SUPPLEMENTAL METHODS

Mice

Male C57BL/6 mice (Jackson Laboratories, Clea Japan, Inc., Tokyo, Japan), at 8–10 weeks of age and 20–25 g body weight, were kept for at least 1 week in a 12-hour light/dark cycle and were fed *ad libitum*. The animal experiment protocol was approved by our institutional review board (# Med-P16-027) and was conducted with strict compliance to the ethical guidelines issued by The University of Tokyo Graduate School of Medicine.

Peritoneal Sepsis

Mice, spontaneously breathing, under general anesthesia with 2%–4% of isoflurane underwent cecal ligation and puncture (CLP). A midline laparotomy was performed and the cecum was identified and meticulously exteriorized. A section 75% from the tip of the cecum was isolated for ligation with 4-0 silk thread. The ligated cecum was then punctured side-to-side with either a 22-gauge or 18-gauge needle to make two holes. A 22-gauge needle was used for mild peritoneal sepsis and an 18-gauge needle was used for severe peritoneal sepsis. After returning the cecum into the peritoneum, the abdomen was closed in two layers (4-0 silk for peritoneal membrane and 3-0 silk for skin), and 1 mL of normal

saline supplemented with 25 mg/kg of imipenem cilastatin (Merck & Co., Inc., Kenilworth, NJ) was administered subcutaneously (E1, E2).

Interferon Beta

Interferon beta (IFNβ, PBL Assay Science, Piscataway, NJ, USA) was diluted to an appropriate concentration in phosphate buffered saline. Either 3 hours prior or 12 hours after CLP, animals received either IFNβ (700,000 U/kg, 200 μl) or the same volume of phosphate buffered saline subcutaneously.

Peritoneal sepsis, survival, and collection of samples

Four groups with different conditions were evaluated: mild sepsis with a 22-gauge needle (mCLPctrl), severe sepsis with an 18-gauge needle (sCLPctrl), severe sepsis with the therapeutic application of IFNβ (sCLPtIFNβ), and severe sepsis with the prophylactic application of IFNβ (sCLPpIFNβ) (Figure 1A). The survival rate and daily body weight were evaluated for all groups over the 6-day study period after CLP (Figure 1B). At 24 hours after CLP, other sets of animals from the four cohorts were euthanized by exposure to an excess concentration of isoflurane and exsanguination. Heparinized blood was drawn for complete blood counts, evaluation of surface antigen expression on circulating neutrophils,

and serum cytokine levels. Subsequently, peritoneal lavage was performed to collect peritoneal exudate cells and to measure peritoneal cytokine levels.

Validation for survival assay

To conduct accurate surgery and treatment to animals, maximum 30 animals / series can be handled. 1 series of experiment consists of 2-3 groups with 7-10 mice / group, which makes it difficult to statistically evaluate the differences in each series because of the limited number of animals per group (n = 7-10/group). To verify the results of survival assay, we carried out the experiment six times. sCLP-tIFNβ had been done four times in parallel, and sCLP-pIFNβ had been done three times in parallel to sCLPctrl. We incorporated all results in analysis. Survival rate in each series varied even in the same condition (mean survival rate of sCLPctrl at 6 series is 24.3 ± 21%). However, in every series, survival rate was greatest in sCLP-tIFNβ followed by sCLPctrl ≥ sCLP-pIFNβ and that ranking did not change throughout the series.

Based on these results, we believe that our results in survival experiments should not be biased by inter series variation.

Peritoneal lavage, cytology, bacterial burden

Mice were euthanized with excess isoflurane followed by exsanguination. For peritoneal lavage, a 23-gauge needle was inserted into the peritoneal space through the lower lateral quadrant and 5 mL of ice-cold Roswell Park Memorial Institute (RPMI) supplemented with 1 mM EDTA was injected, the stomach was massaged several times, and the needle withdrawn. This procedure was repeated two times. Peritoneal lavage fluid (PLF) was placed in sterile vials and serially diluted at equal volumes for culture. Ten-fold serial dilutions of PLF were prepared and plated, and plates were incubated for 24 h at 37°C, after which colonies were counted.

The PLF was centrifuged at 300 × g at 4°C for 5 minutes. The supernatant was used for cytokine analysis after passing it through a 0.22-μm filter and stored at −80°C. The pellet was resuspended in 1 ml of RPMI with L-glutamine, 25 mM HEPES containing 10% fetal bovine serum and 100 U/mL of penicillin, and 100 μg/mL of streptomycin and the total cell number was measured with a hemocytometer. Cytospin samples were prepared by centrifuging the suspensions at 350 rpm for 10 minutes and cell differentials were determined by counting at least 400 leukocytes by Diff Quick Stain (Sysmex Corporation, Hyogo, Japan). Lavaged cells consisted of over 90% neutrophils for all cohorts with CLP (data not shown).

Phagocytic capacity of peritoneal exudate cells

Overall, 2.5 × 10^8 PECs/well in RPMI with Pen/Strep, 10% FBS were plated on a 98-well culture plate for 2 hours and 50 μg/mL of pHrodo™ Green *E. coli* BioParticles® Conjugate (IncuCyte® pHrodo® Green E. coli BioParticles®, Essen Bioscience, Inc. Ann Arbor, MI) was applied. The plates were kept in an incubator with 5% CO_2, at 37°C, with 100% humidity for up to 24 hours and time-sequence changes in fluorescent intensity were measured using the IncuCyte® ZOOM Live-Cell Analysis System (Essen BioScience, Ann Arbor, MI) according to the manufacturer's instructions.

Cell surface antigen quantification of circulating neutrophils

Under anesthesia with inhaled isoflurane, blood was collected by orbital bleeding into heparinized microcentrifuge tubes. Complete blood counts (CBC) of peripheral blood were measured with an automated hematology analyzer MEK-6458 (Nihon Kohden, Tokyo, Japan) (Figure 1B).

Neutrophils from individual mice were characterized for CXCR2 expression levels using flow cytometry. White blood cells in heparinized whole blood were stained with fluorescein isothiocyanate (FITC)-labeled Ly-6G antibody (Cat No. 130-093-138, Miltenyi Biotec K.K.

Koto-ku, Tokyo, Japan) and phycoerythrin (PE)-labeled CD182 (CXCR2) antibody (Clone; SA044G4, Cat. No. 149304, BioLegend, San Diego, CA) on ice.

Red blood cells were lysed with PharmLyse™ (BD Biosciences, San Jose, CA) and excess antibodies were washed away. CXCR2 expression on neutrophils ($Ly6G^{high}$) was evaluated with a flow cytometer (BD Accuri™ C6, BD Biosciences, San Jose, CA).

Cytokine analysis

Biological fluid (serum, peritoneal lavage fluid, and media supernatant) samples were evaluated for cytokines/chemokines using a BD™ Cytometric Bead Array (CBA, Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Cytokines (IL-6, KC, TNF-α, MCP-1, and IL-10) were measured using a flow cytometer (BD Accuri™ C6, Becton, Dickinson and Company).

Peritoneal macrophage phagocytic capacity

A peritoneal macrophage phagocytic assay was performed with a phagocytosis assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions. After 5×10^5 cells/500 µL/well cells were incubated in 24-well plates for various time periods, IgG opsonized latex beads were added and cells were maintained for 24 hours with 5% CO_2, at 37°C. Cells were scraped, washed with RPMI media and

analyzed using a flow cytometer. Phagocytic capacity was expressed as the mean fluorescent intensity of macrophage populations gated with a cytogram.

mRNA expression of peritoneal macrophage stimulated with LPS and / or IFNβ

Murine peritoneal macrophage-derived cell line RAW264.7 (American Type Culture Collection, Manassas, VA, USA) were seeded at 3 x 10^5 cells/2mL/well in the culture medium (RPMI with Pen/Strep, 10% FBS) for overnight and stimulated with LPS with or without co-incubation with 15,000U/mL of IFNβ. After incubation, RNA was extracted using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA) and cDNA was generated using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions.

Cells were harvested at 3, 12, 24 hours after LPS stimulation to evaluate time-course change of each genes.

To mimic *in vivo* situation, cells were first stimulated with LPS for 12 hours, and then washed with 1mL x 2 with culture medium, kept unstimulated for 2 hours, and re-stimulated with LPS for 3 hours accompanied with or without IFNβ. Another set of cells were first incubated with IFNβ for 3 hours before LPS stimulation for 3 hours. Expression of suppressor of cytokine signaling (SOCS)-3, p21 and IL-6 was quantified for each culture condition with TaqMan®

Gene expression assay (Applied Biosystems, Foster City, CA, USA) using StepOne™ Real-Time PCR System according to manufacturer's instructions. Expression value for each gene was calculated by normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and fold change in expression to unstimulated control was determined for each condition. Each assay ID for TaqMan® Gene expression assay is as follows; *Gapdh*; Mm99999915_g1, *Il6*; Mm00446190_m1, *Socs3*; Mm00545913_s1, cyclin-dependent kinase inhibitor 1A (*p21*); Mm04205640_g1. Concomitantly, IL-6 secreted into the culture media was evaluated with ELISA described above.

References

E1. Rittirsch D, Huber-Lang MS, Flierl MA, Ward PA. Immunodesign of experimental sepsis by cecal ligation and puncture. *Nat Protoc*. 2009;4:31-6.

E2. Enoh VT, Lin SH, Lin CY, Toliver-Kinsky T, Murphey ED, Varma TK, Sherwood ER. Mice depleted of alphabeta but not gammadelta T cells are resistant to mortality caused by cecal ligation and puncture. *Shock*. 2007;27:507-19