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13. ABSTRACT (Maximum 200 Words)

Rats exposed to advanced composite materials (ACM) combustion atmospheres develop a time-dependent pulmonary inflammation characterized by the influx of polymorphonuclear neutrophils (PMNs) into the lumen of the lung, epithelial permeability changes and inflammatory cytokine release. Mice with defective tumor necrosis factor alpha (TNF-α) gene produced significantly less interleukin-1 beta (IL-1β) and IL-6 while mice with defective IL-1β gene produced significantly more TNF-α with on effect on IL-6. Both strains had significantly fewer BALF cells and PMNs but no effect on epithelial permeability. The use of antibodies towards TNF-α and IL-1β had no effect on epithelial permeability, BALF IL-1β or TNF-α. IL-1β antibodies did not affect BALF cell numbers but did significantly increase PMNs. No effect was noted on IL-6 levels with anti-IL-1β. TNF-α antibodies significantly increased the BALF cell numbers and IL-6 but significantly decreased the percentage of PMNs in the BALF. In conclusion, gene silencing was effective in reducing the inflammation (TNF-α > IL-1β) from ACM combustion atmospheres while pretreatment with TNF-α or IL-1β antibodies under the current protocol is ineffective.

14. SUBJECT TERMS
Cytokines, lung, ACM, BALF

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TABLE OF CONTENTS

Cover Page----------1

SF 298----------2

Table of Contents----------3

Introduction----------4

Body----------4

Key Research Accomplishments----------31

Reportable Outcomes----------31

Conclusions----------31

References----------32

Salary Support----------32

INTRODUCTION

The use of advanced composite materials (ACM) in the replacement of metallic counterparts has increased in both the civilian and military sectors. ACM parts can be found in planes, ships, submarines and personnel carriers. A significant advantage of ACM over metals is the weight reduction. Unfortunately, unlike metallic parts, ACM burns. The toxicity of such combustion atmospheres is largely unknown. It is the subject of this project to determine the toxicity of such atmospheres to the respiratory system in an animal model.

BODY

Year 1

Characterization of pulmonary injury

Exposure of rats to ACM combustion atmospheres resulted in a significant influx of inflammatory cells into the airway lumen (Figure 1). With the exception of day 7, all time points revealed significant increases in cell number when compared to controls. Cell differentials revealed significant increases in the percentage of PMNs in the BALF of ACM groups on day 1 and day 3. The remainders of the time points were comparable to controls (Figure 2). To assess pulmonary epithelial permeability changes, albumin was measured in the BALF. Albumin levels in the ACM groups were significantly greater than controls for all time points with the exception of day 156 (Figure 3). Alkaline phosphatase (AP) was measured to assess epithelial type II lung cell injury. No significant elevations of AP were noted in any of the ACM groups as compared to controls (results not shown). Lactate dehydrogenase (LDH), an intracellular enzyme, was used to assess generalized cellular injury. Again, no significant elevations of LDH were noted in any of the ACM groups as compared to controls (results not shown).

To evaluate the cellular responses to ACM exposure, several inflammatory cytokines were measured in the BALF. One such cytokine was tumor necrosis factor alpha (TNF-α). TNF-α was significantly elevated in all ACM groups as compared to controls (Figure 4). In addition, interleukin-1 beta (IL-1β) and interleukin-6 (IL-6) were measured. IL-1β was significantly increased over controls on days 1 and 21 while comparable to controls the remainder of the time points (Figures 5). IL-6 was significantly elevated on day 1 while comparable to controls at all other time points (Figure 6). To evaluate the global response of the lung, the same cytokines were measured in lung homogenates. TNF-α in the lung homogenates was significantly higher on day 3 only (Figure 7). Similar results were found for IL-1β and IL-6 (Figures 8 and 9) with the exception that on day 21, the control groups were significantly higher than the treatment groups. Lung histology revealed no gross pathology. Particle laden mononuclear cells were noted in the ACM groups only.

Year 2

Effect of selected gene silencing on pulmonary injury

The initial research in year 1 revealed that the cytokines TNF-α and IL-1β were significantly increased in the ACM groups as compared to controls. Based on the increase in cell number, PMNs, albumin and cytokines, the time point selected for comparison of treatment effects was day 1. Mouse strains were selected on the basis of the silenced genes and were used to determine the biological responses dependent upon those genes. Mice were exposed to the ACM combustion atmosphere similar to the rats in year 1 but using a lower amount of ACM material (30 gms). The genetically altered mice were compared to their original genetic controls. All mice were exposed to ACM combustion atmospheres so that differences are the result of the altered gene. With the TNF-α silenced strain, BALF cell counts were significantly lower as compared to controls (Figure 10). In addition, PMNs were significantly lower in this group (Figure 11). Analyses of the BALF cytokines revealed significantly lower levels of both IL-1β and IL-6 when compared to controls (Figures 12 and 13). Albumin levels in the BALF were comparable to controls (Figure 14).

In a separate exposure, IL-1β gene defective mice were used and compared to their normal genetic controls. BALF cell counts were significantly reduced in the genetically altered strain as compared to controls (Figure 15). The BALF cell differentials revealed significantly fewer PMNs in the IL-1β group (Figure 16). Interestingly, the measurement of TNF-α in the BALF revealed significantly higher levels in the IL-1β mice as compared to controls (Figure 17). BALF IL-6 was comparable between the two groups (Figure 18). BALF albumin levels were comparable to controls (Figure 19).

Effect of in vivo protection by selected antibodies on pulmonary injury

To determine the effectiveness of pretreatment with the appropriate anti-cytokine, rats were given an intra-peritoneal injection using either 1 ml of rabbit anti-rat TNF-α or rabbit anti-rat IL-1β. Control rats received similar injections of rabbit pre-immune serum. The three groups were exposed to the ACM combustion atmosphere for 1 hour and 24 hours later evaluated for pulmonary inflammation and injury. The use of anti-IL-1β resulted in BALF cell counts that were similar to that of controls. In contrast, anti-TNF-α resulted in significantly higher BALF cell counts (Figure 20). The effects on cell differentials between the three groups were mixed. Anti-IL-1β significantly increased the percentage of PMNs in the BALF while anti-TNF-α significantly decreased them (Figure 21). The BALF levels of TNF-α were comparable between the groups (Figure 22). Likewise, no changes were noted in the BALF levels of IL-1β (Figure 23). In contrast, IL-6 levels in the BALF were significantly elevated when compared to controls (Figure 24). Lastly, albumin levels in the BALF were comparable between the three groups (Figure 25).

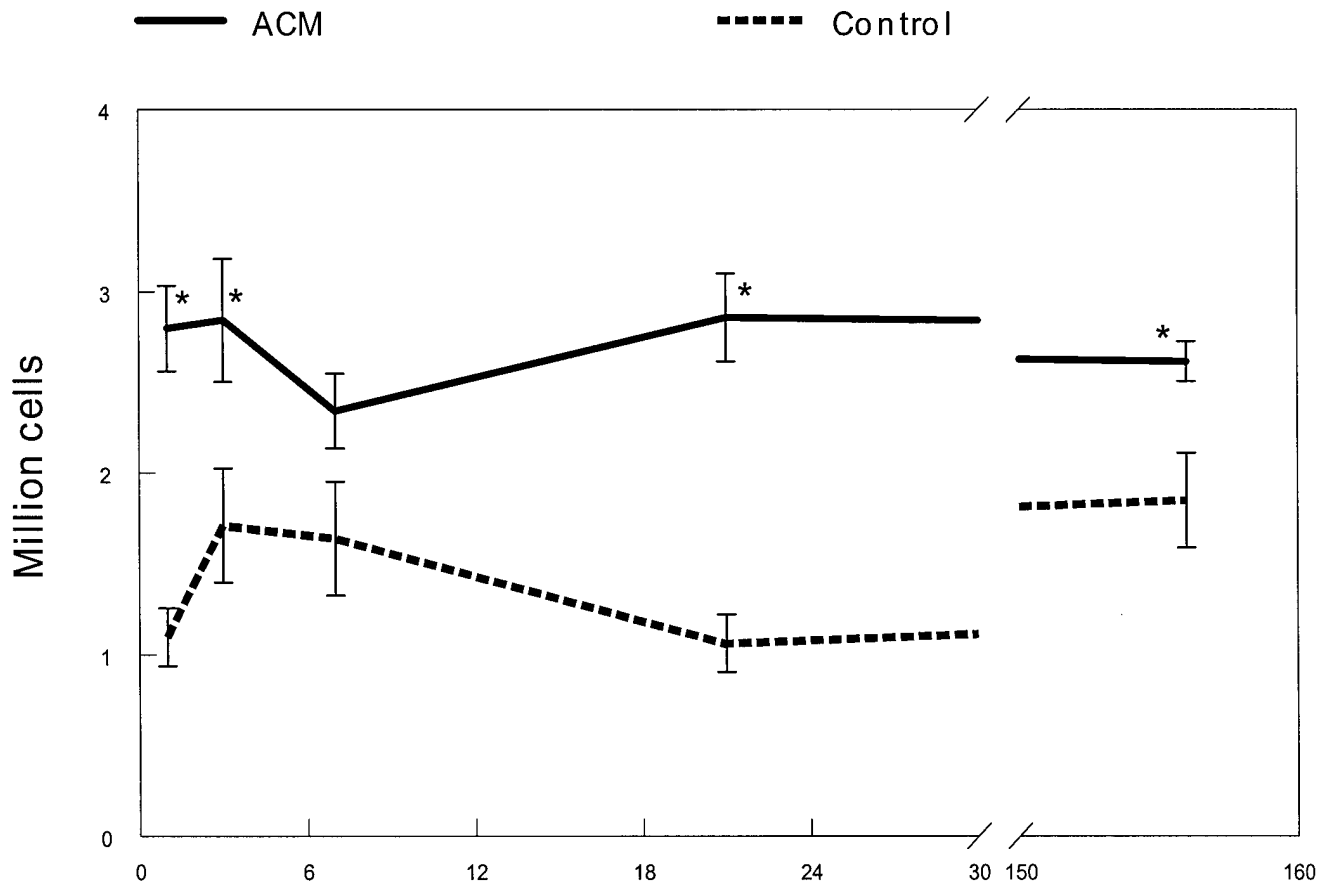

Figure 1. A comparison of the BALF cell counts between rats exposed to ACM combustion atmospheres and rats exposed to filtered air. Results are expressed as means ± SEM. * indicates significant difference from control, $p < 0.05$.

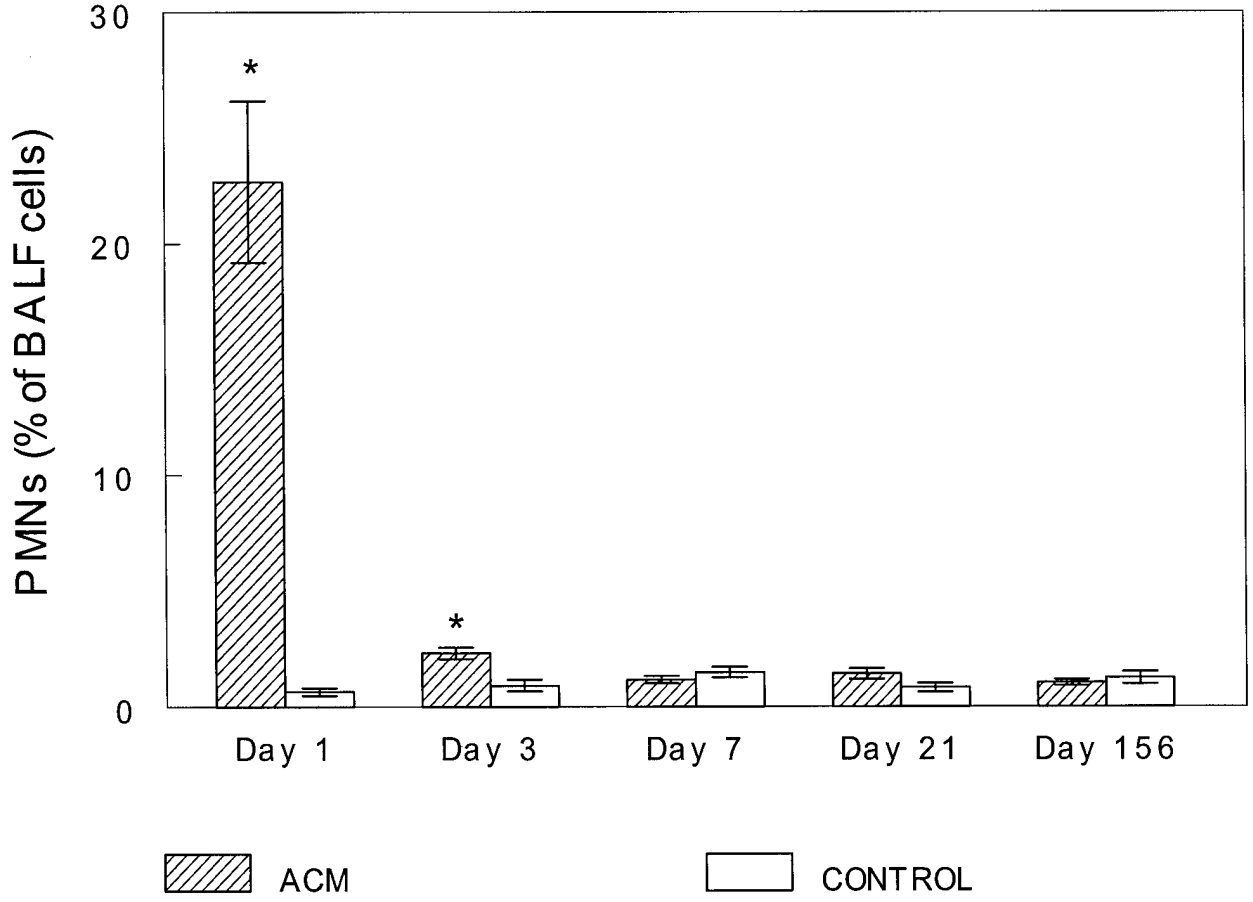

Figure 2. A comparison of the percentage of PMNs in the BALF from rats exposed to ACM combustion atmospheres and those exposed to filtered air. Results are expressed as means ± SEM. * indicates significant difference from control, $p < 0.05$.

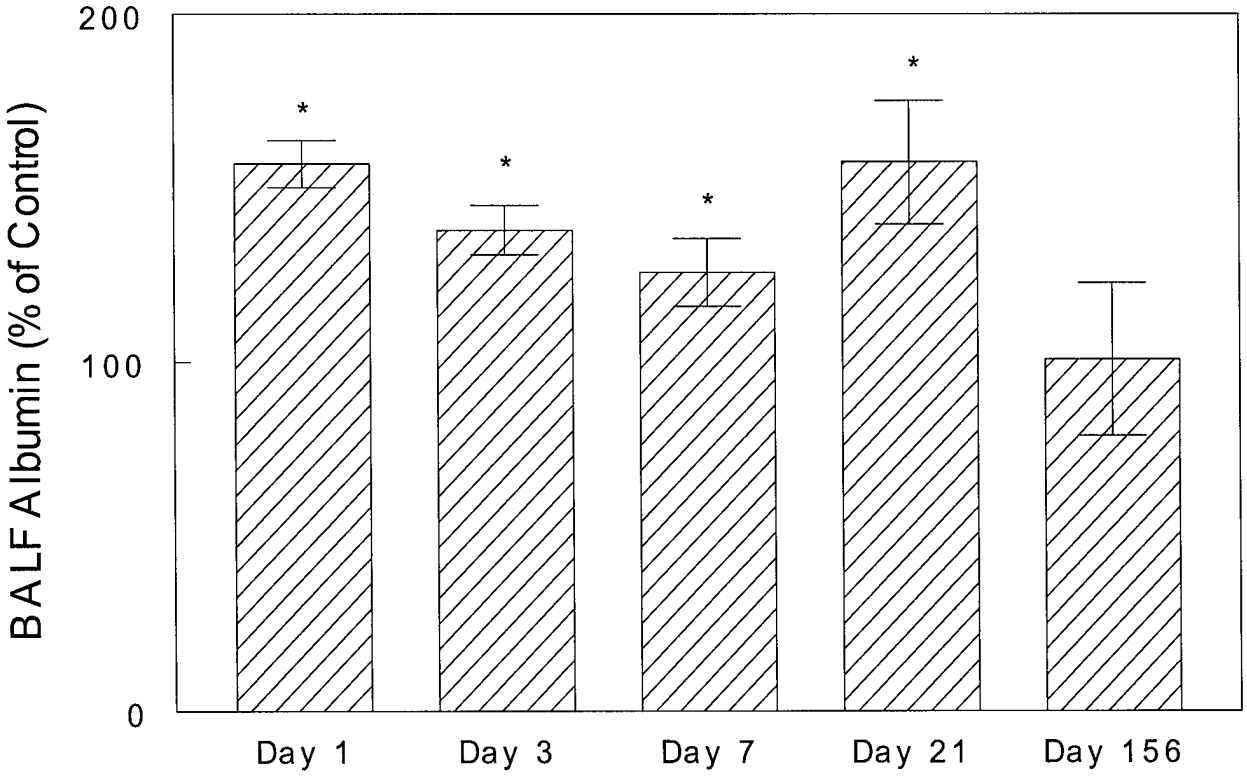

Figure 3. A comparison of the BALF albumin levels from rats exposed to ACM combustion atmospheres. Results are expressed as means ± SEM of the percentage of control values. * indicates significant difference from control, $p < 0.05$.

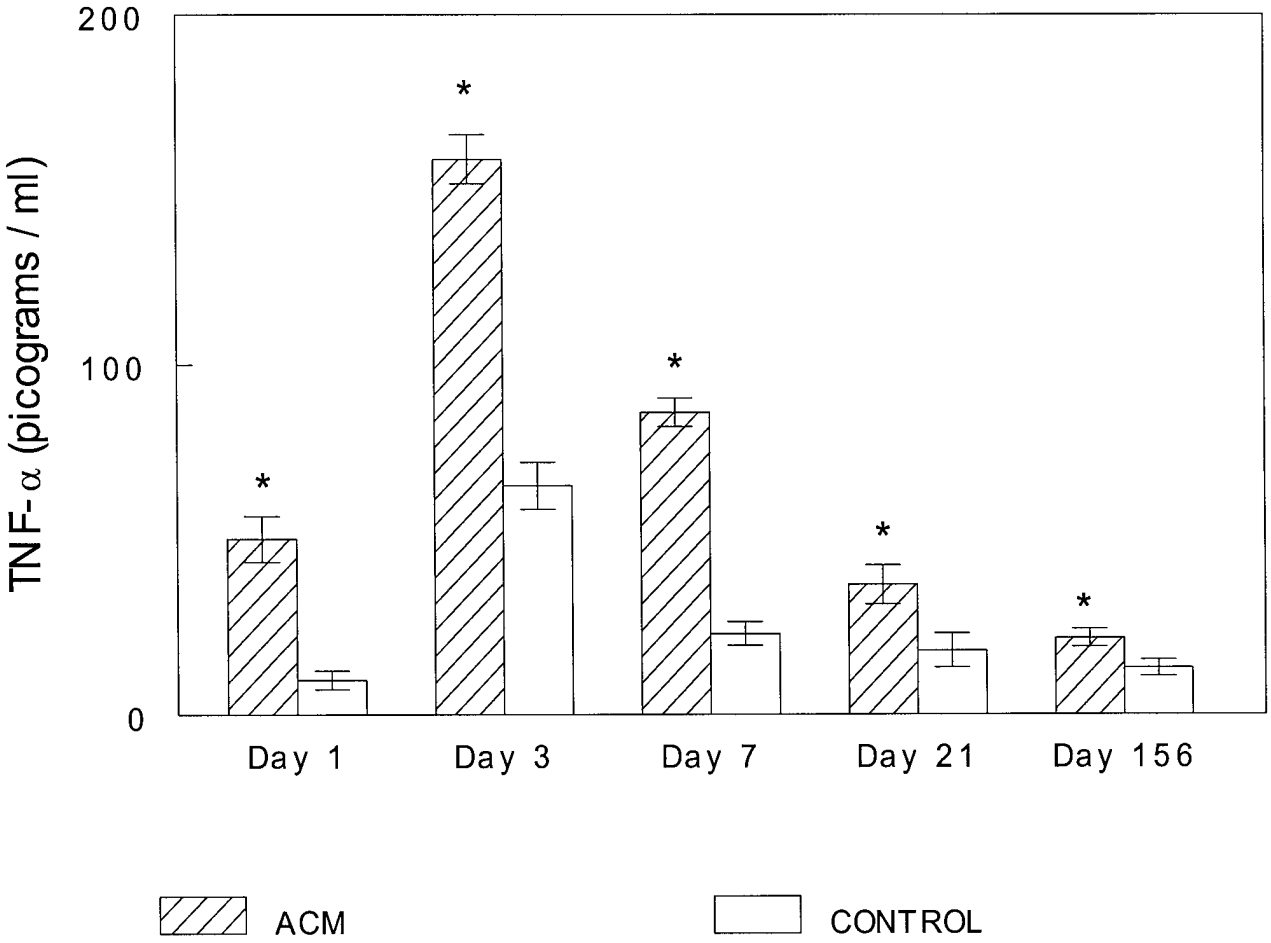

Figure 4. A comparison of the BALF TNF-α levels between rats exposed to ACM combustion atmospheres and filtered air. Results are expressed as means ± SEM. * indicates significant difference from control, $p < 0.05$.

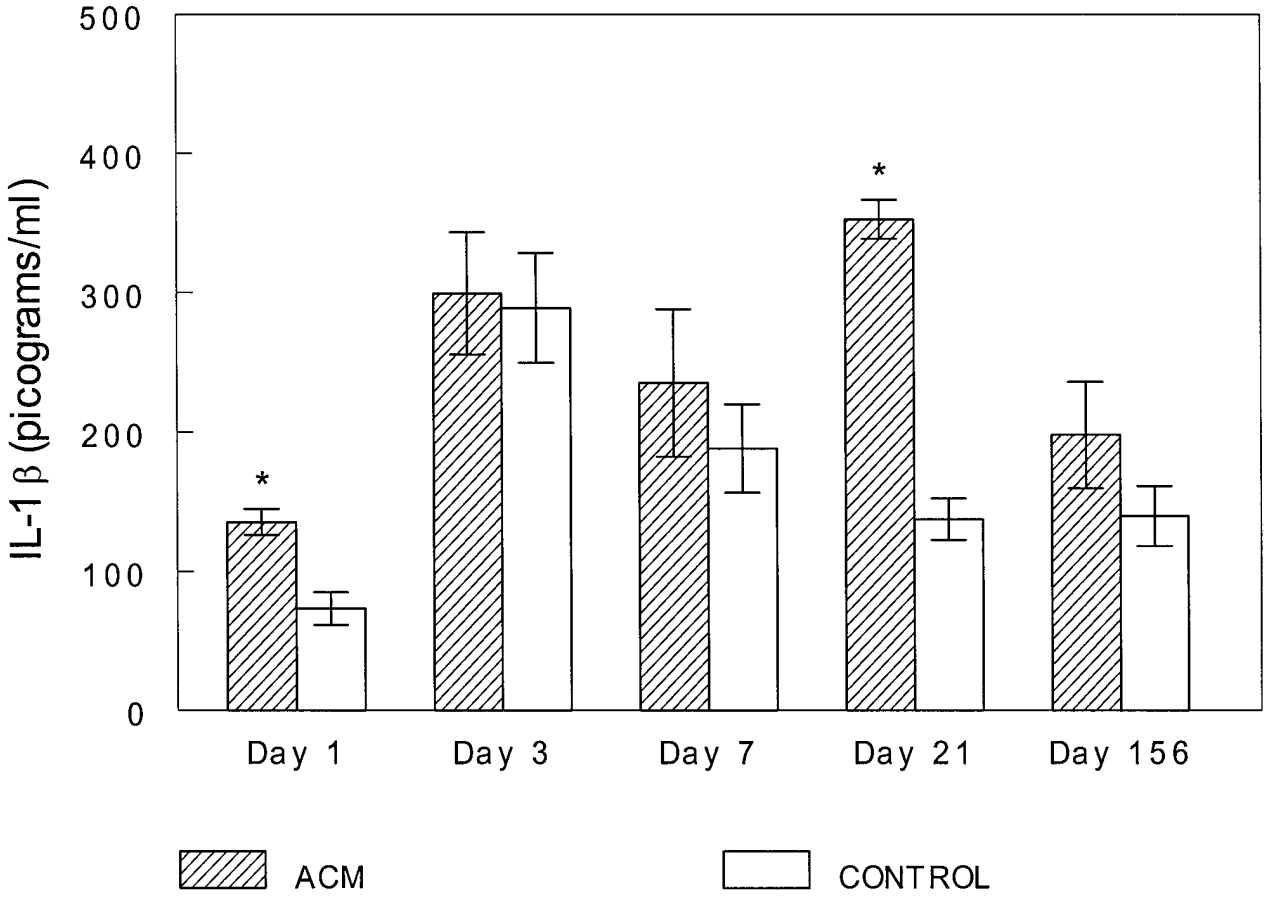

Figure 5. A comparison of the BALF IL-1β levels between rats exposed to ACM combustion atmospheres and filtered air. Results are expressed as means ± SEM. * indicates significant difference from control, $p < 0.05$.

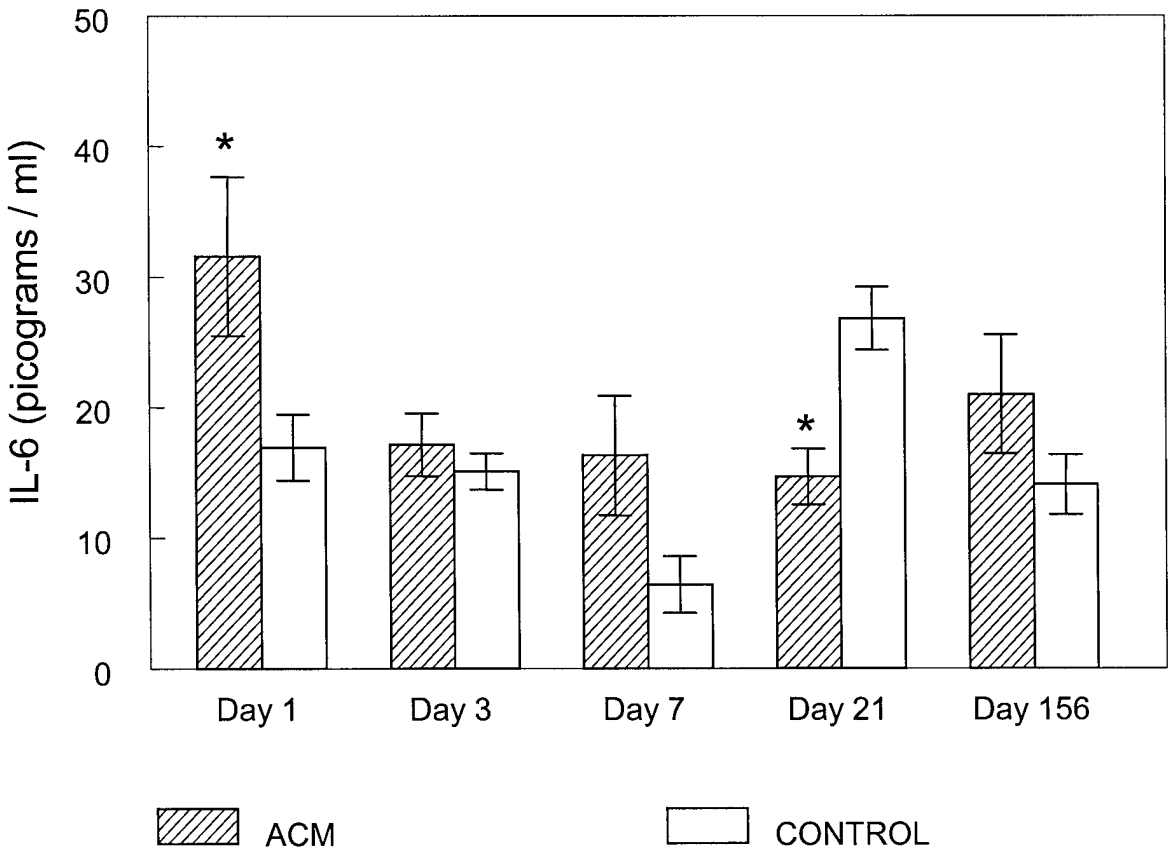

Figure 6. A comparison of the BALF IL-6 levels between rats exposed to ACM combustion atmospheres and filtered air. Results are expressed as means ± SEM. * indicates significant difference from control, $p < 0.05$.

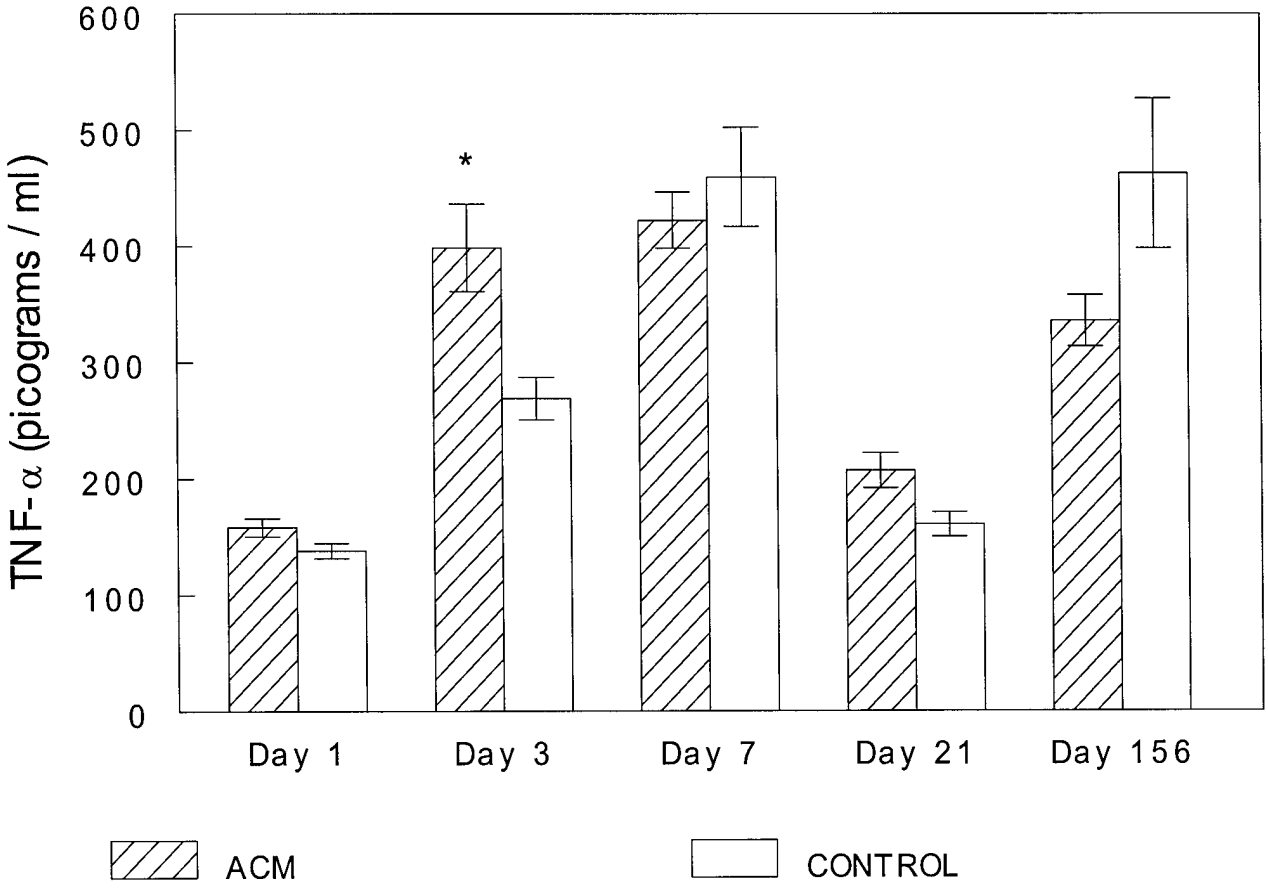

Figure 7. A comparison of the lung homogenate TNF-α levels between rats exposed to ACM combustion atmospheres and filtered air. Results are expressed as means ± SEM. * indicates significant difference from control, $p < 0.05$.

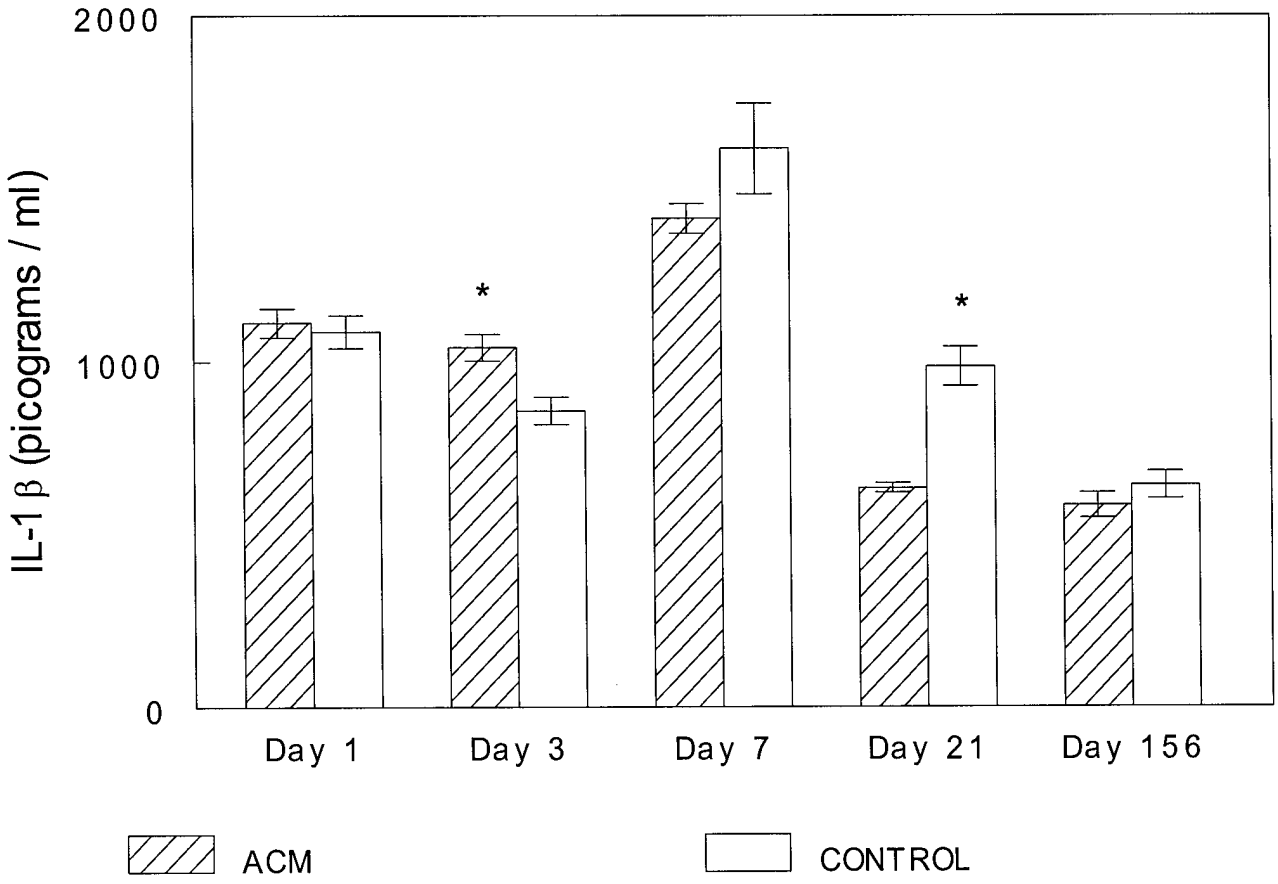

Figure 8. A comparison of the lung homogenate IL-1β levels between rats exposed to ACM combustion atmospheres and filtered air. Results are expressed as means ± SEM. * indicates significant difference from control, $p < 0.05$.

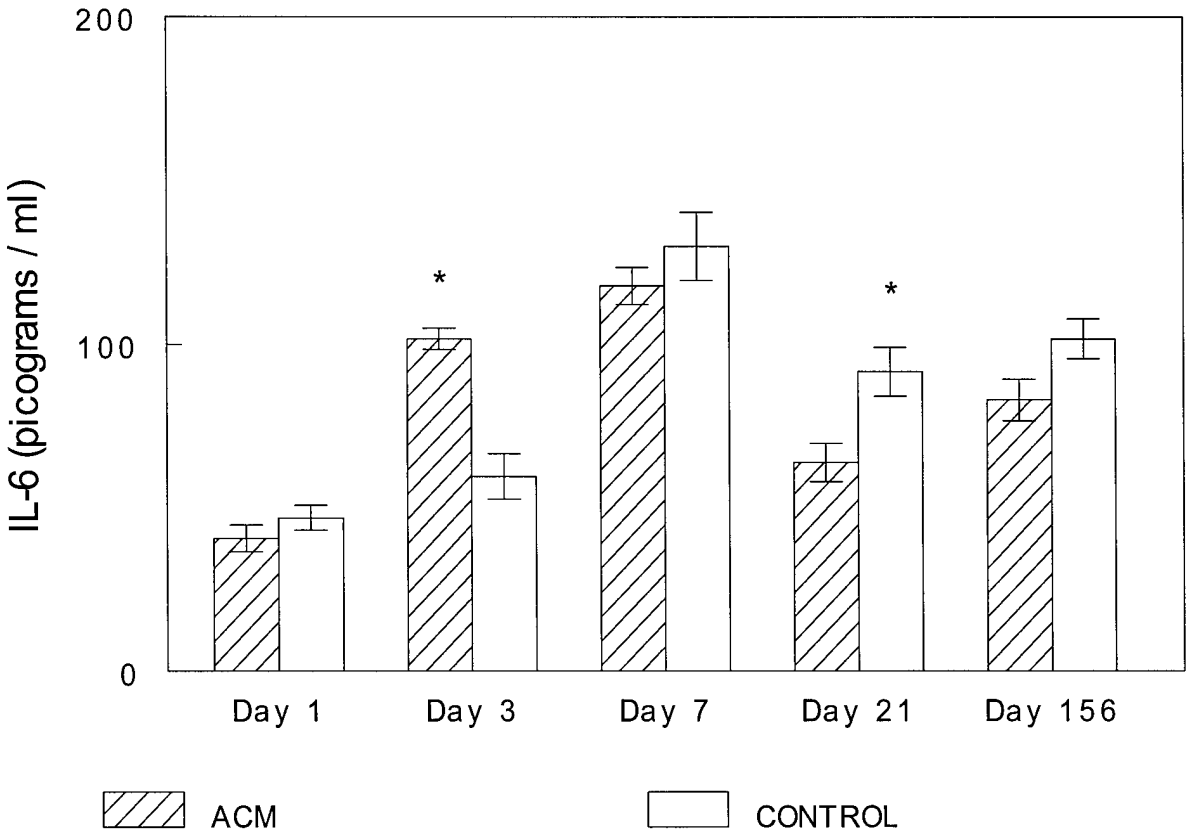

Figure 9. A comparison of the lung homogenate IL-1β levels between rats exposed to ACM combustion atmospheres and filtered air. Results are expressed as means ± SEM. * indicates significant difference from control, $p < 0.05$.

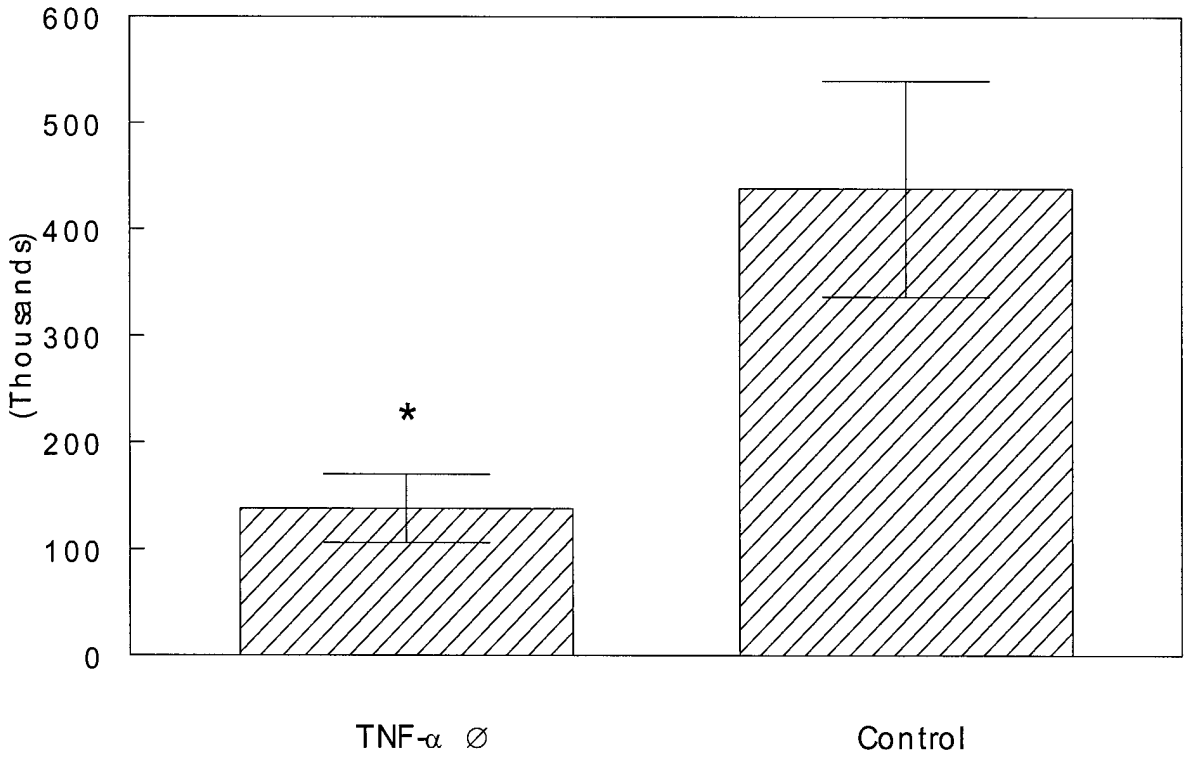

Figure 10. A comparison of the BALF cell counts between rats injected with anti-TNF-α and with pre-immune control serum prior to exposure to ACM combustion atmospheres. Results are expressed as means ± SEM. * indicates significant difference from control, $p < 0.05$.

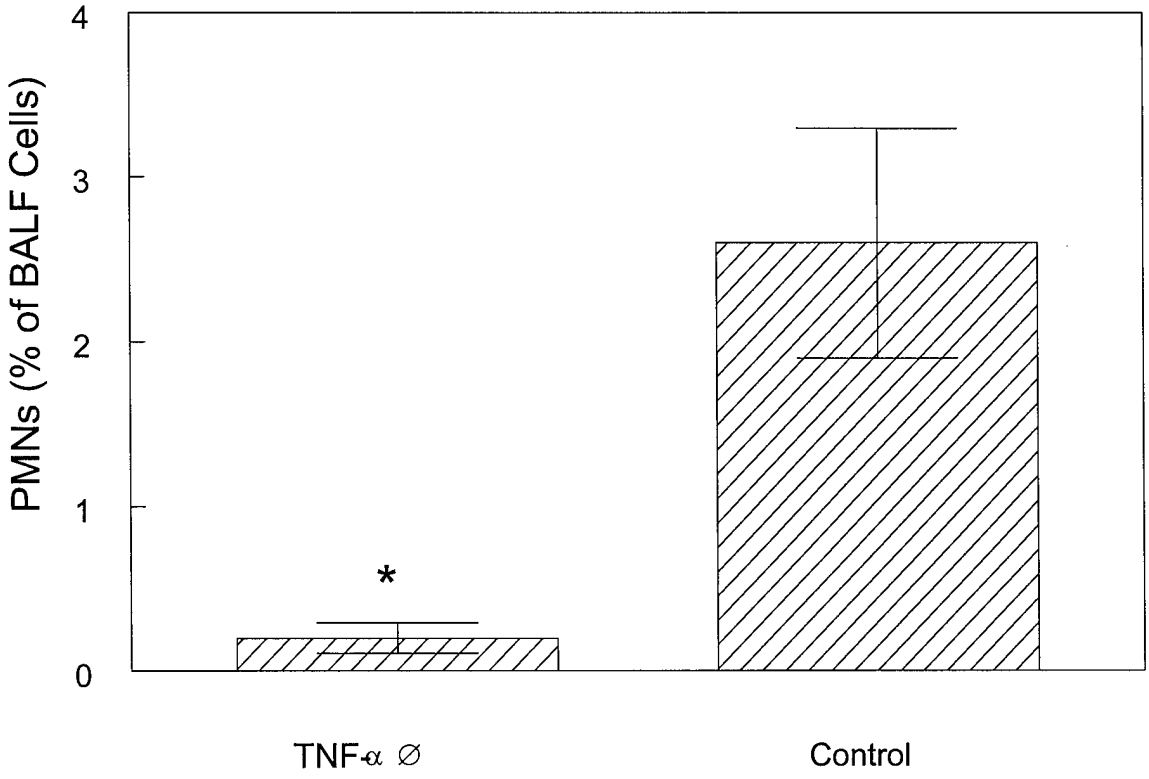

Figure 11. A comparison of the percent of PMNs in the BALF between rats injected with anti-TNF-α and with pre-immune control serum prior to exposure to ACM combustion atmospheres. Results are expressed as means ± SEM. * indicates significant difference from control, $p < 0.05$.

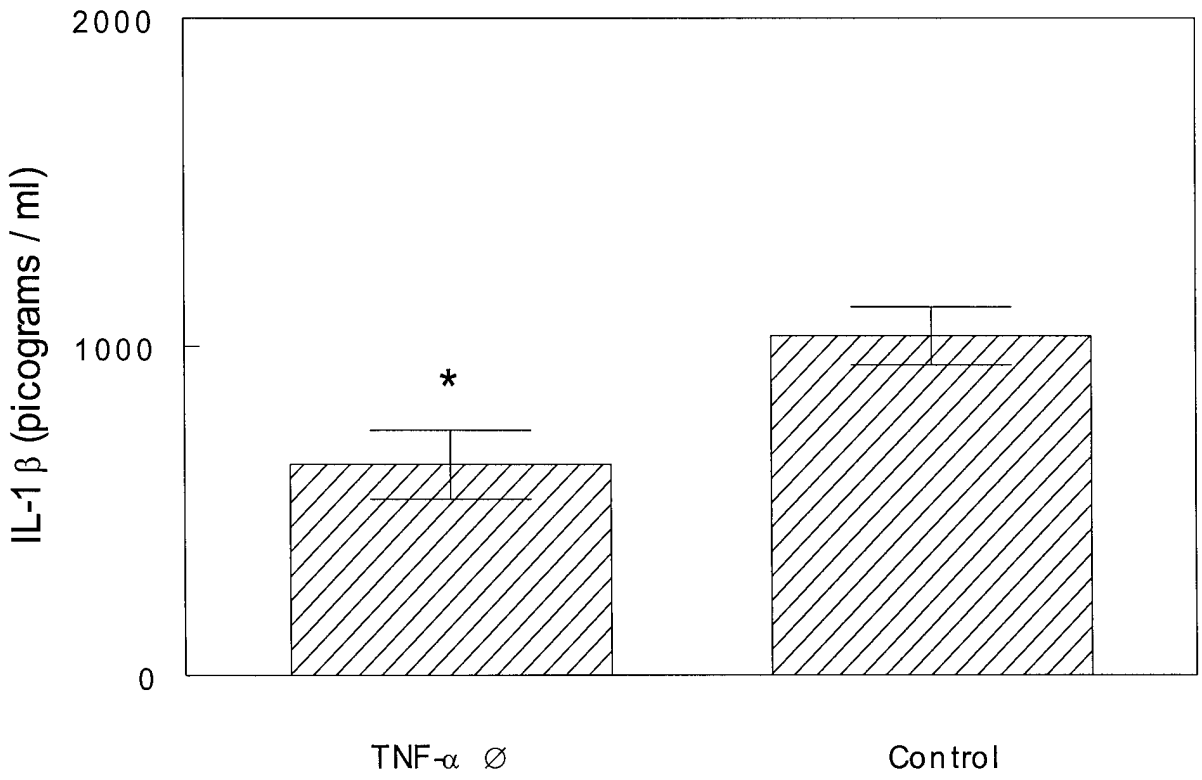

Figure 12. A comparison of the BALF IL-1β levels between rats injected with anti-TNF-α and with pre-immune control serum prior to exposure to ACM combustion atmospheres. Results are expressed as means ± SEM. * indicates significant difference from control, $p < 0.05$.

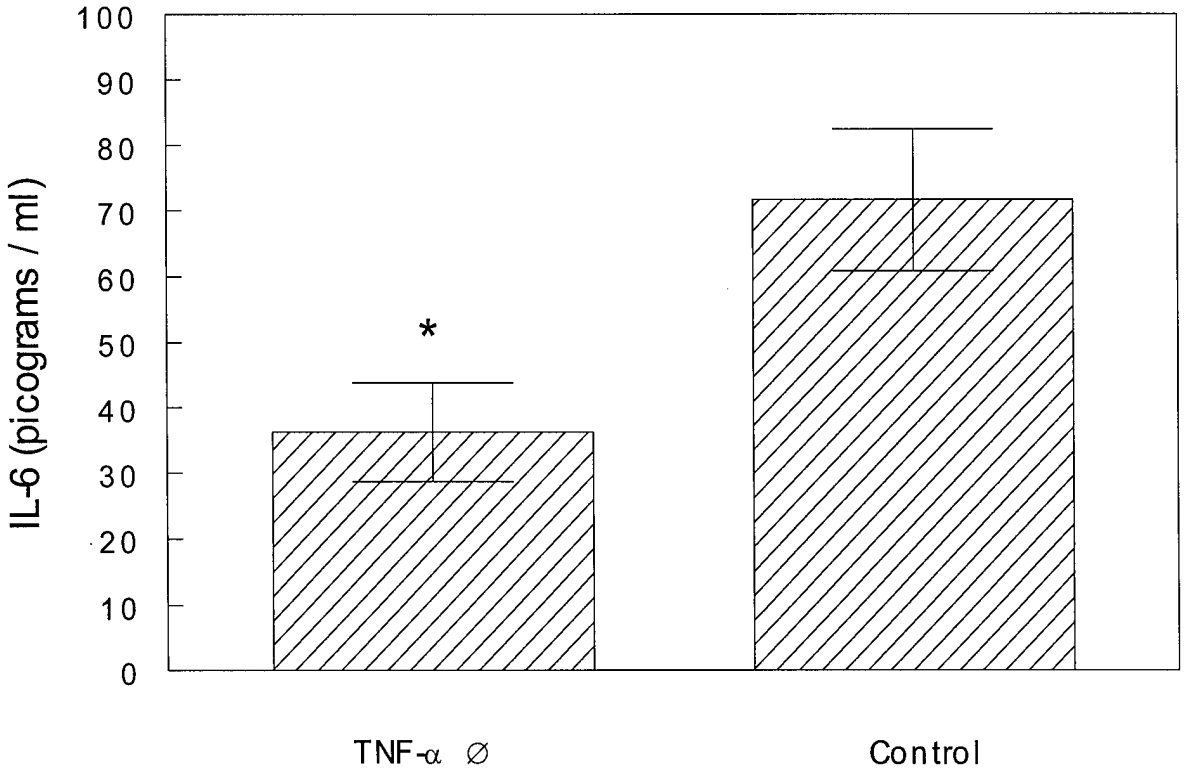

Figure 13. A comparison of the BALF IL-6 levels between rats injected with anti-TNF-α and with pre-immune control serum prior to exposure to ACM combustion atmospheres. Results are expressed as means ± SEM. * indicates significant difference from control, $p < 0.05$.

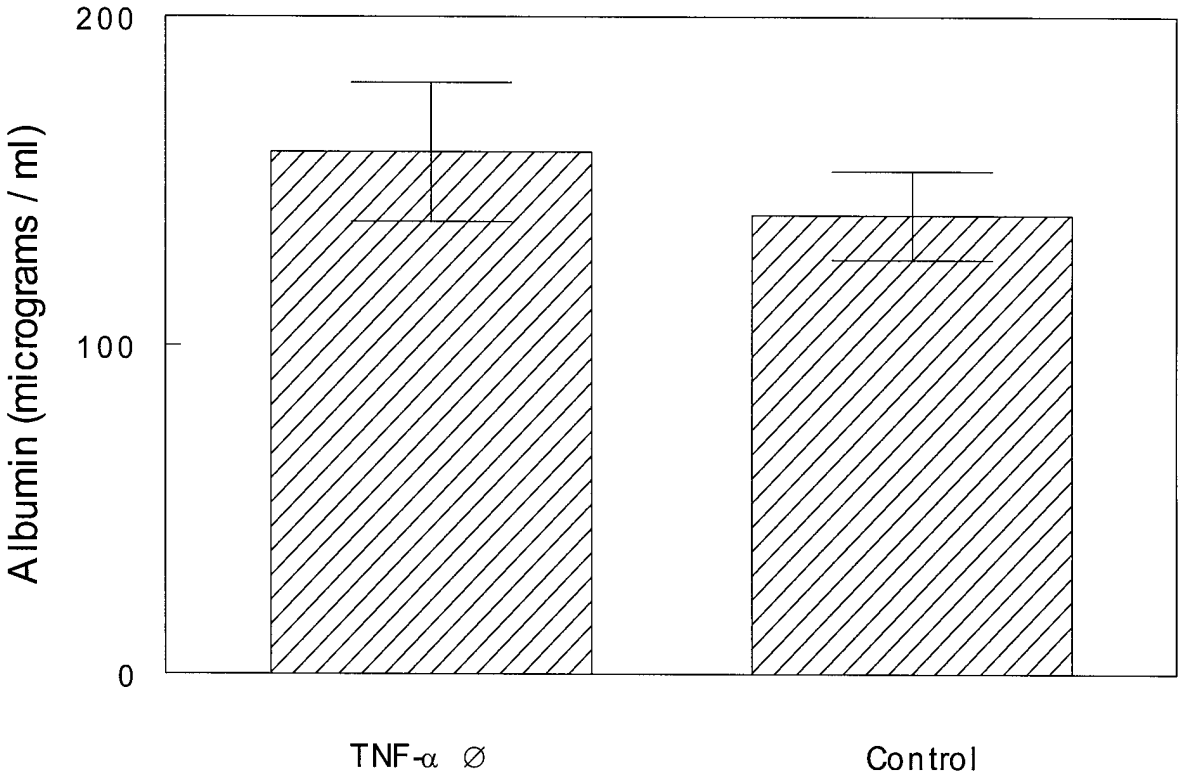

Figure 14. A comparison of the BALF albumin levels between rats injected with anti-TNF-α and with pre-immune control serum prior to exposure to ACM combustion atmospheres. Results are expressed as means ± SEM.

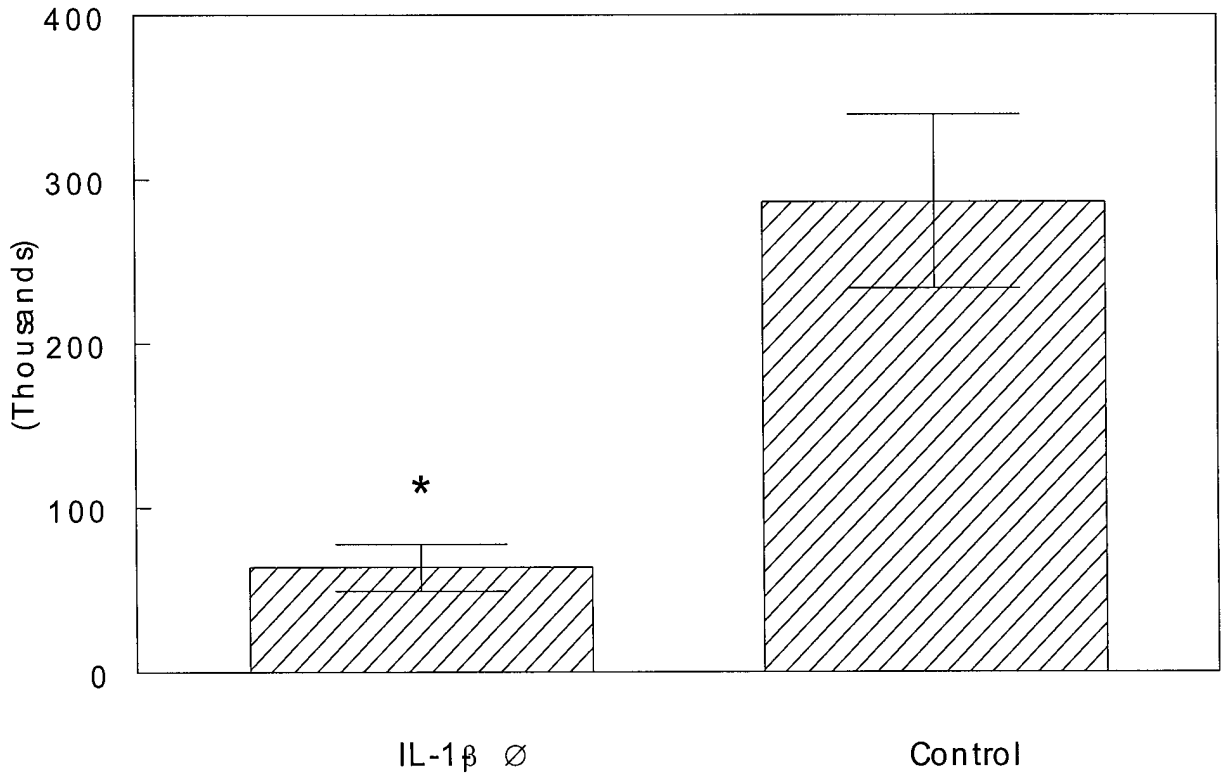

Figure 15. A comparison of the BALF cell counts between rats injected with anti-IL-1β and with pre-immune control serum prior to exposure to ACM combustion atmospheres. Results are expressed as means ± SEM. * indicates significant difference from control, $p < 0.05$.

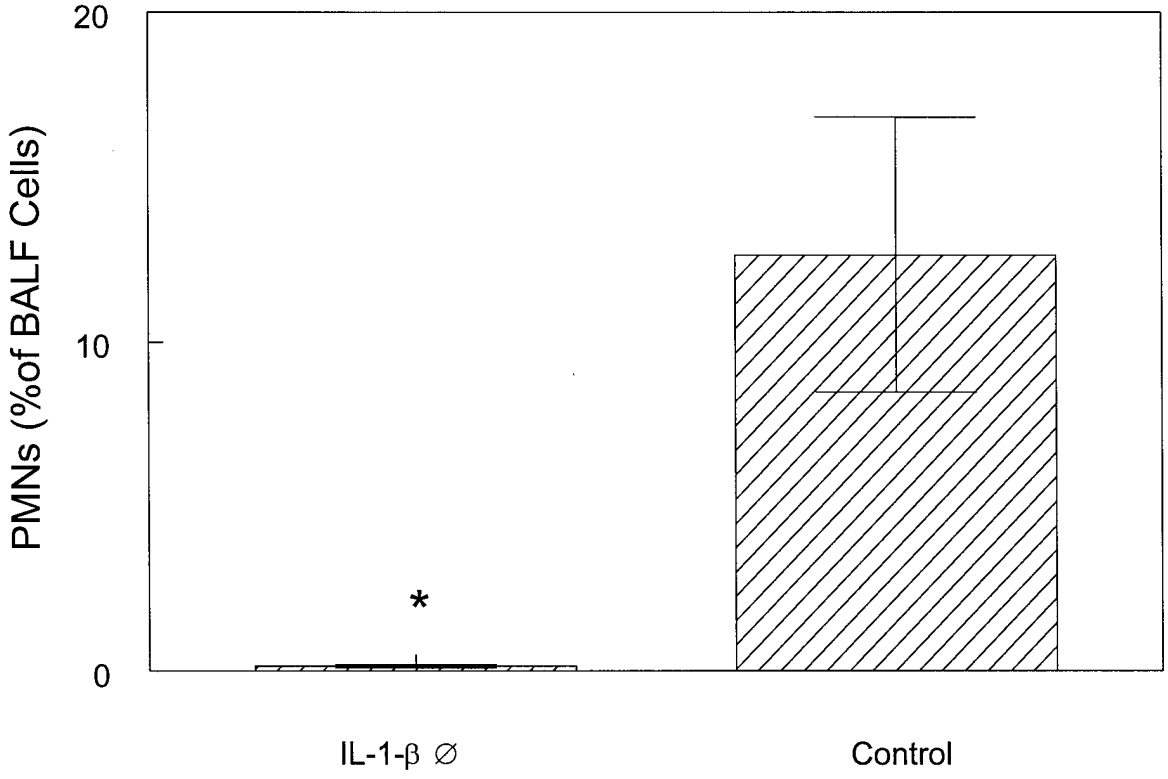

Figure 16. A comparison of the percent of PMNs in the BALF between rats injected with anti-IL-1β and with pre-immune control serum prior to exposure to ACM combustion atmospheres. Results are expressed as means ± SEM. * indicates significant difference from control, $p < 0.05$.

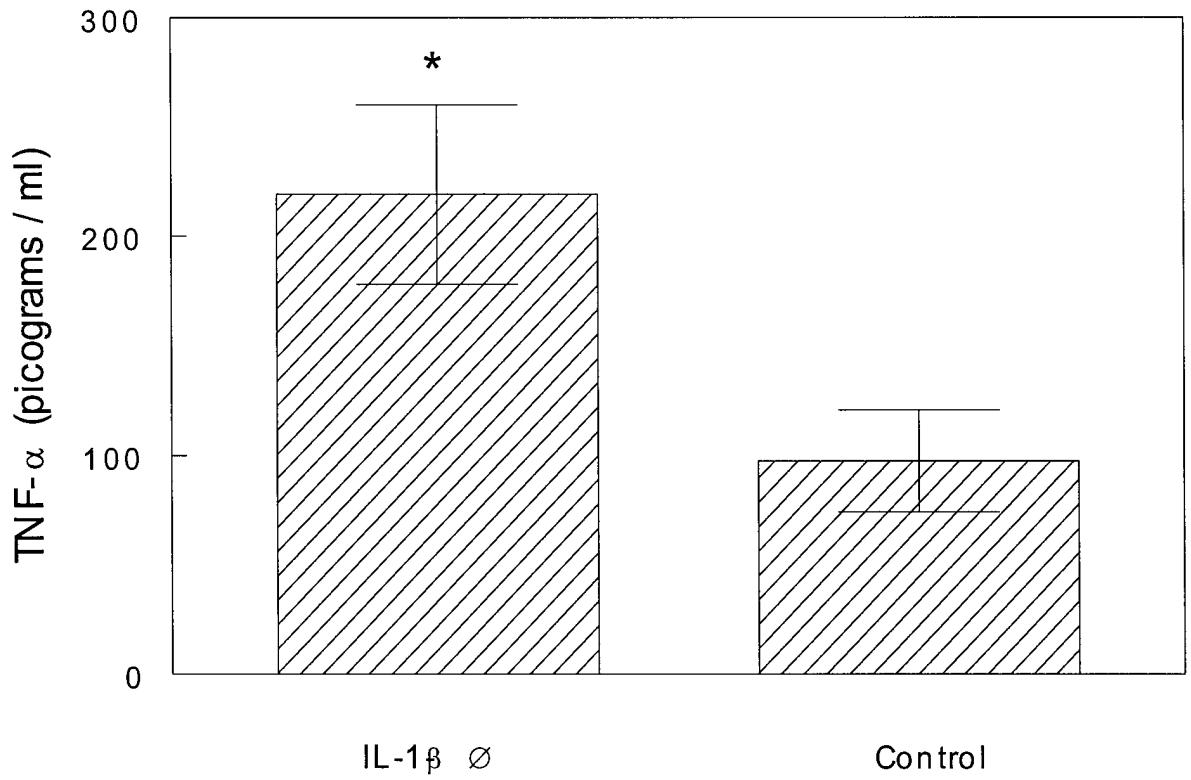

Figure 17. A comparison of the BALF TNF-α levels between rats injected with anti-IL-1β and with pre-immune control serum prior to exposure to ACM combustion atmospheres. Results are expressed as means ± SEM. * indicates significant difference from control, $p < 0.05$.

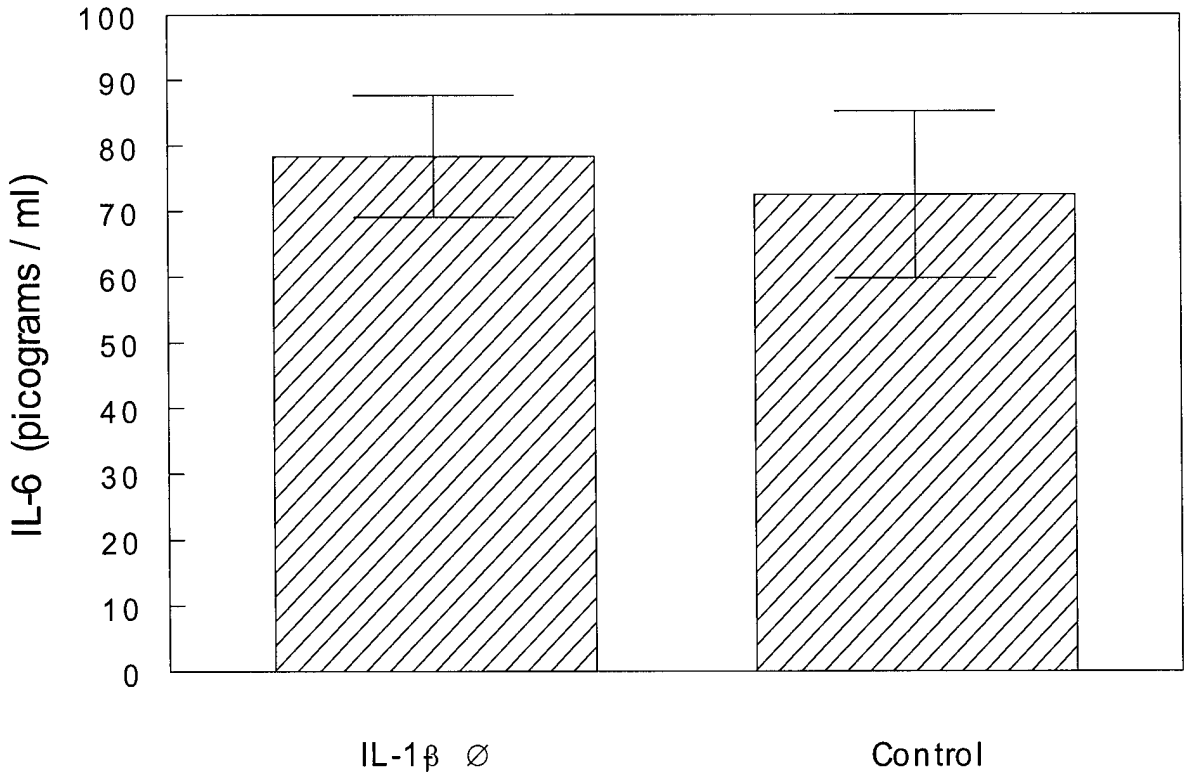

Figure 18. A comparison of the BALF IL-6 levels between rats injected with anti-IL-1β and with pre-immune control serum prior to exposure to ACM combustion atmospheres. Results are expressed as means ± SEM.

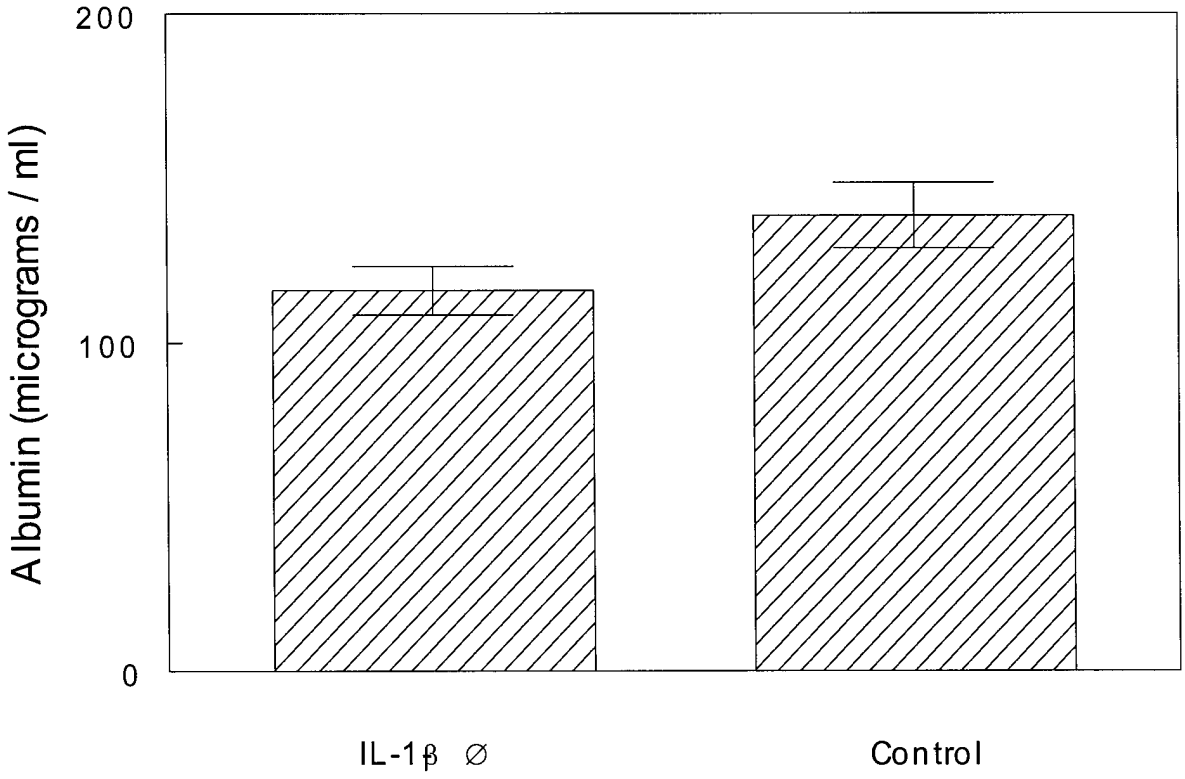

Figure 19. A comparison of the BALF albumin levels between rats injected with anti-IL-1β and with pre-immune control serum prior to exposure to ACM combustion atmospheres. Results are expressed as means ± SEM.

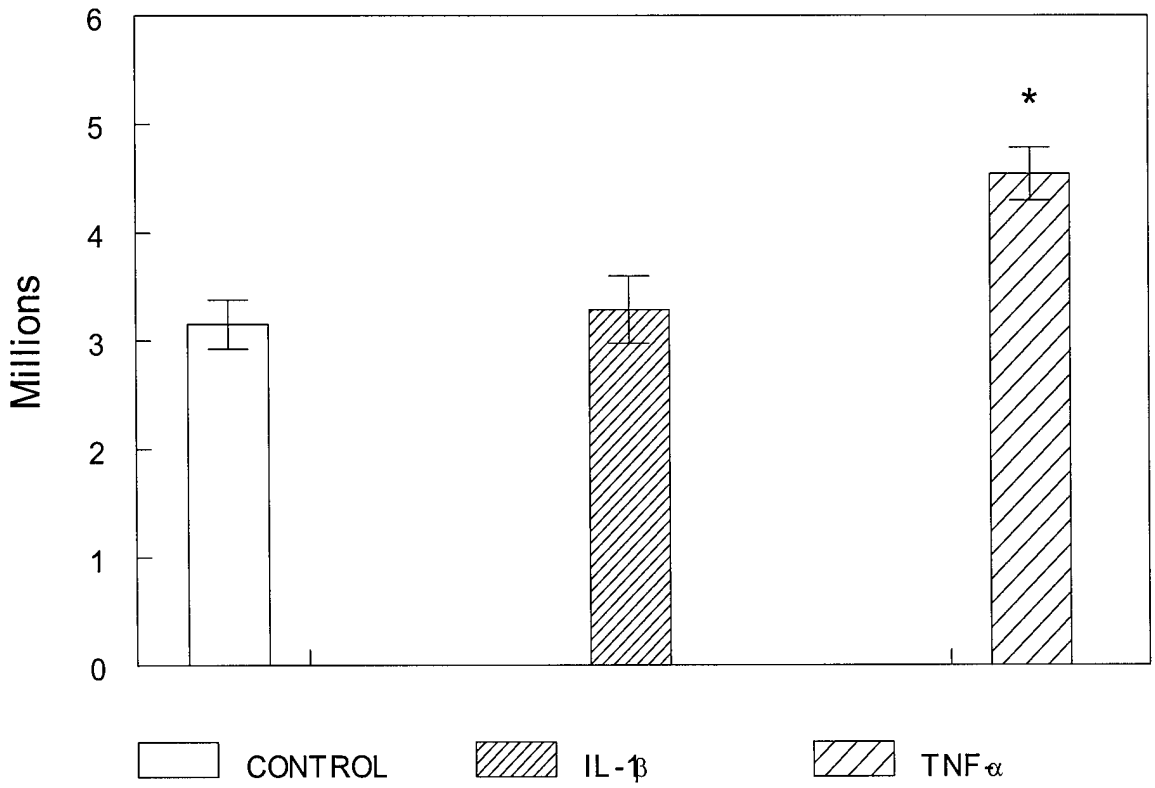

Figure 20. A comparison of the BALF cell counts between rats injected with pre-immune control serum and anti-IL-1β or anti-TNF-α serum prior to exposure to ACM combustion atmospheres. Results are expressed as means ± SEM. * indicates significant difference from control, $p < 0.05$.

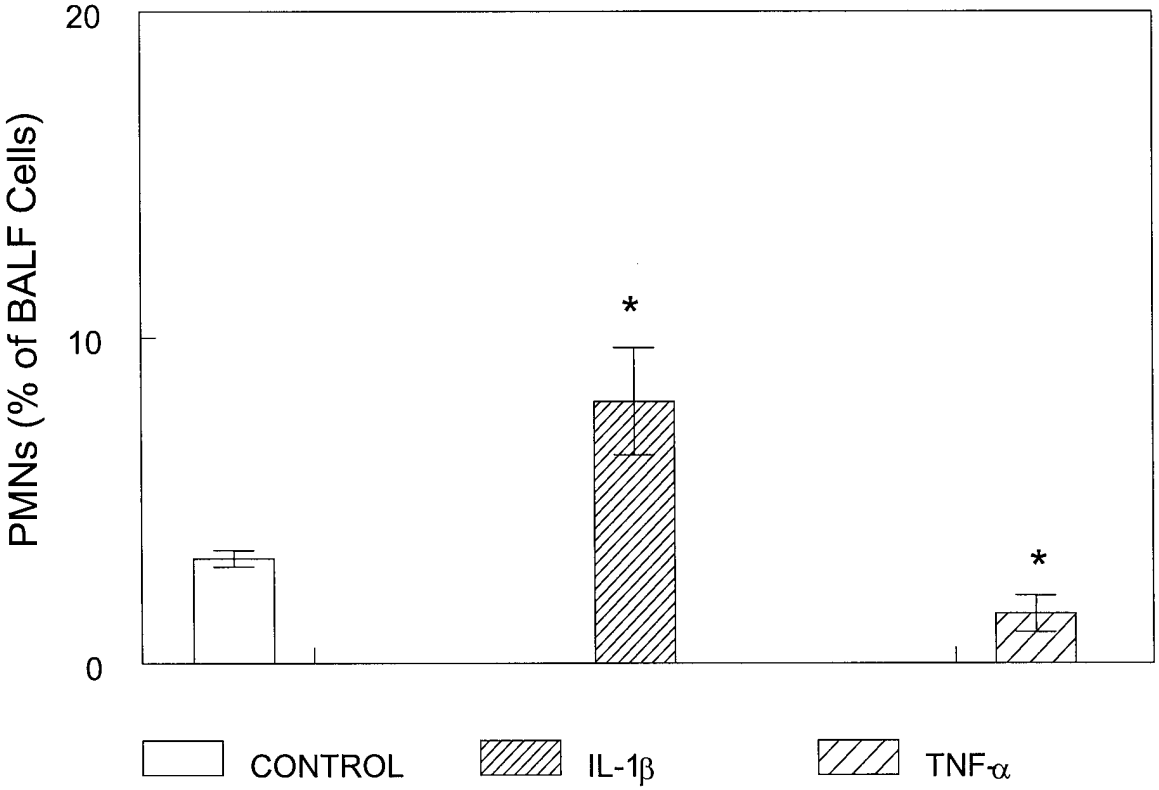

Figure 21. A comparison of the percentage of PMNs in the BALF between rats injected with pre-immune control serum and anti-IL-1β or anti-TNF-α serum prior to exposure to ACM combustion atmospheres. Results are expressed as means ± SEM. * indicates significant difference from control, $p < 0.05$.

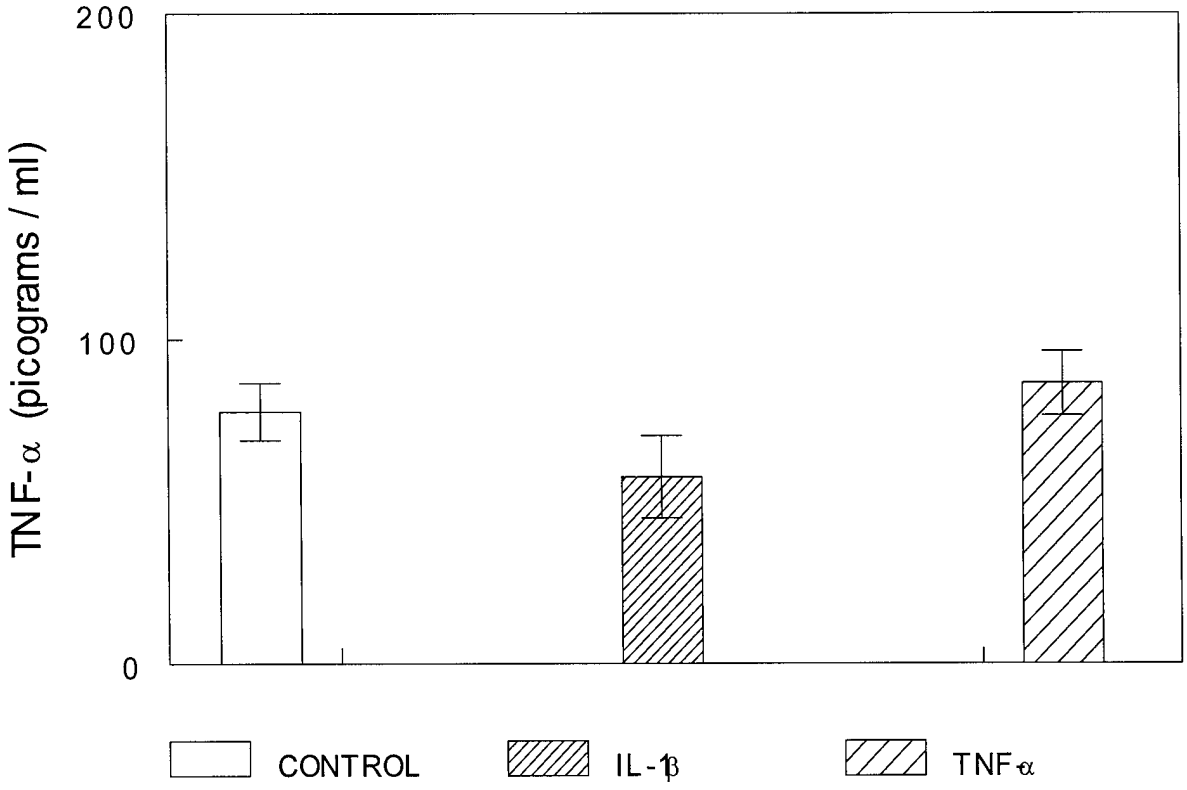

Figure 22. A comparison of the BALF TNF-α levels between rats injected with pre-immune control serum and anti-IL-1β or anti-TNF-α serum prior to exposure to ACM combustion atmospheres. Results are expressed as means ± SEM.

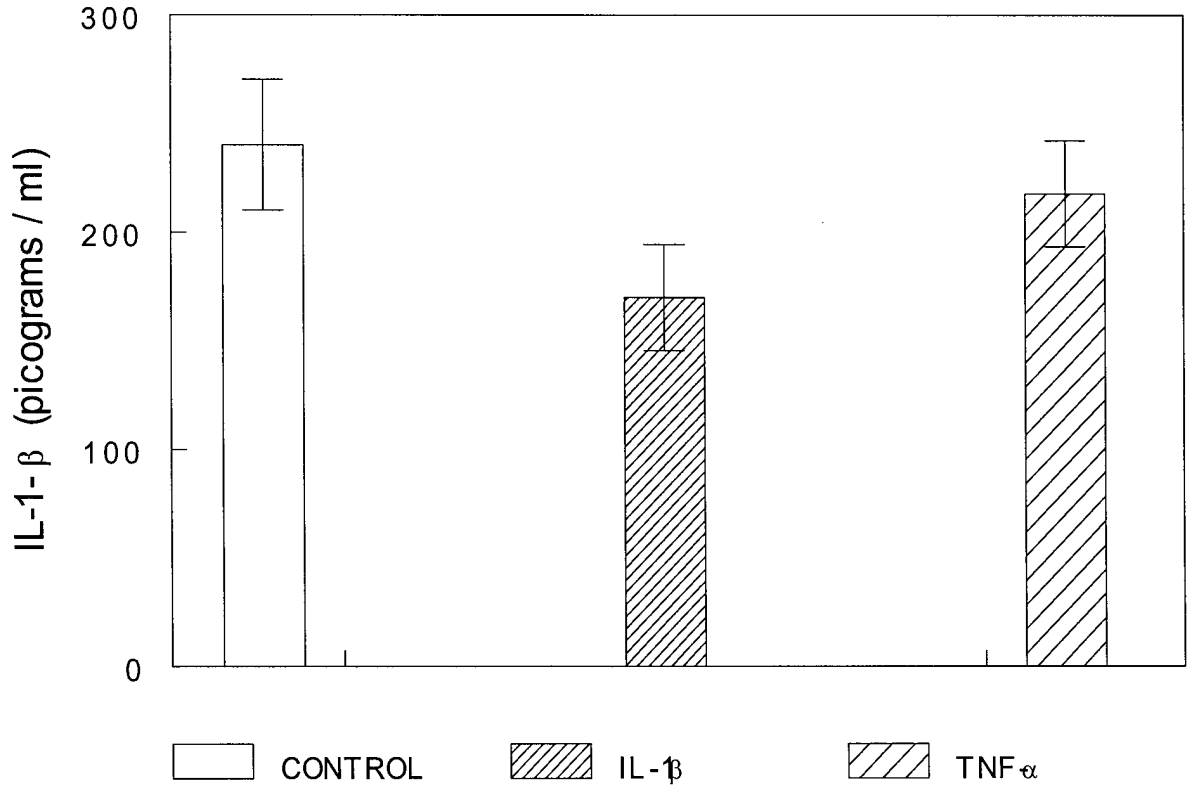

Figure 23. A comparison of the BALF IL-1β levels between rats injected with pre-immune control serum and anti-IL-1β or anti-TNF-α serum prior to exposure to ACM combustion atmospheres. Results are expressed as means ± SEM.

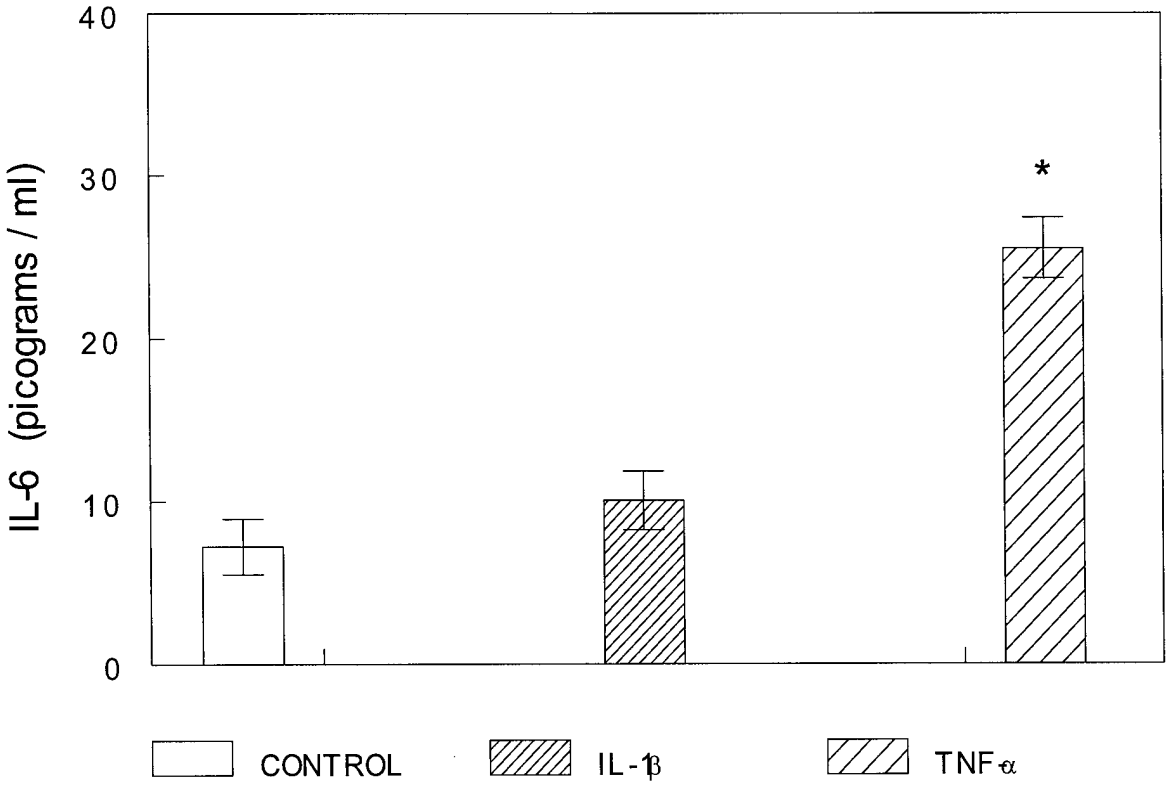

Figure 24. A comparison of the BALF IL-6 levels between rats injected with pre-immune control serum and anti-IL-1β or anti-TNF-α serum prior to exposure to ACM combustion atmospheres. Results are expressed as means ± SEM. * indicates significant difference from control, $p < 0.05$.

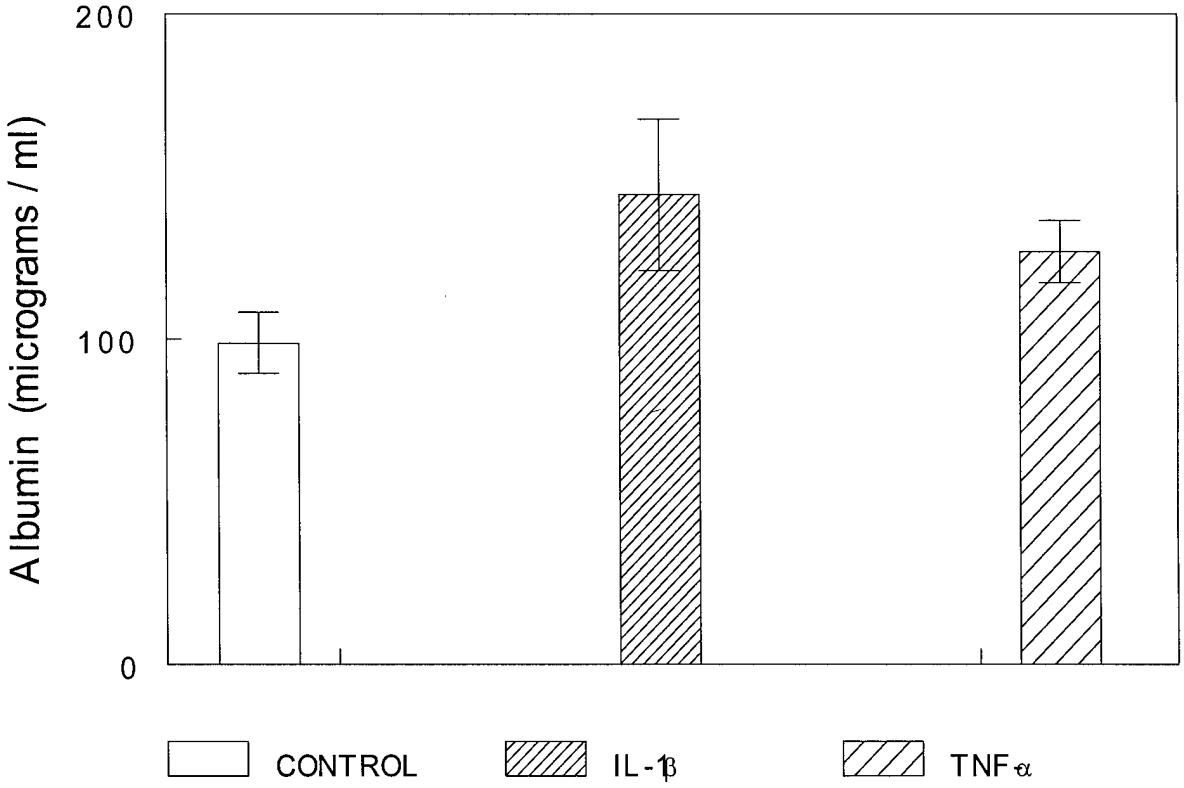

Figure 25. A comparison of the BALF albumin levels between rats injected with pre-immune control serum and anti-IL-1β or anti-TNF-α serum prior to exposure to ACM combustion atmospheres. Results are expressed as means ± SEM.

KEY RESEARCH ACCOMPLISHMENTS

- Characterized the pulmonary toxicity of ACM combustion atmospheres in the rat model
- Determined the effect of selective gene silencing of TNF-α and IL-1β on the pulmonary toxicity from ACM combustion atmosphere exposure
- Evaluated the effectiveness of antibody pretreatment on the pulmonary toxicity of ACM combustion atmospheres

REPORTABLE OUTCOMES

Abstracts and Presentations

Pulmonary Toxicity of Advanced Composite Material Combustion Atmospheres In Rats. PG Reinhart, EC Kimmel, DL Courson, JE Reboulet, AE Jung, and JT Murray. Society of Toxicology, Salt Lake City, Utah. 2003.

Pulmonary Toxicity of Advanced Composite Material Combustion Atmospheres In Rats. PG Reinhart, EC Kimmel, DL Courson, JE Reboulet, AE Jung, and JT Murray. Toxicology and Risk Assessment Conference, Dayton, Ohio. 2003.

Manuscripts

In progress

CONCLUSIONS

A single 1 hour exposure of Fischer 344 rats to ACM combustion atmospheres resulted in the development of pulmonary toxicity. This was characterized by an increase in pulmonary epithelial permeability, an influx of inflammatory cells including macrophages and PMNs, and the release of inflammatory cytokines TNF-α, IL-1β and IL-6. Epithelial permeability changes were persistent with significant increases in BALF albumin maintained through day 21. The time course for resolution of the inflammatory cell influx was dependent on the cell type involved. PMNs gradually diminished to control values by day 7. In contrast, the recruited macrophages persisted in the airway lumen for nearly every time point examined resulting in significantly greater numbers of cells in the BALF when compared to controls.

The production of cytokines is regulated by a complex network of signals from a variety of cells. A significant source of cytokines is the macrophage [1;2]. TNF-α, an inflammatory cytokine was significantly higher in all of the ACM combustion groups as compared to controls. Since greater numbers of macrophages remained in the lung in the ACM groups, it is likely that the source of the TNF-α is the activated macrophages that have persisted to phagocytose the particulate matter from smoke exposure.
The gene silencing experiments were only partially successful in ameliorating the pulmonary toxicity from ACM combustion atmospheres. While the loss of IL-1β was able to reduce the influx of inflammatory cells, it had little effect on the release of cytokines. In contrast, the loss of TNF-α resulted in both a reduction in inflammatory cells and release of IL-1β and IL-6. Neither treatment had any effect on the epithelial permeability changes indicating that the mechanism of permeability changes is independent of inflammatory cell influx.
The results of the antibody pretreatment experiments indicate that a single injection immediately prior to exposure was not sufficient to provide lung protection. Several reasons may account for the differing results in the antibody verses gene silencing experiments. One reason may be that the timing of the injection was not optimal. Another reason may be that the dose of antibody given was insufficient to provide protection. Finally, the clearance of the antibody may have played a part in the lack of protection provided.

The results of this study indicate that the pulmonary injury resulting from single exposure to ACM combustion atmospheres is comprised of two components; one being the epithelial permeability changes and the other being the cellular response of inflammation. It appears that the epithelial changes are independent of the cellular influx of inflammatory cells as well as their cytokines TNF-α, IL-1β or IL-6. In contrast, IL-1β and TNF-α played a significant role in the cellular influx of inflammatory cells into the lung following ACM combustion exposures. Future experiments should focus on the epithelial injury components which possibly may be the result of oxidant release, and the optimized use of the antibodies for TNF-α and IL-1β.

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2. L. M. Hulten et al., *Transplantation* 66, 364-369 (1998).

SALARY SUPPORT

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Jim Reboulet	Tech
Dave Courson	Tech