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C5a promotes development of experimental lupus nephritis which can be blocked with a specific receptor antagonist

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The MRL/lpr murine SLE model has widespread complement activation and deposition of complement fragments in affected tissues. The potent anaphylatoxin C5a has the potential to play a key role in the pathogenesis of lupus nephritis. We found that renal expression of C5aR mRNA and protein was significantly increased in MRL/lpr mice compared to control MRL/+ mice. To examine the role of C5a signaling through C5aR, a specific small molecule antagonist (a) of C5aR was administered continuously to MRL/lpr mice from 13 to 19 wks of age. Littermate controls were given vehicle alone. The progressive impairment in renal function exhibited in the control group was prevented by C5aRa treatment. Infiltration of neutrophils and macrophages into kidneys was significantly reduced in animals treated with C5aRa compared to controls. Furthermore, renal expression of IL-1β and MIP-2 mRNA as well as the extent of apoptosis were significantly decreased with blockade of C5aR, indicating their dependence upon signals delivered through C5aR. Thus, pharmacological blockade of C5aR reduces disease manifestations in experimental lupus nephritis. These data support an important role for the C5a anaphylatoxin in lupus nephritis, and that blockade of C5aR represents a potentially viable treatment for human lupus nephritis.

Introduction

Complement can be activated through the classical, alternative and lectin pathways. C3 is cleaved upon complement activation with generation of C3a and C3b, followed by C5 activation and production of C5a and C5b [1–3]. The generation of C5b begins the non-enzymatic assembly of the C5b-9 membrane attack complex, which can result in cellular death or activation following membrane insertion [4]. The complement system is believed to be centrally involved in the pathogenesis of SLE. In patients with SLE, complement is systemically consumed, as evidenced by a decrease in circulating complement levels. In addition, deposition of complement components occurs in diseased organs, particularly the kidney [5].

The MRL/Mp-Tnfrsf6^{lpr/lpr} strain (MRL/lpr) is a commonly used mouse model of human SLE. The MRL background confers autoimmune susceptibility, while the lymphoproliferation (lpr) gene results from a retroviral insertion in the Tnfrsf6 (Fas) gene [6], leading to nearly complete absence of the apoptosis-promoting Fas protein [7]. These mice share many features of

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Abbreviations: **a:** antagonist · **BUN:** blood urea nitrogen · **ds:** double-stranded · **GN:** glomerulonephritis · **IF:** immunofluorescence · **MRL/lpr:** MRL/Mp-Tnfrsf6^{lpr/lpr} · **q:** quantitative

human SLE, including the development of high levels of autoantibodies to a variety of native antigens such as double-stranded (ds) DNA. Hypocomplementemia and deposition of complement activation fragments in glomeruli suggest that complement activation also contributes to the pathogenesis of the proliferative and sclerosing glomerular disease evident in these mice. The relevance of complement to experimental lupus nephritis is also supported by our previous studies in which we systematically inhibited C3/C5 activation in MRL/*lpr* mice, which led to a reduction in renal disease and prolonged survival in complement-inhibited mice compared to controls [8, 9].

The C5a anaphylatoxin is a 77-aa fragment derived by cleavage of the C5 α-chain [10]. C5a binds its specific G protein-coupled R, C5aR, on a variety of cells, including those of myeloid origin such as neutrophils and monocytes [11–13]. C5aR activation on these cells can contribute to the inflammation in many diseases, such as the reverse Arthus reaction [14] and sepsis [15]. Of relevance to the kidney is the finding of C5aR in cultured renal mesangial and proximal tubular epithelial cells [16–18], and that C5a signaling through C5aR has been shown to be important in the tubulointerstitial injury in a model of immune complex-mediated glomerulonephritis (GN) [19]. As for humans, the expression of C5aR has been observed to be increased in the kidneys of patients with lupus nephritis [20], supporting its potential relevance in disease pathogenesis. Yet, direct studies on the role of C5a and C5aR in the pathogenesis of lupus nephritis have not been performed.

In this study, we first clarified that the expression of C5aR was increased not only in diseased kidneys but also prior to the onset of disease in MRL/*lpr* mouse kidneys. These results suggest that activation of C5aR is involved as a primary event in the pathogenesis of this disease and not just a secondary phenomenon, and that signaling of C5aR, particularly in the milieu of complement activation present as autoimmunity becomes manifest, may be relevant to lupus nephritis. To evaluate this directly, a specific antagonist (a) of C5aR was administered to MRL/*lpr* mice from 13 to 19 wks of age, which led to a reduction in renal disease.

Results

C5aR is upregulated in MRL/*lpr* mouse kidneys

To provide insight into a potential role for C5a in the development of lupus nephritis, we first evaluated the expression of C5aR mRNA and protein. As shown in Fig. 1A, even at 6 wks of age, which is before the
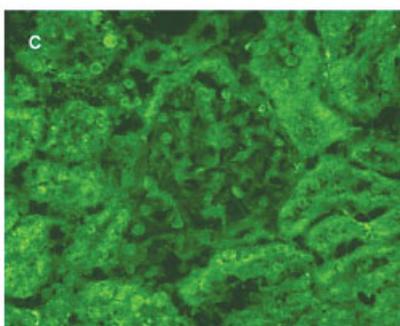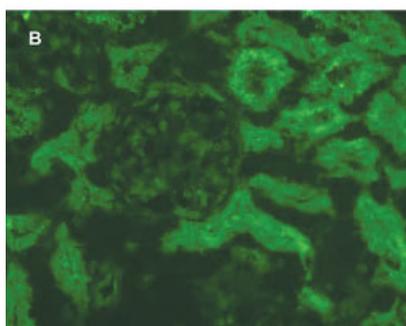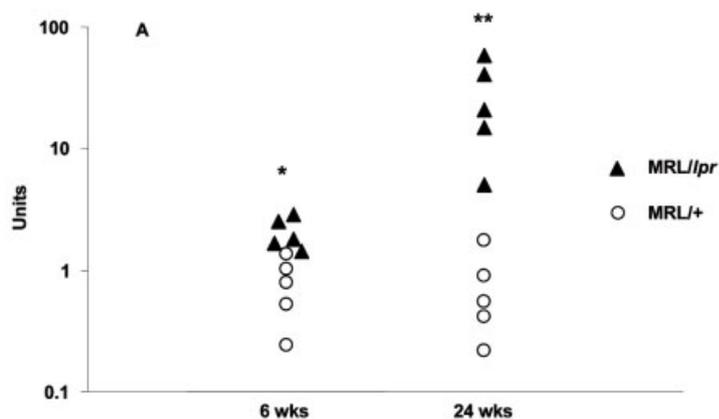

Fig. 1. C5aR is up-regulated in the kidneys of MRL/*lpr* mice before and after development of renal disease. By qRT-PCR, C5aR mRNA was increased in the renal cortices of 6- and 24-wk-old MRL/*lpr* mice compared to MRL/+ mice (A). *p=0.0061, **p=0.046 *vs.* MRL/+. Using IF staining C5aR is seen to be mainly expressed in tubular cells of MRL/+ mice (B), while in MRL/*lpr* mice, it is up-regulated and expressed in glomerular cells and infiltrating cells (C). Original magnifications, ×400.
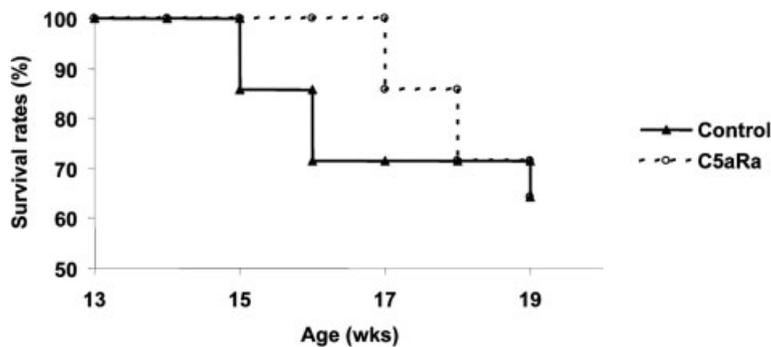
Fig. 2. Blockade of C5aR with antagonist delays early mortality in MRL/*lpr* mice. All MRL/*lpr* mice treated with C5aRa survived until 17 wks of age compared with 10 of 14 littermate MRL/*lpr* controls receiving vehicle. *p*=0.031 between the two groups.

development of lupus nephritis, MRL/*lpr* mice had significantly elevated C5aR mRNA expression compared to age-matched MRL/+ strain controls. As the disease progressed, C5aR mRNA expression also rose markedly, whereas it remained at a relatively low level in age-matched MRL/+ mouse kidneys.

The up-regulation of C5aR in lupus nephritis was also confirmed at the protein level by immunofluorescence (IF) staining. In MRL/+ mouse kidneys, C5aR was found in tubules and some glomerular cells (Fig. 1B), while it was significantly increased in both distribution and intensity in MRL/*lpr* mouse kidneys, not only in tubules and glomerular cells, but also in infiltrating leukocytes (Fig. 1C).

Blockade of C5aR delays mortality and renal disease in MRL/*lpr* mice

Given the significant up-regulation of C5aR in lupus kidneys before and through the disease development, we hypothesized that C5a is actively involved in the pathogenesis of lupus nephritis. To observe the effects of antagonizing C5aR in lupus nephritis, we treated MRL/*lpr* mice with a cyclic hexapeptide antagonist of C5aR, acetyl-Phe-(Orn-Pro-D-cyclohexylalanine-Trp-Arg) (denoted here as C5aRa) [21]. Because of the relatively long-term nature of this disease, subcutaneous mini-osmotic pumps were used to deliver C5aRa or vehicle continuously, thereby achieving steady-state concentrations and avoiding the need for frequent injections. Twenty-eight male littermate MRL/*lpr* mice were randomly and equally divided into C5aRa and control groups. Starting at 13 wks of age, at which time autoimmunity had already begun and renal disease was evident (see below), mice received 1 mg/kg/day of C5aRa dissolved in 50% DMSO in saline; controls were treated identically, except that the 50% DMSO vehicle solution did not contain C5aRa. The dose of 1 mg/kg/day was chosen based on its utility in other mouse models [15]. Serum and urine samples were collected biweekly. C5aRa was administered from 13 to 19 wks of age, which is the key 6-wk phase in which lupus nephritis in MRL/*lpr* mice undergoes marked transfor-

mation from mesangial proliferation to diffuse endocapillary and often extracapillary (crescentic) proliferation. In a minority of mice of these ages, glomerulosclerosis develops, which leads to death of the animal from renal failure. As shown in Fig. 2, this spontaneous death started at 15 wks of age in control mice, while it was significantly delayed until 17 wks in C5aRa-treated animals (*p*=0.031 by Chi square test). However, by the conclusion of the study at 19 wks of age, mortality was equivalent in both groups, illustrating that blockade of C5aR delayed, but did not prevent, this mortality.

Aside from the indirect approach of following mortality as an indicator of renal failure, the presence and magnitude of renal disease in these mice was followed continuously with blood urea nitrogen (BUN) and albuminuria measurements. Consistent with the mortality in control MRL/*lpr* mice, their BUN levels rose significantly over time (Fig. 3), while the BUN levels in C5aRa-treated animals were significantly lower at every time point measured (*p*=0.027).

Similar results were found with albuminuria measurements. At the beginning of the study, all animals had abnormal albuminuria (range 0.05–2.08 mg/mg creatinine). The progressive increase in albuminuria over time was significantly suppressed in C5aRa-treated mice compared with the controls (*p*=0.022). At the conclusion of the study, albuminuria was 9.0 ± 3.6 and 3.0 ± 1.3 mg/mg creatinine in control and C5aRa-treated groups, respectively. Thus, blockade of C5aR with a specific antagonist reduces renal disease in MRL/*lpr* mice, as shown by these two parameters of renal function.

Renal histopathological findings

At 19 wks of age, MRL/*lpr* mice developed endocapillary and extracapillary proliferative GN as well as segmental sclerosis/hyalinosis (Fig. 4A, B), which was quantified using a semiquantitative scoring system (Table 1). While the extent of GN and glomerulosclerosis was higher in the control group compared to the C5aRa-treated group, these differences did not reach statistical significance.
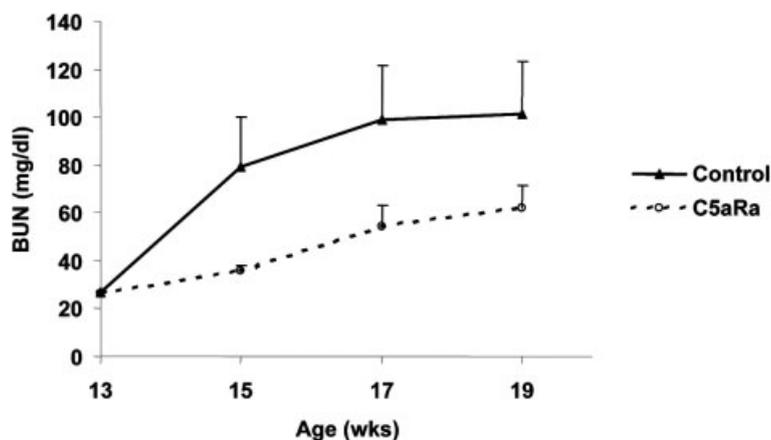
Fig. 3. Renal function is preserved in MRL/*lpr* lupus mice treated with C5aRa. Shown are cumulative biweekly BUN measurements from MRL/*lpr* mice receiving C5aRa or vehicle from 13 to 19 wks of age. *p*=0.027 between the two groups.
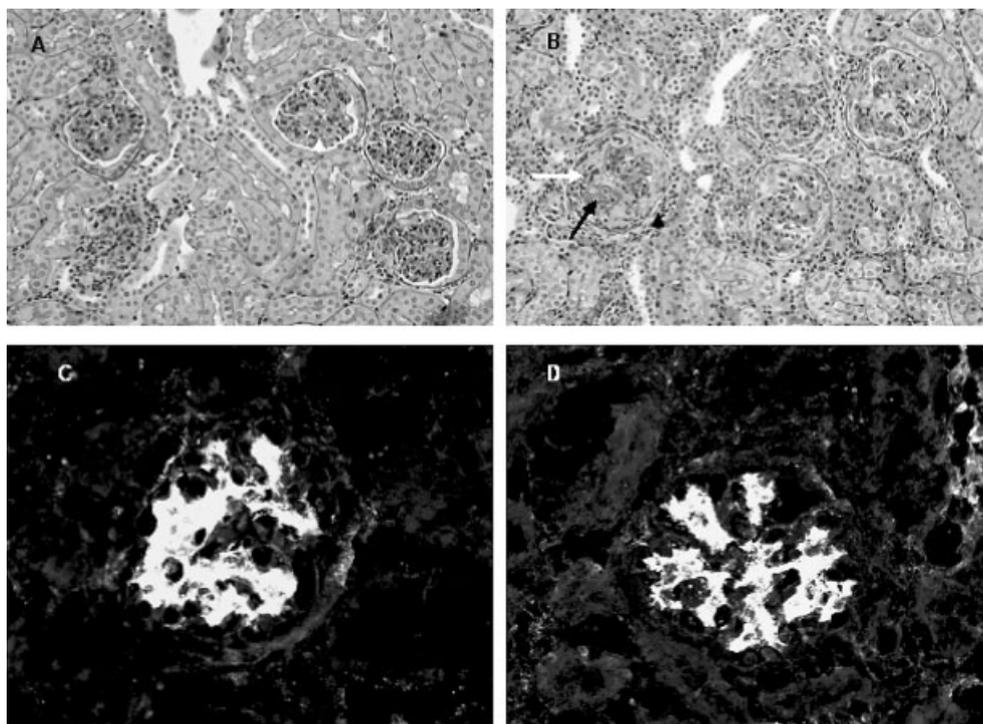

Fig. 4. Histopathological manifestations of disease in 19-wk-old MRL/*lpr* mice. Light microscopic (A, B) and immunofluorescence (C, D) features are shown that are representative of C5aRa (A, C) and vehicle-treated (B, D) groups, as scored in Table 2. There is diffuse proliferative GN (white arrowhead), infiltration with mononuclear cells (black arrowhead), hyalinosis/sclerosis (black arrow) and crescent formation (white arrow), which are reduced by C5aRa treatment. Extensive mesangial and capillary wall deposition of IgG is seen in both groups. Original magnifications, (A, B) ×200, (C, D) ×400.

The deposition of both Ig and C3 in kidneys was also evaluated by IF microscopy given their relevance to both complement activation and lupus nephritis. In 19-wk-old MRL/*lpr* mouse kidneys, there was extensive mesangial and capillary wall deposition of immunoglobulins and C3, as shown in Fig. 4C and D for IgG staining. As expected, C5aR blockade did not affect the distribution or the intensity of C3, IgG, IgA and IgM deposition in MRL/*lpr* mouse kidneys (Table 1).

Since neutrophils and macrophages contribute to the inflammation in lupus nephritis and both express C5aR, the possibility that C5aRa treatment affected the extent of their infiltration was also examined. Both neutrophil and macrophage infiltration into kidneys was significantly reduced after 6 wks of C5aRa treatment compared to controls (Fig. 5, *p*=0.004 and 0.018 for neutrophils and macrophages, respectively).

Autoimmune features are unaffected by C5aR antagonism in MRL/*lpr* mice

Development of anti-dsDNA Ab and circulating immune complexes are key features of autoimmunity in MRL/*lpr* mice, which have the potential to be affected by products

Table 1. Renal pathological findings in MRL/*lpr* mice treated with C5aRa[a]

	GN	GS	C3	IgG	IgA	IgM
Control	2.4 ± 0.7	1.3 ± 0.3	3.4 ± 0.3	3.4 ± 0.2	3.1 ± 0.2	3.4 ± 0.1
C5aRa	1.7 ± 0.4	0.8 ± 0.4	3.1 ± 0.3	3.5 ± 0.2	2.9 ± 0.2	3.1 ± 0.2

[a] Shown are semiquantitative scores (0–4) for the extent and severity of histological indices of renal disease and IF staining in glomeruli of MRL/*lpr* mice treated from 13 to 19 wks with C5aRa or vehicle control. GN, glomerulonephritis, GS, glomerulosclerosis. *n*=9 per group. There were no statistical differences between the two groups in any of the variables.
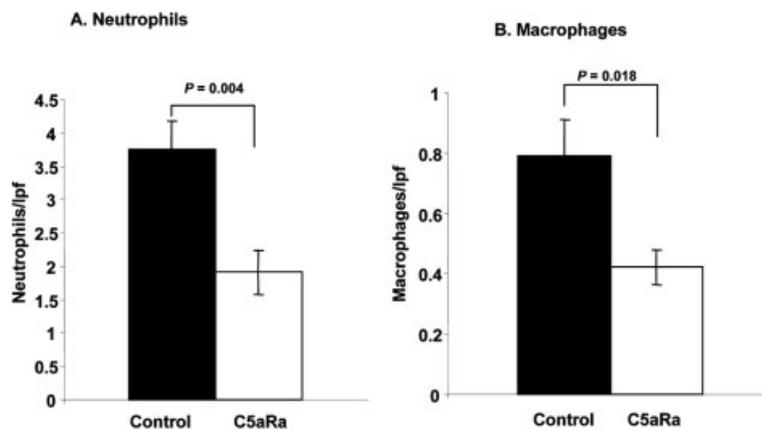

Fig. 5. Renal infiltration with neutrophils and monocyte/macrophages is reduced in MRL/*lpr* mice treated with C5aRa from 13 to 19 wks of age. Average neutrophil (A) and monocyte/macrophage (B) counts per low-power field (×200) from each group are shown.

of complement activation, including C5 [22, 23]. At 13 wks of age, all MRL/*lpr* mice had detectable serum anti-dsDNA Ab, while sera from age-matched MRL/+ and BALB/c mice were negative in this assay. During the 6 wks of treatment, anti-dsDNA Ab rose in both groups without any statistical difference (data not shown). Similar results were found with measurements of circulating immune complexes. Thus, blockade of C5aR in MRL/*lpr* mice did not appear to affect underlying autoimmunity at least as indicated by these two critical components of the lupus disease process.

Effect of C5aRa on renal cortical expression of inflammatory mediators

To provide insight into the potential effects of C5aR in MRL/*lpr* mice, renal cortical expression of IL-1β, IL-2, IL-18, IFN-γ, TNF-α, TGF-β1, ICAM-1, CCR2/MCP-1R, CCL5/RANTES, CCL8/MCP-2, and CXCL2/MIP-2 were evaluated by quantitative (q) RT-PCR. These mediators have been shown to be relevant to lupus nephritis and/ or C5a signaling in past investigations [14, 24–29] and/ or in our unpublished gene array data as being up-regulated in MRL/*lpr* mouse kidneys compared to MRL/ + controls. Preliminary studies were performed with four representative animals from each group. Surprisingly, from these 11 inflammatory mediators, only IL-1β and MIP-2 were identified in these initial studies as being different between the two groups (data not shown). To confirm these changes, all nine surviving

animals from each group were studied. As shown in Fig. 6, renal cortical mRNA expression for IL-1β (32.5 ± 8.2 and 11.1 ± 2.6 U for control and C5aRa groups, respectively, *p*=0.017) and MIP-2 (230 ± 78 and 68 ± 30 U for control and C5aRa groups, respectively, *p*=0.027) were significantly reduced by C5aR antagonism.

Blockade of C5aR reduces renal apoptosis in MRL/*lpr* mice

Renal apoptotic cells were assessed and quantified using the TUNEL technique. In control animals, apoptotic cells could be seen both in the interstitium and in and around glomeruli (Fig. 7A), while in C5aRa-treated animals the number of apoptotic cells was significantly reduced (Fig. 7B) which was formally quantified (Fig. 7C, *p*=0.045 between groups). The relevance of apoptosis to lupus nephritis in this study is supported by its strong correlation with renal histopathological measurements and macrophage infiltration (Table 2).

Discussion

The C5a anaphylatoxin signaling through its C5aR has the potential to contribute to the inflammation present in a variety of disease processes in which complement activation is present. These include immune complex-induced peritonitis [30], experimental arthritis [31],
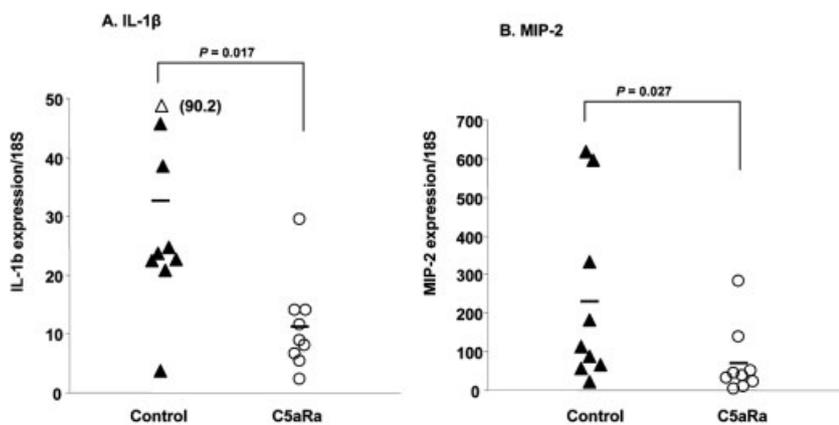
A. IL-1β / **B. MIP-2**

Fig. 6. C5aR antagonism reduces renal expression of IL-1β and MIP-2 mRNA in MRL/*lpr* mice. qRT-PCR data are presented for individual mice (C5aRa, empty circles; control, black triangles) with mean values depicted by horizontal lines in each group. Data are presented as expression units relative to 18S RNA measured in the same sample. The value for IL-1β expression in a single control animal (white triangle) was off the scale and the numeric value is presented in the graph.
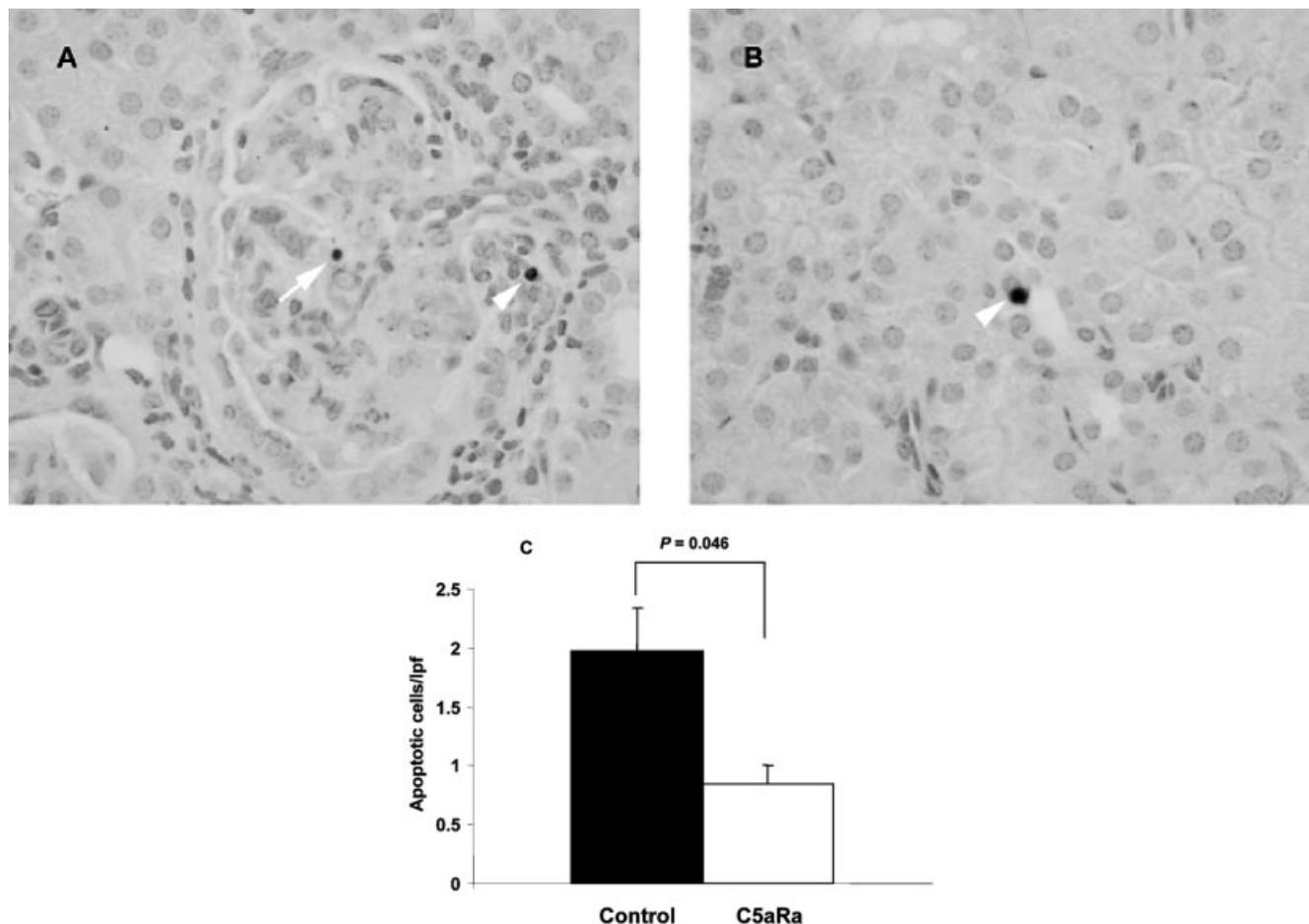

Fig. 7. Blockade of C5aR leads to reduced apoptosis in the kidneys of MRL/*lpr* mice. MRL/*lpr* mice receiving vehicle alone from 13 to 19 wks of age had increased numbers of apoptotic cells in the renal cortex in (arrow) and outside (arrowhead) of glomeruli (A). In animals treated with C5aRa, there was a reduction in the number of apoptotic cells (B), as formally scored in renal cortices of mice from both groups (C). Original magnification, ×400 (A, B).

sepsis [32] and anti-phospholipid syndrome [33]. C5aR mRNA has been found to be up-regulated in glomeruli of patients with lupus nephritis, supporting its relevance in the human disease [20]. Here we have shown that the overexpression of both C5aR mRNA and protein in MRL/

lpr mouse kidneys started before the onset of detectable disease, and increased as autoimmune disease progressed, suggesting a potential role for C5a and C5aR signals in the pathogenesis of this disease.

Table 2. Correlation analysis between kidney apoptotic cell number and renal disease measurement data[a]

	GN score	Glomerulosclerosis	Glomerular crescents	Macrophage infiltration
r	0.765	0.806	0.857	0.951
p	0.027	0.016	0.007	0.000

[a] In each column, the first value is the correlation coefficient and the second is the *p* value. The GN score was a semi-quantitative assessment of glomerulonephritis, glomerulosclerosis and glomerular crescents represent the percent of glomeruli involved, and macrophage infiltration is given as the number of renal cortical macrophages per low-power field.

To study this potential role of C5aR in experimental lupus nephritis, we used acetyl-Phe-(Orn-Pro-D-cyclo-hexylalanine-Trp-Arg), a cyclic hexapeptide antagonist of C5aR. This antagonist is potent towards mouse C5aR with IC$_{50}$ values of 0.5 nM [15], and has been successfully used in a diversity of acute and chronic animal models such as sepsis and endotoxemic shock, and arthritis [15, 34, 35]. The capacity for relatively long-term use as needed in lupus nephritis models was an attractive feature for our studies. Here we utilized a dose of 1 mg/kg/day shown to be effective in a chronic rodent arthritis model [35]. However, rather than utilize multiple i.p. or s.c. doses of C5aRa, we opted to use osmotic mini-pumps to deliver C5aRa continuously. We do acknowledge that such continuous delivery is not directly feasible in human SLE, other than in the short term.

The MRL/*lpr* mouse is a well-studied murine model of human SLE which spontaneously develops proliferative GN as early as 12 wks of age, followed by a histological disease evolution that spans the spectrum in humans (from WHO classes II through VI). Renal failure is believed to be the main cause of death, occurring in half of the mice by 20–24 wks of age [36, 37]. Blockade of C5aR with the specific C5aRa peptide significantly reduced renal disease in MRL/*lpr* mice, as reflected by lower BUN and albuminuria levels compared to control animals, which further translated into delayed mortality in these mice. Similar findings were observed by our group when complement activation was inhibited at the level of C3/C5 convertases in MRL/*lpr* mice [8, 9, 38], indicating that complement is involved in the pathogenesis of lupus nephritis, not only through generation of pro-inflammatory C3a and C3b, and the cell injuring/activating C5b-9 membrane attack complex, but also through the additional product of C5 activation, the C5a anaphylatoxin. Similar findings to ours have recently been observed by backcrossing the MRL/*lpr* strain into the C5aR-deficient strain generated by Wetsel *et al.* [33], including protection from functional renal disease and a delay in mortality (M. Braun *et al.*, C5a receptor deficiency attenuates T cell function and renal disease in MRL^{lpr} mice; submitted for publication), thereby providing further evidence for the relevance of C5a signaling through C5aR in lupus nephritis.

Renal pathological manifestations of lupus nephritis were lower in the C5aRa-treated animals, yet these differences did not reach statistical significance comparing the nine surviving animals from each group. Possible explanations for this include that advantages in terms of survival, BUN and albuminuria in C5aRa-treated animals observed over the 6-wk study are more sensitive for reflecting disease progression than a single measurement of renal pathology. In context with these other data, it is possible that the pathological changes were delayed at times prior to the 19-wk time when we terminated the study. Finally, C5a and other components of the complement system surely contribute to the complex pathogenesis of lupus nephritis, but other inflammatory systems may operate independently from complement activation.

The effect of C5aR blockade on generation of autoantibodies, immune complex clearance and the binding of glomerular immune complexes and complement activation products were also evaluated. As expected, anti-ds-DNA Ab and circulating immune complex levels and renal deposition of IgG, IgA, IgM and C3 were equivalent in C5aRa-treated and control groups, suggesting that the reduced renal disease and prolonged survival in MRL/*lpr* mice due to C5aR blockade was independent of immune complex generation, metabolism and deposition in glomeruli and the ensuing complement activation.

Given the beneficial effects of blocking C5aR signaling in MRL/*lpr* mice, further investigations were conducted to provide insights into this phenomenon. First, renal infiltration with two types of inflammatory cells, neutrophils and macrophages, was found to be significantly reduced by C5aRa treatment. Relevant inflammatory mediators were also evaluated. Among the 11 mediators examined, IL-1β and MIP-2 were reduced in C5aRa-treated animals compared with controls. The importance of IL-1β to lupus nephritis is indicated by its up-regulation in the kidneys of lupus mice [39–41]. IL-1β induces endothelial expression of ICAM-1 and VCAM in diseased MRL/*lpr* mice [42], while administration of small amounts of IL-1β accelerated renal disease and mortality in diseased NZB/W mice [41]. MIP-2 was found to be up-regulated in MRL/*lpr* mice compared to MRL/+ mice in our unpublished

microarray studies, which is circumstantial evidence for its relevance in lupus. In current studies, MIP-2 mRNA was reduced by C5aR blockade. Similar reduction of MIP-2 by C5aR blockade was found in a murine ischemia-reperfusion model [43], suggesting that inhibition of C5a can reduce inflammation through down-regulation of MIP-2.

The role of apoptosis in SLE is being increasingly appreciated. Past studies have observed increased numbers of apoptotic cells in SLE, which can be explained both by an increase in the rate of apoptosis [44] as well as decreased clearance of apoptotic cells [45]. Furthermore, the quantity of apoptosis correlates with SLE disease activity [46, 47]. Although the Fas-Fas ligand pathway for apoptosis is not operative in MRL/*lpr* mice, other members of the TNF receptor superfamily are functional, particularly TNFR1 [48]. The complement system can play a direct role in the induction of apoptosis as well as clearance of apoptotic cells. For example, C1q-deficient mice develop higher titers of autoantibodies and GN with greater number of glomerular apoptotic bodies due to impaired clearance [49]. In a mouse sepsis model, C5aR antagonism almost completely inhibited the activation of caspases-3, -6 and -9, as well as reduced C5a-dependent apoptosis of thymocytes [50]. In our study in MRL/*lpr* mice, use of the same C5aRa as in the sepsis model led to significantly reduced renal apoptosis, suggesting that reduction of C5a-dependent apoptosis may contribute to the beneficial effect of C5aRa seen in this model. Furthermore, we found that apoptosis is strongly correlated with renal disease severity in MRL/*lpr* mice, supporting the relevance of apoptosis to lupus nephritis. The striking correlation with monocyte/macrophage numbers ($r=0.951$) is particularly interesting, and is in keeping with the observations of Lenda *et al*. [51, 52], in which macrophage-directed apoptosis appears to be relevant in models of progressive tubulointerstitial damage, such as obstructive nephropathy, and in these same MRL/*lpr* mice.

In conclusion, in this study we have observed reduced renal disease and prolonged survival in MRL/*lpr* mice by blocking C5aR activation with a cyclic hexapeptide antagonist. Behind these beneficial clinical effects, renal infiltration with neutrophils and macrophages was inhibited, which may be related to down-regulation of two pro-inflammatory mediators, IL-1β and MIP-2. In addition, renal apoptosis was also found to be significantly decreased, indicating the potential role of C5a-dependent apoptosis in the development of lupus nephritis. The evident role of C5a signaling through its C5aR in the pathogenesis of experimental lupus nephritis supports the use of comparable C5aR antagonism as therapy for human SLE and lupus nephritis.

Materials and methods

Animal study and experimental design

To evaluate C5aR expression at different stages of lupus nephritis, MRL/*lpr* and MRL/+ mice (Jackson Laboratories, Bar Harbor, ME) were killed at 6 and 24 wks of age for tissue harvest (five per group). To observe the effects of antagonizing C5aR in lupus nephritis, we treated MRL/*lpr* mice with a cyclic hexapeptide antagonist of C5aR, acetyl-Phe-(Orn-Pro-D-cyclo-hexylalanine-Trp-Arg) [21]. Because of the relatively long-term nature of this disease, s.c. Alzet mini-osmotic pumps (Durect, Cupertino, CA) were used to deliver C5aRa or vehicle continuously, thereby achieving steady-state concentrations and avoiding the need for frequent injections. The Alzet model 2001 was chosen for weekly delivery based on the solubility and stability of C5aRa from pilot data (not shown). Twenty-eight male littermate MRL/*lpr* mice were randomly and equally divided into C5aRa and control groups. Starting at 13 wks of age, mice received 1 mg/kg/day of C5aRa dissolved in 50% DMSO in saline; controls were treated identically, except that C5aRa was not included in the pumps. The dose of 1 mg/kg/day was chosen based on its utility in other mouse models [15]. Serum and urine samples were collected biweekly. At 19 wks of age, a time at which most, if not all, unmanipulated MRL/*lpr* mice have severe renal disease, all surviving animals were killed for tissue harvest.

Laboratory data

BUN and urinary albumin excretion were measured biweekly during the 6 wks of treatment. BUN and urinary creatinine concentrations were measured with a Beckman Autoanalyzer (Beckman Coulter, Fullerton, CA). Urinary albumin concentrations were measured with a mouse albumin ELISA kit (Bethyl Laboratories, Montgomery, TX) as described previously [53]. Urinary albumin is presented as the ratio of urinary albumin to creatinine concentrations (mg/mg). As in humans, urinary albumin excretion is approximately 0.025 mg/mg creatinine in normal mice [54].

Serum anti-dsDNA Ab were measured with an ELISA as previously described [9]. First, methylated BSA (Sigma-Aldrich) was used to coat 96-well plates, followed by calf thymus dsDNA (Sigma-Aldrich). Serial dilutions of sera were incubated for 2 h at room temperature, followed by horse-radish peroxidase (HRP)-conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and OPD peroxidase substrate (Sigma-Aldrich). The A_{450} was then measured. Sera from several 24-wk-old MRL/*lpr* mice were pooled and served as control. The anti-dsDNA antibodies are presented as relative units by plotting against the standard curve. Sera from 24-wk-old MRL/+ and BALB/c mice were used as negative controls.

ELISA was also used to measure circulating immune complex levels as described previously [8]. In brief, 96-well plates were coated with human C1q (Quidel, San Diego, CA). Serially diluted serum samples were incubated, followed by HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Sera from several 24-wk-old MRL/*lpr* mice were pooled and served as positive controls.

Table 3. Primers used in quantitative RT-PCR

Genes[a]	Primer 1 (Forward)	Primer 2 (Reverse)
C5aR	5′-GATGCCACCGCCTGTATAGT-3′	5′-ACGAAGGATGGAATGGTGAG-3′
IL-1β	5′-GCCCATCCTCTGTGACTCAT-3′	5′-AGGCCACAGGTATTTTGTCG-3′
RANTES	5′-GTGCCCACGTCAAGGAGTAT-3′	5′-GGGAAGCGTATACAGGGTCA-3′
MIP-2	5′-CACCAACCACCAGGCTAC-3′	5′-GCCCTTGAGAGTGGCTATGA-3′
IL-2	5′-CCCACTTCAAGCTCCACTTC-3′	5′-ATCCTGGGGAGTTTCAGGTT-3′
IL-18	5′-ACGTGTTCCAGGACACAACA-3′	5′-ACAAACCCTCCCCACCTAAC-3′
ICAM-1	5′-CGCAAGTCCAATTCACACTGA-3′	5′-CAGAGCGGCAGAGCAAAAG-3′
TNF-α	5′-CCGATGGGTTGTACCTTGTC-3′	5′-GTGGGTGAGGAGCACGTAGT-3′
TGF-β1	5′-TTGCTTCAGCTCCACAGAGA-3′	5′-TGGTTGTAGAGGGCAAGGAC-3′
MCP-1R	5′-AGAGAGCTGCAGCAAAAAGG-3′	5′-GGAAAGAGGCAGTTGCAAAG-3′
MCP-2	5′-GGAAGCTGTGGTTTTCCAGA-3′	5′-CTTCCAGCTTTGGCTGTCTC-3′
IFN-γ	5′-ACTGGCAAAAGGATGGTGAC-3′	5′-TGAGCTCATTGAATGCTTGG-3′
18S	5′-ATGGCCGTTCTTAGTTGGTG-3′	5′-CGCTGAGCCAGTCAGTGTAG-3′

[a] RANTES, regulated upon activation, normal T cell expressed and secreted; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein.

Circulating immune complex levels were quantified by plotting against the standard curve and presented as relative units.

Tissue preparation and histological staining

For IF microscopy, kidney sections were snap frozen in 2-methylbutane cooled on dry ice and kept at –80°C until use. For light microscopy and *in situ* apoptotic cell detection, kidney sections were fixed in 10% buffered formalin and embedded in paraffin and stored at room temperature.

For light microscopy, 4-μm sections of formalin-fixed paraffin-embedded tissue were stained with periodic acid-Schiff. The extent of GN and glomerulosclerosis was graded in a semiquantitative (0–4) manner, and the percentage of glomeruli with sclerosis/hyalinosis and crescents was assessed as previously described [8, 9, 55]. All slides were scored by a renal pathologist (M. H.) masked to group.

For IF microscopy, 4-μm cryostat sections were fixed in ether-ethanol and stained with FITC-conjugated antibodies to mouse C3, IgG, IgA (Cappel, Aurora, OH) and IgM (Sigma-Aldrich). The staining intensity and distribution was semi-quantitatively scored from 0 to 4 in a blinded manner as previously described [53].

C5aR, neutrophils and monocyte/macrophages were also detected by IF staining. After blocking with 10% normal goat serum, slides were incubated with a rabbit antibody specific to the N terminus of mouse C5aR [56], followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG (highly cross-adsorbed and lacking reactivity with mouse IgG, Molecular Probes, Inc, Eugene, OR); or incubated with rat anti-mouse neutrophil antibody (Serotec, Oxford, UK) followed by FITC-conjugated goat anti-rat IgG (absorbed with mouse Ig, Serotec) in 10% normal mouse serum for neutrophils; or stained directly with FITC-conjugated rat F4/80 monoclonal antibody (Serotec) for macrophages. To quantify cells in each mouse kidney section, neutrophils and monocyte/macrophages were counted in at least 20 low-power fields (200×) in a masked fashion as described by others [43].

For *in situ* apoptotic cells detection, to detect apoptotic cells in tissue sections, the TACSAXL-Blue™ label *in situ* apoptosis detection kit (Trevigen, Inc. Gaithersburg, MD) was used according to the manufacturer's instruction. Briefly, paraffin-embedded sections were deparaffinated and rehydrated. The free 3′-OH ends of nuclear DNA fragments were labeled with BrdU using terminal deoxynucleotidyl transferase. Sections were then incubated with biotinylated anti-BrdU antibody and streptavidin-HRP. A positive control treated with nuclease and unlabeled negative experimental controls were also included. Apoptotic cells, which stained intensively blue, were counted by a masked observer in at least 20 low-power fields per animal.

qRT-PCR

Total RNA from renal cortex was extracted and cDNA produced as described previously [38]. qRT-PCR was performed using QuantiTect SYBR Green RT-PCR Kit (Qiagen Inc., Valencia, CA) on an ABI 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Expression data were normalized to 18S RNA. The primers used are provided in Table 3.

Statistical analysis

Data are expressed as mean ± SEM and were analyzed using Minitab (version 12, State College, PA) and Stata (version 8, Stata Corp., College Station, TX) software. The Chi square test was used when comparing survival rates between the two groups over time. Repeated-measures ANOVA was used when comparing measures in the two groups over time. For the comparison between two groups at one time point, *t*-testing

was used for parametric data and Mann-Whitney testing was used for non-parametric data. Potential correlations among variables were determined by calculating Pearson product moment correlation coefficients and their *p* values.

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