Immunoglobulin gene alterations in normal and neoplastic B cells
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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 (IgVH/IgVL) 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 a 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\(_{L}\)-69/JH4-* or *V\(_{L}\)-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.\(^24\) 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.\(^26-27\) 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 CD5.10 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/l of each dNTP, 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_H-CDR3 analysis:
PCR amplification of the IgV_H-CDR3 regions used a forward primer in framework region 3 (FR3) in combination with reverse primers specific for JH, Cµ, Cδ, Cy, Cα 2 and Cε (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 \( V_k \)-family specific primers in combination with a \( C_k \)-primer. 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 \( V \)base\(^{30} \) and IMGT/V\(^{31} \) immunoglobulin databases to obtain the \( V \)DJ\( _H \) 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 ×400. 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’-ATGCTCTTCCACCTCGAACC-3’; CXCL10-fw 5’-GG AACCTCCAGTCTCGACC-3’; CXCL10-rev 5’-CAGCTCTGTTGTTGCTCCAT
CC-3’; IL-12-fw 5’- ATTGAGGTCATGGTGGATGC-3’; IL-12-rev 5’-AATGCTGGCATTTTTGCACGGC-3’; IL-4-fw 5’-TGCCTCCAAGAACACAACTG-3’; IL-4-rev 5’-AACGTACTCTGGTGGCTTC-3’; Actin-fw 5’-CATGGACAAAATCTGGCACCA-3’; Actin-rev 5’-CCACTGCACACTTCATGATGGAG-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 (CXCL10), 25 (actin) or 30 (IFN-γ, 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\textsubscript{H}-family-specific leader primers or FR2/FR3 primers in combination with J\textsubscript{H}6 or constant region primers. Of 21 PCMZLs the IgV\textsubscript{H}-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\textsubscript{H}-CDR3 sequence and shared mutations. The V\textsubscript{H} germline gene could be determined for 11 out of 21 PCMZLs, demonstrating that five cases used V\textsubscript{H}1 and six used V\textsubscript{H}3. The results of the IgV\textsubscript{H} analyses are summarized in Table 1. Similarly, V\textsubscript{K} family leader primers combined with a C\textsubscript{K} primer, identified the kappa-rearrangement for seven PCMZLs: Three used V\textsubscript{K}1, one used V\textsubscript{K}2 and three used V\textsubscript{K}3 (Table 2). Mutation frequencies within the IgV\textsubscript{H} 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\textsubscript{L} sequences. Analysis of the R/S ratios in the FR regions of IgV\textsubscript{H} according to Chang and Casali\textsuperscript{35} established that 3 of 8 PCMZLs were significantly below the ratio that would be expected in case of random mutation.

**IgV\textsubscript{H}-CDR3 repertoire.**

IgV\textsubscript{H}-CDR3 amino acid sequences from this study, but also those published by Bahler et al.\textsuperscript{27}, Roggero et al.\textsuperscript{26}, and three sequences obtained from our previous study\textsuperscript{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 V<sub>3</sub>-30/ J<sub>H</sub>4 rearrangement. The IgV<sub>H</sub>-CDR3 sequence of CM21 was homologous to those of 5 chronic lymphocytic

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

<table>
<thead>
<tr>
<th>Patient-number</th>
<th>Ig isotype (RT-PCR) rearrangement</th>
<th>No. of R/S mutations</th>
<th>R/S ratio FR</th>
<th>CDR3 sequence</th>
<th>CDR3-length</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM01</td>
<td>Cγ4</td>
<td>25</td>
<td>0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CAR ATYSSEQYDFDSSSYLDV WGKG</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Cα</td>
<td>33</td>
<td>2.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CAG VDYSDSGRHYFSASYFDV WGKG</td>
<td>19</td>
</tr>
<tr>
<td>CM03</td>
<td>Cγ1/Cα</td>
<td>20</td>
<td>2.6</td>
<td>CAG GGMVSTIPIDY WGKG</td>
<td>11</td>
</tr>
<tr>
<td>CM04</td>
<td>Cμ/CD</td>
<td>19</td>
<td>2.3</td>
<td>CAG DVIILMIFSFRSGDFS WGKG</td>
<td>18</td>
</tr>
<tr>
<td>CM06</td>
<td>Cγ/CC</td>
<td>nd</td>
<td>nd</td>
<td>CAR GKTAVGAPGYYFDY WGKG</td>
<td>15</td>
</tr>
<tr>
<td>CM07</td>
<td>Cγ/CC</td>
<td>nd</td>
<td>nd</td>
<td>CAR VRLDSPY(S/A)FAY WGKG</td>
<td>11</td>
</tr>
<tr>
<td>CM08</td>
<td>Cγ4</td>
<td>43</td>
<td>1.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CAS LRMISDRGFDC WGKG</td>
<td>11</td>
</tr>
<tr>
<td>CM10</td>
<td>Cγ</td>
<td>41</td>
<td>2.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CGR VLNPAHRPIGLAY WGKG</td>
<td>16</td>
</tr>
<tr>
<td>CM11</td>
<td>Cα</td>
<td>4</td>
<td>nd</td>
<td>CAR AVDFDSSSSSF WGKG</td>
<td>12</td>
</tr>
<tr>
<td>CM13</td>
<td>Cμ</td>
<td>12</td>
<td>1.3</td>
<td>CVR GYSIGWYDALAR WGKG</td>
<td>13</td>
</tr>
<tr>
<td>CM15</td>
<td>Cγ1/Cε</td>
<td>24</td>
<td>3.6</td>
<td>CAR ENPRHDVDI WGKG</td>
<td>10</td>
</tr>
<tr>
<td>CM19</td>
<td>Cγ</td>
<td>nd</td>
<td>nd</td>
<td>CAR ESGGAARMGRNYYYYMDV WGKG</td>
<td>20</td>
</tr>
<tr>
<td>CM20</td>
<td>Cγ</td>
<td>nd</td>
<td>nd</td>
<td>CAR HSAEADVEED WGKG</td>
<td>12</td>
</tr>
<tr>
<td>CM21</td>
<td>Cγ</td>
<td>nd</td>
<td>nd</td>
<td>CAR ETNYDSWTGPSHYYFDLWGKG</td>
<td>20</td>
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<tr>
<td>CM22</td>
<td>Cα</td>
<td>nd</td>
<td>nd</td>
<td>CAR GSGDYKTVKDYEDAFD WGKG</td>
<td>17</td>
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<tr>
<td>CM29</td>
<td>Cγ</td>
<td>nd</td>
<td>nd</td>
<td>CAR GVDYDFDLWGKG</td>
<td>9</td>
</tr>
<tr>
<td>CM34</td>
<td>Cγ</td>
<td>nd</td>
<td>nd</td>
<td>CAR DLLLWVGCLDY WGKG</td>
<td>12</td>
</tr>
<tr>
<td>CM35</td>
<td>Cγ</td>
<td>nd</td>
<td>nd</td>
<td>CAR GSHLIGGTIASFD WGKG</td>
<td>14</td>
</tr>
<tr>
<td>CM37</td>
<td>Cγ/CC</td>
<td>nd</td>
<td>nd</td>
<td>CAR LNYVLHLGRLIEGANTNGMDV LGQG</td>
<td>22</td>
</tr>
<tr>
<td>CM43</td>
<td>Cμ/CD</td>
<td>nd</td>
<td>nd</td>
<td>CAR APFLGVDFFDP WGKG</td>
<td>11</td>
</tr>
<tr>
<td>CM44</td>
<td>Cγ</td>
<td>38</td>
<td>1.4</td>
<td>CAR LQRRGLQGYLEY FGQG</td>
<td>13</td>
</tr>
</tbody>
</table>
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 ($V_{H1}-2$ 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 2 IgV L sequence analysis of cutaneous MZBCLs.

<table>
<thead>
<tr>
<th>Patient- IgV L-rearrangement (IMGT/V)</th>
<th>IgV L-rearrangement (Vbase)</th>
<th>No. of mutations</th>
<th>CDR3 sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM01 V3-15<em>01/J2</em>01</td>
<td>DPK21/humkv328h5</td>
<td>6</td>
<td>CQQ YNNWPPY TFGQG</td>
</tr>
<tr>
<td>CM03 V1-5<em>03/J1</em>01</td>
<td>L12a/PCRdil6-5+ of HK201</td>
<td>5</td>
<td>CQQ YGTYSW TFGQG</td>
</tr>
<tr>
<td>CM04 V3D-15<em>01/J2</em>01</td>
<td>L16/humkv31es, DPK21/humkv328h5+</td>
<td>14</td>
<td>CQQ YDTWPSY AFGQG</td>
</tr>
<tr>
<td>CM07 V1-5<em>01/J3</em>01</td>
<td>HK102/V1+</td>
<td>16</td>
<td>CQQ FNTFPSY TFGQG</td>
</tr>
<tr>
<td>CM08 V2D-28<em>01/J2</em>02</td>
<td>DPK15/A19.</td>
<td>20</td>
<td>CMO GLIQIPY TFGQG</td>
</tr>
<tr>
<td>CM11 V3-20<em>01/J1</em>01</td>
<td>DPK22/A27</td>
<td>28</td>
<td>CHQ YGRPG TFGQG</td>
</tr>
<tr>
<td>CM15 V1D-39<em>01/J5</em>01</td>
<td>DPK9/O12</td>
<td>10</td>
<td>CQQ SYSRPP TFGQG</td>
</tr>
</tbody>
</table>

### Table 3 Rheumatoid factor IgV H-CDR3 homology of cutaneous and other MZBCLs.

<table>
<thead>
<tr>
<th>N</th>
<th>RF homology a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastric MZBCL 97</td>
<td>11 (11%) b</td>
</tr>
<tr>
<td>Salivary gland MZBCL 32</td>
<td>13 (41%)</td>
</tr>
<tr>
<td>Pulmonary MZBCL 19</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Other extranodal MZBCL 4</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Splenic MZBCL 32</td>
<td>1 (3%)</td>
</tr>
<tr>
<td>Cutaneous MZBCL 33</td>
<td>1 (3%)</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
It has been established that virtually all extranodal and splenic MZBCLs express CXCR3 \cite{3-6,37}. 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-\(\gamma\), 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.

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.

**Tumor environment**

It has been established that virtually all extranodal and splenic MZBCLs express CXCR3 \cite{3-6,37}. 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-\(\gamma\), 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
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-\(\gamma\) (\(p=0.001\)) 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. This finding is in accordance with previous immunohistochemical studies, although we are the first to demonstrate IgE expression in PCMZL.

Five of the resolved PCMZL IgV\(H\) genes comprised the V\(H\)1 germline gene and six contained the V\(H\)3 germline gene. We previously reported the usage of V\(H\)1, V\(H\)3 and V\(H\)4 and Franco et al. reported V\(H\)2 and V\(H\)6 usage in PCMZLs. These results do not confirm an exclusive usage of V\(H\)3 family members in the Ig-rearrangements of PCMZLs, as was reported by Bahler et al. Analysis of the IgV\(H\) mutations showed that 3 out of 8 PCMZLs had R/S ratios significantly below those expected if mutation had been random. Including previously described cases, 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%).

A total of 33 IgV\(H\)-CDR3 sequences i.e. 21 obtained in this study, 3 from our previous study, 1 from Roggero et al. and the 8 cases published by Bahler et al., 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 IgV\(H\) repertoire bias, as was found in salivary gland and gastric MZBCLs; there was no IgV\(H\)-CDR3 homology between the PCMZLs. Fifteen IgV\(H\)-CDR3 amino acid sequences matched IgV\(H\) sequences from Genbank, including one case that was homologous to a rheumatoid factor. Previous findings reported by Bahler et al. 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.41,42 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.43-45 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.3-5,37,46 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 \textit{B. burgdorferi} infection, and Roggero et al.26 also reported a \textit{Borrelia}-associated PCMZL expressing IgM. Like \textit{H. pylori} in the gastric mucosa, \textit{B. burgdorferi} evokes a Th1 type of response, supportive for the extranodal MZBCL-like phenotype of these two \textit{Borrelia}-associated PCMZLs.47-49 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 (\textit{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 \textit{Borrelia}-associated PCMZLs, if available. In contrast, most of the PCMZLs differ from other extranodal MZBCLs, as they possess switched lgs, 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


