Runt-related Transcription Factor 1 (RUNX1) Binds to p50 in Macrophages and Enhances TLR4-triggered Inflammation and Septic Shock*[S]

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An appropriate inflammatory response plays critical roles in eliminating pathogens, whereas an excessive inflammatory response can cause tissue damage. Runt-related transcription factor 1 (RUNX1), a master regulator of hematopoiesis, plays critical roles in T cells; however, its roles in Toll-like receptor 4 (TLR4)-mediated inflammation in macrophages are unclear. Here, we demonstrated that upon TLR4 ligand stimulation by lipopolysaccharide (LPS), macrophages reduced the expression levels of *RUNX1*. Silencing of *Runx1* attenuated the LPS-induced IL-1β and IL-6 production levels, but the TNF-α levels were not affected. Overexpression of *RUNX1* promoted IL-1β and IL-6 production in response to LPS stimulation. Moreover, RUNX1 interacted with the NF-κB subunit p50, and coexpression of *RUNX1* with *p50* further enhanced the NF-κB luciferase activity. Importantly, treatment with the RUNX1 inhibitor, Ro 5-3335, protected mice from LPS-induced endotoxic shock and substantially reduced the IL-6 levels. These findings suggest that RUNX1 may be a new potential target for resolving TLR4-associated uncontrolled inflammation and preventing sepsis.

Toll-like receptor 4 (TLR4)[4] signaling has critical roles in regulating the inflammation response against pathogens, especially Gram-negative bacteria (1). However, excessive inflammation can cause tissue damage, leading to sepsis and death. Sepsis often occurs in patients with infection and is the leading cause of death in hospitalized patients (2). It is accompanied by overly exuberant inflammatory responses (3). Previous studies have demonstrated that TLR4-deficient mice are resistant to lipopolysaccharides (LPS)-induced septic shock (4), and other surface receptors, including CD11b (5) and VEGFR-3 (6), also regulate TLR4 signaling-associated sepsis. Currently, exploring new effectors as potential targets to prevent sepsis are still under extensive investigation. Macrophages play critical roles in sepsis by regulating inflammation (7, 8). Macrophages can recognize pathogens through Toll-like receptors and produce various proinflammatory cytokines through transcription factor activation, such as nuclear factor κB (NF-κB) (9). The NF-κB family consists of five members: p50/p105, p52/p100, p65 (RelA), RelB, and c-Rel. They share an evolutionarily conserved Rel homology region and can form heterodimers or homodimers (10). The precursors of p50 subunit and p52 subunit are p105 and p100, respectively (11). However, both p50 and p52 lack a transcription activation domain, and they may inhibit transcription unless they interact with other NF-κB family members or other transcription factors containing a transcription activation domain (10). The p50-p65 heterodimer is the classical NF-κB family member (12).

The runx family, another group of key transcription factors, is composed of RUNX1 (also known as acute myeloid leukemia 1 (AML1)), RUNX2, and RUNX3 (13). In humans, *RUNX1* is one of the genes most frequently altered by chromosome translocation and point mutations in acute myelogenous leukemia (14). In addition to participating in the regulation of cell cycle (15), proliferation (16), apoptosis (17), and ribosome biogenesis (18), RUNX1 is functionally related to the immune system. RUNX1 is critical in inducing the production of many genes in immune cells, such as *IL-2* (19), *IL-3* (20), macrophage colony-stimulating factor 1 receptor (*CSF1R*) (21), *CSF2* (22), and *CD4* (23). RUNX1 has been reported to be associated with autoimmune diseases, including rheumatoid arthritis (24) and systemic lupus erythematosus in humans (25). Previous studies suggest that RUNX1 mediates transactivation of *Rorc* to promote T_H17 differentiation and interacts with RORγt to induce IL-17 production (26). Interestingly, RUNX1 is required for the optimal expression of *Foxp3* in natural regulatory T cells and

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[4] The abbreviations used are: TLR4, Toll-like receptor 4; BCL3, B-cell CLL/lymphoma 3; C-Rel, v-rel avian reticuloendotheliosis viral oncogene homolog; IκBζ, nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor ζ; IRF3, interferon regulatory factor 3; NF-κB, nuclear factor-light-chain enhancer of activated B cell; PEMs, primary mouse peritoneal macrophages; RelB, v-rel avian reticuloendotheliosis viral oncogene homolog B; RUNX1, Runt-related transcription factor 1; SOCS, suppressor of cytokine signaling; qPCR, quantitative PCR.

also interacts physically with FOXP3 (27). Moreover, RUNX1 deficiency in CD4$^+$ T cells causes lethal lung inflammation (28). Although a role for RUNX1 has been suggested in T cells, it remains unclear about how RUNX1 regulates TLR4-mediated inflammation.

Previous studies reported that RUNX1 could promote STAT3 activation and inhibit the expression of *SOCS3/4* in epithelial cancer (29). The SOCS family, especially SOCS3/4, plays a negative regulatory role in TLR4-mediated inflammation (30). In this study, we asked whether RUNX1 played vital roles in TLR4-mediated inflammation. We found that RUNX1 interacted with the NF-κB subunit p50 to enhance the production of inflammatory cytokines in macrophages, such as IL-1β and IL-6, and a RUNX1 inhibitor, Ro 5-3335, could protect mice from LPS-induced shock. Our data identify RUNX1 as a positive regulator in macrophage inflammatory responses via the p50 pathway.

Results

Knockdown of Runx1 Expression Reduces the Production of Proinflammatory Cytokines in LPS-stimulated Macrophages—To explore the role of *RUNX1* in macrophages, we first examined whether RUNX1 expression was regulated in response to TLR4 agonist (LPS) stimulation at the mRNA and protein levels. RUNX1 was expressed in the human monocytic cell line, THP-1, mouse macrophage-like cell line, RAW264.7, and primary mouse peritoneal macrophages (PEMs) (Fig. 1, *A–C*). The purity of CD11b$^+$ F4/80$^+$ PEMs was analyzed by FACS (supplemental Fig. S1). In response to LPS stimulation, *RUNX1* expression at the mRNA level was significantly reduced in all these cells (Fig. 1, *A–C, i.e.* ~6 h). We also detected a substantial decrease of RUNX1 at the protein level in these cells following LPS treatment (Fig. 1*D*, supplemental Fig. S2, *A–B*). To determine whether other stimuli affect the expression of *Runx1* in macrophages, macrophages were stimulated with IL-1β, IL-6, or TNF-α. However, these cytokines did not modulate *Runx1* mRNA and protein expression in PEMs (supplemental Fig. S3). These results demonstrate that *RUNX1* is constitutively expressed in macrophages, and TLR4 activation decreases RUNX1 levels in macrophages.

To study the role of RUNX1 in primary macrophages, we used specific siRNA to knockdown *Runx1* in PEMs. The mRNA levels of *Runx1* decreased by 70% in the siRunx1-transfected PEMs compared with the negative controls (Fig. 1*E*). The knockdown efficiency was also confirmed by Western blot analysis (Fig. 1*E*). Next, we analyzed the effects of *Runx1* silencing on the production of inflammatory cytokines in macrophages. In response to LPS, the siRunx1-transfected PEMs produced diminished inflammatory cytokine expression at the mRNA level, including *Il-1β* and *Il-6*, but not *Tnf-α*, compared with the controls (Fig. 1*F*). We also examined IL-6 concentrations using an ELISA kit, and we confirmed that Runx1-silenced PEMs produced lower IL-6 levels than control cells at the indicated times (6, 12, and 24 h) (Fig. 1*G*). In addition to *Il-1β* or *Il-6*, the mRNA levels of other LPS-dependent genes including *Il-12b*, *Csf2*, and *Ccl3* were also decreased in the siRunx1-transfected PEMs (supplemental Fig. S4, *A–C*).

Regulation of IL-6 and IL-1β Production by RUNX1 Overexpression or Treatment with a RUNX1 Inhibitor—To further confirm the effect of RUNX1 on TLR4-induced production of proinflammatory cytokines, we overexpressed *RUNX1* in RAW 264.7 cells. Upon LPS treatment, RAW 264.7 cells that overexpressed RUNX1 had profoundly enhanced *Il-6* and *Il-1β* expression levels, but not *Tnf-α*, compared with control cells that overexpressed GFP (Fig. 2*A*). Additionally, *RUNX1* overexpression also induced RAW 264.7 cells to secrete more IL-6 into the supernatant, as detected by ELISA (Fig. 2*B*).

As the RUNX1 inhibitor, Ro 5-3335 has been previously well described (31), we next examined whether it could inhibit TLR4-triggered production of proinflammatory cytokines in macrophages. As shown in Fig. 2, *C–E*, Ro 5-3335 substantially blocked the expression of *Il-6* at the mRNA and protein level in THP-1 cells, RAW 264.7 cells, and PEMs. We also found that Ro 5-3335 could inhibit the expression of *Il-6* in PEMs stimulated with the TLR2 agonist, PGN, but not the TLR3 agonist, poly(I:C), or the TLR9 agonist, CpG (supplemental Fig. S5). These findings indicate that RUNX1 might positively regulate the TLR4 signaling pathway and proinflammatory cytokine production in macrophages.

RUNX1 Synergizes with the NF-κB Subunits to Enhance TLR4-triggered Inflammation—MAPKs and NF-κB signaling have critical roles in the induction of inflammatory cytokine production (32, 33). To reveal the function of RUNX1 in the TLR4 signaling pathway, we first analyzed the phosphorylation levels of MAPKs and NF-κB. Fig. 3, *A–B*, shows that *Runx1* knockdown did not affect the ERK, JNK, p38, or p65 phosphorylation levels in PEMs. Additionally, we did not detect IκBα degradation differences between the *Runx1*-silenced and wild type PEMs (Fig. 3*B*). Moreover, the RUNX1 inhibitor, Ro 5-3335, did not affect p65 phosphorylation levels or IκBα degradation in PEMs (supplemental Fig. S6).

Because RUNX1 is predominantly located in the nucleus (34), it is possible that RUNX1 might physically interact with or cooperate with other transcription factors in the nuclei to regulate inflammation. In agreement with this speculation, we observed that treatment with the NF-κB inhibitor, BAY 11-7082, strictly abrogated LPS-induced IL-6 and IL-1β production in the GFP control or RUNX1-overexpressing macrophages (Fig. 3*C*). Next, we examined the effect of RUNX1 on NF-κB activation using a dual-luciferase reporter assay. Although *RUNX1* expression alone did not induce activation of the reporter gene in 293T cell, co-expression of *RUNX1* and the NF-κB family members, *p105*, *p50*, or *p65*, significantly increased the NF-κB reporter activity compared with co-expression of the *GFP* control (Fig. 3*D*). The RUNX1 inhibitor, Ro 5-3335, partly reversed the synergized effect between RUNX1 and p50, p65, or p105 (Fig. 3*E*).

RUNX1 Interacts with the p50 Subunit of NF-κB—We next investigated whether RUNX1 could interact with p105, p50, or p65. 293T cells were transiently transfected with *RUNX1* together with *p105*, *p50*, *p65*, or the *GFP-myc* vector control. Cell lysates were immunoprecipitated with anti-p50/p105, p65, or anti-Myc antibodies. We noticed that RUNX1 preferred to be coprecipitated with p50, compared with the other NF-κB family members including p105 and p65 (Fig. 4*A*). Immunopre-
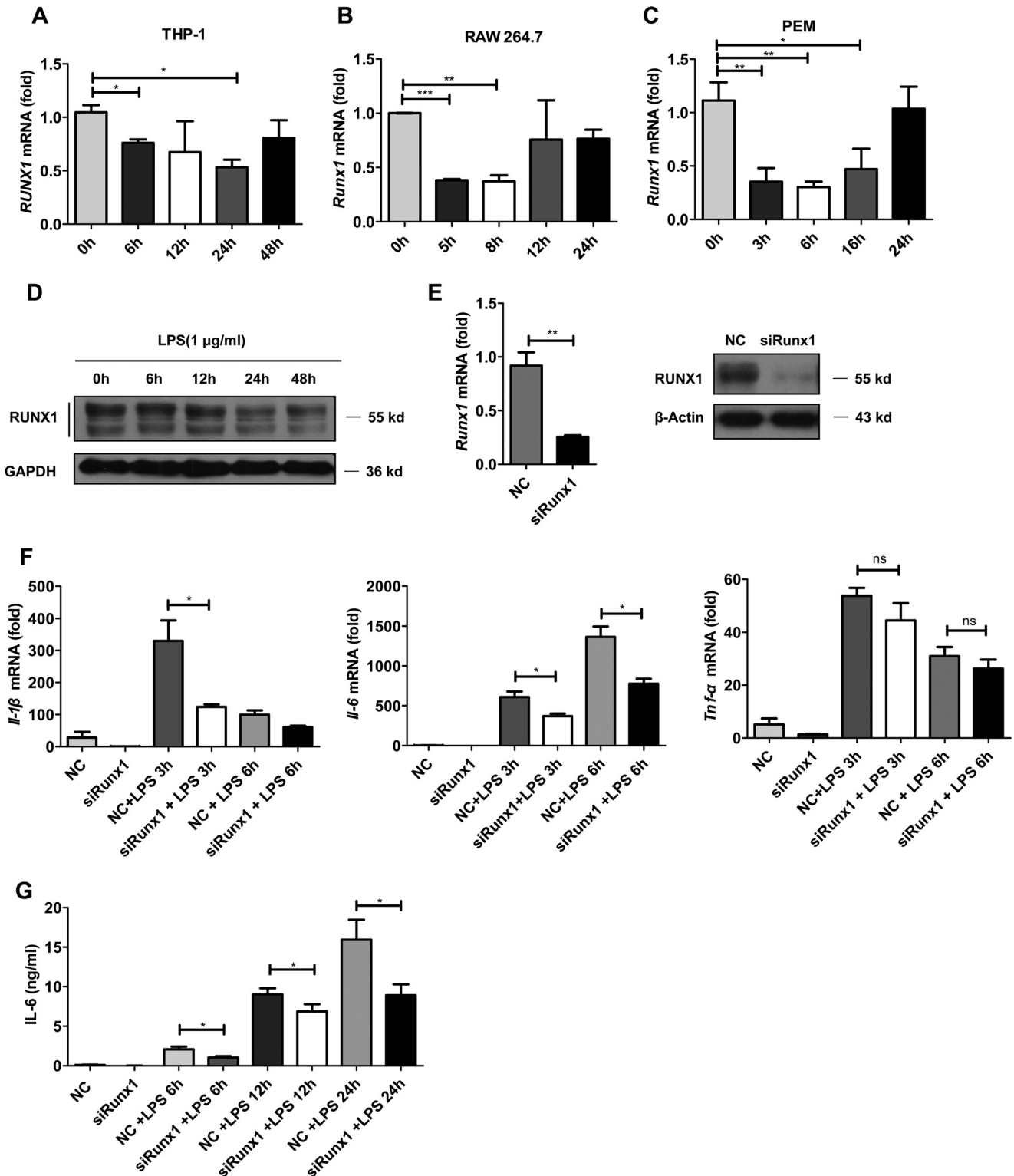
FIGURE 1. **Knockdown of *Runx1* expression inhibits proinflammatory cytokine production in LPS-stimulated macrophages.** THP-1 cells (*A* and *D*), RAW 264.7 cells (*B*), and PEMs (*C*) were stimulated with LPS at different doses for the indicated time points. The *RUNX1* expression levels were detected by immunoblotting or RT-qPCR. The data are shown as the mean ± S.D. of a representative experiment. *E–G*, PEMs were transfected with *Runx1* siRNA or nonspecific siRNA using the Lipofectamine RNAiMAX for 48 h, and the knockdown efficiency was measured by RT-qPCR and immunoblotting (*E*). These PEMs were then stimulated with 1 μg/ml of LPS for the indicated times. The *Il-1β*, *Il-6*, and *Tnf-α* mRNA levels were measured by RT-qPCR (*F*), and the supernatants IL-6 levels were determined by ELISA (*G*). The data are shown as the mean ± S.E. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

cipitation using an anti-RUNX1 antibody was also performed, which indeed pulled down p50 (Fig. 4*B*). Next, we asked whether RUNX1 interacted with the endogenous p50, and con-

firmed the endogenous interaction between RUNX1 and p50 in THP-1 cells (Fig. 4*C*). The interaction between RUNX1 and p50 was not significantly changed by LPS treatment in RAW 264.7
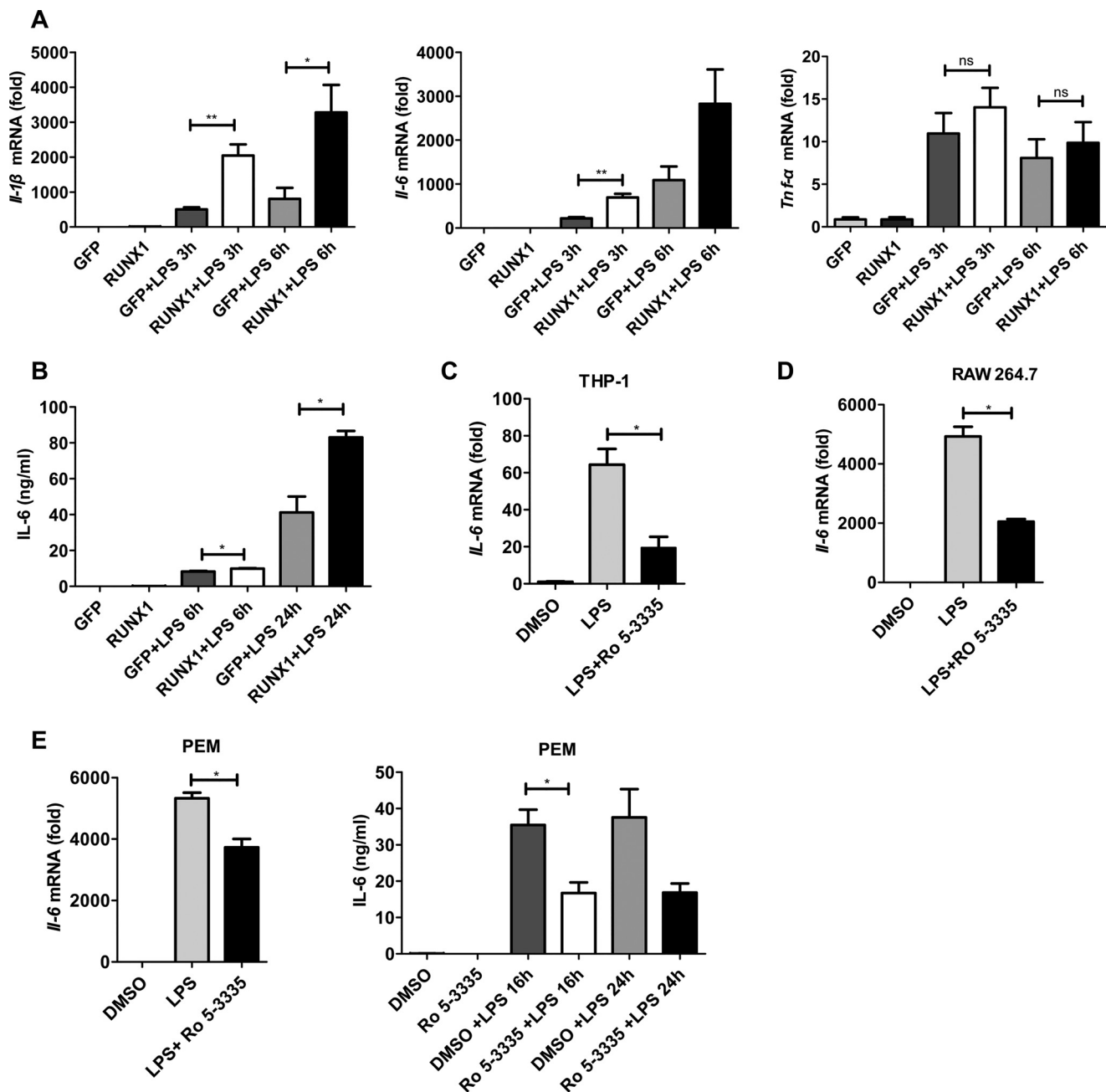
FIGURE 2. **Regulation of IL-6 and IL-1β production by RUNX1 overexpression or treatment with a RUNX1 inhibitor.** *A* and *B, RUNX1-GFP* or a *GFP* vector control (6 μg) were co-transfected with pCL-10A1 (6 μg) into 293T cells. The retroviral supernatants were harvested and used to infect RAW264.7 cells, which was followed by FACS sorting of the GFP$^+$ cells. These stable RAW264.7 cells that overexpressed RUNX1 or GFP were stimulated with 100 ng/ml of LPS for the indicated times. The *Il-1β*, *Il-6*, and *Tnf-α* mRNA levels were measured with RT-qPCR (*A*), and the IL-6 levels in the supernatants were determined with ELISA (*B*). The data are shown as the mean ± S.E. of three independent experiments. THP-1 cells (*C*), RAW 264.7 cells (*D*), and PEMs (*E*) were stimulated with LPS (100 or 1000 ng/ml) for 4 h in the absence or presence of the RUNX1 inhibitor, Ro 5-3335 (50 μM). IL-6 levels were measured by RT-qPCR or ELISA. The data are shown as the mean ± S.D. of a representative of three independent experiments. *, $p < 0.05$; **, $p < 0.01$.

at early time points (30 or 60 min), which overexpressed RUNX1 (Fig. 4*D*). These findings suggest that RUNX1 might regulate TLR4 signaling and proinflammatory cytokine production by binding to p50.

We further investigated whether RUNX1 could affect the recruitment of p50 to the *Il-6* promoter using a ChIP analysis. Consistent with previous reports (35, 36), the levels of p50 on the *Il-6* promoter were unchanged in macrophages in response to LPS treatment, and the RUNX1 inhibitor did not affect the recruitment of p50 to the *Il-6* promoter

(supplemental Fig. S7, *left panel*). However, the RUNX1 inhibitor greatly reduced the levels of RNA polymerase II binding to the *Il-6* promoter (supplemental Fig. S7, *right panel*). These results support the role of RUNX1 in regulating *IL-6* transcript in macrophages after LPS stimulation.

We then sought to determine which domain of RUNX1 could interact with p50. Different truncations of RUNX1 were constructed, including the N-terminal fragment (R1–242), the C-terminal fragment (R243–453), and the Runt domain (R50–178) (Fig. 4*E*). We noticed that all of these truncations could
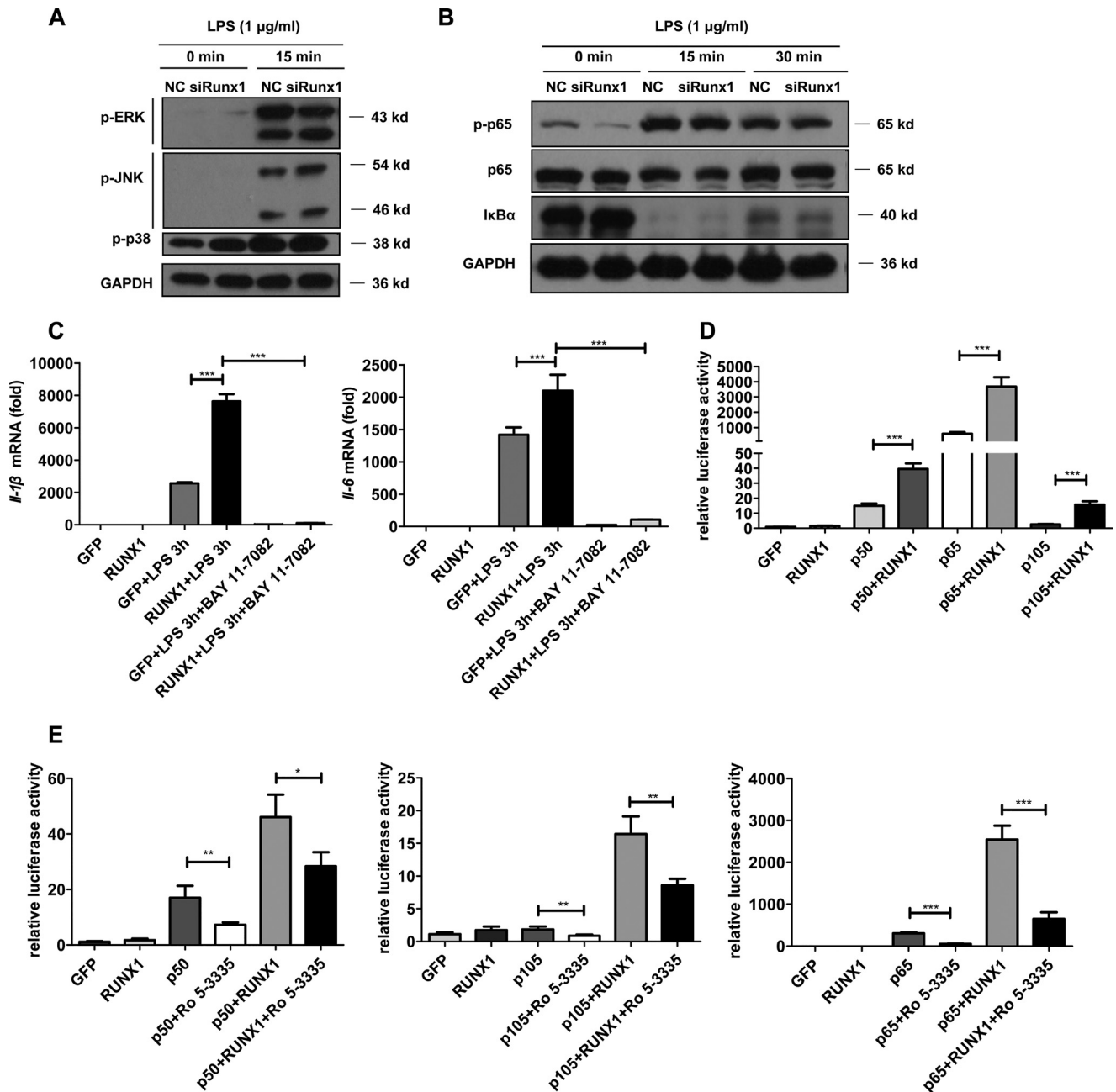
FIGURE 3. **RUNX1 synergizes with the NF-κB subunits to enhance TLR4-triggered inflammation.** *A* and *B,* PEMs were transfected with *Runx1* siRNA or nonspecific siRNA, then stimulated with 1 μg/ml of LPS for the indicated times. Cell lysates were extracted for immunoblotting to detect the phosphorylated ERK, P38, JNK, or p65 levels as well as the total IκBα and p65 levels. The data are shown as a representative of two independent experiments. *C,* the stable RAW 264.7 cell lines that overexpressed *RUNX1* or *GFP* were stimulated with 100 ng/ml of LPS for 3 h in the presence of the NF-κB inhibitor, 10 μM BAY 11-7082, followed by detection of *Il-1β* and *Il-6* by RT-qPCR. *D* and *E,* plasmids expressing *RUNX1* or *GFP* were co-transfected with plasmids expressing *p50*, *p65*, or *p105* into 293T cells in the presence of an NF-κB luciferase reporter plasmid and a *Renilla* luciferase plasmid. After 24 h, the cell lysates were assayed with a dual luciferase reporter assay system (*D*). Alternatively, after 8 h, Ro 5-3335 (50 μM) or DMSO was added for 18 h to examine the luciferase readings (*E*). The data are shown as the mean ± S.E. of at least two independent experiments. *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001.

interact with p50 (Fig. 4*F*). This interaction is similar to the interaction between RUNX1 and Ets-1; both N-terminal and C-terminal fragments of RUNX1 can bind to Ets-1 (37). To further determine which domain of RUNX1 was responsible for NF-κB activation, we overexpressed different truncations of RUNX1 in RAW 264.7 cells. Following LPS stimulation, the C-terminal fragment and the full-length RUNX1 showed enhanced IL-6 expression, but the N-terminal fragment showed no significant effect (Fig. 4*G*). These results indicate

that RUNX1 is dependent on its C-terminal region to augment TLR4-NF-κB activity.

A RUNX1 Inhibitor Protects against LPS-induced Septic Shock in Vivo—Excessive TLR4-NF-κB activation is critical for the induction of sepsis that is accompanied by exacerbated inflammatory responses (6, 7). To investigate the *in vivo* role of RUNX1 in inflammation, we utilized a murine endotoxic shock model via intraperitoneal injection of a lethal dose of LPS. LPS induced death in over 80% of mice at 20 h, whereas
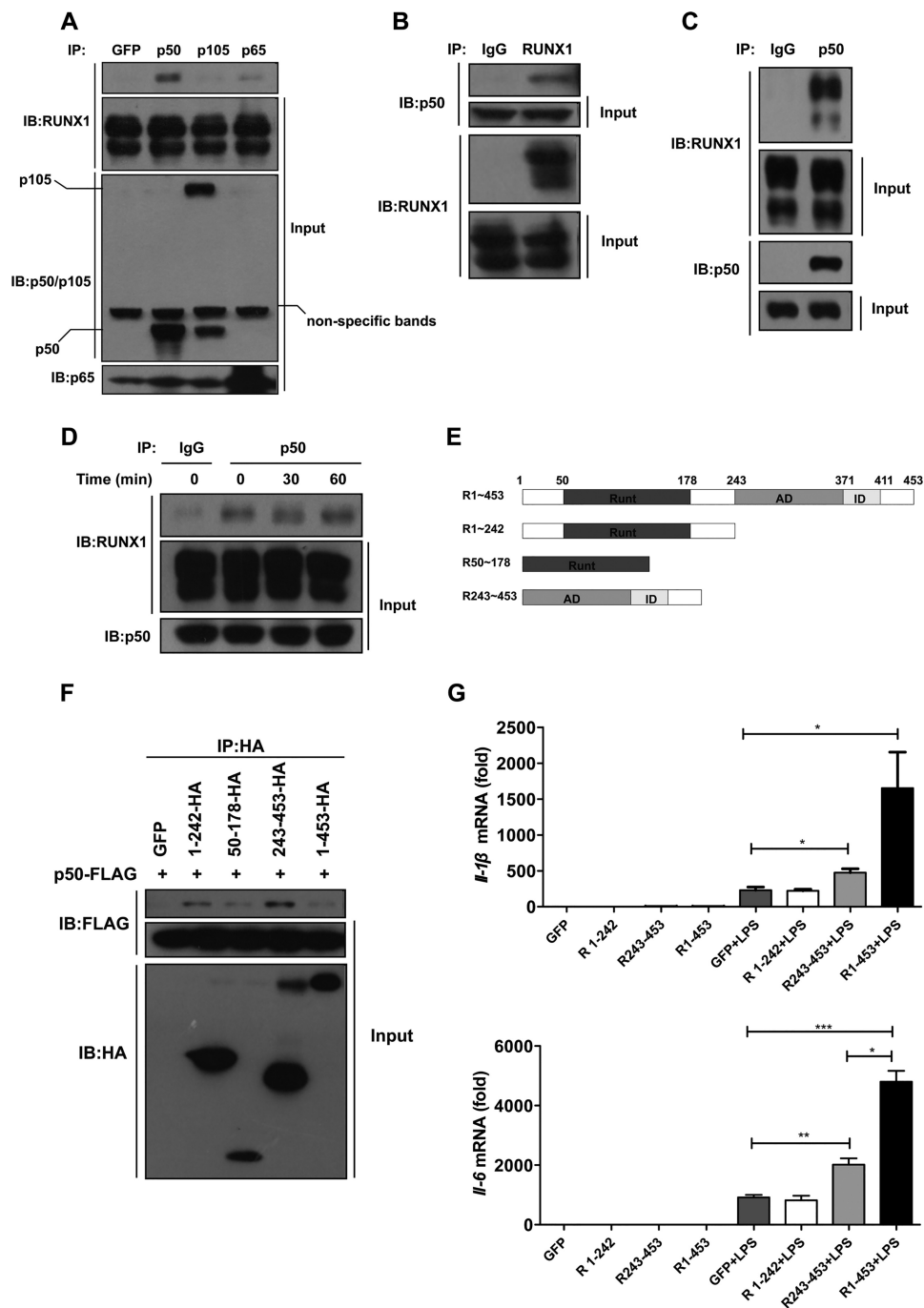
FIGURE 4. **RUNX1 interacts with the p50 subunit of NF-κB.** *A, RUNX1* was co-transfected with plasmids expressing *GFP-myc*, *p50*, *p105*, or *p65* into 293T cells. The cell lysates were immunoprecipitated (*IP*) with the indicated antibodies followed by immunoblotting (*IB*) with an anti-RUNX1 antibody. *B,* 293T cells were co-transfected with *p50* and *RUNX1*, and cell extracts were immunoprecipitated with an anti-RUNX1 antibody, followed by immunoblotting with the indicated antibodies. *C,* THP-1 cell lysates were immunoprecipitated with an anti-p50 antibody followed by immunoblotting with an anti-RUNX1 antibody to detect the endogenous interaction between RUNX1 and p50. *D,* RAW 264.7 cells that overexpressed *RUNX1* were stimulated with LPS for 30 and 60 min, then cell lysates were immunoprecipitated with an anti-p50 antibody followed by immunoblotting with an anti-RUNX1 antibody. *E,* the RUNX1 truncations are listed (*Runt*, Runt domain; *AD*, activation domain; *ID*, inhibition domain). *F,* 293T cells were transiently co-transfected with *p50-FLAG* and *HA*-tagged *RUNX1* truncations (R1~242, R50–178, R243–453 or R1–453), then cell lysates were immunoprecipitated with an anti-HA antibody, followed by immunoblotting with an anti-FLAG antibody. The results are shown as a representative of three independent experiments. *G,* the stable RAW264.7 cells overexpressing *GFP* or the different *RUNX1* truncations (R1~242, R243–453, or R1–453) were stimulated with 100 ng/ml of LPS for 6 h. The *Il-1β* and *Il-6* mRNA levels were measured with RT-qPCR. The data are shown as the mean ± S.E. of two independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

the RUNX1 inhibitor, Ro 5-3335, significantly improved the mouse survival rates at 20 h, 48 h, or longer time points when used at 5 mg/kg (Fig. 5*A*). We further examined the expression of proinflammatory cytokines in various organs and

serum in these mice. Unexpectedly, we observed no significant differences in the *Il-1β*, *Il-6*, or *Tnf-α* mRNA levels in the liver, spleen, lungs, or serum IL-6 concentrations at 3 h after the Ro 5-3335 intervention when compared with the
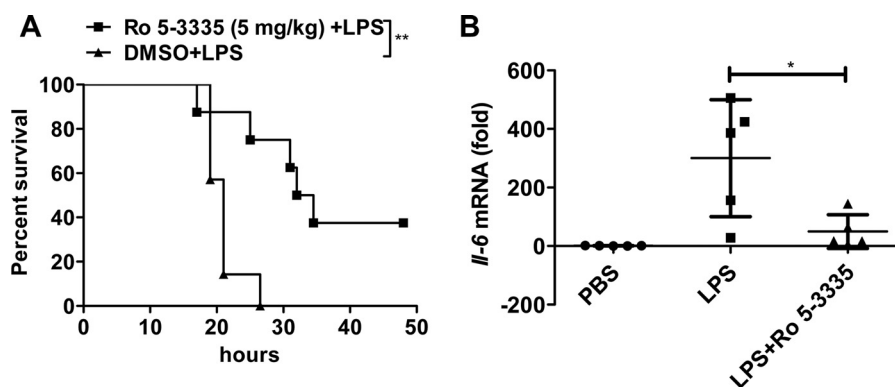
FIGURE 5. **A RUNX1 inhibitor protects against LPS-induced septic shock *in vivo*.** LPS (20 mg/kg) was used to induce septic shock in C57BL/6 mice in the presence or absence of the RUNX1 inhibitor, Ro 5-3335. The survival rates of these mice were recorded (*A*), and the *Il-6* mRNA levels in the kidneys were detected by RT-qPCR (*B*). The data are shown as the mean $\pm$ S.D. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$.

DMSO control mice.[5] However, the *Il-6* mRNA levels were selectively decreased in the kidneys of the treated mice at a later stage (*i.e.* 24 h) (Fig. 5*B*). These results indicate that the RUNX1 inhibitor may resolve IL-6 production in the kidneys and protect against LPS-induced septic shock.

Discussion

Excessive TLR4-NF-κB signaling is closely related to uncontrolled inflammation, tissue damage, and septic shock (38, 39) and is critical to identifying new key regulators in the TLR4-NF-κB pathway, as they could be potential targets that prevent sepsis. RUNX1 is a master transcription factor in hematopoiesis (40) and plays indispensable roles in T cells and autoimmune diseases (28, 41, 42). However, its role is still unknown in the regulation of TLR4-NF-κB signaling in macrophages.

In the current study, we found that *RUNX1* mRNA and protein expression levels decreased following LPS stimulation in macrophages. Silencing of *Runx1* and treatment with the RUNX1 inhibitor, Ro 5-3335, attenuated the expression of *Il-6* and *Il-1β*, but not *Tnf-α*, in mouse peritoneal macrophages, RAW 264.7 cells, and human THP-1 cells. In agreement with this, RUNX1 overexpression enhanced the production of TLR4-induced IL-6 and IL-1β. These findings indicate that RUNX1 is a positive regulator of the TLR4 pathway for the induction of the inflammatory response. Our study is consistent with previous findings that RUNX1 could promote STAT3 phosphorylation by inhibiting *SOCS3/4* expression in epithelial cancer cells (29), and STAT3 is a major signal component in IL-6 signaling (43). Although another recent study reported that RUNX1 inhibits NF-κB signaling by blocking the activity of the IκB kinase in leukemia (44), we detected no differences in IκBα degradation and p65 phosphorylation after silencing *Runx1* or inhibition of RUNX1 activity in LPS-treated murine primary macrophages. These contradictory observations might be due to the different cell types that were used and treated with different experimental methods.

In our study, we also determined whether RUNX1 affected expression of the MyD88-independent genes, such as *Ifn-β* and *Ccl5*. No significant difference of their expression was detected

at the mRNA levels in RAW 264.7 cells overexpressed by either *GFP* or *RUNX1* (supplemental Fig. S8, *A* and *B*). In agreement with this, RUNX1 did not affect the IRF3 phosphorylation levels (supplemental Fig. S8, *C* and *D*). These findings suggest that RUNX1 may not affect type I interferon production.

In agreement with the finding that RUNX1 is located predominantly in the nucleus (34), coexpression of *RUNX1* with NF-κB family members *p50*, *p105*, or *p65*, enhanced the NF-κB luciferase activity. However, we observed that RUNX1 specifically interacted with p50, but not p65, and LPS did not affect the interaction between RUNX1 and p50 at early time points. This might explain why RUNX1 did not affect the *Tnf-α* expression levels in macrophages, since p50-deficient macrophages induce normal *Tnf-α* expression in response to LPS stimulation (45). Previous studies have demonstrated that p50 lacks a transcriptional activation domain (10, 46), and p50 needs to interact with other transcription factors or transcription coactivators, including p65/RelA, RelB, C-Rel, BCL3, and IκBζ (10), for the induction of its downstream target genes. Our study provides the first evidence that RUNX1 binds to p50 and synergizes as a transcriptional coactivator for the production of IL-6 and IL-1β in macrophages. As noticed, the C-terminal fragment of RUNX1 was responsible for NF-κB activation. Since the C-terminal fragment of RUNX1 contains the activation domain, it suggests that p50 might coordinate with the RUNX1 activation domain to activate gene expression. Interestingly, RUNX1a, an isoform of RUNX1, contains a highly conserved N-terminal fragment of RUNX1 (*i.e.* 1–242 amino acids) (13). RUNX1a promotes hematopoietic lineage commitment from human pluripotent stem cell (47) and was overexpressed in acute leukemia (48). Because NF-κB plays critical roles in hematopoietic differentiation and leukemia (49, 50), it is interesting to ask whether RUNX1a might bind to p50 to regulate hematopoietic differentiation and acute leukemia. Nevertheless, no ortholog of human RUNX1a exists in mice (51). Additionally, our data indicates that the N-terminal fragment of RUNX1 was not responsible for NF-κB activation in macrophages. This suggests that RUNX1a cannot compromise the role of RUNX1 in macrophages for the interaction with p50.

Although RUNX1 did not affect the recruitment of p50 to the *Il-6* promoter, it is indispensable for the recruitment of RNA polymerase II to the *Il-6* promoter. This indicates that RUNX1

might act as a transcriptional coactivator of p50 for the production of IL-6 in macrophages.

Another implication of our findings is the *in vivo* function of the RUNX1 inhibitor, Ro 5-3335, in preventing LPS-induced septic shock. The RUNX1 inhibitor, Ro 5-3335, substantially improved the survival rates in the LPS-induced sepsis model. Indeed, we observed a significant reduction of *Il-6* levels at the later stage in mouse kidneys after the Ro 5-3335 treatment. This may be due to the facts that IκBζ binds to p50 and that this is dependent on p50 to selectively promote *Il-6* transcription, not *Tnf-α*, in macrophages (35). As septic shock is the leading cause of acute kidney injury and IL-6 is a vital mediator of acute kidney injury (52, 53), we propose that the RUNX1 inhibitor may protect against LPS-induced septic shock by attenuating IL-6 production and blocking acute kidney injury. RUNX1 might be a new potential therapeutic target for resolving TLR4 signaling that is related to acute excessive inflammation or septic shock.

Experimental Procedures

Reagents and Mice—Antibodies against RUNX1, p50/p105, p65, and IκBα were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against p-p65, p-p38, p-ERK, p-JNK, and p-IRF3 were obtained from Cell Signaling Technology (Danvers, MA). Antibodies against GAPDH were obtained from Beyotime Institute of Biotechnology (Shanghai, China). Anti-HA, anti-Myc, anti-β-Actin-HRP, and anti-FLAG antibodies were purchased from Sigma. Anti-CD11b-FITC and anti-F4/80-APC antibodies, as well as ELISA kits for IL-6 were from eBioscience (San Diego, CA). ChIP-grade antibody against p50 was purchased from Abcam (Cambridge, UK). The EZ-ChIP^{TM} Chromatin Immunoprecipitation Kit was obtained from Merck Millipore (Billerica, MA). Murine IL-1β, IL-6, and TNF-α were obtained from PeproTech (Rocky Hill, NJ). PGN, CpG, and poly(I:C) were purchased from InvivoGen (San Diego, CA). The RUNX1 inhibitor, Ro 5-3335, was purchased from Tocris Bioscience (Bristol, UK). The NF-κB inhibitor, BAY 11-7082, was purchased from Beyotime Institute of Biotechnology. Highly purified LPS from *Escherichia coli* O55:B5 were obtained from Sigma. Male C57BL/6 wild type mice (8–10 weeks old) were purchased from the Model Animal Research Center of Nanjing University, Nanjing, China.

Cell Culture—Primary mouse PEMs from C57BL/6 wild type mice were prepared as described previously (6). PEMs, RAW 264.7 cells (kind gifts from Dr. B. Sun, SIBCB, CAS, Shanghai, China), and 293T cells (kind gifts from Dr. J. F. Chen, SIBCB, CAS, Shanghai, China) were cultured with complete DMEM containing 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml). Human THP-1 cells (kind gifts from Dr. B. Ge, Tongji University, Shanghai, China) were maintained in complete 1640 medium supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml). For macrophage induction, THP-1 cells were stimulated with 100 ng/ml of phorbol 12-myristate 13-acetate for 24 h and allowed to rest for 8–12 h. PEMs were obtained from mice that received an intraperitoneal injection of 2.5–3 ml of 3% Brewer thioglycollate medium for 4 days, and the purity of CD11b$^+$F4/80$^+$ macrophages was analyzed by FACS (supplemental Fig. S1).

Plasmids—The MigR1-*RUNX1* plasmid was a gift from Dr. Shi Jingyi (Shanghai Institute of Hematology). *HA*-tagged *RUNX1* and various *RUNX1* truncations (54) (R1~242, R50–178, and R243–453) were inserted into the XhoI-EcoRI site of the mammalian expression vector, pcDNA3.1 or MigR1. Human *p65* (a kind gift from Dr. Z Zhou, SIBCB, China), *p50*, and *p105* were also inserted into the XhoI-EcoRI site of the vector, pcDNA3.1. The primers are listed in the supplemental information (supplemental Table S1).

Retroviral Transduction—To generate retroviral particles, MigR1-*RUNX1*/*RUNX1* truncations (R1~242, R243–453)-*GFP* (6 μg) were co-transfected with pCL-10A1 (6 μg) into 293T cells by the calcium phosphate method. After 24 h, the retroviral supernatants were collected to infect RAW264.7 cells, and the cells were passaged three times, followed by flow cytometry sorting of GFP$^+$ cells.

siRNA Transfection and RT-qPCR—PEMs (5×10^5) were transfected with 20 nM siGENOME SMARTpool mouse *Runx1* siRNA (Dharmacon, Lafayette, CO) or with 20 nM nonspecific siRNA using the Lipofectamine RNAiMAX kit (Invitrogen) according to the manufacturer's instructions. The siRNA sequences are listed in the supplemental information (supplemental Tables S2 and S3). Total RNA was extracted with RNAiso reagent (TaKaRa Ltd, Kyoto, Japan). cDNA was generated from 1 μg of RNA using Moloney MLV transcriptase (Promega) and examined with quantitative RT-qPCR with SYBR Green Master Mix on a CFX-96 machine (Bio-Rad). siRNA sequences or the primers for detecting *Il-1β*, *Il-6*, and *Tnf-α* were used as described previously (6), and are listed in supplemental Table S4. To determine the specificity of siRNA, expression of the unrelated proteins, including *Wdr7*, *Asap1*, *Naip2*, and *Vps13b*, were detected by RT-qPCR (supplemental Fig. S9).

Cell Stimulation and Cytokine ELISA—PEMs, RAW 264.7, and phorbol 12-myristate 13-acetate-differentiated THP-1 cells were seeded in 12-well plates and stimulated with LPS (100 or 1000 ng/ml) for 3, 6, 12, or 24 h in the absence or presence of 50 μM Ro 5-3335 or 10 μM BAY 11–7082, which was added as a pretreatment for 1 h. The supernatants were collected for cytokine analysis using a commercial ELISA kit according to the manufacturer's instructions.

Luciferase Assay—293T (5×10^5) cells were co-transfected with 0.1 μg of expression plasmid (*p50*, *p65*, or *p105*) and 0.25 μg of NF-κB luciferase reporter plasmid containing 1/20 *Renilla* luciferase plasmid with 0.8 μg of pcDNA3.1-*RUNX1* or pcDNA3.1-*GFP* plasmid using the Lipofectamine 2000 reagent. The cells were recovered for 24 h and measured with a dual luciferase reporter assay system (Promega). For the Ro 5-3335 intervention experiment (31), briefly, 293T cells were transiently co-transfected with 0.1 μg of expression plasmids encoding *p50*, *p105*, or *p65* plus 0.8 μg of pcDNA3.1-*RUNX1*. After 6–8 h, the medium was removed and 50 μM Ro 5-3335 or DMSO was added into the new medium. Then, 18 h later, the cell lysates were assayed with an NF-κB luciferase assay.

Coimmunoprecipitation and Western Blot Analysis—293T cells (5×10^6) were co-transfected with 6 μg of pcDNA3.1-*p50*/*p105*/*p65* plasmid and 6 μg of pcDNA3.1-*RUNX1-HA* or *HA*-tagged various *RUNX1* truncations, and the whole cell lysates were collected to perform co-immunoprecipitation

experiments with the indicated antibodies. The endogenous interactions between RUNX1 and p50 were examined by co-immunoprecipitation in RAW 264.7 or THP-1 cells. PEMs, THP-1, or RAW 264.7 cells were seeded in 12-well plates and stimulated with LPS (1000 ng/ml) for 3, 6, 12, or 24 h, and the whole cell lysates were subjected to immunoblotting with anti-RUNX1 antibodies.

Chromatin Immunoprecipitation—RAW 264.7 cells were stimulated with 100 ng/ml of LPS for 5 h in the absence or presence of 50 μM Ro 5-3335. ChIP experiments were performed with the EZ-ChIP™ kit according to the manufacturer's instructions. To measure enrichment, the purified DNA was quantified by qPCR with the primers covering NF-κB binding site of the murine *Il-6* promoter used as described previously (55). The primers are listed in the supplemental information (supplemental Table S5).

LPS Shock Model and Ro 5-3335 Treatment—Male C57BL/6 mice (8–10 weeks old) received an intraperitoneal injection of the RUNX1 inhibitor, Ro 5-3335 (5 mg/kg), with 5% DMSO in PBS or 5% DMSO in PBS for 3 h, following by an intraperitoneal injection with a lethal (20 mg/kg) or sublethal dose (10 mg/kg) of LPS to induce LPS shock as described previously (56). All the mice were observed and the survival rates were calculated. Serum, liver, lung, kidney, and spleen samples were collected at the indicated time points for cytokine measurements.

Statistical Analysis—Statistical analysis was performed with GraphPad Prism 5, and statistically significant differences were determined by a two-tailed Student's *t* test with 95% confidence intervals.

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