

Strain Differences in Alveolar Neutrophil Infiltration and Macrophage Phenotypes in an Acute Lung Inflammation Model

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Pulmonary infection is a major cause of mortality and morbidity, and the magnitude of the lung inflammatory response correlates with patient survival. Previously, we have shown that neutrophil migration into joints is regulated by arthritis severity quantitative trait loci (QTLs). However, it is unclear whether these QTLs contribute to the regulation of lung inflammation in pneumonias. Therefore, to more clearly define the factors regulating acute inflammatory responses in the lung, we examined two inbred rat strains, DA and F344, that differ in these QTLs and their susceptibility to joint inflammation. Staphylococcal cell wall components lipoteichoic acid (LTA) and peptidoglycan (PGN), administered intratracheally, significantly increased the numbers of neutrophils retrieved in the bronchoalveolar lavage fluid (BALF). F344 had approximately 10-fold more neutrophils in the BALF compared with DA ($P < 0.001$) and higher BALF concentrations of total protein, tumor necrosis factor- α and macrophage inflammatory protein 2. LTA/PGN administration in DA \times F344 congenic strains (*Cia3d*, *Cia4*, *Cia5a*, and *Cia6*) resulted in inflammation similar to that in DA, demonstrating that the genes responsible for the differences in pulmonary inflammation are not contained within the chromosomal intervals carried by these congenic strains. Alveolar macrophages (AMs) isolated from naive F344 stimulated *in vitro* with LTA/PGN produced significantly higher levels of keratinocyte-derived chemokine and macrophage inflammatory protein 2 than alveolar macrophages from DA rats. The differences were related to differential mitogen-activated protein kinase phosphorylation. We conclude that the factors contributing to inflammation can be site and challenge dependent. A better understanding of site-specific inflammation may lead to more effective treatment of acute lung inflammation and injury.

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INTRODUCTION

Pulmonary infection is a major cause of mortality and morbidity (1), particularly in patients who are critically ill (2,3). The innate immune response plays a critical role in host defense. This concerted host reaction to bacteria is influenced by the complex interplay between genetics, epigenetics and the environment and can be organ specific (4–7). *Staphylococcus aureus* is a leading cause of hospital-acquired and community-

associated pneumonia and is typically associated with severe disease (8–10). Although many studies have led to a better understanding of the epidemiology, resistance mechanisms and potential virulence, there is still a paucity of knowledge about the mechanisms involved in the *in vivo* host immune response to *S. aureus* infections (11).

Lipoteichoic acid (LTA) and peptidoglycan (PGN) are components of the cell wall of Gram-positive bacteria, such as

S. aureus, and can stimulate the generation of proinflammatory cytokines and activate leukocytes *in vitro* and *in vivo* (12–16). LTA and PGN can also induce lung inflammation in mice (16) and F344 rats (17). F344 rats have previously been shown to be arthritis resistant, whereas DA rats are susceptible to this inflammatory disease (18). Results of previous studies have demonstrated that neutrophil and macrophage migration into an inflammatory site, such as the synovial-like air pouch injected with carrageenan, is genetically regulated (19). Specifically, arthritis-susceptible DA rats have greater neutrophil and macrophage migration into the synovial inflammatory site than arthritis-resistant F344 rats (19). The difference in neutrophil migration in that

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model is regulated by the arthritis-severity quantitative trait loci (QTLs) *Cia4* and *Cia6* (19), located on rat chromosomes 7 and 8, respectively. Moreover, other arthritis QTLs, such as *Cia3d* and *Cia5a*, have also been shown to regulate joint inflammatory responses (18,20).

Neutrophils and macrophages are critical to the pathogenesis of acute lung injury (21,22), rheumatoid arthritis (23,24) and other inflammatory diseases. Cytokines, produced by neutrophils and macrophages such as tumor necrosis factor- α (TNF- α), interleukin-8 (IL-8), macrophage migration inhibitory factor (MIF) and high mobility group box 1 (HMGB1) mediate arthritis damage and acute lung injury (25–30). These data suggest that arthritis and acute lung injury share some inflammatory mechanisms. However, it remains unclear whether the genetic differences between DA and F344 rats, in particular in arthritis QTLs, are associated with differences in the regulation of lung inflammation.

In the present study we used a pneumonia model in which lung inflammation was induced by Gram-positive bacterial components. We examined the differences in pulmonary inflammation between DA and F344 rats and investigated whether these differences are regulated by arthritis QTLs and what other factors may influence differences in lung inflammation.

MATERIALS AND METHODS

Reagents

LTA and PGN from *S. aureus* (Sigma-Aldrich, St Louis, MO, USA) were dissolved in 0.9% saline to the target concentration, stored at -20°C , and used within 60 d.

Animals

All experiments involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Feinstein Institute for Medical Research.

Arthritis-resistant F344 and arthritis-susceptible DA rats were purchased from

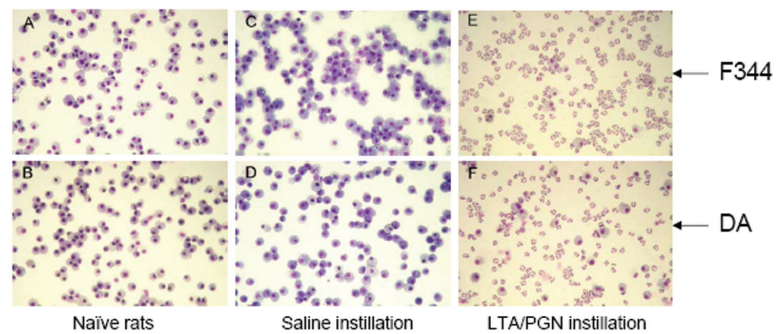


Figure 1. The BALF cell populations. (A) and (B) show cells directly lavaged from naïve F344 and DA rats. These macrophages were used in the *in vitro* studies. (C) and (D) were lavaged from F344 and DA rats receiving saline instillation, and show a predominance of macrophages. (E) and (F) show cells lavaged from lungs F344 and DA, respectively, 18 h after instillation of LTA/PGN, and show the influx of neutrophils.

Harlan Sprague Dawley (Indianapolis, IN, USA). DA.F344(*Cia3d*), DA.F344(*Cia4*), DA.F344(*Cia5a*) and DA.F344(*Cia6*) congenics were generated as previously described (18,20). Briefly, arthritis QTLs were introgressed from F344 into DA background rats through 8 to 10 genotype-guided backcrosses followed by at least 5 intercrosses. All the animals were housed in a pathogen-free environment, under standard light and dark cycles, with free access to food and water.

In Vivo Experimental Protocol

Rats were males 8- to 14-wks old and were F344 (n = 7), DA (n = 7), or the congenic strains DA.F344(*Cia3d*), DA.F344(*Cia4*), DA.F344(*Cia5a*) and DA.F344(*Cia6*) (n = 5/congenic strain) with body weights of 201–305 g. Rats were anesthetized with isoflurane. The tracheas were surgically exposed and LTA 10 $\mu\text{g}/\text{kg}$ and PGN 33 $\mu\text{g}/\text{kg}$ (a dose that we have shown previously to induce lung inflammation [31]) were instilled intratracheally. The animals were allowed to recover from anesthesia and have access to food and water *ad libitum*. After 18 h, the rats were euthanized and the lungs were lavaged twice with saline (0.9% w/v, 35 mL/kg/lavage). The bronchoalveolar lavage fluid (BALF) from each lavage was collected into separate tubes. The tubes were centrifuged and the supernatant stored at -80°C . Only the

supernatant from the first tube was used for measuring chemokines, cytokines and protein content, whereas BALF from both tubes was used for cell counting. After lavage, lung tissue was removed and immediately frozen in liquid nitrogen for myeloperoxidase (MPO), keratinocyte-derived chemokine (KC) and macrophage inflammatory protein 2 (MIP-2) quantification. Control F344 rats and DA rats (n = 5/group) were instilled with the same volume of 0.9% saline and then treated as described above.

In Vitro Alveolar Macrophage Chemokine Response to LTA and PGN

For the *in vitro* studies, F344 and DA rats (n = 5/group) were euthanized without prior instillation. The lungs were lavaged 5 \times with saline (0.9% w/v, 35 mL/kg/lavage). The BALF was centrifuged, and cells were mixed with 0.5 mL 0.2% saline for 10 s to lyse any erythrocytes. The cells were then resuspended in 10 mL Hanks' Balanced Salt Solution (HBSS) and recentrifuged, and the macrophage pellet was collected. The resulting macrophages were 99% pure, as determined by HEMA 3 Stain Set stain (Fisher Scientific, Kalamazoo, MI, USA) (Figure 1A, B), and were 98% viable, based on trypan blue exclusion. The cells were resuspended at 2.5×10^5 cells/mL in RPMI 1640 with 1% fetal bovine serum and 1% penicillin/streptomycin.

Macrophages from each animal were distributed to three different wells at 1 mL/well in 24-well low-attachment cell culture plates (Corning Costar, Cambridge, MA, USA) and cultured in medium, as described above, to which LTA 0.5 $\mu\text{g}/\text{mL}$ + PGN 1.65 $\mu\text{g}/\text{mL}$, LTA 1.0 $\mu\text{g}/\text{mL}$ + PGN 3.3 $\mu\text{g}/\text{mL}$ or an equal volume of 0.9% saline (control) was added, for 6 h at 37°C in 5% CO_2 . The cell culture medium was then centrifuged and the supernatant stored at -80°C for the chemokine assay.

In Vitro Alveolar Macrophage Mitogen-Activated Protein Kinase Activation Study

To explore the differences in mitogen-activated protein kinase (MAPK) activation between these two strains, alveolar macrophages (AMs) lavaged from F344 and DA strains were stimulated by LTA and PGN *in vitro* (LTA 1.0 $\mu\text{g}/\text{mL}$ + PGN 3.3 $\mu\text{g}/\text{mL}$). The whole cell lysates were collected at different time points (0, 1 and 6 h). The MAPK pathway markers (phosphorylated extracellular signal-regulated kinases 1 and 2 [ERK1/2] and P38) were determined in the cell lysates by Western blot. In addition, total ERK1/2 and total P38 protein and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control were determined by stripping the same membrane after the phosphorylated forms had been assessed. Toll-like receptor 2 (TLR2) levels in AMs from F344 and DA were also determined by Western blot. Antibodies used for Western blots (phosphor-P38, total-P38, phosphor-ERK1/2 and GAPDH) were obtained from Cell Signaling Technology (Danvers, MA, USA). Total ERK1/2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). TLR2 antibody was purchased from Abcam (Cambridge, MA, USA).

In Vitro MAPK Inhibition Study

To further detect the role of MAPK ERK1/2 and P38 pathways in the regulation of chemokine production, the effects of MAPK inhibition on KC and

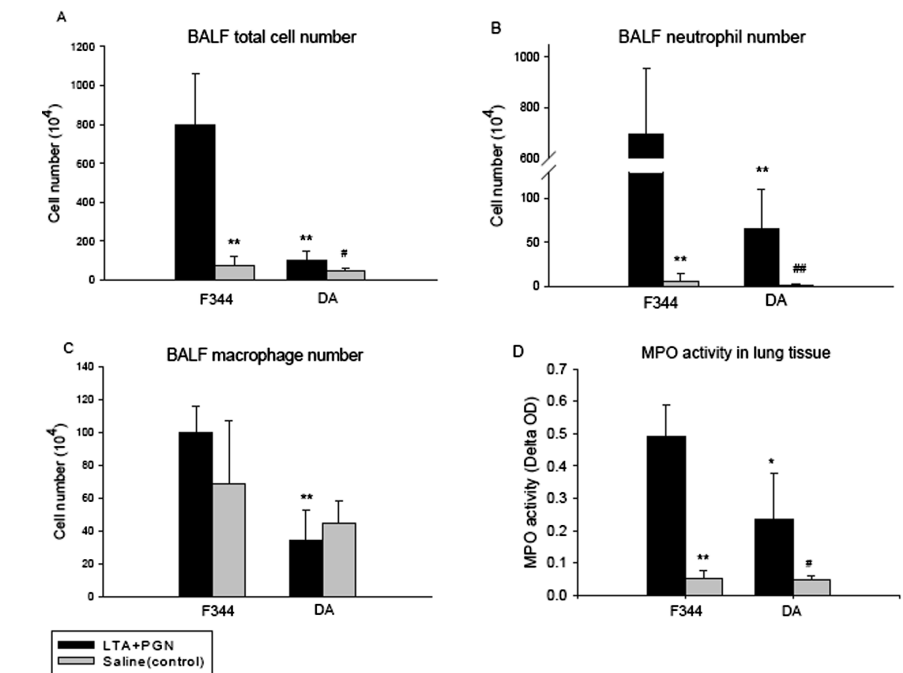


Figure 2. Differential cellular inflammatory response in the lung between F344 and DA rats. Following intratracheal instillation of LTA/PGN, both strains (saline control: $n = 5/\text{strain}$; LTA/PGN: $n = 7/\text{strain}$) show increased total numbers of cells in the BALF (A). This increased cell number is predominately due to a neutrophil influx to the lung (B). Although F344 rats had more macrophages in the BALF than DA rats following either saline or LTA/PGN instillation, there was no significant change in the number of macrophages in either strain owing to LTA/PGN (C). MPO was used as an indicator of accumulation of neutrophils within the lung tissue. F344 rats had higher MPO activity in lung tissue than DA rats, following LTA/PGN instillation (D). Bars indicate mean \pm SD. ** $P < 0.01$ versus F344 treated by LTA/PGN; * $P < 0.05$ versus F344 treated with LTA/PGN; ## $P < 0.01$ versus DA treated with LTA/PGN; # $P < 0.05$ versus DA treated with LTA/PGN.

MIP-2 production were examined. AMs were prepared by using the method described above. The specific inhibitors for p38 (SB-203580; EMD Chemicals, Gibbstown, NJ, USA) and ERK1/2 (PD-98059; Calbiochem, San Diego, CA, USA) were added to the AMs of F344 and DA rats 30 min prior to LTA/PGN stimulation (LTA 1.0 $\mu\text{g}/\text{mL}$ + PGN 3.3 $\mu\text{g}/\text{mL}$). After 6 h, cell culture medium was collected. KC and MIP-2 in the medium were measured by using an ELISA method as described below. To exclude the possibility that the inhibitors may have caused cell damage, a cytotoxicity study was performed at the same inhibitor doses and contact time, and the culture medium was assessed for lactate dehydrogenase (LDH) activity (Takara Bio, Shiga, Japan).

BALF Total Cell and Differential Cell Counts

BALF cells were mixed with 0.5 mL of 0.2% saline for 10 s to lyse any residual erythrocytes, and the cells were then resuspended in 10 mL HBSS. The total cell number retrieved in the BALF was determined with a hemocytometer. Slides were prepared using a Shandon cytocentrifuge (Shandon Scientific, London, UK), and cells were stained with an HEMA 3 Stain Set (Fisher Scientific) for differential cell counting. Counts were made on at least 200 cells per slide by an observer blinded to the grouping.

Cytokine and Chemokine Assays

TNF- α , KC and MIP-2 were measured by enzyme-linked immunosorbent assay (ELISA) using commercial assay kits

(TNF- α : R&D Systems, Minneapolis, MN, USA; KC and MIP-2: Antigenix America, Huntington Station, NY, USA) according to manufacturers' instructions.

MPO Activity Measurement

MPO was measured by using a commercially available assay kit (CytoStore, Calgary, Alberta, Canada) according to the manufacturer's instructions. Briefly, lung tissues were weighed and sample lysis buffer was added at 12 mL per gram of tissue, followed by tissue homogenization. Supernatant from each sample homogenate was collected. Tissue sample supernatant (20 μ L) was combined with 200 μ L of the mixture of chromogen and H₂O₂. Absorbance at 450 nm was measured immediately after addition of chromogen, and again after a 60-sec interval. The absorbance changes during the 60-sec interval were used as a measure of the MPO activity in the sample.

Protein Content Assay

BALF protein concentration was assayed by using the Coomassie (Bradford) Protein Assay Kit (Pierce Company, Rockford, IL, USA). The total protein content was calculated as the product of concentration and BALF volume.

Statistics

Data are presented as mean \pm SD. Student *t* test or one-way ANOVA was used to evaluate the statistical significance of the results. Differences with a *P* value <0.05 were considered significant.

RESULTS

F344 Rats Have Higher BALF and Lung Tissue Neutrophil Infiltration following LTA and PGN Instillation Than DA Rats

Both F344 and DA rats instilled with LTA and PGN showed significant increases in total BALF cell number compared with controls instilled with saline (Figure 2A). However, the cell recruitment into the alveolar space was eight-fold greater in F344 rats than in DA rats

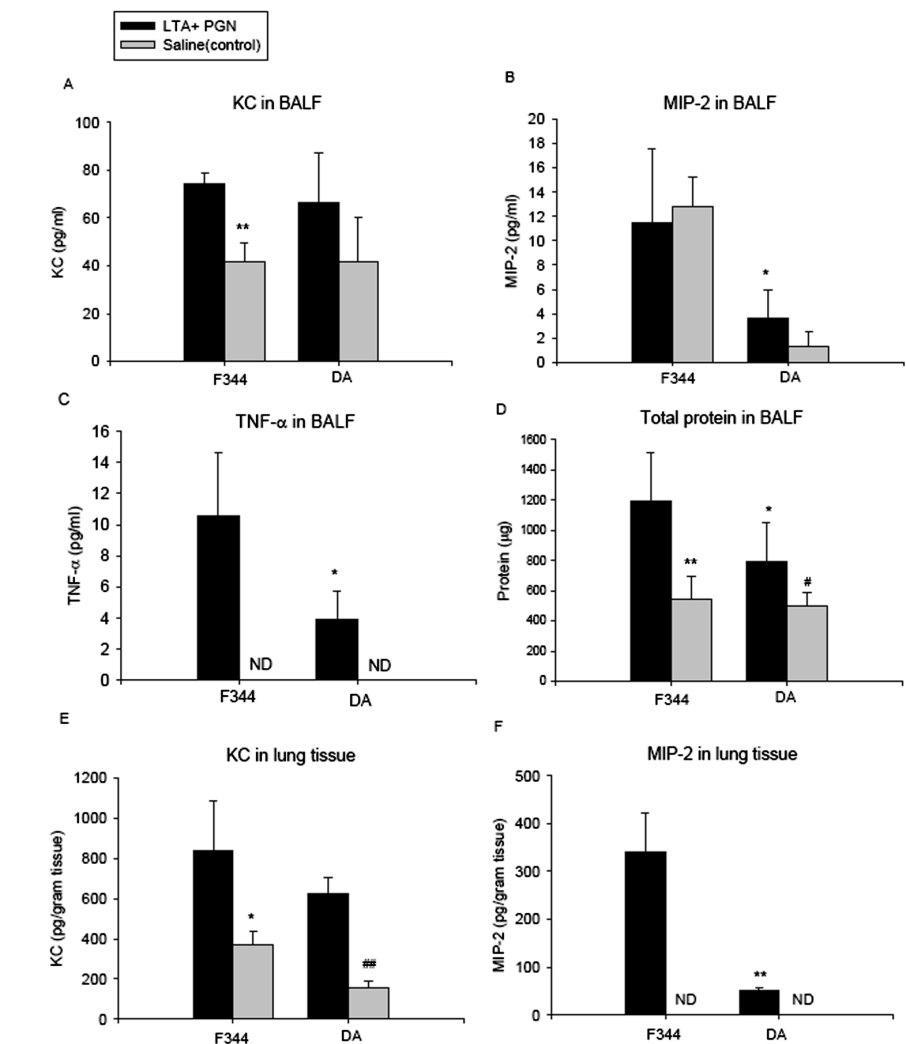


Figure 3. Lung responses to LTA/PGN instillation. F344 rats showed greater inflammatory responses and lung injury to LTA/PGN than DA rats. Following LTA/PGN instillation, there was increased accumulation of cytokines in lung alveolar (A, B, C) and tissue (E, F) samples from both strains. Accumulation of MIP2 (B), TNF- α (C) and protein (D) in the BALF, and MIP-2 in lung tissue (F) were significantly higher in F344 rats, indicating a more robust response and increased damage. Bars indicate mean \pm SD. ***P* < 0.01 versus F344 treated with LTA/PGN; **P* < 0.05 versus F344 treated with LTA/PGN; ##*P* < 0.01 versus DA treated with LTA/PGN; #*P* < 0.05 versus DA treated with LTA/PGN; ND, not detectable.

(mean \pm SD: F344 $7.9 \pm 2.6 \times 10^6$; DA $1.0 \pm 0.4 \times 10^6$; *P* < 0.001) (Figure 2A). The increase was predominantly due to neutrophil influx (BALF neutrophil counts: F344 $7.0 \pm 2.6 \times 10^6$ versus DA $6.6 \pm 4.5 \times 10^5$; *P* < 0.001) (Figure 2B and Figure 1E, F). Nevertheless, macrophage numbers in the BALF did not change significantly following LTA/PGN instillation in either strain. To determine

whether DA neutrophils had been recruited to the lung, but had not crossed into the alveolar space, lung tissue was assessed for neutrophil content based on postlavage MPO assay. However, the lung tissue MPO activity was significantly higher in F344 rats compared with DA rats (*P* < 0.05) (Figure 2D). This demonstrates that the overall recruitment of neutrophils to the lung following

LTA/PGN instillation was significantly different in these two strains.

Inflammatory Mediators in BALF Higher in F344 than DA Rats Following LTA/PGN Stimulation

Concentrations of TNF- α in BALF and MIP-2 in both BALF and lung tissue following LTA/PGN instillation were significantly higher in samples from F344 rats than in the equivalent samples from DA rats under the same conditions (Figure 3), suggesting increased inflammatory responses in F344 compared with DA. In addition, the total protein in the BALF from F344 rats was higher than from DA rats following challenge, indicating increased tissue damage.

Higher Baseline Numbers of AMs in BALF, and Enhanced *In Vitro* AM Response in F344 Compared with DA

We next examined the number of AMs in naïve (untreated) rats. The baseline number of AMs in the BALF was significantly higher in F344 than DA (mean \pm SD cell counts: $15.4 \pm 5.2 \times 10^5$ versus $8.2 \pm 2.3 \times 10^5$; $P = 0.022$, Figure 4C).

AMs from each strain were adjusted to the same concentration (2.5×10^5 cells/mL) and stimulated with LTA and PGN *in vitro*. After 6 h, F344 AMs produced significantly higher concentrations of KC and MIP-2 than DA AMs (Figure 4A, B). These results demonstrate that F344 not only has increased baseline numbers of AMs, but also its AMs produce increased amounts of neutrophil chemoattractants.

The DA.F344(QTL) Congenic Strains Have Similar Inflammatory Response to LTA and PGN as Their Background DA Strain

In previous studies we have shown that the specific arthritis QTLs *Cia4* and *Cia6*, located on rat chromosomes 7 and 8, respectively, mediate the difference between DA and F344 in neutrophil migration into a synovial-like cavity in response to carrageenan (19). Therefore, we assessed whether either of these QTLs, as well as two additional arthritis

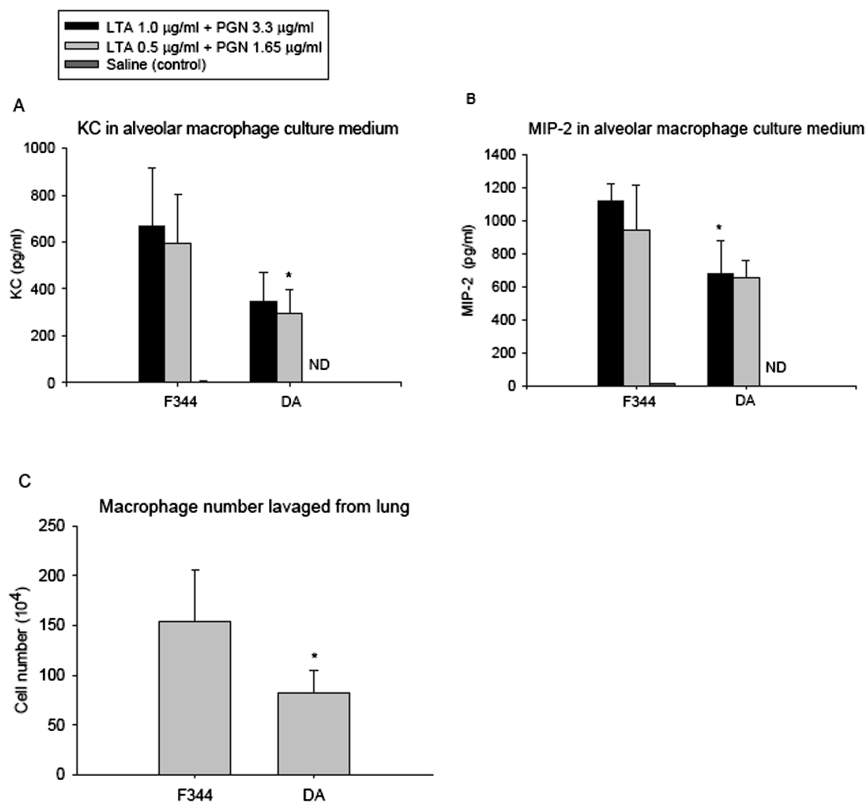


Figure 4. LTA/PGN stimulated accumulation of chemokines in culture medium of isolated primary macrophages. In the absence of LTA/PGN, KC and MIP-2 accumulation was very low or nondetectable. LTA/PGN induced a dramatic accumulation of KC and MIP-2 in AM culture medium. AMs isolated from F344 rats accumulated significantly more of the neutrophil chemokines KC and MIP-2 per cell than macrophages isolated from DA rats (A, B). Furthermore, the number of resident AMs retrieved from naïve F344 rats was significantly greater than that from naïve DA rats (C). Data represent individual studies performed on macrophages isolated from five rats from each strain. Bars indicate mean \pm SD. * $P < 0.05$ versus F344 corresponding group. ND, nondetectable.

QTLs, *Cia3d* and *Cia5a*, might account for a genetically regulated difference in lung inflammation induced with LTA and PGN. Following instillation of LTA/PGN, all four congenic strains (DA.F344[*Cia3d*], DA.F344[*Cia4*], DA.F344[*Cia5a*] and DA.F344[*Cia6*]) developed lung inflammatory responses similar to their DA background, including BALF total cell number, BALF neutrophil counts, BALF macrophage counts, BALF protein content and lung tissue MPO activity (Figure 5). These results suggest that these arthritis and inflammation QTLs do not explain the differences between lung inflammation detected in F344 and DA rats.

Differential MAPK Pathway Responses to TLR2 Stimulation in AMs from F344 and DA Strains

To further explore the mechanism responsible for the strain differences in KC and MIP-2 release by AMs after LTA + PGN stimulation, we focused on the TLR2-MAPK pathway, which is considered to be a major pathway in the regulation of chemokine production during TLR2 stimulation (32,33).

There were similar levels of TLR2 in the AMs from these two strains ($P = 0.69$). Furthermore, there were no significant differences between the strains, in baseline total ERK1/2 ($P = 0.30$) and total p38 ($P = 0.36$). However, the activated forms,

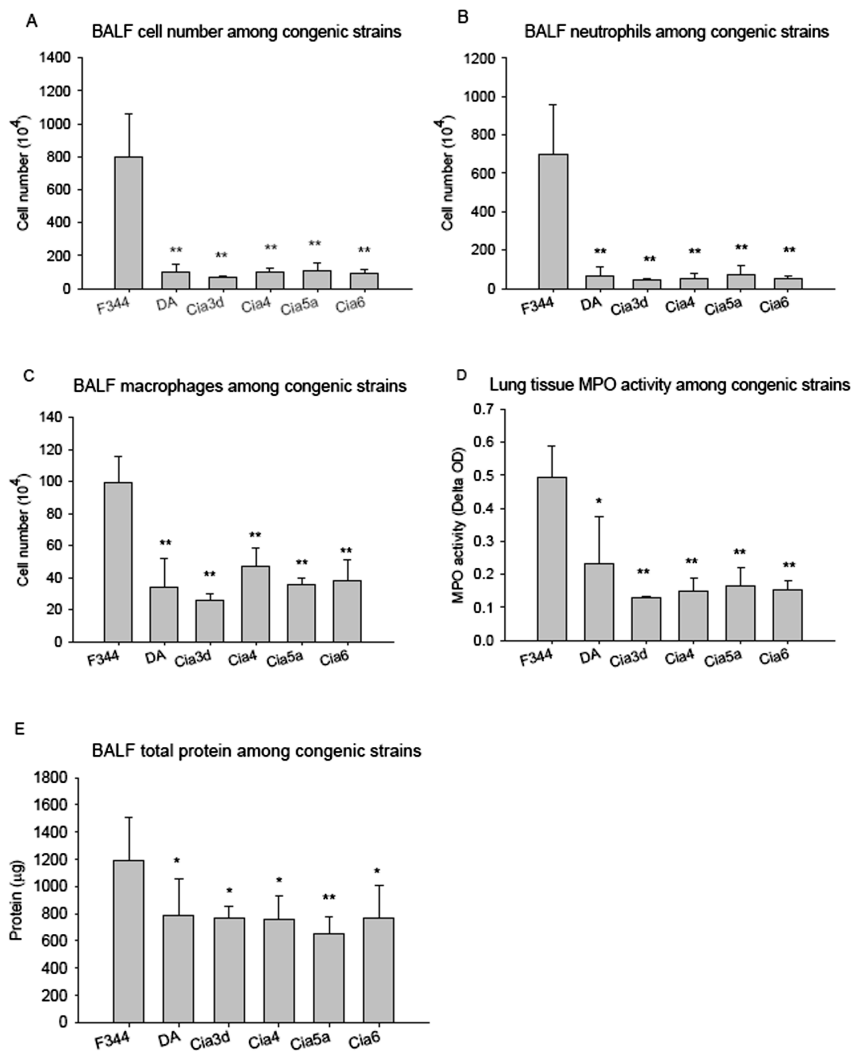


Figure 5. Differential LTA/PGN-induced lung inflammation between F344, DA, and their congenic strains. Following LTA/PGN challenge, the total number of cells (A), number of neutrophils (B), number of macrophages (C) and total protein (E) in the BALF, and MPO activity in the lung tissue (D) were all significantly higher in samples from F344 than DA rats. There was no statistical difference between any of the four congenic strains and their background DA strain ($n = 5$ /congenic strain). Bars indicate mean \pm SD. ** $P < 0.01$ versus F344; * $P < 0.05$ versus F344.

that is, the phosphorylated p38 and phosphorylated ERK1/2 levels were significantly higher in the AMs from F344 rats, after LTA + PGN stimulation (Figure 6). Similar results were achieved from three independent repeats. For F344 versus DA at 1-h and 6-h time points, the p-/t-p38 ratios were 1.47 ± 0.20 -fold and 1.83 ± 0.52 -fold higher ($P = 0.015$ and 0.049 , respectively) and the p-/t-ERK1/2 ratios were 3.09 ± 1.24 -fold and 2.59 ± 0.33 -fold higher ($P = 0.044$ and 0.001). These results

indicate that different activation (phosphorylation) of the MAPK pathway, but not the total TLR2 expression or total MAPK member expression, is associated with the different chemokine production between these two strains in this AM *in vitro* LTA + PGN stimulation model.

Effects of MAPK Inhibition on AM Chemokine Production

To further examine the role of MAPK pathway in the regulation of chemokine

production, effects of MAPK inhibition on KC and MIP-2 production were assessed. In our pilot study, both p38 inhibitor SB-203580 and ERK1/2 inhibitor PD-98059 showed a dose-dependent inhibition of LTA/PGN stimulated KC and MIP-2 production. The minimum doses that can produce maximum inhibitory effects (SB-203580 $20 \mu\text{mol/L}$ and PD-98059 $50 \mu\text{mol/L}$) were then chosen and used for subsequent experiments. Results showed that both SB-203580 and PD-98059 decreased LTA/PGN-induced KC and MIP-2 production (Figure 7A, B). In addition, if the two inhibitors were used in combination, this led to further inhibition of KC and MIP-2 production, compared with the LTA/PGN-stimulated group (Figure 7A, B). The cytotoxicity study showed that both the single and combined administrations did not increase cytotoxicity (LDH release) compared with the control group (Figure 7C).

DISCUSSION

Lung inflammation and pneumonias are major and common causes of morbidity and mortality, particularly in cases associated with *S. aureus* (10). Neutrophils have a central role in acute lung inflammation (34,35), but the local factors regulating neutrophil influx into the lung remain incompletely understood. In this study, we used a model of acute lung inflammation induced by LTA and PGN, components of the Gram-positive bacteria cell membrane that activate macrophages via TLR2 (16,36,37), to explore the factors regulating neutrophil influx into the lung. We demonstrated that the autoimmunity- and arthritis-susceptible DA rats and the resistant F344 rats developed acute pulmonary inflammation induced by LTA and PGN, as indicated by increased numbers of neutrophils and high levels of inflammatory mediators in the BALF. Unexpectedly, F344 rats had significantly higher numbers of BALF neutrophils and macrophages, and their macrophages produced increased levels of neutrophil chemotactic factors such

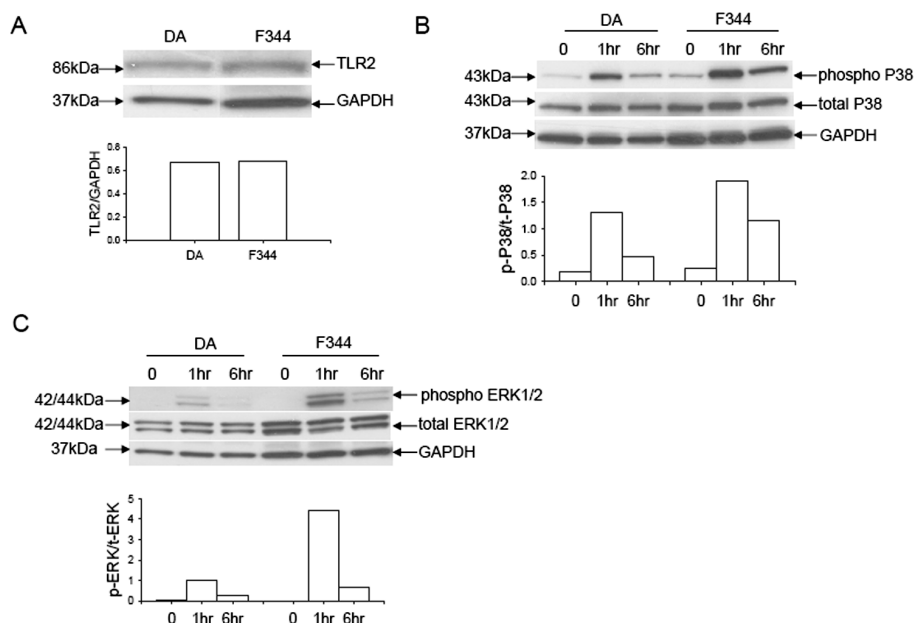


Figure 6. Differential MAPK pathway response to TLR2 stimulation in AMs from F344 and DA strains. AMs from F344 and DA rats were treated with LTA and PGN (LTA 1.0 $\mu\text{g}/\text{mL}$ + PGN 3.3 $\mu\text{g}/\text{mL}$) *in vitro*. Cell lysates were obtained at different time points. Basal level of TLR2 (A), and total P38 and ERK1/2 expressions (B, C) were similar in the two strains. However, the phosphorylation of both P38 and ERK1/2 were higher in the F344 strain (B, C). Similar results were achieved from three independent repeats. The p-P38/t-P38 ratios in F344 rats were 1.47 ± 0.20 - and 1.83 ± 0.52 -fold higher than DA rats after 1 and 6 h of stimulation, with $P = 0.015$ and $P = 0.049$, respectively. Similarly, the p-/t-ERK1/2 ratios in F344 are 3.09 ± 1.24 and 2.59 ± 0.33 fold higher than DA after 1 and 6 h of stimulation, $P = 0.044$ and 0.001 .

as KC and MIP-2. These observations differ from those previously described in studies of acute inflammation in the synovial-like air pouch using carrageenan challenge (19,20), raising the possibility of tissue-specific regulation of inflammatory responses, with different cell types (for example, AMs versus synovial macrophages and synovial fibroblasts) implicated in the tissue responses to injury. Specifically, these two studies used different stimuli. LTA/PGN stimulate TLR2 to induce the inflammatory responses. Our results indicate that LTA/PGN stimulate the TLR2-MAPK-chemokine pathway leading to increased chemokine production and finally increased neutrophil infiltration. However, carrageenan is a high molecular weight sulfated polygalactan, stimulating inflammatory responses through different pathways than LTA/PGN. It has been reported that car-

rageenan induces IL-8 through a distinct Bcl10 pathway(38). Reactive oxygen species (39), lipoxygenase (40,41), and TLR4 (42) may also be involved in carrageenan-induced inflammation. In addition, these two studies explored inflammation at the different tissue sites. The lung has its own specific inflammatory cell population distinct from the joints. In the current study we found that lung residential cells, AMs, are particularly associated with the different inflammation in these two strains. It has been reported that macrophages from different sites have different inflammatory responses (43). We believe that the combination of different inflammatory stimuli activating different cell-signaling pathways at different stimulatory sites may account for the strain differences observed between this study and previous synovial-like air pouch studies (19,20).

Our observations demonstrate that neutrophil migration into the lung, and AM activation in response to bacterial products, are different between these two strains. In an attempt to determine the location of the trait-regulatory genes, we tested four congenic strains generated between DA and F344 (DA.F344[QTL] congenics) and known to regulate arthritis (*Cia3*, *Cia4*, *Cia5a* and *Cia6*) and acute inflammatory responses in the synovial-like air pouch model injected with carrageenan (*Cia4* and *Cia6*) (19). However, all four congenics had BALF findings consistent with their background genome (DA) and excluding a contribution by the introgressed F344 intervals, which suggests that the differences in lung inflammation were not due to genes contained within these chromosomal intervals carried by these congenic strains.

Our previous studies with F344 and DA rats demonstrated that the two strains have similar peripheral blood neutrophil numbers and similar neutrophil chemotaxis in response to chemoattractants (19). These findings suggest that neutrophil responses themselves do not cause the differences in the BALF cell numbers. To explore the reason why more neutrophils migrate to lung in F344 rats, we focused on differences within AMs. The AM is a resident lung-specific cell population and has been reported to play a key role in acute lung injury caused by sepsis, hemorrhage, mechanical ventilation and aspiration (44–48). AMs are one of the first cell types to encounter lung pathogens, or in this study, the instilled bacterial products LTA and PGN. Our results demonstrate that there are differences in both the number of cells present, and in the quality of the AM response between the DA and F344 strains. BALF from F344 rats contained approximately 50% more AMs than from the DA strain. In addition to the higher numbers of AMs in the F344 strain, these cells also showed higher KC and MIP-2 production per cell following LTA and PGN

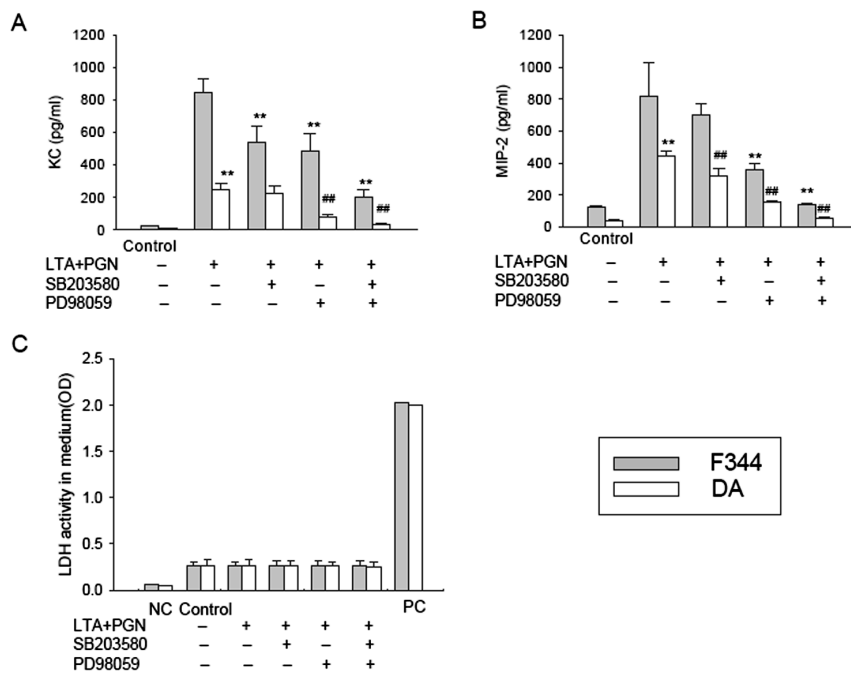


Figure 7. P38 and ERK1/2 pathway inhibitors inhibit LTA/PGN-induced KC and MIP-2 production. AMs were lavaged from naïve F344 or DA rats and cultured *in vitro*. Prior to the LTA/PGN stimulation, the ERK1/2 inhibitor P38 inhibitor SB203580 (20 $\mu\text{mol/L}$), PD98059 (50 $\mu\text{mol/L}$), or both of them were added and incubated with AMs for 30 min. Then LTA/PGN stimulation (LTA 1.0 $\mu\text{g/mL}$ + PGN 3.3 $\mu\text{g/mL}$) was added. KC and MIP-2 production in culture medium were measured after 6 h of LTA/PGN stimulation. Blockade of both ERK1/2 and P38 phosphorylation blocked the majority of KC and MIP-2 production (A, B). The reduction in chemokine accumulation was not due to cytotoxicity as assessed by LDH accumulation in the culture medium (C). NC, negative control (culture medium only); PC, positive control (cell treated by 1% Triton X-100). Data represent individual studies performed on AMs isolated from six rats from each strain. Bars indicate mean \pm SD. ** $P < 0.01$ versus F344 LTA/PGN group; ## $P < 0.01$ versus DA LTA/PGN group.

during *in vitro* stimulation. KC and MIP-2, (also known as CXCL1 and CXCL2), belong to the CXC chemokine family and are major neutrophil chemoattractants acting via the neutrophil surface receptor CXCR2 (49,50). Neutralization of CXC chemokines (49) or their receptor (45,51–55) results in reduction of neutrophil recruitment into the lung following a variety of stimuli. AMs are a major source of chemokines in the alveolar space and respond directly to bacterial products such as bacterial lipopolysaccharide and gram-positive cell wall products such as LTA (50) and toxins such as enterotoxin A (56). In our study, LTA and PGN stimulation resulted in a dose-dependent elevation of KC and MIP-2 production, with F344

AMs producing approximately 50% more MIP-2 and 100% more KC than DA AMs. The significantly increased production of chemokines by F344 AMs may explain, at least partially, the different lung neutrophil influx detected between the two strains.

To further explore the mechanism involved in increased chemokine production by F344 AMs, we focused on the major chemokine production pathway during LTA + PGN stimulation, that is, the TLR2-MAPK pathway (32,33). Our results show that there are no differences in the basal expression of TLR2 and total MAPK members (p38 and ERK1/2) between the two strains. However, the phosphorylation of p38 and ERK1/2 was significantly higher in the F344

group after LTA + PGN stimulation. Furthermore, our results also showed that both p38 and ERK1/2 inhibitors can inhibit LTA/PGN-induced KC and MIP-2 production. If both of these two MAPK pathways are inhibited at the same time, a substantial inhibition of chemokines is achieved, which includes almost 100% of stimulated MIP-2 production and 75% of stimulated KC production. This observation indicates that the p38 and ERK1/2 pathways are two major cell-signaling pathways in LTA/PGN-induced KC and MIP-2 accumulation. The differences in MAPK pathway activation in these two strains can, at least partially, explain the differences in KC and MIP-2 production by AMs following LTA/PGN stimulation.

In conclusion, we have shown that there are significant differences in the regulation of pulmonary inflammation induced by bacterial products in the two different strains of rats. In particular, the differential regulation can be seen in neutrophil infiltration to the alveolar spaces. These differences are associated with different AM phenotypes and, specifically, differences in MAPK signaling pathways. The data presented should lead to a better understanding of the compartmentalization of the lung inflammatory response and tissue-specific regulation of the immune responses. Further study of these particular influences on the inflammatory processes may lead to more effective treatment of acute lung inflammation and injury.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

REFERENCES

- Mizgerd JP. (2006) Lung infection—a public health priority. *PLoS Med.* 3:e76.
- O'Brien JM, Jr., Ali NA, Abercrombie SK, Abraham E. (2007) Sepsis. *Am. J. Med.* 120:1012–22.
- Taira BR, et al. (2009) Ventilator-associated pneumonia in pediatric trauma patients. *Pediatr. Crit. Care Med.* 10:491–4.
- Sokka T, Abelson B, Pincus T. (2008) Mortality in rheumatoid arthritis: 2008 update. *Clin. Exp. Rheumatol.* 26:S35–61.
- Barnes KC. (2005) Genetic determinants and ethnic disparities in sepsis-associated acute lung injury. *Proc. Am. Thorac. Soc.* 2:195–201.
- Garcia JG, Moreno Vinasco L. (2006) Genomic insights into acute inflammatory lung injury. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 291: L1113–7.
- Raz E. (2007) Organ-specific regulation of innate immunity. *Nat. Immunol.* 8:3–4.
- Richards MJ, Edwards JR, Culver DH, Gaynes RP. (2000) Nosocomial infections in combined medical-surgical intensive care units in the United States. *Infect. Control Hosp. Epidemiol.* 21:510–5.
- Styers D, Sheehan DJ, Hogan P, Sahn DF. (2006) Laboratory-based surveillance of current antimicrobial resistance patterns and trends among *Staphylococcus aureus*: 2005 status in the United States. *Ann. Clin. Microbiol. Antimicrob.* 5:2.
- Vincent JL, et al. (2006) Sepsis in European intensive care units: results of the SOAP study. *Crit. Care Med.* 34:344–53.
- Ventura CL, et al. (2008) *Staphylococcus aureus* elicits marked alterations in the airway proteome during early pneumonia. *Infect. Immun.* 76:5862–72.
- Wang JE, et al. (2004) Peptidoglycan of *Staphylococcus aureus* causes inflammation and organ injury in the rat. *Crit. Care Med.* 32:546–52.
- Overland G, et al. (2003) Lipoteichoic acid is a potent inducer of cytokine production in rat and human Kupffer cells in vitro. *Surg. Infect. (Larchmt)*. 4:181–91.
- Hoogerwerf JJ, et al. (2008) Lung inflammation induced by lipoteichoic acid or lipopolysaccharide in humans. *Am. J. Respir. Crit. Care Med.* 178:34–41.
- Hattar K, et al. (2006) Lipoteichoic acid (LTA) from *Staphylococcus aureus* stimulates human neutrophil cytokine release by a CD14-dependent, Toll-like-receptor-independent mechanism: Autocrine role of tumor necrosis factor- α in mediating LTA-induced interleukin-8 generation. *Crit. Care Med.* 34:835–41.
- Leemans JC, Heikens M, van Kessel KP, Florquin S, van der Poll T. (2003) Lipoteichoic acid and peptidoglycan from *Staphylococcus aureus* synergistically induce neutrophil influx into the lungs of mice. *Clin. Diagn. Lab. Immunol.* 10:950–3.
- Lin X, et al. (2007) Age-dependent changes and the increased injurious potential of macrophage migration inhibitory factor in sepsis. *Am. J. Resp. Crit. Care Med.* 175:A696.
- Remmers EF, et al. (2002) Modulation of multiple experimental arthritis models by collagen-induced arthritis quantitative trait loci isolated in congenic rat lines: different effects of non-major histocompatibility complex quantitative trait loci in males and females. *Arthritis Rheum.* 46:2225–34.
- Laragione T, et al. (2007) The arthritis severity quantitative trait loci Cia4 and Cia6 regulate neutrophil migration into inflammatory sites and levels of TNF- α and nitric oxide. *J. Immunol.* 178:2344–51.
- Brenner M, et al. (2005) The non-MHC quantitative trait locus Cia5 contains three major arthritis genes that differentially regulate disease severity, pannus formation, and joint damage in collagen- and pristane-induced arthritis. *J. Immunol.* 174:7894–903.
- Lee WL, Downey GP. (2001) Neutrophil activation and acute lung injury. *Curr. Opin. Crit. Care.* 7:1–7.
- Kindt GC, Gadek JE, Weiland JE. (1991) Initial recruitment of neutrophils to alveolar structures in acute lung injury. *J. Appl. Physiol.* 70:1575–85.
- Pay S, et al. (2006) Expression of CXCR-1 and CXCR-2 chemokine receptors on synovial neutrophils in inflammatory arthritides: does persistent or increasing expression of CXCR-2 contribute to the chronic inflammation or erosive changes? *Joint Bone Spine.* 73:691–6.
- Weissmann G. (2004) Pathogenesis of rheumatoid arthritis. *J. Clin. Rheumatol.* 10:S26–31.
- Miller EJ, Brelsford WG. (1993) Interleukin 8: the major neutrophil chemotaxin in a case of pseudogout. *J. Rheumatol.* 20:1250–2.
- Kurdowska A, et al. (1996) Anti-IL-8 autoantibodies in alveolar fluid from patients with the adult respiratory distress syndrome. *J. Immunol.* 157:2699–706.
- Yang H, Wang H, Czura CJ, Tracey KJ. (2005) The cytokine activity of HMGB1. *J. Leukoc. Biol.* 78:1–8.
- Morand EF, Leech M. (2005) Macrophage migration inhibitory factor in rheumatoid arthritis. *Front. Biosci.* 10:12–22.
- Matsuda N, Nishihira J, Takahashi Y, Kemmotsu O, Hattori Y. (2006) Role of macrophage migration inhibitory factor in acute lung injury in mice with acute pancreatitis complicated by endotoxemia. *Am. J. Respir. Cell Mol. Biol.* 35:198–205.
- Takahashi K, et al. (2009) Macrophage CD74 contributes to MIF-induced pulmonary inflammation PMC2681459. *Respir. Res.* 10:33.
- Linge HM, et al. (2009) Role of Macrophage Migration Inhibitory Factor in a Murine Model of Lung Inflammation. *Am. J. Respir. Crit. Care Med.* 179:A5651.
- Thobe BM, et al. (2007) The role of MAPK in Kupffer cell toll-like receptor (TLR) 2-, TLR4-, and TLR9-mediated signaling following trauma-hemorrhage. *J. Cell. Physiol.* 210:667–75.
- Re F, Strominger JL. (2001) Toll-like receptor 2 (TLR2) and TLR4 differentially activate human dendritic cells. *J. Biol. Chem.* 276:37692–9.
- Janardhan KS, Sandhu SK, Singh B. (2006) Neutrophil depletion inhibits early and late monocyte/macrophage increase in lung inflammation. *Front Biosci.* 11:1569–76.
- Strieter RM, Lukacs NW, Standiford TJ, Kunkel SL. (1993) Cytokines. 2. Cytokines and lung inflammation: mechanisms of neutrophil recruitment to the lung. *Thorax.* 48:765–9.
- De Kimphe SJ, Kengatharan M, Thiemermann C, Vane JR. (1995) The cell wall components peptidoglycan and lipoteichoic acid from *Staphylococcus aureus* act in synergy to cause shock and multiple organ failure. *Proc. Natl. Acad. Sci. U. S. A.* 92:10359–63.
- Lien E, Ingalls RR. (2002) Toll-like receptors. *Crit. Care Med.* 30: S1-S11.
- Borthakur A, Bhattacharyya S, Dudeja PK, Tobacman JK. (2007) Carrageenan induces interleukin-8 production through distinct Bcl10 pathway in normal human colonic epithelial cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* 292: G829–38.
- Bhattacharyya S, Dudeja PK, Tobacman JK. (2008) Carrageenan-induced NF κ B activation depends on distinct pathways mediated by reactive oxygen species and Hsp27 or by Bcl10. *Biochim. Biophys. Acta.* 1780:973–82.
- Yoo S, Han S, Park YS, Lee JH, Oh U, Hwang SW. (2009) Lipoxigenase inhibitors suppressed carrageenan-induced Fos-expression and inflammatory pain responses in the rat. *Mol. Cells.* 27:417–22.
- Zweifel BS, et al. (2008) A rat air pouch model for evaluating the efficacy and selectivity of 5-lipoxygenase inhibitors. *Eur. J. Pharmacol.* 584:166–74.
- Bhattacharyya S, et al. (2008) Toll-like receptor 4 mediates induction of the Bcl10-NF κ B-interleukin-8 inflammatory pathway by carrageenan in human intestinal epithelial cells. *J. Biol. Chem.* 283:10550–8.
- Zhu YN, et al. (2006) Differential expression of inducible nitric oxide synthase and IL-12 between peritoneal and splenic macrophages stimulated with LPS plus IFN- γ is associated with the activation of extracellular signal-related kinase. *Int. Immunol.* 18:981–90.
- Farley KS, et al. (2006) Effects of macrophage inducible nitric oxide synthase in murine septic lung injury. *Am. J. Physiol. Lung Cell Mol. Physiol.* 290: L1164–72.
- Maus U, et al. (2002) The role of CC chemokine receptor 2 in alveolar monocyte and neutrophil immigration in intact mice. *Am. J. Respir. Crit. Care Med.* 166:268–73.
- Lomas-Neira J, et al. (2006) Role of alveolar macrophage and migrating neutrophils in hemorrhage-induced priming for ALI subsequent to septic challenge. *Am. J. Physiol. Lung Cell Mol. Physiol.* 290: L51–8.
- Frank JA, Wray CM, McAuley DF, Schwendener R, Matthay MA. (2006) Alveolar macrophages contribute to alveolar barrier dysfunction in ventilator-induced lung injury. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 291: L1191–8.

48. Nader ND, McQuiller PS, Raghavendran K, Knight PR. (2007) The role of alveolar macrophages in the pathogenesis of aspiration pneumonitis. *Immunol. Invest.* 36:457–71.
49. Reutershan J, Ley K. (2004) Bench-to-bedside review: acute respiratory distress syndrome - how neutrophils migrate into the lung. *Crit. Care.* 8:453–61.
50. Puneet P, Mochhala S, Bhatia M. (2005) Chemokines in acute respiratory distress syndrome. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 288: L3–15.
51. Hirayama S, Shiraishi T, Shirakusa T, Higuchi T, Miller EJ. (2006) Prevention of neutrophil migration ameliorates rat lung allograft rejection. *Mol. Med.* 12:208–13.
52. Hayashi S, Yatsunami J, Fukuno Y, Kawashima M, Miller EJ. (2002) Antileukinate, a hexapeptide inhibitor of CXC-chemokine receptor, suppresses bleomycin-induced acute lung injury in mice. *Lung.* 180:339–48.
53. Lomas-Neira JL, Chung CS, Grutkoski PS, Miller EJ, Ayala A. (2004) CXCR2 inhibition suppresses hemorrhage-induced priming for acute lung injury in mice. *J. Leukoc. Biol.* 76:58–64.
54. Miller EJ, Cohen AB, Peterson BT. (1996) Peptide inhibitor of interleukin-8 (IL-8) reduces staphylococcal enterotoxin-A (SEA) induced neutrophil trafficking to the lung. *Inflamm. Res.* 45:393–7.
55. Lin X, et al. (2005) Alpha-chemokine receptor blockade reduces high mobility group box 1 protein-induced lung inflammation and injury and improves survival in sepsis. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 289: L583–90.
56. Miller EJ, Nagao S, Carr FK, Noble JM, Cohen AB. (1996) Interleukin-8 (IL-8) is a major neutrophil chemotaxin from human alveolar macrophages stimulated with staphylococcal enterotoxin A (SEA). *Inflamm. Res.* 45:386–92.