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Shirit Einav, Olga O. Pozdnyakova, Minghe Ma and Michael C. Carroll

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Complement C4 Is Protective for Lupus Disease Independent of C3

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The role of complement C3 in mediating systemic lupus erythematosus (SLE) was examined using a double-knockout C3null·C4null Fas (CD95)-deficient mouse model. Results from this study reveal significant lymphadenopathy, splenomegaly, elevated titers of anti-nuclear Abs and anti-dsDNA Abs, an increased number of anti-dsDNA-producing cells in ELISPOT assay, as well as severe glomerulonephritis in the double-deficient mice. Based on these clinical, serological, and histological parameters, we find that autoimmune disease in the double-knockout group is similar in severity to that in C4null lpr mice, but not to that in C3null lpr mice. The development of severe SLE in the absence of both classical and alternative complement pathways suggests that it is the absence of C4, and not the presence of C3, that is critical in SLE pathogenesis. Thus, complement C4 provides an important protective role against the development of SLE. The Journal of Immunology, 2002, 168: 1036–1941.

Systemic lupus erythematosus (SLE) is a relatively common autoimmune disorder, occurring in 20–130 of every 100,000 people in the United States (1). The association between the complement system and SLE is contradictory. While complement is a mediator of inflammation, complement deficiency predisposes to the development of SLE. Inherited complement C1q or C4 deficiencies, whether partial or complete, confer a high risk to develop SLE (2–5), whereas C3 deficiency is only rarely associated with SLE-like illness (2, 6, 7).

Two models have been proposed to explain the role of complement in lupus. The first is based on the hypothesis that complement plays a substantial role in the clearance of immune complexes and/or apoptotic debris (8, 9), which is thought to be impaired in lupus. Apoptotic bodies are a potential major source of lupus self-Ags, and failure to remove them could lead to inappropriate production of pathogenic autoantibodies (10). Support for a role for early complement in clearance of apoptotic debris comes from the finding that C1q binds directly to apoptotic blebs (11) and that mice deficient in C1q or C4 have defects in the clearance of injected apoptotic thymocytes (12). Moreover, mice deficient in C1q (9) or C4 (13) spontaneously develop autoantibodies against nuclear Ags. The presence of increased apoptotic bodies in the affected glomeruli of C1q-deficient mice (9) supports this model.

A second, nonexclusive model proposes that the innate immune system, including complement, is protective against lupus by enhancing negative selection of self-reactive B cells. According to this hypothesis, innate proteins enhance the localization of lupus Ags such as dsDNA and nuclear proteins within the primary lymphoid compartment. Efficient presentation of self-Ags results in elimination or editing of potential self-reactive B cells (14). Thus, deficiency in innate proteins such as serum amyloid protein (15), DNase I (16), natural IgM (17, 18), Clq (19), or C4 (13) could lead to an escape from negative selection by self-reactive B cells and their potential activation in the periphery in the presence of cognate T cell help and lupus Ag.

Support for this latter model comes from a study with C4null CR2null, and C3null mice crossed with mice bearing transgenes specific for both anti-hen lysozyme and a soluble form of lysozyme Ag (20). This study found that C4 and CR2, but not C3, are critical in maintaining tolerance to the self-Ag (21). Further support comes from studies with a complement-deficient lpr model. Mice homozygous for the lpr (lymphoproliferation) mutation are Fas (CD95) deficient. Deficiency is associated with a lupus-like disease, the severity of which is dependent on background genes (22, 23). C57BL/6·lpr and C57BL/6 × 129 lpr mice have a mild form of the disease, with only minimal renal involvement (24). Significantly, deficiency for C4 and CR2, but not C3, in combination with lpr on the C57BL/6 × 129 background results in increased severity of lupus-like disease, including renal involvement (21).

The results in the lpr model raise the question: does C3 deficiency protect against lupus? C3 may be required for Ag clearance or tolerance, but the effect is negated by its alternative role as a mediator of inflammation or enhancer of humoral immunity (25). Accordingly, C3-deficient mice might be lupus susceptible, as are C1q- and C4-deficient mice, but fail to develop the disease because of an impairment in inflammation or their ability to mount an immune response. Interestingly, kidney disease is not C3 dependent in the MRL/lpr murine lupus strain (26) or the (NZB/NZW)F1 strain, where disease appears to be mediated by Ab and the FcyR (27). C3 also does not appear to be required for spontaneous disease in C1q-deficient mice (19). By contrast, an intact alternative pathway is required for full pathogenesis in MRL/lpr mice (28). In this study MRL/lpr mice deficient in factor B developed a mild disease phenotype. Susceptibility to lupus disease is multigenic; therefore, the production of autoantibodies and the mechanism of pathology are likely to vary among different strains of mice.

To test the hypothesis that C3 deficiency is protective against the development of lupus, C3null lpr mice were crossed with C4null lpr mice. The resulting double-knockout group was found to have...
SLE disease similar in severity to that reported in the C4null lpr group. These findings demonstrate that C3 is not essential to the development of a lupus-like phenotype in lpr mice; therefore, the absence of increased severity of disease is not due to its role in inflammation or the humoral immune response.

Materials and Methods

Mice

C3null and C4null mice on a mixed C57BL/6 × 129/Sv background were bred with C57BL/6lpr/lpr mice purchased from The Jackson Laboratory (Bar Harbor, ME). Double-knockout C3null/C4null lpr mice were then obtained by breeding C3null lpr with C4null lpr. Genotyping was performed by PCR (29). Complement-sufficient lpr mice were used as controls. All experiments were performed on age- and sex-matched mice maintained under specific pathogen-free conditions.

Immunofluorescence for detection of autoantibodies

Anti-nuclear Abs (ANA) and anti-dsDNA Abs were measured in the serum by indirect immunofluorescence using HEP-2-coated slides and Crithidia lucilae substrate slides (The Binding Site, Birmingham, U.K.), respectively. Slides were first incubated for 20 min with serial dilutions of mouse serum in PBS. After PBS washing, FITC-labeled goat anti-mouse IgG (Sigma-Aldrich) was added for 20 min. Slides were then washed, mounted with Toluene acrylic resin mounting medium (Stephen Scientific, Riverdale, NJ), and viewed by fluorescent microscopy. Titers were reported as inverse values of the last positive dilutions, compared with the 1/40 dilution of wild-type controls. Mean values were calculated for each group.

ELISA for anti-dsDNA Abs

Anti-dsDNA Abs were detected in the serum by modified ELISA, performed as previously described (23, 24, 26, 28, 30). Subsequently, 96-well polystyrene microtiter plates (Immunon; Dynex Technologies, Chantilly, VA) were serially incubated overnight at 4°C with avidin (Sigma-Aldrich), and photobiotinylated circular or linear plasmid DNA were prepared according to the manufacturer’s protocol (Sigma-Aldrich). Wells were washed three times with PBS and 0.02% Tween (PBST) between each step. After blocking with 1% BSA/PBST for 1 h at 37°C, plates were incubated for 2 h at 37°C with serial dilutions of mouse serum in 1% BSA/PBST. Alkaline phosphatase (AP)-conjugated goat anti-mouse whole molecule IgG Abs (Sigma-Aldrich) diluted to 1/500 in 1% BSA/PBST were incubated for 1 h at 37°C. AP substrate (Sigma-Aldrich) was added, and OD was read 30 min later. The anti-dsDNA IgG concentration was approximated using a standard curve obtained from serial dilutions of a mouse serum with known IgG concentration and from standard IgG product (Southern Biotechnology Associates, Birmingham, AL). Mean values were calculated for each group. As a negative control serum samples were applied to plates coated with the Ag or with anti-IgG.

ELISPOT assay for anti-dsDNA AFCs

An ELISPOT assay for quantitation of anti-dsDNA Ab-forming cells (AFCs) was performed as previously described (31). Cell suspensions were prepared from fresh spleens, lymph nodes (LN), and bone marrow (BM) on a Ficol-Paque gradient (Amersham Pharmacia Biotech, Uppsala, Sweden) in DMEM (Life Technologies, Grand Island, NY). Serial dilutions of these suspensions, starting with 105 cells/well, were incubated overnight at 37°C in a 5% CO2 air incubator on 48-well polystyrene plates (Costar, Cambridge, MA) previously coated with avidin (Sigma-Aldrich) and photobiotinylated plasmid DNA and blocked with 1% BSA/PBST. The cells were washed away, and plates were overlaid with AP-conjugated goat anti-mouse IgG Abs (Sigma-Aldrich) diluted to 1/500 in 1% BSA/PBST for 1 h at 37°C. The AP substrate was maintained at 37°C, and 2.3 mM 5-bromo-3-chloroindolyl phosphate solution (Sigma-Aldrich) in 2-amino-2-methyl-1-propanol buffer (Sigma-Aldrich) mixed with 0.6% aurose (Sigma-Aldrich). After overnight incubation at 4°C, blue spots were routinely read by light microscopy under ×100 magnification. Each spot represents an Ab producer by an individual B cell that bound to the plate. Controls included cells incubated on plates without Ag or anti-IgG and DMEM alone without cells. Results represent either the number of anti-dsDNA IgG-secreting cells per 106 cells applied or per total number of IgG-secreting cells (determined in the various organs by using anti-IgG-coated plates). The number of splenic anti-dsDNA IgG-secreting cells was also calculated per total number of splenic lymphocytes. Mean values were calculated for each group.

Kidney staining

Kidneys were quick-frozen in OCT for immunofluorescence staining. Cryosections, 5 mm thick, were stored 24 h or longer at −80°C and then fixed with m-periodate-lysine paraformaldehyde fixative (32) for 30 min. After blocking with 5% milk (Carnation instant milk; Nestle, Solon, OH) in PBST for 30 min, slides were incubated for 1 h with FITC-labeled goat anti-mouse whole molecule IgG (Sigma-Aldrich) or anti-mouse C3 or C4 diluted to 1/200 in 1% BSA/PBS. All stages were performed in a humid dark chamber at room temperature. Slides were washed three times with 1% BSA/PBST between each step. Slides were mounted with Toluene acrylic resin mounting medium (Steps Scientific) and then viewed by fluorescence microscopy. The number of stained glomeruli was counted and divided by the total number of glomeruli seen in 10 high power fields. Mean values for each group were calculated.

Alternatively, kidneys were fixed in 10% buffered formaldehyde (Fisher Scientific, Fair Lawn, NJ) and stained with H&E or periodic acid-Schiff. These sections were classified into four categories based on the degree of glomerular enlargement, hypercellularity, mesangial thickening, hyalin deposition, presence of crescents, and fibrosis using the following scoring system: 0 for <10% glomeruli affected, 1 for 10–30% glomeruli affected, 2 for 30–75% glomeruli affected, 3 for 75–95% glomeruli affected, and 4 for >95% glomeruli affected.

Statistical methods

The significance of mean values was determined by Student’s t test. Values of p > 5% were considered insignificant. Linear correlation was analyzed by a correlation test.

Results

Mass of cervical LN and spleen

Splenomegaly and lymphadenopathy are known manifestations of Fas deficiency and are considered clinical markers for the severity of SLE (22). To estimate the severity of lupus disease in C4null/C3null lpr mice, the mass of cervical LN and spleen was measured and compared with that in the C4null and C3null lpr groups. Females in three age groups, ranging from 10 to 17 wk, were evaluated.

As reported previously, the mass of cervical LNs was significantly greater in C4null lpr mice than in C3null lpr and complement-sufficient (C+/-) lpr controls at 10 and 13 wk (Fig. 1a) (21). At 17 wk the mass was also elevated in C3null mice, but the relative increase was not significant, in contrast to C4null lpr mice. Interestingly, combined deficiency in C3 and C4 did not protect against increased lymphadenopathy. A significant increase in the mass of cervical LNs was observed in the double-deficient mice at both 10 and 13 wk (Fig. 1a). Thus, the absence of lymphadenopathy of the C3null/C4null lpr mice was not explained by a requirement for C3. The effects of C4 deficiency were less pronounced on splenomegaly. However, at 17 wk all three deficient groups developed enlarged spleens relative to C+/- lpr mice (Fig. 1b).

Lupus autoantibodies

A hallmark of lupus is increased ANAs. Previous studies indicated a significant increase in ANA titer in C4null lpr mice relative to C3null and C+/- lpr controls. An explanation for the decreased ANA in C3null lpr mice is that complement C3 is critical for an effective humoral response (25). To test this hypothesis, ANA titers were assayed for each of the four groups of mice by indirect immunofluorescence on HEP-2 slides. As predicted, Ab titers in C4null lpr, but not C3null lpr mice, were significantly elevated relative to those in C+/- controls at each of the three time points. Comparison of the C3null/C4null lpr mice with the single-deficient strains revealed a similarly significant increase in ANAs at 10 and 17 wk. Titers were elevated in double-deficient mice at 13 wk, but the increase was not statistically significant. In general, the mean ANA was slightly greater in C4null lpr mice than in C3null/C4null lpr in each age group, but the increases were not statistically significant (results not shown).
Serum concentrations of anti-dsDNA Abs were elevated in C4 null lpr mice as predicted based on earlier results (21). Likewise, the mean anti-dsDNA titer was elevated in the double-deficient mice but reached statistical significance only in the 17-wk-old mice. In 13- and 17-wk-old mice there was a slight increase in the mean anti-dsDNA titers of C3null C4 null lpr vs C4 null lpr. This finding is in contrast to that for ANAs (Fig. 2b), although the difference was not significant. To confirm the ELISA results, anti-dsDNA Abs were measured using *Crithidia luciliae* slides. An 0.87 correlation was found between the two assays (data not shown). Ten percent of the sera tested were positive for anti-dsDNA Abs measured by ELISA but not by indirect immunofluorescence. Similarly, ELISA results positively correlated with ANA titers (correlation coefficient of 0.73).

ANA patterns in the C4 null and C3 null C4 null lpr groups were highly variable (including nucleolar, nuclear matrix, cytoplasmic speckles, etc.), as opposed to the homogeneous and fine speckles predominantly seen in C+ lpr and C3null lpr groups (Fig. 3). Several sera from the C4-deficient groups demonstrated mixed ANA patterns. In these sera different fluorescence patterns were produced with different dilutions.

In general, deficiency in complement C3 did not appear to impair the autoantibody response to nuclear or dsDNA self-Ags. Therefore, the lack of increased autoantibodies in C3null lpr mice is not explained by an impairment in humoral immunity.

**Anti-dsDNA AFCs**

To determine the frequency of dsDNA-AFCs, cell suspensions were prepared from spleens, LNs, and BM of each group of mice and analyzed by ELISPOT assay on dsDNA-coated plates. Consistent with increased ANA and dsDNA titers, the mean frequency of dsDNA ASCs was elevated in all three lymphoid tissues of C4null lpr mice relative to those in C3 null lpr and C+ lpr (Fig. 4). However, the increase was statistically significant only in spleen and BM. As predicted from the autoantibody titers, lpr mice bearing C3 and C4 deficiency have a significant increase in the frequency of AFCs in all three lymphoid compartments relative to C+ lpr mice. In LNs and BM, an increase in mean AFC was observed in the C3 null C4 null lpr animals relative to C4 null lpr mice, but the difference was not significant. A minimal number of anti-dsDNA-AFCs was observed in wild-type controls (data not shown), while no spots developed in the absence of Ag, cells, or anti-IgG Abs.

To determine whether the relative increase in AFCs was dsDNA specific or represented a general increase in total IgG-producing cells, results were expressed relative to the total number of IgG-secreting cells. Splenic dsDNA-AFCs constituted a significantly

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**FIGURE 1.** Deficiency in C4 alone or in C4 and C3 results in increased lymphadenopathy (a) and splenomegaly (b) in lpr mice. Cervical LNs and spleen from mice, sacrificed at the ages of 10, 13, or 17 wk, were weighed. Each age group from each strain included 5–10 mice (females). Circles represent individual mice; bars represent mean values. *, Statistical significance (p < 0.05).

**FIGURE 2.** Deficiency in C4 or C4 combined with C3 results in increased ANA (a) and anti-dsDNA (b) levels in lpr mice. Mice from each strain were bled at 10, 13, or 17 wk age (10 females in each age group in each strain). ANA titers were measured in the serum by indirect immunofluorescence on Hep-2-coated slides. Anti-dsDNA titers were measured by ELISA and converted to concentrations using a standard curve. Circles indicate individual mice; bars represent mean values, *, Statistical significance (p < 0.05).
higher proportion of IgG-secreting cells in C4 null and C3 null C4 null, but not C3 null lpr, mice compared with C/H11001 lpr controls. Mean values ranged between 8 and 13.6% in the C4-decient strains, while the values for C3 null and C/H11001 lpr controls were 2.5 and 2.9%, respectively. However, C3 null, C4 null, and C3 null C4 null lpr strains exhibited a similar proportion of B cells secreting anti-dsDNA in the BM and LNs compared with that of C/H11001 lpr controls. In all groups the highest proportion of B cells secreting anti-dsDNA was found in the spleen, followed by the LNs and finally BM; proportions tended to increase with age (data not shown). The results suggest that the increase in splenic AFCs is DNA specific and not due to an overall B cell hyperactivity.

Deposition of immune complexes in kidneys
Glomerulonephritis is an important diagnostic and prognostic factor in lupus. Consistent with a previous report by Prodeus et al. (21), immunofluorescent staining of renal cryosections revealed increased IgG deposition in C4 null lpr mice, but not C3 null lpr mice, relative to C/H11001 lpr mice (p < 0.001). IgG deposition in the C3 null C4 null lpr group was similar to that in the C4 null lpr group, i.e., 72 and 65%, respectively (Table I). Affected glomeruli among C+ lpr controls averaged 38%, whereas the incidence among the C3 null lpr group was lower (32%). As expected, no staining was observed in the C3 null C4 null lpr group, and C3 and C4 deposition was absent in C3 null and C4 null lpr mice, respectively. Paraffin-embedded renal sections stained with H&E or periodic acid-Schiff were also evaluated for glomerular abnormalities (Fig. 5). Examination of sections revealed statistically significant (p < 0.02) higher mean pathology scores among C4 null lpr mice compared with C/H11001 lpr controls, while only a minimal increase was observed in the C3 null lpr group. Once again, results for the double-knockout lpr group and C4 null lpr group were similar.

Discussion
Deficiency in C4 accelerates the autoreactive phenotype of lpr mice on the mixed B × 129 background, resulting in increased lymphadenopathy, splenomegaly, anti-nuclear and anti-dsDNA Ab levels, and glomerular injury (21). This acceleration is also evident in the C3 null C4 null lpr mice but not in the C3 null lpr mice. These findings not only implicate a dominant role for C4 in protection against autoimmune disease but also reveal that C3 is not required
The average of the values is similar to that reported in (NZB × NZW)F1 mice, in which clinical parameters of nephritis correlated better with this assay than with serum autoantibodies (35). The relative proportion of B cells producing anti-DNA Abs in the spleens of MRL/lpr/lpr mice was reported previously (36). However, the differential frequency of these cells in the various lymphoid compartments was not discussed. MRL/lpr/lpr mice have a mean value range of 0.76–3% (ss+ds)DNA-AFC per total IgG-secreting cells (36).

Peripheral B cells from lupus patients exhibited values as high as 5%, with mean values of 0.6 and 1% in CD5+ and CD5− B cell subsets, respectively (37). The increased frequency of dsDNA-AFC in the spleens of complement-deficient lpr mice, but not C+ controls, is consistent with findings in human SLE. ELSIOPOT analysis of peripheral blood cells of lupus patients revealed an increased number of DNA-AFC relative to total IgG, in contrast to that observed with environmental Ags (38). This suggests that B cell activation is specific for lupus Ags. By contrast, BM and LN s from the complement-deficient mice exhibited a similar proportion of dsDNA-AFCs as C+ controls despite having a higher absolute number of anti-DNA-secreting cells per 106 cells.

The heterogeneous ANA pattern suggests the presence of auto-reactive B cells directed against a large array of self-Ags in the absence of C4. This is evidenced by the larger variety of ANA staining patterns in C4-deficient lpr strains compared with complement C+ controls (Fig. 3). The mixed ANA pattern demonstrated in several sera of the complement-deficient groups suggests the presence of several autoantibodies in the same mice.

In summary, complement C4 is protective against SLE in B6 × 129 lpr mice. By contrast, despite its role in the immune clearance function, C3, the central component of complement, is not protective. Moreover, the severity of disease in lpr mice with a deficiency in both C4 and C3 is similar to that in C4 lpr mice, demonstrating that the lupus-like phenotype is not dependent on C3.

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