TNF-induced necroptosis is involved in many physiological and pathological processes. Phospho-MLKL is a hallmark of necroptosis. Cecum is a sensitive organ with extensive necroptosis responses to TNF \textit{in vivo}. Here, taking advantage of commercially available mouse TNF and easily accessible reagents and materials, we systematically provide a detailed and highly versatile protocol of detecting necroptosis signaling in mouse cecum by immunohistochemical labeling, which can also be used in other tissues or antibodies in immunohistochemical staining.
Protocol

Detection of necroptosis by phospho-MLKL immunohistochemical labeling

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Summary

TNF-induced necroptosis is involved in many physiological and pathological processes. Phospho-MLKL is a hallmark of necroptosis. Cecum is a sensitive organ with extensive necroptosis responses to TNF \textit{in vivo}. Here, taking advantage of commercially available mouse TNF and easily accessible reagents and materials, we systematically provide a detailed and highly versatile protocol of detecting necroptosis signaling in mouse cecum by immunohistochemical labeling, which can also be used in other tissues or antibodies in immunohistochemical staining.

For complete details on the use and execution of this protocol, please refer to Yang et al. (2020) and Chen et al. (2015).

Before you begin

Solutions are prepared following the recipes in the Materials and equipment section. Solutions which are prepared in advance and can be stored are indicated. A complete list of Materials and equipment required is given in the Key resources table.

Mouse

You should have 8–12 weeks old male or female mice in C57BL/6 background and housed in specific pathogen free (SPF) environment.

Key resources table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Phospho-MLKL (S345) (critical reagent) | Abcam | Cat# ab196436; |
| Chemicals, peptides, and recombinant proteins |        |            |
| Mouse TNF (optional) | Novoprotein | Cat# CF09 |
| Neutral balsam (critical 1) | Sinopharm Chemical Reagent Company (optional) | Cat# 10004160 |
| Paraformaldehyde (critical 1) | Merck Millipore (optional) | Cat# 104005 |
| Ethanol              | Sinopharm Chemical Reagent Company (optional) | Cat# 10009218 |

(Continued on next page)
### Materials and equipment

#### PBS preparation

- **Timing:** ≈ 20 min

### Critical and optional reagents, equipment, software, and suppliers

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Xylene (critical 1) | Sinopharm Chemical Reagent Company (optional) | Cat# 1002341922 |
| Hematoxylin | Fuzhou Maixin Biotech Company (optional) | Cat# CTS-1096 |
| Paraplast | Leica (optional) | Cat# 39601095 |
| K_2PO_4 | Sigma (optional) | Cat# P5655 |
| Na_2HPO_4 | Sigma (optional) | Cat# S7907 |
| KCl | Sigma (optional) | Cat# P5405 |
| NaCl | Sigma (optional) | Cat# S5886 |
| Sodium citrate (critical reagent) | Sigma | Cat# 71402 |
| Tween-20 | Sangon Biotech Company (optional) | Cat# TB0560 |
| 28% ammonia | Sinopharm Chemical Reagent Company (optional) | Cat# 10002118 |

#### Critical commercial assays

- Normal horse serum blocking solution (critical reagent)
  - **Source:** Vector Laboratories
  - **Identifier:** Cat# S-2012

- ImmPRESS HRP Universal Antibody (Anti-Mouse IgG/Anti-Rabbit IgG, Peroxidase) Polymer Detection Kit (critical reagent)
  - **Source:** Vector Laboratories
  - **Identifier:** Cat# MP-7500

- ImmPACT DAB Peroxidase (HRP) Substrate (critical reagent)
  - **Source:** Vector Laboratories
  - **Identifier:** Cat# SK-4105

#### Experimental models: organisms/strains

- C57BL/6J
  - **Source:** Jackson Laboratory
  - **Identifier:** 000664

#### Software and algorithms

- Aperio ImageScope 64 v12.4.0.5043 (optional)
  - **Source:** Leica (optional)
  - **Identifier:** https://www.leicabiosystems.com/cn/digital-pathology/manage/apero-imagescope/

#### Equipment

- Tissue cassettes
  - **Source:** CITOGLAS (optional)
  - **Identifier:** Cat# 80312-3161

- Microtome
  - **Source:** Leica (optional)
  - **Identifier:** Cat# RM2016

- Disposable blade
  - **Source:** Leica (optional)
  - **Identifier:** Cat# 819

- Tissue flotation bath
  - **Source:** Changzhou Haosilin Medical Instrument Company (optional)
  - **Identifier:** Cat# TEC2601

- Slide drier
  - **Source:** Changzhou Haosilin Medical Instrument Company (optional)
  - **Identifier:** Cat# TEC2602

- Microscope
  - **Source:** Olympus (optional)
  - **Identifier:** Cat# CX21

- Aperio VERSA (optional)
  - **Source:** Leica (optional)
  - **Identifier:** Cat# Aperio Versa 200

- Embedding cassette
  - **Source:** Beyotime (optional)
  - **Identifier:** Cat# FS9902

- Syringe 1 mL
  - **Source:** BD (optional)
  - **Identifier:** Cat# 300841

- Semi-enclosed benchtop tissue processor
  - **Source:** Leica (optional)
  - **Identifier:** Cat# TP1020

- Super PAP pen
  - **Source:** Fuzhou Maixin Biotech (optional)
  - **Identifier:** Cat# PEN-0002

- The embedding workstation
  - **Source:** Leica (optional)
  - **Identifier:** Cat# EG1160

- Shaker
  - **Source:** Haimen Kylin-Bell Lab Instruments (optional)
  - **Identifier:** Cat# TS-2

- Pressure cooker
  - **Source:** Guangdong Shunfa hardware Products (optional)
  - **Identifier:** Cat# SFYLG20UX

- Electromagnetic oven
  - **Source:** Midea Group (optional)
  - **Identifier:** Cat# SK2105

- Mouse tail vein injection instrument (optional)
  - **Source:** Nanjing Karwin Biotechnology (optional)
  - **Identifier:** Cat# KW-XXY

### Note:
Except for the labeled critical reagents, all the other labeled optional equipment, software, and suppliers can be replaced by similar equipment, software, and other suppliers.

### Timing

- **Timing:** ≈ 20 min
PBS has to be sterilized by autoclaving and can be stored at room temperature (25 °C) for 6 months.

Wash buffer preparation

- **Timing:** ≈ 5 min

| Wash buffer | Final concentration | Amount |
|-------------|---------------------|--------|
| Tween-20    | 0.1%                | 1 mL   |
| PBS         | n/a                 | 1 L    |
| Total       | n/a                 | 1 L    |

Note: Wash buffer should be freshly prepared on the day of use.

4% paraformaldehyde buffer preparation (critical 1)

- **Timing:** ≈ 16 h

| Wash buffer | Final concentration | Amount |
|-------------|---------------------|--------|
| Paraformaldehyde | 4%            | 40 g    |
| PBS         | n/a                 | Add to 1 L |
| Total       | n/a                 | 1 L    |

Note: (1) 4% Paraformaldehyde buffer must be stored at 4 °C up to 1 month. (2) PFA and PBS mixture can be put in a 56 °C oven overnight (about 16 h), which will make PFA easier to dissolve in PBS.

Citrate antigen retrieval buffer (pH 6.0) preparation (critical 2)

- **Timing:** ≈ 20 min

| Citrate antigen retrieval buffer | Final concentration | Amount |
|----------------------------------|---------------------|--------|
| Sodium Citrate                   | 1.0 mM              | 1.4705 g |
| Adjust the pH to 6.0 with 1 N HCl|                     |        |
| Tween-20                         | 0.05%               | 0.25 mL |
| H₂O                              | n/a                 | Add to 0.5 L |
| Total                            | n/a                 | 0.5 L   |
**Note:** Citrate Antigen Retrieval buffer should be freshly prepared on the day of use.

### 0.08% ammonia

| Wash buffer    | Final concentration | Amount |
|----------------|---------------------|--------|
| 28% ammonia    | 0.08%               | 300 mL |
| Ddwater        | n/a                 | Add to 1 L |
| **Total**      | n/a                 | **1 L** |

**Note:** 0.08% Ammonia can be stored at room temperature (25°C) for 6 months.

△ **CRITICAL:** (1) Paraformaldehyde (PFA), xylene and neutral balsam (which is dissolved in xylene) should always be used with adequate ventilation, preferably in a fume hood. Eyes and skin exposure should be avoided. Follow the safety data sheet when handling these reagents. (2) For preparation of the Citrate Antigen Retrieval buffer, mix to dissolve. Adjust pH to 6.0 with 1 N HCl, then add 0.25 mL of Tween-20, then meter to 500 mL and then mix well. Prepare this buffer fresh, storage too long may cause a bad antigen retrieval and let the staining fail.

### Step-by-step method details

#### TNF injection

**Timing:** 15–30 min

1. Use syringe (1 mL, 25 G) to inject appropriate mouse TNF into the experimental mice through tail vein (i.v) (Critical 1, 2).

△ **CRITICAL:** (1) Intravenous injection requires proficient skills. You need to take more practices before the experiments to avoid injecting drugs into muscle or connective tissues. A suitable volume for intravenous injection is about 200 µL/mice, and TNF can be diluted with PBS or saline. (2) In our lab, mice are injected intravenously with 300 mg/kg TNF (75 mg/mL) diluted in endotoxin free PBS. (Troubleshooting 1)

**Alternatives:** The mouse tail vein injection instrument can help new beginners to inject TNF into the mice through i.v. easier.

#### Tissue fixation and sectioning

**Timing:** ≈ 2 days

1. Generally, cecum samples are harvested at 8 h after TNF injection.
2. Cut the cecum from mouse. Place tissue sample into a labeled 10-mL-tube containing 8 mL 4% paraformaldehyde to fix, and put these tubes on a shaker by 220 rpm shaking for 24–48 h at room temperature (25°C) (critical 1, 2, 3).
3. Cut the fixed cecum open to let all the feces out, and put the cleaned cecum into an embedding cassette, and label it clearly with pencil. (Troubleshooting 2)
4. Transfer tissue cassettes to the Semi-enclosed Benchtop Tissue Processor for dehydration: incubate sequentially in 50%, 70%, 80%, 95% v/v ethanol in water for 25 min each, in 95% v/v ethanol in water twice for 15 min each, in 100% v/v ethanol in water twice for 30 min each, in ethanol and
xylene mixture (1:1 v/v) for 30 min, and then in xylene twice for 20 min each. Perform all the incubation processes at room temperature (25°C).

5. Infiltrate tissues in processor with paraplast tissue embedding medium twice at 60°C for 1.5 h.
6. Select an appropriately sized embedding mold and add molten embedding medium from the embedding workstation so that the mold is approximately three quarters full.
7. Use warmed forceps to transfer the infiltrated tissue into the mold, and then place the mold on the 5°C cold plate of the embedding workstation. Use warmed forceps to keep the tissue in the desired orientation and press the tissue gently to make sure that all the air in the tissue is excluded before the paraffin cools and anchors the tissue in place.
8. Place the labeled tissue cassette lid over the top of the mold and dispense sufficient embedding medium over the lid to fill the mold. Transfer the mold and its lid to the 5°C cold plate for 10 min or until the paraffin has hardened enough for manual release of the tissue block from the base mold.

**Pause point:** After embedded by paraffin, the sample can be stored for at least 6 months at room temperature (25°C).

9. Trim blocks at room temperature (25°C) on a RM2016 microtome: cut 20 μm at a time in a continuous way until the appropriate surfaces of tissue in a block are visible.
10. Put the trimmed blocks in refrigerator for 30 min and then manually section 5 μm thick tissue ribbons using a new disposable microtome blade.
11. Float tissue ribbons in a 42°C tissue flotation bath for 1–2 min and then lift individual tissue sections onto CITOGLAS slides (critical 4).
12. Place slides on a 42°C slide drier for 2 h until sample is free of visible moisture, and make sure there is no water under the tissue. If there is water, please use filter paper to absorb it.
13. Dry slides in a 56°C oven for 30 min and proceed with deparaffinization and immunohistochemical labeling.

**Pause point:** The dry slides can be stored for at least 6 months at room temperature (25°C).

⚠ CRITICAL: (1) The feces of cecum must be cleared after fixed by 4% PFA in order to avoid artificial damages to the epithelium of cecum and the blade. (2) Please make sure all the tissues are immersed in the 4% PFA buffer when the shaker is working. (3) Use at least 15 tissue volumes of 10% PFA to achieve adequate fixation. (4) It is very important to absorb all the water under the tissue otherwise the tissue may detach from the slide under these following steps.

**Deparaffinization and immunohistochemistry**

**Timing:** ≈ 2 days

14. Deparaffinization: incubate three times in xylene for 10 min each, twice in 100% ethanol for 10 min each, 90% v/v ethanol in water for 5 min, 80% v/v ethanol in water for 5 min, 70% v/v ethanol in water for 5 min, and last wash three times for 5 min each by ddwater.
15. Perform antigen retrieval: put the slide in the antigen retrieval buffer in a pressure cooker, and make sure the whole tissue is embedded in the buffer. Put the pressure cooker on an electromagnetic oven, and heat it under 1800 W. After the cooker begins to vent, start timing for 1.5 min and then let the cooker cool to room temperature naturally (critical 1, 2, 3).
16. After antigen retrieval, use a Super PAP Pen to draw a circle around the tissue, and all the following regents and antibodies except the wash buffer are added in the circle (critical 4).
17. Then, slides are washed three times for 5 min on a shaker under 220 rpm condition and then incubated in 50 μL 10% H2O2 v/v in ddwater for 5 min at room temperature (25°C).
18. After three times of 5-min wash by wash buffer on a shaker under 220 rpm condition, these nonspecific binding sites are blocked by 50 μL 2% horse serum for 30 min at room temperature (25°C).
19. Stain with 1.05 μg/mL phospho-MLKL (S345) antibody (dilution rate: 1:1500) in 2.5% horse serum (total volume: 50 μL). Put the side in a slide holder, and then put the side holder in a refrigerator of 4°C for 12 h.

**Note:** The side holder should have some water in side or the antibody dilution may evaporate owing to the refrigerator condition.

20. After phospho-MLKL nurturing, put the slide holder at bench for 30 min to make sure the slide warm up to the room temperature.

21. After three times of 5 min wash by wash buffer on a shaker under 220 rpm condition, incubate slides with ImmPRESS HRP Universal Antibody for 1 h at room temperature (25°C) and then wash the slides three times for 5 min each time by wash buffer on a shaker under 220 rpm condition, and develop the slides with DAB (3,3-diaminobenzidine) for 2 min (critical 5).

22. Use ddwater to wash the slides 3 times for 5 min each time on a shaker under 220 rpm condition.

23. Counterstain the slides with 50 μL Hematoxylin for 1.5 min at room temperature (25°C).

24. After 3 times of 10 s wash each time by ddwater, put the slide in 0.08% ammonia for 6 s, and wash 3 times for 10 s each time by ddwater.

25. Dehydrate slides by incubating them in 80% v/v ethanol in water for 1 min, 90% ethanol for 1 min, twice in 100% ethanol for 1 min, and then twice in xylene for 1 min.

26. Place coverslip over the slides with permanent mounting medium using neutral balsam. Press softly to exclude all the air between the slides and coverslips in and around the tissue (critical 6).

27. Put the neutral balsam to dry in a fume hood at room temperature (25°C) for 12 h.

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**Pause point:** These slides can be stored permanently at room temperature (25°C).

28. Use Aperio VERSA to capture the image and analysis the data (critical 7).

**CRITICAL:** (1) The concentration and pH of the antigen retrieval buffer are critical. The wrong concentration and pH may cause very strong false positive signaling! (Troubleshooting 3) (2) Please count the time of antigen retrieval when the pressure cooker begins to vent. (3) The time course of antigen retrieval is also very important to expose antigenic sites, because too long or too short exposure will cause false positive signaling or signaling loss. (4) The circle drawn around the tissue should not get too close to the tissue or the edge of the tissue might not get enough nurturing of every treatment. (5) When you develop phospho-MLKL, you should do it under the microscope to check the developing status. When you see the signaling of phospho-MLKL, stop the time of development. Generally, it takes 2 min. (Troubleshooting 4 and 5) (6) To exclude the air between the slide and cover slips, you must avoid pressing the tissue too hard which may cause artificial damage to the tissue. (7) The signaling of phospho-MLKL will appear in these damage areas, as described in Troubleshooting 4 and 5. Hence, when you find the damaged area, it will help you find the signaling of phospho-MLKL more quickly and correctly.

**Expected outcomes**

The cecum was damaged when injected with TNF. As Figure 1 shows, we can find submucosa edema, desquamation, and epithelial sloughing in the cecum. The signaling of phospho-MLKL was in the epithelial cell.

**Limitations**

Depending on the cytotoxicity of TNF and environmental impact on mice, you may have to modify the amount of mouse TNF injected, the time of getting cecum sample and of developing phospho-MLKL. The volume of TNF is 150 μL to 300 μL.
Troubleshooting
Described below are some potential problems and recommendations for troubleshooting.

Problem 1
Why do the mice resist to mouse TNF injection?

Potential solution
First, check the housing condition of the mice. All the mice should be fed in SPF condition. Second, you can use L929 or other cell line that are sensitive to TNF-induced cell death to check the cytotoxicity of TNF. Third, dilute the TNF when you inject it to the mice, because long-term exposure of TNF at room temperature (25°C) may decrease the cytotoxicity of TNF.

Problem 2
How do I get the faces out of the cecum after fixation?

Potential solution
Owing to the structure of cecum, it has two blind ends. After fixation, the cecum became very solid and you can cut the two ends (each about 10% of cecum) of the cecum. Then, use scissors to cut the cecum open from its ileocecal flap to its own blind end and forceps to clamp and shake the cecum slowly to let all the faces out.

Problem 3
Why can I not get the pH of citrate antigen retrieval buffer to 6.0?

Potential solution
The pH of antigen retrieval buffer is very sensitive, so you can get 490 mL ddwater in the bottle, then add 20 µL 1 N HCl each time to adjust the pH meanwhile using a magnet rotor to let the HCl dissolve quickly and equally in the buffer. When the pH approaches 6.0, you shall become more careful and add 1 N HCl more slowly and wait till the pH becomes stable and decide to add the HCl or not. Generally, 2–3 mL 1 N HCl is enough to adjust the pH to 6.0 in 500 mL Citrate antigen retrieval buffer. Finally, when the pH value becomes 6.0, add 250 µL Tween-20 and meter volume to 500 mL by ddwater.

Problem 4
Why do I get so many false positive signaling in the cecum after TNF injection?

Figure 1. Immunohistochemical labeling of phospho-Mlkl in the cecum after TNF injection
Cecum of WT mice injected i.v. with 300 mg/kg TNF for 8 h was sectioned and stained with anti-phospho-MLKL antibody. Scale bar, 50 µm.
Potential solution
If you have many false positive signaling, you can try the following methods. First, you shall ensure the concentration and pH of Citrate antigen retrieval buffer. Second, the time course of antigen retrieval must be 1.5 min, because too long or too short time of antigen retrieval will cause false positive signaling. Third, you can reduce the concentration of phospho-MLKL or the developing time of DAB.

Problem 5
Why cannot I get any signaling in the cecum after TNF injection?

Potential solution
First, the damage of the cecum should be observed when TNF had been injected for 8 h. If there is no damage, it means your TNF is not working and please check its cytotoxicity activity as described in problem 1. Second, if there is too much damage of the cecum, it will also make the phospho-MLKL lost. If so, you can collect the samples earlier or reduce the amount of TNF that is injected into the mice. Third, you can also increase antibody concentration of phospho-MLKL. Fourth, you can re-check your antigen retrieval condition, because insufficient exposure of antigenic sites may cause the defect of phospho-MLKL signaling. Fifth, increase the developing time of DAB.

Resource availability
Lead contact
The reagents generated in this study are available with no restriction. Further information and requests for resources and reagents should be directed to the Lead Contact, Jiahuai Han (jhan@xmu.edu.cn).

Materials availability
This study did not generate new unique reagents.

Data and code availability
This study did not generate any unique datasets or code.

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Author contributions
P.H. and T.A. carried out most of the experimental work with help from J.W. and Z.-H.Y.; P.H., T.A., and J.H. designed experiments and interpreted data; P.H., T.A., and J.H. wrote the manuscript; and J.H. conceived and supervised the study.

Declaration of interests
The authors declare no competing interests.

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