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Defined Blocks in Terminal Plasma Cell Differentiation of Common Variable Immunodeficiency Patients

Nadine Taubenheim,* Marcus von Hornung,* Anne Durandy,† Klaus Warnatz,‡ Lynn Corcoran,§ Hans-Hartmut Peter,‡ and Hermann Eibel2*

Common variable immunodeficiency (CVID)3 is the second most frequent primary immunodeficiency in humans with a prevalence of ~1 in 30,000 (1). The major hallmark of CVID is a significant reduction or absence of Abs in the serum of the patients leading to recurrent bacterial infections predominantly of the respiratory and gastrointestinal tracts. Furthermore, an increased incidence of granulomatous inflammation, autoimmune disorders, and gastrointestinal malignancies has been described (2). The syndrome can occur in both sporadic and familial forms and covers a heterogeneous group of disorders of which the underlying molecular bases are largely unknown. Recently, a homozygous deletion in the ICOS gene has been identified in a small group of CVID patients (3). However, in the majority of the patients, the characterization of genetic defects remains elusive. Several functional defects have been described affecting proliferation, activation, and cytokine production in T cells (4, 5). In B cells, early and late differentiation stages may be affected resulting in defective up-regulation of surface molecules such as CD86 and CD70 (6, 7), impaired signaling (8), and somatic hypermutation (SHM) (9), as well as defective formation of memory B cells (10–13).

Class switch recombination (CSR) as well as SHM of the V regions take place in germinal centers (GC) of secondary lymphoid organs and lead to the generation of Ab-secreting plasma cells and Ag-specific memory cells. The GC reaction is initiated by the activation of B cells, which up-regulate BCL-6 and migrate into B cell follicles where they undergo a phase of strong proliferation. At the same time, the specificity of the BCR is modified by SHM, allowing for affinity maturation due to positive selection of B cells for BCRs with the highest affinity.

BCL-6 is a zinc finger protein that acts as a transcriptional repressor. BCL-6 mRNA can be found in a variety of tissues (14), but on the protein level, the expression is mainly restricted to lymphocytes (14–16), with the highest expression in GC centroblasts and centrocytes. PRDM1, encoding the transcriptional repressor Blimp-1, represents a central target gene of BCL-6 (17). Blimp-1 was proposed to be a key regulator of terminal plasma cell differentiation because ectopic expression of the protein was sufficient to drive the differentiation of mature B cells to plasma cells (18).

The defective expression of proteins essential for the differentiation of B cells to GC B cells or plasma cells, such as BCL-6 or Blimp-1, could be responsible for the phenotypic features observed in CVID. In this report, we describe for the first time defects in the GC reaction and plasma cell development in lymph nodes (LN) from CVID patients. We revealed three different stages by which plasma cell differentiation takes place in the control LN and could show distinct blocks in this process in the LN from three CVID patients.

Materials and Methods

Patients

In this study patients were only included with established diagnosis of CVID (ESID criteria, (www.esid.org)). The patients were regularly followed in our outpatient clinic and received monthly i.v. or weekly s.c. replacement therapy, respectively. LN biopsies were from patients who developed during their follow-up regional mediastinal and/or LN swellings to exclude malignant lymphoma. None of the biopsies of the three CVID patients described here showed malignant lymphomas. Control tissues were obtained from patients undergoing LN dissection within the scope of lung cancer treatment.

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3 Abbreviations used in this paper: CVID, common variable immunodeficiency; CSR, class switch recombination; CT, circle transcript; AICDA, activation-induced cytidine deaminase; EGFP, enhanced GFP; GC, germinal center; GLT, germline transcript; HD, healthy donor; LN, lymph node; MFI, mean fluorescence intensity; SHM, somatic hypermutation.
Table I summarizes the clinical and immunological features of the patients. Informed consent was obtained from all patients and controls using biopsy material and peripheral blood for scientific purposes according to a local ethics committee approved research protocol (no. 239/1999).

Reagents

Primary Abs used for immunohistochemistry: anti-IgD-AP (Southern Biotechnology Associates), anti-Syndecan-1 (Serotec), anti-Ki67 (BD Pharmingen), anti-BCL2 and anti-CD23 (DakoCytomation), anti-BOB-1 and anti-CD38 FITC (Caltag Laboratories), anti-IgM-bio (Sigma-Aldrich), anti-CD19 PC5 (IO-Test), SA-allophycocyanin, anti-CD27 PE, anti-CD69 FITC and anti-CD86 PE (BD Pharmingen).

Cell preparation and stimulations

PBMC were isolated from EDTA-blood using Ficoll-Hypaque density gradient centrifugation (PAN Biotech) and cultivated in IMDM (Invitrogen Life Technologies) supplemented with 10% FCS (Linaris). Cells were stimulated with CD40L, using irradiated NIH 3T3 fibroblasts expressing human CD40L under the control of the human EF1α promoter. For BCR stimulation, 6 × 10^5 PBMC were cultivated in 48-well plates in the presence of 1.5 μg/ml anti-IgM-F(ab2) for 24 h. For CSR, 300 U/ml IL-4 (R&D Systems) was added and supernatants were analyzed after 13 days of cultivation by ELISA as described elsewhere (20).

Flow cytometry

PBMC and single cell suspensions of the LN were stained with fluorescence-conjugated Abs. Cells were acquired using a FACS Cavity analyzer, and data were analyzed using CellQuest software (BD Biosciences). Dead cells were excluded by gating on propidium iodide-negative cells and on viable lymphocytes according to their forward and side scatter.

Results

Defective B cell differentiation in GC from CVID patients

In immunohistochemical stainings of LN from CVID patients, we analyzed the expression of different GC and plasma cell markers to detect possible defects during late B cell differentiation.

Large granulomas were detected in the lymph node of one patient whereas in another patient a lymphoma had developed. In the lymph nodes of three patients, we found the GCs to be highly enlarged compared with those found in control tissues. Analysis of Ki67 illustrated that these GC consisted of a high number of proliferating cells, correlating well with high expression levels of the transcriptional repressor BCL-6. Therefore, B cell differentiation in the LN of these CVID patients seems not to be disturbed until the centrocyte stage. At later stages of B cell differentiation, Blimp-1 and Syndecan-1 are expressed in plasma cells and/or in a subset of GC B cells, which are committed to plasma cell differentiation (23). The expression of Blimp-1 and Syndecan-1 in control LN revealed three different subsets of plasma cells that could be further distinguished by their localization in the LN (Fig. 1): Blimp-1^- Syndecan-1^- cells represented a relatively small number of late GC B cells and were found only in the light zone, whereas Blimp-1^- Syndecan-1^- were found both in and around the GC (arrows in Fig. 1). Finally, Syndecan-1^- cells with low expression of Blimp-1 (Blimp-1low Syndecan-1high) were situated in large numbers only outside of GC. Because plasma cell differentiation requires exit from the cell cycle, all of these cells expressed only very low levels of Ki67.

The LN from all patients contained cells expressing Blimp-1 and low levels of Ki67. Expression of Syndecan-1 could be detected only in the LN from patient P3. All Blimp-1-positive cells in the tissues from the patients P1 and P2 were Blimp-1^- Syndecan-1^-, whereas the LN from patient P3 additionally contained Blimp-1^- Syndecan-1^- cells. Thus, Syndecan-1^- cells expressing low Blimp-1 levels (Blimp-1low Syndecan-1high), which were present in the control sections, could not be found in the LN from any of these three CVID patients (Fig. 1A).

Table I. Characteristics of CVID patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/Sex</th>
<th>Bryant et al. (Ref. 35)</th>
<th>Warrnatz et al. (Ref. 10)</th>
<th>Serum IgM (g/L)</th>
<th>Serum IgG (g/L)</th>
<th>% CD19</th>
<th>% SHM In Vivo</th>
<th>Mutations/bp</th>
<th>Clinic</th>
<th>Infections</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>38/F</td>
<td>B</td>
<td>Ia</td>
<td>&lt;0.2</td>
<td>3.2</td>
<td>4.2</td>
<td>NA^b</td>
<td>2.6-6.3</td>
<td>Splenomegaly, enlarged mediastinal LN</td>
<td>Chronic bronchitis, chronic sinusitis</td>
</tr>
<tr>
<td>P2</td>
<td>45/F</td>
<td>B</td>
<td>Ia</td>
<td>0.4</td>
<td>2.1</td>
<td>7.0</td>
<td>0.4</td>
<td>2.9</td>
<td>Splenomegaly, enlarged mediastinal LN, sarcoid-like lesions</td>
<td>Chronic bronchitis, chronic sinusitis</td>
</tr>
<tr>
<td>P3</td>
<td>29/F</td>
<td>B</td>
<td>Ia</td>
<td>0.3</td>
<td>4.0</td>
<td>13.7</td>
<td>2.9</td>
<td></td>
<td>Splenomegaly, enlarged mediastinal and abdominal LN, bronchiectasia</td>
<td>Chronic bronchitis, chronic sinusitis</td>
</tr>
</tbody>
</table>

^bPrior to monthly i.v. IgG substitution.

^b NA, not available.
Quantitative analysis of cell numbers within each Blimp-1\(^{-}\) subset was performed by counting cells expressing Blimp-1 and Syndecan-1 in multiple areas of equal size inside and around GCs. The mean number of Blimp-1\(^{-}\) Syndecan-1\(^{-}\) cells was increased in the GC of CVID patients (P1, 15.5 ± 3.6; P2, 9.0 ± 0.9; P3, 10.3 ± 4.2; control, 5.7 ± 0.6; Fig. 1B), whereas the number of Blimp-1\(^{-}\) Syndecan-1\(^{-}\) cells was lower in LN from P3 and absent in P1 and P2 (P3, 6.3 ± 1.2; control, 15.2 ± 5.1). Blimp-1\(^{low}\) Syndecan-1\(^{high}\) cells were only found in control LN (14.7 ± 3.2) at about equal numbers as Blimp-1\(^{-}\) Syndecan-1\(^{-}\) cells.

Defective formation of memory B cells in CVID patients

Flow cytometric characterization of the LN from the patients showed a significant reduction in the percentage of CD19\(^{+}\) CD27\(^{+}\) memory B cells (28.4 ± 4.1% of LN cells compared with a mean of 59.7 ± 10.9% of LN cells from control tissues; Fig. 1, C and D; p < 0.001).

Thus, besides a defective generation of Blimp-1\(^{-}\) Syndecan-1\(^{-}\) and/or Blimp-1\(^{low}\) Syndecan-1\(^{high}\) cells, impaired generation of memory B cells in the LN from all three CVID patients was found.

The defective development of memory B cells was also detectable in the peripheral blood of the patients (Fig. 1, E and F). Analysis of the IgM and CD27 expression on CD19\(^{+}\) B cells of the patients revealed a strong reduction in the percentage of IgM\(^{+}\) CD27\(^{+}\) B cells (mean value 9.0 ± 2.7% of the CD19\(^{+}\) cells (CVID) vs 18.2 ± 5.7% (healthy donor; HD); p = 0.004; Fig. 1E). The percentage of isotype-switched memory B cells (IgM\(^{+}\) CD27\(^{+}\)) was even more drastically reduced with a mean value of 1.0 ± 0.4% of the CD19\(^{+}\) B cells (CVID) vs 17.8 ± 5.6% (HD) (p < 0.001; Fig. 1E).

Altered responses of patients’ B cells to in vitro stimulation

We could previously show for a subgroup of CVID patients that BCR stimulation leads to an impaired up-regulation of CD86, but not of CD69 (7). To examine defects in the response to Ag encounter and to correlate those data with the immunohistochemical results, we stimulated PBMC from CVID patients P2 and P3 for 24 h with CD40L + anti-IgM and analyzed the surface expression of CD86 and CD69.

The B cells from patient P2 exhibited a lower CD86 expression after stimulation (Fig. 2A, mean fluorescence intensity (MFI) 94.1) whereas on B cells from patient P3 the expression was within the normal range (MFI 284.7; HD range MFI 195–370). The up-regulation of CD69 was found to be much higher on B cells from patient P3 (Fig. 2B; MFI 67) than on B cells from the controls (MFI 17.4–38) or from patient P2 (MFI 22). In terms of CD69 up-regulation, the cells of patient P3 therefore seemed to be more prone to stimulation than the cells of patient P2 or the controls.

After 13 days of stimulation of PBMC via CD40 and the IL-4 receptor, only 19% of the B cells from patient P2 expressed CD38, compared with 58% in patient P3 and 50.9 ± 15.2% in HD (Fig. 2C). The analysis of other activation markers, such as CD27, CD130, or CD180 revealed no difference between the stimulated cells of patients and controls (data not shown).

Generation of SHM

Because the generation of SHM is closely linked to the selection of B cells during memory and plasma B cell formation, we analyzed the frequency and the nucleotide substitution of SHM in CD19\(^{+}\) CD27\(^{+}\) B cells of the patients. As shown in Table I, the SHM frequency was found to be very low in B cells from patient P2, but in normal range (with a normal pattern) in patient P3.

CSR in vitro and in transfer experiments into immunodeficient mice

As a strongly impaired formation of class-switched Abs is a common feature of CVID, we analyzed CSR in vitro and in cell transfer experiments to elucidate possible defects.
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FIGURE 2. In vitro stimulation of PBMC. A and B, PBMC were stimulated for 24 h with CD40L + anti-IgM and expression of CD38 (A) and CD69 (B) was analyzed on the surface of stimulated B cells from patients P2 and P3 and HD, and one representative HD for each were determined. C, PBMC from patients P2 and P3 and one representative HD were stimulated with CD40L + IL-4 for 13 days. Percentages of CD38+ B cells are shown for each dot plot. All stimulation experiments were reproduced at least twice depending on the availability of patient samples.

We determined IgE levels in supernatants of PBMC stimulated with CD40L + IL-4 for 13 days. The PBMC from patient P3 produced normal amounts of IgE (3744 pg/ml), whereas the stimulated cells of patient P2 secreted diminished levels of IgE (994 pg/ml; Fig. 3A).

To further characterize CSR at a molecular level in cells from patient P2, PBMC were stimulated for 5 days with CD40L + IL-4 and expression of AICDA as well as IgE eps-GLT and IgE eps-CT were analyzed. We could not detect IgE CTs, indicating that CSR had not taken place. However, the first steps of CSR initiation were not impaired because AICDA transcript and eps-GLT expression were shown to be normal (Fig. 3B). The low but present amounts of IgE secreted by stimulated B cells from patient P2 may result from low numbers of memory B cells present in the peripheral blood (Fig. 1, E and F), suggesting that the patient might have had B cells able to undergo CSR but lost them over the time and during the time course of the disease.

As the cells of patient P3, but not of patient P2 showed a normal CSR after stimulation in vitro, we further examined the capacity of the cells from these patients to undergo CSR in transfer experiments using immunodeficient mice as recipients. Mixtures of purified peripheral CD4+ T cells and CD19+ B cells (6:1) from patients P2 and P3 and from HD were transferred into the spleens of Rag-2/common-γ-chain-deficient mice. At time points of 7, 14, and 21 days after engraftment, serum concentration of human IgM and IgG were determined. After 3 wk, mice were sacrificed, and spleens were analyzed immunohistochemically for human CD4+ T and CD19+ B cells. Consistent with the in vitro results, transferred cells from patient P3 produced normal amounts of IgG (557 μg/ml vs 503 μg/ml in the control), whereas cells from patient P2 failed to secrete IgG (3 μg/ml) (Fig. 3D). The serum levels of IgM were ~10-fold higher in the mice engrafted with the cells from patient P3 (107 μg/ml) than in control mice (11.2 μg/ml) or those with cells from patient P2 (4.6 μg/ml) (Fig. 3C). In the spleens of all mice, human CD4+ T cells were detected, reflecting a successful transfer in all cases and the presence of the T cells 4 wk after engraftment (data not shown). In contrast, human CD19+ B cells were detectable neither in mice engrafted with cells from HD nor from CVID patients. This might be due to the differentiation of the B cells into Ab-secreting CD19+ plasma cells, which remained undetected, or to a shorter half-life of CD19+ B cells.

The transfer experiments underscore the in vitro switch experiments confirming the inability of B cells from patient P2 to undergo CSR. Conversely, the cells of patient P3 were able to switch in both experiments.

Discussion

CVID is characterized by low concentrations or the lack of Abs. We were interested in analyzing whether any of the Ab deficiencies result from blocks in plasma cell differentiation. To this end, we investigated late B cell differentiation steps in LNs from a subgroup of CVID patients who underwent LN biopsies. Analyzing samples from five patients, we found that the LN from three CVID patients contained large hyperplastic GC consisting of highly proliferating, Ki67+ B lymphocytes. A major fraction of cells in these GC also expressed the transcription factor BCL-6, which is essential for the proliferation of GC B cells. BCL-6 inhibits genes involved in cell cycle control, B cell activation, and B cell differentiation, such as p27kip1, Id2, CD69, or Blimp-1, thereby maintaining the rapid proliferation of GC B cells whereas terminal differentiation into plasma cells is delayed (24). BCL-6 is essential for the GC reaction, because the formation of GC in LN and spleen was absent, and AB affinity maturation was impaired in BCL-6-deficient mice (25, 26). Both GC histology and expression of BCL-6 highly suggest that B cell differentiation is not disturbed up to the centroblast or centrocyte stage in the LN of these patients. This was further underscored by the detection of OCT-2 and BOB-1 expression (data not shown).

Blimp-1 is a master regulator of plasma cell formation as it is both sufficient and required for plasmacytic differentiation (18, 27). Genes repressed by Blimp-1 are associated with proliferation, BCR signaling, CSR or cell cycle, such as BCL-6, c-myc, btk, or
The authors describe plasma cells of the Blimp-1 might provide factors necessary for B cell differentiation in LN. This corroborates the B cell specificity of the defect in patient P2, whereas such as IFN-γ, IL-4, and IL-10 by T cells (data not shown) corroborates the B cell specificity of the defect in patient P2, whereas in P3 the defect seems to affect cells other than B or T cells, which might provide factors necessary for B cell differentiation in LN.

In mice, distinct subsets of plasma cells differentially expressing Blimp-1 and Syndecan-1 have also been reported by Kallies et al. (19). The authors describe plasma cells of the Blimp-1 Syndecan-1- high subset as a less differentiated population than cells expressing both factors (Blimp-1 Syndecan-1- high). At first glance, the model put forward by Kallies et al. suggesting a correlation between increasing expression of Blimp-1 and progressive maturation of plasma cells seems to be inconsistent with our results. However, it should be kept in mind that Kallies et al. used as a tracer for Blimp-1 expression the fluorescence of enhanced green fluorescent protein (EGFP) integrated by homologous recombination into the Blimp-1 encoding Pdrml gene. Because EGFP is a highly stable protein it tends to accumulate within cells resulting in increased light emission (30). Therefore, changes in gene expression are reliably detected only by destabilized EGFP mutants (31). It is therefore not unlikely that Blimp-1 and EGFP may differ in half-lives and turnover rates during plasma cell development, and increasing EGFP fluorescence intensities may not reflect proportionally increasing amounts of Blimp-1 protein. Thus, the EGFP-positive cells described by Kallies et al. may correspond to the Blimp-1 Syndecan-1 high plasma cells detected in the control LN sections. Further, it is also conceivable that the observed differences are due to species differences and/or might reflect the distinct tissues examined.

A previous report ascribed the appearance of Syndecan-1 but Blimp-1- B cells to technical problems arising when the plane of the section does not pass through the center of the nucleus, thereby impeding a detection of Blimp-1 (23). Based on the facts that such cells abundantly surround the GC of control LN and tonsils but completely lack in LN from patient P3, we are putting forward that Blimp-1 Syndecan-1 high B cells represent a discrete B lymphocyte subset rather than a technical artifact. Because Blimp-1 is known to be a transcription factor required for the transition from GC B cells to plasma cells, it is conceivable that Blimp-1 expression is only high at this point and that the Blimp-1 Syndecan-1 high subset represents plasma cells of a later stage, in which Blimp-1 is reduced to a minimum sufficient to maintain the plasma cell stage.

In conclusion, our results define three subsets of plasma cells, according to their differential Blimp-1 and Syndecan-1 expression, in control LN. In LN from the three CVID patients that we analyzed here, we were able to unravel distinct blocks during these stages of plasma cell differentiation. Our data point toward different B cell defects downstream of Blimp-1 that seem to represent essential factors in plasma cell development. Although different subgroups of CVID patients may suffer from other defects or mutations (3, 6, 7) defective plasma cell differentiation is a key finding in CVID and it will be of major interest to further investigate target genes downstream of Blimp-1.
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Disclosures

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