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Akt2 Deficiency Protects from Acute Lung Injury via Alternative Macrophage Activation and miR-146a Induction in Mice

Eleni Vergadi,* Katerina Vaporidi,† Emmanuel E. Theodorakis,* Christina Doxaki,* Eleni Lagoudaki,‡ Eleftheria Ieronymaki,* Vassilia I. Alexaki,* Mike Helms,§ Eumorfia Kondili,† Birte Soennichsen,§ Efstathios N. Stathopoulos,‡ Andrew N. Margioris,* Dimitrios Georgopoulos,‡ and Christos Tsatsanis* 

Acute respiratory distress syndrome (ARDS) is a major cause of respiratory failure, with limited effective treatments available. Alveolar macrophages participate in the pathogenesis of ARDS. To investigate the role of macrophage activation in septic lung injury and identify molecular mediators with therapeutic potential, lung injury was induced in wild-type (WT) and Akt2−/− mice by hydrochloric acid aspiration. Acid-induced lung injury in WT mice was characterized by decreased lung compliance and increased protein and cytokine concentration in bronchoalveolar lavage fluid. Alveolar macrophages acquired a classical activation (M1) phenotype. Acid-induced lung injury was less severe in Akt2−/− mice compared with WT mice. Alveolar macrophages from acid-injured Akt2−/− mice demonstrated the alternative activation phenotype (M2). Although M2 polarization suppressed septic lung injury, it resulted in increased lung bacterial load when Akt2−/− mice were infected with Pseudomonas aeruginosa. miR-146a, an anti-inflammatory microRNA targeting TLR4 signaling, was induced during the late phase of lung injury in WT mice, whereas it was increased early in Akt2−/− mice. Indeed, miR-146a overexpression in WT macrophages suppressed LPS-induced inducible NO synthase (iNOS) and promoted M2 polarization, whereas miR-146a inhibition in Akt2−/− macrophages restored iNOS expression. Furthermore, miR-146a delivery or Akt2 silencing in WT mice exposed to acid resulted in suppression of iNOS in alveolar macrophages. In conclusion, Akt2 suppression and miR-146a induction promote the M2 macrophage phenotype, resulting in amelioration of acid-induced lung injury. In vivo modulation of macrophage phenotype through Akt2 or miR-146a could provide a potential therapeutic approach for aseptic ARDS; however, it may be deleterious in septic ARDS because of impaired bacterial clearance. The Journal of Immunology, 2014, 192: 394–406.

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acute respiratory distress syndrome (ARDS) is a major cause of respiratory failure in critically ill patients, with no effective treatment reported (1). Acute lung injury, the pathologic presentation of ARDS, is characterized by alveolar barrier disruption and lung inflammation (2–7). Two phases of inflammatory response have been recognized in experimental acute lung injury: the acute phase, characterized by inflammatory cell infiltration and increased production of a variety of oxidants, cytokines, and proteolytic enzymes that promote tissue destruction (1, 2) and the resolving phase, during which phagocytosis of debris and alveolar structural repair are taking place (1, 8, 9).

Macrophages play an important role in the pathogenesis of lung injury, initiating the inflammatory response and promoting neutrophil infiltration and tissue damage in the lung (4, 5, 8–13). However, based on environmental stimuli, macrophages can possess the classical (M1) or alternative activation (M2) phenotype (14). Classically activated or M1 macrophages express high levels of inducible NO synthase (iNOS), generate NO, secrete IL-12β, and are prominent in the acute phase of inflammation (14). Genetic depletion or pharmacologic inhibition of iNOS was shown to confer protection against lung injury in several animal models (4, 13, 15–18), suggesting a crucial role for M1 macrophages in the pathogenesis of acute lung injury (ALI). Alternative macrophage activation, or M2, is characterized by high levels of arginase-1 (Arg-1), found-in-inflammatory zone-1 (Fizz1), chitinase-3–like-3 (Ym1), and macrophage galactose C-type lectin 1 and 2 (MGL1, MGL2) (8, 9, 19). M2 macrophages participate in the resolution of inflammation and are known to be beneficial in the outcome of several inflammatory diseases (14, 20–22).

The mechanisms that regulate macrophage activation phenotype are currently being investigated (14, 21, 22). Several signaling pathways, such as STAT-1 phosphorylation, IRF5 upregulation, and SOCS2 and SOCS1 were shown to promote M1 activation (21, 23–25). Also, SOCS3 suppression, STAT6 phosphorylation, and C/EBPβ and IRF4 upregulation were shown to regulate M2
polarization (21, 23, 24). Akt is a family of three serine/threonine protein kinases (Akt1, Akt2, and Akt3) that are important to control cell survival, proliferation, and differentiation (26, 27). We showed that Akt kinases play a key role in the regulation of the macrophage activation phenotype (25, 28, 29). Depletion of the Akt2 isoform in peritoneal macrophages abrogates M1 activation and promotes M2 phenotype by inducing C/EBPβ (22, 25), a transcriptional regulator of Arg-1 expression (30). Akt2 depletion induces C/EBPβ via negative regulation of the microRNA (miRNA) miR-155, known to target C/EBPβ and promote M1 (22, 25, 31). Additionally, TLR4 signaling was shown to play an important role in alveolar macrophage activation in animal models of ALI (12, 32, 33). TLR4 signaling is regulated by the anti-inflammatory miRNA, miR-146a, which targets and suppresses polarization of macrophages to a polarized M2 phenotype (22, 25, 26, 30, 34, 35, 36). Yet, the roles of the miR-146a macrophage activation phenotype and aseptic inflammation have not been investigated.

In the current study, we tested the hypothesis that a prominent M2 macrophage phenotype, such as the one possessed by Akt2−/− mice (22), would be protective in aseptic lung injury. Therefore, we compared the development of HCl acid aspiration–induced lung injury in wild-type (WT) and Akt2−/− mice. Additionally, we examined the molecular mechanisms involved in the regulation of the macrophage activation phenotype, focusing on the roles of Akt2, miR-155, and miR-146a. We also evaluated whether the macrophage activation phenotype can be modulated in vivo by targeting Akt2 or miR-146a. Last, we assessed the biological effect of Akt2 deletion in a septic model of lung injury induced by Pseudomonas aeruginosa to clarify the potential limitations of Akt2 suppression and M2 macrophage polarization under septic conditions in clinical practice.

### Materials and Methods

#### HCl-induced ALI

For induction of ALI, 8–10-wk-old C57BL/6 WT and Akt2−/− mice were anesthetized and intubated orotracheally. HCl solution (0.05 N, pH 1.5), 2 ml/kg diluted in normal saline (NS, 0.9% NaCl), was instilled in the trachea, whereas age- and gender-matched mice received 2 ml/kg NS and served as controls. A bolus of 0.5 ml air was given to ensure that HCl solution reached the distal lung. Mice were then extubated and left to recover from anesthesia with oxygen supplementation. At specific time points following HCl administration, mice were sacrificed; a pressure-volume curve of the respiratory system; protein concentration of BALF was assessed by the BCA method (BCA Protein Assay, Thermo Scientific). IL-6, TNF-α, CXCL-1, and IL-1β protein levels in the lavage fluid were determined using a commercially available sandwich ELISA kit (Quantikine; R&D Systems, Abingdon, U.K.), according to the manufacturer’s instructions.

#### Immunocytochemistry

Cells isolated from BALF were cytencentrifuged and placed on microscope slides. Immunocytochemistry for iNOS, IF5, and Arg-1 was performed by immersing the slides in 4% paraformaldehyde in PBS and incubating with blocking serum, followed by incubation at 4°C overnight with rabbit polyclonal anti-iNOS Ab (Santa Cruz Bio-technology, Santa Cruz, CA), rabbit polyclonal anti-IF5 Ab Cell Signaling Technology, Beverly, MA), or mouse monoclonal anti-mouse Arg-1 (BD Biosciences, Franklin Lakes, NJ). Goat biotinylated anti-rabbit IgG or horse biotinylated anti-mouse IgG (both from Cell Signaling Technology) was used as secondary Ab. FITC-Avidin or Texas Red–Avidin (Vector Laboratories, Burlingame, CA) were used to detect binding of biotinylated primary Abs. Nuclei were counterstained with DAPI (Thermo Fisher Scientific, Waltham, MA).

#### Lung histology and lung injury score determination

For histology purposes, lungs were perfused with PBS through the right ventricle. An incision at the left atrium allowed outflow of the blood. Lungs were inflated intratracheally with 10% formalin at 25 cmH2O pressure, fixed overnight at 4°C, and stored in 70% ethanol before embedding in paraffin. Lung tissue sections of 5 μm were prepared and further deparaffinized and rehydrated. Sections were stained with H&E for histological assessment of lung injury, five independent variables were evaluated—neutrophils in alveolar spaces, neutrophils in the interstitial spaces, hyaline membranes, proteinaceous debris filling the airspaces, and alveolar septal thickening—and weighted to the relevance ascribed to them by Official American Thoracic Society Workshop Report on Features and Measurements of Experimental Acute Lung Injury in Animals (39). The resulting injury score is a continuous value between 0 and 1.

#### Cell sorting and alveolar macrophage isolation

To discriminate alveolar macrophages, cells were stained with FITC–anti-mouse CD45 Ab (BD Biosciences), alphalipocytocin–anti-mouse CD11c Ab (BD Biosciences), or PE-anti-mouse Ly-6G Ab (BioLegend, San Diego, CA) specific for WBCs, alveolar macrophages, or neutrophils, respectively. Cells were evaluated in a MoFlo Cell Sorter (Beckman Coulter, Fullerton, CA), and the percentages of macrophages and neutrophils were analyzed with Summit Software (Summit Software, Fort Wayne, IN). The CD45+CD11c Ly-6G- cells (alveolar macrophages) were sorted further and isolated to purity > 90%.

#### RNA isolation and quantitative PCR

RNA from alveolar or thioglycolate-elicited peritoneal macrophages or total lung was isolated using TRIzol reagent (Life Technologies, Carlsbad, CA). In the case of in vivo–isolated and sorted alveolar macrophages, RNA precipitation was facilitated by the addition of 250 μg/ml RNase-free glucocone (Fermentas, St. Leon-Rot, Germany). One microgram of total DNA-digested RNA was used for cDNA synthesis (Thermoscript RT; Invitrogen, Carlsbad, CA). The SYBR Green method was followed in the PCR reaction. Primer sequences are shown in Supplemental Table I. Ribonose-lipid protein S9 (RPS9) served as the housekeeping gene. Annealing was carried out at 60°C for 30 s, extension was at 72°C for 30 s, and de- naturation was 40 cycles at 95°C for 15 s.

To isolate miRNAs from alveolar macrophages, total RNA was isolated as described above. TaqMan MicroRNA Assays (Life Technologies) were used for cDNA synthesis and quantitative PCR of specific miRNAs. The
miRNA sequence is described in Supplemental Table 1. SmoRNA135 served as housekeeping miRNA. Annealing and extension were carried out at 60°C for 30 s, and denaturation was 40 cycles at 95°C for 15 s in a 7500 Fast Real-Time PCR System (Life Technologies). Analysis of the fold change was performed based on the Pfaffl method (40).

Flow cytometry
Expression of protein levels of iNOS, IL-12β, Arg-1, Fizz1, MGL-1/2, and IL-10 was determined by flow cytometry cell surface and intracellular staining, as previously described (41, 42). Cells isolated from BALF were incubated with Golgi inhibitor (monensin; BD Biosciences), and cell surface staining was carried out by incubation with PerCP-Cy5.5 anti-mouse CD11c (BioLegend), fixation and permeabilization (BD Fixation and Permeabilization Solution Kit; BD Biosciences), and staining with allopurinol-conjugated mAb against murine IL-10, FITC-conjugated mAb against murine iNOS, or PE-conjugated mAb against murine Arg-1 (all from BD Biosciences). Mouse monoclonal anti-mouse Arg-1 (BD Biosciences) and rabbit polyclonal anti-mouse Fizz1 (Abcam, Cambridge, U.K.) were used in separate analyses. FITC goat anti-rabbit IgG (BD Biosciences) or allopurinol-conjugated rat anti-mouse IgG1 (BioLegend) was used as secondary Ab for Fizz1 or Arg-1 staining, respectively. PE anti-mouse MGL1/2 (R&D Systems, Minneapolis, MN) was used for cell surface staining in separate analyses. The proper isotype controls were used in all cases. Flow cytometry data were acquired on a FACSCalibur Legacy Cell Sorter (Beckman Coulter) and analyzed with Summit Soft-ware. Flow cytometry events were gated first based on forward and side scatter and then CD11c+ cells (alveolar macrophages) were selected to evaluate the expression of activation markers.

Nitrite concentration and arginase activity assay
Determination of NO metabolite and nitrite concentration in BALF (based on chemiluminescence reaction) BALF supernatants from control mice and mice with acid-induced lung injury for 12 h were used. A total of 50 μL sulfanilamide solution (1% w/v sulfanilamide, 5% w/v phosphoric acid) was added to an equal volume of sample. Samples were incubated for 10 min in the dark, and 50 μL 0.1% w/v N-(naphthyl) ethylenediaminedihydrochloride was added, followed by a second incubation for 10 min at room temperature in the dark. Absorbance at 550 nm was measured, and the amount of nitrite determined. miRNA sequence is described in Supplemental Table 1. SmoRNA135 served as housekeeping miRNA. Annealing and extension were carried out at 60°C for 30 s, and denaturation was 40 cycles at 95°C for 15 s in a 7500 Fast Real-Time PCR System (Life Technologies). Analysis of the fold change was performed based on the Pfaffl method (40).

Arginase activity: Arginase activity was assessed indirectly by measuring the concentration of urea generated by the arginase-dependent hydrolysis of t-arginine as previously described (43). Alveolar macrophages from animals with acid-induced lung injury (12 h time point) and control mice were harvested, washed, and lysed with 10 mM Tris-HCl (pH 7.4) containing 4.5% (w/v) Triton X-100 and protease inhibitor mixture (Complete; Roche, Basel, Switzerland) for 30 min on a shaker at room temperature. A total of 10 mM MnCl2 was added. Ten microliters of 10 mM MnCl2 was added to 100 μL this lysate, and the enzyme was activated by heating for 10 min at 55°C. Arginine hydrolysis was conducted by incubating the lysates with 100 μL 0.5 M t-arginine (pH 9.7) at 37°C for 60 min. The reaction was stopped with 800 μL H2SO4 (96%)/H3PO4 (85%)/H2O (1/3/7, v/v/v). The urea concentration was measured at 550 nm after the addition of 40 μL α-isonitrosopropiophenone (Sigma-Aldrich, St. Louis, MO) (dissolved in 100% ethanol), followed by heating at 100°C for 30 min. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol urea/min.

Western blot analysis
Macrophage protein lysates were resuspended in SDS-containing loading dye. Twenty micrograms of protein was electrophoresed on 13.3% denaturing polyacrylamide gel prior to wet transfer to 0.45 μm polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). Briefly, after blocking with 5% skim milk in PBS (pH 7.4) containing 0.1% Tween 20 for an hour at room temperature, the membranes were incubated with rabbit polyclonal anti-mouse IRES5 Ab (Cell Signaling Technology), mouse polyclonal anti-mouse TRAF6 Ab (Santa Cruz Biotechnology), or mouse monoclonal anti-mouse B-actin (Cell Signaling Technology) at 4°C overnight. The membranes were then incubated with 40 ng/ml peroxidase-conjugated anti-rabbit or anti-mouse secondary Ab (Santa Cruz Biotechnology), respectively, for 30 min at room temperature, followed by reaction with Lumi-Light ECL substrate (Thermo Fisher Scientific).

Cell cultures and cell transfections
For cell-transfection experiments, peritoneal fluid or BALF from control animals was obtained through instillation of 5 × 1 ml HBSS (without calcium or magnesium) supplemented with 10 mM EDTA and 1 mM HEPES and filtered twice via a 35-μm cell strainer to exclude contami-
the normality test were analyzed using one-way ANOVA with the Bonferroni multiple-comparison post test. Comparison of nonparametric results between groups was performed using the Mann–Whitney U test or the Kruskal–Wallis test with the Dunn multiple-comparison post test, using GraphPad InStat software (GraphPad, San Diego, CA). The p values < 0.05 were considered significant. Results are expressed as mean ± SD or as median (5–95 percentiles), as indicated, and are representative of at least three independent experiments.

**Results**

*Akt2 deficiency protects from the development of acid-induced lung injury*

To determine the role of Akt2 and M2 macrophages in aseptic lung injury, we exposed Akt2<sup>2/2</sup> mice, which possess M2-type macrophages, to acid-induced lung injury. This model induces tissue injury that initiates an aseptic inflammatory cascade. WT mice developed severe lung injury within hours of acid aspiration, which was characterized by decreased lung compliance (Fig. 1A, 1B) and increased protein concentration in BALF compared with control mice treated with NS (Fig. 1C). The severity of lung injury peaked at 12 h postacid aspiration and declined thereafter (Fig. 1B, 1C). Macrophage and neutrophil infiltration increased within 6 h after acid aspiration (Fig. 2A–C). Furthermore, chemokines and cytokines, such as TNF-α, IL-6, CXCL-1, and IL-1β, also accumulated in BALF and reached their highest levels at 6 h postacid aspiration (Fig. 2D). Lung compliance, protein concentration, BALF cell counts, and cytokines returned to baseline at 72 h after acid aspiration (Figs. 1B, 1C, 2A, 2B).

Acid-induced lung injury was less severe in Akt2<sup>2/2</sup> mice compared with WT mice (Fig. 1A–C). A decrease in lung compliance and an increase in BALF protein concentration were observed in acid-treated WT mice when compared to saline-treated mice, which was profound in acid-treated Akt2<sup>2/2</sup> mice (Fig. 1A–C). In addition, the severity of inflammatory cell infiltration (Fig. 2A–C) and the cytokine levels in BALF (Fig. 2D) were lower in Akt2<sup>2/2</sup> mice compared with WT mice.

Histology on lung sections from WT mice at 12 h postacid aspiration demonstrated destruction of normal tissue architecture characterized by thickening of the alveolar walls due to increased cellularity and edema (Fig. 1Db). Proteinaceous debris in the

**FIGURE 1.** Acid aspiration–induced lung injury is reduced in Akt2<sup>2/2</sup> mice. Pressure-volume curve of the respiratory system (A), IC (B), and protein concentration in BALF (C) in WT and Akt2<sup>2/2</sup> mice at several time points after acid (HCl) aspiration compared with NS group. (D) Histological analysis of lung tissue of untreated control WT mice (a), WT mice (b, c), and Akt2<sup>2/2</sup> mice (e, f) at 12 h after HCl administration (H&E stain, original magnification ×400), as well as analysis of acute lung injury score (d). Black arrows in (b) demonstrate thickening of the alveolar walls as the result of increased cellularity. Red arrow in (c) indicates proteinaceous debris in the alveoli. Extravasated RBCs are also depicted. Scale bar, 25 µm. n = 5–8 mice/group. Graphs represent median ± SD. In (d), box shows 5–95 percentiles, horizontal line represents median, and whiskers represent minimum and maximum. *p < 0.05, **p < 0.01, ***p < 0.001, acid treatment versus NS treatment. *p < 0.05, **p < 0.01, ***p < 0.001, Akt2<sup>2/2</sup> versus WT.
alveoli and extravasated RBCs were also observed (Fig. 1De). Lung sections from acid-treated Akt2−/− mice at 12 h postinjury demonstrated less severe lung injury compared with WT mice (Fig. 1Dd). Only mild thickening of the alveolar walls with an inconspicuous presence of inflammatory cells, and focal congestion of capillaries, with mild red cell extravasation, were observed in Akt2−/− mice (Fig. 1De, 1Df).

These results suggested that ablation of Akt2 resulted in reduced manifestations of inflammation in the lung upon acid-induced lung injury.

**Akt2 ablation suppresses M1 polarization and promotes M2 phenotype in acid-induced lung injury**

To determine the inflammatory phenotype of infiltrated macrophages in the model of acid-induced lung injury, we measured the concentration of inflammatory mediators in the BALF and the presence of M1- and M2-type macrophages in the lungs of WT and Akt2−/− mice during the acute phase (12 h following acid aspiration), as well as at the resolution stage (24 h following acid aspiration). In the acute phase of injury, 12 h after acid aspiration, M1 activation of macrophages was observed in WT mice; alveolar macrophages overexpressed iNOS (Fig. 3A, 3C, 3D, 3F) and IL-12β (Fig. 3B, 3E). The expression of M1 markers returned to basal levels at 48 h of injury (Fig. 3A, 3B). The concentration of NO metabolites in BALF was increased in WT mice at 12 h postinjury (Fig. 3G). Expression of M1 markers after acid aspiration–induced lung injury was lower in Akt2−/− mice compared with WT mice (Fig. 3).

The expression of M2 activation markers Arg-1, Fizz1, and Ym1, at 12 h postacid aspiration, was evident in both WT and Akt2−/− macrophages. The levels of Arg-1, Fizz1, and Ym1, as well as arginase activity and MGL1 and MGL2 protein expression, were higher in Akt2−/− macrophages compared with WT ones (Fig. 4A–G, Supplemental Fig. 1A). Arg-1 mRNA was upregulated in WT and Akt2−/− macrophages 12 h following acid aspiration; however, upregulation of its protein and activity were not statistically significant at 12 h, but they were induced further at later time points (24 h following HCl instillation; data not shown). The expression of the anti-inflammatory cytokine IL-10, a characteristic of M2 macrophages, was similarly increased in WT and Akt2−/− macrophages after acid aspiration (Supplemental Fig. 1B), suggesting that its regulation is not under the control of Akt2.

In vitro application of siRNA targeting Akt2 in isolated alveolar macrophages from WT mice resulted in downregulation of Akt2 and concomitant upregulation of C/EBPβ, an M2 polarization–associated transcription factor (22), as well as Arg-1 and Fizz1 (Fig. 4H, 4I), further supporting the effect of Akt2 suppression in promoting M2 polarization in alveolar macrophages.

**Involvement of miR-155 in macrophage-activation phenotype in acid-induced lung injury and the role of Akt2**

Next, we sought to elucidate the mechanisms by which Akt2 deficiency regulates macrophage polarization and protects from acid-induced lung injury. Earlier studies (22, 25) from our group showed that miR-155 plays a central role in promoting M1 activation in macrophages following LPS stimulation and is under the control of Akt2. To investigate the involvement of miR-155 in the regulation of M1 phenotype in our model of aseptic lung injury, we measured miR-155 levels in alveolar macrophages from WT and Akt2−/− mice exposed to acid. We found that the expression of miR-155 did not increase in WT alveolar macrophages at 12 h after acid aspiration, but it was suppressed at 24 h postinjury (Fig. 5A). To exclude miR-155’s involvement in M1 activation in our model, we stimulated alveolar macrophages in vitro...
with BALF collected from WT mice 6 h after acid aspiration. We found that exposure to BALF did not cause an increase in miR-155 levels, although it promoted iNOS expression (Fig. 4C, 4D), suggesting that miR-155 does not contribute to M1 activation of macrophages in this model of aseptic lung injury.

The development of M2 phenotype in WT alveolar macrophages 24 h after acid aspiration was associated with a decrease in miR-155 expression (Fig. 5A). The expression of miR-155 in Akt2−/− alveolar macrophages was lower compared with WT macrophages both at baseline and at 12 h after acid aspiration (Fig. 5B). C/EBPβ levels, a target of miR-155, were inversely correlated with miR-155 levels (Fig. 5B). C/EBPβ expression increased in WT macrophages at 12 h after acid aspiration, and it was higher in Akt2−/− macrophages compared with WT ones (Fig. 5B). These results suggest that, even though miR-155 does not participate in the initial M1 activation of alveolar macrophages in aseptic lung injury, its suppression coincides with the emergence of M2 status.

Akt2-deficient macrophages overexpress miR-146a, which suppresses TLR signaling in acid-induced lung injury

Because macrophage activation and lung inflammation depend on TLR4 signals, we examined the impact of Akt2 ablation on TLR4-signaling components. We found that mRNA levels of TRAF6 and IRF5, but not of IRAK1, three downstream mediators of TLR4 signaling, were increased in WT alveolar macrophages at 12 h after acid aspiration (Fig. 6A, 6B). On the contrary, Akt2−/− macrophages expressed less TRAF6, IRF5, and IRAK1 compared with WT macrophages both at baseline and at 12 h after acid aspiration (Fig. 6A, 6B). Furthermore, application of siRNA targeting Akt2 to isolated alveolar macrophages resulted in downregulation of TRAF6 and IRF5 compared with cells that received nontargeting siRNA (Fig. 6C). IRAK1, TRAF6, and IRF5 mRNA are regulated by the anti-inflammatory miRNA miR-146a (34, 35). Therefore, we evaluated the expression of miR-146a in alveolar macrophages after acid aspiration. miR-146a expression did not change significantly at 12 h after acid aspiration, but it increased at 24 h (Fig. 6E). Interestingly, in Akt2−/− macrophages, the expression of miR-146a was higher compared with WT macrophages prior to acid aspiration, as well as at 12 h and 24 h (Fig. 6E). To further support that suppression of Akt2 results in miR-146a induction, we transfected macrophages with siRNA for Akt2 and found that miR-146a was upregulated (Fig. 6D). Additionally, miR-146a expression was higher, whereas TRAF6 and IRF5 mRNA and protein expression were lower in Akt2−/− peritoneal macrophages compared with WT macrophages (Fig. 6F, 6G). These findings suggested that miR-146a and its targets TRAF6 and IRF5 were affected by Akt2 deletion or suppression.
miR-146a prevents M1 activation in vitro and in vivo and is critical for the protection observed in the absence of Akt2.

To investigate whether miR-146a upregulation is essential for amelioration of the M1 response in Akt2-/- mice, we isolated peritoneal macrophages from WT and Akt2-/- mice; induced M1 polarization by treating them with LPS, a stimulus that promotes macrophage activation; and transfected them with either an miR-146a mimic or an miR-146a inhibitor. As controls, we used cells treated with negative-control miRNA (scramble), cells treated solely with transfection reagent (mock), and cells that remained untreated (i.e., nontransfected).

Akt2-/- macrophages are hyporesponsive to LPS and maintain an M2 phenotype both in vitro and in vivo when mice are subjected to LPS-induced endotoxin shock (22).

Transfection of WT macrophages with miR-146a resulted in suppression of TRAF6 and IRF5. The effect of miR-146a on the suppression of TRAF6 and IRF5 in Akt2-/- macrophages was less prominent compared with WT macrophages (Fig. 7). Inhibition of miR-146a by an miR-146a inhibitor did not affect the expression of TRAF6 or IRF5 in WT macrophages, but it upregulated their expression in the absence of Akt2 (Fig. 7).

Furthermore, miR-146a transfection inhibited LPS-induced iNOS expression in WT macrophages (Fig. 8A) and led to the upregulation of C/EBPβ, Arg-1, and Fizz1 in isolated alveolar macrophages transfected with siAkt2 or non-targeting siRNA (siNeg.C). Cells that received only transfection reagent (mock) or were left untreated (untransfected) were used as controls. Scale bar, 25 μm. n = 5–8 mice/group. Graphs show mean ± SD. In box-and-whisker plots, box shows 5–95 percentiles, horizontal line represents median, and whiskers represent minimum and maximum. *p < 0.05, **p < 0.01, ***p < 0.001, acid treatment versus NS treatment. *p < 0.05, **p < 0.01, ***p < 0.001, Akt2-/- versus WT. MFI, Mean fluorescence intensity of cells gated for CD11c.
suggesting that these molecules are regulated by a different Akt2-dependent mechanism (Fig. 8B–F).

To further confirm that inhibition of Akt2 and induction of miR-146a inhibit M1 activation of alveolar macrophages in vivo, acid-treated WT mice received small interfering RNA against Akt2 (siAkt2) or miR-146a mimic or miR-146a inhibitor intratracheally to modulate Akt2 or miR-146a expression, respectively. Transfection efficiency was confirmed by assessing total lung mRNA levels of Akt2, TRAF6, and IRAK1 (Supplemental Fig. 2B–D), as well as miR-146a mRNA levels (Supplemental Fig. 2F). Distribution of siRNA into the lung parenchyma following its intratracheal administration was assessed by Cy3-conjugated control siRNA (Supplemental Fig. 2D). Because iNOS is the major marker of M1-activated macrophages, we evaluated the effect of siAkt2, miR-146a mimic, or miR-146a inhibitor on iNOS expression. Accordingly, iNOS was significantly suppressed in alveolar macrophages from mice treated with siAkt2 or miR-146a compared with those treated with scrambled RNA (Fig. 8G, 8H), suggesting that in vivo modulation of Akt2 or miR-146a could effectively suppress aseptic lung inflammation induced by acid aspiration. Moreover, administration of miR-146a inhibitor to Akt2−/− mice resulted in partial reversal of iNOS suppression in macrophages from those mice (Fig. 8I, Supplemental Fig. 2E), and it had no significant effect on Arg-1 levels (data not shown), suggesting that miR-146a induction is responsible, at least in part, for the suppressed M1 phenotype of Akt2−/− mice. Arg-1 expression in Akt2−/− alveolar macrophages was not affected by treatment with miR-146a inhibitor. Overall, these findings suggest that induction of M2 macrophages, either by inhibition of Akt2 or induction of miR-146a, may be protective against aseptic lung injury.

FIGURE 5. miR-155 expression in acid-induced lung injury. Expression of miR-155 (A) and its target mRNA C/EBPβ (B) in WT and Akt2−/− alveolar macrophages from mice exposed to acid for 12 or 24 h. miR-155 (C) and iNOS (D) mRNA levels in WT and Akt2−/− alveolar macrophages treated in vitro with BALF from WT mice that received either NS or HCl for 6 h in vivo. Data are mean ± SD for 4–8 mice or samples/group. *p < 0.05, **p < 0.01, acid treatment versus NS treatment. #p < 0.05, ##p < 0.01, Akt2−/− versus WT.

FIGURE 6. TLR4 signaling and miR-146a expression in acid-induced lung injury. mRNA levels of TRAF6, IRF5, and IRAK1 (A) and protein expression of IRF5 (B) in WT and Akt2−/− alveolar macrophages from mice exposed to acid aspiration for 12 h. Scale bar, 25 μm. mRNA levels of TRAF6, IRF5, and IRAK1 (C) and miR-146a in alveolar macrophages treated with siAkt2 or nontargeting siRNA (siNeg.C) (D). Cells that received only transfection reagent (mock) or were left untreated (untransfected) were used as controls. (E) Levels of miR-146a in WT and Akt2−/− alveolar macrophages from mice that received acid for 12 or 24 h. (F) Comparison of miR-146a, TRAF6, and IRF5 mRNA levels in unstimulated WT and Akt2−/− peritoneal macrophages. (G) Protein levels of IRF5 and TRAF6 in WT and Akt2−/− unstimulated peritoneal macrophages. B-actin was used as control. Data are mean ± SD for n = 4–8 mice or wells/group. *p < 0.05, **p < 0.01, ***p < 0.001, acid treatment versus NS. #p < 0.05, ##p < 0.01, ###p < 0.001, Akt2−/− versus WT.
FIGURE 7. Modulation of miR-146a expression in WT and Akt2−/− macrophages in culture. mRNA levels of TRAF6 (A) and IRF5 (B) in WT and Akt2−/− peritoneal macrophages that were transfected with an miR-146a mimic, an inhibitor of miR-146, or a non-targeting control RNA (scramble). Untransfected cells, as well as cells that received only transfection reagent (mock), were used as experimental controls. Data are mean ± SD for n = 4–5 wells/group. On the x-axis, miR-146a represents the miR-146a mimic, and as-miR-146a represents the miR-146a inhibitor (antisense). *p < 0.05, **p < 0.01, ***p < 0.001, LPS treatment versus NS treatment. #p < 0.05, ###p < 0.001, Akt2−/− versus WT.

Effect of Akt2 depletion in a septic lung injury model

Because aspiration-induced lung injury is frequently accompanied by the presence of pathogens, such as bacteria, we investigated whether Akt2 depletion and, therefore, M2 macrophage polarization, affects the response to live bacteria. For this purpose, we inoculated WT or Akt2−/− mice with P. aeruginosa (2 × 107 bacteria/mouse). Lung IC and protein concentration in BALF were similar between WT and Akt2−/− mice (Fig. 9A, 9B), whereas bacterial load (CFU/ml in BALF) was significantly higher in Akt2−/− mice compared with WT mice at 12 h after inoculation (Fig. 9C). Infiltration of macrophages and neutrophils, as well as iNOS expression, was less profound in Akt2−/− mice compared with WT mice (Fig. 9D–F). Macrophages from Akt2−/− mice retained their M2-prone phenotype in septic ALI and expressed more Arg-1 compared with those from WT mice (Fig. 9G). However, IL-6 and TNF-α concentrations in BALF did not differ between Akt2−/− and WT mice (Fig. 9H, 9I), but IL-6 and TNF-α levels appeared significantly reduced in Akt2−/− mice compared with WT mice when normalized to P. aeruginosa CFU (Fig. 9H, I), suggesting that the increase in these cytokines may be due to the increased bacterial load. Histological analysis of lung tissue upon P. aeruginosa infection revealed that both WT and Akt2−/− lung sections have severe distortion of normal lung architecture due to the presence of a dense interstitial and alveolar inflammatory infiltrate composed mainly of neutrophils, macrophages, and lymphocytes (Fig. 9J). However, the parenchymal damage was less severe in Akt2−/− mice compared with WT mice, because they demonstrated less parenchymal consolidation and better preservation of alveoli, probably as a result of reduced lung inflammation (Fig. 9J). Overall, these findings suggest that, although ablation of Akt2 and M2 polarization of macrophages protects from aseptic lung injury, it compromises the response of macrophages to live bacteria.

Discussion

In the present study we show that, in the mouse model of aseptic lung injury, macrophages first exhibit a proinflammatory M1 phenotype, followed by an M2 anti-inflammatory phenotype. Genetic ablation of Akt2 suppresses M1 activation via miR-146a induction, promotes an M2 phenotype, and protects mice from acid-induced lung injury.

ARDS, the devastating clinical syndrome of acute respiratory failure characterized by lung inflammation and alveolar barrier dysfunction, is a major cause of morbidity and mortality in patients in the intensive care unit. Although pneumonia and sepsis are the most common causes of ARDS, several aseptic conditions are associated with ARDS, such as acute pancreatitis, burns, near drowning, multiple trauma, and inhalation injury (1). With no effective treatment available, there is an urgent need to understand and, subsequently, modulate the pathogenesis of lung inflammation.

It is well established that macrophages play a central role in the pathogenesis of ARDS (4, 5, 12, 13). Most of the studies using animal models examined the role of macrophages in LPS-induced lung injury, a model that resembles septic ARDS (11, 18, 20). In this study, we used the model of acid-induced lung injury, a model of aseptic ARDS (2). Similarly to septic lung injury, we found that alveolar macrophages in acid-induced lung injury acquire a classical (M1) activation phenotype in the early phase that is characterized by increased iNOS expression and accumulation of NO metabolites, which are known to contribute to the pathogenesis of ARDS (4, 13–18). Furthermore, we identified the onset of the resolving phase of inflammation, during which suppression of iNOS and predominance of Arg-1, Fizz1, and Ym1 expression, features of M2 polarization, take place. It was reported recently that stem cell–conditioned medium induced M2 polarization and suppressed lung inflammation in a model of LPS-induced lung injury (20), yet no molecular mechanism of M2 induction was suggested. In our study, Akt2-deficient mice exhibited an ameliorated M1 response and an accelerated M2 activation, resulting in significant protection from lung injury and suggesting that early induction of M2 macrophages, via Akt2 depletion, confers protection in the aseptic lung injury model.
Therefore, we sought to investigate the mechanisms involved in the regulation of the macrophage-activation phenotype by Akt2 in this lung injury model. Activation of the PI3K/Akt pathway was demonstrated to mediate anti-inflammatory effects in macrophages (22, 25, 28, 29). The anti-inflammatory actions of the PI3K/Akt pathway are differentially controlled by the two Akt isoforms, Akt1 and Akt2, which also regulate the activation phenotype of macrophages (22). Hence, ablation of Akt2 suppresses LPS responses, promoting an M2 anti-inflammatory phenotype, whereas ablation of Akt1 renders macrophages hyperresponsive to LPS and M1 prone (22). We showed previously that miR-155, a proinflammatory miRNA, is differentially regulated by Akt kinases and plays a central role in M1 polarization of macrophages in several inflammatory models (22). Also, miR-155 was found to be important in the pathogenesis of LPS-induced lung injury (50). Yet, in the aseptic model of acid-induced lung injury, an increase in miR-155 expression was not necessary for the initial M1 polarization of macrophages, whereas a decrease in miR-155 levels was associated with the development of M2 phenotype. These findings suggest that, in this aseptic model, a distinct mechanism regulates macrophage activation.

Various signaling pathways, including STAT1/STAT6, SOCS2/SOCS3, TLR4, and IRF5/IRF4, were reported to regulate macrophage activation (21, 23, 24). Specifically, TLR4 signaling plays a central role in macrophage activation in both septic and aseptic inflammation, including ARDS (32, 33, 51–55), and is critical in the pathogenesis of disease (12, 32, 55). TLR4 signaling is controlled by MyD88- or TRIF-dependent downstream effectors, the signals of which converge for TRAF6 activation and NF-κB nuclear translocation (56). IRAK1 and IRF5 are primarily known to be involved downstream of MyD88 (52), but IRF5 is also involved in TRIF-mediated responses (57). In acid aspiration lung injury, TLR4/TRIF/TRAF6 signaling in lung macrophages was shown to determine the susceptibility to ARDS in vivo (12, 33). Both TRAF6 and IRF5 lead to NF-κB activation, but IRF5 is also an important regulator of iNOS and IL-12β expression and, thus, M1 phenotype (23, 52, 57–60). We found that WT macrophages upregulate TRAF6 and IRF5 gene expression in line with their M1 phenotype, whereas Akt2-deficient macrophages, having an M2 phenotype, exhibit reduced levels of these factors both at baseline and upon acid injury.

miR-146a is an LPS-induced miRNA that plays a critical role in macrophage responses (61). miR-146a is considered an anti-inflammatory miRNA that mutes immune activation initiated by TLR4 by targeting the 3′-untranslated region of TRAF6,
IRAK1, and IRF5 mRNAs (34, 35) and suppressing TLR4-induced NF-κB–regulated gene expression (61–64). In this study, we show that Akt2 deficiency resulted in a significant upregulation of miR-146a, which was of critical importance in suppressing the M1 phenotype. miR-146a transfection in WT macrophages was also able to inhibit iNOS induction, and miR-146a suppression in Akt2−/− mice resulted in upregulation of iNOS expression. It is of interest that introduction of miR-146a in WT macrophages induced expression of the transcription factor C/EBPβ, a master regulator of M2 polarization, indicating that miR-146a induction can promote alternative macrophage activation. However, inhibition of miR-146 in Akt2−/− macrophages did not suppress their M2 phenotype, suggesting that miR-146 is sufficient, but not necessary, for the induction of the M2 phenotype and that additional molecules regulated by Akt2, such as miR-155, maintain M2 activation.

Finally, we show that the pulmonary macrophage-polarization phenotype can be modulated in vivo by targeting local Akt2 or miR-146a expression. Intratracheal administration of siRNA

**FIGURE 9.** *P. aeruginosa* induced lung injury and inflammation in Akt2−/− mice. IC (A), protein concentration (B), and bacterial load (CFU/ml) (C) in BALF from WT and Akt2−/− mice at 12 h after *P. aeruginosa* inoculation (Psa). Neutrophil (D) and macrophage (E) cell numbers in BALF from WT and Akt2−/− mice after *P. aeruginosa* infection (Psa). Protein levels of iNOS (F) and Arg-1 (G) in alveolar macrophages (CD11c+ cells) of WT and Akt2−/− mice with *P. aeruginosa* lung infection assessed by flow cytometry and expressed as mean fluorescent intensity (MFI). Levels of IL-6 (H) and TNF-α (I) in BALF of WT and Akt2−/− mice at 12 h after *P. aeruginosa* inoculation. IL-6 and TNF-α levels are also shown normalized to bacterial load in BALF (CFU ratio = mean CFU/ml of WT mice/CFU/ml of Akt2−/− mice). (J) Histological analysis of lung tissue sections of WT and Akt2−/− mice after *P. aeruginosa* pneumonia (H&E stain) shows severe distortion of lung architecture in both sections as the result of the presence of dense interstitial and alveolar inflammatory infiltrates. Akt2−/− mice demonstrate slightly better preservation of alveoli and less parenchymal consolidation. Scale bars, 50 μm, n = 6 mice/group. In box-and-whisker plots, box shows 5–95 percentiles, horizontal line represents median, and whiskers represent minimum and maximum. *p < 0.05; **p < 0.01; ***p < 0.001, acid treatment versus NS treatment. #p < 0.05, ###p < 0.001, Akt2−/− versus WT.
against Akt2 or of an miR-146a mimic in WT mice exposed to acid-induced lung injury resulted in a significant suppression of iNOS in alveolar macrophages. Because it is well established that suppression of iNOS and subsequent inhibition of M1 activation (4, 13, 15–18), as well as induction of M2 (20), can confer protection in ARDS, the identification of molecules that promote this mechanism in vivo is of the utmost importance. However, Akt2 suppression and M2 macrophages impair the innate immune response against live bacteria, such as *P. aeruginosa*, which limits the use of M2 induction for the protection from gastric acid aspiration–induced lung injury. Simultaneous treatment with antibiotics may overcome such a limitation in a clinical setting.

In summary, this study demonstrates the protective effect of alternative macrophage polarization in acid-induced lung injury and identifies Akt2 and miR-146a as key molecular determinants of alveolar macrophage polarization. Based on these findings, Akt2 and miR-146a appear to be promising therapeutic targets for septic ARDS. However, the impaired bacterial clearance as a result of M2 induction is a major limitation to the use of this therapeutic application in septic ARDS, which represents the great majority of cases in clinical practice either from a pulmonary or nonpulmonary source.

**Disclosures**

The authors have no financial conflicts of interest.

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