Objective—Inflammation contributes to hypertension-induced cardiac damage and fibrotic remodeling. Complement activation produces anaphylatoxins, which are major inflammatory effectors. Here, we investigated the role of complement anaphylatoxins in angiotensin II (Ang II)–induced cardiac remodeling.

Approach and Results—We measured human plasma levels of complement anaphylatoxins in hypertensive individuals and controls and studied the role of complement activation in a mouse model of Ang II–induced hypertension and cardiac injury. We found that complement 5a (C5a) concentration was more elevated in hypertensive individuals than in controls. Infusion of Ang II in mice for 7 days led to increased anaphylatoxin concentration in plasma and perivascular C3b deposition in the heart. C5a receptor (C5aR)–deficient but not C3a receptor–deficient mice exhibited markedly reduced cardiac remodeling and inflammation after Ang II infusion. Pharmacological inhibition of C5a production by an anti-C5 monoclonal antibody produced similar effects to C5aR deficiency. Bone marrow chimera experiments revealed that C5aR expression on bone marrow–derived cells was critical in mediating Ang II–induced cardiac injury and remodeling. The C5aR pathway regulated the expression of adhesion molecules on peripheral monocytes, as well as infiltration and cytokine production of macrophages in the heart.

Conclusions—Complement is activated in hypertensive hearts, and the C5aR signaling pathway on blood monocytes/macrophages plays a pathological role in Ang II–induced cardiac inflammation and remodeling. Therapeutic inhibition of complement may protect patients from hypertension-related heart injury. (Arterioscler Thromb Vasc Biol. 2014;34:1240–1248.)

Key Words: angiotensin II ☺ complement C5a ☺ complement system proteins ☺ hypertension ☺ inflammation

Hypertension induces damage to multiple organs, including the heart, kidney, brain, and eyes.1,2 Cardiac remodeling, characterized by excessive interstitial and perivascular extracellular matrix deposition, leads to increased ventricular stiffness with diastolic heart failure and systolic cardiac dysfunction.3,4 Hypertension-induced cardiac remodeling is a multifaceted process. Elevated angiotensin II (Ang II) causes endothelial cell injury and platelet activation and increases local production of cytokines, chemokines, and infiltration of inflammatory cells. These events are followed by myofibroblast activation and extracellular matrix synthesis, leading to cardiac fibrosis.5 The nature of the inflammatory pathways activated and how they interact with cellular processes and with each other to bring about cardiac remodeling remains to be fully characterized.

The complement system plays a central role in innate and adaptive immunity. Its function in host defense by opsonization and lytic activity, as well as in the clearance of immune complexes and self antigens such as apoptotic cell debris, is well recognized.6 The latter function of the complement links it to systemic autoimmunity, such as lupus.7 In addition, gene mutations or insufficient activity in complement regulatory proteins, leading to excessive complement activation and tissue injury, has been implicated in many human diseases, involving vascular and blood cells.8,9 Of interest, several recent studies have suggested a possible connection between complement activation and hypertension. In a longitudinal cohort study, high concentrations of C3 in plasma were found to be associated with incidence of future blood pressure increase and the development of hypertension.9 Genetic variations in factor H, a key complement regulator, and its related genes may contribute to hypertension risk in the Chinese population.10 In another study, C1q, C3, C3c, and C5b-9 were observed in the renal vessel media, and complement activation and cell...
infiltration occurred before the onset of albuminuria in hypertensive renal damage of a transgenic rat model.11

Although the above association studies are indicative of a possible role of complement in hypertension and its sequelae, the causal relationship between complement activation and hypertension-induced organ injury has not been sufficiently investigated or is a mechanistic understanding of the connection established. In the current study, we measured blood levels of anaphylatoxins in patients with hypertension and investigated the possible involvement of complement activation in a mouse model of Ang II–induced cardiac inflammation and fibrotic remodeling. We conclude that the complement 5a receptor (C5aR) pathway plays a detrimental role in hypertension-induced cardiac inflammation and injury, and therapeutic manipulation of the complement system may benefit patients under these disease settings.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Evidence of Complement Activation in Patients With Hypertension and Ang II–Treated Mice

To evaluate a possible connection between hypertension and complement activation, we measured serum levels of the complement activation products, C3a and C5a, in patients with hypertension and healthy controls. We found that serum C5a but not C3a levels were significantly elevated (2.1-fold) in patients with hypertension (Figure 1A). To study the role of complement in hypertension further, we resorted to an Ang II–induced acute hypertension and cardiac injury model. We infused wild-type (WT) mice with Ang II for 7 days to cause acute blood pressure elevation and then examined local and systemic signs of complement activation. We observed that both C3a and C5a levels in serum were significantly increased at 1 day after Ang II infusion (2.9- and 3.7-fold, respectively) and remained elevated for the duration of 7 days of Ang II treatment (Figure 1B). Similar changes in serum C3a and C5a levels were not observed in sham-treated (infusion of saline) mice (Figure 1B). By immunofluorescence staining, we also detected prominent perivascular deposition of activated C3 fragments (C3b/iC3b) in Ang II–treated but not sham-treated mouse hearts (Figure 1C and 1D), which provided direct evidence of local complement activation in the hearts of Ang II–treated mice. Separately, we examined the expression of complement 3a receptor (C3aR) and C5aR in Ang II–infused mouse hearts by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). As shown in Figure I in the online-only Data Supplement, we detected increased expression of C3aR and C5aR as early as day 1 after Ang II infusion, with the levels peaking on day 3. The increase in C5aR (7-fold on day 3) occurred to a greater degree than in C3aR (2.5-fold), suggesting that the C5aR pathway may play a particularly important role in Ang II–induced cardiac inflammation and remodeling.

C5aR- but Not C3aR-Deficient Mice Are Protected From Ang II–Induced Cardiac Injury and Remodeling

Given the strong evidence of complement activation, we assessed whether anaphylatoxins are involved in Ang II–induced cardiac injury and remodeling. For this purpose, we used mice deficient in C5aR and C3aR and compared their
cardiac gene expression and tissue remodeling responses with that of WT mice. Ang II–induced cardiac injury is characterized by the induction of profibrotic and extracellular matrix genes, including transforming growth factor-β1 (TGF-β1), α-smooth muscle actin (α-SMA), and collagen.1 By immunohistochemistry staining, we confirmed that Ang II infusion in WT mice led to abundant production of TGF-β1 and α-SMA in heart tissues (Figure 2A and 2B). Importantly, we detected greatly reduced positive areas of TGF-β1 and α-SMA staining in Ang II–treated C5aR−/− mouse hearts, but there were no significant changes in C3aR−/− mouse hearts (Figure 2A and 2B). Ang II–treated C5aR−/− mice also displayed ≥80% reduction in Masson-positive areas in the hearts, indicating greatly reduced deposition of collagen, a marker of cardiac fibrosis, whereas Ang II–treated C3aR−/− mouse hearts seemed to be similar to Ang II–treated WT mouse hearts (Figure 2C). These results were further confirmed at the gene expression level. By qRT-PCR, we found that the mRNA levels of α-SMA, Col1a1, and Col3a1 in C5aR−/− mouse hearts were significantly reduced 7 days after Ang II infusion when compared with WT mice (Figure 2D). However, C5aR deficiency did not affect blood pressure, cardiac function, or cardiac hypertrophy of Ang II–treated mice (Figure II in the online-only Data Supplement).

C5aR on Bone Marrow–Derived Leukocytes Is Critical for Ang II–Induced Cardiac Inflammation and Injury

The above experiments demonstrated that C5aR was upregulated in Ang II–induced hypertensive mouse hearts, where it played a key pathological role. By immunostaining and cell sorting analysis, we confirmed C5aR upregulation at the protein level and determined the source of C5aR expression in the hypertensive mouse heart as infiltrating CD11b+ leukocytes (Figure IIIA and IIIB in the online-only Data Supplement). Further flow cytometry analysis with more specific markers revealed that the majority of infiltrated C5aR-expressing cells at days 1 and 7 after Ang II infusion were F4/80+ macrophages, with a smaller fraction being Ly6G+ neutrophils (Figure IIIC in the online-only Data Supplement).

Using Mac-2 as a macrophage-specific marker, we confirmed the increase in macrophage numbers in the hearts of Ang II–infused WT mice. When compared with WT mice, we detected only limited numbers of macrophages in the hearts of similarly treated C5aR−/− mice (Figure 3A). This difference in macrophage numbers between WT and C5aR−/− mouse hearts was confirmed by the relative abundance of CD11b mRNA, as well as that of several macrophage-associated genes such as the proinflammatory cytokine interleukin-1β and the

Figure 2. Complement 5a receptor (C5aR)–deficient but not complement 3a receptor (C3aR)–deficient mice exhibit reduced fibrosis formation after angiotensin II (Ang II) infusion. A, Immunohistochemical (IHC) staining of the profibrotic cytokine transforming growth factor (TGF)-β1 in the hearts of wild-type (WT), C5aR−/−, and C3aR−/− mice infused with Ang II or saline for 7 days (scale bars, 50 μm); the right histogram shows a quantitation of the positive area as a percentage of the total tissue section area (n=3 mice in each group). B, IHC staining and area quantitation of the myofibroblast marker α-smooth muscle actin (α-SMA) as a measure of fibrosis in WT, C5aR−/−, and C3aR−/− mouse hearts (scale bars, 100 μm). C, Masson staining of fibrosis and area quantitation in WT, C5aR−/−, and C3aR−/− mouse hearts (n=6 mice in each group). D, Real-time polymerase chain reaction analysis of α-SMA, collagen I, and collagen III mRNA levels in WT and C5aR−/− mouse hearts (n=3 in each group). *P<0.05 compared with WT mouse groups.
chemokines monocyte chemoattractant protein-1 (chemokine ligand 2 [CCL2]) and macrophage inflammatory protein-1α (CCL3; Figure 3B–3E). Unlike C5aR deficiency, we found that C3aR deficiency did not significantly affect CD11b+ leukocyte infiltration into the hearts of Ang II–infused mice nor did it alter the expression of inflammatory cytokines and chemokines (Figure IV in the online-only Data Supplement).

C5aR has been reported to be expressed on macrophages and neutrophils, as well as other cell types such as endothelial cells under inflammatory conditions.12,13 Although our data showed macrophages to be the dominant C5aR-expressing cells in Ang II–infused mouse hearts, they do not exclude the possibility of a low-level C5aR expression on cardiomyocytes and myofibroblasts that nevertheless could have played a critical role in the cardiac inflammation and injury. To assess the role of C5aR expression on leukocytes in this model, we created C5aR-chimeric mice using bone marrow (BM) transplantation. Successful BM transplantation was determined by tracing splenic C5aR expression using qRT-PCR (Figure 4A). Two months after BM transplantation, mice underwent Ang II infusion. We detected less infiltrating CD45+ leukocytes and lower CD11b mRNA levels in the hearts of Ang II–infused mice receiving C5aR-deficient BM than those receiving WT BM cells, regardless of the genotype of the recipient mice (Figure 4B and 4C; Figure VA in the online-only Data Supplement). Likewise, perivascular fibrosis as assessed by TGF-β–positive or α-SMA–positive area and Masson staining, as well as interleukin-1β, CCL2, and CCL3 mRNA levels, was significantly reduced in mice receiving C5aR-deficient BM than in those receiving WT BM cells (Figure 4D–4I; Figure VB–VD in the online-only Data Supplement). Similar findings were obtained when α-SMA, Col1a1, and Col3a1 mRNA levels in the Ang II–infused chimeric mouse hearts were quantified by qRT-PCR (Figure VE in the online-only Data Supplement). Thus, cardiac inflammation and injury were tracked with C5aR expression on BM cells rather than heart cells.

### C5a-C5aR Pathway Promotes Adhesion and Migration of Activated Macrophages in Ang II–Infused Mice

We next performed experiments to dissect the mechanisms by which the C5a-C5aR pathway on macrophages may have contributed to cardiac inflammation and injury in Ang II–infused mice. Adhesion of macrophages to endothelial cells constitutes the initial step of cell migration. We first examined the levels of adhesion molecules CD11a/lymphocyte function-associated antigen (LFA), P-selectin glycoprotein ligand (PSGL), and CD11b on blood monocytes in response to Ang II infusion. By qRT-PCR, we found that mRNA levels of CD11a/LFA and PSGL on blood monocytes of WT mice were significantly increased after Ang II infusion, whereas that of CD11b remained relatively constant (Figure 5A). Given that blood C5a level was increased after Ang II infusion (Figure 1), we tested the hypothesis that induction of adhesion molecule expression on monocytes of Ang II–infused mice is mediated by C5a. Figure 5B shows that recombinant C5a induced LFA, PSGL, CCL2, and CCL3 mRNA expression in WT but not in C5aR−/− mouse macrophages in vitro. To test the functional significance of increased adhesion molecule expression, we performed cell adhesion assays using isolated blood monocytes from saline and Ang II–infused WT and C5aR−/− mice and cultured endothelial cells. Figure 5C shows that blood monocytes from Ang II–treated WT mice adhered

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**Figure 3.** Complement 5a receptor (C5aR) deficiency reduces macrophage infiltration and cytokines expression in angiotensin II (Ang II)–infused mouse hearts. A, Immunohistochemical staining and area quantitation of the macrophage marker Mac-2 shows that macrophage infiltration was dramatically reduced in the hearts of Ang II–treated C5aR-deficient mice when compared with similarly treated wild-type (WT) hearts (scale bars, 100 μm; n=3 mice in each group). B–E, Real time-polymerase chain reaction shows that mRNA levels of CD11b, interleukin (IL)-1β, monocyte chemotactic protein (MCP)-1/chemokine ligand 2 (CCL2), and macrophage inflammatory protein (MIP)-1α/CCL3 in Ang II–infused C5aR−/− mouse hearts were all significantly reduced at days 1 and 3 when compared with WT mice (n=3 mice in each group). *P<0.05.
to endothelial cells dramatically faster than cells from saline-treated WT mice, and C5aR deficiency significantly impaired monocyte/endothelial interaction. Separately, in a cell migration assay designed to measure chemotactic activity, C5aR–/– macrophages in a lower chamber pretreated with recombinant C5a recruited less macrophages from an upper chamber than similarly treated WT macrophages (Figure 5D).

**Anti-C5 Monoclonal Antibody Treatment Ameliorates Ang II–Induced Cardiac Inflammation and Injury**

Data presented above showed that the C5a-C5aR pathway on monocytes/macrophages plays a critical role in mediating Ang II–induced cardiac inflammation and injury. This finding suggests that complement inhibition may be a potential therapeutic strategy for preventing or treating hypertension-related organ injury. To provide a proof of concept, we treated WT mice with an antismall C5 monoclonal antibody (mAb; BB5.1) 3 hours before Ang II infusion. An irrelevant IgG of the same isotype (MOPC) was used as a control. Figure 6A shows that the number of infiltrating macrophages, as represented by Mac-2–positive cells, in the hearts of Ang II–infused and anti–C5-treated mice were significantly reduced when compared with control IgG-treated and Ang II-infused mice. This was confirmed by a significant reduction in CD45, CD11b, interleukin-1β, chemokine ligand (CCL) 2, and CCL3 mRNA levels in Ang II–treated chimera mouse hearts (n=4 mice in each group) showed their expression to be dependent on C5aR expression on BM cells. G–I, Quantitation of heart areas positive for transforming growth factor (TGF)-β1, α-smooth muscle actin (α-SMA), or Masson staining as measurement of fibrosis in Ang II–treated chimera mice also showed a correlation with C5aR expression on BM cells (n=4–6 mice in each group). In all panels, chimera mice were used 2 months after BMT and were treated with Ang II for 7 days. *P<0.05.

**Discussion**

The connection between inflammation and hypertension-induced cardiac injury is well appreciated, but whether the
complement system, a major innate immune and inflammatory pathway, is implicated in this disease setting has not been well studied. We have provided evidence in the present study that the complement system is overactivated in patients with hypertension when compared with nonhypertensive controls. Using a mouse model of Ang II–induced acute hypertension and cardiac injury/fibrotic remodeling, we further examined signs of complement activation and investigated the contribution of complement anaphylatoxin receptors to the pathological changes caused by Ang II infusion. We found that plasma levels of C3a and C5a were significantly elevated, and there was a marked increase in anaphylatoxin receptor, particularly C5aR, expression in the hearts of treated mice. The latter phenomenon was found to be associated with leukocyte infiltration as indicated by the appearance of a large population of CD45+ cells in the mouse hearts after Ang II infusion. It is notable that although C5aR is known to be highly expressed on neutrophils and macrophages, the infiltrating leukocytes in Ang II–treated mouse hearts consisted primarily of macrophages. Thus, Ang II infusion initiated a strong inflammatory response dominated by macrophages in the mouse hearts, and the C5a-C5aR pathway played a key role in this process.

Consistent with data from Ang II–infused mice, we found that in newly diagnosed hypertensive humans, plasma C5a level was also significantly elevated. However, in contrast to the murine model, we did not observe any changes in plasma C3a levels in patients with hypertension. Given the heterogeneous nature of the human patients, it is perhaps not surprising that we did not see exactly the same complement activation profile as in Ang II–treated mice. C5 activation normally depends on C3 activation to generate C5 convertases, but it is also known that C5a can be generated in the absence of C3.
under inflammatory conditions involving thrombin-mediated C5 cleavage. In a similar example, plasma levels of C5a and C5b-9, but not of C3a, were increased in cases of patients with severe preeclampsia when compared with healthy controls.

The conclusion that C5aR expression on macrophages is important for Ang II–induced cardiac inflammation and fibrotic remodeling is supported by several lines of evidence. First, we demonstrated that C5aR deficiency but not C3aR deficiency ameliorated Ang II–related pathology. Second, pretreatment of Ang II–infused WT mice with an anti-C5 mAb had a similar effect as C5aR deficiency in reversing the disease phenotype. Third, BM chimera experiment revealed that C5aR expression on blood cells rather than resident cardiac cells was responsible for Ang II–induced pathological changes in the hearts. Finally, we found that Ang II treatment led to enhanced expression of adhesion molecules, including LFA and PGSL on peripheral monocytes, which increased their stickiness to cultured endothelial cells, and these effects were recapitulated by C5a stimulation of mouse macrophages in vitro.

It is likely that C5a-mediated macrophages interacted with other cell types and mediators within the cardiac microenvironment to cause pathological changes, such as fibrotic remodeling in Ang II–infused mice. We have previously shown that CD4+ T-cell–derived interleukin-12p35 played a role in regulating the differentiation of M2 macrophages. M2 macrophages promote fibroblast differentiation and fibrosis formation through TGF-β1 production. We have also found that interferon-γ from T cells could stimulate monocyte chemoattractant protein-1 production by macrophages, further promoting macrophage infiltration and cardiac fibrosis through a positive feedback loop. Furthermore, infiltrated macrophages in the hearts stimulate cardiac fibroblasts to produce interleukin-6, which is essential for TGF-β1/Smad activation and cardiac fibrosis as seen in Ang II–treated mouse hearts.

How and by which pathway complement was activated in Ang II–treated mouse hearts remains to be established. In addition to marked elevation of plasma C3a and C5a levels, we observed prominent perivascular C3b deposition in the hearts of Ang II–treated mice, suggesting that complement was primarily activated on tissues surrounding blood vessels. The complement cascade can be initiated through 3 different pathways, namely the classical, lectin, and alternative pathways (APs). The classical pathway is activated by antibody–antigen complexes and the lectin pathway by mannose-binding lectins. Both pathogen-associated molecular patterns and host cell damage-associated molecular patterns can be sensed by these pathways as might occur during pathogen infection or host tissue injury (eg, ischemia reperfusion injury). In contrast, the AP is constantly active at a low level and in the absence of negative control, it is rapidly amplified. Avoidance of AP complement attack by host cells is achieved by membrane-bound or plasma complement regulating proteins, such as decay-accelerating factor and factor H.
evidence of perivascular C3b deposition in the hearts of Ang II–treated mice, we speculate that complement activation may originate from Ang II–induced endothelial injury and platelet activation.\textsuperscript{25,26} Ang II–injured endothelial cells may expose neoantigens to which natural antibodies or lectin can bind and trigger the classical or lectin pathways of complement activation. Similar mechanisms of complement activation have been shown in the setting of ischemia reperfusion injury of endothelial cells.\textsuperscript{27,28} Not mutually exclusive with the possibility discussed above, damaged endothelial cells in Ang II–infused mouse hearts may lose intrinsic membrane complement regulators, such as decay-accelerating factor, Crry, and CD59, or may lose the ability to interact with the key plasma complement regulator factor H,\textsuperscript{29–31} thus allowing AP complement to be amplified unchecked.

Activated platelets may also contribute to complement activation. We have previously described that platelets were activated, and platelet–leukocyte conjugation was formed during Ang II–induced mouse hypertension and cardiac fibrosis, and when mice were pretreated with clopidogrel, cardiac fibrosis was attenuated.\textsuperscript{32} Recent studies have shown that platelets could stimulate complement activation in a variety of pathological settings of inflammation and vascular injury,\textsuperscript{33} and this process is dependent on the expression of P-selectin and gC1qR on the platelet surface, as well as the secretion of chondroitin sulfate from internal platelet stores.\textsuperscript{34,35} P-selectin has been associated with the activation of the AP, whereas gC1qR and chondroitin sulfate activate the classical pathway.\textsuperscript{36–38} The use of gene knockout mice deficient in pathway-specific complement proteins in future studies should help to delineate the complement activation pathways involved in the current disease model.

Undoubtedly, hypertension-related vascular injury and heart disease in human patients are much more complex and heterogeneous than the Ang II–induced mouse model of acute hypertension and cardiac inflammation and injury. Notwithstanding this limitation, our data are in line with a previous report, showing that the C5aR antagonist PMX53 inhibited cardiac inflammation and fibrosis in the DOCA-salt hypertensive rat model\textsuperscript{39} and provide new evidence to support the conclusion that abnormal complement activation is implicated in an increasing number of vascular disorders.\textsuperscript{38–40} Given the evidence of increased complement activation in patients with hypertension, the availability of a clinically approved therapeutic anti-C5 mAb and our proof of concept data show that a surrogate anti-C5 mAb ameliorated Ang II–induced cardiac inflammation and fibrotic remodeling in mice; the role of complement in hypertension-induced vascular injury and heart disease in human patients and the possible benefit of anticomplement therapy in such individuals deserve further investigation.

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Disclosures
None.

Reference
Inflammation contributes to hypertension-induced cardiac damage and fibrotic remodeling. Recent studies have linked abnormal complement activation to several injury-related diseases. Anticomplement drugs, therefore, offer a new therapeutic modality for treating human vascular disorders. In this study, we investigated the role of complement anaphylatoxin C5a in angiotensin II infusion–induced hypertensive cardiac remodeling. We provide evidence that there is increased complement activation in patients with hypertension and in angiotensin II–treated hypertensive mice. Using knockout mouse models, we explored the role of complement anaphylatoxin receptors in angiotensin II–induced acute hypertension, cardiac inflammation, and remodeling. By performing bone marrow transplantation experiments, we found that complement 5a receptor expressed on bone marrow–derived macrophages contributed significantly to these pathological processes. Finally, we showed that inhibition of the terminal complement pathway with an anti-C5 monoclonal antibody also ameliorated angiotensin II–induced cardiac pathology. Collectively, our data suggest a pathological role of complement activation in hypertension-related cardiovascular injury and remodeling, and anticomplement therapy may benefit human patients.
Complement 5a Receptor Mediates Angiotensin II–Induced Cardiac Inflammation and Remodeling

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Complement C5a receptor mediates Ang II-induced cardiac inflammation and remodeling

Materials and Methods

Animals and treatments. C5aR-deficient (C5aR⁻⁻) and C3aR-deficient (C3aR⁻⁻) mice on C57BL/6J background were as described previously. Mice were bred and kept in a specific pathogen-free animal facility of Beijing Anzhen Hospital affiliated to the Capital Medical University. Eight to twelve (8-12) week-old male knockout and age/sex-matched wild-type (WT) littermate controls were used. To induce hypertension, mice were subcutaneously infused with Ang II (Sigma, St Louis, MO) or saline at a 1500ng.kg⁻¹.min⁻¹ dose for 7 days with ALZET miniosmotic pumps (DURECT, Palo Alto, CA). Blood pressure was measured non-invasively every day by the tail-cuff method (Softron BP-98A, Softron, Tokyo, Japan). Cardiac function was evaluated by ultrasonography as described. In some experiments, WT mice were treated twice with a mouse anti-mouse C5 mAb (BB5.1) at a dose of 1 mg/mouse (i.p), first at 2 hours before and then at day 3 after Ang II infusion. Animal experimental protocols were approved by the Animal Care and Use Committee of Capital Medical University.

Measurement of serum C3a and C5a levels. Human serum samples were collected at the Department of hypertension, Beijing An Zhen Hospital with informed consent. Sera were collected from 31 (n=31) newly diagnosed hypertension patients before treatment intervention was initiated, and from 7 (n=7) non-hypertensive individuals undergoing health examination. The study of human subjects was approved by the ethics committee of our hospital. The levels of C3a and C5a were determined by Cytometric Bead Array (CBA, BD Bioscience, Franklin Lakes, NJ) according to the instruction manual. Sera from Ang II or saline infused mice were collected on 0, 1, 3 and 7, and C3a and C5a concentrations were determined by ELISA (Uscn, Wuhan, China) according to the instruction manuals.

Generation of bone marrow chimeric mice. 8 week-old WT and C5aR⁻⁻ male recipient mice were pre-conditioned for two weeks with pH=2.0 drinking water containing 100mg/L levofloxacin and 100mg/L fluconazole. Bone marrow (BM) cells were collected from femurs and tibias of WT or C5aR⁻⁻ mice by needle flushing, and re-suspended in RPMI-1640 /2% FBS / 5U/ml heparin at a density of 1x10⁸ cells/ml and kept on ice. Four hours after irradiation with 10Gy X-rays, recipient mice were intravenously injected with 1×10⁷ BM cells, and then the mice were kept in a specific pathogen-free environment for 8 weeks to reconstitute their BM. Four groups of chimeric mice were generated: WT to WT, C5aR⁻⁻ to WT, WT to C5aR⁻⁻ and C5aR⁻⁻ to C5aR⁻⁻. To evaluate efficiency of bone marrow reconstitution, BM cells were collected from femurs of representative mice at the time of sacrifice, and genomic DNA was isolated and used for genotyping.

Flow cytometry. To prepare periphery blood monocytes (PBMCs) for flow cytometry analysis, red blood cells of periphery blood were lysised by RBC Lysis Buffer (BD
Bioscience) for 5 minutes. The remaining cells were blocked with CD16/32 antibody for 10 minutes, and then incubated with appropriate antibodies diluted in phosphate-buffered saline (PBS) for 30 minutes at 4°C. For the analysis of cells infiltration into heart, the mouse heart was minced into small pieces, and then digested with 200U/ml Collagenase II and 2.4 U/ml Dispase II in PBS at 37°C for 30 minutes. The single cell suspension was filtered, centrifuged and resuspended in PBS. Cells were blocked with CD16/32 antibody and then incubated with antibodies diluted in PBS for 30 minutes at 4°C. All the antibodies used in flow cytometry were listed in Table I. The expression of surface molecules was analyzed by flow cytometry (Beckman Coulter Epics XL.MCL, Miami, FL).

**Cell sorting** For the sorting of CD45⁺CD11b⁺, CD45⁺CD11b⁻, CD45⁻CD31⁺ and CD45⁻CD31⁻ cells from saline and Ang II infused WT heart, the heart tissues were minced and digested to single cell suspension, then the cells were labeled and sorted by CD45 microbeads, LS column and MidiMACS Separators (Miltenyi Biotec., Auburn, CA). The CD45 positive cells were further sorted by CD11b microbeads (Miltenyi Biotec.) for the CD45⁺CD11b⁺, CD45⁺CD11b⁻ cells, and the CD45 negative cells were further sorted by CD31 microbeads (Miltenyi Biotec.) for CD45⁻CD31⁺ and CD45⁻CD31⁻ cells. All the experiments were operated according to the instruction manual. The cells purity sorted by microbeads was examined by flow cytometry.

**Histology and immunohistochemistry.** Mice were euthanized with an overdose of sodium pentobarbital and excised hearts were embedded in paraffin and sectioned at 5μm thickness as described³. Sections were stained with Masson’s trichrome for analysis of cardiac fibrosis. For immunohistochemistry (IHC) staining, tissue sections were incubated with primary antibodies at 4°C overnight after blocking with control serum, followed by incubation with secondary antibodies at 37°C for 30 minutes and detection with 3, 3'-diaminobenzidine (DAB). Images were obtained from each section (ECLIPSE 90i, Nikon, Japan) and analyzed by NIS-Elements Br 3.0 software. Fibrotic area was calculated as a percentage of total myocardial area, and IHC positive staining area was measured in 8-10 regions of each section, averaged and expressed as a percentage of total measured area.

**Quantitative realtime-PCR (qRT-PCR)** Heart tissue mRNA was extracted with Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. 2 μg total mRNA was used for first-strand cDNA synthesis with AMV reverse transcriptase (Promega, Madison, WI). The expression levels of genes were analyzed by qRT-PCR, performed with 2xSYBR mastermix (Takara, Otsu, Shiga) using a BIO-RAD iCycler iQ5 (Bio-Rad, Hercules, CA). All samples were run in duplicate. The DNA primers and cycling conditions are detailed in Table II. Relative expression levels of genes were calculated from cycle threshold values (Ct) using GAPDH as an internal control (gene relative expression= 2^((GAPDH Ct - sample Ct))).

**Bone marrow derived macrophages (BMDMs) preparation.** BMDMs were prepared as previously described³,⁴. Briefly, WT and C5aR⁻/⁻ BM cells were flushed out from femurs and tibias with a 25G needle and filtered. Cells were centrifugation at 1000rpm for 10 minutes and re-suspended. After density centrifugation, cells from the interface of PBS and
Ficoll (HaoYang, TianJin, China) were obtained, washed and re-suspended with growth medium (DMEM high glucose medium supplemented with 10% FBS, 1% penicillin-streptomycin). After plating for 4 hours, non-adherent cells were removed and new growth medium supplemented with 50 ng/ml M-CSF (PeproTech, Rocky Hill, NJ) was added. Cells were cultured in a humidified 5% CO₂ atmosphere at 37°C.

**Endothelial cell culture and mononuclear cell isolation.** Human umbilical vein endothelial cells (HUVECs) were cultured in EBM medium supplemented with 1% antibiotics and 10% FBS at 37 °C under a humidified atmosphere of 5% CO₂. Cells between passage 3 and 7 were used in the experiments. PBMCs were isolated from blood collected from WT and C5aR⁻/⁻ mice with or without Ang II infusion for 1 day by using Ficoll gradients. Isolated cells were re-suspended in EBM medium at a concentration of 1x10⁶/ml for cell adhesion assays.

**Cell adhesion assays.** For monocyte adhesion to endothelia cells, HUVECs were grown to confluence in 48-well plates and treated with recombination TNF-α for 4 hours at 37 °C as indicated. PBMCs labeled with calcein-AM (Invitrogen) were added to each well in the same concentration and incubated for 1 hour. Non-adherent cells were aspirated and the monolayer was gently washed with PBS three times. The fluorescence intensity was measured with the High Content Scanner (Molecular Devices, Silicon Valley, CA) using a MataXpress software.

**Cell migration assay.** Cell migration was quantified in duplicate by use of 24-well Transwell inserts with polycarbonate filters (8-μm pore size) (Corning Costar, Acon, MA). WT BMDMs (1.0 x 10⁴/well) were added to the upper chamber of the insert. The lower chambers were seeded by WT or C5aR⁻/⁻ BMDMs (1.0 x 10⁵/well), which were pretreated with PBS or rC5a (10ng/ml) for 24 hours at 37°C. Cells that had migrated were counted by use of DAPI staining.

**Statistical Analysis.** Data are presented as means ± SEM. Statistical analyses were performed using Student’s t test or one-way ANOVA from GraphPad Prism 5.0 (GraphPad Software, Inc.). A p value of <0.05 was regarded as significant.

### Table I: Antibody list

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Isotype</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>IHC and IF antibodies</td>
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<tr>
<td>C5aR</td>
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<td>Abcam</td>
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<td>Mouse monoclonal</td>
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<td>iNOS</td>
<td>Rabbit polyclonal</td>
<td>1:200</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Flow cytometry antibody</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antibody</td>
<td>Isotype</td>
<td>Dilution</td>
<td>Source</td>
</tr>
<tr>
<td>C5aR-PE Cy7</td>
<td>Rat anti mouse</td>
<td>1:100</td>
<td>Biolegend</td>
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<tr>
<td>CD45 Percp Cy5.5</td>
<td>Rat anti mouse</td>
<td>1:100</td>
<td>BD</td>
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<tr>
<td>CD11b APC Cy7</td>
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<td>BD</td>
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<td>F4/80 PE</td>
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<td>BD</td>
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<td>Ly6G FITC</td>
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<tr>
<td>Secondary antibodies</td>
<td>Conjugate</td>
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<td>Cell Signaling Technology</td>
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### Table II: Quantitative realtime-PCR primer sequences

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<th>Reverse primer</th>
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Complement C5a receptor mediates Ang II-induced cardiac inflammation and remodeling

Supplemental Figure I. Complement receptor C3aR and C5aR were up-regulated in heart after Ang II infusion.

A and B. C3aR (A) and C5aR (B) mRNA levels in Ang II-infused WT mouse hearts at day 0, 1, 3, or 7 were determined by qRT-PCR. Values were normalized to GAPDH. (n=4 in each group). * P<0.05 comparing with day 0.

Supplemental Figure II. C5aR deficiency did not affect blood pressure, cardiac function
or cardiac hypertrophy in Ang II-treated mice. A. Systolic blood pressure was measured by the tail-cuff method at day 0, 3 or 7 after Ang II infusion. B. EF (ejection fraction) value (%) and FS (shortening fraction) value (%) of mice were evaluated at day 7 by cardiac ultrasonography. (n=6 mice per group) C. WGA Staining was used to evaluate cardiac hypertrophy at day 7. ~300 cardiac myocyte cross section areas (CSA) per mouse were measured (scale bars, 50μm).

Supplemental Figure III: Analysis of C5aR-expressing cells in the hearts of Ang II-treated mice. A. Representative IHC staining showing that C5aR-expressing cells are detected in peri-vascular areas in the hearts of WT but not C5aR−/− mice treated with Ang II (scale bars: 50μm). B. CD45+CD11b+ (macrophages/neutrophils), CD45+CD11b− (lymphocytes), CD45−CD31+ (endothelia cells) and CD45−CD31− (fibroblasts and cardiomyocytes) cells from Ang II-infused (7 days) mouse hearts (n=4 in each group) were sorted by magnetic beads, and C5aR mRNA expression was measured by real time-PCR. C. Flow cytometry analysis shows a time-dependent increase in CD45+ leukocyte number in WT mouse hearts after Ang II infusion. Among the CD45+ cells, C5aR expression is primarily detected on F4/80+CD11b+ cells (macrophages) with a smaller population of
Ly6G$^+$CD11b$^+$ (neutrophils) also being detected.

Supplemental Figure IV. C3aR deficiency did not reduce cardiac inflammation in Ang II-treated mice. **A.** IHC staining of Mac-2 as macrophage markers in saline and Ang II-treated mouse hearts showed no difference between WT and C3aR$^{-/-}$ mice (scale bars, 100μm). **B** and **C.** Real time-PCR analysis of CD11b (B) and the cytokines IL-1β, CCL2 CCL3(C) mRNA levels in the hearts of Ang II-treated WT and C3aR$^{-/-}$ mice showed no difference tissue (n=3 mice in each group). Mice were treated with Ang II for 7 days.
Supplemental Figure V. C5aR expression on bone marrow-derived cells contributes to cardiac inflammation and fibrosis. A. Infiltration of inflammatory cells (CD45+) into the hearts of Ang II-treated chimera mice was positively correlated with C5aR expression on BM cells. B-E Cardiac fibrosis as measured by IHC staining of TGF-β1, α-SMA and Masson staining (B-D) or by real time-PCR measurement of α-SMA, Collagen I and Collagen III mRNA levels in the hearts of Ang II-treated chimera mice showed positive correlation with C5aR expression on BM cells (scale bars in B-D, 100μm). Chimera mice treated with Ang II for 7 days. N=4-6 mice in each group.
Supplemental Figure VI: Anti-C5 mAb treatment ameliorates cardiac fibrosis in Ang II-treated mice. A and B, IHC staining of the pro-fibrotic cytokine TGF-β1 (A) or α-SMA (B) in the mouse hearts showed that anti-C5 mAb treatment reduced their expression (scale bars, 100μm for both). C. Masson staining of fibrotic areas confirmed that anti-C5 mAb treatment ameliorated cardiac fibrosis the mouse hearts. Mice were infused with saline or Ang II for 7 days and data are representative of n=4 mice per group.