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Treatment response to isotretinoin correlates with specific shifts in Cutibacterium acnes strain composition within the follicular microbiome

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Protocol

A protocol to detect human CD4$^+$ T cell extracellular traps using scanning electron microscopy

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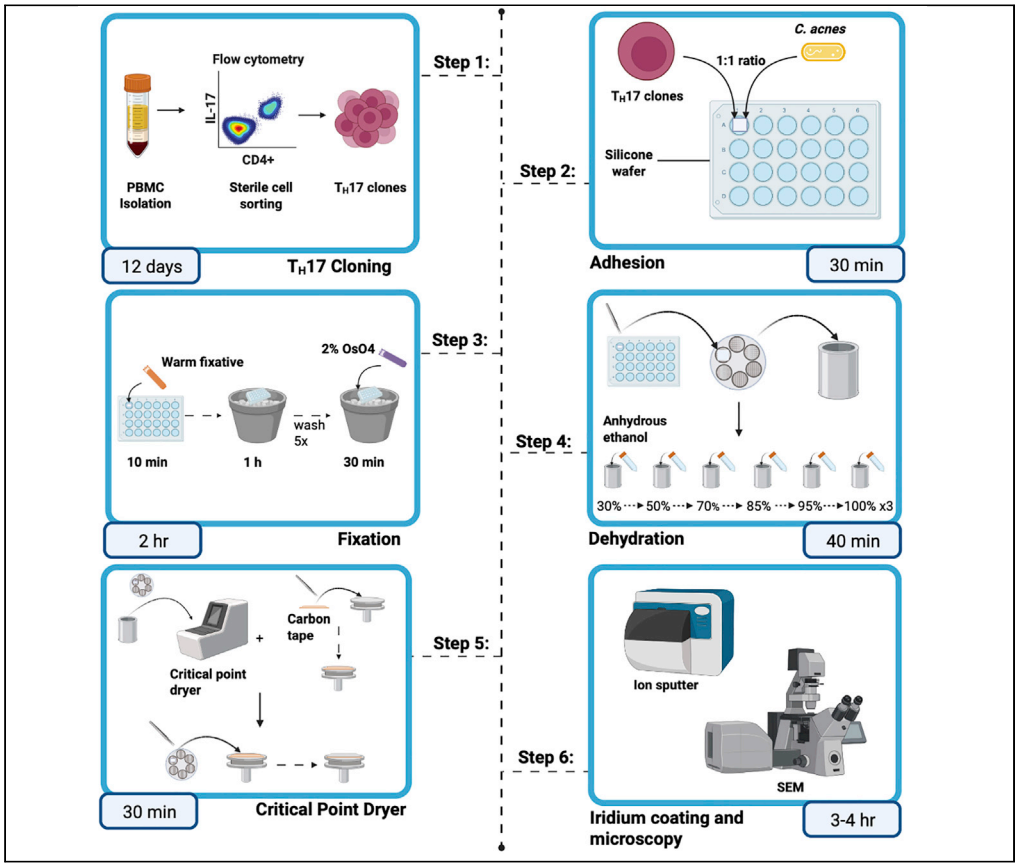

We present a protocol to detect extracellular traps (ETs) induced by *Cutibacterium acnes* in cultured T_H17 clones. We first describe the isolation of *C. acnes*-specific T_H17 clones by sterile cell sorting. We then detail the *in vitro* induction of ETs in T_H17 clones stimulated by *C. acnes* and the imaging of released ETs using scanning electron microscopy. This protocol can be applied to the study of other ETs released by other T cell subsets.

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

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Highlights

Protocol for generation of T_H17 clones from PBMCs

Protocol for stimulation of T_H17 clones to release extracellular traps

Imaging of T_H17 cells and extracellular traps using scanning electron microscopy

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Protocol

A protocol to detect human CD4$^+$ T cell extracellular traps using scanning electron microscopy

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SUMMARY

We present a protocol to detect extracellular traps (ETs) induced by *Cutibacterium acnes* in cultured T_H17 clones. We first describe the isolation of *C. acnes*-specific T_H17 clones by sterile cell sorting. We then detail the *in vitro* induction of ETs in T_H17 clones stimulated by *C. acnes* and the imaging of released ETs using scanning electron microscopy. This protocol can be applied to the study of other ETs released by other T cell subsets.

For complete details on the use and execution of this protocol, please refer to Agak et al. (2021).[1]

BEFORE YOU BEGIN

Institutional permissions

Study approval. This study was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by UCLA IRB (#118-00193). All donors and acne patients provided written informed consent for the collection of peripheral blood and subsequent analysis. Acquire bioethical permissions from relevant institutions.

Radiation safety. All principal investigators/users interested in using the cell harvester (Tomtec MACHIII) are required to send a written request to the radiation safety entity at your institution. After permission is granted, new users are trained by a representative from the manufacturer.

Culture and measuring optical density (OD) of *C. acnes*

Timing: 2 days

1. Obtain *C. acnes* from Biodefense and Emerging Infections Research Resources Repository.
2. Streak Blood agar plates with *C. acnes* glycerol stock, do not let glycerol stock thaw.
   a. Incubate at 37°C for 3–5 days under anaerobic conditions in Reinforced Clostridial Medium (RCM) either in an anaerobic chamber at an $N_2/CO_2/H_2$ ratio of 80:10:10, by volume or in sealed containers (AnaeroPack Rectangular Jar) containing one AnaeroPack sachet.
   b. It will take 3–4 days for adequate growth to occur and colonies to appear.
3. To generate cell cultures of *C. acnes*, pick a single colony using a sterile inoculating loop and inoculate into a tube containing 3 mL of sterile RCM.
   a. Incubate for 3–4 days in AnaeroPack Rectangular Jar as described in step 2 above.
4. To measure OD, take 1 mL of your bacteria and transfer into a labeled Eppendorf tube.

5. Spin down bacteria at 300 × *g* for 5 min.
   a. Dump the supernatant and resuspend in 1 mL of 1× Phosphate buffered saline (PBS).
6. Spin at 300 × *g* for 5 min.
   a. Dump the supernatant and resuspend in 1 mL of 1× PBS.
7. Prepare separate Eppendorf tubes for each strain and create a 1:10 dilution of your bacteria in 1× PBS.
8. Measure OD_{600nm} of bacteria, preferably 0.3–0.5 (Log Phase).
9. To quantify the amount of bacterial/mL, use the equation below:

$$x = \frac{OD_{600} \times \text{dilution factor} \times (1.5 \times 10^7)}{0.2}$$

10. With your concentration of bacterial, divide the number of cells you want to infect by your concentration of bacterial to figure out how much volume you need of your bacteria. This is typically easier when you convert your concentration to bacteria/μL.
    a. Gently sonicate bacteria in sonicating bath for about a minute, mixing occasionally during sonication.
11. Any unused bacteria can be treated with 10% bleach for 10 min and discarded in a biohazard bin.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-human CD4 FITC, monoclonal (1:10 dilution)	Miltenyi Biotec	130-080-501
Bacterial and virus strains		
C. acnes strain HL005PA1	Biodefense and Emerging Infections Research Resources Repository	
Biological samples		
Human blood sample	Adult donors	
Chemicals, peptides, and recombinant proteins		
0.01% Poly-L-lysine	Sigma-Aldrich	P8920
8% Glutaraldehyde	Electron Microscopy Sciences	16020
0.2 M Sodium cacodylate, pH 7.4	Electron Microscopy Sciences	11653
Osmium tetroxide	Electron Microscopy Sciences	20816-12-0
Ethyl alcohol, anhydrous	Electron Microscopy Sciences	64-17-5
CytoStim	Miltenyi Biotec	130-092-172
RPMI 1640	Miltenyi Biotec	130-091-440
Human AB serum, Gemini Bio	Fisher Scientific	50-753-3010
autoMACS Rinsing Solution	Miltenyi Biotec	130-091-222
Propidium iodide	Miltenyi Biotec	130-093-233
Reinforced Clostridial Medium	Oxoid	CM0149B
1× PBS, pH 7.4	Gibco	10010023
Ficoll-Paque Plus	Cytiva	GE17-1440-02
Trypan Blue Solution, 0.4%	Gibco	15250061
DMSO	Sigma-Aldrich	67-68-5
MACS BSA Stock Solution	Miltenyi Biotec	130-091-376
Bovine serum albumin, powder	Sigma-Aldrich	9048-46-8

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
IL-17 secretion assay- Detection kit (PE) human	Miltenyi Biotec	130-094-537
Neutrophil isolation kit	Miltenyi Biotec	130-104-434
Software and algorithms		
FlowJo V7.6	BD Biosciences	Link
Other		
Silicon wafers (5 mmx5 mm)	Ted Pella Inc	16007
Double sided carbon tape	Electron Microscopy Sciences	77817
MACSmix Tube rotator	Miltenyi Biotec	130-090-753
FACS Vantage	BD Biosciences	
Nunc Microwell Terasaki Plates	Thermo Scientific	438733
Mitsubishi AnaeroPack-Anaero	Thermo Scientific	R681001
AnaeroPack 2.5 L Rectangular Jar	Thermo Scientific	23-246-385
Autosamdri 810 Critical Point Dryer	Tousimis	
Harvester 96 MACHIII cell harvester	Tomtec	

MATERIALS AND EQUIPMENT

Reinforced clostridial medium		
Reagent	Final concentration	Amount
Reinforced clostridial medium	3.66%	38 g
H_2O	N/A	1 L
Total	**N/A**	**1 L**
Store at 4°C for up to one month.		

Peripheral blood mononuclear cell (PBMC) culture medium		
Reagent	Final concentration	Amount
Penicillin/Streptomycin (100×)	1×	0.5 mL
L-glutamine (200 mM)	2 mM	5 mL
AB serum	10%	5 mL
RPMI 1640	N/A	39.5 mL
Total	**N/A**	**50 mL**
Store at 4°C for up to one month.		

FACS buffer		
Reagent	Final concentration	Amount
Bovine serum albumin (BSA)	0.5%–1%	10 g
1× PBS	N/A	10 mL
Total	**N/A**	**10 mL**
Store at 4°C for up to one month.		

Detection buffer		
Reagent	Final concentration	Amount
MACS BSA Stock Solution	1×	2.5 mL
autoMACS Rinsing Solution	N/A	47.5 mL
Total	**N/A**	**50 mL**
Store at 4°C for up to one month.		

Alternatives: Prepare a solution containing phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA. EDTA can be replaced by other supplements such as citrate phosphate dextrose, whereas BSA can be replaced by other proteins such as human serum or fetal bovine serum.

T_H17 culture medium

Reagent	Final concentration	Amount
IL-2 (10,000 U/mL)	100 U/mL	0.5 mL
IL-23 (200 ng/mL)	2 ng/mL	0.5 mL
Penicillin/Streptomycin (100×)	1×	0.5 mL
AB serum	10%	5 mL
RPMI 1640	N/A	43.5 mL
Total	**N/A**	**50 mL**

Store at 4°C for up to one month.

Phorbol 12-myristate 13-acetate (PMA) solution

Reagent	Final concentration	Amount
PMA (200 μM)	20 nM	5 μL
AB serum	2%	1 mL
RPMI 1640	N/A	49 mL
Total	**N/A**	**50 mL**

Discard after use.

Fixative

Reagent	Final concentration	Amount
8% glutaraldehyde	2.5%	6.25 mL
0.2 M sodium cacodylate pH 7.4	0.1 M	10 mL
H_2O	N/A	3.75 mL
Total	**N/A**	**20 mL**

Discard after use.

Osmium tetroxide solution

Reagent	Final concentration	Amount
Osmium tetroxide	2%	0.5 g
0.2 M sodium cacodylate pH 7.4	0.1 M	12 mL
H_2O	N/A	12 mL
Total	**N/A**	**24 mL**

Discard after use.
Materials and equipment are listed in the key resources table.

⚠ CRITICAL: Osmium tetroxide (OsO_4) is used as both a fixative and heavy metal stain. OsO_4 is toxic and volatile, therefore all work should be performed in a fume hood using gloves and protective clothing. Handling and waste disposal should be done according to the manufacturer's guidelines.

STEP-BY-STEP METHOD DETAILS

PBMC isolation and stimulation

Timing: 1 day

Isolating of PBMCs from donor blood and stimulating PBMCs for IL-17 secretion for subsequent detection of T_H17 cells.

1. Isolate PBMCs using Ficoll–Paque gradients.
   a. Dilute blood in RPMI 1640 (1:1) and carefully layer 30 mL of blood over 15 mL of Ficoll-Paque Plus and centrifuge at 540 × *g* for 20–25 min at 20°C–25°C with brakes off.
   b. Aspirate plasma without disturbing the interphase.
      i. Collect the interphase and transfer into new 50 mL conical tubes.
      ii. Add RPMI 1640 to bring volume to 50 mL and centrifuge at 650 × *g* for 10 min at 20°C–25°C with brakes on.
   c. Aspirate the supernatant, break the pellet resuspend the pellet in 50 mL of RPMI 1640.
      i. Centrifuge at 300 × *g* for 10 min.
      ii. Aspirate the supernatant and repeat the centrifugation 2 more times.
   d. Aspirate supernatant and resuspend pellet in RPMI 1640 (5 mL RPMI 1640/30 mL of original volume blood; dilute 1:6 to count cells (usually 50 µL stain + 10 µL cells).
      i. If cells are left at 20°C–25°C for 1–2 h, reduce cell concentration to 2 × 10^6/mL to reduce clumping.
      ii. For longer periods, resuspend in 10% human AB serum.
   e. Count cells in hemacytometer with Trypan blue.
      i. Count two sections of 16 squares each, average the two, then multiply by 60,000 (dilution factor × grid size) to determine cells/mL.
2. Culture PBMCs in PBMC medium at 37°C and 5% CO_2.
3. Stimulate PBMCs immediately after isolation with *C. acnes* at 1 multiplicity of infection (1 MOI) in 24-well tissue culture plates. Incubate at 37°C for 16 h.

Pause point: It is possible to start the stimulation of the cells late in the afternoon and perform the Cytokine Secretion Assay the following morning.

Detection of T_H17 cells and sterile cell sorting

Timing: 12 days

Detecting T_H17 cells from fresh PBMCs using the IL-17 Secretion Assay – Detection Kit.

Staining cells for sterile cell sorting

4. Harvest PBMCs cells that were stimulated with *C. acnes* above into Eppendorf tubes and centrifuge at 300 × *g* for 10 min.
   a. Discard the supernatant, break the pellet, and resuspend the cells in 1–2 mL of cold FACS buffer in a 2 mL closable tube per sample.
   b. Repeat the centrifugation step at 4°C–8°C, pipette off supernatant completely.
5. Resuspend the cells in 80 µL of cold RPMI 1640 medium per 10^6 total cells.
6. Pre-mix same volumes of the Catch Reagent, included in the IL-17 Secretion Assay – Detection Kit, and CD4-FITC Antibody (1:10 dilution).
   a. Add 20 µL of this cocktail per 10^6 total cells, mix well, and incubate for 5 min on ice.

Cytokine secretion period

7. Add warm (37°C) RPMI 1640 medium to dilute the cells (1 mL of medium per 10^6 total cells).
8. Incubate cells in a closed tube for 45 min at 37°C under slow continuous rotation by using MACSmix™ tube rotator or turn tube every 5 min to resuspend settled cells.

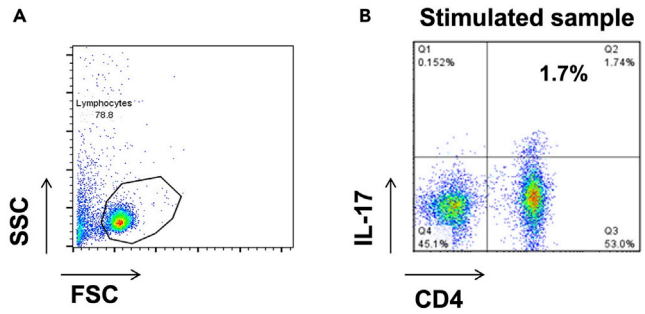

Figure 1. Sterile cell sorting

Peripheral blood mononuclear cells were isolated and stimulated with *C. acnes* for 16 h.

(A) Lymphocyte gate using FSC versus SSC.

(B) Following stimulation, the $CD4^+$ T cells population secreting IL-17 (1.7%) were sterile cell sorted and cloned.

Note: During this step its crucial to prevent contact of cells to avoid cross contamination with cytokines.

a. Put tube on ice.

9. Wash cells by filling the tube with cold detection buffer, centrifuge at 300 × *g* for 10 min at 4°C–8°C. Pipette off supernatant completely.
10. Resuspend the cells in 80 μL of cold detection buffer per 10^6 total cells and add 10 μL of IL-17-PE detection antibody and CD4-FITC antibody.
11. Mix well and incubate for 10 min on ice.
12. Wash cells by adding 2 mL of cold detection buffer, centrifuge at 300 × *g* for 10 min at 4°C–8°C. Pipette off supernatant.
13. Resuspend cells in 500 μL of cold detection buffer and proceed to FACS sorting (Beckton Dickinson FACS Vantage).

Alternatives: Other models of FACs systems can be used.

14. Sterile sort $CD4^+IL17^+$ (T_H17 cells). Exclude dead cells by DAPI staining.

Illustration of analysis

15. Perform one-color cytokine (IL-17) secretion on the stimulated and control PBMCs samples (Figure 1).
    a. Counterstain T cells using CD4-FITC antibody (1:10 dilution).
    b. Stain dead cells with DAPI prior to flow sorting at a final concentration of 0.5 μg/mL.
16. Acquire viable cells by flow cytometry from the stimulated as well as from control sample.
17. Activate a lymphocyte gate based on forward and side scatter (FSC/SSC) properties prior to further gating to exclude non-desired cells and debris (Figure 1).
18. Isolate $CD4^+IL17^+$ (T_H17 cells) using FACS sorting by Beckton Dickinson FACS Vantage.
19. Clone sterile-sorted cells by limiting dilution in Terasaki plates.[2] Clone cells at 0.3 cells/well in the presence of unmatched γ-irradiated PBMCs (1 × 10^6) activated by 2ug/mL Phytohemagglutinin (PHA) in T_H17 media.
    a. After plating, wrap the Terasaki plates in aluminum foil and incubate at 37°C.
    b. Screen Terasaki plates from day 6 post-cloning and transfer growing clones to 96 well flat bottom plates in T_H17 media.
20. Culture cloned T_H17 cells in T_H17 cell medium at 37°C and 5% CO_2.
21. Select for *C. acnes*-specific clones using[3] [H] thymidine proliferation assays by day 8 (Figure 2).
    a. Seed 2.5 × 10^4 cells/well autologous monocytes into 96-well flat-bottom plates.

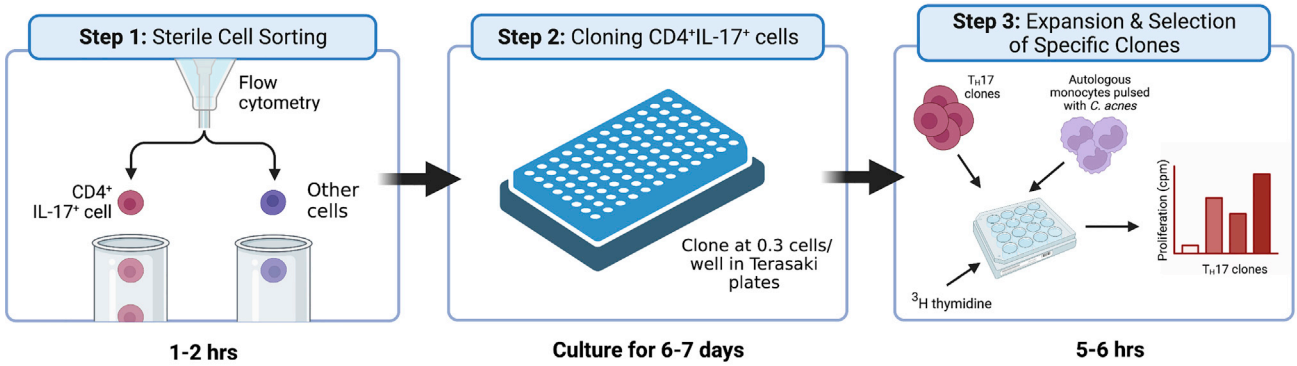

Figure 2. Generation of *C. acnes*-specific T_H17 clones

Peripheral blood mononuclear cells were stimulated with *C. acnes*. After antibody staining, the $CD4^+$ $IL\text{-}17^+$ cells were sterile sorted and cloned and expanded in culture.[2] After cloning, specific clones that proliferated in the presence of autologous monocytes pulsed with *C. acnes* are selected and used for scanning electron microscopy and subsequent functional experiments. Created in Biorender.com.

b. Incubate for 2 h with the *C. acnes* that was originally used as a stimulus to generate the clones. This step allows the monocytes to process and present the *C. acnes* antigens.
c. Wash the monocytes (centrifuge at 300 × *g* for 10 min) to remove any unprocessed bacteria.
d. Seed T_H17 cells clones at a concentration of 5×10^4 cells/well onto the plates containing the autologous monocytes.
e. Add[3] [H] thymidine (1 μCi/well) and incubate for 4 h.
f. Harvest the cells and assay by scintillation counting using Tomtech MACHIII cell harvester. Only the *C. acnes*-specific T_H17 cell clones proliferate during this procedure and are therefore selected for further studies.

⚠ CRITICAL: To avoid potential spills of radioactive waste, users are required to leave the liquid waste in the collection vessel at the cell harvester and be disposed by a trained Radioactive safety officer.

Alternatives: Other models of the cell harvester can be used with appropriate permission and training.

22. Expand the *C. acnes*-specific T_H17 cell clones on days 8–12.

Note: To avoid the effect of long-term culture, use T_H17 cell clones immediately, and/or aliquot and freeze for subsequent functional experiments. Aliquot T_H17 cell clones at a concentration of 1×10^6 cells/mL in freezing medium (10% DMSO in AB serum) into cryogenic vials. Freeze vials in a freezing container, filled with isopropanol, at −20°C to cool at a rate of −1°C/min. Transfer vials to liquid nitrogen after 24 h.

23. Stain for CD4 and IL-17 expression analysis of expanded *C. acnes*-specific T_H17 cell clones with CD4-FITC and IL-17-PE, for example, followed by:
a. Acquisition on BD Biosciences FacsScan.

Alternatives: Other software can be used according to the flow cytometry equipment available.

b. Analysis of CD4 and IL-17 expression using FlowJo software (V7.6). Specific clones should be positive for both phenotypic markers.

Alternatives: Other software can be used according to the flow cytometry equipment available.

Isolation of neutrophils

Timing: 4 h

Isolating neutrophils to use as positive control for neutrophil extracellular trap (NET) formation.

24. Isolate neutrophils using the Neutrophil Isolation Kit following manufacturer's protocol.
25. Use neutrophils as a positive control for NET formation as previously described.[3]
    a. Seed neutrophils at 2 × 10^5 per mL in flat-bottomed 96-well plate.
    b. Stimulate with PMA solution for 80, 100, and 130 min.[3]
    c. Plate neutrophils on silicon wafers and processed as described below for T_H17 clones.

Sample preparation and coating

Timing: 1 h

Stimulating the T_H17 clones with bacteria or PMA without killing the clones may improve release of extracellular traps by clones.

26. Place silicon wafers into a 24-well plate and wash quickly with 100% ethanol 2 times to get rid of any excess silicon dust.
    a. Allow the ethanol to dry off before autoclaving on dry cycle.

⚠ CRITICAL: Silicon wafers come pre-scored and attached to adhesive backing. Gently peel the chip off with sterile tweezers. Make sure to hold the silicon wafers by the edges. Do not touch the surface.

27. Coat silicon wafers (shiny side) with 0.1 mg/mL Poly-L-lysine for at least 2 h at 20°C–25°C in the tissue culture hood or 8 h at 4°C covered.
    a. Rinse 3 times with 1× PBS.

Note: Do not let the wafers dry out. Store the wafers in 1× PBS at 4°C covered. We recommend you coat the silicon wafers the day before you plan to prepare the samples for fixation. Use the coated silicon wafers within one week.

28. Place one sterilized, Poly-L-lysine coated silicon wafer per well in a 24-well plate (shiny, coated side up).
29. Label plate cover with sample designation.
    a. Prepare T_H17 cells/clones and resuspend in T cell media at a concentration of 1 × 10^5 cells/mL.
30. Plate approximately 60–100k of T cells in 1 mL per well of a 24-well plate with a Poly-L-lysine coated silicon wafer in the bottom of each well.
    a. Adhere cells for 30 min.

⚠ CRITICAL: Medium containing cells should also be gently dispersed against the side of the well to avoid direct pressure on the specimen. In addition, cells should be plated to 60%–70% confluence, if the sample is over confluent, you will not get images of single cells. Stimulation and infection should be done on the already adherent cells. Also, ensure that the stimulation is not killing the cells.

31. Prepare bacteria and resuspend in T cell media at concentration of 1 × 10^5 cells/mL.
32. Sonicate the bacteria for at least 4 min to prevent clumping.
33. Infect the adhered T cells with the bacteria at a multiplication of infection (MOI) of one.
    a. From our experience, the induction of extracellular trap formation by bacteria is immediate and therefore a time-course/kinetics for infection is recommended (10 min, 20 min, 40 min and 1 h).
    b. Add enough bacteria preparation in T cell media to cover the silicon wafer.
    c. Proceed to "Sample Fixation" steps after the infection period.

Optional: Positive controls can be prepared. T cells can be stimulated with PMA (10 min, 20 min, 40 min, and 1 h) to determine kinetics of extracellular trap formation and ensure that the stimulation is not killing the cells. Fresh neutrophils can also be isolated as described above and stimulated with IL-8 as a positive control.

Sample fixation

Timing: 6 h

This step cross-links proteins to increase mechanical and thermal stability. Fixing at 4°C may improve the preservation of cells and the released extracellular traps in a natural state.

34. Wash silicon wafers carefully with warm fixative.
    a. Repeat for a total of 3 washes. During the washing steps, leave enough fixative in the well to cover the silicon wafers between each wash to avoid drying of the samples, which adversely affects the ultrastructure.

Pause point: The sample can be stored at 4°C after fixation. When removing the sample from the hood, seal the container with parafilm or tape to avoid exposure of user to hazardous fumes.

35. Add fresh warm fixative to the silicon wafer at 20°C–25°C for 10 min.
    a. Transfer samples on silicon wafers to ice and fix for at least 1 h (fixing at 4°C may improve the preservation of specimens).
    b. Wash with ice-cold 0.1 M cacodylate buffer, pH 7.4 for 2 min.
        i. Repeat for a total of 5 washes. Disperse a slow stream of the wash buffer, ensuring that the silicon wafer does not float, and make sure the specimen remains completely immersed.
36. Add osmium tetroxide solution for 30 min on ice under a chemical hood, away from light.
    a. Wash wafers with ice cold diH_2O for 2 min.
        i. Repeat for a total of 5 washes.
        ii. Transfer the wafers to the critical point dryer sample holder during the last wash.
37. Dehydrate the samples by immersing the entire holder in increasing concentrations of ice-cold ethanol (30, 50, 70, 85, 95%) for 2 min each. Ascending concentration of exchange fluid replaces water in the sample.
    a. Wash the samples with 100% ethanol at 20°C–25°C for 3 min.
        i. Repeat for a total of 3 washes.
        ii. Keep the samples in the ethanol until the next step for a maximum of approximately 15 min. This helps to avoid membrane extraction.

Note: EtOH is hygroscopic, and a freshly opened stock should be used for the final 100% wash to avoid "wet" EtOH which can induce dry artifacts. In addition, letting the samples sit in 100% EtOH for more than approximately 15 min will extract the cell membrane.

38. Dry the samples using the Tousimis Autosamdri 810 critical point dryer (CPD) at the critical point of CO_2 until no more bubbles are visualized.

Note: Critical point drying preserves the surface structure of specimens which could otherwise be damaged due to surface tension when changing from the liquid to the gaseous state.

Note: CPD automated program. The CPD step involves the replacement of exchange fluid by liquid CO_2 (purging) in the sample, followed by then critical drying. The concept of CPD is that high pressure and temperature (above the critical point of CO_2-the CO_2 is called supercritical) there is no gas-liquid line and therefore no phase change, and no surface tension. The CPD program starts from the standard laboratory conditions (20°C, 15 psi), where the sample is bathed in alcohol, followed by a move to a state where CO_2 is liquid ("COOL" and "FILL"). The sample is then purged ("PURGE") to replace the alcohol with liquid CO_2, then moved above the critical point and into the supercritical phase ("HEAT"), and finally brought back down to standard conditions ("BLEED" and "VENT") without ever crossing the liquid/gas line.

39. Mount the silicon wafers on aluminum SEM stubs using double-sided carbon tape.

Note: Samples should be coated with iridium within 24 h.

40. Coat the samples with approximately 5 nm of iridium using an ion-beam sputter coater.

Alternatives: Samples can also be coated with gold, platinum, or palladium.

SEM imaging

Timing: 3–4 h

SEM imaging is carried out in a Zeiss Supra 40VP scanning electron microscope set to 3.5 kV accelerating voltage. There is no one method or condition that assures successful imaging technique and technologies to achieve the best results. Step by step details on how to image are beyond the scope of this protocol. For successful imaging, users are therefore required to take relevant training on how to use the Zeiss Supra 40VP or an available scanning electron microscope at your institution.

EXPECTED OUTCOMES

Our results indicate that we can induce ETs from T_H17 clones in culture (Figure 3). Our protocol can be applied to successfully image ETs released by other T cell subsets.

LIMITATIONS

This protocol is not meant to be exhaustive but offers a starting point for T_H17 cell/clone isolation, selection, stimulation, and imaging by SEM. The protocol does have some limitations. First, although $CD4^+$ T cells are easily accessible within the PBMC population, *in vitro* isolation of antigen-specific T cell clones may be challenging without any prior training. Second, *in vitro* T cell expansion is a very delicate process, as exhausted T cells produce lower amounts of cytokines and antimicrobial proteins and, thus making it difficult to characterize ET formation by SEM.

TROUBLESHOOTING

Problem 1

Clumping of bacteria and T_H17 (Figure 3B).

- T_H17 cells tend to aggregate when the concentration is too high.
- *C. acnes* can also aggregate (form massive clumps).

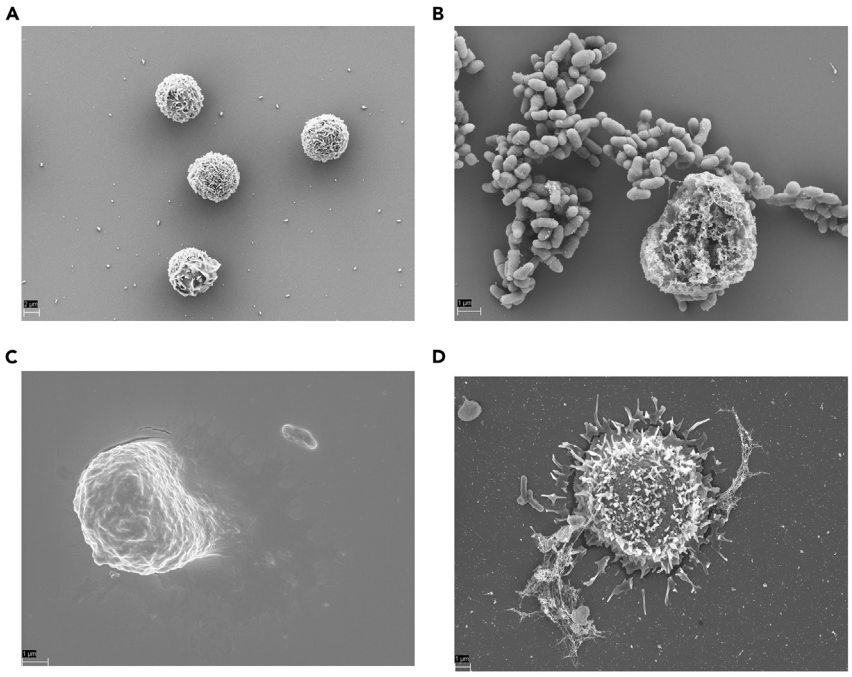

Figure 3. Fixation and imaging of T_H17 clones following infection with *C. acnes*

(A) Successful fixation and imaging.

(B) Failed vortexing and overstimulation of T cells leads to *C. acnes* clumping and T cell death.

(C and D) Over fixation leads to poor images.

- Aggregation/clumping of *C. acnes* can overstimulate T_H17 cells and lead to T_H17 cell death.

Potential solution

- Bacteria should be vortexed for 3–5 min right before adding to avoid clumping and overstimulation of T_H17 cells.
- T_H17 cell concentration should be optimized for the experiment through trial and error.

Problem 2

Overstimulation and expansion of T cells leading to cell death.

- Long-term *in vitro* expansion of antigen-specific T cells can lead to cell death.

Potential solution

- Only short-term *in vitro* expansion of antigen-specific T cells should be done. The entire process from generation to expansion of T_H17 clones takes 8–12 days (Graphical abstract). We refer to the day 12 clones as short-term. After generation, these clones are aliquoted and frozen, and/or used immediately for subsequent functional experiments such as SEM. The cloning and quick expansion allows the recapitulation of the spectrum of the biology present in *ex vivo* T_H17 cells.
- T cells should be used during the resting phase (day 8–12) following expansion as the T cell repertoire may be significantly altered after long-term *in vitro* culture.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, George W. Agak (gagak@mednet.ucla.edu).

Materials availability

This study did not generate any new unique reagents.

Data and code availability

This study did not generate/analyze [datasets/code].

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AUTHOR CONTRIBUTIONS

Conceptualization, G.W.A.; Methodology, G.W.A., N.P.N., and N.O.; Investigation, G.W.A., N.P.N., and K.O.; Writing – Original draft, G.W.A., N.P.N., K.O., and N.O.; Writing – Review & editing, G.W.A., N.P.N., K.O., and N.O.; Funding acquisition, G.W.A; Resources, G.W.A; Supervision, G.W.A.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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