Complement C4 Inhibits Systemic Autoimmunity through a Mechanism Independent of Complement Receptors CR1 and CR2

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Abstract

The complement system enhances antibody responses to T-dependent antigens, but paradoxically, deficiencies in C1 and C4 are strongly linked to autoantibody production in humans. In mice, disruption of the *C1qa* gene also results in spontaneous autoimmunity. Moreover, deficiencies in C4 or complement receptors 1 and 2 (CR1/CR2) lead to reduced selection against autoreactive B cells and impaired humoral responses. These observations suggest that C1 and C4 act through CR1/CR2 to enhance humoral immunity and somehow suppress autoimmunity. Here we report high titers of spontaneous antinuclear antibody (ANA) in $C4^{-/-}$ mice. This systemic lupus erythematosus–like autoimmunity is highly penetrant; by 10 mo of age, all $C4^{-/-}$ females and most males produced ANA. In contrast, titers and frequencies of ANA in $Cr2^{-/-}$ mice, which are deficient in CR1 and CR2, never rose significantly above those in normal controls. Glomerular deposition of immune complexes (ICs), glomerulonephritis, and splenomegaly were observed in $C4^{-/-}$ but not $Cr2^{-/-}$ mice. $C4^{-/-}$, but not $Cr2^{-/-}$, mice accumulate activated T and B cells. Clearance of circulating ICs is impaired in preautoimmune $C4^{-/-}$, but not $Cr2^{-/-}$, mice. C4 deficiency causes spontaneous, lupus-like autoimmunity through a mechanism that is independent of CR1/CR2.

Key words: complement • autoantibody • glomerulonephritis • splenomegaly • immune complex

Introduction

Systemic lupus erythematosus (SLE)[1] is an autoimmune disease characterized by generalized disturbances in T and B lymphocytes and inflammatory damage to many tissues (1, 2). Activated, autoreactive B cells are present in patients with SLE and produce high titers of serum autoantibody to nuclear components. Serum antibody to double-stranded (ds)DNA is an important diagnostic marker for SLE. These antinuclear antibodies (ANAs) can directly attack tissues or form immune complexes (ICs) that elicit inflammation and damage tissues such as the kidney. Either individually or together, both processes can eventually cause organ failure (1, 3, 4).

The etiology and pathogenesis of SLE remain poorly understood. A variety of genetic factors have been linked to SLE (1, 4, 5), and among these, deficiencies in components of the classical pathway of complement carry perhaps the strongest association (1). SLE develops in most individuals with genetic deficiencies in C1 or C4 (6–9). Although genetic deficiencies in complement are rare, acquired deficiencies in complement components are common (8) and characteristic of SLE flares in patients (10–12). These acquired deficiencies are presumed to result from the consumption of complement by ICs (10–12); their impact, if any, in SLE pathogenesis is unknown. Thus, in humans, components of the classical pathway for complement activation, especially C1 and C4, may suppress incipient autoimmunity.

Recently, a similar role for C1q in suppressing autoimmunity in mice was demonstrated by Botto et al. (13). $C1qa^{-/-}$ mice on a mixed B6/129 genetic background spontaneously produced high titers of ANA, whereas wild-type controls generated low levels of autoantibody; consequently, 25% of $C1qa^{-/-}$ mice also exhibited glomerulonephritis (13).

How C1q activity suppresses autoimmunity remains unknown. C1 is the first component in the classical pathway, and one subunit of C1q associates with two subunits of

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[1]*Abbreviations used in this paper:* ANAs, antinuclear antibodies; BCR, B cell antigen receptor; BUN, blood urea nitrogen; GC, germinal center; HRP, horseradish peroxidase; ICs, immune complexes; RU, relative unit; SLE, systemic lupus erythematosus.

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C1r and C1s to form the C1 macromolecular complex (14). C1q binds to the Fc portions of antibodies complexed with antigen. This binding induces enzymatic activity by C1r, leading to the sequential activation of C1s, C4, and C2 to form the C3 convertase (14, 15). The split products of C4 and C3 can attach covalently to proteins (14, 15), and several of these split products—C4b, C4d, iC3b, C3dg, and C3d—are ligands for the CR1 (CD35) and CR2 (CD21) complement receptors (16). Significantly, C4 often mediates the biological activities of C1. For example, phagocytosis and lysis of bacteria (17) are regulated by C1 activity in generating C4 fragments and the formation of the C3 convertase. This interdependence suggests that C1q might suppress autoimmunity through a mechanism also requiring C4, a notion supported by clinical evidence that deficiencies in either C1 or C4 are strongly linked to SLE (6, 7, 9, 18).

How might C1 and C4 suppress the production of autoreactive antibody? Paradoxically, complement promotes specific immunity to T-dependent antigens (16); B lymphocytes express receptors for complement (19), and the temporary depletion of C3 reduces primary antibody responses (20). Also, impaired humoral immune responses are common in individuals with genetic deficiencies in some complement components (21). Recently, mice deficient in complement C1q, C4, and C3 were shown to have diminished antibody and germinal center (GC) responses (22, 23). Diminished antibody and GC responses are also characteristic of *Cr2* knockout mice that are deficient for CR1 and CR2 (24–26). The observation that $C4^{-/-}$ and $Cr2^{-/-}$ mice generate identical patterns of humoral impairment suggests that C4 enhances B cell responses via CR1/CR2 (16).

Three dominant models, not mutually exclusive, have been proposed to explain how C4 interacts with CR1/CR2 to promote humoral immunity. First, CR1/CR2 may focus complement-decorated ICs to follicular dendritic cells, promoting their support of GCs (27–29). Second, complement-decorated antigens may bridge the B cell antigen receptor (BCR) and CR2/CD19 coreceptor to enhance B cell responses (16, 21, 30). Third, complement-decorated antigens may aggregate CR2/CD19 complexes independently of the BCR and elicit CD19 signals that increase B cell responsiveness (31). Similar mechanisms may operate during the late stages of B cell development in bone marrow. $Cr2^{-/-}$ and $C4^{-/-}$ mice do not efficiently anergize B cells expressing autoreactive BCRs (32), and naive B cells from $Cr2^{-/-}$ mice express patterns of V_H gene segment usage different from $Cr2^{+/-}$ controls (26). Similarly, *lpr/lpr* mice bred to be deficient in C4 or CR1/CR2 have accelerated autoimmune disease (32).

These models to explain complement's (C4) role in enhancing humoral immunity and suppressing autoantibody depend on CR1/CR2. This dependence on CR1/CR2 in systemic autoimmunity is consistent with the observation that leukocytes from SLE patients express lower amounts of CR1 and CR2 (33). Similarly, CR1 and CR2 are progressively lost from the surfaces of B cells in MRL/*lpr* mice, even before the onset of autoimmune nephritis (34).

However, if complement's promotion of humoral immunity and suppression of SLE are mediated by CR1/CR2, the absence of clinical associations between genetic deficiencies of C3 and SLE (6, 7) is perplexing, as C3 split products are principal ligands for CR1 and CR2 (16, 21). Indeed, whereas C3-deficient mice have poor primary antibody responses (16), they exhibit good selection against autoreactive B cells and no significant acceleration of *lpr*-induced autoimmunity (32). The weak association of C3 deficiency and autoimmunity does not preclude a role for CR1 and CR2 in self-tolerance, as C4 also generates ligands for CR1 and CR2 (16). The absence of an association does, however, raise the possibility that C4 promotes autoantibody production independently of CR1/CR2 and by a mechanism distinct from complement's immunoenhancing activities.

In this study, we demonstrate that $C4^{-/-}$ mice achieve high levels of spontaneous ANA, splenomegaly, and glomerulonephritis by 10 mo of age. Complete genetic penetrance of C4 deficiency occurs in female mice, but only two-thirds of age-matched males become autoimmune. In contrast, $Cr2^{-/-}$ mice on the same genetic background did not produce significant levels of ANA nor exhibit kidney pathology. Thus, C4 deficiency elicits a lupus-like autoimmunity in mice by processes that do not depend on CR1 and CR2.

Materials and Methods

Mice. $Cr2^{-/-}$ (25) and $C4^{-/-}$ (22) mice were originally established on the 129/Sv genetic background and subsequently bred onto a hybrid B6/129, homozygous Igh^b background as described (26). In brief, $Cr2^{-/-}$ and $C4^{-/-}$ mice were crossed with C57BL/6 mice (The Jackson Laboratory), and F2 offspring were typed for *Cr2* or *C4* (22, 25, 26) and *Igh* haplotypes (26). C57BL/6 and 129 mice share identical MHC haplotypes, the locus most prominently linked to autoimmunity. The two strains differ at *Igh*: B6 mice are Igh^b and 129 mice are Igh^a. As heterogeneity at *Igh* could alter the potential B cell repertoires in these mice, we selected only cohorts of B6/129 mice and their littermates carrying mutant *C4* or *Cr2* alleles that were also homozygous for Igh^b. We designate this background as B6/129.Igh^b or wild type. More than 10 females and males in each cohort were bred to generate experimental and control animals. In some experiments, C57BL/6 mice were also used as normal controls. Mice with a mixed B6/129 genetic background have been used in studies on autoimmunity (13, 35, 36). In some studies, wild-type B6/129 mice exhibit slightly higher levels of background autoantibody than do B6 mice (13). In others, even old B6/129 mice do not have elevated levels of autoantibody (35, 36). MRL-Fas^{lpr} mice were purchased from The Jackson Laboratory. All mice in this study were maintained under specific pathogen–free conditions at the Duke University Medical Center vivarium. Mice were bled at 2, 5–6, or 10 mo of age. All mice were killed at 10 mo of age.

Detection of ANA and Antibody Specific for Native DNA. Slides containing HEp-2 cells and *Crithidia luciliae* were purchased from Sigma-Aldrich and Scimedx Corp., respectively. The presence of IgG ANA and IgG specific for native (n)DNA was deter-

mined by reactivity to HEp-2 (37) or *C. luciliae* (38), respectively. Slides were rehydrated in PBS, pH 7.4, blocked with PBS containing 10% FCS and 0.1% Tween 20 (Sigma-Aldrich), and then washed with PBS containing 1% BSA and 0.1% Tween 20. Serum samples were diluted in this washing solution starting at 1:40 and 1:10 for ANA and anti-nDNA, respectively, and incubated with substrates for 1 h at room temperature. Bound serum IgG was revealed by FITC-conjugated goat anti–mouse IgG (Sigma-Aldrich). Slides were counterstained with Evans blue (Sigma-Aldrich), and examined blindly under a fluorescence microscope. All serum samples that were positive at the starting dilution were serially diluted (1:3 for ANA, 1:2 for nDNA) and titrated to endpoints.

ELISA for Anti-DNA Antibodies. Double-stranded calf thymus DNA (dsDNA; Sigma-Aldrich) was purified by phenol–chloroform extraction and then treated with S1 nuclease (Life Technologies) as described (39) to remove single-stranded (ss)DNA contaminants. To prepare ssDNA, dsDNA was boiled in water for 10 min and diluted in ice cold 1× SSC buffer to 50 μg/ml. dsDNA was also diluted to 50 μg/ml in 1× SSC. Diluted ss- and dsDNA preparations were coated and plates blocked as described (40–42). Serum samples were diluted 1:100 and incubated on DNA-coated plates for 1 h at room temperature. Each ELISA plate included a standard of a serially diluted mAb, TG7-83 (IgG1/λ1; from T.F. Tedder, Duke University, Durham, NC) that avidly binds both ss- and dsDNA. After washing, bound IgG was revealed by horseradish peroxidase (HRP)-conjugated goat anti–mouse IgG (Sigma-Aldrich). HRP activity was determined and analyzed as described elsewhere (26). The TG7-83 standard bound immobilized ss- and dsDNA to an endpoint

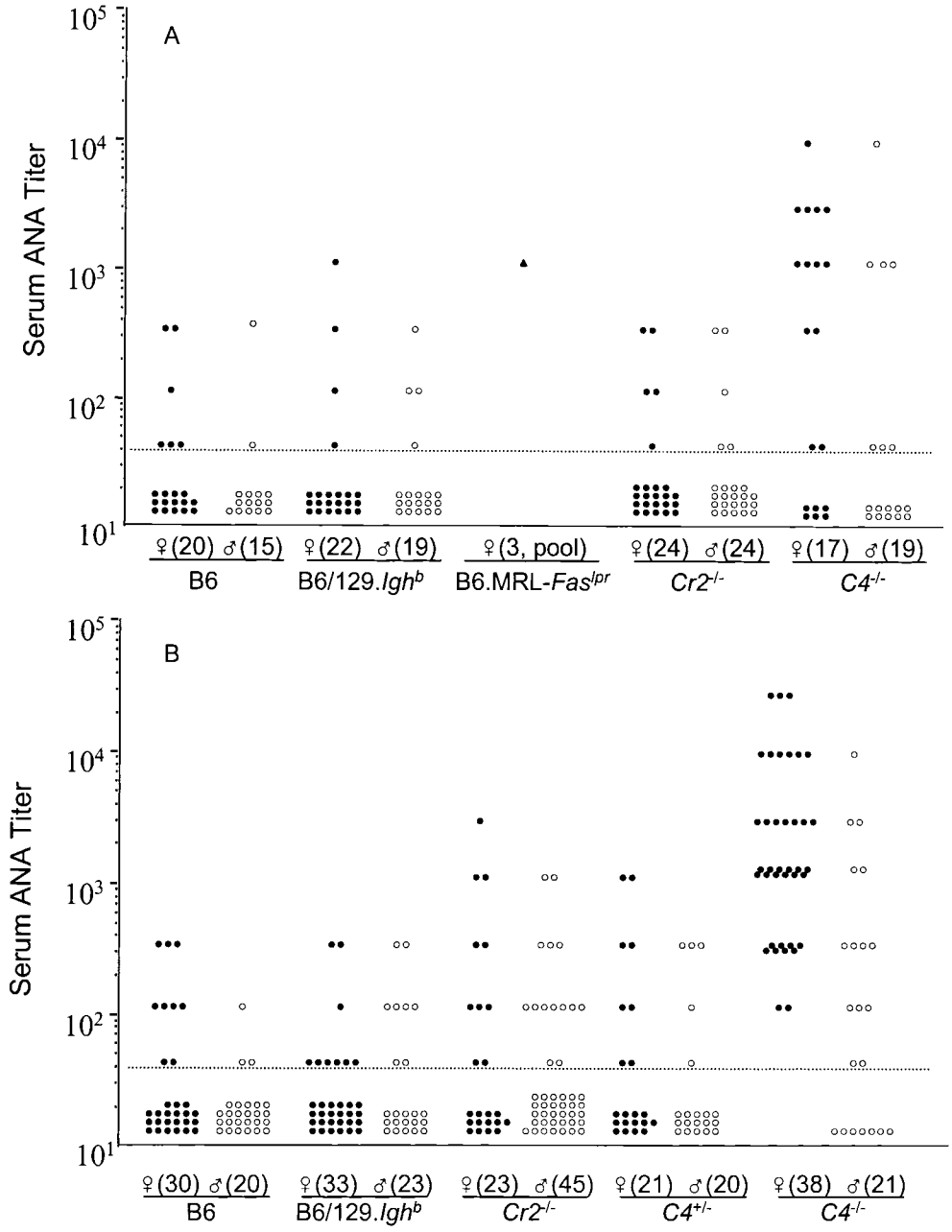

Figure 1. ANA titers in *Cr2*$^{-/-}$, *C4*$^{-/-}$, and control mice at 5–6 (A) or 10 mo (B) of age. Each point represents a serum sample from an individual mouse, except for B6.MRL-*Fas*^{lpr}, which is a serum pool from three mice. Numbers in parentheses following the symbols of sex are indicate numbers of mice. The broken line indicates the cut-off titer (1:40); placement of data points below this line does not indicate differences in titers.

concentration of 25 ng/ml. Serum samples were considered positive if the OD at 1:100 dilution was greater than the TG7-83 endpoint OD on the same plate.

Histopathology of Kidney and Spleen. Kidney and spleen sections were prepared as described (43). Immunohistochemistry for the detection of T and B cells and GCs in spleen sections has been described elsewhere (43). To identify IgG and C3 deposition on kidney sections, acetone-fixed sections were rehydrated in PBS for 20 min and blocked in PBS, pH 7.4, containing 10% normal goat serum (Life Technologies) and 0.1% Tween 20. The sections were then stained at room temperature for 1 h with FITC-conjugated goat anti–mouse IgG (Sigma-Aldrich) or goat anti–mouse C3 antibodies (ICN Biomedicals). After staining, slides were washed, counterstained with Evans blue, and examined by fluorescence microscopy. Some kidney sections were postfixed with 1% paraformaldehyde (Sigma-Aldrich), stained with hematoxylin and eosin (Sigma-Aldrich), and examined by microscopy. Glomerulonephritis was determined according to established criteria (44). Renal function was assessed by measurement of urea nitrogen in serum using a blood urea nitrogen (BUN) rate kit (Sigma-Aldrich).

Flow Cytometry. Splenocyte suspensions were prepared and blocked for FcR-mediated binding (26). Cells were then stained with biotinylated antibodies, followed by staining with streptavidin and antibodies conjugated with fluorochromes. 7-aminoactinomycin D (7-AAD; Molecular Probes, Inc.) was used to identify dead cells. The following antibodies/conjugates were used: biotinylated monoclonal anti–Mac-1, -B220, -CD44, or –TCR-β (PharMingen); FITC-labeled monoclonal GL-7, anti–Gr-1, –TCR-β, or -B220 (PharMingen); PE-conjugated monoclonal anti-B220 or -Fas (PharMingen); and Red 613–labeled streptavidin (Life Technologies).

In Vivo Clearance of Circulating ICs. The IgG2bλ1 mAb, P14.2.14 (from Dr. T. Imanishi-Kari, Tufts University, Boston, MA), which binds (4-hydroxy-3-nitrophenyl)acetyl (NP) with a $K_a \approx 10^6\ M^{-1}$, was used to make ICs. P14.2.14 was mixed with biotinylated NP_{16}-BSA at a 3:1 molar ratio and incubated at 37°C for 1 h. After incubation, the reaction mixture was centrifuged at 12,500 *g* for 10 min; <4% of total protein precipitated. Soluble ICs were injected intravenously into mice. Each mouse received a preparation of ICs formed with 100 μg of antibodies and 13 μg of biotinylated NP_{16}-BSA, arbitrarily designated as 100 relative units (RU). Mice were bled at different times after injection, and levels of IC in plasma were assessed by ELISA. In brief, streptavidin (Sigma-Aldrich) was coated on plates at 10 μg/ml in 0.1 M carbonate buffer, pH 8.8. Plates were then washed and blocked with PBS containing 0.1% Tween 20 and 1% BSA. Plasma samples were serially diluted, added to plates, and incubated for 30 min at room temperature. After washing, bound ICs were revealed with HRP-conjugated goat anti–mouse IgG2b or goat anti–mouse Igλ (Southern Biotechnology Associates, Inc.) at room temperature for 1 h. Plates were then washed, developed, and analyzed as above.

Results

$C4^{-/-}$, not $Cr2^{-/-}$, Mice Produce High Titers of Autoantibody. Cohorts of $C4^{-/-}$, $Cr2^{-/-}$, and control mice were examined for IgG ANA. At 2 mo of age, IgG ANA was not detectable in $C4^{-/-}$ mice ($n = 12$) or in age- and sex-matched wild-type controls ($n = 8$). By 5–6 mo of age, low titers of ANA were occasionally detected (≈20%) in B6/129.*Igh*^{b} and B6 mice (Fig. 1 A). However, more than half (9/17) of age-matched, female $C4^{-/-}$ mice had developed titers of ANA that were comparable to ANA present in sera pooled from B6.MRL-*Fas*^{lpr} mice (Fig. 1 A). At 5–6 mo, some male $C4^{-/-}$ mice exhibited higher levels of ANA than sex-matched controls, but the difference was not statistically significant ($P > 0.05$; χ^2 test). Unlike the C4-defi-

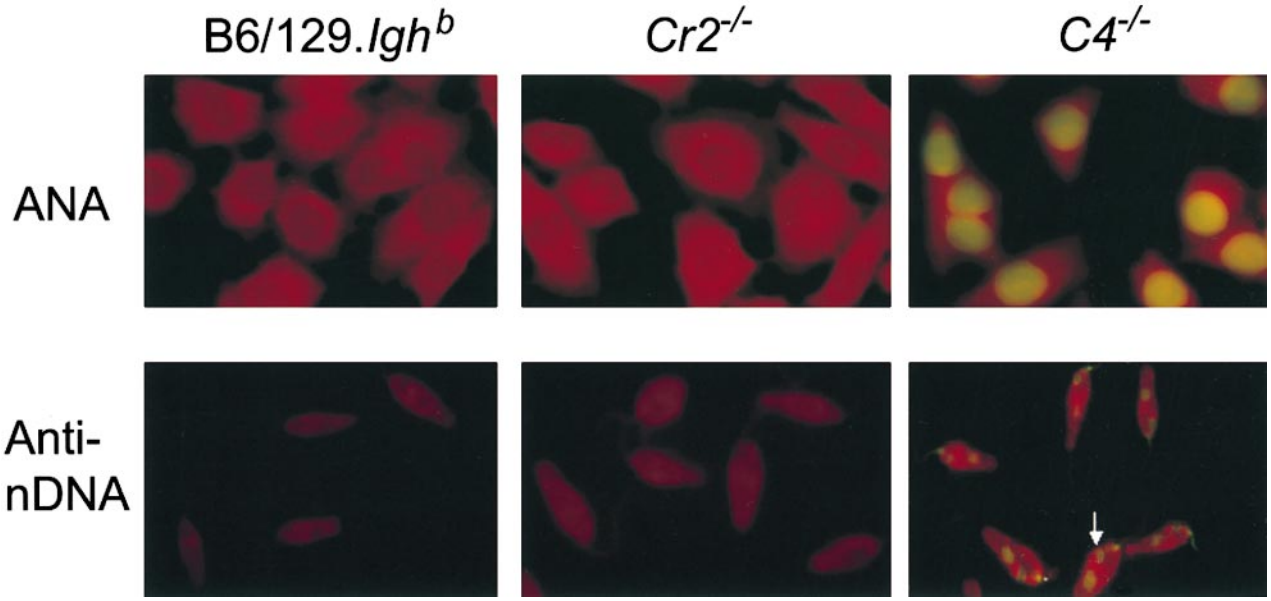

Figure 2. Representative staining for IgG ANA and anti-nDNA serum antibody from B6.*Igh*^{b} (left), $Cr2^{-/-}$ (center), and $C4^{-/-}$ (right) mice. HEp-2 cells were used as substrates for ANA (top panels, ×200 magnification), and *C. luciliae* were used to detect anti-nDNA antibody (bottom panels, ×1,000 magnification). Bound serum IgG was revealed by FITC-conjugated goat anti–mouse IgG (green). Cells were counterstained with Evans blue (red). Arrow indicates serum IgG binding to the kinetoplast of *C. luciliae*, where dsDNA is present in native form.

cient mice, 5–6-mo-old $Cr2^{-/-}$ mice produced only occasional and low levels of serum ANA that could not be distinguished from that present in control groups (Fig. 1 A).

By 10 mo of age, the incidence of ANA in both wild-type and $Cr2^{-/-}$ mice increased by an insignificant amount ($P > 0.05$, χ^2 test), and ANA titers remained equivalent in both groups as well (Fig. 1 B and Fig. 2). $C4^{+/-}$ mice also exhibited background levels of ANA at 10 mo. In contrast, significant titers of ANA, some as high as 1:30,000, were present in all 10-mo-old, female $C4^{-/-}$ mice (Fig. 1 B and Fig. 2). The majority (14/21) of 10-mo-old male $C4^{-/-}$ mice were also positive for ANA, but ANA titers and the frequency of ANA^+ males were significantly lower than those of female littermates (Fig. 1 B).

Antibody specific for DNA is a common component of ANA, and anti-DNA antibody is often used as a diagnostic marker for SLE (1). Therefore, we screened the sera of $C4^{-/-}$, $Cr2^{-/-}$, and control mice for the presence of antibody specific for ss- and dsDNA by ELISA (40–42) and for antibody specific for the native form of dsDNA, nDNA (Fig. 2 and Tables I and II) (38).

In general, the frequencies of reactive sera and specific IgG titers were highest for ssDNA, followed by dsDNA and nDNA, respectively. At 5–6 mo of age, only 5% (1/20) of B6 mice expressed detectable serum titers of any form of anti-DNA antibody, a frequency too low to determine if females were more prone to autoantibody production than males (Table I). Levels of all three types of anti-DNA antibody were modestly higher in age-matched B6/129.Igh^b mice than in B6 mice. Anti–ss- and dsDNA antibodies appear to be more common in female B6/129.Igh^b mice (18%; 4/22) than in males (5%; 1/21) (Table I). At 5–6 mo, the frequency of female (46%; 11/24), but not male (13%; 3/24), $Cr2^{-/-}$ mice with serum anti-ssDNA IgG was significantly higher than that of B6/129.Igh^b controls. However, frequencies of $Cr2^{-/-}$ mice with serum IgG reactive to ds- and nDNA did not differ from those in wild-type animals (Table I). In sharp contrast to the other cohorts of mice, both the frequencies and titers of anti-DNA IgG were generally elevated in female and male $C4^{-/-}$ mice. Between 74% (14/19) and 47% (9/19) of 5–6-mo-old female $C4^{-/-}$ mice developed significant levels of IgG antibody specific

Table I. *Spontaneous Autoantibody Production in 5–6-mo-old Mice*

	B6		B6/129.Igh^b		$Cr2^{-/-}$		$C4^{-/-}$	
	female	male	female	male	female	male	female	male
n	20	15	22	19	24	24	19	17
ANA percent positive*	30	13	18	21	21	21	68	41
Range (titer)‡	0–360	0–360	0–1,080	0–360	0–360	0–360	0–9,720	0–9,720
Median	0	0	0	0	0	0	360	0
χ^2 (*P*)§	ns	ns	–	–	ns	ns	<0.005	ns
Anti-ssDNA percent positive‖	0	0	18	5	46	13	74	41
Range (μg/ml)	0	0	0–50	0–5	0–14	0–4	0–342	0–48
Median	0	0	0	0	0	0	5	0
χ^2 (*P*)	<0.05	ns	–	–	<0.05	ns	<0.0005	<0.01
Anti-dsDNA percent positive‖	5	0	18	5	25	13	53	29
Range (μg/ml)	0–3	0	0–10	0–6	0–14	0–7	0–350	0–30
Median	0	0	0	0	0	0	3	0
χ^2 (*P*)	ns	ns	–	–	ns	ns	<0.05	ns
Anti-nDNA percent positive¶	0	0	5	5	14	14	47	35
Range (titer)	0	0	0–20	0–10	0–20	0–20	0–80	0–40
Median	0	0	0	0	0	0	0	0
χ^2 (*P*)	ns	ns	–	–	ns	ns	<0.005	<0.05

*End-point titers ≤1/40.
‡Samples are arbitrarily assigned a value of zero for statistics purposes if titrated below cut-off values.
§Compared to age- and sex-matched B6/129.Igh^b mice. ns, not significant ($P > 0.05$).
‖Serum concentration ≥2.5 μg/ml.
¶Endpoint titers ≤1/10.

Table II. *Spontaneous Autoantibody Production in 10-mo-old Mice*

	B6		B6/129.*Igh*^{b}		*Cr2*$^{-/-}$		*C4*$^{+/-}$		*C4*$^{-/-}$	
	female	male	female	male	female	male	female	male	female	male
n	30	20	33	23	23	45	21	20	38	21
ANA percent positive*	30	15	27	35	43	31	38	25	100	67
Range (titer)‡	0–360	0–120	0–360	0–360	0–1,080	0–360	0–1,080	0–360	120–29,160	0–9,720
Median	0	0	0	0	0	0	0	0	1,080	120
χ^2 (*P*)§	ns	ns	–	–	ns	ns	ns	ns	<0.00001	<0.05
Anti-ssDNA percent positive‖	13	0	18	13	22	9	10	0	84	29
Range (μg/ml)	0–11	0	0–150	0–32	0–38	0–10	0–4	0	0–286	0–357
Median	0	0	0	0	0	0	0	0	13	0
χ^2 (*P*)	ns	ns	–	–	ns	ns	ns	ns	<0.00001	ns
Anti-dsDNA percent positive‖	7	0	15	4	26	7	14	5	76	14
Range (μg/ml)	0–12	0	0–150	0–30	0–55	0–12	0–5	0–3	0–318	0–260
Median	0	0	0	0	0	0	0	0	5	0
χ^2 (*P*)	ns	ns	–	–	ns	ns	ns	ns	<0.00001	ns
Anti-nDNA percent positive¶	10	0	9	4	9	7	0	0	58	14
Range (titer)	0–20	0	0–20	0–10	0–20	0–20	0	0	0–160	0–80
Median	0	0	0	0	0	0	0	0	10	0
χ^2 (*P*)	ns	ns	–	–	ns	ns	ns	ns	<0.00005	ns

*End-point titers ≤1/40.
‡Samples are arbitrarily assigned a value of zero for statistics purposes if titrated below cut-off values.
§Compared to age- and sex-matched B6/129.*Igh*^{b} mice. ns, not significant ($P > 0.05$).
‖Serum concentration ≥2.5 μg/ml.
¶Endpoint titers ≤1/10.

for ss-, ds-, and nDNA (Table I). At 5–6 mo of age, male *C4*$^{-/-}$ mice also had increased levels of serum anti-ssDNA and -nDNA antibody, but the fraction of positive animals (41%; 7/17, and 35%; 6/17, respectively) and their antibody titers were below that of *C4*$^{-/-}$ females (Table I).

With time, titers of serum autoantibody and the frequency of positive animals increased. These increases are not significant in 10-mo-old B6, B6/129.*Igh*^{b}, and *Cr2*$^{-/-}$ mice but are pronounced in female *C4*$^{-/-}$ animals (Table II). Every (38/38) *C4*$^{-/-}$ female mouse developed IgG ANA by 10 mo of age, and most also had significant titers of serum IgG that bound ssDNA (84%; 32/38), dsDNA (76%; 29/38), or nDNA (58%; 22/38). Autoantibody titers were also significantly higher in 10-mo-old *C4*$^{-/-}$ females, with median concentrations of serum anti-DNA IgG in the range of ≈10 μg/ml (Table II). In contrast, 10-mo-old male *C4*$^{-/-}$ mice exhibited only a modest increase in ANA frequencies and titers and had levels of anti-DNA antibodies that were not significantly different from controls (Table II).

Autoantibody production by female *C4*$^{-/-}$ mice is not an artifact of the mixed genetic background present in these animals. Significant autoimmunity was not present in either female or male 10-mo-old *C4*$^{+/-}$ controls (Table II). Thus, C4 deficiency alone is capable of inducing a potent autoimmune state in B6/129.*Igh*^{b} mice. Realization of this autoimmunity is, however, regulated by sex-specific factors.

Although a significantly higher proportion of female *Cr2*$^{-/-}$ mice exhibited anti-ssDNA IgG antibody at 5–6 mo (Table I), at 10 mo autoantibody levels in *Cr2*$^{-/-}$ mice were not different from B6/129.*Igh*^{b} controls (Table II). Thus, the absence of CR1 and CR2 is not sufficient to cause SLE-like autoimmunity in mice with a genetic background that is a mix of the B6 and 129 strains.

IC Deposition and Glomerulonephritis in C4$^{-/-}$ *Mice.* Consistent with a pattern of SLE-like autoimmunity, 10-mo-old female *C4*$^{-/-}$ mice manifest a striking glomerular pathology with a predominantly mesangial deposition of IgG and C3 and marked enlargement with hypercellularity (Fig. 3). This pattern of glomerulonephritis was detected in half (5/10) of female *C4*$^{-/-}$ mice, but not in *C4*$^{-/-}$ males (0/5). Despite the striking renal histopathology present in 10-mo-old female *C4*$^{-/-}$ mice, we did not detect compro-

mised kidney function. BUN levels in five randomly chosen 10-mo-old female $C4^{-/-}$ and 129/B6.Igh^b mice remained within normal ranges (22.5 ± 2.5 and 20.4 ± 1.6 mg/dl [mean ± SEM], respectively; $P > 0.05$, Student's t test). $C4^{-/-}$ mice in this BUN test cohort were killed, and their kidneys were examined histologically; two exhibited the characteristic glomerular IgG and C3 deposition and mononuclear cell infiltration (Fig. 3). In contrast to $C4^{-/-}$ mice, cohorts of 10 female $Cr2^{-/-}$ and B6/129.Igh^b animals exhibited normal glomerular structure. Significant deposits of IgG or C3 were not observed in the kidneys of wild-type controls and $Cr2^{-/-}$ mice (Fig. 3).

Splenomegaly in C4-deficient Mice. Like other strains of mice that develop spontaneous, systemic autoimmunity (45), female $C4^{-/-}$ animals develop splenomegaly. The spleens of 2-mo-old $C4^{-/-}$ mice (56 ± 2 mg, $n = 12$) are no larger than those of wild-type controls (62 ± 3 mg, $n = 10$). By 10 mo, average spleen weight in B6, B6/129.Igh^b, and $C4^{+/-}$ mice uniformly increased by 40–50% to ~90 mg (Fig. 4). The spleens of $Cr2^{-/-}$ mice were comparably sized at 10 mo of age, with averages of 101 ± 6 and 108 ± 7 mg/spleen in males and females, respectively (Fig. 4). However, increases in 10-mo-old female $C4^{-/-}$ mice were much larger, with an average spleen weight (368 ± 64 mg) about fourfold heavier than that of age-matched controls (90 ± 7 mg; $P < 0.0005$, Student's t test). More than two-thirds (18/26) of 10-mo-old $C4^{-/-}$ female mice had spleen weights at least twice the wild-type average, and approximately one-third (8/26) had spleens greater than or equal to four times larger (Fig. 4). Splenomegaly was present in at least one 10-mo-old male $C4^{-/-}$ mouse, but as a group, male $C4^{-/-}$ mice do not exhibit significant splenic enlargement in comparison to age-matched controls ($P > 0.05$, Student's t test). Indeed, at 10 mo, distributions of spleen weights in B6, B6/129.Igh^b, $C4^{+/-}$, and $Cr2^{-/-}$ mice were not significantly different (Fig. 4). Lymph nodes and Peyer's patches in $C4^{-/-}$ mice appeared comparable in size to all control groups (data not shown).

Analysis of Splenocyte Populations in $C4^{-/-}$ Mice. To determine if splenomegaly in $C4^{-/-}$ mice resulted from the expansion of splenocyte populations, we analyzed spleen cell compartments by flow cytometry. Spleens of 10-mo-old B6, B6/129.Igh^b, and $Cr2^{-/-}$ mice, strains that show no evidence of splenomegaly (Fig. 4), contain similar numbers of nucleated cells (average, 114 ± 14 × 10^6; range, 66–150 × 10^6). Numbers of nucleated splenocytes from five randomly chosen 10-mo-old female $C4^{-/-}$ mice (178 ± 40 × 10^6 per spleen) were moderately (50–60%) increased over controls, even though spleen weights were increased an average of 300%. This observation suggests that vascular congestion plays a significant role in the splenomegaly of $C4^{-/-}$ mice.

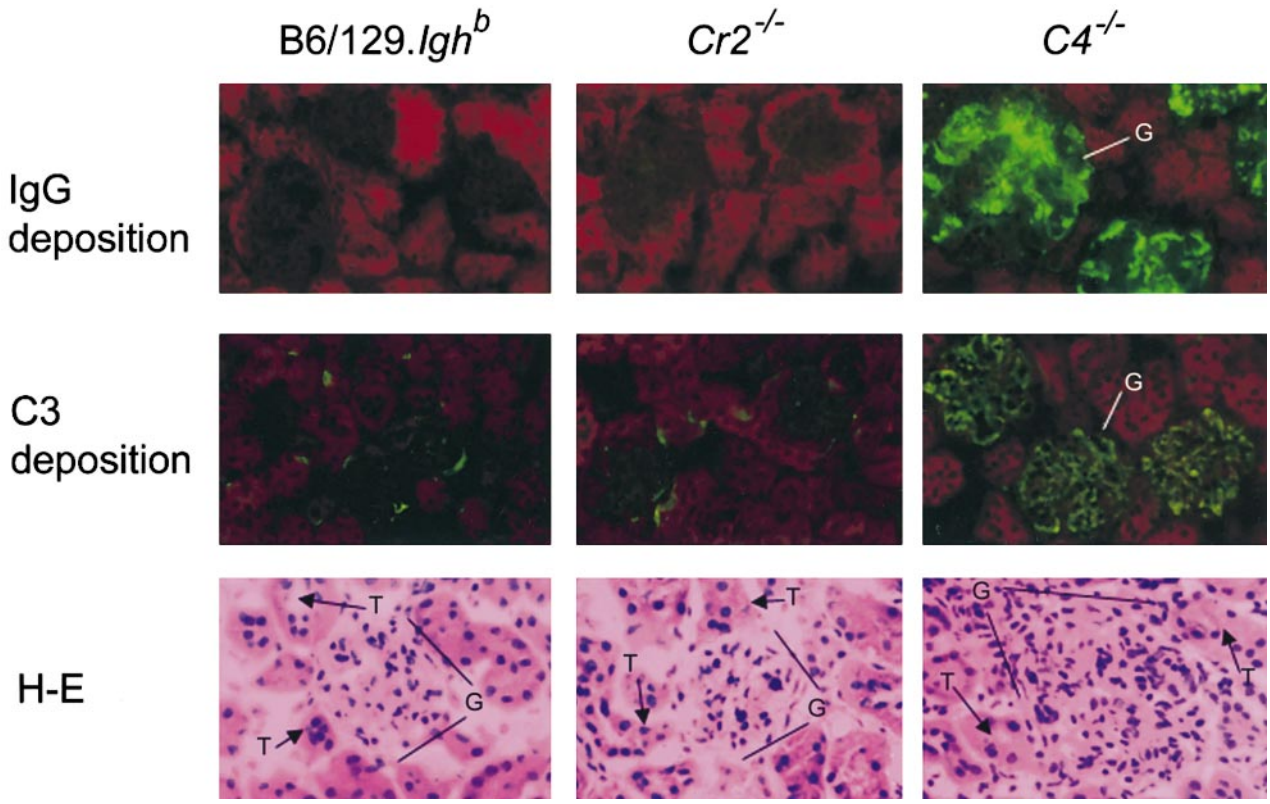

Figure 3. Glomerular IC deposition and glomerulonephritis in $C4^{-/-}$ mice. Kidney sections from 10-mo-old, female B6/129.Igh^b (left), $Cr2^{-/-}$ (center), and $C4^{-/-}$ (right) mice were stained with FITC-conjugated goat anti–mouse IgG (green, top panels, ×200 magnification) or goat anti–mouse C3 (green, center panels, ×200 magnification) and counterstained with Evans blue (red). Glomerular cellularity was examined in sections stained with hematoxylin and eosin (bottom panels, ×400 magnification). G, glomerulus; T, tubule.

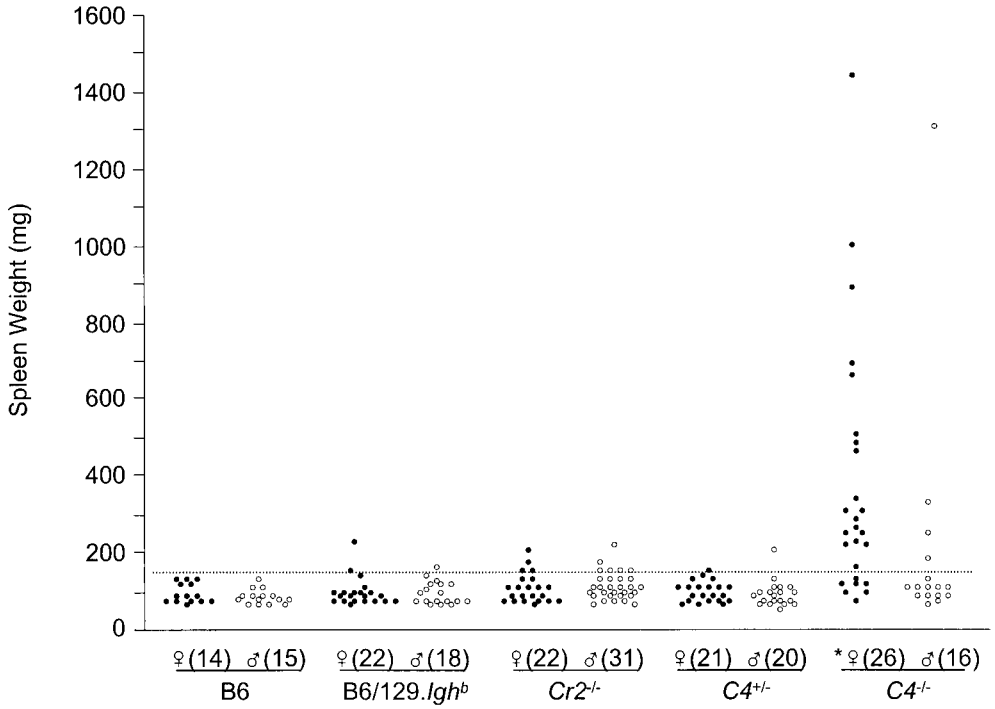

Figure 4. Mice deficient in C4 exhibit splenomegaly. Mice at 10 mo of age were killed and their spleens weighed. The broken line indicates 2 SD above the average weight of spleens in all B6, B6/129.*Igh*^{b}, and *C4*$^{+/-}$ mice. *$P < 0.0005$, Student's *t* test.

Spleens from *C4*$^{-/-}$ and wild-type mice have comparable numbers of B220$^+$ B cells ($77 \pm 23 \times 10^6$ versus $63 \pm 8 \times 10^6$, respectively) and TCR-β^+ T cells ($33 \pm 8 \times 10^6$ versus $30 \pm 2 \times 10^6$) (Table III). However, macrophage (Mac-1$^+$Gr-1$^-$) and neutrophil populations (Mac-1$^+$Gr-1$^+$) (46) were, respectively, two- and fourfold larger in *C4*$^{-/-}$ animals than in age-matched controls (Table III). This increase in inflammatory cells was not evident in *Cr2*$^{-/-}$ mice.

Whereas the numbers of splenic lymphocytes in *C4*$^{-/-}$ mice do not differ from controls, T and B cell populations in 10-mo-old *C4*$^{-/-}$ mice show evidence of cellular activation by increased expression of CD44, Fas, or GL-7 (Fig. 5 and Table III) (47). This activation is not constitutive in *C4*$^{-/-}$ animals, as frequencies of activated lymphocytes in 2-mo-old *C4*$^{-/-}$ mice are not significantly different from age-matched B6/129.*Igh*^{b} controls (Table III). Notably, GL-7 is a marker of GC B cells (48), and we indeed detected spontaneous GC formation in the spleens of *C4*$^{-/-}$ mice by immunohistochemistry (Chen, Z. and G. Kelsoe, unpublished data). This observation implies a role for the GC reaction in the production of autoantibody in these mice (49).

Table III. *Flow Cytometric Analysis of Splenocytes*

	B6	B6/129.*Igh*^{b}		*Cr2*$^{-/-}$	*C4*$^{-/-}$	
Age (mo)	10	2	10	10	2	10
Percent TCR-β^+	23.33 ± 2.30 (ns)	35.45 ± 0.45	26.53 ± 1.53	29.23 ± 2.01 (ns)	37.65 ± 3.18 (ns)	19.06 ± 1.82 (<0.01)
Percent B220$^+$	55.03 ± 0.96 (ns)	47.40 ± 2.83	55.15 ± 1.15	48.98 ± 4.00	46.05 ± 3.18 (ns)	44.16 ± 6.50 (ns)
Percent MAC$^+$Gr-1$^-$	6.85 ± 0.32 (ns)	7.70 ± 0.84	6.09 ± 0.33	6.57 ± 0.58 (ns)	7.07 ± 0.70 (ns)	11.79 ± 1.12 (<0.005)
Percent MAC$^+$Gr-1$^+$	2.47 ± 0.23 (<0.05)	1.86 ± 0.14	1.83 ± 0.21	2.05 ± 0.22 (ns)	2.03 ± 0.41 (ns)	6.57 ± 2.09 (<0.05)
Percent TCR-β^+CD44^{high}	13.20 ± 0.87 (ns)	5.88 ± 1.42	12.30 ± 0.24	9.65 ± 0.19 (<0.01)	5.20 ± 1.46 (ns)	16.58 ± 1.34 (<0.05)
Percent TCR-β^+GL-7$^+$	0.52 ± 0.13 (ns)	0.26 ± 0.06	0.30 ± 0.01	0.24 ± 0.03 (ns)	0.24 ± 0.10 (ns)	1.56 ± 0.28 (<0.005)
Percent TCR-β^+Fas$^+$	2.04 ± 0.19 (ns)	0.51 ± 0.09	1.62 ± 0.22	1.64 ± 0.19 (ns)	0.56 ± 0.14 (ns)	7.67 ± 0.93 (<0.0005)
Percent B220$^+$CD44^{high}	1.59 ± 0.18 (ns)	1.52 ± 0.23	1.33 ± 0.09	0.91 ± 0.09 (<0.01)	0.96 ± 0.11 (ns)	2.34 ± 0.41 (<0.05)
Percent B220$^+$GL-7$^+$	1.27 ± 0.34 (ns)	0.45 ± 0.11	0.67 ± 0.07	0.46 ± 0.10 (ns)	0.42 ± 0.10 (ns)	2.63 ± 0.84 (<0.05)
Percent B220$^+$Fas$^+$	0.31 ± 0.05 (ns)	0.12 ± 0.01	0.23 ± 0.03	0.26 ± 0.03 (ns)	0.13 ± 0.02 (ns)	0.84 ± 0.14 (<0.005)

Percentage of TCR-β^+, B220$^+$, Mac-1$^+$Gr-1$^+$, or Mac-1$^+$Gr-1$^-$ cells are enumerated on a gate of live (7-AAD$^-$) cells. All of the other calculations are on a live lymphocyte gate. *P* values (Student's *t* test, compared to age-matched B6/129.*Igh*^{b} mice) are shown in parentheses; ns, statistically not significant ($P > 0.05$) in comparison to age-matched B6/129.*Igh*^{b} mice. Data represent the mean ± SEM from four to five mice at the age of 10 mo and two mice of 2 mo in each group, randomly chosen from two to three independent litters. All mice are female.

Impaired Clearance of ICs in $C4^{-/-}$ Mice. Complement is implicated in the clearance of ICs (8). Walport et al. (8) have proposed that autoimmunity in complement-deficient patients might result from failure to clear immunogenic ICs. Thus, we tested the role of C4 in the clearance of soluble ICs in vivo at an age (2 mo) when $C4^{-/-}$ mice show no apparent signs of autoimmunity. Cohorts (n = 2–6) of 2-mo-old $C4^{-/-}$, $C4^{+/-}$, $Cr2^{-/-}$, and B6/129.Igh^b mice were injected intravenously with 100 RU of soluble ICs. Levels of IC in plasma (~30 RU/ml) were comparable in all groups at 2 min after injection (Fig. 6). From 2 to 10 min, IC concentrations in plasma decreased about threefold in $C4^{+/-}$, $Cr2^{-/-}$, and wild-type mice; plasma IC levels in $C4^{-/-}$ mice, however, remained almost constant during this period. After 10 min, clearance rates were identical in all groups, including $C4^{-/-}$. The initial delay in IC clearance by $C4^{-/-}$ mice resulted in a persistent, two- to threefold increase in circulating IC levels over that of $Cr2^{-/-}$ mice and B6/129.Igh^b controls. Higher levels of ICs in $C4^{-/-}$ mice remained for as long as 70 min after IC administration (Fig. 6). Rapid clearance of ICs in $Cr2^{-/-}$ mice suggests that CR1/CR2 plays a minor role in eliminating this type of IC from the blood circulation.

Though unlikely, C4 binding to ICs could shield epitopes recognized by the goat anti–mouse IgG detector antibody and consequently reduce the apparent levels of plasma ICs in C4-sufficient mice (Fig. 6). Thus, we repeated the measurements of IC levels using a detector antibody specific for mouse λ L chain; IC clearance rates were virtually identical to those detected by anti-IgG (Fig. 6, inset). IC clearance was similarly impaired in both female and male $C4^{-/-}$ mice (data not shown), implying that if high levels of ICs can break self-tolerance (8), gender-specific factors control the onset of pathological autoimmunity.

Discussion

How does the absence of complement components that enhance humoral immunity promote the production of autoantibody? The conundrum of complement deficiencies causing SLE is so paradoxical (50) that the role of complement per se has been questioned (51). It has been proposed

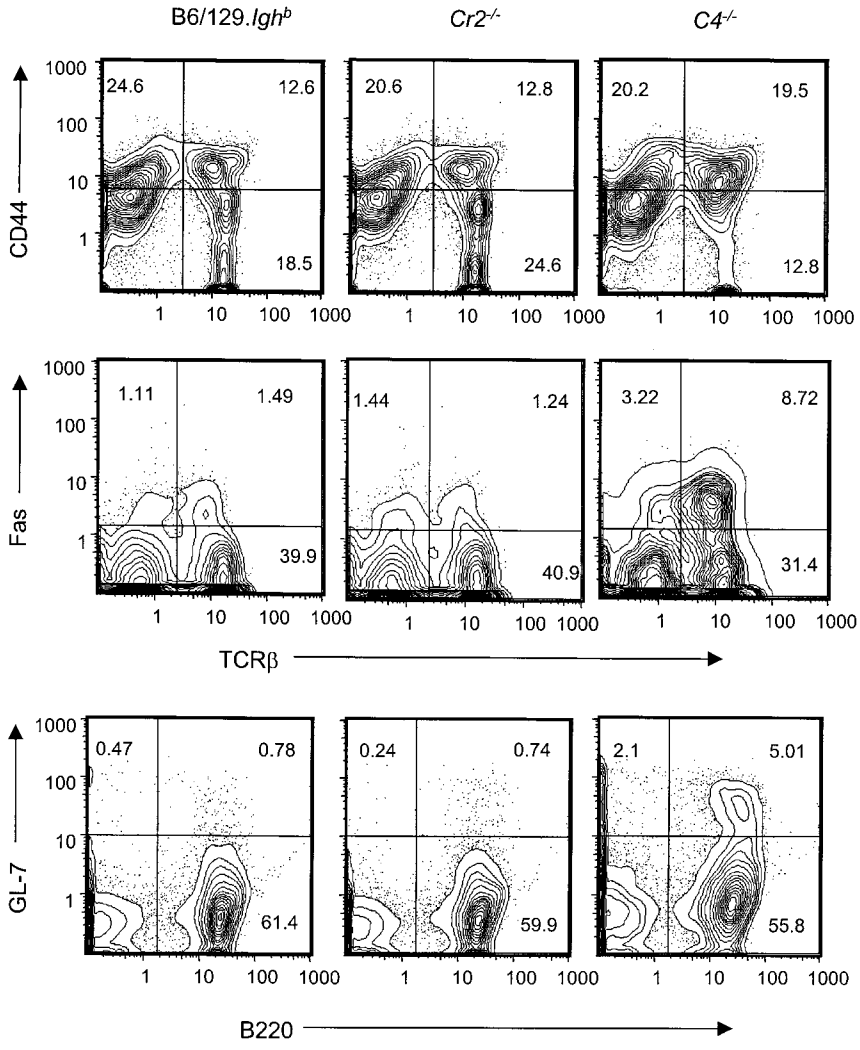

Figure 5. Increased frequencies of activated T and B cells in spleens of $C4^{-/-}$ mice. Splenocytes from 10-mo-old female B6/129.Igh^b (left), $Cr2^{-/-}$ (center), and $C4^{-/-}$ (right) mice were analyzed by flow cytometry. Profiles are generated from a gate for live lymphocytes and are representative of four to five mice in each group (see Table III).

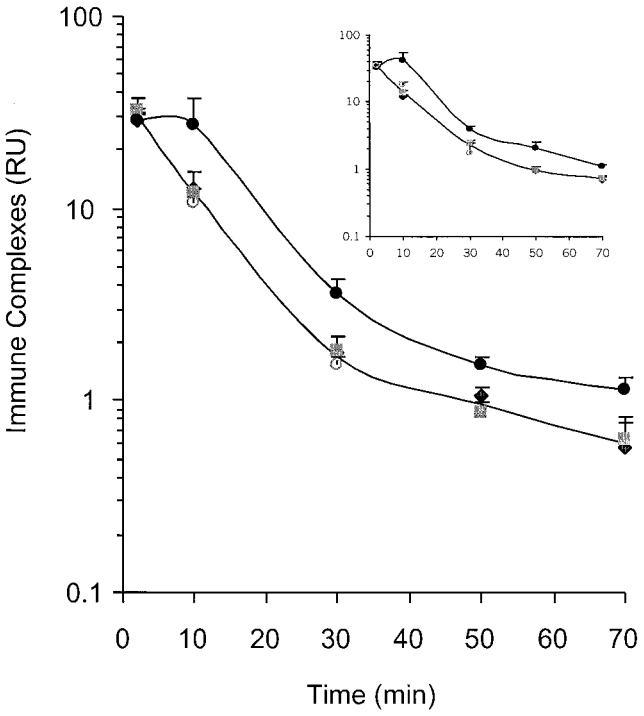

Figure 6. Impaired clearance of ICs in $C4^{-/-}$ mice. Cohorts of B6/129.Igh^b (◆), $Cr2^{-/-}$ (■), $C4^{+/-}$ (○), and $C4^{-/-}$ (●) mice were each injected with 100 RU of ICs through tail veins and bled from orbital sinus at 2, 10, 30, 50, or 70 min after IC injection. IC levels in plasma were assessed by ELISA. Data in the figure were determined with a goat anti–mouse IgG2b detector antibody; inset data were determined with goat anti–mouse Igλ. Upper curves represent the kinetics of IC clearance in $C4^{-/-}$ mice, and lower curves are average clearance rates by B6/129.Igh^b, $C4^{+/-}$, and $Cr2^{-/-}$ mice; these values do not differ significantly from each other ($P > 0.05$; Student's t test). Individual clearance curves for B6/129.Igh^b, $C4^{+/-}$, and $Cr2^{-/-}$ mice are not shown, but IC RU (mean ± SEM) values for each group are presented; each point represents two to six mice. At the time points of 10, 30, 50, and 70 min, IC levels in $C4^{-/-}$ animals differ significantly from B6.Igh^b mice ($P < 0.05$, Student's t test).

that the genetic association of C4 and C2 with lupus only reflects the linkage of polymorphic MHC class I and II genes to the *C4* and *C2* genes (51). This proposal is not supported by the fact that systemic autoimmunity develops in the majority of patients with defective *C1q*, a locus not linked to the MHC (52). Furthermore, *C1qa* knockout mice with a hybrid B6/129 genetic background develop spontaneous autoimmunity, providing direct evidence that C1q deficiency results in the loss of self-tolerance (13). In this study, we demonstrate that $C4^{-/-}$ mice, in contrast to control animals with the same genetic background, spontaneously produce ANA and DNA-specific autoantibody and exhibit histologic glomerulonephritis in an age- and sex-dependent manner. The H-2^b haplotype and the genetic background of B6/129.Igh^b stock are permissive for a humoral autoimmunity caused by C4 deficiency.

The autoantibody in C4-deficient mice is similar to that characteristic of SLE, and the genetic penetrance of autoimmunity in $C4^{-/-}$ mice displays the strong bias for females present in SLE. Case reviews of patients with genetic deficiencies in C4 reveal that 92% (11/12) of C4-deficient females, in contrast to 44% (4/9) of males, were also diagnosed with SLE (6, 7). These values are remarkably similar to the frequencies of 10-mo-old female and male $C4^{-/-}$ mice with ANA (Table II). Factors that segregate with gender modify the suppressive effect of C4 on autoimmunity in both humans and mice (Figs. 1 and 4 and Tables I and II).

In the absence of significant autoimmunity in $Cr2^{-/-}$ mice (Figs. 1–3 and Tables I and II), we conclude that C4 inhibits autoimmunity through a mechanism independent of CR1 and CR2. $Cr2^{-/-}$ mice do not produce levels of serum ANA or anti-DNA antibody significantly above that of normal controls, they have histologically normal kidneys, and they exhibit no splenomegaly or evidence for generalized T and B cell activation (Figs. 1–5 and Tables I–III). These observations are inconsistent with models of autoimmunity that rely on the ability of the CD19/CR2/CD81 coreceptor complex to regulate self-reactive B cells (16, 32, 50). In the absence of strong BCR signals, autoreactive B cells in the bone marrow of $Cr2^{-/-}$ mice might not encounter sufficient antigen concentrations to affect negative selection by apoptotic deletion, receptor revision, or anergy (50). Indeed, the repertoire of peripheral B cells is altered even in $Cr2^{-/-}$ mice with genetic backgrounds that do not promote autoimmunity (26). However, our experiments indicate that the presence of self-reactive B cells is not sufficient for the development of humoral autoimmunity.

What could account for the activation of self-reactive B cells in C4-deficient individuals? Deficiencies in C1q and C4 render mice less able to clear apoptotic cells/debris (13, 53), and C1 was located on the surfaces of apoptotic cells (54). Botto et al. (13) have reported accumulations of TdT-mediated dUTP-biotin nick-end labeling $(TUNEL)^+$ cells in histologically normal glomeruli in $C1qa^{-/-}$ mice. Phagocytosis of apoptotic cells by human macrophages is enhanced by complement-containing sera (55). Mevorach et al. (56) found that apoptotic materials are immunogenic and accelerate the production of autoantibody in mice not prone to autoimmunity. However, the significance of the immunogenicity of apoptotic debris is clouded by reports that phagocytosis of apoptotic cells by dendritic cells renders them tolerogenic (57, 58). Nonetheless, apoptotic cellular debris, especially if associated with microbial products in the form of ICs (18), might activate autoreactive T and B lymphocytes and induce serum antibody specific for self-antigens. Indeed, nucleosome-specific, $CD4^+$ T cells have been identified in lupus-prone mice. Moreover, immunization with nucleosomes enhanced autoantibody production (59).

Complement also acts in the clearance of ICs (18). Our work shows that the clearance of circulating ICs is delayed in $C4^{-/-}$ mice even before the onset of detectable autoimmunity (Fig. 6). Although most ICs were eventually removed from circulation in the absence of C4 (Fig. 6), perhaps by Fc receptors (60, 61), levels of ICs remained modestly elevated in $C4^{-/-}$ mice for as long as 70 min. With time, this delay could cause significant accumulations of ICs.

Several plausible consequences of diminished IC clearance in $C4^{-/-}$ mice come to mind. First, excessive IC deposition could cause inflammatory damage, especially in blood-filtering organs such as kidney and spleen (Fig. 3). Second, inflammatory damage might expose cryptic autoantigens and activate destructive T and B lymphocytes (8). Third, abundant ICs might enhance antigen uptake and activation by antigen-presenting cells, promoting lymphocyte activation, release of inflammatory cytokines, and the abrogation of anergy (8, 18). Guinea pigs deficient in C4 exhibit delayed clearance of particulate ICs (62) and manifest signs of polyclonal B cell activation and a high incidence of IgM rheumatoid factors (63). The blood-filtering function of the spleen may localize IC-induced inflammation, explaining the absence of hypertrophy in lymph nodes and Peyer's patches. ICs and/or the inflammation they induce might also account for the activation of T and B cells in $C4^{-/-}$ mice (Fig. 5 and Table III).

What mediates the role of C4 in immune clearance and autoimmunity? Only deficiencies in C1 (6, 7, 9, 13, 18), C4 (6, 7, 9; Fig. 1–3, Tables I and II), and, to a lesser extent, C2 (6, 7), are strongly associated with spontaneous autoimmunity. In contrast, C3 deficiency does not significantly predispose to autoimmunity in either humans (6, 7) or mice (32). Thus, do C1 and C4 inhibit autoimmunity via their tandem roles in the classical pathway of complement activation or do they act independently? C1 and C4 could act through a receptor(s) for C4 split products, with C1 catalyzing the formation of C4 ligands. Two complement receptors are known to bind C4 fragments, CR1 and CR2 (16). Human CR1 participates in IC clearance; ICs are trapped by CR1 on erythrocytes and transported to the liver and spleen where they are phagocytosed by reticuloendothelial cells (18). C1q and C4b cooperatively increase immune adhesion mediated by human CR1 (64). However, human CR1 contributes little to the phagocytosis of apoptotic cells by macrophages in vitro (55). In our study, mice deficient in CR1 and CR2 clear soluble ICs normally (Fig. 6). This observation carries the caveat that mice have two functional homologues of human CR1, murine CR1 and Crry (65). The *crry* product is proposed to function as a regulatory protein (65), but its homology to CR1 suggests that it might substitute for CR1 in $Cr2^{-/-}$ mice.

If not CR1, CR2, or Crry, what other molecules could mediate the inhibition of autoimmunity by C4? C3-binding proteins distinct from CR1 and CR2 are present on mouse neutrophils and platelets. Although the identities and function(s) of these proteins are not characterized, they may mediate CR1/CR2-independent adherence of ICs. Whether they bind C4 fragments is unknown (66). Recently, CR3 and CR4, but not CR1, were shown to enhance phagocytosis of apoptotic cells (55). However, the primary ligand for CR3 and CR4 is iC3b (67). As C3 is not associated with autoimmunity (6, 7, 32), it is unlikely that CR3 and CR4 are. Alternatively, candidate C1q receptors (68), including the recently cloned C1qRP (69), might affect IC clearance in vivo and thereby inhibit autoimmunity. If this were the case, the association of C4 deficiency and autoimmunity could be explained by the ability of C4 (and perhaps C2) to anchor/stabilize C1 ligands on ICs and/or apoptotic cell debris and promote their adhesion (14, 15, 64). This hypothesis could explain the ordered relationship C1>C4>C2 of deficiencies in components of the classical pathway and risk for SLE (6–8, 18). However, no direct evidence indicates whether these molecules act sequentially or independently to suppress humoral autoimmunity.

We propose a "multiple hit" hypothesis for the induction of humoral autoimmunity in $C4^{-/-}$ mice. First, C4 deficiency may promote autoimmunity by impairing selection against autoreactive, immature/transitional B cells in the bone marrow; evidence suggests that this effect on the B cell repertoire is mediated by CR1/CR2 (32). However, this altered B cell repertoire alone does not lead to serum autoantibody (Figs. 1 and 2 and Tables I and II), although it may accelerate incipient loss of self-tolerance (32). Thus, C4 also acts independently of CR1 and CR2 to promote systemic autoimmunity (Figs. 1–3 and Tables I and II). This effect becomes pronounced with aging and is moderated by sex-linked factors (Figs. 1 and 2 and Tables I and II). A plausible cause of autoimmunity in C4- and perhaps C1-deficient animals is impaired clearance of apoptotic cell debris (53, 55) and ICs (Fig. 6; reference 8). Accumulation of these potential immunogens might break tolerance and activate humoral responses to self-components.

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