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***Streptococcus pneumoniae* and *Pseudomonas aeruginosa* pneumonia induce distinct host responses**

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Abstract

Objective—Pathogens that cause pneumonia may be treated in a targeted fashion by antibiotics, but if this therapy fails, treatment involves only non-specific supportive measures, independent of the inciting infection. The purpose of this study was to determine whether host response is similar following disparate infections with similar mortalities.

Design—Prospective, randomized controlled study.

Setting—Animal laboratory in a university medical center.

Interventions—Pneumonia was induced in FVB/N mice by either *Streptococcus pneumoniae* or two different concentrations of *Pseudomonas aeruginosa*. Plasma and bronchoalveolar lavage fluid from septic animals was assayed by a microarray immunoassay measuring 18 inflammatory mediators at multiple timepoints.

Measurements and Main Results—The host response was dependent upon the causative organism as well as kinetics of mortality, but the pro- and anti- inflammatory response was independent of inoculum concentration or degree of bacteremia. Pneumonia caused by different concentrations of the same bacteria, *Pseudomonas aeruginosa*, also yielded distinct inflammatory responses; however, inflammatory mediator expression did not directly track the severity of infection. For all infections, the host response was compartmentalized, with markedly different concentrations of inflammatory mediators in the systemic circulation and the lungs. Hierarchical clustering analysis resulted in the identification of 5 distinct clusters of the host response to bacterial infection. Principal components analysis correlated pulmonary MIP-2 and IL-10 with progression of infection while elevated plasma TNF α and MCP-1 were indicative of fulminant disease with >90% mortality within 48 hours.

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Conclusions—Septic mice have distinct local and systemic responses to *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* pneumonia. Targeting specific host inflammatory responses induced by distinct bacterial infections could represent a potential therapeutic approach in the treatment of sepsis.

Keywords

Sepsis; Pneumonia; *Streptococcus pneumoniae*; *Pseudomonas aeruginosa*; host response; cytokine

INTRODUCTION

Sepsis is treated using a combination of specific and non-specific therapies. Antibiotic treatment directed against a specific pathogen is a targeted therapeutic approach and results in a favorable outcome in many patients. Simultaneously, generalized supportive care is often initiated. However, when antimicrobial therapy is unsuccessful, patients receive the same non-specific therapy regardless of the inciting infection.

The vast majority of published sepsis trials use entry criteria that do not distinguish between differing types of infection (gram positive, gram negative) but instead rely upon a 17 year-old non-specific definition of sepsis that includes heart rate, respiratory rate, white blood cell count and temperature (1;²). Implicit in the entry criteria for most sepsis trials is the assumption that host response is similar following different inciting infections. In this paradigm, a pathogen-associated molecular pattern (3) or danger-associated molecular pattern (4) is detected by a pattern recognition receptor (5). Even though different pathogen or danger-associated molecular patterns bind to and activate different TLRs, the implication is that a common host response exists, independent of the triggering organism or signaling pathway. Supporting this position is a recent study demonstrating no differences in genome-wide microarray analysis of circulating neutrophils or peripheral blood mononuclear cells between septic patients infected with gram positive or gram negative infections (6;⁷) as well as earlier studies showing a common host response to sepsis (8;⁹). The view that host response is independent of inciting organism has been termed the “generic septic response” (10). In theory, a common host response could be altered via mediator blockade, but this has not proven successful to date in the treatment of human sepsis (11).

The “generic septic response” theory is not universally accepted since some experimental studies have demonstrated differences in gene expression between gram positive and gram negative sepsis (12;¹³). One useful way to study this is to interrogate the host on either a genome-wide RNA level or on a more targeted protein level. This approach has been useful in preliminary studies to discriminate septic patients from those with systemic inflammation (14) and has also been successful in distinguishing between causative organisms in acutely infected children, suggesting a series of mediators can be used for diagnostic purposes (15). Additionally, human volunteers given LPS have a reproducible host response that evolves over 24 hours as subjects go from healthy to sick to healing (16). These studies suggest that the clinical entity “sepsis” may represent a spectrum of related infection-initiated immunological disorders that progress to multiple organ failure and death, and each of these disorders may have a characteristic host response and temporal progression.

To test the hypothesis that there are discrete responses to individual infections as opposed to a common, generic response, we assayed mice given pneumonia with either *S. pneumoniae* or *P. aeruginosa*, the most common cause of gram positive community acquired pneumonia and most common cause of gram negative hospital acquired pneumonia respectively (17;¹⁸).

METHODS

Pneumonia models

P. aeruginosa (ATCC strain 27853) was placed in trypticase soy broth with constant shaking overnight. The resulting culture was centrifuged at 6000g, washed twice with saline, and re-suspended to a density of 0.1 (low dose), or 0.3 (high dose) A_{600nm} . *S. pneumoniae* (strain 99.55, capsular subtype 6A) was placed on 5% blood agar plates overnight, washed, and re-suspended to an absorbance of 0.5 A_{600nm} .

Under halothane anesthesia, mice received an intratracheal injection via a midline cervical incision of one of the following: 20 μ l of *P. aeruginosa* @ 0.1 A_{600nm} ($2-4 \times 10^6$ CFU), 40 μ l *P. aeruginosa* @ 0.3 A_{600nm} ($2-4 \times 10^7$ CFU), or 60 μ l *S. pneumoniae* @ 0.5 A_{600nm} ($2-4 \times 10^7$ CFU). Sham animals were treated identically but received an intratracheal injection of 40 μ l saline. Unless otherwise indicated, all experiments were performed on FVB/N mice. After incision closure, mice received 1 ml of saline via subcutaneous injection for fluid resuscitation. All animal studies were performed in accordance with NIH Guidelines and approved by the Washington University Animal Studies Committee.

Survival studies

Pneumonia was induced by a single investigator, and animals were followed for survival for seven days. The high dose *P. aeruginosa* model has been extensively used in our laboratory (19;²⁰) while the *S. pneumoniae* and the low dose *P. aeruginosa* models were developed for this manuscript. Due to ethical concerns of performing surgery on animals to re-generate a survival curve in a model that has been reproducible in our hands, the portion of the survival curve for the high dose *P. aeruginosa* in figure 1 comes from a previous publication from our laboratory (21) (reprinted with permission from JAMA).

Studies examining the influence of TNF- α were performed on animals given 300 μ g of anti-TNF- α antibody TN3 19.12 (a generous gift of Robert Schreiber, Washington University (22)) three hours prior induction of pneumonia. In order to study the effects of MCP-1 on survival, additional experiments were required to generate survival curves in C57Bl/6 mice that had similar kinetics and 7-day mortality as FVB/N mice shown in figure 1. Doses used were 20 μ l of *P. aeruginosa* @ 0.2 A_{600nm} , 30 μ l *P. aeruginosa* @ 0.3 A_{600nm} , or 20 μ l *S. pneumoniae* @ 0.1 A_{600nm} . Once these experiments were completed, survival studies on MCP-1^{-/-} mice (Jackson Laboratories) were performed.

Cytokines

Mice subjected to the three pneumonia models and sham pneumonia were sacrificed at 6, 12, or 72 hours. To collect BAL samples, the trachea was cannulated with a 22-gauge angiocatheter, and lungs were lavaged with 1 ml of PBS. BAL and blood samples from each mouse were centrifuged for 5 minutes at 6000g. The supernatants were removed and analyzed for soluble inflammatory mediator concentration using a microarray immunoassay measuring IL1b, MIP-2, MCP-1, Eotaxin, IL-18, IFN- γ , MIP- α , TNF- α , IL-6, IL-1ra, IL-10, TNFSF-1, TNFSF-2, IL-2, IL-5, IL-12, IL-13, and RANTES (23). Due to technical difficulties sham values were not obtained in any animal for IL-18, and IL-1ra in both blood and BAL fluid as well as eotaxin in BAL fluid.

Cultures

BAL and blood samples were taken from mice upon sacrifice and diluted serially in saline and plated on blood agar plates. Following incubation at 37° C, plates were examined after 24 and 48 hours for colony counts. Log transformation of calculated colony counts was then used for further analysis (24).

Pattern analysis

Cytokine abundance data was analyzed following importation into SpotFire Decision Site 8.2.1 (Spotfire). Six individual mice were censored from analysis due to technical issues resulting in missing values of either all serum or BAL cytokines. These included three mice given *S. pneumoniae* at 6 hours, one mouse given low dose *P. aeruginosa* at 6 hours and two mice given high dose *P. aeruginosa* at 6 hours. No individual data points were excluded. Values that were below or above the detection limits of the assay were replaced with the lower or upper detection limits to allow for numerical analysis. Hierarchical clustering (UPGMA) was performed on BAL and blood cytokine measurements individually and also together using correlation as the similarity measure. Principal component analysis was performed on cytokine abundance data from three data sets: BAL, blood and combined data. Principal components were calculated using all mice analyzed for hierarchical clustering and averages and standard errors were calculated using either the treatment group identifier or the hierarchical clustering group assignment. Pearson's correlation between individual cytokines and each principal component were calculated to determine whether any of the measured quantities could serve as a surrogate for the principal component.

Blood counts

White blood counts were performed using a Coulter counter (Baker 9000) using 50 μ l of whole blood. Differential cell count was performed by counting 100 leukocytes on a smear with Wright's stain.

Myeloperoxidase assay

Twelve hours after induction of pneumonia, the pulmonary vasculature was perfused with 1 ml PBS, and lungs were frozen in liquid nitrogen (25). Right lower lobe sections were subsequently thawed, weighed, and homogenized in 4 ml of 20 mM potassium phosphate buffer with 0.5 g/dl hexadecyltrimethyl ammonium bromide. Following sonication for 90 seconds, sections were incubated for 2 hours in a 60° C water bath. Samples were then centrifuged and 100 μ l of supernatant placed into 2.9 ml of 50 mM potassium phosphate buffer (pH 6.0) with 0.167 mg/ml O-dianisidine and 0.0005% hydrogen peroxide. Absorbance at 460 nm was measured for 3 minutes. MPO activity per gram of protein was calculated using the rate of change in absorbance over 3 minutes and the protein content of the sample as determined by a modified Bradford assay (26).

Statistics

Data analysis was performed using Prism version 4.0 (GraphPad) and SAS version 9.1. Data are presented as mean $\pm$ standard error of the mean. Survival curves were compared using chi square analysis. Cytokines, quantitative cultures, blood counts and MPO activity were first analyzed using Kruskal-Wallis one-way analysis of variance by ranks. Post hoc pairwise comparisons were conducted using the Mann-Whitney U-test. Cytokine data at each timepoint was compared for all possible groups (ie. sham vs. high dose *P. Aeruginosa*, sham vs. low dose *P. Aeruginosa*, sham vs *S. Pneumoniae*, high dose *P. Aeruginosa* vs. *S. Pneumoniae*, low dose *P. Aeruginosa* vs. *S. Pneumoniae*, high dose *P. aeruginosa* and low dose *P. Aeruginosa*). Individual cytokine levels were not compared between different models at different timepoints. A P value <0.05 was accepted as statistically significant.

RESULTS

Survival

Animals were given one of two doses of *P. aeruginosa* that caused either 96% or 50% seven-day mortality or a dose of *S. pneumoniae* that resulted in a 84% seven-day mortality (Fig. 1A).

Animals died faster following high dose *P. aeruginosa* than *S. pneumoniae*. Mortality following low dose *P. aeruginosa* and *S. pneumoniae* was similar in the first three days ($p=0.59$). There were no statistically significant differences in body weights in mice subjected to any model of pneumonia at either 6 or 24 hours (Figure 1B). Mice given *S. pneumoniae* pneumonia had lower body weights than animals given sham pneumonia 48 and 72 hours following intratracheal injection of bacteria ($p=0.02$ and 0.007 respectively) although no statistically significant differences were seen between animals given low dose *P. aeruginosa* and sham animals at any timepoint.

Bronchoalveolar lavage (BAL) and blood cultures were taken from animals at 6, 12, 48 or 72 hours after infection. Mice given *S. pneumoniae* had approximately 10^7 colony forming units (CFUs)/ml in their airways 48 hours following onset of pneumonia, declining precipitously between day 2 and 3 (Fig 2a). Mice given high dose *P. aeruginosa* had similar bacterial loads in their lungs at 6 hours and a 10-fold increase at 12 hours ($p<0.05$ compared to *S. pneumoniae*). Cultures were not measured at later timepoints in this model because of high levels of mortality at 48 hours. Animals given low dose *P. aeruginosa* had 10-fold fewer bacteria 6 hours after the onset of pneumonia (10^6 CFUs/ml, $p<0.05$ compared to the other infections at the same timepoints). Similar to animals given *S. pneumoniae*, mice infected with low dose *P. aeruginosa* had a substantial decline in pulmonary bacterial load between 48 and 72 hours.

In contrast to local microbial concentrations, animals given *S. pneumoniae* or low dose *P. aeruginosa* had only trace amounts of bacteria detectable in their blood 6, 12 or 48 hours after the onset of pneumonia (Fig. 2b). After 72 hours, however, mice infected with *S. pneumoniae* had significant bacteremia ($>10^4$ CFU/ml blood) while animals infected with low dose *P. aeruginosa* had essentially no bloodborne bacteria. Animals given high dose *P. aeruginosa* had similar low levels at 6 hours with a 10-fold increase in bacterial load 12 hours after onset of pneumonia ($p<0.05$ compared to both other infections at same timepoint).

Cytokine analysis

BAL and blood samples were taken from animals at 6, 12, and 72 hours following onset of pneumonia to measure the local and systemic host response respectively. At 6 and 12 hours post-infection, all animals in all groups were alive, regardless of the ultimate mortality of the pneumonia model used. Animals that received high dose *P. aeruginosa* were not sampled at 72 hours due to greater than 80 percent mortality by this timepoint. Cytokine levels for all models of pneumonia as well as animals that underwent sham operation are listed in table 1 (BAL) and table 2 (blood).

To determine if there were different cytokine levels between pneumonia models, the experimental design allowed for three distinct comparisons of the host response to infection: a) low dose *P. aeruginosa* and *S. pneumoniae*—animals with similar kinetics of mortality over the three days when samples were obtained, b) high dose *P. aeruginosa* and *S. pneumoniae* - animals that would eventually have 96% and 84% seven-day mortality respectively and c) high dose *P. aeruginosa* and low dose *P. aeruginosa* - animals receiving the identical pathogen but at doses that cause differing mortalities.

Pathogens causing similar kinetics of mortality have distinct local and systemic cytokine profiles

Local cytokine production is higher in animals given low dose *P. aeruginosa* in BAL fluid 6 hours after the onset of pneumonia compared to animals given *S. pneumoniae* (Fig. 3a, pro-inflammatory cytokines at top of figure, anti-inflammatory at bottom). This is not a result of lipopolysaccharide (LPS) in gram negative bacteria causing a greater increase in TNF- α , since

this was one of only two cytokines that was higher in mice infected with *S. pneumoniae* at 6 hours. There is a marked temporal shift in the local response to the two infections such that by 72 hours, the vast majority of cytokines are higher in mice subjected to *S. pneumoniae* pneumonia. Importantly, the inflammatory response is not correlated to bacterial colony counts in the lung. At 6 and 12 hours, there are more *S. pneumoniae* bacteria in the lungs than *P. aeruginosa* but higher pro- and anti-inflammatory cytokine concentrations are seen with the latter organism (compare Fig 2a to 3a). Additionally, the late shift toward relative higher cytokine abundance in animals infected with *S. pneumoniae* pneumonia occurs in the setting of a 5 log decrease in bacteria recovered from BAL samples.

Despite having similar (low) levels of bacteremia, systemic concentrations of pro- and anti-inflammatory mediators are higher in animals given low dose *P. aeruginosa* compared to animals given *S. pneumoniae* at 6 hours (Fig. 3b). However, despite a marked increase in bacteria in the blood 72 hours after onset of pneumonia in animals given *S. pneumoniae*, plasma cytokine concentrations are generally similar between both groups. Even though levels of bacteria are more than three logs higher in animals given *S. pneumoniae*, there is not a single mediator that is higher in these animals (compare Fig. 2b to 3b). The local response is therefore markedly different from the systemic response at 72 hours (compared Fig 3a to 3b).

Pathogens causing high seven-day mortality have distinct early cytokine profiles

Both pro- and anti-inflammatory cytokines are generally higher in animals given high dose *P. aeruginosa* in BAL fluid 6 and 12 hours after the onset of pneumonia compared to animals given *S. pneumoniae* (Fig. 3c). This local effect is TNF- α -independent since concentrations of this cytokine are similar in mice subjected to either infection. The inflammatory response is also independent of pulmonary bacterial load since there was not a single cytokine measured that was statistically higher in the lungs of mice infected with *S. pneumoniae* despite having similar concentrations of bacteria at 6 hours (compare Fig 2a to 3c).

There is a similar trend in systemic cytokines. Both pro- and anti-inflammatory cytokines are generally higher in the blood of animals given high dose *P. aeruginosa* 6 and 12 hours after the onset of pneumonia, and at no time is the relative abundance of *S. pneumoniae* higher for any cytokine (Fig. 3d) despite having statistically similar low levels of bacteremia at 6 hours in each group (Fig. 2b). Of note, even when the trend for cytokine abundance is similar between BAL and blood, both the absolute values and ratios may be markedly different between groups. An example is TNFsr-2, which is higher in mice given high dose *P. aeruginosa* in BAL and blood at 6 hours (Fig. 3c and d). TNFsr-2 concentrations in BAL fluid are 1736 and 1185 pg/ml in animals given high dose *P. aeruginosa* or *S. pneumoniae* respectively (ratio 1.5:1), while serum concentrations in the same animals are >100,000 pg/ml and 2034 respectively (ratio >50:1).

Differing doses of *P. aeruginosa* cause distinct early cytokine profiles

Although the bacterial load is higher in the lungs of mice given high dose *P. aeruginosa* compared to low dose, it does not directly correlate to local cytokine abundance (compare Fig. 2a to Fig. 3e). BAL cytokine concentrations are nearly as likely to be higher 6 hours following low dose *P. aeruginosa* as they are following high dose *P. aeruginosa*. Blood concentrations were higher following high dose *P. aeruginosa* for 6 cytokines, higher following low dose *P. aeruginosa* cytokines for 3 cytokines and were not statistically different between the two models for four cytokines (Fig 3f). In contrast to the 6 hour timepoint, by 12 hours cytokine levels were consistently higher in both BAL and blood in high dose *P. aeruginosa*.

Hierarchical clustering of cytokine expression

Figure 3 illustrated that there were statistically significant differences between most cytokines in the different models at each timepoint, and that the magnitude of these differences varied depending on the cytokine, the infection, the body fluid sampled and the timepoint examined. While these data are instructive on a population basis, they do not examine the heterogeneity of the individual response to each challenge. To examine relationships between individual animals, hierarchical clustering of cytokine abundance data from the individual mice was performed (Fig. 4A). Although eight groups of animals were included in the analysis (two infections at three time points and one infection at two time points), only five major nodes were identified.

The principal node separates ten animals in cluster A from the remaining 50 mice. The animals in this group include 5 of 6 mice given high dose *P. aeruginosa* at 6 hours, 4 of 8 of mice given high dose *P. aeruginosa* at 12 hours, and 1 of 7 mice given *S. pneumoniae* at 6 hours. Animals in cluster A are characterized by elevated concentrations of pro-inflammatory cytokines (IL-1b, IL-6, IL-18, MIP- α , TNF- α and TNFsr2) in BAL fluid as well as elevated concentrations of eotaxin, TNFsr2, TNFsr1, and MCP-1 in the blood.

The second node separates 15 mice in cluster B from the remaining 35 animals. These animals include all 6 mice given low dose *P. aeruginosa* at 6 hours, 1 out of 8 mice given low dose *P. aeruginosa* at 12 hours, and all 7 mice given *S. pneumoniae* at 72 hours. These cohorts of mice have a 50-60% mortality rate within 72 hours of the sampling time. However there is no apparent cytokine or combination of cytokines that subdivides either the *P. aeruginosa*-infected or *S. pneumoniae*-infected mice, suggesting that local and systemic cytokines at this time point do not have the capacity to predict which animals will go on to recover or die. Interestingly, the dendrogram on the left of Fig. 4 shows that the 7 *S. pneumoniae*-infected mice have the most similar cytokine expression profiles of all groups of mice in the study. Because of the homogeneity in the *S. pneumoniae*-infected mice, we identified significant differences between *P. aeruginosa*-infected animals and *S. pneumoniae*-infected animals in cluster B (Fig. 4B). As expected based on their pulmonary bacterial burden (Fig. 2), the *P. aeruginosa* infected mice in Cluster B had higher pro-inflammatory cytokines in BAL fluid than did *S. pneumoniae*-infected mice. Four measured proteins were higher in the BAL of *S. pneumoniae*-infected mice -- MIP2, IL-10, TNFsr2 and IL1ra. Surprisingly, although the only mice in Cluster B that were bacteremic were *S. pneumoniae*-infected mice (Fig 2), plasma cytokines were higher in *P. aeruginosa*-infected mice.

The remaining 35 mice clustered into three separate groups. Cluster C contained the 5 remaining animals that received high dose *P. aeruginosa* at 6 and 12 hours that were not in cluster A. Of the 36 measured cytokines, the only one that was significantly different between clusters A and C was systemic TNFsr2.

Cluster D contained 13 mice, all of which were infected with *S. pneumoniae* at either 6 hours (6 of 7 animals) or 12 hours (7 of 11 animals) post-infection. With the exception of a single animal, the clustering algorithm separated the samples obtained at 6 hours from those obtained 12 hours after infection. A small panel of measured components separated these two groups, including marked increases in soluble IL-1 and TNF antagonists in BAL fluid (Fig. 4C). Similar to mice infected with high dose *P. aeruginosa* (clusters A and C), all animals in Cluster D had elevated BAL concentrations of MIP- α .

The final 17 mice in Cluster E contained 7 out of 8 mice infected with low dose *P. aeruginosa* at 12 hours, all 6 mice infected with low dose *P. aeruginosa* at 72 hours and 4 out of 11 mice infected *S. pneumoniae* at 12 hours. No clear pattern in cytokine expression separated these animals despite their markedly different prognoses.

Principal component analysis

Principal component analysis (PCA) is a computational technique that reduces multidimensional data from 1 axis per variable into a lower dimensional representation of that data set viewed from its most informational viewpoint. PCA can reveal the internal structure of a data set to best explain the variance in the data, assuming the data conform to three key assumptions: (1) linearity, (2) that the mean and covariance of the data are important, and (3) that large variances have important dynamics. If the observed data has a high signal-to-noise ratio then the principal components with larger variance usually correspond to interesting dynamics while PC with lower variance corresponds to noise. Data visualization via PCA can illuminate informative dynamics within time-dependent data sets (27).

One particularly informative visualization of PCA of the data outlined above is from principal components 1 and 3 of the combined analysis (Fig. 5A). Principal component 1 separated *S. pneumoniae*-infected animals at 72 hours from the other groups while PC3 separated the 90% 7-day lethality *P. aeruginosa*-infected animals from the other groups. Different time points from the same infection were connected by lines to illustrate hypothetical trajectories of disease progression based on local and systemic cytokine abundance. Interestingly, the hypothetical *S. pneumoniae* and 50% mortality *P. aeruginosa* trajectories become indistinguishable between 6 and 12 hours after infection. PC were also calculated by grouping mice based on which cluster they belonged to in the hierarchical clustering analysis (Fig. 5B). PCA of any cytokine datasets identified statistically significant differences between groups whether classified as treatment groups or clusters (data not shown),

Principal component 1 showed a high correlation with the abundance of MIP-2 ($r^2 = 0.998$) and IL-10 ($r^2 = 0.72$) in BAL (the cytokines whose abundance defines cluster B) whereas principal component 3 correlated with TNF α ($r^2 = 0.84$) and MCP-1 ($r^2 = 0.70$) in the blood and IL-1b in the BAL ($r^2 = 0.64$). By examining BAL MIP2 and plasma TNF α abundance together, four groups emerge (Fig. 5C): (1) Mice that have recovered from infection (i.e. low-dose *P. aeruginosa*-infected mice 72h following onset of pneumonia) have essentially none of these cytokines, (2) mice that have high BAL MIP-2 (*S. pneumoniae*-infected mice 72 hours following onset of pneumonia), (3) mice with high plasma TNF α (*P. aeruginosa*-infected mice that will ultimately have a 90% 7-day mortality) and (4) all other animals (mice with intermediate abundances of local MIP2 in the BAL and systemic TNF α).

Survival studies

To assess the functional significance of relative differences in cytokine levels, survival studies were performed. TNF- α levels were markedly elevated in BAL at 6 hours in *S. pneumoniae* and in both BAL at 6 hours and blood at 12 hours in high dose *P. aeruginosa* (Fig. 6A, B). Treating animals subjected to pneumonia with anti-TNF- α antibody resulted in a marked hastening of mortality in animals where TNF- α levels were high (Fig. 6c, compare kinetics of mortality to Fig. 1) but had no affect in low dose *P. aeruginosa* where local and systemic cytokine levels were lower.

Survival studies were also done on MCP-1 $^{-/-}$ mice, based upon the cytokine's significance in both hierarchical clustering and PCA studies. Cytokine levels were markedly different between BAL fluid and blood (Fig. 7a and 7b). MCP-1 was markedly elevated in the blood of both high dose and low dose *P. aeruginosa* but nearly undetectable in *S. pneumoniae* (Fig. 7b). Despite these differences, there was no survival affect in MCP-1 $^{-/-}$ mice given either high dose or low dose *P. aeruginosa* (where systemic levels were elevated) and a hastening of mortality in MCP-1 $^{-/-}$ mice given *S. pneumoniae* (even though systemic levels were not elevated, Fig. 7c).

Circulating leukocytes

To further define the host response, circulating white blood cell counts were analyzed (Fig. 8a). By 24 hours, all animals with pneumonia had similar decreases in their leukocyte counts despite marked differences in cytokine production ($p < 0.05$ compared to unmanipulated mice, compare Fig 8a. to Fig 3b,d,f). Of note, total circulating white blood cells were lowest when animals had minimal bacteremia, and leukocyte counts returned to normal by three days, even in the setting of marked *S. pneumoniae* bacteremia (compare Fig 2b to 8a). The initial decrease in total white blood cell count was in large part due to a decrease in absolute lymphocyte count in all groups at 12 and 24 hours, independent of type of bacterial infection (Fig. 8b).

In contrast to the similarities in absolute lymphocyte count, there were marked differences in absolute neutrophil counts (Fig 8c). Mice given *S. pneumoniae* had increased circulating neutrophils, mice given low dose *P. aeruginosa* had little change in circulating neutrophils, and mice given high dose *P. aeruginosa* had a marked decrease in circulating neutrophils. To determine whether this could be explained by differential infiltration of neutrophils into lungs of animals with pneumonia, pulmonary MPO assay was performed (Fig. 9). Whether assessed by histology or quantitative MPO activity, there was a substantial increase in pulmonary neutrophils in mice given high dose *P. aeruginosa*, minimal pulmonary neutrophilic infiltration in mice given *S. pneumoniae*, and intermediate levels in animals given low dose *P. aeruginosa*.

DISCUSSION

This study demonstrates that genetically inbred animals have distinct host responses to pneumonia. The inflammatory response is dependent upon both kinetics of mortality as well as ultimate seven-day mortality. Different inocula of the same microbe also cause distinct early host responses but not in a monotonic fashion that might be predicted, since higher bacterial concentrations do not directly correlate to the severity of the inflammatory response. Additionally, the host response is compartmentalized, with substantial variation between local (BAL) and systemic (blood) cytokine profiles.

There is a fundamental disconnect between our results and a) current patient care and b) therapeutic targets of the majority of sepsis clinical trials. Current therapy in sepsis is individualized only as far as targeting specific microbes; however, once antibiotics fail, treatment is non-specific in keeping with the concept of a “generic septic response.” However, if broad-based host responses to infections exist, targeting them may be a rational approach to sepsis therapy that can be undertaken simultaneously to targeting the initiating microbe with antibiotic therapy. To examine this possibility, hierarchical cluster analysis was performed which allowed us to identify 5 distinct host response profiles within the 8 different groups of animals examined. These clusters may have prognostic significance and potential utility for development of targeted therapeutics or diagnostic assays. For instance, all animals that received high dose *P. aeruginosa* were in clusters A and C with the sole difference between the two being a greater than 50-fold difference in systemic TNF α . While there was a high ultimate mortality in each of these groups of animals, it is possible that the difference in TNF α concentrations was linked to rapidity of death. Additionally, a vigorous local inflammatory response appears to correlate with rapid death since animals in clusters A and C would be expected to die in less than 48 hours based upon the survival curves shown in figure 1. Additionally, all animals in Cluster D were infected with *S. pneumoniae* and all had elevated BAL concentrations of MIP- α . We speculate that these mice may be the ones destined to die since 13/18 mice given this bacteria at 6 or 12 hours were in this cluster, and this was very close to the percentage of animals that ultimately died following *S. pneumoniae* pneumonia. The remaining mice infected with *S. pneumoniae* in cluster E had low MIP- α concentrations. Interestingly, local BAL production of MIP- α ceased by 72 hours in animals with *S. pneumoniae* pneumonia (cluster B) even though the majority of those animals would go on to

die as well. It is also remarkable that on the PCA, the most similar cytokine profiles in the entire experiment were from mice subjected to *S. pneumoniae* pneumonia 72 hours after infection. While approximately half of these animals die within 72 hours, there were no differences noted within this entire group of animals.

The lack of correlation between bacterial concentration and host response was surprising. Mice infected with *S. pneumoniae* had a higher pulmonary bacterial load at early timepoints, but mice infected with low dose *P. aeruginosa* had higher cytokine abundance. By 72 hours there was a 10,000-fold decrease in pulmonary bacterial load in mice given *S. pneumoniae*, but despite this drop, local cytokine abundance increased compared to low dose *P. aeruginosa*. Examining blood from the same animals showed higher concentrations of cytokines in mice with low dose *P. aeruginosa* at early timepoints despite similar low levels of bacteremia in both. However, a marked increase in bacteremia in animals given *S. pneumoniae* alone was not accompanied by a change in relative cytokine abundance. The lack of correlation between local and systemic bacterial concentration and the inflammatory response in either compartment suggests that although microbes initiate the host response, it is subsequently modulated, at least partially, independent of the inciting infection and continued presence of infection. A more direct way to examine the correlation between bacterial burden and host response was to compare high dose to low dose *P. aeruginosa*. It was reasonable to predict that giving a substantially higher dose of the same bacteria would lead to a more pronounced inflammatory response, at least in the lungs where the infection was initiated. However, 11 cytokines were different in the two groups 6 hours after the onset of pneumonia, with a near even split - 6 higher in the high dose group, 5 higher in the low dose group. This means that it is at least partially incorrect to assume that the greater the bacterial burden, the more severe the inflammatory response, which has clear implications if attempting to modulate the immune response for therapeutic gain. It should be noted, however, that bacterial counts are only a crude measure of the complex relationship that exists between pathogen and host in sepsis and, in fact, bacterial phenotype may not be an invariant trait, but rather one that undergoes dynamic changes.

The survival experiments demonstrated both the promise and limitations of targeting therapy based upon the host response. While targeting TNF- α and knocking out MCP-1 failed to improve survival in any group regardless of cytokine levels, we were able to identify groups that had worse outcomes with these interventions. If a single model of sepsis alone were used in pre-clinical trials (cecal ligation and puncture often is used for this purpose), it is possible that harmful effects of a therapy in certain subgroups would not be identified. Further, our results show that not only do different organisms lead to different host responses, but severity of illness can also have a profound influence on how the host responds to a specific therapy, even if the inciting organism is the same. It has been postulated that one reason why clinical sepsis trials fail is that animal studies tend to use high mortality models while patient studies use a population that is less sick, which would be expected to behave differently (28). The anti-TNF- α experiments in this study demonstrate a marked worsening of survival in both high mortality groups, with minimal effect in the intermediate mortality group. These results correlate well to two prospective randomized trials of anti-TNF- α antibody in patients, which have shown minimal or no benefit in a population with a baseline mortality of approximately 50% (29;30). However, our results raise the concern that treating those infected with either *S. pneumoniae* or *P. aeruginosa* with a high risk of death may actually be harmful. Of note, previous studies have shown that anti-TNF- α antibody improves survival in rats subjected to lower mortality models of *Escherichia coli* or *Staphylococcus aureus* pneumonia, and we view our results as complimentary to these since we did not examine models of *S. pneumoniae* or *P. aeruginosa* with mortalities of under 50% (31).

We do not have a clear explanation for the survival studies in MCP-1^{-/-} mice. These animals have worsened survival following polymicrobial sepsis (32), and the only effect seen in this study was a hastening of death in animals that did not have a significant increase in sepsis-induced MCP-1. It is possible that different levels of this chemoattractant are necessary depending on the infecting organism or disease severity. However, it is difficult to know if the results seen in a knockout animal with lifelong MCP-1 deletion accurately replicate what would happen if the mediator were targeted in an acute setting.

We do not believe these results are inconsistent with genome-wide RNA microarray analyses of either circulating neutrophils or peripheral blood mononuclear cells that demonstrates no difference between those infected with gram positive and gram negative infections (6;⁷). This is because studies performed specifically on neutrophils or peripheral blood mononuclear cells which would not identify changes in other cell types that might be responsible for the marked differences seen in this study. Further, those studies were performed on a transcriptome level, which would not necessarily identify the changes we found on a translational level. Finally, the mortality rate of patients infected with gram positive or gram negative bacteria ranged from 11-37% in those studies, which is significantly lower than the mortality in all groups examined herein.

Our study has several limitations. Antibiotics were not used in this study because they have been demonstrated to alter the host immune response in both *S. pneumonia* and *P. aeruginosa* pneumonia, which would have complicated interpretation of our results (33;³⁴). However, antibiotics are standard of care in the treatment of sepsis, and their absence limits the clinical relevance of our results. Additionally the host response to sepsis is a dynamic process (27;³⁵) and it is possible that critical information was missed by sampling at only three timepoints. While our study used *S. pneumonia* and *P. aeruginosa* pneumonia as prototypical gram positive and gram negative infections respectively due to their prevalence in septic patients (36), there are marked differences in susceptibility to *S. pneumonia* infections based upon different capsular subtypes and nearly 2000 species of *P. aeruginosa* have been isolated from patients, so it is difficult to determine whether our results are generalizable to either these infections or gram positives and negatives in general (37;³⁸). It also does not study the host response in the absence of overt signs of infection, which may be very significant in light of recent work demonstrating that patients with a high burden of *P. aeruginosa* who do not meet clinical criteria for ventilator associated pneumonia have increased mortality compared to patients with a high burden of *P. aeruginosa* who have evidence of pneumonia (39). Also, the anti-TNF survival curves were performed at a different time than the survival curves in figure 1 without concurrent untreated controls. We therefore cannot exclude the possibility that the results in figure 6 are simply due to the fact that survival can vary between models from week to week, independent of the effect of anti-TNF. Finally, the experiments were performed in mice. While the study allowed for examination of the host response without the confounder of genetic variability and allowed for the ability to precisely titrate each variability examined (kinetics of mortality, seven-day mortality, bacterial concentration), how these results translate to humans is unknown.

Despite these limitations, these results demonstrate that individual infections induce unique host responses. The current paradigm of treating septic patients with supportive care clearly improves outcome in individual patients, but the disease still has an unacceptably high mortality. Our results suggest that the inflammatory host response to sepsis is, at a minimum, dependent upon the inciting organism, the kinetics and severity of infection, the concentration of inoculum, and the time the host response is interrogated. Although there is significant complexity to sepsis as a clinical entity, there appear to be well-orchestrated host responses to infection. The meaning of these responses is yet to be determined.

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Figure 1. Mortality and body weights of mice given different pneumonia models

(A) Animals were followed for survival for seven days after intratracheal injection of *S. pneumoniae* (red, n=24), low dose *P. aeruginosa* (blue, n=20), high dose *P. aeruginosa* (yellow, n=25) or 0.9% saline (black, n=5). The kinetics of mortality are very similar in the first 72 hours between *S. pneumoniae* and low dose *P. aeruginosa*. While mice given high dose *P. aeruginosa* have a higher death rate in the first 72 hours than those given *S. pneumoniae*, their eventual seven-day mortality is similar. Arrows indicate timepoints when cytokine samples were taken in subsequent experiments. Of note, samples taken at 6 or 12 hours were drawn at a timepoint before there was any mortality and samples taken at 72 hours were drawn at a timepoint where there is a similar 50% mortality in *S. pneumoniae* and low dose *P. aeruginosa*. (B) Body weights of animals (n=7-20 mice/experimental group, 5 shams) 6, 24, 48, and 72 hours after induction of pneumonia. There were no statistically significant differences in body weights between mice given sham pneumonia (black) and animals given low dose *P. aeruginosa* (blue) or high dose *P. aeruginosa* (yellow). Weights were lower in mice given *S. pneumoniae* (red) 48 and 72 hours after induction of pneumonia. Note, x axis for survival in panel A is 7 days but x axis for body weights in panel B is 3 days since all subsequent experiments were performed using timepoints ranging from 6-72 hours.

Figure 2. Quantitative BAL and blood cultures

(A) Bacterial counts are similar at early timepoints in BAL fluid from animals given *S. pneumoniae* or high dose *P. aeruginosa* while counts are lower following injection of low dose *P. aeruginosa*. Bacterial concentration drops markedly in BAL fluid between 48 and 72 hours, consistent with animals clearing pulmonary infection. Data has been log transformed for presentation to allow graphical representation of 100,000-fold decrease in lung bacterial burden between these timepoints. (B) Animals given *S. pneumoniae* or low dose *P. aeruginosa* have similar low degrees of bacteremia at early timepoints. There is a marked increase in bacteremia in animals given *S. pneumoniae* between 48 and 72 hours without any change in systemic bacterial concentrations in those given low dose *P. aeruginosa*. Note, the differences in scale on y axis between figures a and b, with substantially higher bacterial concentrations in the lungs than the blood at all points measured except in *S. pneumoniae* at 72 hours.

Figure 3. Relative cytokine abundance in BAL and blood

All panels compare cytokine concentrations between two groups of animals (n=6-11/group/ timepoint) given different models of pneumonia at various timepoints. The presence of a colored horizontal bar indicates that there was a statistically higher level of the measured cytokine in animals given *S. pneumoniae* (red), low dose *P. aeruginosa* (blue), or high dose *P. aeruginosa* (yellow) compared to the other group examined. When no colored horizontal bar is present, cytokine abundance was statistically similar between the two groups examined. Data presented represent 13 of 18 cytokines measured. The five mediators not shown in this figure (IL-2, IL-5, IL-12, IL-13, and RANTES) were excluded either because there were no differences between animals with pneumonia and sham animals or because the majority cytokine levels were below the limit of detection. Raw data for all cytokine levels are shown in tables 1 and 2. (A) Despite having similar mortality at all timepoints measured, the abundance of most pro- and anti-inflammatory cytokines in BAL fluid is higher in mice given low dose *P. aeruginosa* than those given *S. pneumoniae* at 6 hours. However, the pattern reverses nearly completely by 72 hours. (B) Similar to BAL, systemic cytokines are generally higher in mice given low dose *P. aeruginosa* at 6 hours. However, despite the relative increase in cytokine abundance in BAL at 72 hours and the increase in bacteremia seen solely in animals given *S. pneumoniae*, no relative increase in systemic cytokines is noted at this timepoint. (C,D) Despite similar seven-day mortality, relative cytokine abundance is generally higher in animals given high dose *P. aeruginosa*. Not a single cytokine measured was significantly higher in either compartment in animals given *S. pneumoniae*. Cytokine patterns are generally similar between BAL and blood; however, differences exist in multiple mediators such as TNF- α , IL-18 and IL-1 β . It should be noted that IFN- γ levels are at the lower limit of detection in animals given either high dose *P. aeruginosa* or *S. pneumoniae*. (E) BAL samples in animals given either high dose or low dose *P. aeruginosa*. Although there is a higher bacterial load in the lungs of those that received a higher dose, in 5 of 11 cytokines levels where a difference was detected between groups, they were more elevated in those that received low dose bacteria. (F) Although the blood from animals that received high dose or low dose *P. aeruginosa* was more homogenous than BAL, IFN- γ concentrations were higher in animals that received a lower inoculum of bacteria.

Figure 4. Hierarchical clustering of cytokine abundance

(A) All mice with complete cytokine data sets were analyzed simultaneously. Each individual mouse is represented as a row across the figure, showing the abundance of each cytokine in both BAL and blood. Each column represents a single cytokine measured either in the plasma (P) or BAL (B). The columns were ordered based on Pearson's correlation, though no significant relationships among the profiles of individual cytokines were apparent. The columns represent the following cytokines in this order: PIL-5; PIL-2; PIL-12; PIL-13; PIFN-g; PMIP-a; PIL-18; PTNF-a; PIL-1b; PIL-10; BTNFSR1; PIL-6; PMIP-2; PIL-1ra; Peotaxin; BTNFSR2; PTNFSR1; BIL-18; BIL-1b; BIL-6; PMCP-1; BIL-5; PTNFSR2; BTNF-a; BMIP-a; BIL-12; BIFN-g; BIL-13; BRANTES; Beotaxin; BMCP-1; PRANTES; BIL-2; BIL-1ra; BIL-10; BMIP-2. Horizontal bars separate five distinct nodes (A-E) that encompass the eight treatment groups. Mice receiving *S. pneumoniae* are denoted in red while mice receive *P. aeruginosa* are denoted by blue (low dose) or yellow (high dose). The time points where plasma and BAL were acquired are encoded in gray scale saturation. The six hour time point is represented by 25% saturation (light gray), the 12 hour time point is indicated by 50% saturation (dark gray) and the 72 hour time point is indicated by black. Cytokine expression ranged from below limit of detection (green) through the mean value for that cytokine (black) to the highest abundance for that cytokine (red) and as a result this visualization tool is only semi-quantitative. The dendrogram on the left indicates the similarity of adjacent samples. This visualization tool demonstrates the intrinsic variability of the host immunologic response to different infections over time and suggests the identity of cytokines that differentiate across groups. (B) Cytokine abundance data that differentiate the two groups in Cluster B. Cytokines were ranked based on p-value from a Student's t-test comparing the two groups identified in Cluster B by hierarchical cluster analysis. Those with p-values less than 0.05 are shown. Average $\pm$ standard error of the mean are shown for mice infected with *P. aeruginosa* at early time points (blue) and *S. pneumoniae* at 72 hours post-infection (red). (C) Cytokine abundance data that differentiate the two groups within Cluster D. Cytokines were ranked based on p-value from a Student's t-test comparing the two groups identified in Cluster D by hierarchical cluster analysis and those with p-values less than 0.05 are shown. Average $\pm$ standard error of the mean are shown for mice infected with samples taken at 6 hours (light gray) and 12 hours post-infection (dark gray).

Figure 5. Principal component analysis of cytokine abundance data

(A) Samples were grouped according to their infection group and time point. X-axis: PC1 (arbitrary units), Y-axis: PC3 (arbitrary units). Points plotted are the mean value for each PC $\pm$ the standard error of the mean and are labeled with the timepoint when samples were collected. Lines connecting the points for the same infection are shown over time to propose a hypothetical trajectory of cytokine expression that occurs during each infection. Inset demonstrates the close proximity of the low-dose *P. aeruginosa* trajectory and the *S. pneumoniae* trajectory. (B) Each cluster (A-E) identified in figure 4 was also plotted along the same axes as were used in Panel A. In this instance the PC between Panel A and Panel B are identical because PC were calculated using the same data. (C) Local MIP2 and systemic TNFsr2 abundance classify individual mice into four distinct groups (discussed in the text), effectively separating mice destined to die (high systemic TNFsr2, blue) or high local MIP2, yellow) from mice that have cleared infection (low local MIP2 and low systemic TNFsr2, pink). The remaining mice (black) cannot be separated into clinically relevant groups using these criteria.

Figure 6. TNF- α and survival

The pro-inflammatory cytokine TNF- α is elevated in (A) BAL of *S. pneumoniae* and high dose *P. aeruginosa* and (B) blood of high dose *P. aeruginosa*. (C) Anti- TNF- α accelerates mortality in animals where pneumonia induces elevated local or systemic levels of the cytokine but has no impact on survival in animals given low dose *P. aeruginosa* (n=8/group).

Figure 7. MCP-1 and survival

(A) MCP-1 levels in BAL of all groups. (B) Blood MCP-1 levels are markedly elevated in both low and high dose *P. aeruginosa* but not in *S. pneumoniae*. (C) Survival is unaffected in MCP-1^{-/-} mice given either low or high dose *P. aeruginosa* but is accelerated in those subjected to *S. pneumoniae* (n=21-22/group).

Figure 8. Systemic leukocyte response to pneumonia

(A) Total white blood cell counts (n=6-9) are decreased following infection at 12 and 24 hours and increase to basal values 72 hours after low dose *P. aeruginosa*. (B) Absolute lymphocyte counts have a similar trend to total white blood cell counts, with decreases in all groups at all timepoints except 72 hours after low dose *P. aeruginosa*. (C) Absolute neutrophil counts are increased following *S. pneumoniae*, are not significantly changed with low dose *P. aeruginosa* and are markedly decreased after high dose *P. aeruginosa*.

Figure 9. Pulmonary MPO by immunohistochemistry and quantitative assay
Representative histology (n=5-6) in animals given (A) *S. pneumoniae*, (B) low dose *P. aeruginosa* or (C) high dose *P. aeruginosa* shows comparative increasing staining for MPO activity in the three models respectively 12 hours following onset of pneumonia which is confirmed by quantitative assay (D). Micrographs were taken at 200X.

Table 1

BAL

Cytokine	Time (hours)	Sham (pg/ml)	Sp (pg/ml)	Pa-L (pg/ml)	Pa-H (pg/ml)
IL-1β	6	15.1 $\pm$ 2.3 (n = 4) *p = 0.02 vs. Sp **p = 0.006 vs. Pa-L ***p = 0.004 vs. Pa-H	12.1 $\pm$ 0.1 (n = 9) ***p = 0.0002 vs. Pa-L ***p < 0.0001 vs. Pa-H	1809.9 $\pm$ 392.2 (n = 7) ***p = 0.0003 vs. Pa-H	24524.2 $\pm$ 2862.5 (n = 8)
	12	n/a	575.5 $\pm$ 227.8 (n = 11) p = 0.77 vs. Pa-L ***p = 0.0003 vs. Pa-H	24.9 $\pm$ 0.9 (n = 8) ***p = 0.0002 vs. Pa-H	52405.3 $\pm$ 9531.6 (n = 8)
	72	116.1 $\pm$ 101.7 (n = 4) **p = 0.006 vs. Sp p = 0.29 vs. Pa-L	1807.0 $\pm$ 551.9 (n = 7) **p = 0.002 vs. Pa-L	28.4 $\pm$ 4.4 (n = 5)	n/a
MIP-2	6	145.5 $\pm$ 55.0 (n = 4) **p = 0.002 vs. Sp **p = 0.006 vs. Pa-L **p = 0.004 vs. Pa-H	5092.6 $\pm$ 610.1 (n = 10) ***p = 0.0002 vs. Pa-L ***p < 0.0001 vs. Pa-H	38807.0 $\pm$ 9162.6 (n = 7) p = 0.15 vs. Pa-H	18988.1 $\pm$ 2301.2 (n = 8)
	12	n/a	3120.1 $\pm$ 1024.4 (n = 11) p = 0.88 vs. Pa-L ***p = 0.0003 vs. Pa-H	5573.8 $\pm$ 2806.5 (n = 9) **p = 0.002 vs. Pa-H	28003.7 $\pm$ 4115.1 (n = 8)
	72	127.0 $\pm$ 29.4 (n = 4) **p = 0.006 vs. Sp p = 0.11 vs. Pa-L	304097.6 $\pm$ 35058.0 (n = 7) **p = 0.002 vs. Pa-L	24.2 $\pm$ 0.2 (n = 5)	n/a
MCP-1	6	7.4 $\pm$ 7.4 (n = 4) p = 0.05 vs. Sp **p = 0.006 vs. Pa-L **p = 0.004 vs. Pa-H	54.4 $\pm$ 25.4 (n = 10) ***p = 0.0002 vs. Pa-L ***p < 0.0001 vs. Pa-H	322.1 $\pm$ 26.4 (n = 7) ***p = 0.0003 vs. Pa-H	1524.6 $\pm$ 277.6 (n = 8)
	12	n/a	218.1 $\pm$ 64.8 (n = 11) p = 0.49 vs. Pa-L ***p = 0.0003 vs. Pa-H	223.1 $\pm$ 27.8 (n = 9) ***p = 0.0003 vs. Pa-H	1448.1 $\pm$ 131.2 (n = 8)
	72	0.0 $\pm$ 0.0 (n = 4) p = n/a vs. Sp p = n/a vs. Pa-L	1479.6 $\pm$ 470.6 (n = 6) **p = 0.009 vs. Pa-L	264.3 $\pm$ 51.1 (n = 6)	n/a
Eotaxin	6	n/a	12.1 $\pm$ 0.1 (n = 9) ***p = 0.0002 vs. Pa-L	624.8 $\pm$ 124.1 (n = 7) ***p = 0.0003 vs. Pa-H	11.9 $\pm$ 0.1 (n = 8)

Cytokine	Time (hours)	Sham (pg/ml)	Sp (pg/ml)	Pa-L (pg/ml)	Pa-H (pg/ml)
	12	n/a	p = 0.48 vs. Pa-H 11.9 ± 0.1 (n = 11) ***p = 0.0002 vs. Pa-L ***p = 0.0003 vs. Pa-H	1781.3 ± 289.6 (n = 9) *p = 0.02 vs. Pa-H	915.6 ± 296.7 (n = 8)
	72	n/a	394.6 ± 81.7 (n = 7) p = 0.07 vs. Pa-L	113.5 ± 57.3 (n = 6)	n/a
	6	n/a	11.9 ± 0.1 (n = 10) ***p = 0.0001 vs. Pa-L ***p < 0.0001 vs. Pa-H	3483.7 ± 110.2 (n = 7) ***p = 0.0003 vs. Pa-H	1715.0 ± 226.0 (n = 8)
	12	n/a	12.1 ± 0.1 (n = 11) ***p < 0.0001 vs. Pa-L ***p < 0.0001 vs. Pa-H	3180.1 ± 145.3 (n = 9) *p = 0.04 vs. Pa-H	5674.5 ± 1029.0 (n = 8)
	72	n/a	729.2 ± 340.6 (n = 7) p = 0.23 vs. Pa-L	876.5 ± 539.2 (n = 6)	n/a
	6	7.6 ± 4.4 (n = 4) p = 1.00 vs. Sp p = 0.06 vs. Pa-L p = 1.00 vs. Pa-H	11.9 ± 0.1 (n = 10) ***p = 0.0001 vs. Pa-L p = 0.89 vs. Pa-H	874.2 ± 158.7 (n = 7) ***p = 0.0006 vs. Pa-H	11.9 ± 0.1 (n = 7)
	12	n/a	11.9 ± 0.1 (n = 11) ***p = 0.0002 vs. Pa-L p = 0.93 vs. Pa-H	548.4 ± 93.28 (n = 9) ***p < 0.0001 vs. Pa-H	11.9 ± 0.1 (n = 8)
	72	15.6 ± 8.5 (n = 4) **p = 0.006 vs. Sp **p = 0.01 vs. Pa-L	703.6 ± 115.5 (n = 7) p = 0.53 vs. Pa-L	642. ± 108.0 (n = 6)	n/a
MIP-1α	6	426.3 ± 15.3 (n = 4) *p = 0.04 vs. Sp p = 0.06 vs. Pa-L *p = 0.04 vs. Pa-H	38314.5 ± 4160.6 (n = 9) ***p = 0.0002 vs. Pa-L p = 0.11 vs. Pa-H	11007.7 ± 1428.5 (n = 7) ***p = 0.0003 vs. Pa-H	48162.1 ± 1247.1 (n = 8)
	12	n/a	27057.2 ± 5863.7 (n = 11) *p = 0.048 vs. Pa-L p = 0.08 vs. Pa-H	6071.8 ± 1435.3 (n = 9) ***p < 0.0001 vs. Pa-H	42358.0 ± 3306.3 (n = 8)
	72	889.2 ± 235.2 (n = 4) p = 0.06 vs. Sp p = 0.07 vs. Pa-L	4069.7 ± 628.4 (n = 7) **p = 0.001 vs. Pa-L	39.8 ± 15.8 (n = 6)	n/a

Cytokine	Time (hours)	Sham (pg/ml)	Sp (pg/ml)	Pa-L (pg/ml)	Pa-H (pg/ml)
TNF- α	6	42.5 $\pm$ 3.6 (n = 4) **p = 0.003 vs. Sp **p = 0.006 vs. Pa-L **p = 0.004 vs. Pa-H	17225.9 $\pm$ 4665.5 (n = 9) *p = 0.01 vs. Pa-L p = 0.89 vs. Pa-H	4519.0 $\pm$ 1365.1 (n = 7) **p = 0.001 vs. Pa-H	13156.4 $\pm$ 1549.7 (n = 8)
	12	n/a	4792.2 $\pm$ 785.0 (n = 11) p = 0.32 vs. Pa-L p = 0.06 vs. Pa-H	3617.3 $\pm$ 740.0 (n = 9) *p = 0.01 vs. Pa-H	7986.4 $\pm$ 1374.7 (n = 8)
	72	62.2 $\pm$ 9.8 (n = 4) **p = 0.006 vs. Sp p = 0.07 vs. Pa-L	1790.1 $\pm$ 114.6 (n = 7) **p = 0.001 vs. Pa-L	36.2 $\pm$ 7.2 (n = 6)	n/a
IL-6	6	290.8 $\pm$ 182.1 (n = 4) **p = 0.002 vs. Sp **p = 0.006 vs. Pa-L **p = 0.004 vs. Pa-H	2562.7 $\pm$ 475.2 (n = 10) ***p = 0.0004 vs. Pa-L ***p < 0.0001 vs. Pa-H	7486.8 $\pm$ 914.2 (n = 7) **p = 0.002 vs. Pa-H	12694.1 $\pm$ 909.8 (n = 8)
	12	n/a	3763.8 $\pm$ 852.2 (n = 11) *p = 0.03 vs. Pa-L ***p = 0.0003 vs. Pa-H	8129.9 $\pm$ 1282.1 (n = 9) ***p < 0.0001 vs. Pa-H	43991.7 $\pm$ 4996.4 (n = 8)
	72	29.5 $\pm$ 21.6 (n = 4) **p = 0.006 vs. Sp p = 0.29 vs. Pa-L	2965.5 $\pm$ 558.8 (n = 7) **p = 0.002 vs. Pa-L	24.2 $\pm$ 0.2 (n = 5)	n/a
IL-1ra	6	n/a	5212.0 $\pm$ 1015.8 (n = 9) **p = 0.005 vs. Pa-L p = 0.14 vs. Pa-H	12244.7 $\pm$ 2092.0 (n = 7) *p = 0.04 vs. Pa-H	7340.5 $\pm$ 1037.7 (n = 8)
	12	n/a	33621.2 $\pm$ 5382.4 (n = 11) p = 0.45 vs. Pa-L p = 0.39 vs. Pa-H	39829.1 $\pm$ 5655.4 (n = 9) p = 0.07 vs. Pa-H	25691.0 $\pm$ 4642.4 (n = 8)
	72	n/a	44033.0 $\pm$ 9500.9 (n = 7) **p = 0.008 vs. Pa-L	13157.5 $\pm$ 3331.5 (n = 6)	n/a
IL-10	6	1.8 $\pm$ 0.0 (n = 4) p = n/a vs. Sp p = n/a vs. Pa-L p = n/a vs. Pa-H	11.9 $\pm$ 0.1 (n = 10) ***p = 0.0001 vs. Pa-L p = 0.96 vs. Pa-H	23.9 $\pm$ 0.1 (n = 7) ***p = 0.0003 vs. Pa-H	11.9 $\pm$ 0.1 (n = 8)
	12	n/a	11.9 $\pm$ 0.1 (n = 11) ***p = 0.0002 vs. Pa-L	23.9 $\pm$ 0.1 (n = 9) ***p < 0.0001 vs. Pa-H	988.2 $\pm$ 343.0 (n = 8)

Cytokine	Time (hours)	Sham (pg/ml)	Sp (pg/ml)	Pa-L (pg/ml)	Pa-H (pg/ml)
TNFsrI			***p = 0.0003 vs. Pa-H		
	72	40.0 ± 35.4 (n = 4) **p = 0.006 vs. Sp p = 0.26 vs. Pa-L	11311.5 ± 3182.5 (n = 7) **p = 0.001 vs. Pa-L	23.8 ± 0.2 (n = 6)	n/a
	6	335.9 ± 60.9 (n = 4) **p = 0.003 vs. Sp p = 0.65 vs. Pa-L p = 0.68 vs. Pa-H	123.7 ± 15.4 (n = 9) **p = 0.001 vs. Pa-L ***p < 0.0001 vs. Pa-H	281.3 ± 30.1 (n = 7) p = 0.28 vs. Pa-H	337.6 ± 26.9 (n = 8)
	12	n/a	172.8 ± 21.5 (n = 11) *p = 0.01 vs. Pa-L ***p = 0.0003 vs. Pa-H	344.6 ± 54.5 (n = 9) ***p = 0.0006 vs. Pa-H	994.4 ± 241.6 (n = 8)
	72	279.8 ± 57.3 (n = 4) *p = 0.01 vs. Sp p = 0.48 vs. Pa-L	470.5 ± 19.2 (n = 7) **p = 0.001 vs. Pa-L	200.8 ± 23.3 (n = 6)	n/a
TNFsrII	6	1930.8 ± 300.5 (n = 4) *p = 0.01 vs. Sp **p = 0.006 vs. Pa-L p = 1.00 vs. Pa-H	1184.8 ± 183.8 (n = 10) p = 0.23 vs. Pa-L **p = 0.003 vs. Pa-H	879.8 ± 12.7 (n = 7) ***p = 0.0003 vs. Pa-H	1735.7 ± 66.5 (n = 8)
	12	n/a	1762.8 ± 182.8 (n = 11) **p = 0.004 vs. Pa-L **p = 0.006 vs. Pa-H	1001.6 ± 30.4 (n = 8) ***p < 0.0001 vs. Pa-H	2449.6 ± 142.4 (n = 8)
	72	2228.5 ± 564.1 (n = 4) **p = 0.006 vs. Sp p = 0.11 vs. Pa-L	463.8 ± 11.5 (n = 7) **p = 0.001 vs. Pa-L	1229.9 ± 166.8 (n = 6)	n/a
IL-2	6	13.8 ± 0.0 (n = 4) p = n/a vs. Sp p = n/a vs. Pa-L p = n/a vs. Pa-H	12.0 ± 0.0 (n = 10) p = n/a vs. Pa-L p = n/a vs. Pa-H	24.0 ± 0.0 (n = 7) p = n/a vs. Pa-H	12.0 ± 0.0 (n = 8)
	12	n/a	12.0 ± 0.0 (n = 11) p = n/a vs. Pa-L p = vs. Pa-H	24.0 ± 0.0 (n = 9) p = n/a vs. Pa-H	12.0 ± 0.0 (n = 8)
	72	13.8 ± 0.0 (n = 4) p = n/a vs. Sp p = n/a vs. Pa-L	227.8 ± 139.3 (n = 7) p = n/a vs. Pa-L	24.0 ± 0.0 (n = 6) p = n/a vs. Pa-H	n/a
IL-5	6	14.4 ± 4.1	33.0 ± 15.8	12.0 ± 0.0	99.8 ± 15.5

Cytokine	Time (hours)	Sham (pg/ml)	Sp (pg/ml)	Pa-L (pg/ml)	Pa-H (pg/ml)
		(n = 4) p = 0.64 vs. Sp p = n/a vs. Pa-L *p = 0.02 vs. Pa-H	(n = 10) p = n/a vs. Pa-L *p = 0.02 vs. Pa-H	(n = 7) p = n/a vs. Pa-H	(n = 8)
	12	n/a	35.8 ± 11.6 (n = 11) p = n/a vs. Pa-L p = 0.39 vs. Pa-H	12.0 ± 0.0 (n = 9) p = n/a vs. Pa-H	18.8 ± 6.8 (n = 8)
	72	23.1 ± 16.2 (n = 4) p = n/a vs. Sp p = n/a vs. Pa-L	12.0 ± 0.0 (n = 7) p = n/a vs. Pa-L	12.0 ± 0.0 (n = 6)	n/a
	6	4.0 ± 0.0 (n = 4) p = n/a vs. Sp p = n/a vs. Pa-L p = n/a vs. Pa-H	12.0 ± 0.0 (n = 10) p = n/a vs. Pa-L p = n/a vs. Pa-H	219.4 ± 126.7 (n = 7) p = n/a vs. Pa-H	12.0 ± 0.0 (n = 8)
IL-12	12	n/a	16.5 ± 4.5 (n = 11) p = n/a vs. Pa-L p = n/a vs. Pa-H	24.0 ± 0.0 (n = 9) p = n/a vs. Pa-H	12.0 ± 0.0 (n = 8)
	72	32.5 ± 16.6 (n = 4) p = 1.00 vs. Sp p = 0.76 vs. Pa-L	12.4 ± 0.4 (n = 7) **p = 0.001 vs. Pa-L	54.4 ± 30.4 (n = 6)	n/a
	6	9.1 ± 0.0 (n = 4) p = n/a vs. Sp p = n/a vs. Pa-L p = n/a vs. Pa-H	461.3 ± 176.4 (n = 10) p = 1.00 vs. Pa-L p = 0.83 vs. Pa-H	6200.3 ± 5336.3 (n = 7) p = 0.46 vs. Pa-H	401.0 ± 97.1 (n = 8)
	12	n/a	393.3 ± 164.4 (n = 11) p = 0.76 vs. Pa-L p = 0.15 vs. Pa-H	24.1 ± 0.1 (n = 9) **p = 0.008 vs. Pa-H	98.2 ± 86.2 (n = 8)
RANTES	72	107.8 ± 24.7 (n = 4) p = 0.22 vs. Sp p = 0.29 vs. Pa-L	1605.3 ± 502.2 (n = 7) p = 0.07 vs. Pa-L	133.8 ± 109.8 (n = 6)	n/a
	6	427.9 ± 0.0 (n = 4) p = n/a vs. Sp p = n/a vs. Pa-L p = n/a vs. Pa-H	35.0 ± 21.7 (n = 9) ***p = 0.0007 vs. Pa-L **p = 0.004 vs. Pa-H	503.3 ± 206.4 (n = 7) **p = 0.004 vs. Pa-H	119.4 ± 26.2 (n = 8)
	12	n/a	20.4 ± 5.0 (n = 11) ***p = 0.0002 vs. Pa-L	581.1 ± 178.4 (n = 9) p = 0.09 vs. Pa-H	277.8 ± 69.3 (n = 8)

Cytokine	Time (hours)	Sham (pg/ml)	Sp (pg/ml)	Pa-L (pg/ml)	Pa-H (pg/ml)
	72	470.9 ± 119.3 (n = 4) p = 0.23 vs. Sp **p = 0.01 vs. Pa-L	***p = 0.0003 vs. Pa-H 669.2 ± 118.2 (n = 7) **p = 0.001 vs. Pa-L	27.4 ± 3.4 (n = 6)	n/a

Table 2

Blood

Cytokine	Time (hours)	Sham (pg/ml)	Sp (pg/ml)	Pa-L (pg/ml)	Pa-H (pg/ml)
IL-1 β	6	106.2 $\pm$ 25.1 (n = 4) p = 0.57 vs. Sp **p = 0.004 vs. Pa-L p = 0.48 vs. Pa-H	438.5 $\pm$ 266.2 (n = 8) p = 0.80 vs. Pa-L p = 0.34 vs. Pa-H	21.00 $\pm$ 3.000 (n = 8) p = 0.23 vs. Pa-H	1108.8 $\pm$ 422.1 (n = 6)
	12	n/a	890.2 $\pm$ 424.2 (n = 11) p = 0.90 vs. Pa-L p = 0.64 vs. Pa-H	21.00 $\pm$ 3.000 (n = 8) p = 0.72 vs. Pa-H	4408.7 $\pm$ 2636.0 (n = 8)
	72	115.5 $\pm$ 19.3 (n = 4) **p = 0.006 vs. Sp p = n/a vs. Pa-L	10.3 $\pm$ 1.7 (n = 7) p = n/a vs. Pa-L	24.0 $\pm$ 0.0 (n = 6)	n/a
MIP-2	6	183.1 $\pm$ 49.0 (n = 4) p = 0.21 vs. Sp p = 1.00 vs. Pa-L **p = 0.01 vs. Pa-H	542.3 $\pm$ 134.2 (n = 8) p = 0.72 vs. Pa-L ***p = 0.0007 vs. Pa-H	599.9 $\pm$ 306.6 (n = 8) ***p = 0.0007 vs. Pa-H	4785 $\pm$ 962.5 (n = 6)
	12	n/a	800.8 $\pm$ 179.7 (n = 11) *p = 0.04 vs. Pa-L ***p = 0.0005 vs. Pa-H	1897 $\pm$ 435.9 (n = 8) *p = 0.01 vs. Pa-H	13469 $\pm$ 5852 (n = 8)
	72	194.1 $\pm$ 44.4 (n = 4) p = 0.65 vs. Sp **p = 0.01 vs. Pa-L	2931 $\pm$ 1840 (n = 7) p = 0.23 vs. Pa-L	24.17 $\pm$ 0.17 (n = 6)	n/a
MCP-1	6	38.9 $\pm$ 10.6 (n = 4) p = 0.21 vs. Sp **p = 0.004 vs. Pa-L *p = 0.02 vs. Pa-H	104.2 $\pm$ 23.7 (n = 8) **p = 0.003 vs. Pa-L **p = 0.002 vs. Pa-H	237.9 $\pm$ 23.1 (n = 8) **p = 0.002 vs. Pa-H	50000.0 $\pm$ 0.0 (n = 5)
	12	n/a	58.1 $\pm$ 13.4 (n = 11) p = 0.20 vs. Pa-L ***p = 0.0003 vs. Pa-H	87.2 $\pm$ 9.4 (n = 8) **p = 0.002 vs. Pa-H	50000.0 $\pm$ 0.0 (n = 8)
	72	0.0 $\pm$ 0.0 (n = 4) p = n/a vs. Sp p = n/a vs. Pa-L	78.3 $\pm$ 26.3 (n = 7) p = 0.94 vs. Pa-L	70.9 $\pm$ 13.0 (n = 6)	n/a
Eotaxin	6	7856.2 $\pm$ 3276.7 (n = 4) **p = 0.004 vs. Sp	525.6 $\pm$ 284.7 (n = 8) **p = 0.003 vs. Pa-L	2218.9 $\pm$ 348.6 (n = 8) p = 0.85 vs. Pa-H	2013.8 $\pm$ 184.5 (n = 6)

Cytokine	Time (hours)	Sham (pg/ml)	Sp (pg/ml)	Pa-L (pg/ml)	Pa-H (pg/ml)
IL-18		**p = 0.008 vs. Pa-L **p = 0.01 vs. Pa-H			
	12	n/a	292.6 ± 138.3 (n = 11) p = 0.05 vs. Pa-L ***p = 0.0005 vs. Pa-H	1111.5 ± 486.1 (n = 8) **p = 0.007 vs. Pa-H	3915.1 ± 873.6 (n = 8)
	72	3751.8 ± 1058.6 (n = 4) *p = 0.01 vs. Sp p = 0.07 vs. Pa-L	805.8 ± 249.5 (n = 7) p = 0.84 vs. Pa-L	1262.7 ± 579.5 (n = 6)	n/a
	6	n/a	11.9 ± 0.1 (n = 8) ***p = 0.0002 vs. Pa-L p = 0.41 vs. Pa-H	23.9 ± 0.1 (n = 8) *p = 0.04 vs. Pa-H	72.9 ± 60.9 (n = 6)
IFN- γ	12	n/a	420.9 ± 276.4 (n = 11) **p = 0.006 vs. Pa-L p = 0.06 vs. Pa-H	1090.6 ± 521.1 (n = 8) p = 0.72 vs. Pa-H	1820.3 ± 773.5 (n = 8)
	72	n/a	12.1 ± 0.1 (n = 7) **p = 0.001 vs. Pa-L	23.8 ± 0.2 (n = 6)	n/a
	6	108.8 ± 22.9 (n = 4) p = 0.57 vs. Sp *p = 0.048 vs. Pa-L p = 0.76 vs. Pa-H	161.0 ± 80.4 (n = 8) **p = 0.002 vs. Pa-L p = 0.34 vs. Pa-H	838.2 ± 142.8 (n = 8) *p = 0.02 vs. Pa-H	349.2 ± 147.5 (n = 6)
	12	n/a	146.1 ± 63.4 (n = 10) ***p < 0.0001 vs. Pa-L p = 0.96 vs. Pa-H	948.3 ± 137.6 (n = 8) *p = 0.01 vs. Pa-H	246.9 ± 134.2 (n = 8)
MIP-1 α	72	77.5 ± 13.6 (n = 4) p = 0.16 vs. Sp **p = 0.01 vs. Pa-L	214.5 ± 64.5 (n = 7) *p = 0.04 vs. Pa-L	508.0 ± 84.2 (n = 6)	n/a
	6	1274.4 ± 361.1 (n = 4) **p = 0.004 vs. Sp **p = 0.004 vs. Pa-L p = 0.76 vs. Pa-H	12.1 ± 0.1 (n = 8) ***p = 0.0002 vs. Pa-L p = 0.06 vs. Pa-H	6409.7 ± 1104.9 (n = 8) **p = 0.005 vs. Pa-H	1659.4 ± 579.1 (n = 6)
	12	n/a	930.3 ± 501.9 (n = 11) p = 0.05 vs. Pa-L p = 0.21 vs. Pa-H	1561.9 ± 427.4 (n = 8) p = 1.00 vs. Pa-H	2704.5 ± 1124.2 (n = 8)
	72	1136.2 ± 195.6 (n = 4)	841.8 ± 335.4 (n = 7)	340.3 ± 202.9 (n = 6)	n/a

Cytokine	Time (hours)	Sham (pg/ml)	Sp (pg/ml)	Pa-L (pg/ml)	Pa-H (pg/ml)
TNF-α		p = 0.41 vs. Sp p = 0.11 vs. Pa-L	p = 0.53 vs. Pa-L		
	6	40.4 $\pm$ 8.3 (n = 4) p = 0.28 vs. Sp p = 0.46 vs. Pa-L **p = 0.01 vs. Pa-H	24.2 $\pm$ 5.6 (n = 8) *p = 0.01 vs. Pa-L ***p = 0.0007 vs. Pa-H	65.2 $\pm$ 11.4 (n = 8) ***p = 0.0007 vs. Pa-H	203.9 $\pm$ 35.4 (n = 6)
	12	n/a	126.5 $\pm$ 67.6 (n = 11) p = 0.39 vs. Pa-L *p = 0.02 vs. Pa-H	42.2 $\pm$ 6.8 (n = 8) **p = 0.007 vs. Pa-H	278.7 $\pm$ 130.8 (n = 8)
IL-6	72	32.1 $\pm$ 0.0 (n = 4) p = n/a vs. Sp p = n/a vs. Pa-L	61.3 $\pm$ 26.6 (n = 7) p = 0.73 vs. Pa-L	26.9 $\pm$ 2.9 (n = 6)	n/a
	6	231.3 $\pm$ 16.1 (n = 4) **p = 0.004 vs. Sp **p = 0.004 vs. Pa-L **p = 0.01 vs. Pa-H	916.1 $\pm$ 204.6 (n = 8) **p = 0.007 vs. Pa-L **p = 0.001 vs. Pa-H	2570.6 $\pm$ 568.1 (n = 8) p = 0.28 vs. Pa-H	2771.8 $\pm$ 318.5 (n = 6)
	12	n/a	319.0 $\pm$ 95.4 (n = 11) p = 0.39 vs. Pa-L ***p = 0.0003 vs. Pa-H	487.9 $\pm$ 173.2 (n = 8) ***p = 0.0002 vs. Pa-H	14851.3 $\pm$ 5323.2 (n = 8)
IL-1ra	72	36.1 $\pm$ 3.1 (n = 4) p = 0.79 vs. Sp **p = 0.01 vs. Pa-L	109.1 $\pm$ 58.3 (n = 7) p = 0.73 vs. Pa-L	24.2 $\pm$ 0.2 (n = 6)	n/a
	6	n/a	361.4 $\pm$ 110.4 (n = 7) ***p = 0.0003 vs. Pa-L p = 0.14 vs. Pa-H	13519.0 $\pm$ 4691.1 (n = 8) **p = 0.003 vs. Pa-H	1061.5 $\pm$ 359.6 (n = 6)
	12	n/a	851.5 $\pm$ 465.1 (n = 11) *p = 0.01 vs. Pa-L ***p = 0.0006 vs. Pa-H	4460.5 $\pm$ 1394.9 (n = 8) p = 0.08 vs. Pa-H	20624.3 $\pm$ 9818.5 (n = 8)
IL-10	72	n/a	12423.0 $\pm$ 5756.0 (n = 7) p = 0.07 vs. Pa-L	672.8 $\pm$ 349.2 (n = 6)	n/a
	6	309.5 $\pm$ 80.8 (n = 4) **p = 0.006 vs. Sp **p = 0.004 vs. Pa-L p = 0.76 vs. Pa-H	19.2 $\pm$ 7.2 (n = 8) *p = 0.02 vs. Pa-L *p = 0.01 vs. Pa-H	23.9 $\pm$ 0.1 (n = 8) *p = 0.04 vs. Pa-H	403.7 $\pm$ 150.7 (n = 6)
	12	n/a	162.3 $\pm$ 57.98	23.9 $\pm$ 0.1	3859.0 $\pm$ 1366.6

Cytokine	Time (hours)	Sham (pg/ml)	Sp (pg/ml)	Pa-L (pg/ml)	Pa-H (pg/ml)
TNFsrI			(n = 11) p = 0.77 vs. Pa-L ***p = 0.005 vs. Pa-H	(n = 8) *p = 0.01 vs. Pa-H	(n = 8)
	72	247.5 ± 38.9 (n = 4) p = 0.32 vs. Sp **p = 0.01 vs. Pa-L	845.0 ± 537.7 (n = 7) p = 0.23 vs. Pa-L	23.8 ± 0.2 (n = 6)	n/a
	6	352.7 ± 38.6 (n = 4) *p = 0.02 vs. Sp **p = 0.004 vs. Pa-L **p = 0.01 vs. Pa-H	984.2 ± 150.5 (n = 8) p = 0.88 vs. Pa-L p = 0.28 vs. Pa-H	991.6 ± 59.8 (n = 8) p = 0.14 vs. Pa-H	1417 ± 262.9 (n = 6)
	12	n/a	464.1 ± 98.2 (n = 11) p = 0.17 vs. Pa-L ***p = 0.0008 vs. Pa-H	654.8 ± 133.9 (n = 8) **p = 0.002 vs. Pa-H	1788 ± 307.6 (n = 8)
TNFsrII	72	336.2 ± 48.2 (n = 4) p = 0.11 vs. Sp p = 0.61 vs. Pa-L	146.2 ± 63.5 (n = 7) *p = 0.01 vs. Pa-L	449.1 ± 73.4 (n = 6)	n/a
	6	2009.8 ± 190.6 (n = 4) p = 1.00 vs. Sp **p = 0.004 vs. Pa-L *p = 0.02 vs. Pa-H	2034.3 ± 250.6 (n = 7) ***p = 0.0003 vs. Pa-L **p = 0.002 vs. Pa-H	992.6 ± 19.7 (n = 8) **p = 0.002 vs. Pa-H	100000.0 ± 0.0 (n = 5)
	12	n/a	917.8 ± 122.6 (n = 11) p = 0.23 vs. Pa-L ***p = 0.0003 vs. Pa-H	996.3 ± 47.5 (n = 8) ***p = 0.0002 vs. Pa-H	51162.8 ± 18458.9 (n = 8)
	72	1738.2 ± 272.3 (n = 4) **p = 0.006 vs. Sp *p = 0.02 vs. Pa-L	452.4 ± 22.0 (n = 7) **p = 0.001 vs. Pa-L	936.7 ± 50.0 (n = 6)	n/a
IL-2	6	13.8 ± 0.0 (n = 4) p = n/a vs. Sp p = n/a vs. Pa-L p = n/a vs. Pa-H	12.0 ± 0.0 (n = 8) p = n/a vs. Pa-L p = n/a vs. Pa-H	399.2 ± 245.6 (n = 8) p = n/a vs. Pa-H	12.0 ± 0.0 (n = 6)
	12	n/a	110.0 ± 98.0 (n = 11) p = n/a vs. Pa-L p = n/a vs. Pa-H	24.0 ± 0.0 (n = 8) p = n/a vs. Pa-H	12.0 ± 0.0 (n = 8)
	72	13.8 ± 0.0 (n = 4) p = n/a vs. Sp p = n/a vs. Pa-L	12.00 ± 0.0 (n = 7) p = n/a vs. Pa-L p = n/a vs. Pa-H	24.0 ± 0.0 (n = 6) p = n/a vs. Pa-H	n/a

Cytokine	Time (hours)	Sham (pg/ml)	Sp (pg/ml)	Pa-L (pg/ml)	Pa-H (pg/ml)
IL-5		p = n/a vs. Pa-H			
	6	80.8 ± 13.9 (n = 4) *p = 0.048 vs. Sp p = n/a vs. Pa-L p = 0.17 vs. Pa-H	29.7 ± 11.6 (n = 8) p = n/a vs. Pa-L p = 0.85 vs. Pa-H	12.0 ± 0.0 (n = 8) p = n/a vs. Pa-H	37.8 ± 18.1 (n = 6)
	12	n/a	23.5 ± 11.19 (n = 11) p = n/a vs. Pa-L p = 0.83 vs. Pa-H	12.0 ± 0.0 (n = 8) p = n/a vs. Pa-H	19.5 ± 5.2 (n = 8)
IL-12	72	61.5 ± 5.5 (n = 4) p = n/a vs. Sp p = n/a vs. Pa-L	12.0 ± 0.0 (n = 7) p = n/a vs. Pa-L	12.0 ± 0.0 (n = 6)	n/a
	6	132.5 ± 34.3 (n = 4) p = n/a vs. Sp *p = 0.02 vs. Pa-L p = n/a vs. Pa-H	12.0 ± 0.0 (n = 8) p = n/a vs. Pa-L p = n/a vs. Pa-H	36.4 ± 12.4 (n = 8) p = n/a vs. Pa-H	12.0 ± 0.0 (n = 6)
	12	n/a	112.4 ± 100.4 (n = 11) p = n/a vs. Pa-L p = n/a vs. Pa-H	24.0 ± 0.0 (n = 8) p = n/a vs. Pa-H	12.0 ± 0.0 (n = 8)
IL-13	72	111.7 ± 15.8 (n = 4) p = n/a vs. Sp p = n/a vs. Pa-L	12.0 ± 0.0 (n = 7) p = n/a vs. Pa-L	24.0 ± 0.0 (n = 6)	n/a
	6	620.0 ± 126.4 (n = 4) **p = 0.008 vs. Sp *p = 0.03 vs. Pa-L p = 0.11 vs. Pa-H	69.4 ± 48.4 (n = 8) *p = 0.0499 vs. Pa-L p = 0.66 vs. Pa-H	146.6 ± 84.1 (n = 8) p = 0.23 vs. Pa-H	179.9 ± 108.3 (n = 6)
	12	n/a	107.6 ± 79.9 (n = 11) *p = 0.03 vs. Pa-L p = 0.90 vs. Pa-H	24.1 ± 0.1 (n = 8) *p = 0.01 vs. Pa-H	222.8 ± 210.8 (n = 8)
RANTES	72	567.0 ± 64.6 (n = 4) p = 0.32 vs. Sp **p = 0.01 vs. Pa-L	359.6 ± 217.5 (n = 7) p = 0.73 vs. Pa-L	24.2 ± 0.2 (n = 6)	n/a
	6	8999.8 ± 1951.6 (n = 4) **p = 0.004 vs. Sp ***p = 0.004 vs. Pa-L ***p = 0.01 vs. Pa-H	41.7 ± 25.1 (n = 7) *p = 0.03 vs. Pa-L p = 0.73 vs. Pa-H	137.3 ± 69.8 (n = 8) *p = 0.02 vs. Pa-H	31.5 ± 19.5 (n = 6)

Cytokine	Time (hours)	Sham (pg/ml)	Sp (pg/ml)	Pa-L (pg/ml)	Pa-H (pg/ml)
	12	n/a	13.0 ± 1.0 (n = 11) ***p = 0.0003 vs. Pa-L p = 0.90 vs. Pa-H	164.4 ± 71.4 (n = 8) **p = 0.001 vs. Pa-H	16.9 ± 4.9 (n = 8)
	72	7612.5 ± 490.7 (n = 4) p = 0.07 vs. Sp **p = 0.01 vs. Pa-L	66.0 ± 49.8 (n = 6) p = 0.18 vs. Pa-L	52.0 ± 21.5 (n = 6)	n/a