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Adiponectin Attenuates Lipopolysaccharide-Induced Acute Lung Injury through Suppression of Endothelial Cell Activation

Jason M. Konter,* Jennifer L. Parker,† Elizabeth Baez,* Stephanie Z. Li,* Barbara Ranscht,‡ Martin Denzel,‡ Frederic F. Little,* Kazuto Nakamura,‡ Noriyuki Ouchi,‡ Alan Fine,* Kenneth Walsh,† and Ross S. Summer*

Adiponectin (APN) is an adipose tissue-derived factor with anti-inflammatory and vascular protective properties whose levels paradoxically decrease with increasing body fat. In this study, APN’s role in the early development of ALI to LPS was investigated. Intratracheal LPS elicited an exaggerated systemic inflammatory response in APN-deficient (APN−/−) mice compared with wild-type (wt) littermates. Increased lung injury and inflammation were observed in APN−/− mice as early as 4 h after delivery of LPS. Targeted gene expression profiling performed on immune and endothelial cells isolated from lung digests 4 h after LPS administration showed increased proinflammatory gene expression (e.g., IL-6) only in endothelial cells of APN−/− mice when compared with wt mice. Direct effects on lung endothelium were demonstrated by APN’s ability to inhibit LPS-induced IL-6 production in primary human endothelial cells in culture. Furthermore, T-cadherin–deficient mice that have significantly reduced lung airspace APN but high serum APN levels had pulmonary inflammatory responses after intratracheal LPS that were similar to those of wt mice. These findings indicate the importance of serum APN in modulating LPS-induced ALI and suggest that conditions leading to hypoadiponectinemia (e.g., obesity) predispose to development of ALI through exaggerated inflammatory response in pulmonary vascular endothelium. The Journal of Immunology, 2012, 188: 854–863.

Acute lung injury (ALI) is a life-threatening condition whose annual incidence in the United States has steadily increased over the last several decades. Recent data indicate that ~200,000 individuals are affected by this condition each year, and current estimates demonstrate a 30–40% mortality, with even greater numbers left with temporary or permanent disabilities (1).

Although the pathogenesis of ALI is complex, hallmark features include immune and endothelial cell activation, loss of vascular integrity, and accumulation of protein-rich fluid in the airspaces of the lung (2, 3). Specific predisposing risk factors for the development of ALI in the face of systemic stress are incompletely defined. However, recent epidemiological data point to obesity as an important risk factor for development of ALI (4–7). In fact, clinical prediction scores utilizing obesity have stratified obese patients as being at higher risk for developing this condition (8). Whereas certain adipocyte-derived hormones called adipokines contribute to a number of chronic inflammatory conditions, including diabetes and cardiovascular disease, the impact of these hormones on the development of acute critical illness such as ALI is not well understood (9–13).

Adiponectin (APN), a highly abundant adipocyte-derived adipokine (with microgram/milliliter concentrations in serum), circulates as low, middle, and high m.w. complexes. Paradoxically higher serum levels are present in lean, healthy individuals compared with obese and diabetic patients (14). Although APN was initially described as an insulin-sensitizing agent, more recent work has defined its pleiotropic anti-inflammatory and vascular protective role (15–18). For example, clinical studies show an inverse relationship between APN levels and circulating concentrations of proinflammatory markers such as C-reactive protein, IL-6, and TNF-α (19–22). Moreover, experimental studies in mice demonstrate that APN contributes to immune homeostasis in the lung (23) and protects against inflammatory and postischemic injury in other tissue beds, including liver, muscle, brain, and heart (24–27). The immune and vascular protective properties of APN led to speculation that hypoadiponectinemia may play a role in the development of ALI. To evaluate APN’s role in ALI, we used a well-established model of LPS-induced lung injury in mice with targeted APN gene deletion.

Materials and Methods

Mice

All studies were performed using 2-mo-old, gender-matched mice. This time point was selected because it precedes the development of the vascular phenotype in APN−/− mice (28). C57BL/6 APN−/− mice were provided by N. Maeda, T. Funahashi, and Y. Matsuzawa (Osaka University, Osaka, Japan). Wild-type (wt) C57BL/6 mice were obtained from Charles River Laboratories. C57BL/6 T-cadherin–deficient (T-cad−/−) mice were provided by B. Ranscht. All animal experiments were reviewed and approved by Boston University’s Institutional Animal Care and Use Committee. Mice were maintained in a 12-h light, 12-h dark schedule and given food and water ad libitum.
**ALI model**

Alveolar lung injury (ALI) was induced by the administration of a one-time intratracheal (i.t.) injection of 100 μg LPS (1 mg/ml). Delivery of LPS was performed using the tongue-pull maneuver in anesthetized mice. At select time points after LPS administration, lung tissue, serum, and bronchoalveolar lavage (BAL) fluid were obtained for analysis. Lung wet/dry weights were performed, as previously described (28), and BAL protein concentration was measured by Bradford assay and Pierce bicinchoninic acid assay.

**Mouse assessment score**

Following i.t. injection, mice were observed and scored by the mouse assessment score every 4 h (with strict, Institutional Animal Care and Use Committee-approved guidelines for euthanasia). Points were assigned based on four categories of physical appearance, as follows: coat, 1 = smooth; 2 = mild ruffling; 3 = significant ruffling; activity level, 1 = normal (exploring cage), 2 = lethargic (moves slowly without stimulation); 3 = sedentary (moves only with stimulation); respiratory effort, 1 = normal, 2 = labored, 3 = labored, irregular; and posture, 1 = moving or resting normally, 2 = huddled. Scores ranged from 4 (normal) to 12 (most abnormal).

**Immunochemistry**

Immunostaining was performed on lung sections after Ag retrieval using Retrieve A (Zymed, South San Francisco, CA) at 100°C for 20 min and quenching endogenous peroxides with 3% H2O2. Sections were blocked with 2% BSA in PBS, followed by staining with primary anti-CD45, anti-B220, anti-CD3, anti-F4/80 (BD Pharmingen, San Jose, CA), or anti–Gr-1 Ab (R&D Systems, Minneapolis, MN) at room temperature (RT) for 1 h. Sections were washed, and after application of secondary Ab (R&D Systems) tissue were developed using Vectastain ABC (Vector Laboratories, Burlingame, CA) and 3,3′-diaminobenzidine (Vector Laboratories).

**Histology scoring system**

Lung sections were evaluated and scored independently by two members of the laboratory trained in histological assessment and use of the scoring system. For each mouse, three different lobes were examined for the following features: interstitial edema, hemorrhage, and neutrophil infiltration. Each feature could receive a score of 0 (no injury), 1 (minimal injury), 2 (moderate injury), or 3 (severe injury). This was totaled for a given lobe’s score, and the three lobes averaged to generate a score for each mouse, giving a minimum score of 0 and a maximum of 9.

**ELISA**

Nalgene Nunc Maxisorp plates were coated with primary Ab against either IL-6, TNF-α, or IL-10 (R&D Systems) for 1 h at RT and then washed with PBS and 0.5% Tween 20 (PBS-T). After blocking with casein, samples were added to plates for 1 h at RT. Following washing, biotinylated secondary Ab was applied for 1 h, followed by streptavidin-HRP conjugate (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted at 1:20,000. Reaction was developed with 0.01% tetramethylbenzidine dissolved in DMSO and 0.5% hydrogen peroxide and measured using endpoint spectrometry.

**Lung digestion**

Lung tissue from 2-mo-old wt, APN-/-, and T-cad-/- mice was digested with intermittent mechanical disruption using a razor blade and with the enzymes dispase (BD Biosciences, Bedford, MA) and collagenase A (Roche, Basel, Switzerland) for 60 min at 37°C. Following digestion, single-cell suspensions were passed through a 70-μm filter to remove excess debris.

**Flow cytometry and cell sorting**

Prior to flow cytometry analysis, cell suspensions were immunostained with FITC-labeled rat anti-mouse CD45 and PE-labeled rat anti-mouse CD31 or isotype controls (BD Pharmingen, Franklin Lakes, NJ). Immunostained cells were then subjected to flow cytometry cell sorting (MoFlo; Beckman Coulter, Brea, CA) to separate endothelial (CD31+45+) and immune cell (CD45+31+) populations. Cells were sorted into PBS solution and immediately transferred to RLT buffer, and RNA isolation was performed using the Qiagen RNeasy Plus Mini kit (Qiagen, Valencia, CA).

**Real-time quantitative PCR**

Real-time quantitative PCR was performed on cDNA amplified using the WT-Ovation kit (NuGen Technologies, San Carlos, CA). Applied Bio-systems StepOne apparatus was used to measure gene expression for IL-1α, IL-6, Nox2, and E-selectin. The 18S rRNA was used to normalize RNA concentration for each sample.

**Endothelial cell culture studies**

Human pulmonary artery endothelial cells (HPAEcs) were obtained from Lonza (Walkersville, MD) and grown in complete endothelial cell growth media. Cells were pretreated for 24 h with full-length human recombinant APN produced in a mouse myeloma cell line (R&D Systems) at 0, 2, or 10 μg/ml. Following pretreatment, cells were washed and then exposed to either PBS or LPS (Escherichia coli 055:B5; List Biological Laboratories, Campbell, CA) at 100 ng/ml for 24 h, and supernatant was collected for cytokine analysis.

**Western blot analysis**

Homogenized lung tissue was preserved in protease-inhibitor (Roche Complete Mini) solution. Western blot for RelA and serum APN was performed using 20 μg protein; BAL APN was loaded by equal volume, 5 μl/lane. RelA specimens were separated by electrophoresis in a 10% Bis-Tris gel, and APN specimens on a 3–8% Tris-acetate gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane. Primary Ab for β-actin, RelA, phospho-RelA (Cell Signaling, Danvers, MA), or APN (R&D Systems) was applied overnight at 4°C in TBS, followed by secondary Ab staining with anti-rabbit HRP (Millipore, Billerica, MA) for 1 h at RT. Chemiluminescence was performed with Western Lightning detection reagent (PerkinElmer, Waltham, MA).

**Adenoviral studies**

Adenoviral vectors containing the gene for β-galactosidase (Ad-gal) and full-length mouse APN (Ad-APN) were prepared, as previously described (29). Adenovirus was injected (200 μl) into the jugular vein 5 d prior to injection of 100 μg LPS (1 mg/ml). Delivery of LPS was performed using the tongue-pull maneuver in anesthetized mice. At select time points after LPS administration, lung tissue, serum, and bronchoalveolar lavage (BAL) fluid were obtained for analysis. Lung wet/dry weights were performed, as previously described (28), and BAL protein concentration was measured by Bradford assay and Pierce bicinchoninic acid assay.
administration of i.t. LPS. Adiponectin levels were measured by ELISA (B-Bridge International, Cupertino, CA) in serum samples obtained 4 h after i.t. administration of LPS.

Statistical analysis
Statistical significance for difference between groups was assessed using Student’s t test. One-way ANOVA and Student t test were used for Fig. 9A. A cutoff p value, 0.05 was used to determine significance.

Results
Adiponectin attenuates LPS-induced lung injury
ALI was induced in wt and APN−/− mice by administering a one-time i.t. injection of LPS (100 μg). The ability of APN to modulate the physiological response to LPS was examined using a 12-point assessment score based on mouse physical characteristics (Fig. 1A). Results showed that within 4 h of LPS administration, APN−/− mice appeared more ill with increased piloerection and decreased mobility. Assessment scores remained elevated for APN−/− mice at 8 and 24 h after injection (Fig. 1A). This exaggerated systemic response in APN−/− mice was associated with increased serum levels of the proinflammatory cytokine IL-6 and the anti-inflammatory cytokine IL-10 at the 4-h time point (Fig. 1B).

To assess local injury response to LPS, detailed lung histological examination was performed. As early as 4 h after LPS administration, APN−/− mice had more severe ALI histological scores at both 4 and 24 h, based on hemorrhage, interstitial edema, and neutrophil infiltration. Wet:Dry ratio (C) and total BAL protein concentration (D) were increased in APN−/− mice after i.t. LPS when compared with wt mice (n = 4 each group, replicated 2 times for histology and wet:dry, 3 times for BAL protein, *p < 0.05; histological scores were from three mice per group, three lobes examined per mouse). Baseline differences were not observed between wt and APN−/− mice.

FIGURE 2. ALI response in wt and APN−/− mice 4 h after i.t. LPS. A, Lung histological examination 4 h after i.t. LPS demonstrated increased perivascular exudates (arrow), thickened alveolar septa (asterisk), and airspace edema in APN−/− mice (A, B) when compared with lungs of wt mice (C, D). Twenty-four hours after LPS injection, APN−/− mice continued to have more severe injury than wt mice. B, APN−/− mice had more severe ALI histological scores at both 4 and 24 h, based on hemorrhage, interstitial edema, and neutrophil infiltration. Wet:Dry ratio (C) and total BAL protein concentration (D) were increased in APN−/− mice after i.t. LPS when compared with wt mice (n = 4 each group, replicated 2 times for histology and wet:dry, 3 times for BAL protein, *p < 0.05; histological scores were from three mice per group, three lobes examined per mouse). Baseline differences were not observed between wt and APN−/− mice.

FIGURE 3. BAL fluid analysis 4 h after i.t. LPS. A–C, ELISA performed for IL-6, TNF-α, and IL-10 demonstrated no differences in cytokine concentrations between wt and APN−/− mice. D, Differences in BAL cell counts were also not detected at this time point (n = 3 in each group, replicated 2 times, *p < 0.05).
associated with an exaggerated infiltration of immune cells into the airspace compartment. To test this, detailed assessment of lung inflammation was performed 4 h after LPS administration. This time point was selected based on its association with the histological onset of lung injury in APN−/− mice. Surprisingly, analysis of BAL fluid at this early time point did not detect differences in total cell counts or in inflammatory cytokine concentrations (IL-6, TNF-α, IL-10) in wt and APN−/− mice (Fig. 3). These findings led to speculation that APN’s influence on the early injury response is mediated through anti-inflammatory actions occurring outside the airspace of the lung. To explore this further, cellular infiltration at 4 h was examined in lung sections after immunostaining for the pan-hematopoietic marker CD45. CD45+ cells accumulated along vascular endothelium in wt mice. In contrast, CD45+ cells had already infiltrated into lungs of APN−/− mice and were found in clusters scattered throughout the lung parenchyma (Fig. 4A). This observed increase in cellular infiltration was confirmed by morphometric analyses demonstrating a 2.5-fold increase in CD45+ cells in lungs of APN−/− mice at 4 h (Fig. 4B). The infiltrating cells in APN−/− mice were predominately neutrophils based on their staining positive for Gr-1 (Fig. 4C) and negative for B220, CD3, and F4/80 (data not shown). Cellular infiltration was associated with increased activation of the proinflammatory transcription factor NF-κB’s RelA subunit (Fig. 5A), higher levels of proinflammatory cytokines TNF-α and IL-6, and decreased concentration of the anti-inflammatory cytokine IL-10 in lung homogenates of APN−/− mice (Fig. 5B). Taken together, these findings suggest that APN protects against development of ALI at least in part through its ability to suppress inflammation outside the airspace of the lung.

Because APN is known to inhibit immune and endothelial cell activation under basal, nonstressed conditions, we speculated that its ability to protect against the development of ALI is mediated through inhibitory effects on these cell populations. To test this possibility, gene expression was evaluated in primary immune and endothelial cells isolated from lung digests of wt and APN−/− mice.

**FIGURE 4.** Inflammatory cell infiltration 4 h post-i.t. LPS. A, Paraffin-embedded lung sections stained for the pan-hematopoietic marker CD45. Results demonstrated increased cellular infiltration into lung of APN−/− mice 4 h after i.t. LPS. CD45+ cells (brown color) were marginated along blood vessels in wt mice (arrowheads), but had infiltrated into lung parenchyma of APN−/− mice (arrows) at 4 h. B, Morphometric analysis confirmed the increased number of CD45+ cells in lung parenchyma of APN−/− mice (n = 5 in each group, replicated 2 times, *p < 0.05). Differences in CD45+ cells were not observed in lung parenchyma of wt and APN−/− mice at baseline (data not shown). C, Lung sections stained for granulocyte marker Gr-1 show that the majority of cells entering the interstitium of APN−/− mice are granulocytes (arrows). These cells were less abundant in wt mice and were primarily found along blood vessel walls (arrowheads). Baseline differences in anti–Gr-1 staining between sections from wt and APN−/− mice were not noted (data not shown).
4 h after LPS administration. As expected, baseline expression of targeted proinflammatory genes was increased in immune and endothelial cells of APN-/- mice. However, in response to LPS, proinflammatory gene expression was relatively downregulated in immune cells. In contrast, increased expression of IL-6, E-selectin, and Nox2, a regulatory enzyme during oxidative stress, was observed in endothelial cells of APN-/- mice (Fig. 6). These findings suggest that early after LPS administration, endothelial cells are an important target of APN’s anti-inflammatory properties.

FIGURE 6. Quantitative real-time PCR for select proinflammatory genes in endothelial (CD45^-/CD31^+) and immune (CD45^+/CD31^-) cells sorted from lung digests of wt and APN-/- mice. A, In endothelial cells, gene expression for IL-6, Nox2, and E-selectin was increased at baseline and in response to LPS. B, In contrast, expression of IL-6 was increased at baseline in immune cells of APN-/- mice; however, the expression of IL-6, TNF-α, and Nox2 was either the same or decreased in these cells after LPS administration (n = 3 in each baseline group, n = 4 each LPS group, replicated 2 times, *p < 0.05). All values were normalized to cycle threshold of wt mice at baseline.
To establish APN’s ability to suppress endothelial cell activation to LPS, HPAECs were cultured for 24 h in the presence or absence of APN. The state of endothelial cell activation was determined by measuring IL-6 concentration in cell supernatants after exposure to LPS (100 ng/ml) for 24 h. Consistent with an inhibitory effect, APN demonstrated a dose-dependent suppression of LPS-induced IL-6 production in lung endothelial cells (Fig. 7).

To investigate the hypothesis that APN protects against ALI through suppressing endothelial cell activation, we used a unique mouse model of compartmentalized APN deficiency. Mice that are deficient in the APN receptor T-cad display a defect in transport of APN into tissues (30, 31). As depicted in Fig. 8, T-cad−/− mice have elevated serum levels of APN with low concentration in the lung. Thus, to determine whether relative lung APN deficiency is associated with an exaggerated ALI phenotype, we compared the i.t. LPS response in wt, APN−/−, and T-cad−/− mice. Mouse assessment scores measured at 4 h were significantly decreased in T-cad−/− mice when compared with APN−/− mice and were comparable to wt mice (Fig. 9A). Moreover, lung injury, measured by BAL protein concentration and assessed by histological examination, was decreased in T-cad−/− compared with APN−/− mice. In addition, these mice showed decreased lung inflammation as evident by lower IL-6 concentrations and decreased CD45+ cell recruitment into the lung (Fig. 9B–E). These findings suggest that maintenance of serum APN is important in controlling lung inflammation through interactions with pulmonary endothelium.

To evaluate whether increasing serum APN could rescue APN deficiency, we pretreated APN−/− mice with adenoviral vectors containing genes for APN (Ad-APN) or the control gene β-galactosidase (Ad-gal). Serum APN concentrations measured 5 d after i.v. administration of adenovirus were 19.5 ± 11.1 mcg/ml in Ad-APN/APN−/− mice, <0.01 in Ad-gal/APN−/− mice, and 21.2 ± 2.6 mcg/ml in Ad-gal/wt mice. Results of reconstitution studies demonstrated that mouse assessment scores were significantly decreased 4 h after i.t. LPS in Ad-APN/APN−/− mice when compared with Ad-gal/APN−/− mice (Fig. 10). In addition, adenoviral-mediated rescue was associated with improved endothelial barrier function as evidenced by decreased BAL protein concentration in Ad-APN/APN−/− mice. Serum and lung IL-6 concentrations were lower in Ad-APN/APN−/− mice when compared with Ad-gal/APN−/− mice, but this did not reach statistical significance. Taken together, these findings further illustrate the importance of APN in the lung and suggest a potential therapeutic role for this protein in enhancing endothelial barrier function during ALI.

FIGURE 7. Adiponectin’s influence on the LPS response in HPAECs. HPAECs were pretreated for 24 h with PBS or adiponectin at either 2 or 10 μg/ml, and then exposed to LPS for 24 h. Supernatant was collected for measurement of IL-6 concentration. Results demonstrated a dose-dependent reduction in IL-6 response in HPAECs to LPS (n = 3 in each group, replicated 3 times, *p < 0.05).

FIGURE 8. Western blot analysis for APN in serum and in BAL fluid of wt, APN−/−, and T-cad−/− mice (replicated 2 times).

Discussion

The major objective of this study was to evaluate the role of APN in the development of ALI. We demonstrated a novel role for APN in that it protects against the development of ALI and limits the associated systemic response to LPS. These observations in mice suggest a potential mechanism by which human obesity is a risk factor for the development of ALI.

To characterize the mechanisms mediating APN’s protective effects, we focused on the early response to lung injury in APN−/− mice. Unexpectedly, total cell counts and cytokine concentrations in BAL fluid of wt and APN−/− mice were not significantly different at early time points. These findings led us to explore APN’s ability to modulate the ALI response through actions outside the airspace of the lung. We hypothesized that the vascular endothelium of the lung mediates APN’s protective actions in this model because vascular permeability (wet:dry ratio and BAL protein concentration) is increased in APN−/− mice. This hypothesis is supported by observations showing that lung endothelial cells express APN receptors and that APN localizes on the surface of lung endothelium (28). In addition, previous work has shown that targeted deletion of APN promotes an activated endothelial cell phenotype under basal, nonstressed conditions, including upregulation of E-selectin (28). In the current study, we confirmed the baseline upregulation of proinflammatory genes in isolated lung endothelial cells of APN−/− mice and demonstrated further upregulation of IL-6 and other proinflammatory genes in lung endothelial cells early after i.t. LPS. Based on these findings, it is tempting to speculate that increased serum IL-6 levels in APN−/− mice result from endothelial secretion. This is further supported by studies demonstrating that recombinant APN effectively inhibited LPS-induced IL-6 production in primary lung endothelial cells in culture. However, our study does not directly address this potential mechanism of increased circulating IL-6 in LPS-challenged APN−/− mice. Taken together, our findings indicate that APN modulates the ALI response to LPS at least in part through its actions on lung endothelium. Consistent with this
notion, it has been reported that circulating levels of von Willebrand factor, a marker of endothelial injury, are observed in obese patients with ALI (4). Presumably, these patients would have low APN levels at the onset of ALI.

To further test whether APN’s suppression of the early response to LPS is conferred outside the airspace of the lung, a model of relative lung APN deficiency using T-cad−/− mice was used. T-cad is a major APN receptor/binding protein that is expressed on lung endothelium (32). T-cad is unique in that it lacks an intracellular signaling domain, suggesting that it serves predominantly as a docking protein (30, 31), and appears to play an essential role in the transport of APN into the airspace of the lung (30). In this regard, the inflammatory response to LPS in T-cad−/− mice that lack APN in the airspace was similar in all groups 4 h after i.t. administration of LPS. D, IL-6 concentration in lung homogenate was increased in APN−/− mice, when compared with wt and T-cad−/− mice. E, Histology after i.t. LPS shows increased perivascular exudates (arrows) and lung edema and injury in APN−/− mice when compared with both wt and T-cad−/− mice. CD45+ staining (brown color) shows increased tissue infiltration in APN−/− mice when compared with wt and T-cad−/− mice (n = 3 wt and APN−/− mice, n = 5 T-cad−/− mice, all replicated 2 times, *p < 0.05).

Figure 9. Response to LPS in a model of relative isolated lung adiponectin deficiency (T-cad−/− mice). A, Mouse assessment score was decreased in wt and T-cad−/− mice when compared with APN−/− mice at 4 h. B, BAL fluid protein concentration was increased in APN−/− mice when compared with wt and T-cad−/− mice. C, IL-6 concentration in BAL fluid was similar in all groups 4 h after i.t. administration of LPS. D, IL-6 concentration in lung homogenate was increased in APN−/− mice, when compared with wt and T-cad−/− mice. E, Histology after i.t. LPS shows increased perivascular exudates (arrows) and lung edema and injury in APN−/− mice when compared with both wt and T-cad−/− mice. CD45+ staining (brown color) shows increased tissue infiltration in APN−/− mice when compared with wt and T-cad−/− mice (n = 3 wt and APN−/− mice, n = 5 T-cad−/− mice, all replicated 2 times, *p < 0.05).
vascular remodeling in murine models of allergic airway inflammation and chronic hypoxia, and that endothelial dysfunction might underlie the previously described emphysematous phenotype of APN−/− mice (35–37). However, we acknowledge that APN’s protective effects may extend to other vascular beds, as illustrated by observations of exacerbated endothelial cell activation in the systemic circulation during intra-abdominal sepsis in APN−/− mice (38). Furthermore, our data indicate that APN’s actions are not limited to endothelium. For example, we previously found that APN maintains alveolar macrophage quiescence (23).

However, in this study, although we confirmed that unstimulated CD45+ immune cells of APN−/− mice had increased expression of proinflammatory cytokines, we did not find an exaggerated LPS-induced response in these cells at early time points when compared with wt mice. In fact, we found decreased induction of TNF-α and Nox2 in CD45+ cells isolated from APN−/− mice. These findings indicate that APN exerts differential effects on endothelial cells and leukocytes in lung. These effects could be mediated by actions of different APN oligomeric fractions or binding of APN to different cell surface receptors. Future studies investigating the mechanisms mediating APN’s differential effects will be important to develop targeted therapies that selectively suppress activation of endothelial or immune cells in lung.

One other important finding in this study is that APN appears to play a key role in the selective recruitment of inflammatory cells into lung. This is evident from increased neutrophil infiltration into lungs of APN−/− mice. We speculate this may also explain the observed divergence in lung IL-10 levels between wt and APN−/− mice, in that APN may preferentially recruit IL-10–producing immune cells of APN−/− mice when compared with wt mice. IL-6 concentrations in serum and lung were lower in Ad-APN/APN−/− mice when compared with wt mice, but this did not reach statistical significance. IL-6 concentration and total cell counts in BAL fluid were similar in all groups 4 h after i.t. administration of LPS (data not shown; n = 5 all groups, except serum IL-6 analysis n = 3, *p < 0.05).

APN has anti-inflammatory actions that do not require direct cell interactions. For example, APN has been shown in vitro to bind LPS (39). However, if this mechanism played a significant role, decreased levels of BAL cytokines should have been seen in wt versus APN−/− mice 4 h after i.t. LPS. In addition, APN pre-treatment of HPAECs followed by media change prior to LPS challenge was capable of effectively suppressing the LPS response. Both findings suggest that APN-mediated binding and/or sequestering LPS does not represent a major mechanism of action of APN’s modulation of this ALI response.

Another key finding in this study was the observation that restoration of serum APN levels attenuated the response to i.t. LPS. Decreased BAL protein concentration in Ad-APN/APN−/− mice 4 h after LPS administration suggests that APN acts, at least in part, by enhancing endothelial barrier function. This hypothesis is further supported by our in vitro studies demonstrating direct suppressive effects of APN on lung endothelial cells exposed to LPS. Although IL-6 concentration was not significantly different between LPS-injured Ad-APN and Ad-gal/APN−/− mice, we speculate that higher serum APN levels may be required for longer periods to facilitate anti-inflammatory responses in lung. In addition, it is possible that untoward effects of adenoviral infection may have influenced the results of these studies because IL-6 concentration and BAL protein levels were generally higher in adenoviral-exposed mice. Collectively, the rescue studies indicate APN exerts a protective effect on the lung endothelium and suggest a potential therapeutic role for this protein in individuals at risk for ALI.

It is worth mentioning that a recent clinical study from our laboratory demonstrated that high serum levels of APN were associated with increased mortality in critically ill patients with acute respiratory failure (40). This report appears to contradict our results in mice. However, studies in APN−/− mice investigated the role of APN in the development of LPS-induced ALI, in which APN was absent prior to onset of lung injury. In contrast, human studies evaluated the association between APN and mortality in a diverse population (only 21% of which had ALI) with established acute respiratory failure. We speculate that higher APN levels in these patients reflect the host response to critical illness.
and have little or no correlation with serum levels prior to onset of disease. Thus, to confirm findings in mice, future clinical studies will need to investigate the relationship between baseline serum APN levels and risk of developing ALI.

Although our study supports that APN deficiency may be a mechanistic link between obesity and ALI, other adipokines are altered during obesity and may contribute to the pathogenesis of ALI. For example, leptin (41), a highly abundant adipokine, is structurally similar to the IL-6 family of cytokines and demonstrates proinflammatory activity on a variety of cell types. The i.t. administration of leptin in mice has been shown to induce ALI (42). However, clinical studies have not identified a correlation between leptin levels and ALI in human subjects, and this may be explained, in part, by the fact that chronic elevation in serum leptin levels is often associated with resistance at the level of the receptor (42, 43). Future studies examining the role of other adipokines in the development of ALI will be required.

In summary, we identified a previously unrecognized role for APN in limiting the development of ALI in mice. Furthermore, our findings suggest that this effect is mediated in part by APN’s direct action on lung endothelium. Based on this work, we speculate that measuring circulating APN may be important in defining one’s risk for developing ALI.

Disclosures

The authors have no financial conflicts of interest.

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