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ORIGINAL ARTICLE

# Insulin Antagonizes LPS-Induced Inflammatory Responses by Activating SR-A1/ERK Axis in Macrophages

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*Abstract*— Insulin is a key regulator of metabolism and inflammation in the body. However, the mechanism of the anti-inflammatory effect of insulin is not fully understood. In the present study, we investigated the role of the class A1 scavenger receptor (SR-A1), a prototypic member of the pattern recognition receptor family, in the insulin-mediated suppression of inflammatory responses in macrophages. Our murine *in vivo* studies show that insulin can attenuate lipopolysaccharide (LPS)-induced endotoxemia in a SR-A1-dependent manner, and this was consistent with our *in vitro* results which demonstrate that the SR-A1 is necessary for insulin to antagonize the LPS-induced inflammatory responses in macrophages. The effect of SR-A1 on the anti-inflammatory action of insulin might be associated with the activation of the extracellular signal-regulated kinases (ERK) signaling pathway in macrophages. Insulin could inhibit macrophage polarization to a pro-inflammatory phenotype *via* the SR-A1/ERK cascade. Collectively, our results suggest that SR-A1 may be a pivotal element for the anti-inflammation effect of insulin in macrophages.

**KEY WORDS:** insulin; lipopolysaccharide; anti-inflammation; scavenger receptor; ERK pathway.

## INTRODUCTION

Insulin is an essential hormone for regulating metabolic homeostasis. Insulin signaling is not only involved in metabolism but also in the regulation of normal growth and development in insulin-responsive tissues [1–3]. The macrophage is an important type of immune cell in which cellular metabolism and immune functions are closely intertwined. It is known that insulin can suppress endotoxin-induced inflammatory responses in animals and in lipopolysaccharide (LPS)-treated human macrophage-1 cells [4]. The anti-inflammatory mechanisms of insulin may be associated with suppression of the intracellular adhesion molecule-1 and the monocyte chemoattractant protein-1 [5]. However, the intrinsic anti-inflammatory mechanism of insulin in macrophages is yet unknown.

Class A1 scavenger receptor (SR-A1) is a prototypic member of the pattern recognition receptor (PRR) family. Several lines of evidence indicate that SR-A1 functions as a suppressor of inflammatory responses. Yu et al. have reported that SR-A1 attenuates LPS-induced endotoxic shock [6]. The suppressive effect of SR-A1 on inflammatory response is mediated *via* inhibition of the production

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of pro-inflammatory cytokines and nitric oxide [7]. In our previous study, we had determined that SR-A1 exerts anti-inflammatory effect in the obese adipose tissues by shifting macrophages towards M2 polarization [8]. Therefore, we hypothesized that SR-A1 may play a role in the anti-inflammatory action of insulin. In the present study, we demonstrate that insulin strongly inhibits macrophage polarization towards the M1 phenotype which may be associated with the SR-A1-mediated extracellular signal-regulated kinases (ERK) signaling pathway. This newly discovered effect may endow insulin with the ability of anti-inflammation.

## MATERIALS AND METHODS

### Animals

Breeding pairs of wild-type and SR-A1-deficient mice on the ICR background were kindly provided by Dr. Ling WH at the Sun Yat-sen University. Animals were housed under a 12-h light/dark cycle in pathogen-free conditions with free access to mouse chow and water. All animal care and use procedures were in accordance with the guidelines established by the Research Animal Care Committee of the Nanjing Medical University. Male, wild-type, and SR-A1-deficient ICR mice (8 weeks, 25 ± 5 g) were injected intraperitoneally (i.p.) with LPS (10 mg/kg) (*E. coli*, 0111: B4; Sigma-Aldrich, St. Louis, USA) for 8 h to induce LPS shock. To monitoring survival rates, mice were injected (i.p.) with 20 mg/kg of LPS. Mice in the insulin treatment group were injected (i.p.) with insulin (1 U/kg) plus glucose (1 g/kg). The physical condition of the animals was monitored throughout the whole experiment. In the group injected with 10 mg/kg of LPS, no animal became severely ill or died at any time prior to the experimental endpoint. Mice were sacrificed for the experiments, and tissues were isolated and immediately frozen in liquid nitrogen or fixed. Animals were euthanized by placing them into an empty chamber, and then, the chamber was filled with carbon dioxide.

### Cell Culture and Treatments

Primary mouse peritoneal macrophages (PMs) were cultured in DMEM (Gibco, Waltham, USA) containing 10% fetal bovine serum (FBS) (Gibco, Waltham, USA), 100 units/ml penicillin and 100 μg/ml streptomycin, as described previously [9]. After incubation for 24 h and starvation overnight, cells were treated with the indicated concentrations of LPS and insulin (Sigma-Aldrich, St. Louis, USA).

### Materials

Phospho (p)-IRβ, IRβ, p-ERK, ERK, p-AKT, AKT, and p65 antibodies were purchased from Cell Signaling Technology (3024, 9750, 4060, 9272, 9101, 9102, 8242, mouse, 1:1000, Danvers, USA). GAPDH and Lamin B1 antibodies were purchased from Abcam (ab8245, ab133741, mouse, 1:1000, monoclonal, Cambridge, USA). SR-A1 antibodies were purchased from Santa Cruz Biotechnology (Sc-20444, mouse, 1:500, polyclonal, Santa Cruz, USA). PD98059 and LY294002 were purchased from Sigma-Aldrich (St. Louis, USA).

### Western Blot

The proteins were extracted from cells in RIPA buffer (0.5% NP40, 0.1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris HCl (pH 7.4)) containing complete protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). The PVDF membrane was probed with the corresponding primary antibodies. The protein bands were visualized with enhanced chemiluminescence reagents (Thermo Scientific, Rockford, USA) and quantified by using the ImageJ software.

### Quantitative RT-PCR

RNA extraction was performed by employing an RNAiso plus kit (Vazyme Biotech, Nanjing, China) according to manufacturer's instructions. Total RNA was measured using a nano drop, and 500 ng of RNA was used to reverse transcribe to cDNA. Gene expression was analyzed by using SYBR Green, normalized to the levels of GAPDH (ABI Prism 7500 Sequence Detection System; Applied Biosystems, CA, USA).

### ELISA

Mouse serum and cell supernatant concentrations of tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β were measured by ELISA with the corresponding kits from Multi sciences (Hangzhou, China) according to the manufacturer's protocol.

### Statistical Analysis

Data were expressed as the mean ± SEM. Statistical analysis was performed using the GraphPad Prism 7.0 software. Two-group comparisons were analyzed by unpaired Student's *t* test, and multiple-group comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey *post hoc* analysis. Statistical significance was achieved when $P < 0.05$ at 95% confidence interval.

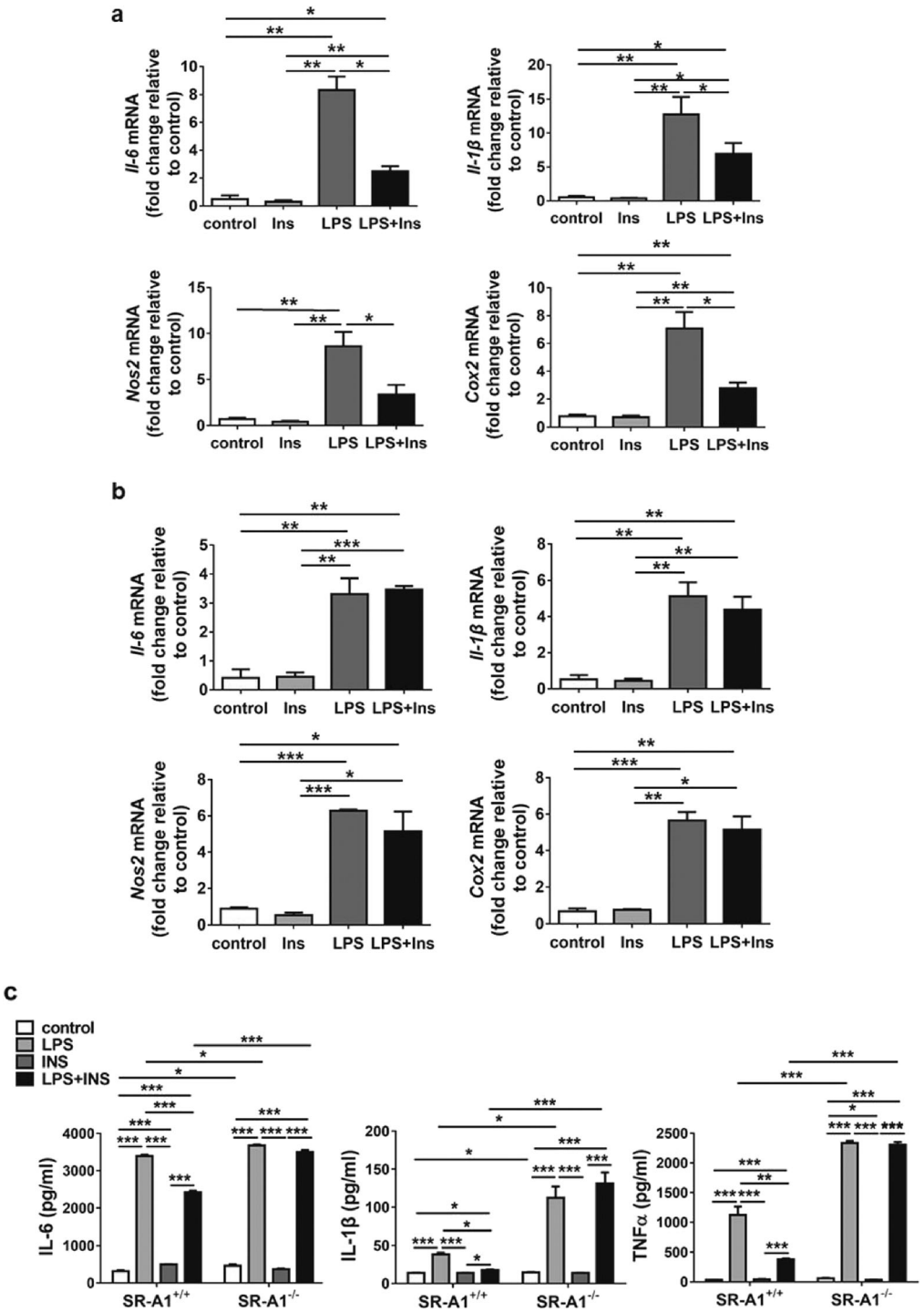
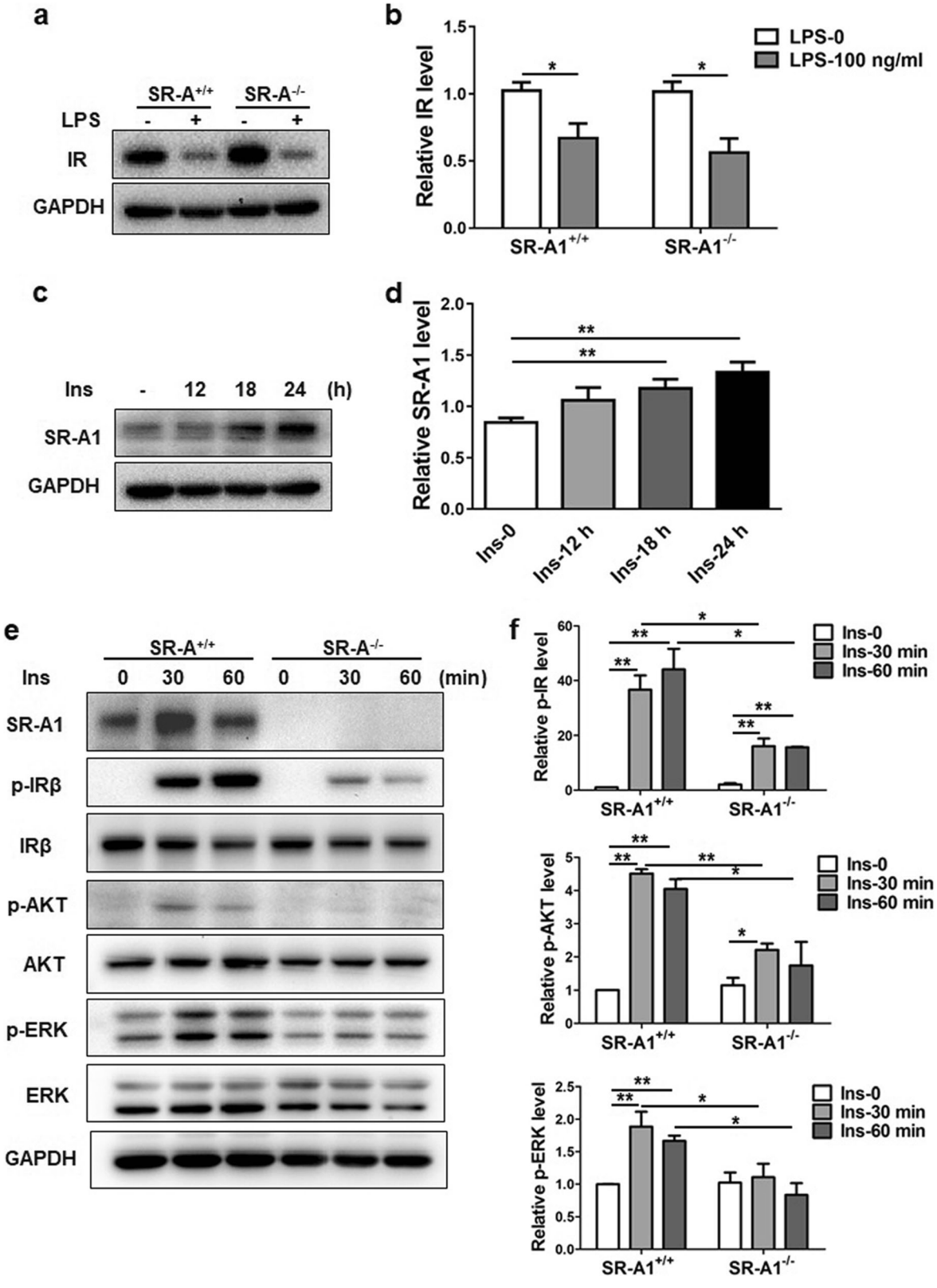



**Fig. 1.** SR-A1 is requisite for the anti-inflammatory activity of insulin in PMs. **a** mRNA levels of *Il-6*, *Il-1β*, *Nos2*, and *Cox2* normalized to the levels of *gapdh* in SR-A1$^{+/+}$ PMs stimulated with insulin (Ins; 100 nM), LPS (100 ng/ml), and LPS plus Ins for 16 h ($n = 3$). **b** mRNA levels of *Il-6*, *Il-1β*, *Nos2*, and *Cox2* normalized to the levels of *gapdh* in SR-A1$^{-/-}$ PMs stimulated with Ins (100 nM), LPS (100 ng/ml), and LPS plus Ins for 16 h ($n = 3$). **c** ELISA analysis of IL-6, IL-1β, and TNF-α in supernatants of SR-A1$^{+/+}$ and SR-A1$^{-/-}$ PMs treated with Ins (100 nM), LPS (100 ng/ml), and LPS plus Ins for 24 h ($n = 3$). Data are expressed as mean ± SEM. *$P < 0.05$; **$P < 0.01$.


a
SR-A+/+
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SR-A1-/-
c
Ins - 12 18 24 (h)
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d
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Ins-0
Ins-12 h
Ins-18 h
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IRβ
p-AKT
AKT
p-ERK
ERK
GAPDH
f
Relative p-IR level
Relative p-AKT level
Relative p-ERK level
Ins-0
Ins-30 min
Ins-60 min
SR-A1+/+
SR-A1-/-

◀ **Fig. 2.** SR-A1 is requisite for insulin-induced IR/AKT and ERK signaling in PMs. **a** Representative Western blots of IR and GAPDH in SR-A1$^{+/+}$ and SR-A1$^{-/-}$ PMs treated with LPS (100 ng/ml) for 0 and 24 h. **b** Densitometric analysis of the western blots ($n = 3$). **c** Representative Western blots of SR-A1 and GAPDH in SR-A1$^{+/+}$ PMs treated with Ins (100 nM) for 0, 12, 18, and 24 h. **d** Densitometric analysis of the Western blots ($n = 3$). **e** Representative Western blots of SR-A1, p-IRβ, IRβ, p-AKT, AKT, p-ERK, ERK, and GAPDH in SR-A1$^{+/+}$ and SR-A1$^{-/-}$ PMs treated with Ins (100 nM) for 0, 30, and 60 min. **f** Densitometric analysis of the Western blots ($n = 3$). Data are expressed as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$.

## RESULTS

### SR-A1 Is Requisite for the Anti-inflammation Effect of Insulin in LPS-Treated Macrophages

It is known that macrophage inflammatory response can be induced by LPS. We found that insulin could dramatically inhibit LPS-induced upregulation of mRNA

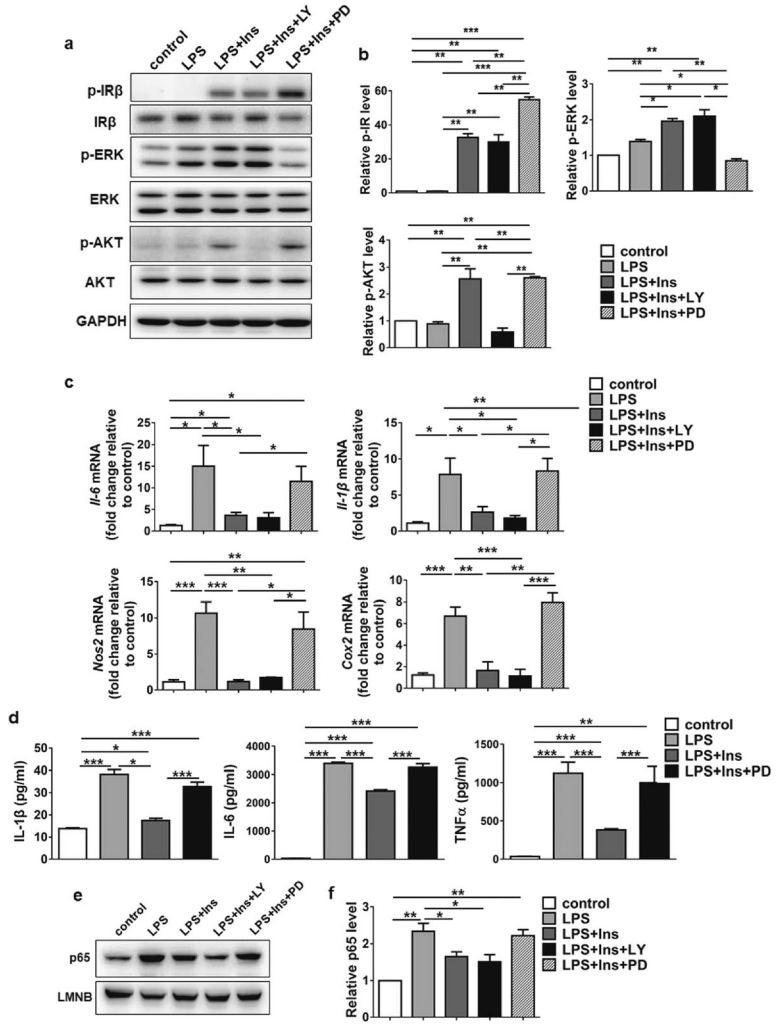


**Fig. 3.** The ERK signaling pathway is critical for the anti-inflammatory effect of insulin. **a** Representative Western blots of p-IRβ, IRβ, p-AKT, AKT, p-ERK, ERK, and GAPDH in SR-A1$^{+/+}$ PMs. The cells were treated without (control) or with an inhibitor ((PD98059 (PD; 10 μM), LY294002 (LY; 10 μM)) for 1 h prior to incubation with LPS (100 ng/ml), LPS plus Ins for 30 min. **b** Densitometric analysis of the western blots ($n = 3$). **c** mRNA expression levels of *Il-6*, *Il-1β*, *Nos2*, *and Cox2* normalized to the levels of *gapdh* in SR-A1$^{+/+}$ PMs treated without (control) or with an inhibitor (10 μM PD, 10 μM LY) for 1 h prior to incubation with LPS (100 ng/ml), LPS plus Ins for 16 h ($n = 3$). **d** ELISA analysis of IL-6, IL-1β, and TNF-α in SR-A1$^{+/+}$ PMs treated without (control) or with 10 μM PD for 1 h prior to incubation with LPS (100 ng/ml), LPS plus Ins for 24 h ($n = 3$). **e** Representative Western blot of p65 and Lamin B1 (LMNB) in SR-A1$^{+/+}$ PMs treated without (control) or with an inhibitor (10 μM PD, 10 μM LY) for 1 h prior to incubation with LPS (100 ng/ml), LPS plus Ins for 90 min. **f** Densitometric analysis of the Western blots ($n = 3$). Data are expressed as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

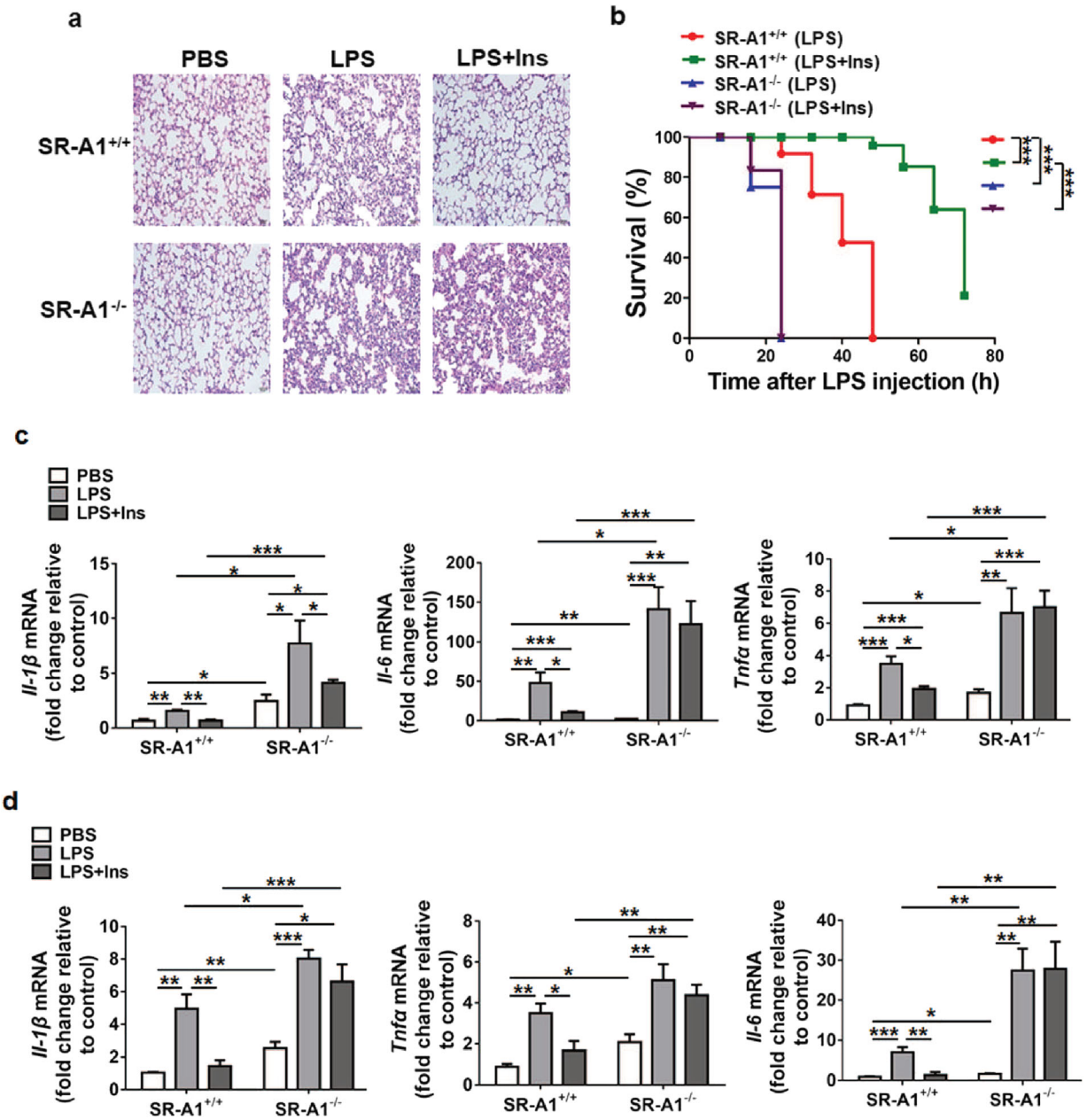


**Fig. 4.** SR-A1 is requisite for the protective effect of insulin against LPS-induced endotoxemia in mice. Wild-type (SR-A1$^{+/+}$) and SR-A1-deficient (SR-A1$^{-/-}$) mice were injected (i.p.) with LPS (10 mg/kg) for 8 h to induce LPS shock (**a**, **c**, and **d**). To monitor survival rates, mice were injected (i.p.) with 20 mg/kg LPS (**b**). Mice in the insulin treatment group were injected (i.p.) with insulin (1 U/kg) plus glucose (1 g/kg). **a** H&E staining of lung tissues; the tissues were excised from SR-A1$^{+/+}$ and SR-A1$^{-/-}$ mice treated with LPS (10 mg/kg), LPS plus Ins (1 U/kg). **b** The survival rates in the SR-A1$^{+/+}$ and SR-A1$^{-/-}$ mice injected (i.p.) with LPS (20 mg/kg), LPS plus Ins (1 U/kg) ($n = 6$). **c** mRNA expression levels of *Il-1β*, *Il-6*, and *Tnf-α* in the lung tissues; the tissues were excised from SR-A1$^{+/+}$ and SR-A1$^{-/-}$ mice treated with LPS (10 mg/kg), LPS plus Ins (1 U/kg) ($n = 6$). **d** mRNA expression levels of *Il-1β*, *Il-6*, and *Tnf-α* in aorta tissues; the tissues were excised from SR-A1$^{+/+}$ and SR-A1$^{-/-}$ mice treated with LPS (10 mg/kg), LPS plus Ins (1 U/kg) ($n = 6$). Data are expressed as mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

levels of inflammatory cytokines and markers, including *Il-6*, *Il-1β*, *Nos2*, and *Cox2*, in cultured murine PMs, though insulin alone did not influence inflammatory responses in macrophages (Fig. 1a). SR-A1 is a PRR primarily expressed in macrophages. To examine whether the SR-A1 pathway is involved in the anti-inflammation effect of insulin in macrophages, we compared the mRNA levels of inflammatory cytokines and markers in SR-A1$^{+/+}$ and SR-A1$^{-/-}$ PMs. As shown in Fig. 1b, the anti-inflammation effect of insulin could be abolished by deletion of SR-A1 in macrophages. Similar results were obtained by measuring the protein levels of inflammatory cytokines (IL-6, IL-1β, and TNF-α) in culture supernatants from SR-A1$^{+/+}$ and SR-A1$^{-/-}$ macrophages (Fig. 1c). These results suggest that SR-A1 is a requisite for the anti-inflammation effect of insulin in macrophages.

### SR-A1 Is Necessary for the Insulin-Activated ERK Signaling and Is Involved in the IR/AKT Signaling Pathway in Macrophages

Insulin activates the IR/AKT and Ras/Raf/ERK signal pathways in macrophages [10]. Yet, the relationship between these signal pathways and SR-A1 is unknown. We demonstrated that LPS treatment downregulated the expression of the IR protein in both SR-A1$^{+/+}$ and SR-A1$^{-/-}$ macrophages (Fig. 2a, b), suggesting that the IR pathway might be suppressed by inflammatory stimuli. Furthermore, insulin upregulated the expression levels of SR-A1 and activated IR, AKT, and ERK signaling in macrophages. Notably, these effects were significantly attenuated in SR-A1$^{-/-}$ macrophages (Fig. 2c–f). Together, these results reveal that SR-A1 may be involved in the insulin-activated IR/AKT and ERK signaling in macrophages.

### ERK Signaling Pathway Contributes to the Insulin's Anti-inflammation Effect in Macrophages

In order to illuminate the anti-inflammatory mechanism of insulin, selective inhibitors for ERK (PD98059) and AKT (LY294002) were used in the study. These two inhibitors could efficiently suppress their respective kinase targets but had no effect on the phosphorylation of IR in macrophages (Fig. 3a, b). Moreover, ERK inhibitor could block the insulin's anti-inflammation effect. Treatment with PD98059 increased the mRNA levels of the inflammatory cytokines and markers (*Il-6*, *Il-1β*, *Nos2*, and *Cox2*) in macrophages, but the AKT inhibitor (LY294002) had dissimilar effects (Fig. 3c, d). These data suggest that the ERK signaling pathway is required for the protection of insulin on LPS-induced inflammation. Consistently, we found that PD98059 (ERK inhibitor) could also block the inhibitory effect of insulin on the nuclear translocation of p65 (Fig. 3e, f), suggesting that ERK signaling can suppress LPS-induced NF-κB activation in macrophages.

Collectively, our results indicate that the ERK but not the AKT signaling pathway may contribute to the inhibitory activity of insulin against LPS-induced inflammation in macrophages.

### SR-A1 Is a Requisite for Insulin's Protection Against LPS-Induced Endotoxemia in Mice

In this study, we also assessed the SR-A1-mediated insulin anti-inflammatory response *in vivo* by utilizing a mouse LPS-induced endotoxemia model. We found that SR-A1$^{-/-}$ mice were more vulnerable to i.p. injection of LPS, with a massive tissue destruction observed in the lung, in contrast to the SR-A1$^{+/+}$ controls (Fig. 4a). Insulin administration could significantly attenuate the LPS-induced endotoxemia and decrease the mice mortality rate. Furthermore, this effect disappeared in SR-A1$^{-/-}$ mice (Fig. 4a, b). Consistently, the expression levels of *Il-1β*, *Il-6*, and *Tnf-α*, either in the lung or in the aorta, undergo similar changes in mice (Fig. 4c, d).

## DISCUSSION

In the present study, we demonstrate for the first time that the anti-inflammatory action of insulin is mediated *via* the SR-A1 pathway in macrophages. This is consistent with the suppressive effect of insulin on the pro-inflammatory transcription factor NF-κB in mononuclear cells [5]. Our findings provide insights on the immuno-metabolic regulatory mechanism of insulin.

Insulin can activate macrophage IR/AKT and Ras/ERK signaling cascades. In accordance with previous studies that had shown that insulin can activate the expression of SR-A1 in dendritic cells [11], we demonstrate that insulin can increase the SR-A1 levels in macrophages. This suggests that there may be a crosstalk between SR-A1 and insulin signaling

pathways. According to our results, SR-A1 in macrophages can enhance the phosphorylation of IR. IR consists of two α and two β subunits that span the plasma membrane of cells. In most cell types, IR is mainly distributed in the caveolae, with very few exceptions [12–14]. The IR in the caveolae can activate the IR/AKT pathway [15, 16]. IR signaling can be impaired by knocking down caveolin-1, a main component of the caveolae [17–19]. SR-A1 is also localized in the macrophage caveolae [9], which enables the spatial approximation required for a crosstalk between IR and SR-A1. The interaction between these two receptors may lead to the upregulation of IR but detailed mechanisms need to be further investigated.

Our previous study has demonstrated that SR-A1 may be useful for the intervention of obesity-associated insulin resistance [8]. The protective effect of SR-A1 may be associated with the ERK signaling activation in macrophages. Furthermore, it has been reported that monocyte IR activity is reduced in humans with systemic insulin resistance [20]. Monocyte-macrophages isolated from diabetic subjects and mice exhibit decreased surface expression and IR activity and diminished insulin-stimulated signaling of IRS2, PI3K, and AKT [21–23]. ERK signaling is one of the metabolism-related signaling pathways that are activated by insulin in cells [1, 24]. The observation that ERK contributed to the insulin/SR-A1-mediated anti-inflammatory signaling suggests that the ERK pathway may play a key role in integrated metabolism and immunity in macrophages. Thus, it may be a useful target for immuno-metabolic intervention for treatment of metabolic diseases.

## FUNDING

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## COMPLIANCE WITH ETHICAL STANDARDS

**Conflict of Interest.** The authors declare that they have no conflict of interest.

**Ethical Approval.** All procedures performed in studies involving animals were in accordance with the ethical standards of the Nanjing Medical University (Permit Number: NJMU/IACUC-1601121).

**Informed Consent.** Informed consent was obtained from all individual participants included in the study.



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