Published in final edited form as:

J Neuroimmunol. 2008 May 30; 196(0): 101–106. doi:10.1016/j.jneuroim.2008.03.007.

C5a is not involved in experimental autoimmune myasthenia gravis pathogenesis

Huibin Qi^a, Erdem Tüzün^a, Windy Allman^a, Shamsheer S. Saini^a, Zurina R. Penabad^b, Silvia Pierangeli^b, and Premkumar Christadoss^{a,*}

^aDepartment of Microbiology and Immunology, USA

^b Department of Medicine, University of Texas Medical Branch, Galveston, TX, 77555-1070, USA

Abstract

C5 deficient mice are highly resistant to experimental autoimmune myasthenia gravis (EAMG) despite intact immune response to acetylcholine receptor (AChR), validating the pivotal role played by membrane attack complex (MAC, C5b-9) in neuromuscular junction destruction. To distinguish the significance of C5a from that of C5b in EAMG pathogenesis, C5a receptor (C5aR) knockout (KO) and wild-type (WT) mice were immunized with AChR to induce pathogenic anti-AChR antibodies. In contrast with C5 deficient mice, C5aR KO mice were equally susceptible to EAMG as WT mice and exhibited comparable antibody and lymphocyte proliferation response to AChR implicating that C5a is not involved in EAMG development.

Keywords

Myasthenia gravis; C5a; C5a receptor; Autoimmunity

1. Introduction

Myasthenia gravis (MG) and experimental autoimmune myasthenia gravis (EAMG) are both classical antibody-mediated diseases, characterized with muscle weakness and triggered by antibodies to acetylcholine receptor (AChR) located at the neuromuscular junction (NMJ) (Christadoss and Dauphinee, 1986; Vincent and Drachman, 2002). Recent years have brought growing awareness of the involvement of the complement system in anti-AChR mediated NMJ destruction in MG and EAMG (Tuzun et al., 2003; Tsujihata et al., 1989). In EAMG, activation of the complement cascade occurs by the classical pathway leading to C3 activation and generation of C3b. Subsequent cleavage of C5 leads to release of C5a and generation of C5b. C5b initiates formation of C5b-9 complex or membrane attack complex (MAC), which is involved in lysis of the postsynaptic NMJ membrane (Tuzun et al., 2003; Tsujihata et al., 1989).

Evidence for the participation of MAC (and therefore C5b) in EAMG pathogenesis comes from multiple sources. MAC has been observed in the postmortem frozen muscle specimens of EAMG mice (Tuzun et al., 2003) and the number of MAC deposits has been reduced in EAMG resistant mice (Tuzun et al., 2003; Tuzun et al., 2006). Further, administration of an antibody against the C6 component of MAC before the passive transfer of anti-AChR antibody has prevented EAMG induction in rats and has inhibited accumulation of MAC

components C6 and C9 (Biesecker and Gomez, 1989). Also, C5 deficient mice immunized with AChR have been shown to be highly resistant to EAMG despite preserved anti-AChR antibody production ability (Christadoss, 1988).

So far, there has been no investigation on the possible participation of C5a in EAMG pathogenesis. It is not clear, for instance, whether EAMG resistance of C5 deficient mice (which are deficient for both C5a and C5b components) is solely due to impaired MAC production caused by the absence of C5b or also to the additional deficiency of C5a associated immune functions. C5a is a major anaphylactic and chemotactic agent and promotes production of cytokines (e.g. IL-1 β , IL-6, IL-12) that are actively involved in EAMG pathogenesis (Morgan et al., 1992; O'Barr and Cooper, 2000). Therefore, inborn deficiency of C5a signaling through C5a receptor (C5aR) might be expected to influence EAMG susceptibility. Hence, to address that question, in this study we examined whether C5a receptor (C5aR) deficient mice were susceptible to MG utilizing an experimental model, EAMG.

2. Materials and methods

2.1. AChR and mice

AChR was purified from the electric organ of *Torpedo californica* by α -neurotoxin affinity column (Wu et al., 1997). Seven- to eight-wk-old C5aR KO mice in the B6 background were kindly provided by Dr. Girardi (Hospital for Special Surgery, NY, NY) and were generated by targeted deletion of the murine C5aR gene. C5aR KO mice were produced by backcrossing to B6 mice for six generations. The mice were determined to be completely C5aR deficient by PCR, Northern Blot and immunohistochemistry analysis. C5aR deficient mice were backcrossed with B6 mice as described in detail (Wenderfer et al., 2005; Girardi et al., 2003). Control wild-type (WT) B6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine, USA). All animals were housed in the viral antibody-free barrier facility at the University of Texas Medical Branch and maintained according to the Institutional Animal Care and Use Committee Guidelines.

2.2. Induction and clinical evaluation of EAMG

All mice were anesthetized and immunized with 20 μ g AChR emulsified in CFA (Difco, Detroit, MI) s.c. at four sites (two hind footpads and shoulders) on day 0 and were boosted with 20 μ g AChR in CFA s.c. at four sites on the back on days 30 and 60. For clinical examination, mice were left for 3 min on a flat platform and were observed for signs of EAMG. Clinical muscle weakness was graded as follows: grade 0, mouse with normal posture, muscle strength, and mobility; grade 1, normal at rest, with muscle weakness characteristically shown by a hunchback posture, restricted mobility, and difficulty to raise the head after exercise, consisting of 30 paw grips on cage top grid; grade 2, mouse showed grade 1 symptoms without exercise during observation period on flat platform; grade 3, dehydrated and moribund with grade 2 weakness; and grade 4, dead. For objective measurement of muscle strength, mice were first exercised with 40 paw grips on cage top grid. Following exercise, mice were made to grasp a grid attached to a dynamometer (Chatillon Digital Force Gauge, DFIS 2, Columbus Instruments, Columbus, OH). The maximal force applied to the dynamometer while pulling the mouse by its tail until it lost its grip on the grid was recorded. Clinical EAMG was also confirmed by i.p. administration of 50 μ l neostigmine bromide, along with atropine sulfate in PBS, and observing improvement in muscle strength.

2.3. RIA to measure muscle AChR content

The total concentration of AChR per mouse carcass was determined according to a previously published method (Wu et al., 1997). Aliquots (0.1 ml) of [125 I] α -bungarotoxin (BTX)-labeled (5×10^{-9} M), Triton X-100 solubilized mouse muscle extracts, with and without benzoquinonium (10^{-3} M) and were mixed with 10 μ l of mouse anti-AChR serum. The resulting complex was precipitated by goat anti-mouse serum and then centrifuged. Radioactivity of the pellet was counted in a Packard gamma counter (Packard Instrument Co., Meriden, CT), and cpm values of samples with benzoquinonium were subtracted from cpm values of samples without benzoquinonium. The results were expressed as picomolar (pM) of [125 I]-labeled BTX-binding sites per gram of mouse carcass.

2.4. ELISA for anti-muscle AChR antibody and isotypes

IgG, IgG1 and IgG2b isotypes to mouse muscle AChR were evaluated by ELISA, using a previously described method (Tuzun et al., 2003). These isotypes were examined due to their established significance in EAMG pathogenesis (Yang et al., 2005). Affinity-purified mouse AChR (0.5 μ g/ml) was coated onto a 96-well microtiter plate in 0.1 M carbonate bicarbonate buffer overnight at 4 °C. Diluted serum samples of 100 μ l (1:500) were added and incubated at 37 °C for 90 min. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG, IgG1 or IgG2b (Caltag Laboratories, Burlingame, CA) (1:1000) were added and then incubated at 37 °C for 90 min. Subsequently, the peroxidase indicator substrate 2,2'-azino-bis-(3-ethylbenzothiazoline 6-sulfonate) substrate (ABTS) solution in 0.1 M citric buffer (pH 4.35) was added in the presence of H_2O_2 , and mixture was allowed to develop color at room temperature in the dark. Plates were read at a wavelength of 405 nm. Normal mouse serum (collected from mice before immunization) was used for the background determination.

2.5. Detection of IgG, C3 and MAC deposits at the NMJ

Sections (10 μ m thick) were obtained from forelimb muscle samples of mice, frozen in liquid nitrogen, and stored at -80 °C. Slides were fixed in cold acetone. After washing with PBS, the sections were incubated with tetramethylrhodamine-conjugated BTX (Molecular Probes, Eugene, OR) (1/500 dilution) for 1 h at room temperature to label the NMJ. Sections were then incubated for 1 h at room temperature with goat anti-mouse IgG (Chemicon International, Temecula, CA), goat anti-mouse complement C3 (ICN-Cappel, Aurora, OH) or rabbit anti-human C5b-9 (MAC) (Calbiochem, San Diego, CA) (diluted 1/1000) to colocalize IgG or complement deposits in NMJ. Anti-IgG and anti-C3 antibodies were FITC-conjugated. For detection of MAC deposits, the muscle tissues were further incubated with Oregon green-conjugated goat anti-rabbit IgG (Molecular Probes). The sections were washed and viewed in a fluorescence microscope (Olympus IX-70).

2.6. Lymphocyte proliferation assay

Inguinal, popliteal, and axillary lymph node cells (LNC) were collected at termination (day 90) of the experiment. Cells (2×10^5 cells/well) were seeded in triplicate into 96-well, round-bottomed microtiter plates in 0.2 ml of RPMI 1640 medium with AChR (2.5 μ g/ml) supplemented with 10% fetal calf serum, penicillin G (100 U/ml) streptomycin (100 μ g/ml), L-glutamine (2 mM), 2-mercaptoethanol (3×10^{-5} M), and HEPES buffer (25 mM). The cells were cultured for 5 days at 37 °C in humidified 5% CO_2 -enriched air, and pulse-labeled with [3 H] TdR (1 μ Ci/well) for 16 h before harvesting. The 3 H incorporation was determined in a Beckman beta scintillation counter (Beckman Coulter Inc., Fullerton, CA). The results are expressed as cpm.

2.7. Immunohistochemical staining for splenic germinal centers

Four-micron-thick sections of 10% formalin-fixed and paraffin-embedded spleens were prepared. Sections were deparaffinized and rehydrated. Following incubation with 3% H₂O₂ in methanol and normal goat serum, antigen retrieval was done with Target Retrieval Solution (Dako, Carpinteria, CA). Sections were then incubated for 30 min with biotinylated-peanut agglutinin (PNA) (Vector Laboratories, Burlingame, CA) diluted 1/250 in DAKO antibody diluents, followed by a second incubation with streptavidin-HRP. Bound conjugates were visualized with DAKO Liquid diaminobenzidine substrate-chromogen for 5 min when a brown color for PNA-positive cells was obtained. Slides were counterstained for 2 min with Mayer's modified hematoxylin diluted 1/5 in distilled water.

2.8. Statistical analysis

To determine the significance of the observed results, three statistical tests were used. Clinical EAMG incidences were compared using the Fisher's exact test, clinical scores were compared using Mann–Whitney *U*-test, and all other parameters were compared using Student's *t*-test.

3. Results

3.1. C5aR KO mice are susceptible to EAMG

C5aR KO (*n*=10) and WT (*n*=10) mice were immunized with AChR in CFA on days 0, 30 and 60. At termination (day 90), 6 of 10 (60%) C5aR KO and 7 of 10 (70%) WT mice had developed EAMG. EAMG incidences of C5aR KO and WT mice were not statistically different ($p>0.05$ by Fisher's exact test). Average clinical scores of C5aR KO (1.4) and WT (1.3) mice did not show statistical difference, either ($p>0.05$ by Mann–Whitney *U*-test). Severe grade 3 muscle weakness was observed in 2 mice in C5aR KO group and 1 mouse in WT group. One mouse each from C5aR KO and WT groups developed severe grade 4 EAMG and died before termination. Average grip strengths of C5aR KO and WT mice declined gradually following 3rd AChR immunization and in line with the clinical data they did not differ significantly throughout the experiment period (Fig. 1A). Also, at termination, muscle AChR levels of C5aR KO and WT mice were comparable (Fig. 1B). These results show that C5aR gene deficiency does not confer mice EAMG resistance and C5a is not involved in EAMG pathogenesis.

3.2. C5aR KO mice have preserved humoral and cellular immune responses to AChR

To evaluate the immune response to AChR in C5aR KO mice, antibody production and lymphocyte proliferation were assessed in AChR-immunized C5aR KO and WT mice (Fig. 2A and C). Fifteen days after 3rd AChR immunization (peak period for anti-AChR antibody), serum anti-AChR IgG, IgG1 and IgG2b levels of C5aR KO mice were comparable to those of WT mice (Fig. 2A). Additionally, seven forelimb muscle sections from each mouse were assessed for antibody and complement deposits. C5aR KO mice displayed equal amounts of NMJ C3, IgG and MAC deposits as WT mice (Fig. 2B and D), suggesting that the immune attack to the NMJ is not impaired in C5aR KO mice. At termination, LNCs from C5aR KO and WT mice were stimulated *in vitro* with or without AChR and lymphocyte proliferation capacity was measured. C5aR KO and WT mice showed comparable proliferative responses to AChR (Fig. 2C). Overall, these results show that impairment of immune functions carried on by C5a-C5aR interaction does not affect the humoral and cellular immune response to AChR, NMJ destruction and consequent EAMG induction in C5aR KO mice.

3.3. C5aR KO mice have higher germinal center counts than WT mice

To further analyze the cellular immune response in C5aR KO mice, lymphocyte maturation in secondary lymphoid tissue was evaluated. For this purpose, spleens from C5aR KO and WT mice were collected at termination and mature lymphocytes in germinal centers were detected by immunohistochemistry using PNA as a marker. The numbers of PNA+ splenic germinal center follicles of C5aR KO mice were significantly greater than those of WT mice (Fig. 3), suggesting that C5a is involved in the recruitment of immune cells to the germinal centers.

4. Discussion

Complement factor C5 is made up of C5a and C5b components. While C5b is primarily involved in MAC formation, C5a has a plethora of immune functions including promotion of leukocyte chemotaxis, enhancement of neutrophil-endothelial cell adhesion and vascular permeability, induction of granule secretion by phagocytes and production of a variety of cytokines all implicated to play pivotal roles in EAMG induction (Szebeni, 2004). Most of these functions are mediated through C5aR, a G-protein-coupled receptor (Szebeni, 2004). Furthermore, C5aR acts synergistically with Fc γ RIII, another EAMG associated factor (Tuzun et al., 2006), to enhance immune complex mediated leukocyte activation and cytokine production (Atkinson, 2006).

While muscle tissues of EAMG mice and MG patients harbor minimal or no inflammatory cells, C5a, as a strong leukocyte recruiter, is involved in immunological diseases characterized by infiltrating inflammatory cells at the target tissue such as rheumatoid arthritis, asthma, ischemia/reperfusion injury and sepsis (Szebeni, 2004). Nevertheless, despite the fact that there is no appreciable NK cell infiltration in the muscle samples of myasthenic mice, NK cell deficient mice are resistant to EAMG presumably due to the lack of type 1 helper T cell response promoted by NK cell dependent cytokines (Shi et al., 2000), suggesting that C5a could also be engaged in EAMG induction by sustaining EAMG related cytokines. Also, mice lacking C5aR have been shown to be partially resistant to autoimmune hemolytic anemia, which is a classical antibody-mediated disease like EAMG (Kumar et al., 2006). In another autoantibody-mediated disease, the antiphospholipid syndrome, Girardi and Romay-Penabad and their respective colleagues demonstrated that C5aR KO mice were resistant to antiphospholipid antibody-mediated pregnancy loss and thrombosis (Girardi et al., 2003; Romay-Penabad et al., 2007).

In a previous experiment, C5 deficient mice were shown to be highly resistant to EAMG with preserved AChR-specific antibody response (Christadoss, 1988). To further dissect the role of C5a in EAMG induction, we immunized C5aR KO mice with AChR and showed that these mice had robust cellular and humoral immune responses to AChR and thus were susceptible to EAMG. Our results suggest that C5 deficiency prevents EAMG induction not through an impairment in the major immunological functions associated with the lack of C5a but rather by deficient MAC production due to absence of C5b. Therefore, pharmacological blockade of C5a signaling through C5aR would not be expected to reduce muscle weakness in MG patients. Our results also suggest that leukocyte chemotaxis and endothelial adhesion do not play significant roles in anti-AChR immune response, NMJ deposit accumulation and subsequent EAMG induction.

Notably, the only immunological parameter that significantly differed between C5aR KO and WT groups was the number of splenic germinal centers. Increased germinal center numbers of C5aR KO mice is compliant with the previously established fact that C5a is a potent chemoattractant for germinal center cells and is involved in the recruitment of activated immune cells from germinal centers to inflammatory sites (Ottonello et al., 1999).

Probably, in the absence of C5a influence, immune cells tend to reside in lymphoid tissues and thus overpopulate the germinal centers. Since cellular infiltration of the muscle tissue is not a part of EAMG pathogenesis, this deficit apparently does not affect EAMG susceptibility.

EAMG mice do not display thymic abnormalities and therefore mouse model of MG does not accurately mimic MG patients with thymic hyperplasia or tumor. C5aR is expressed in early myasthenic thymic tissue (Leite et al., 2007), therefore, assessment of C5a associated functions in MG patients would shed further light to the involvement of C5a in MG pathogenesis.

Acknowledgments

This study is supported by the Muscular Dystrophy Association and AFM (PC), and partially supported by a Research Centers in Minority Institutions_National Institutes of Health grant (G-12-RR03034), a Minority Biomedical Research Support Grant from the National Institutes of Health (GM58268002) and a NIAMS NIH Multidisciplinary Research Center Grant (2P60 AR047785-06) (SSP and ZRP).

Erdem Tüzün was a Myasthenia Gravis foundation Osserman/Sosin/McClure Postdoctoral Fellowship recipient and was an MDA Neuromuscular Disease Research Career Award recipient. Windy Allman is a recipient of a Henry Viets Fellowship from the MG foundation of America and a predoctoral fellowship from AFM.

References

- Atkinson JP. C5a and Fcγ receptors: a mutual admiration society. *J. Clin. Invest.* 2006; 116:304–306. [PubMed: 16453017]
- Biesecker G, Gomez CM. Inhibition of acute passive transfer experimental autoimmune myasthenia gravis with Fab antibody to complement C6. *J. Immunol.* 1989; 142:2654–2659. [PubMed: 2703710]
- Christadoss P. C5 gene influences the development of murine myasthenia gravis. *J. Immunol.* 1988; 140:2589–2592. [PubMed: 3356901]
- Christadoss P, Dauphinee MJ. Immunotherapy for myasthenia gravis: a murine model. *J. Immunol.* 1986; 136:2437–2440. [PubMed: 2419435]
- Girardi G, Berman J, Redecha P, Spruce L, Thurman JM, Kraus D, Hollmann TJ, Casali P, Carroll MC, Wetsel RA, Lambris JD, Holers VM, Salmon JE. Complement C5a receptors and neutrophils mediate fetal injury in the antiphospholipid syndrome. *J. Clin. Invest.* 2003; 112:1644–1654. [PubMed: 14660741]
- Kumar V, Ali SR, Konrad S, Zwirner J, Verbeek JS, Schmidt RE, Gessner JE. Cell-derived anaphylatoxins as key mediators of antibody-dependent type II autoimmunity in mice. *J. Clin. Invest.* 2006; 116:512–520. [PubMed: 16453025]
- Leite MI, Jones M, Strobel P, Marx A, Gold R, Niks E, Verschuuren JJ, Berrih-Aknin S, Scaravilli F, Canelhas A, Morgan BP, Vincent A, Willcox N. Myasthenia gravis thymus: complement vulnerability of epithelial and myoid cells, complement attack on them, and correlations with autoantibody status. *Am. J. Pathol.* 2007; 171:893–905. [PubMed: 17675582]
- Morgan EL, Sanderson S, Scholz W, Noonan DJ, Weigle WO, Hugli TE. Identification and characterization of the effector region within human C5a responsible for stimulation of IL-6 synthesis. *J. Immunol.* 1992; 148:3937–3942. [PubMed: 1602137]
- O'Barr S, Cooper NR. The C5a complement activation peptide increases IL-1β and IL-6 release from amyloid-beta primed human monocytes: implications for Alzheimer's disease. *J. Neuroimmunol.* 2000; 109:87–94. [PubMed: 10996210]
- Ottone L, Corcione A, Tortolina G, Airolidi I, Albesiano E, Favre A, D'Agostino R, Malavasi F, Pistoia V, Dallegri F. rC5a directs the in vitro migration of human memory and naive tonsillar B lymphocytes: implications for B cell trafficking in secondary lymphoid tissues. *J. Immunol.* 1999; 162:6510–6517. [PubMed: 10352266]

- Romay-Penabad Z, Liu XX, Montiel-Manzano G, Papalardo De Martínez E, Pierangeli SS. C5a receptor-deficient mice are protected from thrombophilia and endothelial cell activation induced by some antiphospholipid antibodies. *Ann. N. Y. Acad. Sci.* 2007; 1108:554–566. [PubMed: 17894020]
- Shi FD, Wang HB, Li H, Hong S, Taniguchi M, Link H, Van Kaer L, Ljunggren HG. Natural killer cells determine the outcome of B cell-mediated autoimmunity. *Nat. Immunol.* 2000; 1:245–251. [PubMed: 10973283]
- Szebeni, J. *The Complement System*. Springer-Verlag; New York: 2004. p. 307-344.
- Tsujihata M, Yoshimura T, Satoh A, Kinoshita I, Matsuo H, Mori M, Nagataki S. Diagnostic significance of IgG, C3, and C9 at the limb muscle motor end-plate in minimal myasthenia gravis. *Neurology.* 1989; 39:1359–1363. [PubMed: 2797459]
- Tuzun E, Saini SS, Yang H, Alagappan D, Higgs S, Christadoss P. Genetic evidence for the involvement of Fcγ receptor III in experimental autoimmune myasthenia gravis pathogenesis. *J. Neuroimmunol.* 2006; 174:157–167. [PubMed: 16527362]
- Tuzun E, Scott BG, Goluszko E, Higgs S, Christadoss P. Genetic evidence for involvement of classical complement pathway in induction of experimental autoimmune myasthenia gravis. *J. Immunol.* 2003; 171:3847–3854. [PubMed: 14500686]
- Vincent A, Drachman DB. Myasthenia gravis. *Adv. Neurol.* 2002; 88:159–188. [PubMed: 11908224]
- Wenderfer SE, Ke B, Hollmann TJ, Wetsel RA, Lan HY, Braun MC. C5a receptor deficiency attenuates T cell function and renal disease in MRL^{lpr} mice. *J. Am. Soc. Nephrol.* 2005; 16:3572–3582. [PubMed: 16207826]
- Wu, B.; Goluszko, E.; Christadoss, P. *Experimental Autoimmune Myasthenia Gravis in the Mouse..* In: Coligan, MJ.; Kruisbeek, AM.; Margulies, DH.; Shevach, EM.; Strober, W., editors. *Current Protocols of Immunology*. Vol. 3:8.1. John Wiley & Sons; New York, USA: 1997. p. 1-16.
- Yang H, Tuzun E, Alagappan D, Yu X, Scott BG, Ischenko A, Christadoss P. IL-1 receptor antagonist-mediated therapeutic effect in murine myasthenia gravis is associated with suppressed serum proinflammatory cytokines, C3, and anti-acetylcholine receptor IgG1. *J. Immunol.* 2005; 175:2018–2025. [PubMed: 16034147]

Fig. 1. Grip strength kinetics (A) and muscle AChR content (B) of AChR-immunized WT and C5aR KO mice following 3rd AChR immunization. C5aR KO mice were equally susceptible to EAMG as WT mice ($p > 0.05$ by Student's t -test). Bars indicate standard errors.

Fig. 2.

Serum anti-AChR IgG, IgG1 and IgG2b levels (A), NMJ C3, IgG and MAC deposit counts (B) and AChR-specific lymphocyte proliferative responses (C) of AChR-immunized C5aR KO and WT mice (none indicates lymph node cells cultured in medium with no AChR stimulation, as a negative control). Cellular and humoral responses to AChR were comparable between C5aR KO and WT mice ($p > 0.05$ for all comparisons by Student's t -test). Bars indicate standard errors. IgG, C3 and MAC deposits at the NMJs of AChR-immunized WT and C5aR KO mice (D). Frozen muscle sections were stained for IgG, C3 and MAC (bottom panels, all green fluorescence) and the NMJs were co-localized by BTX (top panels, red fluorescence) (magnification for all, $\times 200$). The immunofluorescence data represent one of 5 sections for each mouse. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. Splenic germinal center counts were elevated in C5aR KO mice (A–C). Spleens from WT (A) and C5aR KO mice (B) were collected at termination and stained with biotinylated-PNA (brown). One representation of 30 samples (3 per mouse) each of C5aR KO and WT mice. Original magnification, $\times 40$. *** indicates $p < 0.001$ by Student's *t*-test, bars indicate standard errors.