FAS INDUCED PULMONARY APOPTOSIS AND INFLAMMATION DURING INDIRECT ACUTE LUNG INJURY

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Material and Methods

Animals: Male mutant mice, expressing non-functional Fas receptor (B6.MRL-*Fas*^{lpr}/J (*lpr*)) or Fas ligand (B6Smn.C3-*Fasl*^{gld}/J (*gld*)), both 8 weeks old, or deficient in monocytes/ macrophages (B6C3Fe *a*/*a*-*Csf1*^{op}/J (*Csf1op*)), 3 weeks old, or their age matched controls (C57BL/6J (*C57*)) (all Jackson Laboratories, Bar Harbor, USA) were used. Animals were allowed free access to water and food before and after procedures. A day-night cycle of 12 hrs was applied. Experiments described here were performed in accordance with National Institutes of Health guidelines and with approval from the Animal Use Committee of Rhode Island Hospital.

Animal Model of Indirect Acute Lung Injury ($ALI_{indirect}$): Acute lung injury was induced as described previously (1-4). In brief, mice were anesthetized with isoflurane and catheters were inserted into both femoral arteries. Anesthesia was discontinued and blood pressure was continuously monitored through one catheter attached to a blood pressure analyzer (BPA MicroMed, Louisville, KY). When fully awake, mice were bled over a 5 to 10 minute period to a mean blood pressure of 30 mmHg (±5 mmHg) and were kept stable for 90 minutes. Immediately after hemorrhage, mice were resuscitated via the catheters with Ringer's lactate at four times the drawn blood volume. Sham-operated controls had their femoral arteries ligated and were restrained for periods of time equal to their hemorrhaged counterparts, but no catheters were inserted and no blood was shed. Polymicrobial sepsis was induced 24 hrs after hemorrhage. Mice were anesthetized with isoflurane, shaved at the abdomen, and scrubbed with betadine. A 1 cm midline incision was made below the diaphragm to expose the cecum. The cecum was ligated, punctured twice with a 22-gauge needle and gently compressed to extrude a small amount of cecal contents through the punctured holes. The cecum was returned to the abdomen, and the incision was closed in layers with 6-0 Ethilon suture (Ethicon, Somerville, NJ). The animals were then resuscitated

with 0.8 ml of lactated Ringer's solution via subcutaneous injection (2-5). In sham-operated controls laparotomy was performed in a similar fashion and the cecum was exposed, however it was neither ligated nor punctured. Importantly, while we only show the Sham group as our control (which we have not found to differ from Hem or CLP alone) for the studies here, we have previously substantially documented that neither HEM nor CLP alone are capable of inducing substantial/consistent increases in indices of lung inflammation, apoptosis or injury alone (1-4,6).

Intratracheal Instillation of Agents: Intratracheal delivery of purified hamster anti-mouse Fas monoclonal antibody Jo2 or the corresponding isotype control at 2 μg/ g body weight (both low endotoxin, azide free) (BD Pharmingen, San Diego, CA) was performed as described previously (2,5). Mice were anesthetized with isoflurane and restrained in supine position with their head reclined. The tongue of the animals was pulled out gently to prevent swallowing and the agent was administered in the oral cavity. Mice were maintained in the described position until the agent was aspirated.

Experimental Groups and Sample Acquisition for *in vivo* experiments: *Lpr*, *gld* or their controls (C57BL/6) were subjected to hemorrhagic shock followed by polymicrobial sepsis 24 hrs thereafter. Blood and lungs were harvested 12 or 24 hrs after the induction of sepsis (i.e. 36 or 48 hrs after HEM). Lungs were gently flushed with 2 ml ice-cold PBS through the vascular system via the right ventricle. Plasma was obtained by centrifugation of blood for 10 minutes, 10000xg at 4°C and frozen at -80°C until analysis was performed. Lung lobes were excised separately, frozen immediately into liquid nitrogen and stored at -80°C until analyses were performed. For survival *lpr* and *C57* animals were subjected to HEM+CLP and survival was assessed for the following 10 days on a daily basis (n=20/ group).

In a second set of experiments (n=4 animals/ group) with identical procedures and time points lungs were instilled *in vivo* with 30 μl/ g body weight of 10% buffered formaldehyde

after exsanguination, excised and stored for 24 hrs in 10% formaldehyde. Afterwards, lungs were paraffin embedded and cut into 5 μm sections for immunohistochemistry or H&E staining.

In a third set of experiments mice were exsanguinated 12 hrs after CLP. The trachea of the animals was carefully exposed and cannulated using a 0.58 internal diameter polyethylene tubing (Becton Dickinson, Sparcks, MD) and 30 μl/ g body weight of ice-cold PBS was carefully instilled into the trachea and recovered. BAL was stored on ice after adding 5 μl of protease inhibitor cocktail (Sigma, St. Louis, MO). Assessment of total lung leak was carried out via quantification of total BAL protein and calculation of the protein ratio between BAL and plasma. To address the degree of $ALI_{indirect}$, BAL/ plasma protein ratio was chosen over the absolute content of protein in BAL to correct for plasma/ BAL protein disturbances caused by the nature of the fixed-pressure model, i.e. controlled shedding of blood followed by resuscitation calculated to four times the shed blood volume.

In a fourth set of experiments (*Csf1op*) macrophage deficient mice and their controls underwent intratracheal instillation of 2 μg/ g body weight of anti-mouse Fas monoclonal antibody Jo2 or isotype control (BD Pharmingen) and were sacrificed 4 hrs thereafter. After exsanguination lungs were excised and snap frozen into liquid nitrogen for further analysis. As shown previously *CSF1op* mice display a marked reduction of alveolar macrophages at 3 weeks of age due to a complete absence of M-CSF in their lungs with no cellular or lung histo/patho-morphological abnormalities (7). The number of mononuclear cells in the bronchoalveolar lavage of the animals in our studies was recorded using a neubauer counting chamber. We found a marked reduction of alveolar macrophages in the bronchoalveolar lavage of IgG treated *csf1op* mice ($2.57\pm0.38 \times 10^5$ cells/ ml Lavage (Mean±SEM)) when compared to *C57* background controls (10.73±1.58).

An overview of the experimental groups is provided in Table 1 of the Supplementary Section.

Preparation of Tissue Samples for Cytokine Measurements: Preparation of lung homogenates for cytokine measurements was performed as described previously (2,8,9). Frozen lung samples were thawed on ice in phosphate buffered saline including 50µl/ ml proteinase inhibitor cocktail for mammalian tissues (Sigma). Samples were then homogenized and sonicated on ice. Subsequently, samples were centrifuged at 12,000 x g at 4°C for 15 min and supernatants were obtained. Supernatants were again centrifuged at 12,000 x g at 4°C for 15 min and supernatants were stored at -80°C until assays were performed.

Cell Culture of Mouse Lung Epithelial Cell Lines: LA-4 (CCL-196, ATCC, Manassas, VA) and MLE 12 (CRL-2110, ATCC) cells were cultured in Kaighn's modification of Ham's F12 medium containing 1.5g/l sodium bicarbonate (ATCC), 15% fetal bovine serum (HyClone, Logan, UT) and 0.1% Gentamicin (Invitrogen, Carlsbad, CA), or HITES medium (ATCC) containing 1.5g/L sodium bicarbonate (ATCC), 2% fetal bovine serum (HyClone), 3.5mM L-glutamine (Invitrogen), 0.005 mg/ml insulin (Sigma), 0.01 mg/ml apotransferrin (Athens Research & Technology, Athens, GA), 30nM sodium selenite (Sigma), 10nM hydrocortisone (Sigma), 10nM beta estradiol (Sigma), 10mM HEPES (Invitrogen), and 0.1% Gentamicin (Invitrogen), respectively, to about 80% confluence in 12 well tissue culture plates (Corning Incorporated, Corning, NY). For some experiments cells were preincubated for 30 minutes with 10µM of the Ras/Raf inhibitor FTI-277 (Methyl {N-[2-phenyl-4-N [2(R)-amino-3-mecaptopropylamino] benzoyl]}-methionate) (Calbiochem, San Diego, CA), 30µM of the MAP (Mitogen-Activated Protein) kinase kinase inhibitor PD98059 (2′-Amino-3′-methoxyflavone) (Calbiochem) or DMSO (dimetyl sulfoxide) (Sigma) as a control. To minimize off target effects, two inhibitors, acting at different levels upstream of Extracellular-Signal-Regulated Kinase (ERK) 1/2 activation/signaling were used. Both inhibitors, PD 98059 and FTI-277 have been demonstrated to possess high specificity (10-12) at the concentrations that we have chosen to use here (13). Subsequently, as we had previously shown that epithelial cells in the lungs of mice subjected to HEM + CLP could be protected

from apoptosis and the development of inflammation by treatment with anti-Fas siRNA (2) we attempted to test if a link between Fas stimulation and the activation of ERK leading to subsequent inflammation existed. To do this, mouse pulmonary epithelial cell lines were incubated with purified hamster anti-mouse Fas monoclonal antibody (Jo2) or purified hamster IgG1 isotype control immunoglobulin (both, BD Pharmingen), both azide free and endotoxin ≤ 0.01 ng/μg protein, for 4 hrs at a concentration of 50 ng/ml. Cell supernatants were harvested for chemokine analysis or cells were collected for assessment of cell proliferation (see below). For other experiments cells were grown to about 80% confluence in 96 well plates (Corning Incorporated) and incubated with Jo2 at 50 ng/ml for 5, 15, 25 and 60 min or IgG and phosphorylation of ERK 1/2 was assessed as described below. No marked differences were found for IgG stimulated cells (0.34±0.02) when compared to non stimulated cells (0.31±0.02). All *in vitro* experiments were repeated at least 3 times.

Cell Proliferation Assay: Cell proliferation was assessed in LA-4 and MLE 12 cells using CyQUANT Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR). In brief, cells were grown to about 80% confluence in 96 well plates (Corning) and stimulated with Jo2 or control for 4 hrs. Supernatants were discarded and cells were incubated with cell lysis buffer including 0.25% of CyQUANT green dye, which exhibits strong fluorescence when bound to cellular nucleic acids, for 5 min. Extinction was read at 485 nm excitation/ 530 nm emission on a fluorescent plate reader (FL500, BioTek Instruments, Winooski, VT) and calculated against a LA-4, or MLE 12 cell standard curve, respectively.

ERK1/2 (Extracellular-Signal-Regulated Kinase) Phosphorylation Quantification: ERK1/2 phosphorylation (activation) was quantified using Cellular Activation of Signaling ELISA KIT (CASE, SuperArray, Bioscience Corporation, Frederick, MD) (14). The assay determines specific phosphorylation of ERK phosphorylation sites at threonine 202 and tyrosine 204 relative to the total amount of ERK present and relative to the cell number per well. In brief, cells were incubated with Jo2 or IgG for 5, 15, 25, and 60 minutes. Cells were

fixed with 4% formaldehyde, washed and quenched with hydrogen peroxide and sodium azide. Antigen was retrieved by micro waving cells for 3 minutes in the presence of antigen retrieval buffer (Superarray). Non-specific binding was blocked by incubating cells for 1 hour with blocking buffer (Superarray). Subsequently, cells were incubated with either anti-phospho- or anti-pan-ERK primary antibody overnight at 4°C, followed by one hour incubation with the corresponding secondary antibody. Absorbance was read at 450 nm and the relative cell density per well was determined using cell staining buffer (Superarray) and reading absorbance at 595 nm. Total ERK phosphorylation was calculated as follows: $(OD_{A450}ERK_{phosho}/ OD_{A595}Cell_{total})/ (OD_{A450}ERK_{total}/ OD_{A595}Cell_{total})$.

Protein Assay: The total amount of protein was quantified using the Bradford dye binding procedure. Briefly, dye reagent concentrate (Bio-Rad, Hercules, CA) was diluted 1:5 with distilled water, and 10 µl of sample (diluted 1:30 in distilled water) were added to 190 µl of diluted dye reagent per well. The color was allowed to develop for 3–5 min and absorbance was read at 595 nm. Protein concentration was determined and calculated against a standard curve.

Chemokine ELISAs: Mouse KC and MIP-2 concentrations in plasma, lung tissue homogenates, BAL and cell supernatants were determined using murine anti-cytokine antibody pairs as well as cytokine standards (both R&D Systems, Minneapolis, MN) as per the manufacture's protocol for basic sandwich ELISA. MCP-1 in cell supernatants was analyzed using mouse MCP-1 BD OptEIA™ ELISA (BD Biosciences). Protein assays were performed, as described above, on tissue homogenates before cytokine ELISAs. Cytokine concentrations are presented as picograms per milligram of protein for each sample.

Cytometric Bead Array: Mouse TNF-α, IL-6, IFN-γ, IL-12, MCP-1, and IL-10 levels were established in plasma and lung tissue homogenates using the cytometric bead array technique (BD™ Cytometric Bead Array Mouse Inflammation Kit, BD Biosciences) according to the manufacturers instruction as described previously (2). In brief, 50 µl of

Mouse Inflammation Capture Bead Suspension and 50 µl PE Detection Reagent were added to the equal amount of sample or standard dilution and incubated for 2 hrs at room temperature in the dark. Subsequently, samples were washed by adding 1 ml of wash buffer and centrifuging at 200xg at RT for 5 min. Samples were analyzed on a BD FACSArray™ bioanalyzer (BD Biosciences) according to the manufacturers instruction. Protein assays were performed, as described above, on tissue homogenates before cytometric bead array.

Myeloperoxidase (MPO) Activity: Lung MPO activity was quantified as described previously (3). In brief, lung tissue was homogenized in 50 mM potassium buffer pH 6.0 with 0.5% hexadecyltrimethylammonium bromide, sonicated on ice, and then centrifuged at 12,000 x g at 4°C for 10 min. Supernatants were then assayed at a 1:20 dilution in reaction buffer (530 nmol/l *o*-dianisidine, 150 nmol/l H_2O_2 in 50 mM potassium phosphate buffer), and read at 450 nm.

Active Caspase-3, FLIP (Fas associated death domain (FADD)-like interleukin-1β-converting enzyme inhibitory proteins) and β-Actin western blotting: Active Caspase-3, FLIP and β-Actin were quantified in lung tissue via western blotting. In brief, 80µg (for Caspase-3) or 50µg (for FLIP) of homogenized lung tissue were electrophoresed on a 18% Tris-Glycine Gel (Invitrogen) and transferred onto a Poly-Vinyliden-Di-Fluorid membrane (Amersham, Piscataway, NJ). Nonspecific binding was blocked with tris buffered saline with Tween (TBST, 0.242%, TrisBase (Sigma), 0.8% Sodiumchloride (Sigma), pH 7.6, 0.1% Tween-20 (Sigma) containing 5% non-fat dry milk (Nestle, Glendale, CA) for Caspase-3 or with phosphate buffered saline with Tween (PBST, 0.8% NaCl, 0.116% Na_2HPO_4, 0.02% KH_2PO_4, 0.02% KCl (all Sigma), pH 7.5, 0.1% Tween-20 (Sigma) containing 5% non-fat dry milk for FLIP. Membranes were incubated with the following antibodies: polyclonal rabbit anti-mouse FLIP (FLICE inhibitory protein) antibody (Cell Signaling Technology Inc., Beverly, MA) at a concentration of 1:1000 or polyclonal rabbit anti-mouse cleaved Caspase-3 (Asp175) antibody (Cell Signaling) at a concentration of 1:500 at 4°C overnight. After

washing membranes were incubated with ECL Rabbit IgG, HRP-linked whole antibody from donkey (Amersham Biosciences) at a concentration 1:10000 for 1 hour at room temperature. After washing, membranes were developed using enhanced chemiluminescence technique (ChemiGlow, Alpha Innotech, San Leandro, CA) according to the manufacturers recommendation. After additional washing Caspase-3 membranes were incubated with polyclonal rabbit anti-mouse β-Actin (Abcam, Cambridge, MA) at a concentration of 1:10000 in PBST containing 5% milk at 4°C overnight and after washing with PBST with ECL Rabbit IgG, HRP-linked whole antibody and subsequently developed as described above. FLIP membranes were treated alike after stripping membranes in buffer containing 2% dodecyl sodium sulfate (Sigma), 62.5mM TRIS HCl, pH 6.7 and 100mM 2-mercaptoethanol (Sigma) for 30 minutes at 55°C and washing in PBST. Protein expression was quantified via densitometry using FluorChem, Version 4.1.0 (Alpha Innotech) and expressed as the ratio of the integrated density values of Caspase-3 or FLIP, respectively, and β-Actin, adjusted to background levels.

Active Caspase-3 Immunohistochemistry: Active Caspase-3 immunohistochemistry was carried out as described previously (2) according to the manufactures protocol (Cell Signaling Technology, Danvers, MA) with minor modifications. In brief, formalin fixed and paraffin embedded lung tissue sections were deparaffinised and antigen retrieval was carried out incubating slides for 10 min in 10mM sodium citrate buffer (Sigma), pH 6.0, at 90°C. Endogenous peroxidase was blocked with 3% H_2O_2 (Sigma) for 10 min and blocking of non-specific binding with 5% goat serum (Pierce, Rockford, IL) for 1hr at RT. Cleaved Caspase-3 (Asp175) antibody (Cell Signaling Technology) was applied in PBS, which included 5% goat serum, overnight at 4°C at a concentration of 1:100. Slides were incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories) for 30 min at RT at a concentration of 8μg/ml, diluted in PBS including 5% goat serum. Slides were then incubated with Vectastain ABC

KIT (Vector Laboratories, Burlingham, CA) for 30 minutes. Staining was visualized with Metal Enhanced DAB Substrate Kit (Pierce). Gills Hematoxylin III was used to counterstain, sections were then dehydrated and Permount (Biomeda, Foster City, CA) was used to coverslip.

M-30 Immunostaining: M30 is a neo-epitope that is revealed when the intermediate filament protein cytokeratin-18 on epithelial cells is cleaved by caspase-3/-6/-9 at Asp396 (15), thus indicating epithelial cell specific apoptosis. M-30 immunostaining was carried out as described previously (2). Antigen retrieval was performed in 10mM Sodium Citrate Buffer, pH 6.0, at 90°C for 20 min. M-30 CytoDeath^{TM} Biotin (Diapharma, Columbus, OH) was used at 1:50 overnight at 4°C. Slides were then incubated with Vectastain ABC KIT (Vector Laboratories) for 30 minutes and then with Metal Enhanced DAB Substrate Kit (Pierce). Gills Hematoxylin III was used to counterstain. Sections were then dehydrated and Permount (Biomeda) was used to coverslip.

TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling): Quantification of TUNEL positive cells was carried out as described previously (16) using the In Situ Cell Death Detection Kit, Fluorescin (Boehringer, Mannheim, Germany). Formalin fixed and paraffin embedded lung tissue sections were deparaffinised and antigen retrieval was carried out by incubating tissue slides with Proteinkinase K (Roche Applied Science, Indianapolis, IN) for 20 minutes at 15 μg/ml. TUNEL reaction mixture was applied for 1 hour at 37°C. For negative controls the transferase enzyme was omitted. Slides for immunohistochemistry of active Caspase-3, M30 and TUNEL were examined as follows: Ten high power fields (HPF) (25 μm^2/ field)/ slide were randomly selected and counted in a blinded fashion (C.S.C) and the number of fluorescent cells per HPF was recorded for statistical analysis. Negative controls were performed by following the exact same protocols but omitting either the first or the second antibody.

Lung Hematoxylin and Eosin (H&E): H&E tissue slides were histopathologically analyzed according to the following criteria as described previously (2): Distension of alveoli, thickening of alveolar septa, perivascular and peribronchial edema and intraalveolar cellular infiltrates. Staining (Core Research Facilities) and analysis (C.S.S.) of H&E sections were performed by a blinded observer.

Statistical Analysis: Results are presented as mean ± standard error of the mean (SEM). The Kolmogorov-Smirnov test (SigmaStat 3.0 (SPSS Incorporated, Chicago, IL)) was used to assess normal distribution of the data prior to performing any given statistical tests. One-Way Analysis of Variance (ANOVA) followed by the Student-Newman-Keuls test as a post hoc test for multiple comparisons was performed to determine significant differences between experimental means. For multiple comparisons TWO-way ANOVA was used after plotting non-linear data onto a logarithmic scale, followed by the Student-Newman-Keuls test. One or two-way ANOVA were used depending on the number of variables tested. Differences in survival were assessed using Fisher's exact test. A p-value < 0.05 was considered statistically significant.

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Table E1

Intervention/ Animals	**Hemorrhagic Shock**	**Polymicrobial Sepsis**	**Intratracheal Instillation**
Indirekt ALI (*C57, lpr, gld*)	+	+	**n/a**
Indirekt ALI Controls (*C57, lpr, gld*)	Sham Procedure	Sham Procedure	**n/a**
Fas induced Inflammation (*Csf1op, C57*)	**n/a**	**n/a**	Jo2 Antibody
Fas induced Inflammation Controls (Csf1op, C57)	**n/a**	**n/a**	IgG Antibody