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Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies

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The complement system plays a paradoxical role in the development and expression of autoimmunity in humans. The activation of complement in systemic lupus erythematosus (SLE) contributes to tissue injury. In contrast, inherited deficiency of classical pathway components, particularly C1q (ref. 1), is powerfully associated with the development of SLE. This leads to the hypothesis that a physiological action of the early part of the classical pathway protects against the development of SLE (ref. 2) and implies that C1q may play a key role in this respect. C1q-deficient (*C1qa*$^{-/-}$) mice were generated by gene targeting and monitored for eight months. *C1qa*$^{-/-}$ mice had increased mortality and higher titres of autoantibodies, compared with strain-matched controls. Of the *C1qa*$^{-/-}$ mice, 25% had glomerulonephritis with immune deposits and multiple apoptotic cell bodies. Among mice without glomerulonephritis, there were significantly greater numbers of glomerular apoptotic bodies in C1q-deficient mice compared with controls. The phenotype associated with C1q deficiency was modified by background genes. These findings are compatible with the hypothesis that C1q deficiency causes autoimmunity by impairment of the clearance of apoptotic cells.

Homozygous deficiency of the first component of the complement system, C1q, is the most powerful human susceptibility gene for the development of autoimmunity. In humans with homozygous C1q deficiency, single base-pair mutations in each of the three *C1q* genes (*a*, *b* and *c*) have been identified[3–8], associated either with absence of detectable protein or expression of reduced levels of dysfunctional protein. We generated C1q-deficient mice by homologous recombination to explore the mechanisms of the link between complement deficiency and autoimmunity. A targeting vector was constructed in which the coding region of the first exon of the *C1qa* gene[9] was disrupted by insertion of a neomycin-resistance cassette (Fig. 1*a*,*b*). The lack of *C1qa* transcripts was confirmed by northern-blot analysis of total RNA from liver and spleen. Homozygous mutant mice had no circulating C1q protein detectable by antigenic assays (ELISA and western blot; Fig. 1*c*) and undetectable functional classical pathway haemolytic activity. Analysis of the offspring obtained from two independent ES cell clones indicated that the disrupted allele was inherited in accordance with normal Mendelian patterns. *C1qa*$^{-/-}$ mice were fully viable and fertile.

We tested the hypothesis that C1q deficiency is associated with an autoimmune phenotype by monitoring cohorts of *C1qa*$^{-/-}$ mice consisting of 40 animals of a mixed genetic background (129/Ola×C57BL/6)F2 and 18 of pure inbred strain (129/Ola). Inbred C57BL/6 (n=23) and 129/Ola (n=59) mice were used as controls. Of the 34 C1q-deficient animals analysed at one year of age, 19 (55%) developed high titres (range: 1/80 to 1/2560) of anti-nuclear antibodies (ANA) compared with 13 of 82 animals (16%) (range: 1/80 to 1/1280) from the two control inbred

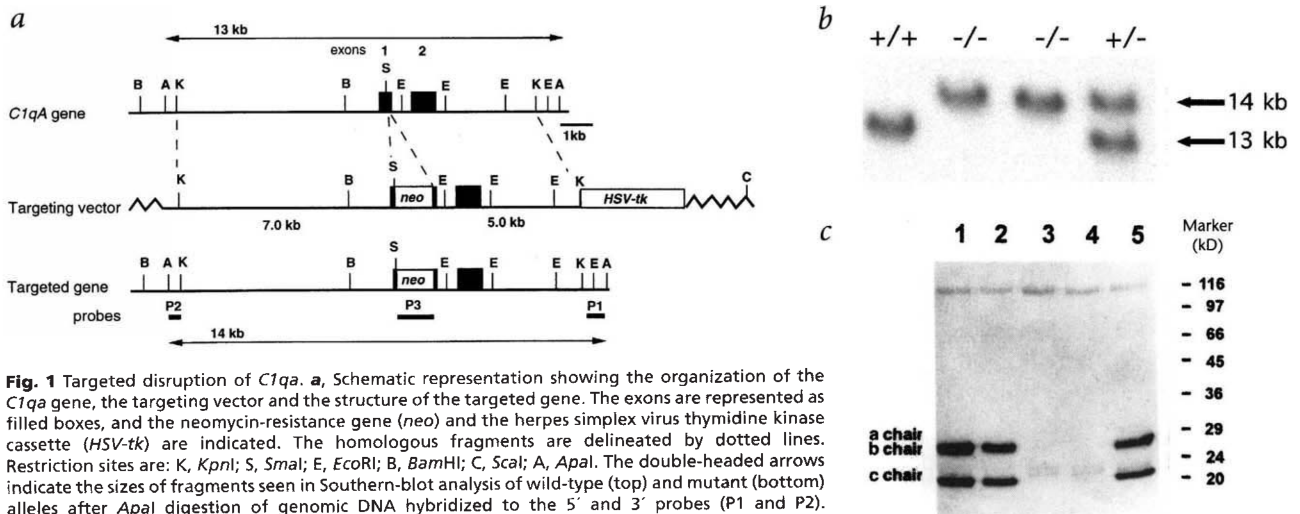

Fig. 1 Targeted disruption of *C1qa*. ***a***, Schematic representation showing the organization of the *C1qa* gene, the targeting vector and the structure of the targeted gene. The exons are represented as filled boxes, and the neomycin-resistance gene (*neo*) and the herpes simplex virus thymidine kinase cassette (*HSV-tk*) are indicated. The homologous fragments are delineated by dotted lines. Restriction sites are: K, *Kpn*I; S, *Sma*I; E, *Eco*RI; B, *Bam*HI; C, *Sca*I; A, *Apa*I. The double-headed arrows indicate the sizes of fragments seen in Southern-blot analysis of wild-type (top) and mutant (bottom) alleles after *Apa*I digestion of genomic DNA hybridized to the 5′ and 3′ probes (P1 and P2). ***b***, Representative Southern blot of *Apa*I-digested DNA from offspring of a cross between animals heterozygous for the disrupted *C1qa* gene. The wild-type allele is 13 kb and the mutant allele is approximately 14 kb due to the insertion of the *neo* gene. ***c***, Western-blot analysis. A polyclonal antibody anti-mouse C1q was used for the precipitation and detection of the protein in the sera from the two wild-type inbred strains 129/Ola (lane 1) and C57BL/6 (lane 5), and from heterozygous (lane 2) and homozygous (lanes 3,4) C1q-deficient animals. The positions of the three chains (reducing conditions) and the marker are indicated.

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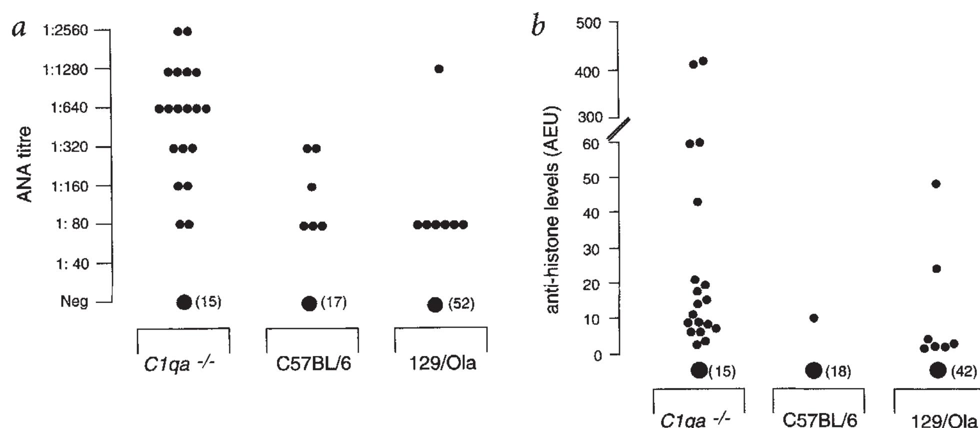
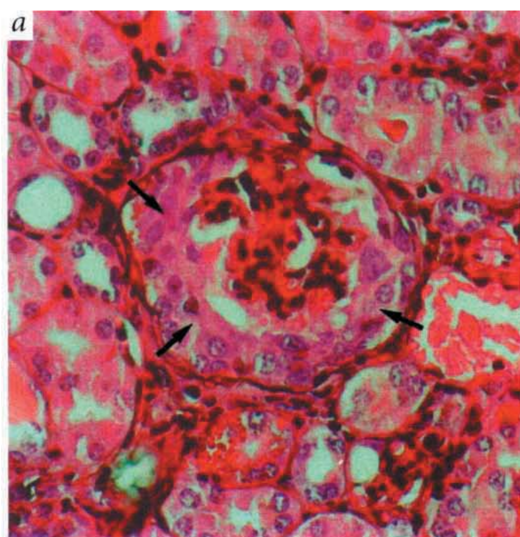


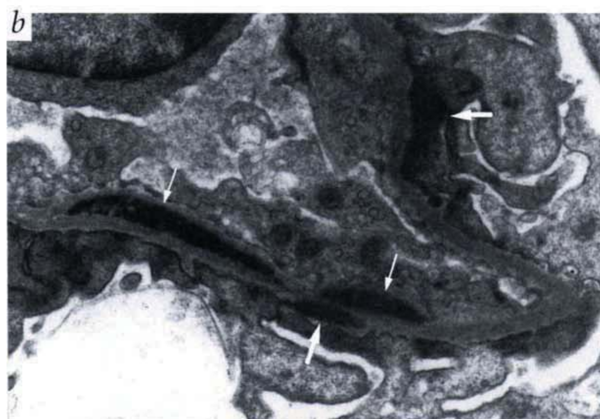
Fig. 2 Autoantibody profiles. ***a***, ANA titres in the C1q-deficient mice (129/Ola×C57BL/6) and in the two inbred strains (C57BL/6 and 129/Ola) at one year of age. Small circles represent one mouse; large circles a variable number of animals as indicated in parentheses. ***b***, Anti-histone antibody levels expressed in arbitrary ELISA units (AEU) relative to a standard positive sample which was assigned a value of 100 AEU. Values higher than 3 AEU (mean + 3SD) were considered positive. The symbols are the same as in *a*.

strains; $P<0.0001$ (Mann-Whitney test; Fig. 2*a*). Similarly, significant differences were observed in the levels of anti-histone antibodies between the C1q-deficient animals and controls (Fig. 2*b*). Total IgG levels were similar in both groups (data not shown). In contrast, none of the 18 $C1qa^{-/-}$ mice on a pure 129/Ola genetic background developed autoantibodies. Six of forty C1q-deficient mice on the hybrid (129/Ola×C57BL/6) background died at four to six months of age with severe proliferative crescentic glomerulonephritis (Fig. 3*a*). The presence of immune deposits in the kidneys was confirmed by silver staining, by immunofluorescence and by electron microscopy which localized the deposits in the subendothelial and subepithelial areas (Fig. 3*b*). C3 deposits in the glomeruli were detected by immunofluorescence using a monoclonal anti-mouse C3 antibody (data not shown). A striking feature of the glomerulonephritis was the presence of multiple apoptotic bodies in diseased glomeruli (Fig. 3*c*). All the remaining animals were killed at one year of age. Among the 40 $C1qa^{-/-}$ mice on the hybrid background, glomerulonephritis was detected in 11 mice (27%). In contrast, no glomerulonephritis was detected in any of the 18 $C1qa^{-/-}$ mice on a pure 129/Ola background ($P=0.01$, Fisher's exact test) or in any of 27 control 129/Ola ($P=0.001$) and 22 control C57BL/6 ($P=0.004$) mice examined from the cohorts.

These initial findings suggested that the expression of autoimmunity in C1q-deficient mice was affected by genetic modifiers. We therefore monitored a second cohort of animals comprising 226 $C1qa^{-/-}$ and 108 control mice (all 129/Ola×C57BL/6) at 3, 5 and 8 months of age (at which point all of them were killed).

Autoantibodies were detected from 3 months of age, and the levels increased progressively (data not shown). Of the $C1qa^{-/-}$ mice, 54% had high titres of ANA at 8 months of age compared with 33% of the controls (Fig. 4; range: 1/80 to 1/2560); $P=0.0038$ (Mann-Whitney test). However, this significant difference in the production of ANA was observed only between the males. Antibodies to the extractable nuclear antigen Sm were detected in 6 of 92 ANA-positive sera from the $C1qa^{-/-}$ mice.





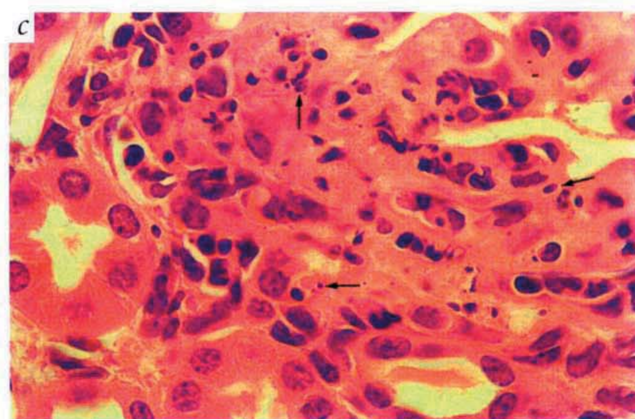


Fig. 3 Glomerulonephritis in C1q-deficient mice. ***a***, Glomerulus showing proliferation of cells in Bowman's space forming a crescent (arrows) (Periodic acid-Schiff methenamine silver, ×420). ***b***, Electron micrograph of part of a glomerular capillary loop showing subendothelial (thin arrows) and subepithelial (thick arrows) electron-dense deposits, ×17,250. ***c***, High-power photomicrograph of part of a glomerulus showing multiple apoptotic bodies. Three examples are indicated by arrows (Periodic acid-Schiff, ×1050).

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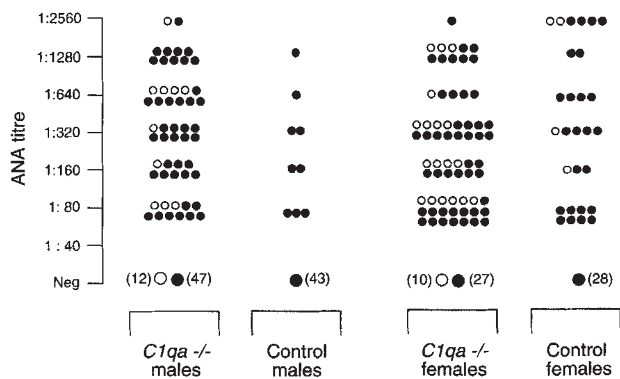

Fig. 4 Autoantibody profiles. ANA levels in 213 C1q-deficient mice and 108 controls at 8 months of age. Females and males are shown separately. Serum samples were titrated to end point. Small circles represent one mouse; large circles, a variable number of animals as indicated in parenthesis. Animals with evidence of glomerulonephritis on histological examination are represented with open symbols; filled circles indicate absence of renal involvement.

Table 1 • Histopathological assessment of kidney sections

Glomerulonephritis		0	I	II	III	IV
Controls	(108)	103	0	1	1	3
C1qa$^{-/-}$	(226)	168	7	27	16	8

None of the 30 ANA-positive controls tested showed antibodies to Sm. In contrast, the titres of autoantibodies to ssDNA, to chromatin and to histone were not significantly different between the gene-targeted and the wild-type mice (data not shown). It appears, therefore, that the background genes that are responsible for the development of low levels of autoimmunity in control hybrids, predominantly females, may cause more severe autoimmunity in the absence of C1q.

Histological examination of the kidneys revealed evidence of glomerulonephritis in 25% of *C1qa*$^{-/-}$ mice compared with 4% of control animals (P<0.0001, Fisher's exact test). Of the animals affected by glomerulonephritis, the five controls were all females, while renal disease was equally present in both sexes of the 58 *C1qa*$^{-/-}$ mice. The sections were scored on a 0–IV scale based on the intensity and extent of the histopathological changes as described previously[10] (Table 1). The glomerulonephritis was again characterized by the presence of multiple apoptotic bodies. Among mice without glomerulonephritis, there were significantly greater numbers of glomerular apoptotic bodies in C1q-deficient mice compared with controls (P<0.0001, Mann-Whitney test; Fig. 5*a*). The number of glomerular apoptotic bodies was greatly increased among mice with glomerulonephritis (Fig. 5*b*). Apoptotic bodies were detected in two of four control mice with grade III or IV glomerulonephritis. However, because only very few control mice developed glomerulonephritis, no statistical comparison was possible with C1q-deficient mice. The other two control mice that developed severe glomerulonephritis showed extensive glomerular fibrinoid necrosis, and apoptotic bodies could not be distinguished. Among the *C1qa*$^{-/-}$ mice, the presence of autoantibodies did not correlate with either the presence or the severity of the renal involvement, while in the control group the number of affected mice was too small for statistical analysis.

Apoptotic cells are cleared rapidly from injured glomeruli[11], and the presence of large numbers of apoptotic bodies in the glomeruli of C1q-deficient mice implies either markedly increased apoptosis or a defect in the clearance of apoptotic cells. The significantly increased numbers of apoptotic bodies in otherwise normal glomeruli in C1q-deficient mice, compared with controls, suggests that this is likely to be a primary defect. This is consistent with the report that C1q binds to apoptotic keratinocytes[12]. Cells undergoing death by apoptosis generate discrete subcellular structures referred to as surface blebs, which contain either nuclear or cytoplasmic constituents, many of which are targeted by autoantibodies in patients with SLE (refs 13–17). It has been proposed that apoptotic cells provide the source of autoantigens which drive the autoimmune response in SLE (refs 15–17). Our observations indicate that C1q may bind to apoptotic cells and promote their physiological clearance. Failure of this process in C1q deficiency may allow the persistence of apoptotic cells which provide the source of autoantigens that stimulate an autoantibody response in genetically susceptible hosts.

Methods

Targeted disruption of the mouse *C1qa* gene. The mouse *C1qa* gene was isolated from a 129/Sv genomic library. The exon-intron organization was determined by restriction endonuclease and DNA sequence analysis. The targeting vector was constructed by inserting the *neo* gene (pMC1Neo-poly (A)$^+$, Stratagene) into an *Sma*I restriction site located in the middle of the first exon. The herpes simplex virus thymidine kinase gene was appended at the 3′ end of the construct, outside the area of homologous recombination, to allow selection against random integration. Two different embryonic stem cell lines were transfected with the targeting vector by electroporation, and G418- and gancyclovir-resistant clones were screened by Southern-blot analysis using diagnostic digestions and probes as shown in Fig. 1. Germline transmission of the disrupted allele was obtained from two independent clones. Homozygous C1q-deficient and wild-type mice used in the analysis were of mixed genetic background (129/Ola×C57BL/6). The care and the use of the animals were in accordance with institutional guidelines.

Complement assays. C1q levels were determined by sandwich ELISA as described[18]. Functional C1q activity was measured by a reconstitution assay using human C1q-deficient serum and sensitized sheep red blood cells. Haemolytic complement activity was determined by a fluid-phase ^{51}Cr-release assay as previously described[19].

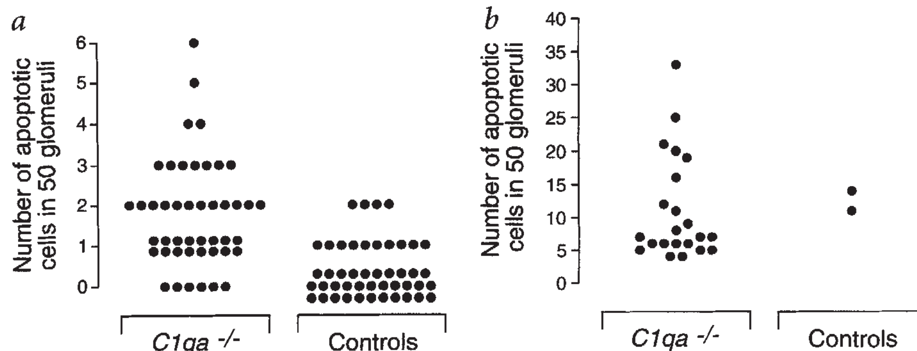

Fig. 5 Apoptotic bodies in glomeruli. ***a***, Number of apoptotic bodies per 50 glomeruli are shown in normal glomeruli of C1q-deficient mice compared with controls. There were significantly more apoptotic bodies in C1q-deficient mice than controls (Mann-Whitney test, P<0.0001). ***b***, Number of apoptotic bodies per 50 glomeruli C1q-deficient mice and controls with Grade III and IV glomerulonephritis. There were significantly more apoptotic bodies in glomeruli of C1q-deficient mice with Grade III and IV glomerulonephritis compared with C1q-deficient mice without glomerulonephritis (Grade 0; Mann-Whitney test, P<0.0001).

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Serological analysis. Mice were bled from the tail vein at regular intervals and serum was stored at −70 °C prior to analysis. IgG ANA levels were measured by indirect immunofluorescence using Hep-2 cells and a fluorescein-conjugated IgG Fc-specific anti-mouse secondary antibody. The serum samples were screened at a 1:80 dilution and the positive samples were titrated to end point. Anti-histone antibody levels were measured by ELISA as described[20]. The serum samples were screened at a 1:50 dilution. The results were expressed in arbitrary ELISA units (AEU) relative to a standard positive sample (derived from a MRL/Mp-*lpr/lpr* mouse) which was assigned a value of 100.

Histology. Kidneys were fixed in 10% buffered formalin and stained with hematoxylin and eosin. Coded samples were scored for glomerulonephritis as described previously[10]. Glomerular hypercellularity was graded on a 0–IV scale in which 0 = no involvement, and grades I–IV = <25%, 25–50%, 51–90% and >90% abnormal glomeruli, respectively. For electron microscopy examination, the kidneys were fixed in glutaraldehyde. Fluorescence microscopy was performed on frozen sections incubated with a monoclonal anti-mouse C3 antibody (Connex) or a polyclonal anti-mouse IgG. Apoptotic bodies were counted in sections coded to obscure their origin. A cell was considered apoptotic when it showed loss of cell volume, chromatin condensation along the nuclear membrane with intensely basophilic staining and/or nuclear fragmentation into spherical structures containing condensed chromatin.

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1. Walport, M.J. & Morgan, B.P. Complement deficiency and disease. *Immunol. Today* **12**, 301–306 (1991).
2. Lachmann, P.J. & Walport, M.J. Deficiency of the effector mechanisms of the immune response and autoimmunity. in *Ciba Foundation Symposium: Autoimmunity and Autoimmune diseases* (ed. Whelan, J.) **129**, 149–171 (Wiley, Chichester, 1987).
3. McAdam, R.A., Goundis, D. & Reid, K.M.B. A homozygous point mutation results in a stop codon in the C1q B-chain of a C1q-deficient individual. *Immunogenetics* **27**, 259–264 (1988).
4. Bowness, P. *et al.* Hereditary C1q deficiency and systemic lupus erythematosus. *Quart. J. Med.* **87**, 455–464 (1994).
5. Petry, F., Le, D.T., Kirschfink, M. & Loos, M. Nonsense and missense mutations in the structural genes of complement component C1q A and C chains are linked with two different types of complete selective C1q deficiencies. *J. Immunol.* **155**, 4734–4738 (1995).
6. Slingsby, J.H. *et al.* Homozygous hereditary C1q deficiency and systemic lupus erythematosus. A new family and the molecular basis of C1q deficiency in three families. *Arthritis Rheum.* **39**, 663–670 (1996).
7. Topaloglu, R. *et al.* Molecular basis of hereditary C1q deficiency associated with SLE and IgA nephropathy in a Turkish family. *Kidney Int.* **50**, 635–642 (1996).
8. Petry, F., Izzet Berkel, A. & Loos, M. Multiple identification of a particular type of hereditary C1q deficiency in the Turkish population: review of the cases andadditional genetic and functional analysis. *Human Genet.* **100**, 51–56 (1997).
9. Petry, F. *et al.* The mouse C1q genes are clustered on chromosome 4 and show conservation of gene organisation. *Immunogenetics* **43**, 370–376 (1996).
10. Jevnikar, A.M., Slinger, G.G., Brennan, D.C., Xu, H.W. & Kelley, V.R. Dexamethasone prevents autoimmune nephritis and reduces renal expression of Ia but not costimulatory signals. *Am. J. Pathol.* **141**, 743–751 (1992).
11. Baker, A.J. *et al.* Mesangial cell apoptosis: the major mechanism for resolution of glomerular hypercellularity in experimental mesangial proliferative nephritis. *J. Clin. Invest.* **94**, 2105–2116 (1994).
12. Korb, L.C. & Ahearn, J.M. C1q binds directly and specifically to surface blebs of apoptotic human keratinocytes. Complement deficiency and systemic lupus erythematosus revisited. *J. Immunol.* **158**, 4525–4528 (1997).
13. LeFeber, W.P. *et al.* Ultraviolet light induces binding of antibodies to selected nuclear antigens on cultured human keratinocytes. *J. Clin. Invest.* **74**, 1545–1551 (1984).
14. Golan, T.D., Elkon, K.B., Gharavi, A.E. & Krueger, J.G. Enhanced membrane binding of autoantibodies to cultured keratinocytes of systemic lupus erythematosus patients after ultraviolet B/ultraviolet A irradiation. *J. Clin. Invest.* **90**, 1067–1076 (1992).
15. Casciola-Rosen, L.A., Anhalt, G. & Rosen, A. Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. *J. Exp. Med.* **179**, 1317–1330 (1994)
16. Rosen, A., Casciola-Rosen, L.A. & Ahearn, J. Novel packages of viral and self-antigens are generated during apoptosis. *J. Exp. Med.* **181**, 1557–1561 (1995).
17. Casciola-Rosen, L.A., Rosen, A., Petri, M. & Schlissel, M. Surface blebs on apoptotic cells are sites of enhanced procoagulant activity: Implications for coagulation events and antigenic spread in systemic lupus erythematosus. *Proc. Natl. Acad. Sci. USA* **93**, 1624–1629 (1996).
18. Trinder, P., Faust, D., Petry, F. & Loos, M. Modulation of mRNA expression and secretion of C1q in mouse macrophages by anti-inflammatory drugs and cAMP: evidence for the partial involvement of a pathway that includes cyclooxygenase, protaglandin E2 and adenylate cyclase. *Immunol.* **84**, 638–644 (1995).
19. Andrews, B.S. & Theofilopoulos, A.N. A microassay for the determination of hemolytic complement activity in mouse serum. *J. Immunol. Methods* **22**, 273–281 (1978).
20. Burlingame, R.W. & Rubin, R.L. Subnucleosome structures as substrates in enzyme-linked immunosorbent assays. *J. Immunol. Methods* **134**, 187–199 (1990).