IGFBP6 orchestrates anti-infective immune collapse in murine sepsis via prohibitin-2-mediated immunosuppression

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Expanded materials and methods

Study Population

Participants in this study included both males and females. For adult cohort, the discovery cohort comprised 91 patients (age ≥ 18 years) admitted to the ICU of the Second Affiliated Hospital of Chongqing Medical University (Chongqing, China). The validation cohort comprised 163 patients (age ≥ 18 years) admitted to the ICU of the First Affiliated Hospital of Chongqing Medical University (Chongqing, China). All participants met the diagnostic Sepsis-3 criteria, defined as life-threatening organ dysfunction caused by a dysregulated host response to infection, with a Sequential [Sepsis-related] Organ Failure Assessment (SOFA) score ≥2, Septic shock was further characterized by persistent hypotension requiring vasopressors to maintain mean arterial pressure (MAP) ≥65 mmHg and serum lactate levels >2 mmol/L (18 mg/dL) despite adequate fluid resuscitation (1). Demographic and clinical data were systematically collected, including age, sex, comorbidities, SOFA scores at admission, infection etiology, microbiological findings (pathogen identification and infection source), therapeutic interventions (mechanical ventilation, vasopressor use, hydrocortisone administration, renal replacement therapy), ICU length of stay, and 28-day mortality. Exclusion

criteria encompassed pregnancy, breastfeeding, active malignancy, prior organ transplantation, HIV infection, autoimmune disorders, or immunosuppressive therapy use (2, 3). Peripheral blood samples were collected at ICU admission and separated by centrifugation (1500g×10 min, 4°C), then stored at -80°C until analysis. Age- and sex-matched non-septic infected patients and healthy volunteers from the same hospitals served as control groups.

For pediatric cohorts, serum samples from 61 patients (<18 years) in the discovery cohort were obtained from Kunming Children's Hospital (Yunnan, China), while the validation cohort (n = 145) was derived from the Children's Hospital of Chongqing Medical University (Chongqing, China). Pediatric sepsis was diagnosed according to the 2024 International Consensus Criteria for Pediatric Sepsis, requiring confirmed infection combined with a Phoenix Sepsis Score (PSS) ≥2 (4). Neonates and preterm infants (gestational age < 37 weeks) hospitalized during the perinatal period were excluded (4). Control groups comprised age-matched non-septic infected children and healthy volunteers.

Animals

Male and female wild type (WT) C57BL/6N mice (6-8 weeks old) and IGFBP6-deficient (*Igfbp6*$^{-/-}$) mice on a C57BL/6N genetic background were procured from the Laboratory Animal Center of Chongqing Medical University (LAC-CQMU) and Cyagen Biotechnology Co., Ltd. (Suzhou, China), respectively. All animals were raised under Specific Pathogen Free (SPF) laboratory of LAC-CQMU. The deletion of *Igfbp6* was verified by polymerase chain reaction (PCR)-based genotyping of tail-derived genomic DNA using two primer sets (TSE041, TSINGKE): Primer set 1: 5'-CTGGACTCTCTGGAAGGAGTG-3' (forward), 5'-ACCAGCCCTTCACTTGTAGCC-3' (reverse), Primers 2: 5'-

CTGGACTCTCTGGAAGGAGTG-3' (forward), 5'-ACTTCACGTTCCACTCAAGGC-3' (reverse). The thermal cycler settings of *Igfbp6* were 3 minutes at 94°C; 35 cycles of 30 seconds at 94°C, 35 seconds at 60°C, and 35 seconds at 72°C; and extension for 5 minutes at 72°C. PCR products were resolved by 2% agarose gel electrophoresis for genotype confirmation.

Sepsis Model

CLP-induced polymicrobial sepsis model was established as described previously (5-7). Briefly, age-matched male and female C57BL/6N mice were anesthetized intraperitoneally (i.p.) with xylazine (4.5 mg/kg) and ketamine (90 mg/kg). A 1 cm midline laparotomy was performed to expose the cecum, which was then ligatured at its external third, and punctured through with a 21-gauge needle (severe CLP, resulting in 0 to 20% survival) or with a 24-gauge needle (resulting in 50 to 60% survival). Sham-operated (control) animals underwent identical laparotomy without cecal ligation or puncture. The cecum was then returned to the abdominal cavity, and the incision was closed in two layers using 5-0 absorbable sutures. Postoperative care included subcutaneous administration of 1 mL prewarmed Ringer's lactate solution containing buprenorphine (0.05 mg/kg) for fluid resuscitation and analgesia. Mice were maintained in a temperature-controlled incubator and monitored every 6 hours for the first 48 hours, followed by 8-hour intervals until study completion. To administer antibiotic therapy, CLP models were given 5 mg/mL meropenem in saline at a dose of 25 mg/kg through intraperitoneal injection immediately post-surgery, and then once a day for 7 consecutive days. Mice exhibiting severe morbidity (e.g., non-responsiveness, labored breathing, or >20% weight loss within 24 h) were euthanized via cervical dislocation under anesthesia. Survival was tracked for 14 consecutive days.

Treatment of mice with rIGFBP6, rCCL2 and agonist

Recombinant murine IGFBP6 was administered i.p. at 2.5, 10 and 50 μg/kg at 1h post-CLP, with PBS serving as the vehicle control. A dose of 25 μg/kg rIGFBP6 was used in all experiments except the survival experiments. For survival experiments, we maintained therapeutic relevance by administering half the initial dose of rIGFBP6 at 24 and 48 hours post-CLP. Recombinant murine CCL2 (rCCL2) was delivered i.p. at 25 μg/kg at 1h post-CLP, with PBS administered to controls. For *in vivo* activation of STAT1, the agonist 2-NP was dissolved in a dimethyl sulfoxide (DMSO) solution (10% DMSO + 90% corn oil) and administrated i.p. immediately following CLP.

Single-bacterial sepsis model

A *S. aureus* - induced peritonitis-related sepsis model was established by i.p. injection of 3×10^8 CFU into male C57BL/6N mice. For the *P. aeruginosa* - induced sepsis, 5×10^7 CFU were injected i.p. into male C57BL/6N mice. Survival was monitored twice daily for 14 days post-infection.

Quantification of bacterial loads

Peritoneal lavage fluids (PLF) were obtained through infusion of 5 mL PBS into the murine abdominal cavity using a sterile syringe, followed by gentle aspiration. Specimens including PLF, blood, and tissue samples were aseptically collected following CLP. All biological samples underwent serial 10-fold dilution in PBS, and plated on blood agar (tryptone soya agar with 5% sheep blood) plates (Thermo Fisher Scientific), and incubated at 37°C for 18h, after which the number of colonies was counted.

Histopathology, immunohistochemistry, and immunofluorescence

Following euthanasia, mice were transcardially perfused with PBS, followed by 4% paraformaldehyde for tissue fixation. Organs were paraffin-embedded and sectioned at 5μm thickness for hematoxylin and eosin (H&E) staining. Histopathological scoring was performed using a standardized four-stage grading system to assess inflammation and tissue damage (8). For immunohistochemistry (IHC) and immunofluorescence (IF), slices were dewaxed and rehydrated, followed by microwave repair of antigens in citric acid buffers (pH 6.0). The specimens were then permeabilized with PBS containing 0.1% Triton X-100, and blocked endogenous peroxidase with 3% H_2O_2. Thereafter, sections were blocked with 5% BSA for 30 minutes at room temperature and incubated with anti-CCL2 antibodies or anti-phospho-STAT1 antibodies overnight at 4°C in a wet box. For immunohistochemistry, sections were incubated with HRP-conjugated secondary antibody and peroxidase TMB substrate kit for 1 hour at room temperature. Hematoxylin was used for nucleus counterstaining. For immunofluorescence, after blocked with 5% BSA, sections and cell slides were incubated with anti-IGFBP6, anti-F4/80, anti-α-SMA, anti-PHB2, anti-STAT1 overnight at 4°C and then incubated with CY3 or FITC conjugated secondary antibody. DAPI was used for nuclear staining. Sections were observed and photographed with an inverted fluorescence microscope (Olympus IX71). Fluorescence integrated density was analyzed using Image J software (version 5.0, NIH).

Epithelial cell culture and treatment

The mouse lung epithelial cell line (MLE-12) and mouse small intestinal epithelial cell line (MODE-K) were maintained in Dulbecco's Modified Eagle Medium (DMEM, 11995065, Gibco) supplemented with 10% fetal bovine serum (FBS, C2910-0500, VivaCell) and 1% penicillin/streptomycin (Gibco BRL) at 37°C under 5% CO_2. For experiments, Cells were

seeded in six-well plates at 1×10^5/mL and cultured in serum-free medium for 12h when the cells reached 80% confluence. After starvation, cells were pretreated with rIGFBP6 (200 ng/mL) for 6 hours, followed by stimulation with inactivated *P. aeruginosa* (MOI = 100).

Characterization of leukocytes

Peritoneal cell suspensions were pelleted and resuspended in PBS. Cytospin slides were prepared and stained with a Wright-Giemsa stain. Cells were washed in FACS buffer (1% BSA in PBS), stained with fluorophore-conjugated antibodies against CD11b, Ly6G, and F4/80, and analyzed using a FACScan flow cytometer (Becton Dickinson, NJ, USA). Data from $\geq 10^5$ events per sample were processed with FC Express software. Gating strategies for macrophage and neutrophil populations are detailed in Supplementary Figure 7B.

Serum biochemistry

Blood was collected in tubes with heparin after cardiac puncture and centrifuged to separate serum. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) were determined with commercial kits (Sigma-Aldrich, St. Louis, MO), using a Hitachi analyzer (Boehringer Mannheim; Germany) according to the manufacturers' instructions.

***In vivo* depletion of macrophages**

Clodronate-encapsulated liposomes were delivered i.p. (200 μL, 5 mg/mL), to deplete macrophages at 48 hours before CLP-induced sepsis. PBS-encapsulated liposomes were delivered in a similar way as a control.

Isolation and culture of primary murine macrophages and neutrophils

Primary peritoneal macrophages were isolated from the peritoneal exudates of C57BL/6N mice

that were pretreated with 3% thioglycolate i.p. for 5 days. The peritoneal exudate cells were harvested via cold PBS lavage, resuspended in DMEM supplemented with 10% FBS, and adjusted to 1×10^6 cells/mL. After 2-hour incubation at 37°C under 5% CO_2, non-adherent cells were removed by PBS washing. Adherent cells (>90% F4/80$^+$ by FCM) were maintained in complete DMEM (6, 9). Bone marrow-derived macrophages (BMDMs) were generated by flushing femurs and tibias with DMEM. Cells were cultured for 7 days in DMEM containing 10% FBS and 50 ng/mL recombinant murine M-CSF, yielding >90% F4/80$^+$ macrophages (10). Bone marrow neutrophils were isolated using a murine neutrophil isolation kit. Purity (>90% CD11b$^+$Ly6G$^+$) was confirmed via FCM as previously described (11).

Macrophage migration assay

Cell migration assays were performed using a 24-well transwell® apparatus with a 0.4 μm pore size (Corning Inc., NY, USA). Approximately 6×10^4 peritoneal macrophage with a volume of 200 μL were loaded into the upper chambers of the trans-well plates. $1x10^5$ MLE-12 or MODE-K with a volume of 600μL conditioned media was collected and added to the lower chambers. After 6 hours of rIGFBP6 pre-stimulation, heat-inactivated bacteria were added to the upper chambers of the trans-well plates. After 12 hours incubation, the cells were fixed with pre-cooled methanol and stained with crystal violet. Non-migrating cells on the upper surface of the membrane were gently removed. The numbers of migrated cells were counted in five randomly chosen fields per insert using Image J software.

Adoptive transfer of macrophages

Peritoneal macrophages were isolated from C57BL/6N WT or *Igfbp6*$^{-/-}$ mice (age 6-8 weeks, male/female 1:1) as described above and treated with 200 ng/mL of rIGFBP6 or PBS for 24

hours to activate the macrophages. The cell viability was calculated by 0.4% Trypan Blue staining solution, and the cell viability was required to be no less than 90%. Cell transfer was performed as previously described (12). In short, 6 hours after CLP surgery, the qualified macrophages treated with rIGFBP6 or PBS were collected, washed twice with PBS, the cell density was adjusted to 1×10^8/mL, and 1×10^7 (100 uL) macrophages were injected intravenously into the mice.

Culture of human monocyte-derived macrophages (HMDM)

Blood was obtained from healthy individuals from the in-house blood donation unit. PBMCs were isolated by density centrifugation using Ficoll-Paque^{TM} PREMIUM 1.084. Isolated PBMCs were incubated with CD14-binding MACS beads for 15 min. Cells were magnetically sorted by positive selection using LS columns (130-042-401; Miltenyi Biotec). Isolated $CD14^+$ cells were differentiated into macrophages using 50 ng/mL M-CSF in RPMI 1640 medium supplemented with 5% heat-inactivated FBS and 2 mM GlutaMAX. Cells were seeded in 24-well plates at 5×10^5 for bacterial infection or cell supernatant acquisition and differentiated into hMDM for 7 d prior to use in experiments. In selected experiments, cells were treated with or without 25 μM of SC79 in the presence or absence of recombinant human IGFBP6 Protein (200 ng/mL) for 4 hours before stimulation with *P. aeruginosa*.

Bacterial phagocytosis assays

Fluorescein isothiocyanate (FITC, Invitrogen)-labeled heat-killed *P. aeruginosa* was first prepared by incubation of *P. aeruginosa* with 0.5 mg/mL FITC for 30 minutes at 37°C. Macrophages (1×10^6 cells) or neutrophils (1×10^6 cells) were incubated with FITC-labeled *P. aeruginosa* at a multiplicity of infection (MOI) of 100 for 30 minutes at 37°C to induce

phagocytosis. Cells were then washed twice with cold PBS, cytoskeleton was stained with TRITC Phalloidin and cell nuclei were stained with 4',6-diamidino-2-phenylindole, followed by visualization using confocal laser scanning microscopy (TCS SP8, Leica).

Bacterial killing assays

Intracellular bacterial killing was determined by incubation of macrophages with live *P. aeruginosa* in the presence or absence of rIGFBP6. Briefly, murine peritoneal macrophages (5×10^5 cells) were infected with live *P. aeruginosa* (MOI = 1:100) at 37°C for 30 minutes. Macrophages were then washed with buffer containing tobramycin (100 μg/mL) to remove extracellular bacteria and subsequently lysed with lysis buffer (Promega). Live intracellular bacteria were quantified by culture of lysates for determination of bacterial uptake (at t = 0 hour) and intracellular killing (at t = 2 hours). Killing rate was calculated from the percentage of colonies present at t = 2 hours as compared to t = 0 hour, as follows: 100 − [number of CFU at t = 2 hours] / [number of CFU at t = 0 hour]. For intracellular bacterial killing by neutrophils, primary murine neutrophils (1×10^6 cells) were infected with live *P. aeruginosa* at an MOI of 100 at 37°C for 30 minutes. The neutrophils were then resuspended in medium containing 100 μg/mL tobramycin to remove extracellular bacteria and subsequently lysed in PBS containing 0.1% Triton 100 for assessment of uptake (t = 0 hour). Additional samples were incubated for 1 additional hour (t = 2 hour) to assess bacterial killing as described above.

Reactive oxygen species (ROS) measurement

Peritoneal macrophages were incubated with heat-inactivated *P. aeruginosa* at an MOI of 100 in DMEM cultured at 37 ºC in 5% CO_2 for 0, 0.5, 1, 2 hours. To measure total intracellular ROS activity, macrophages were treated with the fluorogenic probe DCFH-DA (catalog S0033,

Beyotime) at 5 μM for 30 minutes at 37°C. The medium was then removed, and the cells were returned to prewarmed fresh growth medium. The emitted fluorescence was detected by a fluorescent microplate reader using 490/520 nm excitation/emission filters (Molecular Devices). ROS activity was reported as fluorescence intensity.

Proteomics and analyses

In brief, peritoneal macrophages treated with rIGFBP6 or PBS was sonicated three times on ice using a high intensity ultrasonic processor (Scientz) in lysis buffer (8 M urea, 1% protease inhibitor cocktail). The remaining debris was removed, and the supernatant was collected and the protein concentration was determined with BCA kit. For digestion, the protein solution was reduced with 5 mM dithiothreitol for 30 min at 56 °C and alkylated with 11 mM iodoacetamide for 15 min at room temperature in darkness. The protein sample was then diluted by adding 100 mM TEAB to urea concentration less than 2 M. Finally, trypsin was added for digestion. The peptides were desalted by C18 SPE column. The resulting MS/MS data were processed using MaxQuant search engine (v.1.6.15.0). Tandem mass spectra were searched against the human SwissProt database (20422 entries) concatenated with reverse decoy database. FDR was adjusted to < 1%. This bioinformatics Methods contain GO analysis, COG analysis, pathway functional annotation analysis, and time series analysis. Based on the quantitative results, the differentially abundant proteins between groups were identified, and function enrichment analysis, protein-protein interaction (PPI) analysis, and subcellular localization analysis of the differentially abundant proteins were performed.

Electrophoretic Mobility Shift Assay (EMSA)

The direct interaction between STAT1 and CCL2 promoter was examined using an EMSA/Gel

Shift Kit in accordance with the manufacturer's instructions. Cell nuclear extracts were isolated and incubated with biotin-labeled probes containing the CCL2 consensus sequence. Next, the specific anti-STAT1 antibody was added to the mixture of nuclear extracts and DNA probes. The DNA-protein complexes were transferred onto a nylon binding membrane and detected using a streptavidin-horseradish peroxidase conjugate enhanced chemiluminescence (ECL) detection system. The primer sequence of the CCL2 promoter is shown in Supplemental Table 13.

Western blot

Cells were lysed with RIPA buffer (Beyotime) containing PMSF (Beyotime) and phosphatase inhibitors (Beyotime). The proteins in lysates were denaturated with SDS-PAGE loading buffer (Beyotime) containing β-mercaptoethanol (β-ME) and heated at 100 °C for 10 minutes. Proteins were separated on SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) (Merk-Millipore, Billerica, USA). Membranes were blocked with 5% BSA for 2 h and incubated at 4°C overnight with primary antibodies. Then, membranes were incubated with HRP conjugate secondary antibodies for 1 h and visualized using ECL chemiluminescence analysis by Image Lab 6.0 (Bio-Rad, CA, USA). The relative expression levels of the target proteins were standardized to internal controls while densitometric quantification of band intensities was calculated using ImageJ 5.0 software (NIH, MD, USA).

Co-Immunoprecipitation

Co-Immunoprecipitation (Co-IP) was performed according to the manufacturer's instructions for the Pierce™ Co-Immunoprecipitation Kit. First, the primary antibody was immobilized by coupling it to the resin for 2 hours. Then, the pretreated cell lysate was incubated with the

immobilized antibody at 4°C overnight to form immune complex. Finally, the bound protein was eluted for western blotting analysis.

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

Peritoneal murine macrophages (1×10^5 cells/0.2 mL) were inoculated into a 96-well plate. Various inhibitors at serial concentrations were added to the cells. At 24 hours after incubation, MTT (50 μg; M5655, Sigma) was added to each well and incubated for 2 hours. Viable cells took up MTT and reduced it into dark blue, water-insoluble formazan by mitochondrial dehydrogenase, which reflected the normal function of mitochondria and cell viability. The cells were then lysed with DMSO to yield the color solution. The absorbance at 550 nm was measured to quantify the viable cells.

SiRNA *in vivo* transfection

Trans IT® -QR Hydrodynamic Delivery Solution was used to deliver siRNA to mice (13). Use a restraint device to secure the mouse during the injection, the total volume required was calculated according to mouse weight/10 + 0.1mL. The siRNA and Delivery Solution were mixed and injected through the tail vein at a constant speed within 4-7 seconds.

References:

1. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *Jama.* 2016;315(8):801-10.
2. Santos I, Colaço HG, Neves-Costa A, Seixas E, Velho TR, Pedroso D, et al. CXCL5-mediated recruitment of neutrophils into the peritoneal cavity of Gdf15-deficient mice protects against abdominal sepsis. *Proc Natl Acad Sci U S A.* 2020;117(22):12281-7.
3. Mikacenic C, Price BL, Harju-Baker S, O'Mahony DS, Robinson-Cohen C, Radella F, et al. A Two-Biomarker Model Predicts Mortality in the Critically Ill with Sepsis. *Am J Respir Crit Care Med.* 2017;196(8):1004-11.
4. Schlapbach LJ, Watson RS, Sorce LR, Argent AC, Menon K, Hall MW, et al. International Consensus Criteria for Pediatric Sepsis and Septic Shock. *Jama.* 2024;331(8):665-74.
5. Rittirsch D, Huber-Lang MS, Flierl MA, and Ward PA. Immunodesign of experimental sepsis by cecal ligation and puncture. *Nat Protoc.* 2009;4(1):31-6.
6. Song Z, Zhang X, Zhang L, Xu F, Tao X, Zhang H, et al. Progranulin Plays a Central Role in Host Defense during Sepsis by Promoting Macrophage Recruitment. *Am J Respir Crit Care Med.* 2016;194(10):1219-32.
7. Bai H, Lu Q, Wu C, Xu F, Liu J, Wang K, et al. Bone morphogenetic protein 9 is a candidate prognostic biomarker and host-directed therapy target for sepsis. *Science translational medicine.* 2024;16(732):eadi3275.
8. Achouiti A, Vogl T, Urban CF, Rohm M, Hommes TJ, van Zoelen MA, et al. Myeloid-

related protein-14 contributes to protective immunity in gram-negative pneumonia derived sepsis. *PLoS Pathog.* 2012;8(10):e1002987.

9. Nascimento DC, Viacava PR, Ferreira RG, Damaceno MA, Piñeros AR, Melo PH, et al. Sepsis expands a CD39(+) plasmablast population that promotes immunosuppression via adenosine-mediated inhibition of macrophage antimicrobial activity. *Immunity.* 2021;54(9):2024-41.e8.

10. Zhao T, Zhong G, Wang Y, Cao R, Song S, Li Y, et al. Pregnane X Receptor Activation in Liver Macrophages Protects against Endotoxin-Induced Liver Injury. *Advanced science (Weinheim, Baden-Wurttemberg, Germany).* 2024;11(19):e2308771.

11. Tsuji N, Tsuji T, Yamashita T, Hayase N, Hu X, Yuen PS, et al. BAM15 treats mouse sepsis and kidney injury, linking mortality, mitochondrial DNA, tubule damage, and neutrophils. *J Clin Invest.* 2023;133(7).

12. Lu XJ, Chen J, Yu CH, Shi YH, He YQ, Zhang RC, et al. LECT2 protects mice against bacterial sepsis by activating macrophages via the CD209a receptor. *J Exp Med.* 2013;210(1):5-13.

13. Lewis DL, and Wolff JA. Systemic siRNA delivery via hydrodynamic intravascular injection. *Advanced drug delivery reviews.* 2007;59(2-3):115-23.