Immunoglobulin gene alterations in normal and neoplastic B cells
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In the year of the 150th anniversary of Darwin’s *On the origin of species*, his sketch of the tree of life (front cover) has become quite a familiar picture. This dissertation is not about Darwin, but you might say it is all about evolution. B cell affinity maturation depends on ‘Darwinian’ mutation and selection, as Burnet acknowledged when formulating his “Clonal selection theory of acquired immunity”. Only the B cells that acquire the highest affinity for the antigen will be allowed to survive the germinal centre reaction and reproduce (‘survival of the fittest’). The same way of thinking is applied when discussing cancer. Cancer cells gradually accumulate DNA damage, which, in most cases, will be disadvantageous to the cells. But those cells that acquire mutations enhancing their growth rate or survival will eventually outgrow the others. Could Darwin have suspected how his theory would become fundamental in our way of thinking about general biological principles, when he carefully added the words “I think...”? 
"Immunoglobulin gene alterations in normal and neoplastic B cells"

Dissertation, Febe van Maldegem, University of Amsterdam, The Netherlands

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IMMUNOGLOBULIN GENE ALTERATIONS IN NORMAL AND NEOPLASTIC B CELLS

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aan de Universiteit van Amsterdam
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General introduction

**Innate and adaptive immunity**

**Innate immunity**
The first line of defence against pathogens in the human body is made up of physical barriers like the skin and the mucosal layers of the gastrointestinal and respiratory tracts. When pathogens do succeed in penetrating this barrier, the innate immune system will elicit an initial response. Detection occurs through pattern recognition receptors like the Toll Like Receptors (TLR), expressed by epithelial cells or by more specialised immune cells, e.g. macrophages. These receptors bind general motifs such as polysaccharides or CpG-DNA that indicate the presence of pathogens or otherwise inflicted tissue damage. Upon detection of infection the inflammatory response is launched, mediated by the release of cytokines and chemokines that attract white blood cells like granulocytes and lymphocytes.

**Adaptive immunity**
When the infection cannot be quickly resolved, the adaptive immune system is triggered to initiate a humoral and/or cellular immune response, which requires the activation of antigen-specific T and B cells. Activating signals are provided by specialised antigen presenting cells that take up antigen at the site of inflammation and transport this into the draining lymph nodes where it is presented to the lymphocytes. A wide variety of naive B and T cells, each with their own antigen receptor specificity, reside in the primary follicle of the lymph node in anticipation of encounter with their cognate antigen. In response to the right antigen/T cell receptor (TCR)-match, the T cells become activated and, depending on the kind of cytokines present, differentiate into distinct types of cells; some of which will execute the cellular immune response, others become memory cells or T-helper cells. The latter cell type is required for proper activation of the B cells, which do not only require moderate affinity-binding of the antigen to their B cell receptor (BCR),
a membrane-bound immunoglobulin (Ig), but also co-stimulation by cognate T-helper cells through CD40-CD40L interaction. This bipartite stimulation sets off a germinal centre reaction in the follicle (Figure 1). During several rounds of proliferation, immunoglobulin gene diversification and competition for survival signals, B cells are selected for higher affinity of their BCR to the antigen; a process called affinity-maturation. Depending on the type of defense that is required, the high affinity B cells can subsequently change the isotype of the immunoglobulin (Ig) that is produced. Naive

Figure 1 The germinal centre microenvironment.
Antigen-activated B cells differentiate into centroblasts that undergo clonal expansion in the dark zone of the germinal centre. During proliferation, the process of somatic hypermutation (SHM) introduces base-pair changes into the V(D)J region of the rearranged genes encoding the immunoglobulin variable region (IgV) of the heavy chain and light chain; some of these base-pair mutations lead to a change in the amino-acid sequence. Centroblasts then differentiate into centrocytes and move to the light zone, where the modified antigen receptor, with help from immune helper cells including T cells and follicular dendritic cells (FDCs), is selected for improved binding to the immunizing antigen. Newly generated centrocytes that produce an unfavourable antibody undergo apoptosis and are removed. A subset of centrocytes undergoes immunoglobulin class-switch recombination (CSR). Cycling of centroblasts and centrocytes between dark and light zones seems to be mediated by a chemokine gradient, presumably established by stromal cells in the respective zones (not shown). Antigen-selected centrocytes eventually differentiate into memory B cells or plasma cells. Adapted from Klein and Dalla-Favera, Nat Rev Immunol. 2008.
B cells express a BCR of the IgM or IgD isotype, but after class switch recombination IgG, IgA or IgE antibodies can be secreted, with effector functions ranging from complement activation and microbe opsonisation to stimulating or dampening the activity of other immune cells by binding to their Fc-receptors. After achieving sufficient affinity and the right effector isotype for their BCR, the B cells terminally differentiate into either memory cells or antibody producing plasma cells. The countless antibodies that are produced by the plasma cells, will circulate in the bloodstream and constitute the humoral immune defence. As it takes several days before a full-blown adaptive immune response is obtained after initial encounter with an unknown antigen, there are several forms of memory established to reduce response-time in future infections with the same pathogen. A small number of so-called “long-lived plasma cells” reside in the bone marrow for many years, secreting low levels of antibodies into the blood to maintain a certain titer. In addition the germinal centre reaction has also produced memory B cells (and memory T cells), which can be quickly reactivated upon recognition of the antigen through their high affinity BCRs.

The immunoglobulin gene and its diversifications

Immunoglobulin gene rearrangements
The immunoglobulin heavy (IgV\textsubscript{H}) and light (IgV\textsubscript{L}) chain genes encoding the antigen receptors are products of gene-rearrangements taking place during B cell maturation in the bone marrow. For construction of the IgV\textsubscript{H} gene, a combination is made of genes selected from a large gene pool of “variable”, “diversity” and “joining” genes (Fig 2a), and similarly “variable” and “joining” genes are recombined for the IgV\textsubscript{L} gene.\(^2\) The recombinases RAG-1 and RAG-2 assemble these genes in the presence of TdT, an enzyme which adds non-templated nucleotides to the junction. The most variable region of this sequence, the complementary determining region 3 (CDR3) focussed on the junctions of the VDJ-rearrangement, to a large extent determines the specificity of the immunoglobulin gene. The CDR3 sequence can be regarded unique for each B cell and as such enables studies on clonal relationships between B cells, follow B cells development over time, or identify the malignant B cell population in the case of lymphoma.

Affinity maturation
All B cells together constitute a large repertoire of antigen-receptors capable of recognising most antigens upon first encounter, although this occurs generally with low affinity. To be able to fight an infection efficiently, the B cell receptor undergoes affinity maturation, which, as mentioned above, takes place in the germinal centre. Affinity maturation depends on DNA modifications of the immunoglobulin gene, called somatic hypermutation (SHM) (Figure 2b (i)). During SHM, mutations are introduced into the IgV genes at a frequency much higher than the basal level of replication and repair-errors.
Figure 2 Generation and diversification of functional immunoglobulin (Ig) genes.

a. Functional Ig genes are assembled in developing B cells by RAG (rearrangement activating gene)-mediated gene rearrangement resulting in the fusion of germline V, D (in the case of the heavy-chain locus) and J gene segments. This process does not require an encounter with foreign antigen and results in the production of a primary repertoire of B cells displaying a diversity of Igs on their surface.

b. Diversification of functionally rearranged Ig genes is achieved through three distinct genetic programs, all of which are triggered by activation-induced deaminase (AID)-mediated deamination of dC→dU at sites within the Ig locus. (i) Somatic hypermutation (SHM) of the IgV gene. Following antigen encounter in human and mouse, the integrated V(D)J genes in those cells expressing an antigen-specific IgM antibody (albeit one of low affinity) are subjected to a second wave of diversification, this time by SHM. Iterative alternation of SHM and antigen-mediated selection underpins the production of high-affinity antibodies. (ii) IgV gene conversion. In chickens and rabbits, diversification of the integrated V(D)J gene is achieved by replacing segments of it using neighbouring (and, in the case of chicken, non-functional) IgV genes as template. (iii) Class-switch recombination (CSR). In addition to affinity maturation through iterative mutation of the IgV gene, encounter with antigen also results in CSR, which causes the IgM-expressing B cell to exchange its constant region (IgC) for that of one of the other isotypes (IgG, IgA or IgE). Adapted from Neuberger, et al. Trends Biochem Sci. 2003.
detected elsewhere in the genome. The mutations accumulate in certain hotspots (generally complying to the WRCY consensus, where W = A/T, R = A/G, and Y = C/T), indicating that there is a machinery responsible for actively introducing these mutations. In the year 2000, it was found that an enzyme called Activation-induced Cytidine Deaminase is essential for SHM. By deaminating cytidines to uracil in single-stranded DNA, AID is responsible for the initiating lesion (Figure 3). Direct replication of the U:G mismatch results in transition mutations only (C to T and G to A). Alternatively, the resulting U:G mismatch can be recognised by two distinct repair pathways. On the one hand, uracil-DNA glycosylase (UNG), a member of the base excision repair (BER) pathway, can excise the mismatched uracil followed by error-prone repair at the abasic site. Mice deficient for UNG display a perturbed SHM; the mutation pattern at C:G nucleotides is skewed to transition mutations. Transversion mutations, resulting from DNA synthesis opposite an abasic site generated by UNG, are nearly completely absent.

**Figure 3 DNA deamination and repair.**

DNA deamination model of immunoglobulin gene diversification, emphasizing somatic hypermutation and indicating some of the key enzymes implicated in each pathway. Abbreviations: AID, activation-induced deaminase; Exol, mismatch repair exonuclease 1; IgV, immunoglobulin variable region; NHEJ, nonhomologous end joining; MSH2/6, mismatch recognition proteins; Pol, polymerase; Rev1, Y-family DNA polymerase involved in DNA damage tolerance; TLS, translesion synthesis; UNG, uracil-DNA glycosylase; Xrcc2 and Xrcc3, Rad51 paralog proteins involved in homologous recombination repair. Adapted in modified form from Di Noia and Neuberger, Annu. Rev. Biochem. 2007.
On the other hand, the mismatch repair (MMR) machinery (via the MSH2/MSH6 complex) can process the mismatch by removing several nucleotides. This creates a gap that is subsequently repaired by other error-prone polymerases, which preferentially introduce mutations at A:T pairs (figure 3). Mice deficient for the mismatch repair protein MSH2, give another shift in the mutation pattern: There is a striking bias toward mutation at C:G nucleotide pairs, while normally C:G and A:T mutations are found at equal frequencies. Studies on UNG/MSH2 and UNG/MSH6 double knockout mice, with a defect in BER as well as MMR, have SHM restricted to C:G transition mutations, leaving the “AID-footprint”.

Some vertebrates such as birds and rabbits use an alternative way to diversify their immunoglobulin genes. Unlike humans, chickens have only one functional copy of each \( IgV_H \), \( IgV_L \), \( IgJ_H \), and \( IgJ_L \) gene. Similarly, the \( IgV_H \) locus of rabbits only provide V genes from one family, (\( V_H^3 \)) of which the most proximal is used in 80% of the rearrangements. As a consequence, RAG-mediated recombination of these genes provides only a very limited Ig repertoire. Further diversification is achieved by the process of gene conversion (IGC), in which upstream non-functional pseudo V-genes are recombined into the functional V gene (figure 2b (ii)). This allows for more extensive diversification than somatic hypermutation would be able to provide. AID initiates the recombination event by deaminating cytidines in the \( IgV \)-gene and pseudo V-genes similar to the somatic hypermutation process, but recruitment of recombinational repair factors such as RAD51B, XRCC2/3 and BRCA1 results in DNA homologous recombination rather than mutation.

Isotype switching
After affinity maturation, the B cells may change the Fc-tail of their immunoglobulin, depending on the isotype that is needed. This is accomplished by class switch recombination (CSR); the third immunoglobulin gene alteration that is mediated by AID (figure 2b (iii)). Like with SHM and IGC, dC to dU deamination generates the first lesion and subsequent exonuclease activity by the BER and MMR generate an abasic site. AP-endonuclease is able to open the backbone of the abasic site, generating a single strand DNA break. If this event occurs in close proximity at the opposite strand, a staggered double strand DNA break may occur. Next, the non-homologous end-joining pathway (NHEJ), initiated by ku70/ku80 binding at the site of dsDNA breaks, is responsible for ligation of the two recombining strands.
Activation-induced cytidine deaminase

**AID mutants**

The first evidence that AID is required for SHM and CSR, was provided by the generation of a genetic AID knockout mouse model, which showed a complete defect in SHM and CSR, with a phenotype reminiscent of hyper-IgM patients. Hyper-IgM patients are partially immunodeficient, generally lacking class switched Igs (IgG, IgA and IgE) and most are also unable to generate antibodies with sufficient affinity due to a lack of mutations in the immunoglobulin genes (Figure 5A). Not capable of fighting infections efficiently, their immune-responses are characterised by lymph node hyperplasia, due to the presence of giant germinal centres, and sometimes high levels of serum-IgM. One of the genetic defects underlying this disease was known to involve the \textit{CD40L}-gene, rendering the B cells unsusceptible to T-cell help, but in many cases the genetic defect had been undetermined. In line with the findings on the AID knockout mice, re-examination of these patients revealed that a large fraction of these HIGM-syndromes could be attributed to AID-deficiency. Since then, genetic anomalies of numerous HIGM-patients with varying degrees of deficiency have been mapped to the \textit{AID}-gene, providing some interesting information on the structure-function relationship of the protein (Figure 5A).  

There were some patients in which CSR was defective, while their immunoglobulin genes were mutated. This initiated numerous studies into the requirements of the different domains of AID in SHM and CSR. By means of artificial mutants and \textit{in vitro} readouts for deamination, SHM and CSR, it was found that the C-terminus of AID is required for CSR but dispensable for SHM. This CSR defect was not related to targeting of AID to the Ig switch regions, as sequencing demonstrated AID mediated mutation in these regions. Subsequent studies also found that certain N-terminal mutants lacked SHM activity in cell lines, while \textit{in vitro} CSR-activity was retained. In line with the conserved induction of CSR, \textit{S}$_\mu$ switch region mutation was not affected. Altogether these studies found a partial functional defect while deaminase activity of the mutants was not affected, suggesting an important role for cofactors. In the case of the C-terminal mutants, impaired recruitment of CSR specific repair factors may provide an explanation, while the lack of SHM in the N-terminal mutants might point to a substrate- or locus-specific targeting-factor. On the other hand, most recently a mutation at N51 was found to be able to induce CSR, while lacking any in vitro deaminase activity. Unfortunately, these results remain unexplained thus far.  

Much of the information obtained from naturally occurring and artificial mutants is functionally compared to what is learned from studies on AID orthologues; cytidine deaminases from other species such as yeast or fish, and homologues; other members of the APOBEC-family.
The APOBEC-family of deaminases

Zn-dependent deamination is a conserved feature throughout nature, with enzymes acting on free bases, nucleosides and nucleotides. AID was first described as an APOBEC1 homologue specifically expressed in GC B cells. The APOBEC family of cytidine deaminases is named after the first member to be discovered, apolipoprotein B editing catalytic subunit 1 (APOBEC1), which edits the mRNA of apolipoprotein B (ApoB) leading to a shorter form of the protein (ApoB48 instead of ApoB100). APOBEC2 is one of the earliest family members and highly conserved through vertebrate lineages, yet its function and substrate are unknown. APOBEC2 knockout mice do not have a major distinctive phenotype, and recombinant APOBEC2 has not shown any activity in vitro on DNA, RNA or nucleotides. There are several APOBEC3s (A to H), with APOBEC3G as the most well studied form. APOBEC3G, also known as CEM15, functions as a general innate antiretroviral factor and has received much attention since it was discovered as a factor preventing HIV-infection (if in absence of the HIV virion infectivity factor, Vif). The APOBEC3G deaminase activity is targeted to the retroviral replication intermediates, i.e. copy-DNA. Similar antiviral activities can be conducted by other APOBECs and AID, in vitro/overexpression systems, but it is not known whether such activity is part of their natural function as well. APOBEC4 differs from all the other members of the AID/APOBEC family and may be regarded as a distant or ancestral family member of which the function is unknown. In comparison, the APOBEC-family has a communal structural build (Figure 5E), thought to originate from gene-duplication with the AID-gene (AICDA) as the most likely source. It is characterised by an N-terminal cytidine deaminase motif and a C-terminal pseudocatalytic domain of unknown function, the whole of which is present in duplo in APOBEC3B and 3D-G.

Substrate preference

In parallel to APOBEC1, AID was first assumed to be an RNA-editing protein. DNA mutation would be dependent on an RNA intermediate, activated by AID mediated deamination similar to the ApoB deamination by APOBEC1, encoding a protein responsible for the initiating mutating event. When AID was found to be able to mediate somatic hypermutation in non-Ig genes in fibroblasts and E. Coli, this either required ubiquitous expression of the RNA co-factor, or pointed to direct DNA-deamination by AID. In these experiments, the target sequence preference was similar to the mutation hotspots found for the B cell immunoglobulin genes, supportive for the latter hypothesis. Indeed, numerous in vitro experiments using purified AID now have demonstrated that AID preferentially binds and deaminates single stranded DNA. Direct DNA deamination of the Ig gene by AID would generate a uracil in place of the deaminated cytidine. The finding that hypermutation patterns change in the absence of uracil-DNA glycosylase (UNG), which specifically removes uracils from U:G mismatches in DNA (see above), further supported the DNA deamination model.
Targeting

One of the main questions deals with how AID is targeted specifically to the Ig locus, as the answer will be pivotal to our understanding of the role of AID in lymphomagenesis. Already before the discovery of AID it was found that targeting of somatic hypermutation is linked to transcription, as the Ig promotor region was able to induce SHM onto genes that were normally not mutated. Numerous transgene experiments showed that on the one hand transcription from a heterologous promoter was also able to induce SHM at the Ig locus, correlating with transcription levels, while on the other hand the Ig enhancer was able to induce SHM on any non-Ig transgene. The site of integration did not influence the hypermutability of an Ig transgene. Taken together, it was suggested that certain CIS-acting sequences are required for optimal targeting of the SHM machinery. E-box motifs, the core of the binding site for E12/E47 transcription factors, were identified as putative CIS-acting elements increasing somatic hypermutation activity. More recently a new CIS-element was identified, named “DIVAC” for diversification activator, that is required for hypermutation of the Ig light chain gene and sufficient to activate hypermutation at various non-Ig loci in the DT40 B cell line.

Like SHM, CSR requires transcription at both the switch regions that are to be recombined. The transcripts from these switch sequences are non-coding (sterile transcripts), but G-rich and able to form stable DNA/RNA hybrid structures named R-loops (Figure 4). Such R-loops expose large stretches of single-stranded DNA, increasing accessibility for the hypermutation machinery and possibly also augmenting DNA-instability. In addition, the DNA/RNA hybrids slow down the polymerases, which eventually stall and accumulate in the R-loop structures and in this way increase the time.

Figure 4  Proposed DNA conformation during somatic hypermutation and class switch recombination.

A. The small opening that is formed during transcription of the IgV gene, is thought to provide just sufficient space for AID to act on ssDNA, as concluded from in vitro experiments with artificial DNA substrates.

B. Sterile transcription at the G:C-rich Ig switch region results in the formation of stable RNA:DNA hybrids, called an R-loop. Consequently, the displaced ssDNA strand is an optimal substrate for AID to induce the broad DNA-instability that allows DNA rearrangements to occur.
in which AID can access the DNA. Sterile transcription is induced by cytokines. Each cytokine specifically induces a certain switch region, and as such the cytokine environment of the B cell determines the eventual isotype that will result from the CSR. With the availability of new high-fidelity polymerases, reducing background mutation frequencies in PCR-based gene sequencing, it has become clear that targeting of AID is less restricted to the Ig genes as was thought. An extensive sequencing study in UNG/MSH2/AID single, double and triple-knockout mice revealed that AID introduces mutations genome-wide. Comparisons with mice not or only partly affected in repair, suggests that most of such mutations are normally rescued by high-fidelity repair, whereas some genes appear to be favoured by error-prone repair. This bias in repair can be accounted for some level of SHM “targeting”, however still a significant level of physical AID targeting is required to explain the full SHM-preference. This was underlined by the finding that genes containing E box motifs were enriched among the genes mutated in the MSH2/UNG double-knockout mice.

**Transcriptional regulation of AID**

AID transcription from the *AICDA* gene is induced upon B cell activation in the early centroblasts; through CD40 (T-cell help) in combination with BCR cross-linking or Toll-like receptor (TLR) 4 or 9 activation by LPS or CpG. Independently, engagement of members of the TNF-receptor family (e.g. BCMA and TACI) or binding of the cytokine IL-4 to the IL-4 receptor (IL-4R) can induce AID expression. IL-4 simultaneously promotes germline Ig*ε*-Cε and Ig*ε*-Cε transcription, required for CSR to IgG and IgE respectively. Most of these signals work synergistically; although a single signal can achieve moderate levels of AICDA transcription, usually multiple signals are required to establish full-blown levels of AID expression. The environmental signals are translated into transcriptional activation through several intracellular signalling pathways. The most important signalling route leads via the NF-κB pathway, which is activated upon most of the above mentioned triggers. In addition, IL-4R engagement gives a signal to the JAK/STAT pathway, in particular STAT6. NF-κB and STAT6 can both bind directly to the *AICDA* promoter region, and synergistically promote gene transcription. But, this can only be achieved in the presence of several other transcription factors, of which the most important are PAX5, E2A and HoxC4. These are all important factors in B cell maintenance and together form the “enhanceosome” that mediates trans-activation of the AICDA promoter.

At the end of the germinal centre reaction, when sufficient BCR-affinity has been accomplished, it is again a BCR-signal that terminates AID expression through Ca²⁺/calmodulin mediated inhibition of E2A. Other negative regulators are Blimp1, Id2 and Id3, which act by inhibiting the action PAX5, E2A and STAT6. Altogether the combination of signals via NF-κB and STAT6 pathways, the enhanceosome and negative regulating factors, constitute the tight regulation of transcription that is required to prevent AID expression outside the germinal centre.
**Posttranscriptional regulation of AID**

A relatively recent discovery is that protein expression can be downregulated by microRNAs (miRNAs). Specific basepairing of these small hairpin RNAs with complementary binding sites on the target gene transcripts represses their expression, either by promoting degradation or by suppressing translation. miR-155 was first discovered as a locus involved in lymphomagenesis, and found to be expressed in B cells, T cells, macrophages and dendritic cells. Mice lacking miR-155 are viable and undergo normal central-lympoid development, but exhibit substantial reductions in germinal centre B cells. Furthermore, miR-155 was found up-regulated in B cells undergoing CSR. Among the wide array of potential target genes, AICDA was also found to contain a target site for miR-155 and disruption of this site led to an increase in protein expression and as a result increased CSR. Similarly, AID was found to be a direct target of miR-181b, which is one of the many microRNAs that were found down-regulated in B cells upon LPS + IL-4 stimulation. One can envisage that down-regulation of miR-181b is required to permit AID expression in the GC, while up-regulation of miR-155 is important for curtailment and termination of the GC reaction. Exactly in what degree this form of regulation determines the outcome of the eventual expression levels, will be subject of future study.

Studies related to AID expression in chronic lymphocytic leukemia (CLL) and asthmatic patients, reported the detection of a number of AID isoforms. Even though in none of these cases the expression of these splice variants was found to correlate to prognosis independently, it was suggested that alternative splicing could constitute another form of posttranscriptional regulation of AID expression. Recently, Wu et al. addressed four of these splice variants regarding their expression and functionality in CLL (Figure 5B). Only one splice variant per cell was found to be expressed in both normal and malignant B cells, indicating that splicing of AID was regulated. Furthermore, functional analysis demonstrated that none of the variants was able to induce CSR, while two variants displayed hyper-SHM activity. This split in function, combined with the differential expression of the splice variants, suggests a posttranscriptional form of regulation by means of alternative splicing.

**Regulation at protein level**

Mice transgenic for AID develop T cell tumours and lung epithelium pathology, but not B cell lymphomas. A study of conditional AID-transgenic mice, confirmed that there is some form of regulation that protects B cells from the mutagenic effect of AID. Despite high levels of AID-protein in the B cells, there was no increase in CSR nor SHM activity detectable, suggesting that there is regulation of activity at the protein level. This regulation can be two-fold: (1) requirement of activation for the full function of AID, which would be limiting in the B cells of these mice, and (2) active inactivation of the protein to safeguard against hypermutability. Such regulation can be achieved by posttranslational modifications, such as phosphorylation, by increased turnover, nuclear
in/exclusion, or by cofactors that interfere with AID-activity or are required for activation or proper targeting.

**AID regulation by phosphorylation**

The fact that AID is subject to phosphorylation (Figure 5C) was first discovered when AID, purified from transfected HEK293 cells, was not able to deaminate dsDNA substrates despite the presence of replication protein A (RPA), being in contrast with AID purified from B cell nuclear extracts. HEK293-AID was not able to interact with RPA, because it lacked proper phosphorylation at S38 and T27, mediated by protein kinase A (PKA) which is present in nuclear extracts from activated B cells. Mutation of S38 and T27 also led to a defect in SHM and CSR. More recently it was discovered that S38 mutation can be overcome by an additional T40D mutation, in which the aspartic acid mimics phosphorylation of T40. The T40D variation was found to be present in AID variants from several fish species that, concordantly, do not require S38 phosphorylation for interaction with RPA.

Beside S38 and T27, AID was found to be phosphorylated at Y184, of which the function is not yet known, and at T140, S41 and S43. T140 phosphorylation is not B cell specific, and although protein kinase C (PKC) was able to phosphorylate this site (and S38 as well) in vitro, the kinase responsible for T140 phosphorylation in vivo is not yet known. Like with S38, mutation of T140 affects SHM and CSR, although the defect is relatively mild. S41 and S43 phosphorylation was detected in AID purified from Sf9 insect cells, together with S38 phosphorylation. Functional analysis of phosphorylation null mutants showed that there were no differences in deamination activity and processivity on in vitro transcribed dsDNA substrates (contrasting with previous findings for S38), but there was a striking difference in hotspot motif preference. It is not clear what effect a change in hotspot preference or phosphorylation at S41 and S43 in general will have on SHM and CSR in vivo, but the fact that there is a HIgM patient with a S43P mutation at least demonstrates that the presence of a serine at this position is essential for proper AID function. Reviewed by Basu, et al.

**Cellular localisation of AID**

As shown by immunohistochemistry, AID is predominantly localised in the cytoplasm. To acquire access to its DNA substrates, AID would need to be able to enter the nucleus. Indeed it was found that, like APOBEC1, AID shuttles between cytoplasm and nucleus. This intracellular movement is dependent on a C-terminal nuclear export signal (NES), which interacts with the nuclear exporter CRM1: Deletion or mutation of this C-terminus abrogated nuclear export. Similarly leptomycin B (LMB1), a CRM1 inhibitor, resulted in nuclear accumulation of wildtype AID. Similarly an N-terminal nuclear localisation signal (NLS) was identified, required for shuttling into the nucleus. Interestingly, some NES-mutants were found to hypermutate at a higher frequency than AID wildtype, supposedly attributable to its entrapment in the nucleus, suggesting that
nuclear exclusion is one of the regulatory mechanisms by which AID activity can be limited in order to prevent DNA damage.\textsuperscript{16,56} On the other hand, C-terminal deletion of AID was found to impair CSR, as described above. Though, heterologous NES-swaps demonstrated that this was not directly dependent on cytoplasmic localisation.\textsuperscript{58}

**Proteosomal degradation of AID**

Proteosomal degradation is required to enable termination of reactions in the cell, and with high or variable turnover rates, the activity of a protein can be fine-tuned on demand. For APOBEC3G it was shown that the HIV-1 Vif factor restricts the effectiveness of the APOBEC3G protein by targeting it for proteosomal degradation.\textsuperscript{62-65} Whether this occurs via poly-ubiquitination of Vif or APOBEC3G itself is still under debate.\textsuperscript{59} Similarly AID was found to be subjected to proteosomal degradation, induced by poly-ubiquitination.\textsuperscript{60} Especially the nuclear fraction of the protein underwent increased ubiquitination and turnover, with a half time of 2.5 hr compared to the 18-20 hr for cytoplasmically localised AID. Whether this reflects increased ubiquitination of the nuclear fraction by a specific factor, or rather protection of degradation in the cytoplasm has yet to be determined. Interestingly, the sequence of the NES was found to play an important role in protein stability as well, independent of cytoplasmic localisation, but correlating to CSR activity.\textsuperscript{58} It seems that restriction of nuclear life span can be a way in which the genome is protected from the putatively oncogenic AID.

**Known AID interactors**

Since directed AID activity is thought to depend on function-specific co-factors, much study has been focussed on the identification of the molecular partners of AID (Figure 5D). In line with the dependence on transcription of the target locus, AID was found associated with the transcription complex.\textsuperscript{61} It was however not determined whether this was a direct interaction with a protein in the RNA polymerase II (RNA pol II) or indirectly through binding to associated RNA or DNA.

While AID can deaminate a synthetic dsDNA substrate that forms R-loops upon transcription without the requirement of additional factors in vitro, AID needs assistance to target a substrate that contains ample RGYW motifs but does not form large stable transcription bubble structures. This help is provided by RPA.\textsuperscript{47} RPA is predominantly known for its role in DNA replication, where it binds to the ssDNA of a stalled replication fork and interacts with the RecQ-helicases to enhance unwinding of the replication fork up to several hundred basepairs. This allows entry of repair proteins, such as the base excision repair complex, while maintaining the integrity of the replication fork. The interaction of RPA with AID as well as with the RNA pol II holoenzyme suggests that these proteins travel together with the transcription machinery. RPA provides access to ssDNA within the transcription bubble and functions as an anchor for repair proteins such as UNG after dissociation of AID.\textsuperscript{62,63} The interaction between AID and RPA depends on phosphorylation of AID by another interactor: protein kinase A (PKA) as was discussed before.\textsuperscript{52-54}
AID was also found to be associated with the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), and this interaction was dependent on the C-terminus of AID and the presence of DNA as a co-factor. DNA-PKcs plays a role in the repair of DNA double-strand breaks during CSR, and it was suggested that AID provides the scaffold for DNA-PKcs and in this way mediates its recruitment to the DNA.64

With a yeast two-hybrid screen an isoform of the mouse double minute 2 homologue (MDM2) was identified as another protein that binds to the C-terminus of AID.65 This interaction was not essential for gene diversification in DT40 B cells, on the contrary, a slight increase in gene conversion was detected in MDM2-knockout cells, while overexpression of MDM2 decreased AID activity. This suggested a role for MDM2 in the negative regulation of AID. Possibly, this could occur through ubiquitination-dependent degradation, similar to the E3 ligase activity by which MDM2 mediates regulation of p53 and interestingly, also HIV-1 Vif protein levels.66,67 However, the results were not conclusive in this respect. Eventually, AID protein levels were found to be regulated through ubiquitination (discussed above), but a role for MDM2 herein was not investigated yet.

Figure 5  Schematic overview of AID features.
A. Bar graph representing the frequency and distribution of AID mutations found in HlgM patients. Deletions and insertions were omitted. The x-axis corresponds to the amino acid sequence depicted in (C), the y-axis indicates the number of patients. Data obtained from http://bioinf.uta.fi/AICDAbase/ and http://www.hgmd.cf.ac.uk/ac/index.php.
B. Schematic representation of splice variants of AID. Dotted line depicts the inserted intron and omitted (partial) axons.
C. Graphic of the exon distribution, including the amino acid sequence of human AID. The phosphorylation sites are indicated in purple.
D. “Tag cloud” of AID interactors, roughly positioned at the site of (indirect) interaction. The bigger the “tag”, the better defined and confirmed are the relationships.
E. Predicted AID protein structure, based on crystal structures and modeling of AID homologues APOBEC2 and APOBEC3G. In green, the alpha-helices, in blue the beta-sheets. In yellow are illustrated the large protein domains, based on conserved amino-acid motifs, which are indicated above. The black bar graph is a representation of the conservation of the individual amino acids of in total 35 sequences of AID, APOBEC1, APOBEC2 and APOBEC3A-G orthologs, from Homo sapiens, Macaca fascicularis, Mus musculus, Rattus norvegicus, Gallus gallus, Oryctolagus cuniculus, Oryzias latipes, Takifugu rubripes, and Dirofilaria immitis, aligned to human AID. Sequences were obtained from Conticello, et al.24

a DDX5, RNF126 and Tif5β were presented as newly identified AID-interacting proteins during the February 2009 Keystone symposium “B cells in context”. Unpublished data from Nussenzweig, et al., Papavasiliou, et al., and Reina-San-Martin, et al., respectively.
b RNA polymerase II was found to coimmunoprecipitate with AID, but the site of interaction is unknown.
Figure 5  Schematic overview of AID features.
Again by using a yeast two-hybrid system, the interaction between AID and CTNNBL1 was discovered. In absence of this interaction, either by a deficiency of CTNNBL1 or by disruption of the CTNNBL1-binding site (amino acids 39-43) in AID, antibody diversification was severely diminished in DT40 B cells, whereas enzymatic activity of AID was not affected. This suggests that CTNNBL1 might participate in targeting of AID to the Ig locus. Although the precise function of CTNNBL1 is as yet unknown, its association with factors from the splicing machinery forms another interesting link between AID targeting and transcription.

Lymphomagenesis

B cell lymphoma

B cell malignancies can be found across the whole spectrum of the B cell development stages, ranging from precursor B acute lymphoblastic leukemia (pre-B ALL) to plasmacytomas. Most B cell malignancies grow and metastasize in the lymph nodes, the so called “lymphomas”. The first description of a lymphoma was published by Thomas Hodgkin in 1832, now known as Hodgkin’s disease; characterized by the presence of typical giant cells with multiple nuclei (Reed-Sternberg cells). Nowadays, the WHO classification still makes a distinction between Hodgkin and non-Hodgkin lymphoma, although the latter group now consists of numerous very distinctive lymphomas, which makes this subdivision of little added value. The WHO classification, first formulated in 2001, describes 43 different forms of lymphoma. These lymphomas can be distinguished based on localisation, histology of the tissue, morphology of the cells and immunohistochemistry for markers expressed by the cells. In addition, molecular analysis plays an increasingly important role in the lymphoma diagnosis. The detection of well known chromosomal aberrations by FISH or PCR can distinguish between the lymphoma subtypes, such as the API2/MALT1 t(11;18)(q21;q21) chromosomal translocation that is exclusively found in marginal zone B cell lymphomas and the t(14;18)(q32;q21) IgH/BCL-2 as a hallmark of follicular lymphoma. In addition IgVH sequence analysis can establish whether the tumour can be designated as GC or post-GC derived, based on absence or presence of somatic mutations. In CLL the mutational status has become a very important prognostic tool, as unmutated CLLs generally display a more aggressive course of the disease. Table 1 gives an overview of the most common lymphomas, their typical phenotype and recurrent chromosomal aberrations.

AID in lymphomagenesis

Most of the genomic aberrations found in lymphomas result from the temporary genomic instability during immunoglobulin gene formation or diversification, which is reflected by the site of the chromosomal breakpoints. The t(14;18)(q32;q21) IgH/BCL-2 is a fusion product of the BCL-2 and the immunoglobulin JH gene, caused by erroneous
Table 1  List of the most common B-cell lymphomas with their characteristic immunological phenotype and genetic aberrations.

<table>
<thead>
<tr>
<th>lymphoma</th>
<th>normal B cell counterpart</th>
<th>distinguishing markers</th>
<th>common genetic aberrations</th>
</tr>
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<tbody>
<tr>
<td>B-ALL precursor B acute lymphoblastic</td>
<td>pro - pre B cell (unmutated IgV)</td>
<td>TdT</td>
<td>t(12;21) TEL/AML1</td>
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<tr>
<td>leukemia/lymphoma</td>
<td></td>
<td></td>
<td>t(v;11) MLL rearrangement</td>
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<td></td>
<td></td>
<td></td>
<td>t(1;19) EzA/PBX1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>t(9;22) BCR/ ABL</td>
</tr>
<tr>
<td>MCL mantle cell lymphoma</td>
<td>naive (unmutated IgV)</td>
<td>CD5, cyclin D1, BCL2</td>
<td>t(11;14) CCND1(BCL1)/IgH</td>
</tr>
<tr>
<td>MZBCL Marginal zone B-cell lymphoma</td>
<td>marginal zone B cell (mutated)</td>
<td>CD79a, CD27, BCL2</td>
<td>t(11;18) API2-MALT1</td>
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<tr>
<td></td>
<td></td>
<td>CD5-, CD10-</td>
<td>t(14;18) IgH/MALT1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>t(1;14) BCL10/IgH</td>
</tr>
<tr>
<td>FL follicular lymphoma</td>
<td>germinal centre B cell (mutated IgV)</td>
<td>CD10, BCL2, BCL6</td>
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<td></td>
<td>t(3;14) BCL6/IgH</td>
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<td>t(3;2) BCL6/Igκ</td>
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<td></td>
<td></td>
<td>t(3;22) BCL6/Igλ</td>
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<tr>
<td>DLBCL diffuse large B-cell lymphoma</td>
<td>activated or germinal centre B cell (mutated IgV)</td>
<td>BCL6, MUM-1, CD10</td>
<td>t(14;18) IgH/BCL2</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>t(3;v) BCL6 rearrangement</td>
</tr>
<tr>
<td>BL Burkitt lymphoma</td>
<td>germinal centre B cell (mutated IgV)</td>
<td>CD10, BCL6, Ki67, BCL2^-</td>
<td>t(8;14) c-MYC/IgH</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>t(2;8) Igκ/c-MYC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>t(8;22) c-MYC/igλ</td>
</tr>
<tr>
<td>HL Hodgkin lymphoma</td>
<td>germinal centre B cell (mutated IgV)</td>
<td>CD15, CD30, PAX5</td>
<td>-</td>
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<td>CLL chronic lymphocytic leukemia</td>
<td>memory B cell / B1 cells (unmutated and mutated IgV)</td>
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<td></td>
<td></td>
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<td>del (11q) ATM</td>
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<td>del (17p) p53</td>
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<tr>
<td>LPL lymphoplasmacytic lymphoma</td>
<td>plasmacytoid lymphocytes and plasma cells (mutated IgV)</td>
<td>CD20/CD138, CD38, clgM, CD5-, CD10-</td>
<td>-</td>
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<tr>
<td>MM multiple myeloma</td>
<td>plasma cell (mutated IgV)</td>
<td>CD138, CD38, CD79, CD56, CD19- clgκ / clgλ</td>
<td>(14q) rearrangement/amplif.</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(14q) rearrangement</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>del (11q)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>t(11;14) CCND1(BCL1)/IgH</td>
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Figure 6  AID expression in mature GC-type B cell lymphomas.
Representative examples of tonsil, acute lymphoblastic leukemia, follicular lymphoma, diffuse large B cell lymphoma and chronic lymphocytic leukemia, stained with a monoclonal antibody for AID. AID is predominantly expressed in normal GC B cells and their malignant counterparts (diffuse and follicular lymphoma), while lymphomas with immature or memory B cell phenotype, are negative.
recombination during VDJ-rearrangement. There are also breakpoints found in the Ig variable region (e.g. t(8;14) c-MYC/IgH) and the switch regions (e.g. t(3;14) BCL-6/IgH), implicating SHM and CSR in the generation of these translocations.

In 1998 Pasqualucci et al. reported that the proto-oncogene BCL-6 can be found mutated in 30% of normal GC B cells, but not naive cells, and in lymphomas of GC and post-GC phenotype. Later this finding was extended to other oncogenes in DLBCL, such as PIM1, RhoH, MYC and PAX5, and to other lymphomas. Notably, these genes are also implicated in typical lymphoma associated chromosomal translocations, suggesting a role for aberrantly targeted SHM as initiating event in lymphomagenesis. On the other hand, aberrant class switch recombination was also frequently detected in ABC DLBCL, with numerous mutations, insertions, inversions and deletions detectable in the Sμ region, suggesting that such abnormalities in the regulation of CSR could predispose to chromosomal translocations.

To obtain formal proof that AID is involved in lymphomagenesis, several lymphoma prone mouse strains were crossed with AID knockout mice. The first two studies looked for the occurrence of IL6-transgene- or pristane-induced plasmacytomas in AID-/- background. Although both studies reported a reduction in plasmacytoma outgrowth, their conclusions on the occurrence of the typical c-myc/IgH translocations were contrasting. While these chromosomal translocations were undetectable in IL6tg/AID-/- mice, cells bearing these anomalies were found in the pristane-induced hyperplastic lymph nodes, though these never developed further into a plasmacytoma. Additional studies in vitro demonstrated that AID is able to promote c-myc/IgH translocation in primary B cells, which is greatly enhanced in absence of p53 or factors of the NHEJ pathway normally responsible for proper resolution of the switch region breaks. Multiple additional mouse model studies have been dedicated to the role of AID in lymphomagenesis, but most of these provided merely indirect evidence. Nevertheless, based on the findings in human lymphomas and the accumulating evidence from in vitro and in vivo lymphoma models, it is generally accepted that AID plays a key role in lymphomagenesis, by generating DNA instability during SHM and CSR in the GC reaction.

**AID in lymphoma progression**

We found that AID mRNA and protein was found to be highly expressed in some GC-type B cell lymphomas, like DLBCL, BL and FL (ref and Figure 6). These findings may just be a reflection of the GC-phenotype of these lymphomas. On the other hand, ongoing AID activity may constitute a form of genetic instability that favours transformation of a low grade lymphoma to a high grade lymphoma. On several occasions it was reported that lymphoma prognosis correlates with AID expression. However, expression does not always correlate with ongoing SHM. Possible explanations for this phenomenon include: (i) the SHM machinery has been shut off irrespective of AID expression; (ii) ongoing SHM might not always be detectable in the Ig genes, but rather be aimed “off-target”.
References


65. MacDuff DA, Neuberger MS, Harris RS. MDM2 can interact with the C-terminus of AID but it is inessential for antibody diversification in DT40 B cells. Mol Immunol. 2006;43:1099-1108.


AID splice variants lack deaminase-activity

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http://bloodjournal.hematologylibrary.org/cgi/content/full/113/8/1862-a
To the Editor of Blood,

With particular attention we read the article by Wu et al., dealing with regulation of activation-induced cytidine deaminase (AID) by alternative splicing. We have also studied three alternative AID splice forms, identical to the variants AID-ΔE4, AID-ΔE4a and AID-ivs3 as described by the authors. In accordance with Wu et al. we found that these splice variants did not reconstitute class switch recombination (CSR) in AID knockout mouse splenocytes. In the same assay, dominant negative regulation was tested but not found. We also studied somatic hypermutation (SHM), measuring pGFP*-revertants in NIH-3T3 cells, initially with comparable results as the authors. However, we noticed that the occurrence of revertants correlated with the transcription levels, as measured by mean fluorescence of the IRES-YFP. Linear regression analysis of the GFP frequency in relation to the mean YFP (Figure 1B) shows that all transductants exhibit a similar correlation, except for the empty vector control (LZRS-IRES-YFP), which never reaches a mean YFP intensity alike the others. We concluded that the empty vector is not a suitable control for this assay. Additional controls with the cDNAs of CD79a and CD79b inserted in the vector as mock, yielded a comparable number of GFP cells as the full-length AID (AID-FL) and splice variants (Figure 1A). Similarly, two AID-mutants T150A and F151S both gave rise to GFP cells. As T150A was able to induce CSR it was expected to have SHM-activity, but F151S is a mutant known to have caused HlgM syndrome with a defect in SHM, and should therefore lack GFP cells.

In this SHM-assay, false positive results may arise for several reasons: Klasen et al. previously described the occurrence of GFP-reversion due to the action of viral reverse transcriptase. In addition, we found mutations in YFP, and as YFP is a GFP-derivative this might have contributed to the GFP signal. Induction of endogenous AID may be another complicating factor. Mouse AID was detectable in all samples at very low levels, but additional induction due to signaling of the CD79a and CD79b-ITAM motifs was not observed (data not shown). The absence of “aspecific” GFP cells in the empty vector control may be related to the altered distance of YFP to the promoter, or to the lower transcription/transcript levels.

To circumvent the problems with the SHM-assay mentioned and to settle this issue by a more robust biochemical method, we then used an in vitro deaminase assay as described previously. The results, depicted in Figure 1C, demonstrate that the three splice variants are not able to deaminate the cytidine in the oligonucleotide-substrate and are therefore to be regarded as catalytically inactive.

We also studied the cellular localizations of the AID variants, at first using amino-terminally tagged proteins, which all located to the cytoplasm like the authors described. However, treatment with Leptomycin B, an inhibitor of the nuclear exporter CRM1, was not able to trap AID-FL in the nucleus. In contrast, carboxyl-terminally tagging resulted in nucleo-cytoplasmic shuttling for AID-FL as was described previously, and a disturbed cellular localization for the splice variants (Figure 1D). This result is in agreement with
Figure 1  Deaminase activity and cytoplasmic localization of AID splice variants.

A. Dot plot of the somatic hypermutation assay depicting the frequency of GFP+ revertants in YFP+ NIH-3T3 cells. Each dot represents the measurement of one culture well. Virus stocks were applied in serial dilutions, resulting in a spread of the measurements (also visible in Figure 1B).

B. The same measurements shown in panel A depicted as a function of expression levels of the variants (mean IRES-YFP). Linear regression analysis shows the correlations between mutation frequency and expression levels.

C. In vitro deaminase activity: 100 fmol of a 60-nt FAM-labeled oligo, containing one cytidine located in a hot-spot motif, was incubated with 2 μg recombinant glutathione S-transferase (GST)–AID fusion protein. Subsequent recombinant UDG and NaOH heat treatments resulted in a 30-nt product, which was detected in AID-FL and T150A mutant, but not in the GST control, F151S AID mutant, or with any of the splice variants. Input and appropriate size of the recombinant proteins were verified by Coomassie staining (data not shown).

D. HEK293 cells were transfected with C-terminally tagged AID-GFP fusion proteins, and photographed alive at day 2 after transfection. AID-FL was located in the cytoplasm, but LMB incubation with 10 ng/mL for 3 hours resulted in nuclear accumulation. The AID splice variants display a predominantly nuclear or diffuse localization. Images were acquired with a Leica DM5000B microscope and a Leica DFC500 camera (Leica Microsystems, Rijswijk, The Netherlands) at the original magnification of 200×, and were further processed using Adobe Photoshop 7.
the expectations, since AID-ivs3 and AID-ΔE4 both lack the nuclear export signal and deletion of the α-helix might have dislocated it in AID-ΔE4a, as was also discussed by the authors.

Altogether, these results led us to the conclusion that the AID splice variants are non-functional.

References

Chapter 2

Activation-induced cytidine deaminase splice variants are defective due to the lack of structural support for the catalytic site

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Department of Pathology, Academic Medical Center, University of Amsterdam, The Netherlands.

Submitted
Abstract

Recently, conflicting results were reported on the hypermutation activity of AID splice variants. With the generation of single point mutations, we studied the structure-function relationship of the amino acids that are commonly absent from all described splice variants. The results from these analysis pointed to several amino acids that are required for class switch recombination, without perturbing cellular localisation or nucleocytoplasmic shuttling. A defect in deaminase activity was found to underlie this class switch recombination deficiency. Interestingly, the most debilitating mutations concentrated on hydrophobic amino acids, suggesting a structural role for this part of the protein. Indeed, by generating homologous amino acid replacements, class switch recombination activity could be restored. These results are in agreement with recent reports on the protein structure of the AID-homologue APOBEC3G, suggesting a similar protein composition. In addition, the findings underscore that AID splice variants are unlikely to have preservation of catalytic activity.
**Introduction**

By deaminating cytidines to uracils in the immunoglobulin variable (IgV) genes and switch regions, AID generates the key lesions required for somatic hypermutation and class switch recombination, two processes pivotal in the generation of high affinity antibodies.\(^1\) Although instrumental in adaptive immunity, these genome modifying processes entail the risk of unwanted DNA damage, which is reflected in the various translocations involving the IgV genes and switch regions, characteristic for B-cell non-Hodgkin lymphoma. In addition, aberrant targeting and deregulated expression of AID was described in human B-cell lymphomas.\(^2,3\) Indeed, several mouse models confirmed the oncogenic potential of AID.\(^2\) These findings have raised fundamental questions with respect to regulation of expression, targeting, posttranslational modifications and the structure-function relationship of AID.

Studies related to AID expression have reported the existence of a number of AID splice variants.\(^3-6\) Considering a potential role of alternative splicing in the regulation of AID, functional studies of these isoforms were desired. Recently we and others reported that 3 of these isoforms completely lack class switch recombination (CSR) activity.\(^7,8\) Concordantly, we found that recombinant isoform-proteins were incapable of deaminating a synthetic oligonucleotide substrate *in vitro* and that this deficiency was accompanied by a disturbed cellular localisation. Alternative splicing takes place at the intersection of exon 3-4, reflecting the intrinsic weakness of the splice acceptor site of exon 4. Two of these splice variants, designated AID-ΔE4 and AID-ivs3, lack the entire C-terminus encoded by exon 4 and 5, while a third isoform (named AID-ΔE4a) has a relatively small deletion of the first 10 amino acids (aa) encoded by exon 4. These 10 aa, absent from all 3 splice variants, encode the start of the previously designated “pseudocatalytic site” - in analogy to *E. coli* cytidine deaminase - and constitute a part of the APOBEC-like C-terminal domain (CTD) of AID.\(^9,10\) While the C-terminal amino acids of AID, encoded by exon 5, were found to contain the nuclear export signal (NES), and are absolutely required for CSR but dispensable for SHM, very little is known about the function of the remaining part of the CTD of AID.\(^11,12\) Studies on hyper-IgM patients have described three point mutations leading to single aa changes in this region, i.e. M139V, F151S and R174S; all three lacking both CSR and SHM activity.\(^13\) More recently, T140 was found to be subjected to phosphorylation and mutation of this amino acid also led to a reduced SHM and CSR in vivo.\(^14\) By functionally analysing the effect of mutations introduced in the first 10 aa of exon 4, we have aimed to gain more insight in the structure-function relationship of this part of AID. Interestingly, among these are three putative phosphorylation substrates; two tyrosines (Y144 and Y146) and one threonine (T150).
Materials and methods

AID mutants and vectors:
AID wildtype (AID-WT) and mutants were cloned into the following vectors: for the generation of retrovirus into LZRS-linker-IRE5-YFP (a kind gift from H. Spits); for the nucleocytoplasmic shuttling experiments into pcDNA3.1/CT-GFP-topo (Invitrogen, Carlsbad, CA); and for generation of GST-AID recombinant protein into pGEX5x-1 (GE Healthcare, Little Chalfont, United Kingdom).

Human AID-WT was obtained from Ramos cells, by RT-PCR (the primers are listed in table 1). The generation of AID mutants was established using site-directed mutagenesis: First, two fragments were created by PCR (with Phusion polymerase, Finnzymes, Espoo, Finland), combining the AID-fw primer with a mutant reverse primer or a mutant forward primer.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Forward primer 5′-3′</th>
<th>Reverse primer 5′-3′</th>
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<tr>
<td>AID-fw</td>
<td>CTGGACACCACCATGGGAG</td>
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primer with an AID-rev primer (Table 1). These fragments were then used as input for a second PCR with the AID-fw and -rev primers, creating full length mutant AID. AID-fw/rev1 primers were used for direct cloning into pcDNA3.1/CT-GFP-topo, resulting in a fusion protein of AID and GFP connected through the linker-sequence “QGQFCRYPAQWRPLESR”. The AID-fw and -rev2 primers were used for cloning into LZRS and pGEX, of which the latter primer was equipped with a NotI site to enable easy transfer to the target-vectors. The PCR-products were cloned in Topo 2.1, verified using M13-primer mediated sequencing, and used in subsequent digestion and ligation into the target vectors: BamHI/NotI for ligation into the LZRS and EcoRI/NotI for pGEX5x-1. In the resulting GST-AID fusion proteins GST and AID were linked by “IEGRGIPEFALLDTT”.

**Virus production and titration:**
LZRS vectors containing the AID-encoding sequences were transfected with Fugene 6 transfection reagent (Roche Diagnostics, Almere, The Netherlands) into Phoenix Ecotropic packaging cells (ATCC) to produce supernatant containing the retrovirus. The virus titer was measured by applying the supernatants to 3T3 cells in serial dilutions and in the presence of 10 µg/ml polybrene (Sigma Aldrich, Zwijndrecht, The Netherlands), with subsequent analysis of the percentage of infected cells by flow-cytometry.

**CSR assay**
AID knockout mouse splenocytes were depleted from erythrocytes and sorted for B cells with anti-B220 magnetic beads (Miltenyi Biotec, Utrecht, The Netherlands). Cells were cultured in IMDM with 10% FCS, 1% penicilin/streptomycin, 1% glutamin, BSA (100 µg/ml), 0.04‰ beta-mercaptoethanol, 25 µg/ml LPS (Sigma) and 10 ng/ml mIL-4 (Peprotech, New Jersey, USA). The next day, cells were transduced with equal titeres of the AID-WT, AID mutants, or empty vector as negative control, in RetroNectin coated plates (Takara Bio, Shiga, Japan). On day 3 after transduction, cells were analyzed by flowcytometry, based on YFP expression and immunofluorescence of monoclonal anti-mouse CD45R/B220-APC (RA3-6B2, BD Biosciences) and polyclonal goat-anti-mouse IgG1-PE (Southern Biotech, Birmingham, USA).

**Cytoplasmic localisation**
CT-GFP-topo vectors were transfected into HEK293 cells with Fugene 6 transfection reagent according to manufacturer's instruction. After two days, half of the cells were incubated with 10ng/ml Leptomycin B (Sigma-Aldrich, Saint Louis, MI, USA), for 3-5 hours. AID-GFP was visualised in live cells on a Leica DM5000B microscope at 20x magnification, and recorded with a Leica DFC500 camera.

**In vitro deaminase assay**
pGEX5x-1 vectors were transformed into BL21 Ecoli cells (GE Healthcare) and grown
Figure 1  Functional analysis of AID mutants.

A. The first 10 amino acids of exon 4 were each mutated to alanine for functional analysis. The HIgM mutant F151S was included as well (indicated with an asterix).

B. A representative experiment of class switch recombination, by reconstitution of AID knockout B cells with AID-WT, empty vector and AID mutants. Depicted is the percentage of IgG1+ cells among transduced cells.

C. Relative class switch recombination activity compared to AID-WT (set to 100%). Depicted is the mean of 5 measurements, with the standard error of means illustrated in error bars. Class switch recombination activity was completely absent for the mutants of hydrophobic amino acids Y144A, Y146A, W148A, F151A and V152A.

D. In vitro deaminase activity measured for GST coupled AID-WT and AID-mutants, and GST-only as a control. Deamination at the cytidine in the RGYW hotspot is visible as the 30nt product, after treatment with

E.
until OD 0.6-0.8, at which the bacteria were induced with 1mM IPTG, for 16 hours at 16°C. Concentrated bacteria suspensions were lysed by sonications in the presence of 100 µg/ml lysozyme (Sigma) and 60 U/ml DNase I (Roche). GST-fusion proteins were purified from the lysate with GSTrap HP 1ml columns (GE Healthcare) and dialysed in 20 mM Tris-HCl, pH 7.5, 10 mM NaCl, 0.1 mM DTT, 50% glycerol. Protein concentrations were measured on a NanoDrop ND-1000 Spectrophotometer (Isogen lifescience, IJsselstein, The Netherlands) and confirmed by coomassie staining of DNA-PAGE gels loaded with adjusted protein amounts.

Catalytic activity was assessed using an in vitro deaminase assay, in which 100 fmol of a FAM-labeled 60 nt substrate, containing one cytidine in a RGYW-hotspot motif (FAM-5’-TAAAGGTGAAGAGAGAGAGAGAGAGAAGCTGAAAGAGAGAAGAGAGAAGAGAGTGAAGGAG-3’), was incubated over night at 37°C with 1-2 µg purified protein, in 50 mM Tris, pH 7.5, 100 mM NaCl, and 2 µM MgCl2 in a volume of 10 µl. Uridine excision by recombinant uracil DNA-glycosylase (New England Biolabs, Ipswich, MA, USA) and breakage of the abasic site by NaOH heat-treatment, were performed as described previously by Larijani et al15,16, with the modification that 1 µg RNase A was added to the deamination-reaction17. Deamination products were separated by DNA-PAGE and visualised using the Typhoon Trio imager (GE Healthcare).

Results

AID mutants

We generated AID point mutations within the area of the deletion found in splice-variant AID-ΔE4a; ranging from D143 to V152, i.e. the first 10 amino acids encoded by exon 4, as shown in figure 1A. The F151S mutation previously found in a HIgM patient and mapping to this part of AID, was included as well.

Functional analysis of AID point mutants

To measure CSR activity, retroviral reconstitution of AID knockout mouse B cells and stimulation with LPS and IL-4 was used to specifically induce CSR to IgG1. Like AID wildtype (AID-WT), some of the mutants were able to restore CSR, either completely (F145A) or partially (D143A, C147A, N149A and T150A) (Fig 1B+C). Five mutants recombinant UDG and subsequent alkaline cleavage. AID-WT, C147A, N149A and T150A have preserved deaminase activity, while for GST, W148A and F151S no deaminase-product is evident.

E. Cellular localisation of GFP and AID-GFP fusion products, with and without treatment with the CRM1-inhibitor of nuclear export Leptomycin B (LMB). All AID variants are by default localised in the cytoplasm, but accumulate into the nucleus upon LMB treatment demonstrating functional nucleocytoplasmic shuttling.
appeared completely defective for CSR-activity, i.e. Y144A, Y146A, W148A, F151A and V152A. Several of the mutants were tested for inhibitory effects on AID-WT, using double-transduction experiments, but dominant negativity was not found (data not shown).

Catalytic competence was assessed for several of the mutants using an in vitro deaminase assay, in which the presence of a 30 nt digestion product is a reflection of AID-mediated DNA deamination. Similar to AID-WT, the mutants C147A, N149A, and T150A showed conversion of the substrate, while no activity was found for W148A, F151S and V152A (Fig 1D). In the case of these three mutants, the lack of deaminating activity was in agreement with their null-phenotype for CSR.

Similar to APOBEC1, one of its homologues, AID has been shown to shuttle between nucleus and cytoplasm, mediated by its C-terminal nuclear export signal. This was demonstrated by incubation with the CRM1-exportin inhibitor Lemptomycin B (LMB) resulting in entrapment of AID in the cell nucleus. To see whether the catalytic defect of the mutants coincides with an aberrant cytoplasmic localisation, as was previously found for the splice variants, GFP-tagged AID mutants were expressed in HEK293 cells. AID-WT as well as all mutants showed normal cytoplasmic localisation in untreated cells and nuclear accumulation upon LMB-treatment, demonstrating functional nucleo-cytoplasmic shuttling (Fig 1E).

Interestingly, the mutants with the most severely defective phenotype corresponded to the most hydrophobic amino acids, suggesting a function in maintaining structural integrity. To test this hypothesis, several additional mutants were generated in which substitutions were made to amino acids with similar chemical characteristics, i.e. D143E, Y144F, F145Y, Y146F, W148Y and N149D. Indeed, these homologous mutants were able to rescue partial or complete functionality, as demonstrated by restoration of CSR (Fig 2).

![Figure 2](image)

**Figure 2** Rescue of CSR activity by homologous mutation.

CSR activity can be rescued when replacements are made with amino acids of similar biochemical properties.
Discussion

Recently we showed that three of the reported AID splice variants encode truncated proteins which lack catalytic activity and have abnormal cellular localisation. Alternative splicing takes place downstream of exon 3, a sequence normally encoding a part of to the APOBEC-like C-terminus. Although conserved throughout many of the APOBEC-family members, the function of this domain is still largely unknown. The most C-terminal 10 aa encode a functional NES and mutation of this sequence resulted in a defect in CSR, while SHM remained intact. On the other hand, deletion of 10 amino acids from the N-terminal part of this domain, like in splice variant AID-ΔE4a, was sufficient to give a complete defect in catalytic activity in our experiments. By generating artificial point mutants, we studied the function of this area in more detail. Our results showed that the significance of this sequence is confined to the most hydrophobic amino acids, which, when mutated to the neutral alanine, are defective for CSR and deaminase activity. By introducing point mutations we have generated relatively subtle defects. This has revealed that the CSR deficiency of these mutants is not due to an impairment in cellular localisation or nucleo-cytoplasmic shuttling. Furthermore, the results found for the single aa changes in this study, support our previous findings on the AID splice variants, which all have large C-terminal truncations or deletions that share the absence of the first 10 aa of exon 4, studied here. When converting the hydrophobic amino acids to homologous hydrophobic residues, the functionality was restored, indicating that these amino acids are important in maintaining structural integrity. These results rule out a role for phosphorylation at Y144 and Y146. On the other hand, T150A showed only a partial defect in CSR, which was not directly reflected in the cytidine deaminase assay. Hence, phosphorylation on this residue can not be excluded, moreover as similar results were recently reported for the phosphorylation site at T140.

Sequence comparisons of AID and the APOBECs, demonstrate that the amino acids analysed here are relatively well conserved (Fig 3). Based on the crystal structures of APOBEC2 and APOBEC3G, it can be deduced that they constitute an alpha-helix (α5), positioned parallel to the beta-sheet and opposite to the side containing the DNA-binding and Zn-coordinating motifs. Among the numerous APOBEC3G mutants assayed for mutation frequency in bacteria, five were analogous to the AID mutants presented here: F343A, C346A, W347A, F350A and V351A in APOBEC3G, corresponded to Y144A, C147A, W148A, F151A and V152A in AID, respectively. Only one of these five mutants displayed mutation activity detectable above background, i.e. C346A, while the other four lacked mutation activity, which matches with the outcome of our experiments. The APOBEC3G α5-helix makes extensive stabilizing hydrophobic contacts with the beta-strand platform, and as such supports the catalytic site. Similarly we can conclude that the hydrophobic aa of the α5-helix in the APOBEC-like C-terminus of AID, are of essential structural importance in enabling deaminase activity.

Figure 3 Clustal-W sequence alignment of hAID, the C-terminal domain (CTD) of hAPOBEC3G and hAPOBEC2. In blue and indicated with an asterisk are identical residues, in green, with colon, and in dark-blue, with a period, indicates conservation of strong and weak groups respectively. The 10 amino acids subjected to mutation in this study, are coloured yellow. The predicted secondary structure of APOBEC3G-CTD is illustrated above the sequence.


Chapter 3

Germinal centers in human lymph nodes contain reactivated memory B cells

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http://jem.rupress.org/cgi/content/full/jem.20071006/DC1
Abstract

To reveal migration trails of Ag-responsive B cells in lymphoid tissue, we analyzed IgM-V_{H} and IgG-V_{H} transcripts of germinal center (GC) samples microdissected from three reactive human lymph nodes. Single B-cell clones were found in multiple GCs, one clone even in as many as 19 GCs. In several GCs, IgM- and IgG-variants of the same clonal origin were identified. The offspring of individual hypermutated IgG memory clones were traced in multiple GCs, indicating repeated engagement of memory B cells in GC reactions. These findings imply that recurring somatic hypermutation progressively drives the Ig-repertoire of memory B cells to higher affinities and infer that transforming genetic hits in non-Ig genes during lymphomagenesis do not have to arise during a single GC passage but can be collected during successive recall responses.
Introduction

The humoral immune response relies on mature B cells, each producing a unique immunoglobulin (Ig). After a primary antigenic (Ag) challenge, triggered naive B cells can differentiate directly into plasma cells producing a first wave of specific, low-affinity IgM antibodies (Abs). In parallel, germinal center (GC) reactions are initiated which are critically dependent on T-helper cells and are essential to generate B cells with high-affinity Abs of different classes and to produce memory cells. During the GC reaction, B cells undergo a phase of brisk cell division thereby creating the GC dark zone. These rapidly dividing cells, centroblasts, accumulate nucleotide substitutions in their Ig variable region (IgV) genes, a process designated as somatic hypermutation (SHM). Based on competition for survival signals elicited by native Ag presented at the surface of follicular dendritic cells, B cells with Ig variants with highest affinities obtain growth advantage in the GC light zone. In addition, the Ag-selected B cells may switch Ig class due to a genomic recombination process at the IgH locus by which the rearranged V<sub>H</sub> region is juxtaposed to one of the downstream C<sub>γ3</sub>, C<sub>γ1</sub>, C<sub>α1</sub>, C<sub>γ2</sub>, C<sub>ε</sub> or C<sub>α2</sub> constant region genes. The selected, Ig-affinity matured B cells, either or not class-switched, finally differentiate into Ab-producing plasma cells or memory B cells.

The kinetics of the GC reaction have been extensively studied in rodents after immunization with sheep red blood cells or with haptens coupled to carrier proteins. Immunization experiments with T-cell dependent Ags revealed that recognizable GCs are formed within 4-5 days and are maintained for about 21 days. In spleens from mice immunized with (4-hydroxy-3-nitrophenyl)acetyl coupled to chicken-gamma-globulin (NP-CG), SHM in the GCs was detectable starting from day 8 to reach ~3 mutations on average per IgV<sub>H</sub> gene by day 14. Based on stringent selection, GCs finally become oligoclonal and are reported to contain 3-6 Ag-specific B cell clones on average. In man, in situ analyses on lymph nodes and Peyer’s patches showed that the GCs contained 4-13 B-cell clones with functional IgV<sub>H</sub>. As yet, it is uncertain whether B cells can engage in a GC reaction more than once; in secondary arsonate responses in mice, GC B cells were found to carry higher numbers of somatic mutations in their IgV<sub>H</sub> genes as compared to the primary response whereas affinity-enhancing mutations seemed to appear more rapidly. It remained unclear however whether this was due to accelerated SHM rates or recruitment of memory B cells into these responses. At least two groups have reported that in man the IgV<sub>H</sub> mutation frequencies in both peripheral B cells and intestinal plasma cells increase with age, suggesting repeated rounds of Ag-driven hypermutation.

To gain insight in the expansion and dissemination of Ag-responsive B cells in man, we analyzed the clonal B cell composition of 48 GCs of reactive lymph nodes originating from three donors. We observed that single B cells clones seed into multiple GCs, often located at significant distances and evidence was obtained for active class switch recombination (CSR) of B cell clones within individual GCs. Importantly, in the lymph
nodes of three donors, we encountered the offspring of single, hypermutated IgG clones in multiple GCs, indicative of repeated involvement of Ag-experienced B cells in this unique microenvironment.

**Materials and methods**

**Patient material**

All lymph nodes were fresh-frozen in liquid nitrogen shortly after surgical resection. Lymph node 1 (LN1) was a cervical LN removed from a 4 year-old male suffering from sustained lymphadenopathy, LN2 originated from the arteria hepatica communis region and had been removed from a 75 year-old male suffering from pancreatic carcinoma. LN3 was a cervical LN resected out of a 46 year-old woman suffering from chronic sialadenitis. All LNs contained reactive lymph follicles. Of note, LN2 was purely reactive and did not contain carcinoma cells.

The study was performed in accordance with the ethical standards and approved by the research code committee on human experimentation of our institute.

**Immunohistochemistry**

The immunohistochemical stainings were performed on acetone-fixed cryostat sections using the Powervision® detection system (ImmunoVision Technologies, Daly City, CA). Endogeneous peroxidase activity was blocked with 0.1% NaN₃, 0.3% H₂O₂ in PBS. Visualization of antibody binding was performed with 3-amino-9-ethylcarbazole (AEC) (Sigma, St Louis, MO), 0.03% H₂O₂ in sodium acetate pH 4.9. A monoclonal antibody specific for Ki-67 (MIB-1) (DAKO, Glostrup, Denmark) was used.

**Laser-aided microdissection and cDNA synthesis**

Frozen tissue sections of 10 µm were mounted on polyethylene (PEN) membranes (PALM, Bernried, Germany) and briefly stained with haematoxylin for 1 min, followed by gentle rinsing with tap water and finally with distilled water. After air drying, microdissection was performed using the PALM system. Using the 20X objective, tissue pieces with a diameter of about 50 µm were cut out and catapulted in the cap of a microfuge tube containing 20 µl reverse transcriptase (RT) reaction mix. Next, the tubes were incubated, up side down, in direct contact with a heating block at 42°C for 1 hour, followed by an inactivation at 95°C for 10 min. The RT mix contained: 0.1 mmol/l Pd(N)₆ random primers, 8 U/µl molony murine leukemia virus reverse transcriptase (Invitrogen, Breda, The Netherlands), 1 mmol/l of each dNTP and 1.2 U/µl RNase inhibitor (Roche, Almere, The Netherlands) in 1X first strand buffer (Invitrogen).
IgV_{H} amplification by RT-PCR, cloning and sequencing

IgM-V_{H} and IgG-V_{H} transcripts were amplified using VH family-specific leader primers for the VH1, VH3 and VH4 families of the IgV_{H} genes in combination with C_{µ} and C_{γ} primers, respectively. In some experiments, a fluorochrome (FAM) labeled C_{µ} primer was used to enable automated detection of PCR products by genescanning with capillary sequencing equipment. The PCR was performed with 1 μl cDNA in a 25 μl volume and started with 4 min at 95°C followed by 10 cycles of 1 min at 95°C, 30 sec at 57°C and 1 min at 72°C followed by 40 cycles of 30 sec at 95°C, 30 sec at 55°C and 1 min at 72°C, the reaction was terminated for 6 min at 72°C. The PCR was performed in 1.5 mmol/l MgCl₂ using Platinum Taq polymerase and PCR buffers (Invitrogen) according to the manufacturer’s description. In each RT-PCR run, water controls were included, which were in all cases negative. Moreover, three control samples of the PEN membrane were also tested and turned out to be negative. VH1/VH4-IgV_{H} RT-PCR products were cloned into pTOPO-TA-vectors and transformed into TOP10 bacteria (Invitrogen), to generate molecular IgV_{H} clones. Sequencing on both strands was performed using the big dye-terminator cycle-sequencing kit (Applied Biosystems, Foster City, CA). To identify the IgV_{H} germline gene used and the somatic mutations therein, the sequences were compared to published germline IgV_{H} genes, using the Vbase database and DNAplot on internet (http://www.mrc-cpe.cam.ac.uk).

The Taq error rate in our 50 cycle RT-PCR was experimentally determined in an RT-PCR specific for CD20 using two follicular mantle zone samples of LN1. By sequencing 29 clones, the Taq error rate was found to be ~0.3 bp per 300bp.

Results

Laser-aided microdissection and IgV_{H} amplification of germinal center B-cells

Small tissue samples of 40-80 cells were isolated out of haematoxylin-stained, frozen sections of three reactive lymph nodes (LN) from different donors. In order to distinguish GCs with cycling B cells, adjacent sections were immunohistochemically stained for the proliferation marker Ki-67. We thus microdissected tissue samples of 30, 11 and 16 GCs out of sections of LN1, LN2 and LN3, respectively. As controls, samples from follicular mantle zones (FM) surrounding the GCs and samples from T cell areas (TZ) were collected. IgV_{H} transcripts were amplified by RT-PCR, using VH1-, VH3- and VH4- family-specific leader primers in combination with a fluorochrome-labeled C_{µ} primer, allowing analysis by ‘genescanning’ on automated capillary sequencing equipment. Based on length variability of the complementarity determining regions 3 (CDR3s), the samples generally yielded multiple peaks, representing different B cell clones (not shown). In LN1, we observed in 19 GCs a recurrent 481 bp peak in the VH4-C_{µ} PCR (Fig. 1). In LN3, products of the same lengths were obtained out of GC1 and GC2 in the VH1-C_{µ} PCR (not shown). We thus decided to extensively clone and...
sequence VH4-Cµ PCR products of LN1 and LN2 and VH1-Cµ PCR products of LN3. RT-PCR products which were used for cloning, were generated in parallel using an unlabeled Cµ primer. Depending on the availability of material, also IgG transcripts were amplified, cloned and sequenced.

*The offspring of single B cell clones inhabits multiple follicles*

Out of most samples, IgVH products of different lengths were amplified representing B cell clones with unique IgVH compositions. Interestingly, by PCR and cloning we now identified in LN1 and LN3 as many as 7 distinct IgM clones of which the offspring was detected in more than one GC. In LN3, four IgM-VH1 clones were identified, termed B-µ, C-µ, D-µ en G-µ, which were each present in two GCs (Fig. 2 and Fig. S1). The B-µ clone, found in GC1 and GC2 of LN3 comprised 14 sub-clones (each designated by additive lower case letters Ba-Bn), with amino acid sequence differences in their IgVH-
CDR3 regions. Of this major ‘B’ clone, both IgM- as well as IgG-expressing variants were detected, see below and Figs. S2 and S3. In the two GCs the Bg-µ sub-clone was found. In the 24 GCs isolated from two separate sections of LN1, three recurrent IgM-VH4 clones were identified, designated A-µ, H-µ and I-µ. The H-µ and I-µ clones were each present in two GCs. Most striking was the recurrent A-µ clone, which was traced both by genescanning and sequencing in 19 GCs (Figs. 1 and 2). In all the LN1 samples containing the A-µ clone, two identical nucleotide substitutions in the rearranged V4-30.4 IgVH gene were observed, i.e. one silent (S) mutation at codon 37 of FR2 and one replacement (R) mutation at codon 56 of the CDR2. In 5 of the 19 GCs (GC7, 10, 12, 13 and 25), we

*Figure 2* Localization of recurrent IgM and IgG clones in lymph nodes 1, 2 and 3.

To visualize the proliferating B cells within GCs, the lymph node sections were immunohistochemically stained for Ki67 (red) and subsequently counterstained with haematoxylin (blue). Lymph node 1: The red-filled circles indicate the 19 GCs in which the IgM-VH4 A-µ clone was found. GCs highlighted by a yellow triangle (GC5 and GC25) and by a yellow box (GC11 and GC16) contained the IgM-VH4 H-µ and I-µ clones, respectively. GCs highlighted by a yellow star (GC5, 7, 16, 17 and 20) contained the recurrent IgG-VH4 C-γ clone. Lymph node 2: The red-filled GC4 and GC8 harbored the IgG-VH4 G-γ clone. Lymph node 3: The red-filled GCs harbored the four recurrent IgM-VH1 clones, i.e. the B-µ clone in GC1 and GC2, the C-µ clone in GC2 and GC3 and the D-µ- and G-µ clones, both detected in GC11 and GC13. GCs marked by a yellow star (GC10, 11 and 13) contained the recurrent IgG-VH1 J-γ clone. Samples from follicular mantle zones and T cell areas are indicated by (m) and (t), respectively. Unlabelled bars represent 2 mm.
**Figure 3** Selection of the IgM-VH4 sequences and mutations therein of the recurrent A-\(\mu\) clone in four germinal centers of lymph node 1.

Lollipop shaped symbols indicate nucleotide differences as compared with the V4-30.4 germline IgV\(\mu\) gene. Replacement- and silent- mutations are indicated by closed and open circles, respectively. Gray shaded bars at codons 37 and 56 indicate identical somatic mutations found in all A-\(\mu\) clones. The gray shaded mutation at codon 93 in GC3 and GC5, represents an identical mutation which differed from the codon 93 mutation found in all A-\(\mu\) clones of GC11. The total number of clones in which a particular IgV\(\mu\) mutation pattern was found is indicated by (N\(x\)).

**Figure 4** IgV\(\mu\) sequences and mutations therein of related IgM and IgG clones found within individual germinal centers of lymph nodes 1, 2 and 3.

Lollipop shaped symbols indicate nucleotide differences as compared with the respective germline IgV\(\mu\) genes. Replacement- and silent- mutations are indicated by closed and open circles, respectively. Gray shaded bars covering mutations of IgM and IgG clones indicate identical mutations. The total number of clones in which a particular IgV\(\mu\) mutation pattern was found is indicated by (N\(x\)).

detected clones containing these two ‘basic’ mutations only, whereas in 14 GCs (GC1, 2, 3, 5, 11, 16, 19, 21, 22, 23, 24, 26, 28 and 29), daughter clones with additional mutations were detected (Fig. 3 and Fig. S4). All A-\(\mu\) clones of GC11 contained an extra R mutation at codon 93. A dissimilar mutation at codon 93 was detected in 7 clones
derived from GC3 (sub-clones A) and in one clone from GC5 (sub-clone 1G1). This may be coincidental or due to selection for binding of an identical antigenic epitope. Codon 93 is part of a RGYW motif, the preferential target sequence of the hypermutation machinery (Fig. 3 and Fig. S4). Importantly, the A-µ clone was not detected in the tissue samples from the T cell areas nor from the mantle zones (FM), with exception of the FM7 sample. In the latter, two A-µ clones were detected out of a total of 26 sequenced IgM-V_H clones. Overall, shared mutations between the GC samples were found in 4 of the 7 recurrent clones i.e. the A-µ and H-µ clones of LN1 and the C-µ and G-µ clones of LN3 (Figs. S1 and S4).

**Germinal centers contain isotype-switch variants of individual B cell clones**

Within individual GCs of LN1, LN2 and LN3, a total of 11 IgV_H clones were identified of which both IgM and IgG variants were present. In 8 of these 11 clonotypic IgM/IgG sets, at least one replacement mutation in the IgV_H gene was shared between the IgM and IgG transcripts (Fig. 4). Interestingly, in GC16 and GC19 of LN1, IgG transcripts of the A-µ clone were detected. Small amino acid sequence differences in the IgV_H-CDR3 regions were observed between some of the clonal IgM/IgG sets, i.e. in GC16 (clone A-µ), GC19 (clone D) and in GC20 (clone E) of LN1 (Fig. 4a) and in GC13 (clone F) of LN3 (Fig. 4c). Of note, the IgM and IgG variants of the Bh sub-clone in GC1 of LN3 belong to the already mentioned large clone ‘B’ of which the IgM expressing Bg-µ sub-clone was found in GC1 and GC2. As judged by IgV_H-CDR3 differences, we identified 11 IgM-, 4 IgG- and 1 IgM/IgG-expressing variants of this major ‘B’ clone, which were all found in GC1 and GC2 of LN3 (Fig. 4c and Figs. S2 and S3).

**IgM and IgG expressing B cells have different mutation frequencies**

Out of LN1, LN2 and LN3, we analyzed 48 different GCs, 18 FMs and 3 T-cell areas. Thus, a total of 739 IgM-V_H and 524 IgG-V_H sequences of the GC samples and 370 and 48 IgM-V_H sequences of the FM and T cell area samples were obtained (Table I). The number of unique IgV_H clones detected per GC varied from 1 to as much as 14 (GC1 of LN2) (Tables S1-S3). The average number of mutations of GC IgM clones and of GC IgG clones were 4.1 and 7.4 per IgV_H, respectively (Fig. 5). Of note, a mutation frequency difference was also found when IgM and IgG sequences, either or not clonally related, of individual GCs were compared (Fig. 6). In a minority of GCs only, higher mean numbers of IgV_H mutations were observed in IgM clones as compared to the IgG clones, i.e. in GC1, 24, 25 and 27 of LN1, GC5 of LN2 and in GC10 and 13 of LN3 (Table I). The IgM-V_H clones isolated from the FM samples harbored, as expected, hardly if any somatic mutations, i.e. 0.8 mutations per IgV_H on average. The IgM-V_H clones of the T cell area samples of LN1 harbored on average 3.5 mutations per IgV_H (Fig. 5). When applying an arbitrary cut off of ≤ 1 mutation per IgV_H, unmutated IgM-expressing B cell clones were identified in 24 of the 46 GCs examined (52%). Interestingly, unmutated IgG-expressing B cell clones were identified as well in 8 of the 33 GCs examined (24%). Moreover,
Figure 5  Mean IgV_H mutation numbers of all clones analyzed ordered according to anatomical location in lymph nodes 1, 2 and 3.

FM indicates samples from mantle zones, GC indicates samples from germinal centers and TZ indicates samples from T cell areas. Numbers underneath the base of the bars indicate the number of sequenced clones.

Figure 6  Mean IgV_H mutation numbers of related IgM and IgG clones found within individual germinal centers.

The mean IgV_H mutation numbers of the eleven clonal IgM/IgG sets of figure 4 are shown. M and G indicate related IgM and IgG clones, respectively.
Table I IgV<sub>H</sub> clones found in samples microdissected out of three reactive human LNs.

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<th>GC samples</th>
<th>Amplified Ig transcripts</th>
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<th>Mean no. of IgV&lt;sub&gt;H&lt;/sub&gt; mutations of all clones</th>
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<sup>a</sup> Of GC2, GC3, GC5, GC13, MZ7, and MZ8, two microdissected samples were analyzed.

<sup>b</sup> Of GC1 and GC2, four and two microdissected samples were analyzed, respectively.

<sup>c</sup> Of GC1, two microdissected samples were analyzed.

<sup>d</sup> The microdissected samples of GC17 and FM1–FM5 are indicated in Fig. S5.
LN1: GC5, GC7, GC16, GC17 and GC20, clone C-γ

LN2: GC4 and GC8, clone G-γ

LN3: GC10, GC11 and GC13, clone J-γ
applying the same cut off criterion, we found overall 22% unique, mutated IgM clones in the FMs, 61% mutated IgM and 90% mutated IgG clones in the GCs and 56% mutated IgM clones in the T cell areas, respectively (Tables S1-S6).

The offspring of single hypermutated IgG B cell clones is present in multiple germinal centers

In addition to the IgM clones A-μ, H-μ and I-μ of LN1 and B-μ, C-μ, D-μ and G-μ of LN3, we also encountered three IgG-V\textsubscript{H} expressing clones in multiple GCs of all three LNs. In GC5, 7, 16, 17 and 20 of LN1, the offspring of a heavily mutated VH4-IgG-V\textsubscript{H} clone, termed C-γ, was detected. The daughter C-γ clones contained, in the 5 GCs mentioned, mean numbers of 29.0, 17.5, 16.6, 16.3 and 25.0 mutations, respectively. As many as nine mutations were shared between all the C-γ clones (Figs. 2 and 7). In GC4 and 8 of LN2, a recurrent VH4-IgG-V\textsubscript{H} clone termed G-γ was found. All G-γ clones harbored one shared mutation. Similarly, in LN3 one recurrent, hypermutated VH1-IgG-V\textsubscript{H} clone J-γ was detected in GC10, 11 and 13. All the daughter clones of J-γ harbored, except for 2 clones in GC10 (J-γ sub-clones A2 and A5), two shared replacement mutations in IgV\textsubscript{H}. In addition, some J-γ clones showed differences of their IgV\textsubscript{H}-CDR3 as compared to the consensus CDR3 sequence (J-γ sub-clones A2 and A13 of GC10) (Figs. 2 and 7).

Discussion

Our in situ analyses on the reactive human lymph nodes point out that the B cell response is a highly dynamic process based on entrance and re-entrance of single naive and memory B cell clones in multiple germinal centers. The RT-PCR approach to amplify IgV\textsubscript{H} genes was chosen to be able to discriminate between IgM- and IgG-expressing B cells, information which cannot be obtained by using genomic DNA. We were aware of a potential bias due to intra-GC plasmacytoid cells as they produce disproportionately more Ig per cell. Immunohistochemical stainings indicated that in the GCs of LN1 and LN2, CD138 (syndecan-1) expressing plasma cells were virtually

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure7.png}
\caption{IgV\textsubscript{H} sequences and mutations therein of the recurrent IgG clones found in lymph nodes 1, 2 and 3.}
\end{figure}

The C-γ clone found in five GCs of LN1 is shown in the upper panel, the G-γ clone found in two GCs of LN2 in the middle panel and the J-γ clone found in three GCs of LN3 in the lower panel. Lollipop shaped symbols indicate nucleotide differences as compared with the V\textsubscript{H}-4.4, V\textsubscript{H}-30.4 and the V\textsubscript{H}-69 germline IgV\textsubscript{H} genes, respectively. Replacement- and silent- mutations are indicated by closed and open circles. Gray shaded bars indicate the identical somatic mutations in the different GCs. 2X, two mutations in the indicated codon. The total number of clones in which a particular IgV\textsubscript{H} mutation pattern was found is indicated by (Nx).
absent whereas they were abundantly present in the extrafollicular areas. In LN3, about 50% of the GCs did contain scattered plasmacytoid cells, weakly expressing CD138 (Fig. S6). Intra-GC plasmacytoid cells are, for several reasons, unlikely to be just random follicular immigrants but are rather maturing locally and thus are a direct reflection of the antigen-stimulated GC B cell population. Extra-follicular differentiating plasma cells upregulate CD138 and downregulate receptors that are essential to enter GCs, i.e. membrane bound Ig to interact with antigen and the follicle-attracting chemokine receptor CXCR5. Moreover, they upregulate CXCR4 whose ligand (CXCL12/SDF) is highly expressed in the medullary cords. Finally, the fact that in several GCs isotype switch variants of individual B-cell clones were detected, underscores the solidity of the experimental strategy.

The number of unique VH4- or VH1- expressing clones identified per GC varied between 1 and 14 (GC1 of LN2) (Tables S1-S3). Others have reported numbers of unique clones in human GCs, i.e. ranging between 4-13 and 1-16, whereas in immunization studies with T-cell dependent antigens in the mouse, averages of 3-6 clones per GC have been documented. These combined results are suggestive for a more diverse B-cell repertoire in GCs in man, which still is an underestimation since only one IgVH gene family was analyzed in detail per LN. Furthermore, these findings must be interpreted with caution since in the human system one is not informed on the kinetics and stage of the GC reaction studied.

In LN1 and LN3, we detected 7 distinct IgM-expressing clones which each were present in at least two separate GCs (Figs. 1-3 and Figs. S1 and S2). Most remarkable was the IgM-VH4 A-µ clone that was traced in 19 of 24 GCs in two consecutive sections of LN1 (Fig. 2). It is emphasized that, whereas the A-µ clone was detected in the majority of the GC samples, it was found only in one of the 10 analyzed samples from adjacent mantle zones and not at all in randomly collected samples from the T cell areas of LN1. All the A-µ clones contained two identical mutations and in 14 GCs daughter clones with unique additional mutations were detected. (Fig. 3 and Fig. S4). The finding of the widely disseminated A-µ clone in LN1 is in accordance with studies in mice showing that after a primary immunization with the hapten arsonate, some clonotypic B cells expanded and subsequently populated different follicles in which the daughter cells underwent their own clonal evolution. Extra-follicular proliferation has also been demonstrated in mice immunized with NP-CG. In this system, unmutated and mutated daughter cells of a B cell clone were found in an extra-follicular plasma cell focus and in a neighboring GC, respectively. Thus, two scenarios may explain the presence of IgM clones with shared mutations in multiple GCs, such as the A-µ clone: (i) antigenic activation of a naive IgM precursor B cell, induction of SHM and, after limited proliferation, migration into multiple primary follicles and (ii) extra-follicular re-activation and proliferation of one or more members of a mutated memory IgM B cell and subsequent seeding into various follicles.

As expected, hypermutated IgVH clones were found mainly in the GC samples. By contrast, the mean number of mutations of the IgM clones from follicular mantle zones
did not exceed 0.7 per IgV. Still, the FM samples contained 22% of mutated IgM clones and, conversely, in the GCs as many as 39% of the IgM clones and 10% of the IgG clones were unmutated. These findings are compatible with previous microdissection studies on human lymph nodes\textsuperscript{12,13} and are explained by recent intravital two-photon microscopy studies. In the latter studies, it was demonstrated that trafficking of naive B cells is not completely restricted to the mantle zones as they frequently surpass the GC borders.\textsuperscript{22,23}

We found means of 3.2, 4.2 and 4.8 mutations for IgM clones and 6.4, 5.8 and 9.9 mutations for IgG clones in LNs 1, 2 and 3, respectively (Fig. 5). For tonsillar IgM and IgG B cells, average mutation loads of 5.7 and 9.5 have been reported.\textsuperscript{24} Interestingly, we identified eleven clones of which IgM and IgG isotype variants were present within individual GCs, providing formal proof for active CSR in this environment in man. Shared replacement mutations were identified in 8 out of the 11 paired IgM/IgG clones (Fig. 4). Again, the IgG clones generally contained more mutations as compared to the corresponding IgM clones (Fig. 6). This finding is a priori not expected noticing that the 11 isotype switch variants each originate from single antigen-responsive precursor cells that had resided for equal times in their particular GCs. Isotype-related mutation differences within GCs can, hypothetically, be explained by a non-random process of CSR which is more due at higher IgV-affinities and thus stronger BCR signals. However, the finding of unmutated IgG clones in 8 of the 33 GCs examined, indicates that such an affinity threshold is not absolute.

In three lymph nodes, recurrent hypermutated IgG clones were identified, i.e. in LN1 the IgG-VH4 C-γ clone in 5 GCs, in LN2 the IgG-VH4 G-γ clone in 2 GCs and in LN3 the IgG-VH1 J-γ clone in 3 GCs. Importantly, as many as 9 mutations were shared among the C-γ clones found in the 5 GCs whereas in the J-γ clones retrieved from the 3 GCs, two replacement mutations were common (Fig. 7). This, together with the fact that no corresponding IgM variants were traced, indicate earlier GC passage(s) of precursor clones of C-γ, G-γ and J-γ. These recurrent IgG-expressing clones are thus to be considered as re-activated memory B-cell clones. Repeated GC engagement has been proposed to occur in mice as well. Secondary response to phosphorylcholine-KLH yielded B-cells that were more heavily mutated as compared to primary response B cells, while most of the mutations appeared to be shared among the clones. This latter observation suggested mere expansion of memory B cells within GCs with minimal additional SHM.\textsuperscript{25} Recent intravital two-photon microscopic experiments in mice also indicated that memory B cells are able to join an existing GC, provided they have a competitive advantage in antigen binding affinity.\textsuperscript{31} Repetitive passing of memory B cells through GCs is compatible with (i) the fact that peripheral blood memory B cells on average harbor higher mutation loads as compared to GC B cells in LN and tonsil\textsuperscript{14,26}, (ii) the positive correlation between mean IgV\textsubscript{H} mutation frequencies of memory B cells and age: In young and aged humans, respective mean mutation numbers of 9.7 and 11.5 for IgM and 17.3 and 22.5 for IgG memory B cells have been reported\textsuperscript{17} and (iii) the reported difference in replication history of memory B cells in children and adults, having undergone
approximately 8 and 11 cell divisions, respectively.\textsuperscript{27} In this respect, it can be envisaged that the relative contribution of memory B cells to GC responses increases with age.

It is generally believed that B cells expanding in GCs are at increased risk of genetic derailment. The facts that lymphomas are in majority of B cell origin, of GC or post-GC phenotype and carrying hypermutated $\text{IgV}_H$ genes support the notion that most lymphomas arise during this turbulent differentiation phase. The processes of SHM and CSR, both accompanied by single- and double-stranded DNA breaks, imply genetic instability and are potentially dangerous since they may act also beyond the Ig locus.\textsuperscript{28-30} Indeed, ample evidence is now available that many of the chromosomal translocations specific for the various B cell lymphoma entities are byproducts of these two processes.\textsuperscript{31,32} So far, the implicit presumption has been that the genetic hits necessary for cellular transformation have to occur during the relatively brief proliferation phase of a single GC reaction. Knowing now that memory B cells re-enter secondary follicles, most likely upon renewed antigenic challenge, an alternative scenario of B cell lymphomagenesis can be envisaged. Hence, the transforming genetic hits do not have to occur during the first and only GC passage but can, in parallel to the gain of IgV mutations, gradually accumulate in memory B cells during successive recall responses throughout life. This scenario would explain why the peak incidence of B-NHL is not early in life, when most primary responses occur, but at late adulthood long after establishing the memory B cell repertoire. This pathogenic course is also in accordance with the high $\text{IgV}_H$ mutation frequencies found in all (post) GC B cell lymphomas, i.e. being in the range of those found in peripheral blood memory B cells rather than those of primary GC B cells.\textsuperscript{17,24,26,33-36} Finally, if true one would expect that B cells belonging to expanded memory clones specific for common pathogens would be most at risk and therefore be overrepresented among the various B cell lymphomas. This antigenetic bias should be reflected in the Ig repertoire of (post) GC B cell lymphomas.

**References**

25. Miller C, Sfred J, Kelsoe G, Cerny J. Facultative role of germinal centers and T cells in the
26. Klein U, Rajewsky K, Küppers R. Human immunoglobulin (Ig)M-IgD+ peripheral blood B
cells expressing the CD27 cell surface antigen carry somatically mutated variable region
genes: CD27 as a general marker for somatically mutated (memory) B cells. J.Exp.Med
27. van Zelm MC, Szczepanski T, van der BM, van Dongen JJ. Replication history of B
lymphocytes reveals homeostatic proliferation and extensive antigen-induced B cell expansion.
29. Pasqualucci L, Migliazza A, Fracchiolla N et al. BCL-6 mutations in normal germinal center
B cells: evidence of somatic hypermutation acting outside Ig loci. Proc.Natl.Acad.Sci.USA
31. Bende RJ, Smit LA, van Noesel CJ. Molecular pathways in follicular lymphoma. Leukemia
33. Aarts WM, Bende RJ, Steenbergen EJ et al. Variable heavy chain gene analysis of follicular
lymphomas: correlation between heavy chain isotype expression and somatic mutation load.
34. Bende RJ, Aarts WM, de Jong D, Pals ST, van Noesel CJ. Among B-cell non-Hodgkin's
lymphomas, MALT lymphomas express a unique antibody repertoire with frequent rheumatoid
35. Klein U, Goossens T, Fischer M et al. Somatic hypermutation in normal and transformed
36. Tsuiji M, Yurasov S, Velinzon K et al. A checkpoint for autoreactivity in human IgM+ memory
B cell development. J.Exp.Med. 2006;203:393-400.
37. Aarts WM, Willemze R, Bende RJ et al. VH gene analysis of primary cutaneous B-cell
lymphomas: Evidence for ongoing somatic hypermutation and isotype switching. Blood
The majority of cutaneous marginal zone B cell lymphomas expresses class switched immunoglobulins and develop in a T helper type 2 inflammatory environment

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Abstract

Extranodal marginal zone B cell lymphomas (MZBCLs) arise on a background of chronic inflammation due to organ-specific autoimmunity, infection, or by unknown causes. Well known examples are salivary gland MZBCL in Sjögren’s sialadenitis and gastric MZBCL in *Helicobacter pylori* gastritis. MZBCLs express CXCR3, a receptor for IFN-γ-induced chemokines highly expressed in the chronic inflammatory environment. The immunoglobulin variable heavy/light chain (*IgV<sub>H</sub>/*IgV<sub>L</sub>) gene repertoire of salivary gland- and gastric- MZBCL appears restricted and frequently encodes BCRs with rheumatoid factor (RF) reactivity. Primary cutaneous marginal zone B cell lymphomas (PCMZLs) are regarded as the skin-involving counterpart of extranodal MZBCL. Although PCMZLs have been associated with *Borrelia burgdorferi* dermatitis, PCMZLs generally arise due to unknown causes. We studied an extensive panel of PCMZLs and show that PCMZLs do not conform to the general profile of extranodal MZBCL. Whereas most non-cutaneous MZBCLs express IgM, PCMZL in majority express IgG, IgA and IgE, and do not show an obvious immunoglobulin repertoire bias. Furthermore, the isotype-switched PCMZLs lack CXCR3 and seem to arise in a different inflammatory environment, as compared to other extranodal MZBCL.
Introduction

Extranodal marginal zone B-cell lymphomas (MZBCL), also known as mucosa-associated lymphoid tissue (MALT) lymphomas, generally arise on a background of chronic inflammation. Well known examples are gastric MZBCL associated with Helicobacter pylori infection, and salivary gland MZBCL linked to Sjögren’s syndrome.\(^1\) MZBCLs are composed of heterogeneous populations of small B lymphocytes, including centrocytic and monocytoid cells, plasma cells and sometimes scattered immunoblasts and centroblasts. The tumor cells express CD20, CD22, CD79 and BCL2, and are typically CD5\(^{-}\), CD10\(^{-}\) and BCL6\(^{-}\).\(^2\) Like chronic lymphocytic leukemias (CLLs), but in contrast to all other B-cell non-Hodgkin lymphomas, MZBCLs express the chemokine receptor CXCR3.\(^3,6\) Recurrent genetic aberrations in MZBCL include the t(11;18)(q21;q21) API2-MALT1 and t(14;18)(q32;q31) \(IgH\)-MALT1 translocations, as well as trisomies of chromosomes 3 and 18.\(^7\)

MZBCLs resemble antigen-selected memory B cells, reflected in the expression of mutated immunoglobulin (Ig) genes, mostly of the IgM isotype, and generally with low replacement over silent (R/S) mutation ratios in the framework regions (FRs).\(^3,8\) By studying the complementary determining region 3 (CDR3) sequences of Ig variable heavy chain (\(IgV_{H}\)) genes, we\(^1\) and others\(^8\) have shown that gastric and salivary gland MZBCLs express a restricted B cell receptor (BCR) repertoire with frequent homology to canonical, \(V_{1-69}/JH4\)- or \(V_{3-7}/JH3\)-encoded, rheumatoid factors. Recombinant expression of lymphoma-derived IgM antibodies confirmed this auto-reactivity for IgG in an ELISA.\(^3\)

Primary cutaneous marginal zone B-cell lymphomas (PCMZLs), by virtue of their histology and immunophenotype, are regarded as the skin-involving counterparts of MALT lymphomas.\(^10,12\) In certain areas of Europe, cases with PCMZLs were linked to previous infection with Borrelia burgdorferi, however this was not found among PCMZLs from Asia or the United states.\(^13-17\) Overall, for most PCMZLs the etiology is unknown. Inconsistent findings have been reported for genomic aberrations in PCMZLs. Most remarkable was the near complete absence of the t(11;18) API2-MALT1 translocation in the PCMZLs.\(^7,14,18-22\) Variable frequencies were found for the t(14;18) translocation, including one study reporting MALT1, but also BCL2 as the translocation partner of \(IgH\).\(^22,23\) Additionally, two cases have been reported by Streubel et al.\(^18\) harboring a t(3;14) FOXP1/IgH translocation.

Previously, \(IgV_{H}\) sequences of a total of 14 PCMLs have been analyzed in detail by four independent groups.\(^24-27\) To extend our previous findings on immunoglobulin usage by extranodal MZBCLs, we conducted a detailed Ig gene analysis of an extensive cohort of PCMZLs. Furthermore, we analyzed the inflammatory environment in which the PCMZLs arise. The combined results indicate that the majority of PCMZLs essentially differ from their counterparts at other extranodal sites.
Materials and methods

Patient material
Frozen as well as paraffin-embedded tissues of 17 PCMZLs were obtained from the department of dermatology from the Leiden University Medical Centre, The Netherlands, and paraffin-embedded tissue material of 25 PCMZLs was provided by the department of dermatology from the Medical University of Graz, Austria. Paraffin-embedded material of CM43 was obtained from the Department of Pathology and Laboratory Medicine, University Medical Center Groningen, The Netherlands, and frozen material of CM44 was derived from the department of Pathology at the Academic Medical Center in Amsterdam, The Netherlands. Diagnoses had been established by consensus of national panels of experts on cutaneous lymphoma, applying the WHO-EORTC classification. All lymphomas expressed CD20 (and CD79a), BCL-2, and lacked expression of CD10, and BCL-6 or CD5. Monoclonality had been confirmed in all cases either by immunohistochemistry, PCR, or both. RT-PCR for t(11;18) demonstrated the absence of the API-MALT1 translocation in 11 cases tested (CM01, CM03, CM04, CM05, CM07, CM08, CM11, CM15, CM17, CM35 and CM37). Light chain restriction was established for all cases: CM01, 02, 03, 04, 06, 07, 08, 09, 11, 14, 15, 16, 17, 18, 20, 22, 26, 31, 34, 35, 36, 37, 43 and 44 expressed kappa, while CM05, 10, 13, 19, 21, 23, 24, 25, 27, 28, 29, 30, 32, 33, 38, 39, 40, 41, and 42 expressed lambda.

The study was performed in accordance with the ethical standards and approved by the research code committee on human experimentation of our institute.

RNA isolation and cDNA synthesis
Tissue sections from paraffin embedded material were deparaffinized in xylol followed by ethanol. The dried pellet was dissolved and incubated with 500 µg/ml Proteinase K (Roche Diagnostics, Almere, The Netherlands) in lysis-buffer (10 mM Tris-HCl/pH 8.0, 0.1mM EDTA/pH 8.0, 2% SDS/pH 7.3) at 56°C until complete lysis, after which RNA extraction was performed with TRIZOL. Preceding cDNA synthesis, this RNA was heated for 90 minutes at 70°C.28 Frozen material was directly dissolved in TRIZOL and RNA isolation was performed according to manufacturer’s instructions. The RT mix contained the following: 0.1 mmol/l Pd(N)₆ random primers (Amersham Pharmacia Biotech, Roosendaal, The Netherlands), 8 U/µl molony murine leukemia virus RT (Invitrogen, Breda, The Netherlands), 1 mmol of each dNTP, and 1.2 U/µl RNase inhibitor (Roche) in 1× first strand buffer (Invitrogen). The reaction was performed for 1 hour at 37°C, followed by 10 minutes of inactivation at 95°C.

IgV₄-CDR3 analysis:
PCR amplification of the IgV₄-CDR3 regions used a forward primer in framework region 3 (FR3) in combination with reverse primers specific for JH, Cµ, Cδ, Cy, Cα 24 and Ce (5’- CGGAGGTGGCATTGGAGG -3’). Subsequently a second PCR was performed in
which the appropriate constant region primers for each tumor were combined with any of the \(V_H\) gene family-specific primers. In two cases where cDNA quality was not sufficient to produce a \(V_H\)-family PCR product, a part of the \(V_H\) gene was amplified using an \(FR2\) primer. Immunoglobulin light chain \(V\) (\(IgV_L\)) genes were amplified, using \(V_\kappa\)-family specific primers in combination with a \(C_\kappa\)-primer.\(^{29}\) Reaction-mixture contents and the amplification-programs for the PCRs were performed as described previously; with the exception that Taq Platinum (Invitrogen) was used as polymerase enzyme. Sequencing on both strands was performed using the big dye terminator v1.1 cycle sequencing kit (Applied biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Pseudoclonality was excluded by a second independent PCR, confirming the immunoglobulin sequence of the tumor. The sequences were compared with the Vbase\(^{30}\) and IMGT/V\(^{31}\) immunoglobulin databases to obtain the \(V\)\(\LAMBDA\)DJ\(\LAMBDA\) rearrangement and mutational status.\(^{32,33}\) \(IgV_H\)-CDR3 amino acid sequences were compared to each other and blasted to Genbank (with the blastp-algorithm).\(^{34}\) A sequence was considered to be homologous when (a) sharing at least 75% amino acid sequence homology and (b) a length difference between the CDR3 sequences did not exceed 3 amino acids (maximum gap of 3 aa).\(^3\) \(IgV_H\)-sequences are deposited to Genbank with accession numbers EU835546-EU835559.

**Immunohistochemistry**

CXCR3, CD20 and CD3 expression was visualized on frozen as well as paraffin-embedded tissue sections, with monoclonal antibodies against CXCR3 (clone 1C6, Pharmingen, San Diego, CA), CD20 (clone L26, DAKO, Glostrup, Denmark) and CD3 (clone SP7, Lab Vision, Fremont, USA) in combination with the Powervision\(^\text{TM}\)/poly-HRP detection system (ImmunoVision Technologies, Co, Daly City, CA, USA). Heat induced epitope retrieval was performed on the paraffin sections in Tris/EDTA (tris(hydroxymethyl) aminomethane/ethylenediaminetetraacetic acid) buffer (10 mM/1 mM; pH 9.0) for 10 min at 100°C. 3-Amino-9-ethylcarbazole (AEC) was used as chromogen and haematoxylin for nuclear counterstaining. Images were acquired on an Olympus BX51 microscope in combination with an Olympus DP70 digital camera, at original magnification \(\times400\). Included in the analysis were only those cases in which the B and T cells, visualized with CD20 and CD3 stainings, were discernable as distinct populations, thus enabling an assessment of CXCR3 expression by the tumor B cells.

**Cytokine RT-PCR**

For semi-quantitative RT-PCR the cDNAs from frozen tissues were applied in two dilutions, usually 1:20 and 1:100. Based on satisfactory results in the actin RT-PCR, samples were selected for further analysis. PCR mixture contents were the same as for Ig-PCRs, combined with the following primer-pairs: IFN-\(\gamma\)-fw 5’-GCAGAGCCAAATT GTCTCCT-3’; IFN-\(\gamma\)-rev 5’-ATGCTCTTGCACCTCGAACCT-3’; CXCL10-fw 5’-GG AACCTCCAGTCTCGACACC-3’; CXCL10-rev 5’-CAGCCTCTGATTGGTCCAT
CC-3'; IL-12-fw 5'- ATTGAGGTAGGTGGATGC-3'; IL-12-rev 5'-AATGCTGG CATTTTTGCACC-3'; IL-4-fw 5'-TGCCTCAAAGACACACACTG-3'; IL-4-rev 5'- AACGTACTCTGGTGGCCTTC-3'; Actin-fw 5'-CATGGACAAAATCTGGCACCA CA-3'; Actin-rev 5'-CCACTGCACACTTCATGGGAG-3'. After a hotstart for 4 minutes at 95°C, the first 10 cycles of amplification were performed: Successively 60 seconds at 95°C, 30 seconds at 57°C, and 60 seconds at 72°C. The next 20 \((\text{CXCL10})\), 25 (actin) or 30 \((\text{IFN-\gamma, IL-12, IL-4})\) cycles of amplification consisted of 30 seconds at 95°C, 30 seconds at 55°C, and 60 seconds at 72°C. The reaction was completed for 6 minutes at 72°C. PCR-products were densitometrically scored with Image-Pro Plus v5 (Media Cybernetics, Bethesda, USA) and categorized into 3 levels: no expression (less than 5% of the maximally measured signal in the panel, per gene), little expression (between 5% to 50% of maximum), and a high expression level (a signal higher than 50% of the maximum). By ranking these expression levels 0, 1 or 2, respectively, a maximum ranking of 4 was assigned per patient (being the sum of both dilutions). Statistical significance was determined with a Mann-Whitney rank-sum test.

**Results**

**Immunoglobulin heavy and light chains**

RT-PCRs were performed with IgV<sub>H</sub>-family-specific leader primers or FR2/FR3 primers in combination with \(j_H6\) or constant region primers. Of 21 PCMZLs the IgV<sub>H</sub>-CDR3 sequence of the tumor clone was resolved. Three PCMZLs expressed IgM, of which two co-expressed IgD. Ten PCMZLs expressed IgG and two PCMZLs expressed IgA. Furthermore, three PCMZLs expressed both IgG and IgA and three PCMZLs expressed both IgG and IgE. In each of these six cases, the isotype switch variants were derived of the same precursor clone, as judged by IgV<sub>H</sub>-CDR3 sequence and shared mutations. The \(V_H\) germline gene could be determined for 11 out of 21 PCMZLs, demonstrating that five cases used \(V_H1\) and six used \(V_H3\). The results of the IgV<sub>H</sub> analyses are summarized in Table 1. Similarly, \(V_K\) family leader primers combined with a C<sub>K</sub> primer, identified the kappa-rearrangement for seven PCMZLs: Three used \(V_K1\), one used \(V_K2\) and three used \(V_K3\) (Table 2). Mutation frequencies within the IgV<sub>H</sub> gene varied between 4 and 43 mutations, with a mean number of mutations of 26, while 5 to 28 mutations (mean: 14) were found in the IgV<sub>L</sub> sequences. Analysis of the R/S ratios in the FR regions of IgV<sub>H</sub> according to Chang and Casali\(^{35}\) established that 3 of 8 PCMZLs were significantly below the ratio that would be expected in case of random mutation.

**IgV<sub>H</sub>-CDR3 repertoire.**

IgV<sub>H</sub>-CDR3 amino acid sequences from this study, but also those published by Bahler et al.\(^{27}\), Roggero et al.\(^{26}\), and three sequences obtained from our previous study\(^{24}\), were compared to each other and blasted against Genbank, and analyzed according to previously
defined criteria (see materials and methods). These analyses revealed that there was no 
IgV<sub>H</sub>-CDR3 homology among the 33 PCMZLs. In total, 15 PCMZLs displayed IgV<sub>H</sub>-CDR3 amino acid sequence homology to those of other immunoglobulin sequences 
from Genbank, without any obvious bias. Most homologies matched to immunoglobulin 
sequences from healthy donors. Case 2 from the study of Bahler et al. 27 displayed IgV<sub>H</sub>-CDR3 homology to a rheumatoid factor, both with a V3-30/J<sub>H</sub>4 rearrangement. The 
IgV<sub>H</sub>-CDR3 sequence of CM21 was homologous to those of 5 chronic lymphocytic

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**Table 1 IgVH sequence analysis of cutaneous MZBCLs.**

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<th>Patient-number</th>
<th>Ig isotype</th>
<th>IgV&lt;sub&gt;H&lt;/sub&gt;- rearrangement</th>
<th>No. of mutations</th>
<th>R/S ratio FR</th>
<th>CDR3 sequence</th>
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</tbody>
</table>

R/S ratio FR, replacement/silent mutation ratio in IgV<sub>H</sub> framework regions; aa, amino acids; nd, not determined.

<sup>a</sup> significant according to Chang and Casali. 35

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leukemia cases, which, according to homology criteria of Stamatopoulos et al., can be assigned to homology-subset 7. Pt7 from Aarts et al. was homologous to a gastric MALT lymphoma, although the $V_H$-gene rearrangement did not match ($V_H1$ for pt7 and $V_H3-30$ for the gastric MALT lymphoma). A detailed overview of the results is provided as supplemental table S1, available on the Blood website. Table 3 summarizes the analysis on RF-homology in PCMZLs comparison to other extranodal MZBCLs.

<table>
<thead>
<tr>
<th>Patient- IgV L sequence analysis of cutaneous MZBCLs.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patient- IgV L- IgVL-rearrangement (IMGT/V)</strong></td>
</tr>
<tr>
<td><strong>rearrangement</strong></td>
</tr>
<tr>
<td>CM01</td>
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<tr>
<td>CM03</td>
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<tr>
<td>CM04</td>
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<td>CM08</td>
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<tr>
<td>CM11</td>
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<tr>
<td>CM15</td>
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</table>

<table>
<thead>
<tr>
<th>Rheumatoid factor IgV H-CDR3 homology of cutaneous and other MZBCLs.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N RF homology</strong></td>
</tr>
<tr>
<td>Gastric MZBCL</td>
</tr>
<tr>
<td>Salivary gland MZBCL</td>
</tr>
<tr>
<td>Pulmonary MZBCL</td>
</tr>
<tr>
<td>Other extranodal MZBCL</td>
</tr>
<tr>
<td>Splenic MZBCL</td>
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<tr>
<td>Cutaneous MZBCL</td>
</tr>
</tbody>
</table>

N number of sequences analyzed.  
\[ a \] CDR3 amino acid sequence homology with previously published rheumatoid factors (RF).  
\[ b \] Numbers from previously published analysis, supplemented with newly analyzed cases from Lenze et al. and Sakuma et al.52,53.
Tumor environment

It has been established that virtually all extranodal and splenic MZBCLs express CXCR3. Immunohistochemical staining on PCMZL tissues revealed that a proportion of the infiltrating T cells express CXCR3, however the tumor B cells were negative in all PCMZLs tested, except for CM04, CM13 and CM43 (Figure 1 and Table 4). Next, we studied cytokine expression by performing semi-quantitative RT-PCR. The results depicted in Figure 2A show that in general, MZBCLs have a higher expression of the Th1 type cytokines IFN-γ, CXCL10 and IL-12, in contrast to the class-switched PCMZLs which show more bias towards IL-4 expression, a typical Th2 cytokine. Mann-Whitney

![Figure 1 IgG expressing PCMZLs do not express CXCR3.](image)

Frozen sections of CM09, CM17, CM04, and MALT21 stained with monoclonal antibodies (in red, AEC) specific for CD20, CXCR3 and CD3 (original magnification, x400), and counterstained with haematoxylin. IgG expressing PCMZLs CM09 and CM17 contain CXCR3⁺ T cells, whereas the neoplastic B cells in these tissues are CXCR3⁻. In contrast, CM04, an IgM expressing PCMZL, is positive for CXCR3, like the salivary gland MZBCL MALT21.
scoring of the arbitrary values determined by densitometry of the PCR-products, established that the differences between the two groups were statistically significant for IFN-γ \((p=0.001)\) and IL-4 \((p=0.028)\) (Figure 2B).

**Discussion**

In this study we show that PCMZLs, in spite of histological resemblance, differ from other extranodal MZBCLs. A striking difference is the expression of class switched immunoglobulins by 18 out of 21 analyzed PCMZLs, in six cases with dual isotypes derived from the same clone. In contrast, only ~30% of MALT lymphomas express class switched Igs.\(^3\) This finding is in accordance with previous immunohistochemical studies, although we are the first to demonstrate IgE expression in PCMZL.\(^38-40\)

Five of the resolved PCMZL \(\text{IgV}_H\) genes comprised the \(V_{H1}\) germline gene and six contained the \(V_{H3}\) germline gene. We\(^24\) previously reported the usage of \(V_{H1}\), \(V_{H3}\) and \(V_{H4}\) and Franco et al.\(^25\) reported \(V_{H2}\) and \(V_{H6}\) usage in PCMZLs. These results do not confirm an exclusive usage of \(V_{H3}\) family members in the Ig-rearrangements of PCMZLs, as was reported by Bahler et al.\(^27\) Analysis of the \(\text{IgV}_H\) mutations showed that 3 out of 8 PCMZLs had R/S ratios significantly below those expected if mutation had been random.\(^35\) Including previously described cases\(^24,25\), 46% of the PCMZLs appears to be selected for maintenance of BCR structure, which is somewhat less than we have found in other extranodal MZBCLs (~70%).\(^3\)

A total of 33 \(\text{IgV}_H\)-CDR3 sequences i.e. 21 obtained in this study, 3 from our previous study\(^24\), 1 from Roggero et al.\(^26\) and the 8 cases published by Bahler et al.\(^27\), were analyzed for homology with each other and with sequences in Genbank. Within this relatively large panel of sequences, we were not able to detect an \(\text{IgV}_H\) repertoire bias, as was found in salivary gland and gastric MZBCLs; there was no \(\text{IgV}_H\)-CDR3 homology between the PCMZLs. Fifteen \(\text{IgV}_H\)-CDR3 amino acid sequences matched \(\text{IgV}_H\) sequences from Genbank, including one case that was homologous to a rheumatoid factor. Previous findings reported by Bahler et al.\(^27\) on conserved PS/T or YG/T amino acids encoded by non-templated N-
Figure 2  PCMZLs develop in a distinct inflammatory environment.

A. Semi-quantitative RT-PCR for IFN-γ, CXCL10, IL-12, IL-4 and Actin, on whole tissue samples of 10 extranodal MZBCLs (left) and 14 PCMZLs (right), each sample was tested in two dilutions. The two cases in the middle, CM04 and CM13, represent the IgM+ and CXCR3+ PCMZLs. The lower two panels depict the results of immunohistochemistry for CXCR3, and the Ig isotypes determined by RT-PCR. nd, not determined.

B. Graphical representation of PCR band-intensities as determined by densitometry, in arbitrary values. Differences between extranodal MZBCLs on the left and class switched PCMZLs on the right were significant for IFN-γ (p=0.001) and IL-4 (p=0.028), as determined by a Mann-Whitney rank-sum test.
nucleotide sequences within the CDR3 sequences, which would be a strong argument for similar antigen recognition, were not encountered within our panel of sequences.

Extranodal marginal zone B cell lymphomas generally arise on a background of chronic inflammation, usually of the Th1 type. Th1 cytokines, like IFN-γ and IL-2, are abundantly expressed in the initial chronically inflamed tissues as well as in the eventual tumor environment. Also IFN-γ-induced chemokines like CXCL9 and CXCL10 are expressed by the epithelial and endothelial cells, which attract more Th1 cells expressing CXCR3, the receptor for these chemokines. In agreement with the excess of IFN-γ in the tumor environment, the non-cutaneous MZBCLs express CXCR3, a downstream target of the IFN-γ-induced transcription factor T-bet. Our analyses suggest that most of the PCMZLs develop in a distinct inflammatory environment. The majority of the PCMZLs (90%) lack expression of CXCR3. Of note, the CXCR3+ minority consisted of four IgM+ PCMZLs (CM04, CM13, CM43 and pt6 of Aarts, et al.24). Interestingly, CM13 and CM43 had developed on a background of a *B. burgdorferi* infection, and Roggero et al.26 also reported a *Borrelia*-associated PCMZL expressing IgM. Like *H. pylori* in the gastric mucosa, *B. burgdorferi* evokes a Th1 type of response, supportive for the extranodal MZBCL-like phenotype of these two *Borrelia*-associated PCMZLs. The majority of PCMZLs however seem to reside in a Th2 type cytokine environment, as was supported by the cytokine RT-PCRs. Moreover, the fact that these lymphomas express IgG1, IgG4, IgA and IgE, the latter three of which are typical Th2-dependent isotypes, is compatible with the Th2 inflammatory origin.50,51

The results presented here are suggestive for the existence of two types of PCMZL, most likely related to their pathogenesis. A small subgroup resembles non-cutaneous MZBCLs, being CXCR3+ and IgM+ and potentially (*Borrelia*) infection associated. To confirm these results and to see whether RF-homology is more common among this type of PCMZLs, it would be interesting to study a larger panel of *Borrelia*-associated PCMZLs, if available. In contrast, most of the PCMZLs differ from other extranodal MZBCLs, as they possess switched Igs, lack of RF-homology, do not express CXCR3, and have a cytokine profile more skewed towards the Th2 type. These differences in immunoglobulin repertoire and cytokine environment suggest that PCMZLs do not recognize a similar class of antigens. Further study on the clinical history of this type of PCMZLs might reveal an etiology in the large variety of Th2 type inflammatory conditions of the skin.
References


Chapter 5

Immunological and genetic characterisation of ocular adnexal marginal zone B cell lymphoma

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Submitted
Abstract

Ocular adnexal marginal zone B cell lymphoma (OAMZL) can arise in the connective tissues of the orbit, or from the mucosa associated lymphoid tissue in the conjunctiva. Here we present the immunological and genetic analysis of 20 primary OAMZLs, of which the combined data suggest that they represent a separate group of extranodal marginal zone B cell lymphoma (MZBCL). Immunoglobulin (Ig) sequence analysis demonstrated that OAMZLs have a biased IgV<sub>H</sub> germline gene usage with preference for VH4 family germline genes, and in particular VH4-34. Chlamydia psittaci DNA was not detected, and neither the MZBCL-specific chromosomal translocations, t(11;18) API2/MALT1 and t(14;18) IgH/MALT1. A20 (TNFAIP3), a negative regulator of the NFκB pathway, was found mutated in two cases. Both mutations led to a premature stop codon. Variable nuclear expression of BCL10, NFκB1 and NFκB2 suggests that additional genetic abnormalities may exist in this group of lymphomas, affecting the NFκB pathway. The chemokine receptor CXCR3 was expressed in only a proportion of the cases. Likewise, the integrin receptor α4β7 was found to be variably expressed, with an unexpected lack of correlation to mucosal localisation.
Introduction

Extranodal marginal zone B cell lymphoma (MZBCL) generally arises at sites of acquired mucosa associated lymphoid tissue (MALT). There is a strong association with persistent infections, such as *Helicobacter pylori*, and with autoimmune diseases like Sjögren’s syndrome. \(^1\) Hence, MZBCL are considered to have their origin in chronic inflammation, which may be reflected in skewing of the immunoglobulin (Ig) repertoire; a significant proportion of gastric and salivary gland MZBCLs were found to express B cell receptors (BCR) with striking homology of their Ig complementarity determining region 3 (CDR3) amino acid sequence to known rheumatoid factors (RF). \(^2\) This specificity is preserved over time. Selection against replacement mutations in the framework regions (FR) of the Igs is a common feature of a large part of MZBCLs, suggesting that conservation of a functional BCR is crucial for lymphoma persistence. \(^2\) On several occasions, it has been shown that removal of the source of inflammation, e.g. by antibiotics treatment of *H. pylori* associated gastric MZBCL, also eliminated the lymphoma. \(^2,3\) There are some indications that the MZCBLs may be responsive to the cytokines in their environment. Chronic inflammation from infection with intracellular bacteria or autoimmune diseases like Sjögren’s syndrome, is usually characterised by high levels of IFN-\(\gamma\). \(^4-6\) Similarly, MZBCLs generally contain significant amounts of IFN-\(\gamma\) transcripts, and express the chemokine receptor CXCR3, a direct target of the IFN-\(\gamma\) inducible transcription factor T-bet. \(^7\) Recently we reported that the inflammatory environment of primary cutaneous MZBCL (PCMZL) is not characterised by IFN-\(\gamma\), but rather by IL-4. \(^8\) Compatibly, most of lymphomas do not express CXCR3, and bear class-switched Igs, this in contrast to the IgM expression of most MZBCLs at other sites. The small proportion of the PCMZLs that were CXCR3+, were associated with *Borrelia burgdorferi* and expressed IgM. \(^8\)

The t(11;18) is the most frequently detected chromosomal translocation in MZBCLs. The presence of t(11;18) API2/MALT1 usually excludes the presence of other chromosomal translocations, and it appears to be negatively correlated with RF-specificity. \(^2,9,10\) In addition, t(11;18)\(^-\) cases are unresponsive to treatment with antibiotics, and are not prone to transform into high grade DLBCLs. \(^11,12\) Other recurrent genetic abnormalities include t(1;14) BCL10/IgH, t(14;18) IgH/MALT1, trisomies chr3 and chr18 and gains at 9q34, 11q11-13 and 18q21. \(^9,13,14\) Interestingly, many of these aberrations lead to a constitutive or increased activation of the NF\(\kappa\)B pathway.

Ocular adnexal MZBCLs (OAMZL) have been associated with *Chlamydia psittaci* infection. However, there is debate on the variation between the reported frequencies, which is possibly linked to epidemiological distribution or sensitivity of the detection methods. \(^15-19\) Beside assessing presence or absence of *C. psittaci*, the immunological environment has hardly been explored in this entity. Five studies dealt with the Ig sequences of OAMZL,\(^20-24\) reporting preferential rearrangement of \(V_{H}3\) and \(V_{H}4\) family germline genes. Emphasis has been given on the genetics of OAMZLs, with most recently
the finding that the NFκB inhibitor \textit{A20} (\textit{TNFAIP3}) is a frequent target of somatic mutation and genomic deletions.\textsuperscript{25,26}

Although it has been suggested that immunological stimulation may synergise with genetical aberrations to obtain sustained NFκB activation\textsuperscript{27}, most of the publications on OAMZLs have limited focus to just one of the two factors. In this study, we have addressed the immunoglobulin repertoire, the inflammatory environment and the genetics of 20 OAMZLs. We provide evidence that OAMZL is a distinct entity among MZBCL.

\section*{Materials and methods}

\textbf{Patient material}

Our study group consists of a 20 patients diagnosed as primary ocular adnexal MZBCL, between 1994 and 2008 at the Academic Medical Centre, Amsterdam, the Netherlands, which is a referral center for orbital diseases. This panel contains 8 cases which were previously included in a study on \textit{C. psittaci} in OAMZL.\textsuperscript{28} Sites of origin included orbita (n=13), conjunctiva (n=4) and eyelid (n=3). From patient OM09 several time points were available for analysis: a premalignant orbital lymphoid hyperplasia (OM09A, ‘98), the orbital MZBCL tissue (OM09B, ‘04) and a secondary metastasis with DLBCL transformation in a groin lymph node (OM09C, ‘06). OM20 is a Sjögren-associated lymphoma, previously published as M15.\textsuperscript{3}

Diagnosis was established based on immunohistochemistry, according to the WHO classification: CD3\textsuperscript{−}, CD5\textsuperscript{−}, CD10\textsuperscript{−}, CD20\textsuperscript{+}, CD79a\textsuperscript{+}, BCL2\textsuperscript{−}, BCL6\textsuperscript{−} and cyclinD1\textsuperscript{−}, and for most cases also by exclusion of chromosomal translocations involving BCL1, BCL2, BCL6 and MYC, assessed by split-FISH. The study was performed in accordance with the ethical standards and approved by the research code committee on human experimentation of our institute.

Light chain restriction based on immunohistochemistry was seen for most cases: OM03, 08, 09, 04, 06, 07, 08, 09, 13, 16, 18, 21, 23, 24, 30 and 31 expressed kappa, while OM04, 05, 15, 19, 20 and 29 expressed lambda.

\textbf{RNA isolation and cDNA synthesis}

RNA was isolated from frozen tissue sections with the GenElute mammalian total RNA miniprep kit (Sigma-Aldrich, Zwijndrecht, The Netherlands). The RT mix contained the following: 0.1 mmol/l Pd(N)\textsubscript{6} random primers (Amersham Pharmacia Biotech, Roosendaal, The Netherlands), 8 U/µl molony murine leukemia virus RT (Invitrogen, Breda, The Netherlands), 1 mmol/l of each dNTP, and 1.2 U/µl RNase inhibitor (Roche) in 1× first strand buffer (Invitrogen). The reaction was performed for 1 hour at 37°C, followed by 10 minutes of inactivation at 95°C.
**IgV<sub>H</sub>-CDR3 analysis**

PCR amplification of the V<sub>H</sub>J<sub>H</sub> rearranged Ig sequences used a forward primers in the IgV<sub>H</sub>-gene family-specific leader sequences in combination with reverse primers specific for J<sub>H</sub>, C<sub>H</sub>, Cδ, Cγ, Cα and Ce (5'- CGGAGGTGGCATTGGAGG -3'), as previously published. Reaction-mixture contents and the amplification-programs for the PCRs were performed as described; with the exception that Taq Platinum (Invitrogen) was used as polymerase enzyme. Sequencing on both strands was performed with the big dye terminator v1.1 cycle sequencing kit (Applied biosystems, Nieuwerkerk a/d IJssel, The Netherlands). In each case the tumour-sequence was confirmed in multiple independent PCRs, excluding artifactual pseudo-clonality. The sequences were compared with the Vbase<sup>30</sup> and IMGT/V<sup>31</sup> immunoglobulin databases to obtain the V<sub>H</sub>J<sub>H</sub> rearrangement and mutational status.<sup>32,33</sup>

IgV<sub>H</sub>-CDR3 amino acid sequences were compared to each other and blasted to Genbank (with the blastp-algorithm). A sequence was considered to be homologous when (a) sharing at least 75% amino acid sequence homology and (b) a length difference between the CDR3 sequences did not exceed 3 amino acids (maximum gap of 3 aa).<sup>2</sup>

**Immunohistochemistry**

CD20, CD3, CXCR3, αβ7, BCL10, NFκB1 and NFκB2 were visualised on frozen tissue sections, with monoclonal antibodies against CXCR3 (clone 1C6, Pharmingen, San Diego, CA), CD20 (clone L26, DAKO, Glostrup, Denmark), CD3 (clone SP7, Lab Vision, Fremont, USA), α4β7 (Act-1)<sup>34</sup>, BCL10 (clone 151, DAKO), p105/50 (NFκB1) (Cell Signaling Technology, Danvers, MA, USA) and p100/p52 (NFκB2) (clone 18D10, Cell Signaling Technology), in combination with the Powervision®poly-HRP detection system (ImmunoVision Technologies, Co, Daly City, CA, USA). All frozen tissue sections were fixed with aceton, but in case of immunohistochemistry for BCL10, NFκB1, NFκB2 a secondary fixation with paraformaldehyde was applied as well. 3,3’-Diaminobenzidine (DAB) was used as chromogen for nuclear stainings, and 3-Amino-9-ethylcarbazole (AEC) for the membrane markers, and haematoxylin for nuclear counterstaining. Images were acquired on an Olympus BX51 microscope in combination with an Olympus DP70 digital camera, at original magnification ×400. Included in the analysis were only those cases in which the B and T cells, visualised with CD20 and CD3 stainings, were discernable as distinct populations, thus enabling an assessment of expression by the tumour B cells.

**Cytokine RT-PCR**

Semi-quantitative RT-PCR for IL-4, IFN-γ, and actin were performed as described previously.<sup>8</sup>

**Genetic analysis**

The presence of t(11;18) was tested with RT-PCR as described<sup>15</sup> and/or split-FISH for
MALT1 (18Q), using probes and FISH accessory kit according to manufacturers recommendation (DAKO). t(3;14) FOXP1 was assessed by split-FISH as described previously, using DIG-labelled probes RP11-118O11, -1031N18 and -430J3, and biotin-labelled probes RP11-266O22 and -321A32.35

Mutation analysis for CARD11 (CARMA1) (NM_032415.3) and A20 (TNFAIP3) (NM_006290.2) were performed by RT-PCR and subsequent sequencing of the PCR-product. Primers used for CARD11 amplification: 5'-AAACTGGTGACTGGGAAAGAGC-3' forward and 5'-CCAAAGTCCTGAGAGATGATGG-3', resulting in a product covering exon 3 to 7, encoding the coiled-coil domain and overlapping with the sequence that was found mutated in DLBCL.36 The whole coding sequence of A20 was covered with 4 primer-pairs: fw1: 5'-AGGACTTGGGACTTTGCG-3', rev1: 5'-TGGCCTTCTGAGGATGTTG-3'; fw2: 5'-GGAACTGGAATGATGAATGG-3', rev2: 5'-AGGTTCATGGGATTCTGG-3'; fw3: 5'-CATGAGTACAAGAAATGGCAGG-3', rev3: 5'-TTTCTGCACTTGCTCGG-3'; fw4: 5'-GCCTGTCTCAAGCTGCAC-3', rev4: 5'-ACCATGATGACTGACAGCTCG-3'.

Chlamydia psittaci detection
8 OAMZLs cases were previously tested and found negative for the presence of Chlamydia psittaci DNA.28 Nine of the remaining cases were assessed for the presence of the C. psittaci ompA gene using an internally controlled real-time PCR in the LightCycler 2.0 system as described previously.28,37

Results

IgVH-gene analysis
The clonal tumour IgVH-gene sequence was identified in all 20 lymphomas, generally by using the JH6 reverse primer in combination with one or more of the primers specific for the constant Ig regions. The majority of lymphomas (17/20) expressed non-class switched Igs, i.e. IgVH-CH (IgM) and IgVH-CD (IgD) transcripts. Only 3 cases expressed class switched Igs, either in addition to IgM (OM04 and OM30), or as single isotype (OM20). (Table 1). The mutation frequency ranged from 3 to 53 mutations per IgVH-gene (mean 19). For OM17, the mutation frequency varied between PCR products, ranging from 18-26 mutations per sequence. Analysis of the R/S ratios in the FR regions of IgVH according to Chang and Casali established that for 10 cases this ratio was significantly below what would be expected in case of random mutation in absence of selection. IgVH-gene usage was limited to the VH4 (9 cases), VH3 (9 cases) and VH1 (2 cases) gene families. When we include previously published cases; 26 by Coupland et al.,21 10 from Mannami et al.,23, 8 from Adam et al.,20, 12 cases by Hara et al.,22, and 10 cases recently described by Bahler et al.,24,
it appears that the larger part of OAMZL express IgVH-gene rearrangements using an IgVH-gene from the V<sub>H</sub>3 family (59%) and V<sub>H</sub>4 family (29%) (Supplementary table S1). Within our series of patients, V<sub>H</sub>4 family genes appeared to be over-represented, compared with the frequencies of normal marginal zone (MZ) B cells (45% versus 18%, respectively). However, when the frequencies of all above mentioned studies are combined, V<sub>H</sub>4 gene family usage is still somewhat elevated, but not statistically significant (29%, p=0.150, in a 2-sided Fisher exact test) (Table 2). Interestingly, V<sub>H</sub>4-34 was used in 13 out of 86 (15%) OAMZL rearrangements and V<sub>H</sub>3-74, an IgVH-gene normally seen only in 1.6% of normal MZ B cells, was found in 8.2% of OAMZLs.

Table 1 IgV<sub>H</sub> sequence analysis of 20 ocular adnexal marginal zone B cell lymphomas.

<table>
<thead>
<tr>
<th>Patient-number</th>
<th>Ig isotype (RT-PCR)</th>
<th>IgV&lt;sub&gt;H&lt;/sub&gt;-rearrangement</th>
<th>No. of mutations</th>
<th>R/S FR</th>
<th>CDR3 sequence</th>
<th>CDR3-length</th>
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<td>Cµ/δ</td>
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<td>OM17</td>
<td>Cµ/δ</td>
<td>V&lt;sub&gt;3&lt;/sub&gt;-23/D&lt;sub&gt;2&lt;/sub&gt;-15/JH4</td>
<td>18-26&lt;sup&gt;b&lt;/sup&gt; 1.0&lt;sup&gt;+&lt;/sup&gt;</td>
<td>CAR IGWGG(T/N)PT(D/N)AF(A/D)Y WGQG</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>OM18</td>
<td>Cµ/δ</td>
<td>V&lt;sub&gt;3&lt;/sub&gt;-30/D&lt;sub&gt;3&lt;/sub&gt;-10/JH4</td>
<td>14</td>
<td>1.5</td>
<td>CAR LLQPIITLARFDYGDC WGQG</td>
<td>14</td>
</tr>
<tr>
<td>OM19</td>
<td>Cµ/δ</td>
<td>V&lt;sub&gt;3&lt;/sub&gt;-33/D&lt;sub&gt;1&lt;/sub&gt;-1/JH4</td>
<td>10</td>
<td>1.0</td>
<td>CAR DLQNAFDFY WGQG</td>
<td>9</td>
</tr>
<tr>
<td>OM20</td>
<td>Cα</td>
<td>V&lt;sub&gt;1&lt;/sub&gt;-18/D&lt;sub&gt;4&lt;/sub&gt;-23/JH4</td>
<td>41</td>
<td>1.1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>CAR ATLDLDGYMDF WGQG</td>
<td>11</td>
</tr>
<tr>
<td>OM21</td>
<td>Cµ</td>
<td>V&lt;sub&gt;3&lt;/sub&gt;-23/D&lt;sub&gt;6&lt;/sub&gt;-19/JH4</td>
<td>nd</td>
<td>nd</td>
<td>CAV DSSGWRAIFDY WGQG</td>
<td>13</td>
</tr>
<tr>
<td>OM22</td>
<td>Cµ/δ</td>
<td>V&lt;sub&gt;4&lt;/sub&gt;-59/D&lt;sub&gt;3&lt;/sub&gt;-9/JH4</td>
<td>14</td>
<td>1.0</td>
<td>CAR QRGGGGYDIFTGSHH(D/V) H WGQG</td>
<td>19</td>
</tr>
<tr>
<td>OM24</td>
<td>Cµ/δ</td>
<td>V&lt;sub&gt;4&lt;/sub&gt;-34/D&lt;sub&gt;2&lt;/sub&gt;-15/JH3</td>
<td>11</td>
<td>0.8&lt;sup&gt;+&lt;/sup&gt;</td>
<td>CAS PGYCGSSCYPNGFDI WGQG</td>
<td>16</td>
</tr>
<tr>
<td>OM29A</td>
<td>Cµ/δ</td>
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<td>21</td>
<td>1.0&lt;sup&gt;+&lt;/sup&gt;</td>
<td>CAR GPTMVRGDFSDP WGQG</td>
<td>13</td>
</tr>
<tr>
<td>OM30</td>
<td>Cµ/δ/α</td>
<td>V&lt;sub&gt;4&lt;/sub&gt;-61/D&lt;sub&gt;2&lt;/sub&gt;-8/JH3</td>
<td>8</td>
<td>1.0</td>
<td>CAR EVFADFDI WGQG</td>
<td>8</td>
</tr>
<tr>
<td>OM31</td>
<td>Cµ/δ</td>
<td>V&lt;sub&gt;4&lt;/sub&gt;-30.4/D&lt;sub&gt;4&lt;/sub&gt;-23/JH4</td>
<td>8</td>
<td>5/0</td>
<td>CAR ERCGGSSVEY WGQG</td>
<td>9</td>
</tr>
</tbody>
</table>

<sup>a</sup> insertion of 15 nucleotides of unknown origin

<sup>b</sup> mutation number varied between clones

<sup>c</sup> p<0.05
### Supplemental table S1.
**IgV<sub>H</sub> gene family usage in ocular adnexal marginal zone B cell lymphomas.**

<table>
<thead>
<tr>
<th></th>
<th>V&lt;sub&gt;H&lt;/sub&gt;1</th>
<th>V&lt;sub&gt;H&lt;/sub&gt;2</th>
<th>V&lt;sub&gt;H&lt;/sub&gt;3</th>
<th>V&lt;sub&gt;H&lt;/sub&gt;4</th>
<th>V&lt;sub&gt;H&lt;/sub&gt;5</th>
<th>V&lt;sub&gt;H&lt;/sub&gt;6</th>
<th>V&lt;sub&gt;H&lt;/sub&gt;7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coupland&lt;sup&gt;21&lt;/sup&gt; (Germany)</td>
<td>3</td>
<td>0</td>
<td>14</td>
<td>7</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Adam&lt;sup&gt;20&lt;/sup&gt; (Germany)</td>
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<td>0</td>
<td>6</td>
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<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Mannami&lt;sup&gt;23&lt;/sup&gt; (Japan)</td>
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<td>0</td>
<td>7</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hara&lt;sup&gt;22&lt;/sup&gt; (Japan)</td>
<td>2</td>
<td>0</td>
<td>9</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bahler&lt;sup&gt;24&lt;/sup&gt; (USA)</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>This study (The Netherlands)</td>
<td>2</td>
<td>0</td>
<td>9</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total:</strong></td>
<td>9</td>
<td>0</td>
<td>48</td>
<td>25</td>
<td>3</td>
<td>0</td>
<td>1</td>
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</table>

### Table 2 Frequencies of IgV<sub>H</sub> gene family usage in ocular adnexal marginal zone B cell lymphomas.

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<tr>
<th></th>
<th>V&lt;sub&gt;H&lt;/sub&gt;1</th>
<th>V&lt;sub&gt;H&lt;/sub&gt;2</th>
<th>V&lt;sub&gt;H&lt;/sub&gt;3</th>
<th>V&lt;sub&gt;H&lt;/sub&gt;4</th>
<th>V&lt;sub&gt;H&lt;/sub&gt;5</th>
<th>V&lt;sub&gt;H&lt;/sub&gt;6</th>
<th>V&lt;sub&gt;H&lt;/sub&gt;7</th>
<th>refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal naive B cells (n=368)</td>
<td>18%</td>
<td>0%</td>
<td>50%</td>
<td>24%</td>
<td>5%</td>
<td>0%</td>
<td>1%</td>
<td>a, 43-45,52</td>
</tr>
<tr>
<td>normal MZ B cells (n=258)</td>
<td>10%</td>
<td>0%</td>
<td>66%</td>
<td>18%</td>
<td>3%</td>
<td>1%</td>
<td>2%</td>
<td>a, 43-46</td>
</tr>
<tr>
<td>normal class switched</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>memory B cells (n=447)</td>
<td>16%</td>
<td>0%</td>
<td>54%</td>
<td>22%</td>
<td>6%</td>
<td>0%</td>
<td>1%</td>
<td>a, 44,45,53</td>
</tr>
<tr>
<td>gastric MZBCL (n=74)</td>
<td>12%</td>
<td>1%</td>
<td>64%</td>
<td>22%</td>
<td>2%</td>
<td>0%</td>
<td>0%</td>
<td>2,54-56</td>
</tr>
<tr>
<td>salivary gland MZBCL (n=34)</td>
<td>62%</td>
<td>0%</td>
<td>35%</td>
<td>3%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>2,54</td>
</tr>
<tr>
<td>pulmonary MZBCL (n=17)</td>
<td>12%</td>
<td>0%</td>
<td>59%</td>
<td>29%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>2,54</td>
</tr>
<tr>
<td>splenic MZBCL (n=147)</td>
<td>38%</td>
<td>1%</td>
<td>34%</td>
<td>22%</td>
<td>3%</td>
<td>1%</td>
<td>1%</td>
<td>2,57-59</td>
</tr>
<tr>
<td>cutaneous MZBCL (n=24)</td>
<td>25%</td>
<td>4%</td>
<td>63%</td>
<td>4%</td>
<td>0%</td>
<td>4%</td>
<td>0%</td>
<td>8,29,60,61</td>
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<tr>
<td>ocular MZBCL (n=86)</td>
<td>10%</td>
<td>0%</td>
<td>56%</td>
<td>29%</td>
<td>3%</td>
<td>0%</td>
<td>1%</td>
<td>b, 20-24</td>
</tr>
</tbody>
</table>

<sup>a</sup> Bende et al. unpublished
<sup>b</sup> this study
IgV<sub>H</sub>-CDR3 amino acid sequences were blasted to GenBank. In contrast to MZBCLs from the stomach and lung as previously reported<sup>4</sup>, no obvious pattern of homology, in particular no RF-homology was observed. Some cases (OM03, OM04, OM16, OM19, OM21, OM24, OM30, OM31) showed homology with different B cell chronic lymphocytic leukemia (B-CLL) IgV<sub>H</sub>-CDR3s, which were mostly mutated and not belonging to one of the known homology groups. OM21 and OM24 shared the same V<sub>H</sub>D<sub>H</sub>-rearrangement with their homologous B-CLL.

**Immunological background**

In view of our recent findings on the group of PCMZL, we assessed the expression of CXCR3 by immunohistochemistry. Although the great majority of OAMZL in this panel expressed IgM, nearly two-third of the cases were CXCR3 negative, as demonstrated by immunohistochemistry (Figure 1 and Table 3). Semi-quantitative RT-PCR showed low

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**Figure 1** Expression of CXCR3 and α<sub>4β7</sub> in ocular adnexal marginal zone B cell lymphomas.

The membrane markers CXCR3 and α<sub>4β7</sub> are variably expressed among ocular adnexal MZBCL, with no obvious correlation to mucosal localisation (conjunctiva). Depicted are examples of double negative, double positive and single positive cases, with CD20 and CD3 staining to enable distinction in expression by tumour B cells and infiltrating T cells. Original magnification 400×.
expression levels of both IFN-γ and IL-4, this in contrast to other MZBCLs and PCMZLs, respectively (Figure 2). *C. psittaci* DNA, as assessed by QRT-PCR, was detected in none of the cases (Table 3). In our series, 10 out of 17 OAMZLs expressed α4β7, while 28 out of 30 other MZBCL were α4β7+ (Figure 1 and Table 3).

### Table 3 Immunological environment and phenotype of ocular adnexal marginal zone B cell lymphomas.

<table>
<thead>
<tr>
<th>Patient</th>
<th>C. psittaci</th>
<th>CXCR3</th>
<th>α4β7</th>
<th>site of origin</th>
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<tbody>
<tr>
<td>OM03</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>conjunctiva</td>
</tr>
<tr>
<td>OM04</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>orbit</td>
</tr>
<tr>
<td>OM05</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>eyelid</td>
</tr>
<tr>
<td>OM08</td>
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<td>-</td>
<td>nd</td>
<td>conjunctiva</td>
</tr>
<tr>
<td>OM09B</td>
<td>-</td>
<td>-</td>
<td>nd</td>
<td>orbit</td>
</tr>
<tr>
<td>OM11</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>orbit</td>
</tr>
<tr>
<td>OM12A</td>
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<td>+</td>
<td>orbit</td>
</tr>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>orbit</td>
</tr>
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<td>+</td>
<td>orbit</td>
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<td>orbit</td>
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<td>OM19</td>
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<td>orbit</td>
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<tr>
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<td>23/25</td>
<td></td>
</tr>
</tbody>
</table>

nd  not done
ni  not informative

a  Including previously reported cases2,8,50
b  Including previously reported cases2,48,50
Genetic aberrations

The typical MZBCL translocation t(11;18) AP1/MALT1 was not detected among the 18 OAMZLs tested (Table 4). Chromosomal translocations involving the IgH and FOXP1 genes have been reported for some OAMZLs, but none of the 11 cases tested here harboured this translocation (Table 4). Mutation analysis of the coiled-coil domain of the MALT1 interactor CARD11 did not reveal any aberrancy (Table 4). For A20 (TNFAIP3), a negative regulator of the NFκB pathway, two patients were found to carry a mutation, both leading to a premature stop codon (Table 4 and Figure 3).

Figure 2 Ocular adnexal marginal zone B cell lymphomas have little cytokine expression in the tissue.
Semi-quantitative RT-PCR for IL-4 and IFN-γ, show that OAMZL have relatively low or no expression of these cytokines, while cutaneous MZBCLs typically express IL-4 and other extranodal MZBCLs are characterised by high IFN-γ levels.

Figure 3 A20 mutation in ocular adnexal marginal zone B cell lymphomas.
Two cases were found to bear a mutation in the A20 coding sequence, both leading to a premature stop codon. The traces illustrate the double peaks that indicated the presence of a mutation. A schematic representation of the A20 protein with its functional domains (OTU, ovarian tumour domain; ZF, zinc-finger domain), provides reference to the sites of mutation.
Table 4 Genetic alterations in ocular adnexal marginal zone B cell lymphomas.

<table>
<thead>
<tr>
<th>Patient</th>
<th>MALT FISH</th>
<th>FOXp1 FISH</th>
<th>CARD11 mutation</th>
<th>A20 mutation</th>
<th>BCL10 IHC</th>
<th>NFκB1 IHC</th>
<th>NFκB2 IHC</th>
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<td>-</td>
<td>+</td>
<td>+/-</td>
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<tr>
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nd, not done
ni, not informative
IHC, immunohistochemistry
Nuclear BCL10, NFkB1 and NFkB2 were assessed immunohistochemically (Table 4 and Figure 4). Weak nuclear BCL10 expression was observed in OM03, moderate and strong expression was seen in OM05 and OM12A respectively (Figure 4), while for the other cases nuclear expression was not detectable. Nuclear NFkB1 signal was seen in the majority of OAMZLs, but the staining was weak as compared to t(11;18) + MZBCL controls and most DLBCL. Only OM12A presented with relatively strong nuclear NFkB1 expression (Figure 4). Nuclear and cytoplasmic NFkB2 was mostly observed in scattered foci throughout the tissue, often co-localising with T-cell rich area's suggestive of localised activation, e.g. by CD40 stimulation. Homogenous nuclear NFkB2 staining on the majority of tumour cells was only seen in OM12A and OM15, and in the latter only weak.
Discussion

MZBCLs generally arise in a background of chronic inflammation and despite strong association with particular pathogenic infections and autoimmunity disorders, the antigenic specificity of the lymphomas is generally unknown. One exception is the RF reactivity we found for a significant proportion of gastric and salivary gland MZBCLs. MZBCLs present with mutated BCRs, with the characteristics of selection against replacement mutations in the framework regions, suggesting a dependence on structural integrity of the BCR. These features were also present in the lymphomas described here. In addition, a skewed IgVH-gene usage is regarded as a potential indication of selective pressure towards recognition of certain epitopes. Overall, the results from 5 studies, including the 20 cases presented here, suggest that VH family members are more often used in OAMZLs, in comparison to normal marginal zone B cells, although this was not found to be statistically significant. Recently, Bahler et al. presented a study of 10 ocular adnexal MZBCLs from the USA which showed a restricted usage of VH4-34 (3 cases, 30%); a gene segment that has been associated with autoimmunity and specificity for the red blood cell i/i antigens. Identical frequencies for VH4-34 were previously found by Mannami et al. among ten patients in Japan. The panel of 20 ocular MZBCLs from The Netherlands presented here, also included 5 cases with VH4-34 (25%). However, in other studies the frequencies for VH4-34 were different: 2/26 cases (8%) from Germany by Coupland et al., 0/8 cases from Germany by Adam et al., and 0/11 cases from Japan by Hara et al. Altogether, 13 of in total 86 (15%) OAMZL used VH4-34, which is significantly higher than the frequency in normal MZ B cells (3.5% n=254). It has been argued that the differences in IgVH-frequencies could represent geographical variations in the presence of particular antigens, like the variation that is seen for the C. psittaci association. However, it is difficult to correlate these two parameters from separate studies, since both vary considerably throughout the geographical areas. Our study and that of Bahler et al., by combining both analyses within the same patient groups, present a skewed Ig repertoire in absence of C. psittaci. A screen for homology of the IgVH-CDR3 amino acid sequences with other Ig sequences on Genbank did not reveal any obvious IgVH-CDR3 homology, and in particular no RF-homology. Some cases appeared to be homologous to mutated B-CLL cases, which might point to a common antigen, yet to be determined.

As it has been shown that CXCR3 is a direct target of the IFN-γ inducible transcription factor T-bet, we speculated that CXCR3 expression on the MZBCLs is induced by the IFN-γ detected in the tissue. The ocular MZBCLs presented here, seem to lack abundant expression of either IFN-γ or IL4. CXCR3 expression was found in one third of the cases, and as such appears unrelated to IFN-γ expression or bacterial infection (as far as known). Possibly, the CXCR3 expression by the OAMZLs is a remnant of an earlier infection, as it has been shown that memory cells, once CXCR3+, maintain expression despite decreasing IFN-γ levels over time.

Previously, the majority of MZBCL were found express the α4β7 integrin. In the study by Liu et al., α4β7 was predominantly found in gastrointestinal MZBCL, whereas
only in a minority of OAMZLs. In the current series, 10/17 OAMZLs expressed α4β7. Unexpectedly, the expression did not correlate with mucosal localisation of the tumours, as 2/3 conjunctival MZBCLs were α4β7−.

The characteristic genetic aberrations in MZBCL, t(11;18) API2/MALT1, t(14;18) IgH/MALT1 and t(1;14) BCL10/IgH involve the NFκB pathway. Most recently, other molecules from the NFκB pathway have been implicated in lymphoma: The positive regulators CARD11 (CARMA1), TRAF2, TRAF5, MAP3K7 (TAK1) and TNFRSF11A (RANK) were found mutated in DLBCL36,51 and the negative regulator A20 (TNFAIP3) was found to be a target of large chromosomal deletions and inactivating mutations in both DLBCL51 and MZBCL, in particular OAMZL13,26. In our genetic screen, 2 inactivating A20 mutations were detected, but no translocations involving MALT1 or BCL10, nor mutations in CARD11. The observed weak expression of p50 but not p52, suggests that some additional cases may have an underlying genetic aberration involving the classical but not the alternative NFκB pathway. Especially OM12A is a suspect candidate for an NFκB activating genetic aberration, as suggested by the strong nuclear expression of all three nuclear markers. Unexpectedly, OM16 and OM29A did not show nuclear expression of p50, despite their A20 mutation. We do not know whether these A20 mutations are accompanied by deletion of the other allele, or are haplo-insufficient. Neither is it known what the exact effect of A20 mutations is on the NFκB pathway. The two-fold enzymatic activity of A20 has been shown to be required to terminate the signal transduction via RIP. Mutation of A20 would lead to a prolonged signal upon activation of the pathway, but perhaps does not provide a constitutive signal by itself. The finding that some DLBCL patients with A20 deficiency also carried a mutation in another gene of the NFκB pathway, suggests that A20 mutation alone does not attain maximal NFκB activation.51 Interestingly, OM16 and OM29A both express Ig with Vh4-34 in the rearrangement and appear selected for preservation of functionality, as judged by the R/S ratios. This suggests that BCR signals may be required to synergise with the defect in negative regulation by A20, in order to obtain full-blown NFκB activation. This would then be in contrast with the mutually exclusive findings of t(11;18) and RF-homology in gastric, salivary gland and pulmonary MZBCLs, and the usual absence of other genetic abnormalities in t(11;18)− cases.2,9 The level of NFκB activation that results from the API2/MALT1 seems sufficient to drive the lymphoma by itself.

References

2. Bende RJ, Aarts WM, Riedl RG, de Jong D, Pals ST, van Noesel CJ. Among B cell non-Hodgkin’s lymphomas, MALT lymphomas express a unique antibody repertoire with


Chronic inflammatory disease, lymphoid tissue neogenesis and extranodal marginal zone B cell lymphomas

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Abstract

Chronic autoimmune or pathogen-induced immune reactions resulting in lymphoid neogenesis are associated with development of malignant lymphomas, mostly extranodal marginal zone B cell lymphomas (MZBCLs). In this review we address (i) chemokines and adhesion molecules involved in lymphoid neogenesis, (ii) the autoimmune diseases and pathogens which are associated with development of B cell lymphomas, (iii) the molecular mechanisms involved in the initiation and progression of MZBCL and (iv) ‘potential’ mouse models for MZBCL.
Lymphoid tissue neogenesis and ectopic germinal center formation

Inflammation is a local response to cellular injury and is initiated by macrophages and local epithelial and/or stromal cells that sense microorganisms and cell damage by pattern recognition receptors, i.e. the Toll-like receptors (TLRs), soluble intracellular NOD-like receptors and RIG-like helicases. The triggered cells respond by secretion of a plethora of inflammatory mediators such as histamine, prostaglandins, leukotrienes, platelet-activating factors and typical pro-inflammatory chemokines and cytokines like IL-1β, IL-6, IL-8 (CXCL8) and TNF. These mediators, and in particular TNF, lead to endothelial activation and vasodilatation followed by a local efflux of circulating leukocytes. The first leukocytes arriving on site are granulocytes which combat the microbial invaders, while monocytes/macrophages clean up dead cells, including apoptotic granulocytes and destructed tissue. In parallel, dendritic cells (DCs) take up and process antigens (Ag) from the intruder, mature and migrate to a local lymph node to set off an adaptive immune response.

Chronic inflammatory conditions, due to improper eradication of pathogens, autoimmune processes or chronic allograft rejections, are associated with the genesis of organized lymphoid tissue. In recent years, a number of key molecular determinants operating during the generation of tertiary lymphoid tissue, have been identified. In the complex sequence of events, TNF is again one of the key molecules as it induces the production of CCL19 and CCL21 (SLC), which are important for the attraction of B- and T-lymphocytes.

The infiltrating lymphocytes switch on expression of membrane-bound lymphotoxin αβ (mLTαβ) when activated e.g. by Ag. High levels of mLTαβ lead to lymphotoxin receptor (LTβ-R) ligation on stromal cells and/or macrophages and induce CXCL13 (BLC) production. The local production of CXCL13 mediates homing of B cells and induces the arrived B cells to further upregulate mLTαβ and probably also TNF. The enhanced interaction of CXCL13-producing stromal cells with the TNF- and mLTαβ-producing B cells promotes differentiation of resident stromal cells into follicular dendritic cells (FDCs) which start expressing characteristic molecules to trap immune complexes i.e. the complement receptors CD21 and CD35 and the FcγR-IIb. Subsequent production of CXCL13 by FDCs establishes a positive feedback loop essential for ectopic lymphoid tissue development, similar to embryonic lymphorganogenesis and normal follicle formation (Figure 1). The importance of LT and TNF in this process has been demonstrated by transgenic expression of TNF, LTα and LTα/β in the pancreas and the kidneys, leading to formation of organized lymphoid tissue including FDC-containing follicles. Transgenic expression of CCL21 alone, resulted in extensive lymphoid tissue development in the pancreas. However, ectopic expression of CXCL12 (SDF), CCL19 or CXCL13 leads to attraction of lymphocytes, some compartmentalization but not to the genesis of FDC-containing follicles.
chronic inflammation formation of tertiary lymphoid tissue containing T and B cell areas
activated endothelium
VCAM1 ICAM1 MADCAM1 PNAD
plasma cell
B-cell area
with GC
IL-2 IFN-γ
B T
macrophage
CD40L and cytokines
T cell dependent initiation of ectopic germinal centers
(self) antigen, CD40L and cytokines
plasma cell
B-cell area with GC
CCL19 CCL21 CCL19 CCL21 CCL13 CCL13
CD20 CD138 CD21 Ki67
Depending on the type of pathogen, i.e. differences in the Ag presentation mode and the combination of co-stimulatory molecules and cytokine signals, Ag presenting cells (APCs) guide T cell differentiation into the direction of T helper type 1 (Th1) or T helper type 2 (Th2) cells. A Th1-polarized response depends on IL-12 and results in IFN-γ producing T cells which also produce IL-2 and TNF and on their turn stimulate macrophages, natural killer (NK) cells and CD8+ cytotoxic T cells. This reaction type mainly generates cellular responses against intracellular pathogens like viruses but also evoke T cell help leading to production of, for example, opsonizing IgG2 antibodies (Ab). A Th2 response is characterized by secretion of IL-4, IL-5 and IL-13, and yields humoral immunity, in particular the generation of plasma cells that secrete high affinity Abs, mainly of IgG1, IgG4, IgA and IgE classes.

In the ectopic follicles, germinal center (GC) reactions may occur (Figure 1). There are no reasons to assume that the biological processes operating in ectopic GCs differ from those in GCs of secondary lymphoid tissues. In secondary lymphoid tissues, GC reactions are initiated after activation of B cells and T cells by native and processed antigenic determinants respectively. The B cells bind and internalize antigenic proteins with their membrane-bound immunoglobulins (mIg or B-cell Ag receptors, BCR) and, after intracellular processing, express Ag-derived peptides in MHC-II molecules at their surface. CD4 T cells, that are activated through cognate interactions with these peptide-MHC complexes, recompense the B cells by providing costimulatory signals (T-cell help) through CD40, CD80, CD86 and cytokine receptors. When properly stimulated at the follicular boundaries, the B cells directly differentiate into short-lived Ab-forming plasma cells or migrate back into the follicle to undergo a phase of brisk proliferation thereby creating the GC dark zone. The rapidly proliferating B cells, termed centroblasts, express high levels of the DNA mutator activation-induced-cytidine-deaminase (AID) and accumulate nucleotide substitutions in their Ig variable (IgV) genes, a process designated as somatic hypermutation (SHM). B cells in the GC are prone to undergo apoptosis except for those which, based on favorable point mutations, obtain higher affinities of their BCRs for the Ag. These high-affinity B cells are selected in the

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**Figure 1**  Lymphoid tissue neogenesis and ectopic germinal center formation.

Upper panels: (a) Chronic inflammation is characterized by high levels of TNF, inducing stromal cells to produce CCL19 and CCL21 which attract B- and T-lymphocytes. (b) The interplay between CXCL13-producing stromal cells and increasing numbers of mLTαβ2 and TNF-expressing B lymphocytes, leads to the development of follicular dendritic cells (FDCs) and subsequent formation of lymphoid follicles. (c) T cells provide specific help to antigen activated B cells via costimulatory cytokines and membrane receptors.

Lower panels: Immunohistochemical stainings on a well-organized lymphoid infiltrate in a minor salivary gland of a Sjögren’s syndrome patient. Highlighted is a B cell follicle including a germinal center using Abs specific for B cells CD20, plasma cells CD138, predominantly follicular dendritic cells CD21 and the proliferation marker Ki67.
GC light zone based on successful competition for survival signals elicited by native Ag that is exposed at the surface of FDC, and by CD40L from GC T cells. In addition, the Ag-selected B cells may undergo class switch recombination (CSR) a process during which the switch (S) region sequence upstream of Cµ-Cδ is recombined with any of the other S region sequences located 5’ of each of the constant region genes Cy3, Cy1, Cα1, Cγ2, Cγ4, Cε and Cα2, thus leading to isotype switching from IgM/IgD to either IgG, IgA or IgE. The Ag-selected B cells, either or not class-switched, will finally differentiate into memory B cells or Ab-producing plasma cells.

Marginal zone B cells

In humans and rodents, distinct populations of recirculating peripheral B cells are being distinguished i.e. naive (B2) or follicular (FO) B cells, naive CD5+ B cells, marginal zone (MZ) B cells and class switched memory B cells (Figure 2). Initial studies in mice and men indicated that CD5-expressing naive B cells frequently display poly-/self-reactivity. However, more recent work in men demonstrated a similar frequency of poly-/self-reactivity between CD5+ and CD5− naive B cells. MZ B cells particularly respond to T-cell independent type 2 (TI-2) Ags, like large polysaccharides of bacterial cell walls and polymeric bacterial flagellin, which by repetitive antigenic epitopes, are able to crosslink BCRs. Naive B2 cells are involved in T-cell dependent (TD) GC reactions, generating plasma cells, secreting high affinity Igs, and CD27+ memory B cells. Recently, we obtained evidence in primary human lymph nodes that isotype-switched memory B cells can re-engage in GC reactions.

The marginal zone (MZ) has originally been defined as an anatomical compartment within the spleen located around primary or secondary follicles and containing B cells with distinct phenotypic and functional characteristics. The MZ of the spleen is believed to be positioned in such a way that it primarily encounters blood borne pathogens. Later, primary mucosa-associated lymphoid tissues (MALT) of e.g. Waldeyer’s ring, Peyer’s patches and appendix, locations known for a significant influx of Ags, were also found to contain a marginal zone. MZ B cells in mice and rats express essentially unmutated IgV genes and are supposed to be non-recirculating. On the other hand, human MZ B cells of both spleen and Peyer’s patches do harbor mutated IgV genes and recirculate (Figure 2). Human splenic MZ B cells are IgM+ IgD− and co-express the B cell markers CD20, CD22 and CD79a/b, the memory B cell marker CD27, the complement receptors CD18/CD11b, CD21 and CD35, and the anti-apoptotic molecule BCL-2. MZ B cells are negative for CD5 and CD23 as well as for the GC markers CD10 and BCL-6. Human MZ B cells, both in tissues and in the circulation, express high levels of CD1c. Expression arrays of splenic and recirculating IgM+ IgD− CD27+ B cells revealed similar profiles, including high expression of CD31, CD44 and IL-6, thus confirming the non-resident nature of MZ B cells in the human.
Figure 2  B cell development in man.
Transitional B cells in the spleen potentially mature into three B cell subsets: (i) CD5⁺ mature naive B cells, (ii) conventional mature naive B2 or FO B cells and marginal zone (MZ) B cells which contain mutated IgV genes possibly acquired in a T-cell independent manner. After antigen recognition, mature naive B2 cells engage in T-cell dependent germinal center (GC) reactions in which SHM and CSR occur, generating high affinity class switched memory B cells and plasma cells (PC).
It is debated where and when human MZ B cells obtain their somatic IgV mutations. There is evidence that this occurs outside of GCs as part of an innate diversification program like in sheep and birds. This is supported by the fact that mutated IgM⁺ IgD⁺ CD27⁺ MZ B cells are also found in CD40L-deficient, hyper-IgM patients lacking GCs and that the Ig repertoire of MZ B cells is as diverse as naive B cells, thus not resembling the highly selected Ig repertoire of class switched IgM⁺ IgD⁻ CD27⁺ B cells. Moreover, AID expression was observed in splenic MZ B cells of children under the age of 2 years, but not in older individuals. Recent data by Scheeren et al. indicated that MZ B cells, with mutated IgV genes, are already present in human fetuses in which no active immune responses are thought to happen. AID expression was found in fetal liver and mesenteric lymph nodes but not in the fetal spleen. Repopulation experiments with human hematopoietic stem cells in Rag2⁻/⁻/γc⁻/⁻ mice showed that IgV-mutated MZ B cells develop in a T cell independent manner. Others think that MZ B cells mutate their IgV genes within GCs and argued that some residual GCs may be present in CD40L-deficient patients. GC formation without T cell help has been described in mice, albeit these GCs were smaller, short-lived and SHM frequencies were low. Moreover, CD40L-deficient patients have significantly lower numbers of circulating MZ B cells, being ~20–25% as compared to healthy individuals, indicative for at least a partial defect in MZ B cell development. According to this scenario, MZ B cells would thus not belong to a distinct developmental lineage, but originate from conventional naive B2 or follicular (FO) B cells. As currently no clue exists with respect to the heterogeneity of the MZ B-cell population, the possibility of multiple developmental routes producing hypermutated B cells with a MZ-like phenotype is not excluded (Figure 2).

It has been demonstrated that about 4% of MZ B cells are responsive to bacterial polysaccharides. Still, a large fraction of MZ B cells may thus have other specificities. In one donor, previously vaccinated with Streptococcus pneumoniae polysaccharide Ag, 2 of the 27 Abs (7%) generated out of the MZ B-cell fraction, specifically reacted with this bacterial Ag. Capolunghi et al. showed, by polyclonal activation of naive and MZ B cells with CpG DNA, anti-S. pneumoniae (PnPS serotype 14) production exclusively by MZ B cells. In children below the age of 2 years, no or limited responses are detected against these TI-2 Ags. After the age of 2, the percentage of IgM⁺ IgD⁺ CD27⁺ MZ B cells in the blood increases, which coincides with the appearance of the anatomical MZ structure in the spleen and with increased humoral responses to TI-2 Ags such as pneumococcal polysaccharides.

Human IgM⁺ CD27⁺ MZ B cells, when compared to IgM⁺ CD27⁻ naive B cells, appear to be selected against poly- and self- reactive BCRs. This selection is associated with a decrease in the average length of the IgVH complementarity determining regions 3 (IgVH-CDR3), which is largely due to deletion of B cells expressing the JH6 gene. Indeed, long IgVH-CDR3s have been associated with self- and poly- reactivity. Upon reversion of somatic IgV mutations to their corresponding germline IgV sequences, these Abs did not regain poly- and/or self- reactivity. This indicates that naive B cells with poly-
/self-reacting BCRs are efficiently excluded from the MZ B cell pool already before the onset of SHM. Also in the rat, selection of naive B cells into the MZ B cell compartment is accompanied by a decrease in IgVH-CDR3 lengths. This selection is most likely driven by self antigens as it is also observed in germ-free rats. Notably, no preferential selection of short CDR3s is observed in conventional class-switched IgG+ CD27+ memory B cells. Surprisingly, it has been described that ~50% of IgG+ CD27+ B cells show poly-/self-reactivity which is generally lost when reverting IgV SHM. Thus, the poly-/self-reactivity of IgG+ memory B cells is due to the accumulation of IgV SHM and not due to intrinsic properties of the CDR3s.

Auto-immune inflammatory conditions associated with B cell lymphomagenesis

A number of chronic autoimmune conditions, organ-specific as well as systemic, is associated with an increased incidence of non-Hodgkin's lymphomas (NHL). Among these, Hashimoto's thyroiditis (HT) and Sjögren's syndrome (SS) are the best examples with increased relative risks of 3 - 67 and 9 - 44 respectively to develop extranodal marginal zone B cell lymphomas (MZBCLs). Moreover, for SS patients a 9-fold increased risk of obtaining a diffuse large B cell lymphoma (DLBCL) has been reported. These lymphomas may develop either de novo or by transformation out of a prior low-grade MZBCL. Systemic lupus erythematosus patients were reported to have 8-fold and 3-fold higher incidences of respectively MZBCL and DLBCL as well. Rheumatoid arthritis (RA) appears to be weakly associated with, non-RA treatment related, development of DLBCL and lympho-plasmacytic lymphoma, with reported odd ratios of 1.8 and 2.5 respectively. A recent meta-analysis however, did not reveal an overall statistically significant association between RA and NHL. Celiac disease is strongly associated with the occurrence of enteropathy-type T cell lymphoma. It is not understood why some autoimmune diseases do and many others like e.g. Crohn's disease, ulcerative colitis, type I diabetes, multiple sclerosis, pernicious anemia and sarcoidosis do not entail increased risks of developing NHLs (Table 1).

Sjögren's syndrome

Sjögren's syndrome (SS) is a systemic autoimmune disease characterized by complaints of dry mouth (xerostomia) and eyes (keratoconjunctivitis sicca). Biopsies of (minor) salivary and lacrimal glands typically show mixed infiltrates consisting of CD4 T cells, some CD8 T cells, macrophages, myeloid and plasmacytoid dendritic cells (DC), B cells (~20%) and plasma cells. In about 20% of the patients the infiltrates contain GCs (Figure 1). Compatibly, lymphocyte attracting chemokines such as CXCL12 and
Table 1  Chronic inflammatory conditions and lymphoma association.

<table>
<thead>
<tr>
<th>Disease or Inflammatory condition</th>
<th>Cause</th>
<th>% of patients with ectopic GCs</th>
<th>Affected tissue</th>
<th>Lymphoma association</th>
<th>Lymphoma sub-types (odd ratios)</th>
<th>references</th>
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<tr>
<td>Rheumatoid arthritis</td>
<td>autoimmune</td>
<td>10-35%</td>
<td>synovial membrane</td>
<td>?</td>
<td>DLBCL (OR: 2), LPL (OR: 3)</td>
<td>7, 48, 53</td>
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<td>Sjogren's sialadenitis</td>
<td>autoimmune</td>
<td>~20%</td>
<td>salivary gland</td>
<td>Yes</td>
<td>DLBCL (OR: 9), MZBCL (OR: 9 – 44)</td>
<td>43, 45-48, 54</td>
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<td>Systemic lupus erythematosus</td>
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<td></td>
<td>systemic</td>
<td>Yes</td>
<td>DLBCL (OR: 3), MZBCL (OR: 8)</td>
<td>48, 52</td>
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<td>Celiac disease</td>
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<td>a</td>
<td>intestinal mucosa</td>
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<td>EATCL (OR: 17)</td>
<td>52</td>
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<td>a</td>
<td>intestinal mucosa</td>
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<td></td>
<td>48, 52</td>
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<td>Ulcerative colitis</td>
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<td>a</td>
<td>colon mucosa</td>
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<td></td>
<td>pancreas</td>
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<td>central nervous system</td>
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<td></td>
<td>systemic</td>
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<td></td>
<td>skin</td>
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<td></td>
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<td>thymus</td>
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<td>Yes</td>
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<td>~30%</td>
<td>arteries</td>
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<td>Conjunctivitis</td>
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<td>eye</td>
<td>Yes</td>
<td>MZBCL (OR: ?)</td>
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<td>Gastritis</td>
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<td>30-85%</td>
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<td>Dermatitis</td>
<td>Borrelia burgdorferi</td>
<td>?</td>
<td>skin/synovial membrane</td>
<td>Yes</td>
<td>MZBCL (OR: ?)</td>
<td>79, 164</td>
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</table>

a In these inflammatory bowel diseases, it is difficult to distinguish between lymphoid neogenesis and hyperplasia of normal mucosa-associated lymphoid tissue
b Early onset myasthenia gravis
CCL21 which mainly attracts T cells and CXCL13 which attracts B cells are abundantly expressed in SS.\textsuperscript{54-58} High expression levels of CXCL13 and CCL21 within inflamed tissues correlated with the extent of inflammatory aggregates, the degree of T cell/B cell compartmentalization, the number of peripheral lymph node addressin (PNAd)-positive high endothelial venules (HEVs) and the presence of FDC-network containing GCs.\textsuperscript{58} Salivary gland epithelial cells of both normal and SS patients produce CCL28, which mediates the homing of CCR10-expressing IgA plasmablasts.\textsuperscript{59} Plasmacytoid DCs are present in SS salivary glands which secrete high amounts of type I IFNs (IFN-\(\alpha\) and \(\beta\)) and IL-6, supporting plasma cell differentiation.\textsuperscript{60} Furthermore, IFN-\(\alpha\) induces the B cell and plasma cell survival cytokine BAFF, which was indeed found to be highly expressed in SS patients.\textsuperscript{60,61} CD4\(^+\) T cells from SS salivary glands express \(\sim 40\)-fold higher mRNA levels of IL-2, IFN-\(\gamma\) and IL-10, as compared to peripheral CD4\(^+\) T cells from SS patients or from healthy controls. In agreement, the IL-4 and IL-5 mRNA levels of the SS CD4\(^+\) T cells were low.\textsuperscript{62} Thus, in general the proinflammatory T-helper cell 1 (Th1)-type cytokines IL-2 and IFN-\(\gamma\) are abundant, in Sjögren's sialadenitis.\textsuperscript{62} Accordingly, the IFN-\(\gamma\) induced inflammatory chemokines CXCL9 (MIG) and CXCL10 (IP-10) are highly expressed in SS salivary glands epithelial cells but not in normal salivary glands.\textsuperscript{56}

A variety of nuclear auto-Ags are humoral immune targets in SS patients. Anti-nuclear Abs, among which are the anti-SSA/Ro and anti-SSB/La Abs, are detectable in 70-85\% of the patients. SSA/Ro52, SSA/Ro60 and SSB/La Ags together form a complex with a small cytoplasmic uridine-rich Y RNA.\textsuperscript{63} Five anti-SSA/Ro human monoclonal Abs of SS patients have been produced, i.e. 2 anti-Ro52 IgM Abs derived from peripheral blood B cells and 3 anti-Ro60 IgG Abs obtained from B cells of affected salivary glands. The IgM anti-SSA/Ro52 Abs were regarded unmutated containing 0 and 3 somatic mutations in their \(IgV_H\) genes, while the IgG anti-SSA/Ro60 Abs were heavily mutated, containing \(>20\) somatic mutations.\textsuperscript{64,65} By immunohistochemistry using biotinylated SSA/Ro52, SSA/Ro60 and SSB/La, evidence was obtained that the anti-SSA/Ro and anti-SSB/La Abs are produced by local plasma cells, most likely generated within the ectopic GCs.\textsuperscript{54} Serum anti-SSA/SSB Ab levels correlated with the presence of ectopic GCs.\textsuperscript{66} By Immunohistochemistry, evidence for AID expression was provided \textsuperscript{67} and by tissue microdissection, formal proof was obtained for the occurrence of clonal B cell expansion and SHM in the ectopic GCs (ref\textsuperscript{68} and our unpublished data). Ig repertoire analysis on cells isolated from crude tissues of parotid and minor salivary glands of SS patients, revealed that most \((\sim 80\%)\) of the infiltrating B cells harbored mutated \(IgV_H\) genes and thus are GC, marginal zone or memory B cells.\textsuperscript{69,70} The combined data strongly suggest local generation and affinity maturation of the auto-Abs.
**Hashimoto’s thyroiditis**

Hashimoto’s thyroiditis (HT) and Grave’s disease represent extremes of a clinical spectrum of typical organ-specific autoimmune diseases, histologically characterized by chronic lymphocytic infiltration. In Grave’s disease, inflammation is generally mild and accompanied by the production of thyrotropin receptor stimulating Abs, resulting in hyperthyroidism. In HT, the infiltrates are more severe and progressive, ultimately causing destruction of the thyroid parenchyma and hypothyroidism. It has been shown that in all HT patients the lymphocytic infiltrate is well-organized containing GCs. T-cell attracting chemokines CXCL12 and CCL21 are produced by the follicular epithelium surrounding HEVs. Ectopic expression of CCL21 in the thyroid of mice lead to the development of lymphoid tissue containing FDCs, resembling HT. In this model, also the IFN-γ inducible inflammatory chemokines CXCL9, 10 and 11 were expressed. Cultures of CD4 and CD8 T cell clones retrieved from human HT patient thyroids yielded high levels of TNF and IFN-γ, whereas hardly any clones produced IL-4. Quantitative RT-PCR on patient’s thyroids revealed high levels of IFN-γ and IL-2, confirming that the Th1 cytokines are highly expressed in HT.

Auto-Abs produced in HT are specific for thyroglobulin (Tg), thyroid peroxidase (TPO) and the thyroid stimulating hormone receptor (TSH-R). The Abs against Tg and TPO are not detected in all patients. Auto-Abs specific for the TSH-R are capable of blocking the activation of this receptor and thus can contribute to the impairment of the thyroid function. Numerous anti-Tg and anti-TPO human monoclonal Abs have been reported, isolated from peripheral blood B cells, thyroid tissue and cervical lymph node tissue. All these human monoclonal Abs were heavily mutated containing >10 mutations per IgVH gene. A correlation has been found between the levels of CCL21, CCL22 and CXCL13 in the inflamed thyroid and the titers of thyroid-specific auto Abs. Biotin-labeled Tg and TPO were shown to bind immunohistochemically to ectopic GCs in HT. The combined data are in support of local generation and affinity maturation of anti-thyroideal Abs.

**Infections indirectly provoking B cell lymphomagenesis**

*Helicobacter pylori*-infection related gastric MZBCL is the most commonly mentioned example of bacterium-driven tumorigenesis but in fact is the only undisputed example. Cutaneous MZBCL has been linked to chronic *Borrelia burgdorferi* dermatitis (Lyme’s disease) in a minority of European patients, but not in cases from Asia or the United States. Recently, an association of *Chlamydia psittaci* and ocular adnexal MZBCL was found by PCR in studies from Italy, South Korea, Germany and Austria. Immunohistochemistry, laser assisted microdissection PCR and electron microscopy further provided evidence that *C. psittaci* was present in monocytes/macrophages within...
the MZBCL. Moreover, *C. psittaci* was cultured *in vitro* from conjunctival swabs and/or PBMCs from 25% of ocular adnexal MZBCL patients. However, the association between *C. psittaci* and ocular adnexal MZBCL could not be confirmed in studies from The Netherlands, Japan and The United States (Florida), suggesting geographical differences regarding this link. Hepatitis C virus (HCV) infection has been inferred in the development of malignant B cell proliferations, in particular splenic and extranodal MZBCLs and DLBCLs. In a large intercontinental study the relative risks for HCV patients to develop MZBCL, DLBCL or lymphoplasmacytic lymphoma were calculated as 2.5, 2.2 and 2.6, respectively. (Table 1)

*H. pylori* is a gram-negative bacterium able to persist during lifetime in the gastric mucosa and is present in ~50% of the world population. *H. pylori* binds tightly to epithelial cells via multiple bacterial surface components. *H. pylori*, like most intracellular bacteria, evoke Th1 immune responses characterized by high IFN-γ levels. IFN-γ targets genes with microbicidal properties such as enzymes that generate NO and O₂ radicals. To circumvent the negative effects of these radicals *H. pylori* produces radical-scavenging enzymes. In addition, *H. pylori* secretes urease to neutralize the local low pH. Most strains of *H. pylori* possess the cag pathogenicity island, including the *CagA* gene. The CagA protein leads to a massive influx of neutrophils by inducing high production levels of the chemotactic factor CXCL8 (IL-8) by epithelial cells. Later, during the chronic phase also T cells, B cells, plasma cells and macrophages are recruited and secondary mucosa-associated lymphoid tissue (MALT) is formed within the gastric mucosa. By in situ hybridization and immunohistochemistry, CXCL13 was found to be expressed in the ectopic primary follicles and mainly in the mantle zones of ectopic GCs. *H. pylori* induces a strong Ab response which does not lead to eradication, but instead may contribute to the tissue damage. Two human anti-*H. pylori* single-chain Ig variable fragment (Ig-Fv) isolated from peripheral blood B cells of a *H. pylori*-infected patient have been reported, each displaying mutated *IgVH* genes (>7 mutations). Lymphoid aggregates with GCs have also been observed in *B. burgdorferi*-induced skin and synovial lesions, and in the liver of ~60% of the patients suffering from chronic HCV infections. In the ectopic GCs of *B. burgdorferi*-induced synovitis, B cell expansion and *IgVH* diversification have been demonstrated.

**Extranodal marginal zone B cell lymphoma**

Extranodal marginal zone B cell lymphoma (MZBCL) of mucosa-associated lymphoid tissue, also designated as MALT lymphoma, appears as heterogeneous infiltrates containing small centrocytic and monocytoid B cells, plasma cells and in some scattered immunoblasts and centroblasts. The growth characteristics of MZBCLs resemble those of the normal MZ of e.g. Peyer’s patches. MZBCLs typically expand around ectopic GCs and are able to invade the epithelium to form lympho-epithelial lesions. Colonization of
ectopic GCs by tumor B cells contribute to a pseudo-follicular growth pattern. Also immuno-phenotypically, MZBCL cells are reminiscent of normal MZ B cells of the spleen and Peyer’s patches: They express the pan-B cell markers CD20, CD22 and CD79a/b, the memory B cell marker CD27, the complement receptors CD18/CD11b, CD21 and CD35, the anti-apoptotic molecule BCL-2, and CD1c/CD1d. MZBCLs do not express CD5 and CD23, nor the GC molecules CD10 and BCL-6. There are as yet no markers by which MZBCLs can be unequivocally identified. MZBCLs have a low tendency to disseminate systemically, a feature which explains why in majority these malignancies can be controlled by local treatments alone. About 30% of MZBCLs disseminate, which, due to expression of the mucosal homing integrin α4β7, most often involves other mucosal sites or regional lymph nodes. Interestingly, in both lymph nodes and spleen, MZBCL cells also tend to expand peri-follicularly, in accordance with their MZ B cell properties.

As outlined, the chronic inflammatory conditions enabling MZBCL development are generally of Th1 type, characterized by high IFN-γ levels. IFN-γ receptor binding leads, via STAT1 activation, to induction of genes encoding microbicidal proteins and to induction of the transcription factor T-bet which on its turn induces, among other genes, expression of the chemokine receptor CXCR3. CXCR3 is the specific receptor for the IFN-γ induced chemokines CXCL9 (MIG), CXCL10 (IP-10) and CXCL11 (ITAC). Indeed extranodal and splenic marginal zone B cell lymphomas almost invariably express CXCR3 and T-bet. High expression of CXCL9 has been demonstrated in histiocytes, fibroblasts and endothelial cells of thyroid and gastric MZBCLs. We recently reported that within the group of MZBCLs, most cutaneous MZBCLs are distinct as they arise in a Th2 background and, in accordance with this immunological context, lack CXCR3 and T-bet and carry isotype-switched Igs. Interestingly, a few cases of cutaneous MZBCL did express IgM and CXCR3 like other MZBCLs, and were thus most likely established in a typical Th1 inflammatory environment. Notably, the few B. burgdorferi-infection associated MZBCLs we have studied, also co-expressed IgM and CXCR3.

Genetic alterations in low and high grade extranodal marginal zone B-cell lymphomas

Recurrent chromosomal translocations identified in MZBCLs are t(11;18) (API-2/MALT1), t(14;18) (IgH/MALT1), t(1;14) (BCL-10/IgH) and t(3;14) (FOX-P1/IgH) (Table 2). Except for the t(11;18), these translocations involve IgH loci, like most translocations in other mature B cell lymphomas. The t(11;18) is extraordinary since it does not involve the Ig locus and encodes a fusion protein which is constituted by the amino-terminal portion of API-2 and the carboxyl-terminal of MALT-1. The over-expression of either BCL-10 or MALT-1, but also the API2-MALT1 chimeric protein, cause constitutive activation of the canonical NF-κB signaling pathway.
### Table 2  Chromosomal translocations of MZBCL.

<table>
<thead>
<tr>
<th>Translocation</th>
<th>Stomach</th>
<th>Lung</th>
<th>Salivary gland</th>
<th>Intestine</th>
<th>Ocular adnexae</th>
<th>Skin</th>
<th>Thyroid</th>
</tr>
</thead>
<tbody>
<tr>
<td>t(11;18) API2/MALT</td>
<td>(57/256) 20%</td>
<td>(63/168) 40%</td>
<td>(3/144) 2%</td>
<td>(13/41) 35%</td>
<td>(10/134) 7%</td>
<td>(4/140) 3%</td>
<td>(1/29) 3%</td>
</tr>
<tr>
<td>t(14;18) IgH/MALT</td>
<td>(2/118) 2%</td>
<td>(6/70) 9%</td>
<td>(5/72) 7%</td>
<td>(0/28) 0%</td>
<td>(11/79) 15%</td>
<td>(7/107) 7%</td>
<td>(0/11) 0%</td>
</tr>
<tr>
<td>t(1;14) BCL10/IgH</td>
<td>(0/118) 0%</td>
<td>(2/70) 3%</td>
<td>(1/72) 1%</td>
<td>(2/28) 7%</td>
<td>(0/79) 0%</td>
<td>(0/94) 0%</td>
<td>(0/11) 0%</td>
</tr>
<tr>
<td>t(3;14) FOXP1/IgH</td>
<td>(9/267) 3%</td>
<td>(0/125) 0%</td>
<td>(0/91) 0%</td>
<td>(0/25) 0%</td>
<td>(4/146) 3%</td>
<td>(2/36) 6%</td>
<td>(3/25) 12%</td>
</tr>
</tbody>
</table>

a The t(14;18) IgH/MALT was only detected by Streubel et al. (130) in 7 of 51 cutaneous MZBCL
b The t(3;14) FOXP1/IgH was only detected by Streubel et al. (126) in 4 of 20 ocular adnexae MZBCL, in 2 of 20 cutaneous MZBCL and in 3 of 6 thyroid MZBCL

t(11;18) references: 80 and 130-133, 137-139; t(14;18) references: 130-133; t(1;14) references 130-133; t(3;14) references 126, 131 and 147-150
is found in 5-15% of pulmonary, salivary gland and ocular adnexae MZBCLs. About 5% of intestinal and pulmonary MZBCLs harbor the t(1;14) (Table 2). More recently, three novel IgH translocations in non-gastric MZBCL involving, ODZ2, CNN3 and JMJD2C have been described, of which ODZ2 and JMJD2C were found recurrently.

The t(11;18) is present in ~40% of pulmonary- and in ~20% of gastric- MZBCLs and is virtually absent in MZBCLs of the salivary gland (Table 2). Gastric MZBCLs harboring the t(11;18) were found to be associated with CagA-positive strains of H. pylori. CagA induces activation of neutrophils releasing reactive oxygen species. It has been hypothesized that these are the genotoxic conditions which are instrumental in

**Figure 3  Scenarios of multistep development of gastric MZBCL.**

Chronic *H. pylori* (HP) infection induces lymphoid tissue neogenesis. As a result of both direct and indirect stimulation infiltrating B cells will undergo active proliferation. Direct antigenic stimulation can be accomplished by auto-Ags like IgG-containing immune complexes, by bacterial or other, unknown, Ags. Indirect stimulation is provided by *H. pylori*-specific T cells. Due to the acquisition of genetic damage B cells may obtain growth advantage. Gastric MZBCL with t(11;18) grow autonomously, do not respond to *H. pylori* eradication, but rarely progress to DLBCL. Gastric MZBCL with trisomy 3 and/or 18 and/or having extra copies of the *MALT* gene have a more aggressive clinical behavior. Following inactivation of the tumor suppressor genes *TP53* or *CDKN2A* or due to mutation in oncogenes, possibly by aberrant SHM, MZBCL may transform to DLBCL.
generating the t(11;18).\textsuperscript{137} The assembled literature indicates that t(11;18)-carrying MZBCLs generally possess a limited degree of additional chromosomal imbalances, are non-responsive to \textit{H. pylori} eradication therapy and are not prone to transform into high grade DLBCLs.\textsuperscript{50,51,140-142} T(11;18)-negative gastric MZBCLs with a high degree of genomic imbalances were also associated with \textit{H. pylori} independency.\textsuperscript{143} Trisomies of chromosome 3, 12 and 18 are observed in t(11;18)-negative gastric (20%), pulmonary (40%), ocular adnexae (40%) and salivary gland (60%) MZBCLs.\textsuperscript{130,144} Interestingly, in MZBCLs concurrent gains at 8q24, 9q34, 11q11-13 and 18q21 are frequent.\textsuperscript{144,145} The gains of these loci appear to target genes whose products stimulate the NF-\kappa B pathway (i.e. TRAF2 and CARD9 at 9q34, RELA at 11q11-13 and MALT-1 at 18q21) and the cell cycle (Cyclin D1 at 11q12-13) (Figure 3).\textsuperscript{145} Gain of 6p and loss of 6q23 was specifically found in ocular adnexal MZBCL in \textasciitilde{}25\% of the cases.\textsuperscript{144,146} High resolution tile-path array CGH indicated that 6p gains were centered at the TNF locus at 6p21.33 with NF-\kappa B inhibitor-like 1, TNF, LT\alpha and LT\beta as putative target genes.\textsuperscript{144} The loss of 6q23, consistently deleted the TNF-induced protein 3 also known as A20 at 6q23.3.\textsuperscript{144,146} FISH assays further confirmed the occurrence of A20 deletions in MZBCLs of the ocular adnexa (19\%), salivary gland (8\%) and thyroid (11\%) but not in MZBCLs of lung, stomach, skin and small intestine. A20 is a potent inhibitor of NF-\kappa B signaling which is required for termination of TNF- and TLR- induced NF-\kappa B activation. A recent study showed that both MALT1 and API2-MALT1 can inactivate the A20 inhibitor by proteolysis, which further implicates A20 in the pathogenesis of MZBCL.\textsuperscript{144}

The t(3;14)(\textit{FOX-P1}/IgH), deregulating expression of the forkhead box P1 (FOX-P1) transcription factor, was initially reported by an Austrian study in as much as 4 out of 20 (20\%) ocular adnexae MZBCLs and in 3 out of 6 (50\%) thyroid MZBCLs.\textsuperscript{126} However, in more recent studies by North American and German groups, this translocation was not detected in series of 133 and 122 MZBCLs, respectively.\textsuperscript{131,147} Also others did not detect the t(3;14) in series of 126 ocular adnexae and 19 thyroid MZBCLs.\textsuperscript{131,147,149} On the other hand, Goatly et al.\textsuperscript{148} reported the t(3;14) in 8 out of 188 (4\%) gastric MZBCLs (Table 2). Strong nuclear FOX-P1 expression has been found, irrespective of the t(3;14) or \textit{FOX-P1} copy number changes, in \textasciitilde{}30\% of MZBCLs.\textsuperscript{148,150} Sagaert et al.\textsuperscript{150} recently described five MALT lymphomas with strong nuclear FOX-P1 expression, one with the t(3;14) and four having trisomy 3 and 18, which all transformed into an aggressive, ABC-type DLBCL.\textsuperscript{150} Finally, there is little dispute that t(3;14) is prevalent in a subset of DLBCLs, with extranodal presentation and having the activated B cell-like (ABC) expression profile.\textsuperscript{147,149,151}

Although the precise frequencies of transition of the various low-grade MZBCLs into DLBCLs are not clear in literature, ample evidence exists that MZBCLs can transform particularly into ABC-type DLBCLs rather than into GC-type DLBCLs; (i) trisomy 3 has been observed as a characteristic alteration in both MZBCLs and ABC-type DLBCLs\textsuperscript{49,51,152}, (ii) MZBCLs with high nuclear FOX-P1 were documented to progress into ABC-type DLBCLs\textsuperscript{150}, (iii) the majority of genomic alterations in t(11;18)-negative MZBCLs is also found in ABC-type DLBCLs\textsuperscript{49,152}, (iv) both MZBCLs and ABC-type DLBCLs are
characterized by constitutive NF-κB signaling, (v) DLBCLs which still contain a low-grade lymphoma component are in majority of the ABC type, (vi) primary gastrointestinal DLBCLs show a similar expression profile as gastrointestinal MALT lymphoma and (vii) the majority of rheumatoid arthritis-associated DLBCLs are of the ABC type.

The molecular mechanisms underlying MZBCL progression are as yet ill-defined. A number of genetic alterations has been associated with histologic transformation such as allelic loss and mutation of TP53 and hyper-methylation or deletion of CDKN2A (p16-INK4A, ARF). Furthermore, several chromosomal gains and losses are associated with transformation. Since most MZBCLs express mutated IgV genes with intra-clonal sequence variation, proving previous and suggesting continued exposure to the SHM machinery, a role for the DNA mutator AID in MZBCL transformation cannot be excluded. However, immunohistochemical expression analyses showed that AID is detectable only in a minority of the cases. Accordingly, several investigators demonstrated variable, but generally low AID mRNA expression levels in MALT lymphomas. On the other hand, in ~50% of DLBCL, several proto-oncogenes, including PIM1, PAX5, RhoH/TTF and cMYC are targeted by aberrant SHM. Sequence analysis of MALT lymphomas revealed that 75% of low-grade MZBCLs and 100% of low-grade MZBCLs with a DLBCL component contained mutations in one or more of these oncogenes. In the latter group, higher frequencies of aberrant SHM were found as compared to pure low-grade MZBCLs, supporting the concept of AID-mediated lymphoma progression.

**BCR specificity of MZBCL**

The general idea is that MZBCLs still depend on environmental stimuli and on antigen-receptor ligands. IgVH and IgVL gene sequence analyses have revealed that in spite of high mutation loads the overall structure of the Ig is being preserved. Apparently, selective forces prevent the outgrowth of BCR- MZBCL mutants. Nearly 80% of early stage H. pylori-associated gastric MZBCLs, but also a proportion of cutaneous and ocular adnexal MZBCLs is curable by bacterial eradication alone. Similarly, IFNα-2b treatment can cause regression of HCV-associated MZBCL. In vitro culture experiments with gastric MZBCL cells have revealed that the tumor B cells do not respond to H. pylori directly, but instead depend for their survival on stimuli provided by intra-tumoral, H. pylori-specific T cells. We have recently produced soluble recombinant Abs derived from gastric and other MZBCLs, and indeed did not observe any reactivity with H. pylori bacteria. Alternatively, it appeared that ~10% of gastric, and as much as ~40% of salivary gland MZBCLs, expressed V1-69/JH4- and V3-7/JH3-encoded BCRs with strong IgVH-CDR3 amino acid sequence homology to canonical rheumatoid factors (RF). Among an extensive panel of B-NHLs, this RF homology was unique for MZBCL. Indeed 7 out of 10 recombinant MZBCL-derived
Abs showed strong \textit{in vitro} binding activity to immobilized human IgG.\textsuperscript{118} MZBCLs with high affinity IgG-specific BCRs may thus continuously be stimulated by Ab-Ag immune complexes, like IgG-opsonized \textit{H. pylori} in chronic gastritis or IgG-chromatin and/or IgG-SSA/SSB-RNA in Sjögren’s sialadenitis. The IgG-reactive BCRs may also capture and internalize Ab-Ag complexes and activate TLR9 and/or TLR7 by autologous or bacterial CpG DNA or by autoantigen-associated RNA, consequently potentiating the NF-\textit{κ}B pathway (Figure 4). Synergistic effects of BCR and TLR9 or TLR7 engagement have originally been shown in the mouse by T cell independent activation of IgG-reactive B cells, using IgG-chromatin or IgG-RNA complexes.\textsuperscript{171,172}

Intriguingly, none of 8 previously published\textsuperscript{118}, nor 12 newly analyzed MZBCLs (ref \textsuperscript{170} and H. Inagaki, personal communication) that harbored the t(11;18), express BCRs with RF homology or reactivity. Moreover, the frequency of RF-BCRs, being ~40% of salivary

\textbf{Figure 4}  MZBCL proliferation depends on constitutive NF-\textit{κ}B signaling provoked either by combined CD40/BCR/TLR9 signaling or by the API2-MALT1 fusion protein.

Gastric- and salivary gland MZBCL, lacking t(11;18), depend on CD40L and other T-help factors, together with the (RF-specific) BCR and/or TLR7/9 NF-\textit{κ}B signals. MZBCL with constitutive NF-\textit{κ}B signaling due to t(11;18), do not depend on T-helper factors, BCR nor TLR signaling.
gland, ~10% of gastric and <1% of pulmonary MZBCLs, inversely correlates with the t(11;18) frequencies found in these entities (Table 2). This tentative inverse relation suggests that t(11;18)+ MZBCLs do not depend on BCR (and perhaps neither on CD40 and TLR7/9) signals for their expansion since constitutive NF-κB activation is already guaranteed due to the expressed fusion protein (Figures 3 and 4). The facts that (i) t(11;18)+ gastric MZBCLs are resistant to *H. pylori* eradication therapy and that (ii) within the overall group of MZBCLs, t(11;18) and trisomy 3 harboring cases were from patients without underlying autoimmune diseases, support this hypothesis. Of note, the finding that t(11;18)+ MZBCLs lack RF-BCRs, indicates that this genetic aberration occurs independent to the selection process favoring this specificity.

**Mouse models of marginal zone B cell lymphoma**

As yet a limited number of mouse models has been generated aiming at MZBCL development. In general, four categories of potential ‘MZBCL’ models can be discerned; (i) mice with transgenic expression of genes involved in lympho-organogenesis, (ii) mice chronically challenged with *Helicobacter* species, (iii) mice carrying MZBCL-specific gene alterations and (iv) mice with chronic or uncontrolled T-cell mediated B cell activation.

As delineated in the first chapter, transgenic expression of the key molecules LT and TNF results in augmented lymphoid tissue neogenesis. Similarly, transgenic expression of B- and T-lymphocyte-attracting chemokines initiates formation of ectopic lymphoid tissues, presumably also via the LT/TNF axis. Although it could be argued that in these transgenic animals the continued ectopic lymphoproliferation would ultimately lead to cellular transformation, development of MZBCLs has not been described. This may be due to the fact that in these mice chronic antigenic stimulation has not been assayed.

In mice infected with *Helicobacter* species, the occurrence of organized lymphoid tissues in and beyond the gastric mucosa has been described. Oral infection of A/J mice with *Helicobacter sp.* leads to development of hepatic inflammatory lesions containing HEVs, the production of CCL21 and CXCL13 and influx of B and T cells. Infection of BALB/c mice with *Helicobacter felis* resulted in a massive influx of B cells and lympho-organogenesis in the stomach. It was reported that after 23 months of infection, 25-75% of these mice developed low- or intermediate-grade MZBCLs. In these mice, regression of the infiltrates after anti-bacterial therapy was also demonstrated. Other investigators have infected BALB/c mice with different *H. heilmannii* isolates originating from human and animal hosts. MZBCLs developed in ~25% of the infected mice. The lymphoma prevalence was dependent on the origin of the infecting isolates and the duration of infection. Finally, infection of C57BL/6 mice with *Candidatus H. heilmannii* resulted in the development of gastric MALT lymphoma in 100% of the mice after 6 months. It is noted that in all these infection protocols, the
diagnosis of MZBCLs was based on morphological grounds alone i.e. the presence of centrocyte-like cells and lympho-epithelial lesions, but not on the assessment of monoclonality by Ig gene rearrangement assays nor on any other molecular genetic analyses.174;175

Transgenic FVB mice expressing the API2-MALT1 fusion gene, driven by the SRα promoter under control of the Eμ Ig heavy chain enhancer, specifically triggered the expansion of splenic MZ B cells. However, the expression of the API2-MALT1 fusion protein alone was not sufficient for the development of lymphomas over a period of 50 weeks.179 Immunization of these mice with complete Freund’s adjuvants induced the loss of splenic compartmentalization and a poly- or oligo-clonal lymphoid hyperplasia which gradually disappeared after cessation of the antigenic stimulation.180 P100−/− mice with signal-independent activation of the noncanonical NF-κB pathway had markedly elevated MZ B cell numbers and a disturbed spleen microarchitecture.181 BAFF overexpression, activating the noncanonical NF-κB pathway in BAFF-Tg and BAFF-Tg/TNF−/− mice, resulted in increased survival and accumulation of transitional T2 and MZ B cells.182,183 Interestingly, the BAFF−/− mice showed a high incidence of B cell infiltrates with histological features of extranodal MZBCL, but again not substantiated by molecular analyses.183 Mice with constitutively active IKK2, enhancing the canonical NF-κB pathway, showed a mild B cell hyperplasia rather due to prolonged survival than to proliferation. Cell proliferation was dramatically enhanced when the IKK2 B cells were stimulated via the BCR or TLR4/9.184 Mice with B cell-specific expression of a chimeric CD40-latent membrane protein 1 (LMP1) protein, showed an increased number of FO- and MZ- B cells in secondary lymphoid organs. The constitutive CD40-like signaling via the cytoplasmic LMP-1 tail in the B cells induced activation of the noncanonical NF-κB pathway, but also of the MAP kinases, Jnk and ERK.185 Interestingly, in mice of >12 months, oligo- and mono-clonal B cell lymphomas developed at a high incidence. These B cell lymphomas did not resemble human MZBCLs as they did not express CD21.

Some mouse models nicely underscore the role of chronic T cell help in the development of B-cell lymphomas. For example, in Igλ transgenic mice with B cells presenting Igλ idiotype (Id)-derived peptides on MHC class II, the transferral of Id-specific CD4 T cells resulted in B-cell lymphoma development after approximately 40 weeks. The lymphomas resembled MZBCLs as they expressed CD21, CD35, CD1d and IgM. Moreover, the lymphomas were shown to be mono-/bi-clonal, harbored some somatic IgVH mutations and had major cytogenetic aberrations.186 In mice deficient for the autoimmune regulator (Aire) gene showed a high frequency of MZBCLs after 15-24 months. The B-cell lymphoproliferations were shown to be oligoclonal in the spleens of 4 out of 9 mice and displayed a MZ B cell phenotype with low IgD and high CD1d.187 Interestingly, Aire is a recently discovered transcription factor that is expressed in thymic medullary epithelial cells and plays a key role in central tolerance induction.188 Thus, in these mice MZBCL development is most likely related to chronic help from autoreactive T cells.
In conclusion, the assembled literature points towards a key role of constitutive NF-κB signalling in MZBCL development. This requirement is fulfilled by the combination of persistent BCR triggering, chronic T-cell help and TLR stimulation elicited by chronic infection or autoimmunity. In the ectopically formed lymphoid tissue, these physiological stimuli can be overruled by genetic alterations which guarantee constitutive NF-κB signalling, thus making the cells less dependent on the environmental stimuli.

References


113. Beima KM, Miazgowicz MM, Lewis MD et al. T-bet binding to newly identified target gene promoters is cell type-independent but results in variable context-dependent functional effects. J.Biol.Chem. 2006;281:11992-12000.


121. Auer IA, Gascoyne RD, Connors JM et al. t(11;18)(q21;q21) is the most common translocation in MALT lymphomas. Annals of Oncology 1997;8:979-985.


142. Liu H, Ye H, Ruskone-Fourmestraux A et al. T(11;18) is a marker for all stage gastric MALT lymphomas that will not respond to H. pylori eradication. Gastroenterology 2002;122:1286-1294.


144. Chanudet E, Ye H, Ferry J et al. A20 deletion is associated with copy number gain at the TNFA/B/C locus and occurs preferentially in translocation-negative MALT lymphoma of the ocular adnexa and salivary glands. J.Pathol. 2008


147. Haralambieva E, Adam P, Ventura R et al. Genetic rearrangement of FOXP1 is predominantly detected in a subset of diffuse large B-cell lymphomas with extranodal presentation. Leukemia 2006


154. Davis RE, Brown KD, Siebenlist U, Staudt LM. Constitutive nuclear factor kappaB activity


156. Barth TF, Barth CA, Kestler HA et al. Transcriptional profiling suggests that secondary and primary large B-cell lymphomas of the gastrointestinal (GI) tract are blastic variants of GI marginal zone lymphoma. J.Pathol. 2007;211:305-313.


169. Miklos JA, Swerdlow SH, Bahler DW. Salivary gland mucosa-associated lymphoid tissue


Antigen receptors and somatic hypermutation in B-cell Chronic Lymphocytic Leukemia with Richter’s transformation

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Abstract

**Background and objective:** Activation-induced cytidine deaminase is essential for somatic hypermutation and class switch recombination of the immunoglobulin genes in B cells. It has been proposed that aberrant targeting of the somatic hypermutation machinery is instrumental in initiation and progression of B-cell non-Hodgkin’s lymphomas. In this study, we investigated the B-cell receptor and the role of the somatic hypermutation machinery in B-cell chronic lymphocytic leukemias (B-CLL) prior and after transformation to a lymphoma of a higher malignancy grade (Richter’s transformation).

**Design and Methods:** We investigated the activity of the somatic hypermutation machinery in 9 B-CLL and secondary diffuse large B-cell lymphomas by measuring the expression of Activation-induced cytidine deaminase, in combination with mutation analysis of immunoglobulin (Ig) and non-Ig genes. Furthermore, the structure of the antigen receptors of B-CLL known to have developed a Richter’s syndrome (RS B-CLL) was analyzed by comparing the most variable region of the Ig, the CDR3 region, to CDR3 sequences present on GenBank.

**Results and Interpretation:** Ig variable heavy chain ($IgV_H$) gene studies revealed that Richter’s transformation occurs almost exclusively in unmutated B-CLL. Furthermore, AID expression and somatic hypermutation activity of most RS B-CLL were found higher than those of control (non-transforming) B-CLL. Finally, comparison of the $IgV_H$-CDR3 regions showed a remarkable amino acid sequence homology between 2 RS B-CLL of our panel and 2 RS B-CLL described in literature.

**Conclusion:** The combined findings suggest a role for the Ig gene diversification apparatus during Richter’s transformation and show that distinct RS-B-CLL may recognize recurrent antigenic epitopes.
Introduction

Richter’s syndrome (RS) is the rare occurrence of a histologically and clinically aggressive secondary lymphoid malignancy in a patient with B-cell chronic lymphocytic leukemia (B-CLL)\(^1\). In approximately 3–5 % of B-CLL cases, a lymphoma of a higher malignancy grade develops, reducing the mean disease-free survival to 6 months\(^2\). Usually the high-grade lymphoma is classified as a diffuse large B cell lymphoma (DLBCL) and less commonly as a Hodgkin’s lymphoma\(^3\).

B-CLL is characterized by an accumulation of long-lived, monoclonal CD5\(^-\) CD23\(^-\) mature B cells that express low levels of membrane-bound immunoglobulin\(^1\). About 50% of the B-CLL harbor somatic mutations in their immunoglobulin variable heavy chain (\(IgV_H\)) genes\(^4\). Although the difference in mutation status suggests a different cell of origin, gene expression profiling revealed that mutated and unmutated B-CLL are both most similar to normal memory B cells\(^5\). B-CLL express an \(IgV_H\) gene repertoire clearly distinct from the \(IgV_H\) repertoire of normal B cells of any lineage or maturational stage\(^4\). It has been reported by others and us that \(~19\) % of B-CLL, mostly unmutated, express \(IgV_H\)-CDR3 amino acid sequences homologous with CDR3 regions of other B-CLL (so called inter-B-CLL CDR3 homology)\(^7\)–\(^11\). As yet, at least 8 B-CLL \(IgV_H\)-CDR3 homology groups have been defined\(^10\). The occurrence of highly homologous B-cell receptors among B-CLL strongly suggests that they recognize a limited set of distinct antigenic determinants.

It is unknown to what extent the somatic hypermutation machinery is active in B-CLL. It is generally assumed that B-CLL have a low tendency to acquire additional mutations over time\(^12\). Accordingly, the overall expression level of the enzyme that is essentially required for both somatic hypermutation and class switch recombination, i.e. Activation-induced cytidine deaminase (AID)\(^13\)\(^\text{,}^14\), is very low in blood-derived B-CLL samples as compared to those of purified germinal centre (GC) B cells\(^15\)\(^\text{,}^16\). It has been reported that only a small fraction (<1%) of circulating B-CLL cells, particularly of the \(IgV_H\)-unmutated subgroup, expresses AID\(^17\). However, in whole lymph node samples, where the CD40-expressing B-CLL cells are in close contact with CD40L-expressing CD4\(^+\) T cells, overall AID expression was found to be higher\(^18\). In accordance, \textit{in vitro} stimulation of B-CLL cells by CD4\(^+\) T cells and anti-B-cell receptor antibodies, induces somatic hypermutation in the \(IgV_H\) genes\(^19\).

It has been proposed that promiscuous targeting of the somatic hypermutation machinery may be an initial event in the development of a number of DLBCL\(^20\). It is not known whether this mechanism also applies to progression of low-grade B-non Hodgkin’s lymphomas (B-NHL). In the current study, we analyzed the B-cell receptor and the process of somatic hypermutation in a panel of B-CLL with documented transformation. Our data suggest restricted B-cell receptor specificities and an active somatic hypermutation machinery in B-CLL undergoing Richter’s transformation.
Material and methods

Patient material
All lymphomas were diagnosed according to the WHO classification system. Lymph node material of RS1, RS8 and the control B-CLL was freshly frozen in liquid nitrogen directly after surgical removal. Immunohistochemical analysis of RS1 and RS8 revealed that more than 80% of the tissue consisted of tumor cells. Of all other RS cases and of the peripheral blood samples of the control B-CLL, cell suspensions were frozen in 20% DMSO (Merck, Darmstadt, Germany) in FCS (Invitrogen, Breda, The Netherlands). This study was conducted in accordance with the ethical standards in our institutional medical committee on human experimentation, as well as in agreement with the Helsinki Declaration of 1975, revised in 1983.

FACS analysis and cell sorting
The following monoclonal antibodies (mAb) were used for FACS analysis: PE-conjugated anti-CD23 (clone EBVCS-5; Dako, Glostrup, Denmark), PE-conjugated or FITC-conjugated anti-CD5 (clone L17F12; Dako) and APC-conjugated or PerCP-Cy5.5-conjugated anti-CD19 (clone SJ25C1; Becton Dickinson Biosciences, Erembodegem-Aalst, Belgium). The following polyclonal antibodies were used: FITC-conjugated anti-Igκ, anti-IgM, anti-IgD and anti-IgA, PE-conjugated anti-Igλ, and anti-IgG (polyclonals from Southern Biotechnology Associates, Birmingham, AL). FACS analyses revealed that the cell suspensions of RS3, RS9, RS10, RS11c and RS12 consisted of >90% tumor cells, whereas the peripheral blood sample of RS11a consisted of 60% tumor cells. Of RS4 and RS6, the CD5+, CD19+ tumor cells were isolated using a FACS-Aria (BD Biosciences) cell sorter what resulted in more than 97% pure tumor samples. Germinal centre (GC) B cells were sorted as described previously.

Immunohistochemistry
AID was visualized in formalin-fixed, paraffin-embedded tissue sections using a rat monoclonal antibody. After deparaffination, blocking and antigen retrieval the slides were incubated overnight at 4°C with the primary antibody (1:1000), followed by application of an HRP-conjugated rabbit-anti-rat antibody (1:200, Dako). Subsequently, biotin-free tyramide signal amplification (Dako CSAII kit) enabled detection of AID, which was visualized with Nova Red (Vector). A hyperplastic tonsil functioned as a positive control, omission of the primary antibody as a negative control.

Monoclonal antibodies specific for CD5 (Lab vision, Neomarkers, Fremont, CA), CD23, BCL6, and Ki67 (all from Dako) were used. Antibody detection was performed with the Powervision system (ImmunoVision Technologies, Daly City, CA) and succeeded by peroxidase visualization with 3,3’-diaminobenzidine (DAB) (Sigma), 0.03% H₂O₂ in Tris-HCl pH 7.6.
RNA and DNA were isolated using the Trizol reagent (Invitrogen, Breda, The Netherlands) according to the manufacturer’s description. First-strand complementary DNA (cDNA) was synthesized as described previously albeit 5’-(dT)_{14}-d(A/G/C)-d(A/G/C/T)-3’ primers were used.

Amplification, cloning and sequencing

IgV\textsubscript{H} transcripts were amplified using a mixture of forward primers located in the FR1 regions of the IgV\textsubscript{H} gene families V\textsubscript{H}1 to V\textsubscript{H}6 or alternatively in the FR3 region of V\textsubscript{H}1 to V\textsubscript{H}6\textsuperscript{23} in combination with one of the FAM-labeled reverse primers located in C\textsubscript{\mu}, C\textsubscript{\delta}, C\textsubscript{\alpha} or C\textsubscript{\gamma} regions\textsuperscript{24}. The PCR reaction was performed as described previously using a 30 cycles program\textsuperscript{24} and run on an ABI PRISM 3100 automated sequencer in the presence of either 1pM ROX500 or 1pM ROX1000 marker (Applied biosystems, Warrington, UK). Results were analyzed using the program Genescan analysis (Applied Biosystems). When a monoclonal tumor population was present, IgV\textsubscript{H} amplicons were cloned into the pTOPO-TA vectors and transformed into TOP10 bacteria according to the manufacturer’s description (Invitrogen) and 8 to 16 clones were sequenced of each lymphoma. Sequencing on both strands was performed by an ABI PRISM 3100 automated sequencer (Applied Biosystems) using the big dye-terminator cycle-sequencing kit (Perkin Elmer Corporation). The consensus IgV\textsubscript{H} sequence is defined as the nucleotide sequence that is shared by more than 50% of the clones. Nucleotide alterations that are present in less than 50% of the clones are considered as intraclonal variation. Of note, according to our nomenclature, nucleotide alterations that are present in multiple clones (confirmed nucleotide differences) but are present in <50% of the clones are thus still regarded as intraclonal variation. The amount of intraclonal variation (ICV) was calculated as the mean number of nucleotide differences per clone compared to the consensus IgV\textsubscript{H} sequence and was considered significant when it was higher than the Taq error rate. To determine the Platinum Taq error rate of our experimental design, 48 clones of HPRT were sequenced using the primers 5’TTCCTCCTCTCGAG CAGTCAGC3’ and 5’GCGATGTCAATAGGACTCCAGATG3’. These clones were generated according to the same PCR and cloning procedures as used for the IgV\textsubscript{H} genes. The Taq error frequency thus established is 0.2 per 300 bp.

BCL6 was amplified using the primers 5’CCGCTGCTCATGATCATT3’ and 5’CAGACTCGAGTCTTCCCCATGGATCCACC3’; PIM1 was amplified and sequenced using the primers 5’AGCAGCAGCAACCCACTG3’ and 5’CTCTCCCCACGTG GAAATCC3’. The PCR mixtures contained 1 × pfX amplification buffer, 1U platinum pfX DNA polymerase (Invitrogen), 1mM (BCL6) or 2.5mM (PIM1) MgSO\textsubscript{4}, 0.2mM of each dNTP, 0.5mM of each primer and 1 × enhancer solution.

Both BCL6 and PIM1 PCR reactions started with 3 minutes at 94°C, followed by 39 cycles of 30 seconds at 94°C, 1 minute at 55°C and 1 minute at 68°C. The reaction was terminated for 4 minutes at 68°C. BCL6 and PIM1 amplicons were cloned as described...
above and 12-24 clones were sequenced. BCL6 was sequenced using the primers 5’CCGCTGCTCATGATCATT3’ in combination with 5’GCAAGCGAGAAAAGAGGAA3’ and 5’GTACGCGTTGTGATCTCTCT3’ in combination with 5’CAGACTCGAGTCTTCCATGATCCAC3’. BCL6 and PIM1 were amplified from DNA. Since DNA harbors two alleles of all genes, the consensus BCL6 and PIM1 sequence was defined as the nucleotide sequence that is shared by more than 25% of the clones.

QRT-PCR reactions

Quantitative RT-PCR analyses were performed using a LightCycler (Roche, Almere, The Netherlands). AID was amplified using the primers 5’AGAGGCGTGACAGTGCTACA 3’ and 5’TGTAGCGGAGGAAGAGCAAT 3’ matching sequences in the 3’ end of exon 2 and 5’ end of exon 3, respectively. All reported AID splice variants are detected by this PCR (but not discriminated) except for the splice variant lacking the 3’ end of exon 2 and whole exon 3 en 45. β-Actin was amplified using the primers 5’GGATGACAGGAGATCACTG 3’ and 5’CGATCCACACGGAGTACTTG 3’. The PCR reactions for both AID and β-actin were performed in a volume of 10 µl containing 2 µl cDNA, 1 µl FastStart DNA MasterPLUS SYBR Green I mix (Roche) and 0.5 pM forward and reverse primers. The PCR protocols to amplify AID and β-actin started with 95°C for 6 minutes, after which 40 cycles of amplification were performed, i.e. successively 10 seconds at 95°C, 5 seconds at 60°C (AID) or 61°C (β-actin) and 5 seconds at 72°C (AID) or 8 seconds at 72°C (β-actin). Melting curve analysis was performed to check for PCR specificity. Starting concentrations of mRNAs and PCR efficiencies for each sample were calculated using the LinRegPCR computer program as described before26. Results are expressed as ratios of the calculated values of AID and β-actin.

In vitro stimulation of B-CLL cells

B-CLL cells were cultured for four days in 24-wells plates (Costar, Corning NY, USA). Each well contained 2 × 10^4 B-CLL cells and 1 × 10^5 L cells as a control or 1 × 10^5 CD40L-transfected L cells and 400 U/ml IL4 (Strattmann, Hannover, Germany) with and without anti-IgM (clone MH15/1)(Sanquin, Amsterdam, The Netherlands) coupled CNBR-activated sepharose beads (Amersham biosciences, Uppsala, Sweden). As a positive control peripheral blood B cells of healthy volunteers were stimulated with each experiment.

Results

B-CLL with Richter’s transformation

Tumor samples of 9 B-CLL that underwent clinical and histological progression to a DLBCL were analyzed. RS1, RS3, RS4 RS6 and RS10 presented as a monoclonal population of small CD5+, CD19/CD20+, sIg<sup>low</sup> B-CLL cells which over time transformed
into a DLBCL (Table 1). RS8 already showed signs of transformation at presentation with, next to small tumor cells, a subpopulation of centroblast-like cells with abundant basophilic cytoplasm and irregular nucleoli. In a lymph node sample of RS8 one year later, the percentage of centroblast-like cells had clearly increased. RS9, RS11 and RS12 have been described previously as case 9, case 3 and case 8 respectively27.

Table 1  9 B-CLL with clinical and histological progression to DLBCL.

<table>
<thead>
<tr>
<th>RS</th>
<th>time⁹</th>
<th>diagnose</th>
<th>Source</th>
<th>CD5</th>
<th>CD23</th>
<th>Ig class</th>
</tr>
</thead>
<tbody>
<tr>
<td>RS1a</td>
<td></td>
<td>B-CLL</td>
<td>nasopharynx</td>
<td>+</td>
<td>+</td>
<td>IgM, IgD</td>
</tr>
<tr>
<td>RS1b</td>
<td>20</td>
<td>DLBCL</td>
<td>nasopharynx</td>
<td>+</td>
<td>+</td>
<td>IgM, IgD</td>
</tr>
<tr>
<td>RS3a</td>
<td></td>
<td>B-CLL</td>
<td>LN</td>
<td>nd</td>
<td>nd</td>
<td>IgM, IgD</td>
</tr>
<tr>
<td>RS3b</td>
<td>5</td>
<td>DLBCL</td>
<td>LN</td>
<td>nd</td>
<td>-</td>
<td>IgM, IgD</td>
</tr>
<tr>
<td>RS4a</td>
<td></td>
<td>B-CLL</td>
<td>BM</td>
<td>+</td>
<td>+</td>
<td>IgG</td>
</tr>
<tr>
<td>RS4b</td>
<td>14</td>
<td>B-CLL/DLBCL</td>
<td>PB</td>
<td>+</td>
<td>nd</td>
<td>IgG</td>
</tr>
<tr>
<td>RS6a</td>
<td></td>
<td>B-CLL</td>
<td>LN</td>
<td>+</td>
<td>+</td>
<td>IgM, IgD</td>
</tr>
<tr>
<td>RS6b</td>
<td>86</td>
<td>DLBCL</td>
<td>LN</td>
<td>+</td>
<td>+</td>
<td>IgM, IgD</td>
</tr>
<tr>
<td>RS8a</td>
<td></td>
<td>B-CLL/DLBCL</td>
<td>LN</td>
<td>+</td>
<td>-</td>
<td>IgG</td>
</tr>
<tr>
<td>RS8b</td>
<td>4</td>
<td>B-CLL/DLBCL</td>
<td>LN</td>
<td>+</td>
<td>+</td>
<td>IgG</td>
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<td>RS9a</td>
<td></td>
<td>B-CLL</td>
<td>PB</td>
<td>+</td>
<td>+</td>
<td>IgM, IgD</td>
</tr>
<tr>
<td>RS9b</td>
<td>2</td>
<td>DLBCL</td>
<td>colon</td>
<td>+</td>
<td>+</td>
<td>IgM, IgD</td>
</tr>
<tr>
<td>RS10a</td>
<td></td>
<td>B-CLL</td>
<td>PB</td>
<td>+</td>
<td>+</td>
<td>IgM, IgD</td>
</tr>
<tr>
<td>RS10b</td>
<td>10</td>
<td>DLBCL</td>
<td>PB</td>
<td>+</td>
<td>+</td>
<td>IgM, IgD</td>
</tr>
<tr>
<td>RS11a</td>
<td></td>
<td>B-CLL</td>
<td>PB</td>
<td>+</td>
<td>+</td>
<td>IgM, IgD</td>
</tr>
<tr>
<td>RS11c</td>
<td>13</td>
<td>DLBCL</td>
<td>BM</td>
<td>+</td>
<td>+</td>
<td>IgM, IgD</td>
</tr>
<tr>
<td>RS12a</td>
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<td>B-CLL</td>
<td>PB</td>
<td>nd</td>
<td>nd</td>
<td>IgD</td>
</tr>
<tr>
<td>RS12b</td>
<td>50</td>
<td>DLBCL</td>
<td>PB</td>
<td>+</td>
<td>+</td>
<td>IgD</td>
</tr>
</tbody>
</table>

BM indicates bone marrow; LN, lymph node; nd not done; PB, peripheral blood.

⁹ Time interval in months between samples

RS9, RS11 and RS12 are previously described as Case 9, Case 3 and Case 8²⁷
**IgV\textsubscript{H} genes and CDR3 regions of B-CLL with Richter’s transformation**

To establish the clonal relationship between the tumor populations at presentation and after relapse, the rearranged IgV\textsubscript{H}DJ\textsubscript{H} genes were amplified by RT-PCR and sequenced (Table 2). In all RS cases, the B-CLL and DLBCL cells proved clonally related. Interestingly, sequence analysis revealed that the IgV\textsubscript{H} genes of all 9 RS cases in our panel were unmutated (< 2% consensus mutations) (Table 4). The consensus IgV\textsubscript{H}DJ\textsubscript{H} sequences of most of the RS cases remained unaltered over time. However, RS4 and RS6 had each acquired an additional consensus mutation (i.e. a mutation found in more that 50% of the molecular clones) in their respective IgV\textsubscript{H} genes after transformation (Table 4). RS4 also acquired an additional replacement mutation in the third complementary determining region (CDR3) after transformation in more than 50% of the clones (Table 2).

The IgV\textsubscript{H}-CDR3 region is the most hypervariable region of the Ig and is considered to contribute most to its antigenic specificity. Nevertheless, ~19% of B-CLL, mostly

<table>
<thead>
<tr>
<th>RS</th>
<th>V\textsubscript{H}</th>
<th>D</th>
<th>J\textsubscript{H}</th>
<th>CDR3 (no. amino acids)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RS1</td>
<td>V\textsubscript{H}1-69 (V\textsubscript{H}1.2)</td>
<td>3-10 (fr.1)</td>
<td>J\textsubscript{H}6b</td>
<td>GGRQELLWGFEDYYYYGMDV (21)</td>
</tr>
<tr>
<td>RS3</td>
<td>V\textsubscript{H}4-4b (V\textsubscript{H}4.22)</td>
<td>5-12 (fr.1)</td>
<td>J\textsubscript{H}4b</td>
<td>GLNIVATGDY (10)</td>
</tr>
<tr>
<td>RS4a</td>
<td>V\textsubscript{H}4-39 (DP79)</td>
<td>6-13 (fr.1)</td>
<td>J\textsubscript{H}5b</td>
<td>NSGYSSWFRGYSWFD (17)</td>
</tr>
<tr>
<td>RS4b</td>
<td>V\textsubscript{H}4-39 (DP79)</td>
<td>6-13 (fr.1)</td>
<td>J\textsubscript{H}5b</td>
<td>NSGYSSWFRGYSWFD (17)</td>
</tr>
<tr>
<td>RS6</td>
<td>V\textsubscript{H}5-51 (DP73)</td>
<td>n.a.</td>
<td>J\textsubscript{H}2</td>
<td>RPLQWPLERYWYFDL (15)</td>
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<tr>
<td>RS8</td>
<td>V\textsubscript{H}3-30/30.5 (DP49)</td>
<td>3-22 (fr.2)</td>
<td>J\textsubscript{H}6c</td>
<td>GGDYYDSSSGLYYYYYYMDV (22)</td>
</tr>
<tr>
<td>RS9</td>
<td>V\textsubscript{H}1-69 (DP10)</td>
<td>2-21 (fr.2)</td>
<td>J\textsubscript{H}4b</td>
<td>VAVGAYCGGDCYWREYFDY (20)</td>
</tr>
<tr>
<td>RS10</td>
<td>V\textsubscript{H}3-74 (DA8)</td>
<td>3-16 (fr.2)</td>
<td>J\textsubscript{H}3b</td>
<td>DAWRPAPAYDYV (14)</td>
</tr>
<tr>
<td>RS11</td>
<td>V\textsubscript{H}3-11 (DP35)</td>
<td>3-09 (fr.2)</td>
<td>J\textsubscript{H}5b</td>
<td>DSVWYYDLTGSPQLVSYNWDF (24)</td>
</tr>
<tr>
<td>RS12</td>
<td>V\textsubscript{H}1-8 (DP15)</td>
<td>n.a.</td>
<td>J\textsubscript{H}2</td>
<td>ASSYDSGDDYYYYSLCLL (16)</td>
</tr>
</tbody>
</table>

CDR, complementary determining region; n.a., the D gene was not assigned.
Table 3  Homology between IgVH-CDR3 amino acid sequences of the 8 RS B-CLL and IgVH-CDR3 amino acid sequences present on GenBank.

<table>
<thead>
<tr>
<th>RS</th>
<th>Reference</th>
<th>CDR3 homology</th>
<th>Patient/Clone a</th>
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<tr>
<td>RS1</td>
<td>this study</td>
<td>1 normal B cell clone</td>
<td>ya0208</td>
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<tr>
<td>RS4a</td>
<td>this study</td>
<td>1 RS</td>
<td>CLL57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 B-CLL</td>
<td>CLL8, CLL9 and CLL202</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 normal B cell clone</td>
<td>1HI81</td>
</tr>
<tr>
<td>RS4b</td>
<td>this study</td>
<td>1 RS</td>
<td>CLL57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 B-CLL</td>
<td>CLL8, CLL114, CLL202, CLL209</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 normal B cell clone</td>
<td>1HI81</td>
</tr>
<tr>
<td>RS8</td>
<td>this study</td>
<td>1 RS</td>
<td>case 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 B-CLL</td>
<td>CLL32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 anti-polysacc. of N. Meningitis Ab</td>
<td>SC15</td>
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<tr>
<td>case 3</td>
<td>Matolcsy et al.</td>
<td>1 RS</td>
<td>RS8 (this study)</td>
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<tr>
<td></td>
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<td>1 EBV B cell in AITL</td>
<td>case2</td>
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<td>1 anti-polysacc. of N. Meningitis Ab</td>
<td>SC15</td>
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<td>1 anti-natural Sm Ab</td>
<td>BUD94</td>
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<td>1 anti-Rota virus Ab</td>
<td>RVI-22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 normal B cell clones</td>
<td>102-17 and MBT-159</td>
</tr>
<tr>
<td>B-CLL</td>
<td>Ghiotto et al.</td>
<td>1 RS</td>
<td>RS4a/b (this study)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 B-CLL</td>
<td>CLL39, CLL114, CLL209, CLL7, CLL8 and CLL9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 normal B cell clones</td>
<td>1HI81 and SC77U-44</td>
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<tr>
<td>RS 3557</td>
<td>Matolcsy et al.</td>
<td>2 normal B cell clones</td>
<td>2CB4N2, A29A29</td>
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<tr>
<td>B-CLL</td>
<td>Aoki et al.</td>
<td>1 B-CLL</td>
<td>YarVH b</td>
</tr>
<tr>
<td>case 2</td>
<td>Ohno et al.</td>
<td>1 normal B cell clone</td>
<td>PBT-16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 anti-Staphylococcal protein A Ab</td>
<td>4D5</td>
</tr>
</tbody>
</table>

Ab indicates antibody; AITL, angioimmunoblastic T cell lymphoma.

a GenBank accession numbers: ya0208, AB067329; CLL57, X84339; CLL8, AY486198; CLL9, AY486207; CLL202, AY268373; 1HI81, Y09249; CLL114, AY268372; CLL209, AY300037; CLL32, AY486216; SC15, AF115134; BUD94, Z46379; RVI-22, AY686908; 102-17, AF028108; MBT-159, U32960; CLL39, X84336; CLL7, AY486206; SC77U-44, AF174118; 2CB4N2, AY671324; A29A29, AF460484; YarVH, AF099199; PBT-16, U3220; 4D5, PH1650.

b B-CLL YarVH was 73% homologous to B-CLL4 instead of at least 75% like the other cases in this table.
unmutated, express CDR3 sequences with homology to CDR3s of other B-CLL (inter-B-CLL CDR3 homology), which suggests that a limited set of distinct antigenic determinants is recognized by these B-CLL. This prompted us to investigate the IgV\textsubscript{H}-CDR3 region of B-CLL known to have developed a Richter's syndrome (henceforth called RS B-CLL). The IgV\textsubscript{H}-CDR3 amino acid sequences of the RS B-CLL described in this study and all RS B-CLL described in literature were compared to all CDR3 amino acid sequences available on GenBank (Table 3). For this purpose, we used the NCBI Protein-Blast program with the option “search for short nearly exact matches” (BLASTP2.2.6[apr-09-2003]) as reported previously\(^\text{10}\). Briefly, CDR3 regions consisting of at least 7 amino acids were analyzed. An IgV\textsubscript{H}-CDR3 sequence was considered to be homologous to other CDR3 sequences (i) if sharing at least 75 % amino acid sequence homology. (ii) A length difference between the CDR3 sequences was allowed if not exceeding 3 amino acids (maximum gap of 3 amino acids).

Of the 18 RS B-CLL that were thus studied, 8 (44 %) fulfilled our criteria for CDR3 homology with CDR3 amino acid sequences present on GenBank (Table 3). The CDR3 regions of these 8 RS cases were homologous to the CDR3 regions of 12 normal B-cell clones and 9 B-CLL without reported transformation (Table 3). These latter B-CLL all expressed unmutated IgV\textsubscript{H} genes, except B-CLL \textit{YarVH}\textsuperscript{28} whose IgV\textsubscript{H} genes harbored 5 mutations. Interestingly, we also observed IgV\textsubscript{H}-CDR3 amino acid homology among different RS B-CLL. RS8 expressed a V\textsubscript{H}3-30/D3-22/J\textsubscript{H}6 rearrangement and the IgV\textsubscript{H}-CDR3 amino acid sequence showed \(\approx75\%\) homology to the CDR3 sequence of RS \textit{case 3} of which the V\textsubscript{H}DJ\textsubscript{H} rearrangement was unfortunately not described\(^\text{29}\). In addition, the IgV\textsubscript{H}-CDR3 amino acid sequence of RS8 showed 68% homology to the IgV\textsubscript{H}-CDR3 amino acid sequence of RS \textit{5577}\(^\text{30}\) although it must be noted that the latter expressed a V\textsubscript{H}3-74/D3-09/J\textsubscript{H}6 rearrangement (Figure 1). RS4 showed IgV\textsubscript{H}-CDR3 amino acid homology (\(\approx75\%\)) with RS B-CLL\textit{57}\(^\text{31}\). RS4 and RS B-CLL\textit{57} not only expressed the same V\textsubscript{H}DJ\textsubscript{H} rearrangement (i.e. V\textsubscript{H}4-39/D6-13/J\textsubscript{H}5) but also the same Vk012/02 -Jk1 gene rearrangement (data not shown). Based on the IgV\textsubscript{H}-CDR3 homology that is observed between B-CLL, 8 homology groups have been defined\(^\text{10}\). B-CLL\textit{57} is an IgG\textsuperscript{+} B-CLL that shows homology with 4 other unmutated V\textsubscript{H}4-39 expressing IgG\textsuperscript{+} B-CLL\(^\text{11}\). The IgV\textsubscript{H}-CDR3 region of the first time point of RS4 (RS4\textit{a}) was homologous to 2 B-CLL of this homology group (B-CLL57 and B-CLL202). Remarkably, due to an extra mutation in the IgV\textsubscript{H}-CDR3 region, the DLBCL of RS4\textit{b} (RS4\textit{b}) shared homology with a total of 4 B-CLL of this CDR3-homology group (B-CLL57, CL202 and additionally CL114 and CLL209\(^\text{11}\)) (Figure 1). This B-CLL subgroup, previously denoted by us as homology group 6, is thus extended by our IgG\textsuperscript{+} RS4 and now includes a total of 6 B-CLL, 2 of which underwent Richter’s transformation over time. In conclusion, these data demonstrate that Richter’s transformation occurs preferentially in unmutated B-CLL. Furthermore, we found that the most hypervariable region of the Ig gene, the CDR3 region, was highly homologous among distinct RS B-CLL cases.
The IgV\textsubscript{H}-CDR3 amino acid sequence of RS\textsubscript{4} is homologous to the IgV\textsubscript{H}-CDR3 amino acid sequence of B-CLL\textsubscript{57}. Both RS B-CLL expressed the same V\textsubscript{H}D\textsubscript{JH} gene rearrangement. The IgV\textsubscript{H}-CDR3 amino acid sequence of RS\textsubscript{8} is homologous to the IgV\textsubscript{H}-CDR3 amino acid sequences of case 3 and 3557. Whereas RS\textsubscript{8} expressed the same V\textsubscript{H}D\textsubscript{JH} gene rearrangement as case 3, it differed from the V\textsubscript{H}D\textsubscript{JH} gene rearrangement of 3557. Amino acids are depicted by the single letter code.

FR3 and FR4 indicates framework region 3 and 4;
N amino acid encoded by the non-templated nucleotides;
D gene segment;
JH gene segment;
| identical amino acid;
|– similar amino acid;
× dissimilar amino acid;
Hom percentage of homologous amino acid;
Id percentage of identical amino acid;
Gap length difference in amino acid of the compared IgV\textsubscript{H}-CDR3 sequences.

**Figure 1** IgV\textsubscript{H}-CDR3 amino acid sequence homology of RS\textsubscript{4} and RS\textsubscript{8} with IgV\textsubscript{H}-CDR3 of 3 previously described RS B-CLL.
Endogenous and induced expression of Activation-induced cytidine deaminase

Next, we analyzed the role of the somatic hypermutation machinery during Richter’s transformation. To this end, we quantitatively measured the expression of AID in 5 RS B-CLL before and after transformation and compared these to the expression levels of peripheral blood samples of 15 control B-CLL and of sorted tonsillar germinal center B-cell fractions (Figure 2). AID expression was not quantifiable in any of the 9 (3 IgM+, 6 IgG+) mutated B-CLL, nor in 6 unmutated B-CLL (IgM+). Interestingly, 4 of 5 RS B-CLL did express measurable levels of AID, although the AID/β-actin ratios were clearly below the ratios observed in germinal center B cells (Figure 2).

![Figure 2](image-url)

**Figure 2 Relative AID expression levels of RS B-CLL and control B-CLL before and after stimulation.** Quantitative RT-PCR analysis of AID and β-actin was performed on peripheral blood samples of a panel of 15 B-CLL without reported transformation and 5 B-CLL that transformed to a DLBCL. To induce AID expression, the B-CLL and RS samples were cultured for three days in the presence of IL4 and CD40L. RSA indicates the tumor sample before Richter’s transformation and RSb indicates the tumor sample after transformation to a DLBCL. Each dot represents the average value of at least three AID/β-actin ratio measurements.

It has been described that CD40 engagement induces AID expression in B cells. To investigate if the malignant cells were still responsive to environmental stimuli with respect to their AID expression, 3 RS B-CLL (RS3, RS4, RS6) and 14 control B-CLL were cultured for three days on either untransfected or CD40L-transfected L cells in the presence of IL4 and anti-IgM coupled sepharose beads. As positive controls, peripheral blood B cells and an EBV B cell line were used. Under these conditions, AID expression was increased in healthy donor peripheral blood B cells, the EBV B-cell line (data not shown) and in the mutated (3 IgM+ and 6 IgG+) and unmutated (6 IgM+) B-CLL. However, the stimulated RS B-CLL expressed significantly higher levels of AID, both before and after transformation, as compared to both control B-CLL groups (Figure 2). To further investigate the role of AID in Richter’s transformation, the expression of this
protein was visualized immunohistochemically in paraffin-embedded tissue sections of two RS patients (RS1 and RS8), of 11 lymph node samples of control B-CLL and of a tonsil (Figure 3). In the latter, AID was found within the germinal center blasts and in scattered extrafollicular centroblast-like cells (not shown), as was previously reported by Greiner et al.\(^ {22} \). In accordance with the mRNA expression data of blood-derived B-CLL samples, in 9 out of 11 B-CLL lymph node specimens no AID-expressing cells were present. In one unmutated control B-CLL, sporadic AID-expressing paraimmunoblasts were found in some proliferation centers (Figure 3, upper panels). In another control B-CLL, more AID-expressing centroblast-like cells were observed. However, since in this biopsy scattered residual germinal centers were present we could not exclude that these centroblast-like cells were GC related (data not shown). No QRT-PCR data were available on these patients to confirm these findings. In contrast to the control B-CLL and in accordance with the QRT-PCR data, AID-expressing cells were present in both RS cases of which lymph node material was available, i.e. RS1 and RS8 (Figure 3, middle and lower panel). It is noted that in both tumors, cytoplasmic AID expression was never observed in the small B-CLL cells but was confined to the centroblast-like cells. Immunohistochemical staining for BCL6, CD21 and BCL2 excluded the presence of residual germinal centers in the tissues (data not shown).

![Figure 3](image_url)  
**Figure 3** AID protein expression in RS8 and control B-CLL.  
Haematoxylin&Eosin, Ki67, AID and CD5 stainings on lymph node material of an unmutated B-CLL (upper panel), RS8a (middle panel) and RS8b (lower panel). Small B-CLL cells are in all cases negative for AID, whereas proportions of blastoid cells of RS8a and RS8b show clear cytoplasmic AID staining. Magnification ×25.
Somatic hypermutation in IgV\textsubscript{H} and non-Ig genes in B-CLL undergoing Richter's transformation

Since both quantitative RT-PCR and immunohistochemistry demonstrated that AID is expressed in RS B-CLL, we searched for evidence that the somatic hypermutation machinery indeed has been active in the tumor cells. Individual molecular clones of the amplified IgV\textsubscript{H} genes of RS1, RS3, RS4, RS6, and RS8 were sequenced. The degree of intraclonal sequence variation in IgV\textsubscript{H} was compared with that of 6 unmutated and 9 mutated B-CLL without reported transformation. We found significant intraclonal variation (i.e. a mutation frequency higher than the Taq error rate determined in our laboratory) in several IgV\textsubscript{H} mutated- and unmutated B-CLL and in 2 of the 9 RS B-CLL (RS4\textsubscript{a} and RS6\textsubscript{b}). The degree of intraclonal variation was low with a mean of 0.3 nucleotide differences per clone (Table 4). The observed nucleotide differences were present in only a minority of the clones. Finally, the nucleotide differences that accounted for the ICV in the RS B-CLL were non-confirmed and present in single clones only. The IgV\textsubscript{H} genes are not the only genes that can be targeted by the somatic hypermutation machinery. Since BCL6 and PIM1 are described to be mutated in DLBCL as well\textsuperscript{20}, we amplified, cloned and sequenced these genes in selected RS B-CLL (Table 4). Of RS1, RS3, RS4 and RS8, 790 basepairs (bp) downstream of the transcription initiation site of BCL6 were analyzed. This region includes part of the first intron (position 358 to 1148 according to GenBank AY189709). In RS4 and RS8 one polymorphism (G→C) at position 754 was found in all clones. The consensus sequence of RS1 already harbored one mutation (C→T) at position 897 before Richter's transformation. Interestingly, after transformation an additional (T→C) mutation was found in the consensus sequence at position 1075. Although RS1 and RS3 showed a low degree of intraclonal variation in BCL6 (0.4 and 0.5 per 300 bp per clone respectively, all nucleotide alterations were non-confirmed and found in single clones only), this was significant and higher than the intraclonal variation observed in their IgV\textsubscript{H} genes (≤0.2 per IgV\textsubscript{H} gene). No intraclonal variation was observed in BCL6 of RS4 and RS8. For PIM1, 600 bp downstream of the transcription initiation site was analyzed in RS1 and RS8 (position 859 to 1623 according to GenBank AF386792). In both RS B-CLL the consensus sequence harbored a polymorphism (C→G) at position 1039. Neither mutations nor intraclonal variation were found in this region in either of these lymphomas. Taken together, quantitative RT-PCR and immunohistochemistry both demonstrated that AID is expressed in RS B-CLL. Furthermore we observed a low but distinct degree of ongoing hypermutation in either the IgV\textsubscript{H} genes or BCL6, indicating that the hypermutation machinery indeed has been active during Richter's transformation.
Table 4  Mutations and intraclonal variation of IgV\textsubscript{H}, BCL6 and PIM\textsubscript{1} in 5 RS B-CLL and 15 control B-CLL.

<table>
<thead>
<tr>
<th>RS</th>
<th>AID/β-actin x 10\textsuperscript{-6}</th>
<th>IgV\textsubscript{H} mut\textsuperscript{a}</th>
<th>IgV\textsubscript{H} ICV\textsuperscript{b}</th>
<th>BCL6 mut\textsuperscript{c}</th>
<th>BCL6 ICV\textsuperscript{b}</th>
<th>PIM\textsubscript{1} mut\textsuperscript{c}</th>
<th>PIM\textsubscript{1} ICV\textsuperscript{b}</th>
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<tbody>
<tr>
<td>RS1a</td>
<td>36.1</td>
<td>0</td>
<td>≤ 0.2 (12)</td>
<td>1</td>
<td>0.4 (12)</td>
<td>o\textsuperscript{e}</td>
<td>≤ 0.2 (16)</td>
</tr>
<tr>
<td>RS1b</td>
<td>nd</td>
<td>0</td>
<td>≤ 0.2 (11)</td>
<td>2</td>
<td>0.4 (7)</td>
<td>o\textsuperscript{e}</td>
<td>0 (6)</td>
</tr>
<tr>
<td>RS3a</td>
<td>0.9</td>
<td>2</td>
<td>≤ 0.2 (9)</td>
<td>0</td>
<td>0.5 (23)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>RS3b</td>
<td>0.9</td>
<td>2</td>
<td>≤ 0.2 (16)</td>
<td>0</td>
<td>0.5 (12)</td>
<td>nd</td>
<td>nd</td>
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<tr>
<td>RS4a</td>
<td>10.1</td>
<td>0</td>
<td>0.3 (12)</td>
<td>o\textsuperscript{f}</td>
<td>0.3 (17)</td>
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<td>nd</td>
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<tr>
<td>RS4b</td>
<td>140.0</td>
<td>1</td>
<td>≤ 0.2 (23)</td>
<td>o\textsuperscript{f}</td>
<td>≤ 0.2 (16)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>RS6a</td>
<td>nd</td>
<td>0</td>
<td>≤ 0.2 (12)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
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<tr>
<td>RS6b</td>
<td>50.0</td>
<td>1</td>
<td>0.3 (12)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
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<tr>
<td>RS8a</td>
<td>8.3</td>
<td>0</td>
<td>≤ 0.2 (23)</td>
<td>o\textsuperscript{f}</td>
<td>≤ 0.2</td>
<td>o\textsuperscript{e}</td>
<td>≤ 0.2 (7)</td>
</tr>
<tr>
<td>RS8b</td>
<td>5.0</td>
<td>0</td>
<td>≤ 0.2 (23)</td>
<td>o\textsuperscript{f}</td>
<td>≤ 0.2</td>
<td>o\textsuperscript{e}</td>
<td>≤ 0.2 (9)</td>
</tr>
<tr>
<td>CLL M\textsuperscript{d}</td>
<td>0.7</td>
<td>17</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>CLL UM\textsuperscript{e}</td>
<td>0.2</td>
<td>0</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
</tbody>
</table>

CLL M\textsuperscript{d}, indicates CLL cases with >2% IgV\textsubscript{H} mutations; CLL UM, <2% IgV\textsubscript{H} mutations; ICV, intraclonal variation; mut., mutation; nd, not determined.

\textsuperscript{a} IgV\textsubscript{H} mutations are defined as nucleotide differences present in more than 50% of the clones, as compared to the germline sequence.

\textsuperscript{b} The intraclonal variation is indicated as the mean number of nucleotide differences observed per ~300 nucleotides per clone. In parentheses the number of clones that were sequenced.

\textsuperscript{c} BCL6 and PIM\textsubscript{1} mutations are defined as nucleotide differences that were present in ≥25% of the sequenced clones, as compared to the BCL6 and PIM\textsubscript{1} sequences published on genbank (AY189709 and AF386792 respectively).

\textsuperscript{d} Values are the average of 9 mutated B-CLL.

\textsuperscript{e} Values are the average of 6 unmutated B-CLL.

\textsuperscript{f} A polymorphism was found in BCL6 at position 754.

\textsuperscript{g} A polymorphism was found in PIM\textsubscript{1} at position 1039.
Discussion

To our knowledge, a total of 97 transformed B-CLL have so far been described. In 74 B-CLL patients (76%), the secondary lymphoma was classified as a DLBCL whereas in 23 B-CLL patients (24%) a Hodgkin’s lymphoma developed. Overall, in 67 of the 97 RS cases (69%), the high-grade lymphomas were of the same clonal origin as their low-grade precursors. Clonality was in most studies assessed by southern blot analyses. IgV<sub>H</sub>-CDR3 sequences of only 9 of these 67 RS B-CLL were available. Here we present 9 additional RS B-CLL in which the high-grade lymphomas were all of the same clonal origin as the preexistent B-CLL. Interestingly, of these altogether 18 RS B-CLL, 16 belonged to the unmutated subgroup, indicating that Richter’s transformation occurs almost exclusively in this subset of B-CLL. It is noted that this finding does not necessarily account for the well documented poor prognosis of the unmutated B-CLL subset, since Richter’s transformation is a rare phenomenon.

Previously, several groups including ours have reported that the IgV<sub>H</sub>-CDR3 amino acid sequences of a significant fraction of B-CLL are highly homologous, particularly within the group of unmutated B-CLL. This type of homology is unique for the group of B-CLL, and was not found within extensive cohorts of follicular lymphomas, DLBCLs, Burkitt’s lymphomas and multiple myelomas. This suggests that a proportion of B-CLL recognizes recurrent antigenic epitopes. Of the 18 RS B-CLL analyzed in this study, the CDR3 region of as many as 8 (44%) displayed homology to IgV<sub>H</sub>-CDR3 amino acid sequences present on GenBank. This frequency is not higher than that observed within the group of unmutated B-CLL (44%)<sup>10</sup>. More remarkable is the finding that the IgV<sub>H</sub>-CDR3 amino acid sequence of 5 of these 8 RS B-CLL (i.e. RS4, B-CLL<sup>57</sup> and RS8, case<sup>29</sup>, RS 3557<sup>30</sup>) exhibited inter-RS group homology. Both RS4 and RS8 express an unmutated IgG. In fact, all RS B-CLL with CDR3 regions homologous to that of RS4 expressed unmutated IgG and all have a reported aggressive clinical course. Furthermore, whereas RS4 shared CDR3 homology with 2 of such IgG<sup>+</sup> B-CLL before transformation, an additional mutation in the CDR3 region of RS4 after transformation resulted in CDR3 homology with a total of 4 of these IgG<sup>+</sup> B-CLL. Altogether, these findings point towards selective forces that favor outgrowth and possibly also progression of tumor (sub)clones with B-cell receptors of restricted specificities. Identification of the antigens involved may further clarify the biological mechanism underlying tumor progression and provide tools for therapeutic intervention. Alternatively, IgV<sub>H</sub> gene analyses may thus be of value to identify B-CLL with a poor biological behavior.

Our quantitative RT-PCR demonstrated that in peripheral blood samples of the control B-CLL AID levels did not exceed background levels. Our findings seem to contradict several papers reporting on AID expression in B-CLL. In most of these studies however, AID mRNA expression was measured by non-quantitative RT-PCRs and by consequence the actual expression levels are difficult to judge. Limiting dilution assays revealed that less than 1% of the B-CLL cells express AID and accordingly
quantitative AID mRNA measurements demonstrated that the expression levels found in
the B-CLL were in all cases less than 5% of those found in GC cells\textsuperscript{17,55}. In addition,
western blot analyses showed that AID protein could not be detected in B-CLL regardless
of their \textit{IgV}\textsubscript{H} mutation status\textsuperscript{16}. In contrast to our findings in B-CLL, AID expression was
quantifiable in most of the RS B-CLL at presentation. After transformation, the AID
levels varied considerably among the different RS B-CLL. Moreover, in the transformed
stage AID expression seemed less influenced by \textit{in vitro} CD40 stimulation, suggesting
that the tumor cells are more autonomous. In general, the AID mRNA expression data
were highly compatible with the observed AID protein expression in tissues as assessed by
immunohistochemistry. In lymph node material of 9 of the 11 control B-CLL (mutated
and unmutated), no AID expressing cells were observed, not even in the proliferation
centers. In both RS cases that were histologically analyzed, scattered AID-expressing
tumor cells were present. These AID-positive cells all had a blastoid appearance, whereas
the small tumor cells were devoid of AID expression. It is not clear whether the AID-
expressing cells are B-CLL cells activated by CD40L and IL4 or represent already
transformed cells. In conclusion, our analyses indicate that in B-CLL the presence of
significant numbers of AID-expressing cells is exceptional, whereas in RS B-CLL AID is
more abundantly expressed and in fact may predict an aggressive clinical course.

Sequence analyses of \textit{IgV}\textsubscript{H} and \textit{BCL6} demonstrated low but significant degrees of
intraclonal variation in the RS B-CLL. In both genes, the nucleotide alterations were not
equally spread over the different molecular clones, but clustered in a fraction of the
clones. This suggests that the somatic hypermutation machinery is active in a minority of
the B-CLL cells only, which fits the AID staining results. It is noteworthy that in two of
the four RS B-CLL, the degree of intraclonal variation in \textit{BCL6} was twice as high as the
intraclonal variation observed in the \textit{IgV}\textsubscript{H} genes. Moreover, in the \textit{IgV}\textsubscript{H}-unmutated RS1
an additional consensus mutation was obtained in \textit{BCL6} during transformation, whereas
the \textit{IgV}\textsubscript{H} gene remained unaltered. This finding is remarkable since in normal GC B cells
the \textit{BCL6} mutation rate is 10-100 times lower than the mutation rate in \textit{IgV}\textsubscript{H}\textsuperscript{57-59}.\nMutations in \textit{BCL6} have been reported in \textit{IgV}\textsubscript{H}-mutated\textsuperscript{58-62} and \textit{IgV}\textsubscript{H}-unmutated B-
CLL\textsuperscript{63}. It thus seems that at least in these RS B-CLL, the somatic hypermutation
machinery, and most likely AID, can target \textit{BCL6} (and potentially other non-Ig genes)
while leaving \textit{IgV}\textsubscript{H} unaffected. This, together with the observation that AID expression,
either spontaneous or induced, is higher in RS B-CLL and increases during
transformation suggests a role for this genetic diversification mechanism during the
ongoing transformation of the RS B-CLL.
References

15. Smit LA, Bende RJ, Aten J, Guikema JE, Aarts WM, van Noesel CJ. Expression of activation-induced cytidine deaminase is confined to B-cell non-Hodgkin’s lymphomas of


Discussion

Is there a role for RNA in somatic hypermutation and class switch recombination?

As set forth in the introduction, AID was discovered as a homologue of APOBEC-1, and was first assumed to be an RNA-editing protein. Since then, however, ample evidence has been provided that AID can act directly on DNA, as demonstrated in various experimental in vitro and cell culture systems. Moreover, when SHM is stripped from both pathways of repair that are involved (i.e. mismatch repair and base excision repair, in UNG/MSH2 and UNG/MSH6 knockout mice), this reveals the C:G transition mutation as the basis of SHM, which is fully compatible with the deaminase footprint of AID. Hence, the general consensus now implies direct DNA deamination by AID as the basis of SHM and CSR. Nevertheless, there is a number of recent findings that relate AID to RNA, which will be discussed here.

There are a few alternative models, involving RNA intermediates. Tasuku Honjo, the discoverer of AID, aims his efforts at substantiating an RNA deamination role for AID. He recently found that AID indirectly interacts with mRNA at its C-terminus, which is reminiscent of APOBEC1 binding its target (ApoB-) mRNA via the APOBEC1 complementation factor (ACF).

In addition, the same group claimed to have identified an AID mutant (N51A) which lacked DNA-deamination activity while retaining some CSR activity. They considered this to be supportive for the alternative model in which RNA editing could provide the tools for CSR, however they omitted providing evidence for RNA-editing activity by AID. In fact, in their first report on AID, they mention a lack of RNA-binding and deamination capacity for AID. In agreement, Bransteitter et al. did not detect any deaminase activity on ssRNA or RNA hybridised with DNA.

Edward Steele does not refute DNA-editing by AID, but finds support for an RNA-intermediate in the fixation of mutations in the genome. In his model, AID mutates
cytidines in the Ig gene, prior to transcription. After transcription, the nascent mRNA undergoes adenosine-to-inosine (A-to-I) deamination, supposedly by ADAR1. Error-prone reverse transcriptase activity by Pol-η is then required for copying the mRNA back into DNA, followed by invasion/integration of the genome to incorporate the new mutations. The arguments for this model are based on complex and detailed analyses of mutation patterns and as such reach far beyond the scope of this discussion.5

Neither of the two previous models receive broad support. As such, a potential role for RNA in the AID-mediated Ig gene alterations, will be discussed from the perspective of the standard DNA deamination model. The most longstanding relationship between AID activity and RNA is the requirement for transcription. As addressed in the introduction, somatic hypermutation activity is correlated to the site of transcription and transcription levels, presumably by exposing the ssDNA template. Although it was found that a small stretch of ssDNA similar in size to a transcription bubble gives sufficient space for AID to act, there have been numerous reports on secondary structures that provide more stable and larger openings in the DNA, such as R-loops formed during CSR (see figure 4 of the general introduction). R-loops arise when a nascent G-rich transcript is allowed to pair with the displaced DNA-strand during transcription elongation.6 Transcripts from the “non-coding” strand were detected for both the variable
region and the switch regions of the immunoglobulin genes, which would allow targeting of both strands. However, this finding was recently disposed as a PCR artefact by others.

Normally, newly generated gene-transcripts are capped and loaded with numerous messenger ribonucleoproteins (mRNPs) that will prevent the occurrence of such DNA:RNA hybrids. They guide the (pre-)mRNA through splicing and eventually nuclear export (Figure 1). Mutations in the mRNP-assembling THO-complex, display increased AID mediated mutation and recombination in yeast. Similarly, genome instability in the presence of AID is increased when the Thp1-Sac3-Sus1-Cdc31 complex is affected, supposedly due to the prevention of R-loops by its role in transcription elongation and mRNA export. Hence, down-modulation of the transcription elongation and mRNA export can be envisaged to have a role in facilitating Ig diversification (Figure 2).

Figure 2  Transcription-associated recombination.
A. Numerous ribonucleoproteins and splicing factors associate with the nascent RNA behind the elongating RNA polymerase (RNAP), in order to protect it from hybridising with the displaced DNA strands during transcription.
B. When co-transcriptional formation of an optimal mRNA–particle complex is impaired, the RNA can hybridise with its template DNA strand forming a transient R-loop. The single-stranded non-translated strand region can either be more susceptible to damage or to the formation of DNA secondary structures that can compromise RF progression. This can occur in THO-complex mutants in yeast, and in splicing factor ASF (also known as SF2)-depleted DT40 and HeLa cells, but also at natural loci such as in the switch (S) regions of immunoglobulin (Ig) genes. (Adapted and modified from Aguilera and Gómez-González, Nat Rev Genet. 2008)
In eukaryotes, loading of the THO-complex depends on the presence of the splicing machinery on the mRNA (Figure 1). In agreement, RNA splicing seems important for AID-activity as well. It was found that switching to the γ1 switch region requires the presence of an intact I-exon splice donor site. Similar to mutations in the THO-complex, disruption of the ASF/SF2 splicing factor leads to genome instability. More recently, AID was found to interact with CTNNBL1 (also discussed in the general introduction). The function of this protein is still unknown, yet its co-immunoprecipitation with members of the splicing complex is suggestive for a role in cotranscriptional mRNA processing. The interaction between AID and CTNNBL1 was needed for proper antibody diversification. In analogy, DDX5 was identified as one of the AID-interactors and its knockdown impaired CSR. DDX5 is a helicase that, beside being a transcriptional regulator, has also been implicated in the pre-mRNA processing and alternative splicing. Further characterisation of the components of the cotranscriptional mRNA processing complexes, in particular during SHM and CSR, may provide more insight in the interplay between AID, mRNA, splicing and nuclear export.

To end with, there is the (in-)direct interaction of AID with RNA. Although, Honjo et al. noticed that the interaction with mRNAs at its C-terminus occurred most likely via a co-factor, Bransteitter et al. found that removal of the RNA by Rnase is required to permit DNA-deamination activity in vitro. What the function of RNA binding in vivo would be, is unknown. Interestingly APOBEC-3G, which deaminates cytidines in retroviral copy-DNA, was found to interact with RNA as well. It is currently under debate whether that RNA may attribute to the encapsidation of APOBEC3G into the HIV-I virion.

In conclusion, there seems to be a relationship of AID with RNA, transcription, elongation, splicing and nuclear export, although the precise mechanisms are still elusive. Future investigations in this field may prove rewarding in exposing an additional layer of regulation of Ig gene diversification.

References


Appendix

Effects of processing delay, formalin fixation and immunohistochemistry on RNA recovery from formalin-fixed paraffin-embedded tissue sections

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Abstract

Contemporary pathology involves an emerging role for molecular diagnostics. Current tissue handling procedures (i.e. formalin fixation and paraffin embedment) have their origin in the aim to obtain good tissue morphology and optimal results within immunohistochemistry. Unfortunately, formalin fixation and paraffin embedment (FFPE) is notorious for its poor RNA conservation capacities. In this study, we have examined the impact of the individual steps in tissue handling processes on the RNA extractability, quality and usability for RT-PCR. It was found that a prolonged pre-fixation time (i.e. the time between tissue dissection and fixation) has a measurable impact on RNA integrity when analyzed with the Agilent Bioanalyzer. Surprisingly however, the deteriorated RNA quality hardly had any consequences for RT-PCR yields. Furthermore, we assessed the optimal fixation time for RNA preservation, and we found that an RNA heating step, preceding cDNA synthesis, significantly increases the RNA template length. Finally, we provide a protocol for RNA isolation from immunohistochemically stained FFPE tissue sections. Thus, by applying alterations to tissue handling procedures, archival formalin-fixed and paraffin-embedded tissues become well suitable for RNA based molecular diagnostics.
Introduction

Advances in molecular biology have great impact on our understanding of human disease and consequently have modernized diagnostic and therapeutic medicine. In clinical pathology, the advent of molecular diagnostics is already well established and with it the use of nucleic-acid based assays has significantly increased. Archival formalin-fixed and paraffin-embedded (FFPE) tissue specimens are the most widely available resource for such analyses. The FFPE procedure, historically aimed at tissue conservation and acquisition of optimal morphology, is not necessarily compatible with optimal preservation and extractability of nucleic acids. Genomic DNA can generally be isolated in sufficient quantities out of FFPE tissue. The size of the analyzable DNA fragments obtained however does not exceed ±300-500 base pairs, posing limitations to the molecular analyses that can be performed. This limited template size is often considered to be the result of strand fragmentation.

Similar restraints are true for RNA. In addition, compared to DNA, RNA is much more susceptible to ex-vivo tissue handling and to the various processing steps inherent to current pathological practice. This is due to the intrinsic short half-life of RNA molecules, the presumed abundance of RNase enzymes, and the smaller size of RNA strands which makes them more sensitive to elution during washing. In spite of these limitations, the use of messenger RNA (mRNA) or copy DNA (cDNA), instead of genomic DNA, as molecular template has considerable advantages. Messenger RNAs represent the genes actively transcribed and therefore offer direct information on the actual molecular make-up of cells or tissues. When expressed, the copy number of a gene transcript in a cell is at least in the order of thousands instead of the two DNA copies, implying that less sensitive PCR protocols are demanded. For example, clonality determination and B-cell receptor sequencing in lymphoma is less complicated when cDNA is available. Detection of the tumor clone is easier because often the immunoglobulin gene is highly expressed, and noise caused by the presence of silenced non-functional rearrangements is reduced.1

A qualitative advantage of cDNA-based analysis is that, due to exclusion of the often sizeable non-coding intron regions in mRNA, consecutive exons can be amplified in one piece. Thus, entire coding regions of genes can be covered by a limited number of RT-PCRs. For mutation detection it is less elaborate to sequence cDNA instead of DNA, as for the latter each exon must often be amplified individually, requiring many primer sets. For example, P53 consists of 11 exons, which can be covered by just four sets of primers when sequenced on cDNA.2 cDNA is particularly useful in the search for chromosomal translocations in which the breakpoint-regions may span extensive genomic lengths, and are therefore not suitable for amplification by DNA-PCR. Yet, an RT-PCR on the fusion gene transcripts may provide the means by which the resulting fusion product can be identified.3-6 In addition to somatic mutations or translocations, essential gene products can also become altered in their normal activity or regulation due to alternative splicing
events. An increasing number of recent reports have linked aberrant splicing with prognosis in cancer, recently reviewed elsewhere.\(^7\) Although the identification of underlying mutations in splice sites will still require DNA sequencing, swift screening for the expression of alternative splice variants is can be carried out using RT-PCR.

In the past years, a number of papers have appeared reporting on specific steps regarding the acquisition of mRNA out of FFPE tissues and its applications.\(^8-20\) Here, a systematic analysis was made of the influence of tissue handling and the successive processing steps of daily clinico-pathological practice on the extractability and quality of messenger RNA (mRNA). In addition we provide a protocol, for successful RNA isolation from immunohistochemically stained FFPE tissue sections. These findings may aid in optimal RNA extraction from routine archival tissue material, to be used in RT-PCR based molecular diagnostics.

**Material and methods**

**Tissue handling**

Fresh tissue specimens of three livers, obtained directly after hemihepatectomy, and ovarium carcinoma tissue were left unprepared in phosphate-buffered saline (PBS, pH7.4) for various periods, up to 48 hrs, at either 4°C or room temperature (RT), after which the samples were snap-frozen in liquid nitrogen and stored at -80°C until RNA isolation (not more than five months). The same protocol was applied to ovarium carcinoma tissue as a control.

Fresh tissue specimens (1.0\(\times\)1.5 cm) of normal stomach mucosa were fixed in 4% formaldehyde, phosphate-buffered at pH 7.0 (Klinipath, Duiven, The Netherlands) for 2-72 hrs (standard protocol: 16-24 hours) at room temperature before further processing in a Tissue-Tek VIP\(^\text{TM}\) 5 Vacuum Infiltration Processor (Sakura, Zoeterwoude, The Netherlands) according to the following procedure: 5.5 hrs, 50% alcohol; 4 hrs, 70% alcohol; 6.5 hours, 96% alcohol; 7.5 hrs, 100% alcohol; 2 times 1 hr, 100% alcohol; 3 times 1.5 hrs, xylene; 3 times 1.5 hours, paraffin; and finally embedded in paraffin.

This study was conducted in accordance with the ethical standards in our institutional medical ethical committee on human experimentation, as well as in agreement with the Helsinki Declaration of 1975, as revised in 2000.

**Total RNA- and mRNA-isolation**

Out of 10-20 frozen sections of 10 \(\mu\)m, total RNA was isolated using TRIzol reagent (Invitrogen, Breda, The Netherlands) according to manufacturer’s instructions and dissolved in RNase-free water.

Of the FFPE tissue, 10-20 sections (10 \(\mu\)m) were deparaffinized in xylol for 10 minutes. Xylol was replaced by 3 washes with 100% ethanol. After careful removal of the ethanol, the tissue was air-dried for 5 min. The pellet was dissolved and incubated with
500 µg/ml Proteinase K (Roche Diagnostics, Almere, The Netherlands) in lysis-buffer (10 mM Tris-HCl/pH 8.0, 0.1mM EDTA/pH 8.0, 2% SDS/pH 7.3) at 56°C for 2 hrs. In case of incomplete lysis an extra aliquot of Proteinase K was added and tissue was again incubated for 2 hrs at 56°C. Subsequently, RNA was isolated with TRIZol as described above, with the small alteration that precipitation was done in the presence of 1 µl of glycogen to visualize the pellet (20 mg/ml, Roche). Finally, the RNA was dissolved in TE (1mM Tris-HCl/0.5mM EDTA, pH 8.0).

For mRNA-isolation, 10 sections of 10 µm of the frozen material were used in the TRIZol RNA isolation procedure. Subsequently, mRNA was isolated using the Micro-FastTrack™ Kit (Invitrogen) according to manufacturer’s instructions.

**Quality control of RNA using the Bioanalyzer**

The RNA that was used for quality measurements was subjected to an additional purification step, using RNEasy MinElute cleanup columns (QIAGEN, Venlo, The Netherlands). Total RNA and mRNA concentrations were measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, U.S.A.). After adjustment of the concentration to 50-200 ng/µl using RNase-free water, 1 µl was loaded on a RNA 6000 Nano LabChip and run at a 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands). The RNA integrity number (RIN) was calculated using the 2100 Expert software (demo version B.02.02SI238).21,22

**RT-PCR**

Prior to complementary DNA (cDNA) synthesis, RNA from frozen as well as FFPE tissues, was heated at 65°C for 10 minutes. Alterations in this heating step occurred at 65°C, 70°C and 80°C for 10 min, 2 hrs and 6 hrs.

For complementary DNA (cDNA) synthesis, the RNA was incubated with 2.5 nmol random hexamer pd(N)₆ primers (Amersham Pharmacia Biotech, Roosendaal, The Netherlands), for 10 minutes at 65°C. After cooling on ice, the reaction mixture was added to a final volume of 25 µL. It contained 200 units of M-MLV (Moloney Murine Leukemia Virus) reverse transcriptase (RT) (Invitrogen), 8 mM of dithiothreitol (DTT, Invitrogen), 1 mM of each dNTP (Amersham), 1× first strand buffer (50 mM of Tris-HCl, pH 8.3; 75 mM of KCl; 3 mM of MgCl₂, Invitrogen), and 15 units of RNase inhibitor (Applied Biosystems, Foster City, CA, U.S.A.). The reaction was allowed for 1 hour at 37°C. Subsequently, the enzyme was inactivated during 10 minutes at 95°C.

The resulting cDNA was used in PCRs combined with primers sets specific for 3 different house-keeping genes; hypoxantine phosphoribosyltransferase (HPRT1), porphobilinogen deaminase (PBGD) and beta-2-microglobin (β2-M). Lengths of the PCR products varied between 157 bp and 753 bp (see Table 1). PCRs were performed in a volume of 25 µl containing 1× PCR-buffer (20mM Tris-HCl (pH 8.4), 50 mM KCl, Invitrogen), 1.25-1.5 mM MgCl₂ (Invitrogen), 200 µM dNTP’s (Amersham), 1 unit Platinum Taq polymerase (Invitrogen) and 8 pmol of each primer. The PCR program
consisted of a first denaturing step at 95°C for 4 minutes, followed by 40 cycles of successively 30 seconds denaturing at 92°C, 45 seconds annealing at 55, 60 or 65°C (see Table 1) and 45 seconds extension at 72°C, and a final extension step of 5 minutes at 72°C.

**Immunohistochemistry**

To test the effects of immunohistochemical staining procedures on RNA integrity, the staining protocol was stopped at each consecutive step, after which the section was scratched off the glass and subjected to RNA isolation and RT-PCR as described before. Tissue sections (4 µm) were mounted on glass slides either by using serum or in a warm water bath. Sections were deparaffinized in xylol and ethanol, blocked for endogenous peroxidase activity by immersion in 0.3% H2O2 in methanol for 20 min. Heat induced epitope retrieval (HIER) was performed in Tris/EDTA buffer (10 mM/1 mM; pH 9.0) for 10 min at 120°C, while pepsin pre-treatment was performed at a concentration of 0.25% in 0.01 M HCl/pH 2.0 for 15 min at 37°C. Nonspecific binding sites were blocked with 5% normal goat serum or BSA in PBS, with BSA-c (Aurion, Wageningen, The Netherlands) or with serum-free protein block (DAKO) for 10 min. A mouse monoclonal antibody against vimentin (clone V9, DAKO Netherlands B.V., Heverlee, Belgium) was diluted in PBS or in normal antibody diluent.

<table>
<thead>
<tr>
<th>Sequence (3’-5’)</th>
<th>Gene</th>
<th>cDNA position</th>
<th>Condition PCR</th>
<th>Product size</th>
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<tr>
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<td>156-175 (exon 1)</td>
<td>60°C</td>
<td>338 bp</td>
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<tr>
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<tr>
<td>tggcctgtgattagttagtg</td>
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<td>60°C</td>
<td>227 bp</td>
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<tr>
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<tr>
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<td>770-794 (exon 9)</td>
<td>1.25 mM MgCl2</td>
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</tr>
</tbody>
</table>
(Scytek Laboratories Inc., Logan, Utah, USA). Secondary, the Powervision+poly-HRP detection system (ImmunoVision Technologies, Co, Daly City, CA, USA), or alternatively, Rabbit and Swine-anti-mouse polyclonal antibodies - biotin labeled (DAKO) in combination with streptABComplex (DAKO) were applied. 3,3-Diaminobenzidine (DAB), 3-Amino-9-ethylcarbazole (AEC), Fuchsine acid, Fast-Red and Fast-Blue (all obtained from Sigma-Aldrich, Zwijndrecht, The Netherlands) were used as chromogen and haematoxylin as counterstaining.

**Results**

*Effects of delayed tissue handling on RNA quality*

To analyze the effects of pre-fixation time on RNA quality we chose to use normal liver tissue, assuming that this tissue is relatively sensitive to postponed processing because of the high metabolic activity of the tissue on one hand, and the abundance of degradation proteins on the other hand. In concordance with the results published by Godfrey et al., no consistent differences in the quantity of isolated total RNA was observed between the samples (data not shown). Figure 1a shows the progressive, time-dependent degradation of total RNA isolated from one of the three livers, visible as the gradual loss of the 28S ribosomal RNA (rRNA) band and accumulation of degraded RNA below the 18S band. When RNA qualities were depicted in RNA integrity numbers (Fig. 1b), the curves showed that, although the starting qualities (t=0) of RNA from the three livers differed significantly, shown in the electropherograms (insets), the course of RNA decay under influence of the different storage conditions was similar among the three livers. When the results of the three livers were averaged, it became apparent that there was a protective effect on RNA quality when tissues had been kept at 4°C as compared to room temperature, however, this difference was not significant before 12 hours of storage (Fig. 1c).

> **Figure 1**  Effects of RNA recovery after delayed tissue processing.

RNA was isolated from normal liver and an ovarium carcinoma, which had been left unprepared for 0 to 48 hrs, at either 4°C or room temperature (RT), and subsequently snap-frozen.

A. A gel-like image of total RNA isolated from liver 1.

B. Plotted is the RNA integrity number (RIN) for three liver specimens in time; each curve representing three independent RNA isolations from one liver specimen. Insets: Electropherograms depicting the RNA qualities at start of the experiments.

C. The relative RNA integrity number (%RIN), with t=0 set to a 100%, averaged for the three livers.

D. The RNA integrity number (RIN) of RNAs isolated from ovarium carcinoma in time.

E. Electropherogram of purified mRNA, isolated from fresh frozen tissue and from tissue that had been left unprepared for 24 hours at room temperature.
Figure 1
The tissues left at room temperature continued degrading their RNA progressively, while decay of the samples kept at 4°C seemed to level off to a near steady-state of RNA quality. Similar experiments on tumorous tissue (ovarium carcinoma) as a control indicated that the velocity of RNA degradation is also determined by the tissue type; The overall RNA quality from the control tissue was much better than the RNA isolated from the livers. More strikingly, there was no significant RNA degradation detectable up to 18 hours, not even when the tissue was stored at room temperature (Fig. 1d). Of importance, the effect of delayed tissue handling on the quality of RNA was also clearly measurable when purified messenger RNA (mRNA) was analyzed. The mean size of the mRNA fragments decreased with longer pre-fixation times, visible in the electropherogram as a shift to the left (Fig. 1e).

Effects of delayed tissue handling on RT-PCR
To study the effect of delayed tissue handling on RNA template length, we performed RT-PCRs for various housekeeping genes on the RNA isolated from the frozen specimens of liver 1, for each time point mentioned above. To our surprise, the maximum PCR product with length of 750 bp, could be amplified from all samples, irrespective of storage conditions (Fig. 2) The single exception within these results was the tissue specimen that had been left unprepared at room temperature for 48 hours. Here, the amplifiable template was not above 331 bps.

**Figure 2** Consequences of delayed tissue handling, at either room temperature (RT) or at 4°C, for RNA template length.
RT-PCR was performed on commonly used housekeeping genes: HPRT (750 bp, 500 bp and 225 bp), PBGD (350 bp) and β2M (150 bp). For location of the primers, see table 1.
Influence of fixation time and RNA preincubation temperature on cDNA quality

In sheer contrast to the frozen tissue, a tissue sample that was formalin-fixed and paraffin-embedded, did not show the distinctive 28S and 18S rRNA bands, not even after immediate processing. In addition, the electropherogram showed that the mean length of RNA isolated from FFPE tissue, was severely reduced (Fig. 3a). RT-PCR, aimed at housekeeping genes, indicated that an 8 to 16 hour fixation was optimal, in that PCR products of maximal lengths could be acquired (Fig. 3b). After 2 hrs of (most likely incomplete) fixation, merely smaller mRNAs were amplifiable. Similar results were obtained for tissue that had been fixed for 72 hrs.

In another series of experiments, the influence of RNA heating preceding the RT-PCR on the length of the amplifiable products was evaluated. FFPE-isolated RNA was subjected to several temperatures for different periods of time, prior to cDNA synthesis. The analyses demonstrated that heating of RNA for 2 hrs at 70°C yielded significantly better results than the commonly used 10-minute incubation at 65°C (Fig. 3c, lower panel). Incubation at temperatures above 70°C or for longer periods adversely affected RNA quality (data not shown).

These experiments were repeatedly carried out for different tissues. While the absolute length that could be amplified varied among the samples, the optimum fixation period of 8-16 hrs was consistently observed within each experiment. Similarly, preincubation of isolated RNA for 2 hrs at 70°C prior to cDNA synthesis was optimal in every experiment.

Influence of individual steps of immunohistochemical staining procedure on RNA retrieval.

We had experienced that RNA recovery from paraffin sections, stained according to our standard immunohistochemical procedure, was impossible. Therefore, all steps of the staining procedure were tested for their effects on RNA extractability. Figure 4a illustrates the absence of any PCR product, right after the blocking step. Further examinations showed that addition of serum, either used for mounting on slides, as a blocking reagent, or as a supplement to the antibody diluents, is a major negative factor. Analysis of RT-PCR after application of several commonly used staining reagents, showed that also the visualization method can be of great influence, with AEC giving the best results. Thus, RNA is well extractable from immunohistochemically stained tissue sections, provided that: i) mounting slides is performed using a water bath; ii) a blocking step is omitted or, when inevitable, performed with BSA-c instead of serum; iii) antibody dilutions are made in PBS instead of commercially available diluents; and iv) the eventual staining is carried out using AEC. Figure 4b lists all materials and treatments tested, and their influence on RNA recovery.
Figure 3  Effect of formalin fixation and paraffin embedment (FFPE) on RNA integrity and determination of an optimal fixation time.

A. Electropherogram of RNA isolated from fresh frozen tissue (upper panel) or directly formalin fixed and paraffin embedded tissue (lower panel).

B. Tissue samples were fixed for 2 - 72 hours, and subsequently paraffin embedded. RT-PCR for housekeeping genes was performed on RNA isolated from these samples.

C. RT-PCR for housekeeping genes, on RNA which was isolated from FFPE tissues, fixed for 2, 8 or 72 hours, and subjected to varying heating steps prior to the cDNA synthesis.
Formalin-fixation and paraffin-embedment results in chemical cross-linking of proteins and nucleic acids, thereby preventing RNA degradation mediated by endonucleases present in the tissue. Still, retrieval of good quality RNA from FFPE tissues is often difficult to achieve. Despite the rising efforts to set up frozen tissue archives in many clinical centers, it is common practice that FFPE tissue is the only material available for (molecular) diagnostics. We evaluated the influence of the individual steps of tissue handling procedures on RNA extractability and integrity, and searched for improvements in the RNA isolation protocol. When discussing the topic of RNA isolation, two dogmas generally emerge. First, the tissue material should be fixed or frozen as soon as possible, or should be kept cold to prevent RNA degradation by RNases. Secondly, RNA isolated from FFPE material is largely fragmented and therefore not very useful for molecular assays that require certain template lengths.

To study the effects of delayed tissue handling, we chose liver material assuming that such active tissue full of enzymes, is one of the most difficult tissues to preserve the RNA.
This assumption appeared more true than expected, since two out of three livers used in the delayed tissue handling-experiment did not yield good quality RNA, not even from the samples that had been frozen directly after surgical dissection. This result contrasted with the constant quality of the RNA in the ovarium carcinoma samples. RNA decay in these livers presumably started before they were included in our experiment. Liver tissue is metabolically active, which renders the tissue sensitive to hypoxia, inducing tissue damage and the subsequent release of various degradation enzymes. Extensive ischemia is common during a (hemi-)hepatectomy, with surgery taking 3 to 5 hours. Consequently, during surgery the tissue is exposed to hypoxia and subsequently proteases and nucleases for several hours at body temperature, which is optimal for the enzymatic activity. Indeed, further study of the surgical reports elucidated that liver 1, qualitatively the best of three, had undergone the shortest surgical operation (still 3.5 hours) as compared to the other two livers which were dissected during operations lasting at least 5.5 hours. This finding suggests that warm-ischemia can have dramatic consequences for RNA integrity in the tissue. Nevertheless, with RINs of 8.6, 5.5 and 3.2, representing good, intermediate and poor RNA quality at time point zero, respectively, we were able to measure RNA degradation in time, independent of degradation status at start. And remarkably all three samples showed a similar speed of decay in time. As expected, it was found that prolonged storage of the material before freezing, gradually reduced the RNA integrity when this was measured with the Bioanalyzer. And, cold storage certainly protected the RNA in the tissue from degradation in some degree. However, this protective effect was not found to be significant before the pre-fixation time exceeded 8-12 hours; A delay of tissue handling that normally does not occur in daily practice.

To relate the bioanalyzer results to functional consequences of the RNA decay, we chose to use conventional RT-PCR as measurement, since most of our current molecular diagnostic assays depend on the presence or absence of a PCR-product for readout. We found that the RNA that was isolated from all time points of liver 1, could still serve as template for a successful RT-PCR. Supporting our findings, similar results were obtained in a study by Godfrey et al. that measured the influence of various pre-fixation times on relative gene-expression with quantitative RT-PCR. Altogether, the benefit of keeping tissues at 4°C between dissection and further handling, appears to be relatively limited. Concerning the second dogma, our results unequivocally showed that RNA extracted from formalin-fixed and paraffin-embedded tissues indeed was of inferior quality compared to frozen tissue samples. However, FFPE material is often the only source of RNA for molecular diagnostics. In our attempt to improve RNA recovery from FFPE material, we determined the optimal fixation time. We suspected that insufficient fixation would render the tissue insufficiently protected against the continuing enzymatic degradation, while prolonged fixation could result in irreversible fragmentation. Indeed an optimum was found for fixation time; the best PCR results were obtained when the samples had endured formalin fixation between 8 and 16 hours. As the optimal fixation time depends on the tissue type and specimen size, a standardized fixation protocol
should involve a formalin incubation of at least 4 hours up to a maximum of 24, for regular-size fragments. For core or needle biopsies, the optimal fixation time is probably shorter. These findings may have implications for current tissue handling procedures; e.g. in case of dissections carried out on Fridays: Evidently, it is not desirable to leave the tissue in formalin throughout the weekend.

The finding that RNA yield can be improved by introducing a heating step of 2 hours at 70 degrees before cDNA synthesis is noteworthy. This finding is in agreement with the study of Masuda et al, who showed that formalin treatment of RNA oligos causes mono-methylol addition to the amino bases. In case of extensive fixation, also methylene bridging between two amino groups occurs. Most of the methylol additions may be removed by heat incubation. Hence the improvement of the RT-PCR after a heating step, which contradicts the supposed fragmentation of RNA due to formalin fixation. On the other hand, the methylene bridging is more difficult to break and can therefore cause irreversible restriction of the RNA template length, which may account for the poorer quality of RNA isolated from FFPE as compared to frozen tissue.

In some cases it may be desirable not to use entire tissue sections for RNA isolation, but instead to focus on a certain population of cells that can be identified immunohistochemically. For example, when there are low numbers of tumor cells in the sample, enrichment by laser microdissection may improve analysis. There have been various studies on RNA isolation after immunohistochemical staining, however most were performed on frozen tissue sections and mainly focus on quantitative RT-PCR, for which only short RNA templates are required. In our experience, RNA isolation is not possible after standard immunohistochemistry on FFPE tissue sections. Analysis of the impact of individual steps of the immunohistochemistry protocol on RNA retrieval, revealed that the blocking step and the use of commercially available diluents and amplification kits form the main culprits. Serum turned out to be the constituent that is responsible for this interference, since replacing the serum with BSA-c or omitting it by using PBS-diluted antibodies, greatly improved RNA recovery. Furthermore, best results were obtained when AEC was used as a substrate/chromogen. Using this modified protocol, mRNAs of at least 225 bp can be recovered from immunohistochemically stained FFPE tissue sections.

Obviously, fresh frozen tissue is preferable over FFPE material for its use in RNA based assays. Consequently, increasing numbers of biobanks of non-fixed frozen tissue are being established. Furthermore, alternative alcohol-based fixatives are under development, which will enable improved RNA preservation and retrieval. Still, the main source of material for molecular diagnostics in daily practice comprises of FFPE material. In our study we have shown that with small alterations to current tissue handling and isolation procedures, RNA can be successfully recovered from archival FFPE tissue, i.e. with an acceptable quality for standard RT-PCR based molecular diagnostic assays.
References


Summary

The production of high affinity antibodies is crucial in the combat of pathogenic invaders. Somatic hypermutation and class switch recombination are two DNA modifying processes that take place in the lymph node germinal centres, in order to increase antibody affinity and determine its effector function. However, recurrent subjection of B cells to these immunoglobulin gene alteration processes, due to chronic and recurrent infections or autoimmunity, may lead to accumulation of collateral DNA damage, this way increasing the risk of malignant derailment. This thesis deals with the molecular side of antibody diversification and its role in lymphomagenesis. A putative role for alternative splicing in the regulation of the enzyme responsible for the DNA modifications, i.e. activation-induced cytidine deaminase (AID), is investigated. Structure-function analysis of artificial AID mutants further substantiate this research. The significance of the B cell receptor and its antigen-specificity for lymphoma development, is explored by studying the immunoglobulin sequences of normal germinal centre and malignant lymphoma B cells. Biased immunoglobulin repertoires, in perspective to the genetic and environmental background of these tumours, provide insight in the origin and development of B cell malignancy.
Antistoffen spelen een belangrijke rol in de afweer tegen ziekteverwekkers. Ons basisrepertoire aan antistoffen bestaat uit een grote verzameling membraangebonden immunoglobulines (Ig), ook wel B cel receptoren (BCR) genoemd. Elke BCR heeft zijn eigen specificiteit, maar begint met een lage affiniteit voor het antigen. Na de eerste ontmoeting met het specifieke antigen, raakt de B cel geactiveerd, begint te delen en ondergaat een zgn. kiemcentrumreactie in lymffollikels. Gedurende deze kiemcentrumreactie worden er met hoge frequentie mutaties geïntroduceerd in het immunoglobuline gen (somatische hypermutatie), welke effect hebben op de affiniteit van de BCR voor het antigen. Op basis van competitie voor antigen-bindingssterkte, worden de B cellen met de hoogste affiniteit geselecteerd om te overleven. Deze cellen kunnen vervolgens, via een genomische herschikking, een ander constant gensegment in het immunoglobuline gen brengen (klasse switching), waardoor de staart van het Ig en daarmee de effectie functie van het antistof aangepast wordt aan de behoefte van de immuunrespons. Beide processen in het kiemcentrum grijpen aan op het DNA, wat risico's met zich mee brengt. Mature B cell lymfomen blijken vaak tekenen te vertonen van fouten die zijn opgetreden in deze DNA modificaties.

In **Hoofdstuk 1** is een brief van onze hand, geschreven in reactie op een artikel waarin gerapporteerd werd dat de activiteit van AID gereguleerd wordt door alternatieve splicing van het messenger RNA. Onze brief beschrijft in het kort hoe resultaten in een vergelijkbaar onderzoek juist aantoonden dat de AID splice varianten geen katalytische activiteit meer bezitten en dat daarom een rol voor splicing in de regulatie van AID onwaarschijnlijk is. Verder laten we zien dat de methode waarmee in eerste instantie somatische hypermutatie activiteit werd gemeten voor de splice varianten, een aantal technische valkuilen heeft die mogelijk verantwoordelijk zijn voor de tegenstrijdige conclusies.

In **Hoofdstuk 2** wordt dieper ingegaan op dat deel van het AID eiwit dat varieert tussen de verschillende splice vormen. Met behulp van door ons geïntroduceerde puntmutaties brengen we subtiele veranderingen aan in het eiwit, om deze mutanten vervolgens te testen op functionaliteit. Hieruit blijkt dat het voornamelijk de hydrofobe aminozuren zijn, die na mutatie een katalytisch defect eiwit opleveren. Dit defect lijkt daarom vooral structureel van aard te zijn. Dit wordt bevestigd door een tweede serie mutanten waarin de hydrofobe aminozuren vervangen worden door aminozuren met vergelijkbare eigenschappen, welke geen of veel minder negatieve effecten blijken te hebben op de enzymatische
activiteit. De resultaten zijn in overeenstemming met eerder gepubliceerde structuurmodellen voor APOBEC3G, een eiwit behorende tot dezelfde familie als AID, met een vergelijkbare structuur. Aan de hand van APOBEC3G wordt vervolgens bediscussieerd hoe dit deel van het eiwit in relatie staat ten opzichte van de katalytische site en hoe dit de eerdere bevindingen voor de AID splice varianten onderschrijft.

Hoofdstuk 3 is een studie van het immunoglobuline repertoire in reactieve lymfeklieren. Via het sequencen van immunoglobuline genen van B cellen geisoleerd uit kiemcentra, laten we zien dat B cellen van dezelfde klonale origine, in meerdere kiemcentra terug te vinden zijn. Daar deze kloon al in gemuteerde vorm over meerdere kiemcentra verspreid gevonden werd, is dit zeer suggestief voor een memory B cel die bij een herhaalde infectie opnieuw geactiveerd is geraakt en wederom een kiemcentrum reactie ondergaat. Dit is een belangrijke bevinding en bracht ons tot een nieuw model voor het ontstaan van laaggradige B cel lymfomen. Als gevolg van chronische of terugkerende infecties ondergaan de B cellen herhaaldelijke kiemcentrum reacties en kunnen zo DNA schade accumuleren tot er ontsporing van de cellen optreedt.

Dat een chronische ontsteking tot het ontstaan van lymfomen kan leiden is duidelijk terug te vinden in de marginale zone lymfomen, welke sterk geassocieerd zijn met bepaalde chronische infecties en autoimmuunziekten. Hoofdstukken 4 en 5 bestuderen marginale zone lymfomen, gelocaliseerd in de huid en het oog, met betrekking tot de immunoglobuline genen om zo een indruk te kunnen krijgen van de rol die het antigen heeft gehad in de ontwikkeling van deze maligniteiten.

Een discussie over het geheel aan genetische en omgevingsfactoren in marginale zone lymfomen en de rol van antigenherkenning door de B cell receptor, wordt uitgebreid gevoerd in hoofdstuk 6, een review van de literatuur over dit onderwerp.

Het onderwerp van hoofdstuk 7 wordt gevormd door een ander laaggradig lymfoom (chronisch lymfoide leukemie) waarbij verondersteld wordt dat antigenherkenning een belangrijke rol speelt in het ontstaan van de aandoening. Deze studie richt zich echter meer specifiek op het proces van transformatie naar een hooggradig lymfoom (diffuus groot-cellig B cel lymfoom), de zogenaamde Richter's transformatie, om daarin de rol van antigenherkenning te bestuderen aan de hand van de immunoglobuline sequenties. Ook de rol van heractivatie van kiemcentrumprocessen in de transformatie wordt verkend, aan de hand van mutatie analyse en bepalingen van de AID expressie.

In de discussie wordt ingegaan op recente bevindingen die suggereren dat er ook een rol is voor RNA in de regulatie van somatische hypermutatie en klasse switching. Hierbij wordt met name de invloed van post-transcriptionele verwerking van nieuwe gentranscripten, zoals splicing en export van RNA uit de kern, bediscussieerd.

De appendix omvat een studie naar de effecten van bewaren, fixeren en inbedden in paraffine van patienten weefsel, op de kwaliteit van het materiaal, met name gelet op de bruikbaarheid van RNA uit dit weefsel voor moleculair diagnostische doeleinden. Vervolgens wordt de methode voor RNA extractie, uit dit soort materiaal, geoptimaliseerd. Het protocol dat uit deze studie voortgekomen is, is o.a. toegepast voor het onderzoek beschreven in hoofdstuk 4.
Febe van Maldegem is geboren op 14 januari 1980, te Goes en groeide op in Kortgene. In 1997 behaalde zij haar VWO diploma aan Het Goese Lyceum, waarna zij Medische Biologie ging studeren aan de Universiteit van Amsterdam. Tijdens haar studie was zij een jaar actief als secretaris van CONGO, de studievereniging van de faculteit Biologie. Haar eerste onderzoeksstage liep zijn op de afdeling Celbiologie en Histologie van professor Van Noorden aan het AMC, onder de begeleiding van Olaf Mook en Wilma Frederiks. Tijdens deze periode werkte zij mee aan het onderzoek naar de rol van matrix metalloproteinases in metastasering van coloncarcinoma. In haar tweede onderzoeksstage bestudeerde zij de expressiepatronen van Dlk-1 in neuroblastoma, onder begeleiding van Vera van Limpt, in de groep van professor Versteeg aan de afdeling Antropogenetica van het AMC. In 2003 behaalde Febe haar doctoraal diploma, waarna zij in maart 2004 haar promotieonderzoek startte aan de afdeling Pathologie van het AMC, onder begeleiding van professor Van Noesel. Onderwerpen van onderzoek waren B cel non-Hodgkin lymfomen en de rol van de B cel receptor en de kiemcentrum-reactie in het ontstaan en de progressie van deze ziekten. Hiernaast bestudeerde zij de moleculaire eigenschappen van Activation-induced cytidine deaminase (AID), het enzym dat verantwoordelijk is voor de DNA-modificaties gedurende de kiemcentrum-reactie. Dit proefschrift is een wetenschappelijk verslag van dit promotieonderzoek, wat verdedigd zal worden op 19 november 2009. In juli 2009 begon zij een post-doctoraal onderzoek in de groep van professor Neuberger aan het MRC Laboratory of Molecular Biology, te Cambridge (UK), met behulp van een Rubicon fellowship van de Nederlandse organisatie voor Wetenschappelijk Onderzoek (NWO).
List of publications


van Maldegem F, Jibodh RA, van Dijk R, Bende RJ, van Noesel CJM. Activation-induced cytidine deaminase splice variants are defective due to the lack of structural support for the catalytic site. Submitted.

Dankwoord

En dan, onmisbaar in elk proefschrift; de waardering voor alle bijdragen van anderen aan het eindresultaat. Want je dacht toch niet dat ik dit allemaal alleen gedaan had?

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Aarti, wat hebben we samen een hoofdbrekens doorstaan met die deaminase assay. Maar het is je wel mooi gelukt! Die data is uiteindelijk essentieel gebleken in ons splice-varianten verhaal. Thera, wat kun jij pipetteren vrouw! Aan de lopende band! Jennifer, het was maar kort, maar onze samenwerking was erg prettig. En wat een drive heb jij Remco, om naast je studie nog wat uurtjes te komen PCR-en elke week. Je leverde een belangrijke bijdrage aan het huidlymfomen stuk! Bedankt voor al je werk aan de ooglymfomen. Alex, Mireille, Jitske, Folkert, Mirjam, Jos, Chris, Jan, en natuurlijk Monique en Esther (S), jullie ook heel erg bedankt voor alle ontelbare kleine dingen die jullie steeds voor mij gedaan hebben.

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