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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 Eibel*‡*

Common variable immunodeficiency (CVID) is a heterogeneous disorder characterized by defective Ab production and recurrent bacterial infections. The largely unknown causes are likely to comprise a diverse set of genetic or acquired defects. In this study, we investigated terminal B cell differentiation in lymph nodes from CVID patients. Up to the germinal center B cell stage, B cell differentiation was normal but terminal plasma cell development was found to be impaired. Using differential Blimp-1 and Syndecan-1 expression in controls, we defined three different plasma cell subsets that correspond to progressive developmental stages locating to different sites in the lymph node. In the CVID patients, we could only detect one or two of these subsets indicating a defective differentiation. Thus, terminal plasma cell differentiation was found to be impaired despite normal expression of Blimp-1. B cells reaching only the first stage of plasma cell differentiation were further unable to undergo isotype switching and to up-regulate activation markers on B cells stimulated in vitro.

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-BCL-6 and anti-CD23 (DakoCytomation), anti-BOB-1 and anti-OCT-2 (Santa Cruz Biotechnology) and anti-Blimp-1 (19). Abs and reagents used for flow cytometry: anti-CD19 allophycocyanin 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(ab)2 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). Expression of activation-induced cytidine deaminase (AICDA), IgE germline transcript (GLT), and SHM in the IgH V regions

Detection of SHM in the IgH V regions

Frequency and characteristics of SHMs in the variable region of the IgM H chain (VH region) was studied in purified CD19^+ CD27^+ B cells as described previously (9). PBMCs were labeled with anti-CD19 mAb and anti-CD27 mAb (Immunotech) and then purified by FACS sorting. The presence of 1.5 g/ml anti-IgM-F(ab)2 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). Expression of activation-induced cytidine deaminase (AICDA), IgE germline transcript (GLT), and circle transcript (CT) was examined as described in the report of Imai et al. (21).

Detection of SHM in vivo

Human T and B cells from PBMC were isolated with CD4-positive selection kit and anti-CD19 PanB magnetic beads, respectively (Dynal).

Purified cells were transferred into Rag-2/−/− common-γ-chain-deficient mice (originally described by Colucci et al. (22)). Within 24 h of their isolation, 1–3 × 10^6 cells in a 50-μl suspension were directly injected into the spleens of the mice (6:1 T/B cell ratio). Blood samples were taken from the tail vein and human IgM and IgG concentrations were measured in the serum by ELISA. Commercial coating Abs, alkaline phosphatase-conjugated detection Abs and standards for IgM and IgG (Jackson ImmunoResearch Laboratories) were used. The assays were developed with AP substrate p-nitrophenyl-phosphate (Sigma-Aldrich). After 3 wk, mice were sacrificed and spleens were taken for immunohistochemical analyses.

Immunohistochemistry

Methanol/acetone (1:1) fixed 10-μm cryosections of LN were treated with 0.1% phenylhydrazine in PBS for 30 min at room temperature to inactivate endogenous peroxidases. Immunohistochemical stainings were then performed according to the procedures specified in Elite Vectastain kits, using the Nova Red, Vector SG, and Vector AP substrates (Vector Laboratories).

Flow cytometry

PBMC and single cell suspensions of the LN were stained with fluorescence-conjugated Abs. Cells were acquired using a FACS Calibur 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 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 centocyte 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^− cells were found both in and around the GC (arrows in Fig. 1). Finally, Syndecan-1^+ cells with low expression of Blimp-1 (Blimp-1^low Syndecan-1^high) 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 therefore 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-1^low Syndecan-1^high), 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) (0.4-2.3)</th>
<th>Serum IgG (g/L) (7-16)</th>
<th>% CD19 (6-19%)</th>
<th>SHM In Vivo</th>
<th>% Mutations/bp (2.6-6.3)</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>Splenomegaly, enlarged mediastinal LN</td>
<td>Chronic bronchitis, chronic sinusitis</td>
<td></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>Splenomegaly, enlarged mediastinal LN, sarcoid-like lesions</td>
<td>Chronic bronchitis, chronic sinusitis</td>
<td></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>Splenomegaly, enlarged mediastinal and abdominal LN, bronchiectasis</td>
<td>Chronic bronchitis, chronic sinusitis</td>
<td></td>
</tr>
</tbody>
</table>

^aPrior to monthly i.v. IgG substitution.

^bNA, not available.
BCR stimulation leads to an impaired up-regulation of CD86, but we could previously show for a subgroup of CVID patients that altered responses of patients’ B cells to in vitro stimulation (Fig. 1).

IgM+ decan-1high cells were only found in control LN (14.7%, P1, P2, and 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-1lowSyndecan-1high 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-1lowSyndecan-1high 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 from patients P2 and P3. 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.
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 (374 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-g-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
AID (28, 29). The mutual inhibition of BCL-6 and Blimp-1 represents a double-negative feedback loop that ensures the exclusive expression of genes necessary at either the GC or plasma cell stage. In control LN, we could observe three different plasma cell subsets by analyzing their Blimp-1 and Syndecan-1 expression. According to these results, we propose a model in which late plasma cell differentiation can be divided into at least three steps defined by their Blimp-1 and Syndecan-1 expression (Fig. 4).

Plasma cell precursors up-regulating Blimp-1 further become Blimp-1/Syndecan-1 double-positive plasma cells and can differentiate into Blimp-1low/Syndecan-1high cells.

Blimp-1-expressing cells were observed in the LN from all three CVID patients. This finding was unexpected because the serum of these patients contains no Abs, suggesting a defect in the differentiation of plasma cells. However, only the LN from patient P3 contained Syndecan-1+ cells, although at lower numbers compared with the control sections; these cells all coexpressed Blimp-1. Thus, only subsets representing the first or second stages of our model of plasma cell differentiation were detected in the LN of these CVID patients. Further, the numbers of Blimp-1+ Syndecan-1− cells in the LN of the patients were higher than in the control LN, which is consistent with our hypothesis of a blocked plasma cell differentiation.

In addition to the arrested plasma cell differentiation, peripheral B cells from patient P2 also showed an impaired activation in terms of CD86 and CD38 up-regulation after in vitro stimulation via the BCR or CD40L + IL-4, respectively. Defective isotype switch was shown in vitro and by cell transfer experiments into Rag-2/common−/− mice, where the cells did not secrete IgG but normal levels of IgM. Further, analysis of SHM in CD19+CD27+ cells from the peripheral blood of patient P2 showed no mutated V regions. In contrast, the defect of patient P3 seems not to interfere with these processes because the cells revealed intact CSR both in vitro and in transfer experiments and exhibited no defective up-regulation of CD86 and CD38 and V regions in CD19+CD27+ cells were found to be mutated both with normal frequency and pattern.

The finding of normal proliferation and production of cytokines such as IFN-γ, IL-2, 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− subset as a less differentiated population than cells expressing both factors (Blimp-1+/Syndecan-1−). 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 Prdm1 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-1low/Syndecan-1high 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-1low/Syndecan-1high 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-1low/Syndecan-1high cells represent plasma cells of a later stage, in which Blimp-1 is reduced to a minimum sufficient to maintain the plasma cell stage.

Terminal plasma cell formation therefore may be impaired even though Blimp-1 is expressed. The blocked differentiation step varied between patients P1 and P2 vs patient P3 suggesting different underlying mechanisms. It will be of interest to examine the functional integrity of Blimp-1, especially seen in patients P1 and P2, where no Syndecan-1− cells were detectable. However, a severe defect of Blimp-1 is improbable because Blimp-1 also has important functions in cells other than plasma cells rendering a deficiency for Blimp-1 embryonic lethal (32). Besides Blimp-1, the expression of XBP-1 is also pivotal for terminal plasma cell differentiation (33). Because XBP-1 acts downstream of Blimp-1, it is conceivable, that the induction of this protein might be impaired in our patients. However, deletion of XBP-1 is embryonic lethal too, because it is an important factor in the unfolded protein response and is essential for the development of fetal hepatocytes (34). Keeping this in mind, it is unlikely that a genetic defect abrogating the function of XBP-1 accounts for the defective terminal plasma cell differentiation in our CVID patients.

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.

**FIGURE 4.** A proposed simple model of late plasma cell differentiation defined by Ki67, BCL-6, Blimp-1, and Syndecan-1 expression.
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Disclosures
The authors have no financial conflict of interest.

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