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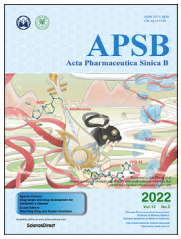

ORIGINAL ARTICLE

IKKβ mediates homeostatic function in inflammation *via* competitively phosphorylating AMPK and IκBα

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Homeostasis;
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Abstract Inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ) is one of important kinases in inflammation to phosphorylate inhibitor of nuclear factor kappa-B (IκBα) and then activate nuclear factor kappa-B (NF-κB). Inhibition of IKKβ has been a therapeutic strategy for inflammatory and autoimmune diseases. Here we report that IKKβ is constitutively activated in healthy donors and healthy $Ikk\beta^{C46A}$ (cysteine 46 mutated to alanine) knock-in mice although they possess intensive IKKβ−IκBα−NF-κB signaling activation. These indicate that IKKβ activation probably plays homeostatic role instead of causing inflammation. Compared to $Ikk\beta^{WT}$ littermates, lipopolysaccharides (LPS) could induce high

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Anti-inflammatory drug;
Phosphorylation

mortality rate in $Ikk\beta^{C46A}$ mice which is correlated to breaking the homeostasis by intensively activating p-IκBα−NF-κB signaling and inhibiting phosphorylation of 5′ adenosine monophosphate-activated protein kinase (p-AMPK) expression. We then demonstrated that IKKβ kinase domain (KD) phosphorylates AMPKα1 *via* interacting with residues Thr183, Ser184, and Thr388, while IKKβ helix−loop−helix motifs is essential to phosphorylate IκBα according to the previous reports. Kinase assay further demonstrated that IKKβ simultaneously catalyzes phosphorylation of AMPK and IκBα to mediate homeostasis. Accordingly, activation of AMPK rather than inhibition of IKKβ could substantially rescue LPS-induced mortality in $Ikk\beta^{C46A}$ mice by rebuilding the homeostasis. We conclude that IKKβ activates AMPK to restrict inflammation and IKKβ mediates homeostatic function in inflammation *via* competitively phosphorylating AMPK and IκBα.

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1. Introduction

Kinases have become attractive targets of drugs to treat multiple diseases during the past 30 years. Hence understanding the multiple functions of these kinases is vital for discovery of new drugs. The IκB kinase (IKK) complex is composed of two serine-threonine kinases, IKKα and IKKβ, and a regulatory subunit NEMO (also known as IKKγ)[1]. By integrating signals from activation stimuli, IKKβ is the predominant catalytic subunit of the IKK complex to activate nuclear factor kappa-B (NF-κB) canonical signaling by phosphorylating IκBα and helix−loop−helix (HLH) motifs is necessary for the activation[2]. Thus, IKKβ has emerged as a molecular target for development of anti-inflammatory drugs due to the importance of IKKβ−NF-κB signaling in inflammation[3]. Many IKKβ inhibitors have been investigated in various pre-clinical models of inflammatory and autoimmune diseases and a handful of phase I/II clinical trials of IKK inhibitors have indeed been performed[4]. However, the efficacy and safety of these inhibitors was critically questioned because IKKβ-deficient mice are embryonic lethal at 14 days of gestation due to massive liver apoptosis[5], and the clinical applications of these inhibitors have not yet been approved[6]. These studies strongly suggest that the function of IKKβ in the inflammation is complicated and needs to be further intensively investigated.

In addition to NF-κB-dependent functions, IKKβ also plays key roles in regulating many physiological functions in immunity and cancer through NF-κB-independent pathways by phosphorylating other key proteins[7,8]. IKKβ phosphorylates X-box binding protein 1 (XBP1) and increases its activity, which mediated hepatic inflammation in glucose homeostasis[9]. Additionally, IKKβ promotes cancer cell metabolic adaptation to glutamine deprivation *via* phosphorylation of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB3) or activation of p53[10,11], indicating that IKKβ is a critical kinase in cell metabolism.

The 5′ adenosine monophosphate-activated protein kinase (AMPK) is a well-known sensor of energy balance in cell metabolism by responding to ATP-depleting processes[12], and IKKβ plays a cardinal important role in the AMPK induced autophagy[13,14]. It was recently reported that IKK can promote cytokine-induced and cancer-associated AMPK activity and attenuates phenformin-induced cell death in liver kinase B1 (*LKB1*)-deficient cancer cells[15]. Intensive research using various cell types indicates that AMPK can suppress inflammatory responses *via* induction of autophagy and inhibition of NF-κB signaling[16]. Hence, the correlation among IKKβ, AMPK, and inflammation needs to be further investigated. In particular, it is crucial to uncover how IKKβ mediates two opposite functions of inflammation and anti-inflammation.

Here we demonstrated that IKKβ kinase domain (KD) can interact with and phosphorylate AMPKα1. In addition, AMPKα1 and IκBα can be competitively catalyzed by IKKβ. In our previous study, we found that IKKβ with cysteine 46 mutated to alanine, $Ikk\beta^{C46A}$ significantly increases its kinase activity[17]. Then $Ikk\beta^{C46A}$ knock-in mice were generated to investigate the endogenous activation of IKKβ in the pathogenesis of inflammation. By using $Ikk\beta^{C46A}$ knock-in mice possessing simultaneous activation of AMPK and IκBα, we demonstrated that IKKβ KD−AMPK−ULK^{S555} axis-induced autophagy restricted inflammation while IKKβ HLH−IκBα−NF-κB signaling simultaneously generated inflammation to mediate the homeostasis. This study may shed light on the potential reasons for the lack of clinical success of IKKβ inhibitors and offer implications in the drug design and therapeutic strategy to treat inflammation.

2. Materials and methods

2.1. Experimental design

The objective of this study was to reveal the interaction between IKKβ and AMPK and then to clarify the homeostatic function of IKKβ in inflammation *via* competitively catalyzing AMPK and IκBα phosphorylation. Immunoprecipitation and liquid chromatography−mass spectrometry (LC−MS) analysis were employed to examine the interaction between AMPK and IKKβ and the sites of AMPK phosphorylated by IKKβ. The balance between IκBα and AMPK was revealed by inhibiting or activating AMPK to disrupt or recovering the homeostasis.

2.2. Chemicals, antibodies, and plasmids

IKKβ, p-IκBα (Ser32/36), p-p65, p65, Atg7, Atg12, Atg5, Atg3, LC3A/B, mammalian target of rapamycin (mTOR), p-mTOR (Ser2448), p-ULK1 (Ser555), p-ULK1(Ser757), ULK1, p-AMPKα1 (Thr183)/AMPKα2 (Thr172), AMPKα, and p62 antibodies were purchased from Cell Signaling Technology (Boston, MA, USA). p-IKKβ antibody were from Abcam (London, UK). A primary antibody against β-actin was from Santa Cruz (Dallas, TX, USA). Macrophage colony-stimulating factor (M-CSF) was from Miltenyi Biotec (Cologne, Germany). Rapamycin [98%

purity, verified by high performance liquid chromatography (HPLC)] was from the MCE (Middlesex County, NJ, USA). Lipopolysaccharides from *Escherichia coli* O55:B5, chloroquine (CQ), trifluoroacetic acid (TFA), and formic acid (FA) were from Sigma (St. Louis, MO, USA). TRIzol reagent and Lipofectamine™ LTX Reagent with PLUS™ Reagent were from Invitrogen (Carlsbad, CA, USA). Tumor necrosis factor (*Tnf-α*), interleukin-6 (*Il-6*), interleukin-1 beta (*Il-1β*), interferon gamma (*Ifn-γ*), and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) primers were synthesized by Life Technologies (Carlsbad, CA, USA). The FastStart Universal SYBR Green Master and Transcriptor First Strand cDNA Synthesis Kits were from Roche (Mannheim, Germany). Dulbecco's modified Eagle's medium (DMEM), minimum essential medium (MEM), trypsin, and fetal bovine serum (FBS) were from GIBCO (Grand Island, NY, USA). The expression of FLAG-tagged IKKβ plasmid was provided by Professor Tom Gilmore (Boston University, Boston, USA). FLAG-tagged IKKβ K44A (lysine 44 mutated to alanine) and HA-tagged AMPKα1 plasmids were obtained from Addgene (Watertown, WA, USA). Single-domain constructs, FLAG-tagged IKKβ kinase, FLAG-tagged IKKβ ubiquitin-binding domain (ULD), FLAG-tagged IKKβ leucine zipper (LZ), and FLAG-tagged IKKβ HLH were generated using standard subcloning procedures. Desired mutant AMPK plasmids were prepared using custom designed oligonucleotide primers. Recombinant IKKβ kinase was from Active Motif (Carlsbad, CA, USA). Recombinant AMPK protein was from Millipore (Darmstadt, Germany). Recombinant IκBα protein was from Signalchem (Richmond, BC, Canada). Compound C and 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) were from Selleck Chemicals (Houston, Texas, USA). Protein Assay Reagent was from Bio-Rad (Richmond, CA). Dithiothreitol (DTT), iodoacetamide (IAA) and carbamide were provided by GE Healthcare (Piscataway, NJ). Trypsin protease was from Thermo Fisher Scientific (Rockford, IL, USA). The solid-phase extraction (SPE) C18 cartridge was from Waters Corporation (Milford, MA, USA). Water with 18.2 MΩ was produced by a Milli-Q Ultrapure water system (Millipore).

2.3. Kinase assays

The *in vitro* kinase assay was performed at 30 °C for 30 min by mixing recombinant IKKβ kinase or immunoprecipitated FLAG-IKKβ with recombinant AMPK protein in kinase buffer (50 mmol/L HEPES, pH 7.3; 15 mmol/L $MgCl_2$; 20 mmol/L KCl; 2 mmol/L EGTA; 1 mmol/L DTT; 100 μmol/L ATP). For experiments involving AMPK competition with IκBα, the kinase assay was performed at 30 °C for 30 min by mixing 10 or 50 ng of recombinant AMPK or IκBα protein with 10 ng of recombinant IKKβ kinase in kinase buffer. Reactions were then analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.4. Transfection

Transfection assays were preformed according to the manual of Lipofectamine™ LTX Reagent with PLUS™ Reagent. In brief, HEK293T cells were cultured in DMEM with 10% FBS and 1% penicillin−streptomycin (Pen−Strep). Then, 2 μg of DNA and 2 μL of Plus Reagent were added to 500 μL of OPTI-MEM Reduced Serum Media (GIBCO) and incubated for 5 min at room temperature. Lipofectamine LTX™ Reagent was added into the above solution and then mixed gently. The mixture was incubated for another 30 min at room temperature to form DNA−lipofectamine LTX Reagent complexes. Finally, the above complexes were directly added into indicated well containing cells and mixed gently. The cells were incubated at 37 °C in a CO_2 incubator for 24 h.

2.5. Immunoprecipitations

The immunoprecipitation assay was performed according to the instruction of FLAG Immunoprecipitation Kit (Sigma). In brief, cells were washed twice with phosphate buffer saline (PBS) and lysed in ice-cold lysis buffer (50 mmol/L Tris-HCl, pH7.4; 150 mmol/L NaCl; 1 mmol/L EDTA; 1% Triton X-100; and 10 μL 100× protease inhibitors per 1 mL). The soluble fractions of cell lysates were isolated by centrifugation at 12,000 × *g* for 10 min. The cell lysates were then added into the anti-FLAG M2 resin and incubated with rotation overnight at 4 °C. Immunoprecipitates were washed 3 times with wash buffer and eluted with elution buffer. Cell extracts or immunoprecipitated proteins were denatured by loading buffer and boiling for 5 min and analyzed by Western blot.

2.6. Protein digestion

For protein sample extracted from gel, the gel was firstly stained by silver staining kit (GE Healthcare) according to the manufacturer's instructions. Then the gel piece excised from silver stained gel was reduced with dithiothreitol (DTT, 10 mmol/L, 30 min, 56 °C). After that, iodoacetamide (IAA, 55 mmol/L, 20 min, at room temperature, in dark) was added. Finally, the gel piece was incubated with 10 ng/μL trypsin in 40 mmol/L ammonium bicarbonate with 10% acetonitrile (ACN) for 30 min on ice and replenished with 40 mmol/L ammonium bicarbonate with 10% ACN at 37 °C overnight. For immunoprecipitated proteins, the process of protein digestion was the same with the description in the previous study[18]. Briefly, the protein contents were firstly quantitated using the detergent compatible (DC) Protein Assay Reagent (Bio-Rad). Then the protein solutions were diluted as 1 μg/μL using water and reduced with DTT (200 mmol/L, 1 h, 37 °C). After that IAA (1 mol/L, 1 h, 37 °C, in dark) was added. The reaction mixture was stopped with DTT (200 mmol/L, 1 h, 37 °C). Samples were diluted with ammonium bicarbonate buffer (25 mmol/L) to the concentration of urea was below 1 mol/L. Finally, the proteins were digested by trypsin overnight (37 °C) with an enzyme-to-protein ratio at 1:50 (*w*/*w*). Ultimately, digests were then purified using a solid-phase extraction (SPE) C18 cartridges (Waters Corporation), and elution was dried by dry nitrogen and stored at −20 °C until analysis.

2.7. Nano-LC−Q-TOF-MS

Peptides from protein digestion were analyzed using an UltiMate 3000 RSLC nano (Thermo Fisher Scientific) system and a Maxis Impact Accurate-Mass Q-TOF-MS system (Bruker Corporation, Billerica, MA, USA) with CaptiveSpray source. The LC conditions were set as previously described[19]. For the MS conditions, positive mode was applied, and the MS parameters were set as follows: end plate offset at 500 V; capillary at 1500 V; dry gas flow at 4 L/min; and dry temperature at 160 °C. The top 10 intensity-binned precursors with charge states $> +1$ were

preferred for charge injection device (CID) MS/MS analysis across the range of m/z 300−1700 window.

2.8. Identification of protein

LC−MS data were processed into peak lists with Bruker Compass Data Analysis software, and then the lists were converted into the Mascot against the Swiss-Prot 51.6 database. The methionine oxidation (M), phospho (C), phospho (D), phospho (H), phospho (R), phospho (ST), and phospho (Y) were selected as variable modifications, while cysteine carbamidomethyl (C) was selected as fixed modification. The parameters selected for database searches were as previously described[18].

2.9. Animals and treatments

The $Ikk\beta^{C46A}$ transgenic mice were reported in our previous study[17]. $Ikk\beta^{C46A}$ knock-in mice and their wild type counterparts were raised in the International Institute for Translational Research of Traditional Chinese Medicine of Guangzhou University of Chinese Medicine (Guangzhou, China). The animals were housed in a pathogen-free barrier facility with a 12-h-light/12-h-dark cycle with free access to food and water. Animal experimental procedures were performed strictly in accordance with animal welfare and other related ethical regulations approved by Guangzhou University of Chinese Medicine (Guangzhou, China). The genotypes of transgenic mice were identified by polymerase chain reaction (PCR) and gene sequencing using the following primers: forward primer: GTGATGTGGGGGTGGTGAGG; reverse primer: TTTGGGCTG AGCTCCTGTCG. The PCR conditions were denaturing at 94 °C for 5 min, 35 cycles of denaturing at 94 °C for 30 s, annealing at 66 °C for 30 s, and elongation at 72 °C for 30 s, and a final elongation at 72 °C for 10 min. For LPS or compound C treatment, mice were intraperitoneally injected with LPS at 15 mg/kg or compound C at 60 mg/kg. For experiment involving AICAR, mice were pretreated with 300 mg/kg AICAR 0.5 h by intraperitoneal injection before challenged with LPS. For experiments involving CQ, mice were treated with 60 mg/kg CQ by intraperitoneal injection. For experiment involving rapamycin, mice were intraperitoneally injected with 4 mg/kg rapamycin before challenged with LPS. For experiment involving berberine (BBR), mice were intragastrically administrated with 50 mg/kg BBR before challenged with LPS. Hypothermia was monitored, and mice were observed for mortality at least twice daily.

2.10. Cell culture

For the isolation and treatment of the bone marrow derived macrophages (BMDMs), bone marrow cells (4×10^6 cells) from $Ikk\beta^{C46A}$ mice or wild-type littermates were cultured and the purity of cells was identified according to previous method[20]. Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors with consent and the study was approved by the Ethic Committee of Macau University of Science and Technology (Macau SAR, China).

2.11. Protein extraction and Western blot analysis

Lung, liver, kidney tissues, or BMDMs were lysed with RIPA buffer to harvest total cellular proteins and were lysed with NE-PER-Nuclear extraction solution (Thermo Fisher Scientific) to collect nuclear protein according to the manufacturer's instructions. The protein concentration was calculated by the bicinchoninic acid (BCA) kit. The extracts were then subjected to electrophoresis in 8% or 15% SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 5% non-fat milk, the membrane was subsequently incubated with primary antibodies and horse radish peroxidase (HRP)-conjugated secondary antibodies. Finally, the antibody-bound proteins on the membrane were examined by using chemiluminescence (ECL) detection system.

2.12. Enzyme-linked immunosorbent assay (ELISA)

The serum of mice or cellular supernatant was collected at indicated time points. The amount of pro-inflammatory cytokines including TNF-α, IL-6, IL-1β, and IFN-γ in these samples were determined by ELISA kits (R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.13. RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA of the lung homogenates and BMDMs was isolated with TRIzol reagent according to the manufacturer's instructions. The RT reactions were performed according to the instruction manual of the transcriptor First Strand cDNA Synthesis Kit (Roche). Quantitative PCR was performed with an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Waltham, MA, USA). The levels of *Tnf-α*, *Il-6*, *Il-1β*, and *Ifn-γ* mRNA were normalized to the levels of *Gapdh* mRNA. The sequences of PCR primers were listed in Supporting Information Table S1.

2.14. Histopathological analysis

The lung tissues of the mice were dissected, and the tissues were fixed with 4% paraformaldehyde and embedded with paraffin. The sections (5 μm) were sliced and stained with haematoxylin and eosin (H&E). Six randomly selected high-power fields (HPFs, 400×) in each section were observed using a LEICA DFC310 FX inverted microscope (Leica, Wetzlar, Germany).

2.15. Statistical analysis

All data were expressed as the mean ± standard error of mean (S.E.M). The significance of the differences was analyzed by one-way analysis of variance (ANOVA) or t-test using GraphPad Prism software (San Diego, CA, USA). Values of $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ were considered statistically significant.

3. Results

3.1. The homeostatic role of IKKβ is correlated to p-AMPK and p-IκBα expression

It is well known that LPS-induced IKKβ activation mediates inflammation *via* up-regulation of IκBα−NF-κB signaling[21]. However, we observed that the PBMC isolated from some healthy donors constitutively expressed IKKβ activation without exhibiting inflammation (Supporting Information Fig. S1), indicating that IKKβ activation is not sufficient to generate inflammation and the complicated roles of IKKβ in inflammation need to be further elucidated. In our previous study, we constructed $Ikk\beta^{C46A}$ plasmid

and generated *Ikkβ^{C46A}* knock-in mice which exhibited more intensive kinase activity compared to *Ikkβ^{WT}*[17]. We then found that the increased kinase activity of IKKβ^{C46A} is attributed to possess much more *trans*-autophosphorylation sites in the recombinant protein compared to IKKβ^{WT} (Supporting Information Table S2). Interestingly, we did not find the significant difference between *Ikkβ^{C46A}* mice and wild type littermates in inflammation although IKKβ−NF-κB signaling pathway was obviously activated in the organs of *Ikkβ^{C46A}* mice compared to wild type littermates (Supporting Information Fig. S2A−S2C). These results indicated that IKKβ probably plays homeostatic role by regulation of alternative mechanism to restrict inflammation occurrence mediated by IKKβ−NF-κB canonical pathway activation.

In our study, we noticed that LPS clearly induced IKKβ−NF-κB signaling activation in the BMDMs and livers of *Ikkβ^{C46A}* mice, compared to those derived from *Ikkβ^{WT}* mice (Fig. 1A and B). The mRNA expression and secretion of pro-inflammatory cytokines were also up-regulated in BMDMs of *Ikkβ^{C46A}* mice (Fig. 1C and D). In agreement with the results, we found that severe inflammation was generated in LPS-treated *Ikkβ^{C46A}* mice compared to wild type littermates by showing dropped body temperature, increased inflammatory exudates in lung tissues, up-regulated mRNA expression level, and secretion of indicated cytokines (Fig. 1E−H, Supporting Information Fig. S3). We then analyzed the results of H&E and found macrophages are one of predominant cell populations infiltrating in the lung of the septic mice, indicating that the lung-resident macrophages probably were activated in sepsis.

Notably, we observed that 80% *Ikkβ^{C46A}* mice treated with 15 mg/kg LPS died within 120 h. On the contrary, all *Ikkβ^{WT}* mice treated with same dosage of LPS well survived although those inflammatory responses were observed (Fig. 1I). Interestingly, BBR, an IKKβ inhibitor targeting on IKKβ Cys179[22], could not substantially rescue the mortality of *Ikkβ^{C46A}* mice (Fig. 1J).

To explore the underlying mechanism, we analyzed the signals associated with inflammation. Our results demonstrated that LPS substantially suppressed AMPK phosphorylation in livers of *Ikkβ^{C46A}* mice, compared to those from *Ikkβ^{WT}* mice (Fig. 1K). Other signals correlated to inflammation were not significantly changed (Supporting Information Fig. S4). These results indicated that the IKKβ mediated homeostasis is correlated to p-AMPKα and p-IκBα expression and LPS heavily disrupted the homeostasis to induce the mortality of *Ikkβ^{C46A}* mice (Fig. 1L).

3.2. *IKKβ-mediated homeostasis is interrupted by inhibition of AMPK resulting in IκBα−NF-κB activation to induce inflammation*

To determine whether the balance between AMPK and IκBα probably is existed and required for maintain homeostasis under physiological status, we analyzed the expression of p-AMPK and p-IκBα−NF-κB expression in *Ikkβ^{C46A}* mice. We found that p-AMPK and p-IκBα−NF-κB expression were clearly and simultaneously increased in *Ikkβ^{C46A}* mice compared to *Ikkβ^{WT}* mice (Fig. 2A). We then addressed whether AMPK inhibition can break the homeostasis and in turn activate IκBα−NF-κB to generate inflammation in *Ikkβ^{C46A}* mice as well as *Ikkβ^{WT}* littermates. We found that AMPK inhibitor compound C increased mortality, dropped body temperature, increased inflammatory exudates in lung tissues, and up-regulated production of IFN-γ and IL-6 in *Ikkβ^{C46A}* mice (Fig. 2B−E, Supporting Information Fig. S5). The reduced body temperature, lung inflammation, and increased cytokines secretion were also observed in *Ikkβ^{WT}* mice treated with compound C, although the survival of these mice was not affected (Fig. 2B−E), indicating the degree of inflammation mediated by compound C is correlated to suppression of p-AMPK expression. In concert with our results *in vivo*, IκBα phosphorylation was increased when AMPK function was deprived by compound C in the liver tissues derived from *Ikkβ^{C46A}* mice (Fig. 2F), and the same trend could be found in the BMDMs silenced by *Ampk* siRNA (Fig. 2G). These results affirmed our hypothesis that AMPK phosphorylation counterpoised IκBα−NF-κB activation to maintain physiological status of *Ikkβ^{C46A}* mice (Fig. 2H). It is worth noting that IL-1β and TNF-α were not affected in the assay and the underlying mechanism needs to be further investigated although IFN-γ and IL-6 production probably correlated to recruitment of IKKβ to the JAK1 complex[23].

In line with the role of AMPK in autophagy induction, the inflammation was also induced by CQ in *Ikkβ^{C46A}* mice (Supporting Information Fig. S6A−S6D). CQ also generated mild inflammatory responses in *Ikkβ^{WT}* mice due to only induction of IL-6 secretion (Fig. S6A−S6D). These results implied that IKKβ−AMPK induced autophagy contributed to preventing inflammation occurrence in *Ikkβ^{C46A}* mice (Fig. S6E). Notably, compound C induced inflammation is more severe than CQ, suggesting that AMPK plays the vital role in IKKβ-mediated homeostasis.

3.3. *IKKβ KD interacts with and phosphorylates AMPKα1^{Thr183}*

To analyze the correlation between IKKβ and AMPK, we performed LC−MS assay and found that AMPKα1 and α2 was the co-precipitated proteins in IKKβ pull-down experiments (Fig. 3A and Supporting Information Table S3). We then co-expressed FLAG-IKKβ and HA-AMPKα1 in HEK293T cells and pulled down FLAG-IKKβ to validate the interaction between IKKβ and AMPKα1. As shown in Fig. 3B, IKKβ not only interacted with but also phosphorylated AMPKα1. Furthermore, we applied IKKβ^{WT}, IKKβ^{C46A}, and IKKβ^{K44A}, a catalytically inactive mutant of IKKβ[24], to determine whether IKKβ interacts with and phosphorylated AMPKα1 at Thr183 depending on its kinase activity. Immunoprecipitation assay and *in vitro* kinase assay results demonstrated that IKKβ phosphorylated AMPKα1 at Thr183 and this catalytic capability of IKKβ on AMPKα1 depended on its kinase activity (Fig. 3C and D), suggesting that the recruitment and phosphorylation of AMPKα1 relies on the kinase activity of IKKβ. To further characterize the role of different IKKβ motifs in interaction with AMPK, we constructed various truncated plasmids of *IKKβ* containing KD, LZ, ULD, and HLH. The results of immunocytochemistry showed that IKKβ KD and IKKβ HLH co-localized with AMPK in HEK293T cells (Fig. 3E). We then further utilized truncated constructs of IKKβ to co-immunoprecipitate AMPK, and found that only the IKKβ KD construct could successfully interacted with AMPK besides full-length IKKβ (Fig. 3F), indicating that IKKβ KD is the primary domain that associated with and phosphorylated AMPKα1 at Thr183.

3.4. *IKKβ competitively phosphorylates IκB$\alpha^{Ser32/36}$ and AMPKα1^{Thr183}*

We then employed LC−MS to examine the sites of AMPKα1 phosphorylated by IKKβ. Our results showed that Ser184 and Thr388 could be phosphorylated (Fig. 4A). To validate the results, we generated the mutations of the putative phosphorylation

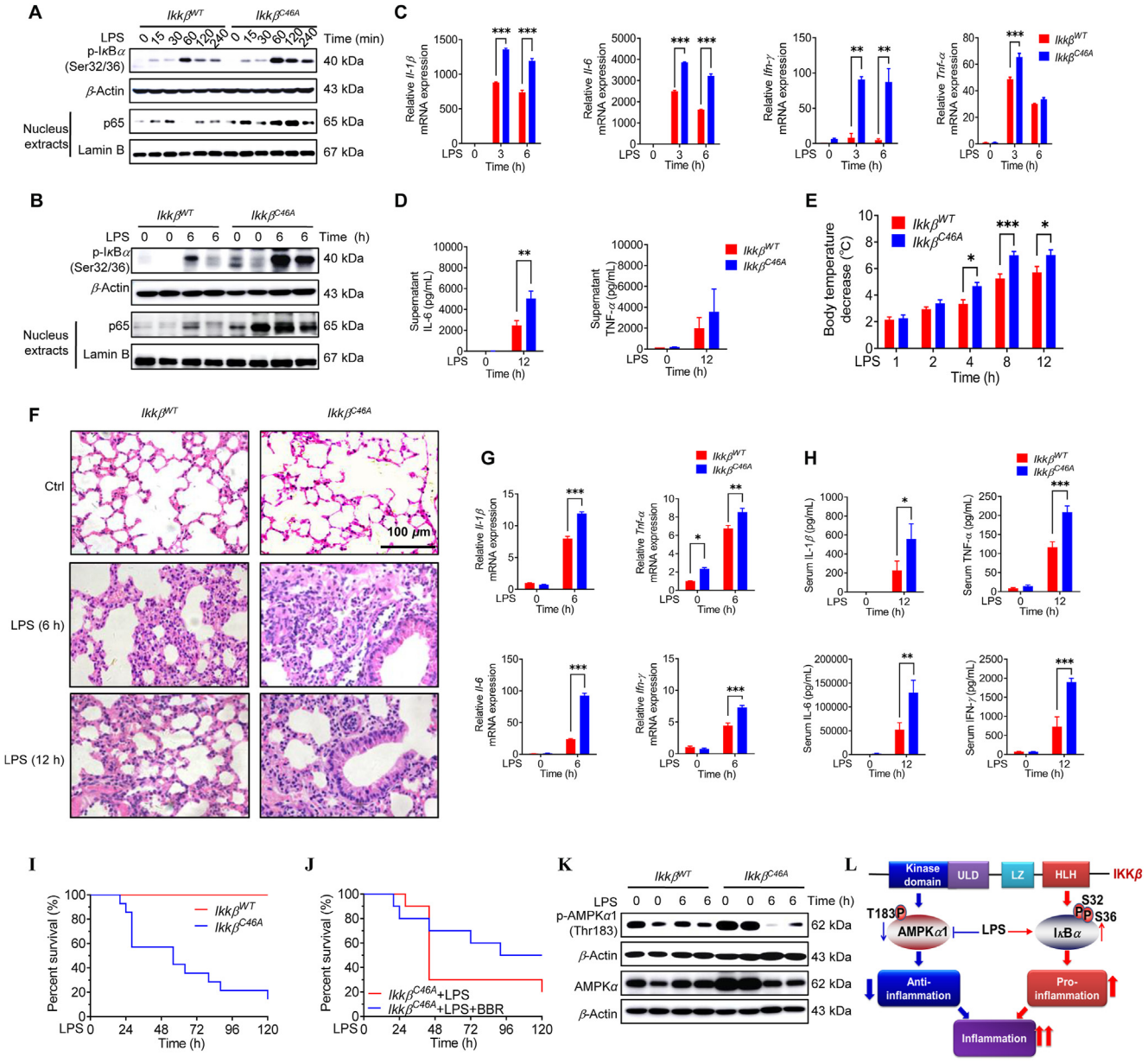

Figure 1 $Ikk\beta^{C46A}$ knock-in aggravates LPS-induced mortality by simultaneously inhibiting AMPK phosphorylation and facilitating IκBα−NF-κB activation. (A, B) The expression of p-IκBα and p65 nuclear expression in the BMDMs and livers of the $Ikk\beta^{WT}$ and $Ikk\beta^{C46A}$ with or without LPS treatment for indicated time points. (C, D) The indicated cytokines mRNA expression levels and secretion were examined in BMDMs. BMDMs were isolated from the $Ikk\beta^{C46A}$ and $Ikk\beta^{WT}$ mice and stimulated with or without 100 ng/mL LPS ($n = 3$). (E−H) Body temperature ($n = 14$), histopathological images of the lung tissues ($n = 3$), the indicated cytokines mRNA expression levels and secretion in the lungs or serum ($n = 3$−4) of the $Ikk\beta^{C46A}$ and $Ikk\beta^{WT}$ mice with or without 15 mg/kg LPS treatment. (I) The mortality of $Ikk\beta^{C46A}$ mice intraperitoneally administrated with or without LPS treatment was observed every 4 or 8 h for 120 h ($n = 14$). (J) The effect of IKKβ inhibitor on the mortality of the $Ikk\beta^{C46A}$ mice treated with LPS ($n = 10$). (K) The expression of AMPK and p-AMPK in the livers of the $Ikk\beta^{WT}$ and $Ikk\beta^{C46A}$ mice with or without LPS treatment. (L) The schematic showing that LPS induces the intensive phosphorylation of IκBα and dephosphorylation of AMPK to generate inflammation *in vivo*. Data are expressed as mean ± S.E.M. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

sites including Thr183, Ser184, and Thr388 into alanine ($AMPK\alpha1^{T183A}$, $AMPK\alpha1^{S184A}$, and $AMPK\alpha1^{T388A}$). We found that IKKβ interacted with AMPKα1 at Thr183, Ser184, and Thr388 (Fig. 4B). Importantly, IKKβ KD was the dominant domain to mediate the interaction (Fig. 4C). We also found that mutations of Ser184 and Thr388 of AMPKα1 significantly decreased $AMPK\alpha1^{Thr183}$ phosphorylation level (Fig. 4D). Since phosphorylation of AMPKα1/α2 at Thr183/Thr172 in the activation loop is required for AMPK activation[25], our results indicated that AMPKα1 Thr183 activation depends on phosphorylation at Ser184 and Thr388 which is probably mediated by IKKβ.

Since IKKβ phosphorylates AMPKα and IκBα relying on KD and HLH, respectively, we hypothesized there is competition between

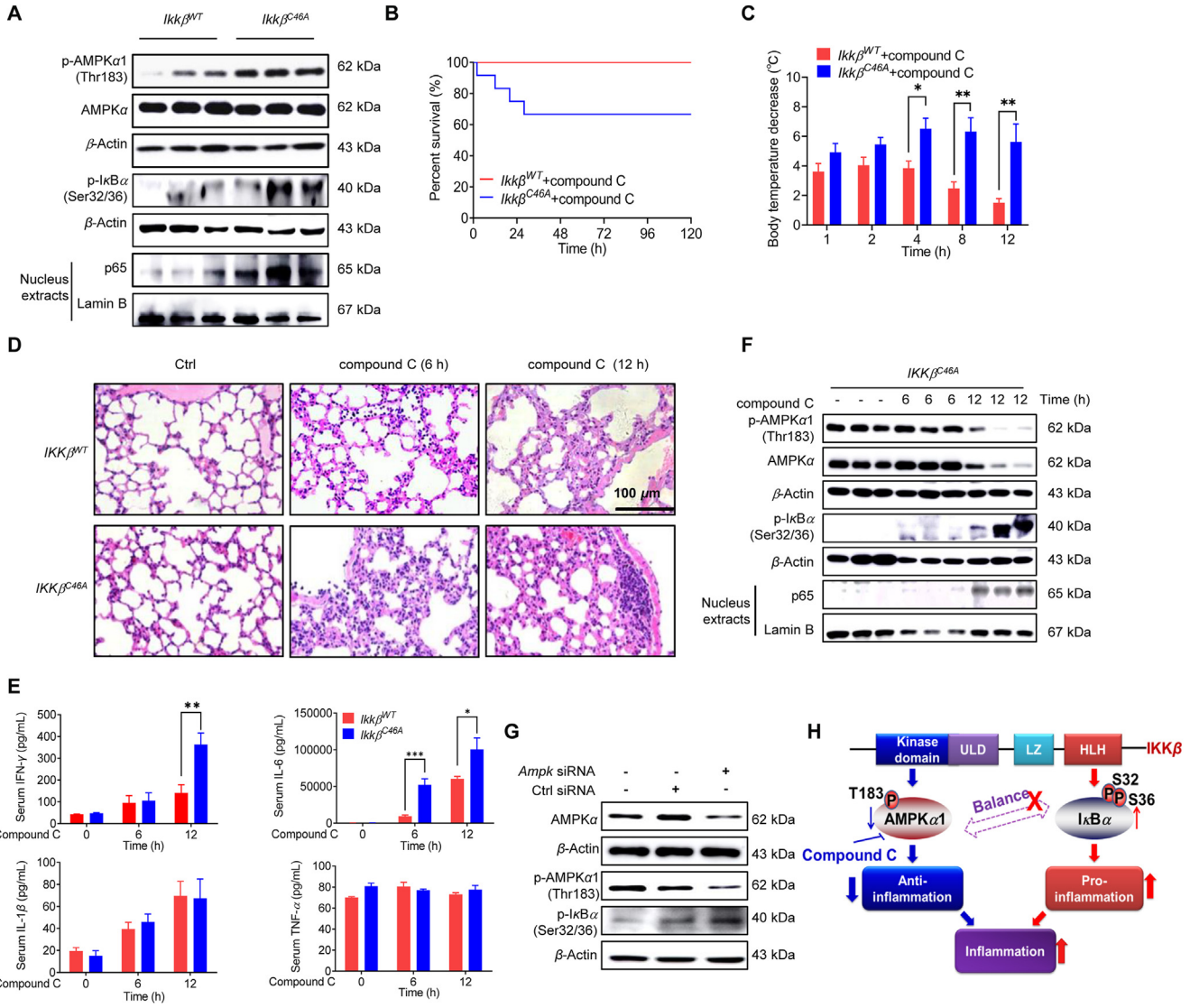

Figure 2 Inhibition of AMPK results in IκBα−NF-κB activation-induced inflammation. (A) The expression of indicated proteins in the livers of the *Ikkβ*^{WT} and *Ikkβ*^{C46A} mice. (B−E) The mortality ($n = 10$), body temperature ($n = 12$), histopathological images of lung tissues and the concentrations of indicated cytokines in the serum of the *Ikkβ*^{C46A} mice with or without compound C treatment ($n = 5$). (F) The expression of indicated protein in the livers of the *Ikkβ*^{C46A} and *Ikkβ*^{WT} mice with or without compound C treatment. (G) The expression levels of p-IκBα, p-AMPK, and AMPK in BMDMs derived from the *Ikkβ*^{C46A} mice upon *Ampk* knockdown. (H) The schematic showing that AMPK phosphorylation counterpoises IκBα−NF-κB activation to maintain homeostasis of *Ikkβ*^{C46A} mice. Data are expressed as mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

AMPKα and IκBα to be catalyzed by IKKβ. We thus performed *in vitro* kinase assays by incubating different ratios of AMPKα and IκBα with IKKβ. We discovered that IκBα and AMPKα indeed were competitively catalyzed by IKKβ in one-loss-the other-gain manner (Fig. 4E and F). These results suggested that IKKβ phosphorylates AMPKα *via* KD to restrict inflammation, whereas it phosphorylates IκBα *via* HLH to induce inflammation (Fig. 4G).

3.5. *Activation of AMPK rebuilds homeostasis of IKKβ to the rescue LPS induced mortality of Ikkβ*^{C46A} *mice*

Because IKKβ activation mediates homeostasis instead of inflammation by competitively phosphorylating IκBα and AMPKα, LPS induces inflammation probably is due to disruption of the homeostasis by activating p-IκBα and suppressing p-AMPK rather than activation of IKKβ. We then proposed that AMPK activation could rebuild the homeostasis to attenuate LPS-induced mortality of *Ikkβ*^{C46A} mice. As shown in Fig. 5A−D and Supporting Information Fig. S7, AMPK agonist AICAR greatly alleviated mortality and inflammation in LPS-treated *Ikkβ*^{C46A} mice by decreasing body temperature, ameliorating lung inflammation, and reducing secreted cytokines. As our expectation, inhibition of p-AMPK by LPS could be reversed by AICAR in *Ikkβ*^{C46A} mice. Simultaneously, LPS induced IκBα phosphorylation and nuclear translocation of NF-κB was inhibited by AICAR in *Ikkβ*^{C46A} mice (Fig. 5E). In agreement with the *in vivo* results,

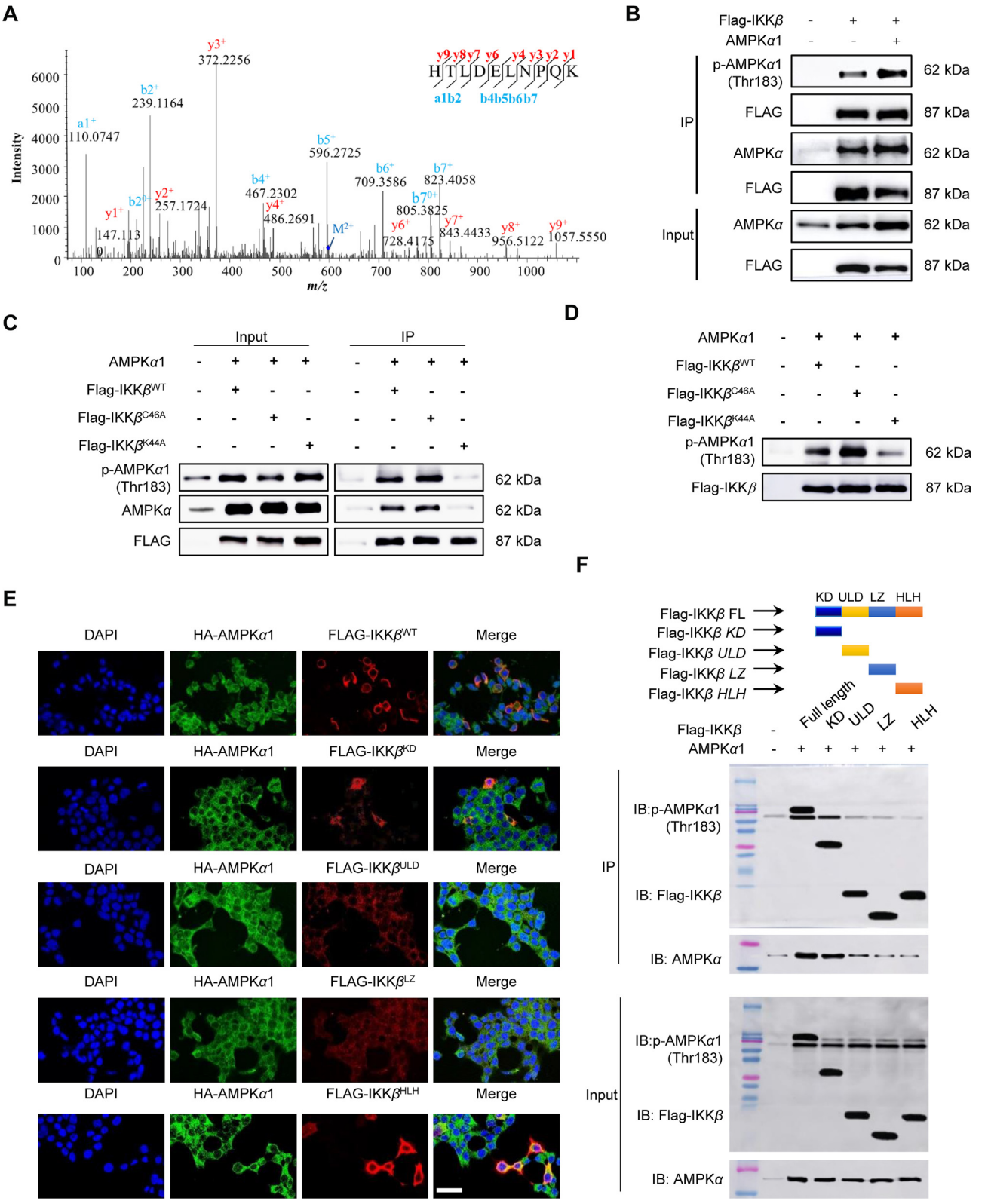

Figure 3 IKKβ kinase domain (KD) interacting with and phosphorylating AMPKα is correlated to the IKKβ kinase activity. (A) IKKβ interacting with and phosphorylating AMPKα1 was determined by LC−MS. (B) IKKβ interacting with and phosphorylating AMPKα1 at Thr183 was determined by immunoprecipitation and Western blot with the indicated antibodies. (C) AMPKα1 recruitment and phosphorylation at Thr183 depended on IKKβ activity. (D) AMPKα1 phosphorylated at Thr183 depended on IKKβ activity *in vitro*. (E) AMPK co-localized with IKKβ full-length (IKKβ FL) and indicated truncated IKKβ constructs were analyzed by immunofluorescence staining. Scale bar: 50 μm. (F) AMPKα1 interacted with and was phosphorylated by FLAG-IKKβ KD.

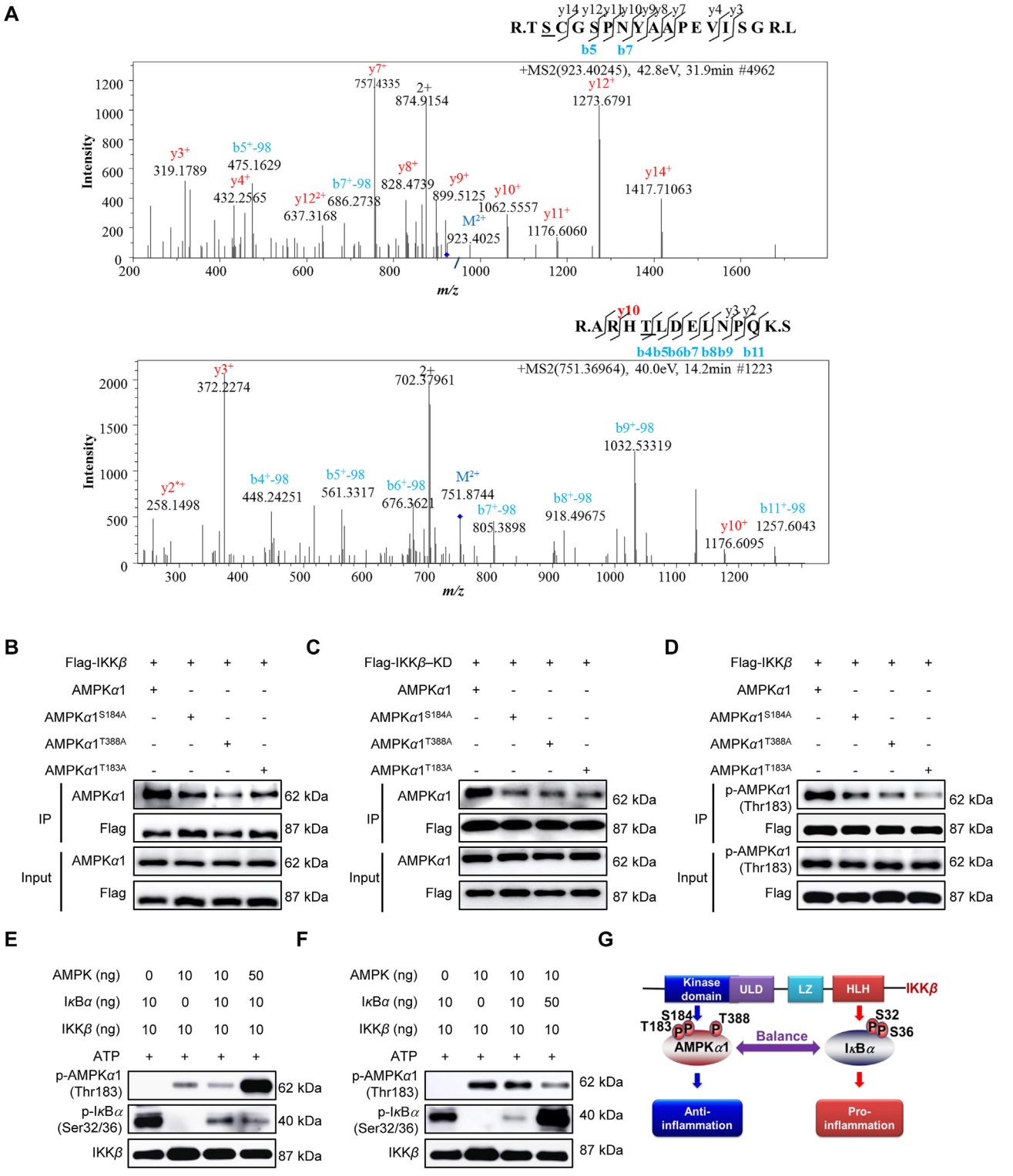

Figure 4 IKKβ KD interacts with and phosphorylates AMPKα1 at S184 and T388, and AMPK competes with IκBα to be phosphorylated by IKKβ. (A) IKKβ phosphorylating AMPKα1 at Ser184 and Thr388 was determined by LC−MS. (B) IKKβ interacted with AMPKα1 at Thr183, Ser184, and Thr388. (C) IKKβ KD interacted with AMPKα1 at Thr183, Ser184, and Thr388. (D) AMPKα1 phosphorylation at Thr183 depended on phosphorylation of Ser184 and Thr388 which was mediated by IKKβ. (E, F) AMPKα1 and IκBα were competitively with each other to be phosphorylated by IKKβ. (G) The schematic showing that the dual and opposite role of IKKβ restricts and induces inflammation by phosphorylating AMPK and IκBα.

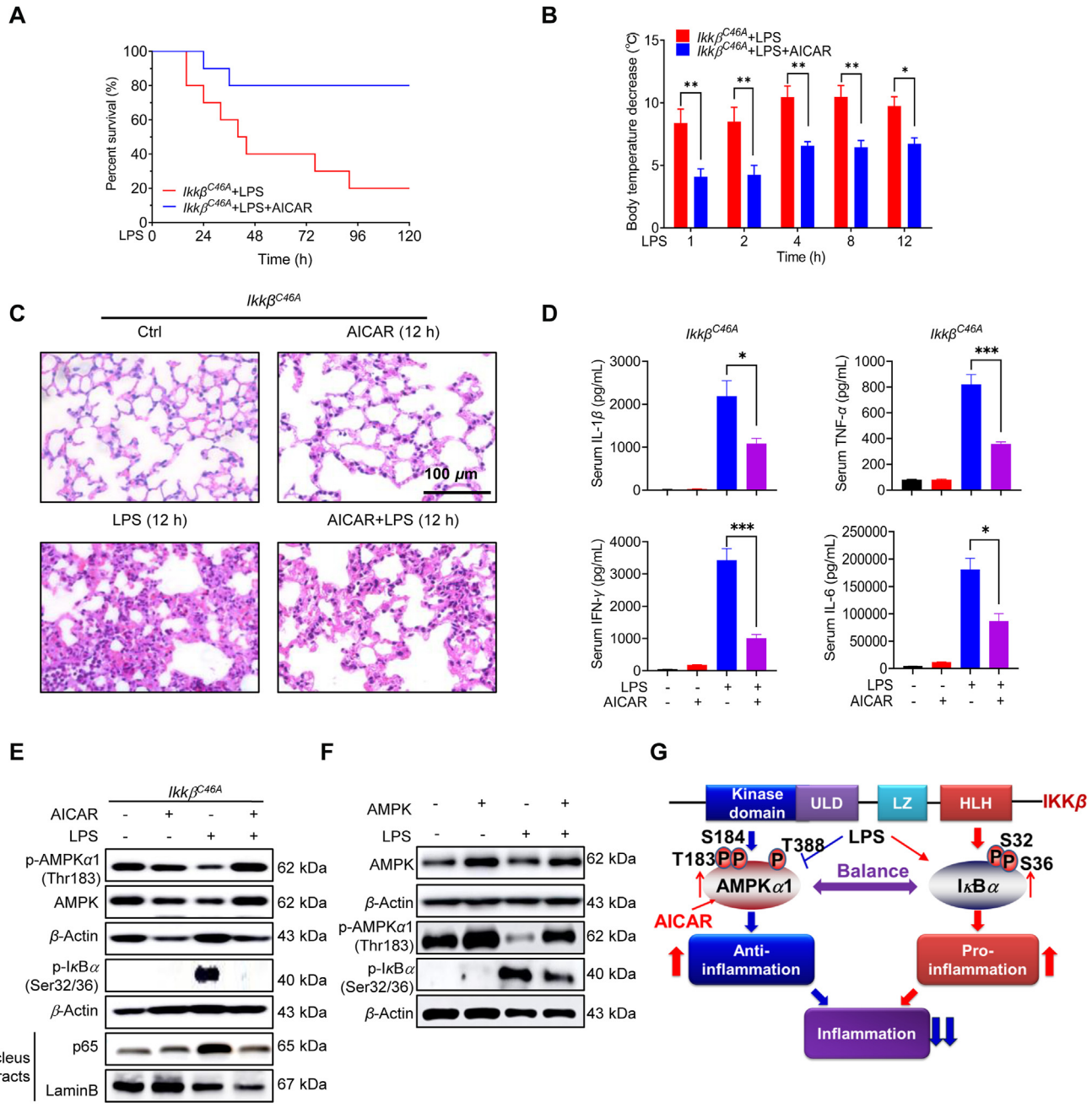

Figure 5 AMPK agonist rescues LPS induced mortality of *Ikkβ*^{C46A} mice. (A) The mortality of *Ikkβ*^{C46A} mice was monitored every 4 or 8 h for up to 120 h after intraperitoneally administrated with 15 mg/kg LPS in the presence or absence of 300 mg/kg AICAR ($n = 10$). (B, C) Body temperatures and histopathological images of lung tissues of the *Ikkβ*^{C46A} mice with the indicated treatments ($n = 10$). (D) The secretion of indicated cytokines in the serum of the *Ikkβ*^{C46A} mice with indicated treatments ($n = 5$). (E) The expression levels of p-AMPK, p-IκBα, and p65 in the livers of the *Ikkβ*^{C46A} mice with indicated treatments. (F) The expression levels of p-AMPK, p-IκBα, and AMPK were determined, followed by transfection of AMPKα1 plasmid into BMDMs isolated from the *Ikkβ*^{C46A} mice and then treated with or without 100 ng/mL LPS. (G) The schematic showing that AMPK agonist induces the transition from inflammatory to physiological status. Data are expressed as mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

LPS inhibiting AMPKα phosphorylation and up-regulating IκBα phosphorylation could be prevented by overexpression of AMPKα1 in BMDMs derived from *Ikkβ*^{C46A} mice (Fig. 5F). These results indicated that the balance between AMPKα and IκBα is existed to maintain the physiological status and activation of AMPK reconstructs the homeostasis to the rescue LPS-induced severe inflammation (Fig. 5G).

3.6. Induction of autophagy attenuates LPS-induced mortality of Ikkβ^{C46A} *mice*

A previous study has reported that IKK complex contributes to the induction of autophagy involved in AMPK[13]. In line with these results, we also found that *Ikkβ*^{C46A} significantly induced autophagy *via* suppression of p-mTOR and p-ULK1^{S757}, activation of

p-ULK1^{S555} and degradation of ULK1 independent of Atg 3, 5, 7, and 12 expressions (Fig. 6A–C). These results indicated that induction of autophagy helps to maintain the homeostasis of *Ikkβ*^{C46A} mice and may result from AMPK activation in *Ikkβ*^{C46A} mice. In coincide with the results, we found increased autophagy in the BMDMs from *Ikkβ*^{C46A} mice compared to wild type littermates, and the autophagy could be inhibited by LPS (Supporting Information Fig. S8A). Hence, we further explored that autophagy induction by rapamycin treatment partially rescued the mortality of LPS-induced *Ikkβ*^{C46A} mice (Fig. 6D), although rapamycin presents a less effect than AMPK agonist. In concert with the result of AMPK agonist treatment, decreasing body temperature, ameliorating lung inflammation, and reducing secreted cytokines were also observed after rapamycin treatment in LPS-induced *Ikkβ*^{C46A} mice (Fig. 6E–G and Fig. S8B). Taken together, our results suggested that autophagy may partially

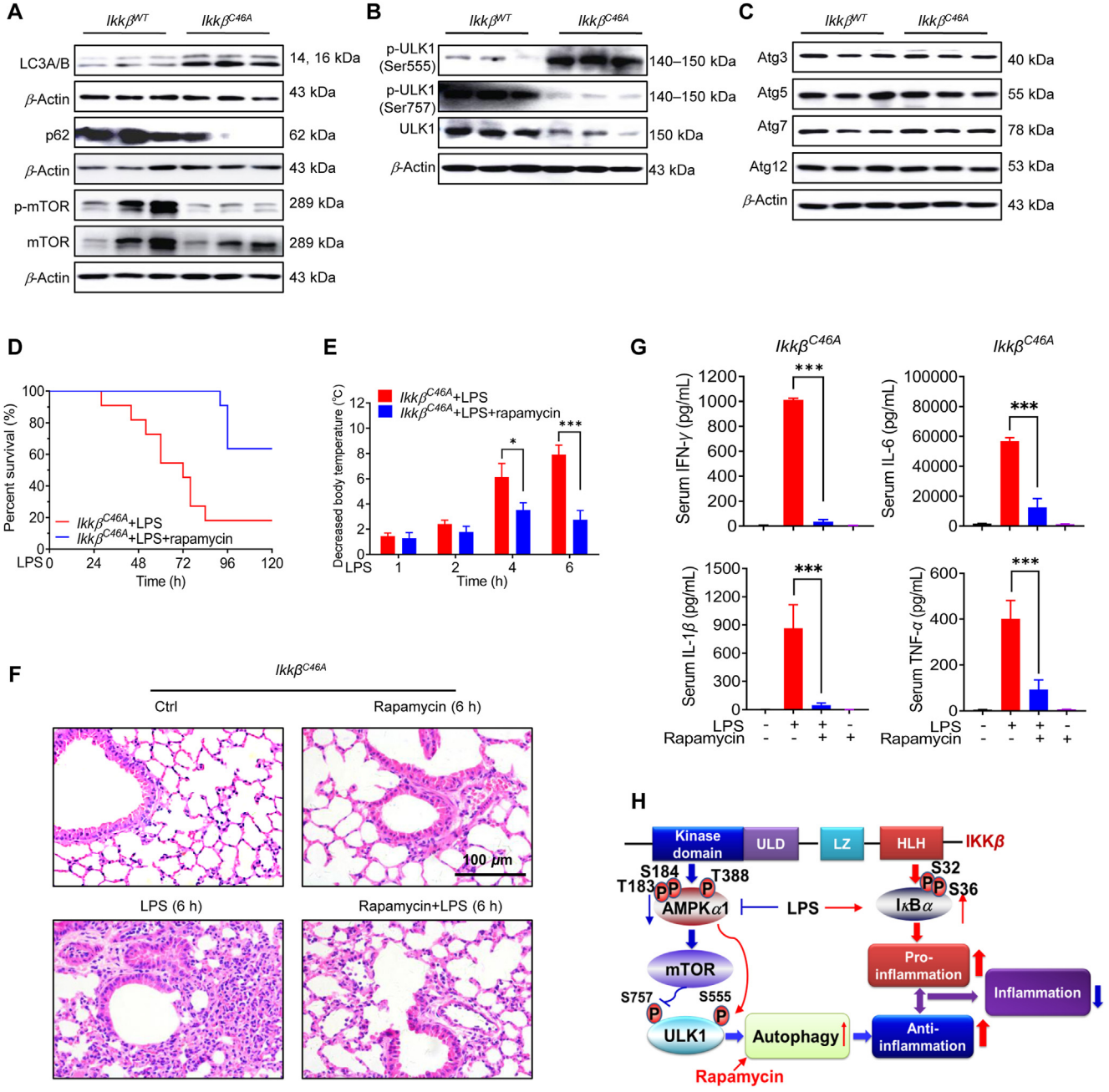

Figure 6 Rapamycin attenuates LPS-induced mortality of *Ikkβ*^{C46A} mice. (A–C) The expression levels of LC3 A/B, p62, mTOR-ULK signaling, and autophagic proteins in liver tissues of the *Ikkβ*^{C46A} mice and *Ikkβ*^{WT} mice. (D) The mice were intraperitoneally administrated with 15 mg/kg LPS in the presence or absence of 4 mg/kg rapamycin and monitored for mortality every 4 or 8 h for up to 120 h ($n = 11$). (E) Body temperatures of the *Ikkβ*^{C46A} mice with indicated treatments were examined ($n = 7$). (F) Histopathological images of lung tissues of the *Ikkβ*^{C46A} mice with indicated treatments. (G) The secretion of indicated cytokines in the serum of *Ikkβ*^{C46A} mice with indicated treatments ($n = 4$). (H) The schematic showing that autophagy may partially counterbalance the activation of IKKβ–IκBα–NF-κB mediated by LPS and in turn suppress inflammation. Data are expressed as mean ± S.E.M. $^*P < 0.05$, $^{***}P < 0.001$.

counterbalance the activation of IKKβ−IκBα−NF-κB mediated by LPS and in turn suppresses inflammation (Fig. 6H). Collectively, IKKβ directly modulates AMPK phosphorylation and in turn activates ULK^{S555}-autophagy, and induction of autophagy partially recovers IKKβ-mediated homeostasis to attenuate LPS induced inflammation.

4. Discussion

Due to the crucial role of IKKβ in inflammation by initiating IκBα−NF-κB signaling pathway[26], inhibition of IKKβ is an attractive strategy for development of anti-inflammatory drugs[27,28]. However, the clinical trials of many IKKβ inhibitors, such as MLN-120B, IMD-2560, and SAR-113945, have been terminated due to problems of efficacy, safety, and non-selectivity[29−31]. Therefore, discovery of new IKKβ-selective inhibitors to treat inflammatory and autoimmune diseases has largely declined in the past 10 years[32].

Although much attention has been focused on the NF-κB-dependent functions of IKKβ, IKKβ can regulate NF-κB-independent mechanisms to mediate inflammation, apoptosis, cell proliferation, and metabolic homeostasis by phosphorylating SNAP-23, PFKFB3, IRF7, p53, and BAD[10,11]. It was reported that the inhibitors of Tpl2 which is activated by IKKβ could selectively inhibit the production of pro-inflammatory cytokines more than IKKβ inhibitors, because IKKβ is activated by many ligands and not just inflammatory irritation[32], indicating that the complicated role of IKKβ in inflammation need to be explored.

It was also reported that IKKβ induces autophagy *via* activation of AMPK[13], and accumulated evidences indicated that AMPK restrains inflammation through stimulating autophagy and inhibiting NF-κB activation[33]. Accumulating literatures described how activation of AMPK suppresses inflammatory responses whereas inhibition of AMPK activity is associated with increased inflammation[34,35]. Due to the key role of AMPK in inflammation, several pharmacological AMPK activators have been identified to inhibit inflammation, including metformin and AICAR[35,36]. These results indicate that IKKβ generates inflammation *via* activation of IκBα−NF-κB and simultaneously restrains inflammation which is correlated to activation of AMPK. Hence, these contradictory results probably correspond to the unsuccess of IKKβ inhibitor to be developed as anti-inflammatory drugs and intensively mechanistic study is desired.

In our previous study, we have identified an IKKβ inhibitor ellipticine targeting Cys46 *in vitro* and *in vivo*[20]. During processing the study, we found *Ikk*β^{C46A} mice did not exhibit inflammation phenotypes although they possessed IKKβ activation. On the other hand, we also analyzed the samples from health donors and found some of them harboring IKKβ activation. These surprising phenomena indicate that IKKβ activation plays homeostatic function rather than mediating inflammation.

We then found LPS induced the increased mortality of *Ikk*β^{C46A} mice compared to wild type mice by highly concurrent inhibition of AMPK and activation of IκBα−NF-κB signaling. These results suggest that there may be a direct correlation among AMPK, IKKβ, and inflammation. Indeed, we demonstrated that IKKβ KD can interact with and phosphorylate AMPKα1 at Thr183, Ser184, and Thr388. Importantly, we showed that IKKβ can competitively phosphorylate AMPKα1 and IκBα. And the homeostasis mediated by IKKβ could be interfered by AMPK inhibitor and autophagy inhibitor. We further discovered that mortality induced by LPS could be greatly rescued by AMPK agonist AICAR and partially by autophagy inducer rapamycin through restoring the homeostasis. Therefore, we reckon that IKKβ plays a dual and opposite role in inflammation *via* simultaneously phosphorylating AMPK and IκBα to maintain the homeostasis *in vivo*.

As we observed in this study, the homeostasis mediated by IKKβ was interfered with LPS *via* decreasing AMPK activation and increasing IκBα phosphorylation to induced severe inflammatory responses in *Ikk*β^{C46A} mice. Interestingly, we found that BBR, an inhibitor targeting IKKβ Cys179[22], could suppress the inflammation while its anti-inflammatory effect cannot be compared with AICAR, the agonist of AMPK. This suggests that activation of AMPK is better strategy than inhibition of IKKβ to recover the inflammation induced by LPS.

IKKβ activity controls basal expression of autophagy in mammal cells independent of NF-κB, and the cells lacking IKKβ fail to induce autophagy in response to cellular starvation[37]. In coincided with the report, we observed that the autophagy visibly increased in *Ikk*β^{C46A} mice. In light of the effect of rapamycin in autophagy induction[38], we found rapamycin could ameliorate the death of *Ikk*β^{C46A} mice induced by LPS, while the effect of rapamycin was less than AICAR. Accordingly, CQ induced inflammation in the mice although the inflammatory symptoms were mild compared to compound C. Since IKKβ activation mediates autophagy by involving AMPK[13], AMPK may play the key role in IKKβ-mediated homeostasis. It was also reported that AMPK activation by AICAR or metformin inhibited NF-κB activation by decreasing IKKβ-dependent IκBα phosphorylation to mediate anti-inflammation[39,40], while there are no indications that IKKβ could directly interact with AMPK to restrain inflammation. Our study has filled this gap by showing IKKβ can concurrently phosphorylate AMPK and IκBα and play dual inflammatory/anti-inflammatory roles. Hence our novel findings may provide new insights into anti-inflammatory drug discovery.

Previous studies have shown that LKB1 and CaMKKβ are the two major upstream kinases capable of phosphorylating Thr183, which is critical for significant activation of AMPK[41]. In this study, we not only found that IKKβ is another important kinase to phosphorylate Thr183 for activation of AMPK, but also demonstrated that phosphorylation of Thr183 could be influenced by Ser184 and Thr388. Notably, all the three amino acid residues interact with IKKβ KD, implying that IKKβ is important to activate AMPK. Although we have demonstrated Ser184 and Thr388 of AMPK have been phosphorylated when IKKβ was overexpressed in the cells, we have not successfully made monoclonal antibody of Ser184 and Thr388 to validate the capability of IKKβ to phosphorylate the two sites of AMPK by *in vitro* kinase assay. Intensive studies are required to elucidate the function of AMPK phosphorylation at Ser184 and Thr388 and further understand the role of AMPK in the inflammatory diseases.

On the other hand, the HLH domain of IKKβ is essential to phosphorylate IκBα[42], implying that IKKβ probably generates inflammation *via* phosphorylation of IκBα (HLH domain dependent) and restricts inflammation *via* phosphorylation of AMPK (kinase domain dependent). Indeed, our results clearly demonstrated there is competition between AMPK and IκBα to be phosphorylated by IKKβ. This provides the underlying mechanism to elucidate AMPK activation suppressing p-IκBα−NF-κB signaling[43].

It is noteworthy that wild type mice exhibited mild inflammatory responses compared to *Ikk*β^{C46A} mice, which probably is correlated to relatively low activation level of IKKβ−p-AMPK−ULK1^{S555}−autophagy in the mice. These results suggested that IKKβ plays the dual and opposite functions in inflammation, providing a scientific explanation why IKKβ inhibitors have repeatedly failed in clinical trials.

5. Conclusions

In the current study, we have provided the first direct evidence that IKKβ restricts inflammation *via* phosphorylation of AMPK to counterpoise IκB−NF-κB activation. Breaking the homeostasis by suppression of AMPK could result in activation of p-IκBα−NF-κB and inflammatory responses. This balance could be extremely broken by LPS to induce severe inflammatory responses *via* concurrently inhibiting AMPK phosphorylation and up-regulating IκBα phosphorylation. Subsequently, these severe inflammatory responses induced by LPS could be remarkably rescued by activation of AMPK rather than inhibition of IKKβ. The link between IKKβ and AMPK we established here may mechanistically explain the long-standing conundrum of the correlation among IKKβ, AMPK, and inflammation.

Our study revealed that IKKβ is the upstream kinase to phosphorylate AMPK and mediates its homeostatic function by competitively catalyzing phosphorylation of AMPK and IκBα to restrict and generate inflammation, respectively. Hence, the study not only explores the potent deficiency of IKKβ as drug target but also demonstrates activation of AMPK may be a better strategy than inhibition of IKKβ for development of anti-inflammatory drugs.

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Author contributions

Ting Li and Liang Liu conceived the study, supervised experiments, and wrote the manuscript. Juan Liu and Yuxin Zhuang performed experiments, analyzed data, and assisted in manuscript writing. Qiang Wu, Jianlin Wu, Meixian Liu, Yue Zhao, Zhongqiu Liu, Caiyan Wang, Linlin Lu, Yingjiao Meng, Kawai Lei, Xiaojuan Li, Qibiao Wu, Elaine Lai-Han Leung, and Zhengyang Guo performed experiments and analyzed data.

Conflicts of interest

The authors declare that they have no conflict of interests.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2021.09.012.

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