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Human resistin promotes neutrophil pro-inflammatory activation, neutrophil extracellular trap formation, and increases severity of acute lung injury

Shaoning Jiang[*], **Dae Won Park**[*,†], **Jean-Marc Tadie**[*,‡], **Murielle Gregoire**[#], **Jessy Deshane**[*], **Jean Francois Pittet**[§], **Edward Abraham**[¶], and **Jaroslaw W. Zmijewski**

[*]Department of Medicine, University of Alabama at Birmingham, Birmingham, AL 35294-0012, USA

[§]Department Anesthesiology, University of Alabama at Birmingham, Birmingham, AL 35294-0012, USA

[†]Division of Infectious Diseases, Korea University Ansan Hospital, Ansan 425-707

[‡]Service des Maladies Infectieuses et Réanimation Médicale, Centre Hospitalier Universitaire, Rennes, France

[#]INSERM, UMR U917, Université de Rennes 1, Rennes, France

[¶]Office of the Dean, Wake Forest University School of Medicine, Winston-Salem, NC 27157.

Abstract

Although resistin was recently found to modulate insulin resistance in preclinical models of type II diabetes and obesity, recent studies have also suggested that resistin has proinflammatory properties. In these studies, we examined if the human specific variant of resistin affects neutrophil activation as well as the severity of LPS-induced acute lung injury (ALI). Because human and mouse resistin have distinct patterns of tissue distribution, experiments were performed using resistin humanized 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*$^{+/-/-}$, compared to control *Rtn*$^{-/-/-}$ neutrophils. Expression of human resistin inhibited the activation of AMP-activated protein kinase (AMPK), a major sensor and regulator of cellular bioenergetics that is also implicated in inhibiting inflammatory activity of neutrophils and macrophages. In addition to the ability of resistin to sensitize neutrophils to LPS stimulation, human resistin also enhanced neutrophil extracellular trap formation. In LPS-induced ALI, humanized resistin mice demonstrated enhanced production of pro-inflammatory cytokines, more severe pulmonary edema, increased NET 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 may be a target for future therapies aimed at diminishing the severity of acute lung injury and other inflammatory situations where neutrophils play a major role.

Correspondence: Jaroslaw W. Zmijewski, Ph.D. Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine University of Alabama at Birmingham 901 19th St. South, BMRII 304. Birmingham, AL 35294. Phone: (205) 934-7793 | Fax: (205) 934-7437 | zmijewsk@uab.edu.

Keywords

AICAR; human resistin; inflammation; acute lung injury

INTRODUCTION

Resistin is a secretory cysteine-rich protein that belongs to the FIZZ (Found in Inflammatory Zone) protein family, and is characterized as an insulin resistance factor found in mice model of type II diabetes and obesity (1-4). Recent studies have shown 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 (T2DM) (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 resistins 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 expression of rodent resistin which is limited only 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). Whereas intraperitoneal administration of purified murine resistin has modest inflammatory effects in mice, marked increase in the severity of liver injury only resulted 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 pro-inflammatory activation, often accompanied with 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, have established the important link between metabolism, neutrophil activation, and inflammation (27-29). Although AMPK is a major metabolic sensor and regulator of energy production (30, 31), AMPK can also inhibit NF-κB associated signaling in TLR2 or TLR4-stimulated neutrophils and macrophages (27, 32). Moreover, mice that received the AMPK activators metformin or 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, in the present studies we examined the hypothesis that interactions between human resistin and AMPK result in increased neutrophil pro-inflammatory activation and enhances 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 ($RTN^{-/-/}$) as well as humanized resistin mice ($hRTN^{+/-/-}$), that exclusively expressed the human variant of resistin.

MATERIALS AND METHODS

Mice

The "humanized resistin mice" ($hRTN^{+/-/-}$) and the resistin knockout mice ($RTN^{-/-/-}$) were generated by Dr. Mitchell A. Lazar as previously described (5). Briefly, the transgenic mice on the C57BL/6 background with expression of human resistin under the control of CD68 promoter were bred to C57BL6 resistin knockout mice ($RTN^{-/-/-}$) to generate mice that express human resistin but are lack of murine resistin (5). Wild type C57BL/6 mice were purchased from the National Cancer Institute-Frederick (Frederick, MD, USA). Male mice, 10 to 12 weeks of age, were used for experiments. The mice were kept on a 12-h light-dark cycle 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 resistin in serum of ARDS and septic patients

The study protocol was approved by the local ethics committee (CHU 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, France. These patients were compared to healthy volunteers. 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). 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) was from Enzo Life Science (Plymouth Meeting, PA, USA). Phorbol myristate acetate (PMA) and *E. coli* 0111:B4 endotoxin (LPS) was purchased from Sigma-Aldrich (St. Louis, MO). Custom antibody mixtures (Abs) and negative selection columns for neutrophil isolation were from StemCell Technologies (Vancouver, British Columbia, Canada) whereas antibodies to phospho-Thr172-AMPKα and total AMPKα, and NADPH oxidase subunit p-$p40^{phox}$ were obtained from Cell signal (Danvers, MA). Histone 3 and β-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti- citrulline-histone 3 antibody and mouse

monoclonal antibody to histone 3-FITC were obtained from Abcam (Cambridge, MA). Emulsion oil solution containing 4',6-diamidino-2-phenylindole (DAPI) was from Vector Laboratories (Burlingame, CA). Sytox Green probe were 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 antibodies specific for the cell surface markers F4/80, CD4, CD45R, CD5, and TER119 (StemCell Technologies, Vancouver, BC, Canada,) for 15 minutes at 4°C followed by incubation with anti-biotin tetrameric antibodies (StemCell Technologies) for 15 minutes. The complex of anti-tetrameric antibodies and cells were then incubated with colloidal magnetic dextran iron particles (StemCell Technologies) for an additional 15 minutes at 4°C. The T cells, B cells, red blood cells, 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 cytospin preparations, was consistently greater than 98. Viability of purified bone marrow neutrophils was determined after trypan blue staining and was consistently greater than 95%. Human neutrophils were isolated from the peripheral blood of healthy donors using CD16 microbeads magnetic cell sorting kit (MACS, Miltenyi Biotechnology, San Diego, CA)) according to manufacturer's instructions. The CD16-positive cells (neutrophils) were collected and were suspended in RPMI 1640 medium.

Purification and culture of peritoneal macrophages

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

ELISA

Human resistin, TNF-α and MIP-2 were measured in serum, neutrophil culture media or bronchoalveolar lavages 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 minutes. Equal amounts of proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred onto polyvinylidene fluoride (PVDF) membranes (Immobilon P; Millipore, Billerica, MA). The membranes were probed with specific antibodies as described in the figure legends followed by detection with HRP-conjugated goat anti-rabbit IgG or anti-mouse IgG. Bands were visualized by enhanced chemiluminescence (Super Signal; Pierce

Biotechnology, Rockford, IL) and quantified by AlphaEaseFC software (Alpha Innotech, San Leandro, CA).

Measurement NETs-derived DNA

Bone marrow neutrophils (2×10^5 cells) were seeded in Costar 96-well black plates (Corning, MA) in the presence of 0.5% fetal bovine serum 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 (10^6 cells) for indicated time at 37°C and then free DNA in culture medium was measured using time dependent fluorescence of Sytox Green probe (Fluostar OPTIMA spectrophotometer, (BMG LABTECH Microplate Readers, Alexandria, VA, USA), at an excitation wavelength of 492 nm and an emission wavelength 530 nm. To measure release of DNA in the lung of mice, BAL fluid (50 μl) was incubated with 50 μl of Sytox Green (5 μM) for 10 minutes 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 figure legends. Next, cells were gently washed with PBS and incubated with paraformaldehyde (4%) for 30 minutes at room temperature. Cells were subsequently incubated with PBS/BSA (3%) for 30 minutes at room temperature, and anti-histone 3 - FITC labeled antibody for additional 30 minutes. 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 (model LSM 710 confocal microscope; Carl Zeiss Micro Imaging, Germany) provided by the High Resolution Imaging Facility at the University of Alabama at Birmingham (33).

Acute Lung Injury Model

Acute lung injury was induced by intratracheal administration of 2 mg/kg LPS in 75 μl of PBS as previously described (27, 34, 44-46). With this model, ALI is characterized by neutrophil infiltration into the lung interstitium and airways, development of interstitial edema, and increased pulmonary pro-inflammatory cytokine production, with the greatest degree of injury being present 24 hours after LPS exposure (27, 47). ALI was induced in $hRTN^{-/-/-}$ or $hRTN^{+/-/-}$ mice by intratracheal instillation of LPS. At 24 hours after LPS administration, bronchoalveolar lavages (BAL) was obtained by cannulating the trachea with a blunt 20-gauge needle and then lavaging the lungs three times with 1 ml of iced PBS. Samples were subjected to ELISA or Western blot analysis. In additional experiments, lungs were processed to paraffin sections followed by hemotoxylin/eosin 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 then dried in an oven at 80°C for 7 days 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 Student's *t* test for comparisons between two groups. Multigroup comparisons were performed using one-way ANOVA with Tukey's *post hoc* test. A value of *P* less than 0.05 was considered significant.

RESULTS

Resistin is increased in the circulation of patients with ARDS and sepsis

As shown in Figures 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 and sepsis (Figures 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). In order to delineate the effects of human resistin on neutrophil pro-inflammatory activation and development of ALI, we utilized “humanized resistin mice” (*hRTN*$^{+/-/-}$), i.e. mice that express human, but are deficient in murine resistin (5). As shown in Figure 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 was also detected in the serum of unmanipulated *hRTN*$^{+/-/-}$ mice (Figure 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 (Figure 1C).

Human resistin inhibits AMPK activation and enhances pro-inflammatory activity of LPS-stimulated neutrophils

To determine the effects of human resistin on neutrophil activation, *hRTN*$^{-/-/-}$ or *hRTN*$^{+/-/-}$ neutrophils were incubated with or without LPS followed by measurement of cytokines in culture media. As shown in Figure 2A, expression of human resistin significantly increased TNF-α and MIP-2 production by LPS-treated neutrophils. Of note, in spite of 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 pro-inflammatory 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 pro-inflammatory responses of many cell types, including neutrophils and macrophages (27). As shown in Figure 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 Figures 2C and D, treatment with AICAR increased phosphorylation of AMPK to a greater extent in $hTRN^{-/-/-}$ as compared to that found in $hTRN^{+/-/-}$ neutrophils. These data suggest that human resistin increases neutrophil sensitivity to LPS-induced cytokine production through inhibiting AMPK activation.

Human resistin stimulates neutrophil extracellular trap formation

Extensive NETs formation has been shown to perpetuate inflammation and cardiovascular complications (25, 50). Although NETs were also 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 with or without recombinant human resistin. Experiments were also performed in the presence of isolated platelets, as platelets have previously been shown to stimulate netosis (22, 51). Neutrophils were also incubated with PMA, an effective activator of NET formation (22, 23, 52). Images in Figure 3A show that exposure of neutrophils to human resistin resulted in NETs formation, as evidenced by DNA staining with DAPI and direct immunodetection of histone 3 with FITC-labeled anti-H3 antibodies. In particular, the appearance of extracellular histone 3 co-localized 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 (Figure 3B) (53). Western blotting also revealed significant increases of extracellular histone 3 in culture media that were collected from resistin-treated neutrophils (Figure 3C). Of note, after 18 hours of culture, the amount of free DNA in the media was significantly increased in $hRTN^{+/-/-}$ as compared to $hRTN^{-/-/-}$ neutrophils (Figure 3D).

AMPK inhibition promotes resistin-induced NETs formation

Recent studies have shown that NADPH oxidase and reactive oxygen species were implicated in the formation of NETs (52, 54). As shown in Figure 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 $p40^{phox}$ (Figure 3F). These results suggest that human resistin induces NETs 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 (Figure 3G). Of note, inclusion of the AMPK activator AICAR diminished resistin-induced increases in histone 3 citrullination (Figure 3H). These results suggest that crosstalk between resistin and AMPK signaling is an important regulatory mechanism involved in neutrophil netosis.

Increased severity of LPS-induced acute lung injury in humanized resistin mice

To explore the effects of human resistin on acute lung injury, *hRTN*$^{+/-/-}$ and control *RTN*$^{-/-/-}$ mice were subjected to intratracheal (i.t.) instillation of saline (control) or LPS. In spite of increased concentrations of resistin in serum and bronchoalveolar lavage (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*$^{+/-/-}$ as compared to *RTN*$^{-/-/-}$ mice. In particular, increased wet to dry ratios, indicative of more severe interstitial pulmonary edema, were present in LPS treated *hRTN*$^{+/-/-}$ mice (Figure 4A). Compared to resistin deficient mice (*RTN*$^{-/-/-}$), exposure to LPS increased the numbers of total white cells and neutrophils in bronchoalveolar lavages isolated from LPS treated *hRTN*$^{+/-/-}$ as compared to control mice (Figure 4B). Histological analysis of the lungs showed increased tissue damage and enhanced neutrophil infiltration in *hRTN*$^{+/-/-}$ compared to h*RTN*$^{-/-/-}$ mice after LPS exposure (Figure 4D). Although modest amounts of resistin were present at baseline in BAL from *hRTN*$^{+/-/-}$ untreated mice, significant increases in human resistin concentrations were present in the BALs of LPS-treated *hRTN*$^{+/-/-}$ mice (Figure 4C). Compared to TNF-α and MIP-2 concentrations found in BALs from LPS treated *hRTN*$^{-/-/-}$ mice, even higher concentrations of these cytokines were present in LPS-treated *hRTN*$^{+/-/-}$ mice (Figures 5A and B). *hRTN*$^{+/-/-}$ mice also showed increased markers of NET formation in the lungs after LPS exposure, including free DNA (Figure 5C) and enrichment of HMGB1 (Figure 5E) and histone 3 (Figure 5D) in BALs. These results indicate that human resistin contributes to a more robust pro-inflammatory pulmonary response in LPS-induced acute lung injury.

Although exposure of humanized resistin mice to LPS was associated with more severe acute lung injury than in wild type mice, such results may reflect the absence of mouse resistin in the *hRTN*$^{+/-/-}$ mice. Therefore, to more directly determine the contribution of human resistin to severity of ALI, wild type mice were given intratracheal (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 Figures 6A and 6C, significantly higher numbers of total white cells and neutrophils as well as greater elevations in TNF-α and MIP-2 were found in the BALs of mice given both human resistin and LPS as compared to LPS alone. Of note, intratracheal administration of human resistin alone had negligible effects on BAL white cells or cytokine levels (Figures 6A, 6B, and 6C). In additional experiments, we examined the effects of human resistin on AMPK phosphorylation in the lung. As shown in Figure 6D, intratracheal administration of human resistin or LPS alone resulted in modest decrease in AMPK phosphorylation, and exposure to LPS and human resistin produced even greater decrease in AMPK phosphorylation. Of note, previous studies have shown that inhibition of AMPK activation potentiated the proinflammatory effects of LPS (55).

Activation of AMPK *in vivo* partially attenuates LPS-induced acute lung injury in humanized resistin mice

As previous studies have shown that pharmacologically induced activation of AMPK with AICAR diminished LPS-induced ALI (27), we examined if 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 hours followed by intratracheal injection of LPS (i.t., 2 mg/kg), and then were sacrificed 24 hours later to determine the severity of lung injury. As shown in Figures 6E and F, administration of AICAR resulted in decreased neutrophil accumulation in the lungs as well as diminished levels of BAL TNFα compared to saline treated mice.

DISCUSSION

In these studies, we have shown that human resistin enhances neutrophil pro-inflammatory 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 pro-inflammatory 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, in spite of the presence of considerable amounts of human resistin in the circulation under basal conditions in *hRtn*$^{+/-/-}$ mice, only minimal increase in cytokines was found in bronchoalveolar lavages. 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 has been reported in mice given resistin and LPS (19). Overall, these results suggest that resistin contributes to pro-inflammatory 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. Of note, previous studies have shown that crosstalk between TLR4 induced cellular activation and other TLR-independent signaling cascade is an important mechanism for enhancement of pro-inflammatory responses in neutrophils (56-59).

Several possible mechanisms may be involved in the ability of human resistin to increase neutrophil sensitivity to LPS. Among these are potentiation of TLR4/NF-κB signaling (60), alteration in insulin signaling (4, 6), and inhibition of AMPK activation, which has been 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 inflammatory responses, decreased activation of AMPK in *hRtn*$^{+/-/-}$ as compared to *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

bereberine diminished TLR4 induced activation of neutrophils, macrophages, and endothelial cells (27, 33, 62). The severity of endotoxin-induced acute lung injury was decreased in mice treated with either metformin or AICAR (27, 45, 63). Of note, AICAR partially attenuates LPS-induced acute lung injury in humanized resistin mice. AMPK activation has also been shown to be associated with improvement of vascular integrity in murine models of ALI and of airway re-modeling 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 also resulted in enhanced phosphorylation of the NADPH oxidase subunit p-40phox and neutrophil extracellular trap formation (NETs), 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 were shown to be coupled with more robust inflammatory response (22, 50, 65). For example, HMGB1 has been shown to contribute to the development of more severe ALI or sepsis (65). Similar to resistin, HMGB1 itself has modest pro-inflammatory 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 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 have shown that NADPH oxidase is linked to activation of granular proteases and generation of NETs, independently of TLR4 engagement (68). As AMPK has also been shown to affect neutrophil NOX2 activity (69), the ability of resistin to diminish AMPK phosphorylation provides a plausible regulatory mechanism for NOX2-induced NETs formation.

Our results indicate that human resistin primes neutrophils to release greater amounts of proinflammatory mediators, such as TNF-α and MIP-2, increases NET formation, and enhances the severity of ALI. Concentrations of resistin similar to those present in the plasma of *hRtn*$^{+/-/-}$ mice have been found in the circulation of critically ill patients, with the highest concentrations of resistin present in patients with sepsis, an important predisposing condition for the development of ALI (70, 71). Previous preclinical studies have shown that increased expression of human resistin correlated with the appearance of other inflammatory biomarkers, including IL-6, IL-12, CRP or SOCS3 (16, 18, 72). Although extrapolation of potential mechanisms of organ dysfunction from animal models to life-threatening human conditions, such as sepsis or ALI, need to be confirmed in clinical trials, our results obtained using humanized resistin mice not only indicate that resistin has an important contributory role in neutrophil activation and ALI, but also suggest that resistin may be a potential therapeutic target in patients with organ system failures, such as ALI.

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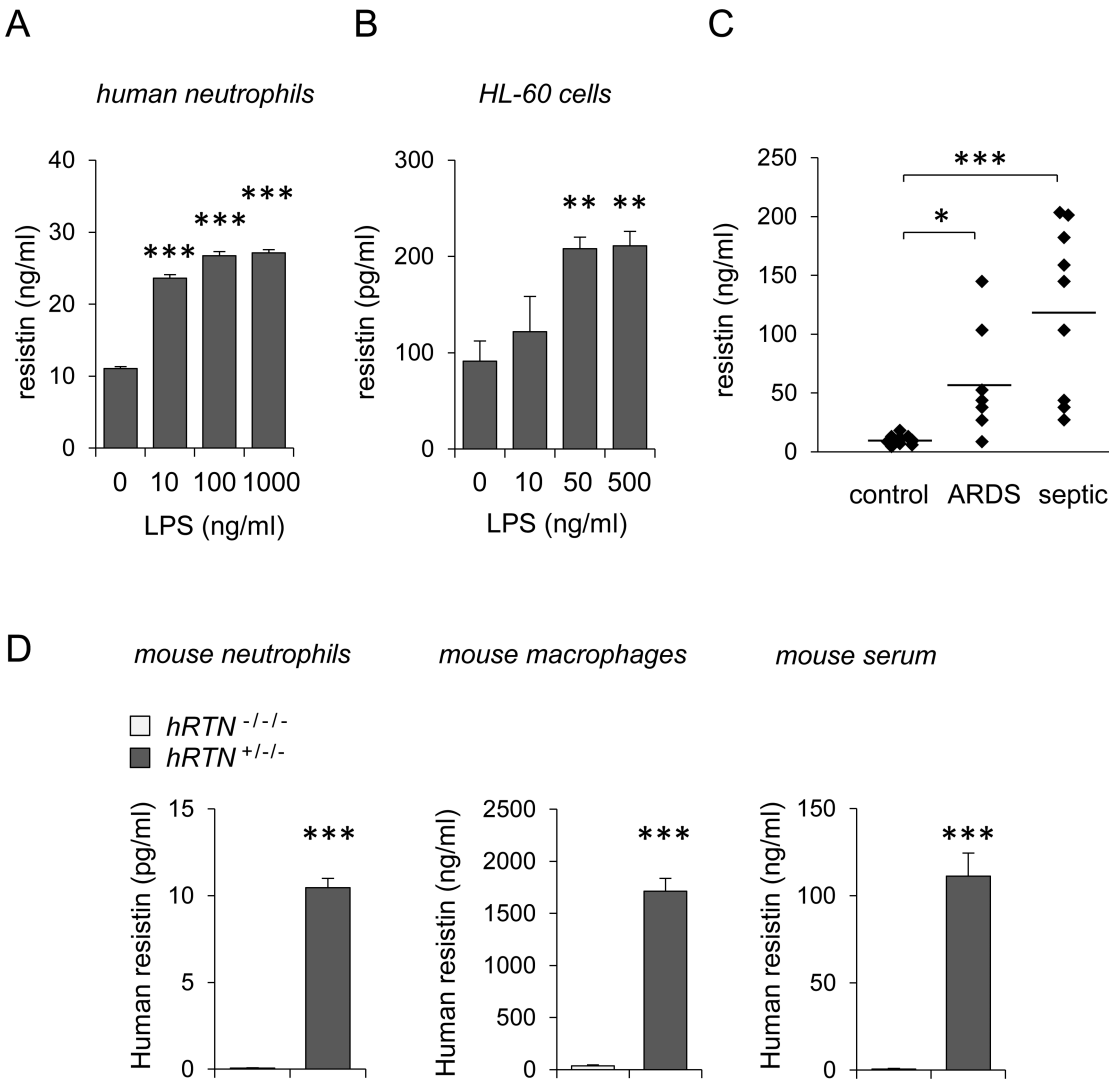

Figure 1. Resistin is expressed by LPS-stimulated neutrophils, humanized resistin mice and in critically ill patients

(**A** and **B**) Human resistin was measured in the culture medium of LPS-treated peripheral human neutrophils or differentiated HL-60 cells. (**A**) Neutrophils were incubated with indicated concentrations of LPS for 4 hours (Means ± SD, $n = 3$, *** $P < 0.001$). (**B**) HL-60 leukocytes were differentiated into the surrogate PMNs using 1.3% DMSO treatment over 5 days. Resistin in the culture medium of HL-60 cells was determined after exposure to LPS for 24 hours (Means ± SEM, $n = 4$, ** $P < 0.01$). Panel (**C**) shows amount of human resistin in plasma of normal (control), ARDS and septic patients (Means, $n = 7$-13, * $P < 0.05$, *** $P < 0.001$). (**D**) Human resistin was measured in the media of bone marrow neutrophils and peritoneal macrophages cultured in serum free conditions for 4 hours. Resistin was also measured in the serum of murine resistin deficient ($hRTN^{-/-/-}$) and humanized resistin mice ($hRTN^{+/-/-}$). Mean ± SD, $n = 3$, *** $P < 0.001$.

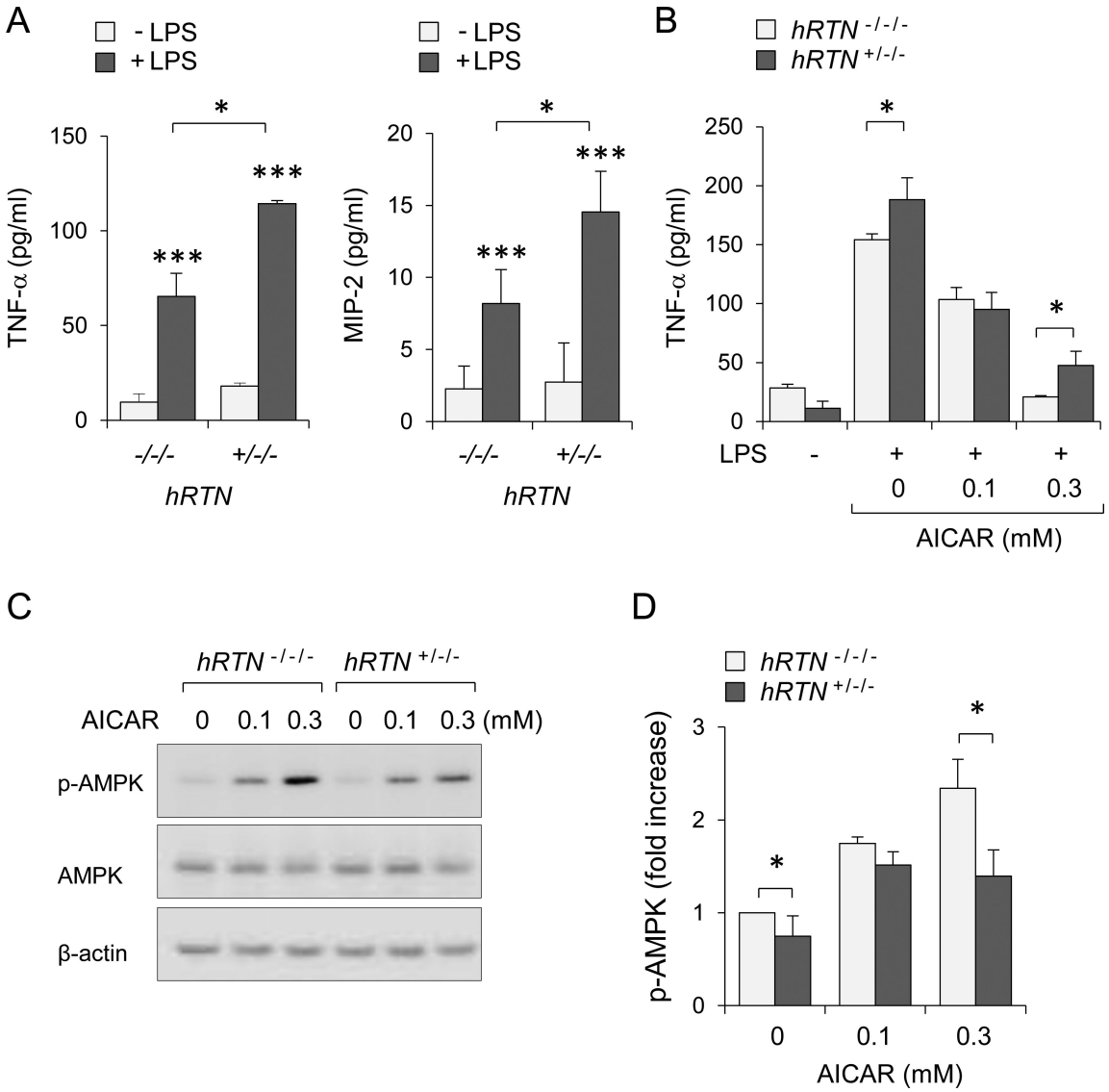

Figure 2. Human resistin diminished AMPK activity and increased neutrophil sensitivity to LPS challenge

(**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 hours and media were subjected to ELISA (Means ± SD, $n = 3$, *** $P < 0.001$ compared to untreated or * $P < 0.05$ compared LPS-treated $hRTN^{-/-/-}$ and $hRTN^{+/-/-}$). Panel (**B**) shows amount of TNF-α in culture media obtained from $hRTN^{+/-/-}$ or $RTN^{-/-/-}$ neutrophil that were pretreated dose-dependently with AICAR for 60 minutes followed by exposure to LPS (0 or 30 ng/ml) for additional 4.5 hours. Mean ± SD, $n = 4$, * $P < 0.05$. (**C** and **D**) Representative Western blots and optical densitometry show phopho-Thr172-AMPK, AMPK, and β-actin obtained from $hRTN^{+/-/-}$ or $RTN^{-/-/-}$ neutrophils treated with AICAR (0, 0, 0.1 or 0.3 mM) for 60 minutes. Mean ± SEM, $n = 3$ - 4, * $P < 0.05$.

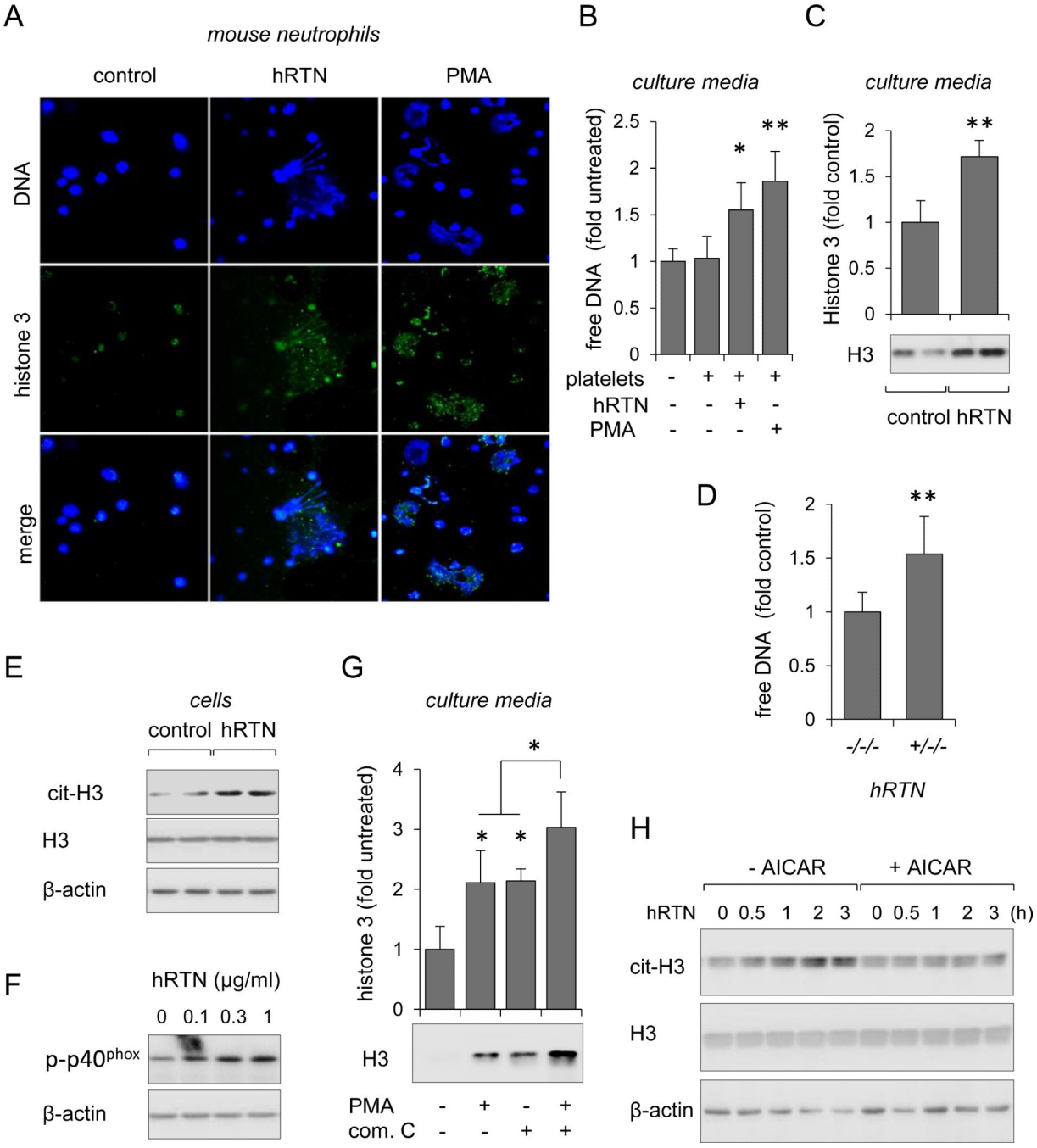

Figure 3. Human resistin induces formation of neutrophil extracellular traps

(**A** and **B**) Representative images (**A**) and quantitative data show amount of DNA (**B**) and histone 3 associated with NETs formation. Bone marrow neutrophils isolated from wild-type C57BL/6 mice were incubated with purified human resistin (hRTN; 1 μg/ml) or PMA (100 nM) for 3 hours in the presence of platelets. DNA, blue; histone 3, green. Means ± SD, n = 3, * $P < 0.05$ or ** $P < 0.01$ compared to untreated. (**C**) Representative Western blot and optical bend densitometry show the amount of histone 3 in the culture media obtained from neutrophils treated with purified hRTN (1 μg/ml) for 3 hours (Means ± SD, n = 3, ** $P <$

0.01). Panel (**D**) shows amounts of free DNA in culture media of neutrophils obtained from *hRTN*$^{-/-/-}$ or *hRTN*$^{+/-/-}$ mice. *hRTN*$^{-/-/-}$ *or hRTN*$^{+/-/-}$ neutrophils were cultured for 16 hours in RPMI 1640 media and serum (10%) from *hRTN*$^{-/-/-}$ or *hRTN*$^{+/-/-}$ mice. (**E** and **F**) Representative Western blots show (E) levels of citrullinated histone 3, histone 3, and β-actin in neutrophils after treatment with purified hRTN (1 μg/ml) or (**F**) levels of p-p40^{phox} in neutrophils treated dose-dependently with hRTN for 3 hours. (**G**) Western Blot analysis of extracellular histone 3 in culture media of neutrophils subsequently treated with Compound C (10 μM) for 30 minutes followed by incubation with PMA (100 nM) for 3 hours. (Means ± SD, n = 3-5, # $P < 0.05$ compared to non-treated control; * $P < 0.05$). Panel (**H**) shows amount of citrullinated histone 3, histone 3, and β-actin in neutrophils treated with AICAR (0 or 0.3 mM) for 60 minutes and then inclusion of purified hRTN (1 μg/ml) for indicated time.

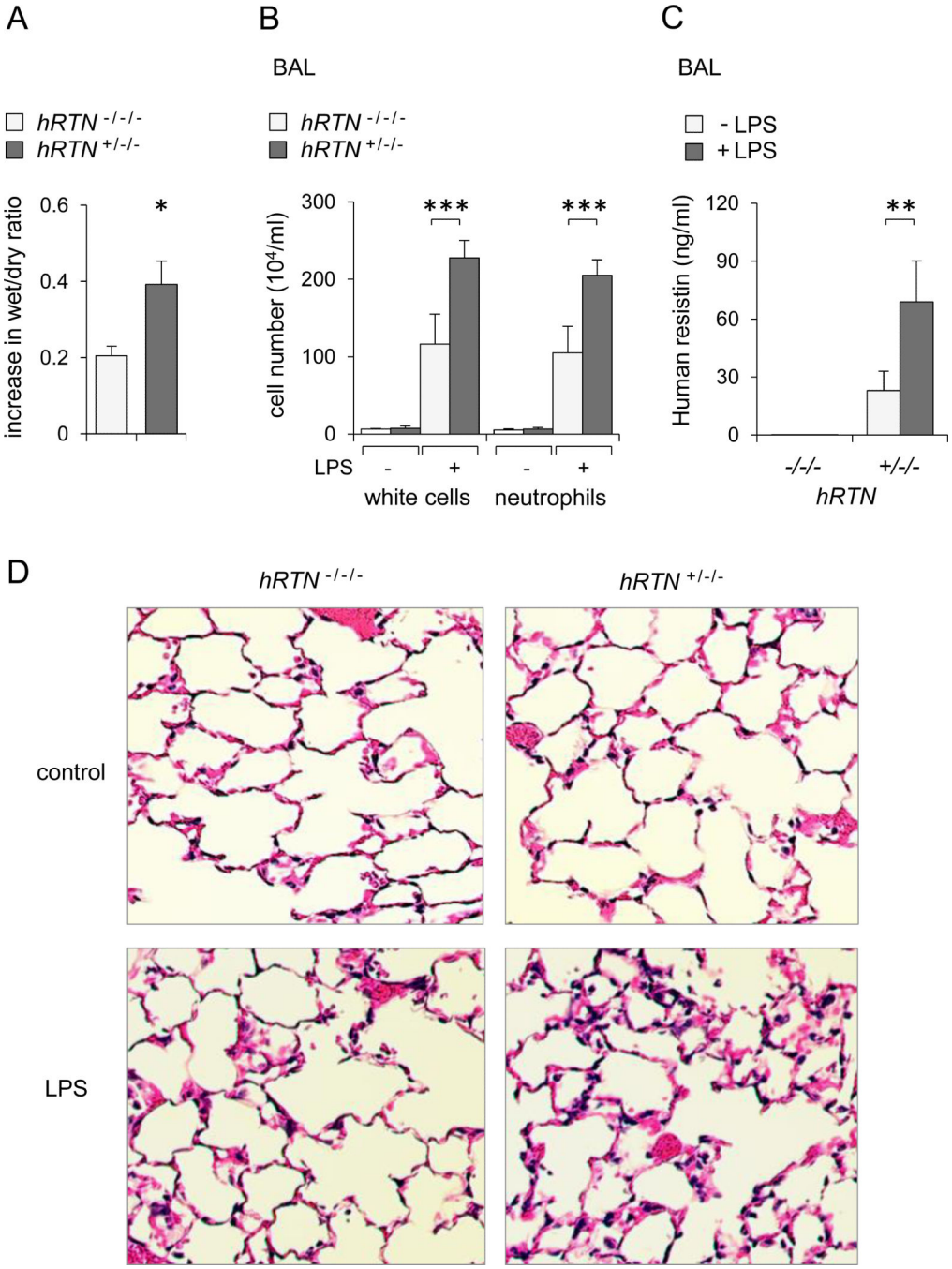

Figure 4. Human resistin increases severity of LPS-induced acute lung injury

hRTN$^{+/-/-}$ or control *RTN*$^{-/-/-}$ mice were subjected to intratracheal application of LPS (0 or 2 mg/kg) for 24 hours. Panel (**A**) shows lung wet-to-dry ratios measured 24 hours after LPS administration. Fold increase above values present in mice receiving saline alone are shown (Means ± SEM, n = 4-5, * $P < 0.05$). Panel (**B**) shows numbers of total white cells and neutrophils in BAL fluid from saline (-LPS) or LPS (+LPS) treated mice (Means ± SD, n = 3-6, *** $P < 0.001$). (**C**) Human resistin was measured in BALs of *hRTN*$^{+/-/-}$ or *hRTN*$^{+/-/-}$ mice treated with saline (-LPS) or LPS (+LPS) 24 hours previously (Means ±

SD, $n = 5$, ** $P < 0.01$). Panel (**D**) shows representative H&E staining of lung sections obtained from control (saline) or LPS-treated *hRTN*$^{+/-/-}$ or *RTN*$^{-/-/-}$ mice.

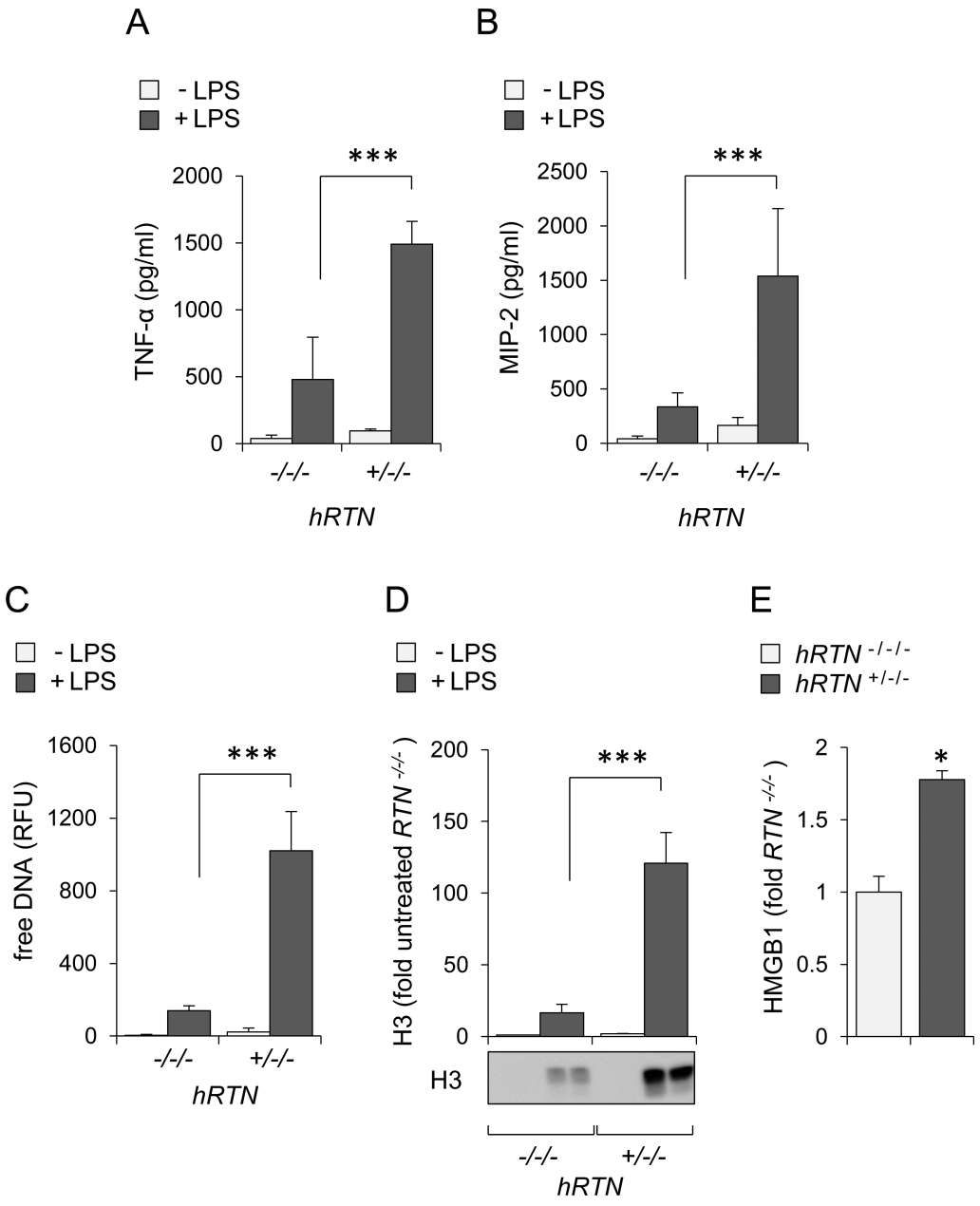

Figure 5. Human resistin increases production of pro-inflammatory mediators, including NETs-associated histone 3 and HMGB1 in BALs of humanized resistin mice

The levels of (**A**) TNF-α, (**B**) MIP-2, (**C**) free DNA, (**D**) extracellular histone 3, and (**E**) HMGB1 was determined in BAL fluids obtained from $hRTN^{+/-/-}$ or $RTN^{-/-/-}$ mice that were treated with saline (-LPS) or LPS (+LPS; 0 or 2 mg/kg) 24 hours previously. Means ± SD, n = 3-6, * $P < 0.05$, *** $P < 0.001$.

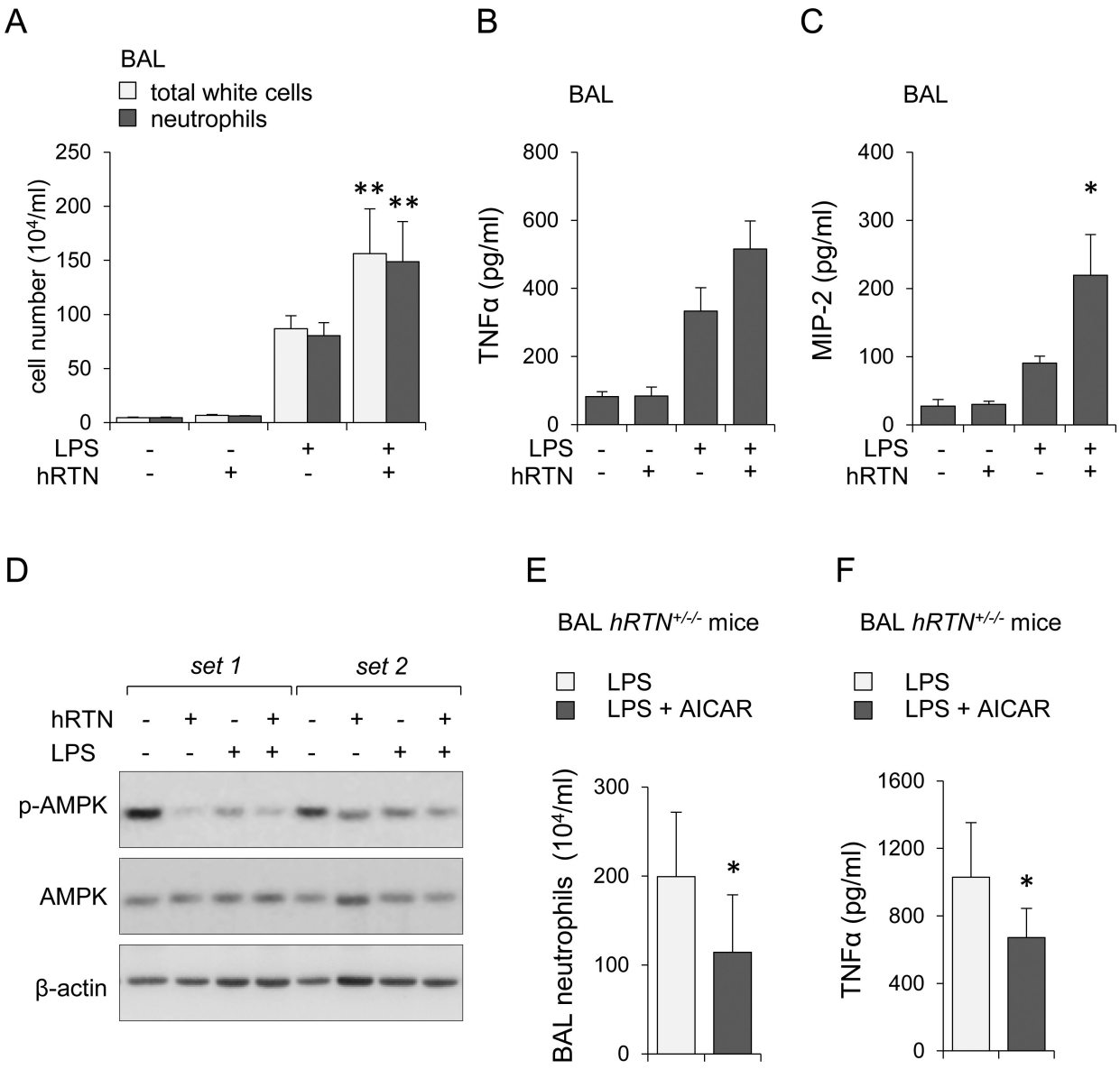

Figure 6. Co-administration of human resistin and LPS increases the severity of ALI

(**A**, **B**, **C,** and **D**) wild type mice were subjected to intratracheal (i.t.) instillation of purified human resistin (0.5 mg/kg), LPS (2 mg/kg), or combined administration of human resistin and LPS, and then BALs obtained 24 hours later. Panels (**A**) and (**B**) show numbers of total white cells, neutrophils and levels of TNF-α and MIP-2 in BAL fluid (Means ± SD, $n = 4$, * $P < 0.05$; ** $P < 0.01$). (**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 hours prior to intratracheal instillation of LPS (2 mg/kg). Numbers of neutrophils (**E**) and levels of TNF-α (**F**) in BAL fluids were measured 24 hours after LPS injection (Means ± SD, $n = 4$, * $P < 0.05$).