Monocytoid B cells: An Enigmatic B cell Subset Showing Evidence of Extrafollicular Immunoglobulin gene Somatic Hypermutation

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Abstract

Monocytoid B cells are IgM⁺, IgD⁻/⁺, CD27⁻ B cells, localized in the perisinusoidal area of the lymph node. These cells are especially prominent in infections such as those caused by toxoplasma and HIV. The ontogeny of monocytoid B cells with respect to B cell maturation is incompletely known. We analysed clonal expansion, somatic hypermutation and expression of activation-induced cytidine deaminase (AID) in monocytoid B cells. Sequence analysis of the rearranged immunoglobulin heavy chain genes amplified from microdissected monocytoid B cell zones with a high proportion of proliferating cells reveals the presence of multiple clones with low-level ongoing mutations (mean frequency: 0.46 × 10⁻² per bp). Mutation analysis of these ongoing mutations reveals strand bias, a preference of transitions over transversions as well as the occurrence of small deletions, as observed for somatically mutated immunoglobulin genes in the human germinal centre. Proliferation, ongoing mutation as well as expression of AID, combined, is evidence that monocytoid B cells acquire the mutations in the extrafollicular perisinusoidal area of the lymph node and pleads against a postgerminal centre B cell origin.

Introduction

Monocytoid B cells are an as yet enigmatic B cell subpopulation, localized in the perisinusoidal area of the lymph node. These cells are morphologically characterized by round to oval nuclei and more abundant cytoplasm, features that are more typical for monocyte-derived cells, hence their name. Whether they are akin to the marginal zone B cells or the circulating IgM⁺, IgD⁻ and CD27⁻ B cells is a matter of discussion [1–3]. Monocytoid B cells are especially abundant in lymphadenitis caused by toxoplasma and HIV infection. The function of monocytoid B cells is as yet poorly understood.

Our previous observations as well as those of others showed that monocytoid B cells have rearranged immunoglobulin genes with a variable number of somatic hypermutations [4–6]. The germinal centre of the B cell follicle in secondary lymphoid tissues is the major site of clonal B cell expansion and somatic hypermutation of rearranged Ig genes [7, 8]. Clonal expansion and somatic hypermutation are essential for affinity maturation of the T cell-dependent humoral immune response [9–11]. A low level of somatic hypermutation in B cells of patients with hyper-IgM syndrome and X-linked lymphoproliferative disease, characterized by the absence of functional germinal centres, suggests that clonal expansion and somatic hypermutation may also occur outside the germinal centre and, perhaps, outside the context of the T cell-mediated immune response [12–17]. In addition, there is also experimental evidence that somatic hypermutation may occur in B cell compartments other than the germinal centre [18, 19]. Furthermore, somatic hypermutation has also been shown in immature B cells prior to antigen stimulation [20–22].

Whether monocytoid B cells acquire somatic mutations in the germinal centre and are therefore postgerminal centre B cells has not yet been investigated and is the subject of this study. We have isolated monocytoid B cells from the perisinusoidal lymph node area and have studied proliferation, immunoglobulin somatic mutation as well as expression of activation-induced cytidine deaminase (AID), an enzyme which is required for somatic hypermutation [23, 24]. The results indicate that monocytoid B cells acquire somatic hypermutations in the perisinusoidal area of the lymph node and therefore questions their postgerminal centre B cell origin.
Materials and methods

Strategy. Three perisinusoidal zones of monocytoid B cells from two lymph nodes were microdissected. Each zone, consisting of about 500 cells, was submitted to multiplex PCR amplification of rearranged immunoglobulin heavy chain genes. The PCR products from each monocytoid B cell area were subsequently cloned into a bacterial vector. About fifty randomly chosen bacterial clones from each of the respective three monocytoid B cell zones were sequenced. Sequences that had an identical CDR3 region of the rearranged IgH gene were considered as clonally related sequences. Mutations were determined by comparison with germline immunoglobulin sequences. Divergent mutations in such clonally related sequences can either be explained by an active somatic hypermutation within a clonal B cell population, that is, a B cell clone with so-called ongoing somatic hypermutation, or, alternatively, by the preferential amplification of sequences and base pair mismatching during the PCR process. Therefore, an equal number of peripheral blood cells, about 500 cells, from a healthy donor were submitted to the same analysis. Normal peripheral blood B cells are not clonally related and thus no ongoing mutations should be detected. In addition, germline sequences were repeatedly amplified with the same multiplex PCR technique. These PCR products were cloned and sequenced. From the latter experiment, a PCR-induced base pair misincorporation error rate could be determined. Because the Taq polymerase error rate is to some degree sequence dependent, the germline genes most used by monocytoid B cells in our analysis were chosen as template for the PCR error control experiment. Using the Taq polymerase error rate thus established, a statistical analysis was performed to evaluate the likelihood that the ongoing mutations observed in the clusters of clonally related sequences were because of polymerase error. For the purpose of this statistical analysis and in view of the low rate of mutations, all sequences showing ongoing mutations within the several clusters of clonally related sequences were grouped together. The ongoing mutations were also further analysed to determine whether or not these mutations were polymerase induced: real somatic mutations show strand bias, whereas polymerase-induced errors do not. Other characteristic features of somatic mutation were also investigated.

Selection of tissues and blood samples. Ten reactive lymph nodes that showed monocytoid B cell hyperplasia were selected from the archive of the department of pathology, Oslo University Hospital. Formalin-fixed paraffin-embedded as well as snap-frozen tissue was available in all cases. These samples were left-over samples after histological diagnosis. All samples were anonymized. The study was approved by the institutional review board. The cause of the lymphadenitis was not identified in nine patients, whereas one case showed serologic evidence of toxoplasma infection (case 2). Two samples were selected for microdissection of monocytoid B cells, and all samples were used for immunohistochemical analysis. For control studies, peripheral blood leucocytes of a healthy adult donor were obtained.

Immunohistochemistry. To analyse monocytoid B cells and cells in their microenvironment, immunohistochemistry on paraffin-embedded tissue sections of the lymph nodes of the ten cases was performed. The following primary antibodies were used: anti-CD3 (Novocastra Laboratories Ltd, Newcastle-upon-Tyne, UK), anti-CD4 (Dako, Glostrup, Denmark), anti-CD8 (Novocastra), anti-CD20 (Novocastra), anti-CD21 (Novocastra), anti-CD23 (Novocastra), anti-CD27 (Dako, Denmark), anti-CD35 (Novocastra), anti-AID (clone EK2-5G9, rat monoclonal antibody, a kind gift of Dr. Niedobitek, Erlangen, Germany) [25], anti-Bcl2 (Dako, Denmark), anti-Bcl6 (Dako) and anti-Ki-67 (Dako). Either the ABC method [26] or the EnVision method (Dako) immunostaining procedures were used according to the instructions of the manufacturer.

Microdissection of monocytoid B cell zones. To help identify monocytoid B cells for microdissection, 5-μm frozen lymph node tissue sections of cases 1 and 2 were stained with a mixture of anti-IgD (Dako), anti-CD23 (Dako) and Ki-67 (mib1; Biogenex, San Ramon, CA, USA). The ABC method was used for immunostaining [26]. All slides were counterstained with Mayer’s haematoxylin. Monocytoid B cells were easily recognized as relatively large cells with abundant clear cytoplasm, localized in the perisinusoidal lymph node area. Microdissection of B cell follicles was avoided by CD23 staining of the follicular dendritic cells and IgD expression of the mantle zone B cells. The combination of morphological analysis with immunohistological staining did allow to dissect a homogeneous area with monocytoid B cells. However, microdissection using these parameters did not exclude the potential contamination with a potential scattered immunoblast or other small, likely memory B cell. Before microdissection, immunostained sections were digested with 10 mg/ml collagenase H (Boehringer, Mannheim, Germany) in PBS for 1 h at 37 °C. Subsequently, monocytoid B cell zones with a respective high and low number of proliferating Ki67-positive cells were microdissected and aspirated using a hydraulic micromanipulator (Narishige MMW-202D, Tokyo, Japan) as previously described [27]. Each isolated cell zone contained a similar number of about 500 cells. The cell zones were dropped in 10 μl of PCR buffer (50 mM KCL, 10 mM Tris–HCL pH 8.4, 0.01% gelatin) containing 200 mg/ml of proteinase K (Qiagen, Hilden, Germany), covered with 50 μl of mineral oil and finally digested at 37 °C for 16 h prior to PCR analysis.
PCR amplification of the rearranged IgH genes. Rearranged IgH genes were amplified using a semi-nested PCR method as previously described [6, 27]. In the first round of the PCR, a mixture of six framework I (FR I) VH family-specific primers and three primers complementary to all JH genes were used. The second round of the PCR was performed in six separate reactions with one of the six VH FR I primers and a mixture of three internal JH primers.

To the digested cells, 30 µl of a master mix (200 mM dNTPs, 2.5 mM MgCl₂, 10 nM of each primer in PCR buffer) was added and heated at 94 °C for 10 min. While at 94 °C, 1.5 U of Taq polymerase (Amplitaq Gold; Perkin Elmer Corporation, Norwalk, CT, USA) diluted in 10 µl of dH₂O was added to the reaction mixtures. The PCR conditions of the first round consisted of one cycle at 95 °C for 2 min, 59 °C for 4 min, 72 °C for 80 s followed by 34 cycles at 95 °C for 90 s, 59 °C for 30 s, 72 °C for 80 s and one final cycle of 72 °C for 5 min. For the semi-nested PCR, performed in a 50 µl volume, 2 µl of the first round product was used as the template. Reagents and reagent concentrations were the same as for the first PCR round, except for MgCl₂ (1.5 mM) and semi-nested primers (100 nM). The second PCR consisted of a total of 35 cycles. The denaturing and extension temperatures as well as the cycling times were identical to those of the first round of PCR. Controls consisted of the B cell line Namalwa DNA and peripheral blood DNA, whereas negative controls were non-template controls and the T cell line MOLT-4 DNA. An aliquot of 8 µl of the PCR products was size-fractionated through a 1.5% agarose gel in 1× TBE buffer.

Cloning and sequencing of the PCR products. All PCR products were concentrated by vacuum drying. The dried products were resuspended in 10 µl of loading buffer and size-fractionated through a 1.5% agarose gel. The appropriate bands were excised, and the PCR products were extracted from the gel with Qiaquick spin-columns (Qiagen) following the manufacturer’s recommendations. The purified products were cloned using the pGem®-T easy Vector system (Promega, Madison, WI, USA) following the recommendations of the manufacturers.

An aliquot of the purified DNA was sequenced in both directions. This was accomplished by the cycle sequencing reaction using the DNA sequencing kit (PE Applied Biosystems, Foster City, CA, USA) [28]. Universal M13 forward and reverse sequencing primers were used (Life Technologies, Paisley, UK). The products of the sequencing reaction were analysed using the Applied Biosystems 377 sequencer (PE Applied Biosystems). The accession numbers of the rearranged IgV sequences are AJ289253–AJ289432.

Sequence and somatic mutation analysis. The identification of the VH, DH and JH germline sequences was performed by sequence comparison to the IMGT database (http://www.imgt.org).

Features of somatic hypermutation such as G/C and A/T bias (strand bias), the ratio of transitions over transversions, the RGYW/WRCY motif bias, the mutability index of various trinucleotides as well as the number of deletions were studied [29–32]. The mutability index for the various trinucleotides was calculated according to Smith et al. [33]. Briefly, the number of mutations affecting a given trinucleotide in all of the obtained sequences was divided by the expected number of mutations affecting that trinucleotide in the absence of bias. The mutability indices thus calculated were compared with the ones published for human VH genes [31]. The percentage of A/T to G/C transitions, a feature typical of Taq polymerase error, was determined [34].

Taq polymerase and multiplex errors. The rate of Taq polymerase error was determined by amplifying control VH2-70 and VH5-51 germline Ig sequences using the same PCR conditions as used for analysing monocytoid B cells. The PCR products were sequenced, and the total number of basepair variations compared with the germline sequence was divided by the accumulated length of all sequences analysed. VH2-70 and VH5-51 germline sequences were chosen as templates for this test because most of the larger clusters of clonally related sequences obtained from monocytoid B cells used these genes. The error rate was found to be 1 × 10⁻³ per bp per cycle, which is comparable to that reported in other studies [5].

Hybrid sequences can be observed when highly homologous sequences are simultaneously amplified in a multiplex PCR. This can be a source of miscalculation of the somatic mutation rate [35–37]. We have quantified these multiplex errors by co-amplifying two known rearranged immunoglobulin sequences of the VH₂ family (VH2-70 and VH2-05) using the same PCR conditions as for the monocytoid B cells. PCR products were cloned and sequenced. The multiplex error rate was 10%. Therefore, all our sequences were carefully screened for the occurrence of these errors. This was achieved by comparing all potential somatic hypermutations with all other germline and mutated sequences obtained from the same PCR. We omitted all sequences for final analysis of which a potentially mutated basepair was homologous with any of the other non-clonally related sequences of the same VH family. This ruled out that hybrid sequences were analysed. However, this screening for errors may have led to a degree of underestimation of the number of true somatic mutations.

Statistical analysis. The likelihood of ongoing somatic mutations versus Taq polymerase-induced sequence errors in clonally related sequences from monocytoid B cells was calculated using χ² statistics with the help of graphical log-linear models (GLM) [38, 39].
A potential pitfall when analysing B cell clones using a multiplex PCR method is the possible non-stochastic amplification of subsets of sequences. This was tested by comparing the number of clonally related sequences obtained from monocytoid B cell zones with those from normal blood B cells, using $\chi^2$ statistics.

To study mutational strand bias, we counted the A, T, G and C mutations for all monocytoid B cell sequences with mutations and separately, for the subset of sequences showing only ongoing mutations. $\chi^2$ statistics was used for analysing the G/C and A/T ratios after normalization of the ratios for the respective frequency of the nucleotides in the respective germline sequences. Additionally, we also tested the transition/transversion ratio. The RGYW and WRCY bias for all mutations and for ongoing mutations was calculated, but not statistically analysed because of the relative scarcity of these motifs.

Results

Immunohistochemistry: a subset of monocytoid B cells expresses AID but not CD27

Results of the immunophenotypic analysis are summarized in Table 1. Focal collections of monocytoid B cells expressed AID and the proliferation-associated marker Ki-67 (Fig. 1A–D). This expression was not seen in all monocytoid B cell zones throughout the lymph node. AID expression was very intense in the cytoplasm of monocytoid B cells, but was also expressed in the nucleus of a subset of cells. Of interest, most AID-expressing cells were proliferating, as indicated by double staining for Ki-67 (Fig. 1E–F). The cellular distribution of AID expression as well as the co-expression of AID with Ki-67 in monocytoid B cells is similar to that reported for germinal centre B cells [40]. With the exception of low expression in a few cells, monocytoid B cells did not express CD27. A variable number of CD4- but not CD8-expressing T cells were admixed with the monocytoid B cells. Also, monocytoid B cells variably expressed CD40. The level of CD40 expression was similar or weaker to that noted for germinal centre B cells. No CD21-, CD23- or CD35-expressing follicular dendritic cells were seen admixed monocytoid B cells.

Monocytoid B cells show clonal expansion and somatic hypermutation

A monocytoid B cell zone with a low number of proliferating cells (<25 per cent), designated zone A, was microdissected from case 1. Two zones with a high number of proliferating cells (>50%), designated zones B and C, were microdissected from cases 1 and 2, respectively. The analysis of the rearranged immunoglobulin heavy chain gene sequences of these monocytoid B cells and control peripheral blood B cells is given in supplementary Tables S1–S4.

Statistical analysis revealed that clonally related sequences obtained from monocytoid B cells in zones B and C, but not in zone A, with the lowest number of proliferating cells, are likely derived from clonally related B cells rather than from non-stochastic amplification of nucleotide sequences ($P$ values 0.0297, 0.01 and 0.356, respectively). True clonal expansion was further supported by the characteristic intraclonal sequence variation, revealing that these have features of somatic hypermutations and not of PCR-induced sequence errors (Table 2); the number of ongoing mutations in monocytoid B cells was significantly higher than the Taq polymerase-induced sequence error ($P = 0.046$), and a G/C mutation ratio indicating strand bias ($P = 0.0005$) was seen. PCR-induced base substitutions are strictly symmetric and do not show strand bias because both strands of the DNA

|         | GC B cells | MC B cells | GC T cells | MCB zone T cells | GC DC | MCB zone DC |
|---------|------------|------------|------------|------------------|-------|-------------|
| AID     | +          | +/−        | NA         | NA               | NA    | NA          |
| CD10    | +          | −          | −          | −                | NA    | NA          |
| Bcl-2   | −          | −/−        | −          | −                | NA    | NA          |
| Bcl-6   | +          | +          | −          | −                | NA    | NA          |
| CD40    | +          | +/−        | NA         | NA               | NA    | NA          |
| CD27    | +          | +/−        | +          | +                | NA    | NA          |
| Ki67    | +          | +/−        | NA         | NA               | NA    | NA          |
| CD21    | −          | −          | NA         | NA               | +     | −           |
| CD23    | −          | −          | NA         | NA               | +     | −           |
| CD35    | −          | −          | +          | −                | NA    | NA          |
| CD3     | −          | −          | −          | +                | NA    | NA          |
| CD4     | −          | −          | −          | +                | NA    | NA          |
| CD8     | −          | −          | −          | −                | NA    | NA          |

GC, germinal centre; MC, monocytoid; MCB, monocytoid B cell; DC, dendritic cell; −/−, variable and relatively low number of cells; +/−, variable but relatively high number of cells; NA, not analysed; AID, activation-induced cytidine deaminase.

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molecule, obviously, function as templates for the polymerase. By contrast, strand bias is a characteristic of somatic hypermutation, the mechanism of which targets primarily the non-transcribed strand \[37, 41\]. In addition, we found an A/T to G/C transition percentage well below the level of that observed for Taq polymerase error, that is, typically above 50% \[34\].

Ongoing somatic hypermutation was found in 11 clusters of clonally related IgH genes. Figure 2 illustrates the genealogical trees of six of the largest clusters of sequences. Further characteristics of the mutations occurring in the monocytoid B cell IgH sequences are summarized in Table 2. As is evident from the table, the mutations, including the ongoing mutations, showed a G/C mutation bias, a predominance of transitions over transversions and the presence of deletions, all characteristics of somatic hypermutations \[41\]. A significant RGYW/WRCY motif bias could not be demonstrated, likely due to the low number of these motifs present in the collective sequences. There were 38 and six mutations observed in those motifs, when all mutations or only ongoing mutations are analysed, respectively. The respective expected numbers are 24 and six if mutations occurred by chance only. Finally, supplementary Table S5 summarizes the mutability indices for a range of trinucleotides observed for all mutations. This analysis was not performed for the ongoing mutations separately, in view of the too low number of mutations for this type of

Figure 1 Immunohistochemical analysis of monocytoid B cells. A variable number of monocytoid B cells (MBC) express Ki-67. More germinal centre B cells express Ki-67 (GC) (A, X200 and B, X400). (B). Monocytoid B cells as well as germinal centre B cells express activation-induced cytidine deaminase (AID) (C, X200 and D, X400, inset X600). The inset highlights nuclear expression as well as cytoplasmic expression in a number of monocytoid B cells. Double staining for Ki-67 (brown) and AID (red) reveals co-expression of AID with Ki-67, both in the dark zone germinal centre B cells (E, X400) and monocytoid B cells (F, X400 and inset X600).
Table 2 Characteristics of the mutations in the rearranged VH sequences of monocytoid B cells.

| Characteristic                        | All sequences | Clusters of sequences with ongoing mutations |
|---------------------------------------|---------------|----------------------------------------------|
| Overall mutation frequency           | 0.35 × 10⁻² per bp | 0.46 × 10⁻² per bp (0.046)                      |
| Mutation frequency of mutated sequences only | 1.33 × 10⁻² per bp | –                                             |
| A/T mutation ratio (strand bias)     | 1.05 (0.84)   | 1.7 (0.27)                                    |
| G/C mutation ratio (strand bias)     | 2.22 (0.0005) | 4.9 (0.00047)                                  |
| Transition/Transversion ratio        | 1.95 (0.00037)| 3.7 (0.00015)                                 |
| RGYW and WRCY bias                   | 1.55          | 1.02                                          |
| Deletions                             | 2             | 1                                             |
| A/T → G/C transitions (%)            | 16            | 30                                            |

Except for the calculation of the mutation frequency, mutations at the same positions in clonally related sequences were only counted once, as mutations were considered not to represent independent events. Also, for all calculations except the mutation frequency, clonally related sequences were considered as one sequence.

*a* All mutations occurring at A or G nucleotides were divided by the mutations occurring at T or C nucleotides, respectively, and were normalized for the relative occurrence of the nucleotides in the sequences.

*b* The total number of transitions observed (G to A, A to G, C to T and T to C) was divided by the total number of transversions observed (G to C, G to T, A to T, A to C, C to G, C to A, T to A and T to G).

*d* The total number of mutations observed in RGYW and WRCY motifs was divided by the expected number of mutations in those motifs.

Analysis. Most mutability indices, except for a minority of trinucleotides, are similar to those published for somatic hypermutations [31].

Discussion

Clonal expansion with somatic hypermutation of rearranged immunoglobulin genes is a characteristic feature of germinal centre B cells [7, 8]. It is the cornerstone of antibody affinity maturation [8–10]. We and others have previously shown that monocytoid B cells, as an as yet poorly characterized B cell population, and marginal zone B cells show a low level of somatic hypermutation of their rearranged IgV genes [6, 42]. We also observed that monocytoid B cells may clonally expand [6]. However, the level of clonal expansion may vary considerably according to the monocytoid B cell zone analysed. Whether somatic hypermutations accumulate during clonal expansion of the monocytoid B cells in situ or are acquired during previous passage through the germinal centre was unresolved. Here, we provide evidence that clonal expansion of monocytoid B cells and somatic hypermutation occur in situ, that is, in the lymph node perisinusoidal area. We do so by demonstrating ongoing mutations of VH genes in proliferating monocytoid B cells, residing in a well-characterized B cell area of the lymph node outside the germinal centre and by showing that these proliferating cells express AID.

We have chosen to perform PCR analysis of IgH genes of microdissected B cell zones instead of performing single-cell PCR analysis. This allowed us to obtain significantly more sequences, necessary for the statistical analysis of our data. This method has also been used by others [5, 43]. In addition, the immunophenotype of monocytoid B cells is not characteristic enough to allow the sorting of cells by flow cytometry for single-cell PCR analysis. A low level of somatic mutation (0.35 × 10⁻² per bp) was detected in monocytoid B cells, as previously shown by our group and others [4–6]. In addition, and importantly, in 11 groups of clonally related IgH sequences from monocytoid B cells, ongoing somatic mutations are present. These mutations are unlikely Taq polymerase errors, but true somatic hypermutations because of a higher frequency (P < 0.05) and increased A/T to G/C transition ratio compared with Taq polymerase-induced mutations [34]. Moreover and importantly, the high normalized G/C mutation ratio observed for the ongoing mutations indicates strand bias. This is a feature of somatic hypermutation, but is not compatible with PCR-induced base substitution that is symmetrical [44]. The fact that the somatic mutations in monocytoid B cells show a G over C nucleotide bias is consistent with one of the strand-biased signatures of the somatic hypermutation process in human Ig genes [37, 44]. The ongoing mutations, as well as all the mutations considered together, in monocytoid B cells display also other characteristics of somatic hypermutation; nucleotide transitions were more frequently observed than transversions and deletions were seen. We also found biased targeting of RGYW/WRCY and defined trinucleotide motifs, other features of the somatic hypermutation process [29–33, 45, 46]. Also, the mutability indices of most of the trinucleotides are similar to those of somatic hypermutations [31]. Some indices are divergent, likely due to the fact that the published indices were derived from non-productively rearranged sequences, whereas ours are derived from all rearranged sequences. The latter represent a mixture of both non-productively as well as productively rearranged sequences. Mutability indices
derived from the productively rearranged sequences do not only reflect intrinsic mutability but also reflect any selective pressure that may have occurred on the cells.

Of importance, we show that proliferating monocytoid B cells express AID as one of the few B cell subpopulations, apart from germinal centre B cells. AID expression was previously also demonstrated in scarce scattered interfollicular cells and a population of immature foetal B cells \[40, 47\]. AID expression is required for somatic hypermutation \[23, 24\]. The expression of AID as well as the presence of ongoing mutations in proliferating monocytoid B cells is evidence that somatic hypermutation has occurred in the perisinusoidal area of the lymph node, from which these cells were isolated, rather than in the germinal centre. Whether these cells acquire additional mutations through subsequent passage in the germinal centre, as suggested by others, has not been addressed in our study \[5\].

**Figure 2** Genealogical trees of clonally related sequences. These sequences are derived from monocytoid B cell zone A, cluster 5 (A), monocytoid B cell zone B, cluster 1 (B), cluster 3 (C) and from monocytoid B cell zone C, cluster 1 (D), cluster 2 (E) and cluster 3 (F), respectively. The basepair changes because of somatic mutation as well as their location with respect to the germline VH gene is given in the circles. The codes above the circles refer to the sequences given in supplementary Tables S1–3. X represents a hypothetical intermediate.
Somatic hypermutation outside the germinal centre has already been suggested before, both in human and mice. Patients with hyper-IgM syndrome as well as patients with X-linked lymphoproliferative disease, who lack germinal centres, do show a low level of somatic hypermutation [12–17]. However, the latter only occurs in a subset of IgM⁺/IgD⁺/CD27⁺ cells, but not in peripheral blood IgM-only or Ig-switched B cells [13]. Weller et al., [4, 13] therefore, argue that a second B cell diversification pathway, separate from that involving the germinal centre and likely involving marginal zone B cells, exists in humans. This seems to be confirmed by the Scheeren et al., [19] using T cell-deficient mice with a humanized immune system. By contrast, Seifert et al. [48] have shown that the majority of IgM⁺, IgD⁺, CD27⁺ B cells carry BCL6 somatic mutations and have used this as an argument for a postgerminal centre cell origin of these cells. It is of interest that the Ig mutation frequency of B cells in patients with the hyper-IgM syndrome (1.4 × 10⁻² per bp) and peripheral blood IgM⁺, IgD⁺, CD27⁺ B cells (0.4 × 10⁻² per bp) is similar to that found for mutated monocytoid B cells in our study (1.33 × 10⁻² per bp, Table 2) [2, 12]. Despite these similarities, monocytoid B cells have a IgM⁺, IgD⁺, CD27⁺ immunophenotype contrasting with the IgM⁺, IgD⁺, CD27⁺ peripheral blood B cell population. Therefore, these two B cell populations are likely different.

The germinal centre of the B cell follicle in secondary lymphoid tissue is the major site of clonal B cell expansion and somatic hypermutation of the rearranged IgV genes. Stimulation by T cells through CD40–CD40 ligand as well as through CD86–CD28 interaction is involved in the induction of somatic hypermutation in the germinal centre [49]. In addition, in vitro studies have shown that cross-linking of the surface Ig and cognate T cell help are required, whereas lipopolysaccharides or the addition of CD40 ligand is not sufficient to induce somatic hypermutation [50–52]. Other as yet unknown signals are likely involved as well [44]. The cellular interactions triggering the somatic hypermutation process in monocytoid B cells are unknown. The microenvironment of the monocytoid B cell zone is different from that of the germinal centre, but may have some features in common with the latter. T helper cells, which are numerous in germinal centres, are also present in the monocytoid B cell zone, although in lesser numbers [1]. Follicular dendritic cells are absent in the monocytoid B cell zone. By contrast, neutrophils, macrophages and sinus-lining cells form part of the microenvironment of monocytoid B cells [1]. Whether these various cell types can contribute to the clonal expansion of B cells and somatic hypermutation clearly needs more study. It is also unknown which types of antigens, if any, trigger the proliferation and somatic hypermutation in monocytoid B cells.

In this study, we confirmed that monocytoid B cells proliferate [5, 6]. However, this proliferation is only focal. The five times lower level of somatic hypermutation in monocytoid B cells compared with that in germinal centre B cells suggests a substantially lower clonal burst size of monocytoid B cells than that of germinal centre B cells, assuming a similar mutation rate between 10⁻³ and 10⁻⁴ per bp per generation [44]. Based on mutation frequency and assuming that monocytoid B cells are selected in a similar way as germinal centre B cells, the clonal burst size of monocytoid B cells should be a few hundreds of cells. By contrast, the clonal burst size of germinal centre B cells is several thousands of cells, according to the mathematical model of Shlomchik et al. [53]. This calculation is in agreement with the lower numbers of proliferating cells observed in the monocytoid B cell zone compared with the germinal centre. Clonal expansion of monocytoid B cells could not be demonstrated in a previous study [42]. Although varying numbers of proliferating monocytoid B cells were observed, these cells were not selected for sequence analysis of IgH genes. The latter, together with the lower number of sequences analysed, may explain why clones of monocytoid B cells could not be demonstrated [42].

In contrast to our previous findings and to the recent findings of Lazzi et al., a smaller number of rearranged IgV genes in monocytoid B cells belong to the VH3 and VH4 families [5, 6] and most belong to VH1 and VH2 families. These differences are likely due to the selection of cases studied here. However, we cannot exclude a certain level of skewage towards certain VH gene families because of PCR amplification. The VH gene usage observed in control peripheral blood B cells indicates, nonetheless, that PCR-induced artefact is minimal. In peripheral blood B cells, VH3 and VH4 family sequences were predominantly found, as expected, and not VH1 or VH2 genes as would be expected if the latter genes were preferentially amplified. Of interest, toxoplasma infection was demonstrated in one of the cases we analysed. It is not excluded that toxoplasma infection contributed to the selection of VH3 family genes. VH2 family genes have been reported to be overrepresented in the antibody repertoire following bacterial antigens [54]. However, no data are available for toxoplasmosis.

The presence of somatic hypermutations has been used to study the cellular origin of human B cell lymphomas [55, 56]. Largely based on the presence of somatic hypermutations and of ongoing mutations, B cell lymphoma has been classified as of pregerminal centre, germinal centre and postgerminal centre B cell origin [55]. While this is a valid concept, it may need to be modified. Our results indicate that the low-grade somatic hypermutation or low-grade ongoing mutation may not necessarily be proof of an origin from germinal centre or postgerminal centre B cells.
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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Sequence and mutation analysis of the rearranged IgV genes of monocytoid B-cell zone A.
Table S2 Sequence and somatic mutation analysis of the rearranged IgV genes of monocytoid B-cell zone B.
Table S3 Sequence and somatic mutation analysis of the rearranged IgV genes of the monocytoid B-cell zone C.
Table S4 Sequence and somatic mutation analysis of the rearranged IgV genes peripheral blood B cells.
Table S5 Mutability indices of the mutated trinucleotides in al sequences derived from monocytoid B cells.

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