$B\beta_{15-42}$ Protects against Acid-induced Acute Lung Injury and Secondary *Pseudomonas* Pneumonia *In Vivo*

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Rationale: **Acute lung injury (ALI) is a serious condition in critically ill patients that predisposes to secondary bacterial pneumonia. Vascular leak is a hallmark in the pathogenesis of ALI. The fibrin-derived peptide $B\beta_{15-42}$ was shown to preserve endothelial barriers, thereby reducing vascular leak. The potential therapeutic role of $B\beta_{15-42}$ in ALI has not been addressed so far.**

Objectives: **To investigate the therapeutic potential of $B\beta_{15-42}$ in ALI and secondary pneumonia induced by *Pseudomonas aeruginosa*.**

Methods: **The effect of the fibrin-derived peptide $B\beta_{15-42}$ was studied in models of ALI, induced either by pulmonary administration of LPS or hydrochloric acid. Lung inflammation was analyzed by quantifying cell influx, cytokine levels, and oxidized lipids. Vascular leak was determined by Evans Blue extravasations and alveolar protein content. In subsequent two-hit studies, mice were infected with *P. aeruginosa* 24 hours after induction of aspiration pneumonitis and effects of $B\beta_{15-42}$ on inflammation, bacterial clearance, and survival were evaluated.**

Measurements and Main Results: **After LPS or acid inhalation, proinflammatory cytokine levels, neutrophil influx, and vascular leak were found diminished in mice treated with $B\beta_{15-42}$. Acid aspiration impaired macrophage functions and rendered mice more susceptible to subsequent *P. aeruginosa* infection, whereas mice that received $B\beta_{15-42}$ during acid aspiration and were subsequently challenged with bacteria displayed reduced inflammation, enhanced bacterial clearance, and ultimately improved survival.**

Conclusions: **The fibrin-derived peptide $B\beta_{15-42}$ exerted protective effects during ALI, resulting in diminished lung injury and preserved antibacterial properties of macrophages, which improved outcome during subsequent *P. aeruginosa* pneumonia.**

Keywords: acute lung injury; inflammation; pneumonia; *Pseudomonas aeruginosa*

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Vascular leak and neutrophil migration are hallmarks of acute lung injury (ALI). Despite high mortality rates, specific therapies to prevent lung injury and inflammation are not available.

What This Study Adds to the Field

The fibrin-derived peptide $B\beta_{15-42}$ prevents vascular leak and protects mice from ALI and secondary *Pseudomonas aeruginosa* pneumonia *in vivo*.

Acute lung injury (ALI) is a serious condition defined as rapid-onset bilateral pulmonary infiltrates and hypoxemia of noncardiac origin (1, 2). Acute respiratory distress syndrome is the most severe form of ALI. With a reported incidence of 79 per 100,000 and an in-hospital mortality of 40%, ALI represents a serious problem among intensive care unit (ICU) patients (3). ALI can develop as a result of direct injury to the lungs, such as during pneumonia, or aspiration of gastric contents, or occur in the course of systemic inflammation, such as during sepsis or after trauma (4). Despite these different etiologies, the pathological features observed in ALI share common findings like protein-rich edema and accumulation of neutrophils (4, 5).

To investigate the molecular mechanisms leading to ALI, a number of animal models have been established. Among them, LPS- and hydrochloric acid (HCl)-induced ALI are known to yield very reproducible results and are characterized by a rapid influx of polymorphonuclear leukocytes (PMNs) and release of proinflammatory cytokines. Although both models ultimately lead to the disruption of endothelial barriers, LPS seems to primarily target the endothelium, whereas HCl has been reported to initially damage epithelial cells (6). Acid aspiration is a widely used model of ALI in mice, as it ideally imitates the pathophysiologic events observed in humans (7–9) by mimicking the clinically relevant event of aspiration of gastric contents in patients with reduced consciousness, referred to as aspiration pneumonitis (10).

Secondary bacterial infection is a frequent and dreaded complication in critically ill patients suffering from ALI (10). *Pseudomonas aeruginosa* is one of the most common pathogens causing nosocomial pneumonia, particularly in ICUs, where intubation favors its colonization, and antibiotic therapy selects multiresistant strains (11). Experimentally it was shown that preceding acid aspiration primes for an exaggerated, and thereby harmful, inflammation to subsequent administration of LPS (12) or bacteria (13). In both reports, administration of LPS or bacteria after instillation of acid led to a dramatic increase in proinflammatory cytokines, such as IL-6, IL-1β, keratinocyte-

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derived chemokine (KC), or macrophage-inflammatory protein (MIP)-2 (13). van Westerloo and colleagues nicely demonstrated that clearance of *Klebsiella pneumoniae* was greatly impaired in mice suffering from preceding acid aspiration (13). In addition, others reported that acid aspiration enhanced bacterial adherence (14) and that gastric acid and particulate aspiration impaired pulmonary bacterial clearance (15). Hence, although ALI itself is considered a life-threatening condition, it furthermore predisposes to nosocomial pneumonia, thus underlining the urgent need for better treatment of ALI.

In 2005 a naturally occurring peptide derived from the N-terminus of the β-chain of fibrin, B$\beta_{15–42}$, was shown to protect from myocardial reperfusion injury in rats due to its capacity to prevent leukocyte migration (16). In a subsequently installed multicenter phase IIa clinical trial, these findings could be confirmed in patients suffering from acute myocardial infarction (17). B$\beta_{15–42}$ significantly reduced the size of necrotic zones in patients with acute myocardial infarction undergoing primary percutaneous coronary intervention (17). In another series of experiments, B$\beta_{15–42}$ was shown to be vasculoprotective in models of vascular leak, such as Dengue hemorrhagic shock or LPS shock (18). It improved survival and reduced vascular leak in a Fyn-dependent manner (18). The antiinflammatory and vasculoprotective features of the peptide prompted us to test its therapeutic potential in the lung.

Although our understanding of pathophysiological mechanisms underlying ALI has improved substantially over the last years, therapeutic advances are missing and treatment recommendations are limited to protective ventilation and supportive care (19). The high lethality and great clinical importance of ALI prompted us to explore the therapeutic potential of B$\beta_{15–42}$ during ALI and secondary bacterial pneumonia.

METHODS

Animals

Pathogen-free 9- to 11-week-old male C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany). All experiments were approved by the local Ethics Committee of the Medical University Vienna and the Ministry of Sciences.

Induction of ALI and Pneumonia

ALI and pneumonia were induced as described previously (20). Briefly, mice were short-term anesthetized by inhalation of isoflurane (Abbott Laboratories, Vienna, Austria), and 50 μl of LPS (*Escherichia coli* O55:B5, 100 ng; Sigma, St. Louis, MO) or *P. aeruginosa* (PA103) at indicated amounts was instilled intranasally. For the induction of acid-induced lung injury mice were anesthetized using ketamine and xylazine and 50 μl of 0.1 N endotoxin-free HCl (Sigma) was injected intratracheally. For more detailed information see the online supplement.

Peptide Preparation and Administration

B$\beta_{15–42\text{-}NH_2}$ (GHRPLDKKREEAPSLRPAPPPISGGGYR-NH_2) was used as a proteolytically stable analog of B$\beta_{15–42}$; random peptide or saline was used as control. Peptides were produced by solid-phase peptide synthesis and purified with reverse-phase high-performance liquid chromatography using nucleosil 100-10C18 columns (Lonza, Brussels, Belgium, and piChem Forschungs- und Entwicklungs-GmbH, Graz, Austria) (16). Mice were treated with 4.8 mg/kg intraperitoneally at t = 0 and t = +1 hour after LPS or acid challenge, respectively. In all experiments lasting longer than 24 hours mice received a third dose at t = +6 hours.

Lung Sampling and Quantification of Colony-forming Units

Whole lungs were harvested and processed as described (21, 22); determination of lung cfu is described in the online supplement.

Determination of Vascular Permeability, Edema, and Histology

Vascular leak was determined using Evans Blue extravasations as described (23). Edema was quantified by determining total protein concentration in bronchoalveolar lavage fluid (BALF) using a protein assay kit (Pierce, Rockford, IL). For lung histology the left lobe was removed and processed as described earlier (21) and paraffin sections were stained with hematoxylin and eosin. The degree of inflammation was scored based on the size of the infiltrate, presence of edema, bronchitis, thrombi, endotheliitis, pleuritis, and perivascular infiltrates by a pathologist blinded for groups. Immunohistochemical staining of interleukin-1 receptor associated kinase (IRAK)-M was done as described (24). Further details are outlined in the online supplement.

BAL and Differential Cell Count

BAL was performed as described previously (21). Cells were enumerated using a hemocytometer and differential cell counts were performed on cytospin preparations stained with Giemsa. The BALF was stored at −70°C for determination of cytokines, protein content, and oxidized lipids.

Protein, Oxidized Phospholipids, and Myeloperoxidase Assays

Tumor necrosis factor (TNF)-α, IL-1β, IL-6, IL-10, KC, MIP-2, and myeloperoxidase (MPO) were quantified in BALF and lung homogenates using specific ELISAs (R&D Systems, Minneapolis, MN and HyCult, Uden, the Netherlands) according to the manufacturers' instructions. Oxidized lipids were measured as described (25). More detailed information is provided in the online supplement.

Phagocytosis Assay

Phagocytosis of heat-killed *P. aeruginosa* (PA103) was assessed in essence as described (26), and is further outlined in the online supplement.

Evaluation of mRNA Levels in Whole Lung Preparations

Semiquantitative mRNA analysis for IRAK-M transcripts in whole lung preparations was done as described (24) and is further outlined in the online supplement.

Statistics

Values are expressed as mean ± SEM. Data between two groups were analyzed using unpaired Student *t* test; for more than two groups one-way analysis of variance followed by Tukey multiple comparison test was used. Differences in cfu counts (nonparametric) were calculated using Mann-Whitney test (two groups) or Kruskal-Wallis test followed by Dunn multiple comparison test (more than two groups). Survival data were analyzed by Kaplan-Meier followed by log-rank test. Criteria for significance for all experiments were *P* less than 0.05.

RESULTS

B$\beta_{15–42\text{-}NH_2}$ Exerts Antiinflammatory Properties within the Lungs

We first analyzed the effects of B$\beta_{15–42\text{-}NH_2}$ during acute pulmonary inflammation and challenged mice with 100 ng LPS intranasally and administered 4.8 mg/kg of the peptide intraperitoneally immediately after LPS challenge and 1 hour later. Control animals received saline or control peptides, respectively. After 6 hours we enumerated cells in BALF where we found significantly reduced numbers of neutrophils in mice treated with the peptide (Figure 1A). In line with the decreased influx of PMNs we observed significantly reduced levels of proinflammatory cytokines and chemokines in BALF (Figure 1B) and lungs (Figure 1C) from B$\beta_{15–42\text{-}NH_2}$-treated mice. Although TNF-α levels were significantly diminished in the bronchoalveolar compartment, IL-10 concentrations were elevated in lungs of B$\beta_{15–42\text{-}NH_2}$-treated animals (Figure 1C).

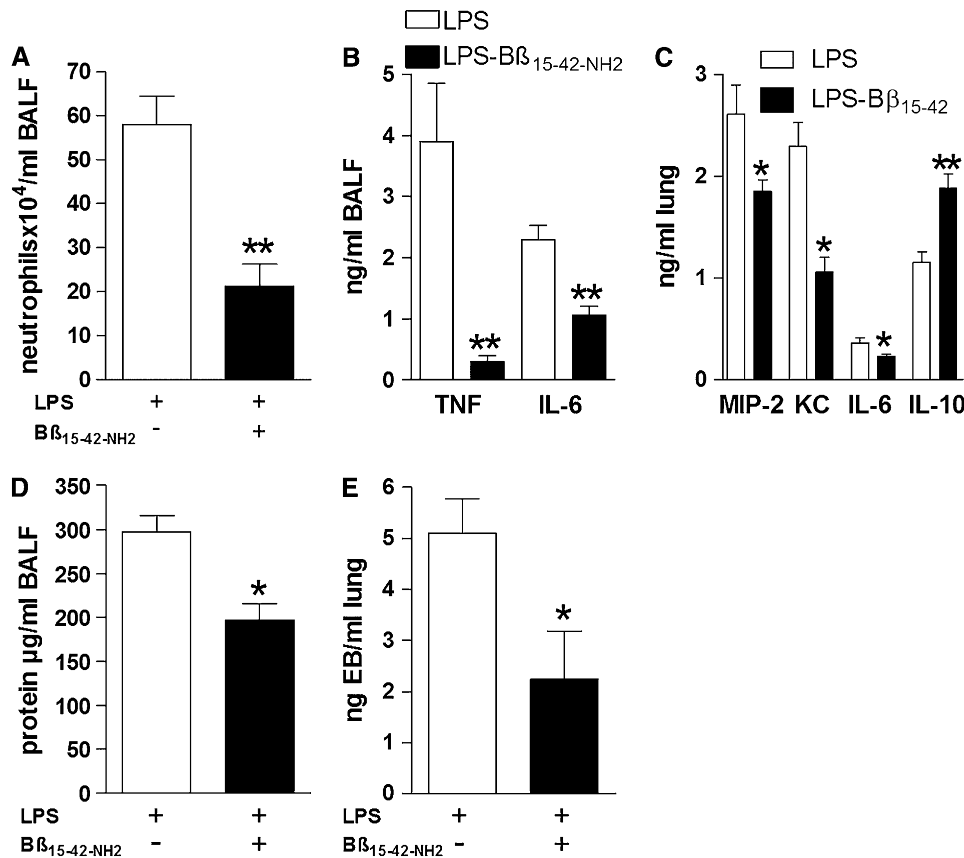

Figure 1. $B\beta_{15\text{–}42\text{-}NH_2}$ exerts antiinflammatory properties in the lung. Mice received 100 ng LPS intranasally and $B\beta_{15\text{–}42\text{-}NH_2}$ or carrier (NaCl), respectively, intraperitoneally at t = 0 and t = +1 hour. After 6 hours (*A*) polymorphonuclear leukocyte influx was assessed by cytospin preparations of bronchoalveolar lavage fluid (BALF), (*B*) cytokines were measured in BALF and (*C*) lung homogenates, and (*D*) total protein content in the BALF. (*E*) For measurement of Evans Blue extravasations mice received 10 μg LPS intranasally and $B\beta_{15\text{–}42\text{-}NH_2}$ or NaCl treatment, respectively, as described above. (*A–D*) n = 6 mice/group, (*E*) n = 4 mice/group. Depicted are representative data out of three independent experiments. Data are mean ± SEM; $^*P < 0.05$, $^{**}P < 0.01$.

Because vascular leak is considered a hallmark of ALI, we also measured total protein contents in BALF of LPS-challenged mice and detected lower protein amounts in $B\beta_{15\text{–}42\text{-}NH_2}$-treated animals (Figure 1D). Finally, we were able to confirm that $B\beta_{15\text{–}42\text{-}NH_2}$ treatment reduced vascular leak within the pulmonary compartment by illustrating diminished Evans Blue extravasations during LPS-induced ALI (Figure 1E). Hence, these data demonstrate that $B\beta_{15\text{–}42\text{-}NH_2}$ exerts potent antiinflammatory effects within the respiratory tract and attenuates vascular leak during ALI *in vivo.*

$B\beta_{15\text{–}42\text{-}NH_2}$ Dampens Acid-induced Lung Inflammation

Having established that $B\beta_{15\text{–}42\text{-}NH_2}$ reduces inflammation and improves vascular barrier function during LPS pneumonitis *in vivo*, we next aimed for a model in which these findings could be used for therapeutic purposes and decided to extend our studies to acid-induced ALI, which more closely reflects the situation seen in ICU patients. For this purpose we administered 50 μl of endotoxin-free 0.1 N HCl intratracheally, treated mice with $B\beta_{15\text{–}42\text{-}NH_2}$ or saline as described above, and evaluated mice every 2 hours up to 8 hours. As depicted in Figures 2A–2D, $B\beta_{15\text{–}42\text{-}NH_2}$ treatment resulted in a diminished PMN influx to the bronchoalveolar compartment, reduced levels of proinflammatory cytokines, and decreased total protein concentration in BALF. To investigate the inflammatory response and impact of $B\beta_{15\text{–}42\text{-}NH_2}$ in more detail we then focused on 6 hours after instillation of acid and revealed that treatment with the peptide markedly contained the increase of leukocytes (Figure 2E). A similar pattern was observed for levels of the proinflammatory cytokines in BALF and lung tissue (Figures 2F and 2G). In line with a previous report, which delineated IL-6 as a crucial mediator of acid-induced lung inflammation (25), we found IL-6 highly elevated on acid aspiration, whereas $B\beta_{15\text{–}42\text{-}NH_2}$ treatment resulted in diminished IL-6 levels (Figures 2F and 2G). IL-1β was significantly reduced in lungs of mice that received $B\beta_{15\text{–}42\text{-}NH_2}$ (Figure 2G), which is noteworthy as an earlier report discovered that IL-1R gene deficient mice exhibited an improved bacterial clearance during *P. aeruginosa* pneumonia (27). Similar to what we observed during LPS pneumonitis, $B\beta_{15\text{–}42\text{-}NH_2}$ treatment led to enhanced levels of the antiinflammatory cytokine IL-10; significantly higher IL-10 concentrations were discovered in BALF from the treatment group, and IL-10 levels obtained from lung homogenates were modestly elevated, although differences did not reach significance (P = 0.055) (Figures 2F and 2G). Furthermore, the inhibitory effects of $B\beta_{15\text{–}42\text{-}NH_2}$ on vascular leak during acid aspiration were confirmed by showing reduced total protein concentrations in BALF and diminished Evans Blue extravasations (Figures 2H and 2I). Because acid aspiration–associated generation of reactive oxygen species and subsequent oxidation of pulmonary phospholipids were shown recently to perpetuate ALI *in vivo* (25), we measured BALF levels of oxidized phospholipids using the well-described antibody E06 (28). In accordance with diminished pulmonary inflammation, we observed a tendency toward reduced levels of oxidative epitopes in $B\beta_{15\text{–}42\text{-}NH_2}$-treated animals (Figure 2J), although differences did not reach significance (P = 0.07). Together, $B\beta_{15\text{–}42\text{-}NH_2}$ was able to attenuate lung injury after acid aspiration *in vivo*.

Preceding Acid Aspiration Impairs Bacterial Clearance during *P. aeruginosa* Pneumonia

Arguing that preexisting acid-induced lung damage predisposes patients for subsequent bacterial pneumonia, we hypothesized that attenuation of acid-induced lung injury might improve outcome during secondary bacterial respiratory tract infection.

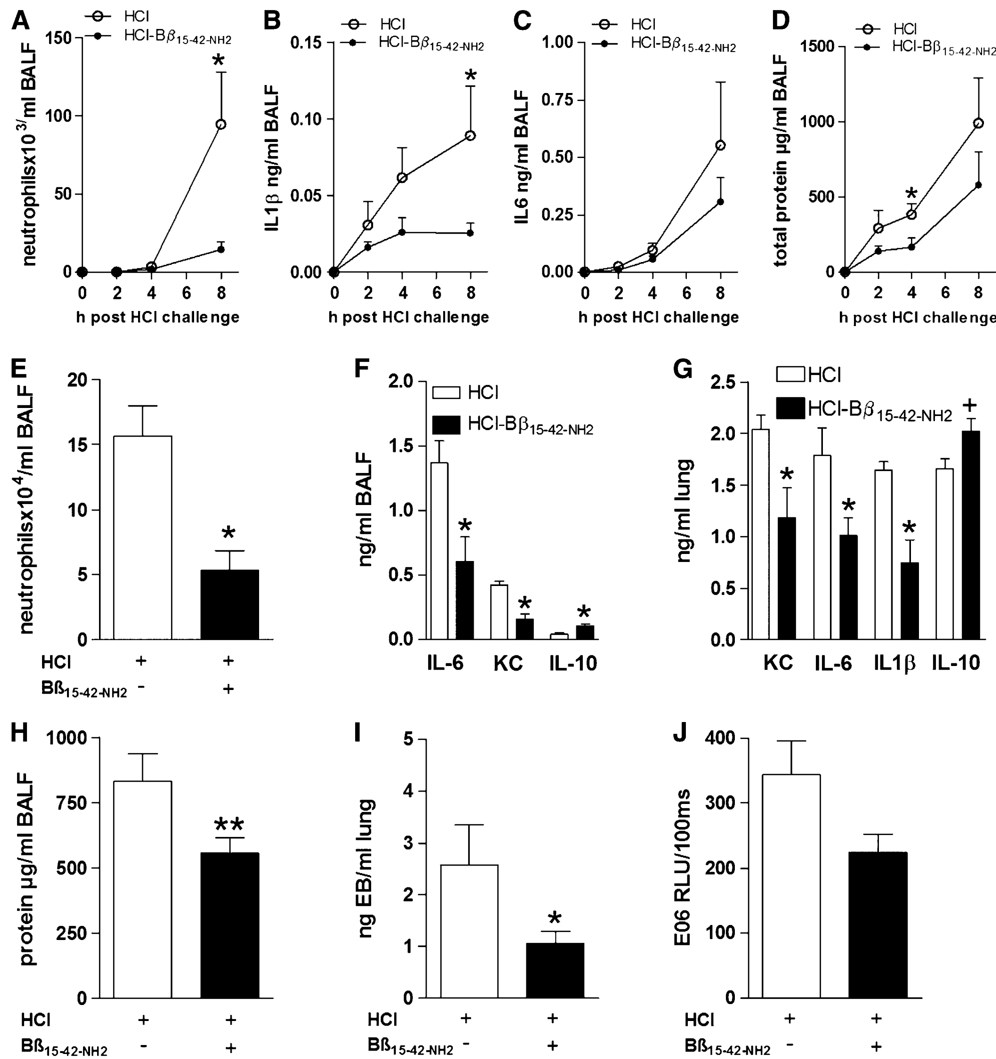

Figure 2. $B\beta_{15-42-NH_2}$ dampens acid-induced lung inflammation endotoxin-free 0.1 N HCl (50 μl) was instilled intratracheally in mice followed by intraperitoneal injection of $B\beta_{15-42-NH_2}$ or carrier (NaCl) at t = 0 and t = +1 hour. Time-course analysis for (*A*) polymorphonuclear leukocyte (PMN) influx, (*B*) IL-1β, (*C*) IL-6 levels, and (*D*) total protein concentration in bronchoalveolar lavage fluid (BALF) are depicted. (*E*) After 6 hours PMN influx in BALF was assessed on cytospin preparations, cytokines and chemokines were measured in (*F*) BALF and (*G*) lung homogenates. (*H*) Total protein content was measured in BALF. (*I*) Evans Blue extravasations were quantified in lung homogenates and (*J*) oxidation epitopes (E06) in BALF. (*A–I*) n = 6 to 8 mice/group; (*J*) pooled data of two experiments (n = 11–13 mice/group). Data shown are one out of two independent experiments and depicted as mean ± SEM; $^*P < 0.05$, $^{**}P < 0.01$.

To test this concept we first attempted to establish that acid aspiration would alter the course of secondary pneumonia induced by *P. aeruginosa*, the most frequently isolated pathogen in hospital-acquired pneumonia (29). For this purpose we induced acid aspiration or administered saline, respectively, and infected all mice intranasally with *P. aeruginosa* after 24 hours. This specific infection time point was chosen after we had determined that acid-induced lung inflammation gradually resolved by 24 hours. Mice were then killed 16 hours after secondary intranasal infection with *P. aeruginosa* and the inflammatory response and bacterial clearance were evaluated. As anticipated, preceding acid aspiration was associated with highly elevated lung levels of proinflammatory cytokines, such as KC, IL-6, IL-1β, or TNF-α, compared with control animals (Figure 3A). Neutrophil influx, which was assessed by measuring lung MPO concentrations, was significantly higher in mice that underwent acid aspiration before pneumonia as compared with saline-treated mice (Figure 3B). Furthermore, despite this exaggerated inflammatory response, bacterial clearance was greatly impaired, leading to almost 1000-fold increased bacterial

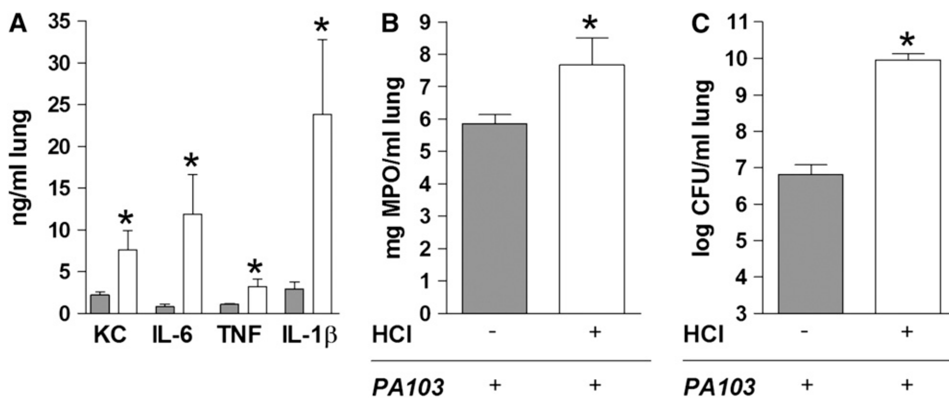

Figure 3. Acid aspiration impairs host response to *Pseudomonas aeruginosa* pneumonia. Mice received 50 μl of 0.1N HCl (HCl group, *open bars*) or sterile NaCl (sham group, *shaded bars*) and 24 hours later 1 × 10^4 cfu *P. aeruginosa* intranasally. (*A*, *B*) Mice were killed 16 hours after bacterial infection and lung cytokines, chemokines, and myeloperoxidase were evaluated. (*C*) Serial dilutions of lung homogenates were plated on blood agar plates to determine bacterial cfu. Data are from n = 8 mice/group and represent mean ± SEM. $^*P < 0.05$.

TABLE 1. PULMONARY INFLAMMATION MARKERS 24 HOURS AFTER ACID ASPIRATION

Lung	Sham	HCl	HCl-B$\beta_{15-42\text{-}NH_2}$
MPO mg/ml	1.2 ± 0.08	1.5 ± 0.02*	1.1 ± 0.01
IL-6 ng/ml	0.28 ± 0.02	0.53 ± 0.13	0.31 ± 0.03
KC ng/ml	1.64 ± 0.06	2.7 ± 0.12*	1.24 ± 0.02
TNF ng/ml	0.23 ± 0.01	0.23 ± 0.02	0.21 ± 0.03
E06 RLU/100 ms	6108 ± 2085	15947 ± 4190	5453 ± 887

Definition of abbreviations: E06 = oxidation epitopes; KC = keratinocyte-derived chemokine; MPO = myeloperoxidase; TNF = tumor necrosis factor.

Mice underwent NaCl (sham) or acid aspiration (HCl) with subsequent B$\beta_{15-42\text{-}NH_2}$ treatment (HCl-B$\beta_{15-42\text{-}NH_2}$). Pulmonary inflammation markers were evaluated after 24 hours. Shown are mean ± SEM of n = 3 mice/group.

* $P < 0.05$ versus sham and HCl-B$\beta_{15-42\text{-}NH_2}$ group.

counts in lungs from mice of the acid-aspiration group, as compared with control animals (Figure 3C). Therefore, preceding acid aspiration impairs host defense mechanisms against secondary *P. aeruginosa* infection.

B$\beta_{15-42\text{-}NH_2}$ Diminishes the Unfavorable, Exaggerated Immune Response to *P. aeruginosa* after Acid Aspiration

We next tested our hypothesis that B$\beta_{15-42\text{-}NH_2}$ treatment would reduce acid-induced lung injury and thus attenuate detrimental effects on the host response to subsequent bacterial pneumonia. For this purpose we repeated previously described experiments, challenged mice with HCl or saline (sham group), respectively, and treated one group of mice that received HCl intratracheally with B$\beta_{15-42\text{-}NH_2}$ intraperitoneally at t = 0 hours, +1 hour, and +6 hours after intratracheal acid administration. Twenty four hours later we ensured gradual resolution of acid-induced lung inflammation and observed that inflammatory markers did not differ between the sham group and HCl-mice that received B$\beta_{15-42\text{-}NH_2}$ (Table 1).

Next we aimed to evaluate the effects of these differences on a second-hit pneumonia with *P. aeruginosa*. We therefore inoculated mice intranasally 24 hours after acid aspiration or sham surgery, respectively, with *P. aeruginosa* and evaluated the host inflammatory response 16 hours thereafter (i.e., 24 h acid aspiration + 16 h infection). On analysis of pulmonary cytokine and chemokine levels, we discovered that B$\beta_{15-42\text{-}NH_2}$ treatment of mice that received HCl resulted in almost identical lung concentrations of MPO, KC, IL-6, TNF-α, and IL-1β as those found in mice that received saline instead of HCl (Figures 4A–4C). Although acid aspiration followed by *P. aeruginosa* infection led to an enhanced inflammatory response, sham-treated mice (i.e., NaCl instead of HCl) and B$\beta_{15-42\text{-}NH_2}$-treated mice (i.e., HCl and B$\beta_{15-42\text{-}NH_2}$) displayed significantly reduced concentrations of proinflammatory mediators and MPO

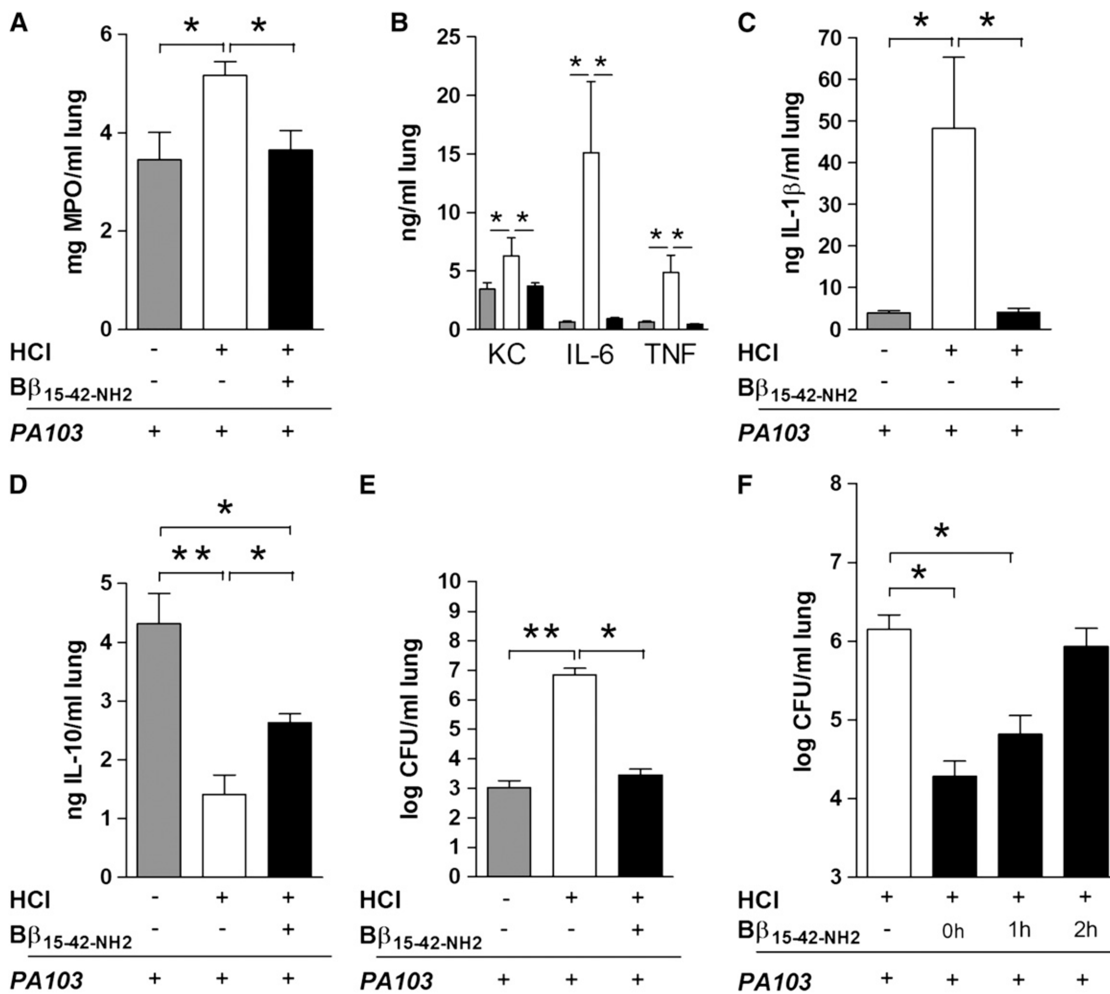

Figure 4. B$\beta_{15-42\text{-}NH_2}$ treatment of aspiration-induced acute lung injury dampens inflammation during secondary *Pseudomonas aeruginosa* pneumonia. Endotoxin-free 0.1 N HCl (50 μl) was instilled intratracheally in mice followed by intraperitoneal injection of B$\beta_{15-42\text{-}NH_2}$ (HCl-B$\beta_{15-42\text{-}NH_2}$) (*solid bars*) or sterile NaCl (HCl) (*open bars*) at t = 0, t = +1 hour, and t = +6 hours. Sham-treated (*shaded bars*) animals received sterile NaCl intratracheally and intraperitoneally (sham group). After 24 hours all three groups were infected with 1 × 10^4 cfu *P. aeruginosa* intranasally. (*A–D*) Sixteen hours after infection mice were killed and myeloperoxidase, cytokines, and chemokines in lung homogenates were evaluated. (*E*) Lung cfu were determined on blood agar plates. Depicted is one out of three independent experiments of n = 6 to 9 mice/group. In (*F*) mice were treated as described above with the exception that B$\beta_{15-42\text{-}NH_2}$ treatment was started at t = 0 hours, t = +1 hour, or t = +2 hours after acid aspiration (*solid bars*). Control mice (*open bars*) did not receive any B$\beta_{15-42\text{-}NH_2}$. All mice were infected with *P. aeruginosa* 24 hours after acid aspiration. (*A–F*) Data are mean ± SEM; *$P < 0.05$, **$P < 0.01$.

levels. In parallel, the antiinflammatory cytokine IL-10 showed the opposite feature, with lowest levels found in mice that underwent acid aspiration before infection (Figure 4D). When enumerating lung cfu we observed striking differences: $B\beta_{15-42-NH_2}$-treated mice that underwent acid aspiration showed an identical bacterial load as sham-treated control animals (NaCl aspiration), whereas in mice that underwent acid aspiration followed by bacterial infection approximately 4-log higher numbers of bacteria were recovered from lungs (Figure 4E). Likewise, systemic bacterial dissemination was found reduced in $B\beta_{15-42-NH_2}$-treated mice. Although 33% of blood cultures were positive in acid-aspiration mice, only 8% and 13% of control or $B\beta_{15-42-NH_2}$ treated mice, respectively, displayed systemic bacterial spread. To finally test if administration of $B\beta_{15-42-NH_2}$ after onset of lung injury still exerts therapeutic effects, we repeated the second-hit study and started peptide treatment at t = 0 hours, t = +1 hour, or t = +2 hours after acid aspiration. Identical to earlier experiments, all mice received additional doses of $B\beta_{15-42-NH_2}$ 1 hour and 6 hours after the first application and *P. aeruginosa* was administered 24 hours after acid aspiration. As depicted in Figure 4F, $B\beta_{15-42-NH_2}$ treatment 1 hour after the initial injury still exerted beneficial effects and resulted in significantly improved bacterial clearance.

In line with enhanced bacterial outgrowth and proinflammatory cytokine levels, mice of the acid aspiration and infection group exhibited significantly more pronounced signs of lung inflammation and injury as assessed by histopathological scoring of lung slides (Figure 5). Lungs from both control and $B\beta_{15-42-NH_2}$-treated mice showed only residual signs of inflammation. Therefore, improvement of lung barrier function during acid aspiration attenuated detrimental effects of subsequent *P. aeruginosa* challenge *in vivo*.

$B\beta_{15-42-NH_2}$ Treatment Reduces Mortality Due to Secondary *P. aeruginosa* Pneumonia

To ultimately verify the potential therapeutic benefit of $B\beta_{15-42-NH_2}$ treatment during acid-induced ALI followed by bacterial infection, we induced acid or NaCl aspiration in mice, treated one HCl group with $B\beta_{15-42-NH_2}$, followed by *P. aeruginosa* infection, and monitored survival over 4 days. To exclude any potential effect of $B\beta_{15-42-NH_2}$ on the course of bacterial infection itself, we included a second sham group that received $B\beta_{15-42-NH_2}$ together with NaCl aspiration (sham-treated $B\beta_{15-42-NH_2}$). Survival data clearly demonstrated the impact of preceding acid aspiration on secondary pneumonia, as well as the therapeutic role of $B\beta_{15-42-NH_2}$ herein. Although all mice undergoing acid aspiration succumbed to bacterial infection within 44 hours, 50% of control animals (i.e., NaCl aspiration) and 40% of $B\beta_{15-42-NH_2}$-treated acid aspiration mice survived secondary *P. aeruginosa* pneumonia (both control groups and HCl-$B\beta_{15-42-NH_2}$ mice $P < 0.05$ vs. HCl) (Figure 6). $B\beta_{15-42-NH_2}$ administration to sham-treated mice had no effect on the course of bacterial infection. Hence, $B\beta_{15-42-NH_2}$ treatment clearly improved the acid aspiration–induced impairment in host defense mechanisms and decreased death from secondary bacterial pneumonia.

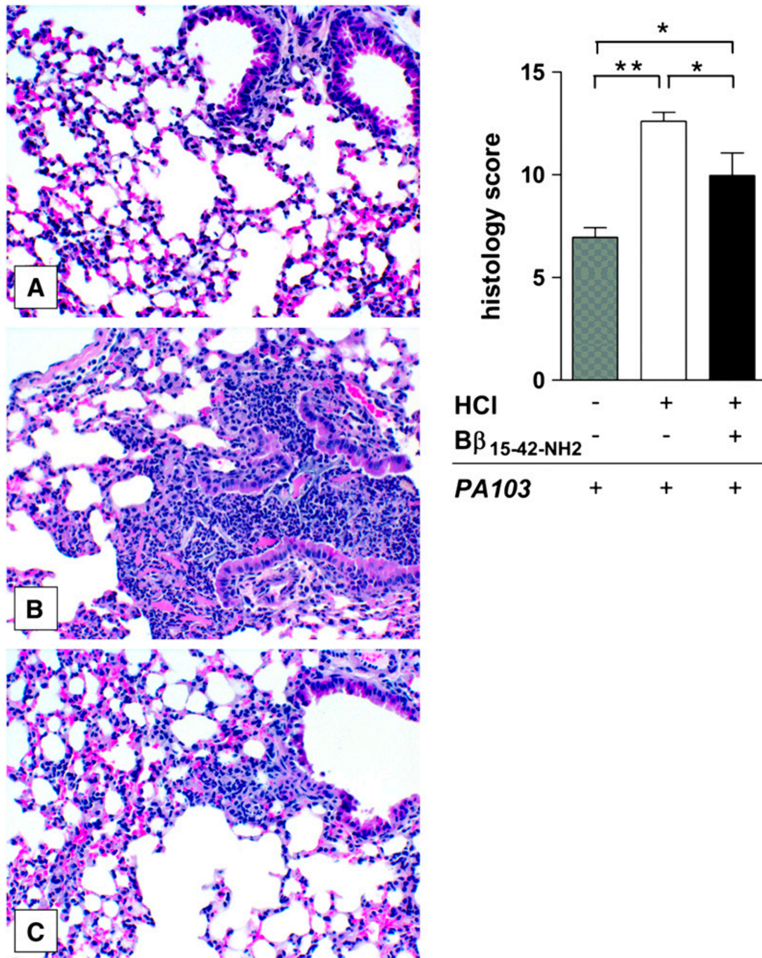

Figure 5. Less severe pulmonary infiltrates in $B\beta_{15-42-NH_2}$-treated mice. Endotoxin-free 0.1 N HCl (50 μl) was instilled intratracheally in mice followed by intraperitoneal injection of (*C*) $B\beta_{15-42-NH_2}$ (HCl-$B\beta_{15-42-NH_2}$) or (*B*) sterile NaCl (HCl) at t = 0, t = +1 hour, and t = +6 hours. (*A*) Control groups received sterile NaCl intratracheally and intraperitoneally. After 24 hours all three groups were infected with 1×10^4 cfu *Pseudomonas aeruginosa* intranasally. Lung sections stained with hematoxylin and eosin were scored as described in the METHODS section by a pathologist blinded for groups and are expressed as inflammation score. Representative slides are shown; magnification ×20. Depicted is one out of three independent experiments of n = 6 to 9 mice/group; data are mean ± SEM; $*P < 0.05$, $**P < 0.01$.

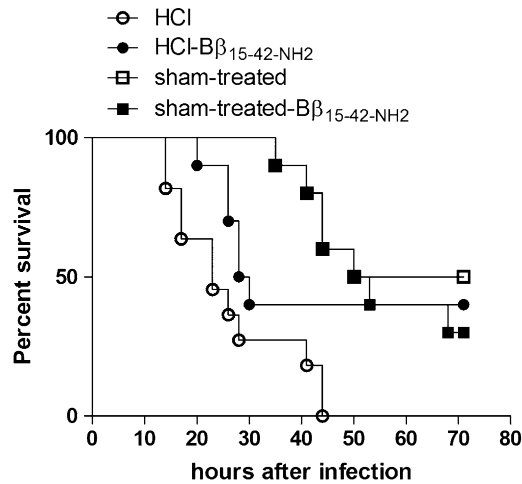

Figure 6. $B\beta_{15\text{–}42\text{-}NH_2}$ improves survival during secondary *P. aeruginosa* pneumonia. Endotoxin-free 0.1 N HCl (50 μl) was instilled intratracheally in mice followed by intraperitoneal injection of $B\beta_{15\text{–}42\text{-}NH_2}$ (HCl-$B\beta_{15\text{–}42\text{-}NH_2}$) or sterile NaCl (HCl) at t = 0, t = +1 hour, and t = +6 hours. Control groups received sterile NaCl intratracheally and intraperitoneally (sham-treated) or NaCl intratracheally and $B\beta_{15\text{–}42\text{-}NH_2}$ intraperitoneally (sham-treated-$B\beta_{15\text{–}42\text{-}NH_2}$). After 24 hours all four groups were intranasally infected with 1×10^4 cfu *P. aeruginosa.* Survival of n = 9 to 11 mice/group was monitored over 72 hours. Control and $B\beta_{15\text{–}42\text{-}NH_2}$-treated mice displayed an improved survival compared with the HCl-group ($P < 0.05$).

Preceding Acid Aspiration Impairs Antibacterial Properties of Alveolar Macrophages

In our efforts to understand how $B\beta_{15\text{–}42\text{-}NH_2}$ treatment improved bacterial clearance during secondary pneumonia despite decreased inflammation, we hypothesized that prevention of ALI might preserve antibacterial properties of phagocytes at the onset of infection. To test this idea we first investigated if acid aspiration *per se* impaired the phagocytic properties of macrophages and repeated acid aspiration studies to isolate primary alveolar macrophages 24 hours after mice received HCl alone or in combination with $B\beta_{15\text{–}42\text{-}NH_2}$ (i.e., at the time when we challenged mice with *P. aeruginosa* in earlier experiments). Isolated primary alveolar macrophages were then studied for their ability to phagocytose *P. aeruginosa ex vivo*. As shown in Figure 7A, preceding acid aspiration significantly impaired phagocytosis of bacteria by alveolar macrophages ($P < 0.001$ vs. sham-treated mice), whereas treatment with $B\beta_{15\text{–}42\text{-}NH_2}$ completely prevented this effect ($P < 0.001$ vs. HCl group). Hence, preceding acid aspiration itself led to impaired antimicrobial properties of alveolar macrophages and attenuation of pulmonary inflammation (as seen in $B\beta_{15\text{–}42\text{-}NH_2}$-treated animals) restored the phagocytic functions of alveolar macrophages. To understand how preceding lung injury interfered with bactericidal properties of phagocytes, we hypothesized that expression of negative regulators, which are required for resolution of inflammation, might concurrently affect the antimicrobial functions of macrophages. We therefore quantified expression levels of IRAK-M in lung homogenates 24 hours after induction of acid aspiration and indeed found significantly enhanced IRAK-M transcript levels in lungs from mice that received HCl, as compared with sham-treated or HCl-$B\beta_{15\text{–}42\text{-}NH_2}$ animals (Figure 7B). In addition, immunohistochemical studies on primary alveolar macrophages disclosed strongest IRAK-M protein expression in cells from HCl-treated mice as compared with HCl-$B\beta_{15\text{–}42\text{-}NH_2}$ animals (Figure 7C). Together, we demonstrated that preceding lung injury increased expression of negative regulators, such as IRAK-M, which was associated with impaired bactericidal properties of alveolar macrophages at the onset of bacterial infection (i.e., 24 h after HCl administration) and might thus explain worsened outcome during subsequent *Pseudomonas* pneumonia.

DISCUSSION

Vascular leak, neutrophil influx, and increase in cytokines at the site of injury are hallmarks of ALI in humans and animals (5, 6, 30). Although important progress has been made in understanding the pathogenesis of ALI over the last years, significant therapeutic implications are still missing. To fill this gap we decided to investigate the potential role of a peptide that has been shown earlier to prevent transmigration of neutrophils and vascular leak in models of myocardial reperfusion injury. This peptide is called $B\beta_{15\text{–}42}$, consists of 28 amino acids, and is a natural plasmin digest of fibrin (16). For studies shown here we have used a proteolytically stable analog, $B\beta_{15\text{–}42\text{-}NH_2}$. We studied different models of ALI and show that treatment with $B\beta_{15\text{–}42\text{-}NH_2}$ diminished lung inflammation *in vivo*. Furthermore, we were able to illustrate that acid-induced ALI impaired antibacterial defense mechanisms and thus primed for an exaggerated inflammatory response to secondary bacterial infection by *P. aeruginosa* and that treatment with $B\beta_{15\text{–}42\text{-}NH_2}$ could attenuate the detrimental effects of preceding ALI *in vivo*. The net result was improved survival from secondary *P. aeruginosa* pneumonia. We suggest that reduced inflammation throughout the course of acid aspiration in animals treated with the peptide, and therefore accelerated regeneration from this injurious event (Table 1) with restored antimicrobial properties (Figure 7A), is responsible for the improved outcome in the second-hit model. To our knowledge, this is the first report that explicitly demonstrates a therapeutic strategy to improve outcome during secondary bacterial pneumonia by diminishing ALI *in vivo*.

Endothelial cells play a central role in the pathogenesis of ALI (31). The biological properties of $B\beta_{15\text{–}42}$ were first described in 2005, when antiinflammatory features of the peptide were identified (16). We have extended these studies and showed recently that $B\beta_{15\text{–}42}$ antagonizes stress-induced RhoA activation (18), which is an integral regulator of endothelial cell contraction by regulating levels of myosin light chain phosphorylation (32–34). Cell contraction and breaking of cell–cell contacts results in gap formation and leak (32, 35, 36). Earlier studies thoroughly investigated the role of myosin light chain kinase 210 (MLCK210), which is abundantly present in endothelial cells, during sepsis and LPS-induced ALI. Using MLCK210 gene-deficient mice or a small-molecule inhibitor approach, respectively, two reports demonstrated diminished lung injury in response to sepsis or LPS and mechanical ventilation *in vivo* (37, 38). Furthermore, genetic studies revealed single nucleotide polymorphisms of the MLCK210 gene to confer susceptibility to sepsis- and trauma-associated ALI in humans (39, 40). These findings are in line with the proposed mode of action of $B\beta_{15\text{–}42}$, namely inhibiting RhoA activation with subsequently reduced myosin light chain phosphorylation (18). The functional importance of this finding was conclusively confirmed by showing reduced vascular leak in LPS-challenged mice that have received $B\beta_{15\text{–}42}$. We hereby extended these findings and focused on the potential therapeutic role of $B\beta_{15\text{–}42}$ during ALI *in vivo*. Using two distinct mouse models of ALI enabled us to show that early treatment with $B\beta_{15\text{–}42}$ efficiently preserved the endothelial barrier and diminished pulmonary inflammation after LPS or hydrochloric acid administration.

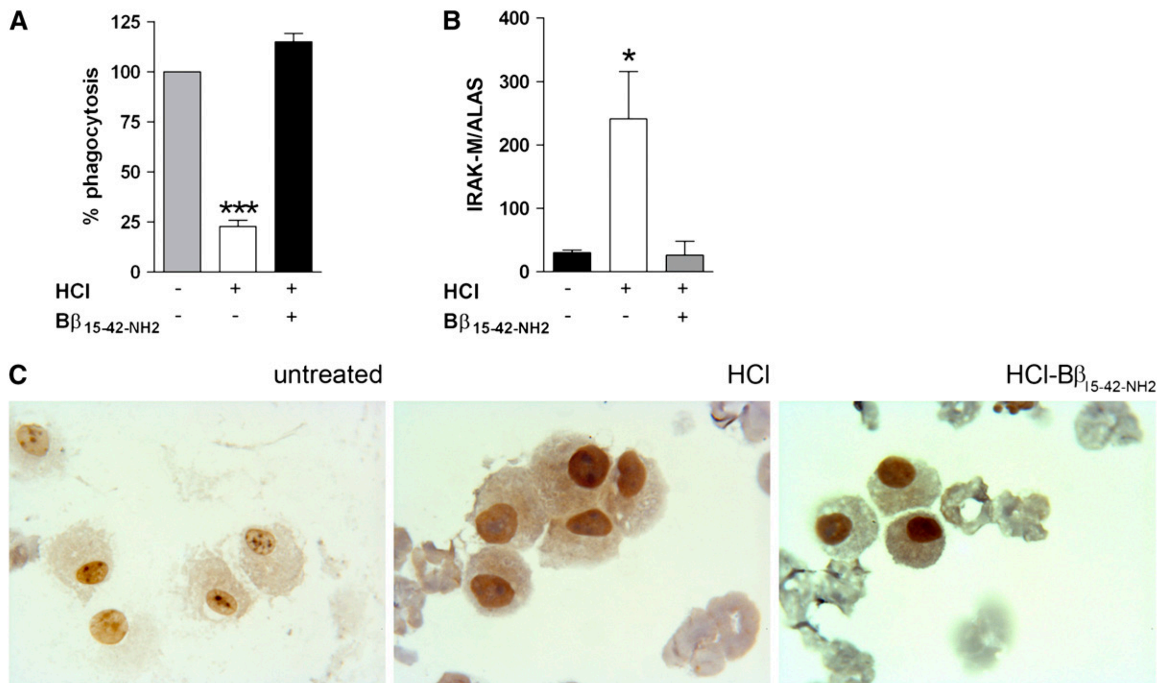

Figure 7. Acid-induced inflammation impairs bacterial phagocytosis by alveolar macrophages. Endotoxin-free 0.1 N HCl (50 μl) was instilled intratracheally in mice followed by intraperitoneal injection of $B\beta_{15–42\text{-}NH_2}$ (HCl-$B\beta_{15–42\text{-}NH_2}$) or sterile NaCl (HCl) at t = 0, t = +1 hour, and t = +6 hours. Control groups received sterile NaCl intratracheally and intraperitoneally (sham-treated). (*A*) After 24 hours alveolar macrophages were harvested, and uptake of fluorescein isothiocyanate–labeled *P. aeruginosa* was assessed by fluorescence-activated cell sorter; (n = 7 mice/group). (*B*) Lungs were harvested after 24 hours to conduct reverse transcriptase–polymerase chain reaction on interleukin-1 receptor associated kinase (IRAK)-M; (n = 3 mice/group). (*C*) Representative slides from immunohistochemical staining of IRAK-M on alveolar macrophages 24 hours after acid aspiration. Values are expressed as mean ± SEM; *$P < 0.05$, ***$P < 0.001$.

Aspiration of acid represents a clinically relevant and useful tool to study ALI (6), as aspiration of gastric contents is a major cause of ALI and is associated with high mortality rates (27, 41). Adding to the poor prognosis, patients with ALI who require mechanical ventilation are at increased risk for secondary bacterial infection (42). The chemical injury by HCl is believed to directly damage airway epithelia, which in turn triggers an inflammatory response followed by edema formation and influx of neutrophils (5, 43–47). Experimentally it has been shown that prior lung injury caused by acid aspiration or during sepsis primes for fatal secondary pneumonia (13, 48). van Westerloo and colleagues observed acid-induced enhanced inflammation to result in worsened outcome during secondary *Klebsiella* pneumonia. In line with these findings, we also observed a tremendously enhanced inflammatory response and impaired bacterial clearance in a model of secondary bacterial pneumonia induced by *P. aeruginosa* after acid aspiration. We moreover disclosed that acid aspiration resulted in impaired bacterial clearance, which was associated with enhanced pulmonary expression of the negative regulator IRAK-M (Figures 7B and 7C). IRAK-M is an inhibitor of TLR-signaling and is involved in the resolution of inflammation (49). Deng and colleagues demonstrated the crucial role of IRAK-M in bacterial clearance of *P. aeruginosa* earlier using a model of sublethal cecal ligation puncture followed by secondary bacterial pneumonia (48). We hereby confirmed and extended these observations by demonstrating that $B\beta_{15–42\text{-}NH_2}$ treatment was able to reduce lung injury, diminish IRAK-M expression, and thus restore antimicrobial properties of alveolar macrophages.

Time-course studies revealed the immediate leakage of proteins into the alveolar compartment and therefore suggest an early involvement of endothelial cells. In parallel, beneficial effects of $B\beta_{15–42\text{-}NH_2}$ were discernable 2 hours after induction of lung injury and ultimately resulted in less pronounced inflammation and thus accelerated resolution. It therefore seems likely that increased IL-10 levels 6 hours after acid aspiration already reflected the early resolution phase, because increased phagocytosis by macrophages of spent cells is associated with release of IL-10 (50). This concept was further confirmed by reduced IRAK-M transcript levels in $B\beta_{15–42\text{-}NH_2}$-treated animals after 24 hours. Hence, $B\beta_{15–42\text{-}NH_2}$-associated attenuation of lung injury expedited resolution and recovery.

To our current knowledge, VE-cadherin is the only transmembrane ligand of $B\beta_{15–42}$. VE-cadherin is expressed on endothelial cells and is of integral importance in regulating endothelial barrier function and inflammation (51). The reduced cytokine levels seen after treatment with $B\beta_{15–42}$ could be a secondary effect of vascular integrity *in vivo*, as incubation of $B\beta_{15–42}$ with LPS-stimulated monocytes, alveolar macrophages, or endothelial cells *in vitro* did not result in an altered release of proinflammatory cytokines (data not shown). Moreover, we could clearly illustrate that $B\beta_{15–42}$ administration to sham-treated mice did not affect host defense mechanisms against *P. aeruginosa in vivo*. Of interest is the observation that even mice that were treated 1 hour after acid aspiration showed improved bacterial clearance in a second-hit model (Figure 4F). Apart from endothelial cells, alveolar epithelial cells importantly contribute to the integrity of the alveolar barrier and are crucially involved in formation and clearance of ALI (52). Although it is tempting to speculate that $B\beta_{15–42}$ also acts on epithelial cells, we currently have no data that would imply a role for $B\beta_{15–42}$ in specifically affecting the epithelial barrier function.

In conclusion, we hereby established tissue-protective properties of $B\beta_{15–42}$ within the pulmonary compartment by in-

vestigating two distinct models of ALI. Our data furthermore indicate that mitigation of ALI can restore antimicrobial properties of alveolar macrophages and thus improve outcome during secondary bacterial pneumonia. Together these results as well as recently published data on the tolerability and efficacy of $B\beta_{15-42}$ in patients undergoing coronary intervention (17) suggest that $B\beta_{15-42}$ might be an attractive therapy to abate harmful consequences of ALI.

Conflict of Interest Statement: U.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.M.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. W.D. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. I.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. B.D. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. I.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. G.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.P. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. C.J.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.R. is the founder and a share holder of Fibrex Medical Inc. Patents include Use of peptides derived from the a α or b β chain of human fibrinogen for the treatment of shock, publication info: BRPI0506148-2006-10-24; Peptides and/or proteins and use thereof for the production of a therapeutic and/or prophylactic medicament, publication info: U.S. patent application 2004019259, DK 1341819T-2006-10-16; Pharmazeutische Zubereitung zur Behandlung von Schock, publication info: AT414097B-2006-09-15; Peptide and/or proteins and their use for manufacture of a therapeutic and/or preventive medicament, publication info: AT329614T-2006-07-15; Pharmaceutical preparation for the treatment of hemorrhagic shock and the sequels thereof A2067/2005; Methods of screening for compounds having antiinflammatory activity, which also prevent vascular leak and edema formation and use thereof, US 11/860, 488/2007. P.P. is the founder and a share holder of Fibrex Medical Inc. Patents include Use of peptides derived from the a α or b β chain of human fibrinogen for the treatment of shock, publication info: BRPI0506148-2006-0-24; Peptides and/or proteins and use thereof for the production of a therapeutic and/or prophylactic medicament, publication info: U.S. patent application 2004019259, DK 1341819T-2006-10-16; Pharmazeutische Zubereitung zur Behandlung von Schock, publication info: AT414097B-2006-09-15; Peptide and/or proteins and their use for manufacture of a therapeutic and/or preventive medicament, publication info: AT329614T-2006-07-15; Pharmaceutical preparation for the treatment of hemorrhagic shock and the sequels thereof A2067/2005; Methods of screening for compounds having antiinflammatory activity, which also prevent vascular leak and edema formation and use thereof US 11/860, 488/2007. S.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript

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