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Original article

B-cell numbers and phenotype at clinical relapse following rituximab therapy differ in SLE patients according to anti-dsDNA antibody levels

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Abstract

Objectives. To correlate the kinetics of B-cell repopulation with relapse after B-cell depletion therapy in SLE patients and address whether variation in relapse rate, B-cell numbers and phenotype are related to anti-dsDNA antibody levels.

Methods. Sixty-one patients with refractory SLE were treated with a standard rituximab regimen. Clinical and serological measures of disease activity and B-cell numbers were assessed. B-cell phenotype was examined in a subgroup of patients by flow cytometry.

Results. Disease relapse was substantially delayed beyond B-cell repopulation, and early relapse was associated with a faster rate of repopulation. At relapse, B-cell numbers were significantly lower than at baseline in patients with high anti-dsDNA antibody levels (>100 IU/ml) but not in patients with low anti-dsDNA antibody levels. Of the patients with high anti-dsDNA antibodies at baseline, levels fell significantly only in those patients who remained in remission after repopulation. Relapse with high anti-dsDNA antibody levels was associated with an increased percentage of $IgD^{-}CD27^{hi}$ plasmablasts, whereas relapse with low anti-dsDNA antibody levels was accompanied by an increased percentage of $IgD^{-}CD27^{-}$ B cells.

Conclusion. Anti-dsDNA antibody levels distinguished two patient groups, which differ in their B-cell number and phenotype at relapse following rituximab, and suggest that different B-cell pathologies exist in SLE. The data imply that B-cell numbers should be kept very low for a sustained period in patients with high dsDNA binding, therefore justifying a more aggressive regimen.

Key words: systemic lupus erythematosus, CD20 antibody, rituximab, anti-DNA antibodies.

CLINICAL SCIENCE

Introduction

SLE is an autoimmune rheumatic disease with heterogeneous clinical manifestations typically characterized by B-cell activation and autoantibodies that target nuclear antigens [1]. In addition to the multiple abnormalities in B cells found in patients with SLE and animal models of the disease, the importance of B cells in this disease has been reinforced by many reports describing clinical and serological improvements in patients with SLE that have been treated with the B-cell-depleting agent rituximab [2–4]. In our cohort, >80% of patients with SLE refractory to conventional therapy responded to their first cycle of rituximab [2]. Surprisingly, randomized control trials have failed to confirm the efficacy of rituximab in SLE [5].

The heterogeneous nature of SLE suggests that the pathogenesis varies between individual patients, which could modify the response to rituximab. The identification and utilization of biomarkers, which may reflect alternate disease mechanisms, could identify which patients are more likely to respond as well as aid in the design of more effective therapies. Anti-dsDNA antibodies are recognized as highly specific diagnostic markers for SLE and human monoclonal anti-dsDNA antibodies have been shown to be pathogenic in recipient immunodeficient

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mice [6]. Anti-dsDNA antibodies are routinely measured to monitor disease activity in SLE, and increases in their titre have been used as a guide to treat lupus patients with conventional therapy before flares are clinically apparent [7, 8]. Moreover, a decrease in anti-dsDNA antibody titres has been associated with a clinical response to rituximab [2, 9]. Approximately 30% of patients with lupus do not have raised levels of anti-dsDNA antibodies, and whether these patients respond differently to rituximab remains unclear.

B-cell homeostasis is significantly disturbed in patients with SLE, which includes an increased population of plasmablasts and double negative ($IgD^{-}CD27^{-}$) B cells [10]. B-cell depletion leads to a profound reduction in all these subsets, with long-term responders appearing to have a relatively immature B-cell compartment following B-cell repopulation [11]. In general, rituximab tends to restore B-cell homeostasis in lupus, although there is considerable variation between individual patients [12]. Indeed, the kinetics of B-cell repopulation in individual lupus patients receiving rituximab and its relationship with disease relapse has not been fully elucidated. We investigated whether these factors could be integrated to understand divergent treatment responses and relate these findings to the timing of disease relapse following rituximab.

Patients and methods

Patients with SLE (all of whom met the revised classification criteria for the disease [13]) were treated on the basis that they had failed to respond to standard immunosuppressive therapy [prednisolone and at least one of the following, percentage of patients in brackets: AZA (70%), CYC (42%) and mycophenolate (26%)]. All had active disease as defined by the classic BILAG index, scoring at least one A or two Bs in one of eight organ-based systems [14]. The treatment regimen included two infusions of i.v. rituximab (1000 mg) 14 days apart with i.v. methylprednisolone (100–250 mg) and i.v. CYC (750 mg), in all but two patients, the day after the first rituximab infusion.

Clinical assessment including disease relapse was determined by an increase in the clinical indices of active disease, based on the classic BILAG index [14]. Patients attended on average every 2 months. Disease activity was graded in eight organ-based systems from an A grade (highest disease activity) to E, the lowest. Patients were deemed to have relapsed if they had one new A grade or two new B grades after rituximab therapy. Anti-dsDNA antibody levels were measured by ELISA (Shield Diagnostics Dundee, UK) (normal <50 U/ml). This study was approved by the University College London Hospitals (UCLH) Ethics Committee and all patients gave written informed consent according to the Declaration of Helsinki. Patient numbers are indicated for each part of the study.

B-cell quantification

B cells were counted in the haematology laboratory of UCLH from 4 ml of blood collected in tubes containing EDTA. PBMCs were extracted and labelled with CD19 antibodies. The $CD19^{+}$ B cells were then measured on a flow cytometer.

Cell separation and flow cytometry

For a subset of patients, PBMCs were extracted using lymphoprep and stained using antibodies for the markers CD19 (APC/PE-Cy7, eBioscience), IgD (FITC, BD Biosciences) and CD27 (PE, BD Biosciences). The levels of expression of these markers were recorded by FACS. The data were analysed using FlowJo software.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 4 Version 4.0c software. Wilcoxon signed-rank test was used to compare paired data before and after rituximab therapy. Mann–Whitney test was used to compare unpaired data between groups. Kaplan–Meier curves were generated to compare time to B-cell repopulation. Log rank test was used to examine differences between the curves. Analysis of correlation was done using linear regression. Fisher's exact test was used to determine the association of renal disease and raised anti-dsDNA antibody levels.

Results

Patients—summary of baseline characteristics

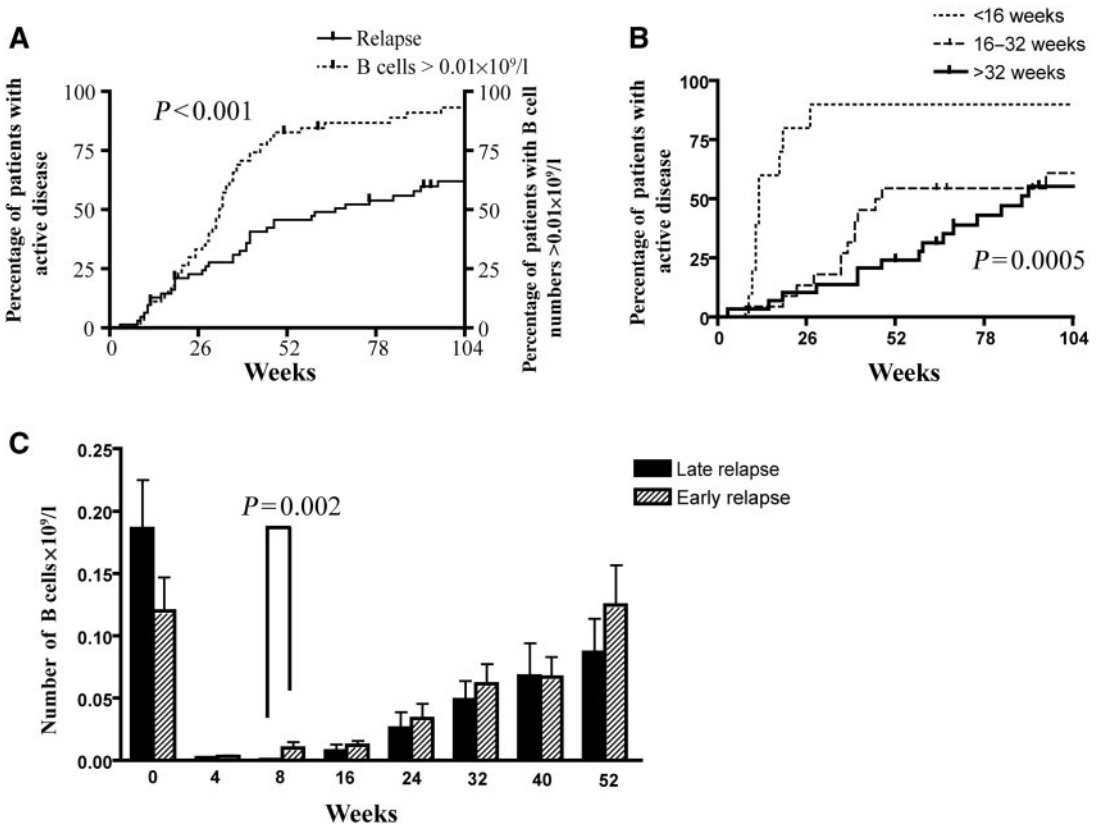
The median age at diagnosis of the patients studied was 23 years (range 10–66 years) and at treatment was 33 years (range 17–73 years). Disease duration prior to B-cell depletion therapy (BCDT) ranged from 0 to 28 years (median 6.5 years). Of the patients treated, 56 were females and 5 were males. Supplementary Table 1 (available as supplementary data at *Rheumatology* Online) describes the active organ involvement at time of treatment, indication for treatment, age and duration of disease at BCDT, and ethnic origin of the patients. There was a significant association between renal disease and elevated anti-dsDNA antibodies (>100 IU/l) in our patient cohort; 20 of 26 patients with high anti-dsDNA antibodies had nephritis compared with 11 of 35 patients who had low or normal anti-dsDNA antibody levels ($P < 0.0007$).

A more rapid rate of B-cell repopulation in patients who relapse earlier

The median B-cell number before treatment was 0.122×10^9/l (range $0.010–0.679 \times 10^9$/l). Following rituximab therapy all patients achieved B-cell depletion, defined as a B-cell count of $<0.01 \times 10^9$/l. The median time to repopulation (defined as a B-cell count of $>0.01 \times 10^9$ B cells/l) following rituximab therapy was 32 weeks and to clinical relapse (defined as a new BILAG A score or two new Bs) was 66 weeks. The rate of repopulation was greater than the rate of relapse ($P < 0.001$) (Fig. 1A).

To examine whether early repopulation was associated with early relapse, patients were divided into three groups according to whether they repopulated before 16 weeks ($n = 10$), between weeks 16 and 32 ($n = 22$), or after

FIG. 1 The rate of B-cell repopulation compared with the time to clinical relapse in patients with SLE following treatment with rituximab therapy. (**A**) Kaplan–Meier curves comparing the time to B-cell repopulation ($>0.01 \times 10^9$ B cells/l) with the time to clinical relapse following treatment with rituximab therapy ($n = 61$). (**B**) Comparison of the clinical relapse rates in patients who repopulate before 16 weeks ($n = 10$), between weeks 16 and 32 ($n = 22$), and after 32 weeks ($n = 29$). Analysis was carried out using the log rank test. (**C**) B-cell numbers are shown from baseline to week 52 between patients who relapse before 18 months (early, $n = 37$) after treatment and patients who relapse (or remain in remission) after 18 months (late, $n = 24$). Columns indicate the mean B-cell number and bars indicate the S.E.M. Differences between groups were analysed by the Mann–Whitney rank-sum test.



32 weeks ($n = 29$). A Kaplan–Meier analysis showed that early repopulation was associated with early relapse ($P < 0.001$) (Fig. 1B). Furthermore, 39 and 45% of patients had inactive disease for >2 years in the group of patients that repopulated between 16 and 32 weeks, and after 32 weeks, respectively, compared with only 10% of patients who repopulated before 16 weeks.

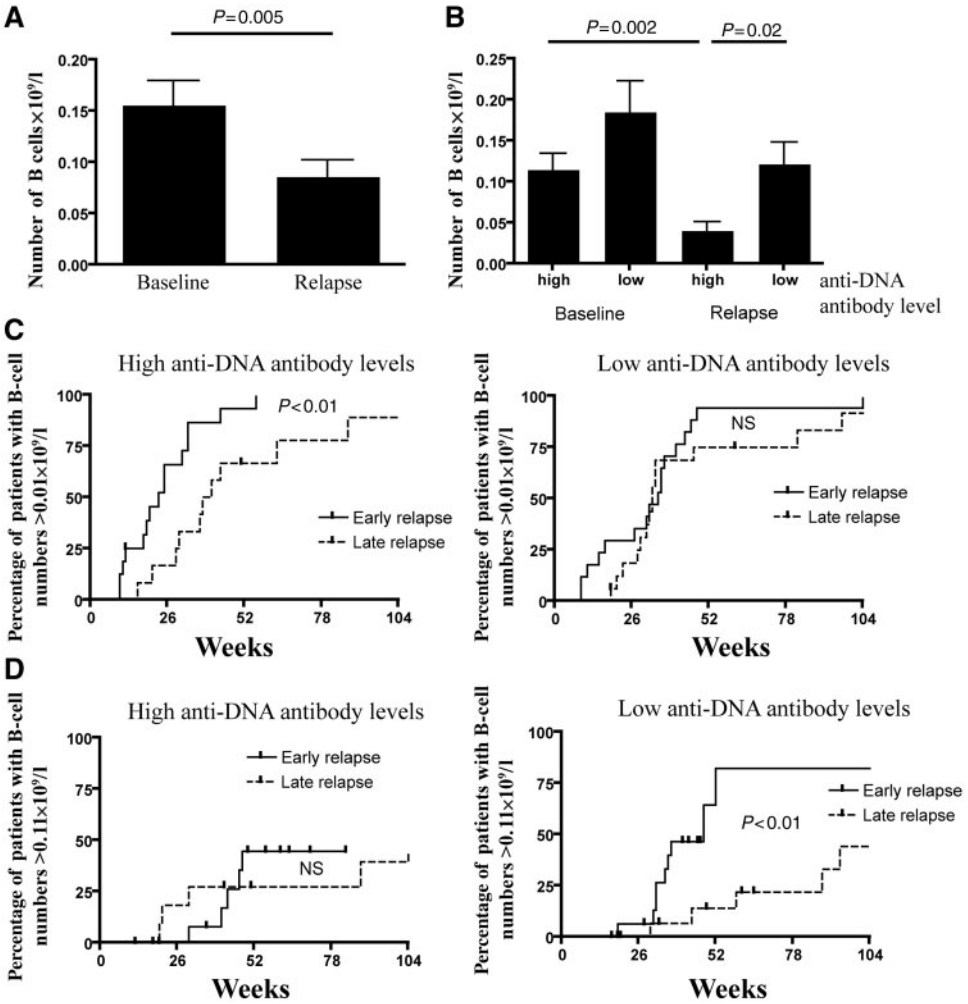
Higher B-cell numbers can be seen as early as 8 weeks following rituximab therapy ($P = 0.002$) in those patients who relapsed early (defined as occurring before 18 months, $n = 37$) compared with patients who relapsed late (>18 months, or remain in remission, $n = 24$) (Fig. 1C).

B-cell numbers at relapse differ according to anti-dsDNA antibody levels

B-cell numbers at relapse were lower than the B-cell numbers at baseline ($P = 0.005$, Fig. 2A). Two distinct patient groups were identified based on anti-dsDNA antibody levels; one with high levels (>100 IU/l, $n = 17$) where relapse occurred with low B-cell numbers and a second with low or normal levels (<100 IU/l, $n = 20$) where relapse occurred with normal or high B-cell numbers (Fig. 2B). Thus patients with levels of anti-dsDNA antibodies >100 IU/l relapsed with lower B-cell numbers than patients with low or normal levels of anti-dsDNA antibodies ($P = 0.02$). In addition, B-cell numbers at relapse were significantly lower than at baseline in patients with high anti-dsDNA antibodies ($P = 0.002$) but not in patients with low anti-dsDNA antibody levels. However, patients with high levels of anti-dsDNA antibody levels at baseline do not relapse earlier even though they relapse with fewer B cells. There was no correlation between B-cell numbers and C3 levels (unpublished data).

We next analysed whether anti-dsDNA antibody levels modified the association between earlier disease relapse and a faster rate of repopulation as shown in Fig. 1. Disease relapse before 18 months (early) was significantly associated with earlier repopulation in patients with high

FIG. 2 B-cell numbers in rituximab-treated patients who relapse with high anti-dsDNA antibody levels compared with those that relapse with low anti-dsDNA antibody levels. (**A**) B-cell numbers at baseline and clinical relapse ($n = 37$). (**B**) B-cell numbers at baseline and relapse divided according to high (>100 IU/l, $n = 17$) and low ($n = 20$) anti-dsDNA antibody levels measured at relapse. (**C**) Kaplan–Meier curves comparing time to B-cell repopulation $>0.01 \times 10^9$ B cells/l and (**D**) $>0.11 \times 10^9$ B cells/l in patients with high or low levels of anti-dsDNA antibodies at baseline who relapse either before or after 18 months. Comparisons of the curves were carried out using the log rank test. NS = $P > 0.05$.



($P < 0.01$) but not low or normal levels of anti-dsDNA antibodies (Fig. 2C). However, repopulation to higher numbers of B cells (0.11×10^9 B cells/l) did not occur any more rapidly in patients with high anti-dsDNA antibody levels in earlier relapsers (Fig. 2D). In contrast, patients with low or normal anti-dsDNA antibody levels who relapsed before 18 months attained B-cell numbers $>0.11 \times 10^9$ B cells/l more rapidly than patients who relapsed after 18 months with low anti-dsDNA antibody levels ($P = 0.01$) (Fig. 2D).

The difference in the median time to repopulation ($>0.01 \times 10^9$ B cells/l) between those patients with high anti-dsDNA antibody levels who relapsed early and those who relapsed later or remained in remission was 13 weeks (25 weeks in the early relapse group, 38 weeks in the late relapse/remained in remission group). Conversely, in patients with low levels of anti-dsDNA antibodies, early relapse was not associated with more rapid repopulation to $>0.01 \times 10^9$ B cells/l. However, this latter group attained higher B-cell numbers ($>0.11 \times 10^9$ B cells/l) earlier than patients who relapsed late or remained in remission. The difference in the median time to repopulation to this higher B-cell level between early and late relapse/remained in remission in patients with low anti-dsDNA levels was >58 weeks [48 weeks (early) compared with >104 weeks (late)].

A decrease in anti-dsDNA antibody levels was associated with remission in patients with high levels at baseline

To determine whether changes in anti-dsDNA antibody levels correlated with clinical relapse following rituximab

therapy, levels of anti-dsDNA antibodies were analysed at four time points: baseline, B-cell depletion, B-cell repopulation and relapse/remission (Fig. 3A). Remission was defined as no relapse within 12 months of repopulation. In patients with high levels of anti-dsDNA antibodies who remain in remission ($n = 9$), there was a significant decrease in levels of anti-dsDNA antibodies between baseline and repopulation ($P < 0.01$). Despite this decrease, anti-dsDNA antibody levels remained significantly higher in these patients compared with those who had low or normal levels at baseline ($P < 0.01$). Levels of anti-dsDNA antibodies did not fall at any time point in patients with high levels at baseline who relapsed ($n = 17$), rather there was a significant increase in anti-dsDNA antibody levels associated with relapse ($P < 0.05$). Overall, there was a larger percentage decrease in the levels of anti-dsDNA antibodies in those patients who remain in remission and who had baseline levels >100 IU/l compared with those who relapsed (Fig. 3B). Correlating the decrease in anti-dsDNA antibody levels with the duration of B-cell depletion indicates that there was a greater decline in anti-dsDNA antibody levels the longer B cells remained $< 0.01 \times 10^9$ B cells/l ($R = 0.42$, $P = 0.003$) (Fig. 3C).

Different B-cell phenotypes correlate with disease relapse

Given that patients with high anti-dsDNA antibody levels relapsed with lower B-cell numbers than patients with low anti-dsDNA antibody levels, we hypothesized that there might be differences in B-cell subsets according to anti-DNA levels in patients during relapse and remission. In a subset of patients ($n = 32$), we analysed the different B-cell subsets in relapsing patients and those who remained in remission. As shown in Fig. 4, patients who had high anti-dsDNA antibodies at relapse ($n = 6$) also had increased percentages of $IgD^{-}CD27^{hi}$ plasmablasts ($P < 0.01$) compared with patients who remained in remission ($n = 9$). In contrast, patients who had low or normal levels of anti-dsDNA antibodies had an increased

Fig. 3 Changes in anti-dsDNA antibody levels following rituximab therapy in relapsing patients compared with those that remain in remission. (**A**) Anti-dsDNA antibody levels are shown at baseline, during B-cell depletion, at B-cell repopulation and at remission or clinical relapse in patients who had either high levels [>100 IU/l, $n = 9$ (remission), $n = 17$ (relapse)] or low levels [$n = 15$ (remission), $n = 20$ (relapse)] at baseline. Columns indicate the mean; bars indicate the S.E.M. (**B**) Percentage change in anti-dsDNA antibody levels after rituximab therapy in patients with high levels at baseline that go into remission or relapse after B cells repopulate. Lines inside the boxes indicate the median; outer borders of the boxes indicate the 25th and 75th percentiles; bars extending from the boxes indicate the range. Differences between the groups were analysed by the Mann–Whitney rank-sum test. (**C**) Correlation between the change in anti-dsDNA antibody levels prior to B-cell repopulation and the time to B-cell repopulation.

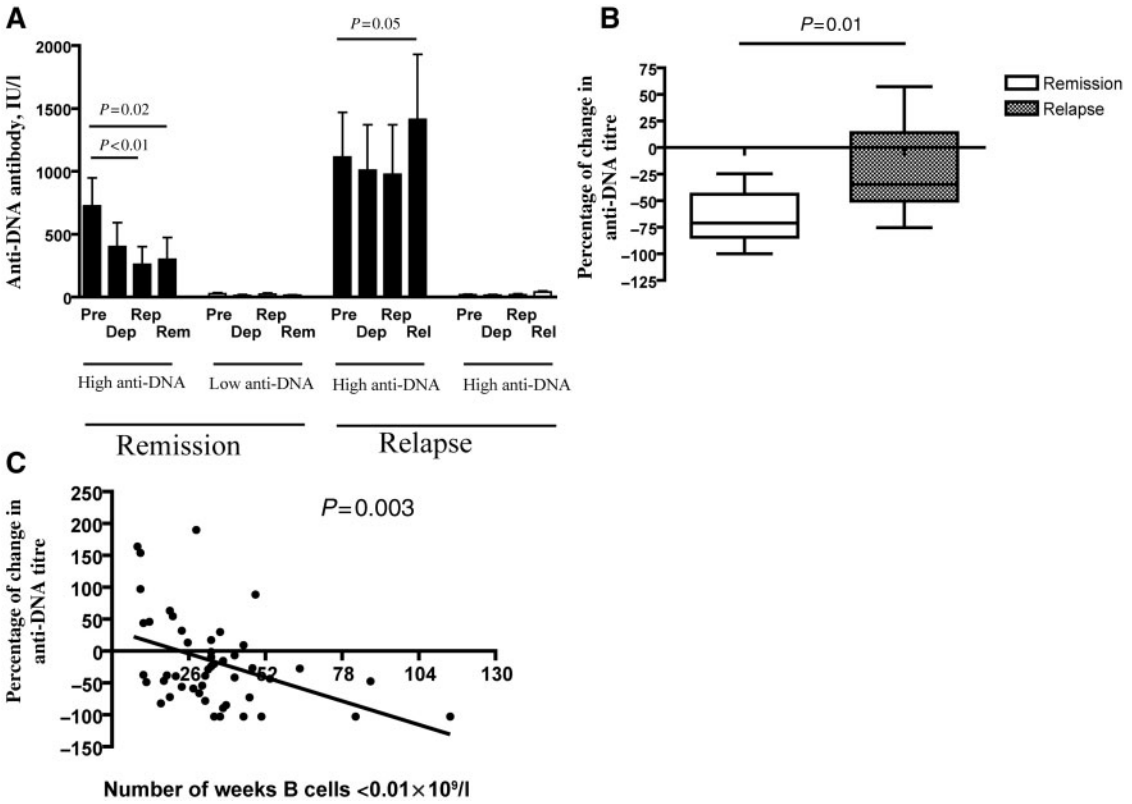
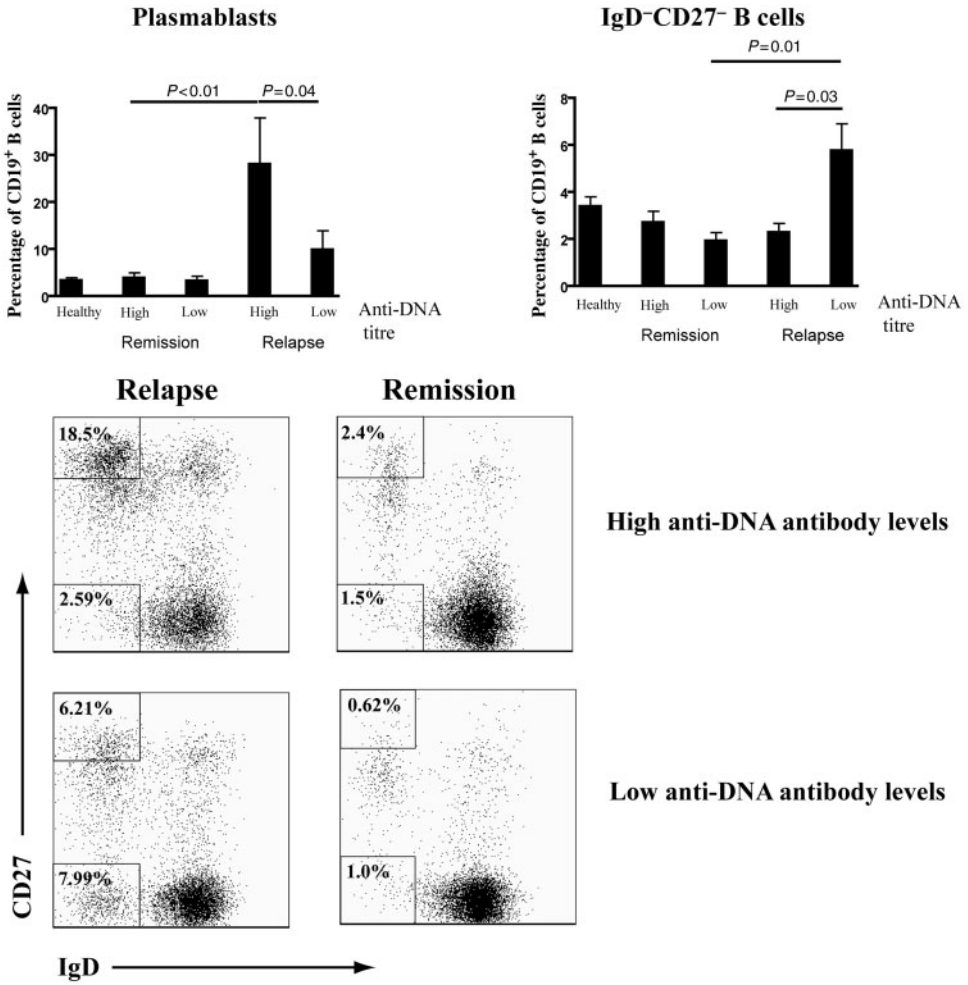


Fig. 4 B-cell subsets at clinical relapse in patients divided according to anti-dsDNA antibody levels. Peripheral blood B cells (gated on CD19) were analysed by flow cytometry for the expression of IgD and CD27. Representative and cumulative data are shown. Percentages of plasmablasts ($IgD^{-}CD27^{hi}$) or double-negative memory B cells ($IgD^{-}CD27^{-}$) in age- and sex-matched healthy controls ($n = 19$) and in patients who are in remission or relapse with high [>100 IU/l, $n = 9$ (remission), $n = 6$ (relapse)] or low anti-dsDNA antibody levels ($n = 7$, remission, $n = 10$, relapse) are indicated.



percentage of $IgD^{-}CD27^{-}$ (double negative) memory B cells at relapse ($n = 10$) compared with patients who did not experience a disease flare ($n = 7$) ($P = 0.01$).

Discussion

Our results indicate that patients with SLE respond differently to BCDT depending on the duration of B-cell depletion, their levels of anti-dsDNA antibodies and the type of B cells that repopulate. Two distinct groups of patients could be defined according to whether they relapsed with high levels of anti-dsDNA antibodies (>100 IU/l) or with normal or slightly raised levels. Most striking were the very low B-cell numbers at which patients with high anti-dsDNA antibody levels relapsed. These numbers were far below the numbers seen during the flare in disease that prompted the initial rituximab therapy, whereas patients with low anti-dsDNA antibody levels relapsed with similar numbers of B cells found before rituximab therapy. Moreover, patients with high levels of anti-dsDNA antibodies relapse later if their B cells remain $<0.01 \times 10^9$ B cells/l for a longer period. Once this low threshold was attained, further increases in B-cell numbers did not distinguish early and late relapsers in patients with high anti-dsDNA antibody levels. These observations could reflect the kinetics of plasma cell repopulation, which was associated with relapse in patients with high anti-dsDNA antibody levels. It is tempting to speculate that fewer plasma cells are required to induce a relapse compared with $IgD^{-}CD27^{-}$ double-negative B cells found in patients with normal or low anti-dsDNA antibody levels, where disease flare was associated with higher B-cell numbers. Thus plasma cells, via the production of anti-dsDNA antibodies, may be more potent inducers of lupus disease than other B-cell subsets.

Whereas the percentage of $CD27^{hi}$ plasmablasts show a strong correlation with anti-dsDNA levels [15], increased $IgD^{-}CD27^{-}$ B cells, though associated with SLE [16], are not consistently correlated with anti-dsDNA antibody levels [17]. It has been suggested that $IgD^{-}CD27^{-}$ B cells arise through a non-germinal centre pathway, though their provenance has yet to be proved. The absence of CD27, which binds to CD70 on activated T cells, could impair the ability of these double-negative B cells to respond to T-cell help and differentiate into plasma cells [18]. Experimental models of SLE have suggested that B cells may also contribute to pathogenesis through antibody-independent mechanisms, perhaps by driving T-cell autoimmunity [19, 20]. Since there was no association between plasma cells and disease flare in patients with normal or minimally raised anti-dsDNA antibody levels, it is possible that $IgD^{-}CD27^{-}$ memory B cells contribute to the pathogenesis of lupus through an antibody-independent mechanism.

SLE has long been associated with B-cell lymphopenia [21, 22], although the underlying mechanisms remain unclear. Although we have interpreted our data that relapse occurs at low B-cell numbers in patients with high anti-dsDNA antibody levels as indicating that plasmablasts are potent inducers of disease, it also possible that these antibodies are directly reducing B-cell numbers, perhaps by inducing apoptosis [23]. Lymphopenia has long been ascribed to the presence of autoantibodies [24], and there is an association between anti-dsDNA antibodies and lymphopenia [25, 26]. One cytokine with the ability to reverse the B-cell lymphopenia in lupus is B-cell activating factor (BAFF), due to its potent ability to maintain B-cell survival, and it is increased in the serum of lupus patients. One report suggested an association between BAFF and high anti-dsDNA antibody levels [27], though this has not been confirmed by others [28, 29]. Although BAFF levels also increase following rituximab therapy, it has not been associated with duration of depletion or disease flare [28]. Strategies that could target both BAFF and rituximab may aid in maintaining low B-cell numbers and preventing disease relapse.

Several different regimens of anti-CD20 therapy have been used in BCDT but the most effective has yet to be established. The results from this study suggest that the optimum treatment regimen could depend upon the baseline levels of anti-dsDNA antibodies. In patients with high anti-dsDNA antibody levels where relapse occurred at lower B-cell levels, a tailored treatment regimen is required to ensure their B-cell numbers remain low. In addition, the longer these patients remain depleted of B cells, the more likely anti-dsDNA antibody levels will fall. The optimum duration of depletion still needs to be established, but is likely to vary between patients. Patients with low levels of anti-dsDNA antibodies tend to flare at higher B-cell numbers, and therefore the target for depletion does not need to be as stringent as patients with high anti-dsDNA antibody levels. Future therapeutic strategies might also target specific B-cell subsets: $CD27^{hi}$ plasmablasts in patients with high levels of anti-dsDNA antibodies, but $IgD^{-}CD27^{-}$ memory B cells in patients with low levels. In conclusion, this study suggests that in therapeutic trials consideration needs to be given to the heterogeneous nature of the disease and the different mechanisms by which B-cell targeted therapy could induce remission.

Rheumatology key messages

- Low B-cell numbers at relapse after rituximab in lupus patients is associated with high anti-dsDNA antibody levels.
- Plasmablasts dominated repopulating B-cells only in lupus patients with high anti-dsDNA antibody levels.
- Anti-dsDNA antibody levels fell only in those rituximab-treated patients who did not relapse.

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Supplementary data

Supplementary data are available at *Rheumatology* Online.

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