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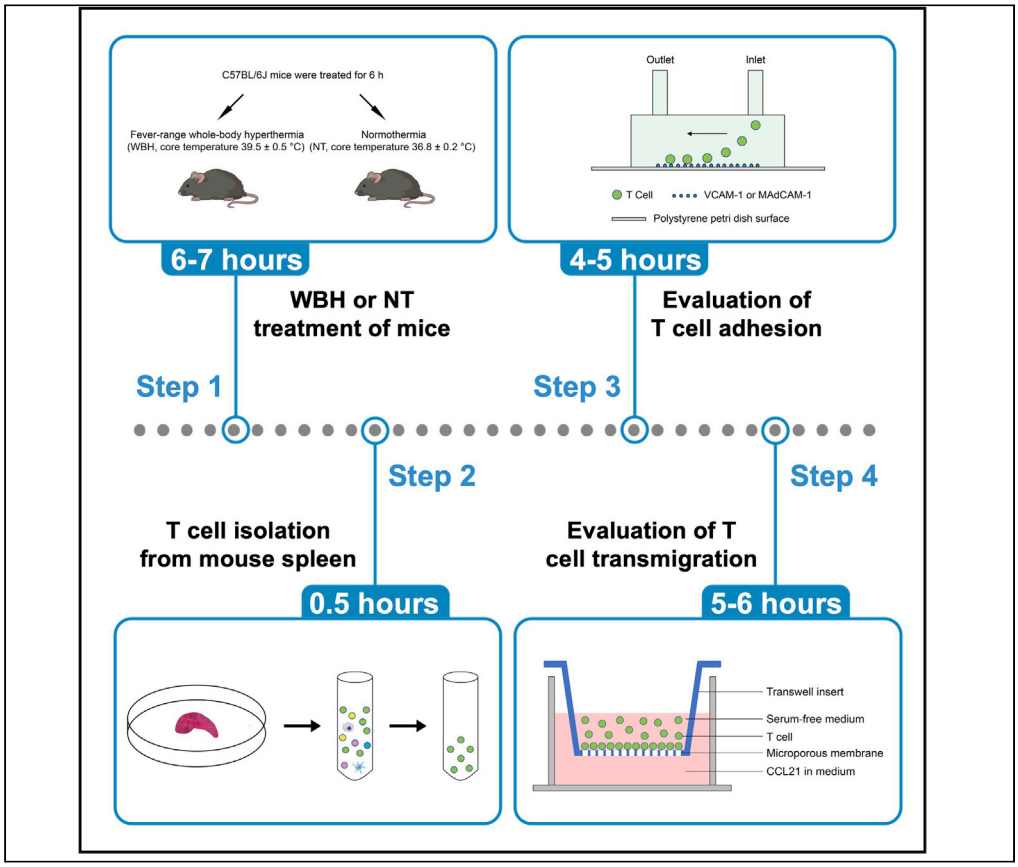


Fever is a complex physiological response enhancing immune surveillance during infection and inflammation. Fever-range whole-body hyperthermia (WBH) treatment can experimentally mimic the febrile condition in mice. Here, we describe a protocol for the treatment of mice with WBH and normothermia. We describe the isolation of T cells from mouse spleen followed by the evaluation of T-cell adhesion and transmigration. This animal model can be applied to studying the dysfunction of the immune system induced by fever.

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## Highlights

Whole-body hyperthermia (WBH) can mimic the febrile condition in mice

We isolate T cells from WBH- or normothermia-treated mice

T-cell adhesion and transmigration assays show dysfunctions caused by fever

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# Protocol for fever-range whole-body hyperthermia (WBH) in mice to study febrile effect on T-cell adhesion and migration

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## SUMMARY

**Fever is a complex physiological response enhancing immune surveillance during infection and inflammation. Fever-range whole-body hyperthermia (WBH) treatment can experimentally mimic the febrile condition in mice. Here, we describe a protocol for the treatment of mice with WBH and normothermia. We describe the isolation of T cells from mouse spleen followed by the evaluation of T-cell adhesion and transmigration. This animal model can be applied to studying the dysfunction of the immune system induced by fever.**

**For complete details on the use and execution of this protocol, please refer to Lin et al. (2019).**

## BEFORE YOU BEGIN

### Mice

Timing: 8–10 weeks

1. C57BL/6J mice were obtained from Jackson Laboratory and maintained under specific pathogen-free conditions.

   *Note:* Mice with distinct background are used in specific assay.

   ⚠ CRITICAL: All animal studies were approved by the Institutional Animal Care and Use Committee of the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (SIBCB-S323-1712-022).

2. Age-matched (8–10 weeks of age) female mice were used in the following assays.

   *Note:* Female mice were usually used in the assay of fever-range whole-body hyperthermia treatment in previous literatures (Appenheimer et al., 2005; Chen et al., 2006; Evans et al., 2001).

### Check equipment

Timing: 1–2 h

3. The environmental chamber (e.g., artificial climate incubator, ZRQ-150, GEMTOP) was pre-set at 38.8°C (Evans et al., 2001; Ostberg et al., 2001). The temperature of environment in the chamber could be stabilized at 38.8°C (± 0.1°C) in 2 h.

⚠ CRITICAL: Environmental temperature was set as 38.8°C according to the previous report (Chen et al., 2006). In the assay conducted by Dr. Chen et al., they monitored the body temperature of mice with a subcutaneously implanted microchip thermotransponder (implanted 1 week or more before WBH treatment) and a programmable data-acquisition system (Bio Medic Data Systems). Under condition, the core temperature of mice was 39.5 ± 0.5°C.

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| DATK32 (10 μg/mL) | ATCC | Cat#HB-294 |
| Chemicals, peptides, and recombinant proteins | | |
| Sodium chloride (NaCl) | Sigma | Cat#S3014-1KG; CAS: 7647-14-5 |
| Potassium chloride (KCl) | Sigma-Aldrich | Cat#P3911-500G; CAS: 7447-40-7 |
| Potassium phosphate monobasic ($KH_2PO_4$) | Sigma | Cat#P5655-500G; CAS: 7778-77-0 |
| Sodium phosphate dibasic ($Na_2HPO_4$) | Sigma | Cat#S5136-1KG; CAS: 7558-79-4 |
| Sodium bicarbonate ($NaHCO_3$) | Sigma-Aldrich | Cat#S6014-500G; CAS: 144-55-8 |
| D-(+)-Glucose | Sigma | Cat#G6152-500G; CAS: 50-99-7 |
| Bovine serum albumin (BSA) | ABCONE | Cat#B24726-250G; CAS: 9048-46-8 |
| EDTA | Sigma-Aldrich | Cat#U3620; CAS: 60-00-4 |
| Calcium chloride dihydrate ($CaCl_2$) | Sigma-Aldrich | Cat#223606-500G; CAS: 10035-04-8 |
| Magnesium chloride hexahydrate ($MgCl_2$) | Sigma | Cat#M2393-500G; CAS: 7791-18-6 |
| Sodium hydroxide (NaOH) | Sigma-Aldrich | Cat#901915-1KG; CAS: 1310-73-2 |
| Paraformaldehyde | Sigma-Aldrich | Cat#V900894-100G |
| DAPI | Sigma-Aldrich | Cat#D9542; CAS: 28718-90-3 |
| Crystal violet | Sigma-Aldrich | Cat#C6158-100G |
| Mouse VCAM-1-Fc | R&D Systems | Cat#643-VM |
| Mouse MAdCAM-1-Fc | R&D Systems | Cat#993-MC |
| Recombinant Mouse CCL21/6Ckine Protein | R&D Systems | Cat#457-6C-025 |
| Critical commercial assays | | |
| EasySep™ Mouse T Cell Isolation Kit | STEMCELL Technologies | Cat#19851 |
| Fetal bovine serum | Sigma-Aldrich | Cat#F0850-50ML |
| RPMI 1640 medium | Sigma-Aldrich | Cat#R8758-500ML |
| Experimental models: Organisms/strains | | |
| Mouse: C57BL/6J (female), 8–10 weeks old | Jackson Laboratory | Cat#JAX:000664; RRID: IMSR_JAX:000664 |
| Software and algorithms | | |
| GraphPad Prism 5.01 | GraphPad | https://www.graphpad.com/ |
| StreamPix 3.61.0.0 | NorPix | https://www.norpix.com/ |
| Image-Pro Plus 6.0.0.260 | Media Cybernetics | http://www.mediacy.com/ |
| Other | | |
| Environmental chamber | GEMTOP | ZRQ-150 |
| Polystyrene petri dish | Greiner | Cat#664160 |
| Circular Flow Chamber Kit | GlycoTech | 31-001 |
| Syringe pumps | Harvard Apparatus | PHD 22/2000 |
| Digital cameras | Pixelink | PL-B623 |
| Transwell chamber | Corning | Cat#CLS3421-48EA |
| Microscope | Olympus | IX51 |
| Fluorescence microscope | Olympus | IX71 |

(*Continued on next page*)

*Continued*

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| 1.5 mL Colorless sterilization centrifuge tube | Axygen | Cat#MCT-150-C-S |
| 15 mL Transparent conical centrifuge tube | GeneBrick | Cat#GP04-6500 |
| 5 mL Polystyrene round-bottom tube | Corning-Falcon | Cat#352054 |

## MATERIALS AND EQUIPMENT

### Stock chemical solutions

- 0.25 M $CaCl_2$ (3.675 g $CaCl_2 \cdot 2H_2O$, to 100 mL with $ddH_2O$; store at 4°C for six months)
- 0.25 M $MgCl_2$ (5.083 g $MgCl_2 \cdot 6H_2O$, to 100 mL with $ddH_2O$; store at 4°C for six months)
- 1 M NaOH (2 g NaOH, to 50 mL with $ddH_2O$; store at 20°C–25°C for one month)
- 0.5 M EDTA, pH 8.0 (73.06 g EDTA, to 500 mL with $ddH_2O$; store at 4°C for six months)

*Note:* Adjust pH of solution with 1 M NaOH while stirring in order to dissolve EDTA powder.

- Coating Buffer, pH 9.0 (0.84 g $NaHCO_3$, to 1 L with PBS; store at 4°C for six months)
- Blocking Buffer (0.2 g BSA, to 10 mL with Coating Buffer; store at 4°C for six months)
- Washing Buffer (0.25 g BSA, 500 μL 0.5 M EDTA, to 50 mL with HBSS; store at 4°C for six months)
- Buffer A (0.25 g BSA, to 50 mL with HBSS; store at 4°C for six months)

PBS 1L

| Reagent | Final concentration | Amount |
|---|---|---|
| NaCl | 136.89 mM | 8 g |
| KCl | 2.68 mM | 0.2 g |
| $KH_2PO_4$ | 1.76 mM | 0.24 g |
| $Na_2HPO_4$ | 10.14 mM | 1.44 g |
| The buffer can be stored at 4°C for six months. | | |

HBSS 1L

| Reagent | Final concentration | Amount |
|---|---|---|
| NaCl | 136.89 mM | 8 g |
| KCl | 5.37 mM | 0.4 g |
| $KH_2PO_4$ | 0.44 mM | 0.06 g |
| $Na_2HPO_4$ | 0.34 mM | 0.048 g |
| $NaHCO_3$ | 4.17 mM | 0.35 g |
| D-(+)-Glucose | 5.60 mM | 1.008 g |
| The buffer can be stored at 4°C for six months. | | |

## STEP-BY-STEP METHOD DETAILS

### Fever-range whole-body hyperthermia treatment of mice

Timing: 6–7 h

1. C57BL/6J mice were injected intraperitoneally with 1 mL sterile 0.9% saline.

   *Note:* The procedure is to avoid dehydration during WBH treatment. See troubleshooting 1.

2. Mice were divided into two groups randomly. One was treated with fever-range WBH (core temperature 39.5 ± 0.5°C) by being placed in an environmental chamber pre-set at 38.8°C for 6 h.


**Figure 1. NT and WBH treatment of mice**

The environmental chambers were pre-set at 22°C or 38.8°C. Mice were placed in the chambers for 6 h to keep the core temperature 36.8 ± 0.2°C or 39.5 ± 0.5°C.

The other normothermia (NT) control mice (core temperature 36.8 ± 0.2°C) were maintained at 22°C for the experimental period (Figure 1).

## T-cell isolation from mouse spleen

Timing: 30 min

Adapted from the manufacturer's protocol, please refer to https://www.stemcell.com/easysep-mouse-t-cell-isolation-kit.html.

3. WBH or normothermia treated mice were sacrificed by $CO_2$.
4. Disrupt spleen in PBS containing 2% fetal bovine serum (FBS). Remove aggregates and debris by passing cell suspension through a 70 μm mesh nylon strainer.
5. Centrifuge at 300 × *g* for 10 min and resuspend at 1 × $10^8$ nucleated cells/mL in 1 mL PBS in 1.5 mL centrifuge tube.
6. Add 50 μL Rat Serum to sample and transfer sample to a 5 mL polystyrene round-bottom tube.
7. Add 50 μL Isolation Cocktail to sample. Mix and incubate at 20°C–25°C for 10 min.
8. Add 75 μL RapidSpheres™ to sample. Mix and incubate at 20°C–25°C for 2.5 min.

*Note:* Vortex RapidSpheres™ for 30 s before adding to sample to make sure particles appear evenly dispersed.

9. Add 1.5 mL PBS to top up the sample. Mix by gently pipetting up and down 2–3 times.
10. Place the tube into the magnet and incubate at 20°C–25°C for 2.5 min.
11. Pick up the magnet, and in one continuous motion invert the magnet and tube, pouring the enriched cell suspension into a 15 mL centrifuge tube.

⚠ CRITICAL: Leave the magnet and tube inverted for 2–3 s, then return upright. Do not shake or blot off any drops that may remain hanging from the mouth of the tube.

## Flow chamber assay

Timing: 4–5 h

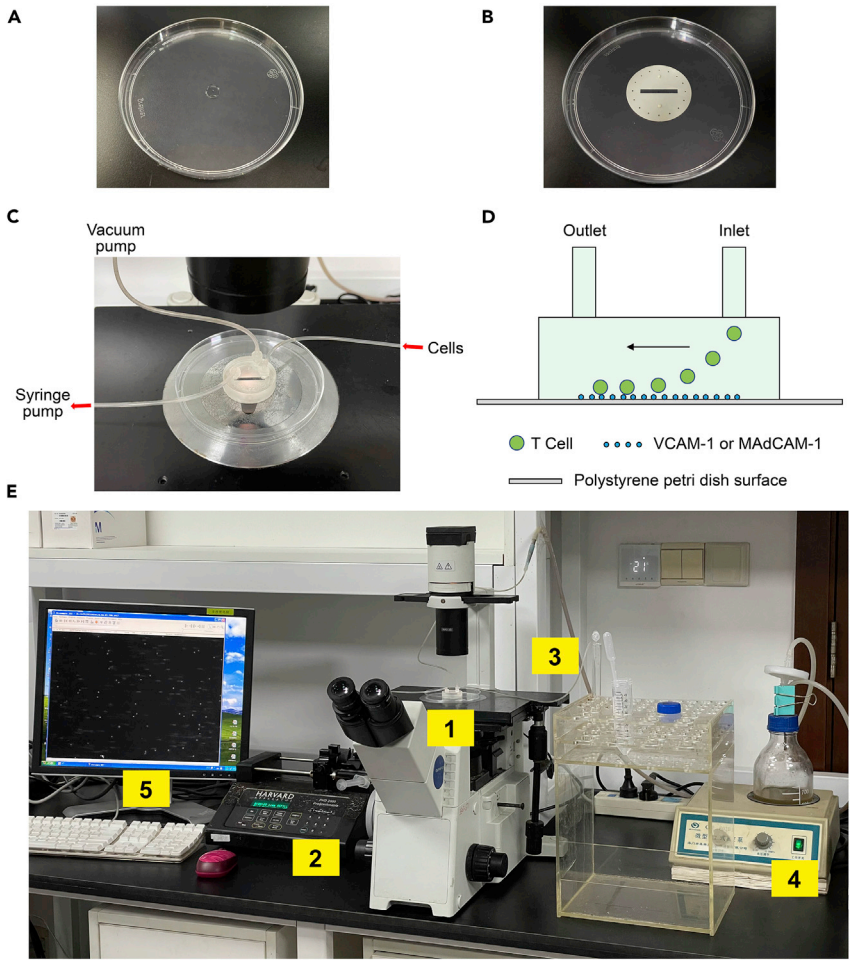


**Figure 2. Flow chamber system**

(A) Mouse VCAM-1-Fc or MAdCAM-1-Fc was coated on a polystyrene petri dish for 1 h at 37°C.

(B) A silicon rubber gasket was covered on the coated protein on polystyrene petri dish.

(C) Photograph of flow chamber system. Cells were diluted in buffer A containing 1 mM $Ca^{2+}$ and 1 mM $Mg^{2+}$ immediately before infusion into the flow chamber at a consistent shear stress of 1 dyn/$cm^2$ for 1 min using a programmable syringe pump.

(D) Schematic diagram of flow chamber system.

(E) Devices for flow chamber system. 1. Flow chamber deck; 2. Programmable syringe pump; 3. Cells in 1.5 mL centrifuge tube; 4. Vacuum pump; 5. Computer used for recording the videos of cell adhesive behavior using StreamPix III software.

12. A polystyrene petri dish was coated with a 5 mm diameter, 20 μL spot of 5 μg/mL mouse VCAM-1-Fc or MAdCAM-1-Fc in coating buffer for 1 h at 37°C (Figure 2A).

*Note:* To study cell adhesion ability of other cell types, the concentration of mouse VCAM-1-Fc or MAdCAM-1-Fc could range from 2 μg/mL to 50 μg/mL. See troubleshooting 2.

13. The spot was washed with blocking buffer and coated with 20 μL blocking buffer for 1 h at 37°C to block non-specific binding sites.
14. Aspirate off blocking buffer, add cover the coated protein on polystyrene petri dish with a silicon rubber gasket (Figure 2B).
15. Cover the gasket with a flow chamber deck and assemble flow system apparatus connecting inlet, outlet, and vacuum lines to the deck. Fill system with media and remove all air from system (Figures 2C–2E).

**Pause point:** The flow chamber system was set up and could be operated after the cell samples were prepared.

16. Isolated T cells were washed twice using washing buffer to eliminate free metal ions and collected by centrifugation at 750 × *g* for 7 min.

*Note:* To guarantee the better status of isolated T cells, steps 12 to 15 could be carried out during fever-range whole-body hyperthermia treatment of mice and T cell isolation. See troubleshooting 3.

17. Cells were washed twice using buffer A to clear away EDTA in washing buffer and collected by centrifugation at 750 × *g* for 7 min.
18. Cells were diluted to 1 × $10^6$/mL in buffer A containing 1 mM $Ca^{2+}$ + $Mg^{2+}$ immediately before infusion in the flow chamber.
19. Cells were infused into flow chamber at a consistent shear stress of 1 dyn/$cm^2$ for 1 min by a syringe pump (Figure 2E). The adhesive behavior was monitored by digital cameras. The videos were analyzed by Image-Pro Plus.

*Note:* α4β7–VCAM-1 binding was disrupted by pre-treating the cells with 10 μg/mL α4β7 blocking antibody DATK32 when examining α4β1-mediated cell adhesion on VCAM-1 substrate. The affinity of VCAM-1 to integrin α4β1 and MAdCAM-1 to integrin α4β7 should be tested in advance. See troubleshooting 4. If there are too many non-specific T cells to the surface of the polystyrene petri dish, the chamber could be washed with 1–2 mL washing buffer by the syringe pump. See troubleshooting 5.

*Note:* The motion of each adherent cell was monitored for 10 s following the initial adhesion point, and two categories of cell adhesion (rolling and firm adhesion) were defined. Adhesion was defined as rolling adhesion if the adherent cells were followed by rolling motions ≥ 5 s with a velocity of at least 1 μm/s; a firmly adherent cell was defined as a cell that remained adherent and stationary for at least 10 s.

### Chemokine-induced transwell migration

**Timing: 5–6 h**

20. Both sides of transwell chambers were coated with 5 μg/mL mouse VCAM-1-Fc or MAdCAM-1-Fc.
21. T cells (2 × $10^6$/mL in 150 μL RPMI 1640 medium) were added to the upper chamber and the lower chamber was filled with 600 μL RPMI 1640 medium with CCL21 (500 ng/mL).
22. After incubation at 37°C for 4 h at 37°C in 5% $CO_2$, cells remaining on the upper surface of the chamber were scraped with a cotton swab, and cells having migrated to the bottom surface were fixed with 2 % formaldehyde at 20°C–25°C for 10 min.
23. Cells were stained with DAPI at 20°C–25°C for 10 min and enumerated by fluorescence microscope.

*Alternatives:* Cells having migrated to the bottom surface could also be stained with 0.5 % Crystal Violet and enumerated by microscope.

*Note:* α4β7–VCAM-1 binding was disrupted by pre-treating the cells with 10 μg/mL α4β7 blocking antibody DATK32 when examining α4β1-mediated cell transmigration across VCAM-1 substrate.

## EXPECTED OUTCOMES

Fever is a highly conserved response to infection or injury in both endothermic and ectothermic species (Evans et al., 2015). Previously, researchers usually treated lymphocytes directly with

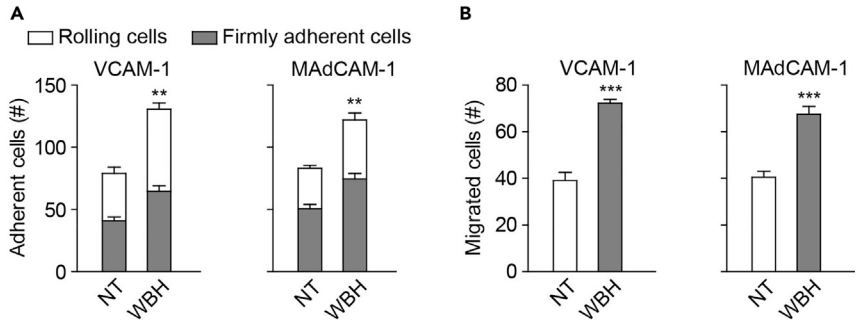


**Figure 3. WBH treatment enhances α4 integrin mediated T cell adhesion and transmigration**

C57BL/6J mice were treated with normothermia (NT) or fever-range whole-body hyperthermia (WBH) for 6 h, and then were sacrificed. T cells were isolated from spleen. α4β7–VCAM-1 binding was disrupted by pre-treating the cells with 10 μg/mL α4β7 blocking antibody DATK32 when examining α4β1-mediated cell adhesion and migration on VCAM-1 substrate.

(A) Adhesion of T cells to immobilized VCAM-1-Fc (5 μg/mL) or MAdCAM-1-Fc (5 μg/mL) substrate in 1 mM $Ca^{2+}$ + $Mg^{2+}$ at a wall shear stress of 1 dyn/$cm^2$.

(B) Transmigration of T cells across VCAM-1-Fc (5 μg/mL) or MAdCAM-1-Fc (5 μg/mL) coated membrane in the presence of CCL21 (500 ng/mL) in the lower chamber.

Data represent the mean ± SEM. ** $p < 0.01$, *** $p < 0.001$ (Student's *t* test). The asterisk in (A) indicates the changes in total adherent cells.

fever-range temperatures and demonstrated that fever could markedly stimulate L-selectin and α4β7 integrin–dependent adhesion of lymphocytes to HEVs (Chen et al., 2004; Evans et al., 2000; Evans et al., 2001). To study the biological function of fever on lymphocyte adhesion and transmigration more physiologically, we'd better treat mice with fever-range whole-body hyperthermia (WBH, core temperature 39.5 ± 0.5°C), and then isolate T cells from mouse spleen to carry out the cell function assay.

Firstly, we examined the effect of fever-range thermal stress on α4β1 or α4β7 integrin-mediated cell adhesion to immobilized VCAM-1 or MAdCAM-1, respectively, under flow conditions in the presence of physiological cations (1 mM $Ca^{2+}$ + $Mg^{2+}$) (Figure 3A). For experiments using VCAM-1 substrate, T cells were pre-treated with α4β7 blocking antibody DATK32 to block α4β7–VCAM-1 binding in order to specifically examine the function of α4β1-VCAM-1 interaction. T cells from WBH mice showed a significant increase in adhesion to immobilized VCAM-1 and MAdCAM-1 at wall shear stress of 1 dyn/$cm^2$ compared with cells from NT mice. In addition, T cells from WBH mice showed significantly enhanced chemokine CCL21-induced transmigration across the VCAM-1– or MAdCAM-1–coated membrane (Figure 3B). Collectively, fever-range thermal stress significantly enhanced α4 integrin mediated cell adhesion and transmigration.

## QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical significance was determined by Student's *t* test using Prism software (GraphPad, version 5.01). The resulting p values are indicated as follows: ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Data represent the mean ± SEM of at least three independent experiments.

## LIMITATIONS

This protocol describes the evaluation of T cell adhesion and transmigration when mice treated with fever-range whole-body hyperthermia or normothermia *ex vivo*. By means of using distinct ligands, researchers could specifically study the biological function mediated by integrins, selectins, chemokine receptors. Whereas, if you want to study the T cell trafficking *in vivo*, T cell distribution in various lymphoid tissues could be examined, and intravital microscopy might also be taken into consideration.

## TROUBLESHOOTING

### Problem 1

Mice were dehydrated and died during WBH treatment (step 1).

### Potential solution

When treated by fever-range whole-body hyperthermia, the lymph nodes of mice swelled up obviously, and in some severe cases mice might die from dehydration or organ failure. To avoid the negative effect, mice were injected intraperitoneally with 1 mL sterile 0.9% saline in advance.

### Problem 2

If researchers want to study cell adhesion ability of other cell types, 5 μg/mL mouse VCAM-1-Fc or MAdCAM-1-Fc might not be sufficient to mediate the binding of cells under flow conditions (step 12).

### Potential solution

The concentration of mouse VCAM-1-Fc or MAdCAM-1-Fc could range from 2 μg/mL to 50 μg/mL, considering different cell types express distinct levels of integrin α4β1 and α4β7.

### Problem 3

The status of isolated T cells was not very well and the following cell adhesion and transmigration could not be carried out successfully (step 16).

### Potential solution

To evaluate the cell adhesion and transmigration of isolated T cells as soon as possible, steps 12 to 15 could be carried out during fever-range whole-body hyperthermia treatment of mice and T cell isolation. Once T cells were isolated from mouse spleen, they could be used in the flow chamber assay and transwell assay directly.

### Problem 4

T cells could not adhere to immobilized VCAM-1 or MAdCAM-1 under flow conditions (step 19).

### Potential solution

No matter integrin ligands (VCAM-1 or MAdCAM-1) were purchased by commercial companies or purified by researches themselves, the affinity of the ligands to integrin α4β1 or α4β7 should be tested in advance. For example, you could investigate the soluble ligand binding ability by flow cytometry.

### Problem 5

Too many T cells adhered to immobilized VCAM-1 or MAdCAM-1 under flow conditions (step 19).

### Potential solution

To block the non-specific binding of T cells to the surface of the polystyrene petri dish, the chamber could be washed with 1–2 mL washing buffer by the syringe pump. EDTA in the washing buffer could chelate the remaining metal ions that might activate integrins. If the non-specific binding still exits, please try to use another clean polystyrene petri dish to coat integrin ligand.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, JianFeng Chen (jfchen@sibcb.ac.cn).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

This study did not generate any unique datasets or codes.

## ACKNOWLEDGMENTS

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## AUTHOR CONTRIBUTIONS

C.D.L. and J.F.C. conceptualized the project and designed the experiments. C.D.L., Z.Y.L., and Y.L. performed the experiments and data analysis. C.D.L. and J.F.C. interpreted the results. The manuscript was drafted by C.D.L. and edited by J.F.C.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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