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# Homozygosity for an allele encoding deacetylated FoxO1 protects macrophages from cholesterol-induced inflammation without increasing apoptosis

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## Abstract

**OBJECTIVE—**Insulin resistance renders macrophages more prone to cholesterol-induced apoptosis by promoting nuclear localization of transcription factor FoxO1. But FoxO1 also decreases macrophage inflammation, raising the question of how is the balance between pro-apoptotic and anti-inflammatory effects determined. We sought to identify the mechanism whereby FoxO1 dampens inflammation without promoting apoptosis. We hypothesized that nutrient-dependent FoxO1 acetylation plays a role in this process.

**METHODS & RESULTS—**We generated knock-in mice bearing alleles that encode constitutively deacetylated FoxO1, and studied the *ex vivo* response of primary peritoneal macrophages. We show that macrophages derived from mice homozygous for constitutively deacetylated FoxO1 alleles retain anti-inflammatory properties in response to free cholesterol loading, without increasing apoptosis. Deacetylated FoxO1 inhibits free cholesterol-induced Akt phosphorylation and increases levels of the Nf-κB precursor p105, decreasing nuclear translocation of Nf-κB p65 subunit, and dampening Mek/Erk activation to prevent inflammation.

**CONCLUSIONS—**Deacetylated FoxO1 regulates p105 to prevent macrophage inflammation without causing apoptosis, suggesting a potential novel therapeutic approach to atherosclerosis through FoxO1 deacetylation.

### Keywords

Genetically altered mice; Mechanism of atherosclerosis/growth factors; Type 2 diabetes; Apoptosis; Cell signaling/signal transduction

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**DISCLOSURES**

The Authors declare that they have no competing financial interest in the work described.

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Complications from atherosclerotic cardiovascular (CV) disease are a leading cause of death [1]. Macrophage inflammation in the vessel wall is a key event in the formation and rupture of atherosclerotic plaques. Various cytokines produced from lesional macrophages contribute to lesion progression [2].

In early atherosclerosis, macrophages take up modified cholesterol-rich lipoproteins, and store their cholesterol largely in esterified form, resulting in "foam cell" formation. As lesions advance, cholesteryl-ester content drops, while unesterified, or "free" cholesterol (FC) rises [3, 4], likely inducing endoplasmic reticulum (ER) stress and expression of transcription factor Chop [5]. The *in vivo* relevance of ER stress-mediated Chop induction to atherosclerosis progression is demonstrated by studies in which *Chop* ablation halved the number of apoptotic lesional macrophages and reduced plaque size in $ApoE^{-/-}$ or $Ldlr^{-/-}$ mice [6]. In addition to ER stress, FC activates the mitogen-activated kinase (Mapk, including Erk1/2, Jnk1/2, and p38) and Ikk/Nf-κB inflammatory pathways, possibly by inducing Tnf-α and Il-6 [7].

Macrophages are insulin-sensitive cells [8]. Defective macrophage insulin signaling predisposes to foam cell formation in insulin-resistant states [9], and impairs the ability of macrophages to relieve ER stress, resulting in greater macrophage apoptosis and plaque necrosis within advanced lesions [10]. In macrophages, FoxO1, FoxO3 and FoxO4 appear to have dual functions, promoting apoptosis in the context of ER stress, but decreasing inflammation in response to FC [11] or LPS [12]. FoxO1 activity is regulated by post-translational modifications to meet the cell's metabolic demand or stress response [13]. Falling nutrient levels activate FoxO1 through dephosphorylation and deacetylation, whereas rising nutrient levels inactivate FoxO1 through Akt-dependent phosphorylation, and Cbp/p300-dependent acetylation [14].

Purpose of this study was to understand the mechanism whereby FoxO1 activation can have effects that are seemingly at odds: more apoptosis and less inflammation. Our goal is to define approaches that leverage the anti-inflammatory actions of FoxO1 for therapeutic ends, without promoting apoptosis. It has been shown that hyperglycemia in type 2 diabetes leads to FoxO1 acetylation [15–17]. Acetylation targets FoxO1 to nuclear PML bodies, where it undergoes deacetylation to become transcriptionally active [15]. Less clear is the effect of FC signaling on macrophage FoxO1, and the role of FoxO1 acetylation in macrophage activation. To answer these questions, we generated knock-in mice bearing alleles that encode constitutively deacetylated FoxO1, and studied the *ex vivo* response of primary peritoneal macrophages to FC challenge. Here we show that deacetylated FoxO1 prevents FC-induced Akt phosphorylation and increases levels of p105, the precursor protein of Nf-κB subunits. p105, in turn, promotes Nf-κB p65 and p50 cytoplasmic retention and dampens Erk activation, decreasing inflammation. The data are consistent with a model in which FoxO1 deacetylation can uncouple inflammation from apoptosis in FC-loaded macrophages.

## METHODS

### Experimental animals

Acetylation-defective FoxO1 knock-in mice ($FoxO1^{KR/KR}$) have been described [18].

### Materials

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### Macrophage culture, FC loading, immunoprecipitation and immunoblotting

We harvested peritoneal macrophages from *FoxO1*$^{+/+}$ or *FoxO1*$^{KR/KR}$ mice by peritoneal lavage 3 days after intraperitoneal injection of 4% thioglycolate, and cultured them in DMEM supplemented with 10% fetal bovine serum and 20% L929 cell-conditioned medium [19]. We loaded cells with FC by incubation in medium supplemented with compound 58035 (10 mg/l) and acLDL (100 mg/l) for the indicated time periods [19]. Protein analysis was carried out as described [11, 16].

### Immunocytochemsitry

We performed FoxO1 immunocytochemstry as described [15]. We measured apoptosis by Alexa 488-labeled annexin V and propidium iodide staining (Vybrant Apoptosis Assay kit, Invitrogen).

### Quantitative RT-PCR and ELISA

We performed quantitative RT-PCR using DyNAmo HS SYBR Green qPCR Kit (Finnzymes) as described [15].

### Adenoviruses

We have described adenoviruses encoding LacZ, GFP-tagged FoxO1-WT and FoxO1-KR, HA-tagged FoxO1-ADA, and nuclear factor-κB (Nf-κB)-luciferase [11, 15, 20, 21].

### Luciferase assays

We measured luciferase activity in peritoneal macrophages transfected with Nf-κB reporter construct using Dual Luciferase reporter Assay System (Promega).

### Statistical analysis

We show data as mean ± SE. We determined statistically significant differences ($P < 0.05$) using unpaired *t*-test or ANOVA with Dunn's *post hoc* test.

## RESULTS

### FC loading induces FoxO1 acetylation in peritoneal macrophages

We examined the effect of FC loading on FoxO1 acetylation and subcellular localization. FC induced FoxO1 acetylation in *FoxO1*$^{+/+}$ but not *FoxO1*$^{KR/KR}$ macrophages (Fig. 1A). The latter also showed increased nuclear FoxO1 in the basal state that was unaffected by FC loading, whereas the number of WT macrophages with predominantly nuclear FoxO1 doubled in response to FC loading (Fig. 1B, C). FoxO1 regulation by FC loading in macrophages is reminiscent of its regulation by high glucose/oxidative stress in pancreatic β-cells [15]. The findings indicate that FC challenge phenocopies nutrient excess (high glucose) to promote FoxO1 acetylation, whereas deacetylation is required to keep FoxO1 in the nucleus.

### Blunted induction of inflammatory cytokines inFC-loaded *FoxO1*$^{KR/KR}$ macrophages

FC loading increased levels of mRNAs encoding *Mcp-1, Tnf-α, Il-6, Il-1β, Rantes, Mip-1α, Mip-1β, Mip-2, Tgf-β1*, and *osteopontin* (encoded by *Spp1*) between 2- and 8-fold in WT macrophages, but these effects were absent (*Mcp-1, Tnf-α, Il-6, Mip-1β, Mip-2*) or blunted (*Rantes, Tnf-α, Il-1β, Mip-2*) in *FoxO1*$^{KR/KR}$ macrophages (Fig. 1D). Levels of *Mip-1α, Tgf-β1*, and *Spp1* were decreased also in the basal state in *FoxO1*$^{KR/KR}$ macrophages (Fig. 1D). In contrast, levels of the anti-inflammatory cytokine *Il-10* were comparable between the two genotypes. The changes in mRNA were paralleled by a fivefold increase in Mcp-1 release in

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the culture medium in WT macrophages that was totally absent in *FoxO1*$^{KR/KR}$ macrophages (Fig. 1E). Release of Tnf-α and Il-6 increased >100-fold in *FoxO1*$^{+/+}$ cells, and was blunted by 40% and 60%, respectively, in *FoxO1*$^{KR/KR}$ cells (Fig. 1F–G).

In contrast, the response of *FoxO1*$^{KR/KR}$ macrophages to the TLR2 agonist zymosan, and to the TLR4 agonist LPS did not show a clear-cut anti-inflammatory pattern and, in some instances (e.g., LPS-stimulated *Il-6*; zymosan- and LPS-stimulated *Tnfα*-induction) were more pronounced in *FoxO1*$^{KR/KR}$ than in *FoxO1*$^{+/+}$ macrophages (Suppl. Fig. IA–B).

## FoxO1-KR doesn't increase FC-induced apoptosis

FC increases macrophage apoptosis by activating the Chop branch of the unfolded protein response [5, 6]. The phosphorylation-defective mutant FoxO1, FoxO1-ADA (in which the three main sites of insulin-induced phosphorylation have been mutated) [22], is constitutively nuclear and exacerbates this effect [11]. As the FoxO1-KR mutant was also predominantly nuclear, we expected it to induce apoptosis. But we failed to detect differences in *Bip* and *Chop* mRNA (Suppl. Fig. IIA), or in the number of apoptotic macrophages in response to FC between *FoxO1*$^{+/+}$ and *FoxO1*$^{KR/KR}$ cells (Suppl. Fig. IIB, C). Apoptosis induced by thapsigargin, an inhibitor of the endoplasmic reticulum $Ca^{2+}$ pump sarcoplasmic/endoplasmic reticulum calcium ATPase (Serca) that promotes ER stress [23], was also comparable between the two cell types, as was expression of type A scavenger receptor (SR-A)–a mediator of acLDL uptake [24]–toll-like receptor-4 (TLR-4), and Tnf receptor 1 (TnfR1), all of which are required for ER stress-induced Mapk activation [25, 26] (Suppl. Fig. IID). These data indicate that FoxO1-KR dampens inflammation without predisposing to apoptosis, a critical difference between it and the phosphorylation-defective mutant.

## FoxO1-KR inhibits FC-induced Akt phosphorylation

In view of the role of Akt in modulating the macrophage anti-apoptotic and anti-inflammatory responses [27], we examined the effects of FC loading on Akt activity in macrophages. In the early stages of FC loading, Akt phosphorylation on Ser-473 and Thr-308 was increased in *FoxO1*$^{+/+}$ macrophages, but this effect was decreased by ~80% in *FoxO1*$^{KR/KR}$ macrophages (Fig. 2A–C). Phosphorylation of the Akt substrate glycogen synthase kinase 3β (GSK3β) was also decreased in *FoxO1*$^{KR/KR}$ macrophages (Fig. 2A, D). In contrast, there was no difference in insulin-induced Akt phosphorylation between the two cell types (Fig. 2E–H). Thus, Akt activation by FC is selectively impaired in *FoxO1*$^{KR/KR}$ macrophages.

## Impaired Mek/Erk activation in *FoxO1*$^{KR/KR}$ macrophages

Macrophage FC loading increases Mcp-1, Tnf-α and Il-6 via Nf-κB and/or Mapk pathways [7] (Suppl. Fig. III). We interrogated the contribution of these two mechanisms to the blunted inflammatory response of *FoxO1*$^{KR/KR}$ macrophages. FC rapidly induced Mek phosphorylation by ~twofold, and promoted biphasic Erk phosphorylation in *FoxO1*$^{+/+}$ macrophages; these responses were significantly attenuated in *FoxO1*$^{KR/KR}$ macrophages (Fig. 3A–C). In contrast, Jnk and p38 phosphorylation increased up to 8-fold and were comparable in both cell types (Fig. 3A, D–E).

## Distinct signaling pathways are elicited by phosphorylation-defective *vs.* acetylation-defective FoxO1 mutants

We have previously shown that the phosphorylation-defective FoxO1-ADA has anti-inflammatory effects in macrophages [11]. As the FoxO1-KR mutant is also predominantly nuclear (although not to the same extent as the phosphorylation-defective mutant), we considered the possibility that the observed effects might simply reflect a gain of FoxO1

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function, and be unrelated to its acetylation state. To investigate this point, we performed *ex vivo* experiments by transducing primary peritoneal macrophages with FoxO1-WT, FoxO1-ADA (phosphorylation-defective) and FoxO1-KR (acetylation-defective), and measured their response to FC loading. FoxO1-KR and WT-FoxO1 inhibited FC induced Akt phosphorylation on Ser-473 and Thr-308, whereas FoxO1-ADA increased it (Supp. Fig. IVA–C). Likewise, FC induced Mek and Erk phosphorylation (Suppl. Fig. IVA, D–E). FoxO1-WT or FoxO1-ADA had no effect on this response, whereas FoxO1-KR blunted it (Suppl. Fig. IVA, D–E). These data indicate that deacetylated FoxO1 has a unique effect that modulates inflammatory pathways in FC-loaded macrophages.

## FoxO1-KR impairs activation of Nf-κB by FC

FC loading activated Nf-κB signaling in macrophages [7,25], stimulating *IκBα* phosphorylation on Ser-32 in a time-dependent manner. This response was nearly absent in $FoxO1^{KR/KR}$ macrophages (Fig. 4A).

*IκBα* phosphorylation triggers its proteasomal degradation, resulting in Nf-κB nuclear translocation [28]. Accordingly, we observed a twofold increase in nuclear content of the Nf-κB p65 subunit following FC loading of $FoxO1^{+/+}$ macrophages (Fig. 4B–D). In contrast, levels of nuclear p65 were unchanged in FC-treated $FoxO1^{KR/KR}$ macrophages (Fig. 4B–D). To correlate these changes with Nf-κB function, we assessed the activity of an Nf-κB reporter gene transduced into $FoxO1^{+/+}$ or $FoxO1^{KR/KR}$ cells. In $FoxO1^{+/+}$ cells, FC induced an ~ 8-fold increase of Nf-κB-luciferase activity, whereas the effect of FC was decreased by 40% in $FoxO1^{KR/KR}$ cells (Fig. 4E).

## Identification of p105 (Nfkb1) as mediator of the anti-inflammatory effects of FoxO1-KR

The combined impairment of Nf-κB and Mek/Erk activities in $FoxO1^{KR/KR}$ macrophages led us to formulate the testable hypothesis that a shared regulator of both pathways was responsible for this reduction. The product of the *Nfkb1* gene, p105, is the precursor of Nf-κB p50 [29], and functions as an IκB-like molecule by sequestering p65, p50, and c-Rel in the cytoplasm [30–32] and preventing their binding to DNA [31]. p105 also inhibits the Mek/Erk pathways by forming stoichiometric complexes with the Mek inhibitor TPL-2/Cot [32]. We detected a fourfold increase in p105 protein levels (Fig. 5A–B), associated with a twofold rise of cytosolic p50 levels in $FoxO1^{KR/KR}$ macrophages (Fig. 5C), consistent with increased extra-nuclear retention of this subunit. In contrast, nuclear levels of p50 increased only by ~30% in $FoxO1^{KR/KR}$ cells (Fig. 5D). The increase of p105 protein was associated with a 30% rise of *Nfkb1* mRNA in $FoxO1^{KR/KR}$ macrophages (Fig. 5E), suggesting that the main mechanism of increased p105 levels is not increased *Nfkb1* transcription. Transduction of FoxO1-KR adenovirus in primary peritoneal macrophages also increased p105 protein levels (Fig. 5F), albeit to a lower extent, probably reflecting the limited efficiency of adenoviral transduction compared to studying a homogeneous population of $FoxO1^{KR/KR}$ macrophages. The increase was independent of changes in *Nfkb1* mRNA (data not shown), consistent with a transcription-independent mechanism. In contrast, FoxO1-WT and FoxO1-ADA had no effect on p105 (Fig. 5F–G). These data indicate that p105 is a specific, albeit indirect (non-transcriptional) target of deacetylated FoxO1.

## Pi3k inhibition mimics the effect of FoxO1-KR on p105

Akt phosphorylates Ikk [33, 34], triggering p105 proteolysis and Nf-κB/Mek/Erk activation. In addition, the Akt substrate GSK3β stabilizes p105 through phosphorylation, preventing its degradation [35]. We reasoned that, if FoxO1-KR increased p105 by inhibiting Akt, its effects should be phenocopied by pharmacological inhibition of Pi 3-kinase (Pi3k). Indeed, the Pi3k inhibitor LY294002 inhibited Akt phosphorylation and GSK-3β phosphorylation, and increased p105 protein levels (Fig. 6A–B) independent of mRNA (Fig. 6C). LY294002 also

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inhibited FC-induced *Mcp-1*, *Tnf-α*, *IL-6*, *IL-1β*, *Mip-1β*, and *Mip-2* expression in macrophages (Fig. 6D). In contrast, *Mip-1α*, *Tgf-β1*, and *Spp1*, whose induction by FC was blunted in $FoxO1^{KR/KR}$ macrophages, were unaffected by LY294002 (Fig. 6D). These data provide indirect support for the hypothesis that the anti-inflammatory effects of FoxO1-KR are mediated by its ability to decrease Akt phosphorylation.

### FoxO1-KR does not act through IκBε, the main target gene of FoxO1-ADA

We have previously shown that phosphorylation-deficient FoxO1-ADA blunts Nf-κB activation by FC and increases IκBε expression [11], leading to p65 retention in the cytoplasm [28, 36–38]. In the light of this precedent, we considered the possibility that FoxO1-KR regulated expression of *IκBα*, *IκBβ*, and *IκBε* (Suppl. Fig. VA). Consistent with prior observations, FC loading increased *IκBε* levels ~fourfold, whereas *IκBα* and *IκBβ* levels were unaffected (Suppl. Fig. VA). However, *IκBε* induction was similar in $FoxO1^{+/+}$ and $FoxO1^{KR/KR}$ macrophages (Suppl. Fig. VA). We also examined the ability of FoxO1-WT, FoxO1-ADA, and FoxO1-KR to regulate the 3 IκBs in primary macrophages. Only FoxO1-ADA significantly increased *IκBε* [11], whereas neither FoxO1-WT nor FoxO1-KR affected any of the 3 *IκB*s (Suppl. Fig. VB). These data support the conclusion that FoxO1-KR inhibits FC-induced Nf-κB activity independent of *IκBε*, and provide more evidence for a deacetylation-specific mechanism of reduced inflammation.

## DISCUSSION

The pathogenesis of inflammation in cardiovascular atherosclerotic disease is multi-factorial, and involves generation of cytokines, free radical formation, hemodynamic stress, hypertension, infections, and accumulation of oxidized phospholipids and 7-oxysterols [39, 40]. FC accumulation in macrophages promotes inflammation through cytokine production [7, 11, 25], leading to increased levels of adhesion molecules and tissue factor [41], and migration of vascular smooth muscle cells [42]. We now show that FC increases FoxO1 acetylation, and that constitutively deacetylated FoxO1 [15] prevents FC-induced inflammation without promoting apoptosis. The latter finding sets this FoxO1 mutant apart from a phosphorylation-defective mutant that we have previously shown to inhibit inflammation AND promote FC-dependent apoptosis [11]. Interestingly, constitutively active Akt prevents FC-induced macrophage apoptosis in insulin-resistant macrophages [11], while of PI3K/Akt inhibition by LY294002 increases it. These findings suggest that FoxO1-KR activates additional anti-apoptotic pathways to offset its pro-apoptotic properties macrophages. As FoxO1 phosphorylation and acetylation are regulated in response to different physiologic cues and disease states [13], the present findings have implications that go beyond the FC loading model.

We propose a novel mechanism to explain the findings (Suppl. Fig. VI). FoxO1 deacetylation blocks FC-dependent Akt phosphorylation, leading to decreased Ikk/Nf-κB and Mek/Erk activation, reduced cytokine expression, and reduced inflammation. We identify the product of the *Nfkb1* gene p105 as a non-transcriptional target of deacetylated FoxO1 that potentially mediates its anti-inflammatory effects. The data provide further evidence that deacetylation fine-tunes FoxO1 function through target gene selection; thus, the phosphorylation-defective and acetylation-defective mutants have opposite effects on Akt phosphorylation and selective effects on *Nfkb1* and *IκBε* expression [11, 21]. Identifying the mechanism of the differential effects on Akt phosphorylation poses a daunting challenge for future work, as does the identification of co-regulatory mechanisms whereby FoxO proteins can regulate gene expression without direct DNA binding [20].

Akt regulates inflammation [33, 43–45] by phosphorylating Ikkα on Thr-23 and activating Nf-kB [45]. Pharmacological inhibition of Ikk increases p105 levels, and inhibits LPS-stimulated

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Nf-κB and Mek/Erk activation in macrophages [30]. On the other hand, p105 gain-of-function fails to affect LPS-induced Nf-κB and Mek/Erk signaling [30], suggesting that this mechanism is specific for certain, but not all inflammatory stimuli. Consistent with this observation, we have observed that *FoxO1*$^{KR/KR}$ macrophages are resistant to FC-induced, but not to Zymosan- or LPS-induced activation of inflammatory cytokines. It is also of interest to note that pharmacological inhibition of Pi3k-Akt mimics the FoxO1-KR effect to raise p105 levels, but has narrower effects on cytokine induction by FC, suggesting that not all effects of FoxO1-KR can be explained by its inhibition of Akt. We propose that decreased Akt activity by FoxO1-KR attenuates p105 proteolysis by two separate mechanisms: Ikk inhibition, leading to Nf-κB and Mapk inhibition; and decreased GSK3β phosphorylation, which has been shown to prevent constitutive processing/degradation of p105 by increasing its stability [35].

The present study confirms the biphasic activation pattern of Mek and Erk [46, 47]. This could be due to delayed activation of certain subsets of Map kinases, or to autocrine signaling by cytokines produced from FC-stimulated macrophages, such as CXC-ligand 4 and IL-10 [48, 49].

The role of FoxO1 in the development of atherosclerosis varies in different cells and tissue types. Insulin receptor-deficient macrophages, in which FoxO1 is constitutively active, display anti-inflammatory responses to FC [11] and LPS [12], but are prone to apoptosis [10]. Conversely, in vascular endothelial cells FoxO1 gain-of-function promotes peroxynitrite and ROS generation [16], predisposing to inflammation. This finding is consistent with the observation that endothelial-cell specific ablation of insulin receptors (a condition associated with increased nuclear FoxO1) in *ApoE*$^{-/-}$ mice worsens atherosclerosis [50], as does Akt1 ablation [51], pinpointing vascular endothelial cells as a key site of atherosclerotic lesion development in insulin resistance.

In conclusion, our study demonstrates that FoxO1 deacetylation is an important regulatory mechanism in cholesterol-laden macrophages, with the potential to uncouple inflammation from apoptosis. As these mechanisms underlie the progression of atherosclerotic plaques from benign to unstable lesions [2], it can be envisioned that treatments promoting FoxO1 deacetylation will favorably affect cardiovascular outcomes in type 2 diabetes.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Non-standard abbreviations and acronyms

| | |
|---|---|
| **LPS** | lipopolysaccharide |
| **FC** | free cholesterol |
| **Mcp-1** | monocyte chemoattractant protein-1 |
| **ILs** | interleukins |
| **TNF** | tumor necrosis factor |
| **Mips** | macrophage inflammatory proteins |
| **Mapk** | Mitogen-activated protein kinase |
| **Nf-κB** | nuclear factor kappa B |

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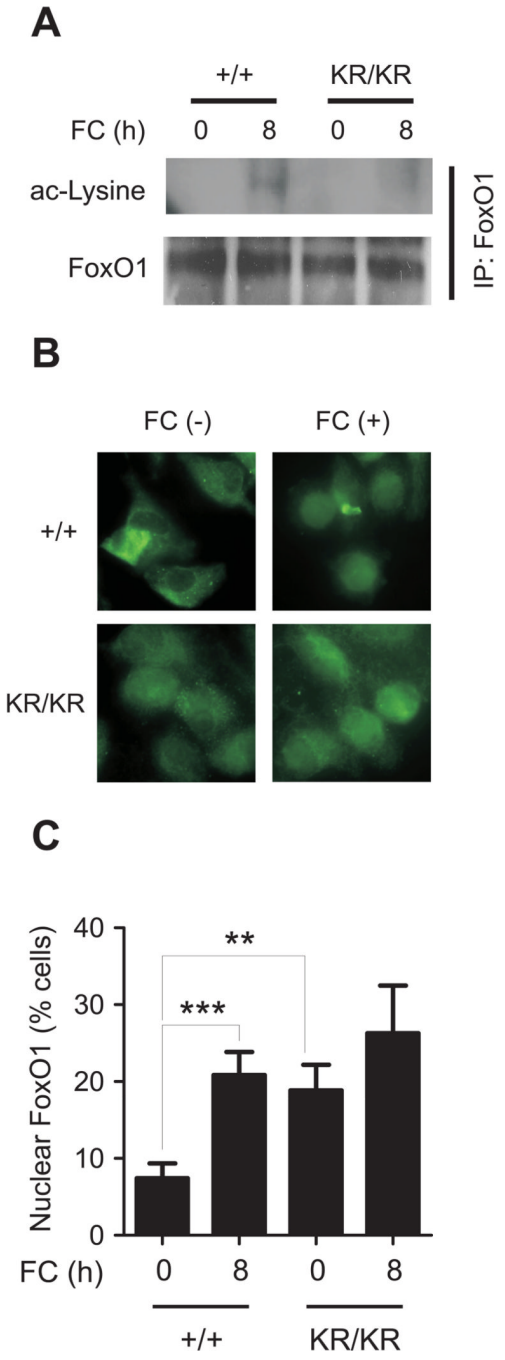


**Figure 1. FC-induced inflammatory cytokine expression and protein secretion in macrophages**

(A) Western blots of FC-loaded *FoxO1*$^{+/+}$ (+/+) and *FoxO1*$^{KR/KR}$ macrophages (KR/KR) macrophages, immunoprecipitated (IP) with anti-FoxO1 antibody followed by immunoblotting with anti-acetyl-lysine (ac-Lysine) or total FoxO1 antibodies. (B) Representative immunohistochemical images of FoxO1 sub-cellular localization. (C) Percentage of cells with nuclear FoxO1. All images are representative of at least 3 independent experiments. Scale bar = 10 μm. ** $P < 0.01$ and *** $P < 0.001$ between the indicated groups. (D) Time course analyses of mRNAs expression in *FoxO1*$^{+/+}$ (+/+, open circles) and *FoxO1*$^{KR/KR}$ macrophages (KR/KR, closed circles) loaded with FC (n=5). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ *vs.* +/+. (E–G) Peptide levels of Mcp-1 (E), Tnf-⟨ (F),

and Il-6 (G) in conditioned medium from macrophages loaded with FC for 24h (n=4). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ between the indicated groups.

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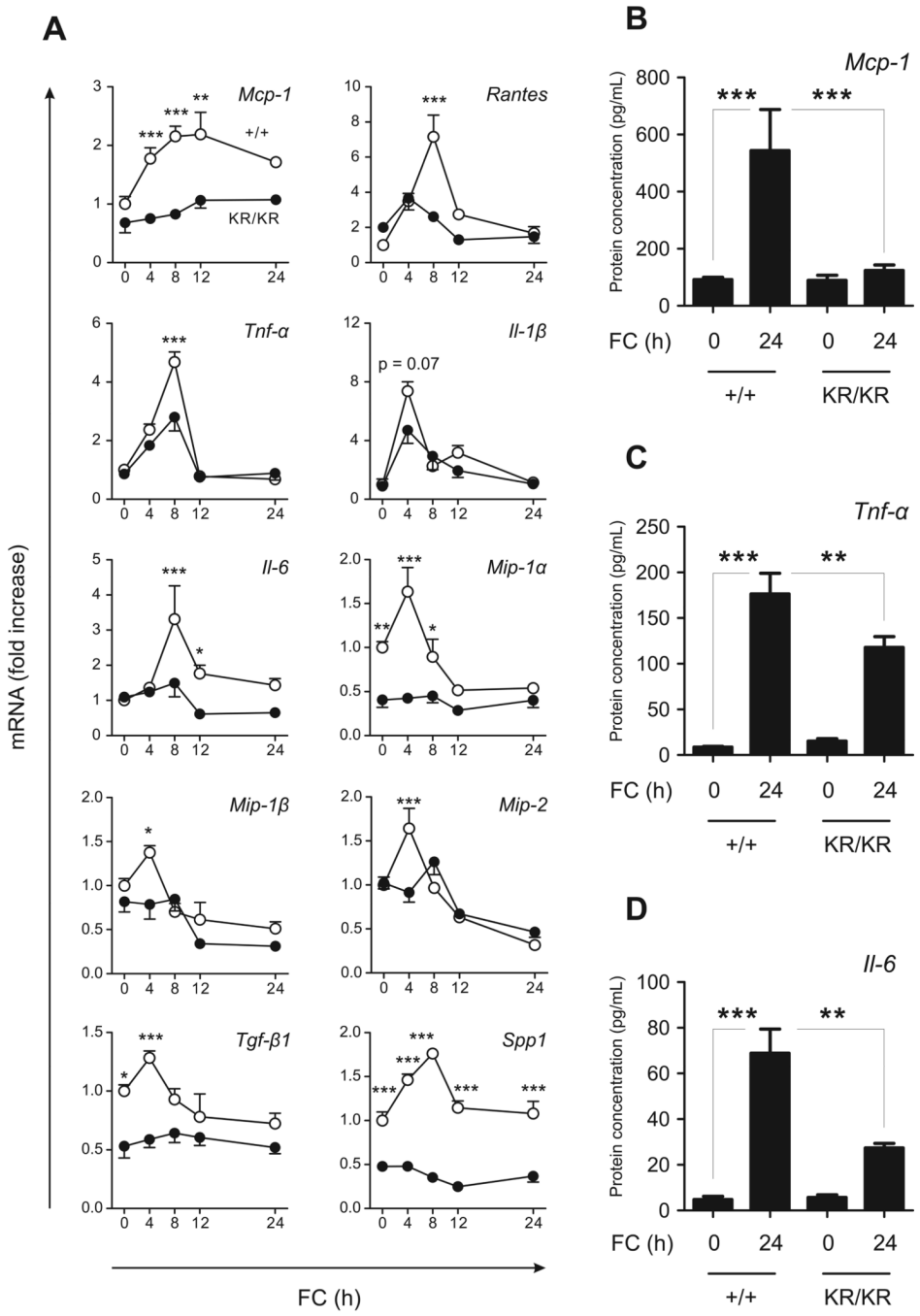


**Figure 2. FC– and insulin–induced Akt and GSK3β phosphorylation in macrophages**

(A) Representative immunoblots and (B–D) quantification of phospho-Akt Ser-473 (B), phospho-Akt Thr-308 (C), and phospho-GSK-3β (D) at the indicated times. (E) Representative immunoblots and (F–H) quantification of phospho-Akt Ser-473 (F), phospho-Akt Thr-308 (G), and phospho-GSK-3β (H) in *FoxO1*$^{+/+}$ (+/+) and *FoxO1*$^{KR/KR}$ (KR/KR) macrophages stimulated with insulin (10μM) for the indicated times. Band intensity was quantified from three independent experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ *vs.* +/+.

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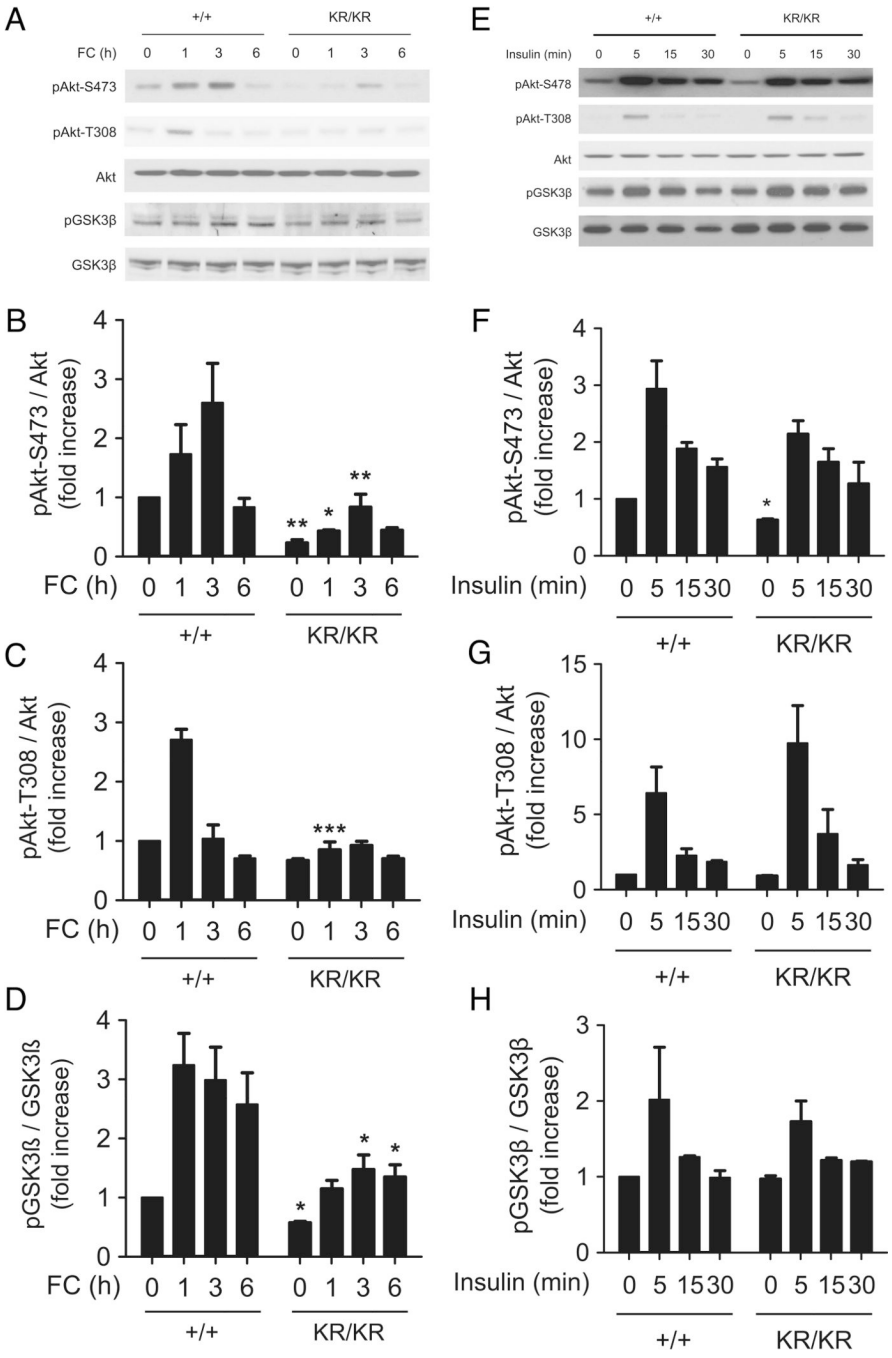


**Figure 3. FC activates Mapk in macrophages**

(A) Representative immunoblots and (B–E) quantification of phospho-Mek1/2 (B), phospho-Erk1/2 (C), phospho-Jnk1/2 (D), and phospho-p38 (E) in *FoxO1*$^{+/+}$ (+/+) and *FoxO1*$^{KR/KR}$ (KR/KR) macrophages loaded with FC for the indicated times. Band intensity was quantified from three independent experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. +/+.

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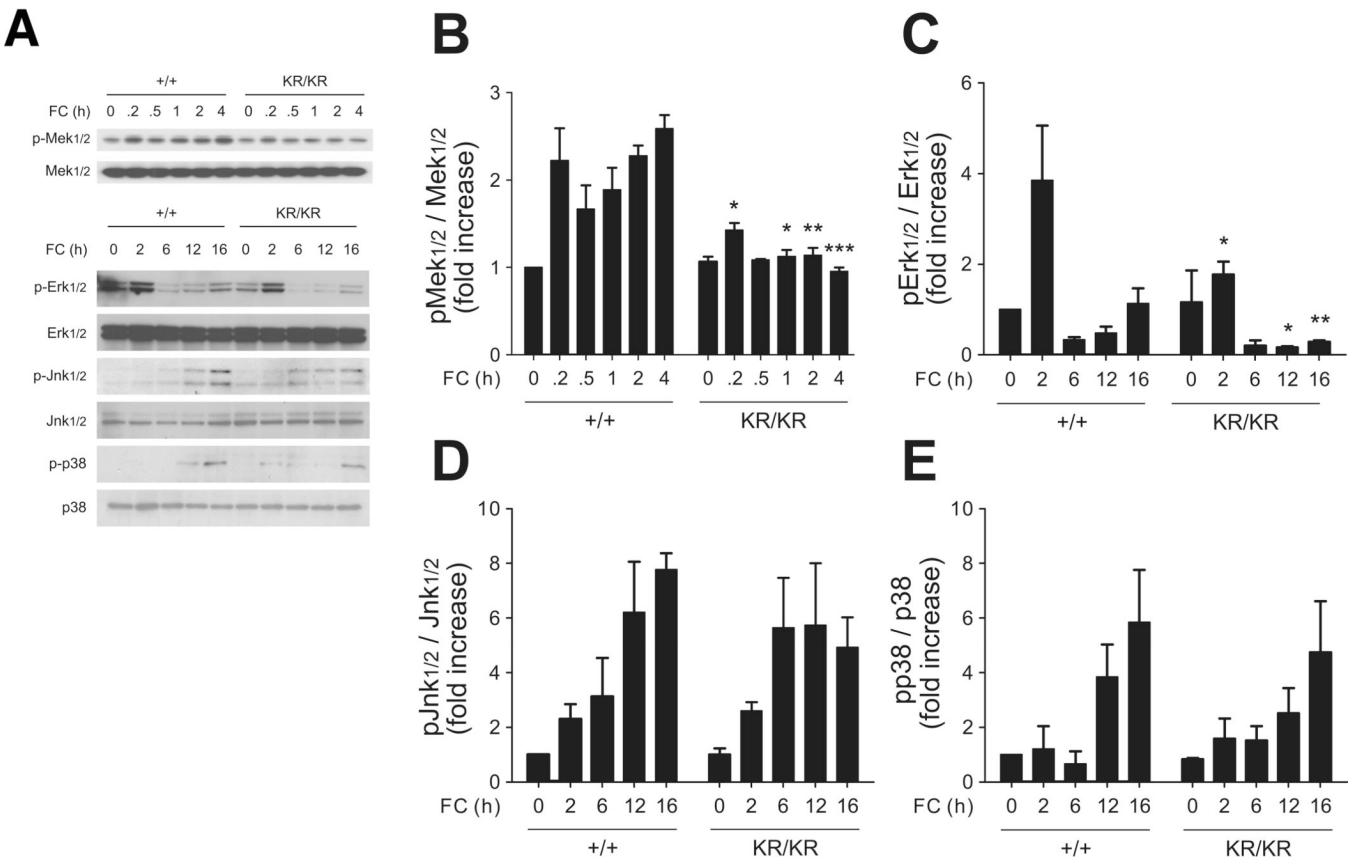


**Figure 4. FC activates Nf-κB in macrophages**

(A) Representative immunoblots of phospho-IκBα (Ser-32) and IκBα in *FoxO1*$^{+/+}$ (+/+) and *FoxO1*$^{KR/KR}$ (KR/KR) macrophages loaded with FC for the indicated times. (B) Representative immunoblots and quantification of (C) cytosolic and (D) nuclear p65. Band intensity was quantified from three independent experiments. (E) Luciferase activity in *FoxO1*$^{+/+}$ (+/+) and *FoxO1*$^{KR/KR}$ (KR/KR) macrophages transduced with recombinant adenovirus carrying an Nf-κB–responsive luciferase reporter construct for 24h followed by FC loading for 8h. NPM: nucleophosmin. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ *vs.* +/+.

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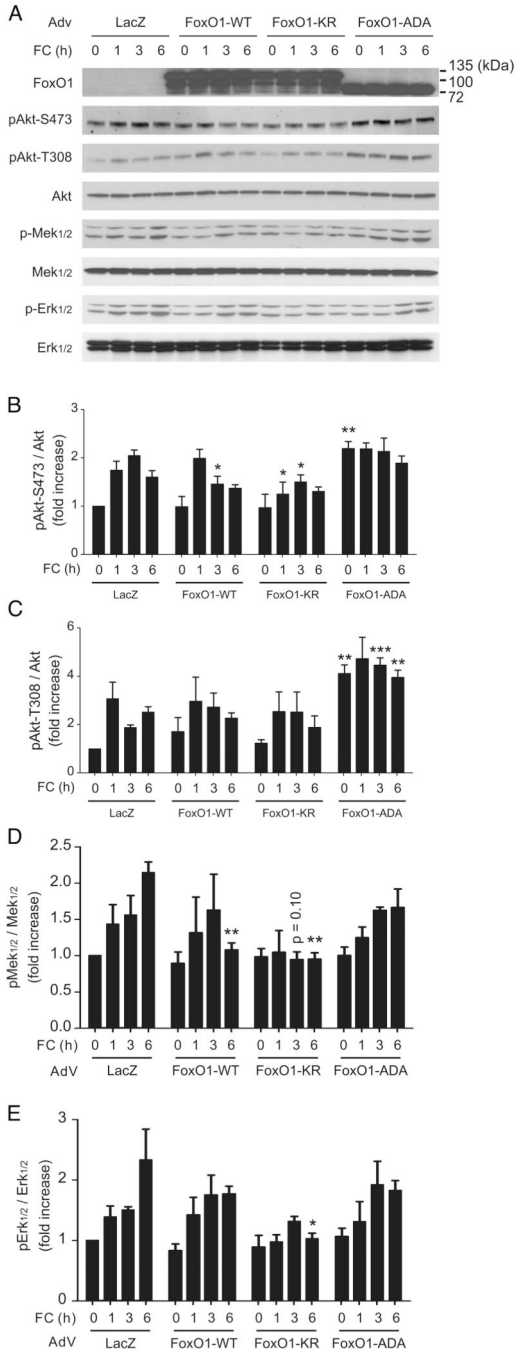


**Figure 5. *Nfkb1* and p105 levels in macrophages**

(A) Representative immunoblots and quantification of cytosolic (B) p105 and (C) p50, and (D) nuclear p105 in *FoxO1*$^{+/+}$ (+/+) and *FoxO1*$^{KR/KR}$ (KR/KR) macrophages loaded with FC for the indicated times. Loading control (α-tubulin and NPM) is shown in Fig. 6B. (E) *Nfkb1* levels in *FoxO1*$^{+/+}$ (+/+) and *FoxO1*$^{KR/KR}$ (KR/KR) macrophages (n=6). (F) Representative immunoblots and (G) quantification of p105 and actin levels in cytosolic fractions from *FoxO1*$^{+/+}$ macrophages transduced with adenovirus (AdV) encoding LacZ, FoxO1-WT, FoxO1-KR, or FoxO1-ADA. Band intensity was quantified from three independent experiments. * $P < 0.05$, and ** $P < 0.01$ *vs.* +/+.

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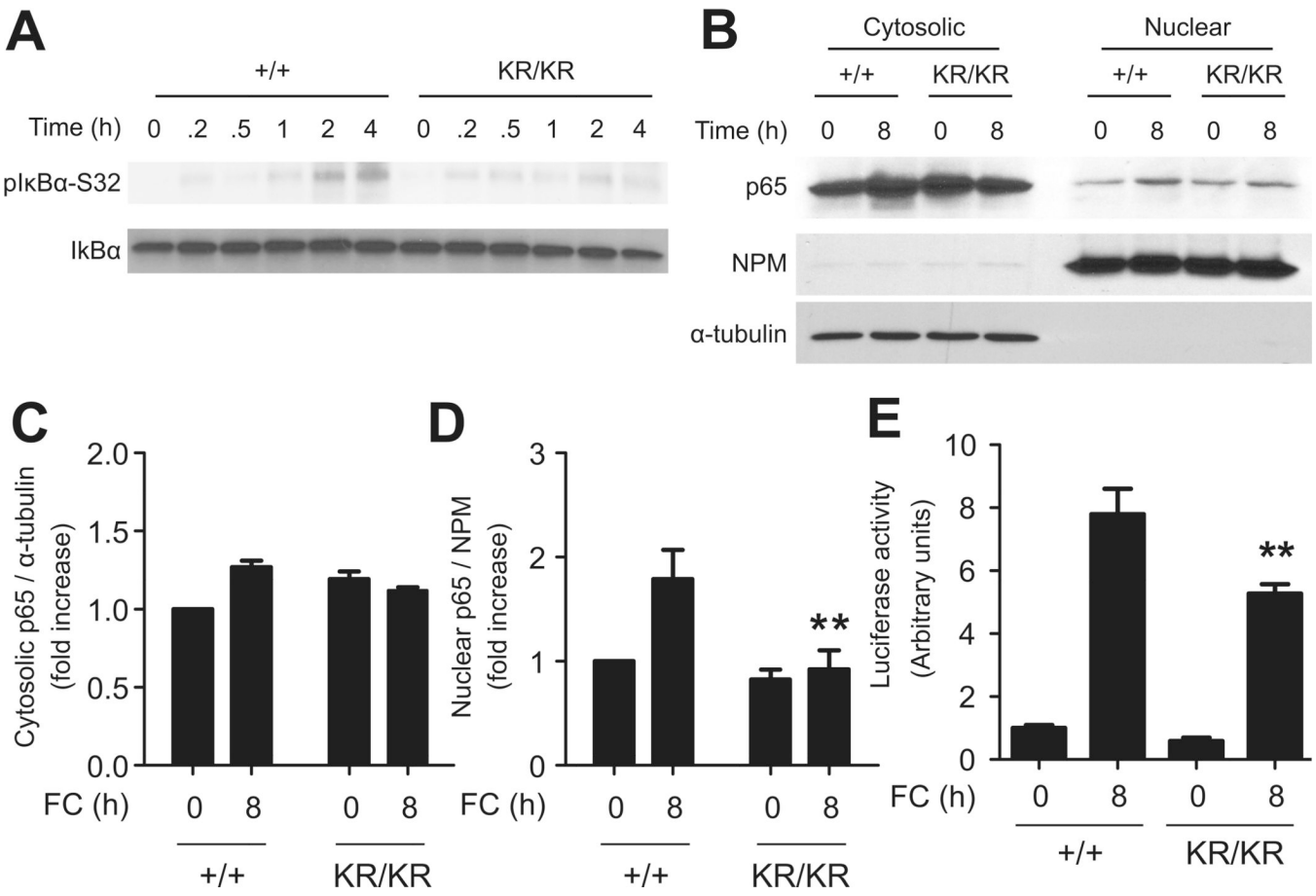


**Figure 6. Levels of p105, *Nfkb1*, and inflammatory cytokines in LY294002-treated macrophages**

(A) Representative immunoblots of p105, phospho-Akt Ser-473, Akt, phospho-GSK-3β and GSK-3β, and (B) quantification of p105 and (C) *Nfkb1* levels in *FoxO1*$^{+/+}$ macrophages treated with LY294002 (LY, 10μM) for 16h. (D) mRNAs encoding inflammatory cytokines in *FoxO1*$^{+/+}$ macrophages pretreated with (LY+, closed circles) or without (LY-, open circles) LY294002 (10μM) for 16h followed by FC loading for the indicated times (n=5). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ *vs.* LY- group.

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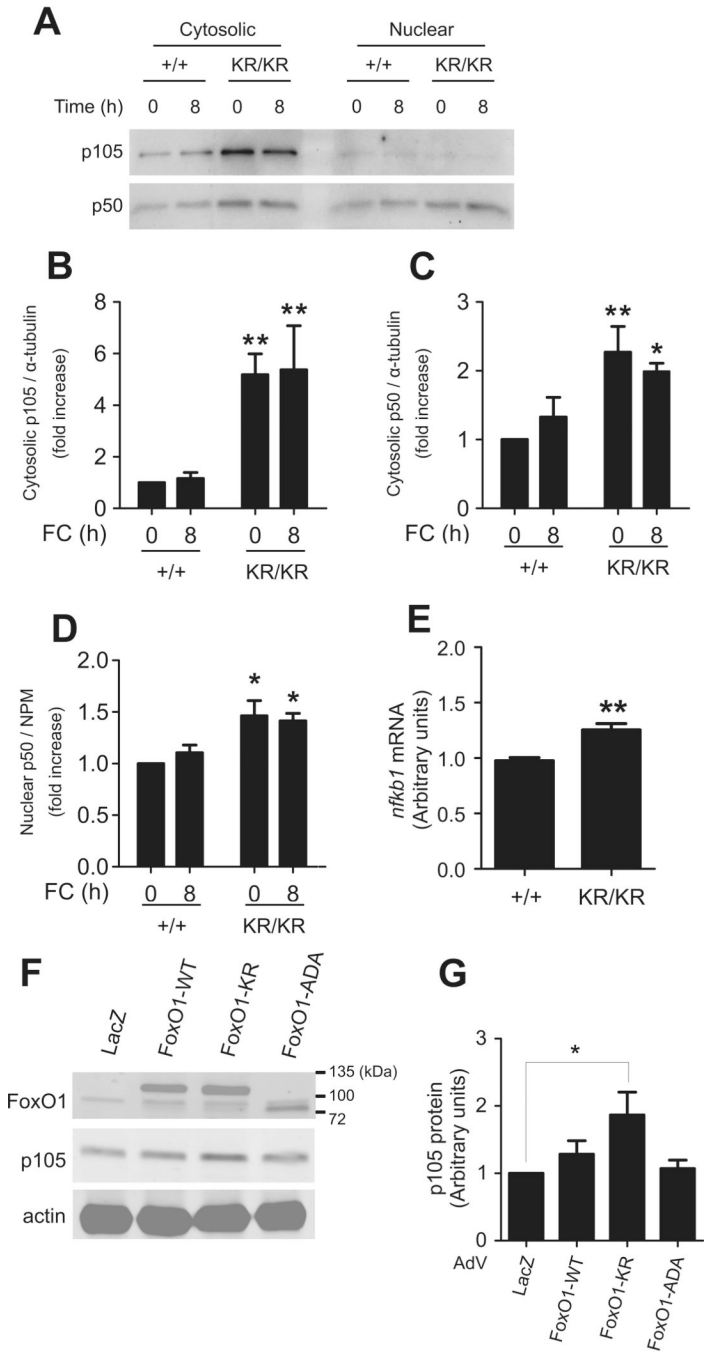


**Figure 7.**

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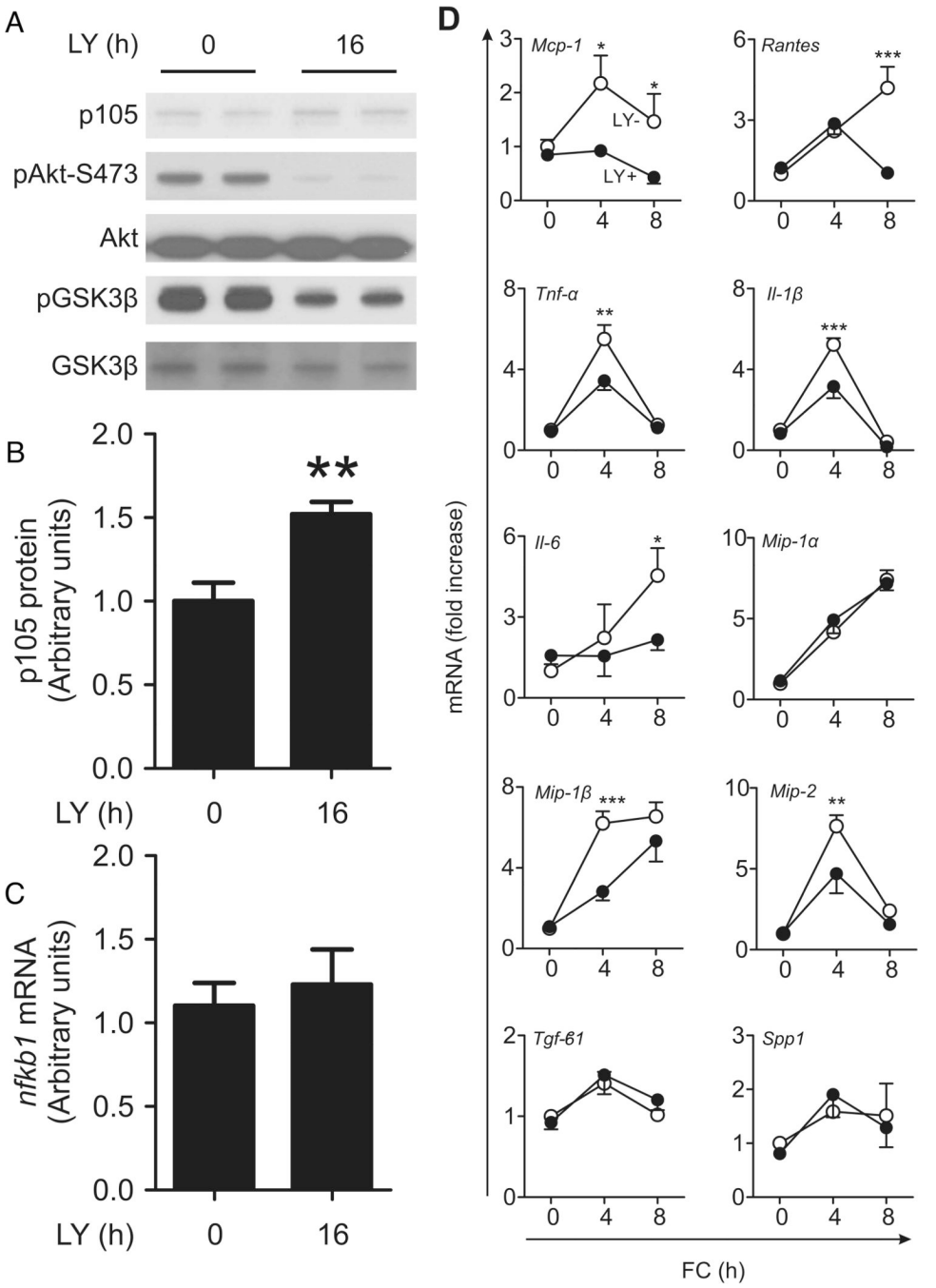


**Figure 8.**