Tracking the Host Response to Infection in Peritoneal Models of Acute Resolving Inflammation

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Running Head "*In vivo* modelling of chronic peritoneal inflammation"

Abstract

Anti-microbial host defence is dependent on the rapid recruitment of inflammatory cells to the site of infection, the elimination of invading pathogens, and the efficient resolution of inflammation that minimises damage to the host. The peritoneal cavity provides an accessible and physiologically relevant system where the delicate balance of these processes may be studied. Here, we describe murine models of peritoneal inflammation that enable studies of competent anti-microbial immunity and inflammation associated tissue damage as a consequence of recurrent bacterial challenge. The inflammatory hallmarks of these models reflect the clinical and molecular features of peritonitis seen in renal failure patients on peritoneal dialysis. Development of these models relies on the preparation of a cell-free supernatant derived from an isolate of *Staphylococcus epidermidis* (termed SES). Intraperitoneal administration of SES induces a Toll-like receptor 2-driven acute inflammatory response that is characterised by an initial transient influx of neutrophils that are replaced by a more sustained recruitment of mononuclear cells and lymphocytes. Adaptation of this model using a repeated administration of SES allows investigations into the development of adaptive immunity, and the hallmarks associated with tissue remodelling and fibrosis. These models are therefore clinically relevant and provide exciting opportunities to study innate and adaptive immunity and the response of the stromal tissue compartment to bacterial infection and the ensuing inflammatory reaction.

Key words Peritonitis, Inflammation, Cytokine, Fibrosis

1 Introduction

Inflammation associated with bacterial infections of the peritoneal membrane (termed peritonitis) is a major cause of treatment failure in patients undergoing peritoneal dialysis in response to renal failure. Using bacterial isolates from a patient with clinical peritonitis we have generated a cell free supernatant of *Staphylococcus epidermidis,* a gram-positive bacterium typically associated with the commensal microflora. Administration of this cell-free isolate to the peritoneal cavity triggers an acute inflammatory reaction [1]. This model has proven remarkably versatile and provides insights into cytokine/chemokine signalling networks, the tracking of immune cell recruitment, activation, and survival within a site of local infection, and mechanisms of inflammatory resolution [2-6]. These investigations have enabled the generation of new hypotheses that may be applied to a range of inflammatory settings or model systems. For instance, the requirement for interleukin-6 (IL-6) signalling in the recruitment and maintenance of leukocyte populations at the site of infection, originally observed in SES-induced peritonitis [5, 7], was readily translated into murine models of inflammatory arthritis [8, 9], where blockade of this pathway was seen to prevent synovitis, and the development of cartilage and bone damage [10, 11]. Insights have also been gained into the molecular events leading to fibrosis, an untreatable condition that is currently the subject of intense research interest [12]. By modifying the protocol of SES delivery, we have developed a model that allows monitoring of adaptive immune memory and the underlining mechanisms responsible for peritoneal tissue damage and fibrosis [12, 13]. Taken together, these studies support the broad relevance of SES-induced murine peritonitis as an adaptable model for understanding inflammatory activation and maintenance, investigating the molecular mechanisms of leukocyte recruitment and clearance, providing opportunities to

explore the relationships between innate and acquire immunity, and how recurrent bacterial infections may contribute to pathogenesis.

Studies have characterised many of the temporal events associated with acute inflammatory activation following SES administration. These include the rapid influx of neutrophils (within 24 hours), their apoptotic clearance, and replacement with a population of mononuclear leukocytes (2-4 days post administration) that include inflammatory monocytes and effector $CD4^+$ T-cell subsets [13]. The model has also been applied to the understanding of immune homeostasis, and proliferative regulation of resident tissue monocytic cells [1, 12, 14]. The very nature of the peritoneal cavity allows analysis of the inflammatory infiltrate (e.g., multi-parameter flow cytometry), the generation of inflammatory mediators (e.g., ELISA; mass spectrometry), and studies of the stromal compartment (e.g., immunoblotting, electrophoretic mobility shift assays, immunocytochemistry, and studies of functional genomics). Thus, the SES-induced model of peritoneal inflammation is highly versatile and amenable to manipulation to allow mechanistic insight into the working of acute inflammation. In this regard, the model shows a number of features reflecting the clinical context in end-stage renal failure patients on peritoneal dialysis and provides opportunities to investigate anti-microbial host defence and tissue damage.

2 Materials

Prepare all solutions using deionized water and analytical grade reagents. Store all reagents at room temperature (unless otherwise specified). Ensure all re-useable glassware is autoclaved before starting. Where appropriate use sterile disposable plastic ware, and good aseptic technique.

2.1 Preparation of Staphylococcus Epidermidis Cell Free Supernatant

1. Bacterial Specimen. Slope of *S. epidermidis* dormant bacteria stored in glycerol at -80 ^{o}C (A clinical strain may be used or an ATCC derived *S. epidermidis* isolate; ATCC-12228).
2. Nutrient Broth No.2: Dissolve 25 g of nutrient broth No.2 per 1 L of deionized H_2O to a total volume of 2 L (2 × 1 L bottles). Autoclaved media might be stored at room temperature for approximately one week.
3. Diagnostic Sensitivity Test (DST) agar: Dissolve 40 g of DST agar in 1 L of deionized H_2O. Autoclave and cool the bottle sufficiently to allow handling. Ensure the agar is fully liquefied. Pour the molten agar broth into petri dishes to an approximate depth of 1 cm. This should be performed in a laminar flow hood using appropriate aseptic techniques. Allow the molten agar to solidify. Wipe any moisture for the petri dish lids with an alcohol swab and seal the petri dishes with parafilm. Plates may be stored at 4 ^{o}C for approximately one week.
4. Tyrode's salt (0.012 M $NaHCO_3$): Dissolve a vial of Tyrode's salt powder (9.6 g) in 1 L of deionized H_2O. Add 1 g $NaHCO_3$ and filter in a Stericup filter bottle. Store in the fridge at 4 ^{o}C and use within one month.
5. Petri dishes 90 mm.

6. 500 mL centrifuge tubes.
7. 125-, 250-, and 500-mL Erlenmeyer flasks.
8. Dialysis tubing MWCO 12-14000 Daltons.
9. Plastic clips for dialysis tubing.
10. Graduated 25 mL sample tubes (universals).
11. MillexGP 0.22 μm filter unit.
12. Phosphate buffered saline (PBS), sterile.

2.2 SES Bioassay

1. 24-well plates.
2. RPMI 1640 supplemented media (RPMI-complete; RPMIc): RPMI 1640, 10 % (v/v) FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 mg/mL streptomycin, 55 mM β-mercaptoethanol.

2.3 Acute Resolving Inflammatory Challenge

1. Syringes (1 mL and 5 mL).
2. Needles (21- and 25-gauge).

2.4 Tracking Innate Immune Responses

1. Fluorescent dye for cellular labelling (e.g., Trace Far Red, Life Technologies-Invitrogen).
2. 3'-(p-aminophenyl) fluorescein.

2.5 Tracking Stromal Tissue Responses in Mice

1. Dissection kit, including scissors.
2. Liquid nitrogen.
3. 1.7 mL Eppendorf tube.
4. Extraction buffer; 50 mM HEPES (pH 7.5), 150 mM NaCl, 25 mg/mL digitonin.
5. Solubilization buffer for cytosolic fractions containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 % (v:v) NP40. For membrane fractions the following buffer is recommended (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.2 % digitonin).
6. LDS sample buffer.
7. BCA Protein Detection Assay.
8. RNA extraction kits.
9. A handheld electric homogeniser.
10. Tris-buffered saline (50 mM Tris-HCl pH 7.5, 150 mM NaCl).
11. Proteinase-K.
12. Ribosomal depletion kit.

3 Methods

Perform the following procedure in a sterile hood using standard aseptic technique. A schematic flow chart summarising the experimental protocol required for the generation of SES is presented in **Fig. 1**.

3.1 Isolation and Culture of S. Epidermidis Single Colonies

Allow all agar petri dishes to reach room temperature before bacterial inoculation.

1. Inoculate agar plate with a loop of dormant *S. epidermidis* bacteria (**Fig. 2**). Streaking of bacteria will ensure isolation of single colonies for subsequent expansion. Incubate at 37 ^{o}C for 48 hours (*see* Error! Reference source not found.).
2. Using a flamed or disposable inoculation loop, capture a single bacterial colony of *S. epidermidis* and transfer it into a 250 mL Erlenmeyer flask bottle containing 50 mL of nutrient broth No.2. Incubate at 37 ^{o}C overnight with agitation at 170 rpm (*see* **Note 2**).
3. Transfer 2 mL of the *S. epidermidis* liquid culture into 500 mL Erlenmeyer flask bottle containing 400 mL of nutrient broth No.2 and incubate overnight at 37 ^{o}C with agitation at 170 rpm (*see* **Note 3**).

3.2 Preparation of SES

1. Visibly check the turbidity of the bacterial culture medium. Transfer the *S. epidermidis* liquid culture into 500 mL centrifuge tubes. Centrifuge at 1800 x *g* for 20 min at 20 ^{o}C.
2. Decant supernatant and fully suspend the bacterial pellet in 50 mL Tyrode's salt. This can be achieved by gentle agitation using an automatic pipette. Centrifuge at 1800 x

g for 20 min at 20 °C. Decant the supernatant and re-suspend Tyrode's salt solution as before. Check the optical density of the suspension on a spectrophotometer at 560 λ. Dilute the bacterial suspension in Tyrode's salt solution to an optical density of 0.5 - 0.6 at 560 nm (corresponding to 5 x 10^8 cfu/mL) (*see* **Note 4**).

3. Incubate the re-suspended culture for 18 - 24 hours at 37 °C. Centrifuge at 5000 x *g* for 30 min at 20 °C. Collect the supernatant and filter using a 0.2 μm Stericup filter bottle system (*see* **Note 5**).
4. Microbiology check 1. SES is a cell free supernatant that is used to induce sterile, resolving inflammation. To ensure that no living bacteria remains in the suspension after the incubation with Tyrode's salt solution, streak a DST agar plate with a 50 μL sample of the filtered suspension. Incubate at 37 °C for 48 hours and assess bacterial growth. The culture should be negative for any microorganism.
5. Dialyze the cell-free extract using deionized water in a ratio of 100 mL/5 L in a cold room at 4 °C (*see* **Note 6**). Prepare the dialysis tubing as instructed by the manufacturer. Knot one end of the dialysis tubing, then pour approximately 50 mL of the cell-free extract into the dialysis tubing and knot the other end. Clip one end so that the tube floats and place it in the water (*see* **Note 7**).
6. Leave for 8 - 12 hours. Change water after 8 - 12 hours and repeat a total of 3 times. Pool the dialyzed extract and divide into 10 mL aliquots in sterile universals. Remove the lids and cover with Parafilm. Freeze the samples at -20 °C overnight (*see* **Note 8**).
7. Lyophilize the SES. Freeze dry the samples overnight or until the water has completely sublimated. Replace the lids of the sterile universals and store at -80 °C. Vials maybe stored for approximately 1 year.

8. Microbiology check 2. To ensure that the lyophilized SES is free of any viable bacteria, reconstitute one aliquot of SES in 400 μL of sterile PBS; pass through a 0.22 μm filter unit and streak on a DST agar plate. Incubate at 37 ^{o}C for 48 hours and assess the growth of *S. epidermidis* in the plate by eye. The culture should be negative for any microorganism.

3.3 Test Validation: SES Bioassay

(Optional): It is recommended that the bioactivity of each SES batch is tested prior to use *in vivo*. Murine peritoneal mononuclear cells will generate inflammatory cytokines (e.g., IL-1β, IL-6, CXCL8, TNFα) in response to SES stimulation. Isolation of these resident tissue cells can be used as a reliable bioassay. Stimulation of these cells with SES triggers the activation of Toll-like receptor-2 (TLR2) [15, 16]. The TLR2 agonist PAM3CSK4 may be used as a positive control.

1. Isolate resident peritoneal mononuclear cells. Perform a peritoneal lavage of C57BL/6 mice with 5 mL of RPMIc using a 5 mL syringe and a 25-gauge needle. Centrifuge at 350 x *g* for 5 min to pellet the cell isolate, remove the supernatant and re-suspend the cells to a concentration of 2 x 10^6 cells/mL (*see* **Note 9**).
2. Plate 1 x 10^6/0.5 mL cells in 24-well tissue culture microtitre plates.
3. SES titration. Reconstitute SES at the ratio of 1 mL/vial in RPMIc and pass through a 0.22 μm filter unit. Prepare serial dilutions of SES (neat, 1/2, 1/4, 1/8, 1/16, 1/32 and 1/64) in RPMIc.
4. Add 0.5 mL of each dilution of SES to each well containing 1 x 10^6 of resident peritoneal mononuclear cells (*see* **Note 10**).

5. Incubate the culture at 37 ^{o}C, 5 % CO_2 overnight. Carefully transfer the culture media to Eppendorf tubes and render cell free by centrifugation (2000 x *g*, 5 min).
6. Carefully transfer the supernatant to a clean tube and store at -80 ^{o}C.
7. Quantify cytokine production using a commercial ELISA (**Fig. 3**).

3.4 In Vivo Administration of SES and Assessment of Acute Resolving Inflammation

1. Reconstitute the SES in sterile PBS at ratio of 0.75 mL PBS/ vial of SES.
2. Filter the reconstituted SES with a 0.22 μm filter unit to a new, sterile tube. Load the 1 mL syringes with the required volume of SES (*see* Error! Reference source not found.).
3. Intra-peritoneal (i.p.) administer 0.5 mL of SES to each mouse (*see* Error! Reference source not found.).
4. At defined time points (0 - 96 hours), euthanize each mouse by an intraperitoneal (i.p.) overdose of anesthetic (240 mg/kg sodium pentobarbital).
5. Dissect out slices (e.g., 1 cm × 1 cm) of the peritoneal membrane, and place in Eppendorf tube for immediate downstream processing on ice (80 mg tissue), or snap-freeze in liquid nitrogen for subsequent -80 ^{o}C storage.
6. These time points (**step 4**) are selected to conduct analysis of the leukocyte infiltrate (e.g., flow cytometry, differential cell count), production of inflammatory mediators (e.g., ELISA, mass spectrometry), and stromal tissue responses (*see* **Note 13**). In wild type mice, a single episode of acute resolving inflammation is characterised by a rapid influx of neutrophils that peaks at 3 - 6 hours, which resolve within 24 hours post stimulation. Neutrophils are replaced by a more sustained population of infiltrating monocytic cells (24 - 72 hours post stimulation), which are characterised by flow

cytometry as F4/80^{int}CD11b^{int} and lymphocytes (including interferon-γ [IFNγ] and IL-17-secreting populations) [1, 4, 5, 7, 17] (*see* **Note 14**).

7. Peritoneal tissue can be harvested to determine gene expression or protein activation within the stromal compartment (e.g., immunohistochemistry, real-time PCR, electrophoretic mobility shift assays, immunoblotting) (*see* **Note 15**).

3.5 Repeated Administration of SES and Profiling of Recurrent Inflammatory Challenge

This acute model can be adapted to a repeat administration model to investigate the cellular and molecular events that lead to the development of memory responses and the onset of inflammation-induced tissue damage (*see* **Note 16**).

1. Deliver SES by i.p. administration at 7-day intervals (day 0, 7, 14 and 21) to mice (*see* **3.4, steps 1** – **3; Note 12**).
2. At each time point (0 - 21 days), as well as at 21 and 28 days after the last administration of SES (i.e., day 42 and 49) (*see* **Note 16**), euthanize each mouse and dissect out peritoneal membrane for immediate use or storage (*see* **3.4**, **steps 4** and **5**).

3.6 Live Model of S. Epidermidis Infection

SES-driven peritonitis provides an extremely versatile model of sterile inflammation. This approach allows investigations of inflammatory regulation without the complications arising from live bacterial infections. However, this model may not be suitable for addressing other questions relevant to the control of infections – e.g., mechanisms of bacterial clearance and dissemination, or systemic responses to local infection. Thus, a modification of the SES acute resolving inflammatory challenge can be achieved using live *S. epidermidis* [18-21].

1. Inoculate an agar plate as before with a loop of dormant *S. epidermidis* bacteria (ATTC 12228 strain). Incubate for 48 hours at 37 ^{o}C.
2. Using a sterile loop, capture a single colony of *S. epidermidis* and transfer it to an Erlenmeyer Flask (125 mL) containing 10 mL of nutrient broth No. 2. Incubate for 6 hours at 37 ^{o}C and 170 rpm agitation. Transfer 60 μL of the liquid culture into a 250 mL Erlenmeyer flask with 60 mL of fresh nutrient broth No. 2. Incubate for 18 hours at 37 ^{o}C and 170 rpm agitation.
3. Assess the desired inoculum size of bacteria (colony-forming units; cfu) by spectrometry. Dilute the bacteria culture to an optical density of 1.1-1.15 at 600 nm (corresponding to 1 x 10^{9} cfu/mL).
4. Wash 3 times in sterile PBS in order to remove the excess of nutrient broth No. 2 and reconstitute to the initial volume.
5. Administer mice with 5 x 10^{8} cfu of *S. epidermidis* (delivered in a volume of 0.5 mL PBS). This bacterial dose is non-lethal and causes no advice changes in body weight. Acute infection and is resolved within 24 - 36 hours.
6. Collect samples at different time points as before. Here, the model can be used to track dissemination of infection into the bloodstream and other organs and can be adapted to investigate the acute phase response and systemic inflammatory outcomes including changes in body temperature.

3.7 Tracking Innate Responses to Infection

Access to the peritoneal cavity provides opportunities to evaluate temporal changes in leukocyte populations (e.g., *via* differential cell counting or multi-parameter flow

cytometry) and inflammatory mediator production (e.g., chemokines, cytokines, lipid mediators and other soluble regulators) using standard immuno-detection assays. However, methods can also be used to track bacterial infection (e.g., clearance or dissemination) using fluorescently labelled bacteria.

1. Prepare an inoculum of *Staphylococcus epidermidis* (*see* **3.6**, **steps 1 – 4**).
2. Re-suspend the bacteria in pre-warmed PBS (to 37 ^{o}C) containing 8 µM Cell Trace Far Red tracker dye. Incubate for 20 min at 37 ^{o}C.
3. Wash 3 times in sterile PBS to remove any excess dye and resuspend in sterile PBS. Fluorescently labelled bacteria can be directly infected into the peritoneal cavity (*see* **3.6, step 5**).
4. At a set time interval (e.g., 3-6 hours following infection), mice are terminated by an approved protocol.
5. Lavage the peritoneal cavity and screen for the phagocytic uptake of fluorescent bacteria using standard flow cytometry or imaging flow cytometry (e.g., using Ly6G immuno-staining to gate infiltrating neutrophils).
6. Extending this approach, the method can also be adapted to evaluate neutrophil phagocytosis in combination with a study of respiratory burst activity. Fluorescently labelled bacteria are administered (i.p.) as described above. However, the peritoneal cavity is now lavaged with 2 mL of RPMI-1640 (serum-free) containing 5 µM of 3'-(p-aminophenyl) fluorescein, a redox sensitive reporter dye that responds to reactive oxygen species.

7. Cells are incubated at 37 ^{o}C for 15 min and immediately transferred to an ice water bath to stop the reaction. Cells are maintained at 4 ^{o}C for all further staining protocols and analysed using standard flow cytometry or imaging flow cytometry (see **Note 17)**.

3.8 Biochemical and Genomic Analysis of Stromal Tissue

SES-induced inflammation stimulates the activation of immune signalling pathways in peritoneal stromal tissues leading to transcription factor activation, chromatin remodelling and changes in gene regulation. Advances in next-generation sequencing methods (e.g., RNA-seq, ATAC-seq, ChIP-seq) now provide exciting opportunities to investigate the stromal tissue response to infection and the mechanisms that support innate and adaptive immunity. Consecutive challenge with SES leads to the development of adaptive immune responses in the peritoneal cavity that include the presence of IFNγ secreting (Th1-polarised) $CD4^{+}$ T-cells. These shift signalling dynamics in the peritoneal membrane to favour pro-fibrotic gene expression programs. Hence the SES model supports the comparative study of acute resolving and non-resolving inflammatory responses. Analysis may be performed at the biochemical level through separation of cytosolic, membrane, and nuclear fractions, or at the genomic level through the isolation of mRNA and nuclear chromatin (**Fig. 4**).

1. Peritoneal membrane sections are excised post-mortem and snap-frozen in liquid nitrogen for downstream processing (80 mg tissue).
2. For biochemical analysis by immunoblot, membrane is pulverised using a pestle and mortar. This may be aided through the parallel administration of small volumes of liquid nitrogen to aid generation of a fine cell powder and/or small tissue fragments.

These are gathered in PBS and centrifuged at full speed in a micro-centrifuge at 4 ^{o}C. The tissue pellet is next re-suspended in the appropriate lysis buffer.

3. To obtain cytosolic and membrane protein pellets for biochemical analysis, extracts are resuspended in extraction buffer, and incubated on ice for 10 min. After centrifugation, pellets are resuspended in the respective solubilization buffers to obtain solubilised cytosolic and membrane fractions.
4. After centrifugation the pellet is resuspended in Tris-buffered saline containing 1 % (w:v) SDS to obtain the nuclear fraction.
5. Aliquots may be mixed with 4 × LDS sample buffer supplemented with 10 % (v:v) β-mercaptoethanol for analysis by SDS-PAGE.
6. For biochemical fractionation (e.g., sucrose gradient or Fast Protein Liquid Chromatography) or enzymatic analysis (e.g., kinase assay, DUB assay) we recommend lysis in the absence of detergents using mechanical stress (e.g., resuspend in water and incubate on ice for 10 min prior to ~20-30 passes through a syringe).
7. For immunoblot analysis protein are quantified using a BCA protein assay. Load ~10 μg protein/well for analysis by SDS-PAGE (based on an 8 - 12 well system).
8. For epigenetic studies, genomic DNA fractions may be subjected to ChIP by diluting (1:4) in Tris-buffered saline and overnight incubation with the appropriate antibody at the dilution factor recommended by the manufacturer. DNA is eluted and prepared for sequencing *via* standard protocols (e.g., Illumina).
9. Total mRNA may be prepared using standard extraction protocols. Extracts of peritoneal membrane (**Fig. 4**) are dissected directly into 1 mL RNA extraction buffer supplemented with β-mercaptoethanol and the tissue dissociated using a handheld

electric homogeniser. Lysates are diluted 1:3 in distilled water and digested in 0.2 mg/mL proteinase-K for 10 min at 55 ^{o}C. Samples are cleared and RNA precipitated in 70 % (v:v) ethanol.

10. Following total RNA extraction, cytoplasmic, mitochondrial, and ribosomal RNA are depleted, and libraries prepared following manufacturer's instructions.
11. The quality and quantity of total RNA or genomic DNA is established using nanodrop and the integrity confirmed on an Agilent 2100 Bioanalyser.

4 Notes

1. The colonies should have a creamy and rounded aspect.
2. The suspension should have substantial turbidity.
3. It is suggested to prepare 4 × 400 mL of culture for each batch in order to obtain around 200 vials of SES at the end of the procedure. At this step, it is recommended to perform a Gram staining to characterise our culture. *Staphylococcus epidermidis* is a Gram-positive coccus. Under the microscope they should appear as rounded cells stained in dark blue following treatment with crystal violet.
4. The spectrophotometer is calibrated against Tyrode's salt solution. Experience shows that a 1/32 dilution gives an optical density of 0.5 - 0.6 at 560 λ. However, it is advisable to perform serial dilutions until the optical density required is achieved.
5. Due to the composition of the SES, the filter may become blocked. If so, replace as necessary.
6. The dialysis is performed to remove the Tyrode's salt from the SES suspension. The semi-permeable membrane (MWCO 12-14000 Daltons) allows Tyrode's salt solute diffusion into dialysis solution.
7. To facilitate the handling of the dialysis membranes, wet them in deionized water before pouring the SES into them. Note that that preparation of dialysis membrane sometimes requires preparation in an EDTA solution. Check manufacturer instructions before commencing.
8. Make a hole in the Parafilm to allow sublimation of the frozen water during the lyophilisation process.

9. Approximately, 2.5-3 x 10^6 resident peritoneal tissue macrophages are isolated from each mouse lavage. Resuspend the cells in 1 mL of RPMIc and count them prior to adjustment to 2 x 10^6 cells/mL.
10. It is advised to prepare a non-stimulated control with 1 x 10^6 cells by adding 0.5 mL of RPMIc alone to 0.5 mL of cells.
11. It is essential to ensure that no bubbles remain in the syringe or the needle before injection. To eliminate this concern always draw more SES suspension than is required for administration.
12. The experiments need to be approved by an institutional animal experimentation ethics committee and be conducted in compliance with the guidelines of the country's governmental bodies that regulate animal experimentation.
13. To ensure optimal cell recovery by peritoneal lavage after SES stimulation, we recommend to lavage the cavity with 5 mL of RPMI. However, in order to assess the levels of inflammatory mediators, we recommend that the lavage is no bigger than 2 mL of PBS so as to avoid extreme dilution.
14. The model is also suitable for the study of resident peritoneal monocytic cells. For example, F4/80^{hi}/$CD11b^{hi}$ monocytic cells, which show a classical disappearance reaction following the initial SES challenge. This population is replenished during the course of the model through proliferative mechanisms [17].
15. The peritoneum is an asymmetrical membrane consisting of different cell types and extracellular matrix components. Therefore, different sample processing is required depending on the downstream applications. For example, a correct orientation of the peritoneal membrane is essential for immunohistochemistry analysis. For protein

quantification or gene expression analysis (e.g., quantitative PCR, RNA-seq), it is recommended to snap-freeze the sample in liquid nitrogen immediately after collection (see outlined protocol).

16. This allows sufficient time for the resolution of inflammation between each SES treatment and the repeat administration promotes the focal development of fibrosis within the peritoneal membrane (day 42 - 49 after the first SES injection) [12].
17. Analysis should be conducted quickly after the initial APF labelling (ideally with 30 min of sample collection). The addition of catalase and superoxide dismutase may be added to confirm the specificity of the fluorescent changes and links to the NADPH oxidase system.

Figure Legends

Fig. 1 Summary schematic of depicting a flow chart of the experimental protocol required for the generation of SES.

Fig. 2 Schematic representation of how to inoculate an agar plate to obtain single bacterial colonies. (**a**) Streak one line in the direction indicated by the arrow with a sterile loop of dormant *S. epidermidis* from the glycerol stock. (**b**, **c** and **d**) Streak four lines in the direction indicated by the arrow using a new sterile loop for each b, c and d.

Fig. 3 Representative plot of the levels of IL-6 produced by murine peritoneal resident cells as a response of different concentrations of SES. SES was reconstituted with 1 mL of RPMIc per vial and filtered (0.22um). SES serial dilutions (neat 1/1, 1/2, 1/4, 1/8, 1/16, 1/32 and 1/64) were prepared with RPMIc as specified in Section 3.3. Blank corresponds to RPMIc alone. For positive control, murine peritoneal resident cells can be stimulated with 10 - 100 pg/mL of IL-1β or 1 – 10 ng/mL of TNFα.

Fig. 4 (**a**) Peritoneal exudates are prepared from lavage samples. These can be used to investigate changes in inflammatory mediator production or leukocyte infiltration. (**b**) Parietal peritoneal membranes are identified as two sections (1 and 2) and can be harvested to evaluate histopathology and changes in genetic parameters. Methods listed in red highlight the types of approaches already used to evaluate inflammatory processes in response to SES or bacterial challenge.

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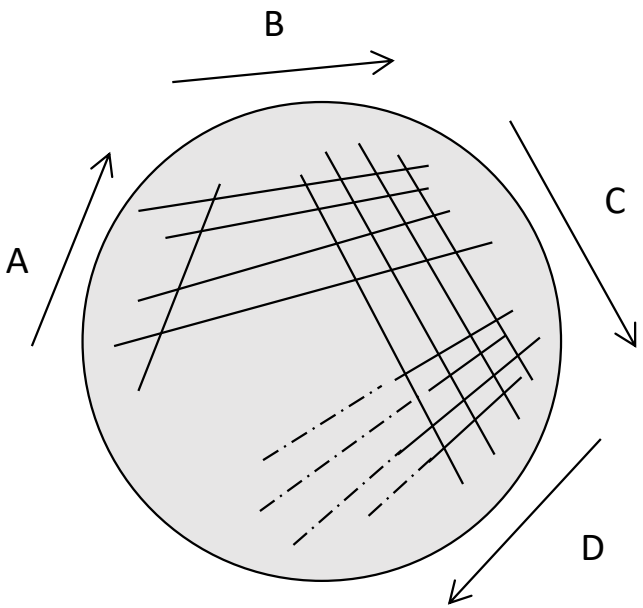

Figure 2. Schematic representation of how to inoculate an agar plate to obtain single bacterial colonies. **A.** Streak one line in the direction indicated by the arrow with a sterile loop of dormant *S. epidermidis* from the glycerol stock. **B, C and D**. Streak four lines in the direction indicated by the arrow using a new sterile loop for each B, C and D.

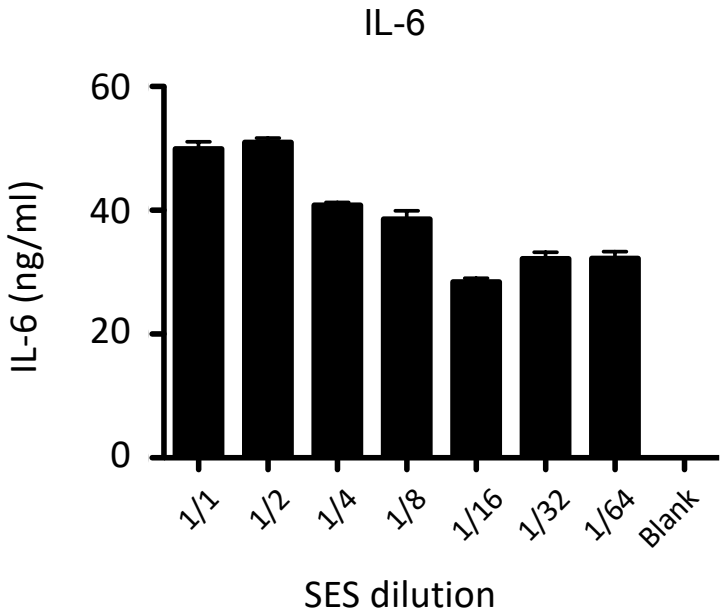

Figure 3. Representative plot of the levels of IL-6 produced by murine peritoneal resident cells as a response of different concentrations of SES. SES was reconstitute with 1 ml of RPMIc per vial and filtered (0.22um). SES serial dilutions (neat 1/1, 1/2, 1/4, 1/8, 1/16, 1/32 and 1/64) were prepared with RPMIc as specified in section 3.3. Blank corresponds to RPMIc alone. For positive control, murine peritoneal resident cells can be stimulated with 10-100 pg/ml of IL-1β or 1-10ng/ml of TNFα

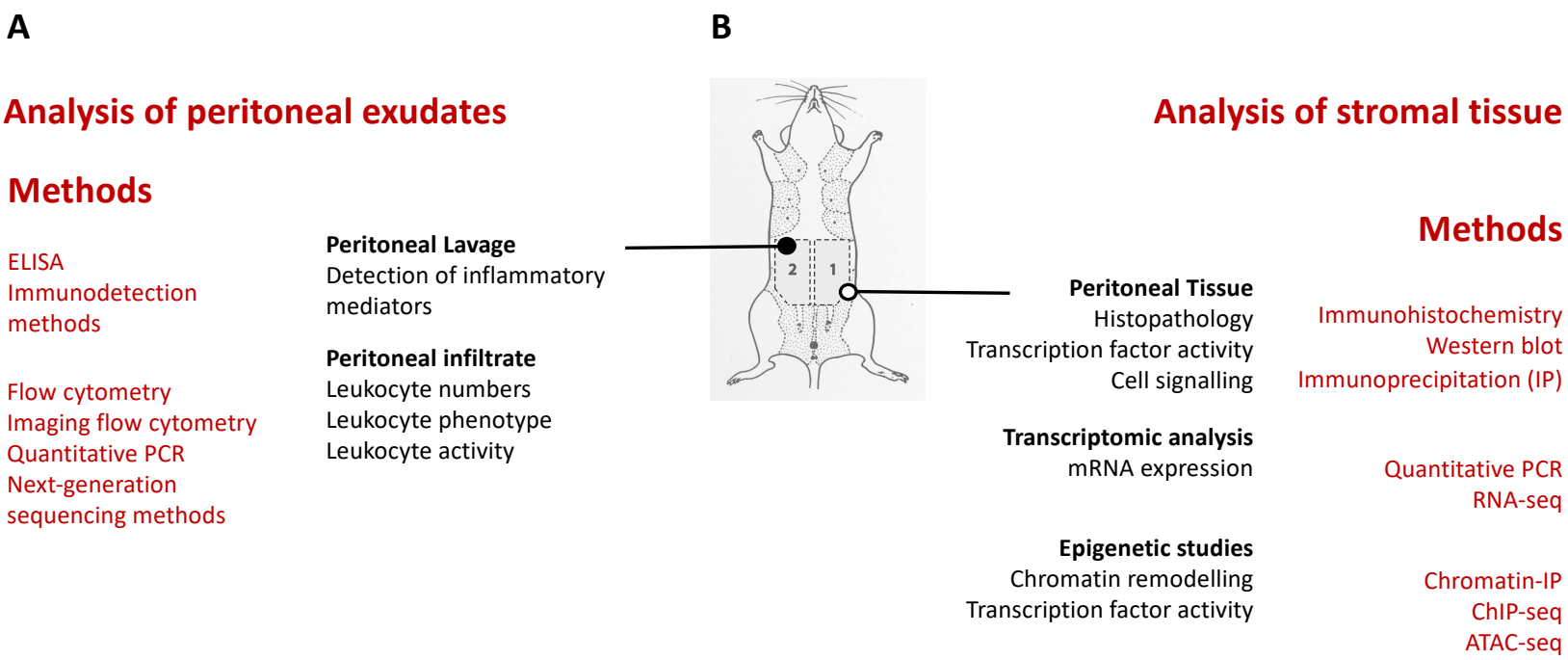

Figure 4. (A) Peritoneal exudate are prepared from lavage samples. These can be used to investigate changes in inflammatory mediator production or leukocyte infiltration. (B) Parietal peritoneal membranes are identified as two sections (1 & 2) and can be harvested to evaluate histopathology and changes in genetic parameters. Methods listed in red highlight the types of approaches already used to evaluate inflammatory processes in response to SES or bacterial challenge.