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Absence of Appl2 sensitizes endotoxin shock through activation of PI3K-Akt pathway (Postprint)

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Abstract

Background: The adapter proteins Appl1 (adaptor protein containing pleckstrin homology domain, phosphotyrosine domain, and leucine zipper motif 1) and Appl2 are highly homologous and involved in several signaling pathways. While previous studies have shown that Appl1 plays a pivotal role in adiponectin signaling and insulin secretion, the physiological functions of Appl2 are largely unknown. Results: In the present study, the role of Appl2 in sepsis shock was investigated by using Appl2 knockout (KO) mice. When challenged with lipopolysaccharides (LPS), Appl2 KO mice exhibited more severe symptoms of endotoxin shock, accompanied by increased production of proinflammatory cytokines. In comparison with the wild-type control, deletion of Appl2 led to higher levels of TNF-alpha and IL-1 beta in primary macrophages. In addition, phosphorylation of Akt and its downstream effector NF-kappa B was significantly enhanced. By co-immunoprecipitation, we found that Appl2 and Appl1 interacted with each other and formed a complex with PI3K regulatory subunit p85 alpha, which is an upstream regulator of Akt. Consistent with these results, deletion of Appl1 in macrophages exhibited characteristics of reduced Akt activation and decreased the production of TNF alpha and IL-1 beta when challenged by LPS. Conclusions: Results of the present study demonstrated that Appl2 is a critical negative regulator of innate immune response via inhibition of PI3K/Akt/NF-kappa B signaling pathway by forming a complex with Appl1 and PI3K.Conclusions: Results of the present study demonstrated that Appl2 is a critical negative regulator of innate immune response via inhibition of PI3K/Akt/NF-kappa B signaling pathway by forming a complex with Appl1 and PI3K.

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Full Text

Absence of Appl2 Sensitizes Mice to Endotoxin Shock Through Activation of the PI3K/Akt Pathway

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Abstract

Background: The adapter proteins Appl1 (adaptor protein containing pleckstrin homology domain, phosphotyrosine domain, and leucine zipper motif 1) and Appl2 are highly homologous and involved in several signaling pathways. While previous studies have shown that Appl1 plays a pivotal role in adiponectin signaling and insulin secretion, the physiological functions of Appl2 are largely unknown.

Results: In the present study, we investigated the role of Appl2 in septic shock using Appl2 knockout (KO) mice. When challenged with lipopolysaccharide (LPS), Appl2 KO mice exhibited more severe symptoms of endotoxin shock, accompanied by increased production of proinflammatory cytokines. Compared with wild-type controls, deletion of Appl2 led to higher levels of TNF- and IL-1 in primary macrophages, and phosphorylation of Akt and its downstream effector NF- B was significantly enhanced. Co-immunoprecipitation revealed that Appl2 and Appl1 interacted with each other and formed a complex with the PI3K regulatory subunit p85 , an upstream regulator of Akt. Consistent with these results, deletion of Appl1 in macrophages exhibited reduced Akt activation and decreased production of TNF- and IL-1 when challenged by LPS.

Conclusions: Our findings demonstrate that Appl2 is a critical negative regulator of innate immune response via inhibition of the PI3K/Akt/NF- B signaling pathway by forming a complex with Appl1 and PI3K.

Keywords: Appl2, Appl1, endotoxin shock, inflammatory cytokines, PI3K, Akt, NF- B

Background

Severe sepsis, or septic shock, represents one of the oldest problems in medicine. In the United States, severe sepsis accounts for 2% of patients admitted to the hospital [1]. Septic shock is caused by severe infection with invading microbes that produce endotoxin from Gram-negative bacteria or analogous molecules from Gram-positive bacteria or fungi. Endotoxins are bacterial lipopolysaccharides (LPS) and constitute the major component of the outer membranes in Gram-negative bacteria. In Gram-negative sepsis, LPS induces profound activation of macrophages and production of potent inflammatory cytokines such as TNF- , IL-1, and IL-6 [2]. These proinflammatory cytokines act on endothelial cells to cause systemic vasodilation, diminished myocardial contractility, endothelial injury and activation, resulting in disseminated intravascular coagulation. This hypoperfusion in turn leads to multi-organ failure affecting the liver, kidneys, and central nervous system.

LPS elicits its biological effects by forming a complex with CD14, LBP, TLR4, and MD-2, which triggers several intracellular signaling pathways to activate NF- B [3]. NF- B is a transcription factor containing five subunits—RelA/p65, RelB, c-Rel, p105/p50, and p100/p52—which function as homo- or heterodimers. In resting macrophages, NF- B dimers reside in the cytoplasm bound to specific inhibitory I B proteins. When stimulated by LPS, I B kinase (IKK) phosphorylates I B, leading to its degradation and thereby releasing NF- B for nuclear translocation, where it facilitates expression of various inflammatory and stress response genes [4-7]. Jun N-terminal kinase, p38 mitogen-activated protein kinase, and Akt pathways have been reported to activate NF- B. Among these pathways, Akt stimulates NF- B by phosphorylating and activating the NF- B p65/RelA subunit through the PI3K/Akt/IKK/I B/NF- B signaling cascade [8-12].

Appl1 and Appl2 are homologous proteins that bind to diverse transmembrane receptors or signaling proteins. Appl1 was originally identified as an intracellular binding partner of the adiponectin receptor and mediates adiponectin-dependent insulin sensitization in skeletal muscle [13,14]. Appl1 plays an essential role in inflammatory responses that depends on cell type. Specifically, adiponectin was shown to either suppress NF- B activity in endothelial cells and adipocytes or activate the NF- B pathway in synovial or cardiac fibroblasts [15-20]. Appl1 also participates in the PI3K/Akt signaling pathway since it was reported to induce phosphorylation of Akt and stimulate transactivation of the p65 subunit of NF- B through IKK [21]. Appl1 transgenic mice showed less peripheral insulin resistance and cardiac dysfunction in response to high-fat diet, with enhanced Akt phosphorylation and glucose uptake in cardiomyocytes [22]. Additionally, Tan et al. reported that Appl1 possesses growth factor-selective effects on Akt signaling in mouse embryonic fibroblasts [23].

On the other hand, the physiological functions of Appl2 are less clear. Whether Appl2 plays a role in inflammatory responses has not yet been studied. However, since several subsequent studies demonstrated that Appl2 down-regulates adiponectin signaling by forming a Yin-Yang regulatory pair with Appl1, it is important to examine whether Appl2 plays a negative regulatory role in inflammation [24,25]. In this study, we generated Appl2 knockout (KO) mice and investigated their response to LPS-induced endotoxin shock. When challenged with LPS, Appl2 KO mice showed more exacerbated symptoms of endotoxin shock. Accordingly, circulating levels and production of proinflammatory cytokines in Appl2 KO mice and macrophages were markedly elevated compared with controls. Further analysis demonstrated that Appl2 modulated the Akt-NF- B signaling pathway, possibly by competitively binding to the p85 subunit of PI3K. Our results suggest that Appl2 functions as a negative regulator of innate immune response via the Akt pathway.

Results

Generation of Appl2 KO Mice To study the role of Appl2 in inflammatory responses, we examined the expression of Appl2 in mouse primary macrophages during acute LPS stimulation. Appl2 expression decreased steadily in response to LPS (Figure 1), suggesting that Appl2 may have distinct functions in inflammatory responses. To better understand its role in inflammation, we generated Appl2 KO mice as described in the Methods. Deletion of Appl2 in these mice was confirmed by Western blot analysis in primary peritoneal macrophages (Figure 2 [Figure 2: see original paper]).

Appl2 KO Mice Are More Prone to Endotoxic Shock When Challenged by LPS To examine the roles of Appl2 in regulating inflammatory responses, Appl2 KO mice were subjected to LPS challenge and their phenotypes were examined. Mice were intraperitoneally injected with indicated doses of LPS, and survival rates were monitored. At a dose of 20 mg/kg LPS, Appl2 KO mice showed more severe symptoms of endotoxin shock and higher mortality compared with WT mice (Figure 3A [Figure 3: see original paper]-B). Survival analysis showed that the survival rate of Appl2 KO mice was significantly lower than that of WT mice after injection. A critical feature of endotoxic shock is disseminated intravascular coagulation, characterized by widespread blood coagulation and vessel hemorrhage, particularly in the kidney. After challenge with indicated doses of LPS, histological examination of kidneys was performed. Appl2 KO mice exhibited severe hemorrhage in their kidneys when WT mice showed no obvious tissue damage at the same LPS dose (Figure 3C). We subsequently measured TNF- and IL-1 serum levels since these are critical proinflammatory cytokines that respond to endotoxic shock elicited by LPS. Compared with WT mice, serum levels of TNF- and IL-1 were higher in Appl2 KO mice (Figure 3D-E).

Increased Production of TNF- and IL-1 in Appl2 KO Macrophages Macrophages are the major target cells of LPS stimulation that produce proinflammatory cytokines. Therefore, we harvested peritoneal primary macrophages and stimulated them with LPS in vitro. After LPS stimulation, cytokine production secreted into the medium was determined by ELISA. We monitored the kinetics of TNF- and IL-1 expression. Compared with macrophages isolated from WT mice, production of TNF- and IL-1 was significantly enhanced in macrophages from Appl2 KO mice upon LPS stimulation (Figure 4A [Figure 4: see original paper]-B). These results suggest that loss of Appl2 enhances proinflammatory cytokine production in primary macrophages in response to LPS challenge.

Enhanced Activation of Akt-NF- B Pathways After LPS Stimulation in Appl2 KO Macrophages We first examined whether LPS could activate the Akt pathway. As shown in Figure 5 [Figure 5: see original paper], phospho-Akt was enhanced in Appl2 KO mouse macrophages compared with LPS-stimulated WT mouse macrophages. We subsequently examined activation of the p65 subunit of NF- B, which can be regulated through the Akt pathway. In macrophages from Appl2 KO mice, phospho-IKK , I B, and p65 were enhanced compared with WT macrophages (Figure 5). These data suggest that Appl2 can regulate inflammation through the Akt pathway for NF- B activation.

The p85 Subunit of PI3K Associates with Appl2 and Appl1 Appl isoforms interact with many different membrane receptors and other proteins. The MyD88-dependent toll-like receptor 4 (TLR4) signaling pathway is a major receptor for LPS in mediating innate immune response [26]. To further pinpoint the molecular mechanism by which Appl2 modulates inflammation, we first examined whether an interaction exists between Appl2 with TLR4 and MyD88. Immunoprecipitation results demonstrated that Appl2 has weak interaction with TLR4 but no interaction with MyD88. Moreover, Appl2 was found to strongly interact with the p85 subunit of PI3K and Appl1. Meanwhile, we also found Appl1 could interact with the p85 subunit of PI3K and Appl2 (Figure 6 [Figure 6: see original paper]).

Appl1 KO Mice Show Less Severe Endotoxic Shock Than WT Mice To better understand their roles in inflammation, we generated Appl1 KO mice (Figure 7A [Figure 7: see original paper]-B). At 30 mg/kg LPS challenge, Appl1 KO mice showed less severe endotoxic shock and lower mortality compared with WT mice (Figure 7C-D). After challenge with indicated doses of LPS, histological examination of kidneys was performed. Compared with WT mice, Appl1 KO mice showed less widespread hemorrhage in their kidneys (Figure 7E) and lower production of TNF- and IL-1 in serum (Figure 7F-G).

Reduced Production of TNF- and IL-1 and Decreased Activation of Akt-NF- B Pathways After LPS Stimulation in Appl1 KO Macrophages We also examined the kinetics of TNF- and IL-1 expression to monitor dynamic changes in cytokine levels at designated periods after LPS challenge. Compared with macrophages from WT mice, production of TNF- and IL-1 was significantly reduced in LPS-stimulated macrophages from Appl1 KO mice (Figure 8A [Figure 8: see original paper]-B). Appl1 was mainly identified to interact with Akt and increase Akt phosphorylation. Phospho-Akt can stimulate transactivation of the p65 subunit of NF- B through I B kinase. Therefore, we examined whether LPS could activate the Akt pathway. As shown in Figure 8C-D, phospho-Akt was attenuated in Appl1 KO mouse macrophages compared with LPS-stimulated WT mouse macrophages. Phospho-IKK , I B, and p65 were also reduced compared with WT macrophages. These data suggest that Appl1 can regulate inflammation through the Akt pathway for NF- B activation.

Discussion

Mounting evidence demonstrates that Appl1 is an important mediator of insulin sensitivity and inflammatory response in adiponectin signaling pathways [27]. However, the cellular functions of Appl2 and its relationships with Appl1 remain poorly understood [24]. To examine the possible involvement of Appl2 in inflammation, we determined Appl2 levels in primary macrophages in response to LPS stimulation. Appl2 expression decreased gradually, suggesting that Appl2 may be involved. Indeed, Appl2 KO mice showed more severe endotoxin shock compared with WT mice when challenged with LPS.

Since the PI3K/Akt pathway is well known to be essential in LPS-induced acute inflammation [28] and Appl1 was shown to regulate Akt activity and substrate specificity [29], we examined the relationship between Appl2 and Akt in inflammatory reactions. We investigated whether the LPS-stimulated Akt pathway could be affected in primary macrophages from Appl2 KO mice. Interestingly, Akt signaling was enhanced in LPS-treated primary macrophages from Appl2 KO mice compared with those from WT mice. These results appear consistent with previous reports wherein Appl1 and Appl2 facilitated Akt activation through Yin-Yang regulation in muscle cells, and phosphorylation of Akt as well as glucose uptake were enhanced in Appl2 KO mice [24,30]. The higher level of Akt activity may be responsible for the enhanced expression of NF- B and subsequent more aggressive inflammatory reaction in Appl2 KO mice challenged by LPS.

TLRs are a class of proteins that play key roles in the innate immune system and digestive system. Activation of TLR signaling through recognition of pathogen-associated molecular patterns leads to transcriptional activation of genes encoding pro-inflammatory cytokines and chemokines via the PI3K/Akt pathway, which in turn controls activation of antigen-specific adaptive immune response [31]. To understand the precise mechanism by which Appl2 regulates inflammation, we hypothesized that Appl2 might interact with the TLR4 receptor because it has been reported to increase Akt phosphorylation in TLR4 high-expression cell lines [32-34]. Contrary to our expectation, binding between Appl2 and TLR4 was not observed. MyD88 is an important protein in TLR signaling pathways that dimerizes with TLR receptors [26], but binding between Appl2 and MyD88 was also not observed. Some studies showed that MyD88 can form a complex with PI3-kinase, an important regulatory protein of Akt [26,35]. Therefore, Appl2 may be involved downstream of the TLR4 pathway. As expected, PI3K apparently interacted with Appl2. These results are consistent with a previous paper wherein Appl2 suppresses insulin signaling and Akt phosphorylation in a PI3K-dependent manner [30]. Our results also showed that Appl2 can interact with Appl1 when challenged by LPS. These findings indicate that a complex likely forms among Appl1, Appl2, and PI3K, and that Appl2 has a negative effect on activation of the PI3K/Akt pathway and therefore on expression of downstream genes involved in inflammation.

In our experiments, Appl2 KO mice exhibited more severe endotoxin shock symptoms and increased production of serum proinflammatory cytokines when challenged with LPS. Although the precise mechanism is not yet known, it is conceivable that absence of Appl2 enhances the interaction between Appl1 and PI3K and hence further activates the Akt signaling pathway and subsequent inflammatory responses. Indeed, deletion of Appl1 exhibited characteristics of reduced Akt activation and decreased inflammatory response.

Conclusions

Findings of the present study show that Appl2 is an important negative regulator in PI3K/Akt-mediated NF- B activation and therefore serves as a target with great therapeutic potential to curb inflammation.

Methods

Generation of Appl1 and Appl2 KO Mice A 4.7 kb genomic fragment upstream of mouse Appl1 exon 17 and a 4.2 kb genomic fragment downstream from Appl1 exon 18 were used as recombination arms in the construction of the targeting vector. A neomycin-resistance gene (neo) expression cassette was inserted between these two regions, resulting in a vector designed to delete the two RNA-binding motifs in exons 17 and 18. For Appl2 targeting vectors, a 3.4

kb and a 3.6 kb genomic fragment upstream and downstream of mouse Appl2 exon 5 were used as recombination arms, respectively. A neo cassette with the loxP sequence was inserted upstream of exon 5, and another loxP site was cloned downstream of exon 5. Both targeting vectors were constructed according to protocol: C57BL/6J embryonic stem (ES) cells were electroporated with the linearized targeting construct [36,37]. After selection by G418, clones with targeted alleles were identified through polymerase chain reaction (PCR) analysis. ES cells with targeted alleles were injected into blastocysts of C57BL/6J mice. Chimeric males were mated with females of the same strain to obtain heterozygous mutant mice. The Appl1 mice were intercrossed to obtain homozygous and wild-type (WT) mice. Then the Appl2-floxed mice were crossed with EIIA-Cre mice, and homozygous Appl2 KO mice were subsequently generated from these crosses.

Animal and Ethics Statement Male Appl1/2 KO mice on a C57BL/6 background and WT mice were housed in standard specific pathogen-free facilities and maintained on a 12 h light-dark cycle at 22±2°C with standard chow (15.9 kJ/g, 10% of energy as fat, 20% as protein, 70% as carbohydrate). Humane endpoints based on animal behaviors were used to minimize suffering in survival and animal studies. All intraperitoneal injections and sacrifices for organ removal and peritoneal macrophage isolation were performed under anesthesia with sodium pentobarbital (50 mg/kg, i.p.). All experimental procedures were carried out in accordance with NIH Guidelines for the Care and Use of Laboratory Animals. Animal experiments were approved by the Animal Care and Use Committee of Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences.

Survival Studies Male mice (8 weeks old) weighing 21±2 g were treated once with intraperitoneal injection of LPS (Sigma-Aldrich). Appl2 KO and WT mice were injected with LPS at 20 mg/kg body weight (n=9). Mice were returned to their cages after LPS injection and closely monitored every eight hours. Mice were given ad libitum access to food and water at all times. When clinical signs of distress or moribund status were recognized, animals were euthanized with sodium pentobarbital (50 mg/kg, i.p.) followed by cervical dislocation. Clinical signs of endotoxin shock include reduced locomotion, diarrhea, piloerection, and body weight loss. The number of surviving mice was recorded at 24 h, 48 h, and 72 h after LPS challenge, and percent survival rates were calculated from the number of animals that survived divided by the total number of tested animals at each time point. Appl1 KO and WT mice were injected with LPS at 30 mg/kg body weight (n=9), and the survival experiment was performed as described above.

Serum and Tissue Samples Appl1/2 KO and WT control mice were injected i.p. with LPS as described. Whole blood samples were collected into tubes containing anticoagulant by tail incision at 1 h, 12 h, 24 h, 48 h, and

72 h after LPS challenge and incubated at room temperature for 15 min prior to centrifugation at 3,000 × g for 20 min. Tail wounds were wiped with 70% alcohol cotton balls to prevent infection. Serum was extracted and stored at -80°C before processing for cytokine assays.

Histochemistry Mice were euthanized by cervical dislocation under anesthesia with sodium pentobarbital (50 mg/kg, i.p.) 24 h after LPS injection, and kidney tissues were fixed in 4% formaldehyde overnight at room temperature immediately after sacrifice. Tissues were paraffinized and sectioned with a microtome, and slides were stained with hematoxylin and eosin (HE) (Sigma) following standard protocols. Sections were examined by light microscopy.

Isolation and Culture of Peritoneal Macrophages for In Vitro Studies Peritoneal macrophages from C57BL/6 mice and Appl1/2 KO mice were collected as previously described [38]. Briefly, peritoneal macrophages were elicited by intraperitoneal injection of 2 ml of 4% thioglycolate (Gibco) in distilled water. After 4 days, elicited macrophages were collected by peritoneal lavage with 10 ml of Hank’ s balanced salt solution (HBSS). Peritoneal lavage fluids were centrifuged at 1500 rpm for 5 min, and cells were resuspended in RPMI-1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 g/ml streptomycin. Equal numbers of peritoneal macrophages derived from three mice were pooled and seeded in three wells (5×10 cells/well). Peritoneal cells were incubated at 37°C in an atmosphere of 5% CO for 3 h to allow macrophages to adhere. Non-adherent cells were removed by washing twice with PBS, and attached cells were used as peritoneal macrophages.

Quantification of Cytokine Expression Serum cytokine levels and cytokines secreted from primary macrophages were examined by ELISA (R&D). Macrophages were stimulated with 1 µg/mL LPS (Sigma) for specified times, and supernatant was collected for quantification of cytokine (TNF- and IL-1) expression.

Western Blot Analysis Peritoneal macrophages were treated with 1 µg/mL LPS for 20 minutes, washed with ice-cold PBS, and proteins were extracted in RIPA buffer (Beyotime). Protein concentration in lysates was determined using a Bradford assay (Bio-Rad). Cell lysates were resolved by SDS-PAGE and transferred onto polyvinylidene fluoride membranes. Membranes were blotted with antibodies to Akt (Cell Signaling), p65 (Cell Signaling), I B (Cell Signaling), IKK (Cell Signaling), phospho-Akt (Cell Signaling), phospho-IKK (Abcam), phospho-I B (Cell Signaling), phospho-p65 (Cell Signaling), Appl1, and Appl2. Appl1 and Appl2 antibodies were from Aiming Xu.

Immunoprecipitation Peritoneal macrophages were treated with 1 µg/mL LPS for 20 min. Cells were washed with PBS, and proteins were extracted with RIPA buffer (Beyotime) at 4°C for 30 minutes. Cells were centrifuged at 10,000 g for 30 minutes at 4°C to pellet and discard cell debris. Cellular protein (100 g) was mixed with 1 g of anti-Appl1 or Appl2 antibody and incubated overnight at 4°C. Then, 10 l of protein G Plus-agarose (Santa Cruz) was added and incubated for another 4 h at 4°C. After incubation, samples were washed three times with lysis buffer. Washed samples were resuspended in SDS sample buffer (Beyotime) and heated at 100°C for 5 min prior to electrophoresis.

Statistical Analysis To examine the survival rate of Appl1/2 KO and WT mice challenged by LPS, Kaplan-Meier analysis was performed using terminal mortality as the endpoint. Data were presented as mean ± SD for statistical comparison of two samples. Student' s t-test was used for evaluation. Survival curves were generated using the Kaplan-Meier method, and their significance was evaluated using the Log-rank test.

Abbreviations

IL-1 : interleukin 1 ; NF- B: nuclear factor B; TNF- : tumour necrosis factor- ; WT: wild-type; Appl1/2: adaptor protein containing the pleckstrin homology domain, phosphotyrosine domain, and leucine zipper motif 1/2; Akt: protein kinase B; IKK: I B kinase; TLR4: toll-like receptor 4; MyD88: myeloid differentiation factor 88; LPS: lipopolysaccharide; p65: nuclear factor NF-kappa-B p65 subunit; PI3K: phosphatidylinositol 3-kinase; p85 : the p85 subunit of PI3K.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Liufeng Mao, Wanhua Lin, Tao Nie, Xiaoyan Hui, Xuefei Gao, Kuai Li, Xiaofeng Tang, and Mengxiao Ding designed and performed the experiments. Liufeng Mao, Wanhua Lin, Tao Nie, and Xiaoyan Hui contributed to writing the manuscript. Peng Li, Yu Wang, and Pentao Liu contributed to discussion of the manuscript. Aimin Xu and Donghai Wu designed the experiments and wrote and edited the manuscript prior to submission.

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References

1. Martin GS, Mannino DM, Eaton S, Moss M: The epidemiology of sepsis in the United States from 1979 through 2000. N Engl J Med 2003, 348:1546-1554.
2. Ulevitch RJ, Tobias PS: Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. Annu Rev Immunol 1995, 13:437-457.
3. May MJ, Ghosh S: Signal transduction through NF-kappa B. Immunol Today 1998, 19:80-88.
4. Brown K, Gerstberger S, Carlson L, Franzoso G, Siebenlist U: Control of I kappa B-alpha proteolysis by site-specific, signal-induced phosphorylation. Science 1995, 267:1485-1488.
5. Chen Z, Hagler J, Palombella VJ, Melandri F, Scherer D, Ballard D, Maniatis T: Signal-induced site-specific phosphorylation targets I kappa B alpha to the ubiquitin-proteasome pathway. Genes Dev 1995, 9:1586-1597.
6. DiDonato J, Mercurio F, Rosette C, Wu-Li J, Suyang H, Ghosh S, Karin M: Mapping of the inducible IkappaB phosphorylation sites that signal its ubiquitination and degradation. Mol Cell Biol 1996, 16:1295-1304.
7. Scherer DC, Brockman JA, Chen Z, Maniatis T, Ballard DW: Signal-induced degradation of I kappa B alpha requires site-specific ubiquitination. Proc Natl Acad Sci U S A 1995, 92:11259-11263.
8. Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, Donner DB: NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature 1999, 401:82-85.
9. Romashkova JA, Makarov SS: NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. Nature 1999, 401:86-90.
10. Sizemore N, Leung S, Stark GR: Activation of phosphatidylinositol 3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NF-kappaB p65/RelA subunit. Mol Cell Biol 1999, 19:4798-4805.
11. Kane LP, Shapiro VS, Stokoe D, Weiss A: Induction of NF-kappaB by the Akt/PKB kinase. Curr Biol 1999, 9:601-604.
12. Madrid LV, Wang CY, Guttridge DC, Schottelius AJ, Baldwin AS, Jr., Mayo MW: Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-kappaB. Mol Cell Biol 2000, 20:1626-1638.
13. Mao X, Kikani CK, Riojas RA, Langlais P, Wang L, Ramos FJ, Fang Q, Christ-Roberts CY, Hong JY, Kim RY, et al: APPL1 binds to adiponectin receptors and mediates adiponectin signalling and function. Nat Cell Biol 2006, 8:516-523.

14. Cheng KK, Lam KS, Wang B, Xu A: Signaling mechanisms underlying the insulin-sensitizing effects of adiponectin. Best Pract Res Clin Endocrinol Metab 2014, 28:3-13.

15. Cheng KK, Lam KS, Wang Y, Huang Y, Carling D, Wu D, Wong C, Xu A: Adiponectin-induced endothelial nitric oxide synthase activation and nitric oxide production are mediated by APPL1 in endothelial cells. Diabetes 2007, 56:1387-1394.

16. Tian L, Luo N, Zhu X, Chung BH, Garvey WT, Fu Y: Adiponectin-AdipoR1/2-APPL1 signaling axis suppresses human foam cell formation: differential ability of AdipoR1 and AdipoR2 to regulate inflammatory cytokine responses. Atherosclerosis 2012, 221:66-75.

17. Ajuwon KM, Spurlock ME: Adiponectin inhibits LPS-induced NF-kappaB activation and IL-6 production and increases PPARgamma2 expression in adipocytes. Am J Physiol Regul Integr Comp Physiol 2005, 288:R1220-1225.

18. Ouchi N, Kihara S, Arita Y, Okamoto Y, Maeda K, Kuriyama H, Hotta K, Nishida M, Takahashi M, Muraguchi M, et al: Adiponectin, an adipocyte-derived plasma protein, inhibits endothelial NF-kappaB signaling through a cAMP-dependent pathway. Circulation 2000, 102:1296-1301.

19. Hattori Y, Hattori S, Akimoto K, Nishikimi T, Suzuki K, Matsuoka H, Kasai K: Globular adiponectin activates nuclear factor-kappaB and activating protein-1 and enhances angiotensin II-induced proliferation in cardiac fibroblasts. Diabetes 2007, 56:804-808.

20. Tang CH, Chiu YC, Tan TW, Yang RS, Fu WM: Adiponectin enhances IL-6 production in human synovial fibroblast via an AdipoR1 receptor, AMPK, p38, and NF-kappa B pathway. J Immunol 2007, 179:5483-5492.

21. Hupalowska A, Pyrzynska B, Miaczynska M: APPL1 regulates basal NF-kappaB activity by stabilizing NIK. J Cell Sci 2012, 125:4090-4102.

22. Park M, Wu D, Park T, Choi CS, Li RK, Cheng KK, Xu A, Sweeney G: APPL1 transgenic mice are protected from high-fat diet-induced cardiac dysfunction. Am J Physiol Endocrinol Metab 2013, 305:E795-804.

23. Tan Y, You H, Wu C, Altomare DA, Testa JR: Appl1 is dispensable for mouse development, and loss of Appl1 has growth factor-selective effects on Akt signaling in murine embryonic fibroblasts. J Biol Chem, 285:6377-6389.

24. Wang C, Xin X, Xiang R, Ramos FJ, Liu M, Lee HJ, Chen H, Mao X, Kikani CK, Liu F, Dong LQ: Yin-Yang regulation of adiponectin signaling by APPL isoforms in muscle cells. J Biol Chem 2009, 284:31608-31615.

25. Barbieri M, Esposito A, Angellotti E, Rizzo MR, Marfella R, Paolisso G: Association of genetic variation in adaptor protein APPL1/APPL2 loci

with non-alcoholic fatty liver disease. PLoS One 2013, 8:e71391.

26. Laird MH, Rhee SH, Perkins DJ, Medvedev AE, Piao W, Fenton MJ, Vogel SN: TLR4/MyD88/PI3K interactions regulate TLR4 signaling. J Leukoc Biol 2009, 85:966-977.
27. Buechler C, Wanninger J, Neumeier M: Adiponectin receptor binding proteins–recent advances in elucidating adiponectin signalling pathways. FEBS Lett 2010, 584:4280-4286.
28. Ngkelo A, Meja K, Yeadon M, Adcock I, Kirkham PA: LPS induced inflammatory responses in human peripheral blood mononuclear cells is mediated through NOX4 and Gialpha dependent PI-3kinase signalling. J Inflamm (Lond) 2012, 9:1.
29. Schenck A, Goto-Silva L, Collinet C, Rhinn M, Giner A, Habermann B, Brand M, Zerial M: The endosomal protein Appl1 mediates Akt substrate specificity and cell survival in vertebrate development. Cell 2008, 133:486-497.
30. Cheng KK, Zhu W, Chen B, Wang Y, Wu D, Sweeney G, Wang B, Lam KS, Xu A: The adaptor protein APPL2 inhibits insulin-stimulated glucose uptake by interacting with TBC1D1 in skeletal muscle. Diabetes 2014.
31. Akashi-Takamura S, Miyake K: Toll-like receptors (TLRs) and immune disorders. J Infect Chemother 2006, 12:233-240.
32. Nomura F, Akashi S, Sakao Y, Sato S, Kawai T, Matsumoto M, Nakanishi K, Kimoto M, Miyake K, Takeda K, Akira S: Cutting edge: endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface toll-like receptor 4 expression. J Immunol 2000, 164:3476-3479.
33. Choe JY, Crain B, Wu SR, Corr M: Interleukin 1 receptor dependence of serum transferred arthritis can be circumvented by toll-like receptor 4 signaling. J Exp Med 2003, 197:537-542.
34. Doan HQ, Bowen KA, Jackson LA, Evers BM: Toll-like receptor 4 activation increases Akt phosphorylation in colon cancer cells. Anticancer Res 2009, 29:2473-2478.
35. Mitsuuchi Y, Johnson SW, Sonoda G, Tanno S, Golemis EA, Testa JR: Identification of a chromosome 3p14.3-21.1 gene, APPL, encoding an adaptor molecule that interacts with the oncoprotein-serine/threonine kinase AKT2. Oncogene 1999, 18:4891-4898.
36. Melton DW: Gene targeting in the mouse. Bioessays 1994, 16:633-638.
37. Wilson RW, Ballantyne CM, Smith CW, Montgomery C, Bradley A, O' Brien WE, Beaudet AL: Gene targeting yields a CD18-mutant mouse for study of inflammation. J Immunol 1993, 151:1571-1578.

38. Lee C, Chun J, Hwang SW, Kang SJ, Im JP, Kim JS: Enalapril inhibits nuclear factor-kappaB signaling in intestinal epithelial cells and peritoneal macrophages and attenuates experimental colitis in mice. Life Sci 2014, 95:29-39.

Figure Legends

Figure 1 [Figure 1: see original paper] Decreased Expression of Appl2 in Primary Macrophages Treated with LPS. (A) Western blot analysis of Appl2 in peritoneal macrophages from WT mice stimulated with 1 µg/mL LPS at specified time points. (B) Densitometric analysis of the above data using NIH Image J software for relative values. *$p < 0.05$, compared with control, n=5.

Figure 2 [Figure 2: see original paper] Generation and Validation of Appl2 KO Mice. (A) Diagram showing the construction of Appl2 KO mouse. (B) Western blot analysis demonstrating the absence of Appl2 protein in macrophages of Appl2 KO mice. Appl2-/-, Appl2 KO mouse.

Figure 3 [Figure 3: see original paper] Appl2 KO Mice Are More Prone to Endotoxic Shock Than WT Mice. (A) Kaplan-Meier survival curves of WT (n = 9) and Appl2 KO (n = 9) mice after LPS challenge (20 mg/kg body weight, intraperitoneal injection). Compared with WT mice, Appl2 KO mice were more susceptible to endotoxin challenge. (B) Photographs of WT and Appl2 KO mice 24 h after intraperitoneal LPS injection. WT mice showed only mild effects, whereas Appl2 KO mice exhibited typical signs of endotoxemia. (C) HE-stained kidney sections from mice at 24 h post-LPS challenge. (D) Mouse serum TNF- level at 60 min after LPS injection. (E) Time course of IL-1 expression in LPS-stimulated Appl2 KO and control mice. Scale bar: 50 m; *$p < 0.05$, compared with WT mice, n=6.

Figure 4 [Figure 4: see original paper] Increased Production of TNF- and IL-1 in Appl2 KO Macrophages. (A) Kinetics of TNF- expression in LPS-stimulated primary macrophages from Appl2 KO and control mice. (B) Kinetics of IL-1 expression in LPS-stimulated primary macrophages from Appl2 KO and control mice. *$p < 0.05$, compared with WT mice, n=5.

Figure 5 [Figure 5: see original paper] Enhanced Activation of Akt-NF- B Pathways After LPS Stimulation in Appl2 KO Macrophages. Peritoneal macrophages derived from peritoneal cells were stimulated with 1 µg/mL LPS for 20 min. Cell lysates were analyzed by western blot. (A) Western blot analysis of peritoneal macrophages from Appl2 KO mice with indicated antibodies. (B) Ratio values of p-Akt/Akt/ -actin, p-IKK /IKK / -actin, p-I B/I B/ -actin, and p-p65/p65/ -actin as determined by densitometric analysis of (A) using NIH Image J software for relative values. *$p < 0.05$, compared with WT mice, n=5.

Figure 6 [Figure 6: see original paper] The p85 Subunit of PI3K Associates with Appl1 and Appl2. Peritoneal macrophages from WT mice were treated with 1 µg/mL LPS for 20 min. Macrophage lysates were immunoprecipitated with IgG, Appl2, and Appl1 antibodies and immunoblotted using antibodies against TLR4, MyD88, Akt, p85 , Appl1, and Appl2, respectively.

Figure 7 [Figure 7: see original paper] Appl1 KO Mice Show Less Severe Endotoxic Shock Than WT Mice. (A) Diagram showing the construction of Appl1 KO mouse. (B) Western blot analysis demonstrating the absence of Appl1 protein in macrophages of Appl1 KO mice. (C) Kaplan-Meier survival curves of WT (n = 9) and Appl1 KO (n = 9) mice after LPS challenge (30 mg/kg body weight, intraperitoneal injection). Compared with WT mice, Appl1 KO mice were less susceptible to endotoxin challenge. (D) Photographs of WT and Appl1 KO mice 24 h after intraperitoneal LPS injection. Appl1 KO mice showed only mild effects, whereas WT mice exhibited typical signs of endotoxemia. (E) HE-stained kidney sections from mice at 24 h post-LPS challenge. (F) Mouse serum TNF- level at 60 min after LPS injection. (G) Kinetics of IL-1 expression in LPS-stimulated Appl1 KO and control mice. Scale bar: 50 m; Appl1-/-, Appl1 KO mouse. *p < 0.05, compared with WT mice, n=6.

Figure 8 [Figure 8: see original paper] Decreased Production of TNF- and IL-1 and Reduced Activation of Akt-NF- B Pathways After LPS Stimulation in Appl1 KO Macrophages. Peritoneal macrophages derived from peritoneal cells were stimulated with 1 µg/mL LPS at specified times, and supernatant was collected for quantification of cytokine (TNF- and IL-1) expression. Cell lysates were analyzed by western blot. (A-B) Time course of TNF- and IL-1 expression in LPS-stimulated primary macrophages from Appl1 KO and control mice. (C) Western blot analysis of peritoneal macrophages from Appl1 KO mice with indicated antibodies. (D) Ratio values of p-Akt/Akt/ -actin, p-IKK /IKK / -actin, p-I B/I B/ -actin, and p-p65/p65/ -actin as determined by densitometric analysis of (C) using NIH Image J software for relative values. *p < 0.05, compared with WT mice, n=5.

Note: Figure translations are in progress. See original paper for figures.

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