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RESEARCH ARTICLE

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Deletion of the *Npr3* gene increases severity of acute lung injury in obese mice

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Abstract

Previous studies have shown that atrial natriuretic peptide (ANP) attenuates agonist-induced pulmonary edema and that this effect may be mediated in part by the ANP clearance receptor, natriuretic peptide receptor-C (NPR-C). Obesity has been associated with lower plasma ANP levels due to increased expression of NPR-C, and with decreased severity of acute lung injury (ALI). Therefore, we hypothesized that increased expression of NPR-C may attenuate ALI severity in obese populations. To test this, we examined ALI in *Npr3* wild-type (WT) and knockout (KO) mice fed normal chow (NC) or high-fat diets (HFD). After 12 weeks, ALI was induced with intra-tracheal administration of *Pseudomonas aeruginosa* strain 103 (PA103) or saline. ALI severity was determined by lung wet-to-dry ratio (W/D) along with measurement of cell count, protein levels from bronchoalveolar lavage fluid (BALF), and quantitative polymerase chain reaction was performed on whole lung to measure cytokine/chemokine and Npr3 mRNA expression. ANP levels were measured from plasma. PA103 caused ALI as determined by significant increases in W/D, BALF protein concentration, and whole lung cytokine/chemokine expression. PA103 increased Npr3 expression in the lungs of wild-type (WT) mice regardless of diet. There was a nonsignificant trend toward increased Npr3 expression in the lungs of WT mice fed HFD versus NC. No differences in ALI were seen between *Npr3* knockout (KO) mice and WT-fed NC, but *Npr3* KO mice fed HFD had a significantly greater W/D and BALF protein concentration than WT mice fed HFD. These findings support the hypothesis that Npr3 may help protect against ALI in obesity.

KEYWORDS
atrial natriuretic peptide, lung inflammation, natriuretic peptide receptor C, obesity

INTRODUCTION

Acute lung injury (ALI) is characterized by increased inflammatory processes caused by various types of lung insult, including bacterial and viral infections. In some patients, ALI leads to the more severe form of acute respiratory distress syndrome (ARDS), which has a hospital mortality rate of approximately 40%.[1,2] In ALI/ARDS, the inflammatory response to lung injury causes increased endothelial permeability, which leads to fluid accumulation in the alveolar space.[3] Under normal physiologic conditions, fluid can be cleared by Na^+/K^+-ATPase pumps, however, in ARDS, damage to the alveolar epithelial barrier disrupts this process and leads to pulmonary edema.[4] Previous studies have shown a link between the development of ALI/ARDS and higher body mass index (BMI).[5,6] Although the prevalence of ALI may be greater in obese patients, some studies suggest that obese patients have less severe disease and better survival.[7,8] However, the mechanism responsible for this observation is not known.

The natriuretic peptides are a family of small proteins characterized by a conserved 17-amino acid loop[9] that plays an important role in a myriad of functions. Atrial natriuretic peptide (ANP) is synthesized and secreted by the cardiac atria and plays a major role in intravascular volume homeostasis.[10] Brain and C-type natriuretic peptide (BNP and CNP) are synthesized in the cardiac ventricles[11], and vascular endothelial cells, and central nervous system,[12] respectively. BNP plays a significant role in enhancing cardiac function,[13] whereas CNP appears to be important in mitigating vascular injury.[14] Studies by our laboratory and by other investigators have shown that ANP has a protective effect against ALI.[15–17] ANP attenuates thrombin[18] and lipopolysaccharide (LPS)-induced barrier dysfunction[19] in pulmonary arterial endothelial cells in vitro and blunts the development of pulmonary edema and inflammation in animal models of ALI.[20] Furthermore, ANP knockout (KO) mice have increased inflammatory markers in bronchoalveolar lavage fluid (BALF)[21] and demonstrate more severe ALI in response to *Staphylococcus aureus*. Similarly, ANP attenuates the NFκB activation induced by hypoxia and LPS in bovine endothelial cells,[22] which may be a result of ANP-dependent PAK1 phosphorylation.[19]

The natriuretic peptides signal through three cell surface receptors- natriuretic peptide receptor-A, -B, and C (NPR-A, NPR-B, and NPR-C).[23] NPR-A has a much greater binding affinity for ANP and BNP than it has for CNP.[24] The reverse is true for NPR-B, whereas NPR-C has a similar binding affinity for all three peptides.[25] NPR-A and NPR-B have an intracellular guanylate cyclase domain.[26] Ligand binding results in activation of the guanylyl cyclase and synthesis of cGMP, which is thought to mediate most of the biological properties of the natriuretic peptides.[27] NPR-C, which is encoded by the *Npr3* gene, has no guanylate cyclase domain[28] and was originally thought to function primarily as a clearance receptor, although it has also been shown to have potential signaling mechanisms via activation of G-coupled proteins or phosphatidylinositol-4,5-biphosphate (PIP_2).[29,30] The receptors that mediate the protective effect of ANP on ALI are not known, however, studies by our lab suggest that both NPR-A and NPR-C may play a role. Previously, we have shown that ANP attenuates LPS-induced pulmonary edema even in the absence of NPR-A.[15] Recently, we found that ANP signaling through NPR-C reduced total neutrophil counts and inflammatory cytokine levels in whole lung and BALF during *Pseudomonas*-induced lung injury in mice.[17] Interestingly, obesity is associated with increased expression of NPR-C in adipose tissue,[31,32] however, the effects of ANP/NPR-C on ALI severity in obesity has not yet been explored.

In this study, we hypothesized that attenuated lung injury in obesity is due to increased expression of natriuretic peptide receptor-C (NPR-C). To test this hypothesis, we assessed severity of lung injury in *Npr3* KO and wild-type (WT) mice fed standard normal chow (NC) or a high-fat diet (HFD) to induce obesity. Interestingly, our findings show that deletion of NPR-C results in more severe lung injury in obese mice but not in mice of normal weights, suggesting that NPR-C may mediate some of the protective effects of obesity on ALI.

MATERIALS AND METHODS

Animals

Male and female *Npr3* KO (0 functional copies of *Npr3*) or WT mice ages 10–18 weeks (bred from Jackson Laboratory NPR-$C^{+/+}$ (WT), strain B6;129-$Npr3^{tm1Unc}$/Mmnc MMRRC ID #: 16) were fed either HFD (60 kCal from fat) or normal chow (NC) (6 kCal from fat) for 12 weeks and subjected to ALI experiments. Genotyping of *Npr3* KO mice was performed as previously described.[17] Briefly, PCR was performed with Nprc5′ (5′-CACAAGGACACGGAATACTC-3′) and Nprc3′ (5′-CTTGGATGTAGCGCACTATGTC-3′) primers (Integrated DNA Technologies) to detect the WT product, while Neo2 primer (5′-ACGCGTCACCTTAATATGC G-3′) was used to amplify the knockout (Figure 2a). Amplification of both products indicated a heterozygous mouse, which were not used in this study. In

addition to PCR, *Npr3* KO mice can be identified via their hunched-back phenotype as previously described.[17,33] Blood was collected via cardiac puncture, centrifuged at 2000 *g* to separate the plasma, which was used to measure ANP levels with the Mouse NT-pro-ANP ELISA kit (LifeSpan Biosciences; LS-F23113), according to the manufacturer's protocol. All animal experiments were conducted in accordance with IACUC-approved protocols at the Providence VA Medical Center.

Bacterial culture

Pseudomonas aeruginosa (*P. aeruginosa*) strain 103 (PA103), was a kind gift from Dr. Troy Stevens at the University of Southern Alabama. PA103 was preserved in CryoCare™ (Thermo Fisher Scientific) before use. Agar plates were prepared by adding 1.5 g agar and 2.5 g of Luria broth to 100 mL ddH_2O. Once agar plates were prepared, *P. aeruginosa* was streaked and grown for 24 h. Bacterial colonies were read with a colorimeter and were used at a concentration of 1×10^6 CFU.

Acute lung injury model

Mice were anesthetized with 2⁻3% isoflurane, and 1×10^6 CFU of PA103 was diluted to a volume of 100 μL with saline and instilled intra-tracheal; 100 μL of saline was used as the control vehicle. 1×10^6 was chosen based on titration studies with three different concentrations of *P. aeruginosa*. After 4 h, mice were euthanized with pentobarbital, lungs were removed, and bronchoalveolar lavage fluid was collected. Lungs were weighed upon excision and again after 72 h after drying at 60°C to calculate their wet-to-dry ratios as a measurement of ALI severity. A schematic of these methods is depicted in Figure 1.

BALF collection/analysis

Lungs were exposed via midline sternotomy. To tie off the left bronchus, a suture was passed under the left lung hilum region. A 23 G needle was inserted into the trachea and 300 μL of sterile phosphate-buffered saline was injected into the lung and aspirated back. The BALF was centrifuged at 2000 rpm for 10 min and stored at −80°C for future analysis. Protein estimation was conducted using a modified Bradford assay at 750 nm absorbance (BioRad). Cell counting was done before centrifugation using a Bio-Rad TC20 cell counter. Readings were taken in triplicate.

Quantitative polymerase chain reaction (qPCR)

RNA was isolated from whole lung tissue using an miRNeasy Mini Kit (Qiagen) and was quantified via NanoDrop (ThermoFisher). cDNA was synthesized from RNA with RevertAid First Strand Synthesis cDNA Synthesis Kit (ThermoFisher) and a C1000 touch thermal cycler (BioRad). PowerUp SYBR green master mix (ThermoFisher) was used with primers (Integrated DNA Technologies) listed in Table 1. qPCR was done on a StepOnePlus Real-Time PCR system (Applied Biosystems). *Npr3* mRNA expression was normalized to housekeeping gene, β-actin, and the geometric mean of the ΔCt values was used to calculate ΔΔCt and subsequent fold changes ($2^{-\Delta\Delta Ct}$). $2^{-\Delta\Delta Ct}$ values were log2 transformed before statistical analysis.

Statistical analysis

Independent samples *t*-test and two-way analysis of variance with Fisher's exact test were performed using Prism GraphPad software.

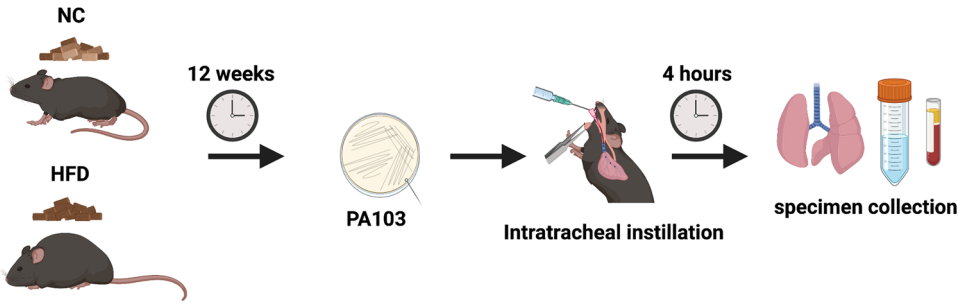

FIGURE 1 Schematic of acute lung injury (ALI) methodology. Wild-type and *Npr3* knockout mice were fed either normal chow (NC) or high-fat diet (HFD) for 12 weeks. Mice then underwent intratracheal instillation of PA103 for 4 h before specimen collection and processing. Created with Biorender.com.

TABLE 1 List of qPCR primers.

Gene	Primer sequence
β-actin	Forward-5′-ACTGTCGAGTCGCGTCCACC-3′
	Reverse-5′-CGATGGAGGGGAATACAGCCC-3′
NPR3	Forward-5′-TTCAGGAGGAGGGGTTGCAC-3′
	Reverse-5′-AGTCTCCACTGGTCATGCCG-3′
IL-6	Forward-5′-TCTGGTCTTCTGGAGTACCA TAGC-3′
	Reverse-5′-GCTTATCTGTTAGGAGAGCA TTGG-3′
TNF-α	Forward-5′-CAGGCGGTGCCTATGTCTCA-3′
	Reverse-5′-GGCTACAGGCTTGTCACTCG-3′
CXCL1	Forward-5′-CACTCAAGAATGGTCGCGAGG-3′
	Reverse-5′-ACAGGTGCCATCAGAGCAGTC-3′
CXCL2	Forward-5′-TCATAGCCACTCTCAAGGGCG-3′
	Reverse-5′-TCAGGTACGATCCAGGCTTCC-3′

Abbreviation: qPCR, quantitative polymerase chain reaction.

RESULTS

Effect of diet on severity of Npr3 expression and *Pseudomonas*-induced lung injury

Compared with NC, 12 weeks of HFD significantly increased body weight in both WT ($p < 0.001$) and *Npr3* KO ($p < 0.05$) mice (Figure 2b,c). The *Npr3* Ko mice given HFD exhibited lower body weights than WT mice on HFD ($p < 0.05$) (Figure 2b,c). There was a trend toward increased NPR-C (*Npr3*) lung expression in WT mice fed HFD and treated with vehicle, but the difference was not statistically significant (Figure 3a). No difference in pulmonary NPR-C (*Npr3*) expression was seen between WT mice fed NC or HFD and treated with PA103 (Figure 3b). This may be because treatment with PA103 significantly increased lung NPR-C (*Npr3*) expression to a similar degree in mice fed both NC ($p < 0.01$) and HFD ($p < 0.01$) (Figure 3c,d). NPR-C (*Npr3*) expression was not measured in *Npr3* KO mice.

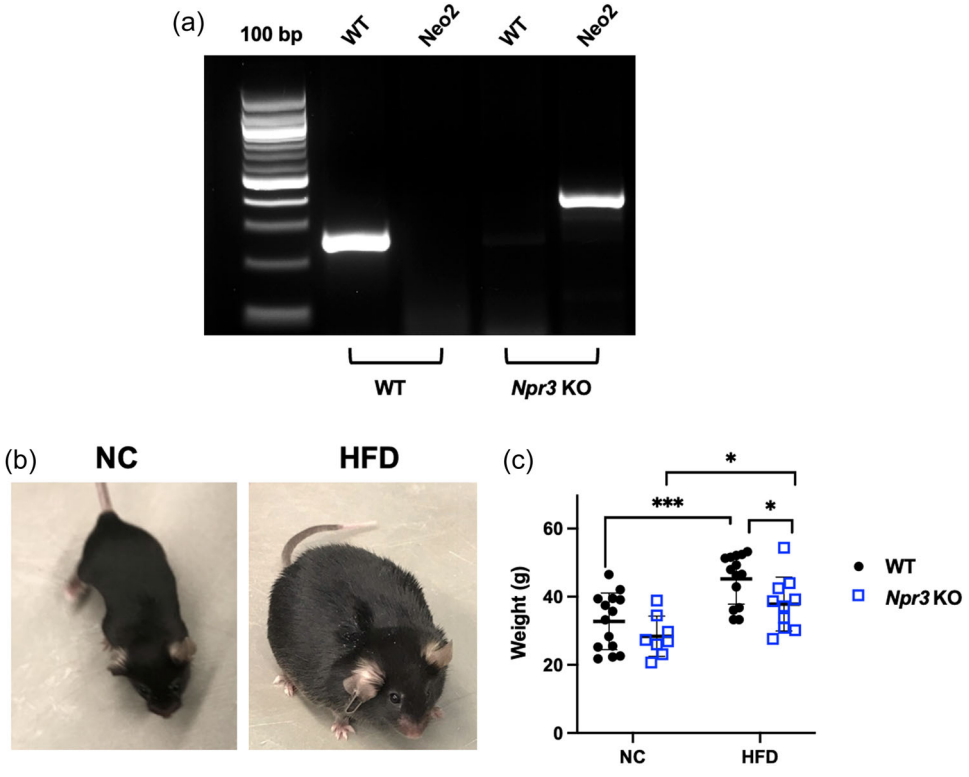

FIGURE 2 HFD induced obesity in WT and *Npr3* KO mice (a) Representative image of PCR gel depicting the genotypic differences between WT and *Npr3* KO mice. Note, WT mice only show a PCR product using the WT-specific primer, while *Npr3* KO mice show a PCR product using only the Neo2-specific primer. (b, c) Mice were fed either NC or HFD for 12 weeks, after which, images were taken and mice were weighed. *$p < 0.05$; ***$p < 0.001$. Data shown are the means ± SEM. HFD, high-fat diet; KO, knockout; NC, normal chow; PCR, polymerase chain reaction; SEM, standard error of mean; WT, wild type.

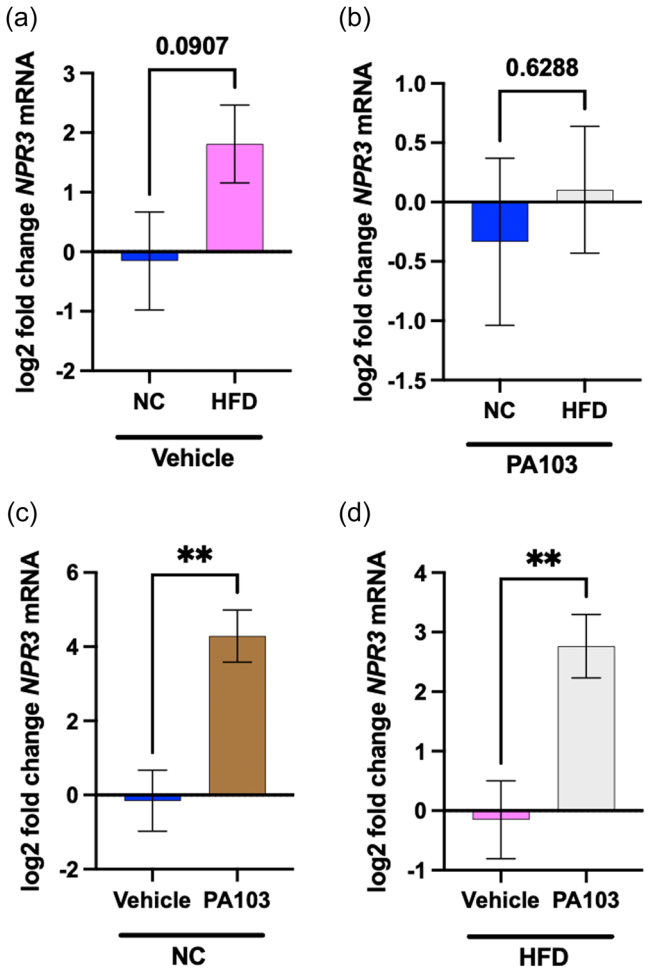

FIGURE 3 Diet does not affect lung NPR-C expression in WT mice. (a) qPCR analysis of NPR-C (*Npr3*) expression between NC (blue bar; $n = 5$) and HFD (pink bar; $n = 6$) vehicle-treated mice. (b) qPCR analysis of NPR-C (*Npr3*) expression between NC (blue bar; $n = 5$) and HFD (gray bar; $n = 7$) PA103-treated mice. (c) qPCR analysis of NPR-C (*Npr3*) expression between vehicle (blue bar; $n = 5$) and PA103 (brown bar; $n = 7$) treated mice fed NC. (d) qPCR analysis of NPR-C (*Npr3*) expression between vehicle (pink bar; $n = 6$) and PA103 (gray bar; $n = 7$) treated mice fed HFD. Ct values were normalized to β-actin, and values shown are log2 transformed fold changes ($2^{-\Delta\Delta Ct}$). Data shown are the means ± SEM. $^{**}p < 0.01$. HFD, high-fat diet; NC, normal chow; NPR-C, natriuretic peptide receptor-C; PA103, *Pseudomonas aeruginosa* strain 103; qPCR, quantitative polymerase chain reaction; SEM, standard error of mean; WT, wild type.

Genetic deletion of *Npr3* increases bacterial-induced lung injury in obese mice

No differences in W/D or BALF protein concentrations were seen between *Npr3* WT and KO mice under baseline conditions (i.e., mice given intratracheal saline). Intra-tracheal administration of PA103 resulted in ALI as demonstrated by increases in W/D and BALF protein concentration compared to mice given intra-tracheal saline (Figure 4a,b). Administration of PA103 did not significantly increase BALF cell counts at the time point studied (Figure 4c), however, mRNA levels of inflammatory cytokines/chemokines IL-6, TNF-α, CXCL1, and CXCL2 were 2-8-fold higher in mice given PA103 than in saline controls, consistent with the development of ALI (Figure 5a–d). In PA103-challenged mice, we found that *Npr3* KO mice fed an HFD had significantly higher W/D compared with WT mice fed HFD ($p < 0.0001$), and *Npr3* KO mice fed NC ($p < 0.01$) (Figure 4a). Similarly, PA103-challenged *Npr3* KO mice fed an HFD had significantly increased BALF protein concentrations compared to WT mice fed an HFD ($p < 0.0001$), and *Npr3* KO mice fed NC (Figure 4b). No significant differences in circulating ANP levels were seen between any of the groups of mice (Figure 4d).

DISCUSSION

Obesity is a well-known risk factor for the development of ALI/ARDS[5,34,35]; however, this does not appear to result in worse clinical outcomes. In fact, a systematic review of 24 different studies found that, while obesity increased the risk of an individual developing ALI/ARDS, it lowered the risk of mortality.[36] Other studies have shown that obese patients with ALI/ARDS require fewer days of mechanical ventilation than normal-weight patients,[7] suggesting a less severe degree of lung injury. Similarly, a recent study showed that coronavirus disease 2019 (COVID-19) patients admitted to the intensive care unit who were obese had a lower incidence of death.[37] This phenomenon, known as the "obesity paradox" is well-documented in ALI/ARDS,[7] however, the mechanism remains poorly understood. Previous studies have found associations between obese patients with ALI and younger age, lower severity of illness as assessed by APACHE III and SAPS II scores, and less severe lung injury assessed by partial pressure of oxygen/fractionation of inspired oxygen (PaO_2/FiO_2) ratio.[38,39] Obese patients with ALI have also shown lower levels of pro-inflammatory cytokines, such as interleukin-6 (IL-6) and −8 (IL-8),[40] which may play a role in protection against severe outcomes by attenuating cytokine storms. Further, Qi et al.[41] found that adipose-derived exosomes, a type of extracellular vesicle, were protective against endothelial barrier disruption by downregulating the TGF-β pathway.[41] Additionally, some investigators argue that obesity mitigates ventilator-induced lung injury by impeding chest wall expansion and reducing transpulmonary airway pressure.[7]

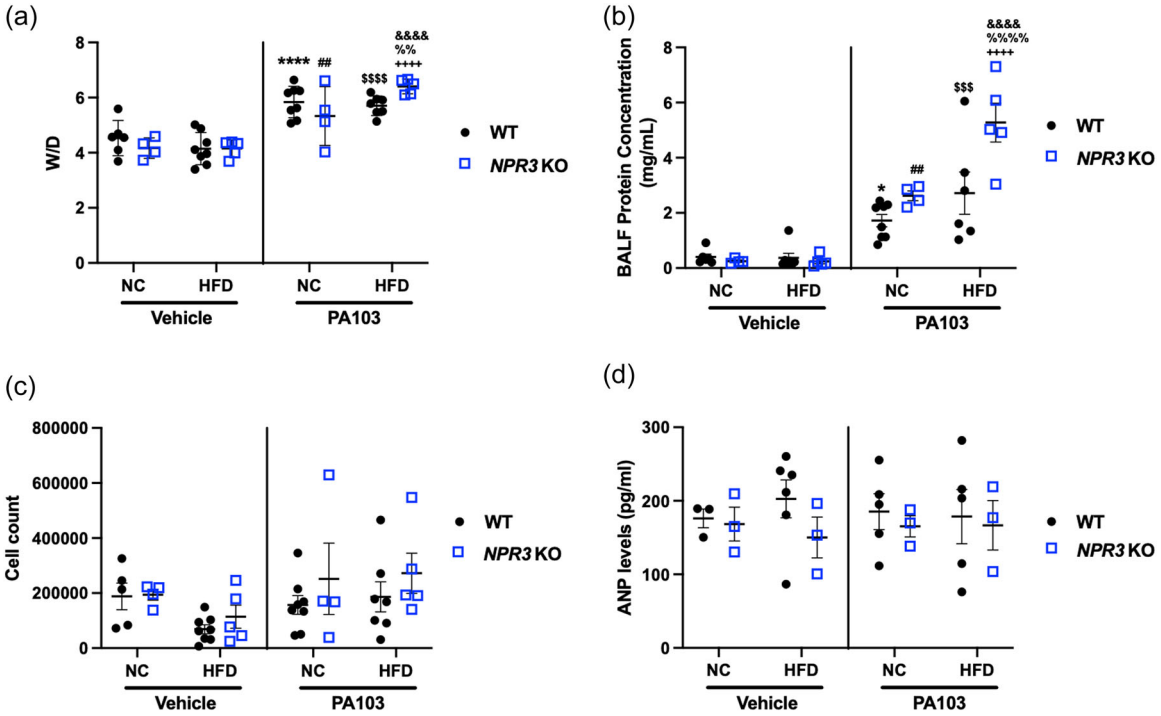

FIGURE 4 Deletion of *Npr3* increases ALI/ARDS severity in obese mice. (a) Lung wet-to-dry weight ratios (W/D) in *Npr3* wild-type and knockout mice (WT, *Npr3* KO) 4 h after treatment with intra-tracheal instillation of saline (vehicle) or *Pseudomonas* strain 103 (PA103). (b) Protein concentration and (c) cell counts in bronchoalveolar lavage fluid (BALF) collected 4 h after intra-tracheal instillation of PA103. (d) Plasma atrial natriuretic peptide (ANP) levels were measured 4 h post-instillation of vehicle or PA103. Note, for all graphs * indicates a statistical difference from NC WT vehicle, # indicates a statistical difference from NC *Npr3* KO vehicle, $ indicates a statistical difference from the HFD WT vehicle, & indicates a statistical difference from HFD *Npr3* KO vehicle, % indicates a statistical difference from NC *Npr3* KO PA103, and + indicates a statistical difference from HFD WT PA103. Data shown are the means ± SEM. $^{*}p < 0.05$; $^{****}p < 0.0001$; $^{\#\#}p < 0.01$; $^{\$\$\$}p < 0.001$; $^{\$\$\$\$}p < 0.0001$; $^{\&\&\&\&}p < 0.0001$; $^{\%\%}p < 0.0$; $^{\%\%\%\%}p < 0.0001$; $^{++++}p < 0.0001$. ALI, acute lung injury; ARDS, acute respiratory distress syndrome; HFD, high-fat diet; NPR-C, natriuretic peptide receptor-C; SEM, standard error of mean.

Obesity has a significant effect on suppressing circulating levels of natriuretic peptides.[42–44] In fact, plasma ANP levels have been found to be 26% lower in obese individuals compared to individuals of a healthy weight.[43] Furthermore, plasma ANP levels increase during ALI[45] and, importantly, obesity has been shown to impair the ability of patients to increase ANP in response to saline load.[46] One mechanism contributing to lower natriuretic peptide levels in obese patients is higher expression of NPR-C. Previous studies have shown that NPR-C is highly expressed in adipose tissue and upregulated in obesity, leading to a "natriuretic handicap" due to greater removal of natriuretic peptides from circulation, and thereby, linking obesity and hypertension.[47–49]

Many groups, including ours, have demonstrated the protective effects of ANP on ALI/ARDS.[15–17,21] Recently, we found that ANP inhibits neutrophil recruitment and release of inflammatory cytokines in ALI and that this effect is mediated by NPR-C.[17] Thus, it is possible that obesity mitigates the severity of ALI by increased NPR-C expression even at the cost of lower circulating levels of ANP. In the present study, we hypothesized that if the protective effects of obesity on ALI are mediated by increased expression of NPR-C, then obese mice with impaired NPR-C expression should develop more severe ALI than obese mice with normal NPR-C expression. To test this hypothesis, we examined severity of ALI in *Npr3* KO and WT mice after feeding them NC or HFD for 12 weeks. Significant lung injury was induced in both genotypes by intra-tracheal administration of PA103, as demonstrated by increases in lung W/D weight, BALF protein concentration, and inflammatory cytokine mRNA expression in the lung, though the lack of cytokine protein expression data is a limitation of the current study and should be explored in future studies. Interestingly, we found that in mice with normal NPR-C expression (*Npr3* WT mice), both PA103 and HFD increased lung expression of NPR-C, although the effect of the latter did not reach statistical significance. Whether or not the increase in pulmonary Npr3 expression in obesity or ALI is protective is unclear.

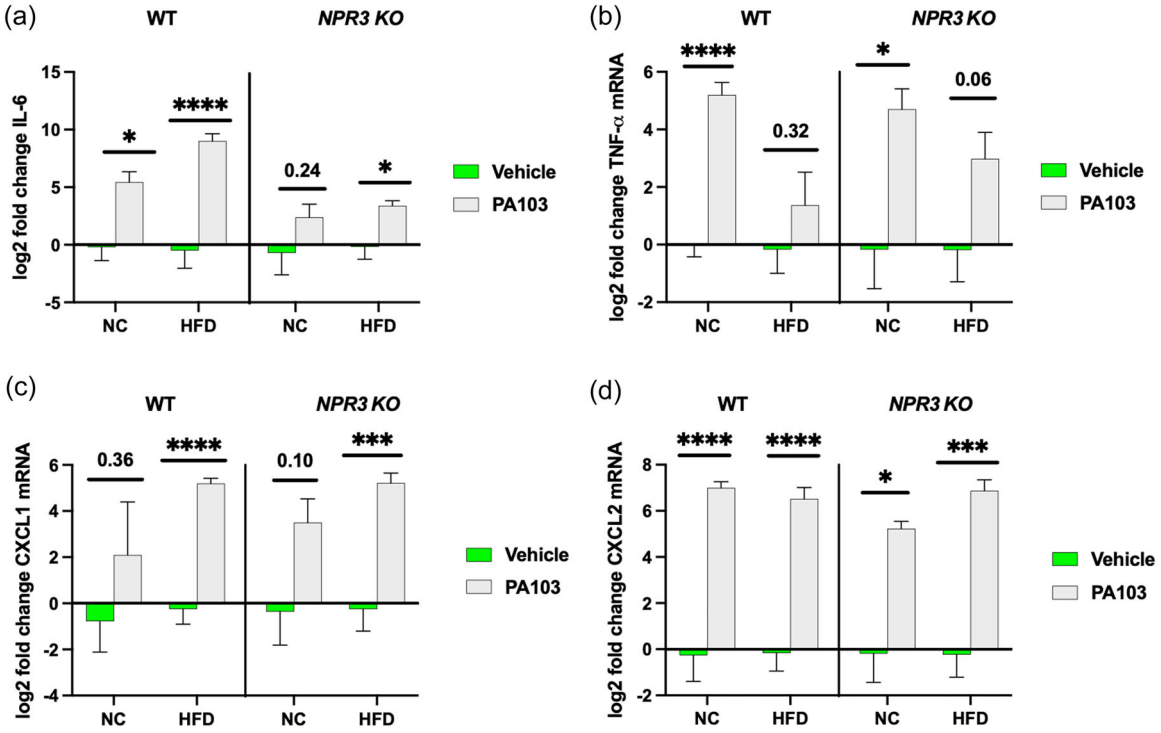

FIGURE 5 PA103 increases cytokine/chemokine expression in lung homogenates. qPCR analysis showing IL-6 (a), TNF-α (b), CXCL1 (c), and CXCL2 (d) mRNA expression between vehicle (gray bar) or PA103 (green bar) treated mice fed NC or HFD. Ct values were normalized to β-actin, and values are expressed as a log2 transformed fold change ($2^{-\Delta\Delta Ct}$) between vehicle and PA103-treated mice. Data shown are the means ± SEM. WT vehicle NC ($n = 5$); WT vehicle HFD ($n = 6$); WT PA103 NC ($n = 7$); WT PA103 HFD ($n = 7$); *Npr3* KO vehicle NC ($n = 3$); *Npr3* KO vehicle HFD ($n = 5$); *Npr3* KO PA103 NC ($n = 3$); *Npr3* KO PA103 HFD ($n = 5$). *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$. HFD, high-fat diet; IL-6, interleukin-6; NC, normal chow; NPR-C, natriuretic peptide receptor-C; PA103, *Pseudomonas aeruginosa* strain 103; qPCR, quantitative polymerase chain reaction; SEM, standard error of mean; TNF-α, tumor necrosis factor-α; WT, wild type.

However, in the absence of functional *Npr3* gene, the severity of PA103-induced lung injury as assessed by lung W/D weight ratio and BALF protein concentration was exacerbated in mice fed HFD. In mice fed NC, no differences in lung W/D weight ratio were seen between *Npr3* KO and WT mice treated with PA103, although there was a nonsignificant trend toward higher BALF protein in KO mice. However, in obese mice treated with PA103, lung W/D weight was significantly higher in *Npr3* KO mice than in WT mice, and BALF protein concentrations were two-fold higher. There were also trends toward higher BALF cell counts in *Npr3* KO than WT mice. Although these results were not statistically significant, it is possible that immune cell populations changed between groups. In our previous study, we found that NPR-C mediated the inhibitory effect of ANP on neutrophil recruitment to the lung in PA103-induced lung injury.[17]

Our hypothesis is that ANP protects against ALI by activating NPR-C. Thus, loss of NPR-C expression in the *Npr3* KO mice should result in greater lung injury. On the other hand, loss of NPR-C could lead to higher circulating ANP levels because NPR-C acts as a clearance receptor. Although plasma ANP levels have not been found to be elevated in *Npr3* KO mice, these mice do exhibit decreased ANP clearance, and we anticipated that ANP levels may rise higher in response to ALI in the KO mice compared with WT mice. However, there were no differences in ANP levels between any of our groups. Thus, it appears that the differences in PA103-induced lung injury in KO versus WT mice (HFD) were due to the loss of receptor and not due to differing ANP levels.

Interestingly, we saw no differences in the severity of PA103-induced ALI between *Npr3* KO and WT mice fed NC. These findings agree with those of our previous study of ALI in *Npr3* KO mice. In that study of normal weight *Npr3* KO and WT mice, PA103-induced the same degree of ALI as assessed by lung W/D weight ratio and BALF protein concentrations, however, in the *Npr3* KO mice, administration of ANP failed to attenuate PA103-induced increases in lung neutrophils and inflammatory cytokines.[17] It is possible that the inhibitory effect of ANP on lung neutrophil recruitment and cytokine levels is not sufficient to

attenuate ALI in normal-weight mice but plays a greater role in ALI during obesity. As mentioned previously, obesity has been associated with lower levels of inflammatory cytokines during lung injury. If this is due, in part, to obesity-related increases in Npr3 expression, then the lack of *Npr3* may have a more pronounced effect on ALI in obese mice. Future studies should focus on the protective molecular mechanisms of NPR-C, as well as its role in the obesity paradox. Additionally, simultaneous inhibition of both NPR-A and NPR-C may be warranted, as the results from our previous study[17] and the current study supports the hypothesis that any protective effects of ANP on ALI/ARDS is not mediated by NPR-C or NPR-A alone.

In conclusion, the findings of the present study suggest that PA103-induced ALI in obese mice may be more severe in *Npr3* KO mice than in mice with normal Npr3 expression. Although we did not find any evidence that ALI was attenuated in mice fed HFD, the greater degree of ALI seen in *Npr3* KO mice compared to WT mice fed, HFD suggests that changes in Npr3 expression during obesity may have important effects on modulating disease progression. Future studies are needed to determine if the effect of NPR-C expression on ALI results in improved survival or more rapid recovery and whether the effect of *Npr3* KO is the result of decreased Npr3 expression in the pulmonary vasculature, alveolar epithelium, or other cell types such as circulating neutrophils or monocytes.

AUTHOR CONTRIBUTIONS

Elizabeth O. Harrington and James R. Klinger: Study conception and design, data acquisition, analysis, and interpretation, drafting and revision of the manuscript, accountability for accuracy, and integrity of the data. Brianna D. Guarino: Data acquisition, analysis and interpretation, drafting, and revision of the manuscript. Christopher D. Dado and Ashok Kumar: Data acquisition, analysis and interpretation, revision, and approval of the manuscript. Julie Braza: Data acquisition, revision, and approval of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

ETHICS STATEMENT

All animal experiments were conducted in accordance with IACUC-approved protocols at the Providence VA Medical Center.

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