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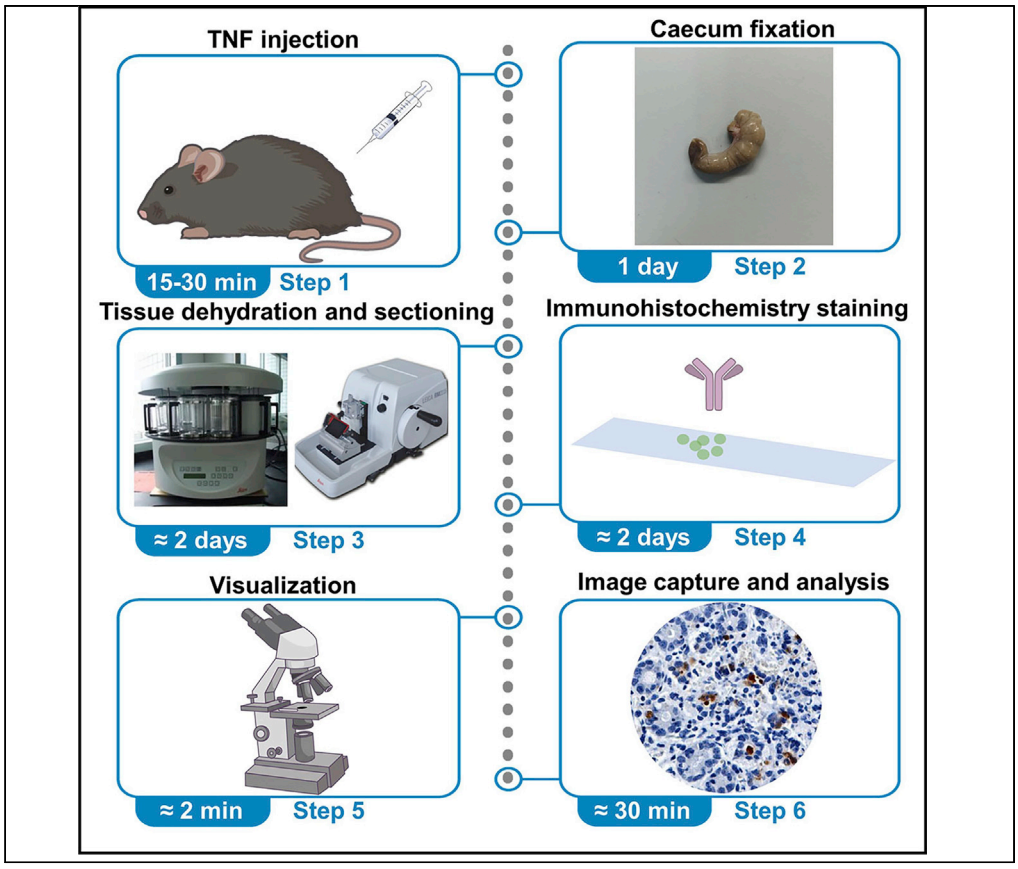
Detection of necroptosis by phospho-MLKL immunohistochemical labeling



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 *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.

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Highlights

Phospho-MLKL is used to detect necroptosis in mouse cecum after TNF challenge

Reliable experimental results with easy-to-obtain experimental materials

The protocol could be applied to other polyphonic tissues or use a different antibody

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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 *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	Novoprotein (optional)	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*)

Continued

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
KH_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)	Vector Laboratories	Cat# S-2012
ImmPRESS HRP Universal Antibody (Anti-Mouse IgG/Anti-Rabbit IgG, Peroxidase) Polymer Detection Kit (critical reagent)	Vector Laboratories	Cat# MP-7500
ImmPACT DAB Peroxidase (HRP) Substrate (critical reagent)	Vector Laboratories	Cat# SK-4105
Experimental models: organisms/strains		
C57BL/6J	Jackson Laboratory	000664
Software and algorithms		
Aperio ImageScope 64 v12.4.0.5043 (optional)	Leica (optional)	https://www.leicabiosystems.com/cn/digital-pathology/manage/aperio-imagescope/
Equipment		
Tissue cassettes	CITOGLAS (optional)	Cat# 80312-3161
Microtome	Leica (optional)	Cat# RM2016
Disposable blade	Leica (optional)	Cat# 819
Tissue flotation bath	Changzhou Haosilin Medical Instrument Company (optional)	Cat# TEC2601
Slide drier	Changzhou Haosilin Medical Instrument Company (optional)	Cat# TEC2602
Microscope	Olympus (optional)	Cat# CX21
Aperio VERSA (optional)	Leica (optional)	Cat# Aperio Versa 200
Embedding cassette	Beyotime (optional)	Cat# FSR902
Syringe 1 mL	BD (optional)	Cat# 300841
Semi-enclosed benchtop tissue processor	Leica (optional)	Cat# TP1020
Super PAP pen	Fuzhou Maixin Biotech (optional)	Cat# PEN-0002
The embedding workstation	Leica (optional)	Cat# EG1160
Shaker	Haimen Kylin-Bell Lab Instruments (optional)	Cat# TS-2
Pressure cooker	Guangdong Shunfa hardware Products (optional)	Cat# SFYLG20UX
Electromagnetic oven	Midea Group (optional)	Cat# SK2105
Mouse tail vein injection instrument (optional)	Nanjing Karwin Biotechnology (optional)	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.

Materials and equipment

PBS preparation

Timing: ≈20 min

PBS	Final concentration	Amount
KH_2PO_4	2 mM	0.27218 g
Na_2HPO_4	10 mM	1.4196 g
NaCl	137 mM	8.00628 g
KCl	2.7 mM	0.201285 g
H_2O	n/a	Add to 1 L
Total	**n/a**	**1 L**

Note: 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
H_2O	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

Timing: ≈5 min

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% H_2O_2 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.

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.

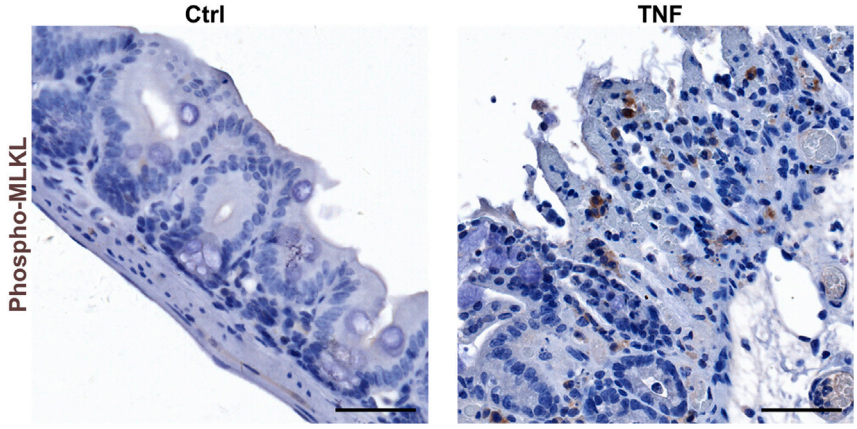

Figure 1. Immunohistochemical labeling of phospho-Mlkl in the cecum after TNF injection
Cecum of WT mice injected i.v. with 300 mg/kg TNF for 8 h was sectioned and stained with anti-phospho-MLKL antibody. Scale bar, 50 μm.

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?

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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