HIPK2 phosphorylates HDAC3 for NF-κB acetylation to ameliorate colitis-associated colorectal carcinoma and sepsis

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Although inflammation is critical for the clearance of pathogens, uncontrolled inflammation also contributes to the development of multiple diseases such as cancer and sepsis. Since NF-κB–mediated transactivation in the nucleus is pivotal downstream of various stimuli to induce inflammation, searching the nuclear-localized targets specifically regulating NF-κB activation will provide important therapeutic application. Here, we have identified that homeodomain-interacting protein kinase 2 (HIPK2), a nuclear serine/threonine kinase, increases its expression in inflammatory macrophages. Importantly, HIPK2 deficiency or overexpression could enhance or inhibit inflammatory responses in LPS-stimulated macrophages, respectively. HIPK2-deficient mice were more susceptible to LPS-induced endotoxemia and CLP-induced sepsis. Adoptive transfer of *Hipk2*$^{+/-}$ bone marrow cells (BMs) also aggravated AOM/DSS-induced colorectal cancer. Mechanistically, HIPK2 bound and phosphorylated histone deacetylase 3 (HDAC3) at serine 374 to inhibit its enzymatic activity, thus reducing the deacetylation of p65 at lysine 218 to suppress NF-κB activation. Notably, the HDAC3 inhibitors protected wild-type or *Hipk2*$^{-/-}$ BMs-reconstituted mice from LPS-induced endotoxemia. Our findings suggest that the HIPK2-HDAC3-p65 module in macrophages restrains excessive inflammation, which may represent a new layer of therapeutic mechanism for colitis-associated colorectal cancer and sepsis.

cytokine storm | colon cancer | HIPK2 | HDAC3 phosphorylation | p65 acetylation

Upon recognizing pathogen-associated molecular patterns, Toll-like receptor (TLR) pathways are activated to produce various proinflammatory cytokines and clear invading pathogens (1). However, uncontrolled inflammation has been implicated in numerous human diseases, including systemic inflammatory response syndrome and common conditions such as diabetes, Alzheimer's disease, and cancers (2, 3). Great efforts have been made to explore drugs that attenuate the uncontrolled inflammation, such as TLR4 antagonist Eritoran tetrasodium (also known as E5564) (4), IL-6 receptor–inhibiting antibody tocilizumab, and anti–IL-6 antibody sirukumab (5, 6). However, limited success has been achieved in the clinical research due to the compensation effect by other TLRs or other cytokines. Given that macrophages are the major innate immune cells located in multiple organs, which are function plasticity to orchestrate proinflammatory and anti-inflammatory responses in various diseases (7), and NF-κB is the pivotal transcription factor for the induction of inflammation in macrophages, identification of key regulators that modulate NF-κB activation in macrophages could effectively ameliorate cytokine storm-related lethal infections or unresolved inflammation-driven cancers.

The mammalian NF-κB family includes five members, NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB, and c-Rel, which form homo- or heterodimers (8), and the most abundant form is the heterodimeric p65-p50 complex. NF-κB has been reported to undergo various post-translational modifications to achieve the maximum activation; for example, the p65 subunit can be modified by phosphorylation, acetylation, ubiquitination, and monomethylation (9–12). In particular, p65 transcriptional activity is tightly and distinctly controlled by p65 acetylation. The acetylation of p65 is reversibly regulated by histone acetyltransferases

Significance

Multiple human diseases including cancer and sepsis are closely related to uncontrolled inflammation. However, there is not much success for clinical therapy to date by targeting specific inflammatory cytokines. Since NF-κB–mediated transactivation in the nucleus is pivotal downstream of various stimuli to induce inflammation, searching the nuclear-localized targets specifically regulating NF-κB activation will provide important therapeutic application. This study has found that HIPK2 restrains NF-κB activation through phosphorylating HDAC3 at serine 374 to inactive HDAC3 deacetylase activity, thus reducing the p65 deacetylation and suppressing inflammation. Our findings reveal a function of the HIPK2-HDAC3-p65 module in macrophages to restrain excessive inflammation, which may represent a therapeutic mechanism for inflammation-related diseases.

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and histone deacetylases (HDACs) including HDAC1, HDAC2 and HDAC3 (10, 13–15). While HDAC1 and HDAC3 target NF-κB through a direct association with p65, HDAC2 regulates NF-κB activity through its association with HDAC1 (13, 16).

In this study, we have identified the role of HDAC3 in p65 deacetylation. HDAC3 regulates gene transcription by modulating the deacetylation of the core histones and some other nonhistone substrates (17). The activity of HDAC3 itself is determined by forming multiprotein complexes with the silencing mediator for retinoid and thyroid receptors (SMRT) or nuclear receptor corepressor (NCOR). In addition, HDAC3 activity can be regulated by phosphorylation and dephosphorylation. For example, HDAC3 serine 424 is phosphorylated by protein kinase CK2 and dephosphorylated by protein serine/threonine phosphatase 4, which promotes or inhibits HDAC3 activity respectively (18). We therefore asked how HDAC3 activity is regulated in macrophages during bacterial infection and whether this contributes to p65-mediated inflammation.

Homeodomain-interacting protein kinase 2 (HIPK2) is a serine/threonine kinase, which is mainly localized in subnuclear speckles. HIPK2 belongs to the evolutionarily conserved HIPK family that contains three closely related members, HIPK1, HIPK2, HIPK3 (19), and HIPK4 (20). As a nuclear protein kinase, HIPK2 binds and phosphorylates a plethora of downstream target proteins and can function as a corepressor or a coactivator depending on its interacting transcription factors. This feature enables HIPK2 to regulate diverse downstream signaling pathways and various biological processes including DNA damage, apoptosis, fibrosis, and angiogenesis by targeting p53, carboxyl-terminal binding protein, transcription factor 3, and myocyte enhancer factor 2C, respectively (21–24). NF-κB is reported as one of the downstream transcription factors of HIPK2 for its up-regulation in HIPK2-overexpressing kidney cells (25). Nevertheless, it remains unclear how HIPK2 regulates NF-κB activation in macrophages against pathogen infection and inflammation-associated cancer.

In this study, we have identified the inducible expression of HIPK2 in inflammatory macrophages, which functions as a negative effector to ameliorate colitis-associated colorectal carcinoma and sepsis. HIPK2 phosphorylates HDAC3 at serine 374 to inhibit its deacetylase activity, which results in p65 acetylation at lysine 218. Consequently, K218 acetylation of p65 suppresses NF-κB activation and inhibits the production of proinflammatory cytokines. Our findings propose a mechanism and function of the HIPK2-HDAC3-p65 module in inflammatory macrophages, which extends our understanding of targeting HDAC3 to ameliorate colitis-associated colorectal cancer and sepsis.

Results

HIPK2 Protects Mice from Colorectal Carcinoma and Is Up-Regulated in Inflammatory Macrophages. Chronic inflammation contributes to the development of many cancers, such as colorectal cancer and hepatocellular carcinoma (HCC) (26). Recently, a group of protein kinases are reported to play dual roles in the development of tumor by both directly blocking tumor cell proliferation and inhibiting immune cell-mediated inflammation. As we previously elucidated, the tumor suppressor serine/threonine protein kinase 4 (STK4, also termed MST1) could also inhibit TLR4-induced production of proinflammatory cytokines in macrophages against inflammation-associated HCC (27). Interestingly, similar to the expression pattern of *MST1*, the tumor suppressor gene *HIPK2* was expressed at higher levels from IL-6^{low} HCC samples than that from IL-6^{high} HCC samples based on the cluster analysis of protein kinases in hepatitis B virus infection–associated HCC patients' samples (Fig. 1*A* and *SI Appendix*, Table S1). To investigate the potential regulatory function of HIPK2 in chronic inflammation-driven cancers, we generated *Hipk2* knockout mice (*SI Appendix*, Fig. S1*A*). The knockout efficiency of *Hipk2* was confirmed by gene sequencing and immunofluorescence staining of the liver and lung tissues (*SI Appendix*, Fig. S1 *B–D*). In line with previous reports showing the developmental defects in *Hipk2*$^{-/-}$ mice (28, 29), we noticed that *Hipk2*$^{-/-}$ mice had reduced body weight and size than those of the age- and sex-matched wild type (WT) and *Hipk2*$^{+/-}$ mice (*SI Appendix*, Fig. S1*E*). Because *Hipk2* is known to be haploinsufficient (30, 31) and *Hipk2*$^{+/-}$ mice grow normally as WT mice, we thus mainly used *Hipk2*$^{+/-}$ mice or macrophages throughout the paper. MC38 colon carcinoma cells were subcutaneously injected into WT and *Hipk2*$^{+/-}$ mice, and tumors grew more rapidly in *Hipk2*$^{+/-}$ mice (Fig. 1 *B* and *C*). Flow cytometric analysis showed that the percentages of macrophages were increased in the tumors of *Hipk2*$^{+/-}$ mice (*SI Appendix*, Fig. S2*A*), while the percentages and numbers of dendritic cells or neutrophils were not changed (*SI Appendix*, Fig. S2 *B* and *D*). Interestingly, the numbers of CD4$^+$ T cells and CD8$^+$ T cells were significantly reduced in the tumors of *Hipk2*$^{+/-}$ mice (*SI Appendix*, Fig. S2*C*). To investigate the potential role of tumor-infiltrating macrophages, *Hipk2*$^{+/-}$ peritoneal exudate macrophages (PEMs) were treated with the MC38 tumor supernatants, which significantly elevated *Il6* transcription levels (Fig. 1*D*). Since AOM (azoxymethane)- and DSS (dextran sulfate sodium)-induced colorectal cancer is a classical chronic inflammation-driven tumor model (32), we then used this model to elucidate the in vivo role of HIPK2 in colitis-associated colorectal cancer. To exclude the influence of other types of HIPK2-deficient cells, we adoptively transferred CD45.2 bone marrow cells (BMs) from either WT or *Hipk2*$^{+/-}$ mice to lethally irradiated CD45.1 mice, followed by the induction of colorectal cancer using AOM and DSS (Fig. 1 *E*, *Left*). We observed more and larger tumors in the colons of *Hipk2*$^{+/-}$ recipient mice in comparison to the WT control (Fig. 1 *E, Right* and *F*). Furthermore, serum IL-6 concentrations in the *Hipk2*$^{+/-}$ recipient mice were much higher than those in WT recipient mice (Fig. 1*G*). Histologically, more low-grade adenocarcinomas were invaded into the colonic submucosa of *Hipk2*$^{+/-}$ recipient mice (Fig. 1*H*). Immunofluorescence analysis of colon tissues showed that HIPK2 expression in F4/80$^+$ macrophages were apparently enhanced after AOM-DSS treatment (Fig. 1*I*). Together, these results suggest that HIPK2 protects mice against chronic inflammation-driven colorectal cancer.

Excessive inflammation initiated by viral or bacterial infection can result in cytokine storm and sepsis. To evaluate the potential role of HIPK2 in acute inflammatory response, we analyzed data of patients with acute respiratory distress syndrome (ARDS) or sepsis in the Gene Expression Omnibus database. *HIPK2* transcription was positively correlated with the anti-inflammatory cytokine *IL10* but was negatively correlated with *IL6* in the peripheral blood samples of patients with ARDS (GSE89953) or sepsis (GSE26440), respectively (*SI Appendix*, Fig. S3 *A* and *B*). To further confirm these findings, we collected peripheral blood mononuclear cells (PBMCs) from sepsis patients and healthy donors to analyze HIPK2 expression. Notably, the percentages of CD14$^+$ PBMCs were significantly increased in sepsis patients (*SI Appendix*, Fig. S3 *C* and *D*), and *HIPK2* transcription was negatively correlated with *IL1B* but positively correlated with *IL10* in PBMCs of sepsis patients (*SI Appendix*, Fig. S3*E*). Furthermore, HIPK2 expression was substantially enhanced in CD14$^+$ PBMCs of sepsis patients (Fig. 1*J*). In line with this, lipopolysaccharide (LPS) stimulation markedly increased the protein levels of HIPK2 in PEMs (Fig. 1*K*). Together, these data indicate that HIPK2 expression is increased in inflammatory macrophages or PBMCs, and it is negatively related to inflammation.

HIPK2 Reduces TLR4/9-Induced Proinflammatory Cytokine Production. As the activation of TLR4 pathway is critical to induce inflammation, we first investigated the role of HIPK2 in TLR4-mediated inflammatory response. Specific small interfering RNAs (siRNAs) were used to knock down *Hipk2* in PEMs (Fig. 2 *A*, *Left*) followed by LPS stimulation or *Salmonella typhimurium* (strain *SL1344*)

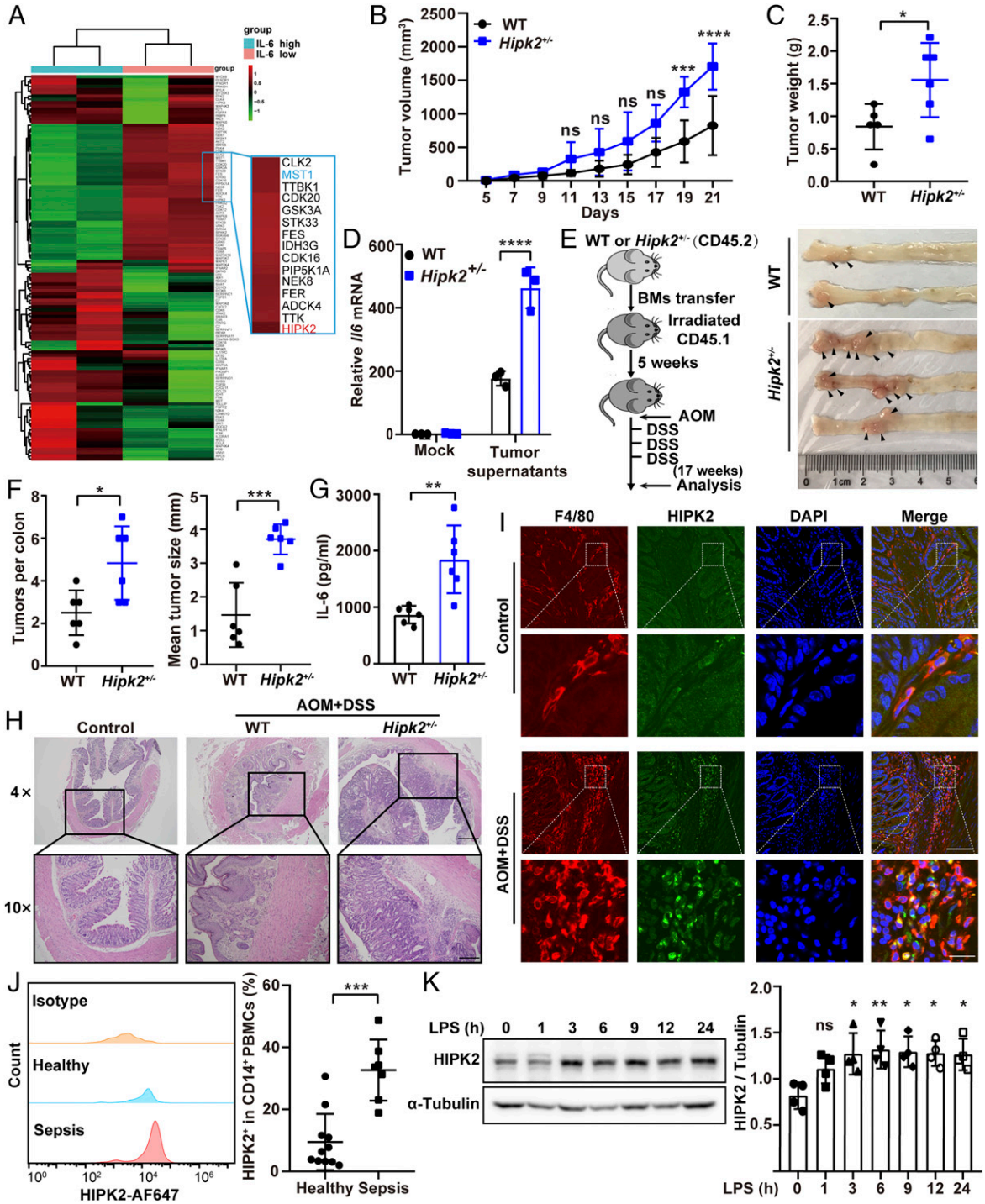

Fig. 1. HIPK2 protects mice from colorectal carcinoma and is up-regulated in inflammatory macrophages. (*A*) Affymetrix microarray analysis of differentially expressed protein kinases in HCC samples of patients with high- or low-serum IL-6 levels. (*B* and *C*) WT ($n = 5$) and *Hipk2*$^{+/-}$ ($n = 6$) mice were subcutaneously injected with MC38 colon carcinoma cells (5×10^5), and the tumor volume (*B*) and tumor weight (*C*) were measured. (*D*) WT and *Hipk2*$^{+/-}$ PEMs were treated with the supernatants of MC38 subcutaneous tumor for 6 h to detect the mRNA levels of *Il6*. (*E–I*) CD45.2$^+$ WT and *Hipk2*$^{+/-}$ BMs were adoptively transferred to the lethally irradiated CD45.1$^+$ recipient mice respectively. A total of 5 wk after reconstitution, mice were injected intraperitoneally with AOM (10 mg/kg) and fed with drinking water containing 2.5% DSS for 7 consecutive days a week later. After three cycles of DSS treatment, mice were euthanized at week 17 (*E*, *Left*). Tumors in the colons were indicated by the arrows (*E*, *Right*), and tumor numbers and size of each mouse were measured (*F*). (*G*) IL-6 concentration in the serum of AOM/DSS-treated WT and *Hipk2*$^{+/-}$ recipient mice was measured. (*H*) Histological analysis of colons was shown by H&E staining. (Scale bar, 500 μm, *Upper*, and 200 μm, *Lower*.) (*I*) Colon tissues from vehicle- and AOM/DSS-treated WT recipient mice were stained by F4/80 and HIPK2 antibodies. (Scale bar, 100 μm, *Upper*, and 20 μm, *Lower*.) (*J*) HIPK2 expression in CD14$^+$ PBMCs from healthy donors or sepsis patients was analyzed by flow cytometry. (*K*) HIPK2 expression was detected in PEMs after LPS stimulation for different time. The relative expression of HIPK2 was normalized to tubulin. Data are from at least three independent experiments (mean ± SD) or representative data (*E* and *H–K*). ns, not significant ($P > 0.05$); *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$, using a two-tailed, unpaired Student's *t* test (*C*, *F*, *G*, and *J*, *Right*) or one-way ANOVA with Holm–Sidak's multiple comparisons test (*K*, *Right*) or two-way ANOVA with Holm–Sidak's multiple comparisons test (*B* and *D*).

infection. Knockdown of *Hipk2* significantly enhanced the messenger RNA (mRNA) levels of *Il6*, *Il1b*, and *Tnfa* (Fig. 2 *A* and *C*) as well as the protein levels of IL-6 and TNF-α (Fig. 2*B*). The macrophage cell lines including murine Raw264.7 cells and human THP-1 cells were constructed to stably overexpress HIPK2, which markedly decreased *Il6* mRNA levels in response to LPS treatment (Fig. 2 *D* and *E*).

To further confirm this phenotype, we prepared HIPK2-deficient macrophages from *Hipk2*$^{+/-}$ and *Hipk2*$^{-/-}$ mice (*SI Appendix*, Fig. S1*F*). *Hipk2*$^{-/-}$ macrophages were used because *Hipk2*$^{-/-}$ mice displayed normal percentages of T cells, B cells, and NK cells in the spleen (*SI Appendix*, Fig. S4 *A* and *B*), normal percentages of macrophages in the peritoneal cavity (*SI Appendix*, Fig. S4 *C* and *D*), and normal development of bone marrow–derived macrophages (BMDMs) as indicated by the comparable levels of *Adgre1* and *Mertk* at each time points (*SI Appendix*, Fig. S4*E*). HIPK2 deficiency promoted *Il6*, *Il1b*, and *Tnfa* transcription (Fig. 2*F*), and IL-6 production (Fig. 2*G*) in BMDMs upon LPS stimulation. Given that HIPK1 and HIPK3 are homologous with HIPK2, we then examined their roles in inflammation. The transcription of *Il6*, *Il1b*, and *Tnfa* was augmented in LPS-stimulated *Hipk1* knockdown PEMs (*SI Appendix*, Fig. S5*A*) but was not affected in *Hipk3* knockdown PEMs (*SI Appendix*, Fig. S5*B*), suggesting that HIPK1 and HIPK2 function similarly to regulate TLR4-induced inflammation. We also determined that HIPK2 deficiency augmented the TLR9 agonist CpG-induced production of *Il6*, *Il1b*, and *Tnfa* (*SI Appendix*, Fig. S5*C*). Taken together, these results have demonstrated that HIPK2 restrains TLR4- and TLR9-triggered inflammation in macrophages.

HIPK2 Deficiency Exacerbates LPS-Induced Endotoxemia and CLP-Induced Sepsis. We next investigated the role of HIPK2 in vivo. HIPK2-deficient mice reached 100% mortality around 20 h post–LPS treatment, which were much earlier than that of WT controls (Fig. 3*A*). Besides, serum concentrations of IL-6, IL-1β, and TNF-α were significantly increased in HIPK2-deficient mice compared to those in WT mice (Fig. 3*B*). Hematoxylin and Eosin (H&E) staining showed severer damages with increased pulmonary interstitial edema and more infiltrating immune cells in the lung of HIPK2-deficient mice (Fig. 3*C*). These observations suggest that HIPK2 deficiency aggravates the severity and sensitivity of mice to LPS-induced endotoxemia.

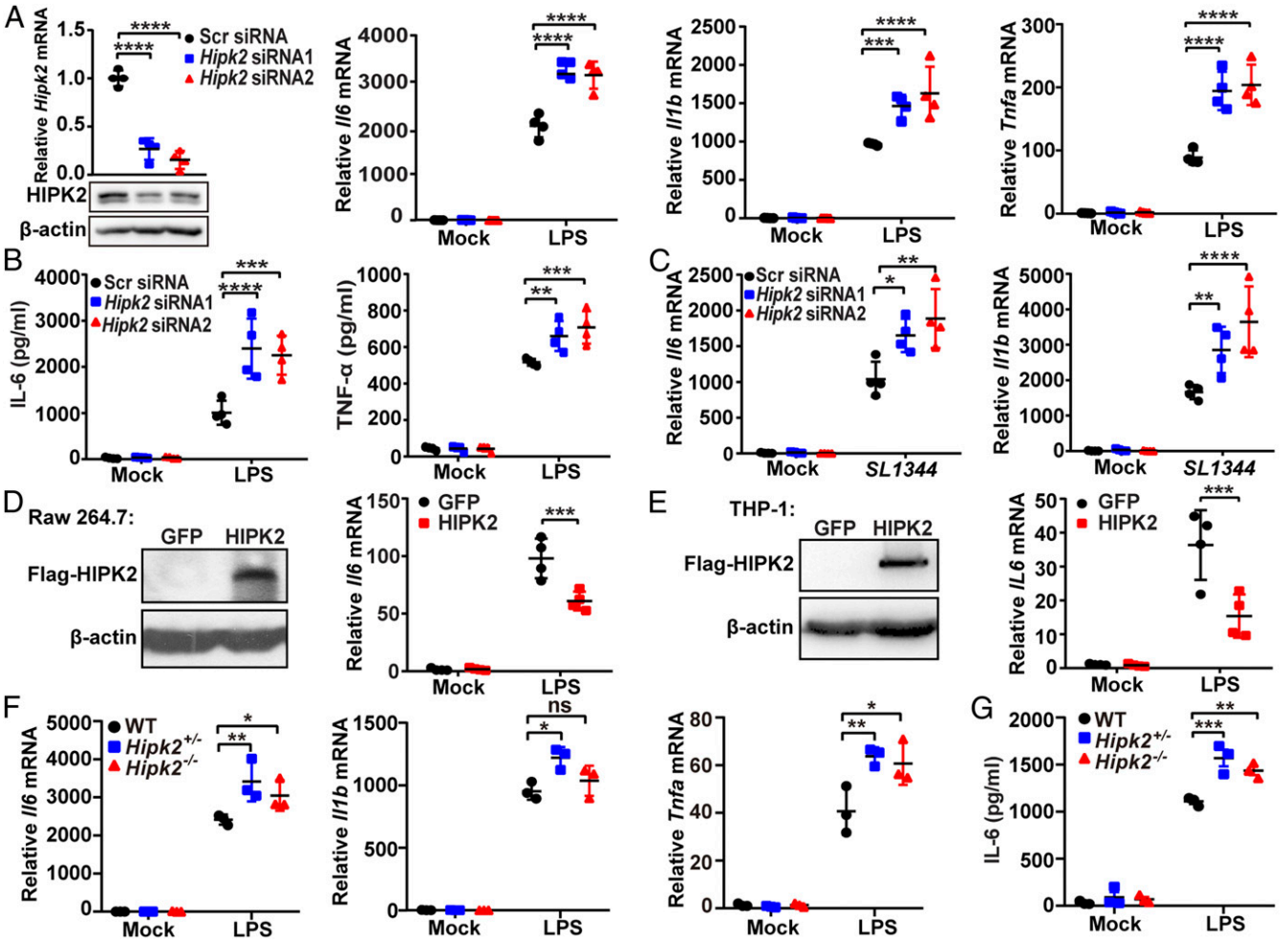

Fig. 2. HIPK2 reduces TLR4/9-induced proinflammatory cytokine production. (*A*–*C*) Specific siRNAs (40 nM) were used to knock down *Hipk2* in PEMs (*A*, *Left*), followed by LPS stimulation or *SL1344* infection for 3 h. *Hipk2* knockdown efficiency, the mRNA levels of *Il6, Il1b*, and *Tnfα* (*A* and *C*), and the protein levels of IL-6 and TNF-α (*B*) were measured. Scr, scramble control. (*D* and *E*) HIPK2-transduced RAW264.7 cells (*D*) or human THP-1 cells (*E*) were generated (*Left*), which were stimulated with LPS for 3 h or treated with PMA followed by LPS stimulation for 6 h to detect *Il6* mRNA levels. (*F* and *G*) WT, *Hipk2*$^{+/-}$, and *Hipk2*$^{-/-}$ BMDMs were stimulated with LPS for 6 h. The mRNA levels of *Il6, Il1b*, and *Tnfa* were detected by qRT-PCR (*F*), and the protein levels of IL-6 were examined by enzyme linked immunosorbent assay (ELISA) (*G*). Data are from at least three independent experiments (mean ± SD). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001, using two-way ANOVA with Holm–Sidak's multiple comparisons test.

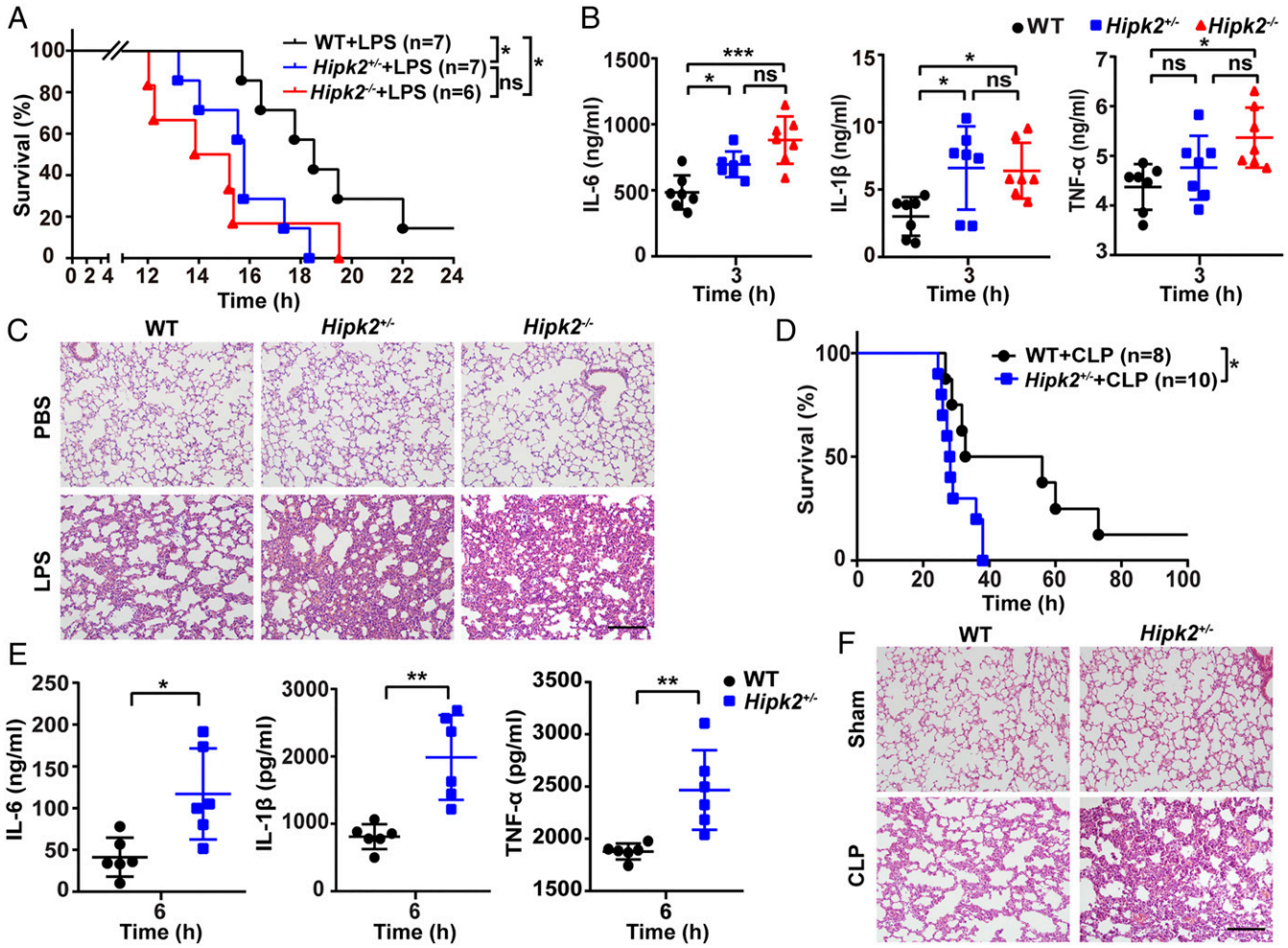

Fig. 3. HIPK2 deficiency exacerbates LPS-induced endotoxemia and CLP-induced sepsis. (*A–C*) WT, *Hipk2*$^{+/-}$, and *Hipk2*$^{-/-}$ mice were intraperitonelly injected with LPS (36 mg/kg). The survival rates were monitored every 3 h for 7 d (*A*). (*D–F*) WT and *Hipk2*$^{+/-}$ mice were subjected to CLP. The survival rates were monitored every 3 h for 7 d (*D*). (*B* and *E*) Serum concentrations of IL-6, IL-1β, and TNF-α were measured 3 h after LPS injection (*B*) or 6 h after CLP treatment (*E*). (*C* and *F*) H&E staining of the lung tissues 12 h after LPS injection (*C*) or 24 h after CLP treatment (*F*). (Scale bars, 200 μm.) Data are from three independent experiments (mean ± SD) or representative data (*C* and *F*). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001, using a log-rank (Mantel–Cox) test (*A* and *D*), one-way ANOVA with Holm–Sidak's multiple comparisons test (*B*), or two-tailed, unpaired Student's *t* test (*E*).

To further test HIPK2 function against bacterial infection–related sepsis, we generated a cecal ligation and puncture (CLP)–induced sepsis model in WT and *Hipk2*$^{+/-}$ mice. Consistently, *Hipk2*$^{+/-}$ mice showed markedly reduced survival time (Fig. 3*D*), substantially enhanced serum concentrations of IL-6, IL-1β, and TNF-α (Fig. 3*E*), and severer lung injury (Fig. 3*F*) compared to WT mice. These observations have demonstrated that HIPK2 could protect mice from CLP-induced sepsis.

HIPK2 Kinase Activity Is Needed to Suppress NF-κB Activation. Since NF-κB signaling is pivotal to induce proinflammatory cytokine production in responding to various stimuli, we investigated whether HIPK2 modulated NF-κB activation. Knockdown of *Hipk2* did not substantially affect IKKα/β phosphorylation or IκBα degradation in LPS-stimulated PEMs (Fig. 4*A*). We therefore tested whether HIPK2 affected NF-κB entry into the nucleus. As shown in Fig. 4 *B–D* and *SI Appendix*, Fig. S6 *A–C*, LPS treatment significantly increased the amount of p65 in the nucleus of HIPK2-deficient PEMs or mouse embryonic fibroblasts (MEFs) by immunoblotting and immunofluorescence assay. To clarify the role of p65 in HIPK2-mediated anti-inflammatory response, *Hipk2* or *p65* were knocked down in PEMs followed by LPS stimulation. Compared to *Hipk2* knockdown alone, double knockdown of *Hipk2* and *p65* failed to up-regulate the transcription of *Il6*, *Il1b*, and *Tnfa* (*SI Appendix*, Fig. S6*D*), indicating that HIPK2 inhibits inflammation mainly through p65.

Since HIPK2 is a serine/threonine protein kinase, we asked whether its kinase activity was required to suppress p65 activation. WT HIPK2 or the kinase dead mutation K221R (containing a lysine-to-arginine substitution at residue 221) were cotransfected with p65 in human embryonic kidney 293T cells (HEK293T cells). The K221R mutant failed to inhibit the NF-κB luciferase activity, revealing a comparable level as that in the control HEK293T cells (Fig. 4*E*). Furthermore, K221R-transduced MEFs was unable to effectively inhibit *Il6* production (Fig. 4*F*) and failed to restrain the nuclear entry of p65 (Fig. 4 *G* and *H*) after LPS stimulation, while a profound inhibitory effect was detected in WT HIPK2-transduced MEFs. We next treated PEMs with inhibitor purvalanol A which blocks HIPK2 autophosphorylation (33). The mRNA levels of *Il6*, *Il1b*, and *Tnfa* were greatly enhanced upon LPS stimulation (*SI Appendix*, Fig. S6*E*). Together, we have demonstrated that HIPK2 depends on its kinase activity to inhibit p65 activation upon LPS stimulation.

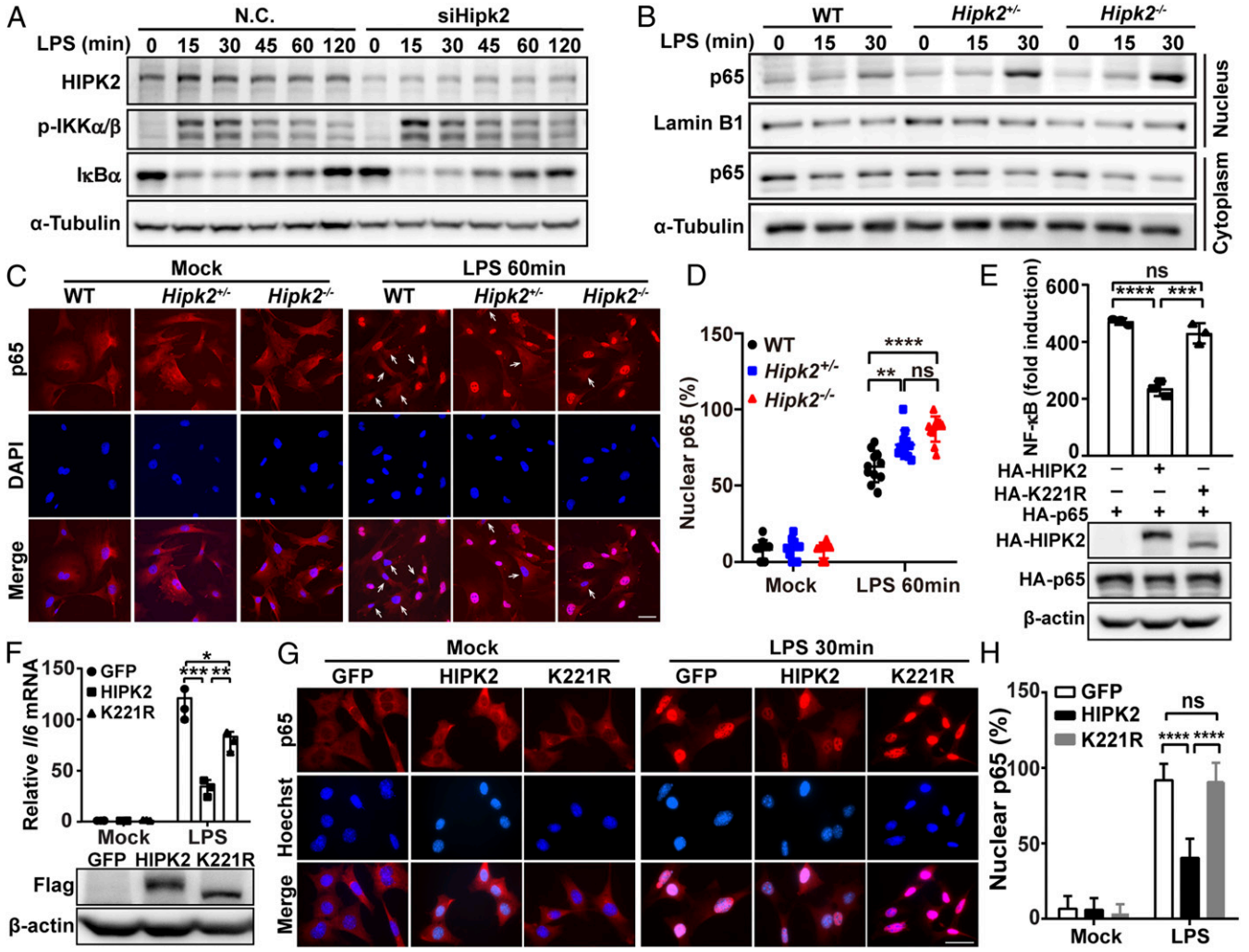

Fig. 4. HIPK2 kinase activity is needed to suppress NF-κB activation. (*A*) The phosphorylation levels of IKKα/β and the degradation of IκB were measured in LPS-stimulated control and *Hipk2* knockdown PEMs. (*B*) The cytoplasm and nucleus were separated from LPS-treated or -untreated WT, *Hipk2*$^{+/-}$, and *Hipk2*$^{-/-}$ primary MEFs to detect the amount of p65. (*C*) WT, *Hipk2*$^{+/-}$, and *Hipk2*$^{-/-}$ MEFs were stimulated with LPS and immunostained with anti-p65 antibody and DAPI to measure the percentages of p65 in the nucleus. (Scale bar, 50 μm.) MEFs without p65 nuclear translocation were indicated by the arrows. (*D*) The percentages of p65 in the nucleus were counted. (*E*) HEK293T cells were transfected with the NF-κB luciferase reporter plasmid and p65, HIPK2, or K221R to measure NF-κB activity. Immunoblots confirmed the expression of the indicated proteins (*Lower*). (*F–H*) MEFs with WT HIPK2 or K221R overexpressing were treated with LPS to detect the mRNA levels of *Il6* by qRT-PCR (*F*) or the nuclear entry of p65 by immunofluorescence (*G*). (Scale bar, 20 μm.) More than 50 cells were counted in each of three independent experiments (*H*). Data are from at least three independent experiments (mean ± SD) or representative data (*A–C* and *G*). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$, using one-way ANOVA with Holm–Sidak's multiple comparisons test (*E*) or two-way ANOVA with Holm–Sidak's multiple comparisons test (*D*, *F*, and *H*).

HIPK2 Phosphorylates HDAC3 at ser374 to Regulate HDAC3 Activity and p65 Acetylation. As the acetylation and phosphorylation of p65 are closely related to its transcriptional activity (15, 34), we asked whether HIPK2 directly bound and phosphorylated p65. No interaction was detected between Flag-tagged HIPK2 and HA-tagged p65 in HEK293T cells (*SI Appendix*, Fig. S6*F*). In addition, *Hipk2* knockdown in PEMs did not obviously affect the phosphorylation levels of p65 after LPS stimulation (*SI Appendix*, Fig. S6*G*). We then examined whether HIPK2 could affect p65 acetylation. Overexpression of HIPK2 in HEK293T cells enhanced the acetylation levels p65, which was further promoted by *SL1344* infection (*SI Appendix*, Fig. S6*H*). Conversely, the acetylation levels of p65 were decreased in LPS-treated *Hipk2*$^{+/-}$ PEMs (*SI Appendix*, Fig. S6*I*). These results indicate that HIPK2 might promote p65 acetylation.

Previous reports imply that HDAC1 and HDAC3 directly bind and deacetylate p65 (13, 16). We did not detect the interaction between HIPK2 and HDAC1 (*SI Appendix*, Fig. S7*A*), but HIPK2 did interact with HDAC3 (*SI Appendix*, Fig. S7 *B* and *C*). We then constructed various truncations of HIPK2 or HDAC3 to map their binding domains. The homeobox-interacting domain (amino acids 510 to 860) and the auto-inhibitory domain (amino acids 860 to 1198) in the carboxyl terminus of HIPK2 interacted with HDAC3 (*SI Appendix*, Fig. S7*B*). Also, the N terminus (amino acids 1 to 320) of HDAC3 interacted with HIPK2 (*SI Appendix*, Fig. S7*C*). Moreover, HIPK2 and HDAC3 showed colocalization in the nucleus of MEFs, and LPS treatment enhanced both the distribution of HIPK2 and its colocalization with HDAC3 (Fig. 5*A*). This result was further corroborated in HIPK2-transduced immortalized BMDMs (iBMDMs) that anti-HA-HIPK2 immunoprecipitation could pull down the increased amount of HDAC3 after LPS stimulation (*SI Appendix*, Fig. S7*D*).

To test whether HDAC3 cooperated with HIPK2 to mediate NF-κB activation, specific siRNAs were used to knock down

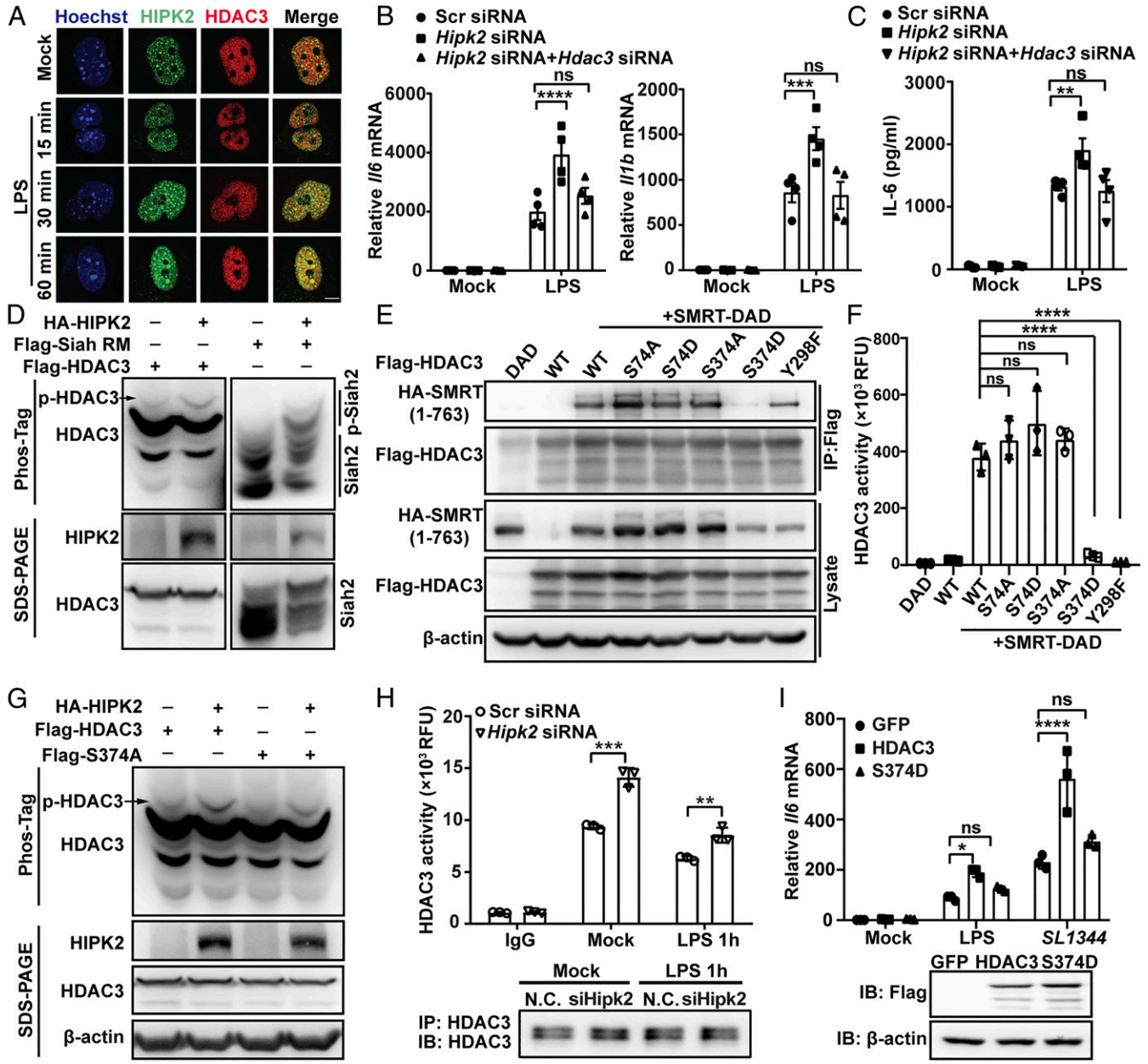

Fig. 5. HIPK2 phosphorylates HDAC3 at ser374 to regulate HDAC3 activity and p65 acetylation. (*A*) Flag-HIPK2–transduced MEFs were treated with LPS, followed by immunostaining with Hoechst, anti-Flag, and anti-HDAC3 antibodies to analyze the colocalization of HIPK2 and HDAC3. (Scale bar, 10 μm.) (*B* and *C*) *Hdac3* and *Hipk2* were knocked down in PEMs with siRNAs (40 nM) as indicated, and the mRNA levels of *Il6* and *Il1b* (*B*) or IL-6 concentrations in the supernatants (*C*) were measured after LPS stimulation for 6 h. (*D*) HEK293T cells were transfected with HDAC3 or Siah2 RM together with or without HIPK2 to detect the phosphorylated HDAC3 and Siah2 by PTMSA. (*E* and *F*) Flag-tagged WT HDAC3 and its point mutations were transfected together with SMRT-DAD (the deacetylase activating domain of SMRT) in HEK293T cells. Cell lysates were immunoprecipitated with anti-Flag antibody to detect the interaction between SMRT-DAD and HDAC3 or its mutants (*E*). The deacetylase activity of HDAC3 was measured by detecting the fluorescent signal of the enzymatically cleaved substrate (*F*). (*G*) WT HDAC3 or S374A mutation was transfected together with or without HIPK2 into HEK293T cells to detect the phosphorylation by PTMSA. (*H*) The deacetylase activity of HDAC3 was detected in LPS-stimulated PEMs with or without *Hipk2* knockdown. (*I*) MEFs with WT HDAC3 or S374D overexpressing were stimulated with LPS or *SL1344* for 3 h to measure the mRNA levels of *Il6*. Data are from at least three independent experiments (mean ± SD) or representative data (*A*, *D*, *E*, and *G*). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001, using one-way ANOVA with Holm–Sidak's multiple comparisons test (*F*) or two-way ANOVA with Holm–Sidak's multiple comparisons test (*B*, *C*, *H*, and *I*).

Hipk2 and *Hdac3* in PEMs. Compared to *Hipk2* knockdown alone, double knockdown of *Hdac3* and *Hipk2* obviously abrogated the augmented mRNA levels of *Il6* and *Il1b* (Fig. 5*B* and *SI Appendix*, Fig. S7*E*) or IL-6 concentrations in the supernatants (Fig. 5*C*) after LPS stimulation. In agreement with this, the HDAC3-specific inhibitor RGFP966 robustly reversed the increased transcription of *Il6* and *Il1b* in *Hipk2* knockdown PEMs upon LPS stimulation (*SI Appendix*, Fig. S7*F*). Taken together, we have elucidated that LPS stimulation promotes the interaction between HIPK2 and HDAC3 in the nucleus to suppress NF-κB activation.

To ascertain how HDAC3 was regulated by HIPK2, the increasing amount of HIPK2 was transfected into HEK293T cells, which did not change the expression levels of endogenous HDAC3 (*SI Appendix*, Fig. S8*A*). Also, HDAC3 expression levels were not

altered in LPS-treated *Hipk2*$^{+/-}$ PEMs (*SI Appendix*, Fig. S8*B*). Considering that the kinase activity of HIPK2 is required to suppress NF-κB activation (Fig. 4 *E*–*H*), we examined whether HIPK2 could phosphorylate HDAC3 by phos-tag mobility shift assay (PTMSA). The E3 ubiquitin ligase Siah2 is a classic substrate of HIPK2, while Siah2 could also induce the polyubiquitylation and degradation of HIPK2 (35). Therefore, the ring mutant of Siah2 (i.e., Siah2 RM), which prevents HIPK2 degradation, was used as a positive control. HIPK2 overexpression caused a significant migration of Siah2 RM, which also induced a shifting band of HDAC3 (Fig. 5*D*). In contrast, purvalanol A treatment reduced the migrating band of HDAC3 in iBMDMs after LPS stimulation (*SI Appendix*, Fig. S8*C*). This indicates that HIPK2 could phosphorylate HDAC3.

To further examine the phosphorylated sites in HDAC3 by HIPK2, HDAC3 immunoprecipitate from HEK293T cells with or without HIPK2 overexpression was detected by mass spectrometry. The serine 74 and serine 374 sites were identified (*SI Appendix*, Fig. S8*D*). To determine whether the two phosphorylation sites of HDAC3 could influence its enzymatic activity, we generated the phosphorylation inactive mutations S74A and S374A and the phosphorylation mimicking mutations S74D and S374D. The deacetylase catalytic mutation Y298F was used as a positive control (36). Previous study has shown that HDAC3's interaction with the deacetylase activating domain (DAD) of SMRT or NCOR is required to engage its catalytic activity (37). Therefore, DAD (amino acids 1 to 763 of SMRT) was coexpressed with HDAC3 or its mutations in HEK293T cells, and only the S374D mutant dramatically reduced the interaction with DAD (Fig. 5*E* and *SI Appendix*, Fig. S8*E*). Moreover, the S374D mutant reduced the deacetylase activity of HDAC3 to a similar level as the Y298F mutant (Fig. 5*F*). When the protein sequences of HDAC3 from human and several nonhuman species were aligned, we noted that S374 is highly conserved (*SI Appendix*, Fig. S8*F*), which manifests that S374 may be an important phosphorylation site of HDAC3 in different species. Furthermore, the intensity of the shifted band in the S374A mutant was less than that of WT HDAC3 when coexpressed with HIPK2 (Fig. 5*G*), indicating that HDAC3 S374 was phosphorylated by HIPK2.

To further validate whether HIPK2 inhibits inflammation through phosphorylating HDAC3 at S374 to suppress its deacetylase activity, immunoprecipitation of HDAC3 and S374D with or without HIPK2 coexpression was performed (*SI Appendix*, Fig. S8*G*). HIPK2 cotransfection markedly reduced HDAC3 activity after *Escherichia coli* infection, which had no effect on S374D activity (*SI Appendix*, Fig. S8*H*). Furthermore, the deacetylase activity of HDAC3 was enhanced in *Hipk2* knockdown PEMs (Fig. 5*H*) but was reduced in HIPK2-overexpressed iBMDMs (*SI Appendix*, Fig. S8*I*). We next examined whether the S374D mutant affected TLR4-induced IL-6 production. Compared to WT HDAC3, the S374D mutant indeed decreased *Il6* transcription after LPS or *SL1344* treatment (Fig. 5*I*). Collectively, these results have demonstrated that HIPK2 binds and phosphorylates HDAC3 at S374 to suppress its deacetylase activity, leading to p65 acetylation and IL-6 production.

HIPK2 Blocks HDAC3-Mediated Deacetylation of p65 K218 to Inhibit Inflammation. Previous studies have reported that the acetylation of p65 at different lysine sites regulates distinct nuclear functions of NF-κB (10). As HIPK2 promoted p65 acetylation (*SI Appendix*, Fig. S6 *H* and *I*), we studied which acetylation site(s) of p65 was affected by HIPK2. Mass spectrometry assay of p65 immunoprecipitate from HEK293T cells with or without HIPK2 overexpression identified the acetylated Lysine 79 and Lysine 218. We constructed the lysine acetylation-mimicking mutations (lysine to glutamine, K79Q and K218Q) and found that K218Q strongly decreased the transcriptional activity of p65, while K79Q had very minor effect (Fig. 6*A*). To further clarify the function of K218, we generated the lysine acetylation-inactivating mutation K218R. Compared to WT p65-transduced MEFs, K218R-transduced MEFs produced more IL-6 at both mRNA and protein levels (Fig. 6 *B* and *C*) and induced more nuclear translocation of p65 after LPS stimulation (Fig. 6 *D* and *E*). Together, we confirmed the inhibitory role of K218 acetylation in NF-κB activation.

Next, it was important to determine whether the acetylation of K218 was regulated by HDAC3. Flow cytometric assay showed that HDAC3 overexpression reduced the acetylation levels of p65 K218, while the S374D mutant exhibited comparable p65 K218 acetylation as the GFP control (Fig. 6*F*). Besides, the K218R mutant substantially enhanced the transcriptional activity of p65, even in the presence of HDAC3 (Fig. 6*G*). These data reveal that HDAC3 reduces the acetylation of K218 through its deacetylase activity. Given that HIPK2 manipulated HDAC3 activity and HDAC3 regulated p65 K218 acetylation, we then analyzed how HIPK2 regulated p65 K218 acetylation. *Hipk2* knockdown PEMs showed reduced p65 K218 acetylation levels after LPS treatment (Fig. 6*H*). This observation was further confirmed in LPS-treated WT and HIPK2-deficient primary MEFs (Fig. 6*I*). Together, we have demonstrated that HIPK2 blocks HDAC3-mediated deacetylation of p65 K218, resulting in the suppression of TLR4-mediated inflammation.

HDCA3 Inhibitors Protect Mice against LPS-Induced Endotoxemia. Since HDAC3 was functionally linked with HIPK2 to regulate TLR4-induced NF-κB activation, we next examined whether HDAC3 inhibitors could protect HIPK2-deficient mice against LPS-induced endotoxemia. As shown in Fig. 7*A*, CD45.1^{+} mice reconstituted with WT or *Hipk2*$^{-/-}$ BMs were treated with HDAC3 inhibitors RGFP966 or MS-275 (Entinostat), followed by LPS challenge. Flow cytometric assay showed that over 95% of peripheral blood cells in the CD45.1 recipient mice expressed the congenic marker CD45.2 after reconstitution (*SI Appendix*, Fig. S9*A*). We also confirmed *Hipk2* deletion in BMs and peripheral blood cells of recipient mice by PCR (*SI Appendix*, Fig. S9*B*) and by real-time qPCR (qRT-PCR) (*SI Appendix*, Fig. S9*C*). Recipient mice reconstituted with either WT or *Hipk2*$^{-/-}$ BMs showed improved survival rates (Fig. 7*B*), alleviated pathologic changes in the lungs and spleens (Fig. 7 *C* and *D*), and reduced serum IL-6 concentrations (Fig. 7*E*) after MS-275 or RGFP966 treatment. These results indicate that the HDAC3 inhibitors could protect WT and *Hipk2*$^{-/-}$ BMs-reconstituted mice against LPS-induced endotoxemia.

In summary, our study has discovered that upon bacterial infection, macrophages enhance HIPK2 expression and interaction with HDAC3, which promotes HDAC3 phosphorylation at S374 and inhibits HDAC3 deacetylase activity, resulting in p65 acetylation at K218 to prevent excessive inflammation. Together, we have proposed that HIPK2 promotes p65 K218 acetylation via inactivating HDAC3, which ameliorates acute cytokine storm-related sepsis or colitis-associated colon cancer (Fig. 7*F*).

Discussion

The persistent presence or amplification of inflammation promotes the development of multiple diseases or exposes patients to a variety of complications (38). Therefore, inflammatory responses should be well controlled at both time and length scales to maintain immune homeostasis. Here, we have identified that the nuclear kinase HIPK2 suppress NF-κB activation in macrophages, which ameliorates infection-related septic shock as well as colitis-associated colon cancer. Considering that HIPK2 also promotes the production of IFNs to enhance antiviral immune response (28), HIPK2 might represent as an evolutionarily conserved protein to effectively clear pathogens without causing cytokine storm during infections. In this scenario, since HIPK2 plays a critical role in the pathogenesis of multiple chronic inflammation-related diseases, including kidney fibrosis, Alzheimer's disease, tumor growth, diabetes, and obesity (25, 39–42), it is critical to further investigate whether limiting inflammation by HIPK2 could provide protection

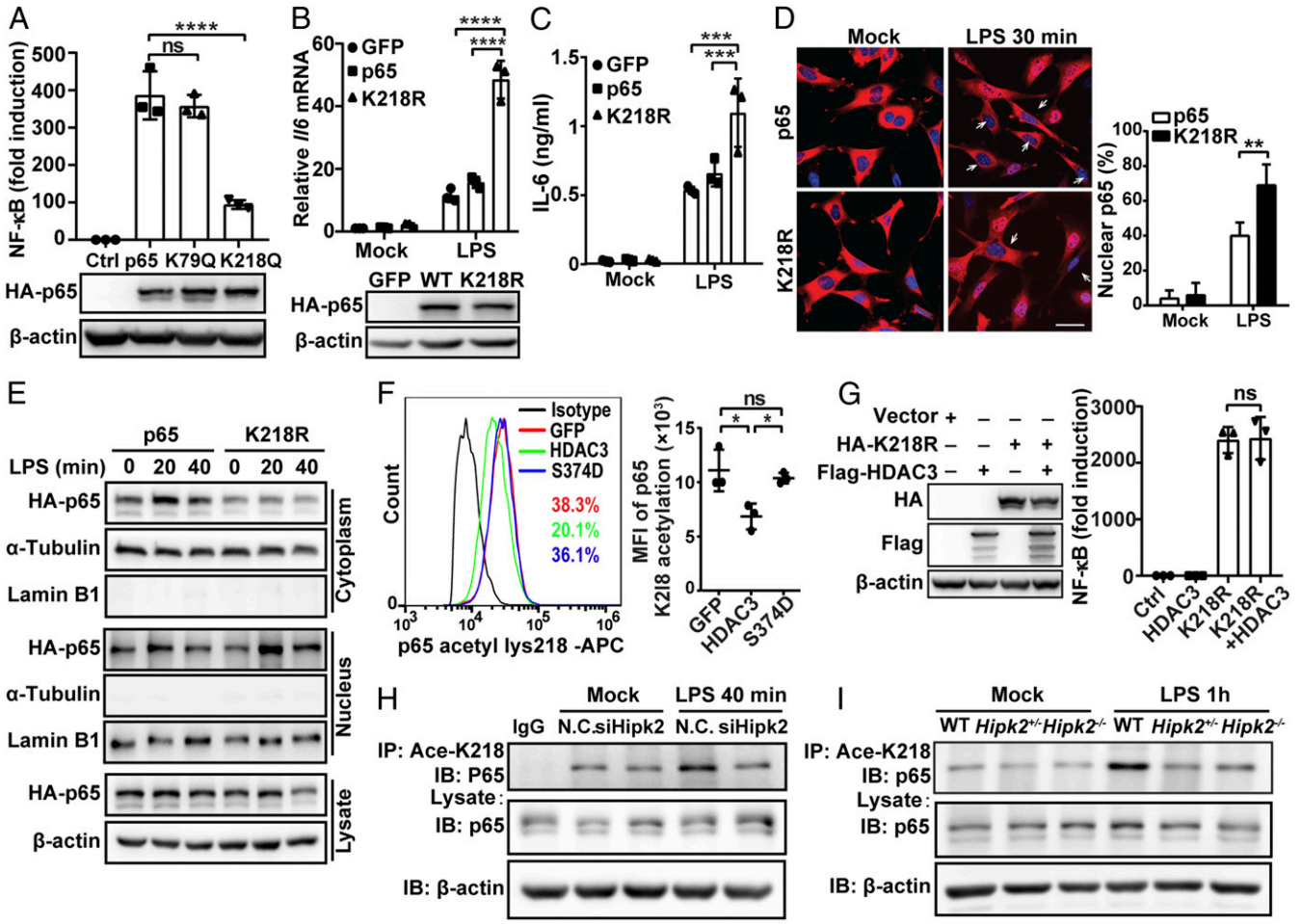

Fig. 6. HIPK2 blocks HDAC3-mediated deacetylation of p65 K218 to inhibit inflammation. (*A*) WT p65, K79Q, and K218Q were transfected with the NF-κB luciferase reporter plasmid into HEK293T cells to measure NF-κB activity. (*B* and *C*) MEFs with WT p65 or K218R overexpressing were stimulated with LPS to measure the production of IL-6 at mRNA (*B*) and protein levels (*C*). (*D*) MEFs with HA-tagged p65 and K218R overexpression were treated with LPS followed by immunostaining with Hoechst (blue) and anti-HA antibody (red) to detect p65 nuclear translocation. (Scale bar, 50 μm.) MEFs without p65 nuclear translocation were indicated by the arrows. More than 50 cells were counted in each of three independent experiments. (*E*) The amount of p65 in the cytoplasm and the nucleus of p65- and K218R-transduced MEFs were measured after LPS treatment. (*F*) The acetylation levels of p65 K218 were detected by flow cytometry in iBMDMs overexpressing WT HDAC3 or S374D mutant. MFI, mean fluorescence intensity. (*G*) K218R and the NF-κB luciferase reporter plasmid were transfected with or without HDAC3 into HEK293T cells to measure the transcriptional activity of p65 by a luciferase assay. (*H* and *I*) *Hipk2* knockdown PEMs (*H*) or knockout MEFs (*I*) were treated with LPS, followed by immunoprecipitation with anti-acetylation K218 antibody and immunoblot with anti-p65 antibody to detect the acetylation levels of p65 K218. Data are from at least three independent experiments (mean ± SD) or representative data (*D–F*, *H*, and *I*). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$, using a one-way ANOVA with Holm–Sidak's multiple comparisons test (*A*, *Upper*; *F*, *Right*; and *G*, *Right*) or two-way ANOVA with Holm–Sidak's multiple comparisons test (*B*, *Upper*; *C*; and *D*, *Right*).

to these diseases. In addition, HIPK2 was reported as a well-known tumor suppressor in tumor cells, and our data suggest it functions as an anti-inflammation effector in macrophages. Consequently, HIPK2 might act as a promising biomarker in inflammation-associated cancers.

A recent study suggests that HIPK2 sustains inflammatory cytokine production by promoting endoplasmic reticulum stress in macrophages, which shows the decreased phosphorylation levels of p65 in LPS-stimulated *Hipk2* knockdown PEMs (43). However, we did not observe significant changes of p65 phosphorylation after *Hipk2* knockdown (*SI Appendix*, Fig. S6*G*), and instead, HIPK2 suppresses inflammation through phosphorylating HDAC3 and increasing p65 K218 acetylation in macrophages. The discrepancy between the two studies is likely due to the different experiment conditions. Nevertheless, our findings are in agreement with previous reports that overexpression of HIPK2 suppresses the expression levels of TNF-α and IL-1β in spinal cord injured rats (44) and attenuates sepsis-mediated liver injury (45).

To date, HDAC3 has not been discovered as a phosphorylation target of HIPK2. In this study, we have demonstrated that the interaction between HIPK2 and HDAC3 was increased in LPS-treated macrophages, and HIPK2 phosphorylates HDAC3 at Ser374 to inactivate its deacetylase activity. So far, only four phosphorylation sites in HDAC3 have been identified to enhance its deacetylase activity, including Ser424 (phosphorylated by protein kinase CK2) (18), Tyr325, Tyr328, and Tyr331 (phosphorylated by proto-oncogene tyrosine-protein kinase Src) (46, 47). In contrast, our study has identified phosphorylation of Ser374 as an inhibitory site to decreases the deacetylase activity of HDAC3. Furthermore, HDAC3 S374 phosphorylation reduces its interaction with the DAD of NCOR/SMRT. These findings indicate a mechanism that combines phosphorylation and protein–protein interaction in regulating HDAC3 activity. In addition, considering that HDAC3 S374 is highly conserved in different species, the mechanism of HDAC3 S374 phosphorylation to reduce HDAC3 activity may exist universally in innate immune cells of many hosts.

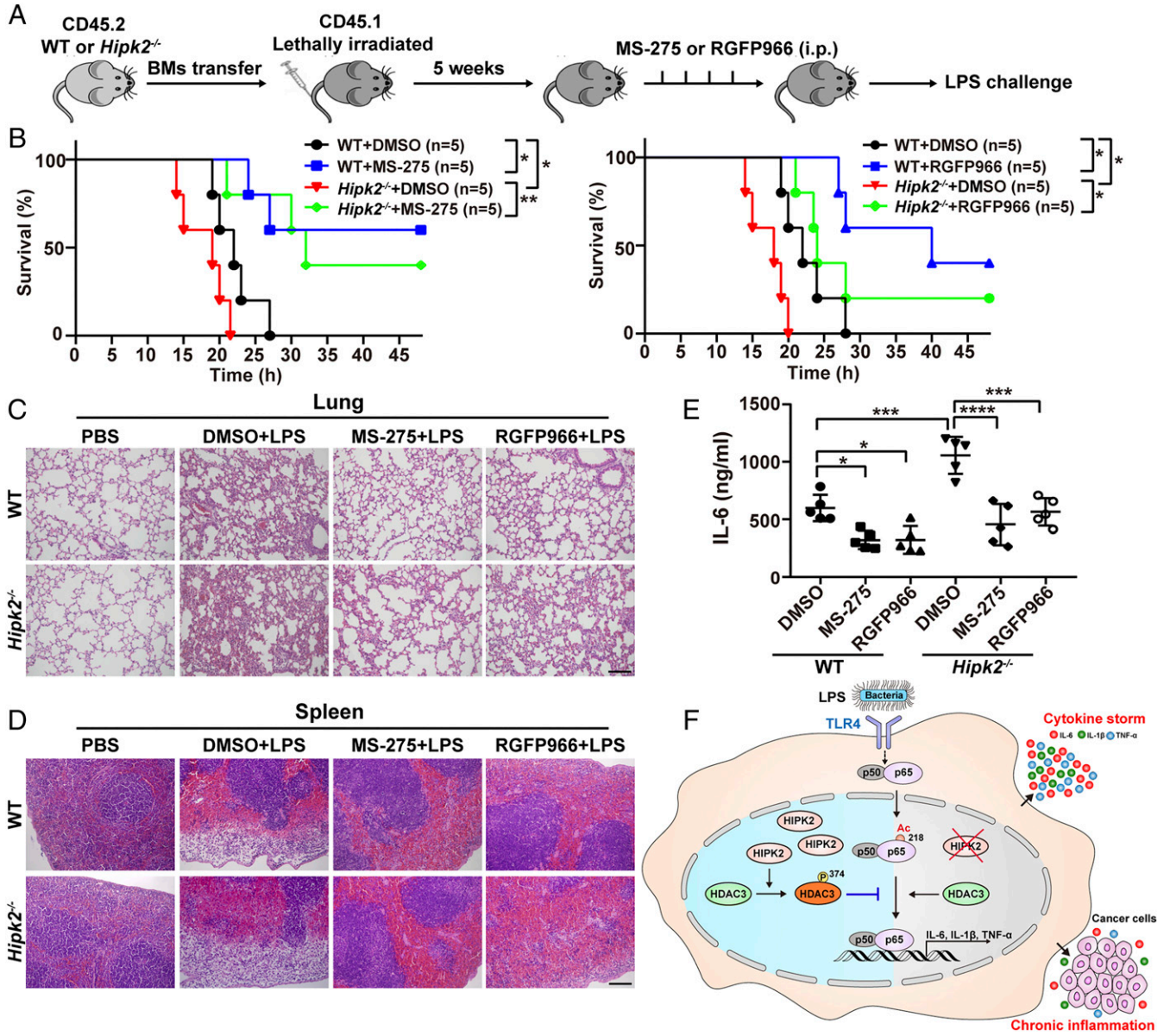

Fig. 7. HDCA3 inhibitors protect mice against LPS-induced endotoxemia. (*A–E*) CD45.2$^+$ WT or *Hipk2*$^{-/-}$ BMs were adoptively transferred to the lethally irradiated CD45.1$^+$ recipient mice respectively. A total of 5 wk later, recipient mice were intraperitoneally injected with MS-275 or RGFP966 (4 mg/kg/d) for 4 d before challenged with LPS (15 mg/kg). (*B*) Survival rates were monitored every 3 h for 7 d. (*C* and *D*) H&E staining of the lung and spleen was performed 24 h after LPS injection (Scale bar, 100 μm). (*E*) Serum concentrations of IL-6 were measured 6 h after LPS injection. Data are from two independent experiments (mean ± SD) or representative data (*C* and *D*). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$, using a Log-rank (Mantel–Cox) test (*B*) or one-way ANOVA with Holm–Sidak's multiple comparisons test (*E*). (*F*) The proposed model. Upon bacterial infection, HIPK2 expression is enhanced in macrophages, which subsequently increases its interaction with HDAC3 and promotes HDAC3 phosphorylation at S374 to inhibit its deacetylase activity, resulting in p65 acetylation at K218 to prevent cytokine storm-related sepsis or chronic inflammation-associated colon cancer.

Acetylation of p65 at distinct sites could differentially regulate NF-κB function. Seven acetylated lysine residues have been identified within p65, including 122, 123, 218, 221, 310, 314, and 315. Acetylation of K310 is required for p65 full activation but does not affect the DNA-binding activity of p65 or the assembly between p65 and IκBα (10). Acetylation of K122 and K123 reduces the DNA-binding activity of p65 and facilitates the removal and nuclear export of p65 by IκBα (15). Acetylation at K314 and K315 in p65 affects neither the nuclear-cytosolic shuttling nor the DNA-binding ability of p65 but differentially regulates the expression of specific sets of NF-κB target genes in response to TNF-α stimulation (48). Notably, acetylation of K221 enhances the DNA-binding ability of p65 and, together with K218 or not, impairs the assembly of p65 with IκBα, leading to prolonged NF-κB response (10). However, the precise function of K218 acetylation was not defined. We demonstrated that K218R mutation could increase the nuclear localization and the transcriptional activity of p65 upon LPS stimulation. According to the crystal structure of p65 DNA-binding domain, the positively charged lysine 218 residue could potentially interact with the negatively charged oxygen atom on the phosphate group of DNA to form hydrogen bond (49). We therefore propose that acetylation of K218 might reduce the DNA-binding activity of NF-κB by neutralizing its positively charged lysine. Together, acetylation of p65 at different lysines might utilize distinct mechanisms to regulate NF-κB function, and site-specific acetylation of p65 represents a

key mechanism to precisely control NF-κB activity when different cell types receive diverse stimuli.

In line with the previous observations that HDAC3 inhibitors could inhibit NF-κB activation and inflammation (50–52), we found that the HDAC3 inhibitors RGFP966 and MS-275 could protect HIPK2-deficient mice from LPS-induced endotoxemia. Similar to the abnormal expression of HIPK2 in the development of many diseases, dysregulation of HDAC3 is also involved in many diseases, including kidney disease (53), Alzheimer's disease (54), and cancers (55). Considering that HDAC3 was the phosphorylation target of HIPK2, we therefore propose to further investigate whether the HDAC3 inhibitors can serve as drug candidates to specifically target these diseases with HIPK2 dysregulation. In addition, since several types of cancers are closely linked with chronic inflammation, and the HDAC3 inhibitor MS-275 is currently in the phase III clinical trial against malignancies, our study may represent a therapeutic mechanism to use the HDAC3 inhibitor for inflammation-related cancers.

Materials and Methods

The Selection of Sepsis Patients. Our study was approved by the Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (Approval TJ-IRB20200720). Patients with infections and Sequential Organ Failure Assessment ≥ 2 are defined as sepsis and as septic shock if also with a vasopressor requirement to maintain a mean arterial pressure of 65 mm Hg or greater and serum lactate level greater than 2 mmol/L (>18 mg/dL) in the absence of hypovolemia (56). A total of 16 patients who met the criteria were enrolled, and the informed consents were signed by themselves or their legally authorized representatives if they were medically incapable. All of the sepsis patients in the trail were infected by gram-negative bacillus, including *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Colon bacillus*, or *Klebsiella pneumoniae*. The most common infection sites in these sepsis patients including the lung, abdomen, urinary tract, and skin/soft tissue. The peripheral blood samples of these patients were collected immediately once diagnosis of sepsis. The clinical characteristics of the sepsis patients were exhibited in *SI Appendix*, Table S2.

More materials and methods used in this article are in *SI Appendix*.

Data Availability. All study data are included in the article and *SI Appendix*.

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