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Short Title: Hypogammaglobulinemia after rituximab in SLE

Pragmatic treatment of patients with Systemic Lupus Erythematosus with rituximab: long-term effects on serum immunoglobulins

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Objective. B cell depletion therapy based on rituximab is a therapeutic option for refractory disease in patients with Systemic Lupus Erythematosus (SLE). The aim of this observational study was to document long-term effects on B cell function by following serum immunoglobulin levels in patients with SLE treated with rituximab in routine clinical practice.

Methods. We included 57 consecutive patients with SLE treated with rituximab and concomitant/sequential immunosuppressants and measured serum total IgG, IgM, and IgA and IgG anti-dsDNA antibodies over a median of 48 months most recent follow-up. Flow cytometry was used prospectively to assess B-cell phenotypes in 17/57 patients.

Results. Twelve patients (21%) had persistent IgM hypogammaglobulinemia (<0.4 g/L) and 3/55 (5%) had low IgG (<7g/L) at most recent follow-up (range 12-144 months). This was not associated with serious adverse events or high anti-dsDNA antibodies (>1000IU/ml; normal<50IU/ml). Factors predictive of low serum IgM included: baseline serum IgM ≤0.8g/L (receiver-operated-curve analysis) and subsequent therapy with mycophenolate mofetil (MMF) (odds ratio=6.8 compared with other immunosuppressants). In patients maintaining normal IgM levels (9/17), the frequency of circulating IgD+CD27+ B cells was significantly higher (*p=0.05*). At 12 months after rituximab, 7/30 SLE patients with baseline anti-dsDNA≤1000 IU/ml had lost seropositivity.

Conclusions. Lower baseline serum IgM levels and sequential therapy with MMF were predictive of IgM hypogammaglobulinemia after rituximab in SLE, but this was not associated with higher levels of anti-dsDNA antibodies or an increased risk of infections. This provides useful directions for clinicians regarding rituximab and sequential immunosuppressive treatment for patients with SLE.

Key words: serum immunoglobulins, rituximab, systemic lupus erythematosus, mycophenolate mofetil, B cell phenotypes, anti-dsDNA antibodies.

Bullet points:

- In SLE patients at long-term follow-up after multiple cycles of rituximab IgG hypogammaglobulinaemia was rare
- rituximab normalized raised IgG in the majority of patients
- Low levels of serum total IgM presented in nearly a quarter of SLE patients and were associated with lower baseline levels, older age and sequential therapy with mycophenolate mofetil
- Low IgM was not associated with persistently high levels of anti-dsDNA or adverse events

INTRODUCTION

Hypogammaglobulinemia can be an important adverse outcome of B-cell depletion therapy with rituximab (a chimeric anti-CD20 monoclonal antibody). Whereas transient hypogammaglobulinemia may not require specific therapy, some patients with B cell malignancies and autoimmune diseases (1) develop persistent hypogammaglobulinemia following rituximab, requiring intravenous immunoglobulin (Ig) replacement therapy, particularly in the context of recurrent infections (2-4). Although anti-microbial antibody responses are relatively robust, the degree of response to challenge with influenza, pneumococcal and tetanus immunogens after rituximab treatment may be impaired. This appears to relate to the degree and duration of B cell depletion in peripheral blood in patients with Rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (5-7).

B cell depletion therapy based on rituximab is as yet unlicensed for SLE, but is used to treat early onset and refractory disease(8-11). However, the probability of, and factors associated with, the incidence of persistent hypogammaglobulinemia after rituximab in the routine clinical setting, has not been explored. Therefore, it is of direct clinical relevance to identify factors that may predict those at an increased risk of developing persistent hypogammaglobulinemia.

Both underlying disease and immunosuppressive therapies may affect serum immunoglobulin levels. In patients with SLE, hypergammaglobulinemia is often present, paradoxically however, hypogammaglobulinemia similar to common variable immunodeficiency occasionally occurs, and may relate to the presence of lymphocytotoxic autoantibodies (12, 13). Selective IgM and IgA deficiency has also been reported (14, 15). Hypogammaglobulinemia may be associated with older age, low IgG at baseline, nephritis (4) and treatment with immunosuppressants including cyclophosphamide and mycophenolate mofetil (MMF) (16-18). A higher cumulative dose or repeated cycles of rituximab, concomitant or sequential use of immunosuppressants appear to increase the risk of persistent hypogammaglobulinemia in ANCA-associated vasculitis and other autoimmune diseases (4). Importantly, long-term persistence of hypogammaglobulinemia and associated adverse events is better appreciated in long-term follow-up studies than short-term clinical trials. Such information could therefore serve to identify those at a higher risk.

B cell hyperactivity, characteristic of SLE, results in excessive production of both polyclonal and autoreactive antibodies (19), even before the onset of clinical disease (20-22). Elevated levels of IgG anti-dsDNA (anti-double-stranded DNA) antibodies are characteristic of SLE and considered pathogenic but may occur independently of hypergammaglobulinemia (23,

24). Immune dysregulation in patients with SLE is at least partly related to changes in the interactions between immune cells within germinal centres and altered trafficking of peripheral blood lymphocytes (25, 26). Some abnormalities in the composition of peripheral B cell phenotypes appear to 'improve' following rituximab, but this may also reflect naïve B cell return, which recapitulates ontogeny (27, 28). Reduced levels of possibly protective natural antibodies of IgM-class have been suggested to be associated with development of anti-dsDNA antibodies in a murine model of SLE (29).

A study of the recovery of B cell subpopulations in patients with SLE who develop hypo-gammaglobulinemia may also relate to the extent of B-cell depletion in the short-term, and the recovery of B-cell subpopulations and/or clones in the long-term, both of which may impact serum immunoglobulin levels. To this end, we investigated whether baseline serum Ig levels, concomitant/sequential immunosuppressants and B cell phenotypes predict the development and/or persistence of hypogammaglobulinemia after rituximab. The relationship between serum Ig levels and anti-ds DNA antibodies was also determined over the course of study.

METHODS

Patients. In this cross-sectional observational study, 57 consecutive patients with SLE, who met the revised American College of Rheumatology classification criteria (30) and treated with rituximab were included. All patients were attending University College London Hospitals (UCLH), U.K and treated according to clinical need. The specific indication for rituximab treatment in this cohort was persistent active disease refractory to conventional immunosuppressive therapies. Clinical notes and laboratory results of all SLE patients treated with rituximab from January 2000 until December 2012 were reviewed retrospectively. As this study was a clinical evaluation, it did not require hospital ethics committee approval and results were compiled from anonymised files. In the cross-sectional B cell phenotype study, collection of blood samples was approved by the UCLH Ethics Committee. Patients gave written informed consent according to the Declaration of Helsinki.

All patients had active disease refractory to hydroxychloroquine and at least two conventional immunosuppressants including azathioprine (AZT), MMF, methotrexate (MTX) or cyclophosphamide (CYC). A majority of patients continued with low dose corticosteroids (CS, prednisolone <10mg/day) but in most cases, the use of other immunosuppressants was stopped until evidence of B cell return (CD19+cells > 5/μl) or started only as required for optimal control of disease activity. A typical rituximab treatment cycle consisted of rituximab, 2 doses of 1g given 1-2 weeks apart in combination with one dose of intravenous cyclophosphamide (750mg). The

clinical response to rituximab in this cohort has been reported previously (31). Clinical and laboratory parameters were analysed during the first cycle of rituximab (up to 12 months) and the most recent time point from all patients, some of who had received multiple cycles of rituximab-based treatment in combination with concomitant and/or subsequent therapy with immunosuppressants to determine longer term effects on the recovery of B-cell subpopulations, serum immunoglobulins (Igs) and autoantibodies.

Clinical and laboratory indices. Serum levels of IgG, IgM and IgA and IgG anti-dsDNA antibody levels were recorded from baseline (before the first infusion of rituximab) up to 12 months after rituximab and also at most recent follow up. Hypogammaglobulinemia was defined by: serum IgG <7 g/L (normal range: 7-16g/L), IgM <0.4 g/L (normal range: 0.4-2.3 g/L); IgA <0.7 g/L (normal range: 0.7-4.0 g/L).

B cell immunophenotyping. B cell phenotypes in whole blood were prospectively characterized by flow cytometry. Samples were stained with fluorescence-tagged antibodies against CD19 (Phycoerythrin-Cyanide dye 7, PECy7), IgD (Fluorescein isothiocyanate, FITC) and CD27 (Phycoerythrin, PE) (BD Biosciences, Oxford, UK). B cells were identified as CD19+ cells in the lymphocyte gated cells. B cell phenotypes were identified as follows: naïve, IgD+CD27-, un-switched memory IgD+CD27+; switched memory IgD-CD27+; double negative (DN) IgD-CD27-.

Statistical analysis. Statistical analysis was performed using Graph Pad Prism version 5.01 (Graph Pad software, San Diego, CA). Matched-pairs signed rank test and Mann-Whitney U test were used to analyze differences in serum Igs between paired and unpaired data, respectively. Spearman's rank test was used to analyze correlations. Fisher's exact test was used to compare the proportions of patients in different groups. Receiver operated curve analysis was used to identify the cut-off value that distinguished patients developing low Igs. Odds ratio was used to express the effect of concomitant and/or subsequent immunosuppressants on the development of hypogammaglobulinemia.

RESULTS

Patient demographics. Clinical features, drug therapies prior to RTX and at most recent follow up and serology are shown for patients with low serum IgM and those retaining normal levels of IgM in Supplementary-tables 1 and 2 respectively. Lupus Nephritis (LN) was diagnosed in 29/57 (51%) patients. Percentages of patients in each group were similar (7/12 (58%) in the low IgM

group and 22/45 (49%) in the normal IgM group). Detailed patient demographics and clinical responses of this cohort of 57 patients have been previously described (31-33). The median age at the time of the first rituximab treatment cycle was 34 years (range 17-74 years). All patients had received previous treatment with at least 2 different immunosuppressants not including corticosteroids, which were continued at a low dose (<10mg/day prednisolone). The median duration of follow-up was 48 months (range 12-144 months).

Serum total Ig and anti-dsDNA antibodies in patients at 12 months follow up post-rituximab. At baseline, 3 patients had low IgM, none had low IgA and one, low IgG (Figure 1A-C). Eleven patients had raised serum IgG levels (>16 g/L). At 12 months follow up after rituximab (n=32), median baseline serum IgG level was 13.9 g/L, which was significantly reduced at 1, 2, 6, and 9 after rituximab ($p<0.05$ for all), but similar to baseline levels at 12 months (median 13.2g/L) (Figure 1A). Median IgM levels however were significantly lower at all time points (Wilcoxon matched-pairs sign rank test; $p<0.005$ for all) with the median serum IgM level at baseline (1.0 g/L) decreasing significantly to 0.71g/L at 12 months (Figure 1B). Median baseline serum IgA level was 2.9 g/L, which fell at 1 and 3 months ($p<0.05$) only (Figure 1C). Percentage change from baseline to 12 months of serum Igs and anti-dsDNA is shown in Figure 2, and we noted a hierarchy in % reduction from baseline with IgM > IgG > IgA (-18.4%; -2.8% ; 10.3%, Figure 2 A, C, D respectively) and remarkable variations in IgG anti-dsDNA levels (Figure 2B).

Serum Ig and anti-dsDNA levels at 12 months post-rituximab: relationship with baseline.

a) Serum IgM

At 12 month follow up, 25% (8/32) of SLE patients had serum IgM levels below the normal range. There was a significant difference between median baseline serum IgM levels in patients who developed low serum IgM levels at 12 months (8/32) and those who did not ($p<0.005$), at 0.5 and 1.0 g/L respectively (Figure 3A).

b) Serum IgG

Only 1 patient had low serum IgG before treatment and at 12 months, only 2 additional patients had levels <7g/L. Figure 4A shows that in patients with IgG hypergammaglobulinemia pre-rituximab (median; 17.9g/L), that there was a significant reduction in median IgG levels between baseline and 12 month follow-up in 11/32 (34%) ($p<0.01$; Wilcoxon matched-pairs signed rank) with levels normalizing in 8 patients. In those with baseline IgGs within the normal range, there was no difference between baseline and levels at 12 months post-rituximab.

c) Serum IgA

Similarly, there was a trend towards higher baseline median serum IgA levels with a reduction in serum IgA levels when compared with baseline median serum IgA levels and with those who did not, at 3.7g/L and 2.7g/L (*p=0.06*) (Data not shown).

d) Anti-dsDNA antibodies

At 12 months, in 8/32 patients (19%) the levels of anti-dsDNA fell to within the normal range but median levels at baseline and 12 month follow-up were similar (169 vs 100 IU/ml; *ns*) (data not shown). In Figure 4B, patients were divided on the basis of having anti-dsDNA titers ≤ or >1000IU/ml. In those with anti-dsDNA levels >1000IU/ml, only 1/9 had levels which fell to within the normal range at 12 months compared with 7/23 (35%) who had levels ≤1000IU/ml at baseline. A significant reduction in median levels after 12 months in patients with titers ≤1000IU/ml, but not in those with titers >1000IU/ml was also noted (Figure 4B).

Effect of rituximab on serum Igs at most recent follow-up. Low serum IgM was present in 12/57 (21%) (Figure 5B) with only 3/55 patients developing low IgG, all of whom had normal pre-treatment levels (Figure 5A). Of 21 patients with raised serum IgG pre-treatment, 15 had normalized at most recent follow-up. The demographics of the 12 patients developing low serum IgM levels following rituximab are shown in Table 1. Interestingly, at 12 months 8/32 (25%) had low IgM and at last follow up a similar proportion, 12/57 (21%), had low IgM, suggesting that accumulated rituximab dose was not necessarily related to development of low serum IgM. There was no difference in sex distribution compared with the whole cohort (data not shown) but they tended to be older (median age 43 years; range 22-59) compared with those maintaining normal IgM levels (median age 32 years; range 21-74) (Mann Whitney U test*; p<0.01*) Three of 56 patients developed low IgA levels (Figure 5C). In 15/40 (38%) patients, anti-dsDNA levels normalized, with the majority (13/15) having had levels ≤1000IU/ml at baseline (Figure 5D). No serious adverse events were observed (31, 33) and none of the patients required intravenous immunoglobulin therapy.

Relationship between low serum IgM and IgG anti-dsDNA antibody levels. We did not find significant correlations between baseline serum IgM and anti-dsDNA levels at baseline or maximal follow-up (data not shown). Further, median serum IgM levels in patients with anti-dsDNA >1000IU/ml and those with levels ≤1000IU/ml (1.1g/L and 0.9g/L respectively) were not significantly different (Mann-Whitney U test; data not shown).

Predictive factors for the development of low serum IgM after rituximab.

IgG hypogammaglobulinaemia was present at long-term follow-up in only 5% of SLE patients. As shown in Figure 3A however, patients with serum IgM levels below 0.4g/L at 12 months after rituximab had significantly lower baseline levels than those with IgM within the normal range at 1year follow-up ($p<0.005$). We employed Receiver-Operated-Curve (ROC) analysis (Supplementary Figure 1A) and found that a pre-treatment serum IgM level ≤0.8 g/L was associated with > 3-fold likelihood ratio for the development of low IgM (<0.4g/L) at 12 months and also at long-term follow-up comparing those with low serum IgM (n=12) and those with normal serum levels (n=43) (Supplementary Figure 1B), with a significant area under the curve of 0.85 (95% confidence interval (CI) 0.7-1.0; $p=0.0002$). This suggested that baseline serum IgM ≤0.8 g/L was associated with a >4-fold increase in the likelihood of developing low IgM (sensitivity 83%; specificity 80%). In accord with this cut-off value, at most recent follow up, we found that 10 of 18 patients with serum IgM ≤0.8 g/L at baseline and only 2 of 37 patients with levels >0.8 g/L developed low IgM (Fisher's exact test; $p<0.0001$).

Effect of sequential therapy with immunosuppressants. The results from our extended data at most recent follow up showed that 12 of 57 patients developed low IgM and 6 of these patients (50%) were treated with MMF at least 6 months after rituximab and 2 months before the time of analysis (Supplementary-table 1). In contrast, only 7 of 43 (16%) patients treated with other immunosuppressants including AZT, MTX and CYC developed low IgM (Figure 3B). The odds ratio for the analysis was 6.8 (CI, 1.66-27.77).

B cell phenotypes in patients with low serum IgM levels. We found that the frequency of un-switched B cells (IgD+CD27+), but not other phenotypes, was significantly lower in patients who developed low IgM after rituximab when compared with those who did not ($p< 0.05$) although a trend for higher frequency of double negative (DN) (IgD-CD27-) memory B cell subpopulation was also apparent (n=8) (Figure 3C). We found no significant difference between sex distribution or age, median times since last rituximab treatment (24 months in the low IgM group, 15 months in those with normal IgM), nor in % B cells, levels of complement-3, cumulative dose of rituximab or anti-dsDNA levels between the groups (Supplementary–tables 1 and 2 and data not shown).

DISCUSSION

In this study, we found that lower baseline IgM levels were predictive of low serum IgM after rituximab and associated with a lower frequency of un-switched memory B cells. Sequential

treatment with MMF after rituximab was also associated with low serum IgM. IgG hypogammaglobulinemia was rare and the majority (71%) of those with raised serum IgG at baseline had normalized at maximum follow-up. Patients with low serum IgM did not experience serious adverse events. In the most recent published results of the cohort from which these patients were derived, we showed that the safety profile was favorable, and infusion related and hypersensitivity reactions were mostly mild to moderate (33).

There was a disparity in the dynamics of fluctuations between isotypes of serum Igs after rituximab. At 12 months and also at long-term follow up, median levels of serum IgG and IgA were not significantly different from those at baseline, with very few patients developing low levels of IgG or IgA. This contrasts with our experience in patients with RA in whom those with lower baseline sIg levels tended to develop persistent IgM and IgG hypogammaglobulinaemia, resulting from an accumulation of incremental decreases after repeat cycles. The incidence of low IgM increased from 9.2%-38.8% and IgG from 11.8%-22.2% of RA patients, after one and 5 cycles respectively (34). In patients with SLE however, our results show that the incidences are much lower after repeat cycles, being for IgM 12/57 (21%) but only 4/57 (7%) developing low IgG, with all retaining IgG levels >5g, and therefore none were treated with IVIG. Interestingly, at 12 months 8/32 (25%) had low IgM and at last follow up a similar proportion 12/57 (21%) had low IgM, suggesting that accumulated rituximab dose was not necessarily related to development of low serum IgM. An important factor influencing serum Ig levels is the balance between synthetic and catabolic rate of different Ig isotypes. IgG catabolism is greater in patients with SLE than in patients with RA whereas IgM catabolism is greater in RA compared to patients with SLE (35).

In patients with ANCA-associated vasculitis (AAV) and TTP, co-therapies such as cyclophosphamaide and plasmapheresis make it difficult to dissect the role of rituximab per se in the development of low serum Igs. None of our patients received plasmapheresis but patients with SLE usually receive a single dose of 750mg of cyclophosphamide, substantially lower than that used in AAV. Comparison between patient groups was also confounded due to lower pre-rituximab serum IgG levels in patients with AAV (4). Of direct clinical relevance, rituximab treatment did not result in significant reductions in serum IgG levels in those with low baseline IgG levels of <6g/L (4). Even allowing for these limitations, we found that incidences of low IgG levels in SLE were markedly less frequent than in patients with AAV and RA.

There was no association between serum IgM levels, or with the development of low IgM, with levels of IgG anti-dsDNA antibodies. Patients who developed low IgM however, had 2 fold-lower median levels of serum IgM before rituximab compared with those without IgM hypogammaglobulinemia at long-term follow-up. Differential effects on Ig classes have also been

described in patients with RA and also in patients with multiple myeloma treated with autologous haematopoietic stem cell transplant (HSCT) and rituximab maintenance therapy (36). Both groups of patients tend to develop low IgM but not G and A. In contrast, some patients with refractory follicular lymphoma treated with rituximab and HSCT developed persistently low IgA and IgG with recovery of IgM levels (34, 37). Underlying disease can therefore influence the development of isotype specific hypogammaglobulinemia.

Serum IgM is derived from both (short-lived) newly generated peri-follicular B cells (CD27-) and from CD27+ (un-switched) marginal zone B cells (38). Differences in specific co-therapies or the regimen used may also therefore account for some of the disparity between the isotypes affected depending on the parent B cells affected. Serum levels of IgA and IgG are largely maintained by long-lived (CD20-) plasma cells, predominantly in the bone marrow. These are therefore not directly targeted by rituximab, and protective immunity is largely maintained, as in RA patients for example (34, 39). It is however difficult to differentiate the direct effects of rituximab preventing formation of new plasma cells from indirect effects through disease control.

We found no difference between time since last rituximab infusion or in cumulative rituximab dose in the subgroup of SLE patients studied for B cell phenotype. In SLE, MMF, but not AZT or HCQ, treatment has been associated with reduced frequency of switched memory B cells and modest decreases in levels of serum Igs and of anti-dsDNA antibodies (40-42). The composition of B-cell subpopulations may vary between individuals with SLE and after rituximab, repopulation appears to recapitulate ontogeny, perhaps further influenced by antigen stimulation (43). We found that the frequency of un-switched (IgD+CD27+) B cells was significantly lower in patients who developed low IgM after rituximab when compared with those who did not. Relative levels of Igs may relate to the composition of B cell pools in bone marrow, lymphoid and inflammatory tissues which differ between individual patients. This is supported by the finding that patients who developed low IgM already had lower baseline levels (44). Un-switched (IgM-committed) B cells are preferentially depleted by rituximab *in vitro*, suggesting a reduced threshold for survival and slow regeneration, of un-switched B cells in SLE (45-47).

Our results indicated a possible association between sequential treatment with MMF after rituximab and low serum IgM. MMF preferentially targets type II inosine monophosphate dehydrogenase, which is up regulated in activated lymphocytes (both B and T lymphocytes) (48, 49). Rituximab preferentially depletes naïve and un-switched B cells, both of which are direct precursors for IgM production. Together with the potential removal of activated naïve and memory cells by MMF, this may explain the profound effect of using a combination of rituximab and MMF on serum IgM levels. Co-therapy with MMF has been associated with higher rate of infections in clinical trial studies using Ocrelizumab (50) and low immunoglobulins were noted in patients

treated with a combination of MMF and Atacicept (51). In the later study, low IgM levels in SLE patients treated with MMF alone in the placebo arm did not recover over the course of study. Our data confirm that caution is needed, when the combination of MMF, with judicious administration of the dose, and rituximab is being considered.

In contrast to IgM, serum IgA, after an initial decrease in median levels, started recovering as early as 2 months after rituximab, approaching baseline levels by 6 months. Early recovery in serum IgA levels suggests that the IgA plasma cell pool was rapidly replenished. It has previously been reported that circulating IgA+ plasmablasts can remain detectable early after rituximab, suggesting resistance to depletion of switched IgA+ precursor-B cells, likely in the mucosal microenvironment and/or early regeneration (52). Serum IgG levels, despite showing a longer 'lag' when compared with the recovery of serum IgA levels, was apparently also sustainable, attaining pre-treatment levels in most patients by 12 months after rituximab. Indeed, rituximab treatment resulted in correction of hypergammaglobulinemia in most patients in our cohort. At long-term follow up, very few (5% of patients) had serum IgG levels below the lower limit of the normal range.

Percentage change from baseline of dsDNA antibodies was highly variable between patients. Differences in patterns of fluctuations in anti-dsDNA antibodies between patients implied a variable contribution from anti-dsDNA committed B cell clones (CD20+) sensitive to B cell depletion and also from long-lived (IgG) plasma cells (CD20-)(53). Autoantibody-committed B-cells are often preferentially removed by rituximab, as has also been shown in patients with RA (39, 54). A significant proportion of patients lost seropositivity to ds-DNA at long-term follow up, however, there was little overall decrease in anti-dsDNA antibodies in those patients with the highest baseline levels, suggesting the presence of a more entrenched autoreactive plasma cell pool.

The limitations of this study were that it was observational and from a single center, and the data were not complete for all time points. However, clinical and laboratory results were available for the majority of patients at most recent follow-up (at least 54/57); complimented by prospective analysis of peripheral blood immunophenotyping of most patients who developed low serum IgM.

Conclusions

Our results showed that hypogammaglobulinemia after rituximab was largely restricted to the IgM class, and was associated with low baseline levels and a lower frequency of un-switched B cells. The development of low serum IgM hypogammaglobulinemia was also associated with sequential mycophenolate mofetil. Monitoring of serum immunoglobulin levels is an important

adjunct to the selection of concomitant/sequential immunosuppressants after B cell depletion therapy. Reassuringly, low IgM after rituximab was not associated in our patients with increased risk of infections. Nonetheless, it would be prudent to continue surveillance of the patients for potential adverse events.

Taken together, the data presented provide new insights into the variability in biological response with rituximab providing useful information for the clinicians using rituximab for SLE.

List of abbreviations

A, Asian; AC, Afro-Caribbean: AZT, azathioprine: CHI, Chinese: C, Caucasian: CS, corticosteroids: DN, double negative: dsDNA, double stranded DNA: HCQ, Hydroxychloroquine: HSCT, haematopoietic stem cell transplant: Ig, immunoglobulin; Igs, immunoglobulins: LN, Lupus nephritis: MMF, mycophenolate mofetil: RA, Rheumatoid arthritis: RTX, rituximab: SLE, systemic lupus erythematosus:

Competing interests

No conflict of interest: VR, GC, LM.

DAI has acted as an Advisor to several companies notably Eli-Lilly; Merck Serono, Pfizer and UCB Pharma. Support offered in lieu of these services is passed onto a local arthritis charity. MJL has received honoraria for participating in meetings from Roche UK, Roche Brazil and Roche Portugal, support for attending conferences from Roche and Chugai UK.

Authors' contributions

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be submitted for publication. Drs Reddy and Cambridge had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Leandro, Reddy, Cambridge

Acquisition of data. Martinez, Reddy, Cambridge

Analysis and interpretation of data. Leandro, Reddy, Cambridge, Martinez, Isenberg

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Figure Legends:

Figure 1. Serum levels of IgG (A), IgM (B) and IgA (C) at intervals up to 12 months after treatment of patients with SLE with rituximab (n=32). Box and whiskers represent median, interquartile range and range. Differences between baseline and subsequent immunoglobulin levels of each class were calculated using Wilcoxon matched-pairs signed rank test with ns = not significant, $p<0.05$*, $p<0.005$** and $p<0.0005$*** as indicated.

Figure 2. Percentage change from baseline in immunoglobulin classes and anti-dsDNA antibodies: Mean and standard deviations of serum IgG (A), anti-dsDNA autoantibodies (B), IgM (C) and IgA (D) at baseline and up to 12 month follow-up after initial cycle of rituximab (administered at Time 0) are expressed as a percentage of baseline (pre-rituximab) levels.

Figure 3. Development of IgM hypogammaglobulinaemia after rituximab: relationship with baseline serum IgM, sequential therapy and B cell phenotype. (A) Results for serum IgM were grouped on the basis of being within the normal range (0.4-2.3g/L) at 12 months (indicated by shaded area) or <0.4 g/L at 12 months. Box and whiskers show median, interquartile range and range with significance between baseline values in each group calculated using the Mann-Whitney U Test. (B) The number of patients who developed IgM hypogammaglobulinemia (IgM<0.4) and who had been treated with mycophenolate mofetil (MMF) (7 of 12) or with other immunosuppressants (7 of 43) following rituximab are shown (odds ratio = 6.8; CI: 1.66 - 27.77). (C) The frequency (%CD19+B cells) of B cell subpopulations in samples available from patients with low (<0.4g/L) (n=8) or serum IgM levels within the normal range (n=9) after rituximab. B cell subpopulations were defined using relative expression of IgD and CD27. Box and whiskers represent the median, interquartile range and range of values and significance calculated using the Mann-Whitney U Test. Significance was at 5% level.

Figure 4. Fluctuations in serum IgG and anti-ds DNA antibody levels in relation to baseline levels. A) Patients (n=32) were grouped on the basis of whether their baseline serum IgG levels were within the normal range or greater than upper limit of normal range (normal range: 7-16g/L; shaded area). Median and range for serum IgG levels for up to 12 months after rituximab are shown. In **B)** IgG anti-dsDNA antibody levels in seropositive patients following rituximab are shown for follow-up of 12 months. Upper limit of positive test was 50IU/ml and shaded area indicates normal range. Results were stratified according to baseline anti-dsDNA antibody levels of ≤1000IU/ml or >1000 IU/ml. Significance between baseline in each group and 12 month values were calculated using Wilcoxon matched-pairs signed rank test (significance level at 5%).

Figure 5. Changes between baseline serum Igs and anti-dsDNA levels in SLE patients at Most Recent follow up. Paired serum Ig levels were available from 54 patients and anti-dsDNA antibody levels from 40 patients. Shaded areas indicate the normal ranges used for each parameter. Values for serum levels of IgG **(A)**, IgG-anti-dsDNA antibodies **(B)**, IgM **(C)** and IgA **(D)** at baseline and at most recent (MR) follow-up (ranging from 12-144 months after initial RTX treatment) are shown. Significance values shown were given by Wilcoxon matched-pairs signed rank tests for values at Most Recent follow-up compared with baseline.

Supplementary-table 1: Patients with low serum IgM (<0.4g/L) post-rituximab (n=12) at most recent follow up. Demographics, ethnic origin, clinical features and drug therapy are shown for individual patients.

Supplementary-table 1: Patients with serum IgM within the normal range (0.4-2.3g/L) post-rituximab (n=45) at most recent follow up. Demographics, ethnic origin, clinical features and drug therapy are shown for individual patients.

Supplementary-table 3: Demographics and laboratory parameters of patients studied prospectively for B-cell phenotype (Figure 3C). Number of cycles of rituximab, co-therapies and serology (C3 and anti-dsDNA antibodies) of 9 SLE patients with immunoglobulin levels within the normal range after rituximab and in 8 patients with SLE who developed low serum IgM levels after rituximab.

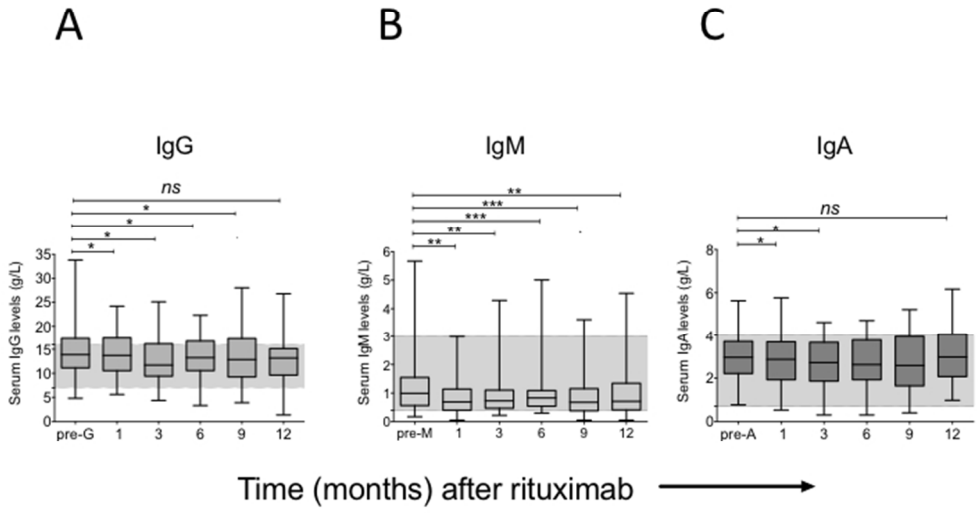

Figure 1. Serum levels of IgG, IgM and IgA at intervals up to 12 months after treatment of patients with SLE with rituximab (n=32). Box and whiskers represent median, interquartile range and range. Differences between baseline and subsequent immunoglobulin levels of each class were calculated using Wilcoxon matched-pairs signed rank test with ns = not significant, $p<0.05$*, $p<0.005$** and $p<0.0005$*** as indicated.

Figure 1
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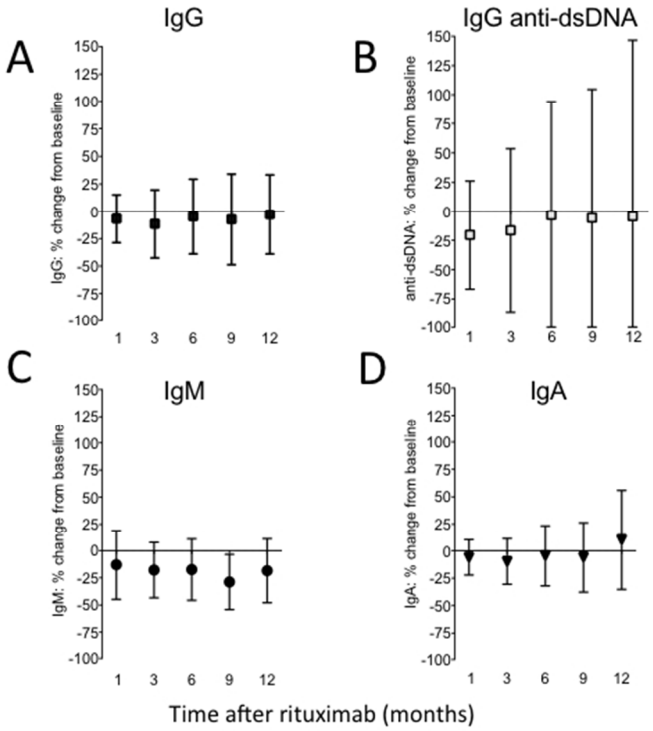

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Figure 2
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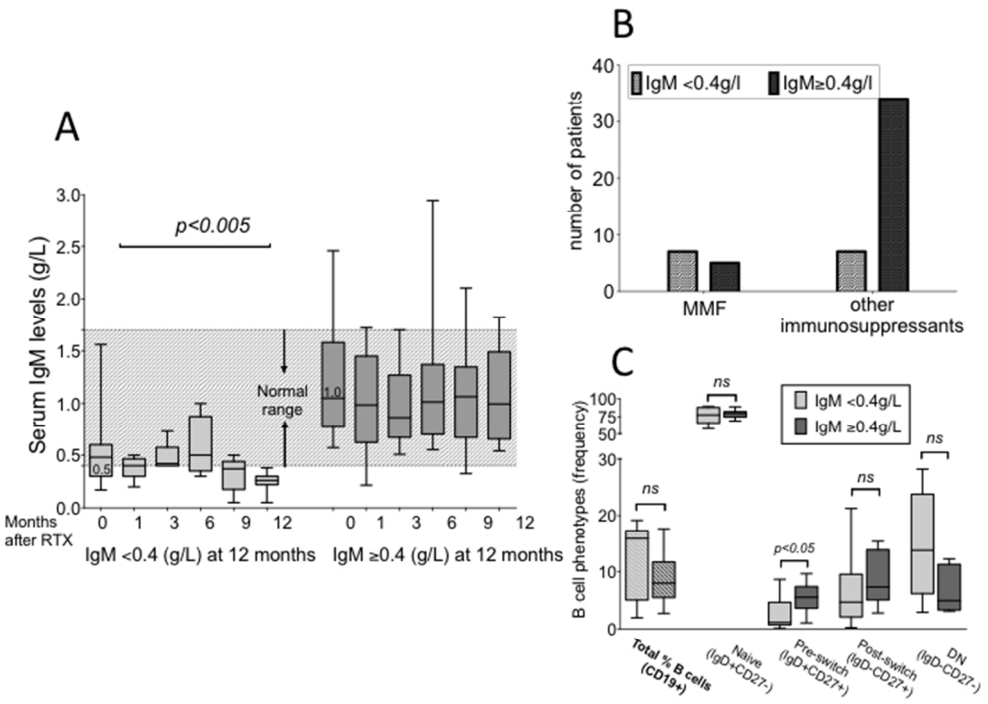

Figure 3
Figure 3. Development of IgM h
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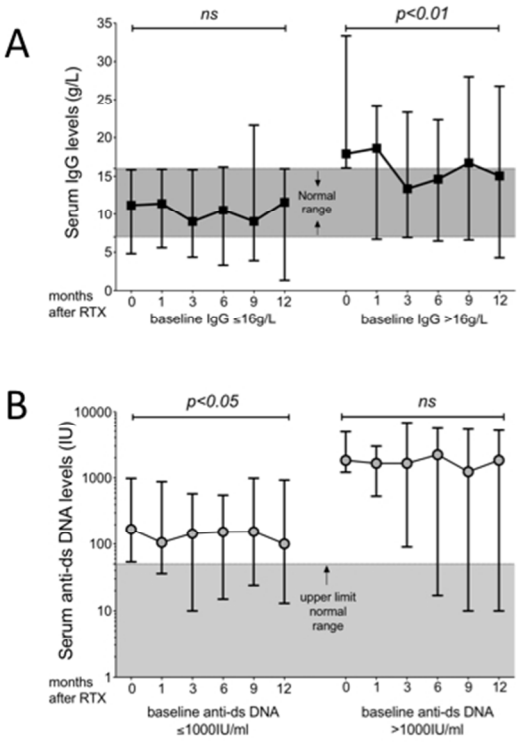

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Figure 4
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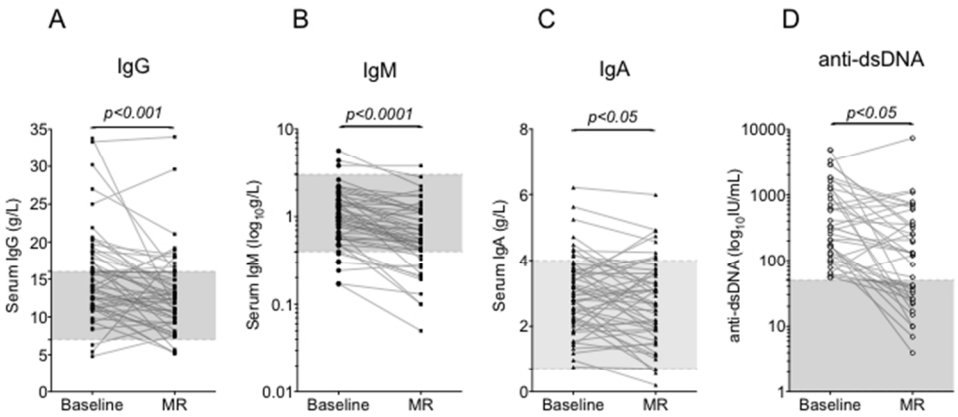

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Figure 5
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Supplementary-table 1: Patients with low serum IgM (<0.3g/L) post-Rituximab

Patient number	Age (years)	Ethnicity	Clinical manifestations	RTX-cycles	Treatment before RTX	Most recent treatment	Serology
2	35	A	Non-renal	1	CS (high), AZA	CS (low), HCQ	ANA,Ro,RNP
11	56	C	Non-renal	1	CS (low), AZA	Nil	RNP
15	53	AC	LN (class 3)	1	CS (low)	CS (low)	RNP
16	33	AC	LN (class 4)	2	CS (low)	CS (low), MMF (1.5g)	ENA-ve
17	42	Ch	LN (class 5)	1	CS (low), MMF (3g), HCQ	CS (low), MMF (2g), Tacro	Ro,La,Sm,RNP
22	51	AC	LN (class 3)	2	CS (low), AZA	MMF (2g)	ENA-ve
30	55	C	LN (class 4)	1	CS (low), MMF (3g), HCQ	CS (low), HCQ, MMF (2g)	ENA-ve
31	50	AC	Non-renal	1	CS (low), MTX	CS (low), Enbrel	Ro
35	45	AC	LN (class 4)	2	CS (low), MMF (1.5g)	CS (low), HCQ	Sm
43	53	AC	Non-renal	1	CS (low), HCQ	HCQ	Ro,La
51	22	AC	LN (class 3)	1	HCQ, MMF (3g)	CS (low), HCQ, MMF (3g)	Ro,La
53	46	C	Non-renal	2	CS (low), HCQ	Nil	Ro

Abbreviations: A, Asian; AC, Afro-Caribbean; C, Caucasian;Ch, Chinese; LN, Lupus nephritis; Rituximab, RTX; dsDNA, (double stranded DNA); C3, complement component-3; HCQ, Hydroxychloroquine; CS, corticosteroids; CS (low), corticosteroids≤7.5mgs/day, CS (low), corticosteroids>7.5mgs/day AZT, azathioprine; MMF, mycophenolate mofetil; Tacro, Tacrolimus; ANA, anti-nuclear antibodies; RNP anti-ribo-nuclear proteins; ENA-ve, negative for anti-extractable nuclear antigens.

Supplementary Table 2: Patients with serum IgM within the normal range (0.4-2.3 g/L) post-Rituximab

Patient number	Age (years)	Ethnicity	Clinical manifestations	RTX-cycles	Treatment before RTX	Most recent treatment	Serology
1	58	C	LN (class 4)	2	CS (high)	CS (low), HCQ	Ro, La
3	28	AC	LN (class 3)	5	Aza, MMF, MTX, CYC	CS (low), HCQ	Ro,La
4	34	A	LN (class 4)	1	Pred, HCQ, MMF (2g)	CS (low), HCQ, AZA	Ro, RNP
5	33	AC	non-renal	1	CS (low), MTX	CS (low)	RNP
6	29	A	LN (class 3)	5	CS (high), HCQ	CS (low), HCQ, Tacro, MMF (1.5g)	ENA-ve
7	26	Ch	non-renal	1	CS (low), HCQ, AZA	CS (low), AZA	RNP
8	31	Ch	non-renal	1	HCQ, AZA	HCQ, AZA	Sm
9	43	C	non-renal	1	CS (low), AZA	HCQ, AZA	ENA-ve
10	58	C	non-renal	1	CS (high)	CS (low)	Ro
12	35	AC	LN (class 4)	1	HCQ, AZA	HCQ, AZA	RNP
13	29	A	LN (class 4/5)	4	CS (low) MMF (2.5g)	CS (low), HCQ	Ro
14	45	AC	LN (class 3)	1	CS (low) MMF (1.5g)	CS (low) MMF (0.5g), Tacr	Sm
18	75	C	non-renal	3	CS (low), MTX	CS (low)	ENA-ve
19	33	A	LN (class 3)	2	CS (low), HCQ, AZA	CS (low), HCQ, MMF (2g)	Ro, Sm, RNP
20	28	C	LN (class 5)	1	CS (low), AZA	CS (low) Tacro	ENA-
21	29	C	LN (class 4)	2	CS (low), HCQ, AZA	CS (low), HCQ, AZA	Ro, Sm, RNP
23	54	A	LN (class 4)	1	CS (low) MMF (1.5g)	CS (low)	RNP
24	29	AC	non-renal	1	CS (high)	CS (low)	ENA-ve
25	30	C	LN (class 4)	1	CS (low), AZA	CS (low) MMF (1g), Tacro	ENA-ve
26	30	Ch	non-renal	1	CS (low), MTX	IFX, MTX	ENA-ve
27	29	A	LN (class 5)	1	CS (low) MMF (2g), HCQ	CS (low), HCQ	ENA-ve
28	25	AC	LN (class 4)	2	CS (low), HCQ, AZA	CS (low), HCQ	Ro
29	42	AC	LN (class 3)	1	CS (high)	CS (low)	Ro, Sm, RNP
32	47	C	non-renal	1	CS (low), HCQ	CS (low), HCQ	Sm
33	33	AC	LN (class 4)	3	CS (low), AZA	CS (low) MMF (2g)	Ro, RNP
34	42	AC	non-renal	2	CS (high)	CS (low)	Ro, Sm, RNP

36	40	C	non-renal	1	CS (low), HCQ	HCQ	ENA-ve
37	30	AC	non-renal	2	HCQ	CS (low), HCQ	Ro
38	27	AC	non-renal	1	CS (low), AZA	CS (low), HCQ	Ro
39	21	A	LN (class 4)	2	CS (low), HCQ, AZA	HCQ	Sm
40	19	AC	non-renal	2	CS (low), HCQ	CS (low), HCQ	RNP
41	51	AC	LN (class 4)	1	CS (low)	CS (low), HCQ	La, Sm
42	40	C	non-renal	2	CS (low), AZA	CS (low), HCQ	Ro, la
44	22	C	non-renal	1	HCQ, MMF (1.5g)	HCQ	Ro, RNP
45	45	AC	LN (class 4)	1	HCQ	nil	Ro La
46	22	AC	LN (class 3)	4	CS (low), HCQ	CS (low) MMF (1.5g)	RNP
47	47	C	LN (class 4)	2	CS (low), HCQ	CS (low), HCQ, MMF (2g)	Sm, RNP
48	35	AC	non-renal	2	HCQ	CS (low)	ENA-
49	35	AC	non-renal	2	HCQ	AZA	Ro, Sm, RNP
50	29	C	non-renal	1	CS (low), HCQ, AZA	CS (low), AZA	Sm, RNP
52	53	C	non-renal	2	HCQ, MTX	HCQ, MTX	Ro
54	72	C	non-renal	2	AZA	CS (low)	RNP
55	25	AC	LN (class 4/5)	1	CS (low) MMF (2g)	CS (low) MMF (1.5g)	Sm
56	26	C	non-renal	2	CS (low) MMF (2g)	CS (low) MMF (1g)	Sm, RNP
57	28		non-renal	2	CS (low) MMF (2g)	CS (low), AZA	ENA-ve

Abbreviations: A, Asian; AC, Afro-Caribbean; C, Caucasian;Ch, Chinese; LN, Lupus nephritis; Rituximab, RTX; dsDNA, (double stranded DNA); C3, complement component-3; HCQ, Hydroxychloroquine; CS, corticosteroids; CS (low), corticosteroids≤7.5mgs/day; CS (high), corticosteroids>7.5mgs/day; AZT, azathioprine; MMF, mycophenolate mofetil; Tacro, Tacrolimus; ANA, anti-nuclear antibodies; RNP anti-ribo-nuclear proteins; ENA-ve, negative for anti-extractable nuclear antigens.

Supplementary Table 3: Demographics and laboratory parameters of patients studied for B-cell phenotype (Figure 3C).

IgA (g/L)	IgG (g/L)	IgM (g/L)	CD19 (%lymphocytes)	RTX-cumulative dose (g)	Post-RTX (months)	Anti-dsDNA (IU/mL)	C3 (g/L)	Co-therapies
Patients with immunoglobulin levels within the normal range after rituximab (n=9)								
2.7	9.6	0.5	17.7	8	9	625	0.8	CS, HCQ
2.7	12.7	0.5	2.7	8	36	27	1.1	CS
3.0	10.9	1.4	9.1	2	9	115	1.2	CS, HCQ
4.6	15.4	0.8	12.5	2	20	13	0.6	CS, HCQ
3.2	13.9	0.7	1.3	2	15	58	1.1	AZT, HCQ
4.6	7.0	1.1	9.5	4	9	17	0.7	HCQ, MMF
4.7	12.2	0.8	6.6	2	72	153	0.8	CS, HCQ
3.4	18.0	0.7	5.2	2	120	975	0.9	MTX, CS
5.2	16.8	1.0	7.0	4	6	103	1.3	CS
Patients who developed low serum IgM levels after rituximab (n=8)								
1.2	7.3	0.2	0.1	2	168	236	1.2	CS, HCQ
2.9	4.3	0.1	2.0	8	24	37	0.9	CS, HCQ
1.7	8.7	0.1	19.2	4	24	2	1.0	CS, HCQ
2.9	11.7	0.4	3.7	2	60	254	0.9	AZT, CS
1.2	14.4	0.3	7.0	4	24	246	1.0	CS, HCQ
1.9	13.7	0.3	6.4	2	10	74	0.8	AZT, CS
0.6	4.3	0.1	15.9	4	48	212	0.8	CS, MMF
1.8	5.2	0.3	16.8	6	24	76	1.4	HCQ, MMF

Abbreviations: Rituximab, RTX; dsDNA (double stranded DNA); C3, complement component-3; HCQ, Hydroxychloroquine; CS, corticosteroids; AZT, azathioprine; MMF, mycophenolate mofetil