Complement-Mediated Macrophage Polarization in Perivascular Adipose Tissue Contributes to Vascular Injury in Deoxycorticosterone Acetate–Salt Mice

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Objective—We have previously shown an increased expression of complement 3 (C3) in the perivascular adipose tissue (PVAT) in the deoxycorticosterone acetate (DOCA)–salt hypertensive model. This study aims to examine the role and underlying mechanism of C3 in PVAT for understanding the pathogenesis of hypertensive vascular remodeling further.

Approach and Results—The role of C3 in macrophage polarization was investigated using peritoneal macrophages from wild-type and C3-deficient (C3KO) mice because we found that C3 was primarily expressed in macrophages in PVAT of blood vessels from DOCA-salt mice, and results showed a decreased expression of M1 phenotypic marker in contrast to an increased level of M2 marker in the C3KO macrophages. Bone marrow transplantation studies further showed in vivo that DOCA-salt recipient mice had fewer M1 but more M2 macrophages in PVAT when the donor bone marrows were from C3KO compared with those from wild-type mice. Of note, this macrophage polarization shift was accompanied with an ameliorated vascular injury. Furthermore, we identified the complement 5a (C5a) as the major C3 activation product that was involved in macrophage polarization and DOCA-salt–induced vascular injury. Consistently, in vivo depletion of macrophages prevented the induction of C3 and C5a in PVAT, and ameliorated hypertensive vascular injury as well.

Conclusions—The presence and activation of bone marrow–derived macrophages in PVAT are crucial for complement activation in hypertensive vascular inflammation, and C5a plays a critical role in DOCA-salt–induced vascular injury by stimulating macrophage polarization toward a proinflammatory M1 phenotype in PVAT. (Arterioscler Thromb Vasc Biol. 2015;35:598-606. DOI: 10.1161/ATVBAHA.114.304927.)

Key Words: complement • macrophage polarization • perivascular adipose tissue • vascular injury

Perivascular adipose tissue (PVAT), surrounding the systemic blood vessels, is thought to be an important, active component of the vasculature, with pivotal roles in vascular health and disease.1–3 Our previous study has demonstrated that complement 3 (C3) deposition notably increased in the PVAT of deoxycorticosterone acetate (DOCA)–salt hypertensive models.4 The complement system is an important component of innate immunity.5,6 On activation of the complement system, the formation of C3 convertases results in the cleavage of the central molecular mechanisms underlying complement-mediated vascular inflammation in PVAT remain poorly understood.

Chronic low-grade inflammation within the PVAT is an important casual factor of vascular disorders.9,10 The infiltration and activation of macrophages found dispersed throughout PVAT are responsible for the inflammatory processes in hypertensive models.11 Two primary macrophage subsets in the adipose tissue have now been well characterized. One subset is termed as classically activated M1 macrophage and the other is considered as alternatively activated M2 macrophage. M1 macrophages produce proinflammatory cytokines, including interferon-γ (IFN-γ), inducible nitric oxide synthases (iNOS), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α). M2 macrophages are linked to the activation of anti-inflammatory mediators, including arginase-1 (Arg-1), found in inflammatory zone-1, IL-10, and chitinase 3-like-3 (YML).12–15 Several reports show that complement activation induced inflammatory factor expression in macrophages.16,17 However, the mechanism underlying complement-mediated...
macrophage activation and their causal roles in hypertensive vascular injury are still not clear. Here, we investigate the role of complements in macrophage activation in PVAT of DOCA-salt hypertensive mice. Through bone marrow transplantation (BMT) approaches, we demonstrate the involvement of complement-mediated macrophage polarization in DOCA-salt hypertension-related vascular inflammation. Our findings show that C3-deficiency results in decreased M1 macrophage phenotype markers and increased M2 macrophage phenotype markers. Bone marrow–specific C3-deficiency ameliorated DOCA-salt–induced hypertensive vascular hypertrophy and fibrosis, associating with shifting macrophage phenotype to less M1 and more M2.

Materials and Methods

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

Results

Complements Are Expressed in Macrophages in the PVAT of DOCA-Salt Hypertensive Mice

To investigate the role of complement expression in hypertensive blood vessels, we used a mouse DOCA-salt hypertension model. As shown in Figure I in the online-only Data Supplement, DOCA-salt treatment resulted in a dramatic increase in media:lumen ratio (Figure 1A) and arterial fibrotic area (Figure 1B), and the fibrotic area in DOCA-salt mice seemed to be more diffused in the adventitia around PVAT. C3 expression in mesenteric arteries was detected by immunostaining. We found that C3 was preferentially expressed in the PVAT rather than mesenteric arterial layers and primarily colocalized with F4/80-positive macrophages (Figure 1A), suggesting that macrophages are the major source of C3 in the PVAT. We also detected significantly increased expression of C3a and C5a, the products of C3 downstream activation (Figure 1B). These results suggest a potential role of complement activation and macrophages in hypertension-related vascular injury.

Complements Regulate Macrophage Polarization

To delineate the role of C3 in macrophages, we first investigated the expression of C3 in peritoneal macrophages with different phenotypes. We found that C3 mRNA expression increased in macrophages polarized toward a proinflammatory M1 phenotype by lipopolysaccharide treatment (Figure 2A), whereas C3 reduced in macrophages polarized toward an anti-inflammatory M2 phenotype by IL-4 treatment (Figure 2B). To assess the function of C3 in macrophage phenotype transition further, we cultured peritoneal macrophages from wild-type (WT) and C3KO mice and analyzed macrophage phenotype marker expression. We found that C3KO macrophages had a significantly decreased expression of M1 markers (IFN-γ, iNOS, IL-6, and TNF-α) after lipopolysaccharide treatment compared with WT macrophages (Figure 2C; Figure IIA in the

![Figure 1](http://atvb.ahajournals.org/)

Figure 1. Complement system is activated in the perivascular adipose tissue (PVAT) of deoxycorticosterone acetate (DOCA)-salt mice. A, Double immunofluorescence analysis of macrophage (anti-F4/80, green) and complement 3 (C3; red) expression in the PVAT of mesenteric arteries from SHAM operation and DOCA-salt hypertensive mice. B, Immunofluorescence staining of C3a (red) and C5a (green) the PVAT of Sham mice (SHAM) and DOCA-salt mice. 4',6-Diamidino-2-phenylindole (DAPI) was used to detect nucleus (n=6 per group). Scale bar, 50 μm. L indicates lumen; P, PVAT; and V, mesenteric artery.
online-only Data Supplement). In contrast, C3 macrophages had an increased expression of M2 markers (Arg-1, found in inflammatory zone-1, IL-10, and YM-1) after IL-4 treatment (Figure 2D; Figure IIB in the online-only Data Supplement).

In addition, we investigated the role of C3 in the activation of key transcription factors involved in macrophage phenotype transition, such as p65 nuclear factor kappa B and signal transducers and activators of transcription 6 (STAT6). C3KO macrophages decreased p65 phosphorylation in response to proinflammatory stimuli (lipopolysaccharide), but increased STAT6 phosphorylation in response to anti-inflammatory stimuli (IL-4; Figure 2E). Furthermore, we performed chromatin immunoprecipitation assays to demonstrate the activity of p65 and STAT6 in vivo. Results showed that p65 binding to the promoter of Tnfα (M1 marker) was decreased, whereas STAT6 binding to the promoter of Arg1 (M2 marker) was...
increased in C3KO macrophages (Figure 2F). These observations together suggest that C3 is critical for macrophage polarization toward the M1 phenotype by promoting nuclear factor kappa B–dependent transcriptional activity.

**Bone Marrow–Specific Deficiency of C3 Alters Macrophage Polarization**

To determine the role of C3 in macrophage polarization and vascular injury in vivo, we performed BMT assays. Transplanted syngeneic recipient WT mice received bone marrow cells isolated from either donor C3KO mice with green fluorescent protein transgenic gene (BMT-C3KO) or age-matched WT mice with green fluorescent protein gene (BMT-WT). The engraftment of donor cells in lethally irradiated recipient mice was confirmed by the presence of donor-derived green fluorescent protein positive cells in the peritoneal fluid (Figure 3A). Six weeks after BMT, the mice were subjected to DOCA-salt hypertensive model. The engraftment was further confirmed with immunofluorescent analysis of green fluorescent protein positive cells (Figure IIIA in the online-only Data Supplement). We also detected greatly reduced positive C3 immnostaining in the PVAT of BMT-C3KO mice (Figure IIIB in the online-only Data Supplement). Flow cytometric analysis showed no significant difference in the percentages of total macrophage (F4/80+) in PVAT between the 2 groups (Figure 3B). Interestingly, there was a notable decrease in the proportion of M1 macrophage (CD11c+CD206−) and an increase in M2 macrophage (CD11c−CD206+) in the PVAT from BMT-C3KO mice compared with BMT-WT mice (Figure 3C). These were further confirmed by immunofluorescent staining, which showed a similar extent of macrophage infiltration (anti-F4/80) in PVAT between the 2 groups. However, M2 marker (Arg-1) was increased, whereas M1 marker (iNOS) was decreased in BMT-C3KO mice compared with BMT-WT mice (Figure 3D and 3E).

**Bone Marrow–Specific Deficiency of C3 Attenuates DOCA-Salt–Induced Vascular Injury**

To examine the expression of inflammatory mediators in PVAT after BMT-C3KO, we performed protein array analysis. As shown in Figure 4A, proinflammatory molecules (IFN-γ, TNF-α, RANTES, IL-12p70, and MCP1 [monocyte chemotactic protein 1]) decreased, whereas anti-inflammatory molecules (IL-4, IL-10, IL-13, and IL-1Rα) increased in the PVAT.
of BMT-C3KO compared with BMT-WT mice. These were further confirmed by the detection of representative genes (IFN-γ, TNF-α, and IL-10) in the PV AT (Figure 4B). Next, we performed histological analyses of the mesenteric arteries and found that vascular hypertrophy (Figure 4C) and fibrosis (Figure 4D) were largely improved in BMT-C3KO mice.

Bone Marrow–Derived C3 Rescues Vascular Injury in C3-Deficient Mice

Although our data pointed to bone marrow–derived macrophages as the dominant C3-expressing cells in DOCA-salt mice, there nevertheless remained the possibility of a low-level C3 expression in adipocytes and vascular cells potentially playing a critical role in the vascular injury. To assess the role of local C3 expression in the PVAT, we used C3KO mice as recipient, which were transplanted with C3KO or WT marrow and subjected to DOCA-salt hypertensive model. Flow cytometric analysis showed that WT BMT (WT-to-C3KO) increased the percentage of M1 and decreased the percentage of M2 macrophage compared with C3KO BMT (C3KO-to-C3KO; Figure IV A in the online-only Data Supplement). Next, we performed histological analyses of mesenteric arteries and found that vascular hypertrophy (Figure IV C) and fibrosis (Figure IV D) were largely improved in BMT-C3KO mice.

C5a Regulates Macrophage Polarization and Aggravates Vascular Injury

The findings with C3KO macrophages pointed to a potential inflammatory response of the components of the complement system downstream of C3. To provide further evidence for this hypothesis, we assessed the active components generated on C3 cleavage, C3a and C5a.7,8 Results showed that C3a peptide agonist (C3a) had no significant effect on macrophage polarization (Figure V in the online-only Data Supplement), whereas C5a peptide agonist (C5a) treatment of peritoneal macrophages displayed higher mRNA levels for M1 markers (IFN-γ, iNOS, IL-6, and TNF-α), and lower levels for M2 markers (Arg-1, found in inflammatory zone-1, IL-10, and YM-1; Figure 5A and 5B; Figure VIA in the online-only Data Supplement). In addition, C5a peptide agonist treatment abolished the beneficial effect of C3KO on macrophage polarization (Figure VIB in the online-only Data Supplement).

We then examined the effects of C5a on vascular injury in DOCA-salt mice. We first detected a greatly reduced deposition of C5a in the PV AT of BMT-C3KO mice compared with BMT-WT mice (Figure VIIA in the online-only Data Supplement). Systemic administration of C5a peptide agonist resulted in an increase in M1 macrophage (F4/80 and iNOS) staining in PV AT (Figure VIIB in the online-only Data Supplement). The percentages of total macrophage and M1 macrophage (F4/80+CD11C+) were higher with C5a peptide agonist treatment than that with control peptide treatment (Figure 5C). C5a peptide agonist also aggravated vascular hypertrophy and fibrosis in DOCA-salt mice (Figure 5D). These findings suggest that complements regulate macrophage polarization and contribute to the DOCA-salt hypertensive vascular remodeling process largely via C5a.

Macrophage Depletion Reduces C3 and C5a Levels and Attenuates Vascular Injury

To determine the role of macrophages in mediating complement expression and vascular remodeling, we investigated the effect of macrophage depletion by clodronate liposomes (Clod)20 on pathological vascular remodeling in DOCA-salt mice. Flow cytometric analysis of the peritoneal fluid revealed that an efficient depletion of F4/80 positive cells was achieved (Figure 6A). Macrophage depletion attenuated the...
induction of C3 (Figure 6B) and C5a expression (Figure 6C) in DOCA-salt mice, as well as vascular hypertrophy and fibrosis (Figure 6D). These observations suggest that the function of the complement system in hypertensive vascular injury is dependent on macrophages.

Hypertension is well established as a stimulus for vascular hypertrophy and remodeling. It was, therefore, important to determine whether complements altered blood pressure in this model. DOCA-salt treatment was shown to cause a significant increase in blood pressure. BMT or systemic C5a peptide agonist treatment did not alter blood pressure in DOCA-salt mice. Macrophage depletion (Clod) resulted in an augmented increase of blood pressure (Figure VIII in the online-only Data Supplement). These results demonstrate that the effects of complements on perivascular inflammation and vascular remodeling are blood pressure-independent.

**Discussion**

In this study, we provide evidence demonstrating that bone marrow–specific deficiency of C3 protects mice from hypertensive vascular injury. A comparison of the expression patterns of phenotype-specific markers in macrophages allows us to conclude that complements modulate M1/M2 polarization, which in turn, contributes to DOCA-salt hypertensive vascular inflammation. Furthermore, we found that C5a but not C3a regulates macrophage polarization and contributes to hypertension-related vascular injury.

Herein, we first show that complements are expressed in the perivascular macrophages and regulate macrophage...
polarization in DOCA-salt hypertensive mice. The involvement of the complement system in the pathological processes of hypertension-induced target organ damage is well appreciated.21 However, the source and distribution of complements have not been well studied in hypertensive vascular disorders. C3 is essential for the critical steps of complement activation, and mainly produced by the liver, also secreted by activated macrophages at inflammation sites and by adipocytes.22 Several reports have suggested that complement molecules are mainly detected around perivascular areas in animal models.7,8 Macrophage infiltration and activation in PVAT are well documented as for loss of anticontractile function in perivascular fat from aldosterone-induced inflammation.11 It is of interest that the recruitment of macrophages after mineralocorticoid/salt administration has previously been suggested as the key mechanism behind the development of cardiovascular injury,11 but the mechanism by which macrophage activation increases in mineralocorticoid administration is still unclear. In this context, it is also worth noting that macrophages generally infiltrate in perivascular tissue and colocalize with complements. We have provided evidence in this study that complement upregulation induces macrophage activation in DOCA-salt mice. Macrophage depletion decreases complement expression, including C3 and C5a in PVAT, which demonstrates that macrophages in PVAT are the main sources of complements in DOCA-salt mice.

Another important issue, whether the complement system is implicated in the macrophage-mediated hypertensive vascular inflammation, has not been well studied. Here, consistent with a previous report,23 we show that C3 mRNA expression is increased in M1 macrophages, but decreased in M2 macrophages. Besides, C3KO peritoneal macrophages exhibit decreased lipopolysaccharide-induced M1 and enhanced IL-4–induced M2 marker expression. More importantly, BMT experiments reveal that complement expression in bone marrow–derived macrophages rather than resident perivascular cells is responsible for DOCA-salt–induced pathological change in blood vessels. Bone marrow–specific deficiency of C3 results in decreased production of inflammatory factors in PVAT and amelioration of DOCA-salt–induced vascular injury. It is probably that mineralocorticoid/salt administration induced complement activation in the monocytes and macrophages, which permeate through the endothelium of blood vessels and infiltrate to PVAT. Complement activation
promotes M1 but attenuates M2 macrophages and interacts with other cell types and mediators within the perivascular microenvironment to cause pathological changes. Although macrophage depletion with clodronate liposomes results in increased blood pressure in DOCA-salt mice, macrophage blockade contributes to complement inhibition and amelioration of vascular injury in DOCA-salt mice. These data are comparable with previous findings, which show that macrophage ablation, through depletion of lysozyme M-positive monocytes or deletion of macrophage colony-stimulating factor, attenuates hypertension-induced vascular dysfunction in animal models. 

Taken together, our data first provide definitive in vivo evidence that complements regulate hypertensive vascular remodeling processes via regulating macrophage polarization in PVAT. However, in addition to macrophages, PVAT includes adipocytes and other cells as well. Although our present work reveals a significant role of complement-mediated macrophage polarization in DOCA-salt–induced vascular injury, the other cells, especially the abundant adipocytes in the PVAT, may also contribute to hypertensive vascular injury via a distinct pathway.

The factors involved in the complement pathway in DOCA-salt–induced vascular injury are also a point of interest. On activation of the complement system, the formation of C3 convertases results in the cleavage of C3 to C3b and C3a. Deposition of C3b on cell surfaces is important for opsonization and phagocytosis. The released anaphylatoxin C3a and C5a that derives from C5 cleavage trigger further immune reactions on binding to their cellular receptors. Previous studies show that C1q, C3a, C3c, C5a and C5b-9 are observed around blood vessels in angiotensin-II–induced hypertension. Among these, C3a and C5a are potent monocyte/macrophage chemoattractants. C3a induces exaggerated growth, a synthetic phenotype and angiotensin-II production in spontaneously hypertensive rats–derived vascular smooth muscle cells. It is also known that C3a can enhance the secretion of lipopolysaccharide-induced IL-1β in monocytes. However, our data suggest that the C3a peptide agonist does not regulate macrophage polarization in vitro, although the role of C3a peptide agonist in perivascular inflammation remains to be investigated. We have found that the C5a peptide agonist enhances M1 polarization and attenuates M2 polarization. C5a peptide agonist administration in vivo aggravates DOCA-induced vascular hypertrophy and fibrosis, accompanied by increased M1 macrophages in PVAT. Our finding is in line with a recent report showing that C5a but not C3a mediates hypertensive cardiac inflammation and remodeling in angiotensin-II–induced hypertension. However, we first provide evidence that the change of macrophage polarization induced by bone marrow–derived C5a in PVAT is the major cellular mechanism for complement-mediated pathological processes in the DOCA-salt hypertensive vascular injury model. Taken together, we propose a model: DOCA-salt administration initiates perivascular macrophages–derived complement cascade activation and subsequently induces C5a production, which promotes M1 inflammatory macrophage recruitment and exacerbates hypertension-related vascular hypertrophy and fibrosis.

In conclusion, our findings provide a novel insight that perivascular immune microenvironment regulated by complement cascade contributes to hypertension-related vascular inflammation, in which C5a-mediated macrophage polarization is crucial for the pathological processes in PVAT. Given the importance of complement-mediated macrophage activation in PVAT, the potential changes of perivascular adipocytes and adipokines (well known to be involved in the regulation of vascular health and diseases) deserve to be investigated further in the future. Notwithstanding the complexity of human hypertension compared with the mouse model, our unique observations indicate an attractive possibility that the complement system could serve as therapeutic tools for inhibiting hypertensive vascular injury.

Acknowledgments

We thank Prof Ren and Dr Jiao for their help with the bone marrow transplantation, Dr van Rooijen for providing clodronate liposomes, and Prof Yu for valuable suggestions and critical reading of the article.

Sources of Funding

This study was supported by National Natural Science Foundation of China (81230071, 81100096, 81100184), National Key Project for Basic Research (2011CB503905), National High-tech R&D Program (2012AA02A516), grants from the Shanghai Municipal Commission of Health and Family Planning (2011Y173).

Disclosures

None.

References


Significance

Accumulating evidence indicates that perivascular adipose tissue is an active component of the vasculature. Recent studies have shown that complement deposition in perivascular area is crucial for several vascular disorders. However, the detailed cellular and molecular mechanism for complement-mediated perivascular adipose tissue dysfunction remains incompletely defined. This study reveals that complement-mediated macrophage activation in perivascular adipose tissue contributes to deoxycorticosterone acetate-salt-induced vascular injury. By performing bone marrow transplantation experiments, we found that complement 3 expressed on bone marrow–derived macrophages contributed to perivascular inflammation. Furthermore, complement 5α administration in vivo accelerated vascular injury via altered macrophage polarization in perivascular adipose tissue. Collectively, our data suggest a pathological role of macrophage-derived complement activation in hypertension-related perivascular inflammation and vascular injury.
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Arterioscler Thromb Vasc Biol. 2015;35:598-606; originally published online January 8, 2015; doi: 10.1161/ATVBAHA.114.304927
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Detailed methods

Animals
C3-deficient (C3KO) mice on a C57BL6/J background were purchased from The Jackson Laboratory. Green fluorescent protein (GFP) transgenic (GFPtg) mice (C57BL/6 background) were obtained from Model Animal Research Center of Nanjing University. GFPtgC3KO mice were generated by crossing GFPtg and C3KO mice. Genotyping was performed to detect the presence of both the GFP and C3 genes. All animal procedures were approved in accordance with institutional guidelines established by the Committee of Ethics on Animal Experiments at the Chinese Academy of Sciences.

Peritoneal macrophage isolation and culture
Peritoneal macrophages were isolated 4 days after an i.p. injection of 3% Thioglycollate Broth (Sigma) from 6 to 7 weeks old wild type (WT) mice and C3KO mice as previously described.1 Macrophages were then cultured in 1640 containing 50 U/mL penicillin/50 µg/mL streptomycin with 10% FBS. M1 macrophage activation was stimulated by 100 ng/ml LPS for 24 hours. M2 macrophage activation was achieved by 10 ng/ml of IL-4 (R&D, 404-ML-010) for 24 hours.

RNA sample preparation and qRT-PCR analyses
Total RNA was extracted from cultured macrophages and adipose tissue using TRIzol (Invitrogen) followed by chloroform extraction according to the manufacturer’s protocol. Aliquots of total RNA were reverse transcribed into single-stranded cDNA by incubation with moloney murine leukemia virus reverse transcriptase (Promega). Real-time qRT-PCR was performed with SYBR Premix Ex Taq kits with ROX (TaKaRa) according to manufacturer’s instructions. Signals were detected on an ABI PRISM 7900 machine (Applied Biosystems). β-actin was used as a standard reference. Reactions were done at 95 °C for 30 sec followed by 40 cycles of 95 °C for 5 sec, 60 °C for 30 sec. Sequences of primers used in this study are provided in Online Table I.

Western blot analysis
The differentiation of 3T3-L1 preadipocytes to adipocytes was performed as previously described.2 Cells were lysed in a radioimmune precipitation assay buffer (RIPA) supplemented with protease inhibitor phenylmethysulfonyl fluoride (PMSF, Sigma-Aldrich) and cocktail (Roche). Protein was run on a 12% SDS page gel and blotted onto PVDF membrane (Millipore) by wet transfer. The blots were first incubated with p-p65 (3033), t-p65 (8242), p-Stat6 (9361) and t-Stat6 (9362) antibodies (CST technology) diluted 1:1000 in blocking buffer containing PBS, 1% Milk, and 0.02% Tween 20. Secondary HPR conjugated antibody in blocking buffer was applied for 2 hours at room temperature. Following 3 washes in PBS containing 0.02% Tween 20 for 10 minutes per wash, the signal was detected by chemiluminescence.

Chromatin Immunoprecipitation
Peritoneal macrophages were subjected to chromatin immunoprecipitation based on EZ-ChIP protocol 17-371 (Millipore).3 Briefly, treatment with 1% formaldehyde was used for cross-linking, and cell lysate was collected and sonicated to shear DNA. Soluble chromatin was rotated for 2 hr
at 4 °C with 100 µl of proteinA-agarose-salmon sperm DNA (Millipore). Precleared lysate was incubated overnight at 4 °C with 2 µg of anti-p65 or anti-Stat6 antibodies. The antibody-protein-DNA complexes were precipitated with 100 µl protein A-agarose beads at 4 °C for 2 h. Complexes were eluted in elution buffer (0.1 mM NaHCO₃ and 1% SDS) before reversal of cross-links overnight at 65 °C under high salt conditions (0.5 M NaCl). After proteinase K digestion, DNA was extracted in 25:24:1 phenol/chloroform/isoamyl alcohol and precipitated overnight in ethanol at -20 °C, and DNA was then eluted in water. The presence of TNFα and Arg1 gene promoter sequences in immunoprecipitated DNA was identified by 35 cycles of PCR using the primer sequences listed in Online Table II. In control samples, primary antibody was replaced with non-immune IgG. All experiments were repeated at least three times.

Bone marrow transplantation and DOCA-salt treatment
Bone marrow transplantation analyses were performed as previously described. Bone marrow was isolated from 5 to 6 weeks old GFPtg or GFPtgC3KO donor mice. Recipient mice (6 weeks old) were subjected to 10-Gy lethal dose irradiation and 4 hours later received 10⁷ bone marrow cells from donor mice. Six weeks later, DOCA-salt hypertension was created as we previously described. Briefly, mice underwent uninephrectomy and a 1-cm incision between the shoulder blades was made through which 50mg DOCA pellets (Innovative Research of America, 21-day release) were implanted subcutaneously. DOCA-salt mice were given water containing 1.0% NaCl and 0.1% KCl. Average systolic blood pressure (SBP) was measured by noninvasive tail-cuff method. For the tail-cuff method, mice were trained 3 days every week, after which SBP was measured during a fixed time period of the day. Three to five measurements were averaged for each mouse.

Histological analysis
Mesenteric arteries from DOCA-salt mice were subjected to measurement of vascular remodeling as described previously. Third-order mesenteric artery including perivascular adipose tissue was isolated and fixed with 4% paraformaldehyde for 24 hours in situ. Vessels were then processed for paraffin embedding, and cut into 4-µm transverse sections for hematoxylin-eosin (H&E) and picrosirius red staining. Morphometric analysis was performed using Image-Pro Plus software to assess vascular hypertrophy by measuring the medium and lumen area. Fibrotic staining was expressed as a percentage of stained areas (red) to the total areas examined.

Immunofluorescence staining
Paraffin sections were first deparaffinized and rehydrated. After boiling in 10 mM sodium citrate buffer to unmask antigens, the slides were blocked in buffer containing 5% normal goat serum, incubated with primary antibody at 4°C overnight, followed by incubation with the fluorochrome-conjugated secondary antibody (Invitrogen). Primary antibodies used were as followings: F4/80 (Rat, eBioscience, 14-4801), iNOS (Rabbit, Santa Cruz), Arg1 (Mouse, BD Biosciences, 610708), GFP (Rabbit, Abcam, ab290), C3 (Goat, Cappel/MP Biomedical), C3a (Rabbit, Comp Tech, A218), C5a (Rabbit, Comp Tech, A221), Mac3 (Rat, BD Biosciences, 550292). The results were analyzed using a laser scanning confocal microscope system (Leica TCS SP5).
Flow cytometry analysis
Single-cell suspensions were prepared as previously described. Mesenteric arteries including adipose tissue were carefully dissected, minced with opposing scalpels, and digested with collagenase (Sigma Aldrich, 0.2 mg/ml) in DMEM with 0.5% FBS at 37°C. After 1 hour of digestion, the digested tissue was filtered through 100 µm cell strainers filters (BD Falcon). Cells were collected by centrifugation (at 300g, 5min), and labeled with fluorochrome-conjugated antibody to F4/80 (1 in 200, AbD Serotec, MCA497A488T), CD11c (1in 100, AbD Serotec, MCA1369A647T), and CD206 (1 in 100, AbD Serotec, MCA2235PET) to differentiate M1 from M2 macrophages. Cells were washed and fixed in 1% paraformaldehyde and acquired on a BD FACSCalibur flow cytometer and with FlowJo software.

Cytokine protein array
Semi-quantitative sandwich-based antibody arrays (RayBio Mouse Cytokine Array G-Series) were used for cytokine profiling according to the manufacturer's instructions. After blocking each array with a blocking buffer, arrays were incubated with PVAT samples. Following extensive washing to remove non-specific binding, the cocktail of biotinylated detection antibodies were added to the arrays. After extensive washing, the array slides were incubated with a streptavidin-conjugated fluor (HiLyte Fluor™ 532, from Anaspec, Fremont, CA). The fluorescent signals were then visualized using laser-based scanner system (GenePix 4000B, Molecular Dynamics, Sunnyvale, CA) using the green channel. To increase the accuracy, two replicates per antibody were spotted, and the averages of the median signal intensities for both spots (minus local background subtraction) were used for all calculations. A hierarchical clustering of samples was performed using Cluster 3.0 software (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm). Heat maps were then generated by using R-project (http://www.r-project.org).

Synthesis of C3a and C5a peptide agonist
The C3a peptide agonist (C3a) and C5a peptide agonist (C5a), which can interact with their respective receptors, were synthesized using Fmoc-based solid phase synthesis as previously described. The amino acids compounds of peptides were as followings: C3a, NYITE LRRQH ARASH LGLAR-COOH, C3a control, EAYKQ RYEDR LELRI ELIG-COOH, C5a, (YSFKP MPLaR), C5a control, (FaPRM KYPAL). Peritoneal macrophages were treated with control peptide or C3a (or C5a, 100 nmol/L), and then were polarized toward a M1 or M2 phenotype. For in vivo experiments, DOCA-salt mice were treated with daily intraperitoneal injections of C5a (10 µg/mouse) or control peptide.

Depletion of macrophages
Clodronate (Clod) and phosphate-buffered saline (PBS) liposomes were kindly provided by Dr. N. van Rooijen (Vrije Universiteit, Amsterdam, The Netherlands). To eliminate macrophages in vivo, mice were injected intraperitoneally with 100 µl liposomes on 2 day before and days 7, 14 after DOCA-salt treatment. Successful macrophage depletion was monitored in peritoneal fluid on day 21 by flow cytometric analysis of F4/80 positive cells.

Statistical analysis
Statistical analysis was carried out using SPSS 19 (SPSS). Comparisons of experimental
groups were analyzed by Student’s t test (two groups) or 1-way ANOVA followed by the post-hoc Dunnett’s test for data with more than two groups (Levene’s tests for equal variance). Dunnett’s T3 test was used as post-hoc test comparison for the analysis of unequal variances (Welch’s and Brown-Forsythe’s test). Values are represented as mean ± SD. The significance level was set at P < 0.05.

References

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Online Table I. Quantitative PCR primers.

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<th>Gene</th>
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<th>Reverse primer sequence</th>
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<tr>
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<td>5’-ctgatattcacaactggtgcgc-3’</td>
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Online Table II. Chromatin Immunoprecipitation PCR primers.
Supplemental figure I Vascular hypertrophy and fibrosis in DOCA-salt hypertensive mice. Representative H&E and picrosirius red staining of cross sections of mesenteric arteries from SHAM and DOCA-salt induced mice (left). Scale bar: 50 μm. Quantification of medium-lumen ratio and arterial fibrotic area (right). * P < 0.05 vs SHAM, t-test (n=6 per group).
**Supplemental figure II** C3-deficiency (C3KO) alters iNOS and Arg1 expression. Peritoneal macrophages were extracted prior to further treatment with 100 ng/ml LPS or 10 ng/ml IL-4 for 24 hr before cell lysis. Lysates were blotted on SDS-PAGE for iNOS, Arg1 and actin expression. The bottom panel shows the statistical analysis of 3 independent experiments. Data are presented as mean ± SD and statistical analyzed by 1-way ANOVA followed by the post-hoc Dunnett’s test.
Supplemental figure III A, Representative Immunofluorescence staining of GFP in PVAT from control (con) and bone marrow transplantation (BMT) mice. B, Representative Immunofluorescence staining of C3 in PVAT from BMT-WT and BMT C3KO mice.
Supplemental Figure IV Bone marrow-derived C3 alters macrophage polarization and rescues vascular injury in C3-deficient mice. A, Single-cell suspensions of mesenteric arteries including adipose tissue from C3-deficient mice with C3-deficient marrow transplantation (C3KO-to-C3KO) and C3-deficient mice with WT marrow transplantation (WT-to-C3KO) were analyzed by flow cytometric analysis with antibodies against F4/80 and CD11c. B, Representative H&E and picrosirius red staining of cross sections of mesenteric arteries from C3KO-to-C3KO and WT-to-C3KO hypertensive mice. Data are presented as mean ± SD, * P < 0.05 vs C3KO-to-C3KO, t-test (n=5 per group).
Supplemental figure V C3a agonist peptide (C3a) has no effect on M1 and M2 phenotype marker expression. Peritoneal macrophages were pretreated with C3a or control peptide (Con) for 18h and then were polarized toward an M1 phenotype with 100ng/ml LPS (A) or M2 phenotype with 10ng/ml IL-4 (B) for 24 hr. Data are presented as mean ± SD and statistical analyzed by 1-way ANOVA followed by the post-hoc Dunnett’s test.
Supplemental figure VI C5a agonist peptide (C5a) alters iNOS and Arg1 expression. A, Peritoneal macrophages pretreated with C5a or control peptide (Con) for 18h were extracted prior to further treatment with 100 ng/ml LPS or 10 ng/ml IL-4 for 24 hr before cell lysis. The right panel shows the statistical analysis of 3 independent experiments. B, Wild type (WT) or C3-Deficient (C3KO) Peritoneal macrophages pretreated with C5a for 18h were extracted prior to further treatment with 100 ng/ml LPS or 10 ng/ml IL-4 for 24 hr before cell lysis. Lysates were blotted on SDS-PAGE for iNOS,Arg1 and actin expression. The right panel shows the statistical analysis of 3 independent experiments. Data are presented as mean ± SD and statistical analyzed by 1-way ANOVA followed by the post-hoc Dunnett’s test.
Supplemental figure VII C5a agonist peptide (C5a) enhances M1 macrophage polarization in the perivascular adipose tissue (PVAT) of DOCA-salt mice. A, Immunofluorescence staining of C5a in the PVAT of BMT-WT and BMT-C3KO DOCA salt mice. B, M1 subtype was analyzed by immunofluorescent analysis of F4/80 and iNOS in the PVAT of C5a treated mice or control peptide (Con) treated mice. Scale bar: 50 μm.
Supplemental figure VIII Tail cuff systolic blood pressure (SBP) was measured in DOCA-salt hypertensive mice.