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Disturbed Peripheral B Lymphocyte Homeostasis in Systemic Lupus Erythematosus[1]

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In patients with active systemic lupus erythematosus (SLE), a marked B lymphocytopenia was identified that affected $CD19^+/CD27^-$ naive B cells more than $CD19^+/CD27^+$ memory B cells, leading to a relative predominance of CD27-expressing peripheral B cells. $CD27^{high}/CD38^+/CD19^{dim}$/surface $Ig^{low}/CD20^-/CD138^+$ plasma cells were found at high frequencies in active but not inactive SLE patients. Upon immunosuppressive therapy, $CD27^{high}$ plasma cells and naive $CD27^-$ B cells were markedly decreased in the peripheral blood. Mutational analysis of V gene rearrangements of individual B cells confirmed that $CD27^+$ B cells coexpressing IgD were memory B cells preferentially using V_H3 family members with multiple somatic mutations. $CD27^{high}$ plasma cells showed a similar degree of somatic hypermutation, but preferentially employed V_H4 family members. These results indicate that there are profound abnormalities in the various B cell compartments in SLE that respond differently to immunosuppressive therapy. *The Journal of Immunology,* **2000, 165: 5970–5979.**

Systemic lupus erythematosus (SLE)[4] is characterized by the production of multiple autoantibodies. Although the pathogenesis of SLE remains enigmatic, autoantibodies against dsDNA and ribonucleoproteins, deposition of immune complexes, complement activation, and leukocyte infiltration are thought to represent a consequence of immune dysregulation in this entity (1–5). Whereas B cell hyperreactivity and spontaneous Ig production by PBLs have been documented in SLE, distinct abnormalities of B cells have not been elucidated in detail. However, it is known that peripheral B cells from SLE patients contain populations that spontaneously produce Ig and also cells that can mature into Ab-secreting cells when cultured in vitro in the absence of obvious activators of B cell differentiation (6–11).

In vivo, Ag-specific activation and differentiation of B cells occur in germinal centers (12–16). Within germinal centers, naive B cells undergo activation, proliferation, somatic hypermutation of rearranged V region genes, Ig isotype switching, and subsequent positive and/or negative selection by Ag (13, 15, 17–19). Within germinal centers, activated B cells mature into Ab-producing plasma cells or, alternatively, become memory B cells. This developmental dichotomy of B cells is reflected by differential expression of a variety of B cell surface Ags, such as surface Ig, CD38, CD20, and CD138 (syndecan-1; see Refs. 14 and 20). In peripheral blood as well as in the bone marrow, memory B cells have been identified in populations of B cells expressing either class-switched Ig isotypes: IgM and IgD, or IgM only (21–25). More recently, $IgD^+/CD27^+$ B cells have been identified as having somatically mutated Ig genes and, therefore, being memory B cells (25). In normal persons, $IgM^+/IgD^+/CD27^-$ naive B cells represent about 60% of the peripheral blood B cell population (25, 26).

In this study, we demonstrate that the frequencies of CD27-expressing B cells were significantly enhanced as a result of a relative and absolute reduction of the total number of naive B cells and a less prominent reduction of memory B cells in the periphery of patients with SLE. A significant population of $CD27^{high}$ plasma cells was identified in the periphery of patients with active SLE. Upon immunosuppressive therapy, the $CD27^+$ B cell population in SLE patients remained stable, whereas the frequencies of naive B cells and $CD27^{high}$ plasma blasts decreased significantly.

Materials and Methods

Patients' material and preparation of samples

Heparinized whole blood (10–20 ml) from patients with various autoimmune diseases (Table I) were obtained from the Department of Rheumatic Diseases, University Hospitals Charite (Berlin, Germany). In detail, we analyzed 13 patients with SLE, fulfilling the criteria revised in 1982 (27), and a group of 9 patients with other autoimmune diseases (2 patients with primary Sjögren's syndrome, 2 patients with polymyositis, 2 patients with progressive systemic sclerosis, 1 patient with polymyalgia rheumatica, 1 patient with polychondritis, and 1 patient with acquired factor VIII resistance) (Table I). Six patients with SLE exhibited a flare at the time of analysis and subsequently underwent immunosuppressive therapy. Two patients had not been diagnosed before (patients 11 and 13), one patient discontinued taking prednisolone 3 wk before the analysis (patient 6), and three patients were taking <10 mg of prednisolone/day (patients 1, 5, and 10) at the time of disease flare. The remaining patients with SLE were being treated with azathioprine (100–150 mg daily) and/or methylprednisolone (12 mg daily) or prednisolone, respectively ($\leq$20 mg daily). As a control, fresh blood from 14 apparently normal healthy blood donors (NHS) were also analyzed. PBMC were prepared as reported previously (24).

Cytometric analysis

Immunofluorescence staining for flow cytometric analysis was performed by incubating PBMC with biotinylated anti-CD19 (SJ25-C1; Southern Biotechnology Associates, Birmingham, AL), anti-CD27 Cy5 (clone 2E4), and either anti-CD38 FITC (clone HIT-2; PharMingen, San Diego, CA.), anti-

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[4] Abbreviations used in this paper: SLE, systemic lupus erythematosus; NHS, normal healthy subject; s, surface.

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Table I. *Demographic data and peripheral B cells in patients and healthy donors, respectively*

Donor	Diagnosis	Sex[a]	Age (years)	WBC (Gpt/l)	Frequency of CD19$^+$ Cells (%)	Total No. of CD19$^+$ Cells (1×10^6 cells/l)
1	Active SLE	f	40	5.51	3.8	209
5	Active SLE	f	43	3.6	6.9	248.8
6	Active SLE	f	36	10.9	4.4	480
10	Active SLE	f	31	5.6	9.7	543.2
11	Active SLE	f	22	9.1	0.7	63.7
13	Active SLE	f	40	4.05	2.6	105.3
2	SLE	f	61	4.25	1.5	64
3	SLE	f	56	9.3	2.6	241.8
4	SLE	f	33	9.61	2.8	269.1
7	SLE	m	41	6.2	0.8	50.0
8	SLE	f	32	12.4	2.1	260.4
9	SLE	m	22	7.0	2.8	196.0
12	SLE	f	53	4.81	3.4	163.5
14	Sjögren's syndrome	f	72	7.2	2.0	144.0
15	Sjögren's syndrome	f	17	5.3	6.3	334
16	Myositis	m	49	9.6	2.4	230.4
17	Myositis	m	55	7.32	5.1	373.3
18	Scleroderma	f	59	10.1	33.3	3363.3
19	Scleroderma	m	55	8.51	6.2	527.6
20	Polymyalgia rheumatic	f	59	7.63	3.4	259.4
21	Polychondritis	f	26	5.64	11.3	637.3
22	Inhibitory hemophilia A	f	64	6.4	5.9	377.6
23	Healthy control	f	57	6.2	9.5	589
24	Healthy control	f	31	4.5	6.9	310.5
25	Healthy control	f	63	9.8	8.2	803.6
26	Healthy control	m	34	5.6	8.5	476
27	Healthy control	m	19	10.2	7.4	754.8
28	Healthy control	m	64	7.3	8.4	613.2
29	Healthy control	m	24	5.9	9.7	572.3
30	Healthy control	m	21	5.2	10.6	551.2
31	Healthy control	m	19	6.3	7.2	453.6
32	Healthy control	f	39	4.9	10.5	514.5
33	Healthy control	m	37	8.4	7.0	588
34	Healthy control	m	38	6.2	6.2	384.4
35	Healthy control	f	35	12.3	9.9	1217.7
36	Healthy control	m	37	6.82	15.1	1029.8

[a] f, Female; m, male.

HLA-DR FITC (clone R30), anti-CD95 FITC (clone CH-11; Immunotech, Marseille, France), anti-CD20 FITC (clone B-Ly1; Southern Biotechnology Associates), anti-human CD138 biotinylated (clone B-B4; Diaclone, Sunnyvale, CA.), anti-human Igκ FITC (G20–193; PharMingen) and λ light chain FITC (JDC-12; PharMingen), anti-human IgG FITC (rabbit anti-human IgG; Dako, Hamburg, Germany), anti-human IgM FITC (rabbit anti-human IgM; Dako), or anti-human IgD FITC (clone IA6-2, mouse anti-human IgD; PharMingen). Incubation with Abs was performed in PBS/0.5%BSA/5 mM EDTA at 4°C for 10 min. Propidium iodide (1 μg/ml; Sigma, Munich, Germany) was added immediately before cytometric analysis to exclude dead cells. Before incubation with streptavidin-PE (0.5 μg/ml; PharMingen), cells were washed twice. For intracellular staining, the cells were fixed in 2% (w/v) formaldehyde (Merck, Darmstadt, Germany) for 20 min at room temperature, washed, and stored at 6–8°C in PBS/0.5%BSA. The cells were then incubated in PBS/0.5%BSA, with or without 0.5% saponin (saponin buffer; Sigma), and fluoresceinated Ab for 10 min at 4°C and then washed in saponin buffer and PBS. For intracellular analyses, anti-IgE FITC (rabbit anti-human IgE; Dako) was used. In addition, anti-CD5 FITC

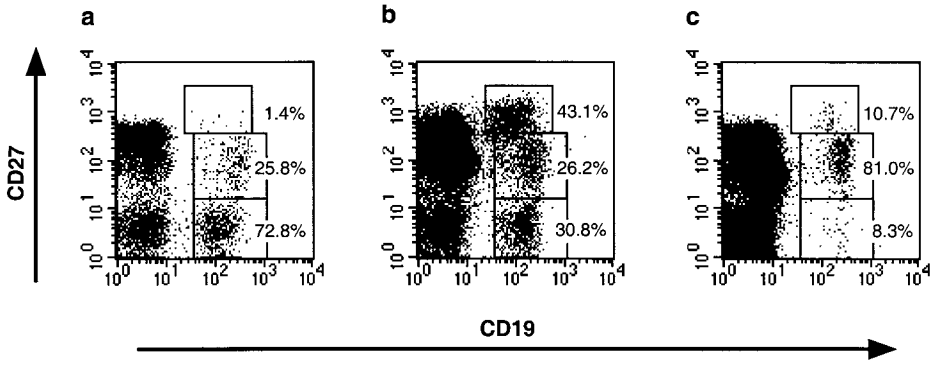

FIGURE 1. Expression of CD27 on CD19$^+$ peripheral B cells from patients with SLE and from a healthy donor. Viable PBMC were gated for analysis according to light scatter and exclusion of propidium iodide. Staining with CD19bio/streptavidin-PE vs CD27 Cy5 is shown for a healthy blood donor (donor 24, *a*) and two patients with SLE (donor 6, *b* and donor 7, *c*). Patient 7 was diagnosed in 1991 and had required dialysis since 1994. He received 5 mg/day prednisolone. Patient 6 was diagnosed in 1998, exhibited a flare, and was not receiving immunosuppression at the time of analysis. Fluorescence gates for the statistical evaluation of CD27$^-$, CD27$^+$, and CD27^{high} B cells are indicated, as well as the frequencies of these populations among B cells.

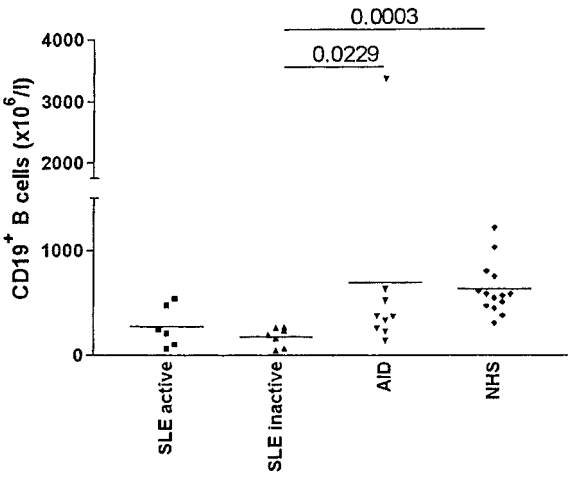

FIGURE 2. Absolute numbers of peripheral B cells were compared between SLE patients with active ($n = 6$) and inactive disease ($n = 7$) as well as in patients of the control groups (AID, patients with other autoimmune diseases ($n = 9$) and NHS ($n = 14$)).

(clone UCHT2, mouse anti-human CD5; PharMingen) was used to characterize the expression of this molecule and CD27. Flow cytometric analysis was performed using a FACSCalibur and CellQuest software (Becton Dickinson, San Jose, CA). Thirty thousand to 200,000 events were collected for each analysis.

Statistical analysis of the data was performed by using GraphPad Prism software (GraphPad, San Diego, CA). Frequencies of B cell populations were calculated using CellQuest software (Becton Dickinson) and differences between blood donor groups were compared using the nonparametric Mann-Whitney *U* test. To analyze the relationship between total white blood cell count and total B cells, the total numbers of B cells of various phenotypes were calculated per milliliter of blood, based on the frequencies of those cells among PBMC, and the total numbers of PBMC. p values <0.05 were considered as statistically significant.

Molecular analysis of V gene usage

For analysis of V_H gene rearrangements, $CD27^-/IgD^+$ cells, $CD27^+/IgD^+$ cells, and $CD27^{high}/IgD^-$ B cells were individually sorted into wells of a 96-well PCR plate into lysing solution (28) (Robbins Scientific, Sunnyvale, CA). For this analysis, PBMC from an untreated SLE patient (patient 10; butterfly rush, nephritis, hypocomplemenaemia, anti-dsDNA titer 1:8 using *Crithidia luciliae* immune fluorescence) were stained with biotinylated anti-CD19, streptavidin-PE, anti-CD27 Cy5, and anti-IgD FITC. Cells were sorted using a FACSVantage (Becton Dickinson).

Rearranged V_HDJ_H gene rearrangements employing specific V_H gene segments were amplified for all V_H families as described previously (28). The PCR error rate for this analysis has been shown to be 10^{-4}/bp (29). After column purification of PCR products (GenElute Agarose Spin Column; Supelco, Bellefonte, PA), they were directly sequenced using the Applied Biosystems Prism Dye Termination Cycle Sequencing kit (Perkin-Elmer, Foster City, CA) and analyzed with an automated Sequencer (Applied Biosystems Prism 377; Perkin-Elmer). Sequences were analyzed using the V BASE Sequence Directory to identify the respective germline V genes, using DNAPlot (University of Cologne/http://www.genetik.uni-koeln.de/dnaplot/) and Sequencher (Gene Codes, Ann Arbor, MI) software. The mutational frequencies of the productively rearranged V_H gene segments obtained from individual cells of the three B cell populations were analyzed with the χ^2 test.

Results

Enhanced frequencies of $CD27^+$ B cells in SLE patients exhibiting a marked peripheral B cell lymphopenia

PBMC of all 13 patients with SLE, 9 patients with other autoimmune diseases, and 14 NHS were analyzed for the expression of CD27 as a marker of memory B cells. Some of these were also analyzed for the expression of surface IgM (sIgM), sIgG, sIgD, CD38, CD138, CD20, CD95, and HLA-DR. The frequencies of $CD27^+/CD19^+$ cells were calculated according to statistical threshold sets in reference to control stainings, as shown in Fig. 1. Patients with inactive SLE treated with azathioprine and/or glucocorticoids had significantly lower total numbers of peripheral B cells regardless of their treatment regimen (Fig. 2) compared with both control groups. When the overall frequency of peripheral B cells expressing CD27 was examined, SLE patients with active and inactive disease had significantly higher frequencies of CD27-expressing B cells than both control groups (Fig. 3). The two control groups did not differ in the overall frequency of CD27-expressing B cells ($p = 0.4310$).

The increased frequency of $CD19^+/CD27^+$ peripheral B cells in patients with SLE results from a reduction of the $CD19^+/CD27^-$ naive peripheral B cell pool and a lesser decline in $CD19^+/CD27^+$ memory B cells

The increase in the frequency of $CD19^+/CD27^+$ peripheral B cells in SLE patients was not caused by an expansion of the $CD19^+/CD27^+$ subpopulation. Rather, it was a consequence of a significantly reduced total number of naive $CD19^+/CD27^-$ peripheral B cells of SLE patients with active ($85 \pm 54 \times 10^6$ cells/l) and inactive disease ($74 \pm 70 \times 10^6$ cells/l) compared with other patients ($546 \pm 941 \times 10^6$ cells/l, $p = 0.012$ and $p = 0.0052$, respectively) and normal controls ($418 \pm 204 \times 10^6$ cells/l, $p = 0.0006$ and $p = 0.0004$, respectively) (Fig. 4). Patients with active disease did not differ in their frequency of naive B cells compared with those without disease flares ($p = 0.7308$, Fig. 4). In contrast, the total number of $CD27^-$ B cells was not significantly lower in patients with other autoimmune diseases than in normal controls ($p = 0.0833$). As shown in Fig. 4, the reduction in these absolute numbers of naive B cells coincided with a reduced frequency of $CD27^-$ B cells in SLE patients compared with both control groups, whereas these frequencies did not differ between the two groups of SLE patients ($p = 0.9452$) or between the control groups ($p = 0.8749$).

Further analysis led to the discrimination of two distinct CD27-expressing B cell populations in peripheral blood: one CD27 at high levels ($CD27^{high}$) and one expressing CD27 less brightly ($CD27^+$). The frequency of $CD27^+$ B cells in patients with other autoimmune diseases did not exceed those found in NHS ($p = 0.5496$, Fig. 5). Moreover, patients with active lupus and those

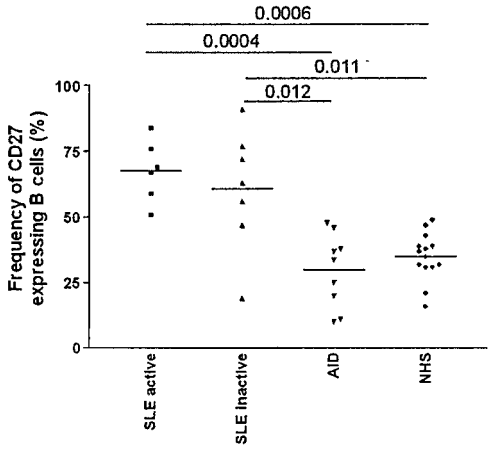

FIGURE 3. Comparison of the frequency of overall CD27-expressing peripheral B cells ($CD27^+$ plus $CD27^{high}$) from patients with active and inactive SLE as well as from individuals of the control groups. The frequencies were determined by cytometric analysis as shown in Fig. 1. The arithmetic statistical mean values are indicated for each group.

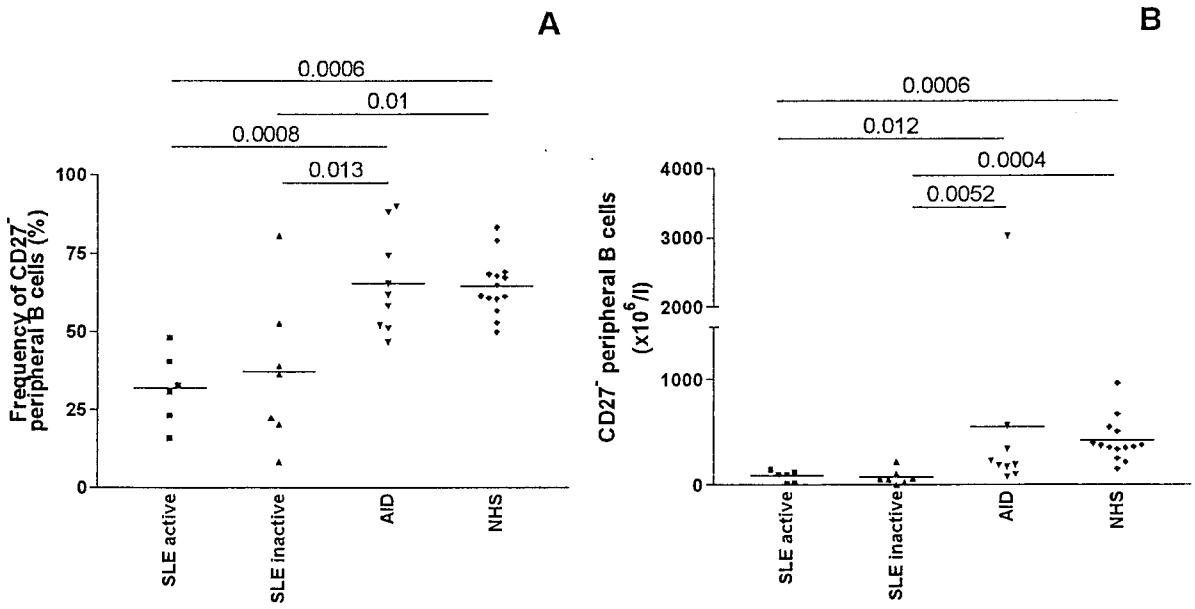

FIGURE 4. *A*, Frequency. *B*, Total number of CD27$^-$ naive B cells from patients with active and inactive SLE as well as from individuals of the control groups.

with inactive treated disease did not differ in regard to the frequency of CD27$^+$ peripheral B cells ($p = 0.2949$). In most patients with SLE (10/13), CD27$^+$ B cells comprised 39.2% or more of the peripheral B cells, with a mean frequency of 43 ± 17% in active and 55 ± 22% in inactive SLE patients. One female patient (donor 4) was exceptional in that only 15.9% of her B cells expressed CD27, a frequency in the range of NHS controls. Interestingly, this patient had delivered a healthy child 1 wk before the analysis and did not manifest any disease activity at that time. Her white blood cell count and levels of complement factors C3 and C4 were normal. Anti-dsDNA Abs were not detectable, although previously she had had a 1:8 titer of anti-dsDNA and 1:80 titer of anti-nuclear Abs (fine speckled pattern).

As opposed to SLE patients, CD27 was expressed on B cells from patients with other autoimmune diseases (mean, 29 ± 14%; highest value, 48.4%) at a similar frequency as found in NHS controls (mean, 34 ± 9%; highest value, 48.9%). Two patients with other autoimmune diseases exhibited frequencies of peripheral CD27$^+$ B cells higher than 39%, a similar frequency as that found in the patients with SLE. This included one patient with Sjögren's syndrome (44.7%, patient 15) and another one with acquired factor VIII resistance and hemophilia A (48.4%, patient 22). Careful analysis of the clinical characteristics of these two patients in comparison to the other patients with autoimmune diseases did not document any significant differences. Most notably, these patients lacked an enhanced frequency of CD27^{high} B cells in the periphery as detected in patients with SLE (see below).

Although the frequency of CD27$^+$ B cells among CD19$^+$ cells was significantly increased in inactive SLE patients compared with patients with other autoimmune diseases ($p < 0.017$) and NHS

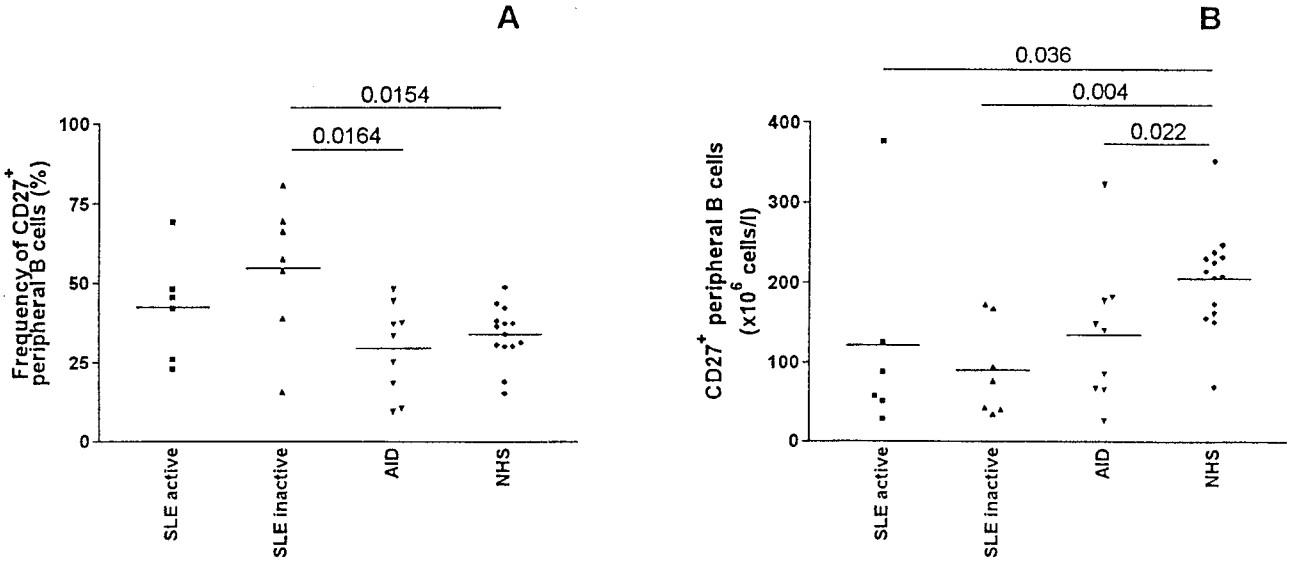

FIGURE 5. *A*, Frequency. *B*, Total number of CD27$^+$ peripheral memory B cells from patients with active and inactive SLE as well as from individuals of the control groups.

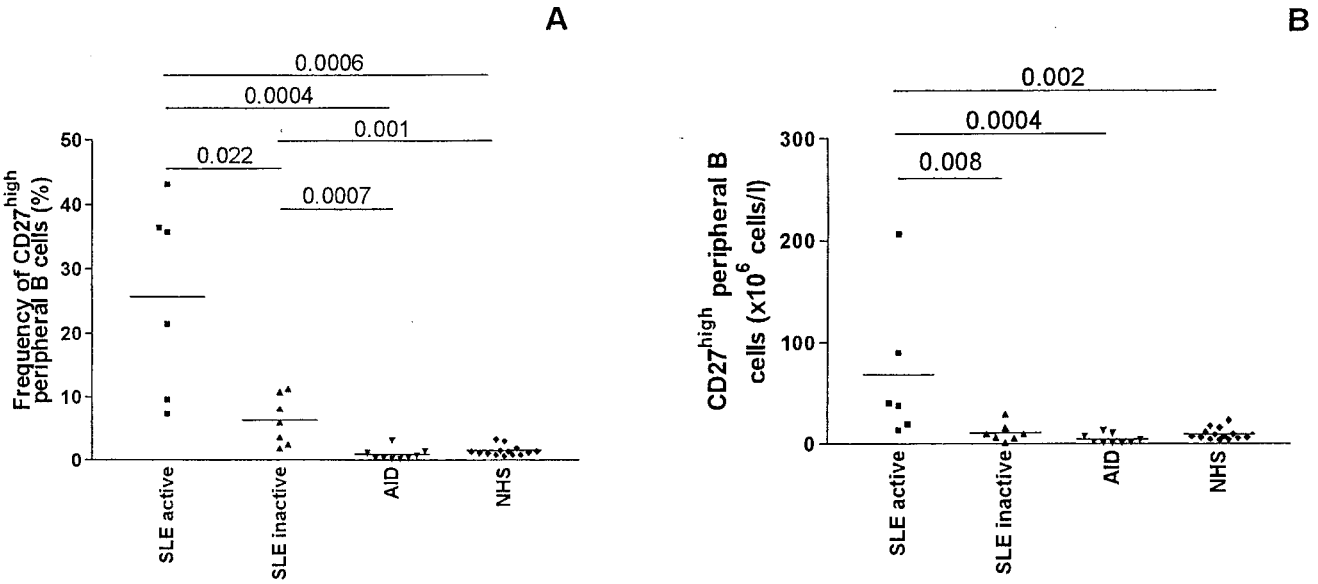

FIGURE 6. *A*, Frequency. *B*, Total number of $CD27^{high}$ peripheral plasma cells in patients with active and inactive SLE as well as from individuals of the control groups

($p < 0.016$, Fig. 5) only, the absolute number of these cells was significantly higher in NHS compared with the other groups analyzed (Fig. 5). Moreover, the absolute number of $CD27^+$ cells did not differ between any of the patient groups. Although the absolute number of $CD27^+$ B cells was diminished in all patient groups, the magnitude of the decrease noted in the SLE patients was markedly less than the decrease in the number of circulating $CD19^+/CD27^-$ naive B cells.

An increase in the frequency and the number of the peripheral $CD27^{high}/CD19^+$ B cell subpopulation is characteristic of active SLE

Patients with active and inactive SLE showed an increased frequency of peripheral B cells expressing high levels of CD27 ($CD27^{high}$, Figs. 1 and 6) in contrast to NHS ($p < 0.0006$ and $p < 0.001$, respectively, Fig. 6) and to controls with other autoimmune diseases ($p < 0.004$ and $p < 0.0007$, respectively). Among the SLE patients with active disease, the mean frequency of these cells was 26 ± 15%, ranging between 7.4 and 43.1% of peripheral B cells, significantly higher than in SLE patients with inactive disease (mean, 6 ± 4%; minimum, 1.9%; maximum, 11.2%; $p < 0.022$). The $CD27^{high}$ B cell subpopulation was uncommon in the blood of NHS (1.4 ± 0.8% of peripheral B cells). In patients with autoimmune diseases other than SLE, such cells were found at frequencies of 0.9 ± 0.9%. The frequencies of $CD27^{high}$ B cells in the seven SLE patients without disease activity were higher than those in patients with other autoimmune diseases ($p < 0.0007$) as well as in NHS ($p < 0.001$). However, the absolute numbers of $CD27^{high}$ B cells were only significantly increased in active SLE patients compared with inactive SLE patients ($p < 0.008$), patients with other autoimmune diseases ($p < 0.0004$), and NHS ($p < 0.002$). It should be pointed out that among the non-SLE autoimmune controls, one of the patients with Sjögren's syndrome was the only one with a significantly increased population of $CD27^{high}$ B cells (3.2%).

$CD19^+/CD27^{high}$ cells express CD38, CD95, HLA-DR, CD138, and intracellular Ig

As shown in Fig. 7, and representative for the SLE patients ana-

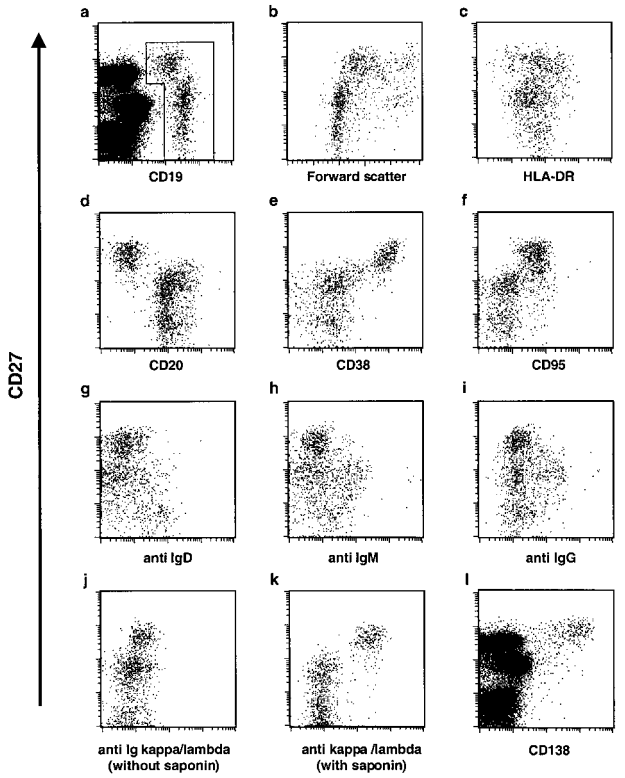

FIGURE 7. Cytometric characterization of peripheral $CD19^+$ cells in a patient with lupus flare. *a*, Viable peripheral mononuclear cells of a patient with a lupus flare (patient 13, Table I), gated according to scatter and propidium iodide exclusion, were stained for CD19-PE and CD27-Cy5. $CD19^+$ B cells were gated for further analysis as indicated. *b*, Staining of CD27 vs forward light scatter, as an indication of cell size. $CD19^+$ B cells, as gated in *a*, were counterstained for HLA-DR, CD20, CD38, CD95, IgD, IgM, IgG (*c–i*), and CD138 (*l*) and are plotted against CD27. Formaldehyde-fixed cells were gated according to scatter and CD19-PE staining and counterstained for CD27 and for Ig light chains with (*k*) or without (*j*) permeabilization of the cell membrane with saponin.

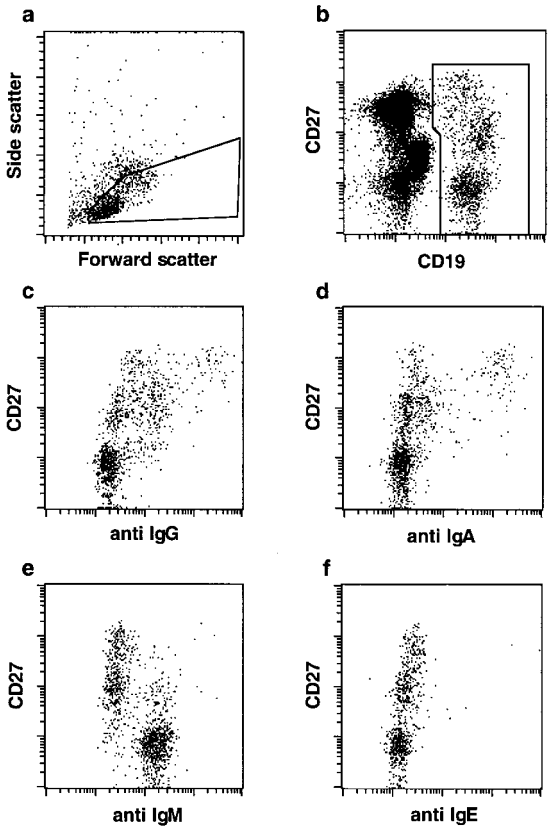

FIGURE 8. Intracellular stainings of B cells characterized according to scatter properties (*a*) and CD19 expression (*b*) for Ig classes demonstrated that CD27^{high} B cells (*b*) express large amounts of IgG (*c*) or IgA (*d*), whereas IgM (*e*) is rarely expressed and IgE (*f*) not at all.

lyzed, the CD27^{high} cells of patients with active disease showed a higher forward scatter than the other B cells (Fig. 7*b*), indicating that they were larger cells. Both CD27$^+$ and CD27^{high}, but not CD27$^-$, cells had distinct subpopulations of even larger cells, which might reflect distinct activation stages. This contention was supported by the HLA-DR staining, which also revealed a heterogeneity among CD27$^+$ and CD27^{high} cells indicative of recent activation of at least some cells. In addition, and unlike CD27$^+$ cells, all CD27^{high} cells expressed lower levels of CD19, high amounts of CD38 and CD138, and no CD20, markers of plasma cells (30). Of note, they also expressed high levels of CD95, expression of which had not previously been described for plasma cells, but rather on early plasma blasts (Fig. 7, *d–f, l;* see Refs. 30 and 31). Few, if any CD27^{high} cells expressed sIgM, IgD, IgG, or Ig light chains (Fig. 7, *g–i*), but all stained intracellularly for κ or λ light chains (Fig. 7*k*). As shown in Fig. 8, most CD27^{high} cells express either IgG or IgA in four SLE patients analyzed. Few IgM- and no IgE-expressing cells were detectable. In the patients analyzed, most CD27$^+$ and CD27^{high} B cells did not express CD5 (data not shown). In summary, the cytometric phenotype of CD27^{high} cells was indicative of plasma cells.

The expression of CD19 by CD27^{high}/CD138 coexpressing B cells was further analyzed in detail. As shown in Fig. 9 and representative for six individuals analyzed, B cells positive for CD138 almost exclusively express CD27^{high} (Fig. 9*a*) and the majority of them coexpress CD19 (Fig. 9*b*), whereas only two-thirds of the CD19^{dim}/CD27^{high} B cells do express CD138 (Fig. 9, *c* and *d*).

CD19$^+$/CD27^{high} and CD19$^+$/CD27$^-$ cells are decreased by immunosuppressive treatment of SLE patients

To determine whether the presence of CD27^{high} B cells in peripheral blood was related to disease activity and/or treatment, we performed a follow-up analysis on two of the patients who initially had a prominent population of these cells in the periphery at the time of flare symptoms and consequently had been treated with immunosuppressive therapy. As seen in Fig. 10, the administration of i.v. methylprednisolone, 1000-mg bolus for 2 days and 500 mg for the successive 3 days, led to a marked reduction of the peripheral CD27^{high} plasma cell subpopulation. Subsequently, the patient received i.v. cyclophosphamide (800 mg bolus) once. Afterward, the patient's condition improved and the CD27^{high} B cells had almost completely disappeared from the periphery and the number of naive B cells was reduced significantly, with CD27$^+$ memory B cells not detectably affected. The phenotype shown in Fig. 10*c* was characteristic of three SLE patients treated with immunosuppressive therapy and without apparent disease activity. Another SLE patient (Fig. 1*c*), treated with hemodialysis, still exhibited smoldering activity including lowered complement factors. This patient still showed an increased frequency of peripheral plasma cells (10.7%) but a low frequency of naive B cells (5.3%).

V_H gene usage and hypermutation in CD27$^+$ and CD27^{high} B cells in SLE

Individual IgD$^+$/CD27$^-$, CD27$^+$/IgD$^+$, and CD27^{high}/IgD$^-$ B cells were sorted by FACS using single-cell deposition. The mutational frequencies of V_H gene segments were determined for the three cell types (Table II). Among productively rearranged V_H genes, there was a significant difference in the mean frequency of mutations between the CD27$^-$ (mutational frequency, 0.4%) and the CD27-expressing B cell populations (mutational frequency, 6.46%, $p < 0.0001$), which is consistent with the classification of CD27$^+$ cells as memory B cells. CD27$^+$ (mutational frequency,

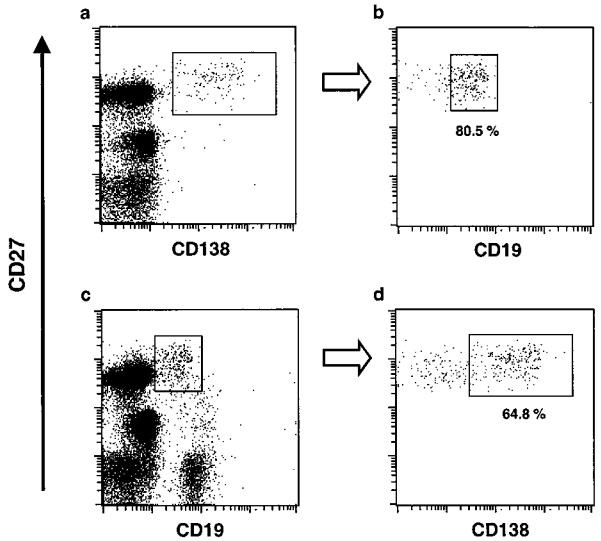

FIGURE 9. Cytometric analysis of coexpression of CD138 (syndecan-1) and CD19 by CD27^{high} cells. Cells were stained with CD19-fluorescein, CD138-streptavidin-PE, and CD27-Cy5 and gated according to forward scatter and exclusion of propidium iodide. Additional gates were set according to staining with CD19, CD27, and CD138. Identical gates were used in *a* and *d* and *c* and *b*, respectively. Almost all CD27^{high} B cells coexpress CD138 (*a*). More than 80% of CD27^{high} B cells also express CD19 (*b*). Gating of CD27^{high}/CD19^{dim} cells for CD138 showed that about two-thirds of these cells express CD138 (*c* and *d*).

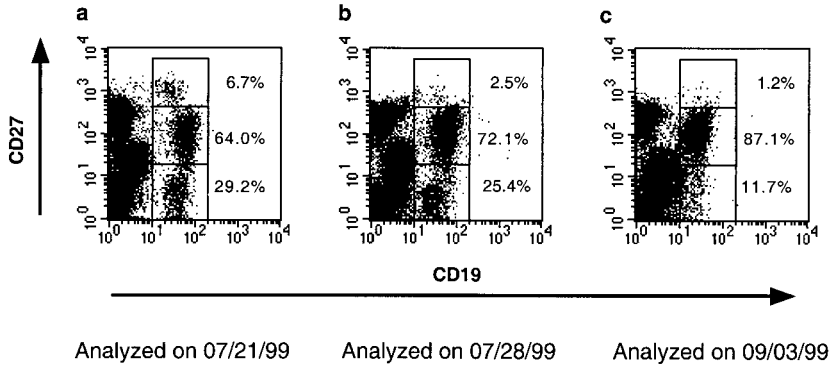

FIGURE 10. Follow-up analysis of peripheral B cells from a patient with SLE after treatment. Viable peripheral mononuclear cells from an SLE patient with a lupus flare at the time of first analysis (patient 10, Table I) were gated for cytometric analysis according to light scatter and exclusion of propidium iodide. Staining of CD19 vs CD27 is shown for peripheral blood obtained at time of flare (*a*), 1 wk later (*b*), and 5 wk later (*c*). At the time of flare, the patient was untreated and initially received i.v. methylprednisolone bolus (1g/day) for 2 days. Dosage was tapered for the succeeding 7 days (until 50 mg/day), with cytometric analysis on day 7 (*b*). Subsequently, the patient received i.v. cyclophosphamide bolus (800 mg). Approximately 5 wk later, the last cytometric analysis was performed (*c*). The populations of $CD27^-$, $CD27^+$, and $CD27^{high}$ cells were gated and their frequencies among $CD19^+$ B cells are indicated.

6.1%) and $CD27^{high}$ cells (mutational frequency, 6.9%) did not differ in their mutational frequencies ($p = 0.191$).

In the B cells of this patient, the ratio of productively rearranged V_H3 and V_H4 genes representing the most frequently used V_H families was remarkably different between $CD27^-$ and $CD27^+$ cells (Table II). Whereas V_H3 genes were preferentially used by B cells expressing IgD but not CD27 (13/14) and no $IgD^+/CD27^-$ B cell used V_H4 gene segments, the latter gene segments were used by some $IgD^+/CD27^+$ memory B cells (2/15) and >50% of $IgD^-/CD27^{high}$ plasma cells (8/15, if the genes used by all cells of a clone were considered as one). With regard to individual genes, the V_H3–23 gene was found most often in $CD27^-/IgD^+$ naive (6/14) and $CD27^+/IgD^+$ memory B cells (4/15), whereas V_H4–34 was amplified from 3 of 15 and V_H4–59 from 3 of 15 $CD27^{high}/IgD^-$ cells. The increase in mutational frequencies coincided with an increase in frequencies of B cells using V_H4 in the productive repertoire. Notably, V_H1 family members were found to be rearranged in all B cell subpopulations. However, productively rearranged V_H1 family members occurred in $CD27^+$ B cells (2/15) and $CD27^{high}$ plasma cells (1/15) only.

In addition, a clonally expanded population within the $CD27^{high}$ plasma cells employing $V_H4-61/D3-09/J_H4$ could be identified with an almost identical CDR3 of 66 bp and a common insertion of 6 inserted bp in CDR1 (Fig. 11). The three rearranged V_H4-61 genes of this clone carried 25 mutations (gene D12 IV V_H4 A1), 44 mutations (gene D12 IV V_H4 F1), and 54 mutations (gene D12 IV V_H4 H3).

Discussion

The current study provides evidence that in patients with SLE there is significant B lymphocytopenia associated with major disturbances in the homeostasis of all three major B cell types, naive and memory B cells and plasma cells. $CD27^+$ memory B cells were the predominant peripheral blood population in SLE, yet they were still present in significantly lower numbers in all patients when compared with normal subjects (active SLE, $122 \pm 130 \times 10^6$ cells/l; inactive SLE, $90 \pm 59 \times 10^6$ cells/l; other autoimmune diseases, $136 \pm 89 \times 10^6$ cells/l; NHS, $205 \pm 64 \times 10^6$ cells/l, all $p < 0.05$ compared with NHS). A distinct population of $CD19^+$ B cells ($CD27^{high}$) expressing CD27 highly, CD38, CD138, and intracellular but not sIg was identified as an expanded population in SLE patients with a lupus flare. It remains to be shown whether these abnormalities of B cell homeostasis are interdependent. Upon immunosuppressive therapy, both the populations of naive $CD27^-$ B cells and $CD27^{high}$ plasma cells were reduced in the SLE patients, whereas the $CD27^+$ memory cell population was apparently not affected.

Extensive work has been devoted to analysis of autoantibody-producing cells and perturbation of T lymphocyte homeostasis in patients with SLE (32; reviewed in Ref. 5). With regard to B lymphocytes, spontaneously activated B cells and polyclonal production of Ig, including autoantibodies, have been repeatedly demonstrated in the peripheral blood and in the bone marrow of SLE patients (reviewed in Refs. 5, 6, and 8–11). The current data are consistent with these findings, indicating that there are expanded numbers of phenotypically defined plasma cells in the blood of patients with active SLE. For the first time, we show here that these plasma cells express high levels of CD27.

CD27 belongs to the TNF receptor family and is expressed preferentially by T cells but also by B cells. CD27 signals after interaction with its ligand, CD70, which is expressed on T cells. CD27/CD70 signaling appears to act at late stages of B cell differentiation, providing a key signal for the maturation of memory B cells into Ig-secreting cells in the germinal center reaction (33–35). Expression of CD27 on B cells is apparently induced in the context of germinal center reactions and is maintained on memory B cells (25). $CD27^+$ B cells in human peripheral blood show extensive somatic hypermutation of their V genes, irrespective of the isotype they express, marking them as descendants of cells activated previously in vivo (25, 36). Here, we confirm this observation and extend it to peripheral $CD27^+/IgD^+$ B cells and $CD27^{high}$ B cells from a patient with a lupus flare.

Of the V_H gene rearrangements from the $CD27^+/IgD^+$ B cells analyzed, 14 of 15 showed mutation rates of 2–15% (overall mutational frequency, 6.1%). In comparison, 10 productively rearranged V_H segments from $CD27^-/IgD^+$ B cells showed an overall mutational frequency of only 0.4%. In peripheral B cells from normal subjects, Klein et al. (25) had observed similar frequencies for $CD27^-$ and for $CD27^+$ B cells. Eleven of 14 V_H regions obtained from $CD27^{high}$ plasma cells were highly mutated (3.4–10.5%; mean, 6.9%), In addition, three cells of a heavily mutated $CD27^{high}$ plasma cell clone expressed the V_H4-61 gene segment with 25–54 mutations. $CD27^+$ and $CD27^{high}$ cells both showed a high degree of somatic hypermutation, but they differed

Table II. *Analysis of productively rearranged V_H gene sequence obtained from individual peripheral B cells*

Gene	V_H Segment	Reading length (bp)	Mutations (*n*)	Sequence Homology[a] (%)
$CD27^-/IgD^+$				
D12 II VH3 E1	VH3-09/DP-31	222	4	98.2
D12 II VH3 G2	VH3-23/DP-47	223	1	99.6
D12 II VH3 E3	VH3-74/DP-53	226	0	100.0
D12 II VH3 F4	VH3-23/DP-47	225	1	99.6
D12 II VH3 G4	VH3-23/DP-47	222	0	100.0
D12 II VH3 D5	VH3-64/DP-61	226	2	99.1
D12 II VH3 B6	VH3-23/DP-47	227	1	99.6
D12 II VH3 A7	VH3-23/DP-47	222	0	100.0
D12 II VH3 C8	LSG 6.1	231	0	100.0
D12 II VH3 E8	VH3-23/DP-47	226	0	100.0
D12 II VH3 A1[b]	VH3-09/DP-31	224	0	100.0
D12 II VH3 C2[b]	VH3-33/DP-50	221	1	99.5
D12 II VH3 E12[b]	VH3-73	222	0	100.0
D12 II VH1 D9[b]	VH1-08/DP 15	219	0	100.0
$CD27^+/IgD^+$				
D12 III VH3 F1	VH3-23/DP-47	227	10	95.6
D12 III VH3 H2	VH3-33/DP-50	223	8	96.4
D12 III VH3 E4	VH3-64/DP-61	226	0	100.0
D12 III VH3 E6	VH3-21/DP-77	224	17	92.4
D12 III VH3 A7	VH3-09/DP-31	224	18	92.0
D12 III VH3 G7	VH3-23/DP-47	224	15	93.3
D12 III VH3 H7	VH3-33/DP-50	218	14	93.6
D12 III VH3 F8	VH3-07/DP-54	209	9	95.7
D12 III VH3 B11	VH3-23/DP-47	225	11	95.1
D12 III VH3 G11	VH3-23/DP-47	224	14	93.8
D12 III VH4 C7	VH4-30.4/DP-78	210	5	97.6
D12 III VH4 G7	VH4-59/DP-71	210	21	90.0
D12 III VH1 A3	VH 1-69/DP 10	218	32	85.3
D12 III VH1 B1	VH 1-46/Hg^{3+}	219	13	94.1
D12 III VH3 C8[b]	VH3-33/DP-50	222	10	95.5
$CD27^{high}/IgD^-$				
D12 IV VH3 E1	VH3-30/DP-49	227	23	89.9
D12 IV VH3 G1	VH3-13/DP-48	223	24	89.2
D12 IV VH3 H3	VH3-21/DP-77	226	2	99.1
D12 IV VH3 A6	VH3-23/DP-47	219	23	89.5
D12 IV VH3 E6	VH3-33/DP-50	209	0	100.0
D12 IV VH4 B1	VH4-59/DP-71	217	12	94.5
D12 IV VH4 G1	VH4-b/DP-67	213	22	89.7
D12 IV VH4 F2	VH4-34/DP-63	203	7	96.6
D12 IV VH4 H2	VH4-59/DP-71	191	11	94.2
D12 IV VH4 F3	VH4-34/DP-63	217	18	91.7
D12 IV VH4 G3	VH4-34/DP-63	202	13	93.6
D12 IV VH1 D2	VH 1-18/DP-14	222	23	89.7
D12 IV VH4 C2[b]	VH4-59/DP-71	180	15	91.7
D12 IV VH4 G2[b]	VH4-04/DP-70	141	11	92.2
D12 IV VH4 A1[c]	VH4-61	253	25	90.1
D12 IV VH4 F1[c]	VH4-61	253	45	82.3
D12 IV VH4 H3[c]	VH4-61	253	55	78.2

[a] Sequence homology and the underlying V_H segments were determined as described in *Materials and Methods.*
[b] Nonproductive V_H rearrangement.
[c] Clonally related V_H gene rearrangements.

in their V_H gene preference. Thirteen of 14 IgH loci of $CD27^-/IgD^+$ B cells and 11 of 15 IgH loci of $CD27^+/IgD^+$ cells used V_H3 genes in VDJ recombination. In $CD27^{high}$ B cells, however, only 5 of 15 IgH loci used V_H3 but 9 genes of the V_H4 family. In addition, the three clonally related cells used the V_H4-61 segment. Preferential usage of V_H4 genes by postswitch cells has been reported by other groups for patients with rheumatoid arthritis (37, 38), whereas V_H3 was most frequently found in naive B cells or in unfractionated peripheral B cells from normal subjects (24, 28, 39). Moreover, the gene V_H4-34 frequently used in the clonally unrelated $CD27^{high}$ cells analyzed here has been reported previously to be involved in the formation of anti-dsDNA Abs in SLE patients (40–42) and to be expanded in patients with disease activity (41). This V_H4 gene encodes cold agglutinins (43–45). It was also frequently used in immune responses of infectious mononucleosis (41, 46). In normal subjects, this particular gene occurred at frequencies of 3.5% among peripheral $CD5^+$ and 3.9% among $CD5^-$ B cells (24) or 3–10.8% among peripheral B cells (44, 45, 47, 48). The high frequency of V_H4-34 usage in peripheral $CD27^{high}$ B cells, the high frequency of such cells in untreated SLE patients, and their disappearance upon successful immunosuppressive treatment imply that $CD27^{high}$ plasma cells expressing this V_H gene rearrangement may be involved in the etiopathogenesis of SLE.

The identification of $CD27^{high}/CD19^+$ B cells as a prominent population of peripheral B cells in patients with active SLE represents a central finding of the present study. These cells express little if any surface, but increased intracellular, Ig compared with $CD27^+$ B cells. Apart from CD27, the $CD27^{high}$ cells express CD38, HLA-DR, and CD95, but not CD20 or CD5 and little

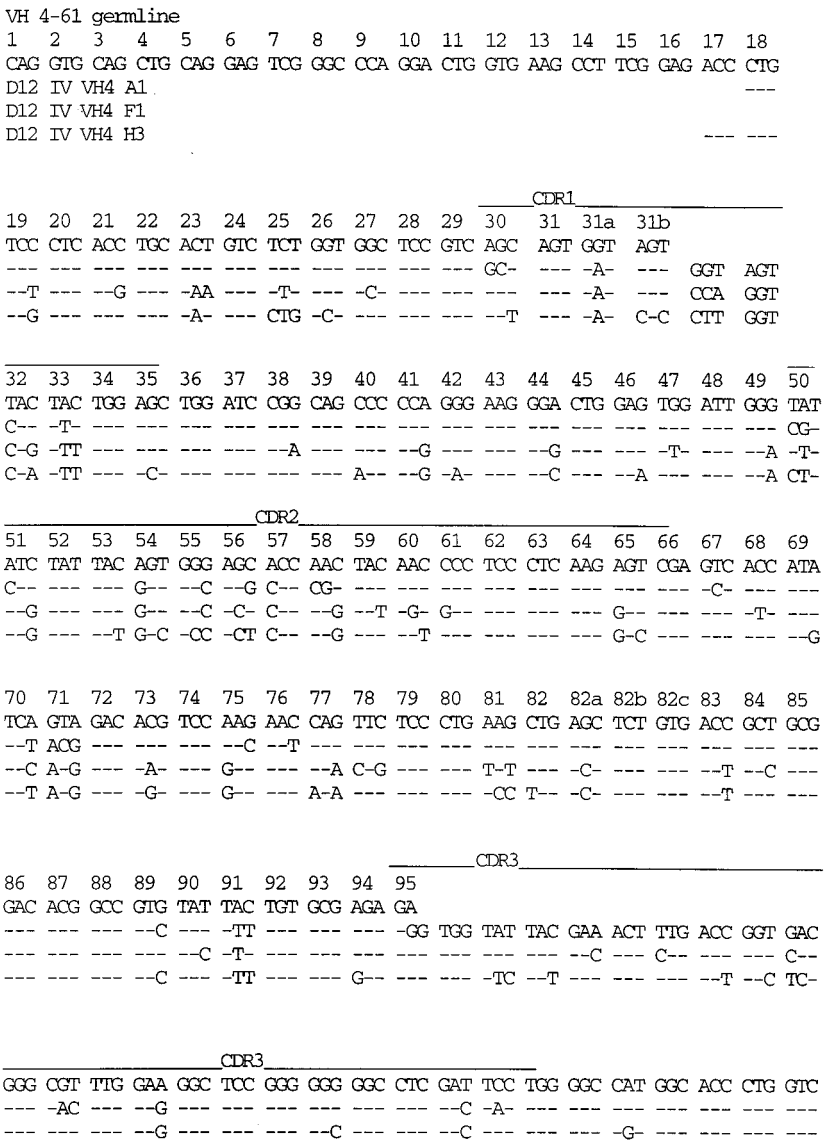

FIGURE 11. V_H gene sequences of the three clonally related B cells obtained from CD27^{high}-expressing peripheral plasma cells from a patient with a lupus flare. These V_H rearrangements shared an insertion of 6 bp in CDR1 and a CDR3 length of 66 bp as well as some common mutations.

CD19. All cells expressing intracellular Ig are CD19^{dim} (data not shown). The expression of CD19 on peripheral plasma cells has been shown before (49, 50) and contrasts with the apparent absence of CD19 on myeloma cells (50, 51). Only two-thirds of them also express CD138$^+$ (syndecan-1). As for CD5, it has been shown that both CD5$^+$ as well as CD5$^-$ B cells (52) obtained from SLE patients can produce anti-DNA Abs. The current data indicate that CD27$^+$ and CD27^{high} B cells are almost exclusively members of the CD5$^-$ B cell population, also in patients with an SLE flare. The expression of CD38, CD138, CD95, and intracellular Ig, down-modulation of CD20 and CD19, and hypermutated rearranged V_H genes identifies these cells as plasma cells (30, 53).

For CD27$^+$ memory B cells, Agematsu et al. (34, 35) have shown that these cells can be induced in vitro to differentiate into Ig-secreting plasma blasts upon stimulation with CD70, IL-2, and IL-10. CD38$^+$ peripheral cells have been shown by Lakew et al. (54) to secrete Ig in vitro spontaneously. Since expression of CD38 and CD27^{high} on peripheral B cells is perfectly correlated, it can be inferred that CD27^{high}/CD19^{dim} cells are Ab-secreting plasma cells.

In summary, the current study provides clear evidence that the expression of CD27 identifies marked disturbances of B cell homeostasis with respect to naive and memory B cells and plasma cells. Notably, a striking B lymphocytopenia and a marked reduction of CD27$^-$ B cells appear to be characteristic of SLE and not only the result of therapeutic interventions. In addition, active SLE is characterized by a marked increase in circulating plasma cells that is dramatically reduced by immunosuppressive therapy. The pool of CD27$^+$ peripheral B cells is less susceptible to immunosuppressive therapy in contrast to the pools of naive B cells and CD27^{high} plasma cells. These results have clear implications for diagnosis and therapy of SLE. Cytometric monitoring of the various B cell populations using CD27 in conjunction with CD19 may provide an important diagnostic parameter for monitoring disease activity in SLE patients.

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