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Human Resistin Promotes Neutrophil Proinflammatory Activation and Neutrophil Extracellular Trap Formation and Increases Severity of Acute Lung Injury

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Although resistin was recently found to modulate insulin resistance in preclinical models of type II diabetes and obesity, recent studies also suggested that resistin has proinflammatory properties. We examined whether the human-specific variant of resistin affects neutrophil activation and the severity of LPS-induced acute lung injury. Because human and mouse resistin have distinct patterns of tissue distribution, experiments were performed using humanized resistin mice that exclusively express human resistin (hRTN+/−) but are deficient in mouse resistin. Enhanced production of TNF-α or MIP-2 was found in LPS-treated hRTN+/− neutrophils compared with control Rtn−/−/− neutrophils. Expression of human resistin inhibited the activation of AMP-activated protein kinase, a major sensor and regulator of cellular bioenergetics that also is implicated in inhibiting inflammatory activity of neutrophils and macrophages. In addition to the ability of resistin to sensitize neutrophils to LPS stimulation, human resistin enhanced neutrophil extracellular trap formation. In LPS-induced acute lung injury, humanized resistin mice demonstrated enhanced production of proinflammatory cytokines, more severe pulmonary edema, increased neutrophil extracellular trap formation, and elevated concentration of the alarmins HMGB1 and histone 3 in the lungs. Our results suggest that human resistin may play an important contributory role in enhancing TLR4-induced inflammatory responses, and it may be a target for future therapies aimed at reducing the severity of acute lung injury and other inflammatory situations in which neutrophils play a major role. The Journal of Immunology, 2014, 192: 4795–4803.

Resistin is a secretory cysteine-rich protein that belongs to the FIZZ protein family and is characterized as an insulin resistance factor found in a mice model of type II diabetes and obesity (1–4). Recent studies showed that resistin plays an important role in regulating glucose homeostasis, as well as the pathophysiology of insulin resistance, in rodents (5). In particular, loss of resistin was shown to improve insulin sensitivity (6), and hyperresistinemia results in insulin resistance that predisposes to type II diabetes mellitus (5). In addition to regulation of glucose homeostasis, including enhancing insulin sensitivity, resistin was recently implicated in the development of cardiovascular disorders. For example, cardiac hypertrophy resulted from expression of resistin in diabetic rat hearts (7). The proposed mechanism of action for resistin’s effects on inducing hypertrophy of ventricular myocytes relates to inhibition of AMP-activated protein kinase (AMPK), a major sensor and regulator of bioenergetics at cellular and organism levels (8, 9). Although mouse resistin RELMα and RELMβ and human resistin have been implicated in inflammation (10–12), variant resistins have distinct patterns of tissue distribution and, therefore, appear to have compartment-specific effects. Unlike the expression of rodent resistin, which is limited to adipocytes, human resistin is primarily produced by macrophages and neutrophils, and significant amounts of resistin are found in the lungs (3, 13, 14). Increased expression of human resistin occurs in immune disorders, including dysregulated inflammatory conditions (3, 13, 15). For example, systemic amounts of human resistin are elevated for prolonged periods in septic patients (16, 17). However, the contributory role of resistin in inflammation and organ injury has not been well characterized. Only high concentrations of human resistin were reported to directly stimulate cytokine production, such as TNF-α, by RAW 264.7 cells (18). Although i.p. administration of purified murine resistin has modest inflammatory effects in mice, a marked increase in the severity of liver injury only occurred when resistin was combined with endotoxin challenge (19).

Neutrophils play an essential role in innate immune and inflammatory responses directed toward eradication of microbial infection (20, 21). However, exaggerated proinflammatory activation, often accompanied by the release of neutrophil extracellular traps (NETs), is frequently associated with collateral tissue damage and organ dysfunction, including development of acute lung injury (ALI) (22–26). Recent studies, including results obtained in our laboratory, established the important link among metabolism, neutrophil activation, and inflammation (27–29). Although AMPK is a major metabolic sensor and regulator of
energy production (30, 31), AMPK also can inhibit NF-κB–associated signaling in TLR2- or TLR4-stimulated neutrophils and macrophages (27, 32). Moreover, mice that received the AMPK activators metformin or 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR) were partially protected from endotoxin-induced ALI (27). Although activation of AMPK before LPS challenge can suppress the inflammatory response in many cell types, little is known about mechanisms responsible for the observed inhibition of AMPK activity during inflammatory responses (27, 33, 34).

Recent studies suggest that resistin is able to diminish AMPK activity (7, 35, 36). Because activation of AMPK has anti-inflammatory functions, we examined the hypothesis that interactions between human resistin and AMPK result in increased neutrophil proinflammatory activation and enhance the development and severity of LPS-induced ALI. Given the well-characterized differences in structure and localization of human and murine resistin, our experiments were performed using mice deficient in rodent resistin (RTN<sup>−/−</sup>) as well as humanized resistin mice (hRTN<sup>+/−</sup>), which exclusively expressed the human variant of resistin.

Materials and Methods

**Mice**

Humanized resistin mice (hRTN<sup>+/−</sup>) and resistin-knockout mice (RTN<sup>−/−</sup>) were generated by Dr. Mitchell A. Lazar (University of Pennsylvania, Philadelphia, PA), as previously described (5). Briefly, the transgenic mice on the C57BL/6 background, with expression of human resistin under the control of the CD68 promoter, were bred to C57BL/6 resistin-knockout mice (RTN<sup>−/−</sup>), which exclusively expressed the human variant of resistin. Wild-type C57BL/6 mice were purchased from the National Cancer Institute-Frederick (Frederick, MD). Male mice, 10–12 wk of age, were used for experiments. The mice were kept under 12-h light–dark cycle conditions, with free access to food and water. All experiments were conducted in accordance with protocols approved by the University of Alabama at Birmingham Animal Care and Use Committee.

**Measurement of resistin in serum of acute respiratory distress syndrome and septic patients**

The study protocol was approved by the local ethics committee (Centre Hospitalier Universitaire Rennes), and written informed consent was obtained from the patient or their closest relative. We studied 27 patients (13 male, 14 female) who were admitted to the Medical ICU at Rennes University Hospital; they were compared with healthy volunteers. Acute respiratory distress syndrome (ARDS; n = 8) was defined using the Berlin definition (37). Septic shock (n = 13) was defined according to internationally accepted criteria (38).

**Materials**

Recombinant human resistin expressed in HEK293 cells was purchased from AdipoGene (San Diego, CA). Compound C was obtained from Millipore (Billerica, MA). AICAR was from Enzo Life Sciences (Plymouth Meeting, PA). PMA and Escherichia coli 0111:B4 endotoxin (LPS) were purchased from Sigma-Aldrich (St. Louis, MO). Custom Ab mixtures (Abs) and negative-selection columns for neutrophil isolation were from STEMCELL Technologies (Vancouver, BC, Canada), whereas Abs to phospho–Thr172-AMPKα and total AMPKα, as well as NADPH oxidase subunit p47<sup>phox</sup>, were obtained from Cell Signaling Technology (Danvers, MA). Histone 3 and β-actin Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti–citrulline–histone 3 Ab and mouse mAb to histone 3–FITC were obtained from Abcam (Cambridge, MA). Emulsion oil solution containing DAPI was from Vector Laboratories (Burlingame, CA). SYTOX Green probe was purchased from Invitrogen (Carlsbad, CA).

**Isolation of neutrophils**

Bone marrow neutrophils were purified using a negative-selection column purification system, as previously described (27, 39). Briefly, bone marrow cell suspensions were isolated from the femur and tibia of a mouse by flushing with RPMI 1640 medium. Negative selection to purify neutrophils was performed by incubation of the cell suspension with biotinylated primary Abs specific for the cell surface markers F4/80, CD4, CD45R, CD5, and TER119 (STEMCELL Technologies) for 15 min at 4˚C, followed by incubation with anti-biotin tetrameric Abs (STEMCELL Technologies) for 15 min. The complex of anti-tetrameric Abs and cells was incubated with colloidal magnetic dextran iron particles (STEMCELL Technologies) for an additional 15 min at 4˚C. The T cells, B cells, RBCs, monocytes, and macrophages were captured in a column surrounded by a magnet, allowing the neutrophils to pass through. Neutrophil purity, as determined by Wright–Giemsa–stained cytopsin preparations, was consistently >98%. Viability of purified bone marrow neutrophils was determined after trypan blue staining and was consistently >95%. Human neutrophils were isolated from the peripheral blood of healthy donors using a CD16 MicroBeads Magnetic Cell Sorting Kit (MACS; Miltenyi Biotec, San Diego, CA), according to the manufacturer’s instructions. The CD16<sup>+</sup> cells (neutrophils) were collected and suspended in RPMI 1640 medium.

**Purification and culture of peritoneal macrophages**

Peritoneal macrophages were elicited in 8–10-wk-old mice using Brewer thioglycollate injected i.p. Cells were collected 4 d after injection of Brewer thioglycollate and plated in 48-well plates (2.5 × 10<sup>5</sup> cells/well) in RPMI 1640 medium.

**ELISA**

Human resistin, TNF-α, and MIP-2 were measured in serum, neutrophil culture media, or bronchoalveolar lavage (BAL) using ELISA kits (R&D Systems, Minneapolis, MN), according to the manufacturer’s instructions and as previously described (40, 41).

**Western blot analysis**

Western blot analysis was performed as described previously (27, 42, 43). Briefly, cell lysates were mixed with Laemmli sample buffer and boiled for 15 min. Equal amounts of proteins were resolved by NaDodSO<sub>4</sub>-PAGE and transferred onto polyvinylidene fluoride membranes (Immobilon; Millipore). The membranes were probed with specific Abs, as described in the figure legends, followed by detection with HRP-conjugated goat anti-rabbit IgG or anti-mouse IgG. Bands were visualized by ECL (Super Signal; Pierce Biotechnology, Rockford, IL) and quantified by AlphaEaseFC software (Alpha Innotech, San Leandro, CA).

**Measurement of NET-derived DNA**

Bone marrow neutrophils (2 × 10<sup>5</sup> cells) were seeded in Costar 96-well black plates (Costar, Corning, NY) in the presence of 0.5% FBS and SYTOX Green (5 μM), a cell-impermeable DNA-binding dye. Platelets were purified from whole blood collected in sodium citrate anticoagulant tubes, and platelet-rich plasma was obtained by centrifugation. The cells were incubated with human resistin or PMA in the presence of platelets (10<sup>5</sup> cells) for the indicated time at 37˚C, and free DNA in culture medium was measured using time-dependent fluorescence of SYTOX Green probe (FLUOSTAR OPTIMA spectrophotometer microplate reader; BMG LABTECH, Alexandria, VA), an excitation wavelength of 492 nm and an emission wavelength of 530 nm. To measure release of DNA in the lung of mice, BAL fluid (50 μl) was incubated with 50 μl SYTOX Green (5 μM) for 10 min, followed by reading SYTOX Green fluorescence.

**Imaging NETs and extracellular histone**

Neutrophils were cultured on poly-l-lysine–coated glass coverslips and treated as described in the figure legends. Next, cells were gently washed with PBS and incubated with paraformaldehyde (4%) for 30 min at room temperature. Cells were subsequently incubated with PBS/BSA (3%) for 30 min at room temperature and anti-histone 3–FITC–labeled Ab for an additional 30 min. Neutrophils were washed with PBS, and samples were mounted with emulsion oil solution containing DAPI to visualize nuclear and released DNA. Confocal microscopy was performed, as previously described, using a confocal laser scanning microscope (LSM 710 confocal microscope; Carl Zeiss MicroImaging, Jena, Germany) provided by the High Resolution Imaging Facility at the University of Alabama at Birmingham (33).

**ALL model**

ALL was induced by intratracheal (i.t.) administration of 2 mg/kg LPS in 75 μl PBS, as previously described (27, 34, 44–46). With this model, ALL is characterized by neutrophil infiltration into the lung interstitium and airways, development of interstitial edema, and increased pulmonary proinflammatory cytokine production, with the greatest degree of injury being
present 24 h after LPS exposure (27, 47). ALI was induced in hRTN−/− or hRTN+/− mice by i.t. instillation of LPS. At 24 h after LPS administration, BALs were obtained by cannulating the trachea with a blunt 20-gauge needle and then lavaging the lungs three times with 1 ml iced PBS. Samples were subjected to ELISA or Western blot analysis. In additional experiments, lungs were processed to paraffin sections followed by H&E staining, as previously described (45).

Wet-to-dry lung weight ratios
Separate groups of mice were used to measure wet-to-dry ratios and for BAL fluid acquisition. The wet-to-dry ratio was determined as reported previously (45, 47). All mice used for lung wet-to-dry weight ratios were of identical ages. Lungs were excised, blotted, and then weighed to obtain the “wet” weight. Lungs were dried in an oven at 80°C for 7 d to obtain the “dry” weight.

Statistical analysis
Statistical significance was determined by the Wilcoxon rank-sum test (independent two-group Mann–Whitney U test), as well as the Student t test for comparisons between two groups. Multigroup comparisons were performed using one-way ANOVA with the Tukey post hoc test. A p value < 0.05 was considered significant.

Results
Resistin is increased in the circulation of patients with ARDS or sepsis
As shown in Fig. 1A and B, resistin was dose dependently released from LPS-treated human neutrophils and from dHL-60 cells, a human cell line that, upon differentiation, resembles primary neutrophils. Elevated amounts of resistin in the peripheral circulation were found in critically ill patients, including patients with ARDS or sepsis (Fig. 1C). In particular, the highest levels of resistin were present in plasma of septic patients.

Expression of human resistin by neutrophils and macrophages isolated from hRTN−/− and hRTN+/− mice
Both human and murine resistin are implicated in the development of insulin resistance (4, 6, 48). However, murine resistin is exclusively expressed in adipocytes, whereas human resistin is primarily expressed in leukocytes (1, 3, 49). To delineate the effects of human resistin on neutrophil proinflammatory activation and development of ALI, we used humanized resistin mice (hRTN+/−) (i.e., mice that express human resistin but are deficient in murine resistin) (5). As shown in Fig. 1D, whereas modest amounts of resistin were produced by hRTN+/− bone marrow neutrophils, much greater amounts of human resistin were present after culture of hRTN+/− peritoneal macrophages. Human resistin also was detected in the serum of unmanipulated hRTN+/− mice (Fig. 1D, right panel). Of note, the levels of human resistin in the serum of hRTN+/− mice were similar to those found in the circulation of septic patients (Fig. 1C).

Human resistin inhibits AMPK activation and enhances proinflammatory activity of LPS-stimulated neutrophils
To determine the effects of human resistin on neutrophil activation, hRTN−/− or hRTN+/− neutrophils were incubated or not with LPS, followed by measurement of cytokines in culture media. As shown in Fig. 2A, expression of human resistin significantly increased TNF-α and MIP-2 production by LPS-treated neutrophils. Of note, despite the production of human resistin, little or no release of TNF-α or MIP-2 was found in unstimulated RTN+/− neutrophils. Such results suggest that rather than directly inducing proinflammatory activation of neutrophils, human resistin “primed” neutrophils for a more robust response upon LPS/TLR4 engagement.

Previous studies suggested that resistin-mediated development of insulin resistance was associated with inhibition of AMPK activation (7, 35). The activation status of AMPK plays an important role in regulating the proinflammatory responses of many cell types, including neutrophils and macrophages (27). As shown in Fig. 2B, exposure to the AMPK activator AICAR dose dependently diminished TNF-α production after LPS stimulation in hTRN−/− neutrophils. However, the inhibitory effects of AICAR were diminished in neutrophils that expressed human resistin. As shown in Fig. 2C and 2D, treatment with AICAR increased

FIGURE 1. Resistin is expressed by LPS-stimulated neutrophils and humanized resistin mice, as well as in critically ill patients. Human resistin was measured in the culture medium of LPS-treated peripheral human neutrophils (A) or differentiated HL-60 cells (B). (A) Neutrophils were incubated with the indicated concentrations of LPS for 4 h. Data are mean ± SD (n = 3). (B) HL-60 leukocytes were differentiated into the surrogate PMNs using 1.3% DMSO treatment over 5 d. Resistin in the culture medium of HL-60 cells was determined after exposure to LPS for 24 h. Data are mean ± SEM (n = 4). (C) Amount of human resistin in plasma of normal (control), ARDS, and septic patients. Data are means (n = 7–13). (D) Human resistin was measured in the media of bone marrow neutrophils and peritoneal macrophages cultured in serum-free conditions for 4 h. Resistin also was measured in the serum of murine resistin-deficient mice (bRTN−/−) and humanized resistin mice (bRTN+/−). Data are mean ± SD (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001.
FIGURE 2. Human resistin diminished AMPK activity and increased neutrophil sensitivity to LPS challenge. (A) Levels of TNF-α and MIP-2 were determined in the culture media of hRTN+/− and hRTN+/− neutrophils. Cells were incubated with LPS (30 ng/ml) for 4 h, and media were subjected to ELISA. Data are mean ± SD (n = 3).(B) Amount of TNF-α in culture media obtained from hRTN+/− or RTN−/− neutrophils that were treated with AICAR for 60 min, followed by exposure to LPS (0 or 30 ng/ml) for 4.5 h. Data are mean ± SD (n = 4). Representative Western blots (C) and optical densitometry (D) show phospho-Thr172-AMPK, AMPK, and β-actin obtained from hRTN+/− or RTN−/− neutrophils treated with AICAR (0, 0.1 or 0.3 mM) for 60 min. Data are mean ± SEM (n = 3–4). *p < 0.05,**p < 0.001.

phosphorylation of AMPK to a greater extent in hRTN+/− neutrophils compared with hRTN+/− neutrophils. These data suggest that human resistin increases neutrophil sensitivity to LPS-induced cytokine production through inhibiting AMPK activation.

**Human resistin stimulates NET formation**

Extensive NET formation was shown to perpetuate inflammation and cardiovascular complications (25, 50). Although NETs also were found in the lungs of mice with LPS-induced ALI (23), the relationship between resistin and NET formation has not been described. To address this issue, bone marrow neutrophils isolated from wild-type mice were cultured on poly-D-lysine–coated glass described. To address this issue, bone marrow neutrophils isolated and cardiovascular complications (25, 50). Although NETs also were found in the lungs of mice with LPS-induced ALI (23), the relationship between resistin and NET formation has not been described. To address this issue, bone marrow neutrophils isolated from wild-type mice were cultured on poly-D-lysine–coated glass coverslips and treated or not with recombinant human resistin. Experiments also were performed in the presence of isolated platelets, because platelets were shown to stimulate NETosis (22, 23, 51). Neutrophils also were incubated with PMA, an effective activator of NET formation (22, 23, 52). Fig. 3A shows that exposure of neutrophils to human resistin resulted in NET formation, as evidenced by DNA staining with DAPI and direct immunodetection of histone 3 with FITC-labeled anti-H3 Abs. In particular, the appearance of extracellular histone 3 colocalized with staining for chromatin DNA, indicative of NET formation. NETosis was further confirmed by measuring the concentrations of free DNA in culture medium using SYTOX Green fluorogenic probe, which becomes fluorescent upon binding to DNA (Fig. 3B) (53). Western blotting also revealed significant increases in extracellular histone 3 in culture media that were collected from resistin-treated neutrophils (Fig. 3C). Of note, after 18 h of culture, the amount of free DNA in the media was significantly increased in hRTN+/− neutrophils compared with hRTN+/− neutrophils (Fig. 3D).

**AMPK inhibition promotes resistin-induced NET formation**

Recent studies showed that NADPH oxidase and reactive oxygen species were implicated in the formation of NETs (52, 54). As shown in Fig. 3E, exposure to human resistin increased the citrullination of histone 3, a process known to stimulate chromatin decondensation prior to deployment of NETs. In addition to chromatin relaxation, Western blotting revealed that human resistin dose dependently increased phosphorylation of the NADPH oxidase subunit p40phox (Fig. 3F). These results suggest that human resistin induces NET formation through mechanisms that are likely to involve assembly of NADPH components that accompany neutrophil priming.

Because resistin inhibited AMPK activation, we next examined whether compound C, an AMPK inhibitor, could affect NETosis. We found that inclusion of compound C in neutrophil cultures enhanced release of extracellular histone 3 to a similar extent as that found after PMA treatment; additive effects were observed when neutrophils were treated with both PMA and compound C (Fig. 3G). Of note, inclusion of the AMPK activator AICAR diminished resistin-induced increases in histone 3 citrullination (Fig. 3H). These results suggest that cross-talk between resistin and AMPK signaling is an important regulatory mechanism in neutrophil NETosis.

**Increased severity of LPS-induced ALI in humanized resistin mice**

To explore the effects of human resistin on ALI, hRTN+/− and control RTN−/− mice were subjected to i.t. instillation of saline (control) or LPS. Despite increased concentrations of resistin in serum and BAL of humanized resistin mice, expression of inflammatory mediators or evidence of lung injury was negligible in saline-treated hRTN+/− or resistin-deficient mice. In contrast, administration of LPS significantly increased lung injury in hRTN+/− mice compared with RTN−/− mice. In particular, increased wet-to-dry ratios, indicative of more severe interstitial pulmonary edema, were present in LPS-treated hRTN+/− mice (Fig. 4A). Compared with resistin-deficient mice (RTN−/−), exposure to LPS increased the numbers of total white cells and neutrophils in BALs isolated from LPS-treated hRTN+/− mice compared with control mice (Fig. 4B). Histological analysis of the
lungs showed increased tissue damage and enhanced neutrophil infiltration in hRTN+/−/− mice compared with hRTN−/−/− mice. Significant increases in human resistin concentrations were present in BALs from untreated hRTN−/−/− or hRTN+/−/− mice. Representative Western blots show levels of citrullinated histone 3, histone 3, and β-actin in neutrophils after treatment with purified hRTN (1 μg/ml) or compounds (Fig. 6E). Levels of p-p40phox in neutrophils treated with hRTN for 3 h (Fig. 6F). Western blot analysis of extracellular histone 3 in culture media of neutrophils treated with compound C (com. C; 10 μM) for 30 min, followed by incubation with PMA (100 nM) for 3 h. Data are mean ± SD (n = 3–5). *p < 0.05. (H) Amount of citrullinated histone 3, histone 3, and β-actin in neutrophils treated with AICAR (0 or 0.3 mM) for 60 min and then inclusion of purified hRTN (1 μg/ml) for the indicated times.

Although exposure of humanized resistin mice to LPS was associated with more severe ALI than in wild-type mice, such results may reflect the absence of mouse resistin in hRTN+/−/− mice. Therefore, to more directly determine the contribution of human resistin to the severity of ALI, wild-type mice were given i.t. instillation of purified human resistin (0.5 mg/kg), LPS (2 mg/kg), or the combination of human resistin and LPS. As shown in Fig. 6A and 6C, significantly higher numbers of total white cells and neutrophils, as well as greater increases in TNF-α and MIP-2, were found in the BALs of mice given both human resistin and LPS compared with LPS alone. Of note, i.t. administration of human resistin alone had negligible effects on BAL white cells or cytokine levels (Fig. 6A–C). In additional experiments, we examined the effects of human resistin on AMPK phosphorylation in the lung. As shown in Fig. 6D, i.t. administration of human resistin...
or LPS alone resulted in a modest decrease in AMPK phosphorylation, and exposure to LPS and human resistin produced an even greater decrease. Of note, previous studies showed that inhibition of AMPK activation potentiated the proinflammatory effects of LPS (55).

**Activation of AMPK in vivo partially attenuates LPS-induced ALI in humanized resistin mice**

Because previous studies showed that pharmacologically induced activation of AMPK with AICAR diminished LPS-induced ALI (27), we examined whether a similar approach affects the severity of pulmonary injury in humanized resistin mice. In these experiments, hRTN+/−/− mice were treated with saline (control, i.p.) or AICAR (i.p., 500 mg/kg) for 4 h, followed by i.t. injection of LPS (2 mg/kg), and were sacrificed 24 h later to determine the severity of lung injury. As shown in Fig. 6E and 6F, administration of AICAR resulted in decreased neutrophil accumulation in the lungs, as well as diminished levels of BAL TNF-α, compared with saline-treated mice.

**Discussion**

In these studies, we showed that human resistin enhances neutrophil proinflammatory responses to LPS stimulation. Similar to the ability of resistin to enhance TLR4-induced neutrophil activation in vitro, more severe LPS-induced ALI was present in mice that express human resistin (hRtn+/−/−). Although previous studies suggested that resistin had proinflammatory action, our results indicate that human resistin itself has only modest effects on TNF-α or MIP-2 production by neutrophils. However, exposure of neutrophils to resistin appears to prime them for enhanced activation when subsequently stimulated by LPS. Similarly, despite the presence of considerable amounts of human resistin in the circulation under basal conditions in hRtn+/−/− mice, only a minimal increase in cytokines was found in BALs. In contrast, the severity of lung injury was significantly increased in hRtn+/−/− mice after pulmonary instillation of LPS. Of note, similar to our results, excessive inflammatory responses in liver and skeletal muscle were reported in mice given resistin and LPS (19). Overall, these results suggest that resistin contributes to proinflammatory responses and the development of more severe organ injury through mechanisms that appear to involve either resistin-mediated priming effects or synergistic interactions with TLR4 and a putative resistin receptor.

Several possible mechanisms may be involved in the ability of human resistin to increase neutrophil sensitivity to LPS. Among

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**FIGURE 4.** Human resistin increases severity of LPS-induced ALI. hRTN+/−/− or control RTN+/−/− mice were subjected to i.t. application of LPS (0 or 2 mg/kg) for 24 h. **(A)** Lung wet-to-dry ratios were measured 24 h after LPS administration. Fold increase above values present in mice receiving saline alone are shown. Data are mean ± SEM (n = 4–5). **(B)** Numbers of total white cells and neutrophils in BAL fluid from saline-treated (-LPS) or LPS-treated (+LPS) mice. Data are mean ± SD (n = 3–6). **(C)** Human resistin was measured in BALs of hRTN+/−/− or hRTN+/−/− mice treated with saline (-LPS) or LPS (+LPS) for 24 h. Data are mean ± SD (n = 5). **(D)** Representative H&E staining of lung sections obtained from control (saline) or LPS-treated hRTN+/−/− or RTN+/−/− mice. Original magnification ×20. *p < 0.05, **p < 0.01, ***p < 0.001.
these are potentiation of TLR4/NF-κB signaling (60), alteration in insulin signaling (4, 6), and inhibition of AMPK activation, which was shown to have anti-inflammatory properties (6, 7, 35, 36). Indeed, our data showed decreased phosphorylation of AMPK, as well as diminished ability of AICAR to activate AMPK in hRtn+/− neutrophils. Consistent with the ability of AMPK activation to inhibit

**FIGURE 5.** Human resistin increases production of proinflammatory mediators, including NET-associated histone 3 and HMGB1 in BALs of humanized resistin mice. The levels of TNF-α (A), MIP-2 (B), free DNA (C), extracellular histone 3 (D), and HMGB1 (E) were determined in BAL fluids obtained from hRtn+/− or RTN−/− mice that were treated with saline (-LPS) or LPS (+LPS; 0 or 2 mg/kg) for 24 h. Data are mean ± SD (n = 3–6). *p < 0.05, **p < 0.001.

**FIGURE 6.** Coadministration of human resistin and LPS increases the severity of ALI. (A–D) Wild-type mice were subjected to i.t. instillation of purified human resistin (0.5 mg/kg), LPS (2 mg/kg), or both, and BALs were obtained 24 h later. Numbers of total white cells and neutrophils (A), as well as levels of TNF-α (B) and MIP-2 (C), in BAL. Data are mean ± SD (n = 4). (D) Representative Western blots showing phospho-Ser172 AMPK, total AMPK, and β-actin in lung homogenates. (E and F) hRTN+/− mice were treated with saline or AICAR (i.p., 500 mg/kg) 4 h prior to i.t. instillation of LPS (2 mg/kg). Numbers of neutrophils (E) and levels of TNF-α (F) in BAL fluids were measured 24 h after LPS injection. Data are mean ± SD (n = 4). *p < 0.05, **p < 0.01.
inflammatory responses, decreased activation of AMPK in hRtn+/− neutrophils compared with hRtn+/+ neutrophils was associated with more robust TNF-α production after LPS treatment. In previous studies, pharmacologic inhibition of AMPK or genetic deficiency resulted in more robust activation of the TLR4/NF-κB-signaling cascade and production of inflammatory mediators in LPS-stimulated neutrophils or IFN-γ-stimulated astrocytes and microglia (61). Similarly, exposure of cells to the AMPK activators metformin, AICAR, or berberine diminished TLR4-induced activation of neutrophils, macrophages, and endothelial cells (27, 33, 62). The severity of endotoxin-induced ALI was decreased in mice treated with either metformin or AICAR (27, 45, 63). Of note, AICAR partially attenuates LPS-induced ALI in humanized resistin mice. AMPK activation also was shown to be associated with improvement of vascular integrity in murine models of ALI and of airway remodeling in preclinical models of asthma (55, 64).

In addition to the ability of human resistin to increase proinflammatory cytokine production by neutrophils cultured with LPS, exposure of neutrophils to resistin resulted in enhanced phosphorylation of the NADPH oxidase subunit p47phox and NET formation, as well as increased extracellular concentrations of the alarmins HMGB1 and histone 3 in association with NETs. Similarly, increased NET formation was found in lungs of hRtn+/− mice subjected to LPS instillation. Although the precise mechanism responsible for the ability of resistin to induce NETs has not been determined, NET formation, as well as release of the intranuclear proteins HMGB1 and histone 3, was shown to be coupled with more robust inflammatory responses (22, 50, 65). For example, HMGB1 was shown to contribute to the development of more severe ALI or sepsis (65). Similar to resistin, HMGB1 itself has modest proinflammatory effects that are potentiated by combination with other inflammatory insults, including TLR2 or TLR4 agonists or IL-1 (66). Our results also suggest that AMPK activation contributes to NET formation. In particular, activated AMPK appears to modulate NET formation through mechanisms that involved inhibition of chromatin decondensation, an essential step that precedes DNA deployment in NETosis (52, 67). Previous studies showed that NADPH oxidase is a decondensation, an essential step that precedes DNA deployment in inflammatory insults, including TLR2 or TLR4 agonists or IL-1 (66).

Disclosures
The authors have no financial conflicts of interest.

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