Generation of C5a in the absence of C3: a new complement activation pathway

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Complement-mediated tissue injury in humans occurs upon deposition of immune complexes, such as in autoimmune diseases and acute respiratory distress syndrome. Acute lung inflammatory injury in wild-type and C3^{-/-} mice after deposition of IgG immune complexes was of equivalent intensity and was C5a dependent, but injury was greatly attenuated in $Hc^{-/-}$ mice (Hc encodes C5). Injury in lungs of $C3^{-/-}$ mice and C5a levels in bronchoalveolar lavage (BAL) fluids from these mice were greatly reduced in the presence of antithrombin III (ATIII) or hirudin but were not reduced in similarly treated $C3^{+/+}$ mice. Plasma from C3^{-/-} mice contained threefold higher levels of thrombin activity compared to plasma from $C3^{+/+}$ mice. There were higher levels of F2 mRNA (encoding prothrombin) as well as prothrombin and thrombin protein in liver of $C3^{-/-}$ mice compared to C3^{+/+} mice. A potent solid-phase C5 convertase was generated using plasma from either $C3^{+/+}$ or $C3^{-/-}$ mice. Human C5 incubated with thrombin generated C5a that was biologically active. These data suggest that, in the genetic absence of C3, thrombin substitutes for the C3-dependent C5 convertase. This linkage between the complement and coagulation pathways may represent a new pathway of complement activation.

Deposition of IgG immune complex in tissues, together with deposition of the complement protein C3, is a hallmark of autoimmune diseases in humans and is also a feature of glomerulonephritic disorders and vasculitis occurring after infectious diseases¹. Extensive studies of IgG immune complex–induced tissue injury in rodents have identified the roles of $Fc\gamma Rs^{2-4}$ and the complement anaphylatoxins (C3a and C5a) as well as their receptors (C3aR and C5aR, respectively)^{5–11}. The use of complement-deficient mice has provided insights into the mechanisms of complement-mediated inflammatory responses, but in the case of $C3^{-/-}$ mice inflammatory responses to IgG immune complexes seem to be largely intact¹², although this is not the case in IgM-mediated models of inflammation¹³.

The anaphylatoxin C5a is a proteolytic fragment of C5 generated by the high-affinity C5 convertase containing C3b in addition to C4b and C2a. Some time ago it was suggested that complement-independent enzymes, such as thrombin¹⁴⁻¹⁶, neutrophil elastase¹⁷ and a macrophage serine protease¹⁸, have C5 convertase (C5a-generating) activity. Similarly, in the absence of C3, C4b2a has C5 convertase activity albeit with very low catalytic efficiency¹⁹. Here we describe that $C3^{-/-}$ mice, like $C3^{+/+}$ mice, develop intense lung injury that is C5a dependent after deposition of IgG immune complexes. This unexpected finding was accompanied by in vitro generation of a solid-phase C5 convertase by plasma from $C3^{-/-}$ mice. In BAL fluids of injured lungs, C5a levels were similar in $C3^{+/+}$ and $C3^{-/-}$ mice, suggesting that activation of complement and generation of C5a can occur in the absence of C3. Our data suggest that thrombin can function as a C5 convertase, providing evidence for a direct linkage between the complement and the clotting pathways. The data suggest a new pathway of complement activation in the absence of C3.

We compared lung inflammatory responses resulting from intrapulmonary deposition of IgG immune complexes after treatment with either preimmune IgG or C5a-specific IgG in Hc+/+ and Hc-/mice by measuring the extravascular leakage of ¹²⁵I-BSA (expressed as the permeability index), neutrophil content and levels of MIP-1a in BAL fluids (Fig. 1a–c). In $Hc^{+/+}$ mice receiving preimmune IgG, the permeability index and BAL neutrophil content rose nearly fourfold and BAL MIP-1 α content rose 15-fold (P < 0.05). In the presence of antibody to C5a, however, albumin leakage was significantly reduced to near baseline levels (P < 0.05), and BAL neutrophil and MIP-1a contents were also reduced significantly (P < 0.05). $Hc^{-/-}$ mice showed much smaller increases in the permeability index and in BAL neutrophil content and MIP-1a levels. These reduced parameters were unaffected in the presence of either preimmune IgG or antibody to C5a. Similar findings for the levels of tumor necrosis factor (TNF)-a and the CXC chemokine MIP-2 in BAL fluids from $Hc^{+/+}$ and $Hc^{-/-}$ mice were also obtained (data not shown). These data suggest that C5a has an important role in lung

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Figure 1 Immune complex-induced injury in C5 and C3 wild-type and knockout mice. IgG immune complex-induced lung injury was initiated in $Hc^{+/+}$ and $Hc^{-/-}$ mice (**a**-**c**) and in $C3^{+/+}$ and $C3^{-/-}$ mice (**d**-**f**), and vascular leakage was assessed (a,d). Mice also received intratracheally either preimmune IgG (pre-IgG) or C5a-specific IgG (anti-C5a IgG) at the time of induction of lung injury. In negative controls (ctrl), intravenous administration of BSA was omitted. Neutrophil content (polymorphonuclear cells, PMN) in BAL fluids (b,e) and content of MIP-1 α in BAL fluids (c,f) were determined in samples obtained 4 h after immune complexinduced lung injury. *P < 0.05 compared to negative controls (ctrl), **P < 0.05 compared to the immediately preceding bar. N.S., not significant compared to the immediately preceding bar. For each bar, n > 5 mice.

inflammatory responses in $Hc^{+/+}$ mice, with greatly reduced responses in $Hc^{-/-}$ mice.

Inflammatory responses were also evaluated in $C3^{+/+}$ and $C3^{-/-}$ mice (**Fig. 1d–f**). In $C3^{+/+}$ mice receiving preimmune IgG, the permeability index and BAL MIP-1 α content rose fivefold, whereas BAL neutrophil content rose threefold. In mice receiving antibody to C5a, the permeability index and BAL neutrophil and MIP-1 α contents were significantly reduced (P < 0.05). We saw the same patterns in $C3^{-/-}$ mice, and these measures of inflammation and injury were markedly suppressed in the presence of antibody to C5a.

We evaluated morphological changes in lungs 4 h after intravenous infusion of BSA to induce IgG immune complex formation with or without antibody to C5a in $C3^{+/+}$ and $C3^{-/-}$ mice (**Fig. 2**). Lung sections from control mice that did not receive BSA (**Fig. 2a,d**) were essentially normal in appearance. In the presence of IgG immune complexes together with preimmune IgG, lungs from both $C3^{+/+}$ and $C3^{-/-}$ mice showed extensive intra-alveolar hemorrhage, neutrophil accumulation and fibrin deposits (**Fig. 2b,e**). In the presence of antibody to C5a, all such features were greatly attenuated in both $C3^{+/+}$ (**Fig. 2c**) and $C3^{-/-}$ mice (**Fig. 2f**).

Generation of C3a, detected by ELISA, was routinely used to screen mouse plasma. Barely detectable amounts of C3a were present in nonactivated $C3^{+/+}$ plasma, but this level rose to nearly 9,000 µg/L in zymosan-activated plasma (**Fig. 3a**). No C3a was detected in activated $C3^{-/-}$ plasma. In subsequent experiments, we activated $C3^{+/+}$ and $C3^{-/-}$ plasma with zymosan, immunoprecipitated with antibody to C5a and then subjected the immunoprecipitate to electrophoresis (SDS-PAGE) and western blot analysis. C5a immunoprecipitated from activated $C3^{+/+}$ or $C3^{-/-}$ plasma was positioned near the 15 kDa marker, consistent with glycosylated C5a (**Fig. 3b**). No such bands for C5a were detectable in nonactivated plasma.

We opsonized zymosan particles with $C3^{+/+}$ or $C3^{-/-}$ plasma to produce a solid-phase C5 convertase (**Fig. 3c**). We then washed the particles and incubated them with human C5. Generation of C5a (determined by ELISA) occurred when we used either $C3^{+/+}$ or $C3^{-/-}$ plasma to generate the C5 convertase, the former producing nearly double the amount of C5a compared to the latter. We subjected the reaction products to western blot analysis (**Fig. 3c**); C5 products were positioned between the 10 and 15 kDa markers, consistent with glycosylated C5a. When particulate IgG immune complexes were similarly opsonized with $C3^{+/+}$ and $C3^{-/-}$ plasma, generation of C5a from human C5 occurred: immune complexes opsonized with $C3^{+/+}$ or $C3^{-/-}$ plasma generated C5a levels of 252 + 5.6 and 156 + 15.5 µg/L, respectively (data not shown).

We performed similar experiments in the absence or presence of ATIII (**Fig. 3d**). On zymosan particles, $C3^{+/+}$ and $C3^{-/-}$ plasma generated C5 convertases that produced C5a levels of approximately 700 and 350 µg/L, respectively, after incubation with human C5. When ATIII was added to either plasma sample before addition of zymosan particles, C5a generation fell 63% and 73% in the case of $C3^{+/+}$ and $C3^{-/-}$ plasma, respectively, suggesting that solid-phase C5 convertase generated on zymosan particles with mouse $C3^{+/+}$ or $C3^{-/-}$ plasma is sensitive to ATIII.

We carried out various experiments to define the nature of the C5 convertase generated by $C3^{-/-}$ plasma and in $C3^{-/-}$ mice. $C3^{+/+}$ and $C3^{-/-}$ mouse plasma samples were subjected to western blot analysis for detection of prothrombin/thrombin after SDS-PAGE. We detected bands between the 97 and 66 kDa markers, consistent with the size of prothrombin; these bands were more intense in samples of $C3^{-/-}$ plasma (**Fig. 3e**). Conversion products of prothrombin were found



Figure 2 Lung injury in $C3^{+/+}$ and $C3^{-/-}$ mice and protective effects of C5a-specific antibody (anti-C5a). Paraffin-embedded lung sections (representative of at least three mice in each group) from $C3^{+/+}$ mice (**a–c**) and $C3^{-/-}$ mice (**d–f**). In lungs from negative control (ctrl) mice (**a,d**), intravenous administration of BSA was omitted. Lungs 4 h after intrapulmonary deposition of either IgG immune complexes in the presence of preimmune IgG (**b,e**) or C5a-specific IgG (**c,f**) stained with hematoxylin and eosin (original magnification, ×40).

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with $C3^{+/+}$ or $C3^{-/-}$ mouse plasma, washed, then incubated with human C5. Supernatant fluids were analyzed by ELISA for human C5a. Inset, supernatant fluids were immunoprecipitated and then evaluated by western blots for C5a. For each bar, n > 10 mice. Western blot in inset is representative of three independent experiments. (d) Solid-phase C5 convertase was generated on zymosan particles. As indicated, ATIII was added to plasma samples before activation. Generation of C5a was measured by ELISA. For each bar, n > 5 mice. (e) Total plasma protein $(1-25 \ \mu g)$ from $C3^{+/+}$ and $C3^{-/-}$ mice was analyzed by western blot analysis for prothrombin and its activation products. Western blot shown is representative of two independent experiments. (f) Thrombin enzymatic activity was measured spectrophotometrically and expressed as units thrombin/25 μ plasma. For each bar, n > 7 mice. (g) Human C5 was incubated with purified human plasma thrombin (0.5–15 units) and reaction products were assessed for C5a (by ELISA) and for neutrophil chemotactic activity. Inset, western blot analysis of C5 generated by thrombin; recombinant human C5a is also shown. Chemotaxis and western blot data are representative of two independent experiments.

between the 45 and 66 kDa markers, and were more intense in samples of $C3^{-/-}$ plasma. Thrombin enzymatic activity measured in $C3^{+/+}$ and $C3^{-/-}$ plasma was approximately three times greater in $C3^{-/-}$ plasma than $C3^{+/+}$ plasma (Fig. 3f). We incubated purified human thrombin with human C5 and then heated it to destroy thrombin activity (Fig. 3g). Using ELISA, we determined the amount of C5a generated after incubation of purified human thrombin with human C5. The amount of C5a generated was proportional to the amount of thrombin added, and no C5a was detected in the absence of thrombin. Because C5a is a chemoattractant for neutrophils, we assessed the ability of the reaction products to induce chemotaxis of human neutrophil. Chemotactic activity approximately paralleled the levels of C5a generated (Fig. 3g). Western blot analysis using antibodies to C5 showed a single band between 10 and 15 kDa, consistent with the size of glycosylated C5a, whereas recombinant (nonglycosylated) human C5a migrated slightly faster, near the 10 kDa marker (Fig. 3g). After deglycosylation of thrombin-generated C5a using PNGase F, matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis showed an 8,269 Da fragment, which is the same as the theoretical mass of human nonglycosylated C5a (data not shown).

Prothrombin and thrombin protein and F2 mRNA as measured in frozen sections and liver homogenates, respectively, from $C3^{+/+}$ and $C3^{-/-}$ livers showed evidence for the presence of prothrombin and thrombin protein in $C3^{+/+}$ liver (**Fig. 4a**), with much more intense staining in $C3^{-/-}$ liver (**Fig. 4b**). Staining in $C3^{-/-}$ liver was abolished if the primary antibody was omitted (**Fig. 4c**). The insets below frames a and b are RT-PCR products for prothrombin/thrombin and *Gapdh*. We performed RT-PCR for these samples and found less intense bands in $C3^{+/+}$ liver homogenates than in $C3^{-/-}$ liver homogenates. *Gapdh* content showed approximately equivalent loading.

We compared lung injury in the IgG immune complex model in $C3^{+/+}$ and $C3^{-/-}$ mice in the presence or absence of ATIII (**Fig. 4d**). In $C3^{+/+}$ mice, the permeability value was reduced by 44% (nonsignificant) in the presence of ATIII, whereas in $C3^{-/-}$ mice treated with ATIII, the value was reduced by 73% (P < 0.01).

We carried out similar experiments involving immune complexinduced lung injury in the presence or absence of hirudin (**Fig. 4e**). Like ATIII, hirudin did not protect against injury in $C3^{+/+}$ lungs, but was markedly protective in $C3^{-/-}$ lungs. We also tested the effects of hirudin in another $C3^{-/-}$ mouse strain (Boston) and found similar protective effects (**Fig. 4e**).

We measured C5a levels in BAL fluids (**Fig. 4f**). Acute lung injury in $C3^{+/+}$ mice caused a threefold rise in C5a levels, to 0.88 + 0.09 ng/ml compared to noninjured $C3^{+/+}$ lungs (0.32 + 0.07 ng/ml). The addition of ATIII or hirudin into the airways of $C3^{+/+}$ mice did not reduce BAL content of C5a. C5a content in uninjured $C3^{-/-}$ lungs (0.27 + 0.04 ng/ml) was low and rose nearly threefold in immune complex–injured lungs. This increase was abolished (to 0.29 + 0.07 ng/ml) in the presence of ATIII or hirudin, suggesting that thrombin is predominantly responsible for generation of C5a in the lungs of $C3^{-/-}$ mice.

There are three known pathways of complement activation (**Fig. 4g**). These pathways converge at C3, leading to generation of C3a and C5a as well as the membrane attack complex (C5b-9). Data presented above indicate that thrombin has C5 convertase activity. Thrombin had limited function as a C5 convertase in $C3^{+/+}$ mice and in $C3^{+/+}$ mouse plasma, but became the dominant C5 convertase in the genetic absence of C3. Whether tissue factor or related factors are activated concomitantly with complement activation to convert prothrombin to thrombin remains to be determined.

Inflammatory lung injury triggered experimentally in rodents by IgG immune complexes, lipopolysaccharide, live bacteria, ischemiareperfusion and other circumstances proceeds along a series of pathophysiological steps^{8,20–24}. These insults trigger complement activation and recruitment of neutrophils together with activation of cellular defenses, leading to release of cytokines and chemokines²⁵. These events ultimately cause recruitment of neutrophils followed by release of destructive proteases and oxidants from phagocytes. It is well established that deposition of IgG immune complexes results in acute tissue injury, involving C5a and C5aR as well as FcRs^{2,3,5,10,20}.



C3a, C5a and their receptors may be involved in the pathophysiology of lung inflammation^{14,26-28}. In the current studies, lungs from $C3^{+/+}$ and $C3^{-/-}$ mice showed full inflammatory responses to intrapulmonary deposition of IgG immune complexes. C5a-specific antibody greatly suppressed these lung inflammatory responses, suggesting that C5a can be generated in the absence of C3. This hypothesis was confirmed by in vitro and in vivo studies. A potent solid-phase C5 convertase activity was generated from both $C3^{+/+}$ and $C3^{-/-}$ plasma (Fig. 3). That $C3^{+/+}$ and $C3^{-/-}$ mice had the same intensity of acute lung injury (Figs. 1 and 2) may be explained by the fact that lung cells are known to synthesize complement proteins, including C5. Our data suggest that C5a produced in injured lungs from $C3^{+/+}$ mice is derived from a C3-dependent convertase, whereas in $C3^{-/-}$ mice C5a is chiefly generated by thrombin, which functions as a C5 convertase. Further, our data indicate that C5a produced by thrombin is functionally active. Overproduction of prothrombin and thrombin in $C3^{-/-}$ mice seems to occur in the liver, as supported by immunostaining results and mRNA measurements (Fig. 4), but the molecular basis for this compensatory response is not clear.

Our studies suggest that a new pathway exists for generation of C5a, especially in the absence of C3, with thrombin acting as a potent C5

Figure 4 Evidence for thrombin as a C5 convertase. Liver frozen sections or liver homogenates from $C3^{+/+}$ and $C3^{-/-}$ mice were evaluated for prothrombin and thrombin protein (a-c) or F2 mRNA (insets). Samples in a-c were counterstained with hematoxylin (original magnification, $\times 100.$) (c) The primary antibody was omitted. (d) IgG immune complex injury was induced in $C3^{+/+}$ and $C3^{-/-}$ mice in the presence or absence of 5 units ATIII given intratracheally with BSA-specific antibody, and the leak of ¹²⁵I-BSA into the lung was measured. For each bar, n = 7. (e) $C3^{+/+}$ and $C3^{-/-}$ mice underwent immune complex injury in the absence or presence of recombinant hirudin given intratracheally. The injury index and protection by hirudin is also shown in a second strain (Boston) of C3-/- mice. For each bar, n > 7. (f) C5a was measured by ELISA in BAL fluids. BAL fluids were obtained 4 h after initiation of the lung reactions and effects of ATIII or hirudin on C5a levels assessed in $C3^{+/+}$ and C3^{-/-} mice. For each bar, n > 7 mice. (g) The various pathways of complement activation, together with the role of thrombin. MAC, membrane attack complex; MBL, mannosebinding lectin; TF, tissue factor.

convertase. It seems that when the delicate balance between the various components of the complement and coagulation pathways is perturbed, as in C3 knockout mice, there exists a compensatory adaptive pathway featuring increased presence of thrombin, which can function as a C5 convertase to generate authentic C5a.

METHODS

Reagents and chemicals. Unless otherwise specified, we purchased chemicals and reagents from Sigma. We obtained purified human C5 from Quidel. We obtained ELISA kits and components for detecting mouse C3a, human C5a and mouse C5a from Cedarlane, DRG Diagnostics and BD

Pharmingen, respectively. We obtained antibody to rodent prothrombin and thrombin from Santa Cruz Biotechnologies.

Mice. $C3^{-/-}$ mice have been previously described²⁹ with no detectable C3 protein in their serum. A second $C3^{-/-}$ mouse strain (also on a C57BL/6 background) was provided by M. Carroll (CBR Institute for Biomedical Research, Harvard Medical School). We confirmed the presence or absence of C3 in zymosan activated sera and plasma using the mouse C3a ELISA kit. We obtained $Hc^{+/+}$ and $Hc^{-/-}$ (B10D2) congenic mice as well as $C3^{+/+}$ mice from Jackson Laboratories. All studies were conducted in accordance with the University of Michigan Committee on Use and Care of Animals.

Cloning and preparation of recombinant mouse C5a. We isolated total RNA from normal mouse liver tissue using Trizol (Life Technologies) and reverse transcribed it using a RT-PCR kit from Promega. We amplified the mouse C5a sequence using the following primers: 5'-CTGTCGCGAGTCAGCCATATGAAC CTGCATCTCCTA-3' (5' primer) and 5'-GTCACATCGCGACACGGATCCT CACCTTCCCAGTTGGAC-3' (3' primer). We subcloned them into the pET 15b bacterial expression vector (Novagen). Recombinant C5a was expressed in pLysS cells (Novagen) and purified over a Ni²⁺ column.

Antibodies to rodent C5a. Rabbit polyclonal affinity purified C5a-specific IgG targeting the carboxyl-terminal peptide region of rat C5a (with the sequence

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CTIADKIRKESHHKGMLLGR, corresponding to amino acid residues 58–77 of rat C5a) blocked neutrophil chemotactic activity of recombinant mouse C5a as well as neutrophil chemotactic activity of glycogen-activated mouse serum (data not shown).

Immunoprecipitation of C5a from activated mouse serum. We obtained blood from mice by cardiac puncture. Where indicated, blood was drawn into syringes containing either nothing, 10 mM EDTA or 10% citrate. We then incubated serum or plasma incubated with zymosan (10 mg/ml) at 37 °C for 2 h and immunoprecipitated C5a as previously described²⁵ or measured by ELISA.

IgG immune complex-induced lung injury. We induced lung injury in mice using previously described methods³⁰. We used at least five mice for each treatment condition.

As indicated, mice received either 40 μ g preimmune IgG or 40 μ g IgG to C5a or 5 units antithrombin III (human) or 5 units of recombinant hirudin mixed with BSA-specific antibody (125 μ g rabbit IgG) immediately before airway instillation. We injected BSA (1.0 mg) intravenously, but did not inject BSA in negative control mice. To measure lung permeability, a trace amount of ¹²⁵I-BSA was injected intravenously. When mice were killed, the pulmonary vasculature was flushed with 2.0 ml PBS and the amount of remaining lung radioactivity was measured as a ratio to radioactivity in 100 μ l blood recovered from the inferior vena cava.

Generation of solid-phase C5 convertase and measurement of C5a. We incubated $C3^{+/+}$ or $C3^{-/-}$ mouse plasma (25 µl) with zymosan particles (1.2 mg in 100 µl) or immune complex particles (10–50 µg) for 30 min at 37 °C. We then washed the particles in Hank Balanced Salt Solution and incubated them with human C5 (10 µg) for 2 h at 37 °C. We analyzed the supernatant fluids by ELISA for generation of C5a according to the manufacturer's instructions (DRG Diagnostics).

Neutrophils and chemokines in BAL fluid. Four hours after immune complex–induced lung injury, mice were killed and BAL fluids collected for neutrophil counts and chemokine levels (as determined by ELISA) according to the manufacturer's instructions (R&D Systems).

Histological analysis. We instilled lungs via the trachea with 10% buffered (pH 7.0) formaldehyde, then surgically removed them, embedded them in paraffin and sectioned them, then stained them with hematoxylin and eosin.

Immunostaining for prothrombin and thrombin and mRNA analysis. For immunostaining, we froze mouse livers in OCT compound (Miles Co.) and obtained frozen sections, stained them overnight with goat thrombin-specific antibody (Santa Cruz Biotechnology; diluted 1:20 in PBS containing 0.1% BSA). We then washed sections in PBS and incubated them for 2 h with horseradish peroxidase (HRP)-conjugated goat-specific donkey IgG (Jackson Immunochemicals) diluted 1:200 in PBS, after which we washed and incubated them with the HRP-specific substrate diaminobenzidine (DAB; Kirkegard and Perry) for 5 min.

For mRNA analysis, we isolated total RNA from mouse livers using the Trizol method (Life Technologies Inc.) according to the manufacturer's instructions. We then performed real-time PCR (using iScript one-step RT-PCR kit with SyBR green from Bio-Rad) with primers for *F2* (5' primer, 5'GTGTGGGGGCCAGCCTTATCAGTG-3' and 3' primer, 5'-CCGCTCTACAAT GGGCAGGTTCA-3') and *Gapdh* (5' primer, 5'-GCCTCGTCTCATAGACAAG ATG-3' and 3' primer, 5'-CAGTAGACTCCACGACATAC-3') using a Bio-Rad analyzer. Threshold cycle values, standard curves and melting curves were generated using the software provided by the manufacturer (Bio-Rad).

ELISA for mouse C5a. We coated ELISA plates with purified monoclonal mouse C5a-specific IgG (5 μ g/ml). After blocking of the nonspecific binding sites with 1% milk in PBS, we added activated serum and recombinant mouse C5a (as a standard) in various dilutions. We subsequently used biotinylated monoclonal mouse C5a-specific antibody (500 ng/ml), followed by incubation with streptavidin-peroxidase (400 ng/ml). We then added *O*-phenylenediamine dihydrochloride, stopped the color reaction with 3 M sulfuric acid and read the absorbance at 490 nm.

Spectrophotometric measurement of thrombin activity. We performed the thrombin assay using S-2238 (H-D-phenylalanyl-L-pipecolyl-L-arginine-p-nitroaniline dihydrochloride; DiaPharma) as a substrate. After thrombin cleavage of the substrate, the amount of p-nitroaniline dihydrochloride formed was measured by absorbance at 405 nm. We performed the assay in the presence of aprotinin, according to the manufacturer's instructions.

Western blot and chemotaxis assay. We performed western blot and chemotaxis assays as previously described¹⁸.

Statistical analyses. Values are expressed as mean + s.e.m., after one-way analysis of variance, individual group means were compared with the Student-Newman-Keuls multiple comparison tests. Differences were considered significant when P < 0.05.

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AUTHOR CONTRIBUTIONS

M.H.-L., J.V.S., F.S.Z., S.M.D., R.L.W., M.A.F., D.R., L.M.H., R.A.W., S.R.M., T.A.N., J.G.Y. and P.A.W. contributed to animal models. J.V.S., F.S.Z., J.D.L. and T.A.N. contributed to ATIII and hirudin experiments. J.D.L. and F.G. contributed to thrombin experiments.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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