AUTOANTIBODIES AGAINST COMPLEMENT C1Q IN SYSTEMIC LUPUS ERYTHEMATHOSUS

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GENERAL SUMMARY

Complement and C1q, the first component of the classical pathway of activation of the complement system, are considered to be involved in the pathogenesis of systemic lupus erythematosus (SLE), a systemic autoimmune disease. In fact, C1q deficiency is the strongest disease susceptibility gene for human SLE. However, most SLE patients do not have primary C1q deficiency, but a substantial number of patients with SLE develop secondary hypocomplementemia with depletion of C1q and its deposition in affected tissues. Furthermore, autoantibodies against C1q (anti-C1q) are frequently found in SLE patients and they are strongly associated with the consumption of C1q as well as the occurrence of severe lupus nephritis.

Anti-nucleosome antibodies as a marker of active proliferative lupus nephritis

To date there is no gold standard to predict severe nephritis in patients with SLE. Like anti-C1q antibodies, anti-dsDNA antibodies and anti-nucleosome antibodies are also considered to be useful markers of severe lupus nephritis. Therefore we aimed to establish the true prevalence of anti-nucleosome and anti-dsDNA antibodies at the time of active proliferative lupus nephritis, and compared this to inactive SLE controls who either had or had not experienced nephritis in the past. Results were compared to anti-C1q that had previously been investigated in the same cohort of patients. In this study, anti-C1q autoantibodies had been shown to be an excellent marker of active proliferative lupus nephritis, which suggests a pathogenic role in SLE. In contrast, our data on anti-nucleosome antibodies and anti-dsDNA antibodies suggest that these autoantibodies are

of limited use in distinguishing patients with active proliferative lupus nephritis from SLE patients without active renal disease.

Autoantibodies against complement C1q specifically target C1q bound on early apoptotic cells

To better understand the pathogenic mechanisms in SLE, we intended to analyze the conditions that lead to an autoimmune response against C1q. Since anti-C1q are known to recognize neoepitopes on bound C1q but not on fluid phase C1q, we aimed to clarify the origin of anti-C1q by determining the mechanism that renders C1q antigenic. We analyzed the binding characteristics of anti-C1q antibodies, such as their ability to recognise C1q bound on different classes of immunoglobulins, on immune complexes and on cells undergoing apoptosis. Interestingly, we did not observe the binding of anti-C1q to C1q bound on immunoglobulins or immune complexes. However, anti-C1q were found to specifically target C1q bound on early apoptotic cells. Our findings provide a direct link between human SLE, apoptosis and C1q. Due to the exceptional presentation of neoantigens by the C1q molecule, our data suggest that early apoptotic cells are a major target of the autoimmune response in SLE.

Anti-C1q autoantibodies do not correlate with the occurrence of nephritis in lupus-prone MRL/MpJ+/+ mice

In SLE patients, a strong correlation between the occurrence of anti-C1q antibodies and lupus nephritis has been demonstrated. However, it is difficult to demonstrate in SLE patients whether anti-C1q titers are predictive for a renal flare. Therefore we conducted a follow-up study of lupus-prone MRL/MpJ+/+ mice with the aim to analyze the occurrence of anti-C1q autoantibodies and their correlation with the onset, type and

severity of nephritis. Despite the abundant and early presence of anti-C1q, they did not correlate with survival and severity of glomerulonephritis, contradicting our initial hypothesis. However, different pathogenic mechanisms in glomerulonephritis in lupus-prone MRL/MpJ+/+ mice and human proliferative lupus nephritis might account for the unexpected observation.

GENERAL INTRODUCTION

The complement system, C1q and SLE

The complement system is part of the innate immunity and one of the major effector mechanisms of antibody-mediated immunity. It has three main physiologic activities: defending against infections, bridging innate and adaptive immunity, and clearing immune complexes and apoptotic cells. Complement proteins are plasma and cell surface proteins that are normally inactive or only transiently active and become activated after they are attached to microbes or antibodies. There are at least three pathways of complement activation: (1) the classical pathway, which is usually activated by binding of the plasma protein complex C1 (composed of the large subcomponent C1q and the two smaller serine proteases C1r and C1s) to certain isotypes of antibodies that have bound to antigens; (2) the alternative pathway, which is activated by direct recognition of certain microbial structures, and (3) the lectin pathway, which is triggered by a plasma protein called mannose-binding lectin (MBL) that binds to mannose residues on microbes and other particles. The pathways are triggered enzyme cascades that all lead to the cleavage of C3 and consecutive activation of the terminal pathway of complement (C5 to C9). The terminal complement pathway, leading to the formation of a membrane-attack complex and the generation of the potent anaphylatoxin C5a, is a unique system that assembles several complement proteins to form a membrane pore (C5b-9) where complement was activated. This pore can cause lysis of a cell or microbe (Figures 1 and 2).

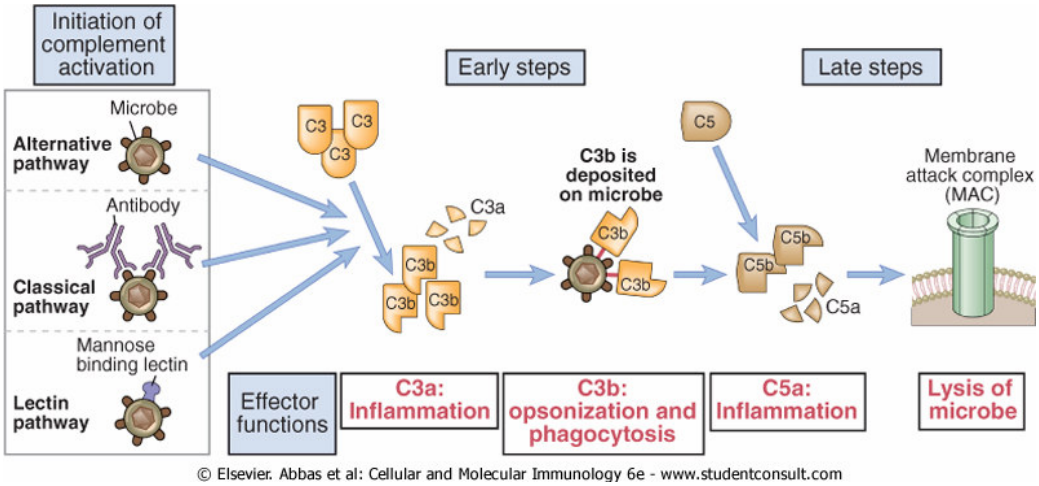

Figure 1. Pathways of complement activation. The activation of the complement system may be initiated by three distinct pathways, all of which lead to the production of C3b (the early steps). C3b initiates the late steps of complement activation, culminating in the production of peptides that stimulate inflammation (C5a) and the formation of the membrane-attack complex, so called because it creates holes in plasma membranes. The principal functions of major proteins produced at different steps are shown.

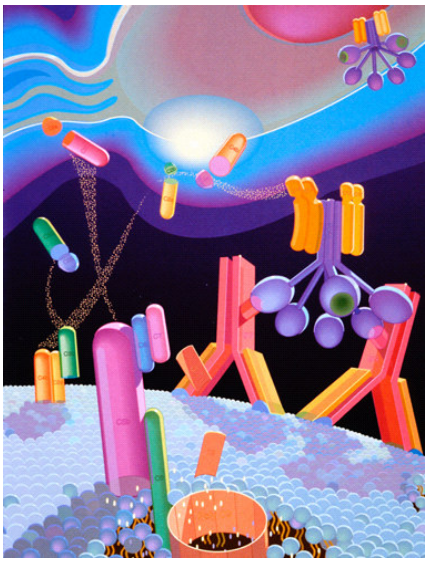

Figure 2. The classical pathway of complement activation. The classical pathway is initiated by the binding of C1 to antigen-complexed antibody molecules, that may be soluble, fixed on the surface of cells (as shown) or deposited on extracellular matrices.

Mammalian cells express regulatory proteins that block complement activation and thus prevent injury to normal host cells, such as membrane cofactor protein, decay-accelerating factor (DAF) or type 1 complement receptor (CR1). Furthermore, the complement system is regulated by several plasma proteins, such as C1 inhibitor, Factor I, Factor H and C4-binding protein (1-3).

Functions of the complement cascade and its components are best understood by looking at complement deficiencies: an autosomal dominant inherited disease called hereditary angioneurotic edema is caused by a deficiency of C1 inhibitor, a regulatory protein of the classical pathway of activation of the complement system. The mediators of edema formation include a proteolytic fragment of C2, called C2 kinin, and bradykinin. C1 inhibitor is also an inhibitor of other plasma serine proteases besides C1r and C1s, including kallikrein and coagulation factor XII, which can promote the formation of bradykinin in their activated form. Thus, C1 inhibitor deficiency not only affects complement but also the kinin system and the coagulation cascade. In contrast to deficiencies of regulatory proteins, deficiencies of components of the membrane attack complex, C3 and components of the alternative pathway are associated with infections. Deficiencies of components of the membrane attack complex are typically associated with *Neisseria* infections, *Neisseria meningitis* in particular, indicating that complement mediated cell lysis is particularly important in the defense against these bacteria. Deficiency of C3 is associated with frequent serious pyogenic bacterial infections (as well as membranoproliferative glomerulonephritis and rashes), indicating the central role of C3 and its importance in the defense against infections. Deficiencies in classical pathway components are less frequent and associated with bacterial infections, but also

with the occurrence of systemic lupus erythematosus (SLE), the archetype of a systemic autoimmune disease. The strength of the association and the severity of the disease are inversely correlated with the position of the deficient protein in the cascade (1-3). Homozygous deficiency of C1q, C1r and C1s, and C4 are strongly associated with susceptibility to SLE, whereas the prevalence of patients with a C2 deficiency is only about 10%. In fact, C1q deficiency is the strongest disease susceptibility gene for the development of human SLE (4, 5). SLE is characterized by the occurrence of a variety of autoantibodies, B-cell hyperactivity and immune complex formation (6, 7). It has been widely accepted that immune complex formation leading to complement activation is associated with tissue injury in SLE. However, evidence is lacking since this hypothesis is based on the association between complement activation and the presence of immune complexes and injured tissue.

Waste disposal hypothesis

A more recent theory on the pathogenesis of SLE is the so called 'waste disposal' hypothesis. This hypothesis assumes that SLE is driven by a defective clearance of dead and dying cells that could become antigenic and provoke an autoimmune response (8-11). Several studies provide support for this hypothesis: mice with a defect in the clearance of apoptotic cells were shown to develop severe autoimmunity with the occurrence of autoantibodies directed against nuclear components, as seen in SLE patients (12). Vice versa, lupus-prone mice were shown to have an impairment of apoptotic cell uptake (13) and macrophages derived from SLE patients were also shown to have a defective uptake of apoptotic cells (14). Furthermore a number of lupus antigens could be located on the surface of apoptotic bodies and apoptotic blebs (15) and it was demonstrated that

injection of an excess of apoptotic cells into healthy mice led to the production of autoantibodies (16). Therefore, it seems that apoptotic cells are the source of autoantigens that drive the autoimmune response in SLE.

Apoptosis and C1q

Independent of the findings mentioned above, C1q has been described to bind to apoptotic cells and to promote their clearance either directly or by complement activation (17-20). One ligand for C1q on apoptotic cells was shown to be phosphatidylserine (21). These reports were supported by the finding that C1q deficient mice have a delayed clearance of apoptotic cells and an accumulation of apoptotic bodies in the glomeruli (22).

C1q and SLE

Interestingly, C1q deficient mice also were shown to develop a lupus-like syndrome suggesting an additional link between C1q and SLE. This link is strengthened by the fact that homozygous C1q deficiency is the strongest disease susceptibility gene in human SLE, suggesting that complement, and especially C1q, is involved in the prevention of autoimmunity through its role in the clearance of dead and dying cells.

However, although hypocomplementemia is frequently found, most SLE patients do not have primary C1q deficiency but other links between C1q and SLE exist. Hypocomplementemia in SLE patients usually is due to consumption of C1q and other components of the classical pathway of complement (23), in particular during flares. In addition, C1q is deposited in affected tissues, such as the skin or the kidney (24-26). Furthermore, in about one third of SLE patients, autoantibodies against C1q (anti-C1q) develop and they are associated with complement consumption (27, 28). Anti-C1q were

shown to strongly correlate with the occurrence of biopsy-proven active lupus nephritis (29) and severe forms of lupus nephritis are rare in the absence of anti-C1q (30). Therefore anti-C1q are believed to have a pathogenic role in SLE, possibly resembling that of hereditary C1q deficiency.

Anti-C1q

Anti-C1q autoantibodies were first recognized in 1971 (31) and identified in 1988 (32, 33). They were mostly found in patients with SLE but the highest titers were observed in patients with the Hypocomplementemic Urticaria Vasculitis Syndrome (HUVS), which is closely related to human SLE (34). No apparent differences between the binding characteristics of anti-C1q from patients with SLE and HUVS could be found (35). Anti-C1q are mostly of the IgG isotype (36, 37) and antibodies of the IgG2 subclass were shown to constitute 5-74% of the total IgG anti-C1q detected (median IgG2-to-IgG1 ratio 1.4:1; the normal ratio of IgG2-to-IgG1 in serum is about 1:4) (38).

Anti-C1q autoantibodies have been reported to bind with high affinity and via the $F(ab')_2$ fragments to the collagen like region of the C1q molecule (32, 33). No cross-reactivity has been identified thus far (39, 40). In addition, our recent analysis of human monoclonal anti-C1q Fab autoantibodies generated by phage display from the bone marrow of an anti-C1q-positive SLE patient suggest that anti-C1q are specific, of high affinity and the result of an antigen-driven immune response (Schaller et al, *submitted*).

Interestingly, anti-C1q cannot be depleted by fluid phase C1q, suggesting that they bind to a neoepitope that is only expressed upon conformational changes that occur when C1q binds to a target structure (32). While in vitro, such a target structure could be, for

example, a polystyrene surface, so far the physiological ligand of C1q leading to the exposure of the epitope of anti-C1q is unknown.

Some of the open questions that have to be addressed at this stage

1. Are anti-C1q a better marker for severe lupus nephritis than other autoantibodies?

   So far there is no gold standard to predict severe nephritis in patients with SLE. Anti-dsDNA antibodies are considered to be the main diagnostic tool for SLE and a useful marker of disease activity. However, not all patients with active lupus nephritis have high and/or increasing titers of anti-dsDNA. More recent studies suggested anti-nucleosome antibodies are a useful marker in the diagnosis of active lupus nephritis (41-43). Anti-nucleosome antibodies were described to be more sensitive and to possess greater diagnostic efficiency than anti-dsDNA antibodies (44-47). Therefore, we aimed to establish the true prevalence of anti-nucleosome and anti-dsDNA antibodies at the time of active proliferative lupus nephritis compared to inactive SLE controls and compared to the occurrence of anti-C1q that had been investigated in the same cohort of patients.

2. What is the physiological ligand of C1q that makes it antigenic, i.e. that leads to the exposure of the neoepitope relevant for the binding of anti-C1q?

   SLE is widely accepted as an immune complex disease and C1q has been reported to be involved in the clearance of immune complexes (IC) (48). In addition it has been shown that C1q expresses neoepitopes when bound to IC

(49). Therefore, it was most likely that C1q bound to immune complexes is able to express neoepitopes that can be targeted by anti-C1q autoantibodies. However, in a situation of an impaired clearance of apoptotic cells, C1q bound on apoptotic cells might also become antigenic and drive the autoimmune response, similar to nuclear components that are normally not exposed to the immune system. Therefore, we aimed to better understand the conditions under which neoepitopes on C1q are exposed and lead to an anti-C1q autoimmune response. This analysis would be of importance since it would also lead to a better understanding of the pathogenic mechanisms in human SLE.

3. Comparison of human and mouse anti-C1q: do mouse anti-C1q also correlate with the occurrence of lupus nephritis as seen in SLE patients? If so, can anti-C1q be used to predict a renal flare?

   In SLE patients a strong correlation between the occurrence of anti-C1q antibodies and lupus nephritis has been demonstrated. Furthermore, a rise in anti-C1q titer was suggested to be predictive for a renal flare but, to date, has not been clearly demonstrated in patients (50). Since such a follow-up study is difficult to perform in patients with SLE (it would require a large cohort of SLE patients followed for many years), but possible in mice with the additional advantage of being able to receive complete histological data, we aimed to analyze a lupus-prone mouse strain (MRL/MpJ) for the occurrence of anti-C1q autoantibodies and their correlation with the onset, type and

severity of nephritis. Since nephritis occurs late in MRL/MpJ mice and in variable degrees of severity, this model of SLE was considered to best resemble the human situation. In addition, our study would also allow us to define whether this strain could be used for further *in vivo* studies of anti-C1q autoantibodies.

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AIM

The aim of my thesis was to investigate the possible pathogenic role of anti-C1q antibodies in SLE and thus contribute to the understanding of the pathogenic mechanisms of SLE and, possibly, related autoimmune diseases. This understanding is of importance for the development of new treatment strategies not only for patients with established severe lupus nephritis but also for the prevention of renal flares in SLE patients.

SECTION 1:

ANTI-NUCLEOSOME ANTIBODIES AS A MARKER OF ACTIVE PROLIFERATIVE LUPUS NEPHRITIS

Abstract

Anti-nucleosome autoantibodies were previously described to be a marker of active lupus nephritis. However, the true prevalence of anti-nucleosome antibodies at the time of active proliferative lupus nephritis has not yet been well established. Therefore the aim of this study was to define the prevalence and diagnostic value of autoantibodies against nucleosomes as a marker for active proliferative lupus nephritis.

In a prospective multicenter study, anti-nucleosome and anti-dsDNA antibodies were determined in 35 adult SLE patients at the time of the renal biopsy demonstrating active class III or IV lupus nephritis and compared to 59 control SLE patients.

Elevated concentrations of anti-nucleosome antibodies were found in 31/35 (89%) patients with active proliferative lupus nephritis compared to 47/59 (80%) control SLE patients. No significant difference between the two groups with regard to the number of positive patients ($p = 0.2$) or the antibody concentrations ($p = 0.2$) could be found. The area under the receiver-operator characteristic (ROC) curve as a marker of the accuracy of the test in discriminating between proliferative lupus nephritis and inactive/no nephritis in SLE was 0.581 (CI 0.47-0.70, $p = 0.2$).

Anti-nucleosome antibodies have a high prevalence in patients with severe lupus nephritis. However, our data suggest that determining anti-nucleosome antibodies is of limited help in the distinction of patients with active proliferative lupus nephritis from SLE patients without active renal disease.

Introduction

Systemic lupus erythematosus (SLE) is characterized by constitutional symptoms, specific inflammatory organ involvement and immunologic abnormalities. The cardinal immunologic finding is the occurrence of a variety of autoantibodies against nuclear, cytoplasmic and cell surface antigens. Recent studies suggested that nucleosomes, the basic element of chromatin and an ubiquitous product of apoptosis, are the primary autoantigen in SLE, responsible for the generation of a number of antinuclear autoantibodies (1,2). Furthermore, complexes of nucleosomes and autoantibodies were observed to bind to the glomerular basement membrane in vivo (3,4). A more recent study of lupus nephritis patients could demonstrate that glomerular electron-dense deposits contain apoptotic nucleosomes. These glomerular basement membrane-associated nucleosomes seem to be the target structure for nephritogenic autoantibodies (5). Independently, anti-nucleosome antibodies were described to be a useful marker in the diagnosis of active lupus nephritis (6-8). They seem to be more sensitive and to possess greater diagnostic efficiency than anti-dsDNA (9-14). These observations are of importance since at presence there is no gold standard to predict severe nephritis in patients with SLE (15). However, the true prevalence of anti-nucleosome antibodies at the time of active proliferative lupus nephritis and their diagnostic value in this situation has not been well established. In this prospective multicentre study we investigated the diagnostic value of anti-nucleosome antibodies as a marker for biopsy-proven active proliferative lupus nephritis.

Methods

Participants

In this prospective multicenter study all consecutive adult SLE patients undergoing renal biopsy for suspected proliferative lupus nephritis were included (16). Patients had to fulfill at least 4 out of the 11 American College of Rheumatology (ACR) criteria (17,18) to be at least 18 years old and to give written informed consent for the study participation. All patients were recruited between August 1998 and October 2006 at the University Hospitals in Basel, Geneva and Lausanne (Switzerland), Madrid University Hospital La Paz (Spain) and Prague Charles University (Czech Republic). Patients were excluded from the study if they were <18 years old, did not give written consent or did not fulfill at least 4 out of the 11 ACR criteria for the diagnosis of SLE. Renal histologies were classified according to the International Society of Nephrology/Renal pathology Society (19). For the present study only patients with class III or IV lupus nephritis were considered. According to the abbreviated version of the classification, combined classes III/V or IV/V were considered as class III or IV, respectively.

Serum samples were obtained at the time of the renal biopsy $\pm$ 7 days and stored at -80°C until further use.

Results obtained in the study population were compared to retrospectively analysed SLE patients without clinical signs of lupus nephritis at any time (n = 36) and SLE patients with biopsy-proven lupus nephritis (classes III or IV) without clinical disease activity at the time of the serum sampling (n = 23). All SLE control patients had to fulfill at least 4 out of the 11 ACR criteria for the diagnosis of SLE and were selected by availability of

sera and data on renal function only. The absence of lupus nephritis in these patients was defined as normal urinalysis results and creatinine levels. Inactivity of pre-existing lupus nephritis was defined as either normal or continuously decreasing or stable values for proteinuria, erythrocyturia (<20erythrocytes/field) and creatinine during the 6 months preceding the serum sampling.

Detection of autoantibodies

Autoantibody concentrations were measured independent from each other and blinded to the clinical state of the patients.

IgG-anti-nucleosome antibodies were measured using a commercial quantitative enzyme linked immunosorbent assay (ORGENTEC Diagnostika GmbH, Mainz, Germany) according to the manufacturer's instructions. The ELISA plate was pre-coated with nucleosomes isolated from human cell cultures. Sera in appropriate dilution were incubated for 30 minutes. Anti-nucleosome antibodies were detected using a HRP conjugated anti-human IgG antibody. The cut-off for a positive test result as determined by the manufacturer (20U/ml) was obtained by testing samples from 133 normal blood donors. The cutoff value was established as the mean value plus 3 standard deviations.

Anti-dsDNA antibodies of all immunoglobulin classes were measured by radio immunoabsorbent assay (Farr-assay) according to the manufacturer's instructions (IBL, Hamburg, Germany). The cut-off for a positive test result as determined by the manufacturer (7 IU/ml) corresponds to the 98 percentile obtained by testing blood samples from 590 healthy donors.

Statistical analysis

Patients groups were analyzed by Mann-Whitney U-test and Chi-square test, and receiver-operator characteristic (ROC) curves generated using GraphPad Prism version 4 (GraphPad Software, San Diego, CA, USA).

Results

Participants and test results

35 of the 40 SLE patients undergoing renal biopsy had class III or IV lupus nephritis. The patient characteristics are summarized in table I.

Anti-nucleosome antibodies were found in 31 of the 35 patients (89%) with biopsy-proven active proliferative lupus nephritis. In the control populations, 19 of 23 (83%) SLE patients with a history of lupus nephritis but without clinical signs of activity at the time of sampling and 28 of 36 (78%) SLE patients without clinical signs of lupus nephritis at any time were positive for anti-nucleosome antibodies ($p = 0.7$). Between the two SLE control populations alone, no significant differences were found with regard to the number of positive patients ($p = 0.7$) and the antibody concentrations ($p = 0.1$), but there was a trend towards higher titers in patients without clinical signs of lupus nephritis at any time. As shown in Figure 1 A, SLE patients with active proliferative lupus nephritis had a non-significant trend towards higher concentrations of anti-nucleosome antibodies when compared to control patients without or inactive lupus nephritis ($p = 0.2$).

In comparison, anti-dsDNA antibodies were found in 33 of the 35 patients with active proliferative lupus nephritis (94.3%) compared to 49 of 58 of SLE control patients without or inactive nephritis (84.5%) ($p = 0.16$). However, as demonstrated in Figure 1 B, titers of anti-dsDNA antibodies in patients with active proliferative lupus nephritis were significantly higher than in SLE controls ($p < 0.001$).

Table I. Patient characteristics.

	Patients with active Lupus nephritis Number (in %)	Patients with inactive Lupus nephritis Number (in %)	Patients without Lupus nephritis Number (in %)
Number of patients	35	23	36
Female/male	28 (80) / 7(20)	20 (87) / 3(13)	32 (89) / 4(11)
Age (median/range)	32/19-68	45/24-65	39.5/19-83
Ethnic groups			
Caucasians	34 (97)	23 (100)	36 (100)
Others	1 (3) asian	--	--
Active lupus nephritis			
Class III	10 (29)	--	--
Class IV	25 (71)		
Hypocomplementemia	33 (94)	7 (30)	12 (33)
ANA positive	35 (100)	22 (96)	34 (92)
Treatment at sampling			
Oral steroids	11 (31)	n/a	n/a
Other immunosuppressants	5 (14)		

Hypocomplementemia = low C3 and/or low C4; other immunosuppressants = azathioprine, mycophenolate mofetil, methotrexate, cyclosporine A or cyclophosphamid; n/a = not available

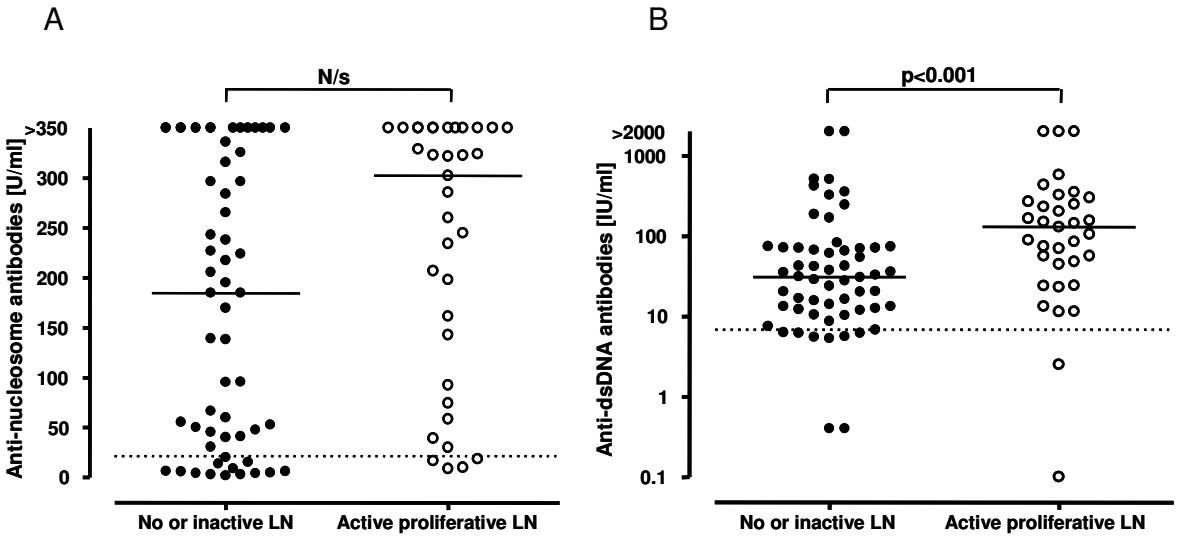

Figure 1. Anti-nucleosome antibodies and anti-dsDNA antibodies in SLE patients with inactive/no lupus nephritis versus active lupus nephritis. A. Anti-nucleosome antibody concentrations in SLE patients with inactive lupus nephritis (LN) at the time of blood sampling and without lupus nephritis at any time respectively versus SLE patients with active proliferative lupus nephritis ($p = 0.2$). **B.** Anti-dsDNA antibody concentrations in SLE patients with inactive lupus nephritis at the time of blood sampling and without lupus nephritis at any time respectively versus SLE patients with active proliferative lupus nephritis ($p < 0.001$).

N/s = not significant.

As a marker for active proliferative lupus nephritis, the anti-nucleosome assay showed a sensitivity of 88.6% and a negative predictive value of 76.5%. The specificity and the positive predictive value were 22.0% and 40.3% respectively. In order to better determine the accuracy of the assay in the discrimination between active proliferative lupus nephritis and inactive/no nephritis in SLE patients a receiver-operator characteristic

(ROC) curve was created (Figure 2 A). The area under the curve as a marker of the accuracy of the test was 0.581 (95% CI 0.465-0.697, p = 0.2).

As a marker for active proliferative lupus nephritis, the Farr assay had a sensitivity of 94.3% and a negative predictive value of 81.8%. However, the specificity of the assay was low (15.5%). The area under the ROC (Figure 2 B) curve as a marker of the accuracy of the test in discriminating between active proliferative lupus nephritis and inactive/no nephritis in SLE was 0.710 (95% CI 0.597-0.821, p < 0.001).

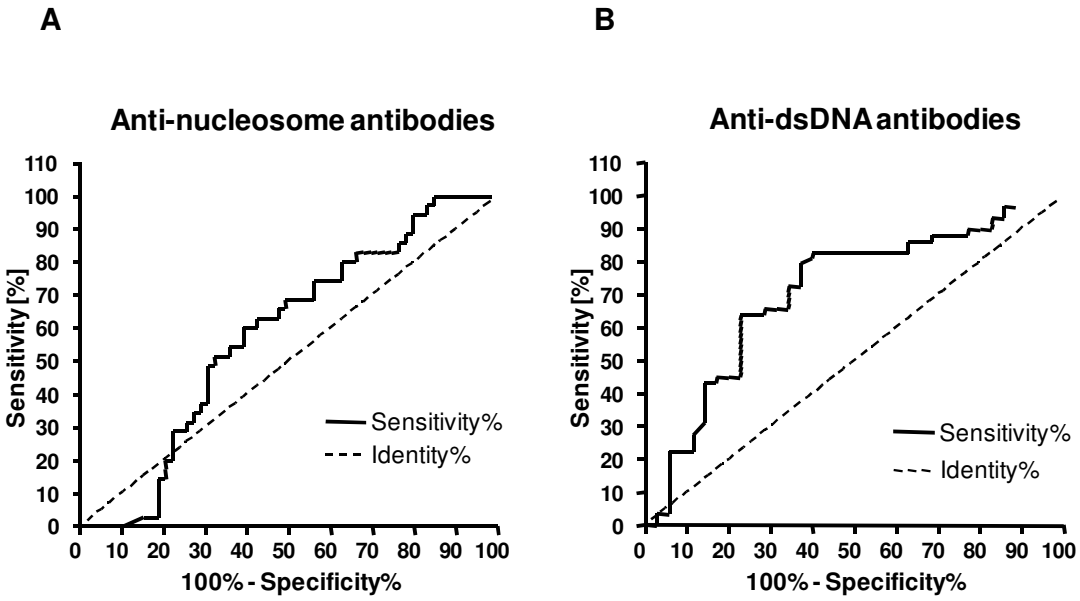

Figure 2. ROC curves for anti-nucleosome and anti-dsDNA antibodies for the discrimination between active and inactive/no lupus nephritis. A. ROC curve for anti-nucleosome antibodies for the discrimination between active and inactive/no lupus nephritis. The curve was generated using anti-nucleosome results from the study population and the SLE control patients shown in Figure 1 (area under the curve = 0.581 95% CI 0.47-0.70, p = 0.2). **B.** ROC curve for anti-dsDNA antibodies for the discrimination between active and inactive/no lupus nephritis. The curve was generated using anti-dsDNA results from the study population and the SLE control patients shown in Figure 1 (area under the curve = 0.710 95% CI 0.60-0.82, p < 0.001).

Discussion

The aim of this study was to determine the prevalence and the diagnostic value of autoantibodies against nucleosomes in the diagnosis of proliferative lupus nephritis. The determination of anti-nucleosome antibodies at the time of the renal biopsy demonstrating active proliferative lupus nephritis was considered to be the ideal tool to address the question since it should allow demonstrating maximum differences in autoantibody concentrations when compared to inactive SLE control patients. In this setting, the anti-nucleosome assay showed a high sensitivity and negative predictive value. However, in contrast to anti-dsDNA antibodies, anti-nucleosome antibodies were not significantly elevated when compared to control SLE patients with inactive disease indicating that the test is of limited help for the diagnosis of proliferative lupus nephritis.

Sensitivity and specificity of the anti-nucleosome assay in our study were in the same range as results obtained in previous studies using different assays and definitions of a positive test result (9,12,20). However, the timing of blood sampling in relation to the renal flare appears to be the most important difference to previous studies. In our study we determined anti-nucleosome antibodies at the time of the renal biopsy demonstrating class III or IV lupus nephritis in order to avoid influences by changes in disease activity or the possibility of a clinical misinterpretation of a renal flare.

A non-significant trend towards higher concentrations of anti-nucleosome antibodies was seen in patients with active proliferative lupus nephritis compared to patients with inactive/no nephritis. Thus, a larger cohort might have unmasked a statistically significant difference between the study populations as it was observed for anti-dsDNA

antibodies. However, our data suggest that determination of anti-dsDNA antibodies also seem not to be an ideal tool for the discrimination between patients with active lupus nephritis and patients without active renal disease. In contrast, in the same setting as the presented study, autoantibodies against complement C1q were shown to be an excellent marker of active proliferative glomerulonephritis in SLE patients with an area under the ROC curve of 0.96 ($p < 0.0001$) (16).

Complexes of nucleosomes and anti-nucleosome antibodies associated with the glomerular basement membrane are likely to be recognized by C1q. As a consequence C1q might become a target for autoantibodies itself leading to either further complement activation with consecutive local tissue destruction or an altered C1q-dependent clearance of the immune complexes (21). Therefore, one might speculate that both anti-nucleosome and anti-C1q antibodies would be required for the development of severe lupus nephritis.

In conclusion, our study shows a high prevalence of anti-nucleosome antibodies in patients with proliferative lupus nephritis but they are of limited help in distinguishing patients with active proliferative lupus nephritis from SLE patients without active renal disease.

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SECTION 2:

AUTOANTIBODIES AGAINST COMPLEMENT C1Q SPECIFICALLY TARGET C1Q BOUND ON EARLY APOPTOTIC CELLS

Abstract

Autoantibodies against complement C1q (anti-C1q) are frequently found in patients with systemic lupus erythematosus (SLE). They strongly correlate with the occurrence of severe lupus nephritis suggesting a pathogenic role in SLE. Since anti-C1q are known to recognize a neoepitope on bound C1q but not on fluid phase C1q, the aim of this study was to clarify the origin of anti-C1q by determining the mechanism that renders C1q antigenic.

We investigated anti-C1q from serum and purified total IgG of patients with SLE and hypocomplementemic urticaria vasculitis as well as two monoclonal human anti-C1q Fab fragments from a SLE patient generated by phage display. Binding characteristics such as their ability to recognise C1q bound on different classes of immunoglobulins, on immune complexes and on cells undergoing apoptosis were analyzed. Interestingly, anti-C1q did not bind to C1q bound on immunoglobulins or immune complexes. Neither did we observe specific binding of anti-C1q to C1q bound on late apoptotic/necrotic cells when compared to binding in the absence of C1q. However, as shown by FACS analysis and confocal microscopy, anti-C1q specifically targeted C1q bound on early apoptotic cells.

Anti-C1q were found to specifically target C1q bound on cells undergoing apoptosis. Our observations suggest that early apoptotic cells are a major target of the autoimmune response in SLE and provide a direct link between human SLE, apoptosis and C1q.

Introduction

Systemic Lupus Erytehmatosus (SLE) is a systemic autoimmune disease characterized immunologically by a variety of autoantibodies, B-cell hypereactivity and immune complex formation (1, 2). Complement, especially C1q the first component of the classical pathway of complement is considered to be involved in the pathogenesis of systemic lupus erythematosus (SLE). This view is based on the following observations: First, almost all patients with C1q deficiency develop a lupus-like syndrome with homozygous C1q deficiency being the strongest disease susceptibility gene for the development of SLE (3, 4). Second, a substantial number of patients with SLE develop hypocomplementemia with depletion of C1q and other components of the classical pathway of complement (5) and C1q is deposited in affected tissues (6, 7) . Last, in about one third of unselected SLE patients autoantibodies to C1q can be detected and they are strongly associated with the occurrence of severe lupus nephritis as well as the consumption of C1q (8-10).

A possible explanation for the strong link between complement C1q and SLE is the so called 'waste disposal' hypothesis (11, 12). This hypothesis assumes that SLE is driven by a defective clearance of dead and dying cells that could become antigenic and drive autoimmunity (13, 14). Experimental support for this hypothesis stems from the fact that mice with defined single gene defect leading to an altered clearance of apoptotic cells develop severe autoimmunity with the occurrence of autoantibodies directed against nuclear components as seen in SLE patients (15). Vice versa, lupus-prone mice were shown to have an intrinsic impairment in apoptotic cell uptake (16) and macrophages

derived from the peripheral blood of SLE patients also had a defective uptake of apoptotic cells (17). In addition, a number of lupus autoantigens could be located on the surface of apoptotic bodies and apoptotic blebs (18) and the injection of an excess of apoptotic cells in healthy mice could induce the production of autoantibodies (19) such as anti-nuclear, anti-ssDNA and anti-cardiolipin antibodies. Therefore, apoptotic cell debris seems to be the source of autoantigens in SLE. Independently, C1q has been described to be involved in the clearance of self-antigens generated during apoptosis by binding specifically to apoptotic keratinocytes and vascular endothelial cells (20-25). Furthermore, an accumulation of apoptotic cells could be observed in kidneys from C1q deficient mice. These findings suggested that in addition to its role in the clearance of immune complexes complement C1q is also involved in the prevention of autoimmunity through a role in the disposal of dead and dying cells.

However, although there are strong links from C1q to SLE, from SLE to apoptosis and from apoptosis back to C1q, no direct link of all three (SLE, C1q and apoptosis) has been established. Furthermore, since most patients with SLE do not have a primary deficiency of complement C1q, the pathogenic link between C1q and human SLE is not well understood.

Low levels of C1q, as often observed in human SLE, are due to the consumption of early components of the classical pathway of complement (5). Secondary hypocomplementemia in SLE is most often associated with autoantibodies against C1q (9, 10, 26, 27). Anti-C1q have been shown to strongly correlate with the occurrence of biopsy-proven active lupus nephritis (9) and severe forms of lupus nephritis in the absence of anti-C1q are rare (28). These findings suggest that anti-C1q have an active

role in the pathogenesis of SLE. Anti-C1q antibodies were first recognised in 1971 (29) and identified in 1988 (30, 31). They were mostly seen in patients with SLE but the highest titres have been observed in patients with the Hypocomplementemic Urticaria Vasculitis Syndrome (HUVS) that is closely related to human SLE (8, 32). In a comparative study no apparent differences between the binding characteristics of anti-C1q from patients with SLE and HUVS could be found (33). The antibodies were mostly of the IgG isotype (34, 35) and, in contrast to the binding of immune complexes, bound to the collagen-like region of C1q (CLR/C1q). The binding was of high affinity and mediated by Fab fragments. As shown for most of the other lupus-autoantibodies, no cross reactivity of anti-C1q with other antigens could be identified (36, 37). In addition, our recent analysis of human monoclonal anti-C1q antibodies generated by phage display from the bone marrow of a patient with SLE suggests that anti-C1q are specific, of high affinity and the result of an antigen-driven immune response (Schaller et al, *submitted*).

Interestingly, anti-C1q bind to a neo-epitope that, due to conformational changes, is only expressed on bound C1q. However, the precise epitope could not yet be identified and the mechanism that renders C1q antigenic had not been clarified. Since SLE is an immune complex disease and since C1q has originally been described to be involved in the clearance of immune complexes (IC), the most likely mechanism is that C1q after having bound to IC expresses one or several neo-epitopes that are targeted by anti-C1q. However, in the context of an impaired clearance of apoptotic material, it is also possible that C1q bound to the surface of apoptotic bodies becomes antigenic itself similar to nuclear components that are normally not exposed to the immune system. Therefore, understanding the conditions under which the neo-epitope of C1q is exposed leading to

an anti-C1q autoimmune response in SLE might provide a better understanding of the pathogenic mechanisms in SLE.

Materials and methods

Human anti-C1q autoantibodies

Patient serum

As a source of anti-C1q we used serum from patients with either SLE or Hypocomplementemic urticarial vasculitis syndrome (HUVS). SLE patients fulfilled at least 4 out of the 11 criteria of the American College of Rheumatology (ACR) (38, 39). HUVS patients had been described previously (8). Sera from healthy blood donors were used as a control.

Purified total IgG

Total IgG was purified from a healthy blood donor and one of the above described patients with HUVS (case 1) having developed mesangioproliferative glomerulonephritis, by Protein G affinity chromatography. This patient had low level ANA (1:80, speckled) and was negative for antibodies against double-stranded DNA, phospholipids and nucleosomes.

Preparation of human Fab anti-C1q antibodies by phage display

As described in a separate study, a bone marrow derived IgG_1 kappa/lambda (IgGκ/IgGλ) Fab phage display library from a SLE patient with high anti-C1q Ab titer had been screened against purified human C1q by ELISA. In short: After five panning rounds, the phagemid DNA from the third and fourth round of panning was prepared and

the gene III fragment removed by restriction enzyme digest, followed by transformation of XL1-blue cells in order to produce soluble IgG_1 Fabs (Schaller et al, *submitted*).

The screening yielded six Fab fragments (Fabs) showing the typical binding characteristics described for anti-C1q: The Fabs exhibited strong binding (affinity showing K_d in the range of 8.4×10^{-8}M to 1.4×10^{-7}M) to the neoepitopes expressed by polystyrene-bound C1q that could be located on the collagen-like region (CLR) of the molecule.

For the experiments presented in this study we used the clones A14 and B8 because they showed the highest signals of the 6 anti-C1q Fabs generated when tested for binding to C1q in ELISA.

Anti-C1q control antibodies

For the detection of deposited C1q different control antibodies were used:

Monoclonal mouse anti-C1q antibodies

Murine monoclonal anti-human C1q antibodies were generated by the immunization of $C1q_a$-deficient mice backcrossed for at least 10 generations on a BALB/c genetic background (kind gift of Prof. Marina Botto, Imperial College, London, UK) with purified human C1q (gift from Bühlmann Laboratories, Schönenbuch, Switzerland; >99% pure as judged by SDS PAGE) in collaboration with Prof. Ton Rolink (Immunology, Department for Biomedicine, University of Basel, Switzerland). In short, two times three mice were immunized subcutaneously with either 20µg C1q alone or 20ug C1q bound on heat aggregated IgG generated from purified total IgG of healthy BALB/c mice. BALB/c mice. One mouse in each of the two groups showing the highest

levels of anti-C1q in serum as judged by ELISA (see below) was sacrificed and the spleen was used to generate hybridomas according to a standard protocol (40).

Antibody secreting hybridoma cells were screened for anti-C1q by ELISA. In short, human C1q was coated overnight on ELISA plates (Nunc, Rosklide, Denmark) at a concentration of 0.5µg/ml. Supernatants of clones were diluted 1:1 in PBS Tween (0.05%), 1% FCS containing 1M NaCl. After incubation plates were washed and bound IgG was detected using biotinylated polyclonal goat anti-mouse IgG (SouthernBiotech, Alabama, USA) and horse radish peroxidase labeled Streptavidin (Jackson ImmunoResearch Europe, Suffolk, UK). Before expansion, anti-C1q producing hybridoma cell lines were selected by limited dilution and repeated testing for anti-C1q production. Nine (Figure 1) clones producing antibodies specific for human C1q could be generated that were able to bind C1q even under high salt conditions, i.e. in the presence of 1 M NaCl. All clones were also positive for murine C1q and showed no cross reactivity as judged by immunohistochemical staining of spleen sections from normal BALB/c mice compared to $C1q^a$ deficient mice (data not shown). Interestingly, whereas the binding of most of the generated murine monoclonal anti-human C1q antibodies to plate-bound C1q could be inhibited by the preincubation with fluid phase C1q, three out of the 14 clones (clones 23D11 and 34A4) could not be inhibited by fluid phase C1q even in antigen excess (Antibody:Antigen ratio in the range of 1:5-20). This lack of inhibition by fluid phase C1q indicated the binding to neoepitopes that are only expressed on bound C1q but not on fluid phase C1q. Binding of antibodies with the strongest signal to plate bound C1q with or without preincubation with fluid phase C1q is demonstrated in Figure 1.

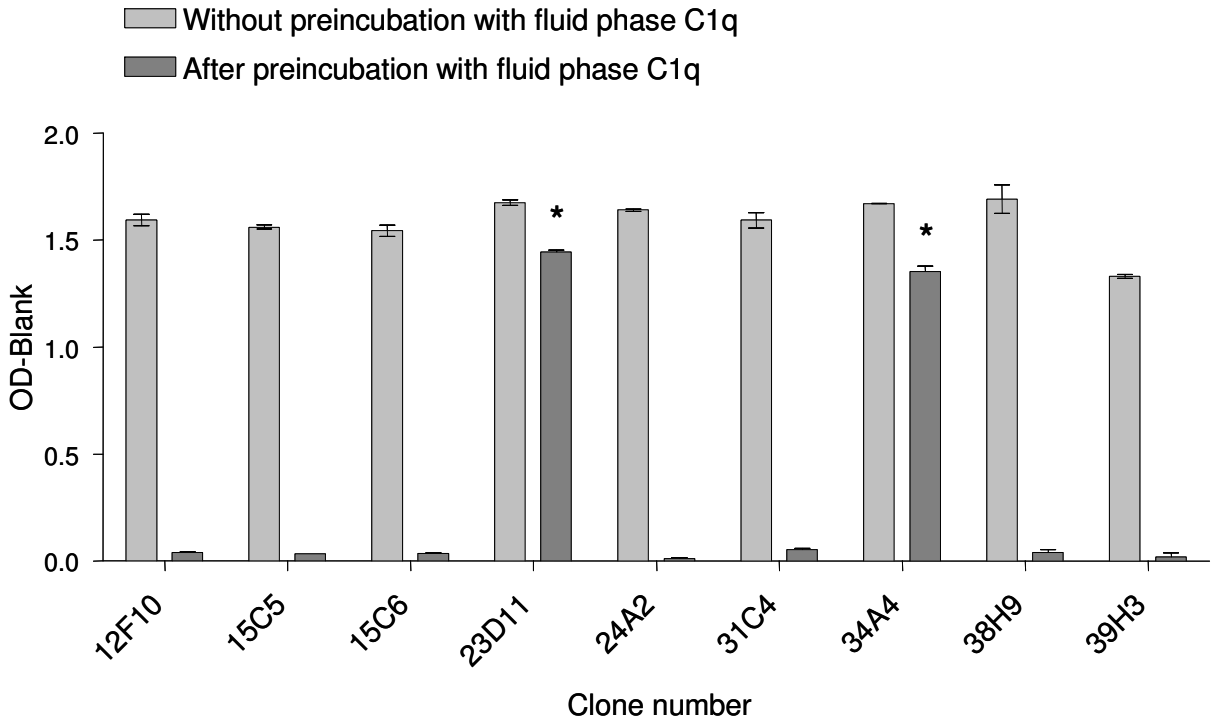

Figure 1: Binding of monoclonal mouse anti-human C1q antibodies to plate-bound C1q with or without preincubation with fluid-phase C1q.

Monoclonal mouse anti-human C1q generated in $C1q_a$-deficient mice were tested for their binding to C1q coated on ELISA plates with or without prior incubation with fluid phase C1q. Binding of most of the anti-C1q antibodies could be inhibited by preincubation with fluid phase C1q, whereas two clones, namely clone 23D11 and 34A4, could not be inhibited by fluid phase C1q even in a 5 to 10 times antigen excess indicated by *. This lack of inhibition by fluid phase C1q suggests the binding to neoepitopes that are only expressed on bound C1q.

For experiments performed in this study clones 12F10 and 23D11 were used. Whereas 12F10 bound C1q independent of its deposition, 23D11 only bound to neo-epitopes expressed on solid-phase C1q. Clones 12F10 and 23D11 were used to verify the successful binding of C1q to immunoglobulins, immune complexes, HAGG, polystyrene

beads and phosphatidylserine in ELISA assays. 12F10 and 23D11 could not be used to verify the successful binding of C1q to dsDNA and nucleosomes due to competition for the same epitopes.

Commercially available anti-C1q antibodies

A murine monoclonal antibody to purified human C1q (Quidel, San Diego, CA, USA) was used in order to verify the successful binding of C1q to nucleosomes and dsDNA in ELISA assays. The same antibody was FITC labeled (FluoroTag™ FITC conjugation kit, Sigma, Missouri, USA) according to the suppliers instructions and used for the detection of C1q on apoptotic cells together with a FITC conjugated rabbit anti-human C1q antibody (DakoCytomation, Glostrup, Denmark).

Binding of anti-C1q autoantibodies to C1q bound on immunoglobulin preparations or immune complexes

Polystyrene ELISA plates (Nunc, Rosklide, Denmark) were coated with either different unspecific monoclonal mouse antibodies IgM, IgA, IgG1, IgG2a, IgG2b and IgG3 at a concentration of 1µg/well (SouthernBiotech, Alabama, USA) or polyclonal goat anti-C1q sera (Quidel, San Diego, USA). Alternatively immune complexes were established by either coating with collagen type 1 (Sigma-Aldrich conc 1mg/ml) at a concentration of 0.0625µg/well in a total volume of 100ul or 0.5% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) in PBS. Precoating was followed by incubation (overnight at 4°C) with a monoclonal mouse anti-human collagen type 1 (Calbiochem, Merck,

Darmstadt, Germany) in a dilution of 1/2000 in 0.2M carbonate buffer (pH 9.6) or polyclonal rabbit anti-bovine albumin (Rockland Immunochemicals, Gilbertsville, PA, USA) in a dilution of 1/250 respectively. In addition to the BSA-anti-BSA complexes as explained before, we also established anti-BSA-BSA complexes by precoating polyclonal rabbit anti-BSA followed by incubation with 0.5% BSA. The plates (exept for the wells with the BSA-anti-BSA and anti-BSA-BSA complexes respectively) were then washed with PBS 0.05% Tween 20, and blocked with 0.5% BSA before incubation with C1q 0.5µg/well for 2 hrs at 37°C. After washing, anti-C1q positive sera of SLE and/or HUVS patients as well as normal human serum (NHS) diluted 1/50 in high salt buffer (1M NaCl, 1% FCS, PBS Tween 20 0.05%) were incubated for 2hrs at 37°C. C1q binding to the constructs was verified using a monoclonal mouse anti-C1q antibody in a dilution of 1/200 (clone 12F10 and 23D11). The detection was carried out with either a biotinylated monoclonal mouse anti-human IgG (SouthernBiotech, Alabama, USA) or biotinylated goat anti-mouse IgG_1 (SouthernBiotech, Alabama, USA) diluted in high salt buffer. Binding was detected by peroxidase-conjugated streptavidin (Jackson ImmunoResearch Europe, Suffolk, UK) followed by the addition of substrate (BD OptEIA, Becton Dickinson, NJ, USA) and a 4M H_2SO_4 stop solution. The absorbance was measured at 450nm (Global medical instrumentation inc. Ramsey, Minnesota, USA). The whole construct but without C1q served as a blank and was established for each construct separately. The results expressed as 'specific OD' were calculated by subtracting the corresponding blank.

In order to verify whether the immune complexes established *in vitro* were able to activate complement, NHS 1/50 was added to the construct and deposition of C4d was detected using a monoclonal mouse anti-human C4d (Quidel, San Diego, USA).

Depletion experiment with polystyrene beads or HAGG

Depletion of anti-C1q from sera of SLE and HUVS patients being highly positive for anti-C1q was performed using C1q bound to heat aggregated IgG (HAGG; IgG was isolated from NHS using a protein G column, then incubated for 30min at 65°C) versus C1q bound to polystyrene beads imitating the binding of C1q to polystyrene ELISA plates (Polybead, Polysciences Inc. Warrington, USA). After preincubation of the sera with HAGG-C1q or beads-C1q, the complexes were removed either by ultracentrifugation at 192'000 *g* for 1 h at 4°C (HAGG-C1q) or by centrifugation at 3200 *g* for 5min (beads-C1q) and the supernatants were tested for anti-C1q activity using a conventional anti-C1q ELISA as described previously (41, 42). The experiments were performed in different dilutions and values of normal human serum were used as blanks. Data is expressed as maximal depletion (in %) obtained when compared to sera depleted with HAGG or beads alone.

Cell populations

Jurkat (human T-cell leukemia) and Molt 4 (human T-cell leukemia) cells were cultured in RPMI 1640 with L-glutamine and 26mM HEPES (Gibco products, Invitrogen Corporation, NY, USA) with 10% fetal calf serum (FCS; Gibco products, Invitrogen Corporation, NY, USA). For the preparation of human peripheral blood T-cells,

peripheral blood mononuclear cells (PBMCs) from healthy donors were cultured in RPMI, 10% FCS, then stimulated with Phytohaemaglutinin (PHA; Remel Europe Ltd, Dartford, UK) (1µg/ml) and IL-2 (Novartis Pharma, Basel, Switzerland) (500U/ml). T-cells were isolated by CD3 positive selection with MACS beads (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany).

Binding of anti-C1q autoantibodies to C1q bound on apoptotic cells

Two million cells/ml were washed twice with RPMI 1640 (Gibco products, Invitrogen Corporation, NY, USA) without FCS in order to remove C1q from culture medium. Cells were then seeded in 6-well tissue culture plates (Falcon® Becton Dickinson, NJ, USA) at a concentration of 5 million/2.5ml per well and irradiated with UV light at 254nm for 3min (stratalinker® 1800, Stratagene, Cedar Creek Texas) after incubation for 2-3 hours at 37˚C and 5% CO_2.

Alternatively, cells were incubated with staurosporine (Biosource, Invitrogen Corporation, NY, USA) at 0.43µM for 6hours at 37°C and 5% CO_2 . After being rendered apoptotic, cells were incubated with 3µg purified C1q (Complement Technologies, Texas, USA) per $3 \cdot 10^5$ cells and 100µl per well in PBS at 37°C for 20min in 96well U-bottom tissue culture plates (Falcon®, Becton Dickinson, NJ, USA). Plates were washed with PBS and incubated with the anti-C1q Fab autoantibodies A14 and B8 (as described above) at 2µg/ml or with purified IgG from sera of an anti-C1q positive HUVS patient or an healthy blood donor at 500µg/ml in PBS for 20min at 37°C. C1q-dependent binding of anti-C1q to apopotic cells was detected by staining with a goat anti-human PE-conjugated $F(ab')_2$ fragment specific for $F(ab')_2$ fragments (Jackson ImmunoResearch Europe,

Suffolk, UK) or goat anti-human IgG specific for the Fcγ fragment PE-conjugated $F(ab')_2$ fragments (Jackson ImmunoResearch Europe, Suffolk, UK) in PBS for 15min at room temperature in the dark. Cells were then washed with PBS and stained with FITC conjugated rabbit anti-human C1q (DakoCytomation, Glostrup, Denmark) or with a FITC labeled murine monoclonal antibody to human C1q (Quidel, San Diego, USA) for 15min at room temperature in the dark. Finally, to identify apoptotic cells, cells were stained with Annexin-V APC (BD Pharmingen, NJ, USA) and 7-AAD (7-amino actinomycin D, BD Biosciences, NJ, USA) in Annexin V binding buffer (BD Pharmingen, NJ, USA) for 15min in the dark. Annexin V binding buffer was added 1:1 to the cells which were then analyzed by flow cytometry within 1 hour. The binding of anti-C1q to viable, early and late apoptotic cells was analyzed. Cells that were incubated with either C1q or Fab anti-C1q autoantibody alone served as controls.

The percentage of cells being double positive for C1q and anti-C1q was compared to the percentage of cells being single positive for anti-C1q in the absence of C1q. The C1q dependent increase in signal was calculated, i.e. 'fold-increase'.

Confocal microscopy

Cells were stained as described above, except that the anti-C1q Fab autoantibody A14 was used at a concentration of 3µg/ml. The cells were fixed in 1% paraformaldehyde (Merck, Darmstadt, Germany) before being placed on microscopy slides (Erie scientific company/Thermo Fisher Scientific, Portsmouth, USA) and dried. VECTASHIELD® mounting medium with DAPI (Vector laboratories, Burlingame, USA) was added and cells were visualized by confocal microscopy with a pinhole of 1.5um.

Anti-C1q autoantibody binding to antigens expressed on apoptotic cells

Binding of the two human anti-C1q Fab autoantibodies (1µg/ml) to C1q bound to double-stranded human placental DNA (Sigma, Buchs, Switzerland) and phosphatidylserine (Fluka, Buchs, Switzerland) was assessed. Furthermore, binding of anti-C1q from patient sera to C1q bound to dsDNA was tested. DNA and phosphatidylserine had been coated onto Costar microtiter plates in 1%BSA-PBS at a concentration of 2 µg/well (DNA) or 5 µg/ml in 100% methanol (phospatidylserine) in a total volume of 100 µl overnight at 37°C (DNA) or at room temperature (phosphatidylserine) respectively. The plates were then washed with PBS-Tween 0.05% and blocked with 3%BSA in PBS for 1h at 37°C before incubation with purified human C1q at 0.5ug/well in PBS for 2hrs at 37°C. The plates were then washed again before adding anti-C1q Fab autoantibodies (1µg/ml) or sera from an anti-C1q positive HUVS patient or a healthy donor (1/50 in high salt buffer). In parallel, deposited C1q was detected by a monoclonal mouse anti-C1q (clone 23D11 or Quidel, San Diego, USA) diluted 1/1000 in PBS to verify the successful binding of C1q. The detection of the antibodies was carried out as described above.

Furthermore, we analyzed if nucleosomes could be the binding partner of C1q allowing exposure of the neo-epitope relevant for the binding of anti-C1q antibodies by using a modified commercially available ELISA kit (ORGENTEC Diagnostika GmbH, Mainz, Germany). As described above, C1q was allowed to bind to the plate pre-coated with nucleosomes isolated from human cell cultures. The binding of anti-C1q Fab autoantibodies or anti-C1q from sera of a HUVS patient or a healthy donor was then

analyzed. The detection of the antibodies and the calculation were carried out as described above.

Statistical analysis

To determine statistical differences between the C1q-dependent binding of anti-C1q preparations to apopototic cell populations, Wilcoxon matched tests (paired, non parametric) using GraphPad Prism version 4 (GraphPad Software, San Diego, USA) were performed.

Results

Anti-C1q autoantibodies do not recognize C1q bound to immunoglobulins or immune complexes

Assuming that neoepitopes of C1q targeted by anti-C1q would be expressed once C1q has bound to immunoglobulins/immune complexes, *in vitro* constructs of immunoglobulins and immune complexes respectively were tested for binding of anti-C1q in the presence or absence of C1q antigen. Specific binding to bound C1q of anti-C1q positive sera diluted in high salt buffer was evaluated by deducing unspecific binding of sera to the construct without C1q (i.e. specific OD). As shown in Figure 2 anti-C1q antibodies recognize C1q neither on monoclonal or polyclonal immunoglobulin preparations (Fig 2A) nor on different immune complexes (Fig 2B and C) in spite of abundant C1q deposition as verified by the binding of a mouse monoclonal anti-C1q (23D11). The lack of binding was not due to a lack of expression of neoepitopes since the mouse monoclonal anti-C1q 23D11 specifically recognizes a neoepitope expressed on bound C1q (see methods).

Furthermore, the immunoglubulins/immune complexes were able to activate complement as verified by C4d deposition suggesting a C1q deposition resembling the physiological binding to immune complexes (data not shown).

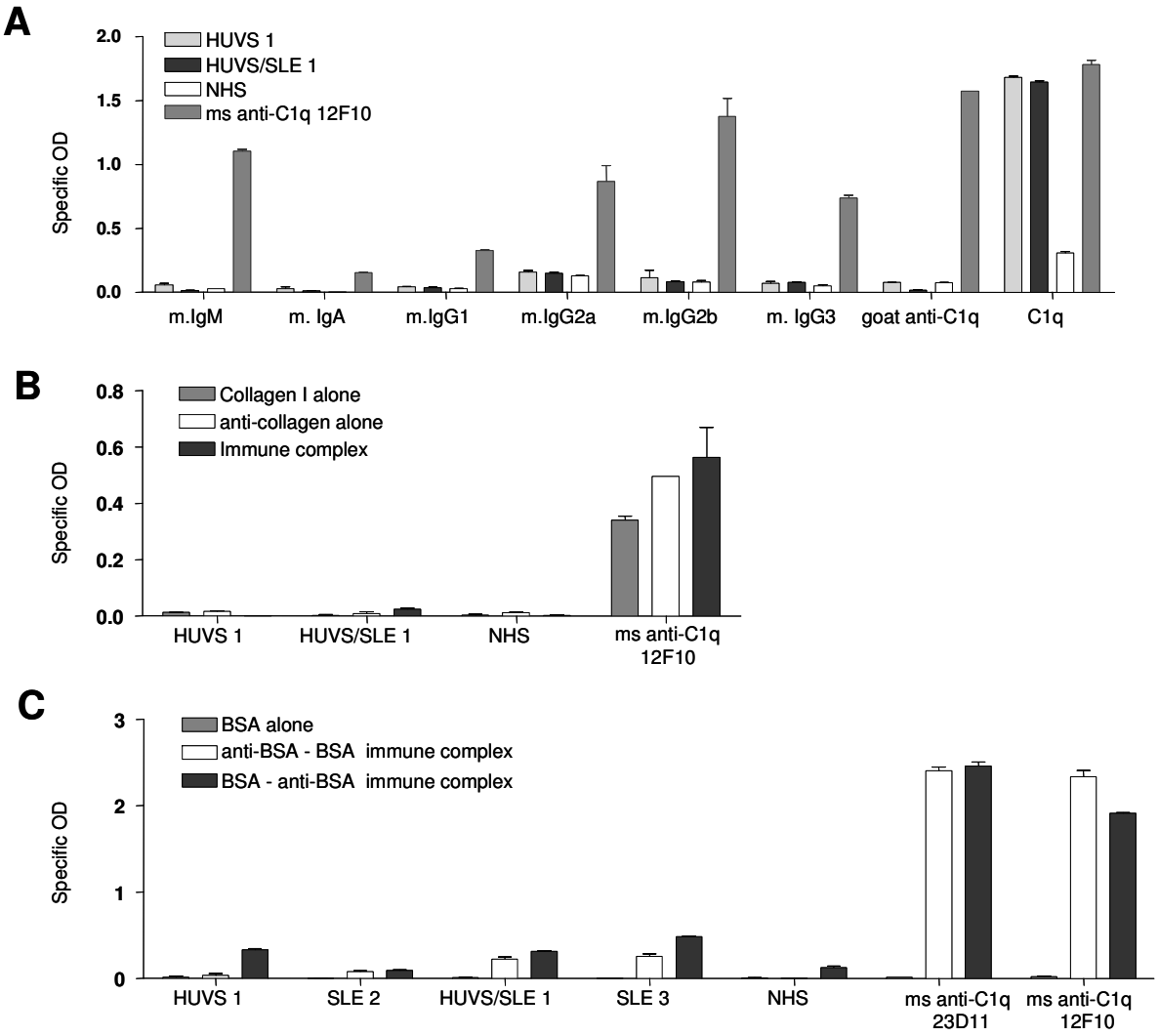

Figure 2: Anti-C1q autoantibodies don't recognize C1q on immunoglobulins or immune complexes.

Purified human C1q was allowed to bind to ELISA plates precoated either with different unspecific monoclonal mouse antibodies (IgM, IgA, IgG1, IgG2a, IgG2b and IgG3) or polyclonal anti-C1q (**A**), or with immune complexes consisting of collagen-anti-collagen (**B**) or BSA-anti-BSA (**C**). The successful binding of C1q to each construct was verified using monoclonal mouse anti-human C1q (clones 12F10 and/or 23D11). Specific binding to bound C1q of anti-C1q positive sera (HUVS 1, HUVS/SLE 1, SLE 2, SLE 3) diluted in high salt buffer was evaluated by deducing unspecific binding of sera to the construct without C1q (i.e. specific OD) and compared to NHS. Anti-C1q positive sera showed no C1q specific binding to C1q bound on immunoglobulins or immune-complexes when compared to controls.

In order to confirm the results described above, we set up depletion experiments with polystyrene beads and heat aggregated IgG (HAGG) that served as a model for immune complexes. Anti-C1q positive sera could substantially be depleted by C1q bound to polystyrene beads, imitating the situation of C1q bound to polystyrene ELISA plates. However, anti-C1q could not be depleted by C1q bound to HAGG although both C1q on polystyrene beads and C1q on HAGG were able to deplete mouse monoclonal anti-C1q 12F10 and 23D11 antibodies (Figure 3).

Additionally, no C1q-dependent binding of anti-C1q to C1q when bound on laminin, heparan sulfate, fibronectin, Peptide 2J and collagen I and IV could be observed, indicating that neither immunoglobulins/immune complexes nor those proteins are able to present C1q such that it can be recognized by anti-C1q autoantibodies (data not shown).

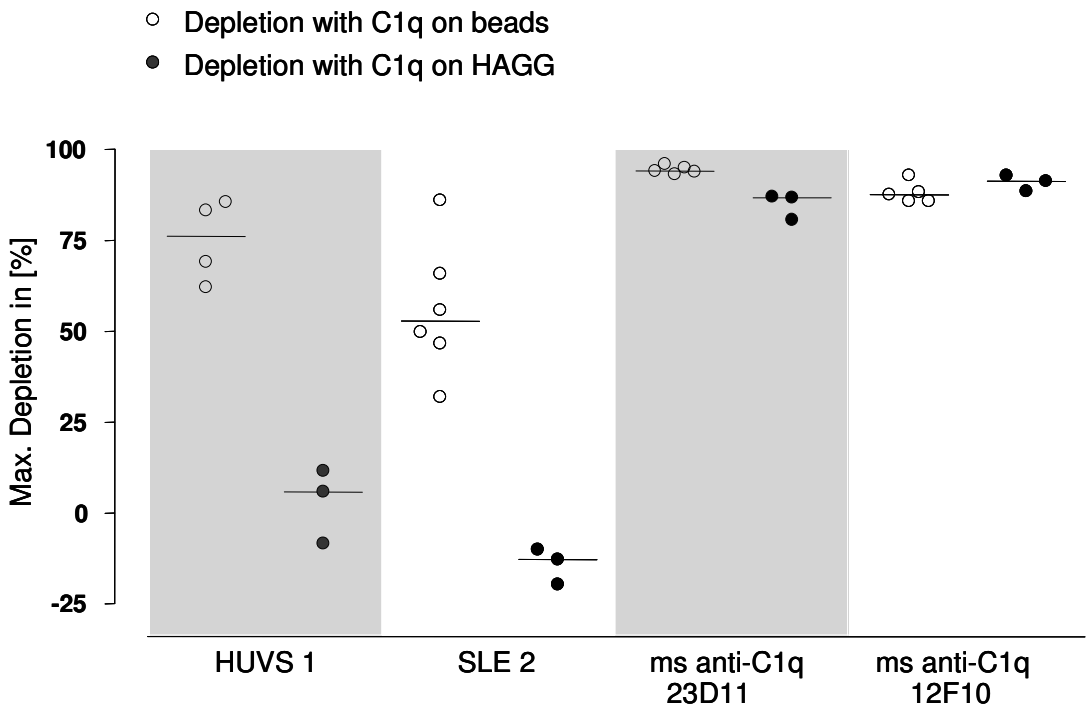

Figure 3: Anti-C1q autoantibodies cannot be depleted by C1q bound to heat-aggregated IgG (HAGG).

Depletion experiments in anti-C1q positive sera (HUVS 1, SLE 2) were performed comparing the successful depletion of anti-C1q from sera by C1q bound to HAGG (HAGG-C1q) versus C1q bound to polystyrene beads (beads-C1q). Monoclonal mouse anti-human C1q (clones 12F10 and 23D11) served as a control. After preincubation with HAGG-C1q or beads-C1q, the complexes were removed by centrifugation and the supernatants tested for anti-C1q activity using a conventional anti-C1q ELISA. The experiments were performed in different dilutions and the maximal depletion obtained compared to sera depleted with HAGG or beads alone, (i.e. maximal depletion in %). Anti-C1q antibodies from sera can be depleted by C1q bound to polystyrene beads, but not by C1q bound to HAGG.

Human anti-C1q Fab autoantibodies recognize C1q on early apoptotic cells

Having shown, that C1q bound on immunoglobulins/immune complexes is not targeted by anti-C1q, C1q bound on apoptotic cells was investigated as a potential target for anti-C1q.

As described by others (21, 43), C1q mostly bound to late apoptotic/necrotic cells (double positive for Annexin V and 7AAD) whereas there were only few C1q positive 'early' apoptotic cells (Annexin V single positive) after preincubation with purified human C1q (Figure 4D). As shown in Figure 5, in a population of Jurkat cells that had been rendered apoptotic by UV light irradiation all cells being still viable (double negative for Annexin V and 7AAD) were negative for C1q and anti-C1q Fab binding. In contrast, we observed binding of anti-C1q Fab autoantibodies A14 and B8 to C1q on early apoptotic (Annexin V positive, 7AAD negative) cells when compared to binding in the absence of C1q. In the presence of C1q, a median of 66.41% cells bearing C1q were also positive for anti-C1q Fab autoantibody A14 (range: 40.42-78.70), whereas a median of 76.0% cells bearing C1q were positive for anti-C1q Fab autoantibody B8 (range: 53.40-91.80). On late apoptotic/necrotic cells, we also observed cells being positive for both C1q and anti-C1q Fab autoantibodies. However, in contrast to early apoptotic cells, late apoptotic/necrotic cells also revealed binding in the absence of C1q antigen suggesting additional unspecific binding (Figure 5).

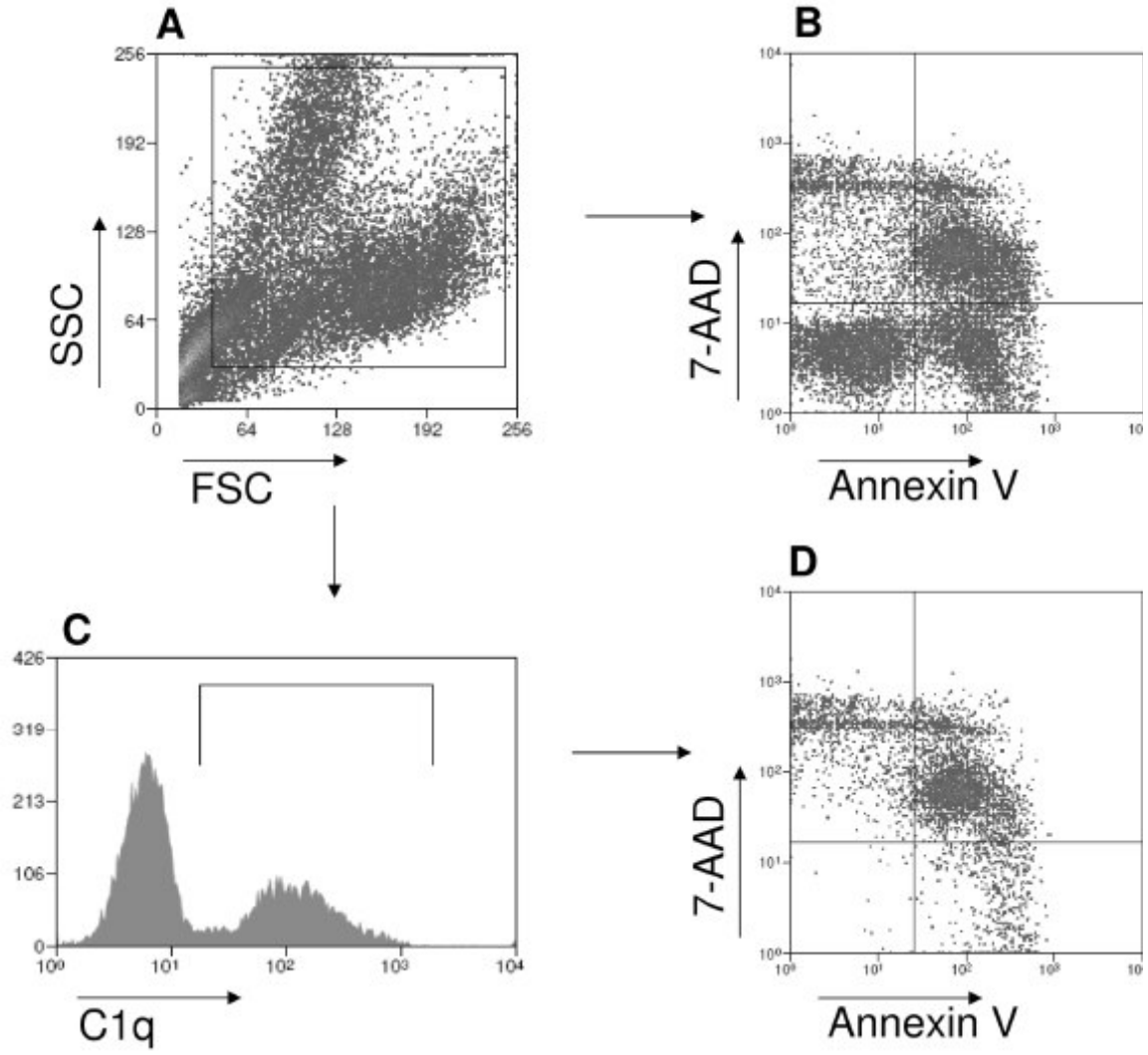

Figure 4: C1q mostly binds to late apoptotic/ necrotic cells but also to early apopotic cells.

Jurkat cells were irradiated and analyzed by flow cytometry. Shown are forward scatter (FSC) and side scatter (SSC) (**A**) as well as stainings to discriminate between viable, early and late apoptotic and necrotic cells, using Annexin V and 7AAD (**B**). Viable cells were gated as double negative, early apoptotic cells were gated Annexin V positive and 7AAD negative. Late apoptotic/necrotic cells were gated double positive for Annexin V and 7AAD. A substantial part of cells stained positive for C1q (**C**). C1q positive cells alone mostly consisted of late apoptotic/necrotic cells (D, upper right quadrant) but also of early apoptotic cells (D, lower right quandrant).

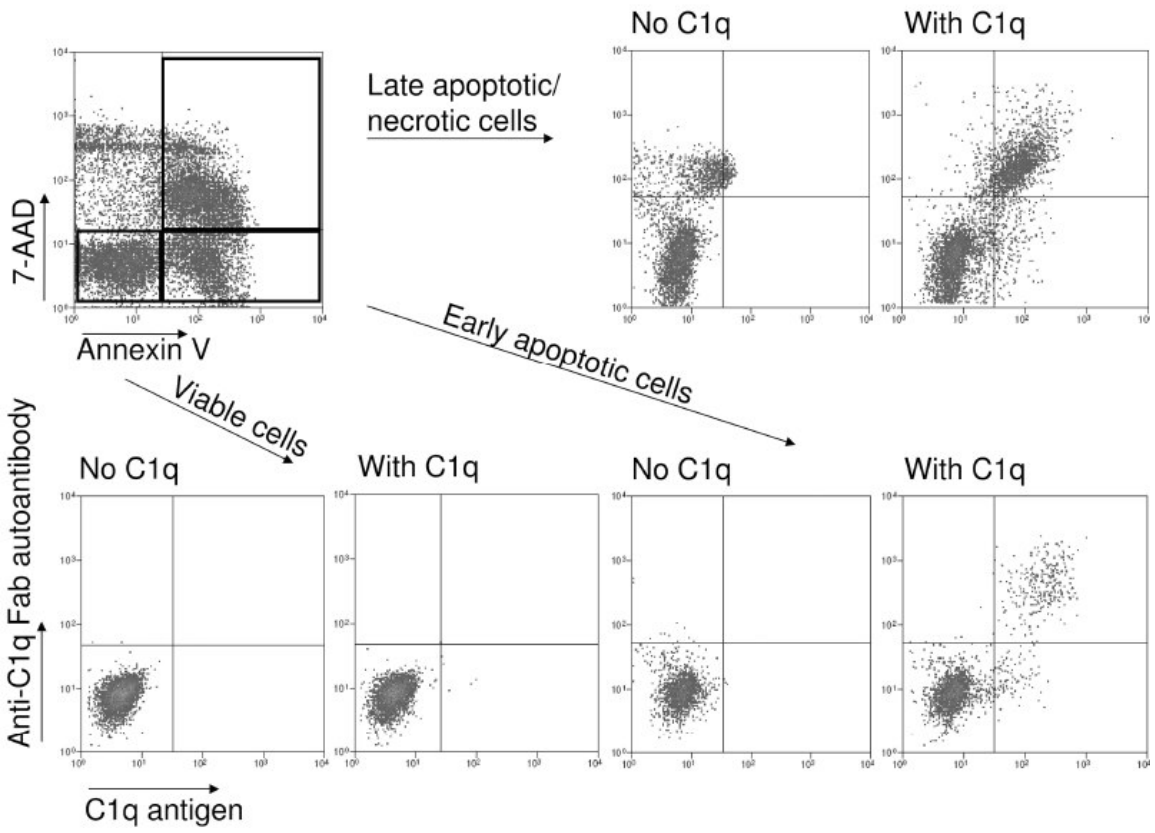

Figure 5: Binding of human anti-C1q Fab autoantibodies to C1q on apoptotic cell populations.

The dot plots demonstrated in the figure exemplify apoptotic Jurkat cells that were incubated with C1q and anti-C1q Fab A14 ('with C1q') or anti-C1q Fab alone ('no C1q'), gated on different cell populations i.e. viable, early apoptotic and late apoptotic/necrotic cells. Viable (Annexin V and 7AAD negative) cells were double negative for C1q as well as for anti-C1q. Within early apoptotic cells (Annexin V positive and 7AAD negative) only a few cells stained single positive for anti-C1q Fab autoantibody alone but in the presence of C1q most cells bearing C1q were also positive for anti-C1q Fab. In contrast, a substantial part of late apoptotic/necrotic cells showed positive staining by anti-C1q Fab in the absence of C1q suggesting a C1q-independent binding to late apoptotic cells. In the presence of C1q most cells being positive for anti-C1q Fab, also stained positive for C1q.

To quantify the C1q-dependent binding of anti-C1q, we calculated the relative increase of binding of A14 and B8 to apoptotic cells in the absence versus binding in the presence of C1q. Whereas A14 and B8 showed clear C1q specific binding to early apoptotic cells, we could not demonstrate C1q-dependent binding to late apoptotic/necrotic cells (Figure 6).

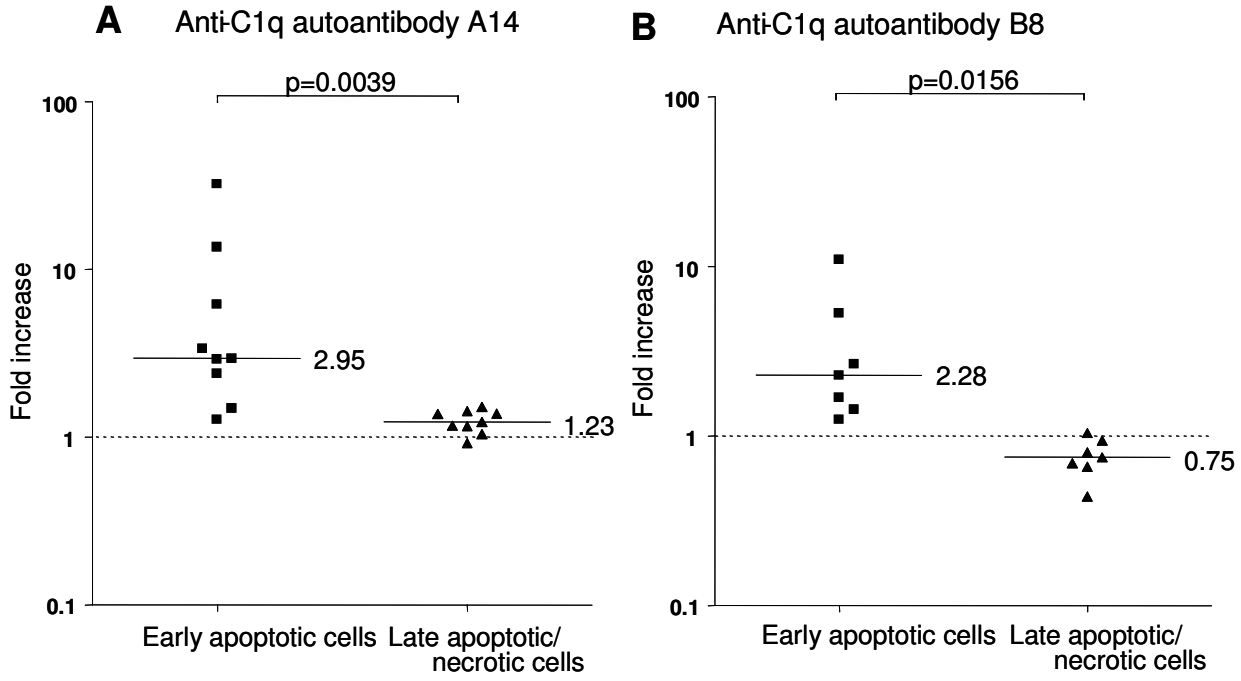

Figure 6: Human anti-C1q Fab autoantibodies specifically target C1q on early apoptotic cells but not on late apoptotic/necrotic cells.

Two anti-C1q Fab autoantibodies (A14 and B8) were analyzed according to the procedure shown in Figure 5. The graphs show the C1q dependent increase in anti-C1q binding comparing early apoptotic versus late apoptotic/necrotic cells. Each point represents a separate experiment. The dotted line indicates a 1-fold increase corresponding to C1q independent binding. Anti-C1q Fab A14 and B8 show C1q specific increased binding to C1q bound on early apoptotic cells, whereas there was no C1q-dependent binding to late apoptotic/necrotic cells.

These results could be confirmed using Jurkat cells being rendered apoptotic with staurosporine (Figure 7A) and by using another T-cell line (Molt 4 cells) as well as using peripheral blood T-cells as a source for apoptotic cells (Figure 7 B and C).

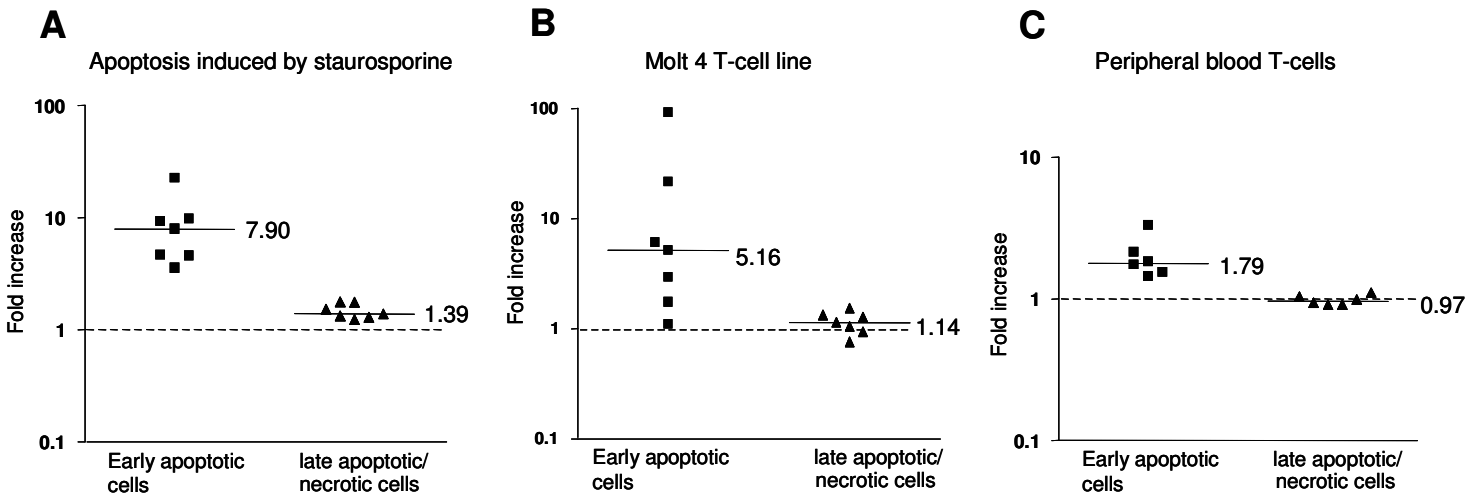

Figure 7: Human anti-C1q Fab autoantibodies target C1q bound to early apoptotic cells generated by staurosporine as well as to early apoptotic Molt 4 and peripheral blood T-cells.

Binding of anti-C1q Fab autoantibody A14 to C1q bound on early apoptotic versus late apoptotic/necrotic cells was analyzed as done for the assay described in Figure 4. Each point represents a separate experiment. The dotted line indicates a 1-fold increase corresponding to the C1q independent binding. **A.** Jurkat cells were rendered apoptotic by staurosporine. The graph shows that C1q is bound specifically by the anti-C1q Fab A14 when bound on early apoptotic cells but not when bound on late apoptotic/necrotic cells. **B.** Anti-C1q Fab A14 shows C1q specific binding to C1q bound on early apoptotic but not to C1q bound on late apoptotic/necrotic Molt-4 T-cells. **C.** Anti-C1q Fab A14 shows C1q specific binding to C1q bound on early apoptotic but not to C1q bound on late apoptotic/necrotic peripheral blood T-cells.

Purified total IgG from an anti-C1q positive HUVS patient also showed C1q specific binding to early apoptotic cells

To confirm the results obtained with human anti-C1q Fab autoantibodies we also tested purifed IgG from a HUVS patient with high titers of anti-C1q antibodies but undetectable anti-dsDNA, anti-phospholipid and anti-nucleosome antibodies compared to IgG from a healthy donor. IgG from the HUVS patient showed significant C1q-dependent binding to early apoptotic cells but again there was no quantifiable C1q-dependent binding to late apoptotic/necrotic cells confirming our results seen with anti-C1q Fab autoantibodies. In contrast, IgG from a healthy donor showed no C1q dependent binding neither to early apoptotic nor to late apoptotic/necrotic cells (Figure 8).

C1q and anti-C1q Fab autoantibodies colocalize on intact apoptotic cells

In order to analyse whether C1q and anti-C1q Fab autoantibodies indeed colocalize on apoptotic cells confocal microscopy was performed. As expected, abundant C1q deposition to dying cells could be observed (Figure 9B). The staining patterns varied between a more patchy staining for C1q on cell debris and more circular staining on apparently intact cells. Interestingly, colocalisation of C1q and anti-C1q Fab autoantibodies was primarily found on apoptotic cells with more circular C1q staining that had an intact cell membrane and an intact nucleus most likely representing cells in early stages of apoptosis (Figure 9D). As control, viable cells stained negative for C1q and anti-C1q Fab autoantibodies (Figure 9B and C).

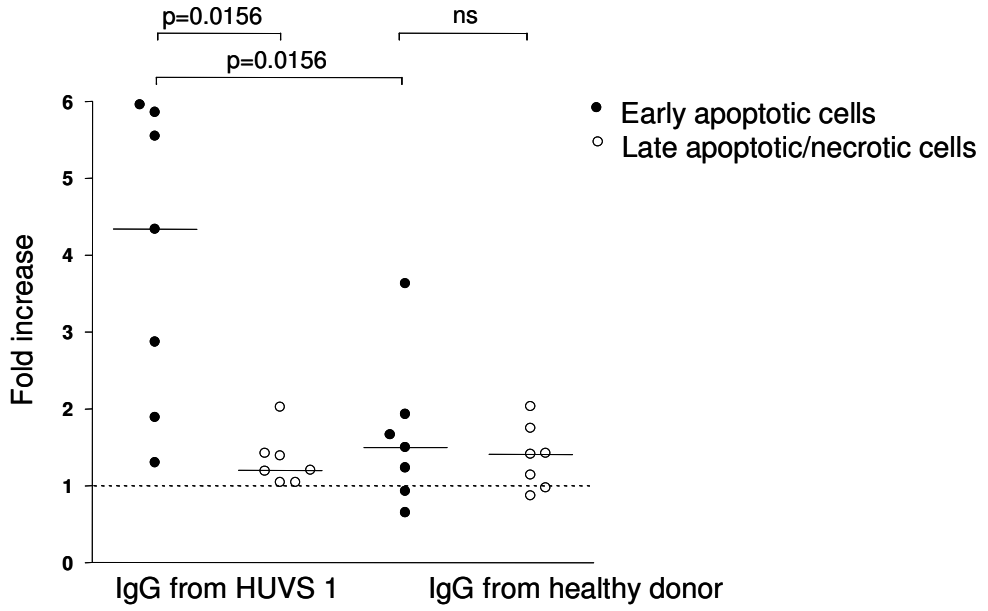

Figure 8: Purified IgG from a HUVS patient shows C1q specific binding to C1q bound on early apoptotic cells.

The graph shows C1q specific binding of total IgG from an anti-C1q positive HUVS patient (HUVS 1) and a healthy donor to C1q bound on early apoptotic versus late apoptotic/necrotic cells. Binding of anti-C1q from total IgG to C1q bound on early apoptotic versus late apoptotic/necrotic cells was analyzed according to the procedure demonstrated in Figure 5. Each point represents a separate experiment. The dotted line indicates a 1-fold increase corresponding to C1q independent binding. Total IgG from a HUVS patient showed binding to C1q bound on early apoptotic cells but not on late apoptotic/necrotic cells. Whereas total IgG from NHS showed no C1q specific binding, neither to C1q bound on early apoptotic nor to C1q bound on late apoptotic/necrotic cells.

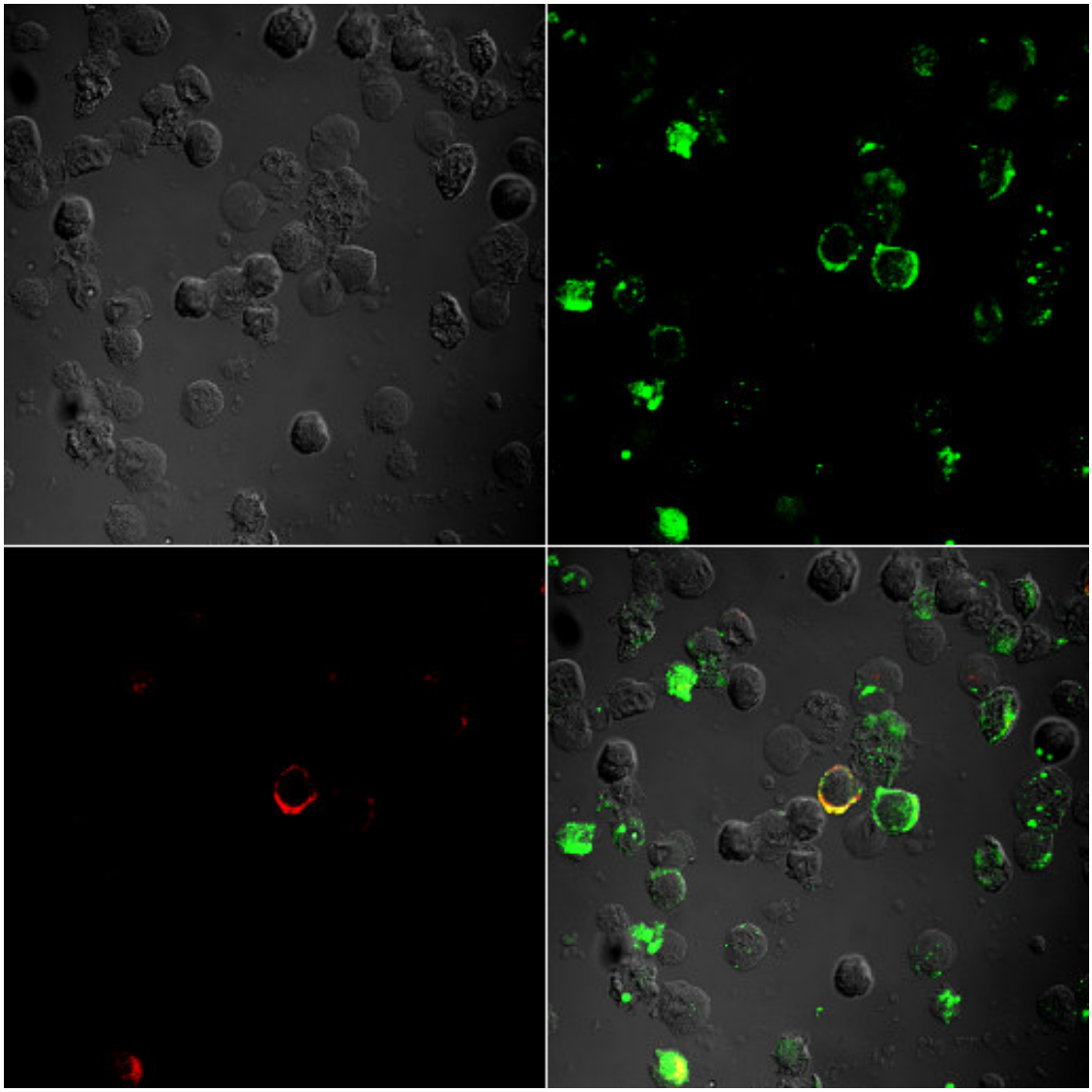

Figure 9: C1q and Anti-C1q Fab autoantibody colocalize on membranes of intact apoptotic cells.

Apoptotic cells were stained for C1q (green) and Anti-C1q Fab autoantibody A14 (red). **A.** Transmission light shows viable, apoptotic and necrotic cells. **B.** C1q (green) binds to apoptotic and necrotic cells. **C.** Anti-C1q Fab autoantibody A14 binds to the membrane of an intact apoptotic cell. **D.** C1q and Anti-C1q Fab autoantibody A14 colocalize on the membrane of an intact apoptotic cell. This double staining could be observed on further intact apoptotic cells and numbers were comparable with the relative amount of C1q positive early apoptotic cells as estimated by FACS analysis (Figure 4). In contrast, cell debris also staining strongly positive for C1q remains mostly negative for anti-C1q Fab autoantibody A14.

Human Fab anti-C1q autoantibodies do not recognize C1q on dsDNA, phosphatidylserine or nucleosomes

In order to elucidate potential receptors of C1q on apoptotic cells that might be responsible for the expression of neo-epitopes relevant for the binding of anti-C1q autoantibodies, we tested the binding of anti-C1q Fab autoantibodies and anti-C1q positive serum from a HUVS patient (undetectable anti-dsDNA, anti-phospholipid and anti-nucleosome antibodies) to C1q bound on phosphatidylserine, dsDNA and nucleosomes. As a control monoclonal mouse anti-C1q clone 23D11 and polyclonal mouse anti-C1q were tested in the same ELISA confirming that C1q was indeed able to bind to those autoantigens. As demonstrated in Figure 10, anti-C1q Fab autoantibodies did not recognize C1q bound to phosphatidylserine nor bound to dsDNA nor bound to nucleosomes, indicating that none of those autoantigens tested is able to present C1q in an anti-C1q suitable conformation. Similarly, no C1q-dependent binding of sera from an anti-C1q positive HUVS patient to nucleosomes and dsDNA could be observed (data not shown).

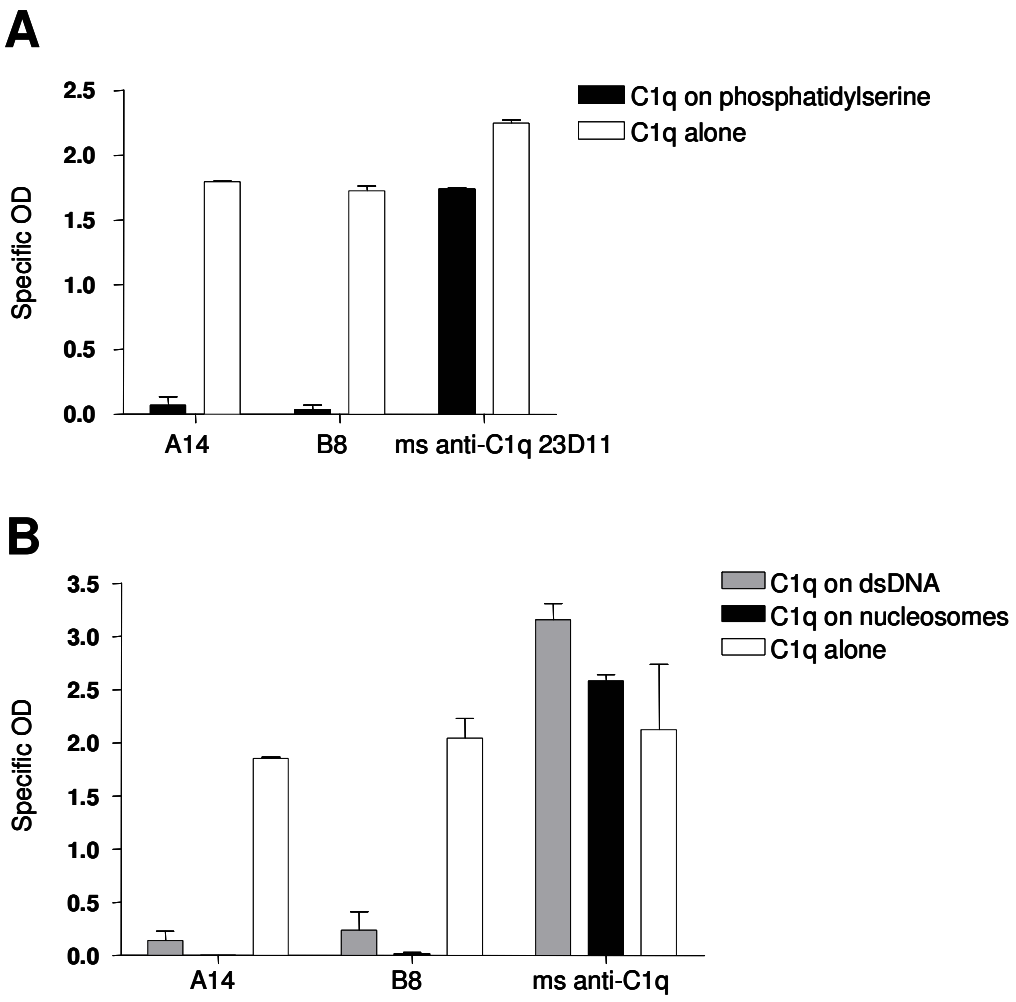

Figure 10: Anti-C1q Fab autoantibodies do not recognize C1q on phosphatidylserine, dsDNA or nucleosomes.

Purified human C1q was allowed to bind directly to ELISA plates (control) or to plates precoated with either phosphatidylserine or dsDNA or nucleosomes. The successful binding of C1q was verified using mouse monoclonal anti-human C1q. The whole construct without C1q served as a blank (specific OD). **A.** Anti-C1q Fab autoantibodies A14 and B8 recognize C1q directly bound to an ELISA plate, but not C1q bound to phosphatidylserine in spite of abundant binding of C1q as demonstrated by the monoclonal mouse anti-C1q control. **B.** Anti-C1q Fab autoantibodies show no C1q specific binding neither to C1q bound on dsDNA nor to C1q bound on nucleosomes.

Discussion

Previous studies suggested a link from SLE to apoptosis, from apoptosis to complement C1q and from C1q back to SLE. These links are based on observations that apoptotic cells are a source of autoantigens and that SLE indeed is associated with a defective clearance of dead and dying cells. Apoptosis links further to complement C1q since C1q has been described to bind to apoptotic cells and to promote the clearance of apoptotic cells. Finally, C1q has a strong link back to SLE, since homozygous C1q deficiency is the strongest disease susceptibility gene for the development of SLE (3, 4). However, most patients with SLE have no primary C1q deficiency. Nevertheless, there are indirect links from C1q to SLE: A substantial number of SLE patients develop hypocomplementemia of the components of the classical pathway of complement including C1q in particular during flares. Second, C1q is well known to be deposited in affected tissues (6, 7). Last, autoantibodies against complement C1q are frequently found in patients with SLE with a strongly increasing prevalence in case of renal flares (28). By the demonstration that anti-C1q from SLE patients specifically target C1q bound on early apoptotic cells we can strengthen the link between complement C1q and SLE and even provide a direct link between all three: SLE, apoptotis and C1q.

So far the origin of anti-C1q autoantibodies was unknown. As demonstrated by two different methods, anti-C1q autoantibodies did not bind to C1q bound on immunoglobulins or immune complexes. This observation was surprising and contradicts current hypotheses on the mechanisms that lead to the conformational changes of C1q required to allow the binding of anti-C1q (42, 44). The lack of binding was not due to a

general lack of expression of neoepitopes on C1q bound to immune complexes. As shown by others (45) and by the successful binding of the neoepitope specific control anti-C1q 23D11, we could demonstrate that binding of C1q to immunoglobulins/immune complexes indeed leads to conformational changes of the molecule. The lack of binding of anti-C1q to these neoepitopes suggests that conformational changes of C1q are complex and strongly dependent on the nature of the ligand.

The observation that anti-C1q specifically recognize C1q bound on early apoptotic cells, as shown by FACS analysis and confocal microscopy, is not surprising. Apoptotic cells have already been described to be a potential source of autoantigens in SLE. For example nucleosomes that are considered a main autoantigen in human and murine lupus are primarily generated by apoptosis (46, 47). Furthermore intracellular and nuclear antigens as targeted in SLE were shown to be translocated to the cell membrane and modified during apoptosis (18). In addition, the injection of apoptotic cells into healthy mice could promote the development of anti-nuclear autoantibodies (19). C1q acts as a bridging molecule between apoptotic cells and macrophages and stimulates the process of ingestion (23). In the context of an altered clearance of apoptotic cells due to mechanisms that are independent of C1q, C1q itself exposing unusual conformational epitopes could become antigenic and give rise to the production of anti-C1q autoantibodies. However, to date there has not been direct evidence for the hypothesis that prolonged exposure of C1q on incompletely cleared apoptotic cells indeed is the origin of the autoimmune response in SLE. The observation that anti-C1q are specifically directed to C1q bound on early apoptotic cells is now providing first evidence for this hypothesis. Even more, the exceptional expression of neoepitopes by C1q and binding characteristics of anti-C1q

strongly support the hypothesis that in general early apoptotic cells are the major target of the autoimmune response in SLE.

We also observed binding of anti-C1q Fab autoantibodies on late apoptotic/necrotic cells that was independent of the presence of C1q, an observation for which we have no explanation other than that cells undergoing late apoptosis or necrosis generate various new epitopes, and therefore this binding might be unspecific. However, we cannot exclude a specific binding of anti-C1q to late apoptotic/necrotic cells targeting structures that remain to be identified. Furthermore, it is possible that C1q-dependent binding of anti-C1q to late apoptotic/necrotic cells had been masked by abundant binding of anti-C1q in the absence of C1q. Since to date the occurrence of late apoptosis has not been demonstrated *in vivo*, in contrast to early apoptotic cells, and since early apoptosis naturally precedes the occurrence of late apoptotic cells, early apoptotic cells are more likely to be the real target of the autoimmune response in SLE. However, we think that the binding of C1q with the consecutive recognition by anti-C1q occurs in an advanced stage of early apoptosis. In line with our observations, it has recently been shown that a subset of SLE patients produces autoantibodies against a Ro 60 apotope that is exposed on the surface of early apoptotic cells. This immune response was shown to be specific for SLE, because the Ro 60 apotope was almost absent in patients with primary Sjörgen's syndrome (48). In contrast to antibodies against the Ro 60 apotope, anti-C1q autoantibodies were shown to strongly correlate with disease activity and are directed against a highly functional molecule. The ability of C1q to sense multiple signals due to its multimeric structure together with the observations that C1q facilitates the clearance of apoptotic cells suggests a central role for C1q in apoptotic cell clearance and thus in

immune tolerance (49, 50). The role of C1q as an actor of immune tolerance has been reinforced by the discovery of its involvement in the biology of dendritic cells (DC) (51, 52). It was shown that C1q is a potent modulator of DC, resulting in cells characterized by an impaired capacity of cytokine production and an impaired upregulation of costimulatory molecules, leading to a limited T-cell response. It was proposed that C1q regulates the threshold of DC activation and thereby prevents hyperactivation of the overall immune response. As a consequence of our findings the binding of anti-C1q autoantibodies to C1q on early apoptotic cells might alter the clearance of apoptotic cells and enhance the underlying pathogenic mechanisms misleading dendritic cells to a proinflammatory immune response against apoptotic cells.

However, the clinical observation that anti-C1q strongly correlate with the occurrence of sever lupus nephritis but not with other organ manifestations remains to be elucidated. Since C1q deficient mice developed a lupus-like disease with accumulation of apoptotic bodies in the glomeruli but not in other organs, one can speculate that glomeruli are particularly sensitive to defects in the clearance of apoptotic cells (53) and therefore might be the loci of predilection for the mechanisms mentioned before. Ongoing studies will have to elucidate the consequences of anti-C1q targeting C1q on early apoptotic cells.

In conclusion, links from SLE to apoptosis, from apoptosis to C1q and from C1q back to SLE are well established. Demonstrating that anti-C1q specifically bind to C1q bound on early apoptotic cells, our data additionally provides a direct link between all three, SLE, C1q and apoptosis. Furthermore, the exceptional expression of neoepitopes by C1q and

binding characteristics of anti-C1q seem to identify early apoptotic cells as a major target of the autoimmune response in human SLE.

Outlook

- Ogden et al (23) showed that C1q stimulated the ingestion of apoptotic cells by ligation of calreticulin on the phagocyte surface which in turn is bound to CD91. Therefore, calreticulin will have to be analysed for being the target of C1q on early apoptotic cells that allows the exposure of neoepitopes relevant to the binding of anti-C1q autoantibodies.

- As demonstrated by Casciola-Rosen et al, apoptotic material represents a source of autoantigens, because intracellular and nuclear antigens are modified during apoptosis and translocated to the cell membrane (18). It was proposed that these autoantigens have in common a vulnerability to oxidative modification. The influence of oxidation on C1q is not known. Therefore, oxidized C1q might be another target that allows the binding of anti-C1q.

- T cells from the peripheral blood of lupus patients as well as other cell types (such as neutrophils or macrophages) will be rendered apoptotic and analyzed for binding of C1q and its recognition by anti-C1q antibodies. In the blood of SLE patients increased levels of apoptotic neutrophils were found and they correlated with disease activity (54). Furthermore, lupus patients have evidence of accelerated macrophage apoptosis, a phenomenon that correlates with disease activity (55-57). Since macrophages themselves are an important source of C1q, macrophages from C1q deficient mice could be analyzed as controls.

- In a phagocytosis assay, consequences of the binding of anti-C1q to C1q bound on early apoptotic cells on the uptake of the cells by macrophages (or immature dendritic cells) will have to be analyzed. Anti-C1q could impair the clearance of apoptotic cells or, maybe more likely, will alter the clearance by engagement of Fcγ-receptors leading to a more inflammatory type of phagocytosis.

- Anti-C1q will be investigated for their ability to activate or block the classical pathway of complement on apoptotic cells.

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SECTION 3:

ANTI-C1Q AUTOANTIBODIES DO NOT CORRELATE WITH THE OCCURRENCE OF NEPHRITIS IN LUPUS PRONE MRL/MPJ +/+ MICE

Introduction

Several inbred mouse strains in which lupus-like autoimmune disease spontaneously develops have provided experimental systems for analyzing the pathogenesis of the disorder (1). Anti-C1q autoantibodies have been detected in all three major mouse models of SLE (2):

The NZB/W (NZB x NZW) F1 mouse model is generally considered to most closely reflect the properties of human SLE. This strain exhibits a strong female gender bias in susceptibility and develops a severe systemic autoimmunity with high titers of anti-nuclear autoantibodies (ANA), culminating in immune complex-mediated fatal glomerulonephritis (3, 4).

In contrast to human SLE, susceptibility to SLE in **BXSB mice** is limited to males, because the presence of a Y chromosome carrying yaa, which interacts with several genes in the BXSB genome, is essential for the initiation of severe systemic autoimmunity. They develop high titer ANA and immune complex nephritis which lead to early mortality (3, 5).

MRL/MpJ-lpr/lpr mice (MRL/MpJ +/+ mice carrying the autosomal recessive lpr mutation of the Fas apoptosis gene) develop progressive, severe lymphadenopathy, multiple SLE-like autoantibodies and hypergammaglobulinemia (6). The failure of the

fas-fas ligand system to delete autoreactive extrathymic T-cells accounts for the accumulation of vast numbers of CD4- CD8-anergic T-cells. The fas mutation is also expressed in B-cells, leading to a failure to delete autoantibody forming cells but not to B-cell proliferation analogous to T-cells (7). Although the fas pathway is an important regulatory pathway in the immune system, mutations in fas are not associated with susceptibility to human SLE but lead to a disease called autoimmune lymphoproliferative syndrome (3, 4). However, MRL/MpJ-lpr/lpr mice, have become the most widely used model of human SLE.

MRL/MpJ +/+ mice are the parent strain of MRL/MpJ-lpr/lpr. Despite carrying the normal Fas gene, MRL/MpJ +/+ mice also exhibit an autoimmune disorder including the occurrence of glomerulonephritis. Symptoms are more severe in female mice, but are manifested much later in life compared to those of MRL/MpJ-lpr/lpr mice or other lupus-prone strains as outlined above.

The highest levels of anti-C1q have been detected in MRL/MpJ-lpr/lpr mice (2). However, this mouse model of SLE was shown not to be ideal to dissect the time course of the disease because of its fast and severe course. At 2 months of age all mice had already elevated anti-C1q antibodies and all mice developed progessive renal damage, thus not allowing demonstrating a direct link between the two. In addition, no correlation between levels of anti-C1q and albuminuria, as a measure for renal injury, was detected (8). Therefore a mouse model with a more delayed and less uniform autoimmune syndrome (**MRL/MpJ +/+)** was chosen (9, 10). Preliminary studies in a small cohort of MRL/MpJ +/+ mice have shown that some but not all of these mice develop elevated titers of anti-C1q as compared to normal BALB/c mice (Trendelenburg M, unpublished

data). Furthermore, anti-C1q could not be detected in MRL/MpJ +/+ mice being C1q deficient. Since in addition glomerulonephritis in MRL/MpJ +/+ mice is a late event and occurs in variable degrees of severity, these mice were considered to be the best model for human SLE.

Materials and methods

Animals and experimental protocol

Female MRL/MpJ +/+ mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) at 4 weeks of age. Balb/c mice were maintained at our animal facility. All animals had free access to water and standard chow. Animal care and experimentation were performed in accordance with the national guidelines (Federal Veterinary Office) for the care and use of laboratory animals. Serum and urine were first collected every month, then every second month. Mice were euthanized according to the national guidelines for the care and use of laboratory animals with a carbon dioxide chamber.

In a first cohort, survival of 30 MRL/MpJ +/+ mice was compared to survival of 12 Balb/c mice. In further cohorts, 26 MRL/MpJ +/+ mice were euthanized at month 11 and 20 mice at month 14. Mice were euthanized with pentobarbital 0.1g/ml, 300-350ul, (corresponds to 30ug/ mouse or 0.6-0.75ug/g bodyweight) followed by axle bleeding and kidneys were collected. As a control, kidneys and spleen from 2 Balb/c mice, 6 week-old, and kidneys from 12 Balb/c mice, 14 month-old, were collected.

Detection of IgG and autoantibodies against complement C1q

When measuring murine anti-C1q antibodies, Hogarth MB et al obtained highly significant correlations between results generated with purified human and mouse C1q used as antigen (2). Therefore, ELISA plates (Nunc, Rosklide, Denmark) for anti-C1q measurements were coated overnight at 4C° with purified human C1q (gift from Bühlmann Laboratories, Schönenbuch, Switzerland; >99% pure as judged by SDS

PAGE) at a concentration of 0.5µg/ml or for determination of total IgG with goat anti-ms Ig (H+L) (SouthernBiotech, Alabama, USA) at a concentration of 2ug/ml. For the anti-C1q ELISA, serum samples were diluted 1:50 in PBS Tween (0.05%), 1% FCS containing 1M NaCl and for the detection of IgG, serum samples were diluted 1/800000 in PBS. After incubation plates were washed and bound IgG was detected using biotinylated polyclonal goat anti-mouse IgG (SouthernBiotech, Alabama, USA) and horse radish peroxidase labeled Streptavidin (Jackson ImmunoResearch Europe, Suffolk, UK). A monoclonal mouse anti-human C1q (generated by immunization of $C1q_a$-deficient mice, clone 23D11) was used to generate a standard curve. Anti-C1q are expressed as U/ml. To calculate the amount of IgG, a standard mouse IgG preparation (SouthernBiotech, Alabama, USA) was used.

Antibody elution from kidneys

The procedure was performed as described before (8). In short, 6 kidneys from 6 mice with high titers of anti-C1q antibodies were pooled, minced and collected in 3ml PBS containing protease inhibitor cocktail (Roche, Mannheim, Germany). The mixture was centrifuged at 3000 rpm for 5 min and supernatants were collected. Pellets were washed and resuspended in 1.5ml elution buffer consisting of 0.1 M glycine-Hcl, 0.15 M Nacl, pH 2.5 and sonicated on ice with 3 bursts of 30s and amplitude of 25. After overnight rotation at 4°C, samples were centrifuged for 10 min at 10000 rpm and supernatants were collected and adjusted to pH 7.5. Samples were then tested by ELISA for the presence of anti-C1q and IgG as described above.

Histological studies

Renal tissue of mice was fixed in buffered formalin 10%, embedded in paraffin and 2µm sections were stained for IgG and C3 as well as nuclei by immunohistochemistry and with periodic acid-Schiff (PAS). Histopathological changes were scored with the help of an experienced histopathologist, being blinded to the code of the sections. To reveal IgG and C3 deposition, sections were deparaffinated and hydrated according to standard protocols. The sections were digested with protease XXIV (0.03% 50ul per section) (Sigma, Missouri, USA) for 5 min (IgG) and 10min (C3) and washed in 100% ethanol. Then they were rehydrated in PBS and blocked with normal goat serum (Vector Laboratories, Burlingame, CA). The sections were incubated overnight in PBS, 1% BSA with biotinylated goat anti-ms IgG 1/1000 (SouthernBiotech, Alabama USA) and rabbit anti-human C3c 1/2000 (DakoCytomation, Glostrup, Denmark), which crossreacts with mouse C3c. After washing in PBS, the sections were incubated for 45 min in PBS 1% BSA with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA). They were washed again and incubated with VECTASTAIN Elite ABC reagent (Vector Laboratories, Burlingame, CA) for 30 min, then washed again and incubated with freshly prepared DAB solution (Vector Laboratories, Burlingame, CA) for 2 min until a suitable color had developed. Counterstaining was carried out with Mayer's hematoxylin (J.T. Baker, Mallinckrodt Baker, Inc., Philipsburg, NJ, USA) for 2 min followed by 2 min each of rinsing in tap water, distilled water, ethanol 70%, 96%, 100% and UltraClear (J.T. Baker, Mallinckrodt Baker, Inc., Philipsburg, NJ, USA). Finally samples were mounted with UltraKitt mounting medium (J.T. Baker, Mallinckrodt Baker, Inc., Philipsburg, NJ, USA). For staining of nuclei, the sections were deparaffinated and hydrated, followed by

staining with bisbenzimide H33258 fluorochrome trihydrochloride (Calbiochem, Merck, Darmstadt, Germany) for 1 min. Then sections were washed 3 times and mounted with Ultrakitt mounting medium (J.T. Baker, Mallinckrodt Baker, Inc., Philipsburg, NJ, USA). Size of glomeruli and number or area of nuclei per glomeruli were determined for 10 glomeruli per mouse.

Statistics

Kaplan Meier curves, column statistics, area under the anti-C1q curves (AUC anti-C1q) and non parametric correlation tests (Spearman) were performed using GraphPad Prism version 4 (GraphPad Software, San Diego, USA).

Results

Survival analysis

First, a survival study of 30 MRL/MpJ +/+ and 12 Balb/c control mice was performed. The onset of death within the MRL/MpJ +/+ group occurred after 5 month and 50% mortality was reached at 18 month. After 22 months all MRL/MpJ +/+ mice were dead. At this time mortality in the Balb/c control mice group was 8.33% (1 out of 12). The difference in mortality between the two mouse strains was significant (by log-rank test, $p < 0.0001$) (Figure 1).

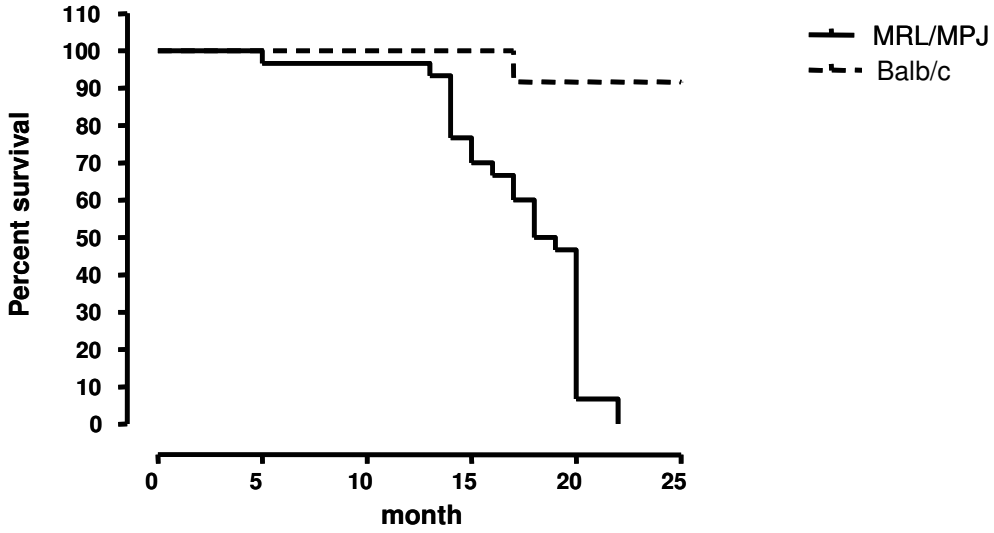

Figure 1. Survival analysis of 30 MRL/MpJ +/+ and 12 Balb/c mice. By 15 months the mortality rate in the MRL/MpJ +/+ group was 30% compared with no mortality observed in the Balb/c wild-type group. (By 22 month the mortality rate in the MRL/MpJ +/+ group was 100% compared to 8.33% in the Balb/c wild-type controls). The survival curves differed significantly (by log-rank test, $p < 0.0001$).

Detection of anti-C1q autoantibodies in serum and kidney eluate

Most of the MRL/MpJ +/+ mice were positive for anti-C1q autoantibodies, but they showed a large variability in titers. Starting at 3 month of age, MRL/MpJ +/+ mice had elevated anti-C1q when compared to Balb/c control mice. Although all mice showed a rise in titer at later time-points, individual mice showed fluctuating anti-C1q titers (Figure 2).

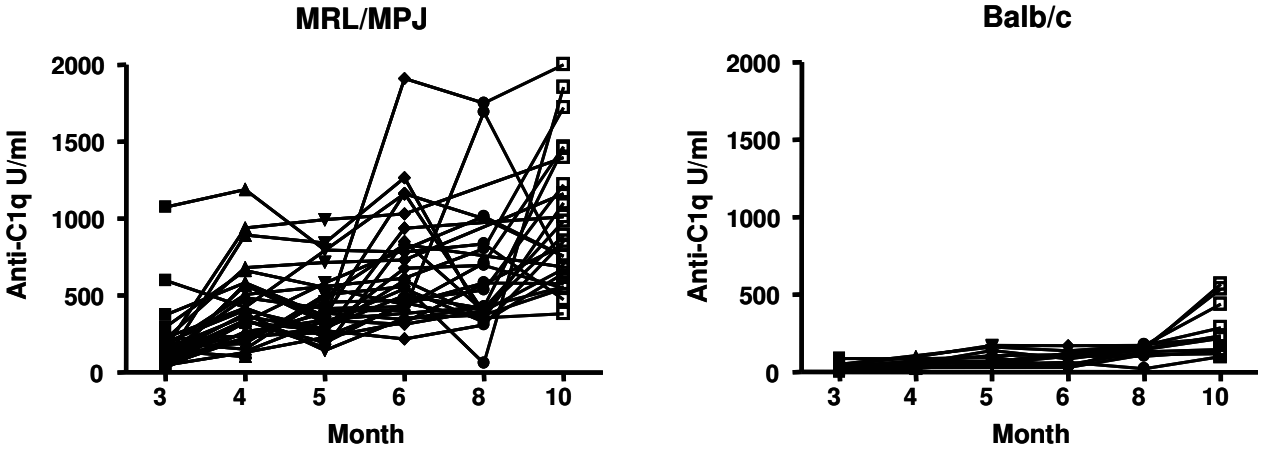

Figure 2. **Anti-C1q autoantibody titers in individual mice over time.** (a) MRL/MpJ +/+ and (b) Balb/c mice. Data are expressed relative to a standard monoclonal ms anti-C1q antibody. In MRL/MpJ +/+ mice, anti-C1q levels strongly varied over time.

Kidney tissue of 14 month-old MRL/MpJ +/+ with high titers of anti-C1q antibodies and Balb/c control mice was eluted to investigate the presence of anti-C1q autoantibodies in renal tissue. Anti-C1q autoantibodies were present in the eluate of kidney tissue from MRL/MpJ +/+ mice, but not in eluates of kidney tissue from Balb/c control mice. When comparing anti-C1q antibodies per µg IgG in the same kidney eluate or corresponding

serum, an enrichment of anti-C1q in the kidney eluate of MRL/MPJ mice was seen (Figure 3).

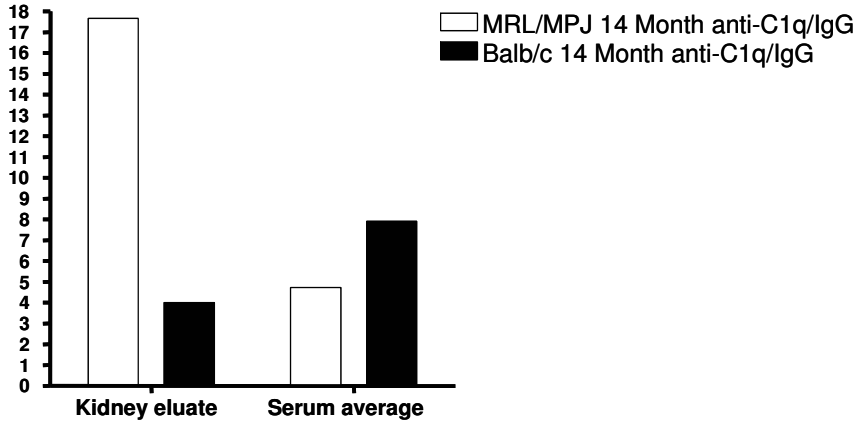

Figure 3. Renal elution of anti-C1q antibodies. Anti-C1q reactivity per μg IgG in eluate and of kidney tissue and serum of MRL/MPJ mice and Balb/c control mice at 14 month of age. An enrichment of anti-C1q autoantibodies is seen in the kidney eluate of MRL/MPJ mice when compared to serum.

Correlation between anti-C1q autoantibodies and renal damage

No correlation between survival and peak anti-C1q levels (p=0.0619) or areas under the anti-C1q curves (AUC anti-C1q; p=0.1139) was found. Renal pathology was assessed by C3 and IgG deposition within glomeruli as well as by determining the size of the glomeruli and the amount of nuclei per glomeruli. When compared with Balb/c mice, MRL/MpJ +/+ mice showed significantly larger glomeruli and more nuclei per glomerulus (data not shown). Some MRL/MpJ +/+ mice had deposition of C3 and/or IgG within their glomeruli with various amounts, whereas others were negative (Figure 4.)

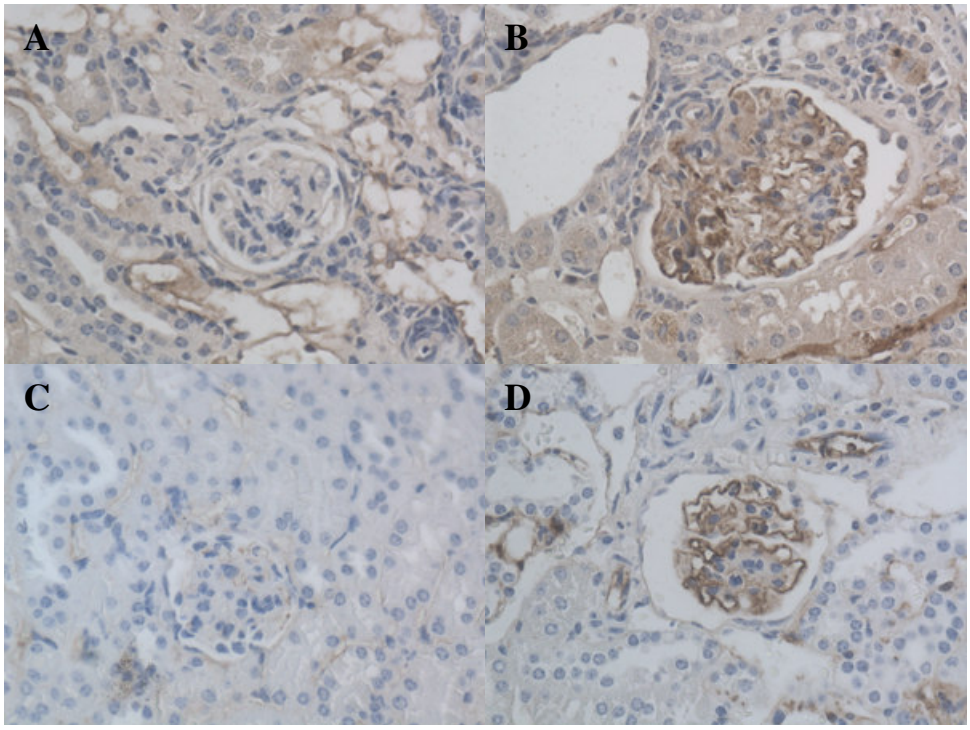

Figure 4. **Deposition of C3 and IgG in renal sections of MRL/MpJ +/+ mice**. MRL/MpJ +/+ mice having deposition of C3 and/or IgG varied in their positivity, whereas others were negative.

A. Glomerulus of a MRL/MpJ +/+ mouse, no deposition of C3. **B.** Deposition of C3 in a glomerulus of another mouse. **C.** Glomerulus without IgG deposition. **D.** Glomerular deposition of IgG. Original magnification x40.

Despite the fact that the majority of mice developed a glomerulonephritis, no correlation between peak anti-C1q levels or areas under the anti-C1q curves and any of these parameters could be detected (Table 1).

Table 1. Correlation of anti-C1q antibodies with parameters of glomerular histology in MRL/MpJ +/+ mice.

	C3 staining	IgG staining	Median glomerular area	Median area of nuclei per glomerulus
Peak anti-C1q level	0.5333	0.4649	0.2224	0.8405
AUC anti-C1q	0.3788	0.8595	0.8057	0.8013

Numbers are p-values for a two-tailed test of correlation (Spearman). Immunohistochemistry stainings of renal sections for C3 and IgG staining were scored, see also Figure 4. Total glomerular area and area of nuclei of 10 glomeruli per mouse were determined.

Discussion

As described for other lupus-prone mouse strains, we also found elevated levels of anti-C1q antibodies in MRL/MpJ +/+ mice starting at 3 month of age. Levels of anti-C1q antibodies varied in their positivity, which allowed the analysis of differences between mice with high levels of anti-C1q and mice with low levels of anti-C1q. 14 month-old mice having high titres of anti-C1q antibodies in serum, were shown to have accumulation of anti-C1q in their kidneys. This finding is in line with the finding of anti-C1q antibodies in renal tissue of MRL/MpJ-lpr/lpr mice having progressive renal damage (8) as well as in post-mortem material of end-stage kidneys from patients with lupus nephritis (11). In addition, some MRL/MpJ +/+ mice exhibited renal abnormalities at the age of 14 months with increased cellularity of their glomeruli as well as IgG and/or C3 deposition within the glomerulus which is in line with previous studies showing some deposition of C3 and IgG already in glomeruli of 7 month-old MRL/MpJ +/+ mice (9, 12). However, no correlation was found between levels of anti-C1q and parameters of renal abnormalities. In addition, levels of anti-C1q did not correlate with overall survival of MRL/MpJ +/+ mice. Therefore, our observational data suggest that anti-C1q seem not to be involved in the pathogenic mechanism of glomerulonephritis in lupus-prone MRL/MpJ +/+ mice and do not support the hypothesis that anti-C1q have a pathogenic role in SLE. However, anti-C1q in human SLE were shown to strongly correlate with proliferative forms of SLE nephritis but not with other forms of glomerulonephritis (13, 14), including the so-called C1q nephropathy that histologically closely resembles SLE nephritis (15, 16). Furthermore, from a morphological point of view, the

glomerulonephritis observed in MRL/MpJ +/+ mice differs from proliferative lupus nephritis. In MRL/MpJ +/+ mice, only slight mesangial proliferation in association with a membranous pattern of immune deposits was seen. Thus, different mechanisms might be involved in the pathogenesis of human lupus nephritis and the nephritis seen in MRL/MpJ +/+ mice. Only large studies on SLE patients that are followed over long periods might be able to answer the question whether anti-C1q are predictive of renal flares.

In conclusion, we could not observe a correlation of anti-C1q with survival and severity of glomerulonephritis in lupus-prone mice. Therefore our data do not support the hypothesis that anti-C1q have a pathogenic role in SLE. However, different pathogenic mechanisms might be involved in the glomerulonephritis of lupus-prone MRL/MpJ +/+ mice and human proliferative lupus nephritis.

Outlook

- Glomeruli of MRL/MpJ +/+ mice will be stained for C1q and C4, components of the classical pathway of complement, as further markers of lupus nephritis. The amount of deposition of C1q and C4 will be analyzed for their correlation with anti-C1q autoantibodies.

- A second cohort of 11 month-old MRL/MpJ +/+ mice will be analyzed for the correlation of anti-C1q with an earlier stage of glomerulonephritis.

- Since a preliminary analysis of IgG1 anti-C1q in MRL/MpJ +/+ mice unexpectedly suggests a protective role in this strain, we will also analyze IgG anti-C1q subclasses for their correlation with survival and severity of nephritis in the MRL/MpJ +/+ mouse cohorts.

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Publications

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Poster presentation: Bigler C, Lopez-Trascasa M, Potlukova E, Moll S, Danner D, Schaller M, and Trendelenburg M. Anti-nucleosome antibodies as a marker of active proliferative lupus nephritis.

XXII International Complement Workshop, 2008, Basel, Switzerland

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