The Lung-Liver Axis: A Requirement for Maximal Innate Immunity and Hepatoprotection during Pneumonia

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Abstract

The hepatic acute-phase response (APR), stimulated by injury or inflammation, is characterized by significant changes in circulating acute-phase protein (APP) concentrations. Although individual functions of liver-derived APPs are known, the net consequence of APP changes is unclear. Pneumonia, which induces the APR, causes an inflammatory response within the airspaces that is coordinated largely by alveolar macrophages and is typified by cytokine production, leukocyte recruitment, and plasma extravasation, the latter of which may enable delivery of hepatocyte-derived APPs to the infection site. To determine the functional significance of the hepatic APR during pneumonia, we challenged APR-null mice lacking hepatocyte signal transducer and activator of transcription 3 (STAT3) and v-rel avian reticuloendotheliosis viral oncogene homolog A (RelA) with *Escherichia coli* in the airspaces. APR-null mice displayed ablated APP induction, significantly increased mortality, liver injury and apoptosis, and a trend toward increased bacterial burdens. TNF- α neutralization reversed hepatotoxicity, but not mortality, suggesting that APR-dependent survival is not solely due to hepatoprotection. After a milder (nonlethal) *E. coli* infection, hepatocyte-specific mutations decreased APP

concentrations and pulmonary inflammation in bronchoalveolar lavage fluid. Cytokine expression in airspace macrophages, but not other airspace or circulating cells, was significantly dependent on APP extravasation into the alveoli. These data identify a novel signaling axis whereby the liver response enhances macrophage activation and pulmonary inflammation during pneumonia. Although hepatic acute-phase changes directly curb injury induced by TNF- α in the liver itself, APPs downstream of these same signals promote survival in association with innate immunity in the lungs, thus demonstrating a critical role for the lung-liver axis during pneumonia.

Keywords: inflammation; acute-phase response; liver; pneumonia

Clinical Relevance

These findings support a functionally significant role for liver activation during pneumonia. A better understanding of the cells and signals controlling the lung-liver axis may have clinical implications for patients with or at risk for pneumonia.

Acute lower respiratory infections are a major public health burden, not only in the United States, but also worldwide. They are responsible for the most infection-related

deaths in the United States (1) and, according to the World Health Organization, account for the greatest burden of disease worldwide (1). Since the

discovery of antibiotics in the mid-20th century, the mortality rate from lung infections has remained virtually unchanged (2), warranting more vigorous

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investigation into the biological signals required for effective host defense. These signals, however, can be deleterious themselves, requiring careful consideration of the balance between pro- and anti-inflammatory processes elicited by invading pathogens. Therefore, a better understanding of the inflammatory response to pathogens may ultimately yield useful and safe interventions.

The acute respiratory distress syndrome is most frequently caused by pneumonia (3). Lower respiratory tract infections result in an innate immune response that is characterized by neutrophilic infiltrates, production of cytokines and chemokines, and an accumulation of proteinaceous edema due, in part, to disruption of alveolar barrier integrity (4). As inflammation ensues in the airspaces, the acute-phase response (APR) is stimulated within the liver (5–7) and is defined by significant changes (either increase or decrease) in plasma protein concentrations of acute-phase proteins (APPs) (8). Examples of APPs include serum amyloid A (SAA), serum amyloid P (SAP), LPS binding protein (LBP), complement factors, and fibrinogen, among others (8, 9). Once the APR is initiated, functionally diverse APPs remodel the circulating proteome (8), at which point they can influence any number of events, particularly at sites of plasma extravasation, such as the pneumonic lung. Their individual functions vary from opsonization of bacteria, leukocyte activation, antiprotease and antioxidant activities, to key mediators in the coagulation pathway (9, 10). However, their collective function remains mostly speculative, including that in the context of lung infections.

Previous studies from our laboratory have shown that critical pulmonary host defense cytokines, IL-6, TNF, and IL-1, are necessary for APR induction during bacterial pneumonia, implicating the liver as a functional conduit through which these factors promote innate immunity (5). More specifically, these cytokine pathways were required for activation of signal transducer and activator of transcription 3 (STAT3) and NF- κ B ν -rel avian reticuloendotheliosis viral oncogene homolog A (RelA) transcription factors that drive APP expression in the liver during pneumonia (5, 11, 12). Based on these findings, we generated a hepatocyte-specific STAT3/RelA double-knockout mouse in which the hepatic APR is unchanged at baseline, but virtually ablated in response to

infection (11). Here, we use this APR-null mouse model to more precisely and comprehensively study the causal relationship between hepatic acute-phase changes and the host response to gram-negative lung infection.

Materials and Methods

Mice

Experiments were performed using the previously described APR-null mouse model (11). Hepatocyte STAT3 and RelA were functionally deleted using the CRE-*LoxP* system. Briefly, mice with homozygous floxed alleles for *Stat3* and *RelA* were crossed with mice expressing CRE-recombinase under transcriptional control of an albumin promoter. This system is well established to specifically and effectively drive gene expression in virtually all hepatocytes by 6 weeks of age (13), and we have previously verified loss of STAT3 and RelA liver protein content using this system (11). Protocols were approved by the Institutional Animal Care and Use Committee at Boston University.

Infections and Bronchoalveolar Lavage

Mice were anesthetized by intraperitoneal administration of ketamine (50 mg/kg) and xylazine (5 mg/kg) and infected by intratracheal instillations of the left lobe with indicated doses of *Escherichia coli* (serotype 06:K2:H1; no. 19138; American Type Culture Collection, Manassas, VA), as previously detailed (14). Mice were killed by isoflurane overdose. Lungs were isolated and bronchoalveolar lavage fluid (BALF) was collected as described previously (12, 15). Supernatants from the first wash were used for protein analyses. Pooled cells from all washes were stained (Diff-Quick; Dade-Behring, Deerfield, IL) for differentials.

Anti-TNF- α Studies

Mice were treated with a 100 μ l, 5 mg/ml bolus of either a control IgG (clone HRPN; Bio X-cell, West Lebanon, NH) or an anti-TNF- α antibody (clone XT3-11; Bio X-cell) by tail vein injection immediately before intratracheal *E. coli*.

Statistical Analysis

Statistical analyses were done using GraphPad Prism 6.0 (GraphPad, La Jolla,

CA). Data are represented as means ($\pm$ SEM), except CFU data, which are illustrated as individual values with medians. Single group comparisons were done using Student's *t* test or the Mann-Whitney *U* test. Multiple group comparisons were made using either a one-way ANOVA or two-way ANOVA, followed by a Holm-Sidak test.

Additional methods, including protein measurements, quantitative real-time PCR, alanine aminotransferase (ALT) measurements, liver histology and morphometry, flow cytometry, blood urea nitrogen measurements, Western blotting, and densitometry, are included in the online supplement.

Results

The APR Is Necessary for Survival during Pneumonia

To determine the role of liver-specific acute-phase changes in response to gram-negative bacterial lung infections, we used our previously established mouse model in which floxed *Stat3* and *RelA* alleles are simultaneously targeted by CRE-recombinase under transcriptional control of an albumin promoter (11). This strategy severs the lung–liver axis, ablating gene expression changes in response to all conditions so far tested (11). Control (CRE[−]) or mutant (CRE⁺) mice were infected intratracheally with 10⁶ CFU of *E. coli*. *E. coli* is among several species of gram-negative enterobacteria that are common and relevant causes of nosocomial pneumonia (16–18). In addition, *E. coli* is a well-established model of gram-negative lung infections in mice (12, 19–21), and was particularly desirable in our current study, due to its ability to be consistently titrated for varying degrees of virulence. *E. coli* causes a self-resolving or severe pneumonia, depending on dose, that causes little to no systemic dissemination and can also elicit significant airspace edema. This enables us to effectively study the influence of exudate APPs on localized lung inflammation and host defense without the potentially confounding effects of disseminated bacteria on liver stimulation. Of particular importance to this study is our previous finding that *E. coli* pneumonia induces a robust liver response that occurs independently of dissemination, and is instead dependent on signaling from IL-6,

TNF- α , and IL-1 (5). We observed that CRE⁺ mice succumbed to the infection at a significantly greater rate than their littermate controls, indicating that hepatic transcription is necessary for survival after what is revealed here as a severe and lethal *E. coli* infection in mutant mice (Figure 1A). To determine the direct relationship between survival and the pneumonia-induced APR, we measured liver messenger RNA (mRNA) induction (Figures 1B and 1C) and circulating concentrations (Figures 1D and 1E) of two representative APPs: SAA and SAP.

As anticipated, control (CRE⁻) mice had a large APP response (liver mRNA and circulating protein) that was completely ablated in CRE⁺ mutants (Figures 1B–1E). Circulating concentrations of two other APPs not shown in Figure 1, lipocalin 2 (LCN2) and LBP, followed an identical expression pattern indicative of APR deficiency (data not shown).

We next sought to determine the ability of APPs to extravasate into infected airspaces, where they would be positioned to directly modulate inflammation and host defense, possibly contributing to lethality in

CRE⁺ mice. Both SAA and SAP protein levels in BALF were markedly elevated in response to infection in CRE⁻ mice (Figures 2A and 2D). As observed in the circulation, SAA induction was significantly reduced in CRE⁺ mice (Figure 2A). Lung mRNA induction of SAA1 was unaffected by genotype (Figure 2B), suggesting that differences in BALF protein concentrations were likely due to differences in delivery from the circulation. This is further evidenced by a direct correlation between BALF and serum SAA concentrations at 30 hours of infection (Figure 2C). SAP,

Figure 1. The acute-phase response (APR) is necessary for survival during a severe *Escherichia coli* pneumonia. Mutant (CRE⁺) mice and littermate controls (CRE⁻) were intratracheally inoculated with 1×10^6 CFU of *E. coli*. (A) Survival data were collected at the indicated time points. Significance was assessed by Mantel-Cox test. (B and C) At the same time points, liver messenger RNA (mRNA) was extracted and serum amyloid A (SAA) and serum amyloid P (SAP) transcript fold induction was determined using quantitative RT-PCR (qRT-PCR). (D and E) Serum was collected and concentrations of SAA and SAP were determined by ELISA. * $P < 0.05$ for overall effect of genotype over the indicated time course as determined by two-way ANOVA ($n = 5$ –8 per group). Data are presented as means ($\pm$ SEM).

Figure 2. Intrapulmonary acute-phase protein (APP) content is influenced by systemic acute-phase changes. CRE⁺ mutant mice and littermate controls (CRE⁻) were intratracheally inoculated with 1×10^6 CFU of *E. coli*. (A and D) At the indicated time points, lungs were lavaged and bronchoalveolar lavage fluid (BALF) SAA and SAP concentrations were determined by ELISA. (C and F) A correlation with a linear regression was performed comparing serum SAA or SAP (from Figure 1) and BALF SAA or SAP concentrations (A or D). Individual values represent those determined across both genotypes after 30 hours of infection. (B and E) Lavaged, infected (left) lobes were homogenized and RNA was extracted. SAA1 and SAP transcript fold induction was determined using qRT-PCR. At the same time points, (G) total BALF neutrophils were enumerated and (H) BALF total protein concentrations were measured by bicinchoninic acid (BCA) assay. * $P < 0.05$ for overall effect of genotype over the indicated time course, as determined by two-way ANOVA ($n = 5-8$ per group). Panels lacking asterisks represent data sets in which the overall effect of genotype did not meet the criteria for statistical significance ($P > 0.05$). (I) After 30 hours of infection, serial dilutions of whole-lung homogenates were plated on blood agar plates, incubated overnight at 37°C, and CFUs were determined. P value is reported versus control mice, as assessed by Mann-Whitney test. Data are presented as means ($\pm$ SEM), except CFU data (I), which are illustrated as individual values with medians.

however, showed no difference in airspace protein levels between genotypes, nor was the correlation between BALF and serum SAP concentrations significant (Figures 2D and 2F). Interestingly, other APPs measured in the airspaces, LCN2 and LBP, followed the same pattern as SAP (data not shown), indicating that lung APP content is only selectively dependent on hepatic responses after a lethal *E. coli* infection. In addition, BALF recruited neutrophil

numbers (Figure 2G) and total protein concentrations (Figure 2H) were determined as indices of injury and inflammation. In both genotypes, the effect of pneumonia was substantial yet equivalent, suggesting that mortality in CRE⁺ mice is not directly attributable to changes in acute pulmonary inflammation. However, there was a trend toward increased pulmonary bacterial burdens in mutant mice after 30 hours of infection (Figure 2I). Due to a potential

survival bias, bacterial burdens were not measured at later time points. Nevertheless, the trend of increasing bacterial burdens in APR-null mice indicates a possible role for the hepatic APR in promoting host defense during a lethal *E. coli* infection.

APR Deficiency Causes Liver Injury in Lethally Challenged Mice

Although CRE⁺ mice showed a trend toward increased lung bacterial burdens,

the observed increase was relatively modest, warranting further investigation into the potential cause of death in mice lacking a hepatic APR. It has been shown that NF- κ B RelA deficiency results in embryonic lethality, due to liver degeneration and hepatotoxicity (22–26). Targeted disruption of RelA in the liver also causes hepatotoxicity in direct response to systemic TNF or LPS, but the importance of inducible RelA (i.e., that observed during the APR) has never been explored in the context of lung infections. We hypothesized that pneumonia-induced mortality is attributable, at least in part, to liver injury in CRE⁺ mice, which lack both RelA and STAT3. To test this, we measured serum ALT levels as a sensitive metric of hepatotoxicity. Although pneumonia had no effect on liver injury in control mice, CRE⁺ mice had significantly increased levels of serum ALT over the course of infection (Figure 3A). This finding was confirmed by histological evidence of hepatocyte death in the absence of STAT3 and RelA (Figure 3D). Interestingly, liver TNF- α mRNA induction was significantly, albeit modestly, higher in CRE⁺ mice (Figure 3B). This finding suggests a cycle whereby both the expression of and response to TNF- α is altered in mutant mice, resulting in liver damage and toxicity. However, the observed difference in TNF- α mRNA was not sufficient to manifest in circulating TNF- α protein changes in the same mice (Figure 3C), making the role of differential TNF- α mRNA expression unclear at present.

To determine if this injury was dependent on TNF- α , which has previously been linked to hepatotoxicity in absence of RelA (22, 26–29), we treated mice with 5 mg/ml of either a neutralizing TNF- α antibody or an IgG control antibody by tail vein injection immediately before intratracheal *E. coli*. As expected, at 24 hours after infection, CRE⁺ mice treated with the control IgG antibody had high levels of circulating ALT (Figure 3F), consistent with our initial results (Figure 3A). In contrast, CRE⁺ mice treated with an anti-TNF- α antibody had serum ALT concentrations that were identical to baseline levels observed in CRE[−] mice (Figure 3F). Histology also confirmed the protective effect of the neutralizing TNF- α antibody, with histopathology far more reflective of CRE[−] mice than that of untreated CRE⁺ littermates (Figure 3D, *top panels*). Lung and blood bacterial burdens

were also measured at 24 hours to confirm that the anti-TNF- α antibody had no adverse effects on host defense, which would confound interpretations of host outcomes. Bacterial killing was unaffected by TNF- α neutralization, regardless of genotype (data not shown).

To determine whether liver injury was specifically linked to apoptosis in APR-null mice, we determined protein levels of cleaved caspase-3, the effector caspase for the apoptotic pathway, 24 hours after intratracheal *E. coli* (Figures 3E and 3G). Densitometric analysis of immunoblots revealed significantly higher levels of cleaved caspase-3 in the absence of hepatocyte STAT3 and RelA that were almost completely reversed with TNF- α neutralization (Figure 3G), suggesting that liver injury was at least partly attributable to TNF-induced apoptosis. These data are consistent with previous reports indicating RelA-mediated protection from TNF-induced liver apoptosis (26). In addition to hepatocyte loss (i.e., injury), hepatocyte replacement could also be compromised in APR-null mice, as supported by studies showing STAT3-dependent liver regeneration (27, 28, 30). To address this, we stained liver sections for Ki67, a nuclear antigen used to detect proliferating cells (Figure 3D, *lower panels*). As expected, we observed few positively stained cells in the CRE[−] control group 24 hours after intratracheal *E. coli*, but there was a marked increase in the percentage of Ki67⁺ cells in IgG-treated CRE⁺ mice that was partially reversed upon TNF neutralization (Figure 3H). Thus, these data do not support a defect in hepatocyte turnover as a cause of liver injury in APR-null mice. Rather, cell proliferation appeared to be downstream of hepatotoxicity, with the greatest amounts of Ki67 staining in areas of the greatest liver injury.

To determine whether liver injury was the sole cause of mortality in mutant mice, we measured survival in APR-null mice treated with either the control or TNF- α antibody, the latter of which reversed all metrics of hepatotoxicity. Surprisingly, mice treated with the neutralizing TNF- α antibody, which had normal liver function and baseline levels of hepatic injury, exhibited no improvement in survival (Figure 3I), suggesting that the physiological significance of the APR extends beyond inducible hepatoprotection. As another insight into the potential cause

of mortality in the APR-null mice, we measured blood urea nitrogen levels as an indicator of kidney function, and there were no differences between genotypes (Figure 3J). The mechanism by which the APR prevents mortality during a severe pneumonia remains speculative.

Liver-Derived Proteins Are Necessary to Promote Pulmonary Inflammation during a Nonlethal *E. coli* Pneumonia

Due to lethality differences and a potential survival bias introduced to the results described in the previous figures, we inoculated mice with a milder dose of *E. coli* (4×10^5 CFU) to more carefully determine the impact of liver acute-phase changes on lung inflammation and host defense. At this dose, we observed only rare mortality with no significant difference between genotypes. The magnitude of the APR itself, as determined by systemic SAA and SAP expression (liver mRNA and circulating protein content), was much milder in response to the nonlethal challenge compared with that described previously in Figures 1 and 2, and remained blunted in mutant mice (Figures 4A–4D). We also observed an increase in airspace SAA and SAP concentrations that was completely absent in CRE⁺ mice (Figures 5A and 5B). Again, this disparity between genotypes was not due to differential local expression of these proteins, as there was no significant decrease in lung mRNA induction between genotypes (Figures 5C and 5D). In association with decreased APP levels, we also observed significantly reduced total protein concentrations and a trend toward less neutrophil recruitment in the BALF of CRE⁺ mice (Figures 5E and 5F, respectively). This possible decrease in airspace neutrophil numbers was not due to greater cell death, as 7-aminoactinomycin D (7AAD)⁺ (dead) neutrophils were unchanged in BALF from APR-null mice 15 hours after intratracheal *E. coli* (Figure 5G). Lung bacteria were virtually cleared in response to this milder inoculum, with no difference observed between genotypes (data not shown). In accordance with decreased pulmonary inflammation, the majority of cytokines measured (IL-6, granulocyte colony-stimulating factor [G-CSF], CXCL1, TNF- α , CXCL2, IL-10, and leukemia inhibitory factor) were significantly decreased in CRE⁺ mice compared with littermate controls after 15 hours of

Figure 3. APR-null mice have severe liver injury. Mutant (CRE⁺) mice and littermate controls (CRE⁻) were infected with 1×10^6 CFU of *E. coli*. At indicated time points, serum and livers were collected. (A) Serum alanine aminotransferase (ALT) levels were measured via colorimetric assay, (B) liver TNF- α mRNA transcript induction was measured by qRT-PCR, and (C) serum concentrations of TNF- α were determined by multiplex bead array. To determine the influence of TNF- α on liver injury, mice were injected intravenously by tail vein with 5 mg/ml of either a control IgG or anti-TNF- α antibody, followed immediately by intratracheal *E. coli* (1×10^6 CFU). (D) At 24 hours after infection, histopathology was assessed by hematoxylin and eosin (H&E)-stained liver sections, and cell turnover was assessed by immunohistochemical staining for Ki67. Black arrows indicate areas of acute liver injury

infection (Figure 5H). mRNA induction of IL-6, TNF- α , CXCL2, CXCL1, and G-CSF were also measured in lung homogenates (Figure 5I). Although only IL-6 and CXCL1 were significantly decreased in mutant mice, CXCL2 and G-CSF also trended toward decreases in mutant mice, corroborating airspace protein levels. Whereas cytokines known to be downstream of IL-17 during pneumonia (e.g., CXCL1/2 and G-CSF) (31) were decreased in mutant mice, IL-17 itself remained unchanged (Figure 5H), supporting the observed phenotype as a T helper type 17-independent phenomenon. These data indicate that the hepatic transcriptional responses facilitate acute pulmonary inflammation, as revealed by a milder, nonlethal pneumonia.

The Liver APR Facilitates Cytokine Expression by Airspace Macrophages

The data above suggest that liver-derived APPs enhance immune responsiveness, demanding a more complete understanding of where this process occurs. For instance, it is plausible that products downstream of the liver APR act upon circulating cells in the blood, recruited and/or resident cells in the infected airspaces, or perhaps both. To determine the effect of the APR on cytokine expression in circulating, recruited, and lung-resident myeloid-derived leukocytes, we used fluorescence-activated cell sorting to isolate cells from blood (Figure 6A) and BALF (Figure 7A), and determined cytokine induction using quantitative RT-PCR. Both circulating monocytes (CD45⁺/7AAD⁻/CD115⁺/CD11b⁺) (32) and neutrophils (CD45⁺/7AAD⁻/Ly6G⁺/CD11b⁺) showed no differences in cytokine induction between genotypes at 15 hours after infection (Figures 6B and 6C). In these cells, cytokine expression varied from undetectable (monocyte CXCL1), to increased (monocyte IL-6 and IL-1 β ; neutrophil CXCL1), to

decreased (neutrophil IL-1 β). In contrast, induction of IL-6 and CXCL1 in airspace macrophages (CD45⁺/7AAD⁻/F4/80⁺/Ly6G⁻) from CRE⁺ mice was significantly decreased after 15 hours of infection compared with macrophages from CRE⁻ control mice (Figure 7B). Induction of IL-1 β was unaffected, which was consistent with unchanged BALF protein concentrations (Figures 5H and 7B). This cytokine induction phenotype was unique to airspace macrophages, as airspace neutrophils (CD45⁺/7AAD⁻/Ly6G⁺/F4/80⁻) showed no differences in gene expression for the cytokines measured (Figure 7C). To determine if the changes in cytokine induction required APP delivery into the airspaces (via serum extravasation), we measured cytokine induction at 1.5 hours after infection, a time preceding loss of alveolar barrier integrity, and thus serum protein (and APP) extravasation. Cytokine mRNA was induced, but was equivalent between genotypes at this early point of infection (Figure 7B), suggesting that changes in cytokines are not intrinsic to local cells, but rather, are due to differences in APP exposure from extravasated serum. Taken together, the data imply a role for airspace macrophage cytokine induction by the APR to induce pulmonary inflammation during pneumonia.

Discussion

Our current results are the first, to our knowledge, to indicate a direct and significant influence of hepatic responses on pulmonary inflammation and host outcome during pneumonia. After a higher inoculum of *E. coli*, hepatocyte STAT3 and RelA are necessary for survival, maximal bacterial clearance, and liver protection. We have shown that this response, likely via RelA, is required to counter TNF-dependent toxicity in the liver itself. On

the other hand, our data after a milder, nonlethal inoculum also suggest an extrahepatic role of the APR, whereby liver-derived products promote macrophage cytokine expression and innate immunity in the lungs.

To date, there have been limited studies investigating the functional role of the hepatic APR during inflammation, yet most suggest an antiinflammatory role (33–35). Sakamori and colleagues (35) showed that inflammatory cytokine production was amplified in hepatocyte STAT3^{-/-} mice after intraperitoneal LPS injection, suggesting that STAT3-dependent APPs limit excessive inflammation during sepsis. Renckens and colleagues (33, 34) demonstrated that a pre-existing APR reduces subsequent immune responses to pulmonary challenges with *Klebsiella pneumoniae* or *Acinetobacter baumannii*. Although this is seemingly contradictory to the present results in which the APR supports cytokine synthesis and inflammation, it is notable that the models involve very different scenarios. Renckens and colleagues induced a premature APR by injection of turpentine before lung infection, whereas we interrogated the influence of hepatic responses elicited by the pneumonia itself. Moreover, the APR being studied here was exclusively being induced in hepatocytes downstream of STAT3 and RelA, as opposed to any and all cells responding to systemic turpentine administration. Recently, our laboratory has also shown that the APR is necessary for survival during a gram-positive *Streptococcus pneumoniae* infection (11). Indeed, these studies and others have now revealed over 1,000 gene changes and numerous biological processes in the liver that are altered during pneumonia, any or all of which could influence disease outcome (6, 11, 36). In those studies, CRE⁺ mice showed decreased complement deposition and opsonophagocytosis of

Figure 3. (Continued). (*top panels*) or a Ki67⁺ cell (*bottom panels*). Representative images are shown for livers collected from at least three individual mice per group. (E) After 24 hours of infection, protein was extracted from liver homogenates and subjected to immunoblot analysis for cleaved caspase-3 expression. Each band represents data from an individual mouse. One of two representative blots with livers from four independent experiments is shown. (F) Serum ALT levels were determined 24 hours after infection as an indicator of the amount of liver injury present. (G) A densitometric analysis of band intensity was performed on immunoblots for cleaved caspase-3. The ratio of cleaved caspase-3/pan actin was compared with the control CRE⁻ group for each sample to calculate the percent control changes in band intensity ratios. (H) A blinded morphometric analysis was performed on liver sections stained for Ki67 from D. Data are expressed as the percentage of all cells that stained positively for Ki67. (I) Survival data were collected through 48 hours of infection. (J) Blood urea nitrogen (BUN) levels were determined at the indicated time points as a metric of kidney function. (A, B, C, and J) **P* < 0.05 for overall effect of genotype, as determined by two-way ANOVA (*n* = 4–9 per group). (F, G, and H) **P* < 0.05 versus control, CRE⁻ group was determined by a one-way ANOVA followed by a Holm Sidak's test ([F and H] *n* = 6–13; [G] *n* = sections from three mice per group, with at least six fields of view analyzed). (I) Significance was assessed by a Mantel-Cox test. Data are presented as means ($\pm$ SEM).

Figure 4. Mildly infected mice have an ablated APR. CRE⁺ mice or littermate controls (CRE⁻) were instilled intratracheally with 4×10^5 CFU of *E. coli*. (A and B) At the indicated time points, liver mRNA was extracted and SAA1 and SAP transcript fold induction was determined using qRT-PCR. (C and D) Serum was collected, and concentrations of SAA and SAP were determined by ELISA. * $P < 0.05$ for overall effect of genotype over the indicated time course, as determined by two-way ANOVA ($n = 5$ –11 per group). (B and D) The effect of genotype throughout the 30-hour observation period did meet the criteria for statistical significance ($P > 0.05$). Data are presented as means ($\pm$ SEM).

pneumococcus, leading to increased bacteremia, suggesting that one function of the APR is to limit dissemination of infection. However, those studies were not specifically empowered to observe effects of the APR in the lungs themselves, due to the highly virulent strain of pneumococcus (11). Using phenotypically distinct inocula of *E. coli* in our current study has enabled us to more precisely identify novel functions of the APR both inside and outside of the lungs during pneumonia.

CRE⁻ mice infected with a severe dose of *E. coli* had a robust APR and high concentrations of circulating APPs, and this response was ablated in the absence of hepatocyte STAT3 and RelA. Interestingly, airspace concentrations of SAA in CRE⁻ mice were significantly increased and reflected those in the circulation, but there was no change from baseline in CRE⁺ mice. There was no difference in mRNA induction in whole-lung homogenates between genotypes, suggesting that SAA in the airspaces resulted from plasma extravasation, which is also evident by a significant correlation between serum and BALF SAA levels. Other APPs tested (SAP, LBP, and LCN2), however, showed no

changes in BALF concentrations between genotypes. This is possibly due to the kinetics of circulating APP changes versus lung barrier integrity. For instance, accumulation of SAP in the airspaces through 24 hours may solely represent equivalent extravasation in both genotypes of serum SAP, which remains mostly unchanged until 30 hours, a point at which injury and protein influx may be receding. SAA, on the other hand, increases more rapidly in the circulation of WT mice, perhaps allowing for earlier representation of this difference in the airspaces. Alternatively, different APPs may have diverse abilities to extravasate into the lungs during infection. There have been multiple reports about active uptake of various serum proteins by alveolar epithelial cells (including albumin and ferritin) to transport them into the alveolar space, and SAA could be another serum protein, the BALF concentrations of which are aided by such transport mechanisms (37–41). Although SAP expression is restricted to the liver, LBP and LCN2 are produced in the lungs by alveolar epithelial cells and neutrophils (42–46), which may contribute to BALF concentrations. Overall, the

relationship between circulating and lung APP content appears to be complex and selective, with multiple factors dictating protein distribution. It is important to note, however, that our model is engineered to specifically indicate the consequences of liver-dependent changes, and these hepatic changes are ultimately responsible for the observed phenotypes.

After a higher inoculum of *E. coli*, hepatocyte STAT3 and RelA are necessary for liver protection. We have shown that this response, likely via RelA, is required to counter TNF-dependent toxicity in the liver itself. CRE⁺ mice infected with a severe *E. coli* pneumonia also had a greater mortality rate than littermate controls, despite equivalent lung injury and inflammation between genotypes. It has long been recognized that mice with complete RelA deficiency are embryonic lethal due to TNF- α -dependent liver injury (22, 23, 26–29). There have also been reports linking STAT3 and liver injury in various models (47–50). In our own studies, mice lacking both transcription factors suffered from TNF- α -dependent liver injury, likely due to dysregulated apoptosis that was reversed by TNF- α neutralization. However, liver regeneration, which has specifically been shown to rely on STAT3 (30), does not appear to be defective in mutant mice during pneumonia, as Ki67 staining was actually increased in the absence of this transcription factor (along with RelA). Interestingly, liver injury was not responsible for the increase in mortality, as hepatoprotection via TNF- α blockade was insufficient to reduce mortality in mutant mice. In addition, kidney function was normal in both groups; thus, the cause of mortality in mutant mice remains elusive. One possibility is that combined changes in both local lung innate immunity and liver injury are together contributing to mortality, whereas neither alone is great enough to impair survival. The degree to which these outcomes and/or other manifestations in the absence of an intact liver response contribute to mortality during pneumonia remains to be determined.

Our milder infection protocol revealed that the APR promotes lung inflammation. CRE⁺ mice lacking the APR have a significant reduction in BALF total protein and cytokine concentrations. These findings were directly associated with marked decreases in BALF APP

Figure 5. The APR facilitates pulmonary inflammation and cytokine production during a mild, nonlethal pneumonia. Mutant (CRE⁺) mice and littermate controls (CRE⁻) were infected with 4×10^5 CFU of *E. coli*, and lungs were lavaged at the indicated time points. (A and B) BALF SAA and SAP concentrations were determined by ELISA. (C and D) Lavaged, infected (left) lobes were homogenized and RNA was extracted. SAA1 and SAP transcript fold induction were determined using qRT-PCR. At the same time points, (E) BALF total protein concentrations were measured by BCA assay and (F) total BALF neutrophils were enumerated. * $P < 0.05$ for overall effect of genotype over the indicated time course, as determined by two-way ANOVA ($n = 5-11$ per group). The effect of genotype throughout the 30-hour observation period did meet the criteria for statistical significance in (C) ($P > 0.05$). (G) Cells from the BALF were collected and stained for total (CD45⁺/Ly6G⁺) or dead (CD45⁺/7-aminoactinomycin D [7AAD]⁺/Ly6G⁺) neutrophils. Percentages of dead neutrophils were calculated from total numbers of neutrophils. Significance versus control group was assessed by Student's *t* test ($n = 3-5$ per group). (H) At 15 hours after infection, BALF cytokine protein concentrations were measured by a multiplex bead array (Luminex). (I) Lavaged, infected (left) lobes were homogenized, RNA was extracted, and cytokine mRNA induction was measured by qRT-PCR. * $P < 0.05$ versus control, assessed by Student's *t* test ($n = 10-11$ per group). CXCL, chemokine (C-X-C) motif ligand; ns, not significant. Data are presented as means ($\pm$ SEM). G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte/macrophage colony-stimulating factor.

Figure 6. Cytokine induction in circulating cells is not affected by the APR. Mutant (CRE⁺) mice and littermate controls (CRE⁻) were infected for 0, 1.5, or 15 hours with 4×10^5 CFU of *E. coli*. Blood was collected, red blood cells were lysed, and cells were stained for fluorescence-activated cell sorting. (A) Circulating monocytes were gated on CD45⁺/7AAD⁻/CD115⁺/CD11b⁺, and circulating neutrophils were gated on CD45⁺/7AAD⁻/Ly6G⁺/CD11b⁺. RNA was extracted, and qRT-PCR was performed to determine induction of IL-6, CXCL1, and IL-1β in both circulating monocytes (B) and neutrophils (C). The overall effect of genotype was determined by two-way ANOVA ($n = 3-6$ per group). Data are presented as means ($\pm$ SEM).

concentrations, without such differences in lung APP mRNA. This, along with the hepatocyte-specific nature of our mouse model, strongly suggests that liver APP gene expression is a primary determinant of APP concentrations in injured airspaces. One curious observation to note, however, is that significant differences in lung APP content (15 h) precede what appear to be little (SAA) to no (SAP) changes in blood protein. This discrepancy between circulating and lung APP content could be related to the timing of the measurements, which were taken after 15 hours. Circulating APP concentrations could be significantly different between genotypes at

earlier time points. Alternatively, early changes in airspace APP concentrations could have been secondary to reduced inflammation in mutant mice, such that reduced levels of SAA and SAP resulted from a global decrease in protein flux. Nonetheless, pulmonary APP content was inherently hepatocyte STAT3 and RelA dependent, as the presence of these two transcription factors was the only difference between genotypes, suggesting that liver-derived APPs are necessary for maximal lung inflammation.

We aimed to identify cell types in the lungs directly targeted by acute-phase changes in the liver by measuring cell-

specific cytokine induction in airspace macrophages and neutrophils, both of which represent important cytokine sources in the lungs (51–53). Excitingly, we found an altered cytokine profile in airspace macrophages, but not in circulating monocytes or neutrophils from any site. The macrophage mRNA changes were consistent with the results observed in whole-lung measurements. To more conclusively determine whether plasma, and hence APP, extravasation was required for APR-dependent cytokine synthesis in macrophages, we also measured this outcome at 1.5 hours of infection, a time sufficient to detect increased cytokine

Figure 7. Airspace macrophages are responsible for APP-induced cytokine changes in mildly infected mice. Mutant (CRE⁺) mice and littermate controls (CRE⁻) were infected for 0, 1.5, or 15 hours with 4×10^5 CFU of *E. coli*. Lungs were lavaged, and cells were collected and stained for fluorescence-activated cell sorting. (A) Airspace macrophages were gated on CD45⁺/7AAD⁻/F4/80⁺/Ly6G⁻, and airspace neutrophils were gated on CD45⁺/7AAD⁻/Ly6G⁺/F4/80⁻. RNA was extracted, and qRT-PCR was performed to determine induction of IL-6, CXCL1, and IL-1β in airspace macrophages (B). Expression of IL-6, CXCL1, and IL-1β was determined relative to CRE⁻ controls in airspace neutrophils (C), as there was no recruitment of neutrophils to the airspaces at baseline or 1.5 hours after infection. (B) * $P < 0.05$ for overall effect of genotype or versus control mice in the same group, as determined by two-way ANOVA followed by Holm Sidak's test ($n = 3$ –6 per group). (C) Significance was assessed by Student's *t* test ($n = 10$ –11 per group). Data are presented as means ($\pm$ SEM).

responses, but preceding alveolar edema. At this early time point, macrophage responses were identical between genotypes, supporting the conclusion that extravasated proteins facilitate macrophage cytokine production in an APR-dependent manner. Interestingly, no such effect was observed in circulating cells, suggesting that the reprogramming of macrophages by acute-phase serum occurs strictly in the alveolar compartment. The specific soluble mediators responsible for linking the hepatic APR to macrophage cytokine responses remain unknown. Multiple APPs can bind and activate macrophages, including, but not limited to, SAA (54–56), SAP (57), LBP (58), and C-reactive protein (59, 60). It is also possible that the net effect of acute-phase changes is determined by a combination of relatively modest influences from numerous factors. Thus, it is possible, if not likely, that our findings cannot be traced to a single protein.

However, the data provide evidence for a cell-specific, functional role of the APR in facilitating macrophage function during pneumonia.

Our data are the first to show that APR signals intrinsic to the liver are hepatoprotective, whereas extrahepatic consequences of the APR are necessary to promote host defense and pulmonary inflammation during pneumonia. Although promoting inflammation can be detrimental in the context of acute lung injury, the liver response to pneumonia is also prodefense and, importantly, prosurvival. This protective nature of the liver is consistent with our own studies (11) and those of others during infection (6, 61). For instance, Weber and colleagues (6) showed that liver-derived cholesterol attenuates the pathogenesis of a common *Streptococcus* virulence factor, pneumolysin. Sander and colleagues (61) observed that hepatic STAT3-dependent

signals (SAA in particular) could attenuate mortality during polymicrobial sepsis, perhaps through mobilization of myeloid-derived suppressor cells. Although neither of these particular examples is likely to explain our own results with gram-negative pneumonia, they importantly put forth additional evidence that liver function is indispensable for modulating the pathogenesis of acute infection. Overall, our results indicate that APPs serve critical roles extending beyond their common use as biomarkers. A better understanding of the lung–liver axis will provide valuable insights into potential therapeutic or diagnostic targets for clinical intervention in patients with or at risk for pneumonia. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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