytic anemia. B cells have been shown to contribute to the immune dysregulation in the *Rag* mouse models.[36, 37] Sera from these mice contained high-affinity anti-dsDNA and tissue-specific autoantibodies, and B cells displayed impaired receptor editing. In addition, these mice had increased serum B cell-activating factor (BAFF), which might rescue autoreactive B-cell clones. This increase in serum BAFF levels was also seen in patients with RAG-, Artemis- and X-linked SCID.[37] Similarly to observations in mice, most RAGD patients did not use the *IGKJ5* gene while the *IGKV* gene usage was normal. This suggests that receptor editing in this group of RAGD patients was slightly impaired, which can either be caused by reduced recombination activity due to the *RAG1* mutation, or by the low B numbers leading to reduced selection against autoreactive B cells. The *IGH* repertoire was investigated for long CDR3s, and increased *IGHV4-34* usage, which are associated with autoreactive antibodies.[47, 48] From the three RAG patients we analyzed, only patient 18 suffered from autoimmunity, which was reflected by and increased *VH4-34* gene usage.

The patients divided into the three main clinical RAGD groups hardly differed in their immunobiological parameters and consequently, we could not find any specific pattern that could explain the different clinical phenotypes. Based on our results and earlier reported data we propose an explanatory model for the development of different clinical phenotypes in RAGD patients with similar mutations (Figure 5). If RAGD results in reduced V(D)J recombination, low B- and T-cell numbers are produced with some (compensatory)

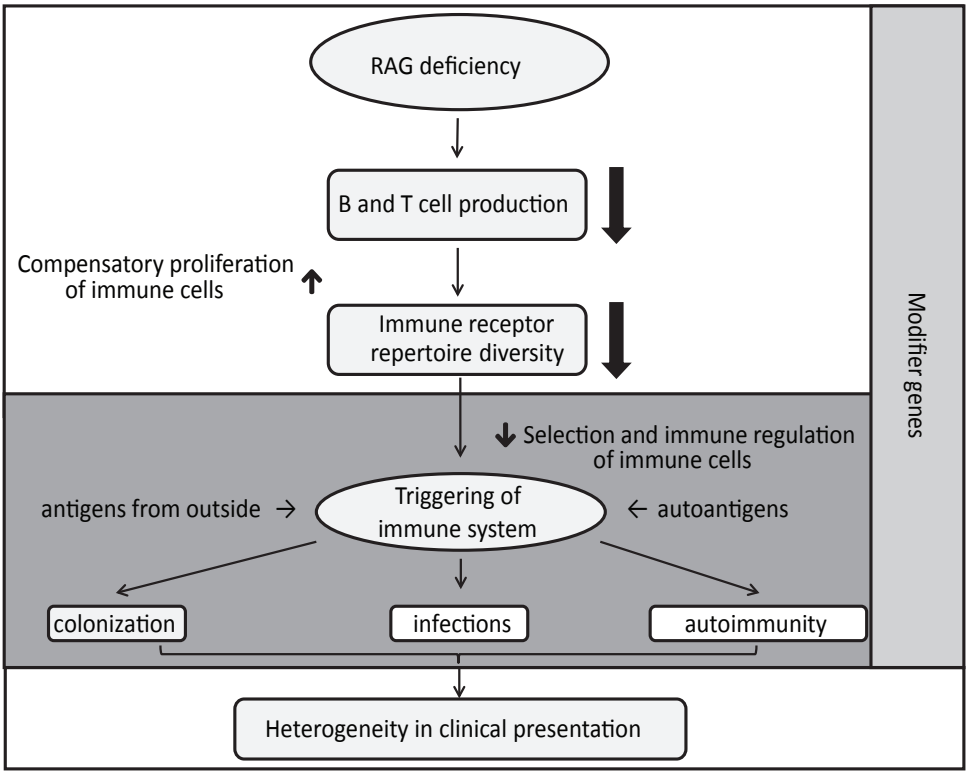

Figure 5. Model for development of clinical phenotype in RAG deficiency. RAG deficiency results in reduced V(D)J recombination leading to fewer B and T cells with a limited repertoire. In an attempt to compensate for the low numbers the B and T cells start to proliferate, but the repertoire remains limited and imbalanced, so that the selection and immune regulation are impaired. Most likely the type of antigenic stimulation together with the incomplete and imbalanced repertoire that has been developed will impact on the eventual clinical phenotype with immune dysregulation problems.

clonal expansion. This expansion might increase the B- and T-cell numbers to even normal levels, but does not change the limited repertoire. In such limited repertoire the selection against auto-reactive cells is impaired. Provided the deficient immune system is not activated, RAGD patients are asymptomatic. However, the inevitably moment that the immune system will be activated by potentially a wide range of different (auto)antigens, the type of antigen and activated effector lymphocyte will have important consequences for the clinical phenotype. In addition, the impaired negative and positive selection of thymic lymphocytes and a reduced number of regulatory T cells might result in autoimmunity when

the patients are exposed to auto-antigens. This phenomenon may occur at any early stage, even *in utero*, illustrated by the fact that patients with OS may have severe erythroderma already at birth, which is unlikely to be triggered by infections. Additionally, directly after birth the skin and gastrointestinal tract become colonized by commensal bacteria, which may trigger chronic diarrhea seen in most RAGD patients. Key steps in the development of a certain clinical phenotype will be the B- and T-cell repertoire, the type of (auto)antigen exposure, the specificity of the antigen receptors and the timing, the cell type involved in the immune activation and the potential influence of genetic variations in modifier genes. Variability in any of these factors might eventually lead to different clinical phenotypes despite similar genetic defect.

In conclusion, this study clearly shows that the type of *RAG1* mutation and the level of residual RAG1 recombinase activity are not the only determinants predicting the clinical phenotype, as previously assumed. The clinical outcome of an individual RAGD patient, depends on a complex interplay between the (limited) immune receptor repertoire, (auto) antigen exposure, the specificity of antigen receptors and the timing and cell type involved in the immune activation. Therefore, the clinical outcome of RAGD patients with similar mutations is extremely difficult to predict.

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Chapter 3.3

Artemis splice defects cause atypical SCID and can be restored *in vitro* by an antisense oligonucleotide

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ABSTRACT

Artemis deficiency is known to result in classical T-B- severe combined in immunodeficiency (SCID) in case of *Artemis* null mutations or Omenn's syndrome in case of hypomorphic mutations in the *Artemis* gene. We describe two unrelated patients with a relatively mild clinical T-B-SCID phenotype, caused by different homozygous *Artemis* splice-site mutations. The splice-site mutations concern either dysfunction of a 5' splice-site or an intronic point mutation creating a novel 3' splice-site, resulting in mutated Artemis protein with residual activity or low levels of wild type *Artemis* transcripts. During the first 10 years of life the patients suffered from recurrent infections necessitating antibiotic prophylaxis and intravenous immunoglobulins. Both mutations resulted in increased ionizing radiation sensitivity and insufficient V(D)J recombination, causing B-lymphopenia and exhaustion of the naïve T-cell compartment. The patient with the novel 3' splice-site had progressive granulomatous skin lesions, which disappeared after stem cell transplantation (SCT). We showed that an alternative approach to SCT can in principle be used in this case: an antisense oligonucleotide (AON) covering the intronic mutation restored wild type *Artemis* transcript levels and NHEJ activity in the patient fibroblasts.

INTRODUCTION

Severe combined immunodeficiency (SCID) is an inherited primary immunodeficiency. Most SCID patients suffer within months after birth from severe opportunistic infections, chronic diarrhea, and failure to thrive. Antimicrobial prophylaxis and immunoglobulin substitution are mandatory in clinical management but curative treatment can only be obtained by allogeneic stem cell transplantation (SCT) and, in an experimental setting, gene therapy.[1-3] Immunologically, SCID is characterized by absence or dysfunction of T-lymphocytes. SCID patients can be divided into two main categories: those with T-B+ SCID (70%), generally resulting from a T cell signaling defect, and those with T-B- SCID (30%), mostly due to a defect in recombination of the variable (V), diversity (D) and joining (J) gene segments.[4, 5]

Differentiation of lymphoid precursors to mature B- and T-lymphocytes requires the rearrangement and expression of genes encoding the immunoglobulins (Ig) or T cell receptors. V(D)J gene segments are recombined to form a functional V(D)J exon. V(D)J recombination is initiated by the lymphoid specific recombination activating gene 1 (RAG1) and 2 (RAG2) proteins. These proteins introduce double strand breaks (DSBs) in the DNA near the recombination signal sequences (RSSs) that flank the V, D and J segments.[6, 7] Subsequently, the DNA DSBs are repaired by the non-homologous end-joining pathway (NHEJ). The DNA DSBs are recognized by the DNA-dependent protein kinase (DNA-PK) complex, which is composed of the DNA-PK catalytic subunit (DNA-PKcs) and the KU70/KU80 heterodimer that directly binds to the DNA ends.[8] Subsequently, the Artemis protein is phosphorylated by DNA-PKcs, and opens the hairpin-sealed coding ends.[9, 10] The coding ends are then further processed by inclusion of palindromic (P) nucleotides due to asymmetric hairpin opening, loss of nucleotides due to exonuclease activity, and addition of non-templated (N) nucleotides by terminal deoxynucleotidyl transferase (TdT).[11] In the final step, the coding ends are ligated by the DNA ligase IV (LIG4)/XRCC4 complex in conjunction with XLF(Cernunnos).[12, 13]

In approximately 70% of T-B-SCID patients mutations are found in the *RAG1* and *RAG2* genes.[14] The majority of the remaining patients show hypersensitivity for ionizing radiation (IR), suggesting a defect in the NHEJ pathway of DNA DSB repair.[15] Mutations in the *Artemis*, *LIG4* and *DNA-PKcs* genes have been identified in these patients.[14, 16-19] Furthermore, mutations in the *XLF* gene have been found

in radiosensitive patients with growth retardation, microcephaly, and immunodeficiency due to profound T- and B-cell lymphocytopenia.[13]

Not all mutations in V(D)J recombination genes give rise to the classical SCID phenotype. Hypomorphic mutations in the *RAG1*, *RAG2* and *Artemis* genes can result in the Omenn's syndrome (OS).[20, 21] Similar to SCID patients, OS patients present in infancy with viral or fungal pneumonitis, chronic diarrhea and failure to thrive. Unlike classical SCID, patients with OS have severe erythroderma, increased IgE levels and eosinophilia.[22, 23] Hypomorphic *RAG1* and *RAG2* mutations have also been reported to cause primary immunodeficiency disease with granulomatous skin lesions.[24] Here we present two SCID patients with different types of *Artemis* splicing defects, both leading to an atypical Artemis-SCID phenotype, characterized by a later onset and milder disease course and in one patient severe localized granulomatous skin lesions.

RESULTS

Case report patient 1

Patient 1 (ID189) is the second daughter of healthy, consanguineous, parents of Turkish descent. She presented at the age of five with two episodes of pneumonia, of which the last one was caused by *Influenza A*. Adenotomy was performed due to frequent upper respiratory tract infections. She experienced chickenpox during infancy with a normal clinical course. The patient was generally in a good clinical condition, with a good psychomotor development and normal growth. The family history revealed one male cousin who had died in Turkey at the age of 15 years from a hematological malignancy. He had been diagnosed with common variable immunodeficiency, suffered from recurrent infections and had been on intravenous immunoglobulin (IVIG) replacement therapy. Another female cousin had been on IVIG therapy for some years as a child, but had apparently grown up to be a healthy adult. Physical examinations showed a healthy girl with no dysmorphic features, no lymphadenopathy or splenomegaly, remarkably small tonsils and diffuse pulmonary wheezing and rhonchus. Antibody responses to pneumococcal polysaccharides and tetanus vaccinations were normal. PCR for Epstein Barr virus (EBV) was positive with incomplete seroconversion (EBV-VCA IgM neg; EBV-VCA-IgG pos; anti-EBNA neg). The antibody titers after previous vaccination against mumps, measles and rubella were in the normal range, IgM anti-Varicella was negative, IgG was positive. Antibodies against CMV and PCR for CMV sequences were

negative. A high resolution computed tomography scan showed minimal brochiectasis with a normal pulmonary function test. On co-trimoxazole prophylaxis, the next two years were uneventful, although signs of mild chronic pulmonary inflammation persisted. Because of an increase in respiratory complaints eventually IVIG was started, which led to a marked clinical improvement. However, at the age of 9 years, she suffered again from frequent respiratory tract infections and increased exercise intolerance. Laboratory tests showed a progressive decrease of B cells and naïve T lymphocytes. SCT was performed at 11 years of age using a matched-unrelated donor and a conditioning regimen containing busulfan, fludarabin and alemtuzumab.

Case report patient 2

This female patient (ID153) of Turkish descent, born from consanguineous parents, presented at the age of 4 years with a history of recurrent upper and lower respiratory tract infections requiring treatment with oral antibiotics. Two years later she developed a severe hypogammaglobulinemia and IVIG substitution was initiated. After start of IVIG, infection episodes became scarce and mild. Notably, although a primary EBV infection was experienced without clinical manifestations, recurrent EBV reactivations, measured by plasma EBV-DNA specific quantitative PCR, could be documented from the age of 7 years onwards. In addition, the patient had an uncomplicated course of chickenpox before the age of 4 years and spontaneously recovered from *Influenza A* infection at the age of 9 years. Specific antibodies against Varicella Zoster Virus and *H. influenzae* (after vaccination in infancy) could be detected. At the age of 5 years she developed skin lesions on the back of her left hand, which steadily progressed into multiple isolated and subsequently confluent lesions on the left hand and lower arm (Figure 1). Histological analyses showed granulomatous inflammation (reported before [25]). Despite extensive and repetitive pathological, microbiological and molecular evaluations, no mycobacterial or fungal pathogens or other infectious agents could be detected. Furthermore, multiple episodes of empirical treatment with tuberculostatic agents or intralesional corticosteroid injections did not affect the progressive behaviour of the lesions. Based on the T- and B-lymphopenia together with the progressive granulomatous skin lesions, the decision was made to treat the patient with allogeneic SCT. After conditioning with busulfan, cyclophosphamide and ATG (thymoglobulin), a 6/6 allele matched unrelated cord blood was infused at the patient's age of 10 years. SCT was considered successful as all blood myeloid and lymphoid cells were from donor origin. Functional immune reconstitution was observed, documented by recovery of naïve T cells and normal B cell

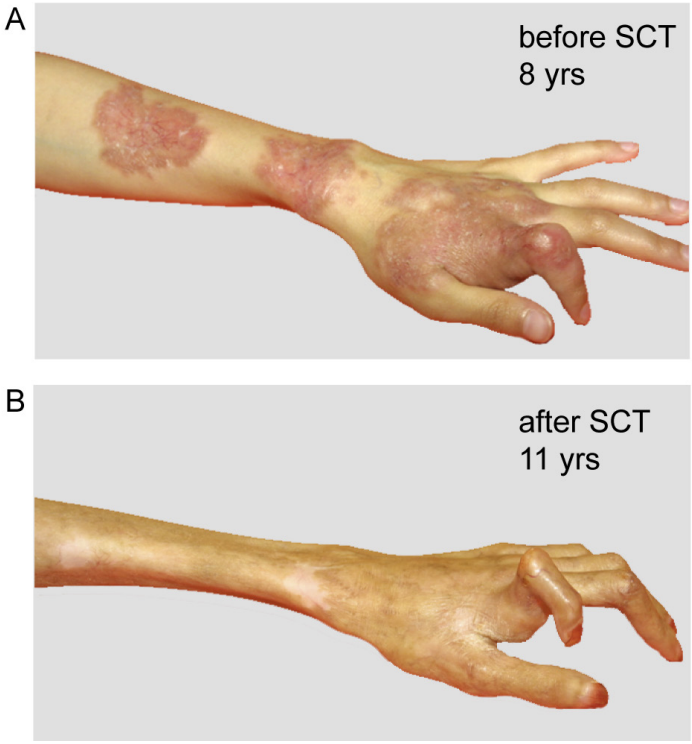


Figure 1. Granulomatous skin lesions. The left lower arm of patient 2 showing granulomatous skin lesions before SCT at the age of 8 years (A) and the regression of the granulomatous skin lesions 1 year after SCT at the age of 11 years (B).

counts, clearance of reactivated EBV, CMV and adenovirus, and regression of the granulomatous skin lesions (Figure 1).

Immunological characteristics

Immunological evaluation in patients 1 and 2 revealed decreased numbers of T cells and B cells, but normal levels of NK cells (Table 1). Within the T cell subsets the numbers of memory and activated T cells were normal, while the naïve (CD45RA+) T cells were low. Apparently, the history of both patients regarding vaccination responses and dealing with viral infections indicates that the T cells present are able to support humoral immune responses and to perform cellular immune functions. The serum immunoglobulin levels in patient 1 were normal, except for the decrease in IgG2 and IgG4 levels (Table 1). In patient 2 the immunoglobulin levels were normal at 4 years of age, but two years later she developed a severe

Table 1. Lymphocyte subsets and immunoglobulin (sub)classes in peripheral blood of patients 1 and 2.

Leukocyte subsets (10^9/L)	phenotype	patient 1 10 yrs	patient 2 8 yrs	healthy controls (5-10yrs) [53]
Lymphocytes		0.6	1.1	1.1-5.9
T cells	CD3+	0.3	0.66	0.7-4.2
	Total CD4+	0.14	0.15	0.3-2.0
	CD4+CD45RA+	0.02	0.01	0.3-1.2
	CD4+CD45RO+	0.12	0.14	0.2-0.6
	Total CD8+	0.15	0.28	0.3-1.8
	CD8+CD45RA+	0.07	0.12	0.2-0.8
	CD8+CD45RO+	0.12	0.18	0.04-0.3
	CD3+TCRγδ+	0.02	0.34	< 0.2
B cells	CD19+	0.02	0.10	0.2-1.6
NK cells	CD16+CD56+	0.09	0.21	0.09-0.9
Immunoglobulin (sub) classes (g/L)		**patient 1 5 yrs**	**patient 2 6 yrs**	**healthy controls (2-7 yrs) [54]**
	IgG1	8.0	2.11	3.5-10.0
	IgG2	0.38	0.44	0.6-3.5
	IgG3	0.31	0.04	0.14-1.3
	IgG4	<0.01	<0.05	<0.3-1.2
	IgM	1.34	0.25	0.5-1.8
	IgA	0.72	0.08	0.1-1.6

hypogammaglobulinemia (Table 1). We analyzed if the B cells could undergo somatic hypermutation (SHM) for affinity maturation of the antigen receptor, by determining the mutation frequency in IgG and IgA transcripts. The overall mutation frequency in patient 1 at 5 years of age was normal, while in patient 2 at 8 years of age the mutation frequency was significantly lower than in age-matched healthy controls (Figure 2). In both patients, the pattern of SHM was suggestive for the potential to produce an antigen-selected B-cell receptor. Despite the low numbers of B and T cells, the infectious episodes in the patients were limited, especially after initiation of IVIG, indicating that the immune system is only partially impaired.

Precursor B-cell differentiation block and increased ionizing radiation sensitivity

Both patients showed a combined immunodeficiency with reduced numbers of peripheral B lymphocytes and naïve T lymphocytes, which is suggestive for a general differentiation defect of cells belonging to the adaptive immune system. Therefore, precursor B-cell differentiation was studied in bone marrow (BM) samples from both patients by assessing the relative distribution of pro-B, pre-B-I, pre-B-II and immature B cells. In healthy children, pro-B and pre-B-I cells constitute

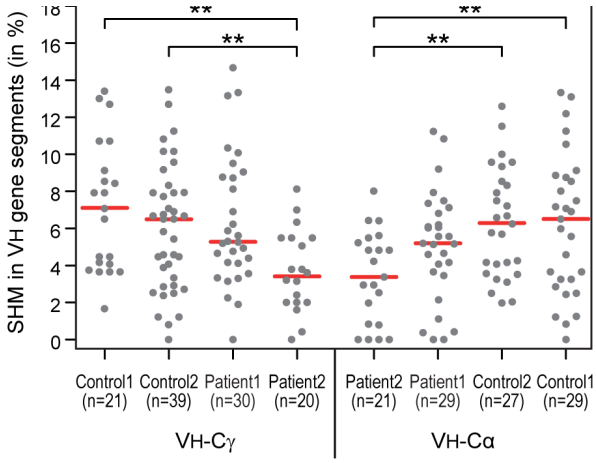


Figure 2. Analysis of somatic hypermutations. Frequencies of Somatic Hypermutations (SHM) in the VH-Cg and the VH-Ca transcripts were normal in patient 1 and significantly reduced in patient 2 compared to two age-matched controls. ** denotes $p<0.01$.

20-25% of the precursor B cells (Figure 3A). Patients with a complete V(D)J recombination defect caused by mutations in *RAG1/RAG2* or *Artemis* only have pro-B and pre-B-I cells and completely lack pre-B-II and immature B cells (Figure 3A). In both patients, the pro-B and pre-B-I cell fractions represented approximately 85% of the precursor B-cells. Analysis of a second bone marrow sample of patient 2 two years later showed an identical pattern, which indicates that the composition of the precursor B cell compartment was stable over time. This composition points towards an incomplete block in precursor B-cell differentiation before the cytoplasmic Igm+ pre-B-II cell stage (Figure 3A), which is characteristic for an incomplete V(D)J recombination defect. Sequence analysis of *RAG1* and *RAG2* revealed no mutations. Subsequently, a clonogenic survival assay was performed to determine whether the patients' fibroblasts, cultured from a skin biopsy were radiosensitive. Fibroblasts from both patients showed increased sensitivity to ionizing radiation (Figure 3B). In contrast PBMNC of patient 2 did not have increased numbers of karyotype abnormalities after low doses of ionizing radiation. Therefore, DSB repair was studied in more detail in patient 2 by counting γ-H2AX foci, a DSB marker, at various time points after irradiation. The γ-H2AX foci disappeared with delayed kinetics in patient 2 and Artemis-deficient fibroblasts (Figure 3C). This resulted in 15% residual foci after 72 hours, suggesting that a comparable level of

unrepairable DSBs remained in patient 2 fibroblasts and in Artemis-deficient cells. These observations were indicative for a defect in DSB repair by NHEJ.

Hypomorphic mutations in Artemis

Artemis is the first candidate gene for NHEJ defects. Therefore, the coding exons and splice-sites of the *Artemis* gene were sequenced. Patient 1 had a homozygous mutation in the 5' splice-site of exon 6 (c.464+1G>A) (Figure 4A). The same mutation was recently described in a patient with an atypical Artemis deficiency with chronic inflammatory bowel disease.[26] This splice-site mutation leads to

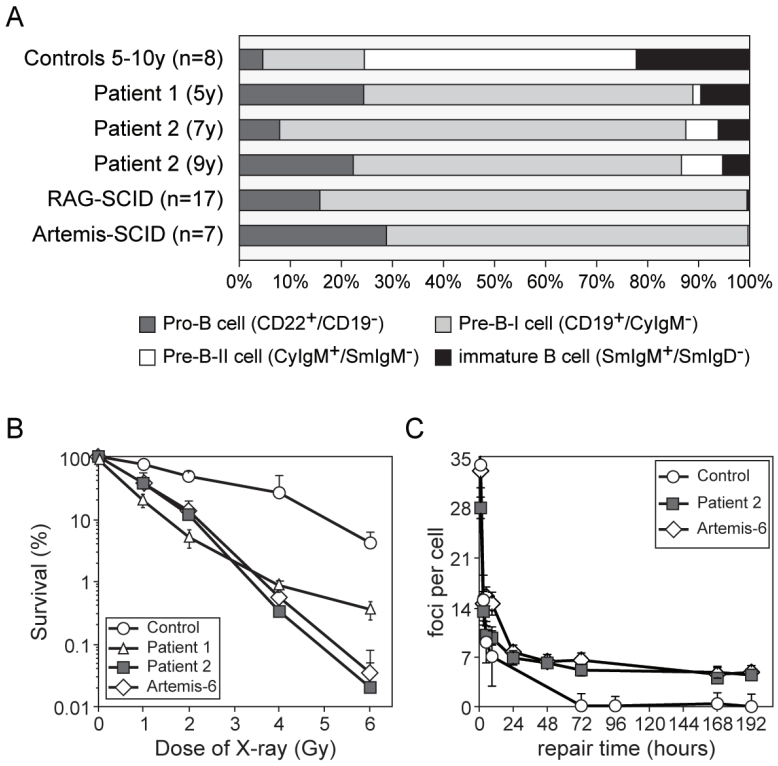
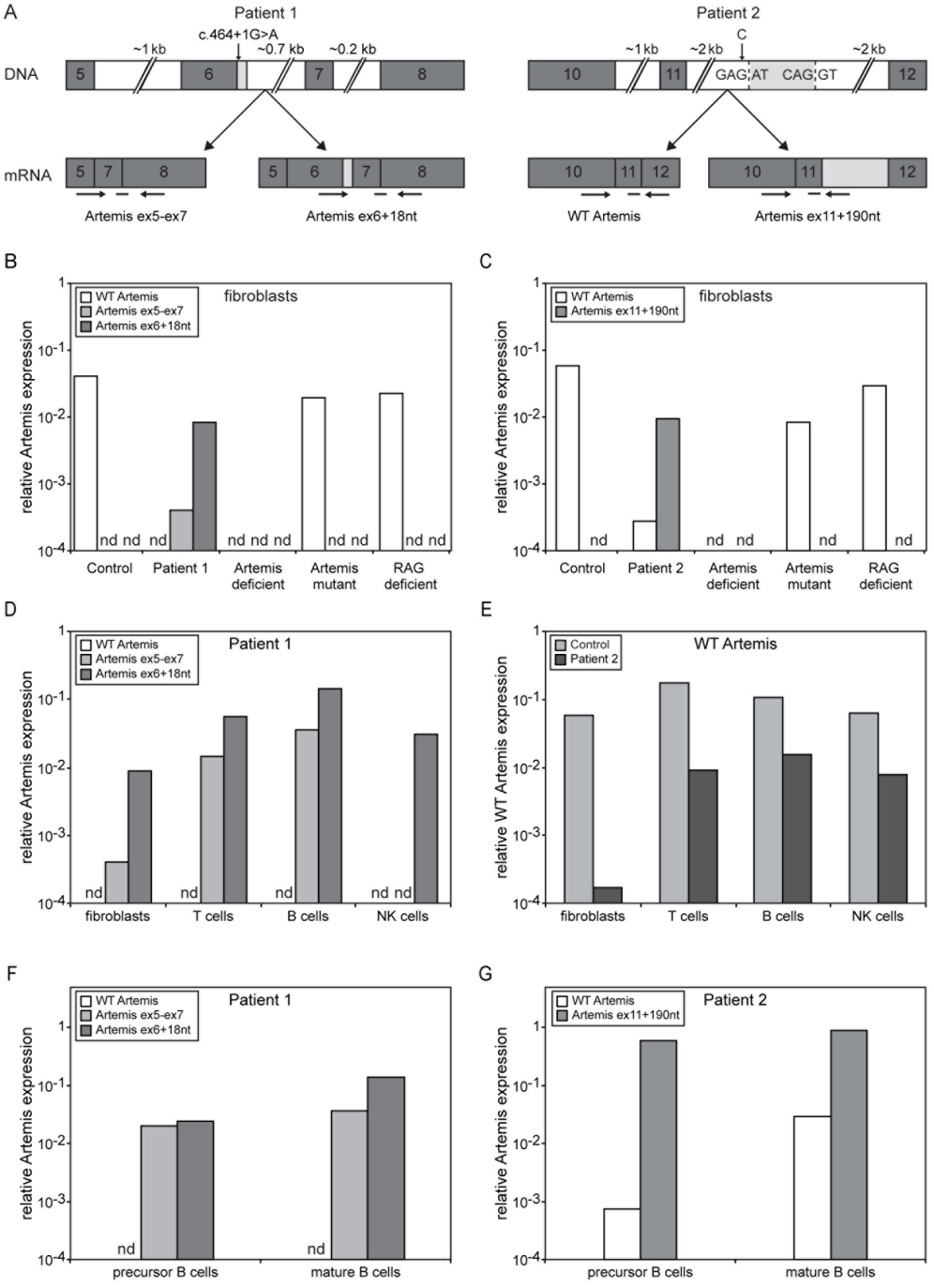


Figure 3. Composition precursor B-cell compartment in bone marrow and DNA DSB repair characteristics. Composition of the precursor B-cell compartment in healthy controls (n=8), patient 1 (5 years) and patient 2 (7 and 9 years), RAG-deficient (n=17) and Artemis-deficient (n=7) patients. Patients 1 and 2 had an incomplete block in precursor B-cell differentiation (A). Clonogenic survival assay showed increased sensitivity to ionizing radiation of fibroblasts of patient 1 and 2 similar as the Artemis-deficient patient. (B). The numbers of γ-H2AX foci per nucleus (average of 40 cells) after radiation with 1 Gy disappeared with delayed kinetics in patient 2 and Artemis-deficient (Artemis-6).[27] fibroblasts compared to control (VH10) fibroblasts (C). Error bars represent the SD from 3 independent experiments.


A
Patient 1
c.464+1G>A
~1 kb
~0.7 kb
~0.2 kb
DNA
5
6
7
8
mRNA
Artemis ex5-ex7
Artemis ex6+18nt
Patient 2
C
~1 kb
~2 kb
~2 kb
GAG;AT CAG;GT
10
11
12
WT Artemis
Artemis ex11+190nt
B
fibroblasts
WT Artemis
Artemis ex5-ex7
Artemis ex6+18nt
relative Artemis expression
Control
Patient 1
Artemis deficient
Artemis mutant
RAG deficient
C
fibroblasts
WT Artemis
Artemis ex11+190nt
Patient 2
D
Patient 1
fibroblasts
T cells
B cells
NK cells
E
WT Artemis
Control
Patient 2
relative WT Artemis expression
F
Patient 1
precursor B cells
mature B cells
G
Patient 2

alternative splicing yielding two aberrant *Artemis* transcripts. In the first transcript (Artemis ex5-ex7) exon 6 is skipped, leading to an in-frame deletion of 34 amino acids of the β–Lact domain (p.M121_R155delinsI). The β–Lact domain is essential for Artemis activity since mutants, lacking parts of the β–Lact domain, could not complement the Artemis defect.[27] In the second transcript (Artemis ex6+18nt) a cryptic 5' splice-site 18 nucleotides downstream of exon 6 was used resulting in the in-frame insertion of six amino acids (p.R155_V156insYWGSYR) (Figure 4A). The 6 amino acids are inserted exactly between the β–Lact and the β–CASP domains, leaving the β–Lact domain intact. Rohr *et al*. showed that the Artemis protein carrying this insertion of 6 amino acids retained residual activity [26]

In patient 2, no mutations were found in the coding exons and splice-sites of the *Artemis* gene. However, polymorphic short tandem repeat markers up- and downstream of the *Artemis* gene showed that this locus was homozygous in the patient. Since the patient's parents are consanguineous, *Artemis* was still considered a candidate gene. Therefore *Artemis* transcripts were sequenced. This revealed a 190-bp insertion (cryptic exon) between exons 11 and 12. Genomic sequence alignment showed that the inserted cryptic exon was part of *Artemis* intron 11, located ~2.0 kb downstream of exon 11 (Figure 4A). Sequence analysis of this part of the intron and the flanking regions in genomic DNA revealed a homozygous nucleotide substitution (c.972+1997G>C), which introduced a new 3' splice-site consensus sequence. Besides this newly formed 3' splice-site a pre-existing cryptic 5' splice-site was used. At the protein level, a stretch of 61 amino acids was inserted after glutamate 324 followed by a stop codon (p.Glu324ins61X). Interestingly, the

Figure 4. *Artemis* mutations and expression of WT and patient-specific *Artemis* transcripts. Schematic representation of the positions of the splice-site mutations in the *Artemis* gene and the WT and patient-specific transcripts that are present in patient 1 (left panel) and patient 2 (right panel). The relative *Artemis* transcript expression was measured by real-time quantitative PCR (RQ-PCR), locations of the primers used in the RQ-PCRs are indicated. For primer and probe sequences see Table 2 (A). Relative *Artemis* expression measured in fibroblasts of control (C5RO), patient 1 or 2, an Artemis-deficient patient (Artemis-5) [27] lacking exon 1 till 3, a patient expressing mutant Artemis (Artemis-8) [27] and a RAG-deficient patient (RAG-SCID-12). WT *Artemis* transcripts were not expressed in fibroblasts of patient 1. For detection of WT *Artemis* transcripts in patient 1 a forward primer overlapping the exon 6-7 border (Artemis ex6ex7 F) in combination with a reverse primer in exon 8 (Artemis ex8R) and the probe TR Artemis ex7ex8 (B). WT *Artemis* transcripts were expressed at low level in fibroblasts of patient 2 (C). T, B and NK cells from patient 1 only expressed patient-specific *Artemis* transcripts (D). In patient 2 the WT *Artemis* transcripts expression was higher in T, B and NK cells compared to fibroblasts (E). The expression levels of the patient-specific *Artemis* transcripts were slightly higher in mature B cells (CD19+IgD+IgM+) compared to precursor B cells (CD19+IgD-) in patient 1 (F), but in patient 2 the WT *Artemis* expression in mature B cells was much higher compared to precursor B cells.

cryptic 190bp exon corresponds to the right arm of an *Alu* element in the antisense direction (*Alu* consensus position 90-279 [28]) and the cryptic 5' splice-site corresponds with *Alu* consensus position 89. Besides the aberrant *Artemis* transcripts, correctly spliced wild type (WT) *Artemis* transcripts were present.

Differential expression of Artemis transcripts in fibroblasts, precursor B cells and mature B cells

To determine the levels of WT and patient-specific aberrant transcripts in different cell types, real time quantitative (RQ) PCR was performed. The location and sequences of the primers and probes used to measure the different transcripts are indicated in Figure 4A and Table 2. Fibroblasts of patient 1 only expressed patient-specific alternative *Artemis* transcripts, but no WT *Artemis* transcripts (Figure 4B). None of the patient-specific *Artemis* transcripts were expressed in control fibroblasts or in fibroblasts of Artemis-deficient or RAG-deficient SCID patients. Fibroblasts of patient 2 expressed both patient-specific *Artemis* transcripts (Artemis ex11+190nt) and WT *Artemis* transcripts, although WT expression was much lower (>300 fold decrease) than in control fibroblasts (Figure 4C). The respective absence or strong decrease in WT *Artemis* transcripts in patient 1 and 2 explains the sensitivity to ionizing radiation of the fibroblasts. WT *Artemis* expression was also absent in sorted T, B and NK cells of patient 1 (Figure 4D).

Table 2. Sequences of primers and probes used for real time quantitative PCR.

primer name	primer sequences
Artemis exon 10 F	GGAGAAAGGAGCAGAAAAACAAA
Artemis exon 12R	TGGATATGCGTTCACAGGACA
Artemis patient 2 R	GGCAATAAAGCGAGACTCCAT
Artemis ex6ex7 F	ACTCCGGGGGCAGAGTCA
Patient 1 ex6nt18 F	ACTCCGGGGGCAGGTACT
Patient 2 ex5ex7 F	ACTGTCCGGGATCAGTTATAGTCA
Artemis ex8R	GCTTCGGACCAGCTCTAAGACT
probe name	**probe sequences**
TR Art exon 11	AGCTCTGTATGAACTCTCTCCAGTCCTCACAA
TR Artemis ex7ex8	ACACTCCTCCCGACTTGGAATTTGGTAAAA

Sequences are given in the 5' to 3' order.

The expression of WT *Artemis* in the sorted lymphocyte subsets of patient 2 was only 10-fold decreased compared to control subsets (Figure 4E). This decrease was much less than the >300 fold decrease of WT *Artemis* transcripts in fibroblasts, and could explain why the fibroblasts of patient 2 were sensitive for ionizing radiation while the PBMCs of patient 2 were not. This difference in WT *Artemis* expression could not be explained by reversion mutations in T, B and NK cells, since these were excluded by sequence analysis of sorted fractions (data not shown). The 10-fold decrease of WT *Artemis* in PBMCs in patient 2 was unexpected, since precursor B cells in bone marrow had a clear V(D)J recombination defect due to improper or insufficient NHEJ activity. Therefore, precursor B cells (pre-B-I, pre-B-II and immature B cells) and mature B cells were sorted from bone marrow to determine *Artemis* transcript levels. The expression level of WT *Artemis* transcripts in precursor B cells was approximately 40-fold lower than in mature B cells derived from patient 2 (Figure 4G). The expression levels of both mutant *Artemis* transcripts in precursor B cells in patient 1 were only slightly lower compared to mature B cells (Figure 4F). In summary, the difference in sensitivity to ionizing radiation between fibroblasts and PBMNC can be explained by the differential expression of *Artemis* transcripts in fibroblasts versus lymphocyte populations and differences in expression in B cell subsets of various differentiation stages.

Effect of the Artemis splice-site mutations on V(D)J recombination

The two patients had different types of *Artemis* splice-site mutations. Patient 1 had a homozygous splice-site mutation resulting in expression of mutant Artemis protein with residual activity, whereas patient 2 had a mutation causing severely reduced expression of WT *Artemis* transcripts. To understand the effects of the different hypomorphic *Artemis* mutations on its function, we studied Artemis-related processes during B-cell differentiation. Artemis is involved in V(D)J recombination, which takes place during precursor B-cell differentiation. Both mutations had effect on V(D)J recombination given the block in precursor B-cell differentiation in bone marrow (Figure 3A). Therefore, we studied V(D)J recombination in more detail by analyzing the coding joints of incomplete *IGH* gene rearrangements (i.e. DH-JH) in DNA isolated from BM mononuclear cells. Both Artemis- and DNA-PKcs-deficient patients have a defect in DNA hairpin opening and show increased P-nucleotides in DH-JH junctions (Table 3).[18, 19, 27] In coding joints of both patients the average number of palindromic (P-) nucleotides per junction was significantly increased to 1.2 in patient 1, and 2.0 in patient 2 compared to healthy controls (0.3 P-nucleotides per junction) (Table 3). These long stretches of P-nucleotide resulted in significantly less deletions compared to controls. The number of non-templated

Table 3. DH-JH junction characteristics of patients 1 and 2 compared with healthy controls, Artemis-deficient patients and a DNA-PKcs patient.

Patient (no. of clones)	DH(del)	P-nucleotides	N-nucleotides	P-nucleotides	(del)JH	total P-nucleotides	total del
Patient 1(25)	3.1	0.7	6.2	0.5	5.1	1.2**	8.2**
Patient 2 (27)	3.9	0.4	4.2	1.6	4.2	2.0***	8.1**
Control (91)	4.5	0.2	9.2	0.1	6.7	0.3	11.2
Artemis (53)[27]	1.9	3.0	4.0	3.8	1.1	6.8***	3.0***
DNA-PKcs (23)[19]	2.3	1.0	4.2	2.0	4.5	3.0***	6.8***

Values represent average numbers nucleotide per junctions. DH(del), average number of nucleotides deleted from the 3' end of the DH gene segment per coding joint given as a negative value; JH(del) average number of nucleotides deleted from the 5' end of the JH gene segment per coding joint given as a negative value; Total del, average of total number of deleted nucleotides per coding junction. Statistical analysis was performed using unpaired T-test. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

(N-) nucleotides was not significantly different from controls. These results show that mutant Artemis with residual activity in patient 1 or low levels of WT Artemis in patient 2 resulted in defective hairpin opening.

Effect of the Artemis mutations on CSR

In mature B cells, class switch recombination (CSR) allows previously rearranged Ig heavy-chain V domains to be expressed in association with a different constant (C) region, leading to production of different isotypes.[29] Artemis has recently been shown to be involved in CSR [23]. Switch (S) junctions resulting from *in vivo* (CSR) events, were cloned and sequenced from B cells of patient 1 and 2. Unique Sμ-Sα1 sequences, representing independent CSR events, were subsequently compared with Sμ and Sα junctions (n=154) from healthy adult controls.[30, 31] Previously described Artemis-deficient patients showed a strong dependence on long micro-homologies and a complete lack of "direct end-joining".[23] The average length of length of microhomology, defined as successive nucleotides that were shared by both the Sμ and Sα regions at the CSR junctions, was not significantly different from control in patient 1 (3.8± 5.2 bp vs. 1.8 ± 3.2 bp in controls) and in patient 2 (2.0 ± 2.7 bp vs. 1.8 ± 3.2 bp in controls). Furthermore, the pattern of Sμ-Sα junctions was indistinguishable from that in controls (Table 4). In summary, expression of mutated Artemis protein with residual enzymatic activity or reduced level of WT *Artemis* had no impact on CSR.

Table 4. Characterization of Sμ-Sα junctions[a].

	Perfectly matched short homology						Total No. of S fragments
	0bp		1-3bp	4-6bp	7-9bp	≥10bp	
	1-bp insertions	No insertions					
Patient 1	3 (17%)	2 (11%)	7 (39%)	3 (17%)	1 (6%)	2 (11%)	18
Patient 2	5 (22%)	7 (31%)	4 (17%)	6 (35%)	2 (9%)	0 (0%)	23
Artemis -/-	6 (11%)*	0 (0%)*	10 (19%)	8 (15%)	9 (17%)	21 (39%)***	54
Controls (1-6 yr)	34 (25%)	24 (18%)	25 (18%)	21 (15%)	11 (8%)	22 (16%)	137
Controls (adults)	39 (25%)	28 (18%)	56 (36%)	15 (10%)	11 (7%)	5 (3%)	154

a). The switch junctions from Artemis-deficient patients were compared with those from age matched controls (1-6 years of age), whereas the switch junctions from patient 1 and 2 were compared with adult controls. Statistically significant differences are bolded. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

In vitro restoration of the Artemis splice defect by antisense oligonucleotide (AON) exon skipping

The defect in patient 2 was caused by a rare intronic mutation that creates a new splice-site leading to exonisation of 190 nucleotides of intronic sequence. We attempted to correct this defect by skipping this cryptic exon. For this purpose, we used antisense oligonucleotides (AONs) to modulate splicing by hiding specific sites essential for exon inclusion from the splicing machinery, without modifying the genome.[32] The AONs used to modulate splicing are different from the oligonucleotides that are used to achieve down regulation of transcripts. The AONs should not activate RNase H, which would degrade the pre-mRNA, and should be able to compete with splicing factors for access to the pre-mRNA. We have applied an AON with 2'-O-methyl ribose groups and a full-length phosphorothioate backbone. This AON is RNase H inactive and has a higher affinity for the target sequence than the 2'-deoxy counterpart. For patient 2 an AON covering the newly formed 3' splice-site in intron 11 of the *Artemis* gene was designed (Figure 5A). Fibroblasts of patient 2 and a healthy control were transfected with a 5'- fluorescein labeled AON. After 24 and 48 hours WT and mutant (Artemis ex11+190nt) *Artemis* transcript levels were measured by RQ-PCR. The levels of WT *Artemis* transcripts in patient 2 were already restored to normal levels within 24 hours after transfection (Figure 5B). In addition, mutant *Artemis* expression was approximately 10-fold decreased. No effects of AON transfection were seen on *Artemis* expression levels in control fibroblasts. Moreover, in the clonogenic survival assay we demonstrated that AON treatment also partially suppressed the sensitivity to ionizing radiation

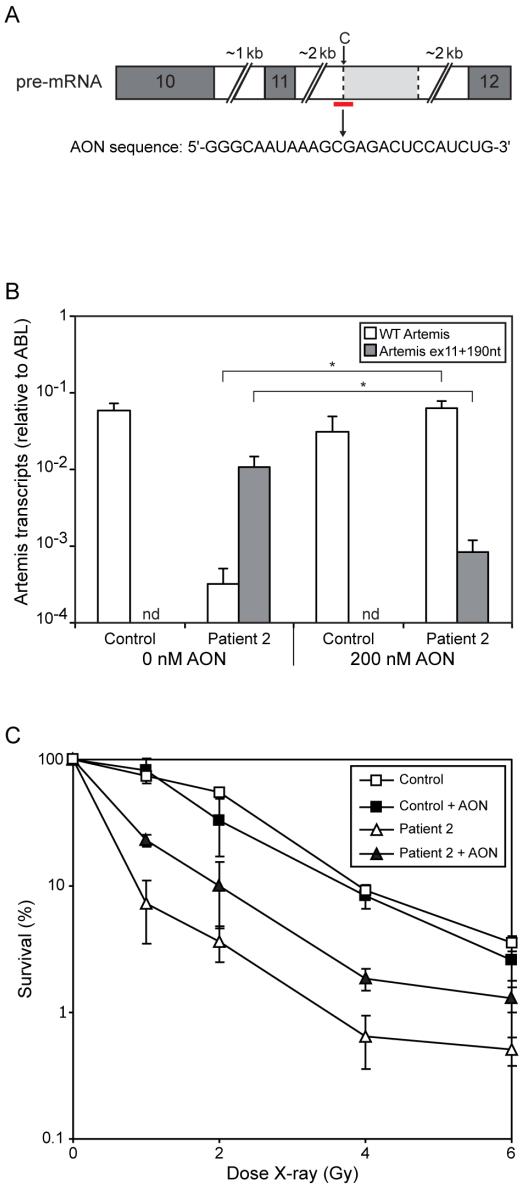


Figure 5. AON partially suppresses the radiation sensitivity in patient 2 fibroblasts. Location of the antisense oligonucleotide covering the G>C nucleotide substitution. The dotted lines represent the splice-sites of the cryptic exon (A). WT *Artemis* transcripts levels in fibroblasts from patient 2 significantly increased 24 hours after transfection with 200 nmol AON compared to transfection without AON, while the patient-specific *Artemis* transcripts significantly decreased. The WT *Artemis* transcript level did not change in control (C5RO) fibroblasts after tansfection with 200 nmol AON (B). Sensitivity for ionizing radiation was partially supressed in fibroblasts of patients 2 after transfection with 100 nmol AON compared to fibroblasts transfected with 0 nmol AON. Transfecting the control (C5RO) fibroblasts did not result in a different sensitivity for ionizing radiation. Error bars represent the SD from 3 independent experiments. * denotes $p<0.05$. (nd) not detectable.

in the patient fibroblasts (Figure 5C). In conclusion, *Artemis* splice-site defect could be restored by AON mediated cryptic exon skipping.

DISCUSSION

In this study we described two unrelated girls suffering from recurrent infections during the first 10 years of their lives. The infectious problems associated with decreased B cell and naïve T cell counts in combination with reduced serum Ig isotype and IgG subclass levels necessitated IVIG therapy. They had normal psychomotor development and growth. Patient 2 suffered from severe localized granulomatous skin lesions. The clinical presentation of both patients was caused by two different types of *Artemis* splice mutations resulting in residual enzymatic activity in one patient and reduced level of WT activity in the other patient

Artemis deficiency normally causes T-B- SCID with opportunistic infections already during the first months of life. Although both patients had an *Artemis* defect, the infectious episodes were limited with a relatively mild course, especially after initiation of IVIG. Apparently, the T cells present were able to induce cellular immune responses and to support humoral immunity. However, due to a low numbers of B cells, naïve T cells and a potentially limited Ig and TR repertoire, clonal selection will be less optimal giving rise to dysregulation in the immune system. This immune dysregulation might underlie the granulomatous skin lesions in patient 2.

Immunodeficiency disease with granulomas has been described previously in patients with hypomorphic mutations in the *RAG1* or *RAG2* genes.[24, 33] Similar to patient 2, repeated tests revealed no micro-organisms in the granulomas of these RAG mutated patients, suggesting that the granulomas were secondary to the impaired immune regulation. The granulomatous skin lesions largely resolved after transplantation and reconstitution of the donor immune system in patient 2, comparable to the patients with hypomorphic *RAG* mutations.[24] Apparently, V(D)J recombination defects with residual recombination activity can give rise to a variety of atypical clinical presentations.[33]

One (c.464+1G>A) of the two *Artemis* mutations was previously identified in a patient with late onset immunodeficiency and inflammatory bowel disease that presented at the age of 9 months.[26] The same *Artemis* genotype resulted in a different clinical phenotype; this might be due to age at the first encounter of infections, genetic background (e.g. modifier genes or epigenetic factors) or environmental factors.[33]

The second mutation (c.972+1997G>C), is a unique homozygous mutation in intron 11 of the *Artemis* gene resulting in exonization of 190 nucleotides from an intronic sequence in *Artemis* transcripts. The mutation corresponds to a nucleotide substitution at position 279 of an *Alu* consensus sequence.[34] Interestingly, it has been shown that mutating this specific guanine nucleotide to any other nucleotide, as is the case in patient 2, results in exonization of the *Alu* sequence.[34] The *Artemis* gene has a higher transposable element content (45%) than the average of the human genome (37%).[35] The most abundant transposable elements found in *Artemis* are *Alu* short interspersed elements (23% of total gene sequence), making *Artemis* prone to exonization of *Alu* elements in mRNA during splicing.

As expected from the V(D)J recombination defect, the expression level of mutant and WT *Artemis* transcripts in patient 1 and 2 was strongly reduced in precursor B cells and fibroblasts. However, mature B cells had only mildly reduced levels of *Artemis* transcripts. The different splicing patterns between fibroblasts, precursor B cells, mature B cells, T cells and NK cells might be caused by cell type and tissue-specific patterns of alternative splicing [36, 37]. However, differences in the various B-cell subsets might also reflect positive selection of a limited number of precursor B-cell clones that had a higher *Artemis* expression and were therefore able to generate a functional BCR and differentiate into mature B cells.

To dissect the functional impact of the hypomorphic *Artemis* mutations, we studied Artemis-related processes during B-cell development. In precursor B cells the reduced Artemis activity or low level of WT Artemis protein resulted in inefficient V(D)J recombination. In mature B cells, Artemis is involved in class switch recombination.[23, 38] Artemis-deficiency results in CSR characterized by strong dependence of long microhomologies in the switch junctions and a complete lack of "direct end-joining". We showed that reduced levels of WT *Artemis* and residual Artemis activity resulted in normal switch junction formation.

Current treatments for SCID patients include SCT [39] and gene therapy for some conditions.[40] However, SCT is still associated with significant treatment related morbidity and mortality, especially when material from unrelated and HLA mismatched donors is used.[41] Gene therapy is currently limited by technical difficulties and the risk of side effects.[42] New approaches for treatment of primary immunodeficiencies have been described, including the use of antisense morpholino oligonucleotides.[43] In this study we used 2'-*O*-methyl modified AONs to restore the expression of WT *Artemis* transcripts, without modifying the genome. AON treatment has also been successfully used in gene correction therapy to restore the reading frame in Duchenne muscular dystrophy patients.[44] Our *in vitro* results

showed the potential for AON treatment as therapeutical approach for patients with specific splice defects.

In conclusion, patients with an *Artemis* defect can present as an atypical SCID with granulomatous skin lesions in the absence of life-threatening infections. Therefore, a NHEJ defect needs also to be considered in patients having reduced number of naïve T cells and B cells in the circulation who presented with milder clinical symptoms than classical T-B- SCID. Furthermore, this study illustrates that AON treatment is a promising approach to treat patients with primary immunodeficiencies and other diseases caused by intronic splice-site mutations.

MATERIALS AND METHODS

Cell samples and flow cytometric immunophenotyping

Peripheral blood, bone marrow and a skin biopsy were obtained with informed consent and according to the guidelines of the local Medical Ethics Committees. Flow cytometric analysis of peripheral blood and bone marrow was performed as previously described.[16, 18, 45]

Cell lines and tissue culture

Primary fibroblasts were cultured from a skin biopsy of patient 1 and 2. Furthermore, fibroblasts from controls (C5RO and VH10), Artemis-deficient patients (Artemis-5 and Artemis-6; both having genomic deletions of exon 1-3 [27]), a patient with mutant Artemis (Artemis-8 (c.1391_1395delGAATC)) and a RAG-deficient patient (RAG-SCID12) (c.1782C>A)) were used. Fibroblasts were cultured in DMEM (BioWhittaker, Walkersville, MD), supplemented with 10% FCS, penicillin (100 U/ml), and streptomycin (100 µg/ml).

Radiation sensitivity assays.

Clonogenic survival assay and the X-ray-induced gH2AX foci assay were performed as previously described. [18, 19, 27]

Sequencing and STR marker analysis

Sequence analysis of genes involved in V(D)J recombination and NHEJ was performed by PCR analysis (for *RAG1* (NCBI M29474), *RAG2* (NCBI M94633), *Artemis/DCLRE1C* (NCBI M94633), *XLF/NHEJ1* (NCBI AJ972687), and *LIG4* (NCBI X83441) or RT-PCR analysis (for *Artemis/DCLRE1C)* of the coding regions with the TaqGold™

amplification system followed by direct sequencing. Primer sequences are available upon request. STR marker analysis was performed as previously described.[19]

Real-time quantitative PCR (RQ-PCR)

Primers and probe (Table 2) were designed to amplify WT or patient-specific *Artemis* transcripts using Taqman-based RQ-PCR. The RQ-PCR was performed on the ABIPRISM 7700 sequence detection system (Applied Biosystems) as described previously.[46]

Analysis of coding joints and SHM

DH-JH junctions were analyzed as previously described.[19, 27] Somatic hypermutation (SHM) was studied in VH3-Ca, VH4-Ca, VH3-Cg and VH4-Cg fragments, amplified from PBMC cDNA and cloned into pGEM-T easy vector (Promega). IMGT nomenclature (http://imgt.cines.fr/) was used to assign the V, D and J segments, and to identify somatic mutations.[47] The mutation frequency was determined for the VH gene segment of each transcript.

Characterization of switch recombination junctions

Genomic DNA was purified from PBMCs using standard methods. The amplification of Sμ-Sα fragments from *in vivo* switched cells was performed as described previously,[30, 48] except that a modified version of *Taq* polymerase (Go Taq, Promega, USA) was used in the PCR reactions. With this modification, a 2-4-fold increase in sensitivity was achieved. The PCR amplified switch fragments were gel purified (Qiagen, Germany), cloned into a modified version of the pGEM-5zf (+) vector and sequenced by an automated fluorescent sequencer in Macrogen (Seoul, Korea). The switch recombination breakpoints were determined by aligning the switch fragment sequences with the corresponding reference sequences (Sμ, X54713; Sα1, L19121; Sα2, AF030305), as described previously.[30, 49]

AON design and transfection

The AON was designed according to the guidelines for AON design using the mfold version 3.2 server program as described before.[50, 51] The AON was synthesized with the following chemical modifications: a 5′ fluorescein group (6-FAM), a full-length phosphorothioate backbone and 2′-*O*-methyl modified ribose groups (Eurogentec, Belgium). Fibroblasts from patient 2 and control (C5RO) were seeded, after 24 hours they were transfected with 200 nmol AON for 3h using 2μl polyethylenimine (PEI) (ExGen500; MBI Fermentas) per μg of transfected AON. At 24h

post-transfection, RNA was isolated using Rneasy minikit (Qiagen, Valencia, CA) and all RNA was used for reverse transcription as described previously.[52]

Statistics

Differences in numbers of P-nucleotides and mutation frequencies in SHM were analyzed using the nonparametric Mann-Whitney *U*-test (1-tailed) and transcripts expression differences were analyzed by the two-tailed T-test for independent samples ($P<0.05$ was considered significant) in the GraphPad Prism program (GraphPad Software). Statistical analysis for the switch junctions was performed using χ^2 test.

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Chapter 3.4

Clinical spectrum of LIG4 deficiency is broadened with severe dysmaturitas, primordial dwarfism and neurological abnormalities

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ABSTRACT

DNA double strand break repair via non-homologous end joining (NHEJ) is involved in recombination of immunoglobulin and T-cell receptor genes. Mutations in NHEJ components result in syndromes that are characterized by microcephaly and immunodeficiency. We present a patient with lymphopenia, extreme radiosensitivity, severe dysmaturity, corpus callosum agenesis, polysyndactily, dysmorphic appearance, and erythema, which are suggestive for a new type of NHEJ deficiency. We identified two heterozygous mutations in *LIG4*. The p.S205LfsX29 mutation results in lack of the nuclear localization signal and appears to be a null mutation. The second mutation p.K635RfsX10 lacks the C-terminal region responsible for XRCC4 binding and LIG4 stability and activity and therefore this mutant might be a null mutation as well or have very low residual activity. This is remarkable since *LIG4* knockout mice are embryonic lethal and so far in humans no complete LIG4 deficiencies have been described. This case broadens the clinical spectrum of LIG4 deficiencies.

INTRODUCTION

The non-homologous end joining (NHEJ) pathway is involved in repair of DNA double strand breaks (DSBs). These can be generated during DNA replication, exposure to exogenous agents such as ionizing radiation, or physiologically during V(D)J recombination, as happens during the early stages of B- and T-cell differentiation to generate antigen specific B- and T-cell receptors. Defects in NHEJ factors result in ionizing radiation (IR) sensitivity, and in defects in V(D)J recombination leading to immunodeficiency. Genetic defects have been described in several NHEJ genes, including *DCLRE1C* (MIM# 605988), *PRKDC* (MIM# 600899), *NHEJ1* (MIM# 611290) and *LIG4* (MIM# 601837).[1-5] To date, 16 LIG4 deficient patients have been described (summarized in Supp. Table S1).[5-14] All patients were IR sensitive, but clinically they can be divided into five distinct disease categories: 1) leukemia, 2) LIG4 syndrome (MIM# 606593), 3) Dubowitz syndrome (MIM# 223370) 4) Omenn syndrome (MIM# 603554) and 5) radiosensitive severe combined immunodeficiency (RS-SCID; MIM# 602450). Here we present a male patient with a new clinical phenotype of LIG4 deficiency characterized by microcephalic primordial dwarfism and neurological abnormalities.

The patient was born with extreme dysmaturity after 37 weeks of gestational age. At the age of 3 months, his height was 43 cm (-7.4 SD), weight 1870 grams (-8.9 SD), and head circumference was 29 cm (-8.9 SD). Besides the dysmaturity, the patient had several dysmorphisms (Figure 1A and 1B) including hypotelorism, small viscerocranium, flat philtrum, thin upper lip, preaxial polydactyly (duplication of distal phalanx of left thumb), brachymesophalangy of the digits V on both hands, and partial cutaneous syndactyly of digits II to V of both feet (Figure 1C and 1D), dysplastic kidneys with bilaterally vesicourethral reflux and urethral valves. Additionally, the patient had the neurological abnormalities, corpus callosum dysgenesia and colpocephaly. At the age of 2 and 4 months he suffered from a *Pseudomonas aeruginosa* and *Enterococcus faecalis* urinary tract infection, respectively, and he tested positive for *P. aeruginosa*, *P. jiroveci*, rhinovirus, norovirus, astrovirus, *Clostridium difficile* and *Candida.* Besides the infectious complications, the first 3 months of life were characterized by feeding difficulties, diarrhea, failure to thrive, cholestatic icterus, tubulopathy, generalized erythema and very dry cracked skin. Initially the patient seemed to recover from the opportunistic infections, but a second episode of an acute sepsis-like syndrome with respiratory insufficiency complicated by severe gastro-intestinal bleeding -probably due to the development of thrombocytopenia- could not be successfully treated; the patient died at the age of 6 months.

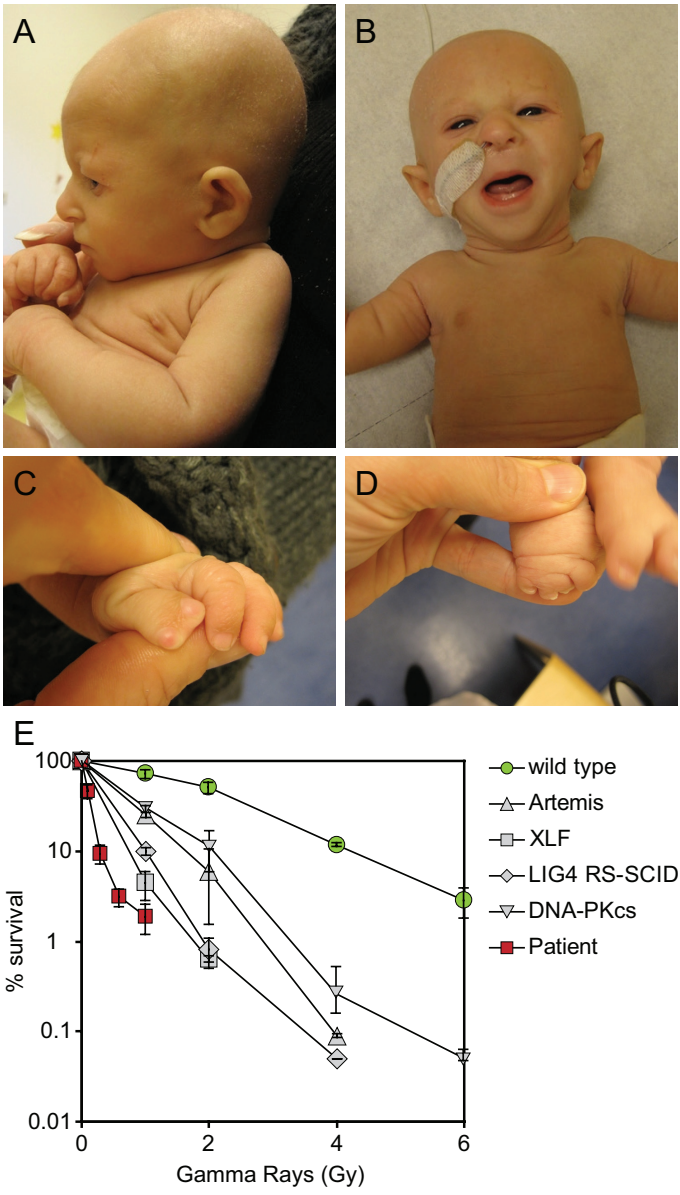

Figure 1. Dysmorphic features of the face, hand and feet and ionizing radiation sensitivity. The patient presented with facial dysmporphisms including beaked nose (A), hypotelorism, small viscerocranium, flat philtrum, thin upper lip (B). In addition, the patient had a duplication of distal phalanx of left thumb, brachymesophalangy of the digits V on both hands (C) and partial cutaneous syndactyly of digits II to V of both feet (D). Clonogenic survival assay of wild type (C5RO) fibroblasts and patients' fibroblasts deficient for Artemis, DNA-PKcs, XLF or LIG4 (LIG4 SCID). The patient was extremely sensitive for ionizing radiation. Each curve represents the mean of at least two independent experiments. Error bars represent SEM (E).

Supplementary Table 1. Overview of LIG4 deficient patients (new and literature)

	Age at diagnosis	Mutations		Radiosensitivity	Malignancy	Microcephaly	Cytopenia	Immunodeficiency	Developmental delay	Reference
Leukemia										
180BR	14y	p.R278H	homoz.	+	leukemia	?	?	-	-	11
LIG4 syndrome										
3703	4.5y	p.R814X	homoz.	++	leukemia	+	+	-	+	6
Patient	14y	p.M249V,	p.K424fs20X	++	leukemia	+	+	+	+	12
411BR	9y	p.A3V,p.T9I p.R278H	homoz.	+	-	(+)	+	+	+	3
2303	46y	p.R580X	p.R814X	+	-	+	+	+	?	3
2304	48y	p.R580X	p.R814X	+	-	+	?	+	?	3
99P0149	9y	p.G469E	p.R814X	+	-	+	+	+	+	3
Case 1	4y	p.delK588	homoz.	?	-	+	+	+	+	13
Case 2	3.5y	p.delK588	homoz.	?	-	+	+	+	+	13
LIG4-4	4 months	p.H282R	homoz.	?	-	+	+	+	+	new
Dubowitz syndrome										
Patient	1y	S205LfsX29	p.R814X	++	carcinoma	+	?	?	?	14
Omenn's syndrome										
Patient	3wk	p.H282L	p.E582DfsX4	?	-	+	?	+	?	9
RS-SCID										
P1	2y	p.H282L	p.K424fs20X	+	+	+	+	+++	-	8
P2	4 wk	p.H282L	p.K424fs20X	+	-	+	-	+++	+	8
P-1	1.5y	p.Q280R	p.K424fs20X	+	-	+	-	+++	-	7
P-2	4 wk	p.Q280R	p.K424fs20X	+	-	+	-	+++	?	7
LIG4-1	1.5y	p.delQ433	homoz.	+	-	-	+	+++	-	5
Patient	17wk	S205LfsX29	K635RfsX10	++	-	+	+	+++	+	new

Immunologic evaluation showed normal numbers of NK cells, very low B cell numbers and increased T cell numbers (Supp. Table S2 and Materials and Methods). The increase in the number of T cells was mainly caused by an increase in the CD8+ T cells probably related to a viral infection. The presence of maternal T-cells was excluded. Immunoglobulin (Ig) G was decreased, which was not secondary to malabsorption, whereas IgM and IgA were normal (Supp. Table S2) and Ig substitution therapy was initiated at the age of 4.5 months.

The clinical presentation, especially the immunodeficiency together with microcephaly was suggestive for a NHEJ defect. Therefore the patient's fibroblasts were tested in a clonogenic survival assay (Supp. Materials and Methods). These were extremely IR sensitive by an order of magnitude c.f. the control at 10% survival (Figure 1E) and even more sensitive than those of LIG4 and XLF deficient patients (three times more sensitive than the control at 10% survival) which are normally more IR sensitive than fibroblasts from Artemis and DNA-PKcs deficient patients (Figure 1E). This result was indicative for a severe NHEJ defect.

Sequencing of the *LIG4* gene (Materials and Methods) showed the presence of two heterozygous single nucleotide deletions in the *LIG4* gene (c.613delT and c.1904delA) (submitted to www.lovd.nl/LIG4). The first deletion was inherited from the mother and resulted in a frame shift and a premature stop codon in the DNA-binding domain (p.S205LfsX29). This mutation was recently described in the LIG4 patient presenting with the Dubowitz syndrome.[14] The mutant LIG4 protein lacks the nuclear localization signal (NLS), the active site, the adenylation domain, the

Supplementary Table S2. Immunophenotyping of the patient

	6 months (x10^9/L)	Reference values (x10^9/L) (Comans-Bitter, et al., 1997)
CD3	5.73	2.4-6.9
CD4	3.38	1.5-5.0
CD4CD45RA/RO	0.10/3.31	RA>RO
CD8	2.41	0.5-1.6
CD8CD45RA/RO	0.10/2.3	RA>RO
CD19	0.06	0.6-3.0
CD56	0.31	0.1-1.3
	4 months (g/L)	**Reference values 5-6 months**
IgG	0.76	2.6-15.2
IgM	0.22	0.07-0.65
IgA	0.85	0.08-0.90

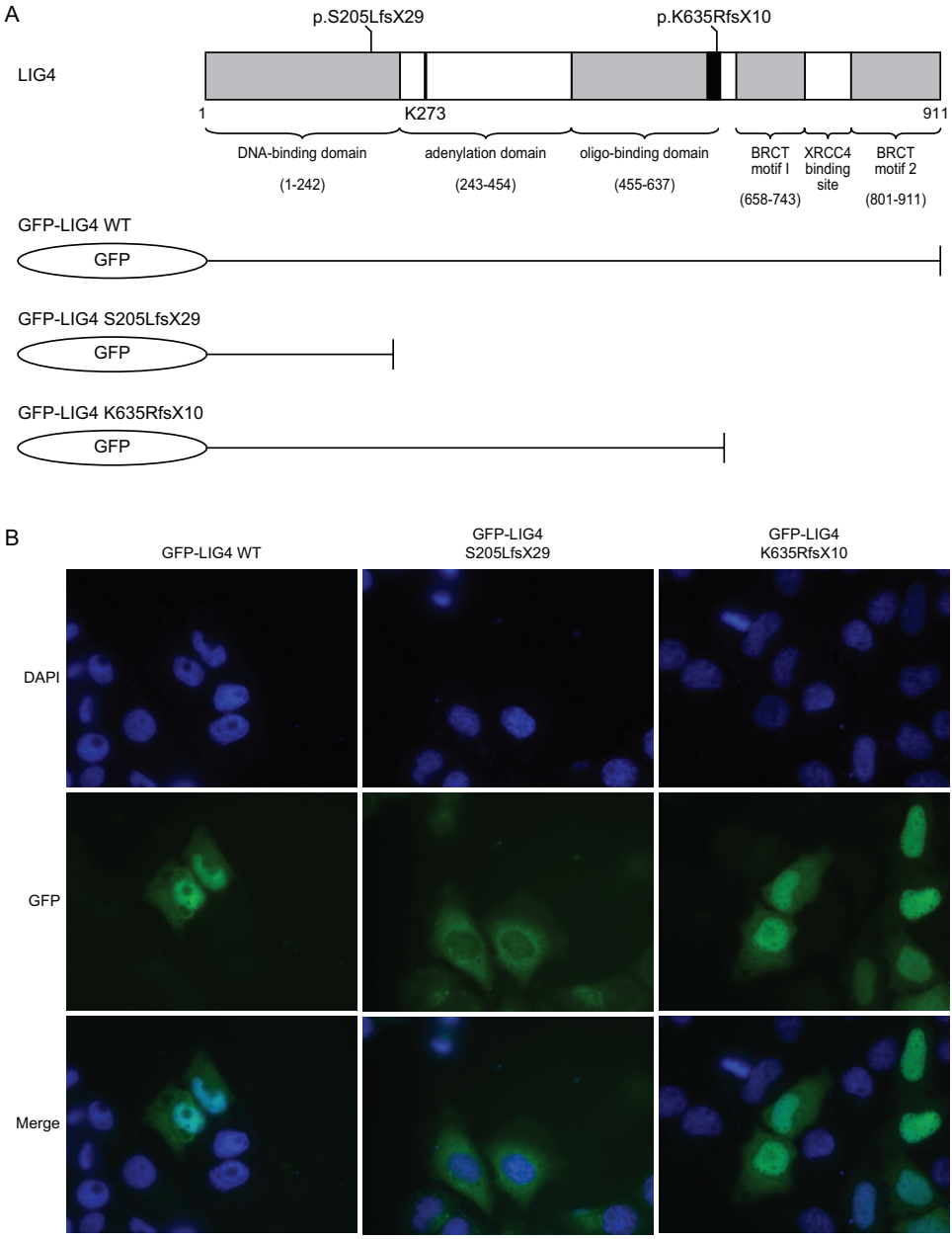

Figure 2. LIG4 mutants and their expression. Schematic representation of the LIG4 protein (NM_001098268.1) and the GFP-LIG4 expression constructs. The different domains, active site (K273) and mutations identified in the patient are indicated. The nuclear localization signal (NLS1 (P_{623}QEKKRK$_{629}$) and NLS2 (A_{630}APKMKKVI$_{638}$)[18] is indicated in black. The numbers between brackets indicated the amino acid position (A). Localization of GFP-LIG4 WT and mutants after transient transfection of U2OS cells (B).

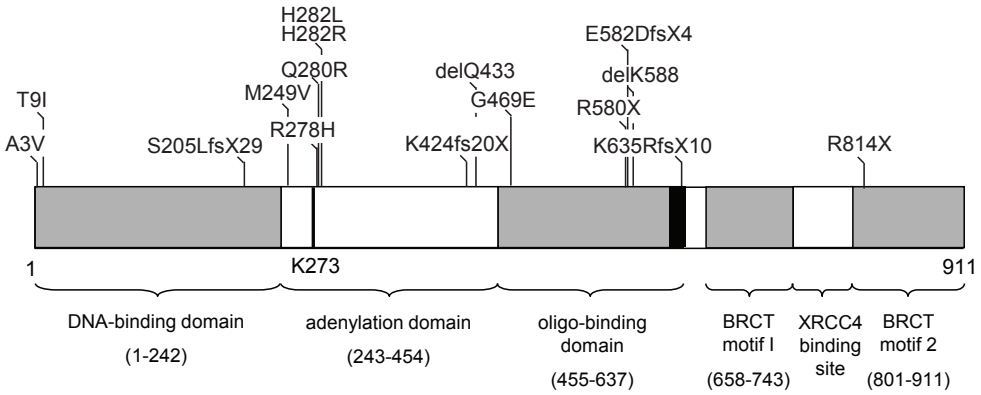

Figure S1. Localization of *LIG4* mutations. Schematic representation of all LIG4 mutations described in literature, the patient described here and our unpublished patient (see Supp. Table S1).

oligo-binding domain, both BRCT motifs and the XRCC4 binding site (Figure 2A). Since, LIG4 exerts its function in the nucleus we investigated the localization of the mutant LIG4 proteins by using green fluorescent protein (GFP) tagged LIG4 expression constructs (Figure 2A and Supp. Material and Methods). In contrast to wild type (WT) LIG4 the S205LfsX29 LIG4 mutant was only expressed in the cytoplasm (Figure 2B), which indicates the S205LfsX29 mutant represents a null mutation.

The second paternally inherited deletion resulted in a frame shift, changing the last four amino acids of the NLS ($K_{635}K_{636}V_{637}I_{638} \rightarrow R_{635}K_{636}L_{637}L_{638}$) without affecting the charge, and a premature stop codon (p.K635RfsX10). In this mutant part of the NLS is retained, but it lacks both BRCT motifs and the XRCC4 binding site, which are necessary for the interaction with Cernunnos/XLF.[15] LIG4 interacts with XRCC4 and forms a 1:2 complex.[16] The interaction with XRCC4 is important since it stabilizes LIG4 protecting it from degradation.[17] This implies that the p.K635RfsX10 mutant has probably very low residual activity or might even be a null mutant.

In our overexpression system, this mutant was still expressed in the nucleus (Figure 2B) and is therefore consistent with the results of Girard *et al* who found that deleting both BRCT motifs and the XRCC4 binding domain (Δ653-911) still resulted in nuclear expression of the mutant LIG4 protein.[18] None of the reported *LIG4* mutations in patients retains the NLS but lacks the XRCC4 interaction domain (Supp. Table S1 and Supp. Figure S1). The p.R814X mutant lacks the BRCT 2 motif, but the NLS and XRCC4 binding site are present.[6, 10] This mutant is expressed in the

nucleus and retained ~10-15% residual double strand ligation activity, but was barely detectable in the patient.[10] The estimated residual activity of this mutant is <1%.[18] The p.R580X mutant lacks the NLS and the XRCC4 interaction domain. Since this mutant is not stably expressed, does not interact with XRCC4 and does not enter the nucleus, it is considered to be a null mutant. Similar to the p.R580X mutant, the p.K635RfsX10 mutant lacks XRCC4-interacting domain,[15] which is necessary for LIG4 stability and protection of LIG4 from degradation.[17] Based on these data and the severity of the clinical phenotype of the patient, we expect that this mutant has even less residual activity than the LIG4 mutants described before and might represent a null mutation. This is remarkable since LIG4 is considered to be essential for humans and Lig4 knockout mice are embryonic lethal.[19, 20] This study shows that *LIG4* mutations affect the immune system or neurological development with different severity.

MATERIAL AND METHODS

Cell samples and flow cytometric immunophenotyping

Peripheral blood and a skin biopsy were obtained with informed consent and according to the guidelines of the local Medical Ethics Committees. Flow cytometric analysis of peripheral blood was performed as described previously (Noordzij, et al., 2003; van der Burg, et al., 2006).

Cell lines and tissue culture

Primary fibroblasts were cultured from a skin biopsy from the patient in addition to fibroblasts from a healthy control (C5RO), Artemis-deficient (Artemis-6) (van der Burg, et al., 2007), XLF deficient (XLF-5) and DNA-PKcs deficient patients (van der Burg, et al., 2009) and a LIG4 SCID patient (van der Burg, et al., 2006). Fibroblasts were cultured in DMEM (BioWhittaker, Walkersville, MD, USA), supplemented with 10% FCS, penicillin (100 U/ml), and streptomycin (100 µg/ml).

Clonogenic survival assay and sequence analysis

Clonogenic survival assay was performed as described previously (van der Burg, et al., 2009). XLF(NHEJ1) (NM_024782.2) and LIG4 (NM_001098268.1) were amplified by PCR and sequenced according to (van der Burg, et al., 2009).

Localization of LIG4 mutants

Mutant GFP-LIG4 expression constructs (S205LfsX29 and K635RfsX9) were made by using the QuickChange site directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) using the GFP-LIG4 WT as the parent plasmid. U2OS cells were transfected using Fugene 6 (Promega, Madison, WI, USA). After 24h cells were fixed with 2% paraformaldehyde and expression was determined using a fluorescence microscope.

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PART 4

Mechanism of repertoire development

Chapter 4.1

XLF deficiency results in impaired generation of junction diversity by TdT

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Manuscript in preparation

Chapter 4.2

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

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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 Evaluation	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.

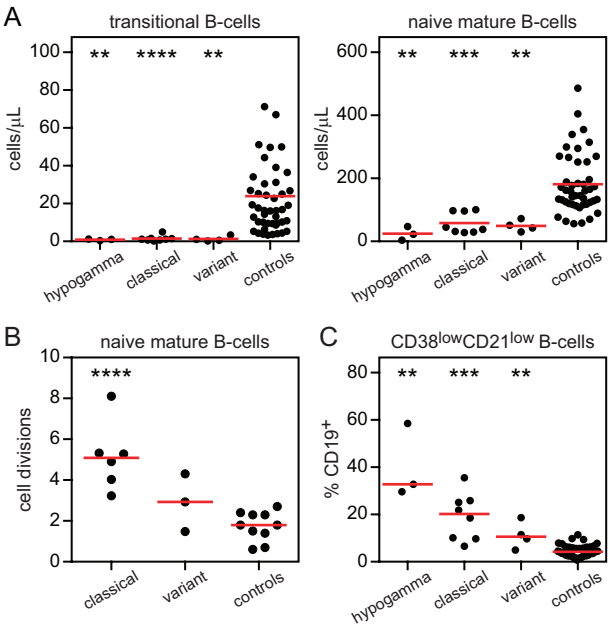

Figure 1. Naive and CD21^{low}CD38^{low} defects in Ataxia Telangiectasia. A. Absolute numbers of blood transitional B-cells (CD19+CD27^{-}CD24^{high}CD38^{high}) and naive mature B-cells (CD19+CD27^{-}CD24^{dim}CD38^{dim}) in three categories of AT patients. **B.** Naive B cell replication history as 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

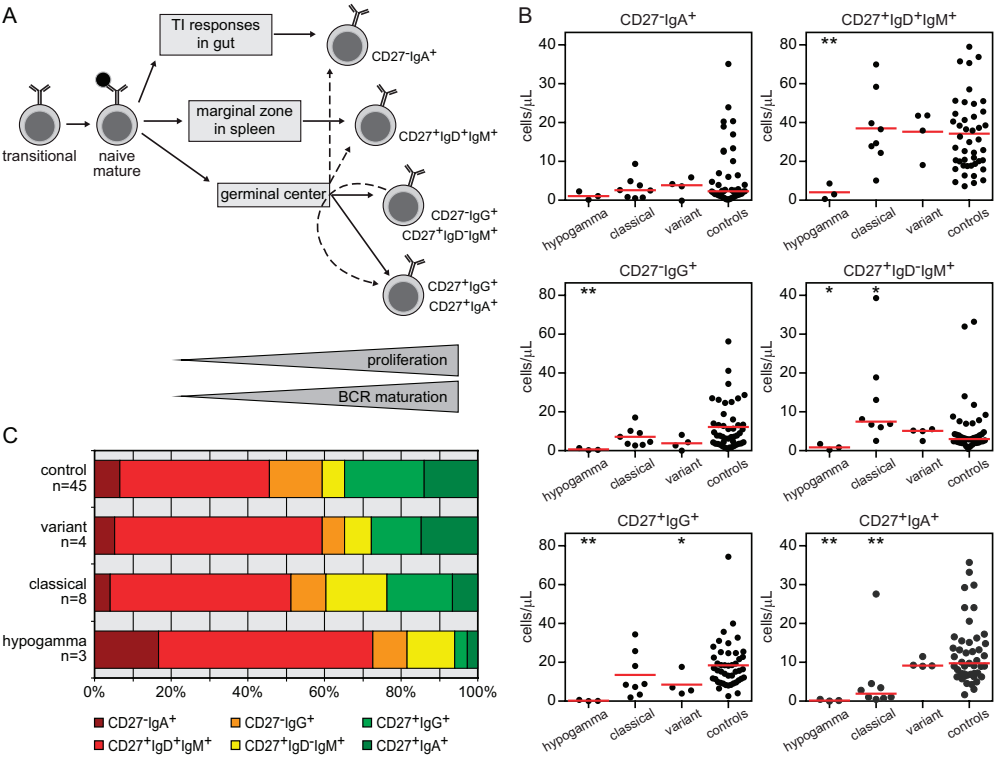

Figure 2. Memory B-cell subset distribution in AT. A. Memory B-cell subsets according to Berkowska et al Blood 2011[16] **B**. Absolute numbers of memory B-cell subsets in three categories of AT patients. **C**. Relative distributions of memory B-cell subsets. Data are compared to normal controls using 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.

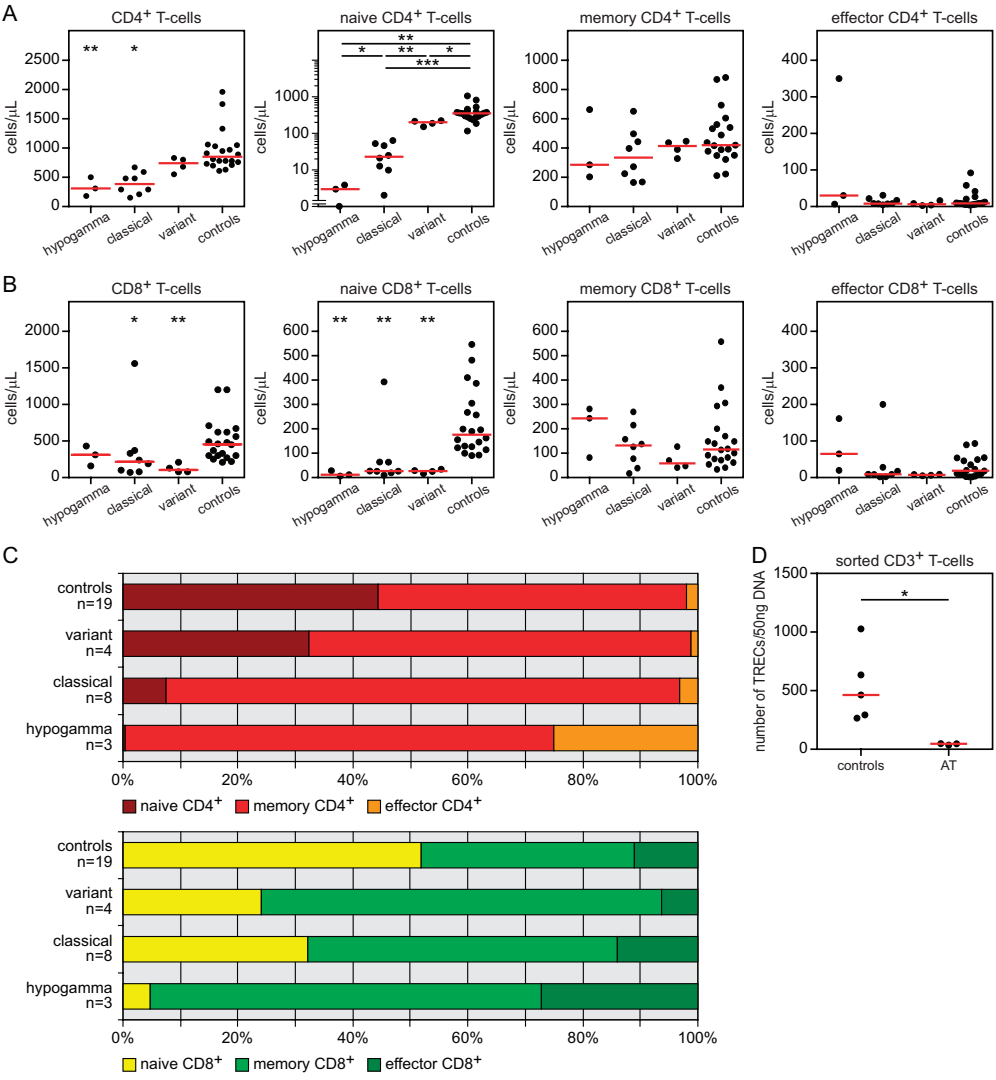

Figure 3. T-cell subset distribution in AT. Absolute numbers of CD4+ (**A**) and CD8+ T-cell subsets in three categories of AT patients **B**. **C**. Relative distributions of T-cell subsets **D**.TRECs in sorted T-cells. Naïve T-cells (CD45RA+CD27+); memory T-cells (CD45RA-CD27+) and effector T-cells (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

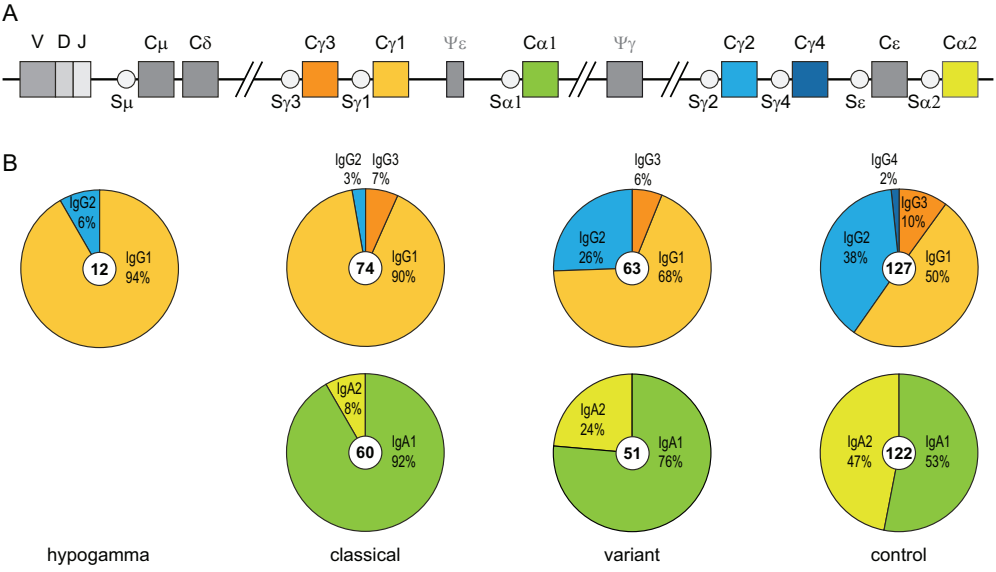

Figure 4. IgA and IgG class switching in *IGH* transcripts of AT patients. A. Schematic representation of the constant regions in the *IGH* locus. **B.** Frequencies of *IGHG2* and *4* and *IGHA2* transcripts in classical AT plus hypogammaglobulinemia, classical AT and variant AT were compared to controls (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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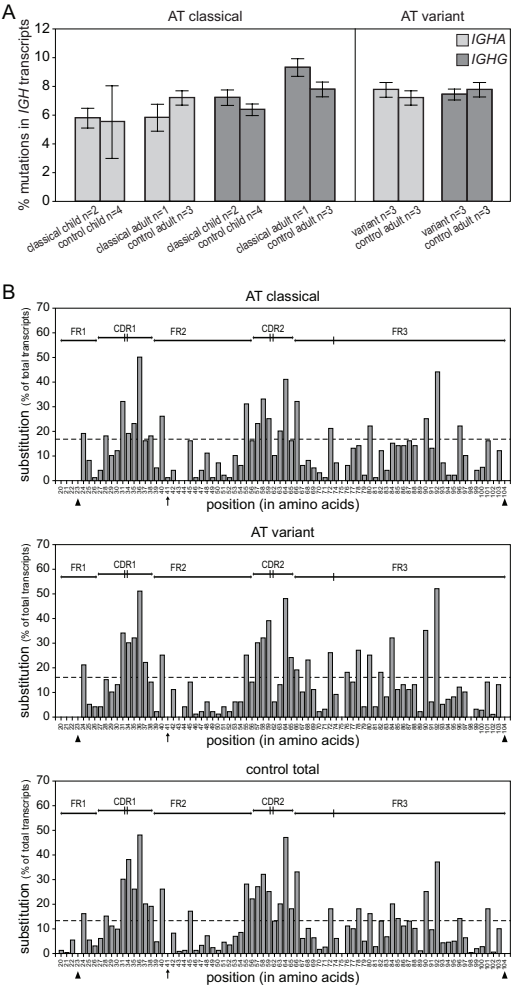
SUPPLEMENTS



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
IGHV4-59*01	IGHD3-22*01	IGHJ4*02	12	tgtgcgagagggagtagtggataccgtacctttgactactgg
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	tgtgcgagagtcactgctcgttactactactactacatggacgtctgg
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	tgtacgagagacttaaagctccaaagcgatagtagtgggagctactcccagtcacatggttttga-tatctgg
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	tgtgcaagagatcgggagtatagcagctcgacttactactactacatggacgtctgg
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	tgtgcaagaggagactatggttcggggagttattataacgatgcctccggaacttttgactactgg
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	tgtgcaagagtaaatgggtacaactggaactacccgccaggggcttttgatatctgg
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	tgtgcaagattggtggcaggggacccttcgtactactttgactactgg
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	tgtgcgagagatctctatgatagtagtggtcgtatatatatccccgaccgtctactgg
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	tgtgcgagagatcggttgggggtatagcagtggcgacggactactgg
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
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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
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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	tgtgcgagagtgcaggctgggatcctctactactactacggtatggacgtctgg
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	tgtgcgagacacgtagtggctacggcccccttatagttactgg
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	tgtgcgagagcttggggaaagtgggagctacccagcgccctttgactactgg
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
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IGHV4-59*01	IGHD2-15*01	IGHJ3*02	10	tgtgcggtggtagctgccggggctttgatatctgg
IGHV3-74*01	IGHD3-16*01	IGHJ3*02	8	tgtgggatccttaatgcttttgatatctgg

Chapter 5

General Discussion

GENERAL DISCUSSION

Severe combined immunodeficiency (SCID) is one of the most life-threatening inherited primary immunodeficiencies (PID). SCID patients present in the first year of life with severe opportunistic infections, chronic diarrhea and failure to thrive. One variant of SCID, T-B-SCID, is caused by a defect in V(D)J recombination. The V(D)J recombination process is required for generation of antigen specific B- and T-cell receptors that form the basis of the adaptive immune system. Important processes during V(D)J recombination are introduction of DNA double strand breaks (DSB) in the immune receptor genes by RAG1 and RAG2 and repair of DSB. Several genetic defects have been found to cause T-B-SCID, such as *RAG1*, *RAG2*, *Artemis, XLF*, *LIG4* and *DNA-PKcs* (see **Chapter 1**). At this moment, there is a delay in making a diagnosis, because detailed insight in the clinical heterogeneity is lacking, and not all candidate genes (i.e. genes that can be mutated in T-B-SCID) are known. Due to the delay in the diagnostic process, adequate treatment is often initiated (too) late, leading to more serious life-threatening infections. The aim of this thesis was to unravel the clinical and immunogenetic heterogeneity of SCID. This chapter discusses methods for the identification of genetic defects and new candidate genes for PID, the spectrum of recombination defects, the role of DNA repair in lymphocyte development, the relevance of accurate diagnostics for treatment choices in SCID patients, newborn screening for SCID, and new approaches to study the immune receptor repertoire.

IDENTIFICATION OF GENETIC DEFECTS

From candidate gene approach to next generation sequencing

The most commonly used method to identify genetic defects in SCID patients is the candidate gene approach.[1] When SCID is suspected, flow cytometric immunophenotyping of the lymphocyte subsets in peripheral blood can be used as a screening assay. Analysis of the B, T and NK cells, can be used to discriminate between T-B+SCID and T-B-SCID. Based on the clinical presentation and immunophenotyping, candidate genes are sequenced using Sanger sequencing. Over 180 different PID-causing genes have been described,[2] from which around 8 genes have been associated with T-B-SCID (Chapter 1).

The candidate gene approach has proven to be successful, but it can be time consuming because in many cases multiple genes have to be evaluated. Over the last three to four years, next generation sequencing (NGS) methods became available, which allows rapid high-throughput sequencing. The advantages of NGS over the candidate gene approach are the large number of genes analyzed in a short period of time, and as the costs for NGS are decreasing, NGS will ultimately become cheaper than Sanger sequencing. Several

approaches can be used to identify the genetic defect by use of NGS, including targeted sequencing of the known PID genes, whole exome sequencing (WES) or whole genome sequencing (WGS).

Targeted approach sequencing of the known PID genes by NGS was recently described by Nijman *et al*.[3] They showed that 161 of the known PID genes are suitable for accurate mutation detection by this method. Targeted sequencing would allow time-effective and cost-effective identification of mutations in PID patients, but novel PID genes cannot be identified.

WES and WGS are unbiased methods to sequence only the coding regions (WES) or the whole genome (WGS). The main advantage of the WES and WGS methods is the identification of novel disease-causing genes. In case of consanguineous families, WES can be combined with homozygosity mapping, in which the genomic region most likely to harbor the pathogenic mutation is identified based on homozygous clusters of single nucleotide polymorphisms.[4, 5]

Pitfalls of the NGS approaches

NGS approaches are good methods to identify new genetic mutations, or mutations in new candidate genes, but there are some pitfalls. First, sequencing of many genes results in detection of many variations. The difficulty is to distinguish the pathogenic variant from the thousands of non-pathogenic variations. This requires good bioinformatics algorithms. Second, not all genes are covered equally. Genomic regions with a high GC content are difficult to sequence. Nijman *et al.* have shown that for the 170 candidate PID genes that were tested, nine genes could not be evaluated, probably because of high GC content or the presence of pseudogenes.[3] In addition, sequences with large structural variations, such as insertions, deletions and translocations, do not align properly to the genome and can therefore be missed. Gross deletions are frequently reported for the PID genes *IGHM*, *BTK* and *Artemis*.[6]

Third, mutations in promoter or regulatory regions and introns resulting in alternative expression or splicing of the genes are generally not targeted by WES and the candidate gene approach. In **Chapter 3.3** an intronic *Artemis* mutation is described resulting in alternative splicing of the *Artemis* gene.[7] This mutation was located 2kb outside the coding exons, and would therefore not have been detected by WES, or the candidate gene approach. This example illustrates the limitations of the techniques and that in certain cases additional assays are necessary to identify the genetic mutation. These include analysis of transcripts, protein expression or functional (*in vitro*) tests.

Identification of new candidate genes

Over the last years, many new PID candidate genes have been described, from which many have been identified by NGS.[8] In **Chapter 2** we described the identification of *DNA-PKcs* as new candidate gene of PID. The mutations in *DNA-PKcs* were not identified by NGS, but via Sanger sequencing after (functional) assays suggesting a DNA-PKcs defect. Irrespective of the applied sequencing approach, after identification of a genetic defect in a potential new candidate gene, the causality between the genetic defect and the (clinical) phenotype should be proven.

Online tools can provide first clues on functionality of the mutated protein, but in the end functional assays are required. In case of our DNA-PKcs$^-$ deficient patient, we identified two homozygous mutations in the *DNA-PKcs* gene, from which one or both could be pathogenic. Interspecies similarities can provide the first clues on protein function, since mutations in conserved regions are more likely to be pathogenic than mutations in non-conserved regions. In addition, the secondary protein structure of proteins can be predicted based on related proteins by online tool such as PredictProtein (www.predictprotein.org), to check whether the mutation changes the secondary structure of the protein. In case of our *DNA-PKcs* mutations, one mutation was found in a non-conserved region, but the other mutation was found in a highly conserved region in a predicted α-helix, suggesting that this mutation was most likely pathogenic. Other tools, like Polyphen (http://genetics.bwh.harvard.edu/pph2), can predict the possible impact of amino acid substitutions on the protein structure. All these prediction tools are only indicative, and functional assays are required to confirm the hypothesis. In case of the *DNA-PKcs* mutation, we could confirm that one mutation was pathogenic by a functional recombination assay and a complementation assay (clonogenic survival assay) (**Chapter 2**).[9]

Can other recombination defects be expected?

For recombination defects, seven candidate genes (*RAG1, RAG2, Artemis, DNA-PKcs, XLF, LIG4, and XRCC4*) are known (see **Chapter 1**). Mutations in the other known NHEJ genes (*KU70, and KU80* have not (yet) been identified. *Ku70*- and *Ku80*-knockout mouse models are viable, and have indeed a SCID phenotype.[10-13] Remarkably, human cell lines with inactivated *KU70* or *KU80* are not viable,[14, 15] indicating that *KU70* and *KU80* are essential for survival in humans. Although functional null mutations in *KU70*, and, *KU80* are unlikely to be found in humans, hypomorphic mutations in these genes cannot be excluded. Therefore, testing for defects in these genes remains valid in radiosensitive-SCID or in patients with immunodeficiency, growth retardation, and/or developmental defects.

SPECTRUM OF RECOMBINATION DEFECTS

Clinical and immunological spectrum of RAG deficiencies

The last decade, it became clear that V(D)J recombination defects not only result in classical SCID[17] or OS[18], but that there is a wide clinical heterogeneity with considerable immunological variation.[19] Especially for the *RAG* genes, many different clinical presentations have been described (Figure 1). Null mutations in RAG result in T-B-SCID, while *hypomorphic RAG* mutations have been associated with a spectrum of clinical and immunological phenotypes.[19] In **Chapter 3.1** we described a patient that has extended the *spectrum of RAG* mutations from severe immune defects to an almost normal condition.[20] This patient presented with idiopathic CD4+ T lympocytopenia caused by hypomorphic *RAG1* mutations. During childhood the patient presented with a single course of extensive chickenpox and moderate albeit recurrent pneumonia, but otherwise she remained disease-free for at least 10 years using prophylaxis.

Recently, Lee *et al* have shown in a comprehensive study that the severity of the clinical presentation correlates with the residual recombination activity of the *RAG1* mutant.[21] They analyzed the expression and recombination activity of 79 human *RAG* mutants and linked these data to the phenotype of the patients. This suggested that the clinical presentation of the patient can be predicted based on the *RAG1* mutation. However, in **Chapter 3.2** we have shown in a group of 22 RAG deficient patients that even a similar *RAG1* mutation can result in different clinical presentations.

The patients presented with "classical" T-B-SCID, OS, or combined immunodeficiency (CID). The main immunological difference was that the OS patients had expansion of the (αβ-)T cells and most of the CID patients had a 'leaky' instead of complete block in the precursor B-cell development. However, these differences cannot fully account for the

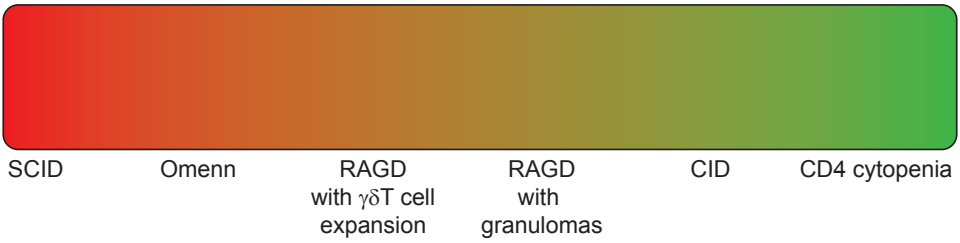


Figure 1. Spectrum of RAG deficiency. RAG deficiency can present in a spectrum of clinical presentations, including typical severe combined immunodeficiency (SCID), Omenn syndrome, RAG deficiency (RAGD) with γδT cell expansion, RAGD with granuloma, combined immunodeficiency (CID), and CD4 cytopenia,

differences in the clinical phenotype. Therefore we hypothesized that hypomorphic mutations in SCID genes result in reduced V(D)J recombination leading to fewer B and T cells with a limited repertoire. In an attempt to compensate for their low numbers the B and T cells start to proliferate, but the repertoire remains limited and imbalanced, so that the selection and immune regulation are impaired. Dependent on the coincidental (limited) repertoire, infection, allergy or autoimmunity can develop, together contributing to the heterogeneity in the clinical presentation.

This model (Figure 2) that we proposed in **Chapter 3.2** holds probably not only true for RAG deficiencies, but for all hypomorphic mutations in genes involved in V(D)J recombination. This is further illustrated by the two patients described in **Chapter 3.3**, who had hypomorphic *Artemis* mutations, and had a mild clinical phenotype. Also mutations in other PID genes like *ZAP70* or *JAK3* can result in different clinical phenotypes.[22, 23]

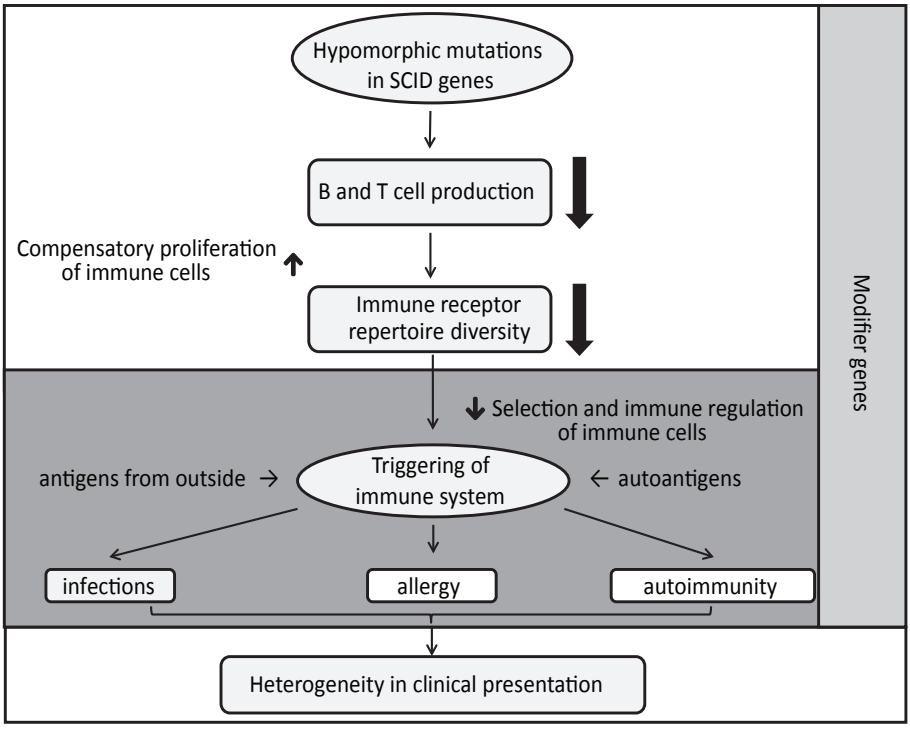


Figure 2. Model for heterogeneity in the clinical presentation of patients with hypomorphic mutations in SCID genes. (for details see text).

Besides immunodeficiency and autoimmunity, the immunological spectrum of RAG deficiencies also includes granulomas,[24-26] which were also found in one of the Artemis-deficient patient described in **Chapter 3.3**.[7] The lesions in this patient steadily progressed into multiple isolated and subsequently a confluent lesion. In none of these SCID patients with granulomas a pathogen was detected. However, the lesions of the Artemis-deficient patient regressed after HCT, suggesting an underlying immunological problem.

In summary, the clinical and immunological spectrum of RAG deficiencies is not only determined by the specific mutation, but also depends on a complex interplay between the (limited) immune receptor repertoire, (auto-) antigen exposure, the specificity of antigen receptors and the timing and cell type involved in the immune activation (Figure 2).

Clinical and immunological spectrum of DNA repair defects

Most of the components of the V(D)J recombination machinery are not solely involved in this process. Only RAG1, RAG2, and TdT are restricted to lymphoid cells. The other proteins are involved in repair of DNA double strand breaks via nonhomologous end joining (NHEJ) or involved in sensing of DNA damage (NBN and ATM). Besides immunodeficiency, patients with mutations in *Artemis*, *DNA-PKcs*, *XLF*, *LIG4*, *NBN* and *ATM* can have additional non-immunological problems such as increased susceptibility for developing malignancies, increased sensitivity to ionizing radiation (IR), and neurological problems (Table 2 and 3 **Chapter 1**).

Patients with defects in DSB repair are sensitivity for IR, but not all patients show the same degree of sensitivity (Figure 3). Patients with Artemis deficiency show a moderate sensitivity for IR compared to XLF- and LIG4-deficient patients (Figure 3). This is consistent with the finding of Riballo *et al.* that Artemis is only necessary for approximately 10% of DNA DSBs induced after IR, but is dispensable for DSBs that can be directly ligated.[27] Artemis is, however, essential for hairpin-opening, which explains the complete block in V(D)J recombination in Artemis-deficient patients.

For *DNA-PKcs* mutations only two patients have been described. Fibroblasts of the first DNA-PKcs deficient patient described in **Chapter 2** had a similar sensitivity to IR as Artemis deficient patients.[9] This DNA-PKcs mutant still had residual kinase activity and the mutation mainly inhibited Artemis activation. However, the second DNA-PKcs mutant described by Woodbine *et al*. had less residual activity and showed very poor cloning efficiency, precluding clonogenic survival analysis.[28] However, it is likely that this DNA-PKcs patient was more sensitive for IR than our patients, since poor cloning efficiency is observed in cells which are very sensitive for IR.[29]

Artemis-deficient patients do not only differ from the other patients with NHEJ defects in sensitivity to IR, but also in clinical presentation. While Artemis-deficient patients have

a severe immunodeficiency, patients with AT, NBS, XLF- and LIG4-deficiency have serious neurological abnormalities and are less immunodeficient.

Patients with AT have severe neurologic abnormalities. They present with progressive neurological impairment and cerebellar ataxia, but clinically the immunodeficiency tends to be mild, although the degree of immunodeficiency is variable ranging from no antibody deficiency to hypogammaglobulinemia, which probably depends on the residual function of ATM. Interestingly, both the classical AT and AT patients with hypogammaglobulenemia had no (detectable) ATM activity. However, the immune system in the patients with hypogammaglobulinemia was more disturbed than classical AT patients. It could be that the current detection method for ATM activity is not sensitive enough to measure low residual activity, or that it does not measure ATM function important for lymphocyte development. ATM is known to function in V(D)J recombination and CSR. The main role of ATM during V(D)J recombination is to stabilize the DNA DSB complex,[30] but it is likely that other proteins such as DNA-PKcs can take over its function. It has been reported that ATM and DNA-PKcs are functionally redundant during signal joint formation.[31]

NBS, LIG4- and XLF-deficient patients generally present with microcephaly, which might be a consequence of extensive apoptosis of newly generated post-mitotic neuronal cells, due to increased occurrence of unrepaired DSBs.[16] However, depending on the residual

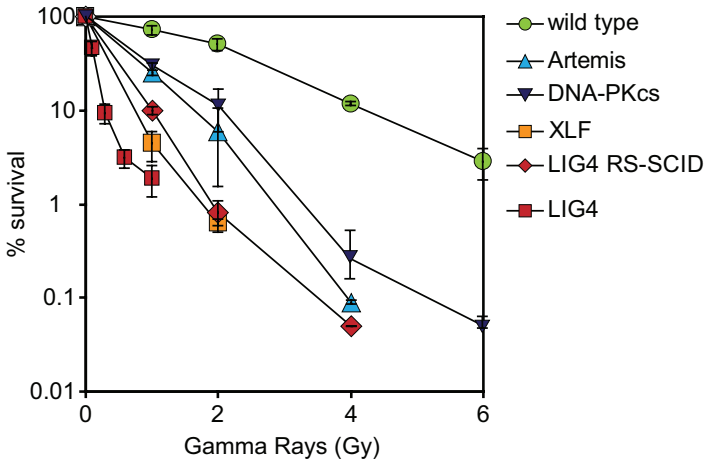


Figure 3. Clonogenic survival assay of patients with NHEJ defect. Fibroblasts cultured from skin biopsies were exposed to increasing doses of irradiation and the percentage survival was determined after 8 days. Fibroblasts from patients deficient in Artemis (Artemis-6),[94] DNA-PKcs,[9] XLF-1 (Chapter 4.1), LIG4 SCID,[40] and LIG4 with primordial dwarfism (Chapter 3.4) are displayed.

activity, the neurological problems of these patients might be more severe. Especially for *LIG4* mutations the diversity in neurological abnormalities is large. Almost all of the LIG4-deficient patients present with microcephaly,[32-38] only in two patients no microcephaly is reported.[39, 40] However, in **Chapter 3.4** we described a LIG4-deficient patient with the most severe neurological abnormalities.[29] Besides microcephaly this patient presented with corpus callosum dysgenesia and colpocephaly. A similar severe neurological phenotype was also observed in the DNA-PKcs-deficient patient described by Woodbine *et al.*[28] This patient presented with microcephaly, seizures, and had profound globally impaired neurological function. This in contrasts to the DNA-PKcs patient described in **Chapter 2** and the DNA-PKcs$^-$ deficient animal models that had no evident neurological phenotype, suggesting that there is more stringent requirement for DNA-PKcs in humans.

In conclusion, the clinical phenotype of patients with DNA repair defects is difficult to predict. Mutations in the same gene can result in a spectrum of immunological and non-immunological phenotypes, and some functions of DNA repair proteins are partly complementary.

TREATMENT OF SCID

Hematopoietic stem cell transplantation

The curative treatment for SCID is allogeneic hematopoietic stem cell transplantation (HCT) and for some specific conditions gene therapy.[41] The first bone marrow transplantation was done in 1968 in a patient with X-linked SCID.[42] Over the years, the long-term survival of these patients improved.[43] Transplantation by use of a geno-identical sibling donor now gives survival of 90%.[44] However the outcome of HCT is dependent on early diagnosis, availability of the compatible donor, type of SCID, preceding co-morbidity, age at transplantation, and the condition regime.[41]

T-B-SCID patients have a poorer outcome after HCT than T-B+SCID. This holds true for both the RAG-deficient T-B-SCID patients as the radiosensitive T-B-SCID patients.[44, 45] For the radiosensitive T-B-SCID patients, this is possibly associated with the generalized radiosensitivity, but it is also possible that the T-B-SCID patients have a later block in thymocyte development, leading to a precursor competition in the thymus.[44, 46] In addition, since many conditioning regimes use alkylating agents that are particularly DNA damaging, radiosensitive T-B-SCID patients require a adapted conditioning regime.

Gene therapy

The main drawback of HCT is the availability of a suitable donor. Therefore, gene therapy is a good alternative. Gene therapy is based on ex-vivo transfer of a transgene via viral

infection to patient-derived hematopoietic stem cells, followed by transplantation back to the patient. Gene therapy for primary immunodeficiencies is now available for patients with ADA-SCID, SCID-X1 (γc deficiency), and Wiskott-Aldrich syndrome.[47] For T-B-SCID, no gene therapy is available at the moment, but gene therapy for Artemis,[48, 49] RAG1,[50, 51] and RAG2[52] are already evaluated in the preclinical phase.

Gene correction

Another more experimental approach is gene correction.[53] This method is similar to homologous recombination, and relies on replacing the DNA region containing the mutation with a fragment of DNA containing wild-type sequence, while leaving the surrounding DNA intact. The DNA DSBs would be introduced at specific DNA target sequences surrounding the gene of interest. This can be done by either homing endonucleases, or zinc finger nucleases, which are artificial fusion proteins.[54-56] The first proofs of principle for this approach was already shown for RAG1 and IL-2 receptor gamma,[53, 56] but further research is necessary.

Modulation of splicing by using antisense oligonucleotides

In **Chapter 3.3** we described the proof of principle of antisense oligo nucleotides (AON) for the correction of an Artemis-splicing defect. AONs are now successfully used in phase II clinical trials in patients with Duchenne muscular dystrophy (DMD).[57] AONs are synthetically modified single-stranded nucleic acids that hybridize to specific sequences on pre-mRNA and can thereby change splicing. In case of our Artemis-deficient patient, the coding exons and the flanking splice-sites were unmutated, but a single nucleotide substitution deep in an intron resulted in alternative splicing of the *Artemis* transcripts. The AON was designed to bind to the site of the intronic mutation thereby blocking the newly formed splice site for the splicing machinery. After addition of the AON to the patient's fibroblasts, the normal *Artemis* splicing was restored and the cells were less sensitive for ionizing radiation.

The success of AON treatment is dependent on the (residual) function of the resulting protein. In case of our Artemis patient, splicing was restored and the wild-type Artemis protein was expressed. In case of DMD patients, AON treatment results in exon skipping, leading to a restoration of the reading frame and the expression of a partially functional protein. SCID is a rare disease, and splice-site mutations do not occur so often, especially not the splice site mutations that are potential candidates for AON treatment. In addition, this approach is hampered by the fact that the AONs need to be continuously administrated. Therefore, AON treatment is probably not suitable for SCID patients, but for other diseases in which splicing needs to be changed AON treatment is a promising approach.

NEWBORN SCREENING FOR SCID

A retrospective study in SCID patients diagnosed prenatally or at birth, because of diagnosis of SCID in a previous sibling or family member, has shown that SCID babies diagnosed at birth have significantly decreased number of infections, are transplanted earlier, and have a improved survival.[58] These studies have shown the necessity of early diagnosis and thereby the relevance of newborn screening for SCID.

Chan and Puck showed that the absence of T cell receptor excision circles (TRECs) extracted from dried bloodspots could be used as an assay for newborn screening for SCID.[59] These δREC-ψJα TRECs are formed during recombination of the TRA locus.[60, 61] During the first stages of TRA rearrangements the TRD locus, which is positioned within the TRA locus, is deleted. This results in the formation of the δREC-ψJα TRECs which can be easily detected by real-time quantitative PCR.[62, 63] TRECs do not replicate and are therefore diluted during proliferation,[62, 64] which makes them suitable markers for the numbers of naive T cells that have recently emigrated from the thymus.[62, 63, 65, 66]

Healthy newborns have TREC numbers equal to 10% of T cells, but adult T cells expand predominantly by proliferation and have therefore lower numbers of TRECs.[67] As a consequence, maternal T cells and oligoclonal expansions of T cells, such as in OS, do not confound the TREC analysis.

Using the TRECs for newborn screening only allows detection of patients with T cell deficiencies. However, excision circles cannot only be used as a marker to measure numbers of naive T cells, but also as a marker for replication history of B cells by using the kappa-deleting recombination excision circles (KRECs).[68] Nakagawa *et al.* were the first to show that KRECs could be amplified from neonatal Guthrie cards and allows identification of patients with B-cell maturation defects like X-linked agammaglobulinemia (XLA).[69] In addition, it has been shown that combined measuring of TREC and KREC is a suitable screening for patients with SCID, XLA, AT and NBS.[70]

However, before SCID can be implemented in the newborn screening, ten criteria should be met (Table 1).[71, 72] The "white paper" on the need for newborn screening for SCID in Europe, has shown that these ten criteria are met.[73] In the next paragraph we will discuss the first five criteria, which are related to the incidence of SCID, treatment, the diagnostic test, and the presence of an asymptomatic stage.

According to criterion 1 the condition should be an important health problem. SCID is an important health problem, the patients die without effective treatment. Although the incidence is not exactly known, it is around 1:50,000-100,000 live births, but the true incidence is probably higher. The lasts years several pilot studies on SCID screening have been performed (reviewed by Buckley[74]) that have screened almost a million newborns. In these studies a total number of 14 SCID and 40 cases of T-cell lymphopenia were identified.

These data suggests that the SCID incidence might be higher than the previously estimated value of 1:50,000- 1:100,000 live births.

SCID can be diagnosed by a suitable test and can be treated with HCT (criteria 2, 3 and 5). According to criterion 4, there should be a recognizable latent or early asymptomatic stage. In most SCID patients infections only start after the first few months of life, because they are partly protected from infection by maternal antibodies. In addition, several studies have shown that the survival significantly increased if treatment is started in the asymptotic phase of the disease.[58] A retrospective study on 43 SCID patients diagnosed in the Netherlands (De Pagter *et al.* manuscript in preparation), showed a high mortality rate (41.4%) caused by fulminant and opportunistic infections. In addition, they showed that SCID is associated with a diagnostic and therapeutic delay, which is believed to reduce the curative treatment outcome.

Hopefully, newborn screening for SCID will soon be implemented in the Netherlands for early diagnosis of SCID, so that treatment can be initiated before clinical symptoms and complications with irreversible organ damage occur.

Table 1. World health organization (WHO) criteria for newborn screening*

	Criteria
1	The condition sought should be an important health problem
2	There should be an accepted treatment for patients with recognised disease
3	Facilities for diagnosis and treatment should be available
4	There should be a recognizable latent or early symptomatic stage
5	There should be a suitable test or examination
6	The test should be acceptable to the population
7	The natural history of the condition, including development from latent to declared disease, should be adequately understood
8	There should be an agreed policy on whom to treat
9	The cost of case finding (including diagnosis) should be economically balanced in relation to possible expenditure on medical healthcare as a whole
10	Case finding should be a continuing process and not a "once and for all" project additionally

*World health organization (WHO) criteria for newborn screening.[71, 72]

DNA REPAIR IN LYMPHOCYTE DEVELOPMENT

Fundamental studies in PID patients with a recombination defect are important for better understanding of the immune system, but also contribute to knowledge on DNA repair. Several DNA repair pathways are known to be involved in the three processes requiring DNA repair during lymphocyte development, i.e. V(D)J recombination, SHM and CSR. These include classical and alternative NHEJ, base-excision repair, and mismatch repair. In addition to these, ATM, NBN,[75] MRE11,[76] RAD50,[77] RNF168,[78] BLM, and LIG1,[79] must be involved in lymphocyte development as well, because mutations in these genes are associated with immunodeficiency (Table 3 **General Introduction**).[80] However, not for all these genes, the exact role in lymphocyte development is known. It is therefore challenging to study the effect of genetic defects in these genes on V(D)J recombination, CSR and SHM. The role of ATM has been described in **Chapter 4.2**. Here, two examples of new studies are discussed.

Role of XLF in V(D)J recombination

In this thesis, we show for the first time a new role of XLF in V(D)J recombination (**Chapter 4.1**). The function of XLF has mostly been studied in DNA repair, but not so much in V(D)J recombination. During DNA repair XLF stimulates LIG4 activity,[81, 82] forms filaments with XRCC4 to keep the DNA ends together in a ligation synapse, [83-86] and is essential for gap-filling by polymerase (pol) λ and polμ during NHEJ.[87] In **Chapter 4.1** we have shown that XLF-deficient B-cells have reduced numbers of N-nucleotides inserted in their IG heavy chain junctions, resulting in shorter CDR3 regions, and consequently resulting in less

Table 2. Junction characteristics of *in vitro* recombination assay

	Deleted nucleotides	N-nucleotides	P- nucleotides
Control (5)	7.4	0.0	0.4
Control + TdT (15)	4.5	2.0	0.4
Control + TdT+ XLF (6)	9.8	1.3	0.0
XLF-deficiency (23)	9.1	0.4	0.0
XLF-deficiency +TdT (12)	8.3	0.5	0.1
XLF-deficiency +TdT+XLF (13)	4.2	0.9	0.3

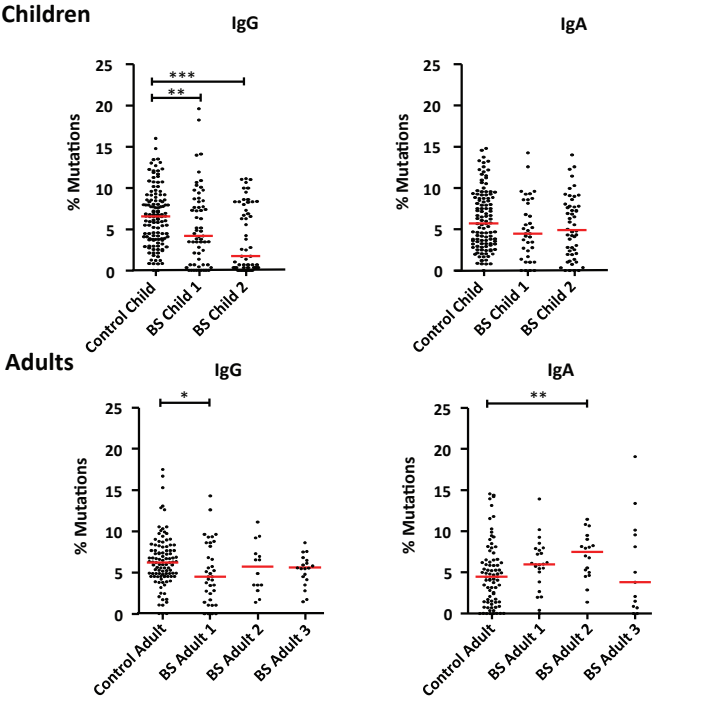
junctional diversity. These data suggest that XLF is essential for addition of N-nucleotides by TdT. This newly described function for XLF is closely related with its function in gapfilling by polλ and polμ.

Polλ, polμ and TdT are members of the polX family of polymerases,[88] Bertocci *et al*. have shown that the three polymerases have specific functions during V(D)J recombination, that result from their regulated recruitment and not from competition between these enzymes.[89] In contrast to TdT, Polμ is a template-dependent polymerase, and is involved in end-processing of IG light chain junctions.[90] Polλ participates in heavy chain rearrangements, in a step preceding the action of TdT.[89] These data strongly suggests that XLF plays an important role in gap filling and nucleotide addition during V(D)J recombination. To confirm this hypothesis, we performed as pilot study an *in vitro* recombination assay in XLF-deficient fibroblasts complemented with and without XLF and TdT and determined the N-nucleotide addition (Table 2). We could show that complementation with both XLF and TdT resulted in an increased number of N-nucleotides compared to complementation with TdT only, but the difference was not significant. This has several causes. XLF-deficient fibroblasts grow poorly and are therefore hard to transfect. This experiment requires transfection of up to five constructs (RAG1, RAG2, XLF and TdT expression constructs and a recombination substrate), which is technically challenging. The number of junctions analyzed is still too low, and the maximum number of N-nucleotides in these *in vitro* recombination assays is relatively low.[90] Therefore, we have to improve our assay to confirm the preliminary results that N-nucleotide addition is restored when XLF-deficient cells are complemented with XLF. This study can form the basis for better understanding of generation of junctional diversity.

Role of helicases in lymphocyte development

Helicases unwind double stranded (ds) DNA, making the strands accessible for replication and repair proteins. RECQ helicases are known to be involved in DNA repair and several other cellular processes.[91] We choose to study patients with Bloom's syndrome (BS) with mutations in the *BLM* gene. BLM is a member of the RECQL gene family of helicases,[92] together with four additional members: RECQL1, WRN, RECQL4 and RECQL5.[93] Bloom's syndrome is characterized by short stature, photosensitivity, facial abnormalities, mental retardation, malignancies, immunodeficiency, and chromosomal instability. Five BS patients were included in our study, three adults and two children. The BS patients have low to normal levels of T and B cells, but most BS patients have an antibody deficiency (Table 3). SHM and CSR seem differently affected in the children and adult patients. The BS children had reduced percentages of SHM in IgG transcripts, while SHM in IgA transcript was normal (Figure 4A). However, CSR seemed to be skewed toward the more proximal genes in both IgG and IgA transcripts (Figure 4B). The adult BS patient showed normal SHM

A


Children
IgG
IgA
% Mutations
Control Child
BS Child 1
BS Child 2
Adults
Control Adult
BS Adult 1
BS Adult 2
BS Adult 3

B

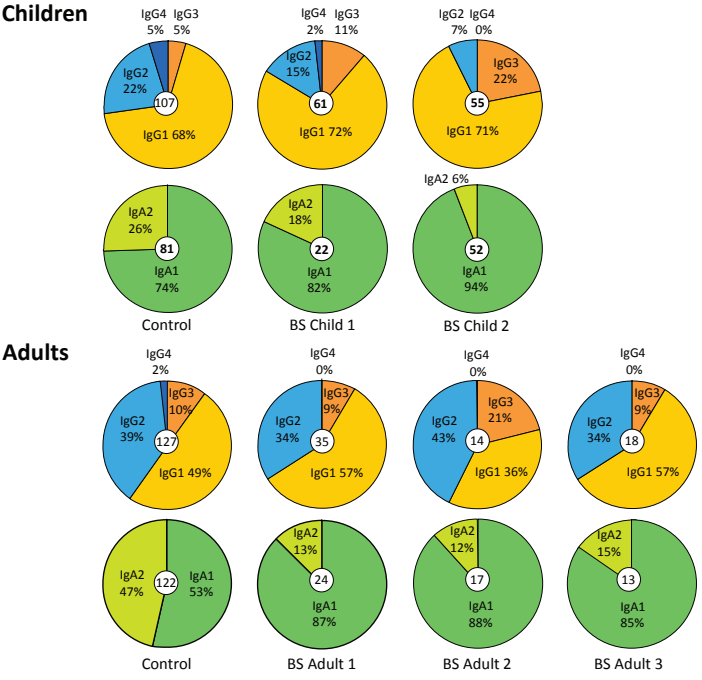

Children
IgG4 5% IgG3 5%
IgG2 22%
107
IgG1 68%
IgG4 2% IgG3 11%
IgG2 15%
61
IgG1 72%
IgG2 7% IgG4 0%
IgG3 22%
55
IgG1 71%
IgA2 26%
81
IgA1 74%
IgA2 18%
22
IgA1 82%
IgA2 6%
52
IgA1 94%
Control
BS Child 1
BS Child 2
Adults
IgG4 2%
IgG2 39%
IgG3 10%
127
IgG1 49%
IgG4 0%
IgG2 34%
IgG3 9%
35
IgG1 57%
IgG4 0%
IgG2 43%
IgG3 21%
14
IgG1 36%
IgG4 0%
IgG2 34%
IgG3 9%
18
IgG1 57%
IgA2 47%
122
IgA1 53%
IgA2 13%
24
IgA1 87%
IgA2 12%
17
IgA1 88%
IgA2 15%
13
IgA1 85%
Control
BS Adult 1
BS Adult 2
BS Adult 3

Figure 4. SHM and CSR in Bloom syndrome patients. A. Percentage of SHM in IgG and IgA transcripts, in children and adult controls and Bloom syndrome (BS) patients. B. CSR in IgG and IgA transcripts, in children and adult controls and Bloom syndrome (BS) patients.

Table 3. Serum immunoglobulin levels in five BS patients

	IgG mg/l	IgA mg/l	IgM mg/l	IgG1 mg/l	IgG2 mg/l	IgG3 mg/l	IgG4 mg/l
BS adult 1	**6.03**	3.04	0.92	**3.73**	1.78	0.36	< 0.05
BS adult 2	**5.32**	1.19	**0.16**	**3.35**	1.08	0.28	0.16
BS adult 3	**6.49**	3.45	1.16	**3.38**	1.73	0.58	< 0.05
BS child 1	6.01	1.45	**0.15**	**3.76**	1.3	0.30	0.24
BS child 2	8.88	0.64	**0.18**	6.68	**0.67**	0.25	< 0.05

Bold numbers indicate reduced values, as compared to age-matched controls

and CSR of IgG transcripts, while CSR was also clearly skewed towards the proximal Cα1 gene in IgA transcripts (Figure 4). These data suggest a role for BLM in B-cell maturation. However, a role for BLM during V(D)J recombination cannot be excluded. The variant AT patients also had low to normal numbers of B cells, but this was caused by compensatory proliferation, and they still showed a reduced diversity of the IGH repertoire (**Chapter 4.2**). Therefore, we will also perform immune receptor repertoire analysis to further investigate this issue in BS patients (see next section).

IMMUNE RECEPTOR REPERTOIRE

Analysis of Ig rearrangements in precursor-B-cells and peripheral B-cells is a powerful tool to get insight into the adaptive immune system of healthy individuals and (immunodeficient) patients. There are several levels of analysis, each providing different information. Junction analysis provides insight in the V(D)J recombination process itself and can be used to identify the defective step in e.g. SCID patients. Analysis of the gene usage can shed light on combination diversity. Finally, with the recent developments in next generation sequencing (such as 454 sequencing), we are now able to make an estimation of the immune receptor repertoire diversity. This has implications for PID patients, but also for other immunological disorders. The different aspects of immune receptor analysis are discussed in this section.

Junction analysis

Analysis of the first rearrangements of the IGH (i.e. DH-JH rearrangements) by cloning and Sanger sequencing, has proven to be informative when studying recombination defects. Abnormalities in the numbers of deletions, N-and P-nucleotides can give clues about defects in the recombination process (Table 4). RAG-deficient patients have a normal distribution of their junctions, because RAG is only important for the induction of the DSBs and not for the processing or the repair. Artemis-deficient patients have increased numbers of P-nucleotides, suggesting asymmetric hairpin opening.[94] In addition, the DNA-PKcs-deficient patient described in **Chapter 2** also had increased numbers of P-nucleotides, indicating a defect in the processing phase of V(D)J recombination. Patients with LIG4-deficiency have increased numbers of deleted nucleotides, probably because of a severe delay in the ligation of the breaks allowing prolonged exonuclease activity.[40]

In **Chapter 3.3** we used junction analysis as a diagnostic tool to identify the candidate gene in an atypical SCID patient without mutations in the coding exons or splice-sties of the *RAG1*, *RAG2*, and *Artemis* genes. Analysis of the DH-JH junctions showed increased the numbers of P-nucleotides, suggesting incorrect hairpin opening by Artemis. Therefore *Artemis* transcripts were sequenced, leading to the identification of the intronic nucleotide substitution resulting in a new splice site.

Table 4. DH-JH junction characteristics

Type of V(D)J recombination defect	Deleted nucleotides	N-nucleotides	P- nucleotides
None			
control	10.2	7.9	0.2
Initiation			
RAG-SCID	12.1	7.7	0.3
Hairpin opening			
DNA-PKcs SCID	6.8	4.2	**3.0**
Artemis-SCID	3.3	4.0	**6.7**
Ligation			
LIG4-SCID	**28.2**	2.8	0.2
XLF-SCID	12.5	**0**	0.2

Aberrant values are indicated in bold.

Junction analysis by using next generation sequencing

All data on the DH-JH rearrangements described in Table 1 is obtained by conventional cloning and Sanger sequencing. This is a laborious method, and the number of unique junctions that can be obtained for analysis is not high (20-100 junctions). However, by use of NGS, more junctions can be studied in a more time-effective and cost-effective way. Since for most of the patients peripheral blood is available, we chose to study the VH-JH junctions. We used the best available primers set, the frame work 1 BIOMED-2 primers.[95] The VDJ junctions were amplified from DNA in a multiplex PCR using VH1-6 subgroup primers and the JH consensus primer, which were adapted for the use for 454 sequencing (Roche).

Since a large number of unique junctions can be analyzed with NGS, better insight into the V(D)J recombination process is obtained. Several parameters of the IGH repertoire can be studied, including the V, D and J gene usage, and the CDR3 length and composition. For example, in **Chapter 4.1** we have showed that the CDR3 regions of XLF-deficient patients were about 10 nucleotides shorter than controls. Increased CDR3 length and VH4-34 usage are associated with autoimmunity. In **Chapter 3.2** we studied the IGH repertoire of three RAG deficient patients and showed that the patient presenting with autoimmunity indeed had an increased CDR3 length.

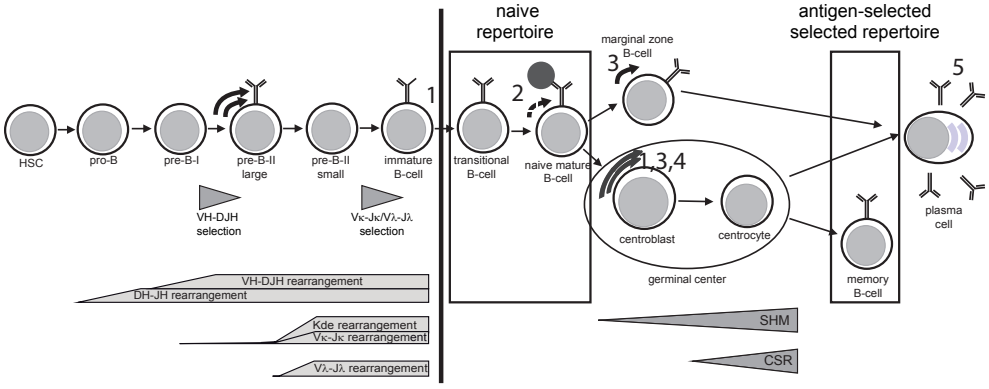


Figure 5. B-cell receptor repertoires. The peripheral B-cell receptor repertoire can be divided into naive and antigen-selected repertoire. The red number indicate the proposed block in differentiation of the CVID patients.

Naive and antigen-selected repertoire

Until recently, we were not able to study the repertoire diversity in depth, but by use of NGS, the repertoire can be studied in much more detail. The B-cell repertoire in peripheral blood can be divided into naive and antigen-selected repertoire (Figure 5). Since the B-cell repertoire changes by SHM and selection processes, it is important to select and sort the correct B-cell population.

The naive repertoire, present in the transitional and naive mature B cells, has not encountered an antigen, and most closely resembles the initial repertoire formed by V(D)J recombination. This repertoire can be used to study V(D)J recombination processes and the diversity of the naive repertoire. The IGH junctions can be amplified from DNA and from RNA. Junctions amplified from RNA are mostly functional, while amplifying junctions from DNA makes it possible to also study the unproductive rearrangements, which have not been selected.

The antigen-selected repertoire is found in cells which encountered antigen, and is therefore different from the initial repertoire, because it has undergone SHM with subsequent selection. Depending on the research question, different B-cell repertoires can be studied. Antigen-selected repertoire is mostly studied at RNA level, by amplifying the transcripts with primers located in or upstream of the V gene and in the constant gene. This enables to study both SHM and CSR, and amplification is less influenced by inefficient primer annealing on positions of mutations in the J gene.

Currently, the antigen-selected repertoire is studied by conventional cloning and Sanger sequencing. The PCR products amplifying the VDJ junctions spliced to the constant region are long, depending on the primers used, between the 500-800 base pairs. Only a few NGS platforms can handle those long reads. In addition, somatic mutations introduced during SHM make the analysis of the antigen-selected repertoire more complicated. Every sequencing platform has a specific error rate, but because somatic mutations are introduced in the IGH junctions, it is difficult to determine if a mutation is a technical error or a somatic mutation. Therefore, new bioinformatics algorithms should be developed.

Large scale analysis of repertoire

The strength of analyzing repertoire by NGS is the number of rearrangements that can be analyzed, but at the same time the amount of data makes the analysis time-consuming. Therefore analysis tools are required that can handle large numbers of sequences. This first step after data gathering is alignment of the rearrangements to the reference sequences and assignment of the genes. The most updated online database with all V, D and J reference sequences can be found in the International ImMunoGeneTics Information system (IMGT).[96] They also offer the tool IMGT/HIGH V-quest, wich assigns the V, D and J genes and gives information about junction characteristics like, functionality, CDR3 length, and

junction composition.[97] This free tool is online available, but the disadvantage is that it cannot be used locally on your computer and implemented in a data pipeline. Another commonly used tool is IgBLAST.[98] This free online tool, which can also be used locally on your computer, can also make use of the IMGT database for reference sequences, but it

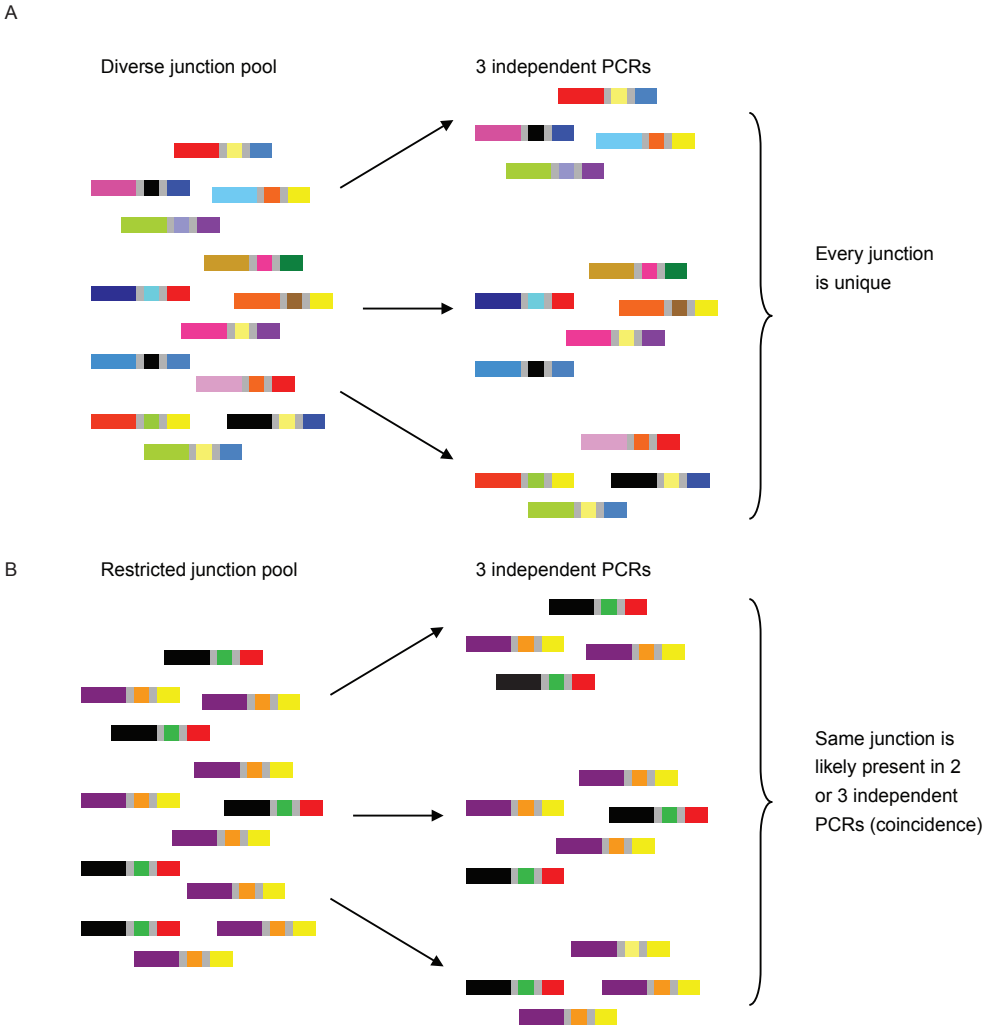


Figure 6. Approach to measure repertoire diversity. A. An immunocompetent individual has a very diverse junction pool. If three independent PCR will be performed on this pool, every junction is likely to be unique. B. An immunodeficient individual has a more restricted junction pool. If three independent PCRs will be performed on this pool, the same junction is likely to be present in more PCR (coincidence).

uses slightly different criteria to determine e.g. the CDR3 region. Therefore the results form IMGT and IgBLAST are not completely overlapping.

Analysis and visualization of the data cannot be done by IMGT or IgBLAST. Not many analysis or visualization tools are available, but the immunoglobulin analysis tool (IgAT)[99] is a useful tool for the visualization of V, D and J gene usage and several CDR3 characteristics. However, we wanted to develop an own pipeline that can visualize the V, D and J gene usage in different ways, but also a tool that is able to calculate the diversity of the repertoire by using coincidences.

Boyd *et al.* developed an algorithm that calculates the IGH repertoire size and diversity.[100, 101] This method is based on the number of unique junctions that are present in parallel PCR amplifications. An immune competent individual has a very diverse junctional pool. If VDJ junctions would be amplified from this pool by e.g. three independent PCRs, none of these junctions would likely to be present in more than one independent PCR (Figure 6A). However, the junctional pool of an immunodeficient individual is not so diverse. Amplifying junctions from such pool, is likely to result in the presence of the same junction in more than one independent PCR (Figure 6B). The occurrence of a similar junction in more than one PCR is called coincidence.

We are currently developing our own analysis and visualization pipeline that will become publically available (Figure 7). The tool can analyze the data via IgBLAST which is integrated into the tool, but it can also handle the IMGT/HIGH V-quest output file. In case of using IgBLAST, NGS data should be transformed into fasta files, which can be uploaded to the custom Galaxy platform.[102-104] In case of using IMGT/HIGH V-quest, files should be uploaded to IMGT/HIGH V-quest and the output files can be uploaded to the custom Galaxy platform. Further processing will be done in the 'R' programming language[105] to generate the tabular and graphical outputs. This tool was already used to generate the visualization and calculation of the coincidences in **Chapter 3.2**, **Chapter 4.1** and **Chapter 4.2**. These (online) analysis tools, make the analysis of large amount of data much more time-effective and visualization of the data easier.

Repertoire diversity in immunodeficient patients

The estimated T and B cell repertoire in immunocompetent individuals is more than 10^{12}. However, defects in V(D)J recombination result in defects in the generation of the BR and TR, leading to (strongly) reduced repertoire diversity. We propose an immune repertoire model, in which the immune status of an individual on a scale between immunodeficient and immunocompetent is determined by the diversity of the immature repertoire and the potential to select and further fine-tune antigen-specific cells that form the peripheral repertoire (Figure 8). We believe that certain immunodeficiencies, like AT, NBS, a subgroup of CVID, and maybe even BS patients have a reduced repertoire diversity. In addition, there

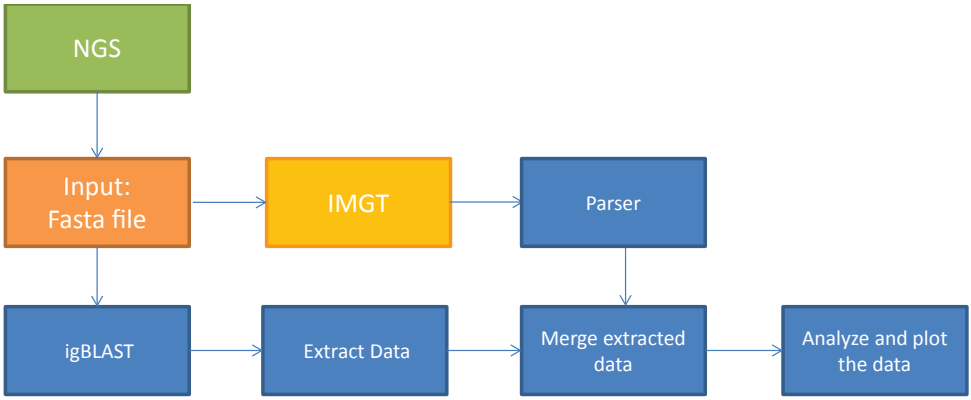


Figure 7. Pipeline for analysis and visualization of IGH rearrangements. From NGS data, fasta files can be extracted. These can either be uploaded into the pipeline (blue) or can be uploaded to the IMGT/HIGH V-quest and subsequently these output file can be uploaded into the pipeline. In the pipeline, the data is extracted or converted and individual PCRs of the same individual can be merged, finally the data is analyzed and visualized. This Repertoire analysis pipeline is being developed by Andrew Stubbs, Michael Moorhouse and David Vetter in collaboration with the Department of Immunology at Erasmus MC, Rotterdam, NL.

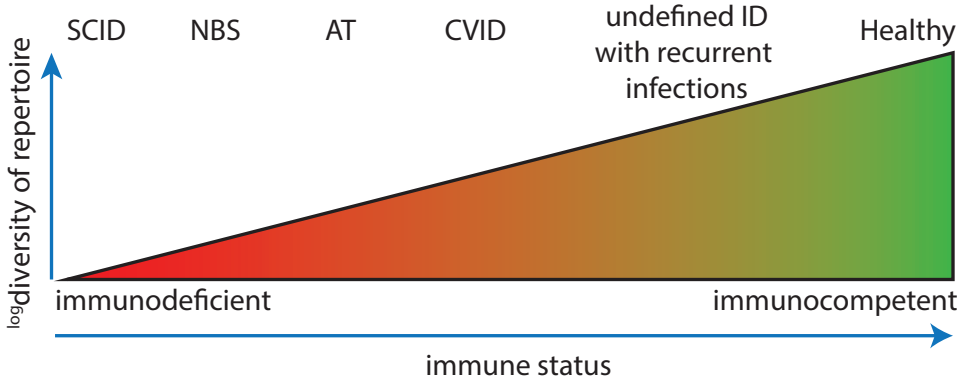


Figure 8. Immune repertoire model. Immune repertoire model. The immune status is dependent on the size and diversity of the immune repertoire. Primary immunodefiencies will be used as disease model. SCID, severe combined immunodeficiency; AT, Ataxia Telangiectasia; NBS, Nijmegen Breakage Syndrome;
CVID, common variable immunodeficiency; ID, immunodeficiency.

is a group of patients with an undefined immunodeficiency but with recurrent infections, who might also have a "gap" in their repertoire.

In **Chapter 4.2,** we studied 15 patients with mutations in the *ATM* gene resulting in classical AT, classical AT with hypogammaglobulinemia, or variant AT. These patients had low numbers of transitional B cells, suggesting reduced B-cell output from the bone marrow. In addition, the naive mature B cells showed increased compensatory proliferation. Together, this suggests a restricted B-cell repertoire. We used the above described approach and amplified the VDJ junctions from naive B cells with three independent PCRs and used next generation sequencing to analyze a large number of junctions. Uniqueness of the junctions was defined by the V, D and J genes and the nucleotide sequence of the CDR3 region. Subsequently, the coincidence of the unique junctions was determined in between the three independent PCRs. The AT patients had increased frequency of coincidences, indicating a reduced naive IGH repertoire.

Similarly to the AT patients, Driessen *et al.* described that a subgroup of CVID patients also had reduced naive-B cells which showed extensive proliferation history.[106] This study encompasses a complete analysis of the peripheral B-cell maturation to get better insight in B-cell defects in CVID. They proposed a classification based on B-cell maturation pathways (Figure 5). Five different groups with proposed immunological defects were identified: 1) B-cell production/DNA DSB repair defect, 2) early-maturation defect, 3) B-cell activation/proliferation defect, 4) germinal center defect, 5) post-germinal center defect. Interestingly, the B-cell maturation pattern from the patients in group 1 resembles the B-cell phenotype of patients with AT **(Chapter 4.2)** and NBS,[107] suggesting that these patients also have a reduction in their naive IGH repertoire. To study the diversity of the repertoire six independent PCRs were performed per sample. Subsequently, the occurrence (coincidence) of a unique junction (defined as unique combination of V, J and amino acid sequence of the CDR3) in these six independent PCR was determined, and the clonality of the repertoire was calculated according to Boyd *et al.*[100] using our data pipeline (Figure 7). In this study two patients per CVID pattern[106], one NBS patients and one control were tested. Per patient 5,000 to 50,000 unique IGH junctions were analyzed. The CVID patients with B-cell pattern 3 to 5 had similar clonality scores as controls and no reduction in the naive repertoire was observed. However, the NBS patients and the CVID patients with B-cell pattern 1 and 2 had a relatively high number of coincident IGH junctions. This resulted in a 10-100 times higher clonality score (Table 5), indicating that these patient indeed have a reduced IGH repertoire. This study confirms that the proposed CVID classification based on B-cell maturation patterns, results in homogenous CVID groups with a similar pathophysiological defect, and that patients from CVID group 1 and 2 are likely to have recombination defect.

Table 5. Coincidences in CVID and NBS patients

		unique junctions						coincidences			
	unique sequences	1	2	3	4	5	6	1	2	3	clonality score
NBS4	16291	1987	1859	3615	3257	3521	2052	14169	1040	14	9.9E-06
CVID1-1	5825	791	696	1102	1173	1034	1029	5779	23		1.7E-06
CVID1-2	21888	3067	2558	1650	4661	5488	4464	21666	102	6	6.2E-07
CVID2-1	18555	3570	2419	3055	3318	3555	2638	18305	125		8.8E-07
CVID2-2	21502	3840	3656	3537	3674	3615	3180	21270	116		6.1E-07
CVID3-1	28813	4938	5314	3947	4677	5732	4205	28787	13		4.1E-08
CVID3-2	33943	5022	6043	4943	6111	7384	4440	33903	20		4.4E-08
CVID4-1	23926	3165	4992	5109	3136	3509	4015	23860	33		1.4E-08
CVID4-2	48057	7844	8495	9267	9489	5865	7097	47982	36	1	4.2e-08
CVID5-1	54103	9860	8091	8161	11667	7134	9190	54045	29		2.5e-08
CVID5-2	43866	7474	7583	6248	8963	6548	7050	43830	18		2.4E-08
control 1	30198	15453	3352	11393				30180	9		3.8E-08

Further applications of repertoire analysis

Next generation sequencing is a powerful tool to study the immune receptors and has many applications. Repertoire analysis can be used to study the V(D)J recombination process, e.g. what is the influence of the recombination signal sequences on the V, D and J gene usage. Furthermore, we now mainly focused on repertoire diversity in immunodeficient patients, but we don't know how the diversity in healthy individuals changes over time. It is speculated that elderly people have a more restricted repertoire. Furthermore, if a link between the site of antigen interaction (CDR3) and peptide interaction can be made, we can study which receptors play a dominant role in immune responses, autoimmunity and allergy.

CONCLUSIONS

The studies described in this thesis indicate that there is considerable clinical and immunological heterogeneity in SCID and other recombination deficiencies caused by DNA repair defects. Early diagnosis and identification of the genetic defect is important for the treatment of the patients. We expect that this will become easier in the near

future if neonatal screening for SCID will be added to the national screening program. In addition, new techniques like targeted sequencing of the known SCID genes, and whole exome/genome sequencing will soon be implemented in PID diagnostics. This will lead to the identification of additional SCID candidate genes and will also give new insights in multi-genetic disorders, such as CVID.

Recombination defects not only result in classical SCID or OS, but there is a wide clinical heterogeneity. Immunologically the patients can present as typical SCID, OS or CID. Null mutations result in typical SCID, but hypomorphic mutations can result in SCID, OS or CID. The clinical phenotype of patients with hypomorphic mutations is hard to predict, since patients with the same gene defect or even similar mutations give rise to a heterogeneity in the clinical presentation. The clinical phenotype of a patient depends on the complex interplay between the (limited) immune receptor repertoire, (auto)antigen exposure, the specificity of the antigen receptors and cell type involved in the immune activation. Patients with DNA repair disorders do not only have an immunological phenotype, but they are also sensitive for ionizing radiation, have increased susceptibility for developing malignancies, and can present with neurological abnormalities.

Studies in PID patients with a recombination defect are not only important for the better understanding of the immune system, but also contribute to knowledge on DNA repair. Besides mutations in NHEJ factors, mutations in other DNA repair proteins are also associated with immunodeficiency. Further research is necessary to unravel the exact role in lymphocyte development for these genes.

Until recently, we were not able to study the immune receptor repertoire in depth. However, NGS finally allows to study the diversity of the repertoire. In this thesis we used a new approach to show that immunodeficient patients have a reduced repertoire diversity. This approach in not only applicable for PID, but can also be used to study repertoire development in immunocompentent individuals, as well as in other more frequent immunological disorders such as allergy and auto-immunity.

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Addendum

Tables with PCR primers and Taqman probes

Abbreviations

Summary

Samenvatting

Dankwoord

Curriculum Vitae

PhD Portfolio

Publications

TABLES WITH PCR PRIMERS AND TAQMAN PROBES

Table 1. PCR primers for short tandem repeat marker (STR) analysis[a]

Gene	Marker	Location	Forward primer (5'-3')	Forward label	Reverse primer (5'-3')	PCR product size	GenBank accession
Artemis	D10S506	upstream	AACACCACACCCAGCCTG	HEX	GATTCACCAACACCACT-GGT	260-331bp	L16275
Artemis	D10S1664	upstream	AACCGTAATAACTTAGGTGCTC	HEX	TGCTGAAGACAGGTAAA-GAG	160-198 bp	Z52497
Artemis	D10S191	upstream	CTTTAATTGCCCTGTCTTC	FAM	TTAATTCGACCACTTCCC	124-152 bp	Z16510
Artemis	D10S674	downstream	GCTGGGCCTAGAGTTTCTT	FAM	GAGGTGGGTGAATCATTT-GA	218-254 bp	G08805
DNA-PKcs	D8S1460	upstream	CACATTCTTGGCCATAGGTT	FAM	TGCAATGTTTCCCTGAGTTT	125-140bp	G10130
DNA-PKcs	D8S359	upstream	TGGCTGAGAACAGTGGTAGG	FAM	AGAATGGGG-TAGTTCCTTCAC	108-130bp	
DNA-PKcs	D8S531	downstream	TTAGGCTGGAGTCCT-GACCTTTTTA	FAM	GGGAACATTGT-CACTTTCACGG	114-120bp	Z23342
DNA-PKcs	AF075268	downstream	AACAATGTTACCCCCGCCT	FAM	GTGTGACACAAGGAAAT-GCC	157 bp	

See Chapter 2 and 3.1

Table 2. PCR primers and Taqmana probes for quantification of expression levels of RAG, Artemis and the in vitro recombination substrate pDVG93

Target	Forward primer	Primer sequence (5'-3')	Reverse primers	Primer sequence (5'-3')	Taqman probe	Probe sequence (5'-3')
RAG1 [a,b]	RAG1_F	TGAGTAATATCAAC-CAAATTGCAGACA	RAG1_R	GGATCTCACCCGGAACAGC	T-RAG1	CCCCAGATGAAATTCAGCACCCACATATTAA
RAG2 [a,b]	RAG2_F	GTTTAGCGGCAAAGATTCA-GAGA	RAG2_R	GTCCATCAAAATTCATCAGTGAGAA	T-RAG2	CAAAAATCTACGTACCATCAGAAACTAT-GTCTCTGCA
Artemis WT[c] exon 10-12	Artemis exon 10F	GGAGAAAGGAG-CAGAAAAACAAA	Artemis exon 12R	TGGATATGCGTTCACAGGACA	TR Art exon 11	AGCTCTGTATGAACTCTCTCCAGTCCTCA-CAA
Artemis mutant[c] ex11+190nt	Artemis exon 10F	GGAGAAAGGAG-CAGAAAAACAAA	Artemis patient 2R	GGCAATAAAGCGAGACTCCAT	TR Art exon 11	AGCTCTGTATGAACTCTCTCCAGTCCTCA-CAA
Artemis WT[c] exon 6-8	Artemis ex6ex7F	ACTCCGGGGGCAGAGTCA	Artemis ex8R	GCTTCGGACCAGCTCTAAGACT	TR Artemis ex7ex8	ACACTCCTCCCGACTTGGAATTTGGTAAAA
Artemis mutant[c] ex5-ex7	Patient 2 ex5ex7F	ACTGTCCGGGATCAGT-TATAGTCA	Artemis ex8R	GCTTCGGACCAGCTCTAAGACT	TR Artemis ex7ex8	ACACTCCTCCCGACTTGGAATTTGGTAAAA
Artemis mutant[c] ex6+18nt	Patient 1 ex6nt18F	ACTCCGGGGGCAGGTACT	Artemis ex8R	GCTTCGGACCAGCTCTAAGACT	TR Artemis ex7ex8	ACACTCCTCCCGACTTGGAATTTGGTAAAA
pDVG93 recombined[d]	DG89	CACAGCAGCGGCCATATC-GAAGGTCG	DG147	TACATTGAGCAACTGACTGAAATGCC	FM23	CTCCATTTTAGCTTCCTTAGCTCCTG
pDVG93 total[d]	DpnI U	GAGCGTCAGACCCCGTAGAA	DpnI L	TAGCTCTTGATCCGGCAAACA	T-DpnI	TTTCTGCGCGTAATCTGCTGCTTGCA

boeckx *et al.* (2002) *Leukemia* **16**:368-375
see Chapter 3.2
See Chapter 3.3
See chapter 3.1 and 3.2

Table 3A. PCR primers used for next generation sequencing of the IGH rearrangements[a]

Gene	A (forward) adaptor	Key	MID (see table b)	Template-specific sequence
VH1 FR1[b]	CGTATCGCCTCCCTCGCGCCA	TCAG		GGCCTCAGTGAAGGTCTCCTGCAAG
VH2 FR1[b]	CGTATCGCCTCCCTCGCGCCA	TCAG		GTCTGGTCCTACGCTGGTGAAACCC
VH3 FR1[b]	CGTATCGCCTCCCTCGCGCCA	TCAG		CTGGGGGGTCCCTGAGACTCTCCTG
VH4 FR1[b]	CGTATCGCCTCCCTCGCGCCA	TCAG		CTTCGGAGACCCTGTCCCTCACCTG
VH5 FR1[b]	CGTATCGCCTCCCTCGCGCCA	TCAG		CGGGGAGTCTCTGAAGATCTCCTGT
VH6 FR1[b]	CGTATCGCCTCCCTCGCGCCA	TCAG		TCGCAGACCCTCTCACTCACCTGTG
Gene	**B (reverse) adaptor**	**Key**	**MID (see table b)**	**Template-specific sequence**
JH cons[b]	CTATGCGCCTTGCCAGCCCGC	TCAG		CTTACCTGAGGAGACGGTGACC

a. See Chapter 3.2, 4.1, and 4.2

b. Van Dongen *et al.* (2003) *Leukemia* **17**:2257-317

Table 3B. Multiplex identifier (MID) sequences[a]

Multiplex identifier (MID)	Sequence MID
MID1	ACGAGTGCGT
MID2	ACGCTCGACA
MID3	AGACGCACTC
MID4	AGCACTGTAG
MID5	ATCAGACACG
MID6	ATATCGCGAG
MID8	CTCGCGTGTC
MID10	TCTCTATGCG
MID13	CATAGTAGTG
MID14	CGAGAGATAC
MID15	ATACGACGTA
MID16	TCACGTACTA

a. See Chapter 3.2, 4.1, and 4.2

Table 4. PCR primers for IGK rearrangements[a]

Gene	Sequence (5'-3')
Vκ1[b]	GTAGGAGACAGAGTCACCATCACT
Vκ2[b]	TGGAGAGCCGGCCTCCATCTC
Vκ3[b]	GGGAAAGAGCCACCCTCTCCTG
Vκ4[b]	GGCGAGAGGGCC-ACCATCAAC
Cκ	ACTTTGGCCTCTCTGGATA

a. See Chapter 3.2

b. Van Zelm *et al.* (2005) *J Immunol* **175**:5912-22

Table 5. PCR primers used for somatic hypermutation and class switch recombination analysis[a]

Target	Primer sequence (5'-3')
VH3 leader	ACCATGGAGTTTGGGCTGAG
VH4 leader	GAACATGAAGCACCTGTGGTTCT
CαL	CGGGAAGACCTTGGGGCTG
Cγ consensus	CACGCTGCTGAGGGAGTAG

a. See Chapter 3.3 and 4.2

LIST OF ABBREVIATIONS

AID	activation-induced cytidine deaminase
ADA	adenosine deaminase
AK2	adenylate kinase 2
A-EJ	alternative end joining
AON	antisense oligo nucleotides
APE	apyrimidimic endonuclease
AT	Ataxia telangiectasia
ATM	ataxia-telangiectasia-mutated
AIHA	autoimmune hemolytic anemia
AIRE	autoimmune regulator
BAFF	B cell-activating factor
BCG	Bacille Calmette-Guerin
BER	base excision repair
BR	B-cell receptor
BcR	B-cell receptor complex
BREC	B-cell receptor excision circle
BS	Bloom's syndrome
BM	bone marrow
XLF	Cernunnos/XRCC4-like factor
CSR	class switch recombination
CID	combined immunodeficiency
CVID	common variable immunodeficiency
CDR	complementary determining region
C	constant gene
CMMR-D	constitutional mismatch-repair deficiency
D	diversity
LIG4	DNA ligase IV
DNA-PK	DNA-dependent protein kinase

DNA-PKcs	DNA-PK catalytic subunit
DN	double negative
DP	double positive
DSB	double strand break
ds	double stranded
DMD	Duchenne muscular dystrophy
EBV	Epstein Barr virus
ESID	European society of immunodeficiencies
Exo1	exonuclease 1
GFP	green fluorescent protein
H	heavy chain
HSCT	hematopoietic stem cell transplantation
ICL	idiopathic $CD4^+$ T lymphocytopenia
ITP	idiopathic thrombocytopenic purpura
ISP	immature single positive
IMGT	ImMunoGeneTics Information system
IG	immunoglobulin
IVIG	intravenous immunoglobulin
IR	ionizing radiation
J	joining
Kde	kappa-deleting element
KREC	kappa-deleting recombination excision cir
MMR	mismatch repair
MNC	mononuclear cells
MID	multiplex identifier
NGS	next generation sequencing
NBS	Nijmegen breakage syndrome
NHEJ	nonhomologous end joining
N	non-templated
NLS	nuclear localization signal
OS	Omenn syndrome

P	palindromic
PB	Peripheral blood
PIKK	phosphoinositidyl 3-kinase related kinase
PID	primary immunodeficiencies
RIDDLE syndrome	radiosensitivity, immunodeficiency, dysmorphic features and learning difficulties
RS	radiosenstive
RAGD	RAG deficiency
RAGD	RAG deficiency
RAG	recombination activating gene
RSS	recombination signal sequences
SCID	severe combined immunodeficiency
STR	short tandem repeat
SP	single positive
ss	single stranded
SHM	somatic hypermutation
SCT	stem cell transplantation
TREC	T cell receptor excision circles
TR	T-cell receptor
TdT	terminal deoxynucleotidyl transferase
TSA	tissue-specific antigen
UNG	uracil-N-glycosylase
V	variable
WES	whole exome sequencing
WGS	whole genome sequencing
WT	wild type
XLA	X-linked agammaglobulinemia

SUMMARY

Every day our body is exposed to many different pathogens. These pathogens or antigens can be recognized and eliminated by our immune system. T- and B-lymphocytes are part of the adaptive immune system. These lymphocytes recognize pathogens with a specific antigen receptor, called the T-cell receptor (TR) and B-cell receptor (BR), respectively.

The antigen receptors are unique for every antigen, therefore an enormous diversity of these specific antigen receptors is needed. To achieve this, the TR and BR have a variable domain that is unique for every T or B cell. This variable domain consists of a "variable" (V), "diversity" (D), and a "joining" (J) gene. For every V, D, or J gene multiple variants are present in the DNA. By recombining different V, D, and J genes many different antigen receptors can be created. This recombination of the antigen receptor genes takes places during T-and B-cell development, and is called V(D)J recombination.

During V(D)J recombination DNA in the antigen receptor loci is cleaved by the so called recombination activating genes 1 (RAG1) and RAG2. Subsequently, the DNA is ligated by a common DNA repair pathway, called non-homologous end joining (NHEJ). The joining of the antigen receptor genes is not precise; nucleotides can be removed or randomly inserted. This causes even more variation of the antigen receptors. The total repertoire of the T- and B- cell receptors is estimated to be more than 10^{12}.

Defects in V(D)J recombination result in a block in B- and T-cell differentiation, leading to severe combined immunodeficiency (SCID). The adaptive immune system of SCID patients is not functioning properly, therefore they have difficulties with protecting the body against bacteria and viruses. Soon after birth SCID patients become very ill and they suffer from recurrent opportunistic infections, protracted diarrhea, and failure to thrive. Without adequate treatment these patients will die. Current treatments for SCID are hematopoietic stem cell transplantation, and in an experimental setting gene therapy.

SCID is an inherited disorder, and is caused by mutations in genes important for V(D)J recombination. Most of these genes are not only important for the immune system, but are also important for the repair of DNA double strand breaks in all cells of our body. Patients with defects in these DNA repair genes are therefore also sensitive for ionizing radiation, but they can also have other defects, such as microcephaly (small head) and growth retardation.

Many mutations identified in the V(D)J recombination genes result in a complete loss of V(D)J recombination activity, these mutations give rise to the classical SCID phenotype. However, some mutations result in residual V(D)J recombination activity, these mutations are called hypomorphic mutations. The clinical phenotype of patients with hypomorphic mutations is not always SCID. These patients can have low numbers of T and B cells.

However, these cells can be autoreactive, and therefore give rise to autoimmunity. The aim of this thesis was to unravel the clinical and immunogenetic diversity of SCID.

In **Chapter 2** we described the identification of a new candidate gene for SCID. This gene, called *DNA-PKcs*, was known to be mutated in horses, dogs and mice suffering from SCID, but mutations in humans were never identified. The patient we described in **Chapter 2** had a classical presentation of SCID, with early onset of recurrent infections, protracted diarrhea and failure to thrive. The DNA-PKcs protein is a large protein with many functions. We were able to show that the mutated DNA-PKcs protein had residual activity, and the main defect was the inability to activate another protein, called Artemis.

The chapters in **Part 3** of this thesis concern the clinical and immunological spectrum of recombination defects. In the last decade it became clear that V(D)J recombination defects not only result in T-B-SCID or Omenn syndrome (OS), but several patients with an atypical presentation have been described in literature. In **Chapter 3.1** we describe a patient with such an atypical presentation. During childhood the patient presented with a single course of extensive chickenpox and moderate albeit recurrent pneumonia, but otherwise she remained disease-free for at least 10 years using prophylaxis. Over time, a subset of T cells, called CD4+ T cells were low, a condition called CD4+ lymhocytopenia. Because the cause of this was not known, this patient was diagnosed with idiopathic CD4+ T lymphocytopenia. The genetic defects underlying this disease were mutations in the *RAG1* gene. This gene is responsible for cutting the DNA during V(D)J recombination. This case extended the spectrum of *RAG* mutations from severe immune defects to an almost normal condition.

In **Chapter 3.2** we studied 22 SCID patients with similar mutations in the *RAG1* gene. Unlike you would expect, the clinical presentation of these patients was not the same, but rather diverse. Some patients presented with the "classical" SCID phenotype, other patients presented with Omenn syndrome (OS) and others had a milder presentation, which we called combined immunodeficiency (CID). Patients with OS also suffer from recurrent infections, protracted diarrhea and failure to thrive, but in addition they have an expansion of T cells that causes an inflammatory skin disease, called erythroderma. The patients with CID were diagnosed after one year of age and they had an expansion of a subset of T cells (γδ T cells) that are normally not so frequent. Different immunological parameters were studied, but no substantial differences were identified that could explain the difference in clinical presentation. Therefore we hypothesized that the clinical outcome of an individual RAG deficient patient, depends on a complex interplay between the (restricted) immune receptor repertoire, (auto)antigen exposure, the specificity of the limited number of antigen receptors, and the timing and cell type involved in the immune activation.

In **Chapter 3.3** we described two unrelated patients, who had a very mild clinical phenotype. During the first 10 years of life they suffered from recurrent infections necessitating

antibiotic prophylaxis and intravenous immunoglobulins, but they did not receive bone marrow transplantation. Initially the genetic defect was not identified in these patients because the coding regions of the SCID genes did not show mutations. However, several diagnostic tools suggested a genetic defect in the *Artemis* gene. This gene is involved in the processing of the DNA breaks during V(D)J recombination. By analysis of the non-coding regions of the *Artemis* gene, we identified mutations that influence the splicing of the *Artemis* gene, resulting in a mutated Artemis protein. This study showed that NHEJ defects should be considered in patients presenting differently from the "classical" SCID patients, and diagnostic tools can be very helpful to identify the genetic defect.

In **Chapter 3.4** we described a patient with a mutation in the DNA repair gene *LIG4*. Patients with mutations in DNA repair genes have, besides the immunological problems, also other problems such as sensitivity for ionizing radiation and neurological abnormalities. This is also described in literature for patients with LIG4 deficiency. However, the patient described in this chapter had a very severe clinical phenotype that has not been described before. The patient was born with primordial dwarfism and several morphological abnormalities. Furthermore, the patient had very serious neurological defects, he did not only had microcephaly, but also parts of the brain were malformed. All mutations in the LIG4 gene described in literature result in residual activity of the protein. However, the mutations identified in this patient result in no or very low residual LIG4 activity. This also explained the very severe clinical phenotype observed in this patient.

The last part of this thesis (**Part 4**) described the mechanism for antigen receptor repertoire development. To be able to cope with many different pathogens there needs to be a large diversity of the immune receptors. This diversity is created by recombination of the different immune receptor genes (combinatorial diversity) and by processing of the DNA (junctional diversity). In **Chapter 4.1** we described the role of the XLF protein on junctional diversity. The XLF protein is involved in the ligation of DNA ends. XLF-deficient patients have microcephaly, and are sensitive for ionizing radiation, however the immunodeficiency is milder. In our study we showed that XLF deficient patients have defects in processing of the DNA during V(D)J recombination, resulting in less junctional diversity.

The total diversity of the B-cell receptors is more than 10^{12}, however with the current methods we were only able to measure the diversity until 10^{3}. Recently, next generation sequencing techniques were developed, which allows sequencing of many sequences in a short time . By using this method we could study the immune receptor repertoire in more detail and identify reductions in the diversity of the immune repertoire, which were not previously identified. In **Chapter 4.2** the immune receptor repertoire of patients with Ataxia Telangiactasia (AT) was studied. This disease is caused by mutations in the *ATM* gene, which is involved in sensing DNA damage. These patients present with neurological problems and have an antibody deficiency. By using NGS to study the immune receptors,

we were able to show that these patients have a reduced diversity of their repertoire in addition to the antibody deficiency.

The studies in this thesis showed the clinical and immunological diversity of recombination defects. They showed that recombination defects do not only present as SCID, but almost in a normal condition. Furthermore, this thesis showed that mutation in DNA repair genes do not only give rise to immunological problems, but also to serious non-immunological problems. Therefore clinicians should be aware that patients with dwarfism and neurological abnormalities can have DNA repair defects.

These studies also showed that the clinical presentation is difficult to predict, and is dependent on multiple factors, including the limited antigen receptor repertoire. By using the innovative NGS technique, we were able to measure this limited antigen receptor repertoire. This does not only give more insight into the V(D)J recombination process, but could in the future also be used to determine to which antigens an individual is protected.

SAMENVATTING

Iedere dag wordt ons lichaam blootgesteld aan verschillende ziekteverwekkers. Deze ziekteverwekkers of andere lichaamsvreemde bestanddelen (antigenen) kunnen worden herkend en geëlimineerd door het immuunsysteem. T en B cellen zijn witte bloedcellen die hierbij een belangrijke rol vervullen. Deze T en B cellen hebben op hun celoppervlak een receptor die antigeen kan herkennen (antigeenreceptor). De B cellen kunnen deze antigeenreceptor ook uitscheiden, dit worden antistoffen genoemd.

Een antigeenreceptor of antistof is specifiek voor een antigeen. Aangezien het aantal mogelijke antigenen enorm is, zijn er minstens zo veel antigeenreceptoren nodig. Om dit te bereiken hebben de antigeenreceptoren een variabel gedeelte dat uniek is voor iedere T- of B-cel. Dit variabele gedeelte bestaat uit een "variable" (V), "diversity" (D), en "joining" (J) gen, of alleen een V en een J gen. Voor elk van deze V, D en J genen zijn verschillende varianten aanwezig in het DNA. Tijdens de T- en B-cel ontwikkeling worden één van deze V, D en J genen aan elkaar gekoppeld, dit proces noemen we V(D)J recombinatie. Door combinaties te maken van de verschillende V, D en J genen ontstaan veel verschillende antigeenreceptoren.

Tijdens V(D)J recombinatie worden DNA breuken geïnduceerd in de antigeenreceptor genen door de RAG1 en RAG2 eiwitten. Vervolgens worden de DNA breuken aan elkaar gezet door een veel gebruikte DNA herstel methode, genaamd non-homologous end joining (NHEJ). Het aan elkaar zetten van de DNA uiteinden gebeurd niet precies, DNA bouwstenen (nucleotiden) kunnen worden weggehaald of willekeurig worden ingevoerd. Dit zorgt voor nog meer variatie van de antigeenreceptoren. De totale variatie (repertoire) van de B en T cel antigeenreceptoren wordt geschat op meer dan een biljoen ($>10^{12}$).

Fouten in het V(D)J recombinatie proces kunnen leiden tot een ernstige gecombineerde immuundeficiëntie (SCID). Patiënten met SCID hebben geen goed functionerend immuunsysteem, en hebben daarom moeite hun lichaam te beschermen tegen ziekteverwekkers. Patiënten met SCID worden snel na de geboorte ernstig ziek. Ze krijgen ernstige infecties waar gezonde kinderen niet ziek van worden (opportunistische infecties), diarree en gedijen slecht. Zonder adequate behandeling overlijden deze patiënten gedurende hun eerste levensjaar. De huidige behandeling bestaat uit beenmerg transplantatie of, in experimentele setting, gentherapie.

SCID is een aangeboren afwijking en wordt veroorzaakt door mutaties in genen die belangrijk zijn voor V(D)J recombinatie. Een groot deel van de eiwitten betrokken bij V(D)J recombinatie zijn niet alleen belangrijk voor het immuunsysteem, maar hebben ook een belangrijke rol in het herstel van DNA schade in alle cellen van het lichaam. Patiënten met een defect in zo'n DNA schade herstel eiwit zijn daardoor gevoelig voor ioniserende

straling, daarnaast kunnen ze andere afwijkingen hebben zoals een klein hoofd (microcefalie) of een groeiachterstand.

Veel SCID patiënten hebben mutaties die er voor zorgen dat er helemaal geen V(D)J recombinatie meer mogelijk is, dit leidt tot het ernstige klinische beeld SCID. Er zijn echter ook mutaties waarbij er nog wel een klein beetje V(D)J recombinatie mogelijk is, dit noemen we hypomorfe mutaties. Het klinische beeld van deze patiënten lijkt niet altijd op SCID. Deze patiënten kunnen nog wel hele lage aantallen T en B cellen hebben, maar het gevaar is dat deze cellen "niet goed zijn" waardoor ze bijvoorbeeld het eigen lichaam aan kunnen vallen. Het doel van dit proefschrift was het ontrafelen van de klinische en immunologische diversiteit van SCID.

In **Hoofdstuk 2** beschrijven we de identificatie van een nieuw kandidaat gen voor SCID. Dit gen, genaamd *DNA-PKcs*, was al geassocieerd met SCID in paarden, honden en muizen, maar mutaties in mensen waren nooit gevonden. De patiënt waarin het nieuwe genetische defect werd gevonden, presenteerde zich als een 'klassieke' SCID patiënt. De patiënt had terugkerende opportunistische infecties, diarree en gedijde slecht. Het DNA-PKcs eiwit is een groot eiwit met vele functies. In deze studie hebben we laten zien dat het gemuteerde DNA-PKcs eiwit nog steeds vele functies goed vervulde, echter kon het niet goed een ander eiwit, betrokken bij V(D)J recombinatie, activeren. Hierdoor konden de DNA breuken niet goed hersteld worden.

De hoofdstukken in **Part 3** van dit proefschrift gaan over de klinische en immunologische diversiteit van recombinatie defecten. In het laatste decennium is het duidelijk geworden dat V(D)J recombinatie defecten niet alleen resulteren in een klassieke SCID presentatie, maar er zijn een substantieel aantal patiënten die zich anders presenteren. In **Hoofdstuk 3.1** beschrijven we een patiënt met een atypische presentatie. Tot haar 10^{e} levensjaar had deze patiënt waterpokken gehad en meerdere longontstekingen, maar verder was deze patiënte niet ziek geweest. Deze patiënt bleek lage aantallen T cellen te hebben. De genetische oorzaken bij deze patiënt waren twee heterozygote mutaties in de *RAG1* genen. Dit gen is betrokken bij het knippen van het DNA tijdens V(D)J recombinatie. Deze casus laat zien dat V(D)J recombinatie defecten zich bijna als normaal kunnen presenteren.

In **Hoofdstuk 3.2** hebben we 22 patiënten bestudeerd die allen een soortgelijke mutatie hebben in het *RAG1* gen. Deze patiënten bleken echter niet dezelfde klinische presentatie te hebben. Sommige patiënten presenteerde zich als "klassieke" SCID patiënten, terwijl andere Omenn syndroom (OS) hadden en een grote groep patiënten had een verschillende atypische presentatie die we samen gecombineerde immuundeficiënties (CID) hebben genoemd. Patiënten met OS hebben naast dezelfde symptomen als SCID ook nog een gedisseminеerde roodheid van de huid. De patiënten met CID hadden een mildere presentatie, ze waren pas na de leeftijd van één jaar gediagnostiseerd, en hadden een groot percentage TRγδ=positieve T cellen, een subgroep van de T cellen die normaal niet

veel voor komen (<5%). We hebben verschillende immunologische parameters bekeken, maar er was eigenlijk geen parameter welke het verschil in klinische presentatie kon verklaren. Daarom denken we dat de klinische uitkomst van RAG patiënten afhangt van een complexe samenhang tussen de gelimiteerde immuun receptor repertoire, (auto)antigeen blootstelling, en de specificiteit van de antigeenreceptoren die aanwezig zijn op het moment dat het immuunsysteem geactiveerd wordt.

In **Hoofdstuk 3.3** hebben we nog twee patiënten beschreven met een atypische klinische presentatie. Deze patiënten waren beiden 10 jaar oud en hadden wel een aantal opportunistische infecties gehad waarvoor behandeling nodig was, maar verder hadden ze geen grote immunologische problemen. In eerste instantie was het moeilijk om bij deze patiënten het genetisch defect te vinden, omdat we geen mutatie vonden in de coderende gedeeltes van de bekende SCID genen. Echter, diverse diagnostische analyses wezen in de richting van het *Artemis* gen. Analyse van het niet-coderende gedeelte van dit gen, wezen uit dat beide patiënten een mutatie hadden die de samenstelling van de *Artemis* transcripten veranderde waardoor een mutant Artemis eiwit aanwezig was in de patiënten. Deze studie laat zien dat patiënten met een V(D)J recombinatie defect zich niet altijd als een "klassieke" SCID patiënt hoeven te presenteren en dat diagnostische analyses erg belangrijk zijn bij het opsporen van het genetische defect.

In **Hoofdstuk 3.4** beschrijven we een patiënt met een mutatie in een DNA herstel gen *LIG4*. Patiënten met mutaties in een DNA herstel gen hebben naast immunologische problemen ook nog andere problemen zoals gevoeligheid voor straling en neurologische afwijkingen. Dit geldt ook voor de patiënten met mutaties in *LIG4* die beschreven zijn in de literatuur. Echter, de patiënt beschreven in dit hoofdstuk had een zeer ernstige klinische presentatie die nog niet eerder beschreven was. De patiënt was veel te klein bij de geboorte en had zogenoemde dwerggroei. Verder had deze patiënt meerdere morfologische afwijkingen, zoals afwijkingen aan de handen en voeten. De neurologische afwijkingen waren ook zeer ernstig bij deze patiënt, hij had niet alleen een klein hoofd, maar ook delen van de hersenen waren niet goed aangelegd.

Alle mutaties in *LIG4* beschreven in de literatuur resulteren in restactiviteit van het eiwit. Echter de mutaties in deze patiënt hebben geen of nauwelijks restactiviteit. Dit verklaart ook het ernstige klinisch beeld. Deze studie laat zien dat mutaties in DNA herstel genen niet direct alleen een duidelijke immunologische presentatie kunnen hebben, maar aanwezig kunnen zijn in patiënten met dwerggroei.

Het laatste gedeelte van dit proefschrift (**Part 4**) gaat over het mechanisme van antigeenreceptor repertoire ontwikkeling. Het immuunsysteem moet ons beschermen tegen vele verschillende ziekteverwekkers, daarom hebben we een grote diversiteit (repertoire) nodig van de antigeenreceptoren. In **Hoofdstuk 4.1** wordt de rol van het eiwit XLF in het creëren van junctie diversiteit beschreven. XLF is een DNA herstel eiwit, wat tijdens V(D)J

recombinatie betrokken is bij het aan elkaar plakken van de twee DNA uiteinden. Patiënten met een mutatie in *XLF*, hebben een milder immunologisch probleem vergeleken met andere SCID patiënten, maar daarnaast hebben ze ook neurologische problemen resulterend in een klein hoofd en zijn ze gevoelig voor ioniserende straling. In deze studie hebben we aangetoond dat patiënten met *XLF* mutaties niet goed willekeurige nucleotiden kunnen inbouwen tijdens het V(D)J recombinatie proces. Dit zorgt voor minder variatie in de antigeenreceptoren.

De totale diversiteit van het B cel repertoire wordt geschat op meer dan 10^{12}, echter met de huidige technieken waren we niet in staat om naar een diversiteit van meer dan 10^3 te kijken. Recent is er een nieuwe techniek beschikbaar gekomen waarbij vele duizenden stukjes DNA in korte tijd worden geanalyseerd, deze techniek noemen we next generation sequencing (NGS). Door gebruik te maken van deze methode, konden we het antigeenreceptor repertoire in meer detail bekijken. In **Hoofdstuk 4.2** hebben we een groep patiënten met Ataxia Telangiectasia bestudeerd. Deze patiënten hebben mutaties in het *ATM* gen, dit gen is betrokken bij het detecteren van DNA schade. Door gebruik te maken van de nieuwe NGS techniek hebben we voor het eerst kunnen laten zien dat deze patiënten een verminderd antigeenreceptor repertoire hebben.

De studies in dit proefschrift werpen licht op de klinische en immunologische diversiteit van recombinatie defecten. Ze laten zien dat recombinatie defecten zich kunnen presenteren als SCID, maar soms ook gepaard gaan met een minimale klinische presentatie. Tevens kunnen patiënten met mutaties in DNA herstel genen ook niet-immunologische afwijkingen hebben. Artsen moeten er dan ook bedacht op zijn dat kinderen met groeiafwijkingen of neurologische aandoeningen een DNA herstel defect kunnen hebben. Deze studies hebben echter ook laten zien dat klinische presentatie moeilijk te voorspellen is, en waarschijnlijk afhankelijk is van meerdere factoren, waaronder het aanwezige gelimiteerde antigeenreceptor repertoire. Met behulp van de innovatieve NGS techniek kunnen we dit gelimiteerde antigeenreceptor repertoire nu in beeld brengen. Dit leidt tot meer inzicht over het V(D)J recombinatie mechanisme en zou in de toekomst ook gebruikt kunnen worden om te bepalen tegen welke ziekteverwekkers een individu is beschermd.

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Gelukkig kon ik het onderzoek naar SCID voortzetten tijdens mijn eerste Masterstage op de afdeling genetica in de groep van Dik van Gent. Het laatste jaar van mijn master, heb ik niet aan SCID gewerkt, maar aan ribosoom deficiënties in het lab van Marieke von Lindren op de afdeling Hematologie.

De afgelopen vier jaar als OIO zijn voorbij gevlogen. Ik heb super veel geleerd, en heb veel leuke verschillende onderzoeken mogen doen. Dit proefschrift had er niet gelegen zonder hulp van veel mensen, die ik hieronder wil bedanken.

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CURRICULUM VITAE

Hanna IJspeert was born in Dordrecht on the 15th of January in 1986. In 2004 she graduated from the secondary school at Develstein College in Zwijndrecht. Thereafter, she studied the Biomedical Sciences at the University of Leiden from 2004 to 2007. During this study she did an internship entitled '*T-B-SCID patient with a new genetic defect caused by a mutation in the Non-Homologous End-Joining pathway'* at the department of Immunology (Erasmus MC, University Medical Center, Rotterdam, The Netherlands), under supervision of Dr. M. van der Burg. After obtaining her bachelor degree in 2007, she started the Master Molecular Medicine at the Erasmus University in Rotterdam from 2007 to 2009. During the first Master internship she conducted research at the department of Cell Biology and Genetics (Erasmus MC, University Medical Center, Rotterdam, The Netherlands) under supervision of Dr. D.C. van Gent. During this internship she worked on a project entitled ' *A missense mutation in DNA-PKcs results in a new type of radiosensitive Severe Combined Immunodeficiency',* which was a continuation of the project of her bachelor internship. During her second Master internship she worked on a project entitled '*Role of ribosomal proteins in erythropoiesis'* at the Department of Hematology (Erasmus MC, University Medical Center, Rotterdam, The Netherlands), under supervision of Dr. M. von Lindern and dr. R. Horos). In 2009 she obtained her Master's degree and started her Phd project entitled '*Scope on SCID; unraveling clinical and immunogenetic heterogeneity of Severe Combined Immunodeficiencies'* at the department of Immunology (Erasmus MC, University Medical Center, Rotterdam, The Netherlands), under supervision of Dr. M. van der Burg and Prof. J.J.M. van Dongen. After completion of her PhD project, she will continue the work as a postdoc in the group of Dr. M. van der Burg at the Erasmus MC in Rotterdam.

PHD PORTFOLIO

Name PhD student:	Hanna IJspeert
Erasmus MC Department:	Immunology and Pediatrics
Research school:	Molecular Medicine
PhD period:	September 2009- March 2014
Promotoren:	Prof. Dr. J.J.M. van Dongen, Prof. Dr. A.J. van der Heijden
Co-promotoren:	Dr. M. van der Burg, Dr. N.G. Hartwig

PhD training

In depth Courses at the Erasmus MC

2010	Molecular Medicine
2010	Biomedical English Writing and Communication
2010	Molecular Immunology
2011	Workshop career orientation
2012	Basic introduction course on SPSS

Seminars and Workshops

2009-2014	Seminars and minisymposia at the department of Immunology
2009-2014	Weekly journal club at the department of Immunology
2010	ESID junior workshop Florence, Italy
2011	Get out of your lab (GOL)- days
2011	Symposium Mucosal Immunology at Erasmus MC

National conferences: oral presentations

19 November 2009	Meeting Department of Pediatrics LUMC *Atypical SCID caused by an Artemis splice defect*
18 December 2009	Dutch Society of Immunology (NVVI) Annual Meeting *Atypical SCID caused by an Artemis splice defect*
23 April 2010	Vergadering sectie pediatrische infectieziekten en immunologie *Atypische SCID met een defect in splicing*
7 December 2010	Minisymposium 'Optical Imaging' *Recruitment of proteins to DNA damage induced by the multi-photon laser*

10 December 2010	Werkgroep immuundeficiencties (WID) *RAG1 mutations resulting in N-terminal truncations give a broad spectrum of clinical phenotypes*
17 December 2010	Dutch Society of Immunology (NVVI) Annual Meeting *RAG1 mutations resulting in N-terminal truncations give a broad spectrum of clinical phenotypes*
14 December 2012	Werkgroep immuundeficiencties (WID) Verbreding van LIG4 spectrum door patiënt met zeer ernstig klinisch fenotype
19 December 2012	Dutch Society of Immunology (NVVI) Annual Meeting Spectrum of LIG4 deficiencies is broadened by a patient with a severe clinical phenotype
18 December 2013	Dutch Society of Immunology (NVVI) Annual Meeting *Antibody deficiency in patients with Ataxia Telangiectasia*

National conferences: poster presentation

4 March 2010	14th Molecular Medicine Day, Rotterdam, The Netherlands Artemis splice defects causes atypical SCID and can be restored in vitro by an antisense oligo nucleotide

International conferences: oral presentations

13 June 2013	Roche 454 sequencing event, Brussels, Belgium *Immune receptor repertoire analysis*
13 November 2013	Roche 454 sequencing event, Mannheim, Germany *Immune receptor repertoire analysis*

International conferences: poster presentations

16 October 2010	14th biannual meeting of the European Society of Immunodeficiencies (ESID), Den Bosch, The Netherlands *Artemis splice-site mutations result in atypical SCID*
5 October 2012	15th biannual meeting of the European Society of Immunodeficiencies (ESID), Florence, Italy *Similar N-terminal RAG mutations give a broad spectrum of clinical phenotypes*
25 August 2013	15th International Congress of Immunology (ICI), Milan, Italy *XLF is required for the generation of junctional diversity by TdT*

Committees

2010-present	PhD committee of the Postgraduate school of Molecular Medicine
2011	Organizing committee of the 15th Molecular Medicine day
2013	Organizing committee of the 17th Molecular Medicine day

Memberships

2010-present	Junior member of the European Society of Immunodeficiencies (ESID)
2009-present	Member of the Dutch Society of Immunology (NVVI)

Teaching at Erasmus MC

2009	Fanconi onderwijs 2nd year medical students (supervision)
2010-2014	Practicum Immunology
2010	Junior Science program
2011	Wintercourse Infection and Immunity II, week 2 (lecture)
2013	Minor 'Ever thought of doing research' (lecture)
2013	Molecular Medicine course (lecture)
2010-2013	Internship supervision (1x bachelor, 2x mast thesis)
2014	Keuzeonderwijs Primaire immuundeficiënties: van kliniek tot genetisch defect (lecture)

Teaching outside Erasmus MC

21 June 2013	Gastcollege Hogeschool Leiden *"Scope on SCID" onderzoek op de grens van de immunologie en de genetica*

Funds

2010	Fund for attendance of the 14th biannual meeting of the European Society of Immunodeficiencies (ESID) from the Trust Fund Association Erasmus University Rotterdam
2013	Travel grant for the 15th International Congress of Immunology (ICI) from the International Congress of Immunology and the Italian Society of Immunology, Clinical Immunology and Allergology (SIICA)

PUBLICATIONS

1. van der Burg M, **IJspeert H**, Verkaik NS, Turul T, Wiegant WW, Morotomi-Yano K, et al. A DNA-PKcs mutation in a radiosensitive T-B- SCID patient inhibits Artemis activation and nonhomologous end-joining. J Clin Invest 2009; 119:91-8.

2. Wiegant WW, Meyers M, Verkaik NS, van der Burg M, Darroudi F, Romeijn R, et al. A novel radiosensitive SCID patient with a pronounced G(2)/M sensitivity. DNA Repair (Amst) 2010; 9:365-73.

3. **IJspeert H**, Lankester AC, van den Berg JM, Wiegant W, van Zelm MC, Weemaes CM, et al. Artemis splice defects cause atypical SCID and can be restored in vitro by an antisense oligonucleotide. Genes Immun 2011.

4. Kuijpers TW, **IJspeert H**, van Leeuwen EM, Jansen MH, Hazenberg MD, Weijer KC, et al. Idiopathic CD4+ T lymphopenia without autoimmunity or granulomatous disease in the slipstream of RAG mutations. Blood 2011; 117:5892-6.

5. Horos R, **IJspeert H**, Pospisilova D, Sendtner R, Andrieu-Soler C, Taskesen E, et al. Ribosomal deficiencies in Diamond-Blackfan anemia impair translation of transcripts essential for differentiation of murine and human erythroblasts. Blood 2012; 119:262-72.

6. Driessen GJ, **IJspeert H**, Weemaes CM, Haraldsson A, Trip M, Warris A, et al. Antibody deficiency in patients with ataxia telangiectasia is caused by disturbed B- and T-cell homeostasis and reduced immune repertoire diversity. J Allergy Clin Immunol 2013; 131:1367-75 e9.

7. **IJspeert H**, Warris A, van der Flier M, Reisli I, Keles S, Chishimba S, et al. Clinical Spectrum of LIG4 Deficiency Is Broadened with Severe Dysmaturity, Primordial Dwarfism, and Neurological Abnormalities. Hum Mutat 2013; 34:1611-4.

8. **IJspeert H**, Driessen GJ, Moorhouse MJ, Hartwig NG, Wolska-Kusnierz B, Kalwak K, et al. Similar recombination-activating gene (RAG) mutations result in similar immunobiological effects but in different clinical phenotypes. J Allergy Clin Immunol 2014.