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Mechanisms of the Hepatic Acute-Phase Response during Bacterial Pneumonia▽

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The acute-phase response is characterized by increased circulating levels of acute-phase proteins (APPs) generated by the liver. During bacterial pneumonia, APPs correlate with the severity of disease, serve as biomarkers, and are functionally significant. The kinetics and regulatory mechanisms of APP induction in the liver during lung infection have yet to be defined. Here we show that APP mRNA transcription is induced in the livers of mice whose lungs are infected with either *Escherichia coli* or *Streptococcus pneumoniae*, and that in both cases this induction occurs in tandem with activation in the liver of the transcription factors signal transducer and activator of transcription 3 (STAT3) and NF-κB RelA. Interleukin-6 (IL-6) deficiency inhibited the activation of STAT3 and the induction of select APPs in the livers of pneumonic mice. Furthermore, liver RelA activation and APP induction were reduced for mice lacking all signaling receptors for tumor necrosis factor alpha and IL-1. In a murine hepatocyte cell line, knockdown of either STAT3 or RelA by small interfering RNA inhibited cytokine induction of the APP serum amyloid A-1, demonstrating that both transcription factors were independently essential for the expression of this gene. These data suggest that during pneumonia caused by gram-negative or gram-positive bacteria, the expression of APPs in the liver depends on STAT3 activation by IL-6 and on RelA activation by early-response cytokines. These signaling axes may be critical for integrating systemic responses to local infection, balancing antibacterial host defenses and inflammatory injury during acute bacterial pneumonia.

Across the socioeconomic spectrum, lung infections result in the loss of more disability-adjusted life years and a greater burden than any other infectious disease, including human immunodeficiency virus/AIDS, tuberculosis, and malaria (28). *Streptococcus pneumoniae* infections account for the highest incidence of community-acquired pneumonia (37), whereas gram-negative enterobacteria such as *Escherichia coli* are a frequent cause of nosocomial pneumonia (1). Successful host defense against these pathogens in the lungs requires an effective innate immune response, consisting of local cytokine production, neutrophil emigration, and extravasated plasma constituents (27).

This local response occurs in tandem with a systemic acute-phase response (APR) typified by altered circulating levels of acute-phase proteins (APPs) (14). The APR first received attention nearly 80 years ago, when a protein capable of reacting with *S. pneumoniae* (C-reactive protein) was discovered in the sera of patients with pneumococcal pneumonia (41). During infection, APPs are synthesized in the liver and circulate at high concentrations in the blood (14). APPs have many functions that may be beneficial to the infected host (14), and deficiencies of select APPs in mice exacerbate infection and injury (8, 47). In humans, APPs can be useful as biomarkers of disease severity during pneumonia (3, 39, 45). To begin refining their utility as diagnostic or prognostic indicators, advancing our understanding of their functional significance as an integrated response to infection, and designing strategies to potentially manipulate APRs to benefit patients, a mechanistic understanding of the APR during pneumonia is needed.

Any of the early-response cytokines (tumor necrosis factor alpha [TNF-α] and interleukin-1 [IL-1]) or IL-6 is capable of inducing APP expression by hepatocytes (5, 23, 40). TNF-α, IL-1, and IL-6 influence APP expression in vivo to variable degrees, seemingly dependent on the initial stimulus inciting an APR (7, 23, 48). During pneumonia, these cytokines are critical for pulmonary inflammation and host defense (20, 21, 29, 42), but whether and how they influence the APR has yet to be determined. In fact, other than its occurrence, very little information is available about the hepatic APR during pulmonary infection. We hypothesized that the induction of APPs in the liver during lung infection requires early-response cytokines (activating RelA) and IL-6 (activating STAT3). We tested this hypothesis using mice with targeted deletions in cytokine signaling pathways and two divergent models of bacterial pneumonia: infection with *E. coli*, which is gram negative and remains compartmentalized to the lung (20), and infection with *S. pneumoniae*, which is gram positive and disseminates from the lung to cause bacteremia (21).

MATERIALS AND METHODS

Mice. Mice lacking a functional gene for IL-6 (*Il6*$^{-/-}$ mice; Jackson Laboratories) and backcrossed 11 generations onto a C57BL/6 background were studied in comparison to wild-type (WT) age- and sex-matched C57BL/6 mice. Triple mutant (TM) mice devoid of all signaling receptors for TNF-α and IL-1 (*Tnfrsf1a*$^{-/-}$ *Tnfrsf1b*$^{-/-}$ *Il1r1*$^{-/-}$) were generated and maintained as previously described on a random hybrid 129/Sv × C57BL/6 background (29). Results obtained from TM mice were compared to those for age/sex-matched WT mice on the same random hybrid genetic background. Experiments incorporating only WT mice were performed with the random hybrid 129/Sv × C57BL/6 back-

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TABLE 1. Primer and probe sequences for real-time RT-PCR[a]

Gene	Forward primer	Reverse primer	TaqMan probe
SAA1	GAGGACATGAGGACACCATTGC	CCAGAGAGCATCTTCAGTGTTCC	AGGAAGAAGCCCAGACCCCACCCT
SAP	CACACTTTTGTTCCACACCCAAG	TCTGAAAGAAGGCTGGTGAAGAC	CTGCTGCTGTCATACCCTGGGCCA
LBP	CTTTGTGATCCTGCCCACCTC	TCAGTCTCACTTGTGCCTTGTC	CCTGTCTTCCGGCTTGGCGTGGTC
IL-6	AGTTGCCTTCTTGGGACTGATG	CAGGTCTGTTGGGAGTGGTATC	AACCACGGCCTTCCCTACTTCACA
TNF-α	TCATACCAGGAGAAAGTCAACCTC	TGGAAGACTCCTCCCAGGTATATG	TGCCGTCAAGAGCCCCTGCCCC
IL-1β	AGTTCCCCAACTGGTACATCAG	TCAATTATGTCCTGACCACTGTTG	ACCTCACAAGCAGAGCACAAGCCT

[a] Primers and probes (with sequences listed in a 5′-to-3′ direction) were designed to amplify an 80- to 200-bp -region within the open reading frames of the following transcripts: SAA1, SAP, LBP, IL-6, TNF-α, and IL-1β.

ground. All experimental protocols were approved by the Harvard Medical Area Standing Committee on Animals and the Institutional Animal Care and Use Committee at Boston University.

Pneumonia. Mice were anesthetized with a mixture of ketamine (50 mg/kg of body weight) and xylazine (5 mg/kg) injected intraperitoneally. Tracheae were surgically exposed and cannulated with a 24-gauge angiocatheter guided into the left bronchus. A 50-μl bolus containing approximately 10^6 CFU of *E. coli* (serotype O6:K2:H1; ATCC 19138) or *S. pneumoniae* (serotype 3; ATCC 6303) in saline was instilled through the angiocatheter into the left lung lobe. The concentration of live bacteria in the instillate was estimated by optical density and verified by plating serial dilutions on 5% sheep blood agar plates.

Real-time RT-PCR. APP and cytokine mRNA contents in the liver, lungs, and/or AML12 cells were quantified using real-time reverse transcriptase PCR (RT-PCR). Mice were euthanized by a halothane or isoflurane overdose at the indicated times after infection. Liver or lung lobes were harvested and preserved in RNAlater stabilization reagent (Qiagen). Total RNA was extracted from livers, lungs, or AML12 cell lysates (see below) using TRIzol (Invitrogen Life Technologies) or the RNeasy minikit (Qiagen). Real-time RT-PCR was performed on 10 ng purified RNA using the iScript One-Step RT-PCR kit for probes (Bio-Rad) and the iCycler iQ real-time detection system (Bio-Rad). In some cases, the reaction was carried out using the Quantifast Probe RT-PCR kit (Qiagen) and the StepOnePlus real-time PCR system (Applied Biosystems). Primers and TaqMan probes (Table 1) were designed using Beacon Designer software (Premier Biosoft International). TaqMan probes were labeled with 6-carboxyfluorescein (5′) and Black Hole Quencher-1 (3′). All mRNA values were normalized to the content of 18S rRNA (20), and expressed as the level of induction (38) relative to the level in uninfected control mice.

SAA protein measurement. Plasma was collected from mice at the indicated times after infection. The serum amyloid A (SAA) protein content was determined by an enzyme-linked immunosorbent assay according to the protocol provided by the manufacturer (Immunology Consultants Laboratory).

Cell culture and siRNA transfection. The murine hepatocyte cell line AML12 (ATCC CRL-2254) was maintained at 37°C under an atmosphere containing 5% CO_2. Complete culture medium contained Dulbecco's modified Eagle medium–F-12 (1:1) supplemented with insulin (0.005 mg/ml), transferrin (0.005 mg/ml), selenium (5 ng/ml), dexamethasone (40 ng/ml), fetal calf serum (10%), penicillin, and streptomycin. For experiments, confluent AML12 cells were subcultured into 24-well tissue culture-treated plates (1.0×10^5 cells/well). After 24 h, cells were transfected in triplicate with RelA small interfering RNA (siRNA) (Dharmacon catalog no. J-040776-05), STAT3 siRNA (Dharmacon catalog no. J-040794-05), or nontargeting (NT) siRNA (Dharmacon catalog no. D-001810-01-05) by using the Dharma*FECT* I transfection reagent (Dharmacon). Following a 48-h transfection period, AML12 cells were stimulated for 2 h with vehicle (0.1% bovine serum albumin in phosphate-buffered saline) or with a cytokine cocktail containing recombinant murine TNF-α, IL-1β, and IL-6 (10 ng/ml each; R&D Systems). At the conclusion of the experiment, cells were washed once with ice-cold phosphate-buffered saline, and lysates were collected in either TRIzol reagent (for RNA analysis; 2 wells/group) or protein extraction buffer (for verification of siRNA-induced knockdown; 1 well/group). Studies were performed on three separate days for a total sample size of three independent experiments, such that a single value ($n = 1$) represents the average of replicates analyzed in one experiment.

Immunoblotting. At the indicated times after infection, liver lobes were collected from euthanized mice and snap-frozen, enabling subsequent processing for both total and nuclear protein extraction as previously described (30, 34). Total protein was also extracted from siRNA-transfected AML12 cells by using a previously established protocol (34). Total and nuclear protein concentrations were quantified using the bicinchoninic acid assay (Sigma), and Western blotting was performed using the NuPAGE gel system (Invitrogen Life Technologies). Polyvinylidene difluoride membranes containing separated proteins were probed with primary antibodies against RelA, Y705-phosphorylated STAT3 (pSTAT3), total STAT3, and β-actin. Primary antibodies were then detected using a horseradish peroxidase-conjugated secondary antibody, and membrane-exposed ECL chemiluminescent films (Amersham Biosciences) were developed using the ECL$^+$ Western blotting detection system (Amersham Biosciences). All antibodies were purchased from Cell Signaling Technology. Densitometry was performed on select STAT3 immunoblots using Image J software (National Institutes of Health). In such cases, pSTAT3 densitometric values were normalized to those of total STAT3. pSTAT3/STAT3 ratios were then calculated as the percentage of the ratio achieved for the indicated control group.

Statistics. Statistical analyses were performed using GraphPad Prism (GraphPad Software) and/or Statistica (StatSoft). Data are presented as means ± standard errors (SE) for the number of samples identified for each figure. Real-time RT-PCR data were calculated as levels of induction and are presented as geometric means ± geometric SE. Comparisons were performed using a Student *t* test or a one- or two-way analysis of variance (ANOVA), followed in

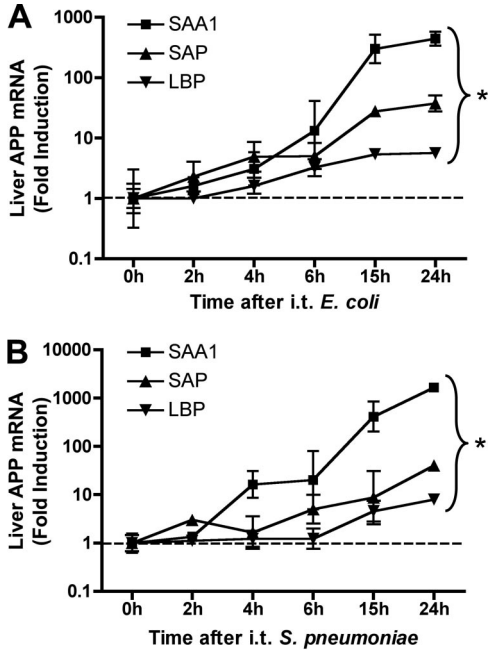

FIG. 1. Bacterial pneumonia induces APP mRNA in the liver. A total of 10^6 CFU of *Escherichia coli* (A) or *Streptococcus pneumoniae* (B) was instilled intratracheally (i.t.) into mice. Livers were collected at the indicated times after bacterial inoculation. SAA1, SAP, and LBP mRNA induction was measured in total-liver RNA extracts using real-time RT-PCR. Levels of induction (versus levels in uninfected mice at 0 h) are expressed as geometric means ± geometric SE ($n = 3$). The asterisk indicates a statistically significant overall effect of infection on mRNA induction as determined by one-way ANOVA ($P < 0.05$).

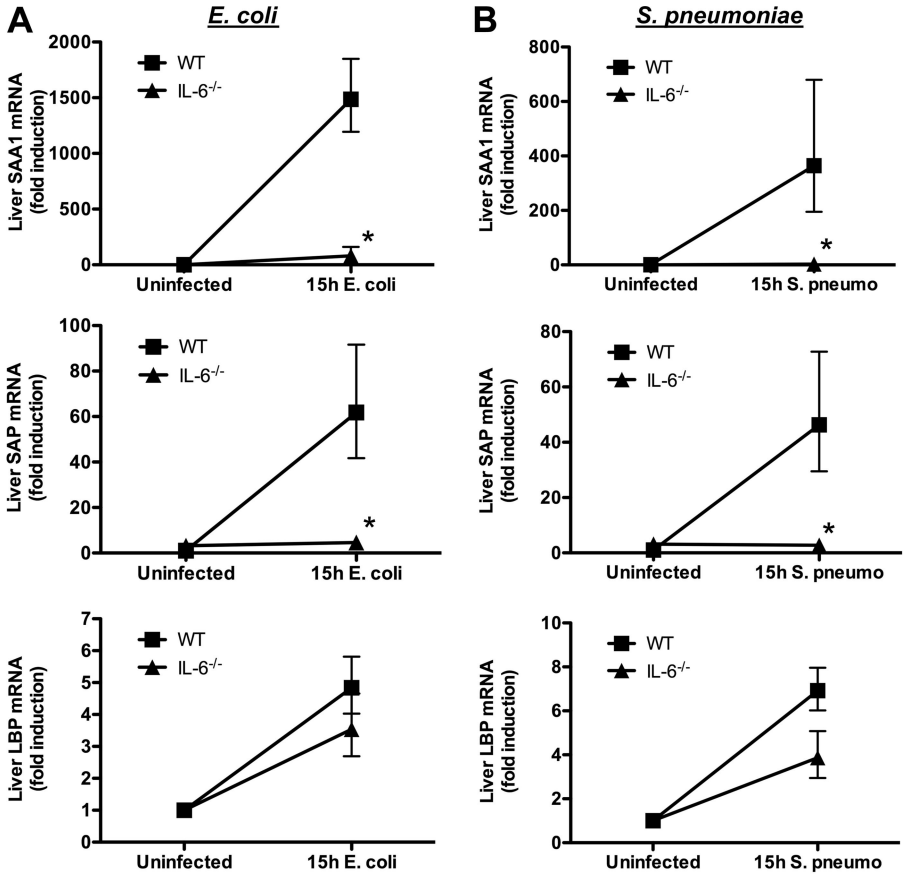

FIG. 2. APP gene induction in the liver during bacterial pneumonia requires IL-6. A total of 10^6 CFU of *Escherichia coli* (A) or *Streptococcus pneumoniae* (B) was instilled intratracheally into WT or IL-$6^{-/-}$ mice. Livers were collected from uninfected (0-h) mice and from mice infected for 15 h. Induction of SAA1, SAP, and LBP mRNAs was measured in total-liver RNA extracts using real-time RT-PCR. Levels of induction (versus levels in WT mice at 0 h) are expressed as geometric means ± geometric SE (*n*, 7 to 9). Asterisks indicate statistically significant differences from WT mice at the same time point as determined by two-way ANOVA followed by a Newman-Keuls post hoc test ($P < 0.05$).

some cases by a Newman-Keuls post hoc test. Data presented on a logarithmic scale were log transformed for analyses. Differences were considered statistically significant at a *P* value of <0.05.

RESULTS

APPs are induced in the liver during pneumonia. To determine the kinetics and extent of the hepatic APR during bacterial pneumonia, we quantified liver mRNA expression of SAA1, serum amyloid P (SAP), and lipopolysaccharide binding protein (LBP) over a 24-h period following an intrapulmonary infection with *E. coli* or *S. pneumoniae*. These APPs were chosen because they are representative of a classic APR (14, 24) and are potentially relevant to antimicrobial host defense (8, 36, 47). As determined by real-time RT-PCR, SAA1, SAP, and LBP mRNA levels in the liver were significantly induced over 24 h in response to both *E. coli* (Fig. 1A) and *S. pneumoniae* (Fig. 1B). These data indicate the presence of an extrapulmonary signaling axis that is sufficient to induce hepatic APP expression in response to a local intrapulmonary infectious challenge.

IL-6 is required for APP expression in the liver. To determine the influence of IL-6 on APP expression in the liver during pneumonia, expression of LBP, SAA1, and SAP mRNAs was determined in IL-6-deficient (IL-$6^{-/-}$) mice 15 h after intratracheal administration of *E. coli* or *S. pneumoniae*, a time point when all APP mRNAs measured were approaching peak values (Fig. 1). Although baseline APP expression was unchanged, SAA1 and SAP mRNA induction was almost completely ablated in IL-$6^{-/-}$ mice during either *E. coli* or *S. pneumoniae* infections (Fig. 2A and B). To determine whether mRNA changes in the liver correspond to changes in circulating APP contents, we also measured plasma SAA protein levels under the same conditions. As with the hepatic mRNA response, SAA plasma protein concentrations were significantly elevated in response to either infection in WT mice, whereas no detectable increase in circulating SAA occurred in pneumonic IL-$6^{-/-}$ mice (Table 2). In contrast to induction of SAA1 and SAP mRNAs, LBP mRNA induction in the liver was not significantly affected by the loss of IL-6. These data demonstrate that IL-6 is essential for the expression of select APPs during pneumonia (e.g., SAA1 and SAP), whereas other APPs (e.g., LBP) can be expressed independently of IL-6 signaling.

Early-response cytokines are required for APP expression in the liver. The influence of early-response cytokines on APP induction during pneumonia was addressed using TM mice

TABLE 2. Effect of IL-6 deficiency on circulating SAA during pneumonia[a]

Treatment	Plasma SAA concn (μg/ml) in mice	
	WT	IL-6$^{-/-}$
No infection	43 ± 4	43 ± 5
E. coli	1,277 ± 356†	48 ± 9*
S. pneumoniae	728 ± 99†	48 ± 7*

[a] Plasma SAA protein concentrations in WT and IL-6$^{-/-}$ mice were determined during pneumonia. Samples were collected from uninfected mice or 15 h after intratracheal inoculation with *E. coli* or *S. pneumoniae*. †, the difference from the level for uninfected mice was statistically significant. *, the difference from the level for WT mice subjected to the same treatment was statistically significant. Comparisons were made using two-way ANOVA followed by a Newman-Keuls post hoc test ($n = 3$ to 5; $P < 0.05$).

lacking all signaling receptors for TNF-α and IL-1 (21, 29). The expression of LBP, SAA1, and SAP mRNAs in TM and WT mice was determined by real-time RT-PCR 15 h after *E. coli* or *S. pneumoniae* inoculation. No significant differences in liver APP mRNA contents were observed between genotypes in the absence of infection. In response to *E. coli* pneumonia, levels of SAA1 and SAP mRNAs in the liver were significantly reduced but not ablated in TM mice (Fig. 3A). After intratracheal *S. pneumoniae* instillation, hepatic mRNA expression of all three APPs, including LBP, was significantly reduced in TM mice (Fig. 3B). Together, these data demonstrate that the early-response cytokines TNF-α and IL-1 are required for maximal expression of APP genes in the liver in response to local intrapulmonary challenges with either *E. coli* or *S. pneumoniae*.

IL-6 is required for hepatic STAT3 activation. Because IL-6 can induce gene expression via the STAT3 transcription factor (17), phosphorylation of STAT3 (pSTAT3) in the liver was analyzed by immunoblotting throughout 24 h of *E. coli* or *S. pneumoniae* lung infections in order to correlate hepatic STAT3 activity with APP gene expression (Fig. 1). In response to intratracheal *E. coli* instillation, STAT3 activation was readily detected in liver protein extracts, with increased immunoreactivity evident as early as 2 h postinfection, peaking between 4 and 6 h, and persisting until the conclusion of the 24-h protocol (Fig. 4A). During pneumococcal pneumonia as well, STAT3 activity was markedly elevated over that in uninfected (0-h) control mice. While pSTAT3 was detectable by 2 h after *S. pneumoniae* infection, the kinetics of the STAT3 response was relatively slower than that caused by *E. coli*: maximal levels were not achieved until 15 to 24 h of infection (Fig. 4A). The

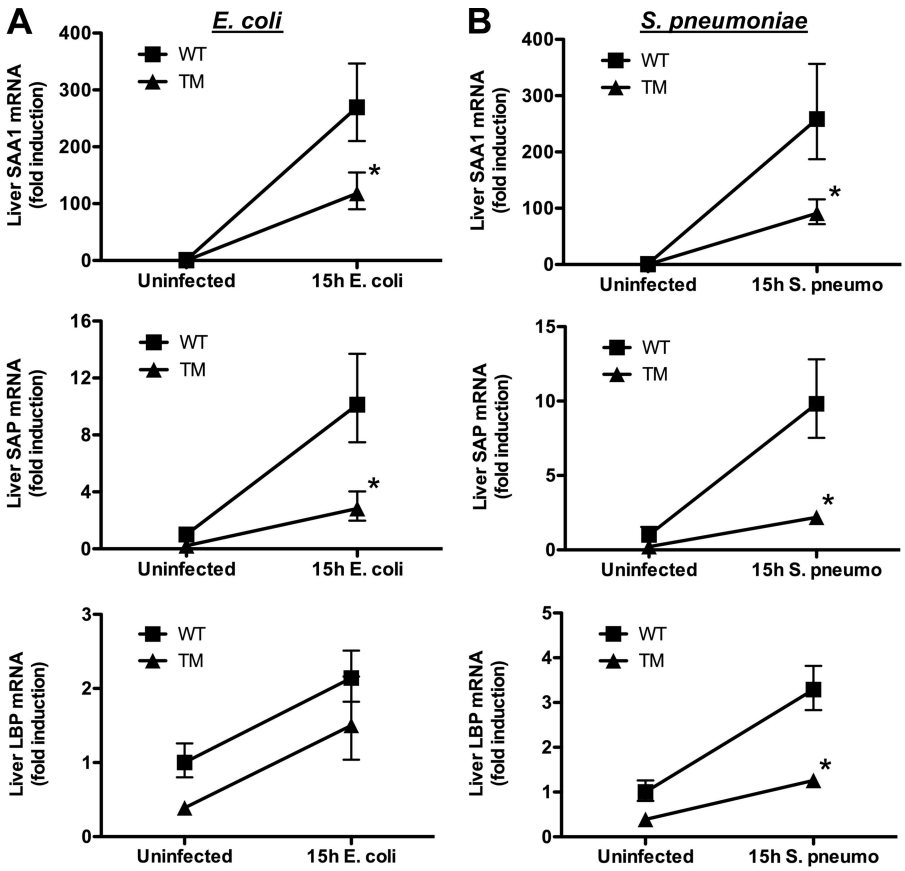

FIG. 3. Induction of APP genes in the liver during bacterial pneumonia requires TNF-α and IL-1 signaling. A total of 10^6 CFU of *Escherichia coli* (A) or *Streptococcus pneumoniae* (B) was instilled intratracheally into WT mice or TM mice lacking all signaling receptors for TNF-α and IL-1. Livers were collected from uninfected (0-h) mice and from mice infected for 15 h. Induction of SAA1, SAP, and LBP mRNAs was measured in total-liver RNA extracts using real-time RT-PCR. Levels of induction (versus levels in WT mice at 0 h) are expressed as geometric means ± geometric SE (n, 7 to 15). Asterisks indicate statistically significant differences from WT mice at the same time point as determined by two-way ANOVA followed by a Newman-Keuls post hoc test ($P < 0.05$).

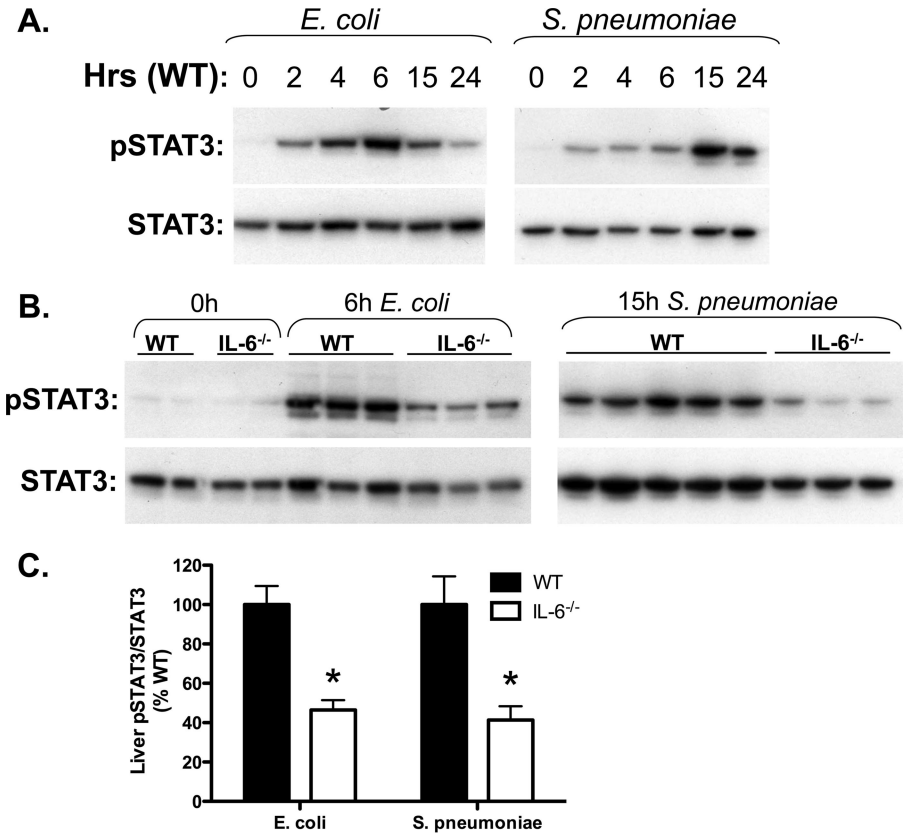

FIG. 4. Bacterial pneumonia induces liver STAT3 activation, which requires IL-6. A total of 10^6 CFU of *Escherichia coli* or *Streptococcus pneumoniae* was instilled intratracheally into WT or IL-$6^{-/-}$ mice. The content of STAT3 phosphorylated on Y705 (pSTAT3) was determined by immunoblotting as a metric of STAT3 activity in total-protein extracts from livers collected at the indicated times of infection. Total STAT3 immunoreactivity was used as a loading control. (A) Representative images from three individual experiments illustrate the kinetics of liver pSTAT3 and total STAT3 immunoreactivity through 24 h of pneumonia. (B) pSTAT3 and total STAT3 contents in the liver are shown for WT and IL-$6^{-/-}$ mice at times of peak STAT3 activation, with each lane representing results from an individual mouse. (C) Densitometric values of pSTAT3 immunoreactivity were normalized to those of total STAT3 and calculated as the percentage of the average pSTAT3/STAT3 ratio in WT mice. Data are means ± SE (n, 3 to 6). Asterisks indicate statistically significant differences from WT mice with the same infection as determined by a Student t test ($P < 0.05$).

modest but detectable increase in the level of pSTAT3 by 2 h correlates with the level of SAA1 mRNA (Fig. 1), which was detectable by PCR as early as 4 h after intratracheal *S. pneumoniae* instillation.

To determine whether STAT3 activation in the liver during pneumonia required IL-6, the hepatic pSTAT3 contents of IL-$6^{-/-}$ and WT mice were compared 6 or 15 h after instillation of *E. coli* or *S. pneumoniae*, respectively. These times were selected to correspond with periods of maximal STAT3 activity in the liver (Fig. 4A). In the absence of infection, pSTAT3 was virtually undetectable in both IL-$6^{-/-}$ and WT mice, with no visible difference between genotypes (Fig. 4B). Six hours after intratracheal *E. coli* instillation, the strong liver STAT3 response to pneumonia observed in WT mice was largely diminished in IL-$6^{-/-}$ mice (Fig. 4B). Similarly, pSTAT3 was nearly undetectable in IL-6-deficient mice after 15 h of pneumococcal pneumonia, in contrast to the robust STAT3 response in the livers of WT mice (Fig. 4B). During both types of infection, lack of functional IL-6 also caused a modest change in total STAT3 levels. Because of this, we performed densitometry to calculate changes in pSTAT3 as a proportion of total STAT3 content in order to quantify the relative phosphorylation status. These data indicate that the percentage of total STAT3 in the liver represented by its active form (pSTAT3) is significantly reduced in IL-$6^{-/-}$ mice in response to both *E. coli* and *S. pneumoniae* (Fig. 4C). Together, these data provide the first evidence of a hepatic STAT3 response to intrapulmonary infection, which occurs in tandem with APP expression. Moreover, the results show that this response is dependent on IL-6 in two distinct infections.

Early-response cytokines are required for hepatic RelA activation. Because the early-response cytokines can induce gene expression via the NF-κB RelA transcription factor (25), we characterized the activation of NF-κB RelA in the liver during lung infections. To do this, RelA immunoblotting was performed on nuclear protein extracts prepared from mouse livers collected throughout 24 h of pneumonia. Nuclear translocation of RelA in the liver was transiently induced in response to *E. coli* pneumonia, with increased immunoreactivity 2 h after inoculation, followed by peak levels at 4 to 6 h (Fig. 5A). RelA activation was also observed in the livers of mice with pneu-

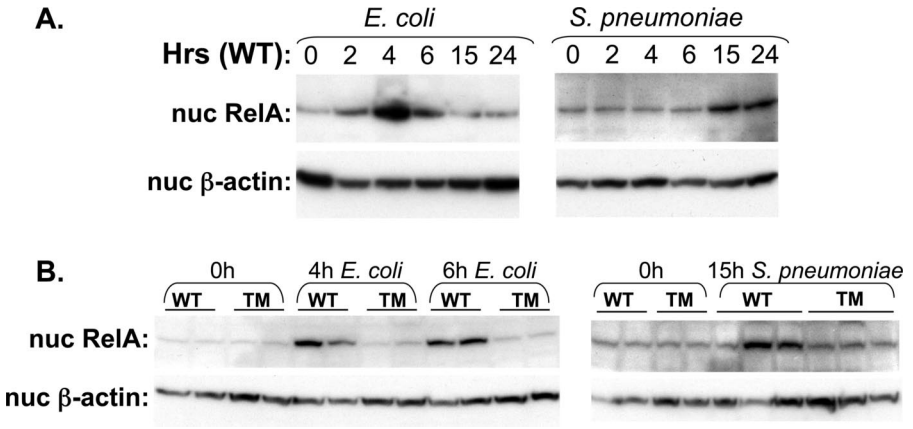

FIG. 5. Bacterial pneumonia induces liver RelA activation, which requires TNF-α and IL-1 signaling. A total of 10^6 CFU of *Escherichia coli* or *Streptococcus pneumoniae* was instilled intratracheally into WT mice or TM mice lacking all signaling receptors for TNF-α and IL-1. Nuclear (nuc) RelA immunoreactivity was determined as a metric of RelA activity in nuclear extracts from livers collected at the indicated times of infection. β-Actin immunoreactivity was used as a loading control. (A) Representative images from three individual experiments illustrate the kinetics of RelA nuclear translocation in the liver through 24 h of pneumonia. (B) Nuclear RelA contents for livers of WT and TM mice at times of peak RelA activation, with each lane representing results from individual mice.

mococcal pneumonia, albeit relatively later (at 15 to 24 h) than that observed in response to *E. coli* (Fig. 5A).

In order to determine whether TNF-α and IL-1 signaling contributed to hepatic RelA activation, this outcome was compared for WT and TM mice. No difference in liver nuclear RelA contents was observed between genotypes in uninfected mice (Fig. 5B). The liver RelA response to *E. coli* pneumonia, however, was completely eliminated in TM mice after 4 and 6 h of infection (Fig. 5B), identified as times of peak RelA activity in Fig. 5A. Similarly, following 15 h of pneumococcal pneumonia, while nuclear translocation of RelA was induced in the livers of most WT mice, this was never observed in TM mice (Fig. 5B). These data demonstrate that RelA activation in the liver correlates with APP induction during pneumonia, and they identify early-response cytokines as a communication network through which this response occurs during each of two distinct types of lung infection.

Lung/liver expression of IL-6 and early-response cytokines is infection dependent. Because the data presented above indicate that IL-6 and receptors for TNF-α and IL-1 are critical links between the lung and the liver during pneumonia, we sought to determine the relative contributions of these two tissues to cytokine synthesis. In response to either *E. coli* or *S. pneumoniae* instillation into the lungs, IL-6, TNF-α, and IL-1β mRNAs were significantly induced in the lungs through at least 24 h of infection (Fig. 6). Interestingly, expression of all three cytokines was also induced in the livers of *E. coli*-infected mice (Fig. 6A), but there were no significant changes in the levels of these cytokine mRNAs in the liver during pneumococcal pneumonia (Fig. 6B). These data indicate that both the lungs and the liver are potential sources of IL-6 and early-response cytokines during *E. coli*-induced pneumonia, whereas during pneumococcal pneumonia, these cytokines are synthesized in the lungs but not in the liver.

STAT3 and RelA are required for cytokine-induced SAA1 expression in hepatocytes. In order to test whether cytokine stimulation of hepatocyte APP expression requires STAT3 and RelA, these transcription factors were knocked down in AML12 cells by using siRNA. AML12 cells are a nontumorigenic mouse hepatocyte line displaying multiple characteristics typical of this cell type, including peroxisomes, bile canaliculus-like structures, and expression of serum proteins (44). Neither SAP nor LBP expression could be induced in AML12 cell cultures, but SAA induction was robust (data not shown). Transfection of AML12 cells with siRNA targeting murine RelA or STAT3 resulted in a nearly complete loss of protein expression relative to that in cells transfected with NT control siRNA (Fig. 7A). Upon validating this loss-of-function approach, we determined the effects of STAT3 and RelA knockdown on APP expression. SAA1 expression was induced by a cytokine cocktail containing IL-6, TNF-α, and IL-1, resulting in an approximately 400-fold increase in SAA1 mRNA contents (Fig. 7B). Knockdown of either RelA or STAT3 was sufficient to nearly eliminate SAA1 induction; the levels of induction did not statistically differ from the baseline. These data demonstrate that RelA and STAT3 are both essential for cytokine-induced SAA1 expression. Moreover, the results implicate these two transcription factors as the direct means through which IL-6, TNF-α, and IL-1 promote hepatic APP synthesis during pneumonia.

DISCUSSION

The innate immune response to lower respiratory tract infections has been studied largely from the perspective of the local lung microenvironment, with remarkably little known about extrapulmonary events that may influence disease outcome. Our present findings help elucidate the kinetics, magnitude, and mechanisms of the hepatic APR during bacterial pneumonia. The observed liver response to lung infections included not only the expression of APPs, the hallmark of the APR, but also the activation of the transcription factors STAT3 and RelA. These transcription factors were both essential to APP induction by hepatocytes stimulated in vitro with cytokines. Finally, our results unequivocally confirm the importance of the cytokines TNF-α, IL-1, and IL-6 in promot-

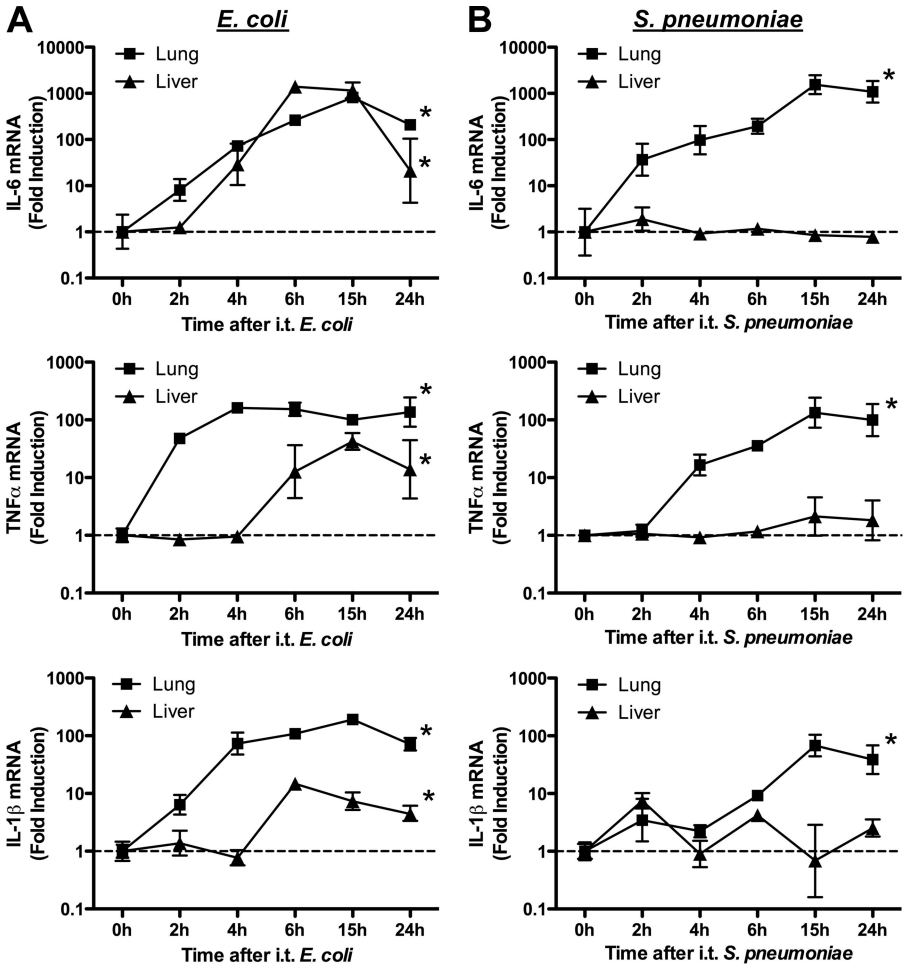

FIG. 6. IL-6 and early-response cytokines are differentially expressed in the lungs and the liver during bacterial pneumonia. A total of 10^6 CFU of *Escherichia coli* (A) or *Streptococcus pneumoniae* (B) was instilled intratracheally (i.t.) into mice. Livers and lungs were collected at the indicated times after bacterial inoculation. Induction of IL-6, TNF-α, and IL-1β mRNAs was measured in total lung and liver RNA extracts using real-time RT-PCR. Levels of induction (versus levels in uninfected mice at 0 h) are expressed as geometric means ± geometric SE (n, 3 to 5). Asterisks indicate statistically significant overall effects of infection on cytokine mRNA induction as determined by one-way ANOVA ($P < 0.05$).

ing the APR, since loss of these signaling pathways had profound effects both on hepatic APP expression and on transcription factor activity in vivo. These findings highlight a communication axis between the lung and liver during pneumonia.

In order to more definitively analyze the APR during pneumonia, we performed every experiment with two different pathogens, *E. coli* and *S. pneumoniae*, both of which are relevant to patients diagnosed with lung infections (1, 37). These two bacteria elicit very different responses in the lungs, with different requirements for host defense mediators and distinct mechanisms of virulence and pathophysiology. In the context of the APR, an important difference between these two organisms is the incidence of bacteremia, which is high during pneumococcal pneumonia but absent in response to our selected dose of intratracheal *E. coli* (10, 11, 20; also unpublished observations). Consequently, initiation of the hepatic APR during *E. coli* pneumonia in the present studies likely resulted from circulating factors produced directly in response to lung infection rather than from bacterial activation of hepatocytes secondary to bacteremia. In addition to circulating host-derived factors, however, it is plausible that disseminated *S. pneumoniae* directly influences hepatic APP expression, since bacteremia can occur in response to lung infections with our selected serotype (type 3) of pneumococcus (10, 11; also unpublished observations).

Despite the different host responses elicited by *E. coli* and *S. pneumoniae* in the lungs, our present results identify IL-6, TNF-α, and IL-1 as critical factors linking pulmonary innate immunity to hepatic transcription factor activation and APP synthesis, regardless of the infectious stimulus. In the case of *E. coli* lung infection, we have previously shown that loss of either IL-6 or early-response cytokine signaling significantly reduces acute pulmonary inflammation, despite relatively normal expression of neutrophil chemoattractants and other proinflammatory mediators in the lungs (20, 29). Activation of STAT3 and RelA is only modestly affected (STAT3) or unaffected (RelA) in the lungs of pneumonic mice lacking IL-6 or TNF-α/IL-1 signaling, respectively (20, 31). In comparison, loss of either cytokine pathway in the current study caused large de-

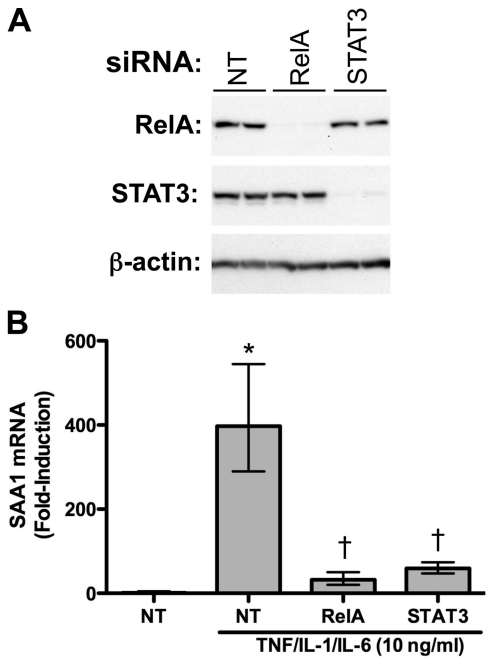

FIG. 7. RelA and STAT3 are required for cytokine-induced APP mRNA induction. The murine hepatocyte line AML12 was transfected with siRNA targeting RelA or STAT3. NT control siRNA was used as a negative control. (A) Immunoblotting was performed for RelA and STAT3 on AML12 total-protein extracts obtained after transfection with NT, RelA, or STAT3 siRNA. β-Actin served as a loading control. Representative images from two of three independent experiments are shown. (B) AML12 cells transfected with NT, RelA, or STAT3 siRNA were stimulated with a cytokine cocktail containing TNF-α, IL-1β, and IL-6 (10 ng/ml each). Real-time RT-PCR was performed on AML12 total-RNA extracts to quantify SAA1 mRNA expression. Levels of induction (versus levels in unstimulated, NT siRNA-transfected cells) are expressed as geometric means ± geometric SE ($n = 3$). Data were collected from three separate experiments, with means from a single experiment representing a sample size of 1. *, statistically significant difference from unstimulated, NT siRNA-transfected cells; †, statistically significant difference from stimulated, NT siRNA-transfected cells. Comparisons were made using a one-way ANOVA followed by a Newman-Keuls post hoc test ($P < 0.05$).

creases in the corresponding transcription factor activity and in APP expression in the liver, implicating extrapulmonary signaling as a major cytokine function during pneumonia. These results link TNF-α/IL-1-induced RelA signaling to liver transcription factor activity and APP synthesis, and they build upon our previous finding that livers from pneumonic TNFR1- and IL1R1-deficient mice have impaired NF-κB DNA binding in response to *E. coli* (31). IL-6, TNF-α, and IL-1 are also critical for promoting acute inflammation and host defense against *S. pneumoniae* in the lungs, for reasons that remain somewhat unclear, particularly in the case of IL-6 (21, 42). In agreement with our findings for *E. coli*-challenged mice, these cytokines were absolutely critical for the APR in the liver during pneumococcal pneumonia, further evidencing a functional role for their presence outside of the lung. Taken together, these data support the postulate that a mechanism by which these cytokines contribute to local lung inflammation and host defense in the lungs may be the induction of APP transcription in the liver.

Our loss-of-function mouse models clearly indicated that IL-6, TNF-α, and IL-1 signaling is a critical liaison between the lungs and liver in response to both gram-negative and gram-positive pathogens. Interestingly, the expression of these cytokines by the liver appears to differ dramatically between these types of infection. During pneumococcal pneumonia, cytokine mRNA expression was increased in the lungs, with no detectable change in the liver, suggesting an endocrine lung-liver axis through which lung-derived cytokines beget a hepatic APR. In contrast, *E. coli*-induced lung infection caused rapid elevations in the levels of cytokine transcripts in both the lungs and the liver, suggesting that either tissue may be an important modulator of downstream APP expression. Elucidating the infection-specific mechanisms and functional significance of liver IL-6, TNF-α, and IL-1 expression is an important goal for future studies.

The prior understanding of mechanisms mediating APR induction during pneumonia has been fragmentary and speculative. Circulating levels of SAP and complement component 3 are significantly reduced in IL-6-deficient mice during pneumococcal pneumonia (42). Our current results strongly suggest that this is due to loss of IL-6-induced STAT3 activity and APP mRNA expression in the liver. Hepatic APP expression in response to intrapulmonary lipopolysaccharide (LPS) is dependent on IL-6 (15, 43) but not on TNF-α (43). This differs somewhat from the findings of the present study, which indicate that the hepatic APR during either of two different bacterial pneumonias requires IL-6 and also TNF-α or IL-1. One possibility to explain this difference is that the biological response to LPS in the lungs differs from that elicited by living bacteria. Alternatively, IL-1 signaling alone may be sufficient to make up for the absence of TNF-α, such that the requirements for these early-response cytokines (each of which activates NF-κB RelA) can be accurately determined only when signaling from both is eliminated. This concept is supported by multiple studies showing a compensatory relationship between TNF-α and IL-1 in response to pulmonary infection (21, 31, 35).

STAT3 and RelA have previously been shown to mediate APP expression in vitro (6, 16, 33). It has also been shown that STAT3 disruption in vivo limits APP expression in response to IL-6 or endotoxemia (4). A limitation of our own in vivo results is that they do not indicate whether STAT3 and RelA are the actual means through which IL-6 and early-response cytokines, respectively, initiate hepatic APP synthesis during pneumonia. To causally link transcription factor activity to cytokine-induced APP expression, we employed an in vitro loss-of-function approach. siRNA-induced knockdown ablated STAT3 and RelA protein expression in the murine hepatocyte line AML12, and loss of either transcription factor prevented cytokine-induced SAA1 mRNA expression. These results suggest that reduced APP synthesis in IL-$6^{-/-}$ and TM mice is a direct result of decreased STAT3 and RelA function. Notably, knockdown of either transcription factor equally decreased and nearly completely eliminated murine SAA1 expression, suggesting that each is essential. This finding supports those of previous promoter-reporter experiments with HepG2 human hepatoma cells, which indicate that human SAA promoter activity is dependent on both RelA and STAT3 (6, 16). Consistent with essential roles for STAT3 and RelA in integrated

responses to microbes in the lungs, STAT3 mutation in humans causes hyper-immunoglobulin E syndrome, characterized by severe lung infections (13, 19, 26), and RelA deficiency in mice renders them susceptible to severe lung infections (2, 35).

The effects of IL-6 and TNF-α/IL-1 deficiencies on the hepatic APR, coupled with the relatively smaller effects of these cytokines on transcription factor activity and other indices of inflammatory signaling in the lungs (20, 29, 42), implicate the absence of lung-liver communication as one possible reason for impaired immunity in these cytokine-deficient mice. However, the true biological significance of the APR during bacterial pneumonia (or in any setting) is unclear (14). Studies of individual APPs have identified relevant immunological roles for multiple APPs. For example, mice lacking a functional gene for LBP have reduced pulmonary inflammation and host defense function in response to intrapulmonary LPS and *Klebsiella pneumoniae*, respectively (8, 9, 12, 22), and SAP-deficient mice have reduced pulmonary and systemic clearance of *S. pneumoniae* (47). Complementarily, exogenous administration and/or overexpression of LBP, SAA, or CRP can be protective against bacterial infection (18, 32, 36). Although these studies demonstrate that particular APPs benefit antibacterial host defense, they do not address the APR specifically or fully. The APR is defined by changes in APP levels compared to baseline rather than by the presence or absence of an APP. Because APP gene targeting eliminates even the often substantive baseline expression of that APP, this strategy elucidates functions of that protein but not the APR. In addition, the APR involves coordinated changes in hundreds of genes (46). Thus, individually targeting select APPs elucidates at best a minuscule fraction of the APR. The true functional significance of the APR remains elusive, and its elucidation is an important research goal that will require new tools or novel approaches.

Based on our current results, we propose that the hepatic APR serves as a downstream and functionally relevant target of inflammatory cytokines synthesized during pneumonia. While the significance of the APR will need to be more specifically and fully defined with future studies, the present data suggest that the APR may be a central aspect of the host defense functions that have been ascribed to TNF-α, IL-1, RelA, IL-6, and STAT3 during pneumonia.

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