C5a Receptor Deficiency Attenuates T Cell Function and Renal Disease in MRL\textsuperscript{\textit{lpr}} Mice

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The development and progression of systemic lupus erythematosus (SLE) is strongly associated with complement activation and deposition. To characterize the role of C5a and its receptor (C5aR) in SLE, C5aR-deficient mice were backcrossed nine generations onto the lupus-like MRL\textsuperscript{\textit{lpr}} genetic background. Evidence is presented that C5aR modulates both renal injury and T cell responses in MRL\textsuperscript{\textit{lpr}} mouse. C5aR-deficient MRL\textsuperscript{\textit{lpr}} mice had prolonged viability, with a mean survival time of 33.0 wk compared with 22.6 wk in control mice. Renal injury was also attenuated in the C5aR\textsuperscript{\textit{+/−}}MRL\textsuperscript{\textit{lpr}} mice. At 20 wk of age C5aR\textsuperscript{\textit{+/−}}MRL\textsuperscript{\textit{lpr}} mice had a complete absence of glomerular crescents and marked reductions in glomerular hypercellularity. There was no difference in the degree of glomerular C3 deposition; however, IgG deposits were reduced in the C5aR\textsuperscript{\textit{+/−}}MRL\textsuperscript{\textit{lpr}} mice. The reduction in glomerular injury was also associated with a four-fold decrease in renal CD4\textsuperscript{+} T cell infiltrates. Whereas there were modest differences in total IgG anti-dsDNA antibody titers, C5aR-deficient mice had 3.5-fold higher levels of IgG1 and 15-fold lower levels of IgG2a anti-dsDNA antibody titers compared to controls. The differences in anti-dsDNA IgG subclasses were associated with reduced CD4\textsuperscript{+} Th-1 responses in the C5aR\textsuperscript{\textit{+/−}}MRL\textsuperscript{\textit{lpr}} mice, including diminished production of IL-12p70, IFN-\textgamma, and increased expression of the Th-2 transcription factor GATA-3. These findings indicate that the C5aR plays a major role in modulating complement-dependent renal injury and Th helper cell Th-1 responses in the MRL\textsuperscript{\textit{lpr}} mouse.


\textbf{S}ystemic lupus erythematosus is an autoimmune disorder wherein the production of autoantibodies, systemic complement activation, circulating immune complexes, and autoreactive T cells are associated with multisystem injury, including nephritis, arthritis, serositis, dermatitis, and blood dyscrasias. Lupus nephritis is mediated in part by local deposition of circulating immune complexes and complement activation products and by the recruitment of circulating leukocytes. The relationship of complement to the pathogenesis of SLE is a complex one. Although systemic complement activation, marked by depression of serum C3 and C4 levels, has been shown to correlate closely with disease activity, genetic deficiencies in the early components of the classical complement pathway, C1 inhibitor, C1q/r/s, C2, or C4, are some of the strongest risk factors for the development of SLE (1). Deficiencies in C3 and terminal complement components are not commonly associated with SLE, and in animal models of lupus, the inhibition of the complement proteins C3 and C5 has been shown to protective (1–3). Taken together these data suggest that complement proteins are likely to participate at several levels to exacerbate or suppress disease severity.

The complement system is a cascade of enzymatic reactions with multiple immunologic effects, including cell lysis, B cell activation, leukocyte recruitment, and clearance of apoptotic cells and immune complexes. The classical, alternative, and lectin pathways converge to generate C5 convertase. The cleavage of C5 results in formation of C5b and C5a. C5a is a small, 11-kD peptide of the complement anaphylatoxin family. C5a has been shown to bind to two receptors, C5aR (CD88) and C5L2. Both C5aR and C5L2 are G protein–coupled seven-transmembrane-spanning receptors of the rhodopsin superfamily (4,5). Whereas the expression and function of C5aR is well characterized, the \textit{in vivo} expression of C5L2 is unclear as is its physiologic significance. C5L2 does not seem to regulate signal transduction as it is not G protein coupled and may act as a decoy receptor competing with CD88 for C5a binding (6–8). Currently, the primary biologic functions of C5a are thought to be mediated through C5aR activation, which has been shown to degranulate mast cells, neutrophils, and eosinophils; enhance smooth muscle contraction; increase vascular permeability; and induce leukocyte chemotaxis (9). C5a/C5aR interactions have been shown to modify the production of IL-12, thus regulating Th-1 cell responses, and to potentiate the production of cytokines such as IL-6, IL-8, and TNF-\textalpha (4,10–12).

The C5aR is expressed abundantly on leukocytes, including
neutrophils, monocytes, eosinophils, and lymphocytes (13–16). Its role in directly modulating lymphocyte responses is an area of intense investigation (17). C5αR is also expressed by a wide range of parenchymal cells, including glomerular mesangial and proximal tubular epithelial cells (18–21). Parenchymal C5αR expression has been shown to be enhanced in areas of acute inflammation, including renal tissue from patients with lupus nephritis (22). C5α has also been shown to alter significantly the renal production of known mediators of kidney disease, including inducible nitric oxide synthase and monocyte chemoattractant protein-1 (19,23).

The MRL/MpJ-Tnfrsf6<sup>−/−</sup> (MRL<sup>−/−</sup>) mouse is a widely used and extensively studied mouse strain that develops a severe spontaneous autoimmune disease similar to SLE (24). The lpr mutation, a retroviral transposon insertion in the FAS gene, results in loss of FAS function and thus a defect in FAS-mediated apoptosis (25). When present on the MRL genetic background, the loss of FAS-mediated apoptosis results in massive lymphoproliferation with expansion of the B220<sup>+</sup> CD4<sup>+</sup> CD4<sup>−</sup> CD8<sup>−</sup> cell population and the generation of autoreactive T cells (26). CD4<sup>+</sup> T cell responses are skewed to a Th-1 cytokine predominance in that IL-12 and IFN-γ production are enhanced (27,28). The ensuing autoimmune disease is characterized by lymphopenopathy, complement activation, severe immune complex renal disease, and 50% lethality by 20 to 24 wk of life (29).

Based on the hypothesis that C5α acting via the C5αR may have a major functional role in mediating disease progression in SLE, mice with a targeted deletion of the C5αR gene were back-crossed onto the MRL<sup>−/−</sup> genetic background. Comparative analysis of immunologic responses and indices of renal injury then was performed between MRL<sup>−/−</sup> control and C5α receptor–deficient MRL<sup>−/−</sup> mice. Experimental data contained in this report support the hypothesis that C5α/C5αR signaling plays a major role in modulating renal injury in the MRL<sup>−/−</sup> model of SLE and suggest that the C5αR is a potential therapeutic target for the treatment of SLE in humans.

**Materials and Methods**

**Mice**

MRL<sup>−/−</sup> (Jackson Laboratories, Bar Harbor, ME) and C5αR<sup>−/−</sup>-C57BL/6 mice maintained in our animal colony were used for back-crossing (30). Genotyping was confirmed by PCR for all F9 mice used in this study (data not shown). These studies were approved by the University of Texas Health Science Center-Houston Animal Welfare Committee.

**Histologic Analysis**

Renal tissue was fixed in PBS buffered 4% formalin, dehydrated, and embedded in paraffin. Four-micron sections were stained with periodic acid-Schiff (PAS). Glomerular injury was graded as follows, with a minimum of 30 glomeruli scored per animal per group: Percentage of glomeruli that contained cellular crescents (>25% of glomerulus effected), percentage of glomeruli with sclerosis involving >25% of the glomerular tuft, and degree of hypercellularity (0 to 3 scale). Tubulointerstitial disease was graded on a 0 to 3 scale as follows: 0, no cellular infiltrates with back-to-back tubules, no evidence of fibrosis; 1, 0 to 5 cells per high-power field (hpf), minimal fibrosis; 2, 5 to 10 cells/hpf with moderate fibrosis; and 3, >10 cells/hpf with marked fibrosis.

**Renal Function**

Serum and urine were obtained from mice at 20 wk of age immediately before histologic analysis. Serum and urine creatinine was determined using a modified alkaline picrate method (Exocell, Philadelphia, PA). Urinary protein concentration was determined by BCA assay and normalized for urinary creatinine concentration. Samples were measured in duplicate with seven to 10 animals per group.

**Immunostaining**

OCT-embedded, snap-frozen, 4-μm sections were stained with the following antibodies: FITC-conjugated donkey anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) and rat anti-murine CD8 and anti-CD4 (eBiosciences, San Diego, CA). The FITC-conjugated goat antimouse C3 (ICN, Aurora, OH) was preabsorbed with mouse IgG before incubation. Control staining was also performed using matched isotypes or IgG (data not shown). With the exception of IgG staining, donkey rhodamine–coupled anti-goat and FITC-coupled anti-rat antibodies were used for the detection of primary antibodies (Jackson Immunoresearch). C3 and IgG staining was scored on a relative scale of 0 to 3. Quantification of anti-CD4 and anti-CD8 staining was graded on the basis of the number of positive cells per glomeruli or per hpf for tubular infiltrates. A minimum of 10 glomeruli and 10 hpf were scored per animal with five to seven animals per group per time point. Histologic and immunofluorescent scoring was performed in a blinded manner.

**Immunophenotyping**

Leukocytes were obtained for FACS analysis from spleens (16 and 20 wk), peripheral blood (20 wk), and cervical lymph nodes (20 wk). Cell populations were characterized with the following markers (eBioscience): CD3 (145-2C11), CD4 (GK1.5), CD8 (53-6.7), CD11b (M1/70), CD19 (6D5), CD25 (PC6.5), CD45 (RB220), CD62L (MEL-14), and GR-1(Ly-6G). A minimum of 10,000 events were collected and analyzed on a FACSCaliber using CellQuest software (BD Biosciences, San Diego, CA). Samples were obtained from five to six mice per group per time point.

**Anti-dsDNA Antibody Titers**

Serum was obtained from 20-wk-old C5αR<sup>−/−</sup> MRL and MRL mice (<i>n</i> = 6 animals/group). Pooled serum from five 20-wk-old C57BL/6 mice served as a negative control. Calf thymus DNA (Roche, Indianapolis, IN) was used to coat 96-well plates and blocked with 5% BSA/PBS. The plates then were washed, and serial serum dilutions were added. After a 2-h incubation at room temperature, the plates again were washed, and horseradish peroxidase–conjugated detection antibody was added (anti-mouse IgG, anti-mouse IgG1, and anti-mouse IgG2a; Jackson Immunoresearch). The plates then were developed, and optical density was determined using a Spectromax M2 microplate reader (Molecular Devices, Sunnyvale, CA). End-point titers were calculated in comparison with pooled negative control serum.

**In Vitro Stimulation**

Whole splenocytes (10<sup>6</sup> cells/ml) were resuspended in complete RPMI and stimulated with either 100 ng/ml LPS (0127:B8; Sigma, St. Louis, MO) and 10 ng/ml IFN-γ (eBiosciences), 0.01% <i>Staphylococcus aureus</i>, Cowan’s strain 1 (SAC) (Calbiochem, San Diego, CA) and 10 ng/ml IFN-γ or with 1 μg/ml soluble anti-CD3 (17A2; eBiosciences). Supernatants were harvested 24 h after stimulation for IL-12p70, IL-10,
and TGF-β1; 48 h after stimulation for IL-4; and 72 h after stimulation for IFN-γ. Total RNA was isolated from whole spleenocytes before and 72 h after stimulation with anti-CD3. First-strand synthesis was performed on 5 μg of RNA using Superscript (Invitrogen, Carlsbad, CA). Quantitative PCR was performed with the following primer pairs using SYBR Green on an ABI 9700 sequence detector (Applied Biosystems): T-bet, CAACACCCCCCTTGGCAGAAG and TCCCCCAAGCAGTTGACAGT; GATA-3, AGAACCAGGCCCCTATCAA and AGTTCGCCAGGATGTC; and IFN-γ, TCAAGTGGCATAGATGTGGAA and GATA-3, AGAACCGGCCCCTTATCAA and AGTTCGCCAGGATGTC. After normalization for glyceraldehyde-3-phosphate dehydrogenase expression, fold changes in RNA expression in comparison with paired RNA samples after stimulation. Purified CD4+ T cells were isolated using two rounds of positive immunomagnetic bead selection (Miltenyi Biotec, Auburn, CA) to achieve >95% purity as determined by FACS (data not shown). CD4+ T cells (10^6/ml) were stimulated with 1 μg/ml soluble anti-CD3, plate bound anti-CD3, or plate bound anti-CD3 and 5 ng/ml soluble anti-CD28 (37.5), ebioscience. Supernatants were harvested 72 h after stimulation for IFN-γ production. Cytokine concentrations were determined by ELISA (Optia Sets; BD-Biosciences) according to the manufacturer’s instructions.

**Statistical Analyses**

Data were analyzed using Sigma Stat software version 3.0 (Jandel Scientific, San Rafael, CA). Survival analysis was performed using log-rank analysis. Distribution and frequency of variables was analyzed by either χ² analysis or Fisher exact testing when appropriate. Comparisons between groups were performed using the t test. P < 0.05 was assumed to be statistically significant.

**Results**

C5aR−/− C57BL/6 mice were back-crossed nine generations onto the MRL [30] genetic background. C5aR−/− MRL [30] mice then were intercrossed to obtain homozygous C5aR−/− MRL [30] (C5aR−/− MRL) mice and C5aR+/− MRL [30] (MRL) controls. These intercrosses resulted in the expected Mendelian ratios of homozygote and heterozygote progeny. Compared with MRL control animals, C5aR−/− MRL had a significantly increased survival, with a mean survival time of 33.0 ± 2.28 wk compared with 22.6 ± 1.2 wk for control mice (n = 13 and 17 mice, respectively; P = 0.003, log rank).

**C5aR−/− MRL mice have improved survival compared to MRL control mice.** C5aR−/− MRL mice (solid line) and MRL control mice (dashed line) were followed for 40 wk to quantify mortality using Kaplan-Meier analysis. C5aR−/− MRL mice had a mean survival time of 33.0 ± 2.28 wk compared with 22.6 ± 1.2 wk for control mice (n = 13 and 17 mice, respectively; P = 0.003, log rank).

Figure 1. C5aR−/− MRL mice have improved survival compared to MRL control mice. C5aR−/− MRL mice (solid line) and MRL control mice (dashed line) were followed for 40 wk to quantify mortality using Kaplan-Meier analysis. C5aR−/− MRL mice had a mean survival time of 33.0 ± 2.28 wk compared with 22.6 ± 1.2 wk for control mice (n = 13 and 17 mice, respectively; P = 0.003, log rank).

C5aR−/− MRL mice have attenuated renal injury and histologic scores.

At 16 wk of age, there was little difference in the severity of histologic injury between MRL control mice and C5aR−/− MRL mice. Both groups had similar degrees of glomerular hypercellularity and glomerular and vascular infiltrates (Table 1). There was a modest increase in the severity of glomerular sclerosis and tubulointerstitial injury in the C5aR−/− MRL mice compared with MRL controls.

By 20 wk of age, there were significant differences in the severity of renal injury in the two groups (Table 1). In the MRL control mice, crescents were seen in 20% of glomeruli, and the remaining glomeruli were markedly hypercellular with varying degrees of glomerular sclerosis (Figure 2, a and c). The degree of sclerosis varied considerably in the MRL control mice. Half of the glomeruli were globally sclerosed, and this may reflect progression from fibrocellular crescents; the remainder were partially sclerotic with no evidence of crescent formation. Few globally sclerosed glomeruli were noted in the C5aR−/− MRL mice (<2%), and this likely is a reflection of the lack of scleromatous crescents. There was severe tubulointerstitial disease with massive mononuclear cell infiltration and tubulointerstitial fibrosis. As opposed to MRL control animals, there was minimal progression in histologic injury in the C5aR−/− MRL mice between 16 and 20 wk of age (Table 1). The C5aR−/− MRL mice had dramatically less glomerular hypercellularity and less tubulointerstitial disease compared with 20-wk-old control animals (Figure 2, b and d). Significant, no glomerular crescents were seen in the C5aR−/− MRL mice. The inflammatory infiltrates surrounding medium and small vessels within the kidney were less intense in C5aR−/− MRL mice than those from MRL control animals (Figure 2, e and f). At 20 wk, the percentage of sclerotic glomeruli was similar in both groups. Functionally, C5aR−/− MRL mice had qualitatively less proteinuria than those from MRL control animals (Figure 2, a and c). At 20 wk, the percentage of sclerotic glomeruli was similar in both groups. Functionally, C5aR−/− MRL mice had qualitatively less proteinuria, although this did not reach the level of statistical significance (P = 0.07); however, serum creatinine concentrations were significantly lower than age-matched control MRL mice (P < 0.01; Table 1).

C5aR−/− MRL mice have reduced glomerular IgG deposition and T cell infiltrates.

Glomerular C3 and IgG deposition was characterized by immunofluorescent microscopy at 20 wk of age (Table 2). There was no significant difference in the degree of C3 deposition between MRL controls and C5aR−/− MRL mice (Figure 3, A and B). IgG staining was seen in both groups along the glomerular capillary loops and the mesangium (Figure 3, C and D). Although the distribution of IgG staining was similar, there was a significantly lower degree of IgG staining in the C5aR−/− MRL mice (Table 2).
T cell infiltrates within the renal parenchyma then were examined by indirect immunofluorescent staining with anti-CD8 and anti-CD4. CD8+ cells composed a minority of the T cell infiltrates and were present in similar numbers in both groups of animals (data not shown). The majority of infiltrating cells in the kidney were CD4+ T cells. At 16 wk of age, there were modest but significant increases in periglomerular and peritubular CD4+ cellular infiltrates in C5aR−/−MRL mice (Table 2). However, at 20 wk of age, there were large increases in the number of periglomerular and peritubular CD4+ cellular infiltrates in MRL control kidneys (Figure 3E). In distinction, in the C5aR−/−MRL mice, the number of CD4+ T cells seemed to decreased from 16 to 20 wk, although this did not reach statistical significance (P > 0.05; Figure 3F). Quantification of the CD4+ infiltrates at 20 wk indicated five-fold fewer periglomerular CD4+ cells and half the number of peritubular CD4+ cells in C5aR−/−MRL kidneys compared with those from MRL control mice (P < 0.005; Table 2).

**C5aR−/−MRL Mice Have Reduced Th-1 Immune Responses**

Given the alterations in T cell infiltrates in the kidneys of C5aR−/−MRL mice, phenotypic analysis of splenocyte populations was performed. The total splenocyte number was similar in all mice assessed. Analysis of T cell subsets of spleens from 16- and 20-wk-old mice revealed increased proportions of CD3−CD4+ cells in C5aR−/−MRL mice compared with MRL control mice (Figure 4). Although there was no difference in the CD8+ T cell population (Figure 4c), CD4+ T cells increased in frequency as C5aR−/−MRL mice aged (Figure 4d). By 20 wk, there was a four-fold increase in the absolute number of splenic CD4+ T cells in the C5aR−/−MRL mice compared with MRL controls (P < 0.005; Figure 4e). There were no significant differences in the absolute number of B220+ CD3−CD4−CD8− T cells or CD8+ T cells between MRL controls and C5aR−/−MRL mice. The expression of CD4+ T cell activation markers (CD62L and CD25) at 20 wk was also similar (data not shown).

The production of anti-dsDNA antibodies was quantified at 20 wk. Although there was a slight decrease in the total IgG end point titer (Figure 5A) in the C5aR−/−MRL mice, this did not reach the level of statistical significance. However, when the end-point titers of specific anti-dsDNA subclasses were measured, C5aR−/−MRL mice had higher end-point titers of IgG1 (3.5 ± 1.0-fold) and lower end-point titers of IgG2a (15.0 ± 6.1-fold) compared with controls (Figure 5B).

For investigating the capacity of C5aR−/−MRL mice to induce Th-1 responses, whole splenocytes were stimulated in vitro with LPS and IFN-γ or SAC and IFN-γ. The production of Th-1 and Th-2 polarizing cytokines was measured by ELISA. There was a significant decrease in the production of IL-12p70 in C5aR−/−MRL mice (Figure 6a). No differences in either IL-10 or TGF-β production were detected (data not shown). In response to anti-CD3 stimulation, splenocytes from C5aR−/−MRL mice produced three-fold less IFN-γ than controls (Figure 6b). IL-4 could not be detected in any of the cultured supernatants. Transcriptional responses linked to Th-1 responses were also inhibited. Anti-CD3-stimulated splenocytes from C5aR−/−MRL mice had reduced IFN-γ and T-bet mRNA expression and higher GATA-3 levels than MRL control mice (Figures 6c). When purified CD4+ T cells were stimulated with anti-CD3 alone or anti-CD3 and anti-CD28, there was no significant difference in IFN-γ production between the C5aR−/−MRL and MRL control mice (Figure 6d).

**Discussion**

The pathogenic role of complement in the MRLpr model is complex. In support of clinical observation that deficiencies in the early classical pathway components predispose to the development of SLE, MRLpr mice deficient in C1q and C4 have significantly worse renal injury and reduced survival compared with controls (31,32). Although human data strongly suggested a pathogenic role for C3 in lupus nephritis, MRLpr mice deficient in C3 were not protected from the development of kidney disease (33). The absence of C3 was not associated with either changes in the degree of lymphadenopathy or differences in T cell subsets. Although there were equivalent levels of autoantibodies and circulating immune complexes in the C3-deficient and control MRLpr mice, the C3-deficient animals had increased glomerular IgG deposition. Although overall renal histologic scores were similar, C3−/−MRLpr mice had earlier and greater amounts of proteinuria. Survival at 24 wk was similar, but there was a trend toward decreased survival in C3−/−MRLpr mice. The increased proteinuria and IgG deposition was thought to be due to the loss of C3-dependent clearance of circulating immune complexes and apoptotic bodies.
Blockade of C3 convertase has also been investigated in the MRL<sup>lpr</sup> mice. The mouse protein Cryst (CR1-related gene/protein y) is an endogenous regulator of C3 convertase and a co-factor for the inactivation of C3b. Cryst transgenic MRL<sup>lpr</sup> mice had decreased glomerular C3 deposition, modestly reduced histologic scores, decreased albuminuria, and prolonged survival (34). Similar results were seen with administration of soluble recombinant Cryst to MRL<sup>lpr</sup> mice (2,34). It is interesting that Cryst-treated mice had higher levels of circulating immune complexes and reduced renal injury, suggesting that alterations in local complement activation were in part responsible for the decrease in renal injury. Therefore, blockade of the complement cascade at the level of C5, preventing the generation of C5a and the formation of the membrane attack complex, should provide significant therapeutic advantages over inhibition of C3 or C3 convertase by avoiding loss of immune complex processing. This hypothesis has been supported by studies in the NZB mouse, in which anti-C5 treatment resulted in decreased proteinuria, less severe renal pathology scores, and increased survival (3). It is unclear, however, whether the effect of C5 inhibi-

**Figure 2.** Histologic renal injury is markedly attenuated in the C5αR<sup>−/−</sup> MRL mice. Representative kidney sections are shown (n = 5 to 7 mice per group per time point). At 20 wk, MRL control mice (a) had diffuse severe tubulointerstitial disease, prominent cellular and fibrocellular glomerular crescents, and leukocytic infiltrates. The severity of interstitial and glomerular lesions was markedly reduced in the 20-wk-old C5αR<sup>−/−</sup> MRL mice (b). This is seen in representative glomeruli from MRL control (c) and C5αR<sup>−/−</sup> MRL mice (d). The intense perivascular infiltrates seen in 20-wk-old MRL control mice (e) were qualitatively less in C5αR<sup>−/−</sup> MRL mice (f).
sure times were used for each antibody at a magnification of 
tative sections from five to seven mice per group. Identical expo-
reduced CD4
or to lack of C5a generation. Here we show that mice that
hibition was due to prevention of complement-mediated cell lysis
ease, suggesting that the dominant effect of C5 inhibition is due to 
loss of C5a/C5aR interactions and not inhibition of C5b-
dependent processes.

Compared with MRL control mice, C5aR−/− MRL mice had signiﬁcantly less glomerular hypercellularity and a complete 
absence of glomerular crescents. Given that the C5aR is highly 
expressed in renal tissue and C5a has been shown to induce 
both mesangial cell and tubular epithelial cell proliferation in
vitro, it is likely that this reduction in cellular proliferation is 
due to the direct effect of C5a/C5aR interactions on renal 
parenchymal tissue. C5a has also been shown to upregulate Fc 
receptor expression, thus enhancing the binding of immune 
complexes to target tissues (35,36). In the NZB/NZW mouse 
model of lupus nephritis, FeRy deﬁciency is associated with 
decreased renal pathology scores, delayed onset of proteinuria, 
and prolonged survival (37). However, FeRy−/− MRL
mice have similar renal disease and survival compared with control 
MRL mice (38). These data, combined with relatively modest 
reduction in glomerular IgG staining in the C5aR−/− MRL mice 
compared with the marked reduction in renal injury, suggest 
that in this model, the effect of C5aR are likely independent of 
Fc receptor expression.

Work by Quigg et al. (39) demonstrated that scCry treatment 
of MRL mice reduced both the accumulation of glomerular 
eextracellular matrix components including collagen 1 and the 
severity of chronic tubulointerstitial injury. These data, com-
bined with our work, suggest that chronic complement activa-
tion promotes the development of renal ﬁbrosis and that C5a 
contributes to this process. Additional support for this hypo-
thesis can be found in work by Welch et al. (40), who demonstrated 
in the horse apoferritin model of chronic immune complex disease 
that mice deﬁcient in C5aR were protected from the development 
of chronic tubulointerstitial disease. The reduction in tubulointer-
stitial ﬁbrosis in the C5aR−/−MRL mice supports this hypothesis; 
however, there was no signiﬁcant reduction in the degree of 
glomerular sclerosis in the C5aR−/−MRL. This suggests that C5a 
is not a primary regulator of extracellular matrix production in the 
glomerulus, and its effects are likely limited to the tubulointerstitial.

An important finding in our study is that C5aR−/− MRL mice 
hibit impaired Th-1–mediated renal injury and immune re-

Table 2. Immunostaining in 16- and 20-wk-old MRL control and C5aR−/− MRL mice

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<th>MRL Ctrl</th>
<th>C5aR−/− MRL</th>
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<tr>
<td></td>
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<td>20 Wk</td>
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<tr>
<td>C3 staining</td>
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<td>Peritubular CD4 staining</td>
<td>4.6 ± 1.2</td>
<td>10.1 ± 0.3c</td>
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aData are mean cell number/glomeruli or per high-power field ± SEM.

bP < 0.05 for 16- versus 20-wk-old C5aR−/− MRL mice.
cP < 0.01, dP < 0.005, for C5aR−/− MRL mice compared with age-matched controls.

Figure 3. C5aR−/− MRL mice have reduced IgG deposition and 
reduced CD4+ T cell infiltrates. MRL control (a) and C5aR−/− 
MRL mice had similar degrees of C3 deposition in the peripheral 
capillary loops as well as in the mesangium (b). Whereas IgG 
deposition was seen in a similar distribution, there was qualita-
tively more IgG in the MRL control mice (c) compared with the 
C5aR−/− MRL mice (d). Numerous periglomerular CD4+ infil-
trates were present in the MRL control mice (e) with a paucity of 
CD4+ cells in C5aR−/− MRL mice (f). The figures are representa-
tive sections from five to seven mice per group. Identical exposure 
times were used for each antibody at a magnification of ×400.
responses. This is illustrated by evidence of prevention of glomerular crescent formation, reduction in CD4⁺ T cell infiltrates, reduced titers of IgG2a anti-dsDNA antibody, and inhibition of IL-12p70 and IFN-γ production. The inhibition of IFN-γ production is more remarkable given that splenocytes from C5aR⁻/⁻ MRL mice had a four-fold higher numbers of CD4⁺ cells than in control spleens. Although there is evidence that human T cells express the C5aR and we have been able to detect low levels, fewer than 10 copies/1000 cells, of C5aR mRNA in purified MRL⁻/⁻ T cells, we have not been able to confirm C5aR protein expression either by FACS or by Western blot (data not shown). Furthermore, the studies of purified CD4⁺ T cells from C5aR⁻/⁻ MRL and MRL control mice indicate that CD4⁺ T cells from the C5aR⁻/⁻ MRL mice have an intrinsically normal ca-

Figure 4. C5aR⁻/⁻ MRL mice have a progressive increase in splenic CD4⁺ T cells. Single-cell suspensions were obtained from whole spleens at 16 and 20 wk. Splenocytes were stained with anti-B220, anti-CD3, anti-CD4, and anti-CD8. The frequency of double-negative T cells (CD3⁻CD4⁻CD8⁻) and single-positive CD4⁺ and CD8⁺ cell populations were analyzed by FACS. Representative scattergrams of CD3⁺ gated splenocytes from 20-wk-old MRL control mice (a) and C5aR⁻/⁻ MRL mice (b) are shown. There was no significant difference in percentage of splenic CD8⁺ T cells in MRL control mice (solid line) and C5aR⁻/⁻ MRL mice (dashed line) at 16 or 20 wk of age (c). There was little change in the percentage of splenic CD4⁺ T cells (d) in the MRL control mice from 16 to 20 wk (solid line); however, there was a significant increase in CD4⁺ T cells in the C5aR⁻/⁻ MRL mice. In terms of absolute numbers (e), there was a four-fold increase in the absolute numbers of CD4⁺ T cells in 20-wk-old C5aR⁻/⁻ MRL mice (□) compared with MRL control mice (■). Data are means ± SE.
pacity to produce IFN-γ, suggesting that the attenuation in Th-1 responses is largely due to indirect effects on T cells via the suppression of IL-12p70 production by host antigen-presenting cells (APC). Although no differences were detected in this model with respect to CD8+ T cell numbers or renal infiltrates at 20 wk, others, however, have suggested that C5aR also has effects on CD8+ T cell function. Kim et al. (41) showed attenuated antigen-specific CD8+ T cell responses in the lungs of mice that were pretreated with a C5aR antagonist before infection with influenza virus. Authors speculated that this might be an indirect effect as a result of C5aR inhibition on migration of APC. Together with our data, these findings suggest that C5aR-dependent alterations in T cell responses are likely APC dependent and may have more generalized implications for complement-dependent modulation of adaptive immune responses.

There are conflicting experimental data on the role of C5a/C5aR signaling and IL-12 production. In vitro stimulation of purified human monocytes with C5a has been shown to suppress IL-12 production; however, inhibition of C5a has been shown to reduce IL-12p70 production from human peripheral blood mononuclear cells (10–12). Moreover, in vitro stimulation of monocytes from C5-deficient mice demonstrated reduced production of IL-12p70 (12). The reduction in IL-12p70 and IFN-γ production seen in the C5aR−/− MRL mice is consistent with C5aR signaling's having a positive role in the production of IL-12 in MRL/lpr mice and supports previously reported findings that loss of C5a/C5aR signaling results in augmented Th-2 responses (12). With respect to ex vivo stimulation, complement components such as C3 and C5 are known to be produced by monocytes, and thus stimulation of mixed cell cultures with known activators of the alternative pathway such as LPS or SAC are likely to generate C5a. Similarly, stimulation of mixed cell cultures with complement-fixing antibodies such as IgG2b anti-CD3 may also induce complement activation and

Figure 5. C5aR−/− MRL mice have increased IgG1 and reduced IgG2 titers of anti-dsDNA antibodies. Serum was obtained from 20-wk-old C5aR−/− MRL, MRL control, and C57BL/6 mice (pooled control). End-point titers for total anti-dsDNA IgG (A), IgG1 (B), and IgG2a (C) were measured using serial serum dilution (x axis). C5aR−/− MRL mice had modest reduction in total anti-dsDNA IgG in comparison with controls; however, end-point titers of IgG were 3.5 ± 1.0-fold higher and IgG2a titers were 15.0 ± 6.1-fold lower in the C5aR−/− MRL mice than in controls (D; P < 0.02). Data are means ± SEM for six samples per group.
C5a generation. This in part may explain the minimal effect seen in pure CD4\(^+/+\) T cell stimulation, as T cells are not recognized as being sources of complement components. With respect to renal disease, glomerular crescent formation is the principal hallmark of severe Th-1–dependent renal injury. Systemic depletion of CD4\(^+/+\) T cells or the inhibition of IL-12 or IFN-\(\gamma\) has been shown to prevent renal injury in murine models of crescentic glomerulonephritis (42–44). The absence of glomerular crescents in the C5aR\(^-/^-\) MRL mice is consistent with the reduction in Th-1 T cell responses in terms of both reduced IL-12p70 and IFN-\(\gamma\) production and CD4\(^+/+\) infiltrates. We found that the reduction in Th-1 responses was associated with an inhibition of the key Th-1 transcription factor T-bet and a relative enhancement of the Th-2 transcription factor GATA-3. Taken together, these data indicate that C5a/C5aR-dependent signaling has a significant impact not only on renal injury but also on influencing T cell function.

In addition to the reduction in Th-1 responses, C5aR\(^-/^-\) MRL mice had a dramatic reduction in CD4\(^+/+\) renal infiltrates and a progressive increase in splenic CD4\(^+/+\) T cells. These findings suggest that C5aR deficiency results in a defect in the recruitment or migration of CD4\(^+/+\) T cells. Although it is possible that this is due to the direct effect of C5aR on T cells, our inability to detect C5aR expression on murine CD4\(^+/+\) T cells suggests that it is more likely that this is an indirect effect. C5a-dependent activation of monocytes and macrophages has been well described, and a loss of C5aR expression by these cells may contribute significantly to the reduced renal recruitment of CD4\(^+/+\) T cells. However, C5a has been shown to upregulate E-selectin, intercellular adhesion molecule-1, and vascular cellular adhesion molecule-1 expression in endothelial cells as well as induce monocyte chemoattractant protein-1 in glomerular mesangial cells, all of which have been shown to be upregulated in lupus nephritis and play major roles in T cell recruitment (19,45). It is probable, therefore, that the reduction in CD4\(^+/+\) T cell recruitment in the C5aR\(^-/^-\) MRL mouse is a result of the loss of C5a/C5aR-dependent activation of both host APC and renal parenchymal tissues.

Finally, the gene encoding the murine C5aR has been mapped to chromosome 7 (3.5 cM). Epigenetic modifiers in this region have been identified by several groups using MRL\(^{lpr}\) mice and other murine lupus models; these include Sle5 (0.5 cM), Lrdm1 (6 cM), Sle3 (15 cM), Lbrv5 (23 cM), Nba5 (23 cM), Lmb3 (28 cM), and Nba3 (31 cM) (46,47). Although it is possible that a polymorphism in a gene linked to the C5aR locus is contributing to attenuation of disease in our mice, there are...
several lines of evidence that suggest that this is not the case. First, B6.Sle3/5 NZMc7 congenic mice develop selective expansion of splenic CD4+ T cells, similar to that seen in the C5aR-deficient mice. B6 mice that lacked the introgressed chromosome 7 interval, which encompasses the C5aR gene, did not develop alterations in splenic T cell population (48). In addition, although Slc3/5 is associated with enhanced T cell responses, no differences in Th-1/Th-2 skewing have been noted in mice expressing this promoter. Second, although the deposition of C3 in C5aR-deficient mice, studies of congenic mice did not demonstrate any impact of this locus on survival. The attenuation in renal injury seen in mice that lack the lmb3 allele was primarily due to reductions in glomerular sclerosis and interstitial fibrosis as opposed to the reductions in mesangial proliferation and crescent formation seen in the C5aR-deficient mice. Although it is extremely difficult to exclude epigenetic effects, clear differences in disease phenotypes suggest that unrecognized confounding effects of these loci are not major contributing factors to the disease attenuation seen in the C5aR−/−MRL mice.

In summary, the findings reported herein support a central role for C5a/C5aR signaling in CD4+ T cell recruitment, enhancement of Th-1 cytokine production, and exacerbation of renal injury in the MRLpr mouse. C5aR−/−MRL mice had higher absolute numbers of CD4+ splenocytes that produced significantly less IFN-γ than controls. The recruitment of these cells was impaired as characterized by a marked reduction in CD4+ renal infiltrates. Although the deposition of C3 in C5aR−/−MRL kidneys is similar to MRLpr controls, there was less parenchymal injury as evidenced by an absence of glomerular crescents, decreased glomerular hypercellularity, and a relative sparing of renal function. These investigations provide strong experimental support linking C5aR activation to disease severity in the MRLpr mouse and indicate that C5a/C5aR plays a significant role not only in mediating renal injury but also in enhancing the induction of CD4-dependent Th-1 responses. These findings provide a rational experimental basis for the potential use of C5aR inhibitors in the treatment of SLE in humans.

Acknowledgments

Supported in part by National Institutes of Health grants AI025011, HL074333 (R.A.W), DK64233 (H.Y.L.), and DK62197 (M.C.B.).

These data were presented in part at the 20th International Complement Workshop, Honolulu, HI, June 2004.

References

7. Otto M, Hawlisich H, Monk PN, Muller M, Klos A, Karp CL, Kohl J: C5a mutants are potent antagonists of the C5a receptor (CD88) and of C5L2: Position 69 is the locus that determines agonism or antagonism. J Biol Chem 279: 142–151, 2004
19. Braun M, Davis AE 3rd: Cultured human glomerular mes-


53. Kong PL, Morel L, Croker BP, Craft J: The centromeric region of chromosome 7 from the MRL mice (Lmb3) is an epistatic modifier of Fas for autoimmunity. *J Immunol* 172: 2785–2794, 2004