

## SUPPLEMENTAL DATA

### Supplemental Methods

#### *Immunoblots*

STAT3 and RelA protein content in livers, lungs, and spleens was assessed by immunoblots as described previously (1, 2) using antibodies purchased from Cell Signaling Technology. Primary antibodies for both proteins were raised against peptides encoded within the floxed portions of *Stat3* and *Rela*, such that only full-length, non-floxed versions of either protein were detectable.

#### *qRT-PCR*

Total liver RNA was isolated and purified using Trizol reagent (Invitrogen) and the RNeasy mini kit (Qiagen), respectively. Real-time RT-PCR was performed on 10 ng of isolated RNA using the TaqMan RNA-to-CT One-Step Kit and the StepOnePlus Real-time PCR system. (Applied Biosystems). Primer and probe sequences were determined using the CLC DNA Workbench (CLC Bio) or Beacon Designer software (Premier Biosoft International). Sequences for SAA1, SAP, LBP, and 18s rRNA are reported elsewhere (1, 3), with the remaining sequences as follows: SAA2 = Fwd 5'-AAGACCCCAATTACTACA-3'; Rev 5'-TAGATAGGAAGCCCAGAC-3'; TaqMan probe 5'-CCCACCCTCAGGACCCCA-3'; LCN2 = Fwd 5'-ATATGCACAGGTATCCTC-3'; Rev 5'-AAACGTTTCCTTCAGTTCA-3'; TaqMan probe 5'-CCACCACGGACTACAACCA -3'. All results were normalized to the quantity of 18s rRNA within each sample and expressed as fold-induction relative to a control group indicated in each figure (4).

#### *Cytokine and Acute phase protein quantification*

SAA and SAP plasma concentrations were quantified by ELISA using kits purchased from Immunology Consultants Laboratory. The SAA ELISA detects and does not differentiate among the multiple SAA isoforms. LCN2 plasma levels were determined by a DuoSet ELISA development system from R&D Systems. For lung protein measurements, homogenates were prepared as previously described (5), and TNF $\alpha$ , IL-6, IL-1 $\beta$ , KC, and G-CSF concentrations were determined using a Bio-Plex Pro Mouse Cytokine Assay (BioRad) and a LiquiChip 200 Workstation (Qiagen).

#### *Microarray*

Total liver RNA was isolated and purified as described above for qRT-PCR. Liver RNA quality was confirmed using an Agilent bioanalyzer and microarrays were performed for each sample using Affymetrix GeneChip Mouse Gene 1.0 ST Arrays. Following data normalization, filtering, and preprocessing, fold changes were calculated between experimental groups. Clustering and heat maps were generated using the Cluster 3.0 and TreeView software ([http://rana.lbl.gov/eisen/?page\\_id=42](http://rana.lbl.gov/eisen/?page_id=42)). Gene Ontology (GO) analysis was performed using the online Database for Annotation, Visualization and Integrated Discovery (DAVID) (6). GO-BP (biological processes) was the database used to determine significantly represented gene categories. The list of potential acute phase

proteins comprised genes belonging to one or more of the following categories: GO:0005615 (extracellular space); GO:0005576 (extracellular region); GO:0044421 (extracellular region part); UniProt: secreted; and UniProt: signal.

### *Bacteriology*

Viable *S. pneumoniae* were quantified in diluted lung tissue homogenates by enumerating colony forming units on 5% sheep blood agar plates as previously described (5).

### *Histology*

Isolated lungs were fixed with 6% glutaraldehyde at 23 cm H<sub>2</sub>O pressure for generation of hematoxylin/eosin (H&E) stained sections. Liver H&E sections were prepared from tissues fixed in 10% formalin.

### *S. pneumoniae viability assay*

Kanamycin-resistant *S. pneumoniae*-Xen10 (Caliper LifeSciences), possessing a stable copy of a modified *Photobacterium luminescens* operon, was resuspended in DMEM supplemented with kanamycin at a concentration of  $2.5 \times 10^6$  CFU/ml. Serum was collected from CRE- and CRE+ mice following 24 hrs of *S. pneumoniae* infection and added to growth medium at concentrations of 0%, 1.25%, 5%, or 20%. 100 $\mu$ l of the bacterial suspension was grown in a 96-well plate at 37°C in an atmosphere containing 5% CO<sub>2</sub>, and luminescence was measured every hour over a 12-hr time course. Each measurement represents that from bacteria incubated with serum from a single mouse.

### *Opsonophagocytosis*

*S. pneumoniae* were labeled with Vybrant DiI labeling solution (Invitrogen). Sera were collected from mice 0 or 24 hrs after i.t. *S. pneumoniae* and diluted in DMEM at varying concentrations to achieve the lowest concentration resulting in  $\geq 25\%$  cells fluorescent following opsonophagocytosis. To do this, fluorescent *S. pneumoniae* were opsonized in serum for 30 min, after which the bacteria/serum mixture was added directly to J774A.1 cells (ATCC) for an additional 1 hr at an MOI of 20 in ultra low-adherence plates. Cells were fixed in 1% formalin and fluorescence per cell was determined using flow cytometry. Bacteria-cell association was eliminated (0% cells fluorescent) when no sera was involved. Furthermore, bacteria-cell association was likely reflective of internalized bacteria since parallel cultures performed at 0°C for all experimental conditions failed to increase J774A.1 cell fluorescence over baseline non-opsonized conditions.

### *C3 deposition*

Serum was collected from mice 24h after i.t. *S. pneumoniae*. Opsonization was achieved by incubating *S. pneumoniae* for 45 min in buffer (HBSS++ containing 2%BSA) supplemented with 1% control or mutant mouse serum. Bacteria were then washed and resuspended in buffer containing FITC-conjugated anti-mouse C3 (1:300; MBL International) for 20 min. The percentage of positively labeled bacteria was determined using flow cytometry. Background fluorescence was based on that exhibited by non-opsonized bacteria, which was identical to that achieved in the absence of antibody.

## Supplemental Tables

Acute Phase Protein	Gene (of 85 total)	Significance of Infection	
		CRE-	CRE+
Alpha 1-antichymotrypsin	Serpina3n	2.40E-04	0.141
	Serpina3m	0.033	0.607
Alpha-1 acid glycoprotein	Orm1	0.001	0.465
	Orm2	0.007	0.132
	Orm3	0.045	0.440
Alpha-2-antiplasmin	Serpinf2	0.026	0.308
C4b-binding protein	C4bp	0.012	0.344
Cxcl1	Cxcl1	2.90E-04	0.691
Factor B	Cfb	0.025	0.357
Fibrinogen	Fgb	0.005	0.695
	Fga	0.010	0.908
	Fgg	0.030	0.568
	Fgl1	0.037	0.777
Haptoglobin	Hp	0.003	0.125
Hemopexin	Hpx	0.009	0.828
Inter-alpha-trypsin inhibitor	Itih3	0.005	0.696
	Itih4	0.008	0.699
Lipocalin 2	Lcn2*	0.002	0.041
Lipopolysaccharide binding protein	Lbp	0.004	0.079
Serum amyloid A	Saa1*	1.73E-05	3.05E-04
	Saa2*	2.41E-05	6.84E-05
	Saa3	0.001	0.236
	Saa4	0.035	0.581
Serum amyloid P	Apcs	0.008	0.718
Transthyretin	Ttr	0.008	0.225

**Table S1:** Acute phase protein genes significantly affected by pneumonia in control CRE- mice are shown as compared to the same transcripts in CRE+ mutant mice lacking STAT3 and RelA in hepatocytes. Significant changes were exclusive to CRE- mice, with the exception of those transcripts denoted by asterisks.

**Table S2**

<b>Gene</b>	<b>Protein</b>
AMIGO3	amphoterin-induced protein 3
APOA2	apolipoprotein A-II
APOA4	apolipoprotein A-IV
APOC2	apolipoprotein C-II
APOF	apolipoprotein F
APOL7A	apolipoprotein L 7a
APON	apolipoprotein N
ARSB	arylsulfatase B
B4GALT1	beta-1,4-galactosyltransferase 1
CALR	calreticulin
CAR14	carbonic anhydrase 14
CCDC134	coiled-coil domain containing 134
CDH1	cadherin 1
CDH22	cadherin 22
CES1	carboxylesterase 1
CGREF1	cell growth regulator with EF hand domain 1
CHRD	chordin
CLEC14A	C-type lectin domain family 14, member a
CMTM6	CKLF-like MARVEL transmembrane domain containing 6
CNPY3	protein canopy homolog 3
COL15A1	collagen, type XV, alpha 1
CPB2	carboxypeptidase B2
CRELD2	cysteine-rich with EGF-like domains 2
CTBS	di-N-acetylchitobiase
DCBLD2	discoidin, CUB and LCCL domain containing 2
DDOST	dolichyl-di-phosphooligosaccharide-protein glycotransferase
DHCR24	24-dehydrocholesterol reductase
DNAJB11	DnaJ (Hsp40) homolog, subfamily B, member 11
DNASE2B	deoxyribonuclease II beta
DPP4	dipeptidylpeptidase 4
DSG1A	desmoglein 1 alpha
DSG2	desmoglein 2
EDEM3	ER degradation enhancing mannosidase alpha-like 3
EFNA1	ephrin A1
EFNA3	ephrin A3
ELTD1	EGF, latrophilin seven transmembrane domain containing 1
ENOX2	ecto-NOX disulfide-thiol exchanger 2
ERO1L	ERO1-like protein alpha
ERP27	endoplasmic reticulum protein 27
F13B	coagulation factor XIII, beta subunit
F3	coagulation factor III
FAS	Fas
FAT3	protocadherin Fat 3
FGF21	fibroblast growth factor 21
FGFRL1	fibroblast growth factor receptor-like 1
FZD8	frizzled homolog 8
GABRB2	gamma-aminobutyric acid (GABA) A receptor, subunit beta 2
GALNT2	polypeptide N-acetylgalactosaminyltransferase 2
GAS6	growth arrest specific 6
GDF9	growth differentiation factor 9

**Table S2 (Contd)**

<b>Gene</b>	<b>Protein</b>
GFOD2	glucose-fructose oxidoreductase domain containing 2
GFRA1	glial cell line derived neurotrophic factor family receptor alpha 1
GHR	growth hormone receptor
GM2A	GM2 ganglioside activator protein
GPI1	glucose phosphate isomerase 1
GPR110 *	G protein-coupled receptor 110
GPR126	G protein-coupled receptor 126
GPR180	G protein-coupled receptor 180
GREM2	gremlin 2
GRN	granulin
H6PD	hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase)
HAPLN4	hyaluronan and proteoglycan link protein 4
HDLBP	high density lipoprotein (HDL) binding protein
HMCN1	hemicentin 1
HMG1L1	high-mobility group protein 1-like 1
HMGB1	high mobility group box 1
HSD17B6	hydroxysteroid (17-beta) dehydrogenase 6
HSPA5	heat shock protein 5
HYOU1	hypoxia up-regulated 1
ICAM1	intercellular adhesion molecule 1
IGFBP7	insulin-like growth factor binding protein 7
IL13RA2	interleukin 13 receptor, alpha 2
IL17RA	interleukin 17 receptor A
IL1R1	interleukin 1 receptor, type I
IL23A	interleukin 23, alpha subunit p19
IL4RA	interleukin 4 receptor, alpha
INHBE	inhibin beta E
INSL3	insulin-like 3
ITGA8	integrin alpha 8
KLK1B4	kallikrein 1-related peptidase b4
LEPRE1	leprecan 1
LIFR	leukemia inhibitory factor receptor
LRG1	leucine-rich alpha-2-glycoprotein 1
LRIG1	leucine-rich repeats and immunoglobulin-like domains 1
LRP1	low density lipoprotein receptor-related protein 1
LRPAP1	low density lipoprotein receptor-related protein associated protein 1
LTBP4	latent transforming growth factor beta binding protein 4
LTBR	lymphotoxin B receptor
LY6D	lymphocyte antigen 6 complex, locus D
MAN2B1	mannosidase 2, alpha B1
MCFD2	multiple coagulation factor deficiency 2
MESDC2	mesoderm development candidate 2
MIF	macrophage migration inhibitory factor
MMP15*	matrix metalloproteinase 15
NID2*	nidogen 2
NRG4	neuregulin 4
OIT3	oncoprotein induced transcript 3
OS9	amplified in osteosarcoma
OSMR	oncostatin M receptor
P4HB	prolyl 4-hydroxylase, beta polypeptide

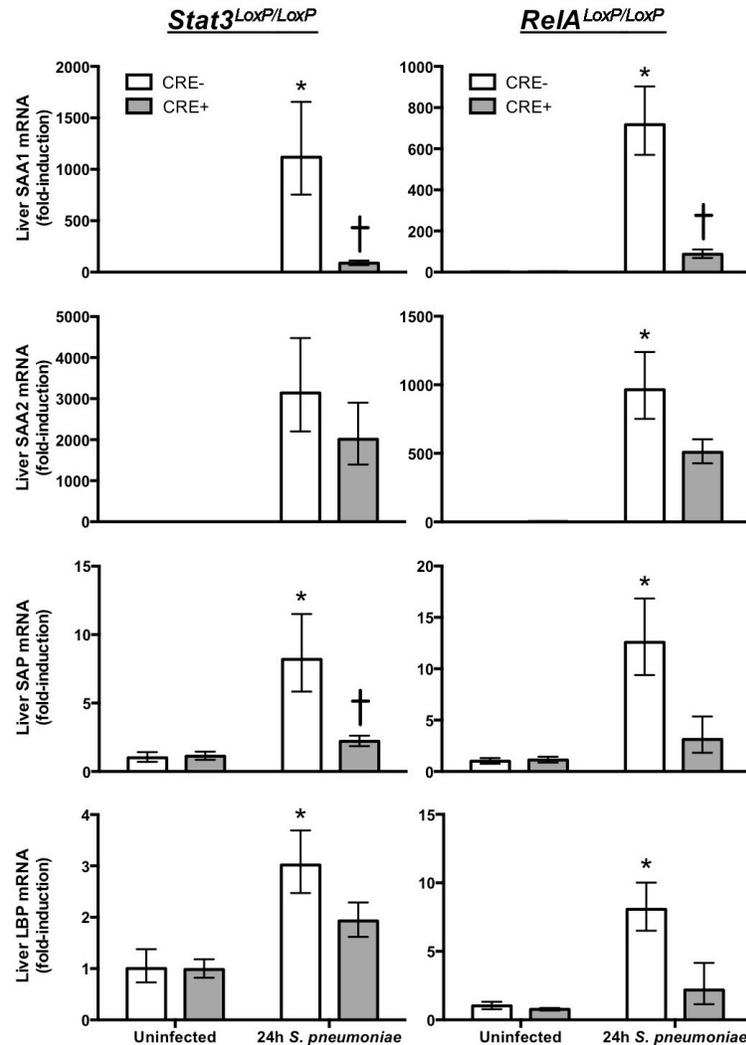
**Table S2 (contd)**

Gene	Protein
PDIA4	protein disulfide isomerase associated 4
PDIA5	protein disulfide isomerase associated 5
PGLYRP1	peptidoglycan recognition protein 1
PIGX	phosphatidylinositol glycan anchor biosynthesis, class X
PLA1A	phospholipase A1 member A
POFUT2	protein O-fucosyltransferase 2
PPIA	peptidylprolyl isomerase A
PRG4	proteoglycan 4
PRRG1	proline rich Gla (G-carboxyglutamic acid) 1
PRTN3	proteinase 3
QSOX1	quiescin Q6 sulfhydryl oxidase 1
RAB4B	Ras-related protein Rab-4B
RARRES2	retinoic acid receptor responder protein 2
RNASET2A	ribonuclease T2A
RPN2*	ribophorin II
SDF2L1	stromal cell-derived factor 2-like 1
SEL1L	protein sel-1 homolog 1
SEMA4B	semaphorin 4B
SEMA4G	semaphorin 4G
SERPINA10	protein Z-dependent protease inhibitor
SERPINA9	serpin A9
SERPIND1	heparin cofactor 2
SLC1A3	excitatory amino acid transporter 1
SLC39A14	zinc transporter ZIP14
SLC46A3	solute carrier family 46, member 3
SMPDL3A	sphingomyelin phosphodiesterase, acid-like 3A
SORT1	sortilin 1
SSR2	signal sequence receptor, beta
SSR4	signal sequence receptor, delta
ST6GAL1	beta galactoside alpha 2,6 sialyltransferase 1
STIM2	stromal interaction molecule 2
SYVN1	synoviolin
TAC1	tachykinin 1
TFPI	tissue factor pathway inhibitor
TGOLN1*	trans-golgi network protein 1
THPO	thrombopoietin
TIE1	tyrosine kinase with immunoglobulin-like and EGF-like domains 1
TIMD2	T-cell immunoglobulin and mucin domain containing 2
TMED10	transmembrane emp24-like trafficking protein 10
TMED3	transmembrane emp24 domain containing 3
TMED9	transmembrane emp24 protein transport domain containing 9
TMEM41A	transmembrane protein 41a
TMEM87B	transmembrane protein 87B
TNFRSF1A	tumor necrosis factor receptor superfamily, member 1a
TNFRSF23	tumor necrosis factor receptor superfamily, member 23
TOR2A	torsin family 2, member A
UGT2A3	UDP glucuronosyltransferase 2 family, polypeptide A3
UGT2B5	UDP glucuronosyltransferase 2 family, polypeptide B5
WNT11	wingless-related MMTV integration site 11

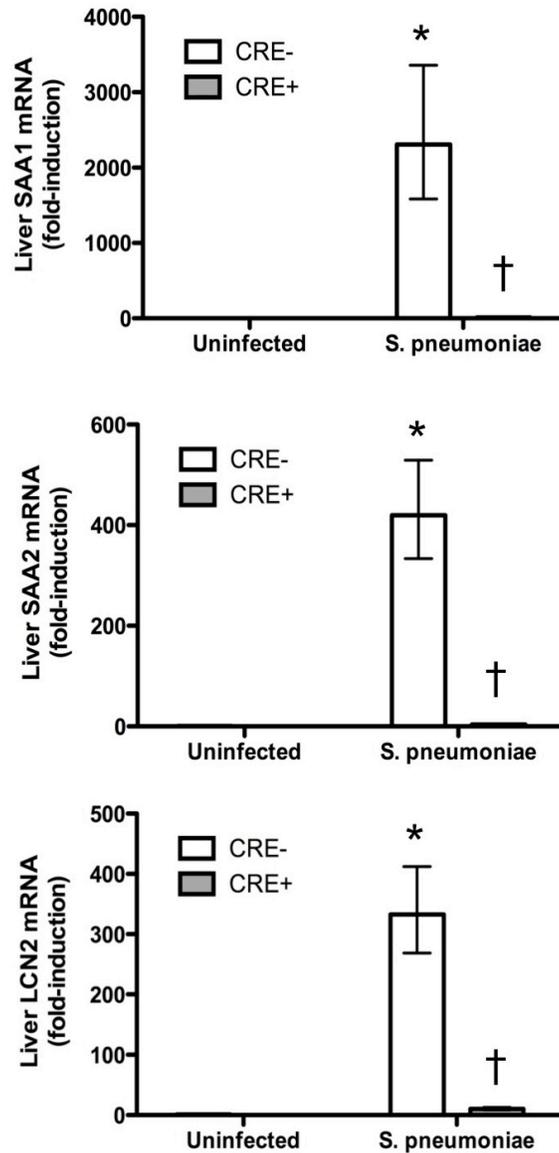
**Table S2:** Potential liver-derived acute phase proteins are shown, as defined by liver transcripts significantly altered during pneumococcal pneumonia and belonging to one or more of the following gene ontology categories: GO:0005615 (extracellular space); GO:0005576 (extracellular region); GO:0044421 (extracellular region part); UniProt: secreted; or UniProt: signal. Significant changes were exclusive to CRE- mice, with the exception of those transcripts denoted by asterisks. These selection criteria cannot

discriminate between membrane-associated proteins (receptors) and those that are truly secreted, but the list provides a basis for future investigation.

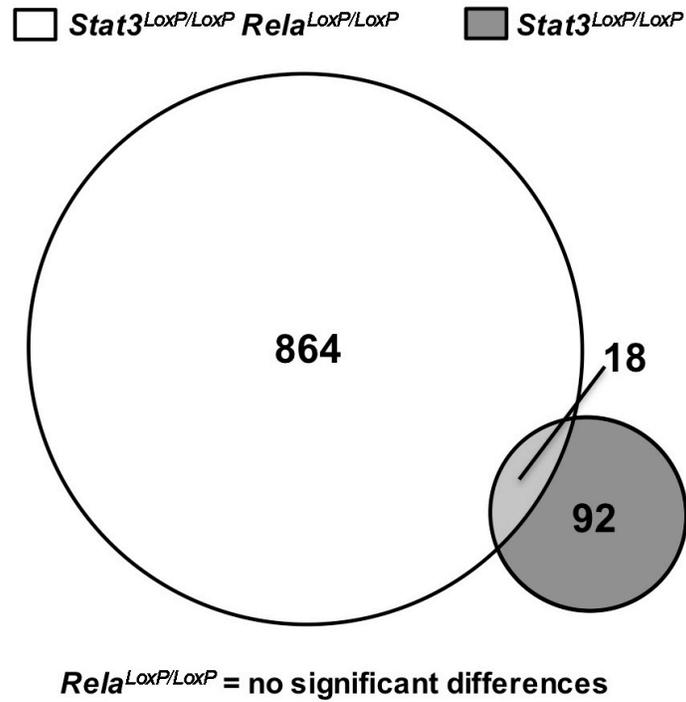
## Supplemental Figures



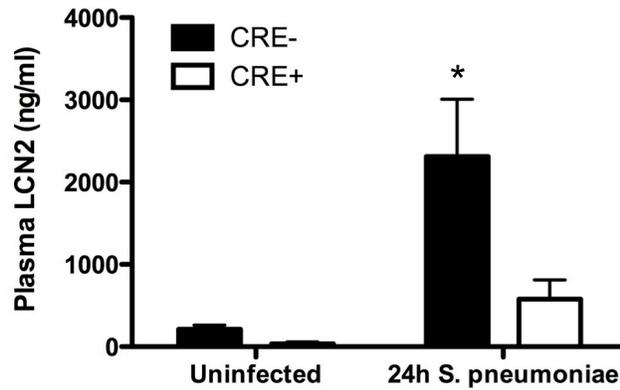
**Figure S1:** Deletion of STAT3 or RelA in hepatocytes has significant yet inconsistent effects on hepatic APP gene expression during pneumococcal pneumonia. qRT-PCR was performed to determine mRNA expression of SAA1, SAA2, SAP, and LBP in uninfected mice and in response to intratracheal (i.t.) *S. pneumoniae* ( $10^6$  colony-forming units). Results were compared between mice expressing (CRE-) or lacking (CRE+) liver STAT3 (left column; *Stat3*<sup>LoxP/LoxP</sup>) or RelA (right column; *RelA*<sup>LoxP/LoxP</sup>). Values represent mRNA fold induction compared to uninfected CRE- mice, expressed as geometric means  $\pm$  geometric SEM (n = 3-9). \*Significant ( $P < 0.05$ ) effect of infection. †Significant ( $P < 0.05$ ) effect of genotype.



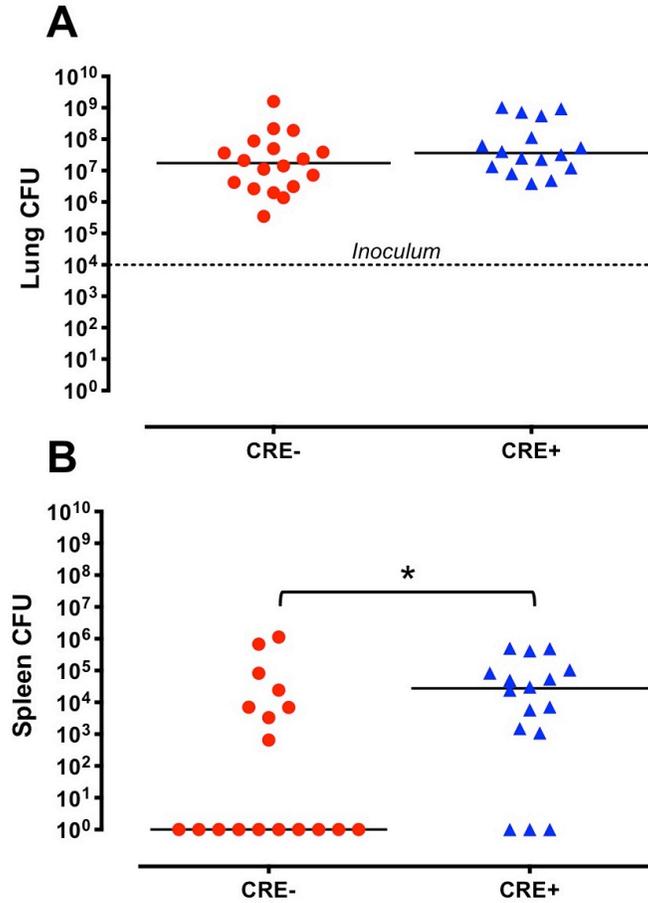
**Figure S2:** STAT3 and RelA in hepatocytes are required for hepatic APP synthesis during pneumonia. qRT-PCR and greater numbers of mice were used to further interrogate the 3 acute phase protein genes identified as partially dependent on STAT3 and RelA in the microarray analyses, SAA1, SAA2, and LCN2. Values represent mRNA fold induction compared to uninfected CRE- mice, expressed as geometric means  $\pm$  geometric SEM (n = 6-10). \*Significant ( $P < 0.05$ ) effect of infection. †Significant ( $P < 0.05$ ) effect of genotype



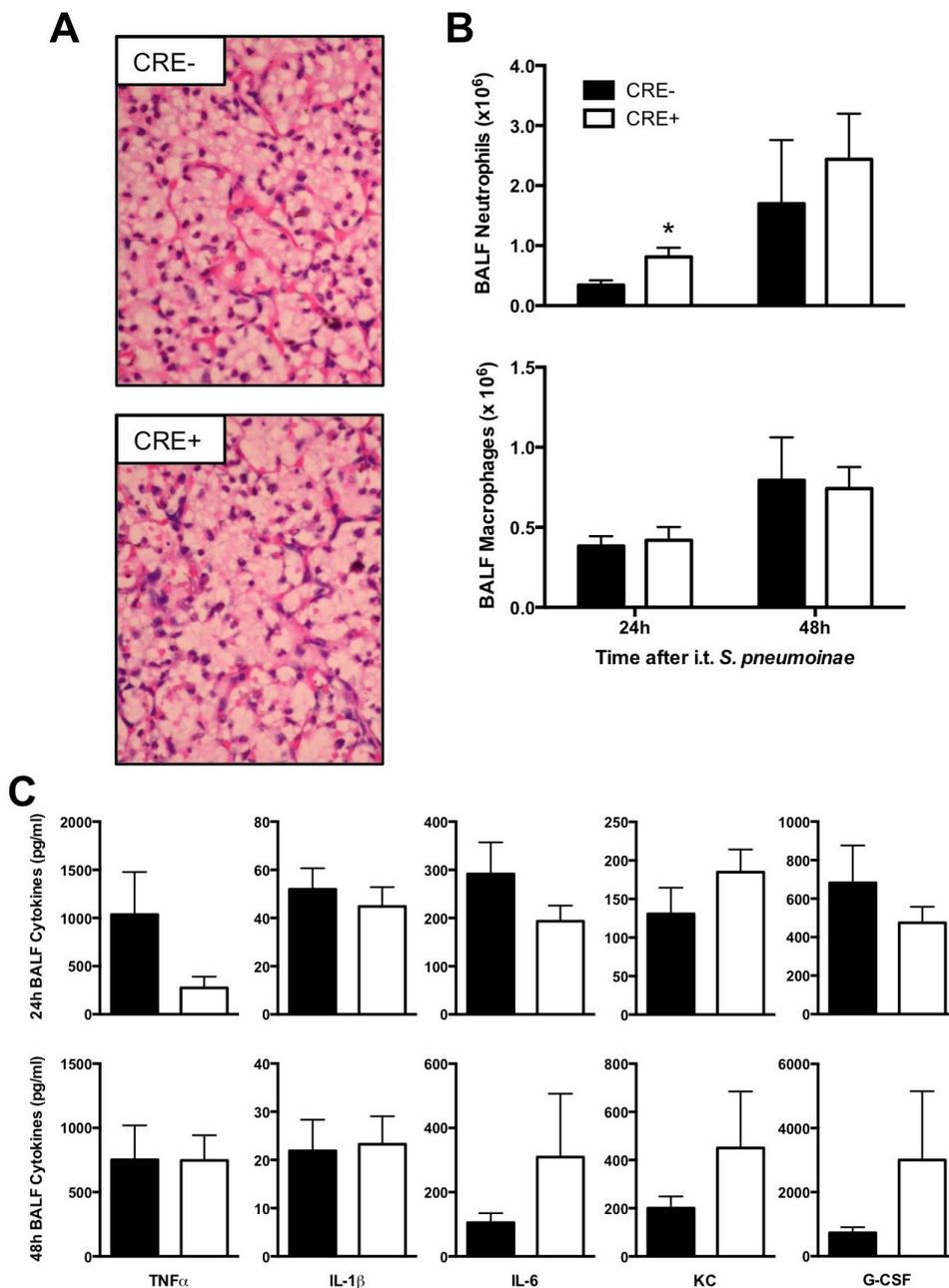
**Figure S3:** Single deletion of STAT3 or RelA has a minimal effect on hepatic gene expression compared to deletion of both factors during pneumonia. The Venn diagram illustrates the scope of hepatic gene changes between CRE- controls and CRE+ mutants lacking STAT3 and/or RelA following 24h of pneumococcal pneumonia (serotype 3; 10<sup>6</sup> CFU). Differences between genotypes were considered statistically significant with FDR < 0.05 (n = 3). No significant differences were detected in mouse livers lacking RelA alone.



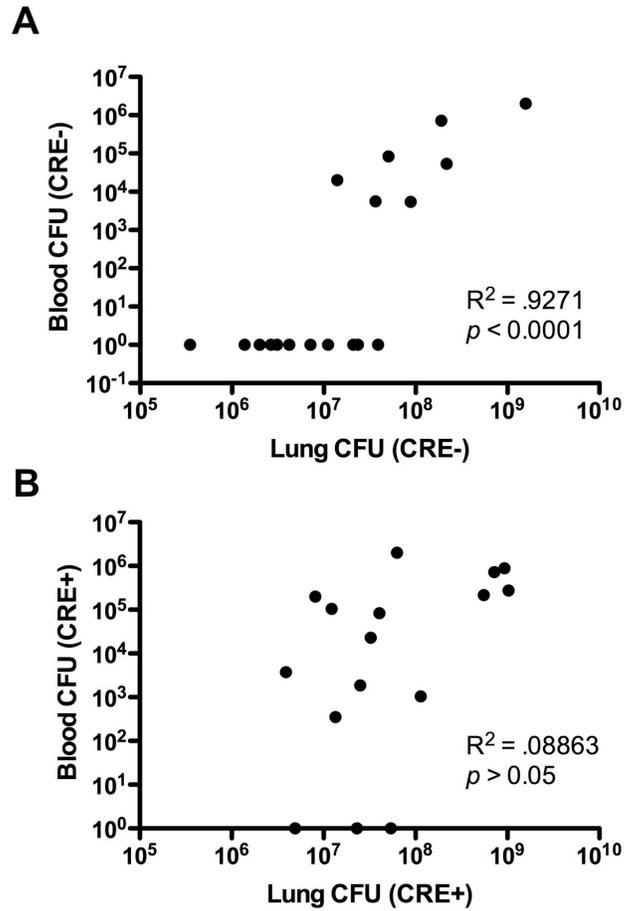
**Figure S4:** Hepatocyte STAT3 and RelA are required for the plasma LCN2 response to pneumococcal pneumonia. Plasma concentrations of SAA and SAP were quantified in samples collected 24 hrs after i.t. *S. pneumoniae* using ELISA and expressed as means  $\pm$  SEM. \* $P < 0.05$  vs uninfected CRE- (n = 3-11).



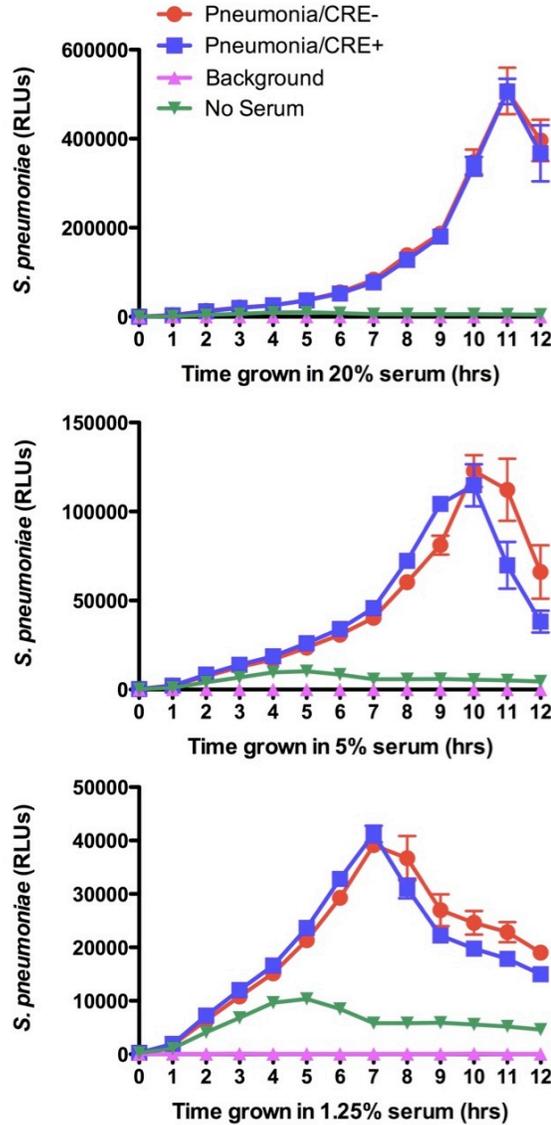
**Figure S5:** Liver STAT3 and RelA are required for blood-borne but not pulmonary host defense during pneumococcal pneumonia. Living *S. pneumoniae* were enumerated in **(A)** lungs and **(B)** spleens collected 48 hrs after intratracheal *S. pneumoniae* serotype 3 ( $10^4$  CFU). Data points represent individual mice, and lines indicate medians (n = 16-18). \*Significant ( $P < 0.05$ ) effect of genotype.



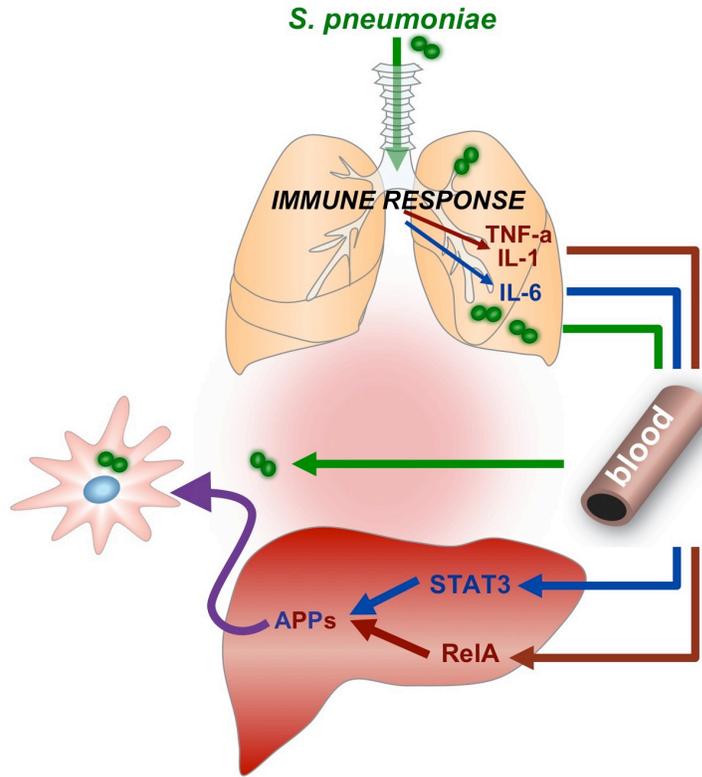
**Figure S6:** Acute pulmonary inflammation is unaffected by the absence of STAT3 and RelA in hepatocytes during pneumococcal pneumonia. **(A)** Representative histological sections (20X magnification) are shown of CRE- and CRE+ mouse lungs following 24 hrs of pneumococcal pneumonia. **(B)** Neutrophil and macrophage counts were determined in bronchoalveolar lavage fluid (BALF) 24 or 48h after i.t. *S. pneumoniae*. **(C)** Protein concentrations of tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), interleukin (IL)-1 $\beta$ , IL-6, KC, and granulocyte colony-stimulating factor (G-CSF) were quantified by cytokine bead array in BALF collected from mice following 24 or 48h of pneumococcal pneumonia. All numeric data are expressed as means  $\pm$  SEM.



**Figure S7:** Bacteremia is associated with lung bacterial burden in CRE- control mice (A), but not CRE+ mutants (B) lacking hepatocyte STAT3 and RelA. Colony-forming units (CFU) of *S. pneumoniae* were enumerated to quantify viable bacterial burden in lungs collected 48 hrs after intratracheal *S. pneumoniae* (serotype 3;  $10^4$  CFU). Data illustrate blood bacteriology as it compares to lung bacteriology in each mouse. N = 16-18



**Figure S8:** Pneumonia does not alter the bacteriostatic or bactericidal capacity of serum. Luciferase-expressing *S. pneumoniae* were incubated in the presence of no serum (green inverted triangles) or serum collected from CRE- (red circles) and CRE+ (blue squares) pneumonic mice. Bacterial growth and viability were monitored by luciferase activity over a 12 hr incubation period. Background (magenta triangle) represents luminescence produced by media alone. Data points represent means  $\pm$  SEM. N = 5-7 mice/group from which serum was collected.



**Figure S9:** STAT3 and RelA in hepatocytes are a critical means through which TNF $\alpha$ , IL-1, and IL-6 induce the acute phase response and limit invasive disease during pneumococcal pneumonia. Individually, STAT3 and RelA have select roles in governing acute phase protein (APP) expression. Together, these transcription factors are entirely responsible for the hepatic response to lung infection, which itself limits the success of disseminated blood pathogens by enhancing opsonophagocytosis.

## Supplemental References

1. Quinton, L.J., Jones, M.R., Robson, B.E., and Mizgerd, J.P. 2009. Mechanisms of the hepatic acute-phase response during bacterial pneumonia. *Infect Immun* 77:2417-2426.
2. Quinton, L.J., Jones, M.R., Simms, B.T., Kogan, M.S., Robson, B.E., Skerrett, S.J., and Mizgerd, J.P. 2007. Functions and regulation of NF-kappaB RelA during pneumococcal pneumonia. *J Immunol* 178:1896-1903.
3. Jones, M.R., Quinton, L.J., Simms, B.T., Lupa, M.M., Kogan, M.S., and Mizgerd, J.P. 2006. Roles of interleukin-6 in activation of STAT proteins and recruitment of neutrophils during *Escherichia coli* pneumonia. *J Infect Dis* 193:360-369.
4. Schmittgen, T.D., Zakrajsek, B.A., Mills, A.G., Gorn, V., Singer, M.J., and Reed, M.W. 2000. Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods. *Anal Biochem* 285:194-204.
5. Quinton, L.J., Jones, M.R., Robson, B.E., Simms, B.T., Whitsett, J.A., and Mizgerd, J.P. 2008. Alveolar epithelial STAT3, IL-6 family cytokines, and host defense during *Escherichia coli* pneumonia. *Am J Respir Cell Mol Biol* 38:699-706.
6. Huang da, W., Sherman, B.T., and Lempicki, R.A. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 4:44-57.