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TITLE:

In Vivo Function of Differential Subsets of Cutaneous Dendritic Cells to Induce Th17 Immunity in Intradermal *Candida albicans* Infection

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SUMMARY:

Here, we demonstrate the *in vivo* function of cutaneous dendritic cell subsets in Th17 immunity of deep dermal *Candida albicans* infection.

ABSTRACT:

The skin is the outermost barrier organ in the body, which contains several types of dendritic cells (DCs), a group of professional antigen-presenting cells. When the skin encounters invading pathogens, different cutaneous DCs initiate a distinct T cell immune response to protect the body. Among the invading pathogens, fungal infection specifically drives a protective interleukin-17-producing Th17 immune response. A protocol was developed to efficiently differentiate Th17 cells by intradermal *Candida albicans* infection to investigate a subset of cutaneous DCs responsible for inducing Th17 immunity. Flow cytometry and gene expression analyses revealed a prominent induction of Th17 immune response in skin-draining lymph nodes and infected skin. Using diphtheria toxin-induced DC subset-depleting mouse strains, CD301b$^+$ dermal DCs were found to be responsible for mounting optimal Th17 differentiation in this model. Thus, this protocol provides a valuable method to study *in vivo* function of differential subsets of cutaneous DCs to determine Th17 immunity against deep skin fungal infection.

INTRODUCTION:

The skin is the outermost barrier organ, which protects the body from invading external pathogens and stimuli[1]. Skin is composed of two distinct layers, including the epidermis—a stratified epithelium of keratinocytes—and the underlying dermis—a dense network of collagen and other structural components. As a primary epithelial barrier tissue, the skin chiefly provides physical barriers and contributes to additional immunological barriers as it contains numerous resident immune cells[2,3]. Among the cutaneous immune cells, dendritic cells (DCs) are a type of professional antigen-presenting cells, which actively take up self- and non-self-antigens and migrate to the regional lymph nodes (LNs) to initiate antigen-specific T cell responses and tolerance according to the nature of antigens[4].

The skin harbors epidermal antigen-presenting cells, namely the Langerhans cells (LCs) and at least two types of DCs, including dermal type 1 conventional DCs (cDC1) and dermal type 2 conventional DCs (cDC2)[5]. Epidermal LCs are of embryonic monocytic origin and maintain their cell number by self-perpetuation under homeostatic conditions[6]. In contrast, dermal cDC1 and cDC2 are of hematopoietic stem cell origin and are continuously replenished by DC-committed progenitors[5]. Cutaneous DCs are characterized by their surface markers, roughly divided into Langerin$^+$ (including LCs and cDC1) and CD11b$^+$Langerin$^-$ populations (mainly cDC2). In addition, this group has revealed that the CD11b$^+$Langerin$^-$ DC population is further classified into two subsets according to CD301b expression[7].

The important functional features of cutaneous DCs are centered on a division of labor, determined mainly by the intrinsic nature of each subset of DCs, *in situ* locations of the DCs, the tissue microenvironment, and local inflammatory cues[8]. These functional characteristics of cutaneous DCs necessitate the investigation of the role of specific subsets of DCs during certain types of immune response of the skin. Upon antigenic stimulation by cutaneous DCs in the draining LNs, naïve CD4$^+$ T cells differentiate into specific subsets of helper T cells, which produce a set of defined cytokines for exerting their effector function[9]. Among the CD4$^+$ helper T cell subsets, interleukin-17 (IL-17)-producing Th17 cells play a crucial role in autoimmune diseases and antifungal immunity[10]. In this regard, cutaneous fungal infection has been a robust model to study Th17 immunity *in vivo*[11,12,13]. When tape-stripped skins are epicutaneously exposed to the *Candida albicans* (*C. albicans*) yeast, epidermal LCs play a pivotal role in driving antigen-specific Th17 differentiation[14].

Protective immunity against intradermal *C. albicans* infection requires innate immunity such as the fibrinolytic activity of fibroblasts and phagocytes[15]. However, little is known about the role of cutaneous DC subsets in establishing Th17 immunity in deep dermal *C. albicans* infection. This paper describes a method of intradermal skin infection of *C. albicans*, which produces local and regional Th17 immune responses. The application of diphtheria toxin (DT)-induced DC subset depletion mouse strains revealed that CD301b$^+$ dermal DCs are crucial for Th17 immunity in this model. The approach described here allows for the study of the Th17 response to deep dermal invasive fungal infection.

PROTOCOL:

NOTE: All animal experiments were approved by the Institution Animal Care and Use Committee (IACUC, Approval ID: 2019-0056, 2019-0055). Six to 8-week-old wild-type (WT) C57BL/6 female mice weighing 18–24 g were used for this study. Some studies were performed using female Langerin-diphtheria toxin receptor (DTR) and CD301b-DTR mice of the same age and weight. Four to six mice were used in each group for an experiment, and the data are representative of three independent experiments. This work was conducted under Biosafety Level 3 conditions, which could also be carried out under Biosafety Level 2 conditions according to institutional guidelines (room temperature 23 °C ± 3 °C, humidity 50% ± 10%).

1. Preparation of *Candida albicans*

NOTE: Experiments in this section were performed in a biological safety cabinet.

1.1. Streak *C. albicans* strain SC5314 onto a yeast-peptone-dextrose-adenine (YPDA) agar plate using an inoculation loop and needle.

1.2. Incubate the plate upside down for 2 days at 30 °C.

NOTE: The YPDA agar plate with *C. albicans* can be stored at 4 °C for up to 1 month.

1.3. Isolate a single colony from the plate for inoculation into 10 mL of YPDA medium in a 50 mL tube using a sterile pipette tip.

1.4. Incubate at 30 °C with shaking at 230–250 rpm for ~17 h.

1.5. Place the yeast suspension in a cuvette, and measure the optical density (OD) at 600 nm every 30 min using a UV-VIS spectrophotometer until the OD_{600} reaches 1.5–2.0.

NOTE: This step may take 16–18 h.

1.6. Spin the yeast suspension at 1000 × *g* for 5 min.

1.7. Discard the supernatant and resuspend the yeast cells in an appropriate amount of sterile phosphate-buffered saline (PBS).

1.8. Count the *C. albicans* cells using a hemocytometer and spin the suspension at 1000 **×** *g* for 5 min.

1.9. Discard the supernatant, and resuspend the *C. albicans* cells in PBS to a concentration of 1×10^7 cells in 40 µL of PBS per footpad.

1.10. For the preparation of heat-killed (HK) *C. albicans*, kill the yeast cells by heating at 65 °C

for 60 min using a heating mixer after determining the cell number.

2. Mouse footpad infection with *C. albicans* [Place **Figure 1** here]

2.1. Anesthetize the mice with isoflurane in an induction chamber until the mice have a slow respiratory rate and show no withdrawal responses to toe or tail pinches.

NOTE: During anesthesia, eye ointment is recommended to prevent dry eyes, especially for anesthesia lasting longer than 5 min.

2.2. Remove the cap from a 31 G needle, and load the 0.3 mL insulin syringe with the prepared *C. albicans* from step 1.9 after mixing the cells.

2.3. Remove the anesthetized mouse from the induction chamber, and gently inject 40 µL of the yeast cells (1×10^7 cells) into the deep dermis of the right footpad for *C. albicans* sensitization.

NOTE: The maximum volume that can be injected into a footpad is 50 µL.

2.4. Withdraw the syringe needle slowly from the injection site.

2.5. Place each mouse alone in a cage until it has fully recovered from anesthesia and then return it safely to the home cage.

2.6. To develop an antigen-specific response to *C. albicans***,** challenge the right footpad of the mice with the prepared HK *C. albicans* via intradermal injection 7 days after the sensitization, as described previously (1×10^7 cells; 40 µL per footpad; repeat steps 2.1–2.5).

2.7. Harvest skin-draining LNs 7 days after *C. albicans* sensitization or lesional footpad tissues 24 h after antigen challenge after euthanasia in a CO_2 chamber.

3. Diphtheria toxin-induced dendritic cell depletion *in vivo*

NOTE: In this study, both Langerin-DTR and CD301b-DTR mice were treated with DT 1 day before and after intradermal sensitization to *C. albicans*.

3.1. Prepare a 10 µg/mL solution of DT in PBS.

3.2. Remove the cap from the needle of a 1 mL insulin syringe, mix the DT, and fill the syringe with the DT.

3.3. Properly restrain the mice in a head-down position.

3.4. Disinfect the ventral side of the mice with 70% ethanol.

3.5. Slowly inject each mouse with 100 µL of 1 µg DT intraperitoneally into the lower left quadrant of the abdomen to deplete specific dendritic cell subsets.

NOTE: Be careful not to damage organs during the injection.

3.6. Wait for 5 s; then, slowly remove the needle.

4. Quantitative real-time polymerase chain reaction

4.1. Place the mouse in a CO_2 chamber until no breathing movement is observed.

4.2. Disinfect the mouse with 75% ethanol, and cut the lesion from the hind footpad skin into small pieces using forceps and scissors.

4.3. Completely immerse the sliced tissues in RNA isolation reagent.

4.4. Homogenize the samples using a tissue homogenizer according to the manufacturer's instructions (2 cycles of 3 min at 30 Hz).

NOTE: Stainless steel beads were used for tissue lysis in this study.

4.5. Spin at 10,000 × *g* for 5 min, 4 °C.

4.6. Carefully transfer the supernatant to a fresh tube.

4.7. Isolate total RNA from the lesional skin using a total RNA isolation kit.

4.8. Determine the RNA concentration using a UV-Vis spectrophotometer.

4.9. Synthesize cDNA using a reverse transcription kit for quantitative real-time polymerase chain reaction (qPCR).

4.10. Perform real-time qPCR with the real-time PCR system by monitoring the synthesis of double-stranded DNA during PCR cycles using green fluorescent dye.

NOTE: In this study, the results were normalized to the level of hypoxanthine-guanine phosphoribosyltransferase (*Hprt*). The primer sequences are listed in **Table 1**, and the PCR protocol is as follows: initial denaturation at 95 °C for 30 s, amplification for 42 cycles (95 °C for 5 s, 60 °C for 30 s).

5. Cell isolation and flow cytometric analysis

5.1. After CO_2 euthanasia, dissect the mice using forceps and scissors, carefully exposing and harvesting the footpad-draining LNs called popliteal LNs, located behind the knee.

5.2. Prepare single-cell suspensions from the footpad-draining LNs of each mouse by filtering the tissues through a 70 µm strainer after homogenization using the plunger of a 3 mL syringe.

5.3. Wash the cells with PBS and spin at 500 × *g* for 5 min at 4 °C.

5.4. Discard the supernatant, and wash the cells with PBS again.

5.5. Spin at 500 × *g* for 5 min at 4 °C.

5.6. Discard the supernatant and resuspend the cells in complete RPMI-10 medium containing 55 µM β-mercaptoethanol, 50 ng/mL phorbol 12-myristate 13-acetate (PMA), and 500 ng/mL ionomycin in a 24 well-plate for T cell stimulation.

5.7. After 1 h, add 10 µg/mL of brefeldin A and 1000x monensin to the cell suspension and culture for an additional 5 h.

5.8. Harvest the cells and wash them with fluorescence-activated cell sorting (FACS) buffer.

5.9. Spin at 500 × *g* for 5 min at 4 °C and discard the supernatant.

5.10. Stain the dead cells with a fixable dead cell-staining dye and incubate for 30 min at 4 °C.

5.11. Wash the samples with FACS buffer and spin them at 500 × *g* for 5 min at 4 °C.

5.12. Discard the supernatant, and stain the cells with fluorochrome-conjugated surface marker antibodies and Fc receptor blocker for 30 min at 4 °C.

5.13. Wash the samples with FACS buffer and spin them at 500 × *g* for 5 min at 4 °C.

5.14. Discard the supernatant and resuspend the pelleted cells in fixation and permeabilization solution for 15–20 min at 4 °C for intracellular staining.

5.15. Wash the samples with 1x washing buffer and spin them at 500 × *g* for 5 min at 4 °C.

5.16. Discard the supernatant, and perform intracellular cytokine staining for 30 min at 4 °C.

5.17. Wash the samples with 1x washing buffer and spin at 500 x *g* for 5 min at 4 °C.

5.18. Resuspend the cells in the appropriate volume (200–300 µL) of FACS buffer.

5.19. Analyze protein expression using flow cytometry.

REPRESENTATIVE RESULTS:

Here, we demonstrated an intradermal infection model of *C. albicans* to study the role of cutaneous DC-mediated Th17 immune response *in vivo*. Following an initial intradermal injection with *C. albicans* into the footpad, the skin-draining LNs were enlarged (**Figure 2A**). During the sensitization period, the ratio of $CD4^+$ to $CD8^+$ effector T cells was notably increased (**Figure 2B**). Additionally, the effector $CD4^+$ T cells abundantly produced IL-17A relative to the effector $CD8^+$ T cells (**Figure 2C,D**). These results indicated that intradermal *C. albicans* infection potently drives IL-17A-producing-$CD4^+$ T cell immunity.

The expression of interferon gamma (*Ifnγ*), *Il4,* and *Il17a* was observed in the challenged footpad skin with *C. albicans* (**Figure 3**). Although the initial sensitization with *C. albicans* led to increased levels of *Ifnγ* and *Il4* mRNA at day 7, there was no increase in *Il17a* at this time point. Importantly, the mice showed a profound elevation of local *Il17a* expression 24 h after *C. albicans* re-exposure. However, the challenge with *C. albicans* did not further increase the mRNA levels of *Ifnγ* and *Il4.* These results indicated that re-exposure to *C. albicans* via the intradermal route, described in this protocol, efficiently induces an antigen-specific IL-17 response in the skin.

Skin DCs migrate and present antigens in the skin-draining LNs after exposure to antigens, initiating antigen-specific immune responses. A DT-induced, DC subset-depleted mouse system was used to determine which DC subsets are responsible for the Th17 immunity to *C. albicans*. Depletion of Langerin$^+$ DCs (LCs and cDC1) resulted in comparable ratios of $CD4^+$ to $CD8^+$ T cells (**Figure 4A–C**) and IL-17A production from $CD44^+$ effector $CD4^+$ T cells in skin-draining LNs (**Figure 4D,E**). Meanwhile, the depletion of $CD301b^+$ cDC2 significantly attenuated IL-17A expression along with a relative decline in $CD4^+$ T cells (**Figure 4A–E**). These findings demonstrate that $CD301b^+$ dermal DCs invoke a protective Th17 immune response against deep dermal *C. albicans* infection *in vivo*.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic diagram of intradermal *Candida albicans* infection model. (**A**) The hind footpads of mice were injected intradermally with 1×10^7 *C. albicans*. After 7 days, the footpads of mice were re-exposed to 1×10^7 HK *C. albicans* by intradermal injection, and the delayed-type hypersensitivity response was measured 24 h after antigen challenge. Local Immune response during *C. albicans* sensitization was analyzed after 7 days in skin-draining LNs. (**B, C**) Images of footpads before intradermal footpad injection with *C. albicans.* (**D, E**) Injection of *C. albicans* into the deep dermis of the right footpad. (**F, G**) Clinical signs of redness and swelling following footpad injection of the right footpad. (**H**) A sketch showing lymphatic pathways from the footpad to popliteal LNs following *C. albicans* injection. (**I**) Exposed popliteal LNs located behind the knee 7 days after *C. albicans* injection and (**J**) without injection. Abbreviations: HK = heat-killed; LNs = lymph nodes.

Figure 2: Intradermal *Candida albicans* infection induces Th17 cell differentiation in skin-draining LNs. (**A**) Representative images of skin-draining LNs in naïve (upper) or *C. albicans*-sensitized mice (lower) 7 days after intradermal injection into the footpad. (**B**) Gating strategies for the effector T cells of skin-draining LNs at day 7 post-infection. (**C**) The proportion of $CD4^+$ to $CD8^+$ effector T cell population in skin-draining LNs 7 days after intradermal sensitization. (**D**)

Representative flow cytometric plots of intracellular IL-17A expression from effector $CD4^+$ T cells or $CD8^+$ T cells in skin-draining LNs 7 days after intradermal infection. (**E**) The absolute cell numbers of IL-17A-producing cells from effector $CD4^+$ or $CD8^+$ T cells. Data are from at least two independent experiments with four to seven mice per group. Error bars indicate mean ± standard error of the mean. ***, $p<0.001$. Abbreviations: LNs= lymph nodes; CD = cluster of differentiation; IL = interleukin; FSC = forward scatter; SSC = side scatter; A = area of peak; TCR = T cell receptor; ns = not significant.

Figure 3: The lesional skin re-exposed to *Candida albicans* characteristically exhibits an antigen-specific IL-17 response. Gene expression analysis of *Ifng*, *Il4*, and *Il17a* in the footpad skin before (7 days after sensitization) and 24 h after HK *C. albicans* challenge via intradermal injection compared to the naïve skin. Data are from at least two independent experiments with four to five mice per group. Error bars indicate mean ± standard error of the mean. **, $p<0.005$. Abbreviations: HK = heat-killed; IL = interleukin; IL = interleukin; *Ifng* = interferon gamma gene; ns = not significant.

Figure 4: $CD301b^+$ dermal DCs drive Th17 immunity against deep dermal *Candida albicans* infection. The skin-draining LNs of naïve, WT, Langerin-DTR, and CD301b-DTR mice with DT treatment were analyzed by flow cytometry 7 days after intradermal *C. albicans* infection. (**A**) Validation for the depletion of specific DC subsets in the epidermis and dermis of WT, Langerin-DTR, and CD301b-DTR mice after DT treatment. (**B**) Gating strategies for the $CD4^+$ and $CD8^+$ T cells of skin-draining LNs at day 7 post-infection. (**C**) The ratios of $CD4^+$ to $CD8^+$ T cells in each experimental group. (**D**) Representative flow cytometric plots of intracellular IL-17A production from $CD44^+CD4^+$ effector T cells in each experimental group. (**E**) The absolute numbers of IL-17A-producing Th17 cells in each experimental group. Data are from at least two independent experiments with five to six mice per group. Error bars indicate mean ± standard error of the mean. **, $p<0.005$; ***, $p<0.001$. Abbreviations: DCs = dendritic cells; WT = wild-type; DTR = diphtheria toxin receptor; DT = diphtheria toxin; CD = cluster of differentiation; LNs = lymph nodes; IL = interleukin; Th17 = T helper 17 cells; ns = not significant.

Table 1: Primer sequences.

DISCUSSION:

This paper describes a method of intradermal *C. albicans* infection that allows the study of the role of cutaneous DCs in Th17 immune response *in vivo*. By applying multiparametric flow cytometric analysis with DT-induced mouse strains, we found that $CD301b^+$ dermal DCs are a crucial cutaneous DC subset for initiating Th17 immunity against deep dermal *C. albicans* infection. Moreover, the results showed that the IL-17-producing T cell response was mainly produced by $CD4^+$ but not by $CD8^+$ T cells, indicating that this model is a faithful model of Th17 immunity *in vivo*.

Previous elegant studies have shown that epicutaneous *C. albicans* infection led to Th17 immunity through IL-6-producing epidermal LCs[14,16]. Moreover, a Th17 response to *C. albicans* epicutaneous infection was specifically induced by the yeast form but not by the hyphal form of

C. albicans, suggesting that the morphology of *C. albicans* is crucial for antifungal Th17 immunity[16]. Therefore, this protocol also utilizes the yeast form of *C. albicans*, which would transform into the hyphal form under nutrition-enriched and higher temperature conditions[17]. It is unclear whether an intradermal injection of *C. albicans* hyphae would induce Th17 immunity. Although previous studies have demonstrated the role of innate immunity against intradermal *C. albicans* hyphal infection that resulted in a high interferon-γ immune response, they did not evaluate the IL-17 response[15,18]. Future studies would be needed to demonstrate differences in Th17 immunity between the yeast and hyphal forms of intradermal infection.

The dermis contains all subsets of cutaneous DCs. In contrast to epicutaneous infection, this protocol shows that $CD301b^+$ dermal DCs are crucial for inducing Th17 immunity against intradermal *C. albicans* infection in the regional LNs. Compared to the epicutaneous infection of *C. albicans,* an intradermal administration route could bypass the persistent exposure of *C. albicans* on the epidermal surface and the resultant epidermal injury mediated by *C. albicans* pseudohyphae invasion[14]. This might induce a lesser degree of epidermal LC activation, which requires IL-1β production and subsequent keratinocyte-derived tumor necrosis factor-alpha (TNF-α) for their migration to regional LNs[19].

Furthermore, an initial intradermal location of *C. albicans* would lead to strong activation and enhanced uptake by dermal DCs. Among the dermal DCs, previous studies have shown that $CD11b^+$ cDC2 and $CD301b^+$ cDC2 mediate a Th17 immune response both in the intestine and the skin[7,20]. The results herein demonstrate the crucial role of $CD301b^+$ dermal DCs for Th17 cell priming in the intradermal *C. albicans* infection model. Thus, this model provides an important experimental protocol to study Th17-centered immunological features of deep dermal fungal infection, which is more prevalent in immunocompromised individuals[21]. The use of immunodeficient mouse strains in this protocol will shed light on the new immunopathogenesis of the deep cutaneous fungal infection.

For a successful experiment to analyze the Th17 immune response to deep dermal *C. albicans* infection, users must be familiar with the anatomical dissection of skin-draining popliteal LNs (**Figure 1**). In addition, users must be trained in multicolor flow cytometric analysis of T cells in the LNs. Advanced knowledge of cutaneous immunology is also required to follow this protocol. The use of this method is therefore ideal to better understand the roles of each cutaneous DC subset during deep fungal skin infections.

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DISCLOSURES:

The authors have no conflicts of interest to declare.

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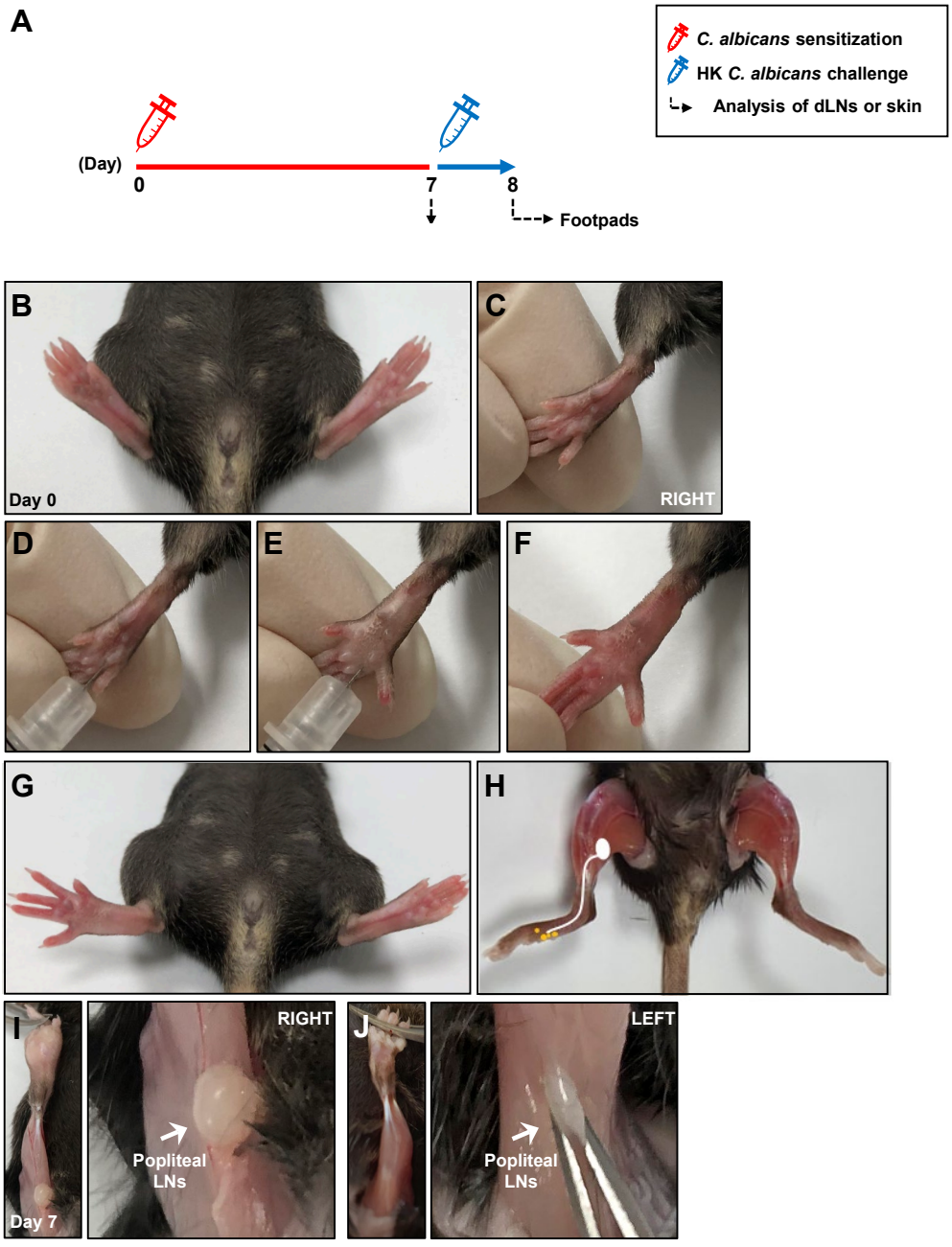
Figure 1



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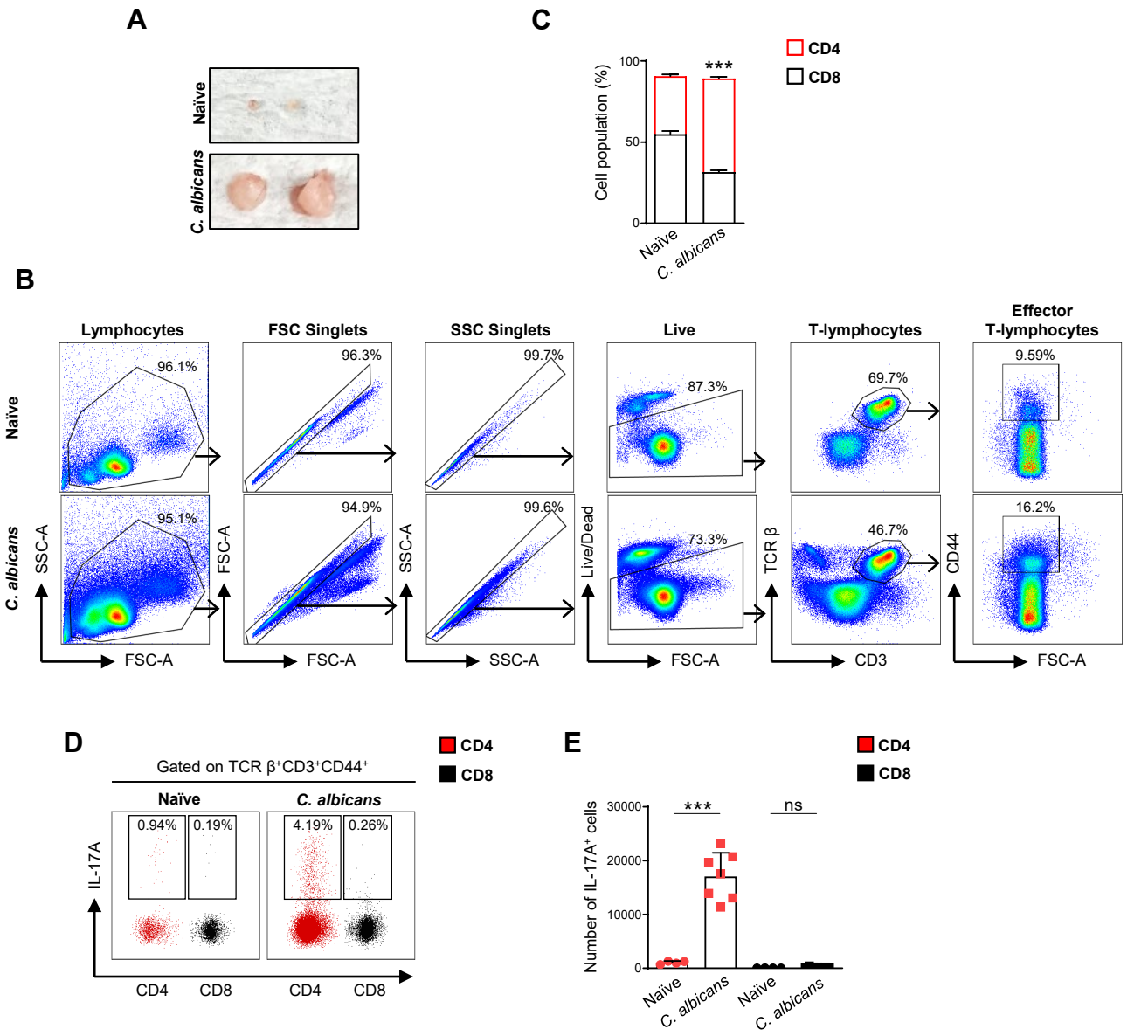

Figure 3

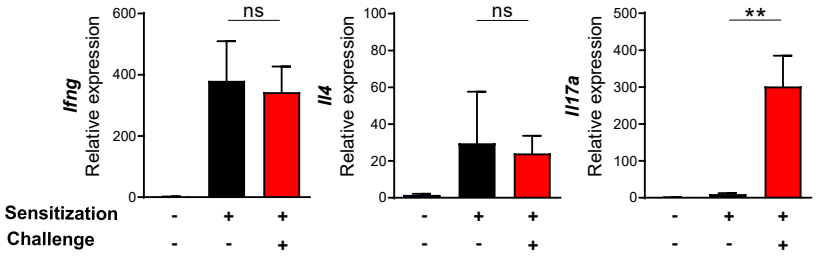

Figure 4

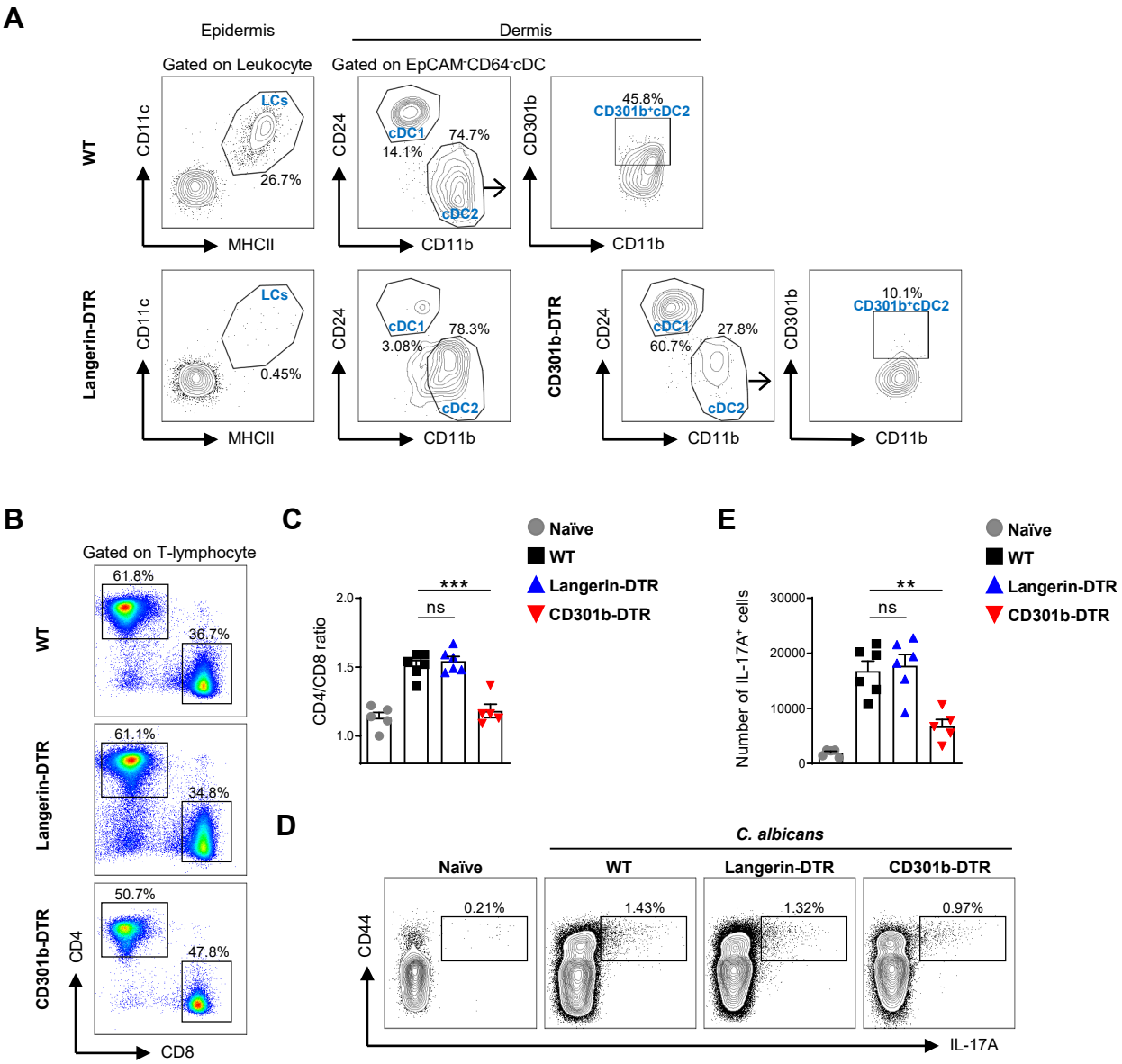

Table 1

Genes	Forward	Reverse
Hprt	TCAGTCAACGGGGGACATAAA	GGGGCTGTACTGCTTAACCAG
Il4	AGATCATCGGCATTTTGAACG	TTTGGCACATCCATCTCCG
Il17a	CAGCAGCGATCATCCCTCAAAG	CAGGACCAGGATCTCTTGCTG
Ifng	GATGCATTCATGAGTATTGCCAAGT	GTGGACCACTCGGATGAGCTC

Vidhya Iyer, Ph.D.
Review Editor
JoVE
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617.674.1888

Re: JoVE62731

Dear Editor,

It is our great pleasure to have an opportunity to revise our manuscript entitled, "In vivo function of differential subsets of cutaneous dendritic cells to induce Th17 immunity in intradermal Candida albicans infection,". We have revised the manuscript according to the suggestion from the editor and expert peer reviewers. We appreciate the time and details provided by each reviewer and have incorporated the suggested changes into the manuscript. I hope that a revised version of the manuscript will still be considered by your journal.

Thank you again for your considerations. I look forward to hearing from you soon.

Sincerely,

Tae-Gyun Kim, M.D., Ph.D.
Assistant Professor
Department of Dermatology, Cutaneous Biology Research Institute, Yonsei University College of Medicine, Seoul, 03722, Republic of Korea

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Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

→ **Done.**

2. Being a video based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Regarding animal treatment in the protocol, please add the following information to the text:

a) Please add details about the mouse strain, sex, age, weight, number etc in the beginning of the protocol.

→ **Done.**

b) What happened to the mice after the study? Please specify the euthanasia method without highlighting any steps.

→ **Done.**

c) Please mention how proper anesthetization is confirmed.

→ **Done.**

d) Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.

→ **Done.**

e) For survival strategies, discuss post-surgical treatment of animal, including recovery conditions and treatment for post-surgical pain.

→ **Our manuscript does not contain survival strategies.**

f) Discuss maintenance of sterile conditions during survival surgery.

→ **Our manuscript does not contain survival surgery.**

g) Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.

→ **Done.**

h) Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered.

→ **Done.**

3) JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript (text, figure legends, figures, tables) and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: TRIzol; TissueLyser; Hybrid-R total RNA kit; NanoDrop; PrimeScript™ RT Master Mix; SYBR Green; Aqua Dead cell stain kit; Cytofix/Cytoperm solution; Perm/Wash buffer; LSR Fortessa flow cytometer etc

→ **We have removed all commercial language in the manuscript.**

4) Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

→ **Done.**

5) Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

→ **Done.**

6) Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a SINGLE LINE space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a one line space between each protocol step and then HIGHLIGHT UP TO 3 PAGES of protocol text for inclusion in the protocol section of the video.

→ **We have added a one line space between each protocol step and highlighted up to 3 pages of protocol text for inclusion in the protocol section of the video.**

7) As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

→ **Done.**

8) Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names.

→ **Done.**

9) Please sort the Materials Table alphabetically by the name of the material.

→ **Done.**

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript by Park et al. described a protocol to induce the differentiation of Th17 cells in mice intradermal infected with Candida albicans. Also the authors used different DTR-induced dendritic cell depletion mice to explore the influence of different cutaneous dendritic cells subsets on Th17 differentiation. Overall, this procedure will benefit relevant researchers to better induce Th17 differentiation in vivo. The relevant key technology is very helpful. However, some key stepsof the protocol in the paper is omitted. A more detailed operation guide is suggested to improve the repeatability of the protocol.

→ **Thank you very much for your kind review of our manuscript.**

Major Concerns:

1. Protocol 2, How many footpads need to be injected per mouse? Does it make a difference of which foot is injected? How to inject? A photo or sketch of the injection will be very helpful.

→ **Thank you for your comment. We used right hind footpad per mouse as it was more convenient for intradermal injection. According to your suggestion, we have added a photo/sketch of the injection in Fig 1.**

2. Protocol 3, How to confirm that the target cells are eliminated by 2 times DTR injection? If not, what is the removal efficiency?

→ **We have added the representative flow cytometric data which showed an efficiency of cell depletion in Fig 4A.**

3. Protocol 5, How to harvest skin-draining lymph? Since this is a key step of this protocol, more details along with sketch are necessary.

→ **Thank you for your comment. According to your suggestion, we have added a photo/sketch of the injection in Fig 1.**

4. Protocol 5, how to gate Th17 cells? Gating strategy will be helpful.

→ **We have added the gating strategy according to your suggestion (Fig 2B).**

Minor Concerns:

1. The dose of Candida albicans in protocol 2 and figure legend is inconsistent.

→ **Corrected.**

2. Figure citation in the result part is not correct.

→ **Corrected.**

3. Figure 3C, it's suggested to separate the gene expression chart by each gene.

→ **We have made separate graphs of the gene expression chart.**

4, Figure 4B, Y-axis should be CD4

→ **Thank you for your comment. As we analyzed IL-17A production from CD44+ effector T cells, we emphasized this in the manuscript (line 279 and line 321) accordingly.**

Again, we sincerely thank you for your valuable comments.

Reviewer #2:

Manuscript Summary:

The authors provide a well described and detailed protocol to study in vivo function of differential subsets of cutaneous DCs by using Langerin-DTR and CD301b-DTR mice and diphtheria toxin treatment. This protocol is useful to study Th17 immune response after intradermal Candida albicans infection.

→ **Thank you very much for your kind review of our manuscript.**

Major Concerns:

Efficiency in DC depletion should be shown by a graph of flow cytometry and/or immunofluorescence.

→ **We have added the representative flow cytometric data which showed an efficiency of cell depletion in Fig 4A.**

Minor Concerns:

Lines 54-55: LC are tisular macrophages with function of dendritic cells. It would be better defining this population as epidermal antigen-presenting cells instead dendritic cells.

→ **Thank you very much for your valuable comment. According to your suggestion, we have revised the sentence accordingly (line 53).**

Line 86: This protocol can be also carry out in Biosafety level 2. This is important to clarify.

→ **Thank you very much for your valuable comment. According to your suggestion, we have revised the manuscript accordingly (line 91).**

In flow cytometry analysis (Figure 2 and Figure 4), gate strategies should be shown.

→ **We have added the gating strategy according to your suggestion (Fig 2B).**

Lines 230: Figure 3 caption: (A) Representative images of footpads in naïve (left) and C. albicans challenged mice (right) via intradermal injection. It is more clear change to: Representative images of footpads in naïve (left) and C. albicans-sensitized mice (right) via intradermal injection (7 days), after 24 h of intrademal challenge with heat killed (HK) C. albicans.

→ **We have changed the photos in Fig 1 according to your comment.**

Line 232: "…after intradermal challenge with C. albicans" change to "…. after intradermal chalenge with HK C. albicans"

→ **Corrected.**

Line 194: Figure 2A and B must be changed to Figure 3A and B

→ **Corrected.**

Line 195: Figure 2C change to Figure 3C

→ **Corrected.**

Lines 207 and 209: Figure 3 must be changed to Figure 4. Figure 3B and C, changed to Figure 4B and C

→ **Corrected.**

Line 71: add references of other fungal cutaneous models: Burstein et al 2018 (10.1016/j.jid.2018.02.042) and Spaber et al 2019 (10.1016/j.chom.2019.02.002.)

→ **We have added above references accordingly.**

Again, we sincerely thank you for your valuable comments.