Anti-MARCKS Peptides Reverse LPS-induced Acute Respiratory Distress Syndrome (ARDS) in Mice

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Abstract

Peptides which inhibit MARCKS protein dramatically reduce sudden neutrophil infiltration in the lung in response to stress. ARDS was induced in mice by intratracheal instillation of LPS. After treatment with inhaled aerosolized anti-MARCKS peptides (BIO-11006 or BIO-10901) with treatment started as long as 36 hrs post LPS instillation (LPS: 5 µg Escherichia coli endotoxin; 2 mg/kg in 50 µl of PBS). Animals were monitored for behavior, at 48 hrs post LPS instillation, after either two administrations of peptide or saline control. At 72 hrs post LPS instillation, animals were sacrificed and lungs lavaged, and total leukocytes, neutrophils, inflammatory cytokines (KC [murine IL-8 equivalent], TNFα) were measured. In addition, NF-κB activation and cytokine gene expression were measured in whole lung homogenates, and lungs fixed for histological examination. Administration of either peptide produced similar effects: Mouse behavior, breathing effort and rate, piloerection, ptosis, "hunching", and activity returned to normal after 2 treatments 12 hrs apart with the inhaled peptide, whereas untreated animals remained moribund. Either peptide caused significant decreases in lung total leukocytes and neutrophils, as well as protein and gene expression of inflammatory cytokines. Activation of NF-κB also was inhibited by peptide inhalation. The results suggest that inhaled aerosolized peptide inhibitors of MARCKS protein could provide therapeutic benefit in patients with ARDS, and might even reverse disease progress if administered after established ARDS.

Materials and Methods

Acute Lung Injury Murine Model using LPS:
5 µg of LPS (*Escherichia coli* endotoxin; 2 mg/kg in 50 µl of PBS) was instilled intratracheally (IT) into female, 8 week old Balb/C mice using routine procedures. Peptides, including MARCKS-N-terminal inhibitors (MANS, BIO-11006, BIO-10901) and controls (RNS) were aerosolized in PBS and administered to the mice via inhalation according to the schedule below. The nebulizer and exposure chamber, which are both relatively small and compact, were placed inside a biological safety cabinet. Aerosol treatment for the mice utilized a TREKS portable aerosol system; Mice (3 per cage) were exposed to the aerosolized peptides for 30 mins starting at each selected time point and again 12 hrs later; each group was exposed to the aerosolized peptide twice.
Groups of 3 mice per cage (n=6 for each time point) were IT instilled with LPS (time 0) and treated with aerosolized: PBS (no peptide ctl); 50µM RNS, BIO-11006, BIO-10901 for 30 min according to the following schedule:
A. Prior to LPS instillation (0 time); again at 12 hrs.
B. 12 hrs after LPS instillation; again at 24 hrs.
C. 24 hrs after LPS instillation; again at 36 hrs.
D. 36 hrs after LPS instillation, again at 48 hrs.
All animals were euthanized at 72 hrs post LPS and underwent bronchoalveolar lavage. Specific indices of lung injury and inflammation were measured as described below. In addition, one lobe was fixed for histological assessment.
Indices of Lung Injury evaluated:
a) Mouse Behavior and Clinical Severity Score: Mice were videotaped for at least 30 sec at 0, 12, 24, 36 and 48 hrs post LPS instillation Videotapes of each cage of mice were analyzed blindly for the following parameters related to disease severity and behavior of the mice; each parameter will be quantified via a scoring of 0 (no disease apparent, normal behavior) to 3+ (Extremely sick) based on the following disease-related parameters:

Materials and Methods

1) Labored breathing/rapid breathing;
2) degree of piloerection;
3) degree of ptosis;
4) degree of "hunching";
5) Assessment of movement in the cage.

b) Amount of lung inflammation was assessed via:
1) Lung histology: Inflammation in parenchymal areas and around airways was assessed blindly by a veterinary pathologist and graded on a semi-quantitative scale of 0 (no inflammation) to +3 (severe inflammation).

2) BALF: total leukocytes; total neutrophils, neutrophil % of leukocytes, proinflammatory cytokine profile (via ELISA: KC, TNFα, IL-6, IL-1β, etc.), cytokine mRNA levels via real time PCR, total protein, were all measured.

Data Analysis
All quantitative data were assessed using standard biostatistical analyses; for multiple comparisons ANOVA with Bonferroni corrections was used to compare treated with untreated mice and to compare treatment with different peptides. Prism statistical software from GraphPad was used for statistical analyses.

Results

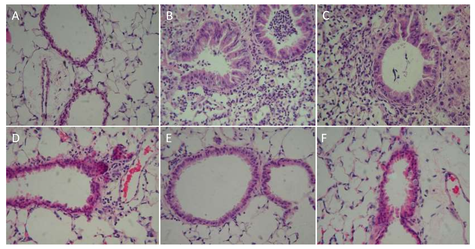

Fig 1. H&E staining: 8 week old Balb/C mice were used in an established acute lung injury (ALI) model with LPS IT as described. Peptides were delivered via inhalation at a concentration of 50 uM into female 8 week old Balb/C mice at different times after LPS instillation. Both BIO-11006 and BIO-10901 peptides prevented development of lung injury and inflammation when administered prior to LPS instillation and reversed lung injury when administered at all time points post LPS instillation
A. PBS only; B. LPS only; C. LPS+RNS (control peptide) ; D. LPS+MANS (parent peptide) ; E. LPS+Bio-10901; F. LPS + Bio-11006 (all peptides given at same time as LPS in this figure).

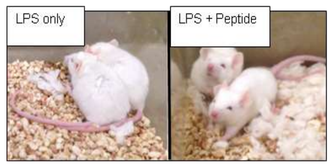

Fig 2. Still photographs taken from videos of mice 48 hrs post LPS exposure. Left: mice treated with LPS only, no peptides. Right: mice treated at 12 and 24 hrs with BIO-11006 aerosol.

Treatment Group	TABLE 2: Physical Condition Characteristics				
	lack of activity	hunched posture	ptosis	piloerection	labored breathing
LPS only	+++	+++	+++	+++	+++
LPS + BIO-11006 0, 12 h	None	None	None	None	None
LPS + BIO-11006 4,16 h	None	None	None	None	None
*LPS + BIO-11006 12, 24 h	None	None	None	+	None
LPS + BIO-11006 24, 36 h	None	None	+	+	None
LPS + BIO-11006 36, 48 h	None	None	+	+	None

Table 1: Blinded analysis of mice by a veterinarian at 48 hrs is shown in Table 2; time of initiation of peptide treatment is indicated under Treatment Group

Results

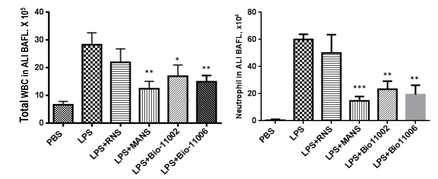

Fig 3. Analysis of BALF: Pretreatment with Peptides. Influx of all leukocytes into the lung (left) and neutrophils (right) is significantly attenuated in mice treated with aerosolized BIO-11006 prior to LPS administration. ** p<0.05; *** p<0.001; compared to LPS treated.

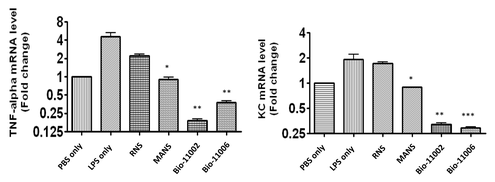

Fig 4. mRNA levels of proinflammatory cytokines (TNF-alpha and KC [murine IL-8 equivalent] are significantly attenuated in mice treated with aerosolized BIO-11006.In this example, mice were instilled with LPS and then treated with BIO-11006 after 4 hrs, and then again at 16 hrs. The mice were euthanized and mRNA expression of KC measured at 72 hrs post LPS via real time PCR. Treatment with BIO-11006 significantly decreased message levels of TNFα and KC. * p<0.05; ** p<0.001; *** p<0.0001; compared to LPS treated.

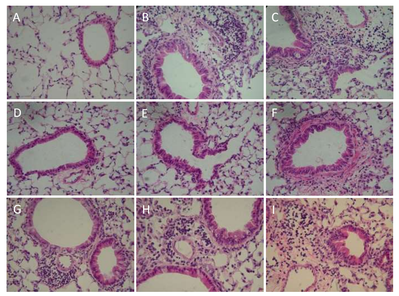

Fig 5. Lung histology at 72 hrs post LPS; peptides administered at various times post LPS. BIO-11006 was administered via inhalation at 50 uM either just prior to (0 hrs) IT instillation of LPS, or 4, 12, 24, 36 or 42 hrs after LPS. The effects of the peptides were obvious, regardless of time of administration after LPS.
A. PBS only (negative control); B. LPS only; C. LPS+RNS; D. Treatment started at 0 hrs; E. Treatment started at 4 hrs; F. Treatment start at 12 hrs; G. Treatment started at 24 hrs; H . Treatment started at 36 hrs; I. Treatment started at 42 hrs.

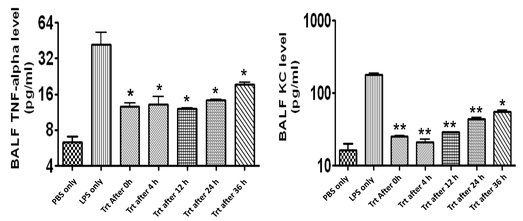

Fig 6: Levels of proinflammatory cytokines (TNFα, KC [murine IL-8 equivalent]) in BALF are significantly attenuated in mice treated with aerosolized BIO-11006, proportional to the time after LPS instillation when peptide treatment was begun.. * p<0.05; ** p<0.001.

Results

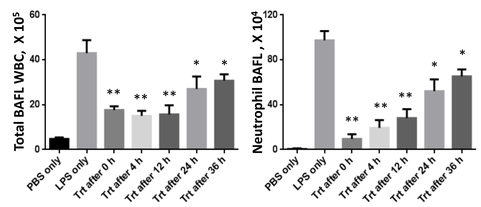

Fig 7. Treatment with aerosolized BIO-11006 attenuates total leukocyte and neutrophil influx into the lung. Numbers of total leukocytes, and especially neutrophils, are proportional to the time post LPS instillation when treatment was initiated, as would be expected. * p<0.05;
** p<0.01

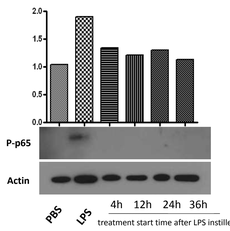

Fig 8. N-terminal MARCKS – inhibitory peptide (BIO-11006) attenuates LPS-induced NF-κB activation in lung, measured at 72 hrs post-LPS. NF-κB activation was assessed vis phosphospho-NF-κB p65 analysis by immunoblotting. Cytoplasmic extracts were prepared from mouse lung tissue. BIO-11006 was administered via inhaled aerosol at the indicated time points post-LPS instillation. Actin was used as a loading control(lower panel).

Conclusions

1. N-terminal MARCKS inhibitory peptides (BIO-11006, BIO-10901) prevent LPS – induced lung injury in mice when administered as an inhaled aerosol prior to LPS instillation.
2. N-terminal MARCKS inhibitory peptides REVERSE LPS – induced lung injury in mice when administered as an inhaled aerosol as long as 36 hrs post LPS instillation.
3. The mechanism by which N-terminal MARCKS inhibitory peptides reverse LPS-induced lung injury in mice involves decreased production of inflammatory cytokines (TNFα , KC [mouse IL-8 equivalent]) and decreased influx of inflammatory cells, especially neutrophils, into the lung.
4. N-terminal MARCKS inhibitory peptides also inhibit LPS-induced NF-κB activation in the lung, suggesting that one mechanism behind the decreased inflammatory cell influx could be decreased production of proinflammatory cytokines.
5. BIO-10901, the metabolite of BIO-11006, has the same efficacy as BIO-11006.

Acknowledgements

Funded by NIH grants R37 HL36982 and R41HL124817

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