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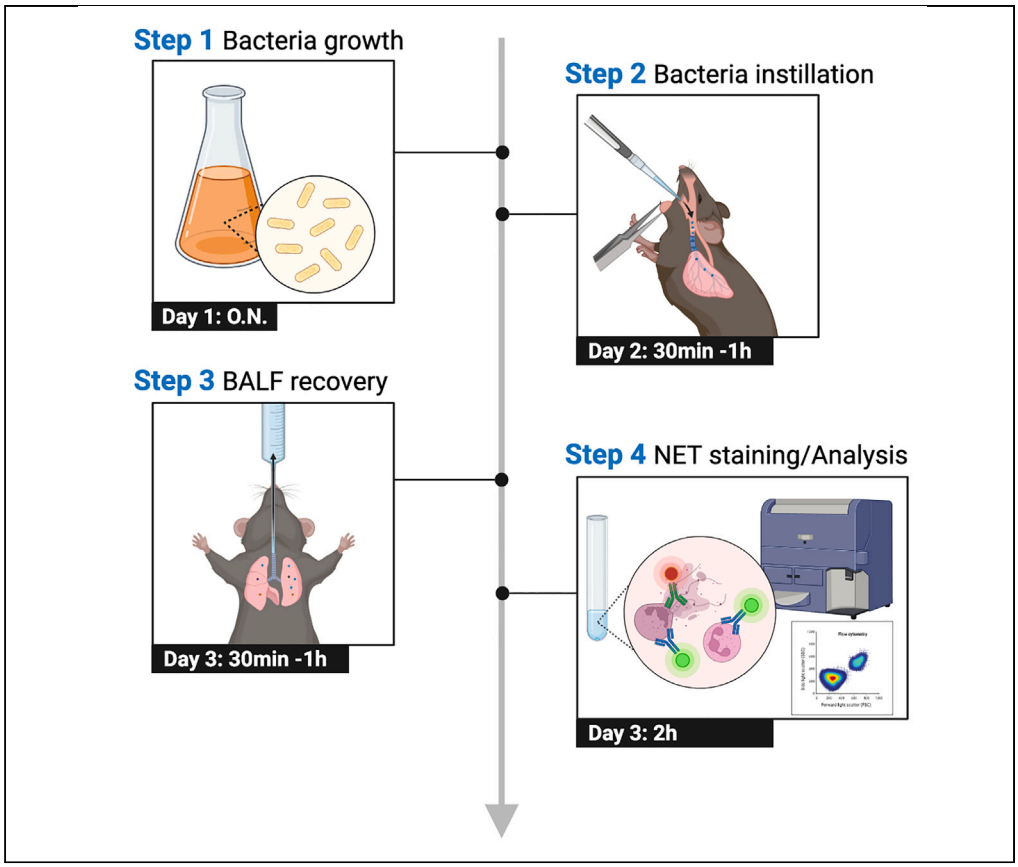
Quantitative cytofluorimetric analysis of mouse neutrophil extracellular traps



The release of neutrophil extracellular traps (NETs) has been involved in numerous infectious and non-infectious diseases. Nevertheless, quantitative analysis of NETs *in vivo* has been challenging. Here, we present a protocol for NET quantification by flow cytometry in the bronchoalveolar lavage fluid (BALF) of mice upon pulmonary infection with *S. aureus*. We describe steps for bacteria growth and instillation and BALF recovery. We then detail staining to quantify the release of NETs and neutrophils recruited to the site of infection.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Analysis of neutrophil extracellular trap formation in a mouse model of lung infection

Step-by-step guide to perform bronchoalveolar lavage (BAL) in mice

Flow cytometry assay of citrullinated histone H3 in BAL neutrophils to assess NETosis

Quantification of absolute numbers of recruited and activated neutrophils in BAL fluid

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Quantitative cytofluorimetric analysis of mouse neutrophil extracellular traps

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SUMMARY

The release of neutrophil extracellular traps (NETs) has been involved in numerous infectious and non-infectious diseases. Nevertheless, quantitative analysis of NETs *in vivo* has been challenging. Here, we present a protocol for NET quantification by flow cytometry in the bronchoalveolar lavage fluid (BALF) of mice upon pulmonary infection with *S. aureus*. We describe steps for bacteria growth and instillation and BALF recovery. We then detail staining to quantify the release of NETs and neutrophils recruited to the site of infection.

For complete information on the generation and use of this protocol, please refer to Poli et al. (2021)[1] and Poli et al. (2022).[2]

BEFORE YOU BEGIN

Neutrophils represent the first line of defense in the case of infectious and sterile injury. They constitute the most abundant immune cell in the blood stream and are the first cells recruited at the damaged site.[3] As all innate immune cells, neutrophils can recognize pathogens through pathogen-recognition receptors (PRRs), and they can activate an appropriate response.[4] Neutrophils exert three major antibacterial functions: degranulation, phagocytosis, and release of NETs. NETs are constituted of long filamentous structures of decondensed DNA covered by citrullinated histone H3 and proteins like neutrophil elastase (NE) and myeloperoxidase (MPO).[5] Although several studies investigated the role of neutrophils and NET release in a number of pathophysiological conditions, the quantification of NETs release *in vivo* has been difficult.

This protocol provides the necessary steps to quantify the number of neutrophils positive for citrullinated histone H3, a hallmark of NET formation, in the bronchoalveolar lavage fluid (BALF) of mice previously infected with *S. aureus*. This protocol uses 6-weeks old, or older, mice and applies to both male and female mice. Moreover, it can be used in mice that have received treatments (i.e., intraperitoneal injections of drugs as we did in[1]). We have also successfully adapted this protocol for detection of neutrophils undergoing NETosis from peritoneal lavage of mice intraperitoneally injected with LPS or *E. coli* (as reported in[1]).

Before starting, prepare.

1. For bacteria growth:

a. Frozen *S. aureus* glycerol stock.
b. Tryptic Soy Broth (TSB).
c. Sterile 250mL Erlenmeyer flask.
d. Sterile 1L Erlenmeyer flask.
e. Plastic cuvettes.

2. For mouse infection:
   a. *S. aureus* culture.
   b. p200 pipette.
   c. Tweezers.
   d. Intubation stand.
3. For BALF isolation:
   a. Tools for mouse dissection (i.e., tweezers and scissors).
   b. Intradermic tube and syringes.
   c. PBS (without Ca^{2+}/Mg^{2+}).
   d. Collecting tubes (FACS tubes).
4. CitH3 staining for flow cytometry:
   a. Staining buffer.
   b. Flow cytometry staining mix.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-citrullinated histone H3 (1:400 dilution)	Abcam	Cat# ab5103 RRID:AB_304752
Anti-rabbit Alexa 568 (1:500 dilution)	Invitrogen	Cat# A11011 RRID:AB 143157
Anti-Ly6G APC (1:200 dilution)	BioLegend	Cat# 127613 RRID:AB_1877163
Anti-CD11b FITC (1:200 dilution)	BioLegend	Cat# 101206 RRID:AB_312789
Anti-CD45 Brilliant Violet 510 (1:200 dilution)	BioLegend	Cat# 103138 RRID:AB_2563061
TruStain fcX (1:100 dilution)	BioLegend	Cat# 101320
Phosphate buffered saline (PBS)	Genesee Scientific	Cat# 25-507
Bacterial and virus strains		
Staphylococcus aureus subsp. *aureus* Rosenbach	ATCC	Cat# 25904
Chemicals, peptides, and recombinant proteins		
DAPI	Invitrogen	Cat# D1306
FACS Lysing Solution	BD Biosciences	Cat# 349202
Intracellular Staining Permeabilization Wash Buffer	BioLegend	Cat# 421002
Triton X-100	Sigma	Cat# X100-5ML
CountBright™ Absolute Counting Beads	Invitrogen	Cat# C36950
Experimental models: Organisms/strains		
Mouse C57BL/6J (Wild type, female, 6–8 weeks)	Jackson Laboratory	Cat# 000664
Software and algorithms		
FlowJo software	Tree Star	FlowJo v10.8 RRID:SCR_008520
Other		
3 mL syringes (no needle)	Fisher Scientific	Cat# 14-955-457
Razor blade	Fisher Scientific	Cat# 12-640
Intradermic PE tubing	BD	Cat# 427425
Disposable cuvettes	Fisher Scientific	Cat# 14-955-127
Tryptic soy broth (TSB)	Sigma	Cat# 22092
FACS tubes	Fisher Scientific	Cat# 14-959-5
BD LSRFortessa	BD Biosciences	N/A

MATERIALS AND EQUIPMENT

Flow cytometry staining buffer		
Reagent	Final concentration	Amount
Intracellular Staining Permeabilization Wash buffer	1 X	5 mL
Triton X-100	1%	500 µL
ddH_2O	N/A	44.5 mL
Total	**N/A**	**50 mL**
This solution can be stored at 4°C for 48 h.		

Flow cytometry staining mix			
Reagent	Stock conc.	Dilution factor	Final conc
αLy6G-APC	0.2 mg/mL	1:200	0.001 mg/mL
αCD11b-FITC	0.5 mg/mL	1:200	0.0025 mg/mL
αCD45-BV510	0.2 mg/mL	1:200	0.001 mg/mL
This solution cannot be stored and needs to be freshly prepared every time.			

STEP-BY-STEP METHOD DETAILS

Bacteria growth

Timing: 1 day

Day 1.

These steps describe how to prepare and grow bacteria for mice infection.

1. Prepare sterile Tryptic Soy Broth (TSB).
2. Scrape a portion of frozen bacteria from the *S. aureus* stock vial using a sterile p200 pipette tip and drop it in a 250 mL Erlenmeyer flask containing 50 mL of TSB.
3. Loosely cover the flask and incubate under shaking at 37°C overnight (O.N.).
4. Add 5 mL of the O.N. grown culture in a 1L Erlenmeyer flask containing 250 mL of TSB.
5. Add 1 mL of bacteria culture in a cuvette and record the initial optical density at 600 nm (OD_{600}) using a spectrophotometer (time zero), then incubate the flask under shaking at 37°C.
6. At 30 min intervals, remove 1 mL of the *S. aureus* suspension and measure its OD_{600} until this value is 0.4, corresponding to 3*10^7 CFU/mL in exponential phase.

⚠ CRITICAL: Accurately determining the concentration of bacteria in the final culture is crucial for the success of the experiments. To achieve this, we recommend using an empirical method to estimate the appropriate OD_{600} value, which corresponds to 3*10^7 CFU/mL. This involves creating a growth curve by collecting samples of the growing bacteria at different time points, measuring their OD_{600}, and then plating them on agar dishes overnight at 37°C to count the resulting colonies the following day.

Mice infection

Timing: 10 mins per mouse

Day 2.

These steps describe how to intratracheally instill *S. aureus* into murine lungs.

7. Centrifuge *S. aureus* culture at 4000× *g* for 10 min and wash twice with PBS. Resuspend at 8*10^8 CFU/mL with saline.
8. Anesthetize the mouse with 2–4% isoflurane accordingly to the procedure approved by your Institutional Animal Care and Use Committee (IACUC). Wait until the animal does not move voluntarily and the breathing rate becomes slow.
9. Arrange the mouse onto an intubation stand.
10. Carefully pull out the tongue with tweezers and instill 80 μL of bacteria suspension (6.4*10^7 CFU/mouse) with a p200 pipette directly into the mouth.
11. While keeping the tongue gently pulled, wait until the mouse swallows and the liquid cannot be seen in the mouth anymore.
12. Place the mouse back in the cage and wait until complete recovery.

Note: If the instillation has been properly done, the mouse will be gasping during the recovery from the anesthesia.

⚠ CRITICAL: The outcome of the experiment is greatly impacted by the quantity of bacteria used. It is therefore recommended to confirm the bacterial count by plating a sample of the cell suspension utilized to infect the mice onto agar dishes and incubating it overnight at 37°C. The following day, confirm the number of CFU used during the experiment.

BALF isolation

Timing: 20 mins per mouse

Day 3.

These steps describe how to obtain the bronchoalveolar lavage fluid (BALF) in mice.

13. 24 h after infection, euthanize one mouse at a time according to the procedure approved by your Institutional Animal Care and Use Committee (IACUC).
14. Place the mouse in a prone position and remove all skin from the lower part of the thorax up to the neck using tools sterilized with 70% EtOH.
15. Expose the trachea by removing all muscles and tissue around it, without damaging or cutting the trachea itself. Put a small pipette tip behind it to better expose the trachea.
16. Carefully open the thorax to display the lungs and cut the diaphragm.

Note: Having an open thorax allows the visualization of the inflation of the lungs, while cutting the diaphragm facilitate the inflation process.

17. Cut halfway the trachea with a razor blade and insert the intradermic tube.
18. Inflate the lungs by injecting 1 mL of PBS through the tube with a syringe.
19. Aspirate with the syringe to recollect the PBS in a FACS tube. Keep on ice.

Note: If the lungs are too small, it is possible to lose some PBS. To avoid losing any material, stop inflating the lungs once they reach full capacity and do not expand anymore.

20. Repeat steps 18–19 twice and collect a total amount of 3 mL of PBS per single mouse.

Note: Record the exact amount of PBS that was recovered in order to normalize the data at the end of protocol.

⚠ CRITICAL: It is particularly important to euthanize the mouse right before the procedure to avoid any rigor mortis.

CitH3 staining for flow cytometry

Timing: 4 h

Day 3.

This section describes steps for the flow cytometry staining of citrullinated histone H3 in neutrophils from BALF.

21. Centrifuge the cells into the FACS tubes at 300× *g* for 5 min at room temperature.
22. Discard the supernatant and resuspend the cells in 100 μL of staining mix. Incubate for 20 min at room temperature and protect from light.

Note: Supernatant can also be conserved and tested for cytokines or other parameter of NETs formation via ELISA kits. We successfully measured the quantity of Citrullinated histone H3 (Cayman, cat#501620) and quantity of myeloperoxidase (MPO)-DNA complexes by using a modified Cell Death Detection ELISA (Roche, cat#11544675001) and we found a positive correlation with the percentage of neutrophil positive for citrullinated histone H3 via flow cytometry.

Note: It is possible to determine lung permeability and damage by measuring albumin concentration in the BALF by ELISA. We successfully quantify albumin in the supernatant of the samples with Albumin Assay kit (Abcam, cat# ab235628).

Note: At this step it is possible to add antibody for staining of other cell populations of interest.

23. Wash the excess of antibody and fix the samples by adding 2 mL of staining buffer and centrifuge the cells at 300× *g* for 5 min at room temperature.
24. Discard the supernatant and repeat step 23.
25. Resuspend the cells in 100 μL of staining buffer containing TruStain fcX (1:100 dilution), to avoid the aspecific binding of the anti-citrullinated histone H3 antibody primary antibody. Incubate 30 min at 4°C.
26. Wash the excess by repeating two times step 23.
27. Stain with primary antibody. Add 100 μL of staining buffer containing anti-citrullinated histone H3 antibody (1:400 dilution). Incubate 1 h at room temperature protected from light.
28. Wash the excess of antibody by adding 2 mL of staining buffer and centrifuge the cells at 300× *g* for 5 min at room temperature.
29. Stain with secondary antibody. Add 100 μL of staining buffer containing anti-rabbit Alexa 568 antibody. (1:500 dilution). Incubate 30 min at room temperature protected from light.

Note: Use a secondary antibody with a bright fluorochrome or the signal could be too faint and could be hard to distinguish positive from negative events.

30. Wash the excess of antibody by adding 2 mL of staining buffer and centrifuge the cells at 300× *g* for 5 min at room temperature.
31. Discard the supernatant and wash the pellet with 2 mL of PBS. Centrifuge the cells at 300× *g* for 5 min at room temperature.
32. Resuspend the cells in 300 μL of PBS.
33. Add 15 μL of CountBright™ Absolute Counting Beads.
34. Acquire the samples with BD LSRFortessa Cell Analyzer and measure the percentage of neutrophils positive for citrullinated histone H3.

Note: If the BD LSRFortessa Cell Analyzer is not available, any other flow cytometer with appropriate lasers and filters can be used.

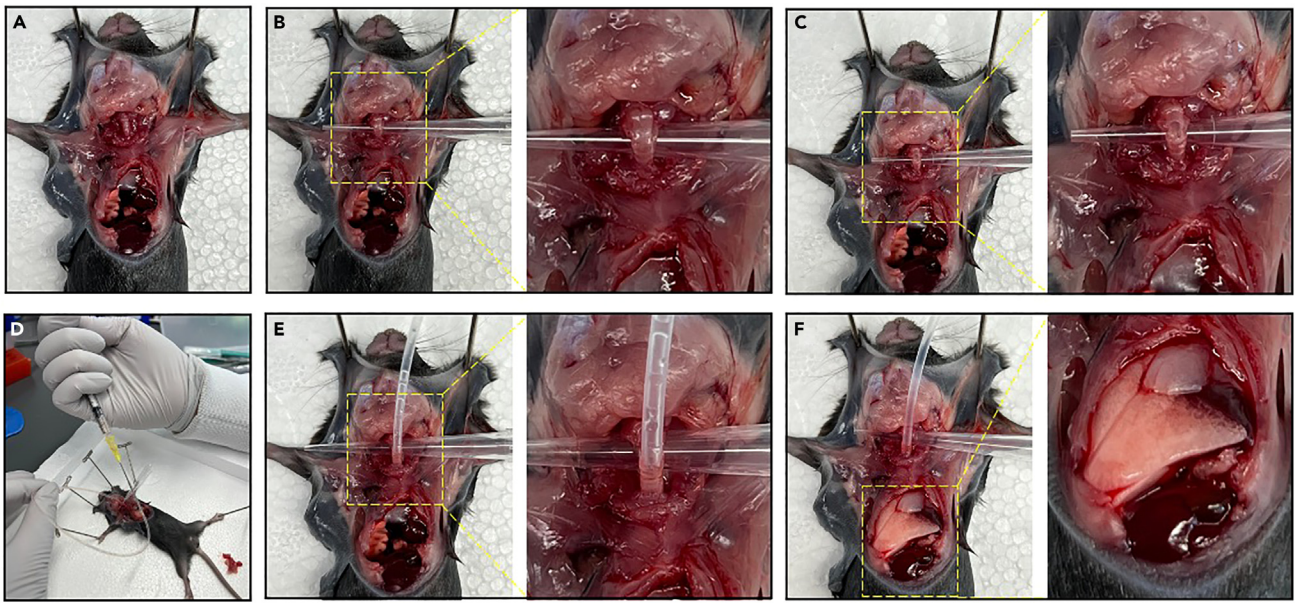

Figure 1. Step by step representation of bronchoalveolar lavage

(A) Carefully expose the trachea by removing muscles and tissues around it.

(B) Put a small tip behind the trachea in order to expose the trachea and create an angle to allow the following cut.

(C) Cut the trachea halfway with a razor blade.

(D and E) Insert in the trachea the intradermic tube connected to a 1 mL syringe filled with PBS.

(F) Inflate the lung by injecting PBS and then gently collect it by slowly pulling the syringe plunger.

35. Analyze the data with FlowJo (see quantification and statistical analysis section for more information).

Pause point: Depending on the type of stimulation and the number of mice, the duration of the protocol may vary. We suggest pausing the protocol after step 24. At this point the samples can be stored at 4°C overnight in staining buffer.

EXPECTED OUTCOMES

In absence of any stimulation, BALF contain a very small number of cells (Figure 1A). Following *S. aureus* instillation, there is a significant recruitment of white blood cells in the BALF, primarily consisting of Ly6G+/CD11b+ neutrophils. The percentage of neutrophils positive for CitH3 is expected to be 20–30% when mice are infected with 6.4*10^7 *S aureus* CFU (Figure 1B).

One crucial step in the release of NETs is the citrullination of histone H3, mediated by peptidylarginine deiminases-4 (PAD4). As control, we suggest using GSK484 PAD4-inhibitor. GSK484 can be administered intraperitoneally (25 mg/kg) 24 h prior to *S. aureus* infection. BALF from GSK484-treated mice infected with *S. aureus* shows a high number of neutrophils with very low positivity for CitH3, allowing the identification of a neutrophil population negative for CitH3 expression (Figure 1C).

It is important to note that any change in the quantity or type of bacteria instilled may affect the number of neutrophils positive for the NETosis marker CitH3.

QUANTIFICATION AND STATISTICAL ANALYSIS

Once the samples have been acquired, the data can be analyzed with FlowJo software. Neutrophil population can be identified as Ly6G + CD11b+ (Figure 2). Then, on neutrophil population identify the CitH3 positive events. Highlight the percentage of neutrophil CitH3+ and the number of events

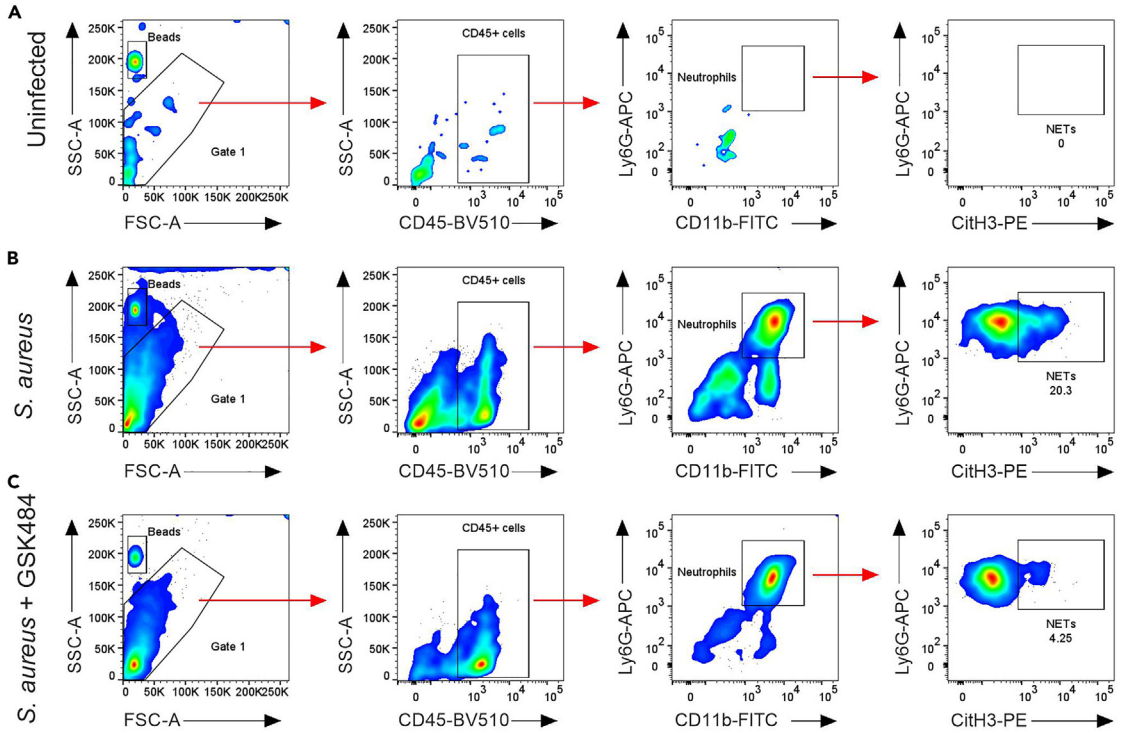

Figure 2. Flow cytometry results of BALF staining

(A) BALF from uninfected mice does not contain cells.

(B) BALF from mice intratracheally infected with *S. aureus* contain high number of cells and neutrophils positive for CitH3 marker.

(C) BALF from mice pretreated with GSK484 and intratracheally infected with *S. aureus* show a strong reduction in neutrophils positive for CitH3.

recorded in the neutrophil gate during sample acquisition (Count of Neutrophils). Identify the signal of the beads (Figure 2) and highlight the number of CountBright™ Absolute Counting Beads events recorded in the beads gate during sample acquisition (Count of Beads). Based on the known concentration of counting beads (beads/μL), to calculate the absolute number of total neutrophils in the sample, use the following formula:

$$Number\ of\ Neutrophil\ in\ BALF\ = \frac{Count\ Neutrophils * Beads\ Concentration}{Count\ Beads} * Final\ Volume$$

Example:

given the following data:

Count of neutrophils = 1337.

Count of beads = 860.

Stock solution concentration of beads = 1040 beads/μL.

Final volume = 315 μL.

To calculate the concentration of beads in FACS tube, use the formula as follow:

$$\frac{\frac{1040\ beads}{\mu l}\ (Stock\ solution) * 15\mu l\ (Volume\ of\ stock\ solution)}{315\ \mu l\ (Final\ Volume)} = 49.52 \Big/ \mu l$$

Next, use the concentration of beads in FACS tube to calculate the absolute number of neutrophils in the FACS tube as follows:

$$\frac{1337\ (Count\ Neutrophils) * \left(\frac{49.52}{\mu l}\right)(Beads\ concentration)}{860\ (Count\ Beads)} * 315\mu l\ (Final\ Volume) = 24251$$

Use the same formula to determine absolute number of neutrophil positive for citrullinated histone H3, as follows:

$$\frac{292\ (Count\ Neutrophil\ Cit\ H3+) * \left(\frac{49.52}{\mu l}\right)(Beads\ concentration)}{860\ (Count\ beads)} * 315\mu l\ (Final\ volume) = 5296$$

LIMITATIONS

This protocol allows the identification of neutrophils positive for CitH3 in BALF of mice previously infected with *S. aureus*. CitH3 is a hallmark of NET release since it is one of the final steps that facilitate chromatin decondensation and DNA release. However, this protocol does not identify DNA released from neutrophils in the extracellular space.

We successfully applied this protocol to the analysis of neutrophils in BALF and peritoneal lavage, but it can be potentially used in other experimental setting in which a high induction of NETs release is expected.

TROUBLESHOOTING

Problem 1

Problems in intratracheal administration of the stimulus (step 10).

Potential solution

It is possible that during intratracheal administration of the stimulus, the mouse does not swallow, and the liquid remains in the mouth. This usually happens when the mouse is not properly anesthetized. After removing the liquid, we suggest putting the mouse back in the isoflurane chamber and wait until the mouse sleeps profoundly. When the breathing rate has slowed down, and the frequency of the thorax movements has diminished, the mouse should be ready to be instilled with the stimulus.

Problem 2

Lungs do not inflate when PBS is injected (step 18).

Potential solution

Double check that the diaphragm has been cut, in order to let lungs expand easily. If the diaphragm is cut, try to insert the tube deeper in the trachea closer to the lungs. Lastly, verify that the trachea is not damaged, and the PBS flows outside it.

Problem 3

Low amount of PBS is recollected after inflation of the lungs (step 19).

Potential solution

Lungs have a limited volume capacity, and the amount of PBS injected is critical. If too much PBS is used to inflate the lungs, some of it can leak out and it is impossible to recollected. To avoid losing any material, slowly inject PBS and stop as soon as the lungs do not expand anymore. Capacity of the lungs usually correlate with age and weights of the mice.

Problem 4

Neutrophils are not detected in the sample (step 34).

Potential solution

In absence of any infection or injury, the BALF does not contain neutrophils. If neutrophils are not detected in mice infected with *S. aureus*, it can be due to errors during intratracheal administration of the bacteria. To verify that intratracheal instillation is performed properly, we suggest practicing with the instillation of a colored solution (i.e., PBS with Evans blue). In this case, if the injection has been properly executed, lungs should become immediately blue.

Problem 5

Neutrophils positive for citrullinated histone H3 are not detected in the sample (step 34).

Potential solution

To clearly identify neutrophils positive for citrullinated histone H3, it is critical that a negative control group is present (i.e., GSK484-treated group, see "expected outcomes" section). If the percentage of neutrophils positive for CitH3 remains low, control the number of bacteria injected and/or try to increase the number of *S. aureus* CFU used.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ivan Zanoni (ivan.zanoni@childrens.harvard.edu).

Materials availability

This study did not generate any new reagent.

Data and code availability

This study did not generate any code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

V.P. and M.D.G. wrote the paper, I.Z. edited the paper, and V.P. and I.Z. conceived the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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