Rheumatology Advance Access published September 30, 2009

 doi:10.1093/rheumatology/kep289

Concise Report

Impaired C3b/iC3b deposition on *Streptococcus pneumoniae* in serum from patients with systemic lupus erythematosus

Fiona Goldblatt[1], Jose Yuste[2], David A. Isenberg[1], Anisur Rahman[1] and Jeremy Brown[2]

Objective. To determine whether opsonization of *Streptococcus pneumoniae* with C3b/iC3b is impaired in serum from patients with SLE.
Methods. The ability of serum samples from 30 patients with SLE, 20 with non-SLE rheumatic diseases (RA, PsA, AS, SS) and 20 healthy controls to opsonize *S. pneumoniae* (strains D39 and Io11697) with C3b/iC3b was assessed using a standardized FACS technique and a FACSCalibur flow cytometer. Results were compared among the three groups using analysis of variance, followed by pairwise comparisons among groups using the Mann–Whitney U-test.
Results. The proportion of bacteria positive for C3b/iC3b was significantly lower in serum from patients with SLE (strain D39: 60.3% ± S.E.M. 2.87, strain Io11697: 55.3% ± 3.8) compared with healthy controls (strain D39: 70.6% ± 2.0, $P = 0.01$; strain Io11697: 67.8% ± 2.6; $P = 0.05$) and non-SLE rheumatic controls (strain D39: 69.8% ± 3.1; $P = 0.03$). For the patients with SLE, there was no association between C3b/iC3b deposition and serum complement levels or measurable classical pathway activity. C3b/iC3b deposition on *S. pneumoniae* was significantly lower in serum from SLE patients with a past history of pneumonia ($n = 3$) compared with those without ($n = 27$; $P = 0.03$).
Conclusions. Opsonization of *S. pneumoniae* with C3b/iC3b was significantly reduced in serum from patients with SLE compared with non-SLE rheumatic disease and healthy controls. Failure to appropriately activate the immune system via complement may contribute to the increased susceptibility of SLE subjects to infections, and may correlate with a risk of pneumonia in a subgroup of SLE patients.

Key words: Systemic lupus erythematosus, Infection, Opsonization, C3, *S. pneumoniae*.

Introduction

Infection is a leading cause of morbidity and mortality for patients with SLE, affecting 11–45% of patients and causing 20–55% of all deaths [1, 2]. A broad spectrum of infectious pathogens has been reported to cause disease in patients with SLE, including unusual opportunistic infections and common pathogens that often behave more aggressively than in the healthy population [3, 4]. The increased susceptibility of patients with SLE to infection is likely to be due to a combination of factors, including underlying immune dysregulation, certain disease manifestations and immunosuppressive therapies.

The Gram-positive bacterium *Streptococcus pneumoniae* is a serious pathogen and a common cause of community-acquired pneumonia, meningitis and septicaemia. Case reports and small series indicate that patients with SLE have an increased frequency and severity of *S. pneumoniae* infections, accounting for 6–18% of all bacterial infections in these patients [1, 4, 5], but the mechanisms underpinning this increased susceptibility have not been clearly defined. Immunity to *S. pneumoniae* is highly dependent on the complement system [6, 7], a major component of the innate immune response. Each complement pathway leads to the formation of a C3 convertase that opsonizes target pathogens with C3b and iC3b derived from the cleavage of the serum protein C3. The classical pathway seems to play a central role for immunity against *S. pneumoniae*, and together with intact IgG2 and splenic function is essential for optimal bacterial opsonization and phagocytosis [7]. Dysregulation of the complement system is important in SLE and defined inherited deficiencies of complement components increase the risk of developing SLE [8]. Classical pathway abnormalities found in patients with SLE could be one mechanism explaining the susceptibility of patients with SLE to *S. pneumoniae* infections through impaired opsonization of bacteria with C3b/iC3b [9–11]. However, to date, there has been a paucity of studies examining the ability of SLE patients' sera to opsonize encapsulated bacteria, and whether functional effects on complement activity could explain some of the increased susceptibility of these patients to infections is not known [10, 12]. Consequently, we have investigated C3b/iC3b deposition on *S. pneumoniae* in serum obtained from our large cohort of British patients with SLE followed at University College London Hospital (UCLH), in comparison with serum from healthy people or those with other autoimmune rheumatic diseases.

Methods

Subjects

The Camden and Islington Community Local Research Ethics Committee granted ethical approval for this study and informed consent was obtained from all participants. Serum samples were collected from three groups of subjects: (i) 30 consecutive consenting patients with SLE attending the lupus clinic at UCLH (all fulfilled the 1997 revised ACR classification criteria for SLE) [13]; (ii) 20 patients fulfilling relevant published criteria for non-SLE rheumatic diseases, who attended other rheumatology clinics at UCLH [RA ($n = 12$); PsA ($n = 4$); AS ($n = 3$); and SS ($n = 1$)]; and (iii) 20 healthy controls without autoimmune disease. SLE disease activity was assessed as per the BILAG index [14] at the time of obtaining the serum sample. High activity was defined as a global BILAG score >6. Clinical information regarding previous serious infections in the patients with SLE was obtained by retrospective review of case notes and electronic database.

[1]Department of Medicine, Centre for Rheumatology, University College London Hospital and [2]Department of Medicine, Centre for Respiratory Research, Royal Free and University College London Medical School, Rayne Institute, London, UK.

Submitted 26 March 2009; revised version accepted 6 August 2009.

Correspondence to: Fiona Goldblatt, Centre for Rheumatology, Department of Medicine, University College London Hospital, 3rd Floor The Windeyer Building, 46 Cleveland Street, London W1T 4JF, UK. E-mail: fgoldblatt@ntlworld.com

C3/iC3b deposition assays

A *S. pneumoniae* capsular serotype 2 (strain D39) used extensively for laboratory studies of pathogenesis and a capsular serotype 23F (strain Io11697; gift from Brian Spratt, Imperial College London), which is commonly associated with clinical disease was used in this study. An established flow cytometry assay was used to detect C3/iC3b deposition on *S. pneumoniae* [6]. Bacterial stocks were thawed and washed by centrifugation at 20 000 *g* for 6 min, followed by resuspension in phosphate buffered saline (PBS). Bacterial aliquots were pelleted and resuspended in 10 μl human sera diluted 1 : 6 in PBS/0.1% Tween-20, incubated for 20 min at 37°C, then washed twice with 500 μl of PBS/0.1% Tween-20. Samples were then combined with 50 μl of FITC-conjugated polyclonal goat anti-human C3 antibody (ICN Cappel, Aurora, OH, USA) diluted 1 : 300 in PBS/0.1% Tween-20 and incubated for 30 min on ice. The bacteria were washed twice in PBS/0.1% Tween-20, fixed in 100 μl of 3% paraformaldehyde (Sigma-Aldrich, Gillingham, Dorset, UK) and resuspended in 100 μl of PBS for flow cytometry analysis. The proportion of bacteria positive for C3 (representing C3b/iC3b deposition on the bacterial surface) and mean fluorescence intensity (MFI) was obtained using a standardized FACS technique and a FACSCalibur flow cytometer (Becton Dickinson, San Jose, USA), collecting data from at least 20 000 bacteria. Calibration with negative controls using bacteria incubated in PBS and then with the FITC goat anti-human C3 antibody (ICN Cappel) was performed prior to each experiment, and is known to give similar results with C3-depleted sera [6]. Samples were blinded and each serum was tested three times and a mean value for the proportion of bacteria positive for C3b/iC3b binding was calculated. Inter-experiment variability was assessed by testing one index SLE sample and one index healthy control sample in all experiments. Statistical analysis was performed using GraphPad Prism software (San Diego, CA, USA). Results were compared among the three groups using analysis of variance, followed by pairwise comparisons among groups using Mann–Whitney U-tests ($P \leqslant 0.05$ significance).

Classical complement pathway functional activity

Human haemolytic complement component activity by the classical pathway was assessed by measuring the radial zones of lysis by serum through an agarose gel containing sheep erythrocytes sensitized with a rabbit anti-sheep erythrocyte antibody (The Binding Site, Birmingham, UK). Twenty patients with SLE (10 with the lowest and 10 with the highest C3b/iC3b deposition on *S. pneumoniae*) were screened. Calibrator and control samples were reconstituted with pre-cooled distilled water, followed by preparation of one-half and one-quarter dilutions of the calibrator in physiological saline. Five microlitres of each calibrator sample and 5 μl of fresh serum samples (in duplicate) were applied to the plate, diffusion was allowed for 18 h at 4°C then plates were incubated at 37°C for a minimum of 30 min. Zones of lysis were measured using a jeweller's eyepiece and a calibration curve plotted. Results were then read from the curve.

Results

Subject characteristics

Of the 30 patients, 23 with SLE were females (76.6%). The sample was representative of the whole cohort attending the specialized SLE clinic at UCLH with regard to age [mean age (S.D.) of 41.5 ± 11.2 years compared with a mean age of the whole cohort 45.3 ± 13.8 years in December 2006] and ethnicity (36% Caucasian, 23.3% Afro-Caribbean and 16.7% Asian). The average ages for patients with non-rheumatic diseases and healthy controls were 58.2 ± 12.8 and 31.6 ± 6.3 years, respectively. Of the non-SLE rheumatic controls, 20% were Asian and 80% Caucasian; whereas, of the healthy controls, 55% were Caucasian, 25% Asian, 10% Afro-Caribbean and 10% Oriental. Of the SLE patients, 27% had a history of renal disease, comparable with 32% in the whole SLE cohort (difference not significant, $0.10 < P < 0.05$ by chi-squared studies). Medication at the time serum was collected is presented in Table 1. The drug regimens are consistent with mildly active SLE, which was also reflected in the fact that the median global BILAG score was 2 and only three patients had global BILAG score >7.

Reduced C3b/iC3b deposition on S. pneumoniae *in serum from patients with SLE*

C3b/iC3b deposition studies were performed using two *S. pneumoniae* strains, D39 and Io11697. There was little inter-experimental variability in the results obtained with the index SLE and healthy samples (<3% for both, data not shown in full). There was a considerable variation in the proportion of bacteria positive for C3b/iC3b in serum from different patients with SLE (range 21–83% for strain D39 and 9–90% for strain Io11697). The range for serum obtained from controls was smaller (healthy controls 55–83% for strain D39 and 49–87% for strain Io11697; non-SLE rheumatic diseases 47–90% for strain D39). Overall, the mean proportion of bacteria positive for C3b/iC3b was significantly lower in serum from patients with SLE (strain D39: 60.3% ± S.E.M. 2.87, strain Io11697: 55.3% ± 3.8) compared with healthy controls (strain D39: 70.6% ± 2.0, $P = 0.01$; strain Io11697: 67.8% ± 2.6, $P = 0.05$; Fig. 1A and B) and to non-SLE rheumatic controls (strain D39: 69.8% ± 3.1, $P = 0.03$; Fig. 1A). There was no significant difference in C3/iC3b deposition on *S. pneumoniae* strain D39 bacteria between non-SLE rheumatic controls and healthy controls ($P = 0.9$; Fig. 1A). The geometric mean for MFI of C3b/iC3b deposition was significantly lower in serum from patients with SLE (strain D39: 113.2 ± 8.9, strain Io11697: 56.9 ± 12.6) compared with healthy controls (strain D39: 137 ± 11.8, $P = 0.09$; strain Io11697: 74.4 ± 9.7; $P = 0.007$). The difference in the geometric mean of MFI with strain D39 was not significantly different between serum from patients with SLE compared with non-SLE rheumatic controls ($P = 0.5$) or non-SLE rheumatic controls and healthy controls ($P = 0.5$). There was an excellent correlation between the results of C3b/iC3b deposition for the two *S. pneumoniae* strains obtained with the same serum ($r = 0.7$; Table 1 and Fig. 1C). As a consequence of this and limited serum availability, C3b/iC3b deposition studies with serum from non-SLE rheumatic controls were performed using only one *S. pneumoniae* strain (D39). C3b/iC3b deposition on *S. pneumoniae* was lower in serum from the small number of SLE patients with a past history of pneumonia ($n = 3$) compared with those without ($n = 27$; 38.9% ± 10.7 and 62.7% ± 2.5, respectively; $P = 0.03$).

For both strains of *S. pneumoniae*, the mean proportion of bacteria positive for C3b/iC3b was not significantly different between SLE patients with and without a history of renal involvement (strains D39 and Io11697, $P > 0.05$, respectively). There was no association between the results for C3b/iC3b deposition and serum C3 levels for both strains (strain D39: $r = -0.04$; strain Io11697: $r = -0.05$; Fig. 1D). Classical complement pathway functional activity was normal in all except four patients and none had reduced C3b/iC3b deposition on *S. pneumoniae* (Table 1). Patients with elevated anti-dsDNA antibody titres (ELISA, Shield Diagnostics, Dundee, Scotland; upper limit of normal in our laboratory = 50 IU/ml) or global BILAG score >6 did not have different serum C3b/iC3b deposition on *S. pneumoniae* than those with normal anti-dsDNA (chi-square, $P > 0.10$) or lower BILAG scores (chi-square, $P > 0.10$). Mean prednisolone doses at the time of obtaining the serum sample were not significantly different between patients whose serum exhibited impaired or normal C3b/iC3b deposition on *S. pneumoniae* (6.1 ± 0.8 and 4.9 ± 1.0 mg/day, respectively;

TABLE 1. Immunological and clinical features of the patients with SLE screened for C3b/iC3b deposition on *S. pneumoniae*

Patient code	Age, years	C3b/iC3b deposition, %		Complement levels, g/l		Recent SLE medications[a]	Global BILAG Score[b]	CP activity[c], CH100 U/ml (392–1019)
		D39	Io11697	C3 (0.8–1.5)	C4 (0.1–0.4)			
S3[d]	49	21.2	9.2	0.45	0.05	Pred 10 mg, MMF	4	520
S16	53	33.0	37.9	1.38	0.34	Pred 5 mg, AZA	2	1037
S29[d]	55	37.6	15.0	1.37	0.21	Pred 10 mg, SMP 500 mg tds	5	520
S7	43	37.8	19.8	0.67	0.13	Pred 10 mg, AZA	5	870
S6	31	39.1	29.9	1.05	0.06	Pred 2.5 mg	4	390
S20	53	47.4	38.9	1.3	0.41	Nil	1	1040
S2	39	50.9	47.6	0.86	NA	Pred 7 mg, AZA	4	1040
S27	36	55.7	56.1	0.84	NA	Pred 5 mg, HCQ	2	620
S11	67	56.6	70.7	1.26	0.25	Nil	1	730
S28	49	57.6	73.4	1.17	0.25	Nil	7	1040
S23[d]	38	58.0	40.0	0.81	0.22	Pred 5 mg, MTX	2	ND
S15	41	58.9	78.3	0.76	0.27	Pred 5 mg, HCQ	7	ND
S1	21	59.1	53.2	0.6	0.05	Pred 5 mg, HCQ, MTX	2	ND
S8	58	60.3	57.8	1.03	0.31	Pred 1 mg	5	ND
S25	57	60.6	31.5	0.96	0.16	Pred 2 mg, AZA	5	ND
S1	33	61.1	38.7	1.26	0.24	HCQ	1	ND
S14	46	63.5	47.8	1.36	NA	Pred 5 mg	2	ND
S9	47	65.1	72.6	0.84	0.08	Pred 3 mg	7	ND
S18	41	65.3	66.5	0.68	NA	Pred 2.5 mg, AZA, HCQ	1	ND
S10	52	68.8	57.2	0.64	NA	Nil	2	ND
S12	32	69.0	54.3	0.89	0.25	Pred 5 mg, HCQ	3	>1040
S17	31	70.2	71.3	1.04	0.15	Pred 5 mg, HCQ	3	500
S26	21	71.4	64.3	0.59	0.10	Pred 5 mg, MMF	2	>1040
S30	37	73.6	79.3	0.72	0.10	HCQ	2	1040
S21	30	74.4	53.7	0.79	0.68	Pred 12.5 mg, HCQ	1	300
S19	39	74.9	73.3	1.63	NA	HCQ	1	>1040
S4	42	76.2	74.8	0.76	0.16	Pred 10 mg, AZA	4	<260
S5	24	77.5	70.5	1.32	0.31	Nil	1	<260
S22	42	81.0	85.5	1.27	NA	Pred 3 mg, HCQ	1	620
S24	39	83.9	90.2	0.87	NA	Nil	1	<260

[a]Daily, unless otherwise specified. [b]Global BILAG score at time sera obtained (active disease >6). [c]Classical complement pathway functional activity. [d]Previous hospitalization with pneumonia (organism not identified). Time period between onset of pneumonia and research serum sample was 4 years for patient S3, 1 year for patient S29 and 2 years for patient S23. ND: not done; C3 and C4: complement components 3 and 4, respectively; NA: not available; Pred: prednisolone; SMP: sulphamethoxypyridazine; MMF: mycophenolate mofetil.

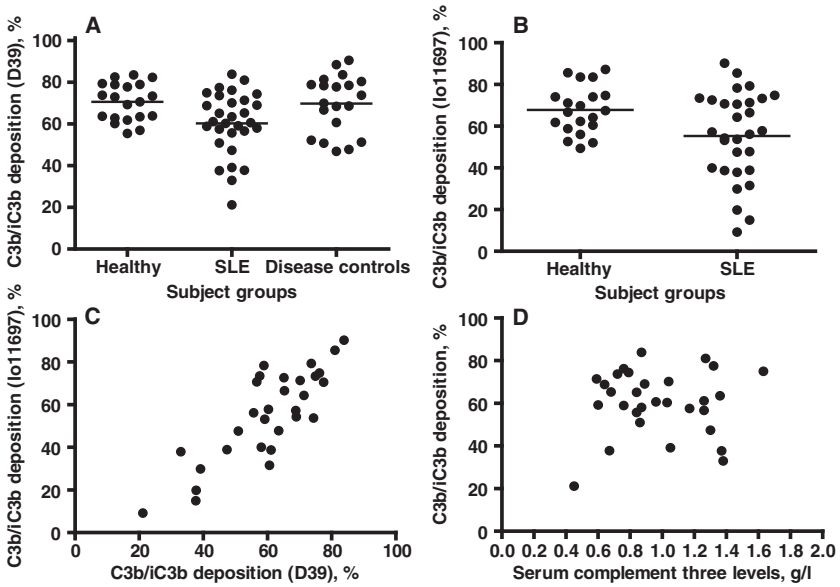

FIG. 1. A flow cytometry assay was used to detect C3b/iC3b deposition on *S. pneumoniae* in human serum from healthy controls, patients with SLE and non-SLE rheumatic controls. Results are expressed as a percentage of bacteria positive for C3b/iC3b. The mean proportion of bacteria positive for C3b/iC3b deposition was significantly lower in serum from patients with SLE compared with the healthy controls when using (**A**) *S. pneumoniae* strain D39 (capsular serotype 2; $P = 0.01$) and (**B**) *S. pneumoniae* strain Io11697 (capsular serotype 23F; $P = 0.05$). The mean proportion of bacteria positive for C3b/iC3b deposition in SLE serum was also significantly lower than non-SLE rheumatic controls [(**A**); $P = 0.03$]. (**C**) Correlation of C3b/iC3b deposition on *S. pneumoniae* strain D39 (capsular serotype 2) and strain Io11697 (capsular serotype 23 F) using serum from patients with SLE ($r = 0.7$). (**D**) Lack of correlation between C3b/iC3b deposition on *S. pneumoniae* strain D39 in serum from patients with SLE and serum C3 levels ($r = -0.04$).

$P > 0.05$). HCQ was more frequently taken by those with normal C3b/iC3b deposition on *S. pneumoniae* compared with those with impaired deposition. There were no differences with any of the other lupus medications (Table 1). Vaccination status against *S. pneumoniae* was unavailable for the SLE samples and known for half of the non-SLE rheumatic controls (60% had received vaccination against *S. pneumoniae*). Mean C3b/iC3b deposition between vaccinated and non-vaccinated controls was not

significantly different (79.7% ± 3.6 and 80.3% ± 1.3, respectively; $P = 0.9$).

Discussion

Patients with SLE frequently develop serious infections, and seem to have a particular susceptibility to *S. pneumoniae*. Both clinical and experimental data demonstrate that complement, particularly the classical pathway, is vital for innate and adaptive immune response to streptococcal pathogens [6, 7]. We demonstrated that deposition of C3b/iC3b on *S. pneumoniae* is significantly reduced in serum from patients with SLE compared with non-SLE rheumatic disease controls and healthy individuals. Given the importance of complement for immunity to *S. pneumoniae*, this may contribute to the increased susceptibility of SLE patients to *S. pneumoniae* and possibly to other pyogenic bacterial infections. In this way, the reduced opsonization of *S. pneumoniae* may reflect a similar mechanism to previously described abnormalities in opsonization causing impaired clearance of apoptotic cells or tissue deposition of immune complexes thought to contribute to the pathogenesis of SLE. The considerable range of results for opsonization of *S. pneumoniae* with C3b/iC3b within the SLE cohort suggests that a subgroup of SLE patients may be at particular risk of *S. pneumoniae* infection. The mechanism(s) underlying why opsonization of *S. pneumoniae* with C3b/iC3b is reduced in sera from SLE patients is not clear. Previous reports have demonstrated impaired phagocytosis of some bacterial pathogens in sera from SLE patients with active disease [10, 12], but we found no correlation of the C3b/iC3b deposition results with global BILAG scores. Hypocomplementaemia and inherited complement deficiencies in SLE have been well described [8], and given the importance of the classical pathway for complement deposition on *S. pneumoniae*, such deficiencies might be expected to explain our results. However, in our study, sera from SLE patients with impaired ability to opsonize *S. pneumoniae* with C3b/iC3b had normal classical pathway activity, and furthermore there was no correlation of total C3 levels with C3b/iC3b deposition results. These data are supported by other investigators who have shown that impaired phagocytosis of *Staphylococcus aureus* in sera from patients with active SLE did not correlate with levels of C3 or classical pathway components [12]. In contrast, other investigators have shown that opsonization defects in sera from SLE patients during periods of disease activity are independent of complement activity and maybe related to immune complex formation [10]. These conflicting data in combination with our results indicate that there are a variety of defects in immunity that can affect SLE patients, and that perhaps these vary between pathogens and with disease activity. Further investigations are required to define the mechanisms underlying the defect in complement deposition on *S. pneumoniae* in sera from patients with SLE. Although it is interesting that sera from patients known to have had a previous episode of pneumonia (the commonest cause of which is *S. pneumoniae*) had reduced ability to opsonize *S. pneumoniae* with C3b/iC3b, there were too few of these patients to draw definite conclusions and further investigation in SLE patients with proven or suspected *S. pneumoniae* infections is required.

In summary, our results demonstrate for the first time impaired deposition of C3b/iCb on *S. pneumoniae* in serum from patients with SLE when compared with healthy individuals and non-SLE rheumatic disease controls. As a consequence, this may result in inadequate activation of the immune system via complement and thereby contribute to the increased predisposition to *S. pneumoniae* infections in some patients with SLE. Testing sera from patients with SLE for impaired opsonization of *S. pneumoniae* may allow those patients most at risk of infection with *S. pneumoniae* (and perhaps other pathogens) to be identified, and consequently improve the targeting of preventative measures.

Rheumatology key messages

- A subgroup of SLE patients has impaired C3b/iC3b opsonization of *S. pneumoniae*.
- Inadequate complement activation of the immune system may increase SLE subjects' susceptibility to *S. pneumoniae* infections.

Acknowledgements

F.G. was supported by The Neil Hamilton Fairley Post Doctoral Fellowship (National Health & Medical Research Council, Australia). J.Y. was supported by the British Lung Foundation project grant (grant P05/3).

Disclosure statement: The authors have declared no conflicts of interest.

References

1 Petri M. Infection in systemic lupus erythematosus. Rheum Dis Clin North Am 1998; 24:423–56.
2 Cervera R, Khamashta M, Font J *et al.* Morbidity and mortality in systemic lupus erythematosus during a 5-year period: a multicenter prospective study of 1,000 patients. Medicine 1999;78:167–75.
3 Petri M, Genovese M. Incidence of and risk factors for hospitalizations in systemic lupus erythematosus: a prospective study of the Hopkins Lupus Cohort. J Rheumatol 1992;19:1559–65.
4 Naveau C, Houssiau F. Pneumococcal sepsis in patients with systemic lupus erythematosus. Lupus 2005;14:903–6.
5 Hill M, Karsh J. Invasive soft tissue infections with *Streptococcus pneumoniae* in patients with systemic lupus erythematosus: case report and review of the literature. Arthritis Rheum 1997;40:1716–19.
6 Brown J, Hussell T, Gilliland S *et al.* The classical pathway is the dominant complement pathway required for innate immunity to *Streptococcus pneumoniae* infection in mice. Proc Natl Acad Sci USA 2002;99:16969–74.
7 Yuste J, Sen A, Truedsson L *et al.* Impaired opsonization with C3b and phagocytosis of *Streptococcus pneumoniae* in sera from subjects with defects in the classical complement pathway. Infect Immun 2008;76:3761–70.
8 Pickering M, Botto M, Taylor P, Lachmann P, Walport M. Systemic lupus erythematosus, complement deficiency, and apoptosis. Adv Immunol 2000;76:227–324.
9 Nived O, Odeberg H, Svensson B. Monocyte in vitro function and systemic lupus erythematosus (SLE). III Bactericidal activity in presence of SLE sera. Scand J Rheumatol 1980;31:53–5.
10 Jasin H, Orozco J, Ziff M. Serum heat labile opsonins in systemic lupus erythematosus. J Clin Invest 1974;53:343–53.
11 Petros D, West S. Overwhelming pneumococcal bacteraemia in systemic lupus erythematosus. Ann Rheum Dis 1989;48:333–35.
12 Nived O, Linder C, Odegerg H, Svensson B. Reduced opsonisation of protein A containing *Staphylococcus aureus* in sera with cryoglobulins from patients with active systemic lupus erythematosus. Ann Rheum Dis 1985;44:252–9.
13 Hochberg M. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arth Rheum 1997;40:1725.
14 Hay E, Bacon P, Gordon C *et al.* The BILAG index: a reliable and valid instrument for measuring clinical disease activity in systemic lupus erythematosus. Q J Med 1993;6: 447–58.