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Complement regulator CD59 protects against angiotensin II-induced abdominal aortic aneurysms in mice

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Abstract

Background—Complement system, an innate immunity, has been well documented to play a critical role in many inflammatory diseases. However, the role of complement in pathogenesis of abdominal aortic aneurysm (AAA), which is considered as an immune and inflammatory disease, remains obscure.

Methods and Results—Here, we evaluated the pathogenic roles of complement membrane attack complex (MAC) and CD59, a key regulator that inhibits MAC, in the development of AAA. We demonstrated that in the angiotensin II-induced AAA model, deficiency of MAC regulator CD59 in *ApoE*-null mice (*mCd59ab*$^{-/-}$/*ApoE*$^{-/-}$) accelerated the disease development, while transgenic over-expression of human CD59 (*hCD59*$^{ICAM\text{-}2+/-}$/*ApoE*$^{-/-}$) in this model attenuated progression of AAA. The severity of aneurysm among these three groups positively correlates with C9 deposition, and/or the activities of MMP2 and MMP9, and/or the levels of phosphor (p)-c-Jun, p-c-Fos, p-IKK-α/β, and p-65. Furthermore, we demonstrated that MAC directly induced gene expression of MMP2 and MMP9 *in vitro*, which required activation of AP-1 and NF-κB signaling pathways.

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Conflict of Interest Disclosures

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Conclusions—Together, these results defined the protective role of CD59 and shed light on the important pathogenic role of MAC in AAA.

Keywords

CD59; complement; complement regulation; MMP2 and MMP9; abdominal aortic aneurysm; signaling pathway

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Abdominal aortic aneurysm (AAA) is characterized by chronic aortic wall inflammation and destructive connective tissue remodeling, including depletion of aortic elastin and fragmentation of medial elastic fibers. AAA is a leading cause of sudden death in aging (> 55 years) men1. Rupture of AAA accounts for 4% of all deaths in people over the age of 65. Risk factors for AAA include age, male gender, atherosclerosis, hypertension, and genetic predisposition though atherosclerosis is considered to be a main cause of AAA2. Enzymatic degradation of elastic lamellae and extracellular matrix (ECM) proteins are underlying characteristics of AAA3. It is well-established that abnormally increased protease activity in aortic tissue plays a critical role in the pathogenesis of aortic dissection and aneurysm. Matrix metalloproteinases (MMPs) such as MMP2 and MMP9 are the predominant proteinases in AAA wall2. In animal models, the development of aneurysms can be suppressed by pharmacologic inhibition (i.e. by tetracycline derivatives) of MMP2 and MMP9, or genetic alterations that eliminate the expression of either proteinase4. Elastolytic cysteine proteases including cathepsins also play a critical role in the development of aneurysm5. AAA is also considered to be an immune and inflammatory disease and macrophages, lymphocytes, and mast cells participate in its development1. However, the role of complement, a main effector of immunity and inflammation, remains unclear.

The complement system consists of about 30 soluble and membrane-bound proteins, and is activated by different sequential activation cascades (the classical, alternative, and lectin pathways) either on the pathogens or in plasma. These pathways converge in one terminal cascade that leads to formation of the membrane attack complex (MAC), a macromolecular pore capable of inserting itself into cell membranes and lysing heterologous cells and bacteria. To protect autologous cells from MAC-mediated attack, an array of complement regulators including CD59 have evolved to restrict complement activation. CD59, a glycosylphosphatidyl inositol (GPI)-linked membrane protein, strongly restricts MAC formation by binding to complement proteins C8 and C9 and preventing C9 incorporation and polymerization.

MAC is also an important mediator of cellular signals that trigger mitogenic effects. Insertion of the MAC allows the release of growth factors such as bFGF and PDGF, and cytokines such as IL-1 and MCP-1, which autocrinally and paracrinically- stimulate proliferation, inflammation, and thrombosis[6, 7]. *In vitro* studies indicate that sublytic MAC activates several cellular signaling pathways such as PI3/Akt (serine/threonine) kinase pathway8, 9, the nuclear factor NF-κB, and the activator protein-1 (AP-1)10, 11.

Extensive evidence from human and animal studies indicates a protective role of CD5912,13 and an atherogenic role of MAC in the pathogenesis of atherosclerosis14. However, only a few human studies (and no animal studies) have examined the role of MAC in the pathogenesis of aneurysm15, 16. Here we investigate the role of CD59 and MAC in the pathogenesis of the AAA induced by angiotensin (Ang II) in $ApoE^{-/-}$ mice, either deficient in mouse CD59a and mouse CD59b ($mCd59ab^{-/-}/ApoE^{-/-}$ described in14,17) or transgenically over-expressing human CD59 (hCD59) in endothelial and circulating cells ($hCD59^{ICAM\text{-}2+/-}/ApoE^{-/-}$ as described in14,18).

METHODS

Animal studies were approved by the Harvard Medical School Institutional Animal Care and Use Committee.

Animal models and AAA production and quantification

Both *mCd59ab*$^{-/-}$ and *hCD59*$^{ICAM\text{-}2+/-}$ mice were in C57BL/6 (B6) genetic background as described in[17, 18]. The hCD59 in *hCD59*$^{ICAM\text{-}2+/-}$ mice is selectively expressed in endothelial cells, macrophages, and platelets[19]. In these transgenic strains, over-expression of hCD59 is effective in providing additional protection against mouse complement[18, 19]. We crossed each of the two strains with *ApoE*$^{-/-}$ mice (B6 background, Jackson Laboratory) to produce *mCd59ab*$^{-/-}$/*ApoE*$^{-/-}$ and *hCD59*$^{ICAM\text{-}2+/-}$/*ApoE*$^{-/-}$ mice, respectively. The successful generation of these compound mice was determined by both PCR methods (for genotyping as described in Supplemental Figure 1A–C), and FACS analysis of mCd59a and mCd59b proteins in *mCd59ab*$^{-/-}$/*ApoE*$^{-/-}$'s erythrocytes (for confirming the physical absence of mCd59a and mCd59b) or hCD59 in the *hCD59*$^{ICAM\text{-}2+/-}$/*ApoE*$^{-/-}$'s platelets [for confirming the over-expression of hCD59 (Data not shown)].

It has been established that the low dose of Ang II used is associated with a significant (30–40%) increase in blood pressure[20]. The high blood pressure may participate in the pathogenesis of Ang II-induced AAA20 and Ang II may contribute to medial macrophage accumulation associated with elastin degradation21.

Since in hyperlipidemic mice, the propensity for the development of AAA is much more common in males than females (as is the case in humans), we selected males as experimental animals. To induce AAA, three-month-old male mice were infused with 1000 ng/kg/min Ang II (Calbiochem, San Diego, California, USA) subcutaneously delivered by Alzet model 2004 osmotic minipumps (DURECT Corp., Cupertino, California, USA) as described in[22], and simultaneously fed a high fat diet (C12108; Research Diets Inc.) for one month23, 24. After fasting the mice overnight, we sacrificed them by CO_2 asphyxiation, drew blood by heart puncture, and stored the sera at −80°C. The aorta was viewed with a dissection microscope. The per adventitial tissue was carefully removed from the aortic wall as described in[22]. The maximal diameter of each aneurysm was measured with a digital caliper. The segment of the aorta from the aortic arch to renal artery was weighed, and one half of the AAA lesion was preserved with OTC and stored at −80°C (for immunoassays). The other half was stored at −80°C for extraction of proteins for western analysis and gelatin zymography. For the detailed procedures of immunofluorescence, histology and gelatin zymography, please see the online-only Data Supplement.

Reporter Assays

pGL2-AP-1-Luc and pGL2- NF-κB-Luc luciferase reporters, with AP-1 and NF-κB response elements cloned into the pGL2-Luc plasmid, respectively, were generous gifts from Drs. Zhu and Rosenfeld[25]. Mouse endothelial cells (2×10^4 each well) were transfected by Lipofectamine 2000 (Invitrogen) with pGL2-AP-1-Luc or pGL2- NF-κB-Luc luciferase reporter plasmid (see details in the online-only Data Supplement).

RNA Interference

Endothelial cells (2.5×10^5 per well, 6-well plates) were transiently transfected using Lipofectamine RNAiMA X (Invitrogen) 5μl/each well with 50nM of mouse p50, mouse c-jun, and siGENOME non-targeting siRNA Pool™ (Dharmacon). The sequences of these siRNA are listed in Supplemental Table 1. After 36 hrs incubation, cells were stimulated

with MAC (C5b6: 24 μg/ml and + for C7, C8 or C9: 24 μg/ml) for an additional 12 hrs, and proteins or RNA were isolated and subjected to western blot and Zymography, or real-time RT-PCR analysis respectively.

Quantitative real-time PCR analysis

Total RNA was isolated from the cultured mouse endothelial cells C166 (ATCC) treated with C5b-9 assembled and siRNA for 12 hours with Trizol reagent (Invitrogen). Real-time PCR was performed using SYBR Green in a real-time PCR machine (iCycler; Bio-Rad). We used the primers (listed in Supplemental Table 2) for detecting p50, c-Jun, MMP2, and MMP9 RNA levels: To normalize expression data, 18s rRNA was used as an internal control gene.

Western Blotting

The proteins were isolated from the mouse AAA or mouse endothelial cells treated with MAC. Primary antibodies for Western blotting including rabbit Phospho-p65 antibody (Cell Signaling), rabbit Phospho-IKK-α/β antibody (Cell Signaling), rabbit Phospho-c-Jun antibody (Cell Signaling), rabbit Phospho-c-Fos antibody (Abcam), rabbit c-Jun antibody (Santa Cruz Biotechology), rabbit p65 monoclonal antibody (Cell Signaling), rabbit IKK-α/β antibody (Santa Cruz Biotechology), rabbit p50 antibody (Santa Cruz Biotechology), rabbit c-Fos monoclonal antibody (Cell Signaling), and mouse anti-mouse β-actin antibody (Santa Cruz Biotechology), which were diluted in blocking buffer, were incubated with the membranes. The final concentrations for these antibodies are listed in Supplemental Table 3 (see details in the online-only Data Supplement).

Statistical analysis

Experimental results are shown as the mean ± SEM. The comparison between two groups or three groups was examined with a nonparametric Mann-Whitney test or nonparametric Kruskal-Wallis test respectively. The details of real-time PCR result analysis are in the supplement materials. *P* values of less than 0.05 were considered significant. The difference in the mortality rate was analyzed by Chi-Square test. All statistical tests with $P< 0.05$ were considered significant.

RESULTS

Protective role of CD59 against AAA development

We used the $ApoE^{-/-}$ either deficient in mCd59a and mCd59b ($mCd59ab^{-/-}/ApoE^{-/-}$) or over-expressing hCD59 in the endothelial cells, macrophages and platelets ($hCD59^{ICAM\text{-}2+/-}/ApoE^{-/-}$) to investigate the role of CD59 and MAC in the AAA. Here, we induced the AAA by the infusion of Ang II to these models for the study of the role of CD59 in the pathogenesis of AAA. $mCd59ab^{-/-}/ApoE^{-/-}$ developed significantly more severe AAA assessed by diameter and weight of the aneurysms (Figs. 1A–C, and supplemental figure 2) and associated with higher mortality rate than $ApoE^{-/-}$ (Fig. 1D). In contrast, the mice transgenic for hCD59 in the endothelial and hematopoietic cells ($hCD59^{ICAM\text{-}2+/-}/ApoE^{-/-}$) developed significantly less severe AAA than $ApoE^{-/-}$ mice (Figs. 1A–D). The incidence rates of the development of AAA in $mCd59ab^{-/-}/ApoE^{-/-}$, $ApoE^{-/-}$, and $hCD59^{ICAM\text{-}2+/-}/ApoE^{-/-}$ mice are 100%, 75% and 75% among the survival mice, respectively. Also, $mCd59ab^{-/-}/ApoE^{-/-}$ mice in the aortas of the AAA had much lower collagen content than $ApoeE^{-/-}$ mice, while $hCD59^{ICAM\text{-}2+/-}/ApoE^{-/-}$ mice had higher collagen content than $ApoE^{-/-}$ mice (Fig. 1E and supplemental figure 3A). An additional difference between $mCd59ab^{-/-}/ApoE^{-/-}$ aneurysms and the others was that $mCd59ab^{-/-}/ApoE^{-/-}$ had more disrupted elastic lamellae than other two group mice (Fig. 1F). In

addition, the cholesterol and triglyceride levels among these groups were not significantly different (Supplemental figure 3B and 3C). The systolic blood pressure among three different group mice after Ang II infusion was not significantly different (Supplemental figure 4), which indicates that either CD59 deficiency or hCD59 over-expression in *ApoE*$^{-/-}$ background mice does not change blood pressure response to Ang II and HFD treatments compared to that in *ApoE*$^{-/-}$. Together, these results suggest that CD59 protects the mice against AAA development.

MAC deposition in the AAA lesions

CD59 is well known as a key inhibitor of MAC formation. To further investigate the underlying mechanisms, by which CD59 protects against the development of AAA, we analyzed MAC deposition in AAA lesions. Staining of the aneurysm sections with anti-C9-specific antibodies revealed that *mCd59ab*$^{-/-}$*/ApoE*$^{-/-}$ mice had significantly more extensive deposits of C9 than *ApoE*$^{-/-}$ mice, and *ApoE*$^{-/-}$ mice has significantly more deposits of C9 than *hCD59*$^{ICAM\text{-}2+/-}$*/ApoE*$^{-/-}$ mice (Fig. 2A and supplemental figure 5A). Analysis of adjacent sections with H&E staining demonstrated that the C9 deposition was both in thrombi (Fig 2B1 and 2C1) and granulation tissue (Fig. 2B2, 2B3, 2C2, and Fig. 3C3). Immunofluorescence staining of AAA showed that *mCd59ab*$^{-/-}$*/ApoE*$^{-/-}$ mice had significantly more macrophages than *ApoE*$^{-/-}$ mice, which in turn had significantly more macrophage cells than *hCD59*$^{ICAM\text{-}2+/-}$*/ApoE*$^{-/-}$ mice (Fig. 2D and supplemental figure 5B). Together, these results are consistent with a pathogenic role of the MAC in the development of AAA in our experimental mice.

MAC up-regulates MMP2 and MMP9 activities

MMP2 and MMP9 play essential roles in the pathogenesis of AAA. Inhibition of and deficiency for MMP2 and MMP9 have been shown to attenuate AAA development in humans and animals [26]. Increased MMP2 and MMP9 activities contribute to degradation of elastin and type IV collagen in tissues including arteries[27], which could explain the greater disruption in elastic lamellae and the lower collagen content in AAA lesions of *mCd59ab*$^{-/-}$*/ApoE*$^{-/-}$ mice (Fig. 1F). For these reasons, we assessed the MMP2 and MMP9 activities in AAA lesions, by gelatin zymography[28]. AAA aortic extracts from *mCd59ab*$^{-/-}$*/ApoE*$^{-/-}$ mice exhibited significantly higher MMP2 and MMP9 activities, while extracts from *hCD59*$^{ICAM\text{-}2+/-}$*/ApoE*$^{-/-}$ mice showed lower activities of than *ApoE*$^{-/-}$ mice (Figs. 3A and 3B).

The fact that MAC deposition and MMP activity correlated with the severity of AAA in our animal models prompted us to investigate whether MAC up-regulates MMP2 and MMP9 activities *in vitro*. MAC treatment increased MMP2 and MMP9 activities in the mouse vascular smooth muscle (VSMC), endothelial and macrophage cells compared to C5b6 alone, which does not lead to MAC assemble, or PBS treatment (Figs. 4A–C). These results indicate that MAC may stimulate the cells and increase MMP2 and MMP9 activities, which may contribute to the development of accelerated AAA lesions in *mCd59ab*$^{-/-}$*/ApoE*$^{-/-}$ mice.

MAC up-regulates MMP2 and MMP9 activities through AP-1 and NF-κB signaling pathways

It has been demonstrated that sublytic MAC induces increased activity of c-Jun NH2-terminal kinase JNK1 in many cells (including endothelial cells and smooth muscle cells29) and activation of NF-κB and AP-110، 11. In order to assess the effect of MAC on these signaling pathways, we analyzed the expression of the phosphorylated c-Jun (p-c-Jun), c-Fos (AP-1 components), p65 (an NF-κB component), and IKK-α/β (a NF-κB regulator) in the AAA lesions of three groups of mice or in the endothelial cell cultures challenged by MAC *in vitro*. Western blot assay revealed that the AAA in *mCd59ab*$^{-/-}$*ApoE*$^{-/-}$ mice had

significantly higher levels of p-c-Jun, p-c-Fos, p-IKK-α/β, and p-p65, while AAA from *hCD59*$^{ICAM\text{-}2^{+/-}}$*/ApoE*$^{-/-}$ mice had significantly lower levels of than *Apoe*$^{-/-}$ mice (Fig. 5A). In contrast, total levels of c-Jun, IKK-α/β and p65 did not differ among the three groups (Supplemental figure 6A). This indicates that the activation of these signaling pathways may play a critical role in the development of AAA in our models. Consistent with these results, we also demonstrated that MAC assembly in mouse endothelial cells directly induced significantly higher levels of p-c-Jun, p-c-Fos, p-IKK-α/β, and p-p65 than C5b6 alone *in vitro* (Fig 5B and supplemental figure 6B), confirming the previous finding that MAC activates these signaling pathways *in vitro* [11, 30, 31], These results, together with the facts that AP-1 is composed of c-Jun and c-Fos, and p65 is a major component of NF-κB [32, 33], indicate that MAC is capable of triggering signaling pathways upstream of AP-1 and NF-κB.

To directly evaluate whether MAC up-regulates the activity of AP-1 or NF-κB, we used a firefly luciferase reporter gene assay to measure their transcriptional activity upon MAC assembly on endothelial cells. The reporter gene activity is directly under the control of AP-1 and NF-κB response elements. As shown in Figure 5C, MAC induced significantly higher levels of AP-1, and NF-κB transcriptional activity than C5b6 alone or PBS treatment (Fig. 5C). These results indicate that MAC assembly on the cells transcriptionally up-regulates AP-1 and NF-κB.

Furthermore, to investigate whether AP-1 and NF-κB signaling pathway activations are involved in the up-regulation of MMP2 and MMP9 mediated by MAC *in vitro*, we used RNA interference against c-Jun, and p50, another component of NF-κB, to suppress these pathways. The up-regulation of MMP2 and MMP9 mRNA and proteins mediated by MAC was attenuated by both p50 (Fig. 5D) and c-Jun RNAi (Fig. 5E). RNAi efficacy was validated at the level of both mRNA and protein (Supplemental figures 7A and 7B). Together, these results suggest that MAC-activated c-Jun and NF-κB signaling pathways participate in the up-regulation of MMP2 and MMP9, which may in turn contribute to the pathogenesis of AAA in our animal models.

DISCUSSION

These results we reported here provide strong support for a protective role of CD59 against the development of AAA and indicate that MAC may indeed play a critical role in the pathogenesis of AAA. The role of complement and MAC in AAA has only been suggested in human studies and has not extensively been studied in experimental models. Patients with vasospasm showed a twofold increase in plasma C3d levels when the spasm occurred, whereas no significant changes in C3d concentration could be detected in aneurysm patients without spasm or in patients with hematoma unrelated to aneurysm rupture34. Specific gene array analysis revealed that increased gene expression was associated with proteinase, reactive oxygen species, growth factors, chemokines, complement, adhesion molecules, and apoptosis in both the intima and the media of aneurysmal walls[35]. Moreover, immunohistochemistry studies of human saccular cerebral artery aneurysm (SCAA) demonstrated that MAC localized consistently in a decellularized layer in the outer wall, and were found in all SCAA samples. The percentage of MAC-positive area relative to the total SCAA wall surface area was greater in ruptured than in un-ruptured SCAAs[16].

Moreover, a recent study demonstrates that C3a and C5a play a critical role in the development of elastase-induced abdominal aortic aneurysm[36]. Together, these data suggest that complement activation and MAC formation are involved in aneurysm wall degeneration and rupture. We used genetic strategies to investigate the role of MAC in AAA. We identified a critical role of CD59 in protecting against AAA and demonstrated that MAC

may play a critical pathogenic role in AAA. It is notable that the deficiency of CD59 results in mild complement-mediated hemolytic anemia and platelet activation[17, 37, 38]. The hCD59 in *hCD59*$^{ICAM-2}$ is expressed not only in endothelial[18] but also in circulating cells including macrophages (see supplemental figure 8) and platelets[14] because the hCD59 transgenic in *hCD59*$^{ICAM-2}$ mice is under the control of ICAM-II[19]. The protective effects in *hCD59*$^{ICAM-2+/-}$*/ApoE*$^{-/-}$ mice against the development of AAA are contributed by the expression of hCD59 in these cells. However, the cellular mechanism of hCD59 protective role remains unclear. In addition, CD59 also has a complement-independent function in regulating NK, B and T cell activities[39, 40], which may also contribute to the AAA. Thus, the mechanisms underlying CD59 complement independent function and MAC-mediated cellular dysfunction in the development of AAA require further investigation.

Cellular response to MAC formation can be classified into two groups (lytic and sublytic) along a response continuum. Lytic MAC formation results in colloidoosmotic swelling and lysis of the target41. Sublytic MAC formation results in release of growth factors and cytokines, activation of cellular signaling, and and/or reprogramming of gene expression. When the pore opens transiently, it can generate significant changes in membrane permeability and internal composition of autologous cells without compromising their viability. Thus, lytic MAC can induce lethal colloido-osmotic swelling in cells such as gram-negative bacteria and heterologous erythrocytes. On the other hand, sublytic MAC mediates non-lethal physiological and/or pathological responses and activates many signaling pathways in autologous cells7. These cellular signaling pathways include 1) elevated Ca^{2+} via Ca^{2+} influx through transient pore in cells, which partially activates PKC and other cellular signaling pathways42; 2) G protein coupled-activation of Ras, Raf-1, MEK, ERK-1 pathway and increased activities of ERK-1, c-jun NH2-terminal kinase JNK1 and p38 MAPK in many cells including endothelial cells and smooth muscle cells30; 3) activation of the PI3/Akt kinase pathway9, 30; 4) activation of NF-κB and AP-1 signaling pathways11, 31; and 5) activation of the JAK1/STAT3 pathway[29]. Among these signaling pathways, c-Jun and NF-κB activation pathways have been demonstrated to play a critical role in the AAA *in vivo*[43, 44]. Here, we demonstrated that AAA lesions in *mCd59ab*$^{-/-}$*/ApoE*$^{-/-}$ had significantly higher, while that of *hCD59*$^{ICAM-2+/-}$*/ApoE*$^{-/-}$ had significantly lower levels of p-c-Jun p-c-Fos, p-IKK-α/β, and p-65 than those in *ApoE*$^{-/-}$. The fact that the activation of AP-1 and NF-κB signaling pathways positively correlated with the severity of the AAA among our experimental mice confirms some of these findings and further suggests that MAC is an important of signaling activator and may play a critical role in the pathogenesis of chronic inflammatory diseases such as AAA.

Whether MAC induces up-regulation of MMP2 and MMP9, and which MAC-triggered signaling pathways are involved in the pathogenesis of AAA have not been examined up to now. Here, we demonstrate that 1) MAC induces the production of MMP2 and MMP9, 2) MMP2 and MMP9 activities were correlated with the severity of AAA, 3) MAC-induced up-regulation of MMP2 and MMP9 activities is related to the activation of AP-1 and NF-κB signaling pathways. Together, these results shed light on the important pathogenic role of MAC in the AAA, and point towards the molecular mechanism of MAC-activated signaling pathways in the development of aneurysm and atherosclerosis.

Extensive evidence indicates that MMP2 and MMP9 play essential roles in the pathogenesis of AAA[26] and the primary source of MMP-9 production is the macrophages in human and mouse AAA tissues[45, 46]. In the AAA lesions of *mCd59ab*$^{-/-}$*/Apoe*$^{-/-}$ mice, the macrophage content is significantly increased as shown in Figure 2D and Supplemental figure 5B. This is consistent with the severity of AAA and higher levels of MMP2 and MMP9 in these lesions. We previously demonstrated that MAC induced endothelial dysfunction in *mCd59ab*$^{-/-}$*/ApoE*$^{-/-}$, which contributes the atherogenesis[14]. MAC also

mediates the endothelial cell apoptosis *in vitro* [47]. Furthermore, we document that the supernatant obtained from MAC-treated endothelial cells triggers the migration of both MAC-treated and non-MAC treated macrophage *in vitro* (Supplemental figure 9A). The increase of macrophage migration may result from the releases of growth factors and cytokines induced by sublytic MAC assemble in endothelial cells, as we reported previously[6, 7]. Of note, the deficiency of CD59 in mice does not result in the decreased number of monocytes (Supplement figure 9B). Together, these findings let us to conclude that increased MMP expression in the aorta with AAA could be caused by 1) increased macrophage infiltration, 2) up-regulation by increased MAC tissue levels or some combination of these two mechanisms.

CLINICAL PERSPECTIVE

Aneurysm including abdominal aortic aneurysm (AAA) is also considered to be an immune and inflammatory disease. The complement is a main effector of the immune response and inflammation. However, the role of complement in the aneurysm pathogenesis has not been extensively investigated. The complement system is activated by three activation cascades, which lead to formation of the membrane attack complex (MAC). MAC is a macromolecular pore capable of inserting itself into cell membranes and lysing heterologous cells and bacteria, and an important mediator of cellular signals including the nuclear factor NF-κB, and the activator protein-1 (AP-1) that trigger mitogenic effects. To protect autologous cells from MAC, an array of complement regulators including CD59 have evolved to restrict complement activation. CD59 strongly restricts MAC formation. Here, we demonstrated that in the angiotensin (Ang) II-induced AAA model, deficiency of CD59 in Apoe-null mice accelerated the AAA development, while transgenic over-expression of CD59 attenuated the AAA progression. The severity of aneurysm positively correlates with C9 deposition, the activities of MMP2 and MMP9, and the levels of phosphorylated (p)-c-Jun, p-c-Fos, p-IKK-α/β and p-p65. Furthermore, we demonstrated that MAC directly induced gene expression of MMP2 and MMP9 *in vitro*, which depended on the activation of AP-1 and NF-κB signaling pathways. Together, these results shed light on the important pathogenic role of MAC in aneurysm, point towards the molecular mechanism of MAC-activated signaling pathways in aneurysm, and suggest inhibition of MAC may provide a novel approach for the treatment/prevention of aneurysm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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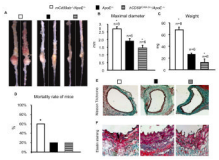

Figure 1. Protective role of CD59 in AAA

A. Representative aneurysm lesions from *mCd59ab*$^{-/-}$/*ApoE*$^{-/-}$, *ApoE*$^{-/-}$ and *hCD59*$^{ICAM\text{-}2+/-}$/*ApoE*$^{-/-}$ (see supplemental figure 2 for full presentation of aneurysm lesions). **B–D**.: The difference in the maximal diameters (mm) (b), the weight of aneurysm (c) and mortality rates among the experimental mice infused by Ang II and simultaneously fed a HFD for one month. The data are displayed by mean ± sem. *P<0.05 *vs ApoE*$^{-/-}$. The difference in the mortality rate among the groups after one month Ang II and HFD treatment was analyzed by X^2 test. **E**. The representation of Massons trichrome stain demonstrated that lower collagen content in the aneurysm lesions from *mCd59ab*$^{-/-}$/*ApoE*$^{-/-}$ and higher collagen contents in aneurysm lesion of *hCD59*$^{ICAM\text{-}2+/-}$/*ApoE*$^{-/-}$ compared to those from *ApoE*−/− (see supplemental figure 3A for quantitative analysis of the result). (**F**). The representative picture of elastin staining demonstrated that the medial elastin was more degraded in the aneurysm lesions of *mCd59ab*$^{-/-}$/*ApoE*$^{-/-}$ mice, and less degraded in the aneurysm lesions of *hCD59*$^{ICAM\text{-}2+/-}$/*ApoE*$^{-/-}$ and *ApoE*$^{-/-}$ mice.

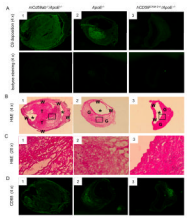

Figure 2. Characterization of aneurysms

A. C9 deposition in the AAA lesions of mice (see supplemental figure 5A for quantitative analysis of the result). **B and C: Representative image of AAA H&E staining**. B1. The wall (W) of the aorta is severely diluted. Most of lumen (*) has been occluded by a thrombus (T) shown in high magnification (X20) in Fig. C1. It is within the thrombus that C9 staining (Fig. 2A1) is most intense. A2. The much of wall (W) of the aorta has been thickened by proliferative granulation tissue (G), fibroblasts, and collagen, some of inflammatory cells and edema fluid shown in higher magnification (X20) Fig. C2. A3. Much of the wall (W) has been thickened by granulation tissue (G), which in this case is less edematous than in figure C3, shown in higher magnification (X20). **D**. CD68 for the detection of macrophage content in lesion areas (see supplemental figure 5B for quantitative analysis of the result).

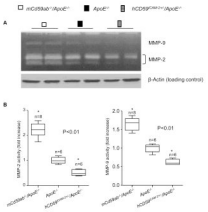

Figure 3. MMP2 and MMP9 in the mouse AAA wall

A. Gelatin gel zymography to detect MMP activity in aortic tissue extracts from different groups of mice (Top panel). The same amount of total proteins from each group was demonstrated by β-actin detection (Bottom panel). **B**. Densitometric analysis of MMP activity changes compared to the density of MMP activity in $ApoE^{-/-}$ mice. The data from the different mice (n) are represented by boxplots. **P*<0.01 *vs* $ApoE^{-/-}$.

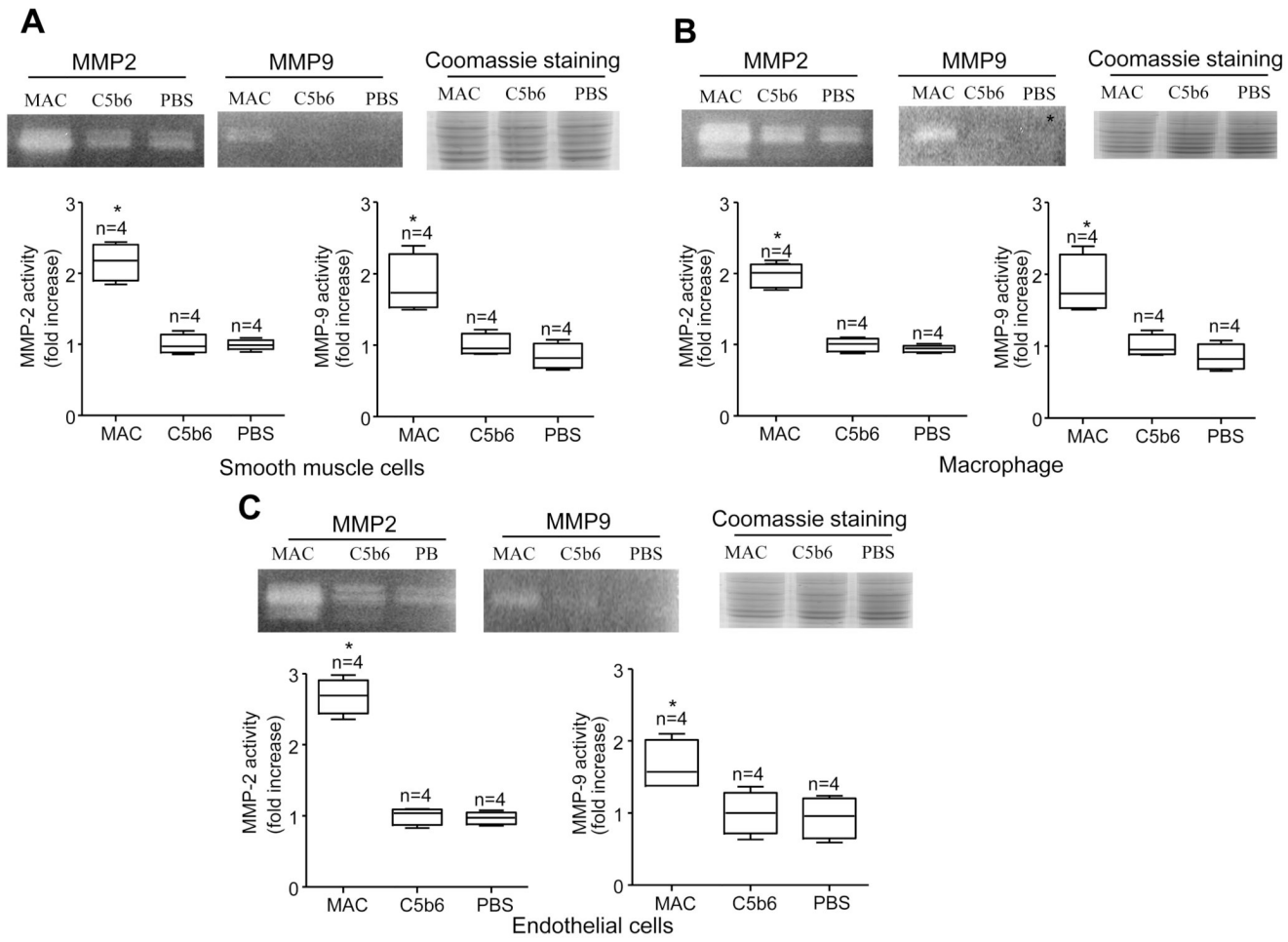

Figure 4. MAC treatment increases MMP activities in mouse cell lines

A–C. Activities of MMP2 or MMP9 from the cell extracts of the mouse smooth muscle (A), macrophage (B), and endothelial cells (C) 12 hours after MAC treatment (assembled by C5b6: 24 μg/ml and + for C7, C8 or C9: 24 μg/ml) on the cells. The MMP activities were examined by gelatin zymography. Coomassie staining of SDS-PAGE showed equal protein loading. Fold changes in the MMP activities treated by MAC assemble compared to that treated with C5b6 alone. The data from the independent experiments (n) are displayed by boxplots. *$P<0.05$ *vs* C5b6.

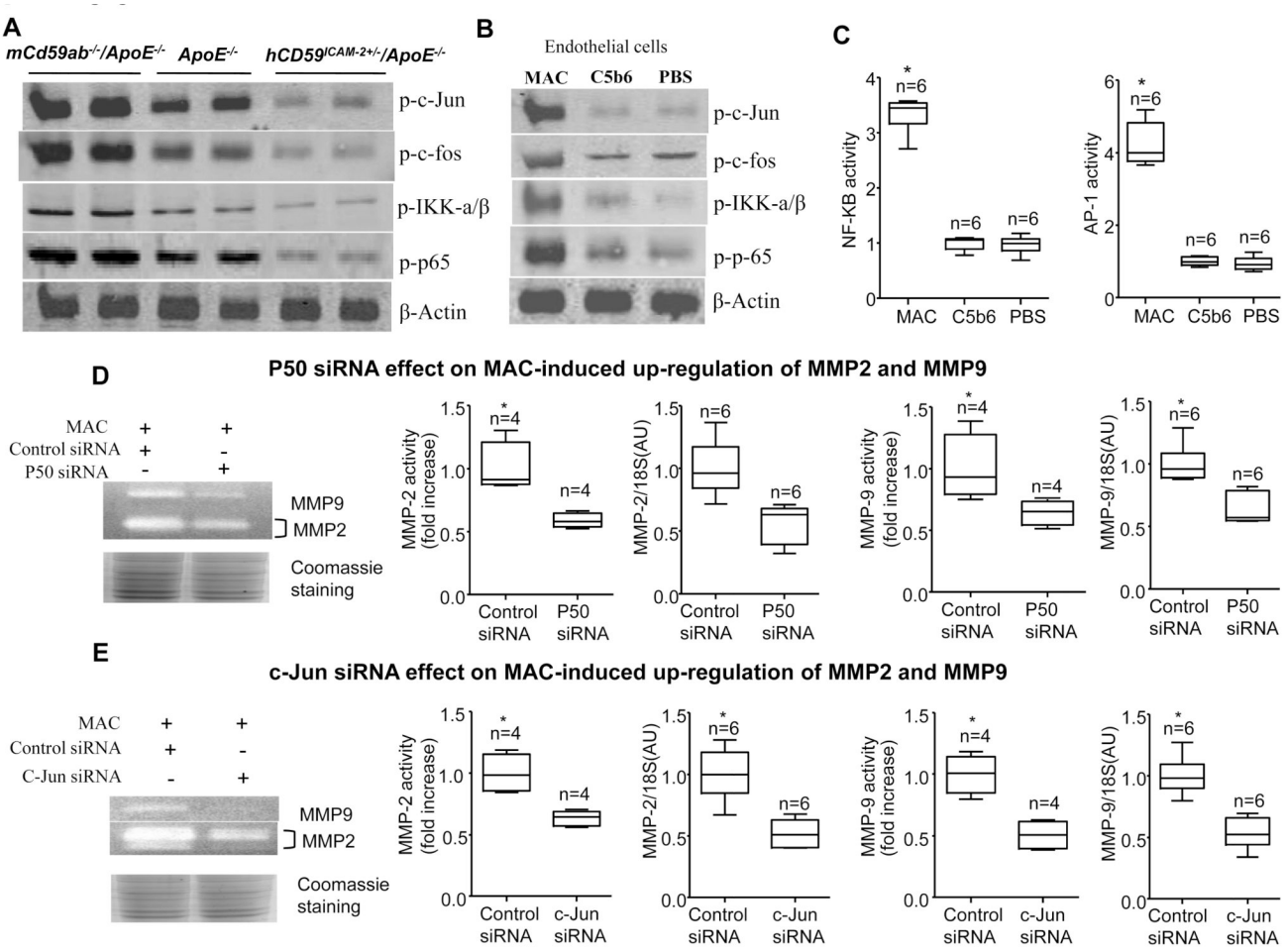

Figure 5. MAC induces MMP2 and MMP9 activities by activating AP-1 and NF-κB transcription factors

A. Representative western blot shows the levels of phosphorylated c-Jun, c-Fos, p65, and IKK-α/β in three groups of mice treated with angiotension-II and fed HFD for 28 days. This experiment was repeated from the AAA lesions of five mice from each group. **B**. Western blot analysis of MAC induced the levels of phosphorylated c-Jun, c-Fos, p65, and IKK-α/β levels in mouse endothelial cell lines (western blot). **C**. MAC induced AP-1 and NF-κB transcription factor activities in mouse endothelial cell lines (reporter assay). Fold changes in these transcriptional factor activities treated by MAC assemble compared to that treated with C5b6 alone. The data from the independent experiments (n) are displayed by boxplots. **P*<0.01 *vs.* C5b6. **D** and **E**. Effects of p50 (**D**) and c-Jun's (**E**) siRNA on MAC-induced up-regulation of MMP2 and MMP9. Coomassie staining of SDS-PAGE showed equal protein loading. MMPs activities were examined by gelatin zymography and were quantified by densitometric analysis. MMPs mRNA levels were detected by real time-PCR. Specific primers detected for MMP2 or MMP9 were listed in supplemental table 2. Fold changes in the MMP activities or levels with specific siRNAs treatment compared to these treated with control siRNA. The data from the independent experiments (n) are displayed by boxplots. **P*<0.05 *vs* control siRNA.