Kinase AKT1 Negatively Controls Neutrophil Recruitment and Function in Mice

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Kinase AKT1 Negatively Controls Neutrophil Recruitment and Function in Mice

Guangwei Liu,* Yujing Bi,† Ruoning Wang,‡ Bo Shen,§ Yan Zhang,* Hui Yang,* Xiao Wang,* Huanrong Liu,* Yun Lu,* and Fei Han*

Neutrophils are critically involved in host defense and inflammatory injury. However, intrinsic signaling mechanisms controlling neutrophil recruitment and activities are poorly defined. In this article, we showed that protein kinase AKT1 (also known as PKBα) is the dominant isoform expressed in neutrophils and is downregulated upon bacterial infection and neutrophil activation. AKT1 deficiency resulted in severe disease progression accompanied by recruitment of neutrophils and enhanced bactericidal activity in the acute inflammatory lung injury (ALI) and the Staphylococcus aureus infection mouse models. Moreover, the depletion of neutrophils efficiently reversed the aggravated inflammatory response, but adoptive transfer of AKT1−/− neutrophils could potentiate the inflammatory immunity, indicating an intrinsic effect of the neutrophil in modulating inflammation in AKT1−/− mice. In the ALI model, the infiltration of neutrophils into the inflammatory site was associated with enhanced migration capacity, whereas inflammatory stimuli could promote neutrophil apoptosis. In accordance with these findings, neutralization of CXCR2 attenuated neutrophil infiltration and delayed the occurrence of inflammation. Finally, the enhanced bactericidal activity and inflammatory immunity of AKT-deficient neutrophils were mediated by a STAT1-dependent, but not a mammalian target of rapamycin–dependent, pathway. Thus, our findings indicated that the AKT1–STAT1 signaling axis negatively regulates neutrophil recruitment and activation in ALI and S. aureus infection in mice. The Journal of Immunology, 2013, 191: 2680–2690.

Abbreviations used in this article: ALI, acute inflammatory lung injury; BALF, bronchoalveolar lavage fluid; BM, bone marrow; DKO, double KO; FCM, flow cytometry; KO, knockout; MMP-9, matrix metalloproteinase 9; MPO, myeloperoxidase; mTOR, mammalian target of rapamycin; ROS, reactive oxygen species; shRNA, short hairpin RNA; WT, wild-type.

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gen-free conditions. Sex-matched littermate mice 6–8 wk of age were mainly used for experiments. Complete chimeras were generated by transferring 1–2×10^8 bone marrow (BM) cells from either wild-type (WT) or AKT1KO mice into lethally irradiated mice (13, 23). Experimental protocols were approved by the Animal Ethics Committee of Fudan University, Shanghai, China.

mAb and flow cytometry
For flow cytometry (FCM) analysis of surface markers, anti-CD45R/B220 (RA3-6B2), anti-CD11b (M1/70), anti-F4/80 (BMS8), anti-Gr-1 (RB6-8C5), anti-Ly6G (1A8), anti-CD4 (GK1.5), anti-CD45.1 (A20), and anti-CD45.2 (104) were obtained from eBioscience. The anti-CD11b (M1/70), anti-CD45 (TU116), anti-CD11c (HL3), and anti-CD11F (AF598) were obtained from BD Biosciences; anti-CD5 (30-F11), anti-Bcl-2 (Bcl/10L4), and anti-Bax (6A7) were obtained from BioLegend; anti-CXCR2 (242216) was obtained from R&D Systems; anti-CD3 (145-2C11) and anti-CD19 (1D3) were obtained from Miltenyi Biotec; anti–matrix metalloproteinase 9 (MMP-9) (ab119906) was obtained from Abcam; anti-MPO (2D4) was obtained from Hyctul Biotech; anti–Bcl-1x (2764) and anti– caspase 3 (9661) were obtained from Cell Signaling Technology.

For detection of phosphorylated signaling proteins, purified cells were activated with LPS (Sigma-Aldrich), were immediately fixed with Phosflow Perm Buffer (BD Biosciences), and were stained with PE or allophycocyanin directly conjugated to Ab to Erk phosphorylated at Thr202 and Tyr204 (D6C7) and anti–phospho-AKT1 (Ser473) (D9E), anti–phospho-AKT1 (Thr308) (C31E5E), or S6 phosphorylated at Ser235 and Ser236 (D57.2.2E). FCM analysis was performed as described previously (24).

Histopathological grading of LPS-induced lung injury
Experimental acute inflammatory lung injury (ALI) was induced by i.v. injection of LPS (5 μg/g), as described previously (25, 26). The histopathology was assessed in a blinded manner with respect to which group or mouse was reviewed, using a modified histological scoring system, as previously described (25, 27). Four easily identifiable pathological processes were scored on a scale of 0–4: 1) alveolar congestion, 2) hemorrhage, 3) leukocyte infiltration or aggregation of neutrophils in air space or the vessel wall, and 4) thickness of the alveolar wall. A score of 0 represented normal lungs; 1, mild, <25% lung involvement; 2, moderate, 25–50% lung involvement; 3, severe, 50–75% lung involvement; and 4, very severe, >75% lung involvement. An overall score of LPS-induced lung injury was obtained based on the summation of all the scores, and data were shown as the mean ± SD. For immunohistochemistry, slides were stained for neutrophils via the immunoperoxidase method with rat anti-mouse Gr-1 mAb (BD Biosciences).

Isolation of neutrophils from BM cells
Neutrophils from BM cells were isolated and were fractionated on a Percoll gradient (28, 29). The enriched neutrophil fraction was recovered at the interface of 65% and 72% Percoll. In some cases, neutrophils were sorted using anti-Ly6G/CD11b mAbs.

Real-time PCR
RNA was extracted with an RNase Kit (QIAGEN), and cDNA was synthesized with SuperScript III Reverse Transcriptase (Invitrogen). A LightCycler 480 (Roche, Basel, Switzerland) real-time PCR system was used for quantitative PCR, with primer and probe sets from Applied Biosystems (Carlsbad, CA); results were analyzed with SDS 2.1 software. The cycling threshold value of the endogenous control gene (Hprt, encoding hypoxanthine guanine phosphoribosyl transferase) was subtracted from the cycling threshold value of each target gene to generate the change in cycling threshold (ΔCT). The expression of each target gene is presented as the “fold change” relative to the expression in WT control samples (2^ΔΔCT), as described previously (30). The primers used in the current study are summarized in Table I.

Immunoblot analysis
Immunoblot analysis was done as described (13) with the following Abs: Ab to AKT1 (ab7929, Abcam), AKT2 (ab13991, Abcam), CXXC1(API151; Millipore), CXXC1L2 (SAB2501255; Sigma-Aldrich), p-STAT1 (9167; Cell Signaling Technology), STAT1 (9172; Cell Signaling Technology), and anti–β-actin (AC-18; Sigma-Aldrich).

Oxidative burst assay
Respiratory burst was determined as described (31). Neutrophils isolated from BM cells were incubated in the presence of 1 μM dihydroxydihemoglobin (Sigma-D1054; Molecular Probes) during stimulation with PMA (Sigma-F8139). Samples were incubated at 37°C for 15 min before immediate FCM analysis. Neutrophils were defined by staining CD11b and Ly6G Ab.

Analysis of neutrophil degranulation
Analysis was performed as described previously, with minor modifications (32). Neutrophils at a concentration of 2–3×10^6 cells per milliliter were plated on wells in the presence or absence of 100 ng/ml TNF-α. After 1 h incubation at 37°C, cells were spun, and the supernatants were removed for measurement of release of MPO and reagent peroxidase (MPO). MPO release was measured with an MPO activity assay kit (E33856; Invitrogen). MMP-9 release was analyzed by zymogram gel electrophoresis (Invitrogen). Gels were stained with Coomassie blue dye and quantified using an Alphaimager.

Bacterial culture
Samples of lung tissue and bronchoalveolar lavage fluid (BALF) were collected. Different dilutions of the obtained suspensions were plated on heated agar plates and incubated at 37°C for 48 h, and then bacterial CFU were counted (23).

In vitro bactericidal activity by neutrophils
BM neutrophils were sorted as described previously (13). S. aureus organisms were grown overnight at 37°C, washed in PBS, and counted. Suspension of S. aureus containing a bacterial concentration corresponding to 10^6 CFU was incubated with or without 5×10^4 neutrophils in flat-bottom 96-well plates (Costar) in a total of 200 μl RPMI 1640 medium at 37°C in 5% CO2 for 3 h. For phagocytosis experiments, neutrophils were collected and blocked with anti-mouse FcγR mAb (2.4G2) and stained with anti-Ly6G–FITC. After washing with cold PBS three times, the phagocytosis percentages of gated Ly6G+ cells were determined by FCM. For S. aureus survival experiments, some wells were treated with 0.01% Triton X-100 in water for 5 min to lyse neutrophils, then washed with 100 μl PBS. Surviving S. aureus CFUs were determined as described previously (24, 33).

In vivo bactericidal activity by neutrophils
WT or AKT1KO mice were injected with 5 ml air in the central back region on day 0. On day 3, the air pouches were reinflated with 3 ml sterile air. On day 6, S. aureus were grown to 5×10^6 CFU and then washed and diluted in sterile PBS to 2×10^6 CFU. Air pouches were injected with 0.5 ml bactericidal culture. At the indicated time points, the infected mice were sacrificed and the air pouches were lavaged with HBSS. Cells were counted and stained with anti-mouse CD11b–PE and anti-mouse Ly6G–FITC, and propidium iodide for analysis on a FACScan. The number of CD11b+Ly6G+ cells on live (propidium iodide–negative) neutrophils was counted. The live bacteria from the air pouch were plated to quantify the number of live CFUs, normalized to the initial number of bacteria put into the air pouch. In addition, supernatants from the air pouch lavage were used to measure MPO and MMP-9 release. Some neutrophils were collected for real-time PCR analysis of MPO and MMP-9.

Depletion of neutrophils
Neutrophil depletion was achieved using anti-mouse Gr-1 mAb (clone RB6-8C5; BioXCell), as described previously (34). Briefly, mice received an i.v. injection of anti-mouse Gr-1 mAb 24 h prior to in vivo experiments. Control mice received equivalent amounts of isotype control Ab in sterile PBS. Depletion of neutrophils was confirmed by staining for circulating neutrophils with anti-Ly6G mAb.

Adaptive transfer of BM neutrophils
Neutrophil adoptive transfer was performed as described previously (35). Isolated BM neutrophils were transplanted into mice that were challenged
Materials and Methods

and shown as mean described in trophils were stimulated by LPS (+) or PBS (–). Right trophils from BALF were counted and shown as mean 4–7, from one of four (A, C–E) or two (B and F–H) independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, compared with the indicated groups.

**FIGURE 1.** The downregulated expression of AKT1 in neutrophils during activation and AKT1 deficiency promotes inflammatory lung injury and bactericidal capacity. (A) Quantitative PCR analysis of AKT isoform expression isolated from BM sorted CD11b+Ly6G+ neutrophils stimulated by LPS, TNF-α, and IL-18. Right. The dose effect of LPS on AKT1 mRNA expression in the CD11b+Ly6G+ neutrophils was assayed by quantitative PCR analysis. (B) WT mouse neutrophils were stimulated by LPS (+) or PBS (–) for 2 h, and the AKT1 and AKT2 proteins were analyzed by immunoblot. (C–E) ALI of AKT1KO mice induced by LPS. (D) H&E staining of lung paraffin sections was done, and representative pathological images are shown. Right. Histological score was evaluated as described in Materials and Methods. Three random sections per lung and five lungs per group were used. (D) Infiltrating neutrophils from BALF were counted and shown as mean ± SD. MPO activity of lung tissue was shown in (E). (F–H) The mouse S. aureus lung infection in vivo model was used as described in Materials and Methods. A total of 2 × 10^8 S. aureus organisms were delivered to mice via the intranasal route. (F) H&E staining of lung paraffin sections was done, and representative pathological images are shown. Right. Histological score was evaluated as described in Materials and Methods. (G) Infiltrating neutrophils from BALF were counted and shown as mean ± SD. (H) The number of live bacteria (CFU/ml) in the lung is shown. Data are shown as mean ± SD, n = 4–7, from one of four (A, C–E) or two (B and F–H) independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, compared with the indicated groups.

**Table I.** Primer sequences used for real-time PCR assays

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<tr>
<th>Gene Names</th>
<th>Primer Sequences (5′→3′)</th>
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<td>AKT1</td>
<td>Forward: CTTCTTGAGAGCTAAGCAGG&lt;br&gt;Reverse: CGAGGACATCTTCTCAGAG</td>
</tr>
<tr>
<td>ACT2</td>
<td>Forward: CTTACGTCAGCTGCTACAGAG&lt;br&gt;Reverse: AGCTCAAGACGAGGAGGAA</td>
</tr>
<tr>
<td>ACT3</td>
<td>Forward: CTCCACTGGAACAACTAGG&lt;br&gt;Reverse: AGTCAGTGATCTTGATGTGGC</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Forward: TGGGAAACACAGCTGTCAGG&lt;br&gt;Reverse: CCACTAGGAGCAGGAGGAA</td>
</tr>
<tr>
<td>IL-6</td>
<td>Forward: GCAATGGCCATTCTGATGATG&lt;br&gt;Reverse: AAGGACTCCTGGCTTTCTCT</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward: CTTAAGCTCGCCCCCAACACC&lt;br&gt;Reverse: GCACTCGCTCCGAGATGCT</td>
</tr>
<tr>
<td>TGF-βI</td>
<td>Forward: GCTTACATTGGGACCCAAACAC&lt;br&gt;Reverse: AGTACAGCCCCAAAATGGTTAAG</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Forward: ATAGAGGAGGCCCCATACAGG&lt;br&gt;Reverse: GTGTACACCCACATTTGACG</td>
</tr>
<tr>
<td>MPO</td>
<td>Forward: TCCCACTCGCAAGGCTTC&lt;br&gt;Reverse: AGATGCCCCAAGTCCTAGG</td>
</tr>
<tr>
<td>HPRT</td>
<td>Forward: CCTAGGCTTTGTTATGGGTCG&lt;br&gt;Reverse: CGAAATACCTGGTCAGTCTC</td>
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**Induction of sterile peritonitis**

Mice were injected i.p. with 3 ml thioglycollate broth (Sigma-70157; Sigma-Aldrich) or PBS as a control. After 4 h, peritoneal exudate cells were harvested by peritoneal lavage with 10 ml cold PBS, as described previously (36).

**STAT1 knockdown with RNA interference**

As described previously (13), a gene-knockdown lentiviral construct was generated by subcloning gene-specific short hairpin RNA (shRNA) sequences into lentiviral shRNA expression plasmids (pLpaL3.7). Lentiviruses were harvested from culture supernatant of 293T cells transfected with shRNA vector. Sorted BM-derived neutrophils were infected with recombinant lentivirus, and GFP-expressing cells were isolated using fluorescence sorting 48 h later. The STAT1 expression was confirmed using immunobots. The sorted neutrophils with either control or shRNA vectors were used for functional assay.

**Statistical analysis**

All data are presented as the mean ± SD. The Student unpaired t test for comparison of means was used to compare groups. A p value < 0.05 was considered statistically significant.

**Results**

**AKT1 expression in neutrophils is downregulated during inflammatory activation**

The mRNA and protein expression of different AKT isoforms in neutrophils was determined by quantitative PCR (primers summarized in Table I) and immunoblot following LPS stimulation. We found that AKT1, but not AKT2 and AKT3, is promptly down-

by S. aureus, as described above. The success of transplantation was confirmed by FCM analysis of peripheral blood leukocytes or inflammatory infiltrating cells in the local site, with Abs against CD45.1 or CD45.2, depending on the origin of transplanted cells. The migration of transplanted neutrophils into the back air pouch of recipient mice was determined by FCM analysis with anti-Ly6G mAb.

**Transwell assays**

Chemotaxis was evaluated according to the procedure described (15), using a 24-well microchemotaxis plate (Corning, Corning, NY) in which the chambers were separated by polycarbonate membrane. DMEM containing 0.01% BSA or CXCL1 (30 nM) was placed in the lower chamber. Purified

AKT1KO and WT BM neutrophil suspensions (5 × 10^5 cells in 500 μl) were placed in the upper chamber. Chambers were incubated at 37˚C with 5% CO2 for 2 h. The results are expressed as the mean number of neutrophils per well and are representative of triplicate measurements. In some assays, cells were pretreated with inhibitors for 30 min, as indicated.
regulated in a dose- and time-dependent manner following LPS stimulation (Fig. 1A, 1B and data not shown). This result indicated that AKT1 might be an important intrinsic modulator for neutrophil activation in inflammation. In addition, AKT1 mRNA expression was also downregulated by inflammatory cytokines, such as TNF-α and IL-8 (Fig. 1A). However, anti-inflammatory cytokines IL-10 and TGF-β did not alter AKT1 expression (data not shown). These results reveal that the downregulation of AKT1 expression in neutrophils might be specific to inflammation.

**AKT1 deficiency enhances the neutrophil inflammatory response in acute inflammation**

Next, we used AKT1KO mice to determine the role of AKT1 in neutrophils. In AKT1KO mice, AKT1 expression is eliminated via gene targeting (Supplemental Fig. 1A), as previously reported (21). Real-time PCR analysis demonstrated efficient deletion of AKT1, but not AKT2 and AKT3, in BM neutrophils (Supplemental Fig. 1A). Two in vivo mouse models, the ALI and *S. aureus* infection models, were applied to test the role of AKT1 in acute infectious inflammation. In the ALI model, histopathological assessment of leukocyte infiltration and MPO activity, a specific enzyme that shows the activity of neutrophils, was used to determine the severity of lung injury. Histological assessment of the inflamed lung showed increased numbers of infiltrating leukocytes (Fig. 1C), with a higher pathological score in AKT1KO mice than in WT mice. Consistently, AKT1KO mice showed an increased neutrophil infiltration in BALF and lung (Fig. 1D, and Supplemental Fig. 1B, 1C) and significantly higher MPO activity in lung (Fig. 1E), both indicating the presence of more infiltrating neutrophils in the inflamed site. Thus, AKT1KO mice showed more severe immune-mediated ALI than did WT mice following LPS challenge. In the *S. aureus* infection model, consistent with the idea that AKT1 deficiency enhances neutrophil-mediated inflammatory response, histological assessment of the inflamed lung showed increased numbers of infiltrating leukocytes with higher pathological score (Fig. 1F), increased numbers of infiltrating neutrophils in BALF (Fig. 1G), and reduced bacterial survival (Fig. 1H) in the lung of AKT1KO mice compared with WT mice. Collectively, AKT1 deficiency promotes the development of acute inflammation.

**AKT1 regulates the inflammatory responses via myeloid-derived cells**

Next, we created AKT1KO→WT, WT→WT, WT→AKT1KO, and AKT1KO→AKT1KO BM chimeras to determine whether AKT1 regulates the inflammatory response via myeloid-derived cells. In the ALI model, more severe pathological lesions, more inflammatory cell infiltration, and higher MPO activity (Fig. 2A) were found in the AKT1KO→WT group or AKT1KO→AKT1KO group than in the WT→AKT1KO and WT→WT groups. Moreover, significantly increased lung weight with a higher ratio of wet/dry lung (Fig. 2B) was observed after challenge by LPS, as well as more infiltrating leukocytes (Fig. 2C) in the AKT1KO→WT group compared with WT→WT groups, indicating that the myeloid-derived cell AKT1 is involved in the inflammatory lung injury. Among the infiltrating inflammatory cells, more neutrophils, but not macrophages and T or B cells (Fig. 2C), were found in the BALF of the AKT1KO→WT group. Next, we assessed the potential role of AKT1 in regulating neutrophil function in the chimeras. The mouse *S. aureus* lung infection in vivo model was used, and the number of live bacteria (CFU/ml) in the lung is shown. Data are shown as mean ± SD, n = 4–6 (A–C) or n = 3 (D, E), from one of three (A–C) or two (D, E) independent experiments. ***p < 0.001, compared with the indicated groups.
We analyzed the production of reactive oxygen species (ROS) and cytokines and the phagocytic activity of neutrophils in chimeras. Following stimulation, neutrophils produced significantly enhanced ROS (Supplemental Fig. 2A) and proinflammatory cytokine TNF-α and IL-1β (Supplemental Fig. 2B). Consistent with these observations, the enhanced phagocytic activity of neutrophils from AKT1KO→WT chimeras was confirmed in vitro (Supplemental Fig. 2C). In addition, the viability of S. aureus cocultured with AKT1KO neutrophils is lower than that of WT→WT chimeras (Supplemental Fig. 2C). Cytokine TNF-α production in macrophages and IFN-γ and IL-4 production in T cells in chimeras were also analyzed with intracellular staining by FCM, but no significant changes were observed (data not shown). These findings collectively reveal that AKT1 signaling is critically involved in regulating neutrophil functional activities. To further exclude the involveoment of T cell– and B cell–mediated adaptive immunity, we generated AKT1, Rag1 double KO (DKO) mice and transferred either DKO BMs or Rag1 KO BMs into a Rag1KO host. In keeping with our earlier results, the transfer of DKO BMs into a Rag1 host resulted in more severe pathological injury, more inflammatory cell infiltration, and higher lung MPO activity (Fig. 2D), compared with control. In addition, we used a similar chimeric experiment with the S. aureus infection model. Clearly, AKT1Rag1KO→Rag1KO chimeras, compared with Rag1KO→Rag1KO chimeras, showed lower survival of S. aureus (Fig. 2E). Collectively, our results suggest that AKT1 regulates the inflammatory responses via myeloid-derived cells.

**AKT1-deficient neutrophils are responsible for the modulation of inflammatory immunity**

To characterize the leukocyte subset involved in mediating acute inflammation, we compared infiltrating leukocytes in BALF, lung, blood, BM, and lung local draining lymph nodes in the ALI model and the S. aureus infection model. Before inflammation induction, no significant differences were observed between the experimental groups (Fig. 3A, 3B; Supplemental Fig. 1B, 1C; and data not shown). However, following ALI and S. aureus infection, macrophages (CD11b+F4/80+) and neutrophils (CD11b+Ly6G+) represented the predominant infiltrating cell populations in BALF and lung (Fig. 3A, Supplemental Fig. 1B, 1C). We further found that the inflammatory lungs isolated from AKT1KO mice contained significantly more neutrophils in the ALI (Fig. 3A, Supplemental Fig. 1B, 1C) and S. aureus infection (Fig. 3B) models. In addition, no significant difference in cell death and cell proliferation was noted in freshly isolated WBCs or BALF cells from WT and AKT1KO mice (Supplemental Fig. 3). However, LPS can significantly promote more apoptotic cell death of AKT1KO than of WT neutrophils in vitro (Supplemental Fig. 3A). In addition, LPS can alter the neutrophil apoptotic-related Bax and caspase 3, but not Bcl-xl and Bcl-2 expression (Supplemental Fig. 3B), as

**FIGURE 3.** AKT1-deficient neutrophils responsible for orchestrating inflammatory lung injury and enhanced bactericidal activity. (A) The BALF CD11b+Ly6G+ neutrophils in mice challenged by LPS were determined by FCM; a representative figure is shown. Right, Absolute CD11b+Ly6G+ neutrophil numbers in lung and peripheral blood were summarized. Data are shown as mean ± SD, n = 5, from one of three independent experiments.*p < 0.05, ***p < 0.001, compared with the control group or the indicated groups. (B) The BALF CD11b+Ly6G+ neutrophils in the mouse lung infection model challenged by S. aureus were determined by FCM; a representative figure is shown. Right, Absolute CD11b+Ly6G+ neutrophil numbers in the lung were summarized. Data are shown as mean ± SD, n = 5, from one of two independent experiments. ***p < 0.001, compared with the indicated groups. (C, D) Depletion of neutrophils from AKT1KO mice pretreated with anti-Gr1 mAb (RB6-8C5) significantly alleviated the lung tissue injury; the typical histological image is shown (upper), and histological scores (lower) are summarized in (C). Depletion of neutrophils was confirmed with FCM in (D). (E) Host defense response to bacterial infection in vivo. Depletion of neutrophils from AKT1KO mice pretreated with anti-Gr1 mAb significantly alleviated the bacterial infection. WT or AKT1KO mice with dorsally located air pouches were infected with S. aureus. The number of live bacteria (CFU/ml) in the air pouch by 6 h post infection is shown. Data are shown as mean ± SD, n = 4, from one of three independent experiments. ***p < 0.001, compared with the indicated groups. (F) Adoptive transfer of CD11b+Ly6G+ cells (CD45.2+; donor cells) from AKT1KO mice significantly decrease the survival percentage of bacteria, compared with indicated groups. The donor cells (CD45.2+) were confirmed with FCM (upper), and bacterial survival (lower) was summarized. Data are shown as mean ± SD, n = 3–5, from one of two independent experiments. ***p < 0.001, compared with the indicated group.
Next, we tested the role of neutrophils in the inflammatory responses by depleting neutrophils via anti-Gr1 mAb RB6-8C5, as described in Materials and Methods (28). Efficient depletion of CD11b+Gr1+ cells was confirmed by FCM or leukocyte count (Fig. 3D and data not shown). In the ALI model, depleting Gr1+ cells in AKT1KO recipients clearly reversed the inflammatory injury and histological score (Fig. 3C). Similarly, in the S. aureus infection model, depleting Gr1+ cells in AKT1KO recipients clearly reversed the survival of S. aureus (Fig. 3E). Importantly, adoptive transfer of the sorted CD11b+Ly6G+ cells (donor cells) from AKT1KO mice significantly decreases the survival of S. aureus, compared with WT, although it also can decrease the survival of S. aureus (Fig. 3F). Thus, these data suggested that AKT1 downregulation in neutrophils is required for protection against acute inflammation.

**AKT1 deficiency promotes neutrophil recruitment through targeting the CXCL1/2–CXCR2 axis**

Next, we asked how AKT1 controls neutrophil recruitment. To address this question, the inflammatory lung tissue and serum chemokine expression was determined with immunoblot and ELISA. Following LPS injection, the expression of CXCL1 and CXCL2, chemokines mediating neutrophil migration, is increased in AKT1KO mice compared with WT mice (Fig. 4A). In addition, the cell surface expression level of CXCR2 in AKT1KO neutrophils is higher in BALF in the ALI model (Fig. 4B). Then, we applied a 3% thioglycollate-induced peritonitis, a well-established in vivo model for studying neutrophil migration (36, 38), to determine the altered migration of AKT1KO neutrophils. A significantly higher number of recruited neutrophils, with a higher CXCR2 expression, were observed in the peritoneal exudate cells of AKT1KO mice compared with WT mice (Fig. 4C). Consistent with these results, AKT1KO neutrophils showed enhanced migration capacity compared with WT neutrophils in in vitro Transwell cell migration assay (data not shown). In addition, the blockage of CXCR2 expression in neutrophils almost completely reverses the enhanced recruitment of AKT1KO neutrophils in the in vitro Transwell migration assay (data not shown) and the in vivo peritonitis model (Fig. 4D) as well as the ALI model (Fig. 4E–G). Treatment with anti-CXCR2 mAb significantly reversed the orchestrated inflammatory responses in AKT1KO, but not in WT, mice in the ALI model (Fig. 4E–G). Thus, our results suggested that AKT1 deficiency promotes neutrophil migration in inflammation by targeting the CXCL1/2–CXCR2 axis.

**FIGURE 4.** AKT1 deficiency increases expression of chemokines that mediate neutrophil recruitment in inflammation. (A) Expression of chemokines CXCL1 and CXCL2 in neutrophils isolated from BALF was determined by immunoblot following ALI Right. Serum CXCL1 and CXCL2 expression was analyzed with ELISA. Data are shown as mean ± SD, n = 4, from one of two independent experiments. *p < 0.05, **p < 0.01, compared with the indicated groups. (B) CXCR2 expression of neutrophils in BALF in WT or AKT1KO mice following ALI was analyzed with FCM, and mean fluorescence intensity (MFI) of CXCR2 was summarized. Data are shown as mean ± SD, n = 4, from one of three independent experiments. ***p < 0.001, compared with indicated groups. (C and D) Sorted WT and AKT1KO CD11b+Ly6G+ neutrophils were labeled with CFSE and PKH, respectively, and were then i.v. injected into syngeneic host WT mice for in vivo migration assays. (C) More AKT1KO neutrophils (PEN) were detected in the 3% thioglycollate (TG)-injected peritoneal cavity than WT cells in the same host. Representative results are shown. Middle, Recruited neutrophil absolute cell numbers were summarized. Right, Expression of CXCR2 in recruited neutrophils was determined by FCM, and MFI was summarized. Data are shown as mean ± SD, n = 4, from one of two independent experiments. ***p < 0.001, compared with indicated groups. (D) CD11b+Ly6G+ neutrophils were isolated from WT and AKT1KO, pretreated with anti-CXCR2 mAb or IgG isotype control 1 h prior to labeling with CFSE and PKH, respectively, and were then i.v. injected into syngeneic host WT mice for in vivo migration assays. Recruited WT or AKT1KO neutrophil absolute cell number was calculated. Data are shown as mean ± SD, n = 3, from one of two independent experiments. ***p < 0.001, compared with the indicated groups. (E–G) WT and AKT1KO mice received anti-CXCR2 mAb or IgG isotype treatment 1 h prior to ALI induction. The inflammatory lung tissue injury and histological score (E), as well as the infiltrating neutrophil number in BALF (F) and lung MPO activity (G), are shown. Data are shown as mean ± SD, n = 5. *p < 0.05 ***p < 0.001, compared with indicated groups.
AKT1 deficiency potentiates the neutrophil bactericidal activity

To further determine the potential role of AKT1 in regulating neutrophil function, we analyzed the production of ROS, granule proteins, and cytokines. Following stimulation, AKT1KO neutrophils produced significantly more ROS (Fig. 5A), granule proteins MMP-9 and MPO (Fig. 5B), and proinflammatory cytokines TNF-α, IL-6, and IL-1β, but not TGF-β (Fig. 5C). In agreement with the above results, the increased phagocytic activity of AKT1KO neutrophils was confirmed in vitro (Fig. 5D). In addition, the survival of S. aureus cocultured with AKT1KO neutrophils is lower than that of WT (Fig. 5D). In a nonlethal skin abscess model of S. aureus infection, in which S. aureus clearance is mediated predominantly by neutrophils within the first 48 h of infection, AKT1KO→WT complete chimera neutrophils were recruited to the infected air pouch more efficiently than were WT→WT chimera control (Fig. 5E). AKT1KO→WT complete chimera mice showed significantly more clearance of the bacteria by 12 h, whereas the S. aureus titer remained high in WT→WT chimera (Fig. 5E). Moreover, the mRNA expression of granule proteins, including MMP-9 and MPO, in neutrophils was significantly higher in AKT1KO→WT complete chimera mice than that in WT→WT chimera (data not shown). This finding nicely correlates with the enhanced degranulation response of AKT1KO neutrophils (Fig. 5B). Taken together, these results indicate that AKT1 acts as an intrinsically important negative modulator of neutrophil bactericidal ability in inflammation.

AKT1 regulates neutrophil recruitment and function independent of mTOR

To investigate the mechanisms that mediate AKT1 function, we assessed the phosphorylation of signaling molecules AKT, ERK, JNK, p38, mTOR, and transcriptional factor STAT1 and STAT3 by FCM in BM-derived neutrophils following LPS stimulation. LPS stimulation resulted in lower phosphorylation levels of AKT at Ser473 and Thr308 residues in AKT1KO neutrophils. In contrast, LPS stimulation resulted in higher phosphorylation in STAT1 and S6 ribosomal protein, a well-established target of the AKT–mTOR pathway, but not in ERK, JNK, p38, and STAT3 in AKT1 KO cells compared with WT cells (Fig. 6A). Although mTOR has been shown to promote the neutrophil function (14, 39), the inflammatory cytokine production, neutrophil migration, and bactericidal activity were not affected by the mTOR inhibitor rapamycin (Fig. 6B–E). Rapamycin efficiently blocked the phosphorylation of S6 (Fig. 6F). Together, our results indicated that AKT1 controls neutrophil recruitment and function independent of the mTOR pathway.

AKT1 regulates neutrophil recruitment and function through inhibiting the STAT1 pathway

Next, we examined the role of STAT1 in neutrophils. The STAT1 inhibitor MTA significantly reverses the phenotypes of AKT1KO neutrophils, as suggested by the increased bacterial survival in neutrophils (Fig. 7A), the reduced number of transmitted neutrophils...
in the in vitro Transwell assay for neutrophil migration (Fig. 7B), the decreased cytokine IL-1β expression (Fig. 7C), as well as the lowered production of MMP-9 (Fig. 7D, 7E) in AKT1KO neutrophils. In contrast, the treatment with STAT3 inhibitor SI-201 results in no effects on the function of AKT1KO cells. Importantly, the above results with STAT1 inhibitors were confirmed using STAT1 shRNA (Fig. 7F–I). Thus, our results suggested that AKT1 regulates neutrophil recruitment and function through inhibiting the STAT1 pathway.

Discussion

Our data support a model in which kinase AKT1 is required for modulating neutrophil recruitment and function in acute inflammation in mice (Supplemental Fig. 4). We found that the AKT1 expression in neutrophils was downregulated during infection and was negatively correlated with the inflammatory activity of neutrophils in acute inflammation. Using the AKT1 KO model, we showed that AKT1 negatively regulates neutrophil migration and bactericidal activity with a neutrophil-intrinsic manner. Therefore, this study identified one previously unknown negative regulatory pathway for neutrophil defense immunity (Supplemental Fig. 4).

Neutrophils are central mediators of inflammatory responses, contributing to the initiation and resolution of inflammation. Using two in vivo inflammation models, we showed that the abrogation of AKT1 markedly promotes the acute inflammatory injuries and potentiates the neutrophil bactericidal capacities, indicating a critical role for AKT1 in regulating neutrophil-mediated acute inflammation. Emerging evidence suggests a role for PI3K/AKT signaling in the regulation of acute and chronic inflammatory processes (14, 40–44). These findings led to the prospect of considering PI3K or AKT isoforms as promising drug targets for the modulation of inflammatory and autoimmune disorders, cancer, and transplantation (14). However, AKT1 deficiency resulted in enhanced atherosclerosis in the APOE−/− mouse model (16). Furthermore, AKT1−/− mice were more sensitive to LPS-induced endotoxin shock and to dextran sulfate sodium–induced colitis and did not develop endotoxin tolerance, whereas AKT2−/− mice were more resistant to the above pathological insults (17). Although AKT2−/− neutrophils exhibited decreased cell migration, granule enzyme release, and O2− production (18), the role of AKT1 in neutrophils remains unknown. The AKT1−/− complete chimera model was used in this investigation to differentiate the myeloid from nonmyeloid and radioresistant-derived cell effects (45). Combining the complete chimeras and a variety of in vivo inflammatory models, we showed that AKT1 negatively controls neutrophil recruitment and activation in acute inflammation.

Neutrophil chemoattractant factors, including CXC chemokines and inflammatory mediators, released by resident cells (46, 47), recruit neutrophils to challenge sites during the initiation of infection and inflammatory response. In our acute lung inflammation model, the lung tissues expressed higher levels of CXCL1 and CXCL2 in either AKT1KO mice or WT mice reconstituted with AKT1KO-derived cells, compared with control mice. Compared with WT controls, significantly higher levels of CXCR2 (the receptor of CXCL1 and CXCL2) expression in AKT1KO neutrophils were observed upon LPS stimulation. Importantly, the blockage of

FIGURE 6. AKT1 deficiency promotes neutrophil recruitment and activity independent of mTOR. (A) BM-derived neutrophils of WT and AKT1KO mice were treated with LPS or medium for the indicated time. Cells were analyzed for the phosphorylation of p-AKT, p-S6, p-p38MAPK, p-ERK, p-JNK, p-STAT1, and p-STAT3. Representative results are shown from one of three independent experiments. (B) Expression of inflammatory cytokines, including IL-6 and IL-1β, in the BALF neutrophils of WT or AKT1KO mouse ALL, with or without rapamycin treatment (7.5 mg/kg), was determined by real-time PCR. Data represent the mean of at least two independent experiments with n = 4. (C) BM-derived neutrophils isolated from WT and AKT1KO mice and pretreated with rapamycin (100 μM) for 4 h prior to in vitro neutrophil Transwell migration analysis was performed, as described in Materials and Methods. Data are shown from one of two independent experiments. (D) Neutrophils isolated from WT or AKT1KO mice and pretreated with or without rapamycin (100 μM) for 4 h prior to in vitro culture with S. aureus. The percentage of phagocytic neutrophils was determined by FCM in (D). S. aureus survival was assayed after coculture of S. aureus with WT or AKT1KO neutrophils in (E). Experiments were performed in triplicate. Representative results are shown from one of two independent experiments. (F) BM-derived neutrophils isolated from WT and AKT1KO mice and pretreated with or without rapamycin for 4 h prior to LPS stimulation. Cells were analyzed for the phosphorylation of p-S6, p-STAT1, and p-STAT3. Representative results are shown from one of three independent experiments.
CXCR2 with anti-CXCR2 mAb significantly reverses the pathological injury and neutrophil recruitment in AKT1KO mice compared with WT mice. It is thought that LPS exposure triggers CXCL1/2 expression in lungs, which serves as a potent chemoattractant for neutrophil migration into the lungs by recognizing CXCR2, causing widespread inflammation and tissue injury. However, LPS also can downregulate CXCR2 expression by causing receptor desensitization and internalization (48). After stimulation by LPS, AKT1KO still showed higher expression of CXCR2 than WT controls. This finding suggests that AKT1 KO could inhibit the desensitization and internalization of CXCR2 (Fig. 4B and data not shown), contributing to the recruitment of neutrophils in all. Collectively, our data suggest that CXC1/2–CXCR2–dependent recruitment of neutrophils is one of the major reasons for the increased number of neutrophils in inflammatory sites in AKT1KO mice.

Neutrophils play a fundamental role in mediating the innate immune response against pathogens through local recruitment, phagocytosis, and production of inflammatory cytokines (1, 49). AKT1KO neutrophils display enhanced migration, phagocytic ability regarding *S. aureus*, and enhanced production of proinflammatory cytokines. In line with the hyperactivity of neutrophils in vitro, AKT1KO mice were resistant to *S. aureus* infection and were more susceptible to neutrophil-mediated acute inflammation in ALI. Upon LPS stimulation, the phosphorylation of S6 and STAT1, but not STAT3, ERK, p38MAPK, and JNK, was markedly enhanced in AKT1KO neutrophils compared with WT controls. The mTOR inhibitor, rapamycin, could not reverse the recruitment and bactericidal activity in AKT1KO neutrophils. However, the STAT1 chemical inhibitor MTA, or shRNA-mediated knockdown of STAT1, is sufficient to reverse the hyperactivity of AKT1KO neutrophils when stimulated with LPS. Consistent with previous reports showing the critical role of STAT1 signaling pathways in innate immunity (50–52), our data suggested that the enhanced bactericidal activity of AKT1KO neutrophils is due to increased STAT1 activity.

In summary, our data support a model in which AKT1 acts as a key negative regulator of the migration and function of granulocytic cells. Our studies also implicate AKT1 as a promising target for the therapeutic intervention of modulating neutrophil function in related disorders and vaccines.

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Disclosures

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