TITLE

Th17 cells differentiated with mycelial membranes of *Candida albicans* prevent oral candidiasis.

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One sentence summary

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ABSTRACT

Candida albicans is a human commensal and causes opportunistic infections. Th17 cells provide resistance against mucosal infection with *C. albicans*; however, the determination of T cell antigens remains little known. Our final goal is to find out the effective T cell antigens of *C. albicans* responsible for immunotherapy against candidiasis. Here, we prepared some fractions including cytosol, membrane, and cell wall from each yeast and mycelial form-cell. Proteins derived from a membrane fraction of mycelial cells effectively induced differentiation of $CD4^+$ T cells to IL-17A producing Th17 cells. To confirm immunological response *in vivo* of the proteins from mycelial membrane, we performed adoptive transfer experiments using *ex vivo* stimulated $CD4^+$ T cells from IL-17A-GFP reporter mice. Mycelial membrane-differentiated $CD4^+$ Th17 cells adoptive transferred intravenously prevented oral candidiasis by oral infection of *C. albicans*, compared with control anti-CD3-stimulated $CD4^+$ T cells. They were confirmed in the clinical score and the number of neutrophils on the infected tissues. These data suggest that effective T cell antigens against candidiasis could be contained in the membrane proteins fraction of mycelial cells, as the design of novel vaccination strategies against candidiasis at our next stage.

Keywords: *Candida albicans*; mycelial membranes; Th17; oral candidiasis; adoptive transfer

INTRODUCTION

Candida albicans is a commensal of the oral cavity and the gastrointestinal and genitourinary tracts of healthy individuals and causes an opportunistic fungal infection in immunocompromised individuals. HIV-infected people frequently develop oropharyngeal candidiasis (OPC) as an opportunistic fungal infection (Fidel 2006). On the other hand, Conti *et al.* (2009) reported that Th17-deficient and interleukin (IL)-17R-deficient mice experiences severe thrush. Moreover, deficiency of IL-17 immunity in humans also develops into OPC (Puel *et al.* 2011). *C. albicans* specific $CD4^+$ T cells which produce cytokines including IL-17 were loss early in HIV-infected people (Hu *et al.* 2013, Goupil *et al.* 2014). Thus, it is well known that the Th17 cells provide resistance against mucosal infection with *C. albicans*.

Studies of the pathogenicity of *C. albicans* have been focused on the interaction between the organism and host cells. The growth of *C. albican*s is dimorphic depending on environmental factors (Gow *et al.* 2011, Kashem *et al.* 2015). *C. albican*s hyphae adhered to epithelial cells induce clathrin or E-cadherin endocytosis, then penetrate into the epithelial cells (Phan *et al.* 2007, Moreno-Ruiz *et al.* 2009, Zhu *et al.* 2012). *C. albicans* cells are recognized by the C-type lectin receptors including dectin-1 and -2 of the host cells and killed by phagocytes including neutrophils and macrophages. Phagolysosomes in phagocytes function by killing pathogens with many stresses. However, *C. albicans* cells in patients survive under some mechanisms and evade being killed by phagocytes (Erwig and Gow 2016). *C. albicans* has many pathways that respond to host stresses (Enjalbert *et al.* 2006, Patterson *et al.* 2013, Danhof and Lorenz 2015). For example, the *ATO* (ammonia transport outward) gene family that encodes putative acetate and ammonia transporters associates with phagosome neutralization (Okai *et al.* 2015). Neutralization of acid in phagolysosome could induce filamentation of *C. albicans* yeast cells in macrophages, then damage the host cells. The function of RAB proteins as central regulators involved in phagosome maturation is dysregulated by the hyphal formation of *C. albicans* in macrophage (Okai *et al.* 2015). These results suggest that the yeast-to-hyphal transition in *C. albicans* is important for pathogenicity. On the other hand, there is only little information about *Candida*-derived helper T cell antigens that focus on Th17 differentiation. An epitope isolated as MHC class II-bound ligands which were naturally processed in dendritic cells infected with *C. albicans* was an Als1/Als3 (proteins encoded by the

agglutinine-like sequence gene family)-derived peptide (Bär *et al.* 2012). A 15-mer peptide of ADH1 protein (alcohol dehydrogenase as one of mannoproteins) stimulated IL-17A production from the *C. albicans*-specific T cell hybridoma (Trautwein-Weidner *et al.* 2015). Some antigens were reported from a view of vaccine. Cell wall-associated proteins including Eno1(enolase), Fba (fructose-bisphosphate aldolase), Gap1 (glyceraldehyde-3-phosphate dehydrogenase), Hwp1 (hyphal wall protein-1), Met6 (methyltetrahydropteroyltriglutamate), and Pgk1 (phosphoglycerate kinase) were tested as vaccines combining β-mannan and peptide epitopes against candidiasis (Xin *et al.* 2008). The recombinant N terminus of Sap2 (secreted aspartyl proteinase 2) as a virulence factor was also tested as a mucosal anticandidal vaccine (Sandini *et al.* 2011). These tested proteins are mainly included in cell surface proteins or members of glycolytic pathway.

We focus on finding the novel T cell antigens of *C. albicans* recognized by the T cell receptor of $CD4^+$ T cell based on Th17 differentiation. Here, we prepared some fractions from each yeast and mycelial form-cell lysates by glass bead disruption to determine candidate for effective T cell antigens in proteins extracted from whole cells of *C. albicans*. Proteins that involved a membrane fraction from mycelial cells effectively induced differentiation of $CD4^+$ T cells to Th17 cells. Moreover, oral candidiasis in a murine model was prevented by adoptive transferred Th17 cells which were *ex vivo* stimulated with the mycelial membrane proteins.

MATERIALS AND METHODS

Fungal strain and growth conditions

Candida albicans SC5314 (Gillum *et al.* 1984) was grown on YPD agar plates (1% yeast extract, 2% Bacto-peptone, 2% glucose and 1.5% agar) for 18 h at 37°C. Yeast cells were harvested from colonies using sterilized scrapers, and washed with phosphate buffered saline (PBS) using sterilized cellulose nitrate filters (1.2 μm pore size, Sartorius-stedim, Gottingen, Germany). To obtain mycelia, 5×10^6 yeast cells of *C. albicans* were inoculated in 50 mL of 20% fetal bovine serum (FBS) medium in a disposable plate, then incubated for 24 h at 37°C. Mycelia were harvested and washed with PBS using sterilized cellulose nitrate filters (8 μm pore size, Sartorius-stedim, Gottingen, Germany). Both form cells were pooled at -80°C to be crushed physically.

C. albicans GFP was constructed using plasmid pGFP-ACT1 (Umeyama *et al.* 2005)

linearized with *Stu*I which was introduced into the *CaRP10* locus of *C. albicans* ura- strain CAI4. Yeast cells were transformed by the modified lithium acetate method of Umeyama *et al.* (2005). The strain was used for experiments *in vivo* because it is possible to confirm inoculum cells easily.

Preparation of cell fractions

The procedure of *C. albicans* cell fractions is outlined in Fig. 1B. Each harvested yeast cell and mycelium was frozen at -80°C, then crushed immediately with a cold mortar and pestle. The frozen crushed powders were mixed with protease inhibitor solution (nacalai tesque, Kyoto, Japan) and glass beads, and then disrupted using a Multi-Beads Shocker (YASUI KIKAI, Osaka, Japan) based on the method of Munro *et al.* (2007). The homogenates except glass beads were centrifuged for 20 min at 6,000g. The supernatant was used to obtain a cytosolic fraction and a membrane fraction. The pellet was used to obtain a cell wall fraction. The supernatant was centrifuged for 60 min at 105,000g based on the method of Mora-Montes *et al.* (2008). The high-speed supernatant was used as a cytosolic fraction. The high speed-pellet was used as a membrane fraction. To obtain membrane proteins from membrane fractions, the fractions were treated with 1.5 % final concentration of octylglucoside at 4°C for 1 h and then the detergent was removed from the fractions using Pierce detergent removal spin columns (Pierce Biotechnology, IL, USA). A cell wall fraction was obtained by washing the homogenate 5 times with 1 M NaCl to remove non-covalently linked proteins and intracellular contaminants based on the method of Munro et al. (2007). The cell wall fraction was boiled for 5 min twice, then freeze-dried. Two types of cell wall protein were isolated from a freeze-dried cell wall fraction based on the method of de Groot *et al.* (2004) and Sorgo *et al.* (2013). One of them was obtained to release glycosylphosphatidylinositol-dependent proteins (GPI-proteins) from a cell wall fraction by incubating with undiluted HF-pyridine (Tokyo Chemical Industry, Tokyo, Japan) at 0°C for 17 h. The other one was obtained to release mild alkali-sensitive proteins by incubating with 30 mM NaOH at 4°C for 17 h. A heat-kill treatment of whole cells was performed to boil for 5 min twice. Heat-killed samples were freeze-dried, and used at 10μg dry weight ml^{-1} for immunological response experiments. Regarding heat-killed mycelial samples, they were frozen and crushed roughly with a cold mortar and pestle, then collected with PBS prior to freeze-drying.

Protein determination

Protein was measured by the method of Bradford (1976) using BSA as a standard.

Electrophoresis

SDS-PAGE was carried out following standard protocols (Laemmli 1970), and proteins were stained by silver stain standard protocols (Switzer RC 3rd *et al.* 1979).

Mice

C57BL/6N mice (Kyudo, Saga, Japan) and C57BL/6-*Il17a*^{tm1Bcgen}/J mice (The Jackson Laboratory, Bar Harbor, ME) were purchased and bred under specific pathogen-free conditions at Fukuoka Dental College. Six- to 8-week old male and female mice were used for experiments. All animal experiments were done according to the guidelines of the committee of Ethics of Animal Experiments of Fukuoka Dental College.

Isolation and culture of dendritic cells (DCs)

Bone marrow (BM) cells were harvested from femurs and tibias of B6 mice. BM cells (1 x 10^6 cells well^{-1}) were cultured at 37°C in a 12-well plate in a total volume of 2 mL in the presence of 10 ng mL^{-1} of GM-CSF (PeproTech). On day 3 and day 5, each culture was split into two 1 mL wells, and added 1 mL fresh medium containing 10 ng mL^{-1} of GM-CSF to the original and new wells. On day 7, the cells were harvested and used as BM-derived dendritic cells (BMDCs).

Isolation of CD4$^+$T cells and stimulation with *C. albicans* cell fractions

CD4$^+$ T cells were magnetically isolated from lymph nodes and/or spleen cells by a previously described method (Hashimoto *et al.* 2017). Magnetic sorting was performed using Dynabeads Mouse CD4 (Invitrogen, Thermo Fisher Scientific, MA, USA) followed by treatment with DETACHaBEAD Mouse CD4 (Invitrogen). CD4$^+$ T cells (1×10^6 well^{-1}) were co-cultured in a 24-well plate with mitomycin C-treated BMDCs (0.5×10^6 well^{-1}) in a total volume of 2 mL in the presence of *C. albicans* cell fractions for 6 days. The differentiated T cells were washed and restimulated with 50 ng mL^{-1} phobol myristate acetate (PMA; Sigma) and 500 ng mL^{-1} ionomycin (Sigma) in the presence of 10 μg mL^{-1} Brefeldin A (Sigma) at 37°C for 4 h. The cells were stained

with anti-CD4 antibody (Ab) (APC, clone RM4-5, BD Bioscience) and permeabilized with 0.1% saponin (Nacalai Tesque). Intracellular cytokines were stained with anti-IL-17A Ab (PE, clone TC11-18H10, BD Bioscience) and anti-IFNγ□Ab (FITC, clone XMG1.2, BD Bioscience). All data were obtained using FACSCalibur (BD Bioscience).

Murine oral candidiasis model and adoptive transfer of *ex vivo* stimulated CD4$^+$ T cells

The murine oral candidiasis model was used in some modified methods described previously (Takakura *et al.* 2003, Ishijima *et al.* 2012, Nagao *et al.* 2017). However, immunosuppressed condition and antibiotics administration were avoided in this experiment. To verify the absence of commensal fungi in mice, stools were obtained before every mouse experiment and cultured using BD CHROMagar Candida plates (BD, NJ, USA). On the day of oral infection, mice were anesthetized by intramuscular injection into the femur with 14.4 mg kg^{-1} of chlorpromazine chloride. The whole surface of a mouse tongue was smeared 20 times with 2.0×10^9 cells mL^{-1} of *C. albicans* cell suspension using cotton swabs. After a 3-day infection period without feed, a clinical score was given using the assessment of the degree of oral infection by Hise *et al*. (2009). To measure viable *Candida* cells on mouse tongues, the tongues of the mice were aseptically collected after euthanasia, weighed, and homogenized with 1 mL PBS for 1 min using Power Masher II (Nippi, Tokyo, Japan), then diluted 10- to 1000-fold in PBS. Each dilution was cultured in triplicate using BD CHROMagar Candida plates. For adoptive-transfer experiments, IL-17A-GFP donor mice were orally inoculated with alive *C. albicans* cells (2×10^8 cells mouse^{-1}) or PBS using sonde syringe. CD4$^+$ T cells from lymph nodes and spleen cells of the donor mice were cultured with BMDCs and mycelial membrane proteins on day 10 after 1° infection. In anti-CD3 Ab stimulated experiments, CD4$^+$ T cells were isolated from the donor mice given PBS instead of *C. albicans*, and followed by co-culturing with BMDCs and anti-CD3 Ab (clone 145-2C11, BD Bioscience). On day 6, dead CD4$^+$ T cells in the culture were removed using Lympholyte-M (Cedarlane, NC, USA). Donor CD4$^+$ T cells (1- 2×10^6 cells mouse^{-1}) were adoptive transferred to recipient mice (C57BL/6N mice) intravenously 1 day before oral candidiasis experiments.

Histology

Periodic Acid-Schiff (PAS) staining for oral fungal infection was performed 3 days after infection with *C. albicans* on formaldehyde-fixed frozen sections with commercial PAS staining kit (Muto Pure Chemicals, Tokyo, Japan). Images were captured with a microscope (KEYENCE BZ-9000, Osaka, Japan). Sections were analyzed at 100× and 400× magnification. The number of polymorphonuclear leukocytes (PMNs) were pathologically evaluated in microscopic fields, and expressed as the number per mm^2. Images of unstained yeast cells and mycelial cells in Fig. 1A were captured with the same microscope at ×1000 magnification.

Statistical analysis

Statistical significance was determined by a two-tailed Student's *t*-test.

RESULTS

Cell fractions from yeast cells and mycelial cells of *C. albicans*

Unstained yeast cells and mycelia of *C. albicans* were used in this study as shown in Fig. 1A. Yeast cells were harvested from colonies on YPD agar plates after being cultured for 18 h at 37°C. Many budding yeast cells were observed. Mycelia were collected from 20% FBS medium following a 24 h culture at 37°C. Both form cells were fractionated as described in Materials and Methods resulting in supernatant (Sup-CL), cytosolic fraction, membrane proteins (MP), cell wall proteins treated with NaOH (CWP-NaOH) and cell wall proteins treated with HF-pyridine (CWP-HFp) (Fig. 1B). Proteins involved in each fraction were analyzed by SDS-PAGE (Fig. 1C). There was no distinctive difference between proteins from yeast and mycelial fractions.

Differentiation of $CD4^+$ T cells to Th17 cells by cell fractions from *C. albicans*

To determine the effective cell fractions that preferentially differentiated into Th17 cells, we examined whether proteins involved in these fractions induce C57BL/6 naive $CD4^+$ T cells isolated from peripheral lymphoid organs toward Th17 cells. In yeast cell fractions, none of the fractions differentiated toward Th17 cells, compared to heat-killed whole cells as a control (Fig. 2A and B). However, as shown in Fig. 2C, a considerable population of $CD4^+$ T cells (9.0%) produced IL-17A but not IFN-γ with mycelial membrane proteins (mycelial MP) fraction, compared to heat-killed cells (4.2%) and

other fractions (0.7–4.4%; Fig. 2C). Furthermore, mycelial MP induced IL-17A production significantly more than heat-killed cells ($P < 0.05$; Fig. 2D). These results indicate that IL-17A producing $CD4^+$ T cells could be efficiently differentiated by MP fraction in mycelial form of *C. albicans*, but not in yeast form. Therefore, we focused on the mycelial MP, as the effective T cell antigens of *C. albicans* responsible for immunotherapy against candidiasis.

Th17 cells differentiated with mycelial MP prevented murine oral candidiasis

Mycelial MP induced the IL-17A production *in vitro* in Fig. 2. To test whether mycelial MP could be the effective T cell antigens in order to prevent murine oral candidiasis, we used mice adoptive transferred Th17 cells which were *ex vivo* stimulated with mycelial MP (Fig. 3A). To readily identify the IL-17A producing $CD4^+$ T cells, we used fluorescent IL-17A-GFP reporter mice for this experiment. As previously reported (Bär *et al.* 2012), $CD4^+$ T cells only show weak response in producing IL-17A when stimulated by *C. albicans* antigen in uninfected mice, whereas cells from infected mice show significant response. To increase the proportion of Th17 cells which were adoptive transferred, IL-17A-GFP donor mice were infected orally with 2×10^8 yeast cells $mouse^{-1}$ using a feeding needle for mice. Control mice were taken 200 μL of PBS $mouse^{-1}$. Ten days later, $CD4^+$ T cells from 1° infected IL-17A-GFP mice were cultured with dendritic cells and mycelial MP (MP-stim). $CD4^+$ T cells from uninfected IL-17A-GFP mice were cultured with dendritic cells and anti-CD3 Ab (Anti-CD3-stim). None of the mice exhibited detectable carriage of *C. albicans* based on the data of fungus culture in a stool taken before the oral infection (data not shown). Then 6 days later, the $CD4^+$ T cells that depleted dead cells in culture were adoptive transferred to C57BL/6 recipient mice intravenously. To avoid any influence on adoptive transferred cells, the recipient mice were not treated with immunosuppressive and antibacterial agents. On the day of oral infection, recipient mice were anesthetized by intramuscular injection. Then *C. albicans* cell suspension at 2×10^9 cells mL^{-1} were swabbed on the whole tongue. Assessments of IL-17A-GFP^+ expression of adoptive transferred T cells were shown in Fig. 3B. T cells stimulated with mycelial MP contained ~50% IL-17A producing T cells, whereas anti-CD3-stimulated T cells only contained ~1% IL-17A producing T cells. The activity of an antigen involved in mycelial MP was dependent on its presentation by

MHC class II because IL-17A production in response to mycelial MP was blocked when an anti-MHC class II Ab was added to the cultures (data not shown). As shown in Fig. 3C, the severity of the oral infection was assessed in accordance with clinical scores by Hise *et al.* (2009) 3 days after the oral infection. Clinical severity was significantly lower in recipient mice stimulated by mycelial MP compared with positive control mice (candidiasis) and anti-CD3-stimulated recipient mice. Surface conditions of the tongues of each group were shown in Fig. 3D. Tongues were removed aseptically from each group of oral candidiasis model mice to quantify fungal burden of the tongue (Fig. 3E). Recovered fungal burden of the tongue was not significantly different among three groups. Three groups of oral candidiasis model mice were showed similar weight loss for 3 days (Fig. 3F). Assessment of IL-17A expression in adoptive-transferred $CD4^+$ T cells isolated from the cervical lymph nodes (CLN) and the lymph nodes except CLN of recipient mice was shown in Fig. 3G. When $CD4^+$ T cells from mycelial MP-stimulated mice were adoptive-transferred to recipient mice and followed by an infection with *C. albicans*, the $CD4^+$ T cells that involved Th17 cells gathered more in CLN compared with other lymph nodes of recipient mice. These results indicate that Th17 cells differentiated with mycelial membranes of *C. albicans* prevent oral candidiasis.

Histological evaluation of *C. albicans* infection

To histologically visualize the oral candidiasis, sections of the mouse tongue in four groups, including an uninfected control group, were stained with PAS to detect *C. albicans*. Robust mycelial invasions into the intraepithelial layer were observed in sections of anti-CD3-stimulated group as well as the candidiasis group (Fig. 4A). Importantly, mycelial invasions into the intraepithelial layer were rarely detected in sections of MP-stimulated group (Fig. 4A). Furthermore, the number of neutrophils infiltrated into the intraepithelial layer in a section of MP-stimulated group showed a significant reduction compared with candidiasis ($P < 0.01$) and anti-CD3-stimulated ($P < 0.01$) groups (Fig. 4B). On the other hand, there was also a little reduction in the number of neutrophils in anti-CD3-stimulated group ($P < 0.05$) compared with the candidiasis group, despite of the lack of significance in the difference between them in the clinical score (Fig. 3C). These results indicate that Th17 cells differentiated with mycelial MP prevent oral candidiasis with infiltration of neutrophils.

DISCUSSION

It is well known that Th17 cells producing the cytokines IL-17 and IL-22 function as anti-fungal T cells (Medzhitov 2007, Hernández-Santos and Gaffen 2012, Becattini *et al.* 2015). Als1/Als3- or ADH1-derived peptides were reported as *C. albicans* T cell antigens (Bär *et al.* 2012, Trautwein-Weidner *et al.* 2015). These proteins localize to the fungal cell wall. We focus on finding a novel responsible T cell antigen of *C. albicans* to T cell receptor of $CD4^+$ T cells which indicates more effective antigens for Th17 differentiation compared to whole cells of *C. albicans*. From this perspective, we fractionated cell wall, cytosol, and membrane from yeast cells and mycelial cells of *C. albicans*. Proteomic analysis of cytoplasmic and surface proteins from yeast cells, mycelia, and biofilm of *C. albicans* by Martínez-Gomariz *et al.* (2009) showed that the differential abundance of proteins was found between biofilm and planktonic cells and between yeast cells and mycelia. Moreover, the analysis showed that the differential abundant cytoplasmic proteins affected on several processes and functions. Therefore, the differential abundance of proteins would be included among cell fractions in Fig. 1. However, it has been remained unclear what kind of protein effectively respond to $CD4^+$ T cells. Here we recognized that there are different responses to naive helper T cells among cell fractions. Membrane proteins fraction of mycelial *C. albicans* (mycelial MP) induced more IL-17A production than heat-killed whole cells *in vitro* (Fig. 2C and D). An active protein in mycelial MP would be a mycelial unique protein or a protein existing in larger amount in mycelia than in yeast cells.

To confirm the effectiveness of mycelial MP *in vivo*, we have provided a murine model of oral candidiasis. Some murine models of oral candidiasis have already been reported. When investigations, including our previous study, for antifungal activity *in vivo* were performed, mice were treated with immunosuppression and antibacterial drugs (Kamai *et al.* 2001, Takakura *et al.* 2003, Ishijima *et al*. 2012, Nagao *et al*. 2017). In investigations on *C. albicans* cell functions and cell products, mice treated with immunosuppression and without antibacterial drugs were used (Solis and Filler 2012, Moyes *et al.* 2016, Richardson *et al.* 2017). In immunological researches, mice treated with antibacterial drugs and without immunosuppression were used (Hise *et al.* 2009). Recently, it is thought that the use of antibiotics causes modulation of the interactions between the microbiota, especially the gut microbiota, and the immune system (Russell

et al. 2013, Shankar *et al.* 2015, Shen and Wong 2016, Wheeler *et al.* 2016, Iliev and Leonardi 2017). Here we used mice treated without immunosuppressed medicines and antibiotics administration using swabs of yeast cells on tongues at high concentration of cells. As shown in Fig. 3E, the oral fungal burden of mice infected with *C. albicans* was 10^5-10^6 CFU per gram of tongue after 3 days of infection. This result was typically comparable to previous reports (Conti *et al.* 2009, Solis and Filler 2012). Clinical severity of recipient mice transferred Th17 cells which were stimulated by mycelial MP in Fig. 3C was reduced, however, the CFUs of *C. albicans* in tongues of three groups in Fig. 3E were not significantly different. These results and histological data in Fig. 4A show that the yeast-to-hyphal transition in *C. albicans* is more important for pathogenicity than the cell number. Kashem *et al.* (2015) reported that *C. albicans* yeast cells are required for Th17 cell responses. Therefore, it would be considered that Th17 cells transferred in the model mice were activated in mechanisms of response to *C. albicans* yeast cells swabbed on tongues, then controlled morpho-type switching of *C. albicans*. Similar weight loss among three groups showed in Fig. 3F could be governed by skipping feed for 3 days.

It is possible to investigate by using adoptive transfer experiments whether antigen specific T cells respond to pathogen products *in vivo*. Here $CD4^+$ T cells from IL-17A-GFP donor mice differentiated with mycelial MP of *C. albicans* were adoptive transferred to recipient mice, followed by an oral infection of *C. albicans*. We assessed the number of IL-17A producing T cells in CLN and draining lymph nodes except CLN detected with GFP by flow cytometry day 3 after infection (Fig. 3G). Migration of the adoptive transferred $CD4^+$ T cells which were stimulated with mycelial MP were observed more in CLN compared to in other lymph nodes. Hernández-Santos *et al.* (2013) reported that $CD4^+$ T cells adoptive transferred in lymphocyte-deficient Rag1–/– recipient mice mediated a local response of tongue in oral infection with *C. albicans*. Therefore, our results show that antigen-specific $CD4^+$ T cells in oral infected mice migrate to regional lymph nodes, follow mediate a local response of mucosal tissue.

In the anti-CD3-stimulated and candidiasis control groups, pathological sections of a tongue that occurred candidiasis showed robust mycelial invasion and neutrophil infiltration into the intraepithelial layer of tongues (Fig. 4A and B). Candidalysin discovered by Moyes *et al.* (2016) were secreted by *C. albicans* mycelia during invasion

on oral epithelial cells in animal models of mucosal infection. It was reported that Candidalysin drove neutrophil recruitment at the vaginal mucosa (Richardson *et al.* 2017). Therefore, abundant neutrophil infiltration observed in Fig. 4B would be recruited with Candidalysin secreted by robust mycelia. On the other hand, pathological sections of mycelial MP stimulated group reduced oral candidiasis and observed few neutrophil infiltration (Figs. 4A and B). As reported by Conti *et al.* (2016), it is considered that IL-17A produced by Th17 cells would stimulate the antimicrobial peptides including β-defensin produced at oral epithelial cells. Therefore, by such as mechanisms through IL-17A signaling on oral epithelial cells, the mycelial transition of swabbed yeast cells of *C. albicans* would be inhibited, and oral candidiasis would be prevented.

In summary, this study demonstrates that proteins from a mycelial membrane proteins fraction are inducible to differentiate $CD4^+$ T cells to Th17 cells. Moreover, the antigen specific Th17 cells are possible to protect oral infection with *C. albicans*. On the base of these findings, we are going to determine effective T cell antigens from the mycelial membrane proteins of *C. albicans* responsible for designing novel vaccination strategies against candidiasis at our next stage.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: ST, TC, JN, YT.

Performed the experiments: ST, TC, JN, SI, YN, KA-M, KY, KT.

Analyzed the data: ST, TC, JN, YN, KA-M, HK, YT.

Wrote the paper: ST, TC, JN, YT.

Led the research: YT.

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FIGURE LEGENDS

Figure 1 Cell fractions from yeast cells and mycelial cells of *C. albicans.* (A) Unstained yeast cells and mycelial cells of *C. albicans* SC5314 used for cell fractions were viewed at 1000× magnification. All bars indicate 20 μm. (B) Preparation of cell fractions. Sup-CL; supernatant of cell lysate, MP; membrane proteins fraction, CWP-NaOH; cell wall proteins treated with NaOH fraction, CWP-HFp; cell wall proteins treated with HF-pyridine fraction. (C) SDS-PAGE of *C. albicans* proteins solubilized from each cell-fractions. All fractions were freeze-dried and adjusted at 10 μg μL^{-1}. M; molecular weight marker. Abbreviations in Fig. 1C are the same in Fig. 1B.

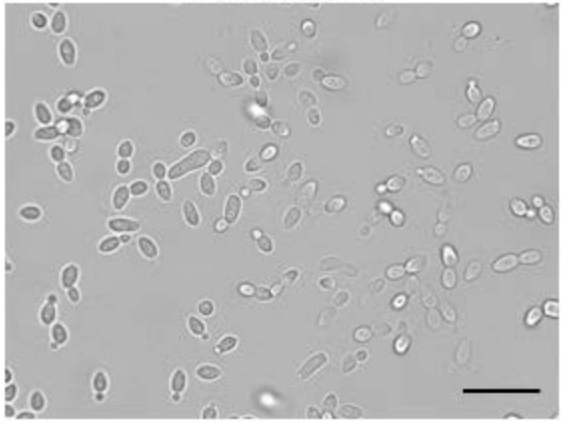
Figure 2 Analysis of *C. albicans* cell fractions involved $CD4^+$ T cell antigens by flow cytometry. （A and C）Naive $CD4^+$ T cells were magnetically isolated from peripheral lymph nodes and spleen cells. Cytokine production was analyzed by intracellular cytokine staining and flow cytometry. (B and D) Each bar represents the mean and SD of independent 4 experiments per group. *, $P < 0.05$ by a two-tailed Student's *t*-test. Cell fractions from yeast cells and mycelial cells were prepared as shown in Fig. 1B. Abbreviations in Fig. 2A to 2D are the same in Fig.1B.

Figure 3 Th17 cells differentiated with mycelial MP prevented oral candidiasis in mice. (A) Timeline of infection model. The first (1°) infection in donor mice was orally inoculated with *C. albicans* or PBS using a feeding needle. $CD4^+$ T cells from lymph nodes and spleen cells of donor mice were cultured with BMDCs and mycelial membrane proteins on day 10 after 1° infection. On day 6, donor $CD4^+$ T cells were adoptive-transferred intravenously to recipient mice, followed by experiments of oral candidiasis 1 day later. (B) IL-17A expression in $CD4^+$ T cells from IL-17A-GFP mice for adoptive-transfer was detected by flow cytometry. $CD4^+$ T cells stimulated with mycelial MP are shown as MP-stim (right), whereas $CD4^+$ T cells stimulated with anti-CD3 Ab are shown as anti-CD3-stim (left). Independent experiments were repeated twice. A representative result is shown. (C) Mean clinical severity score of recipient mice adoptive-transferred $CD4^+$ T cells. Assessment of clinical severity of oral candidiasis by Hise *et al.* (2009) was used. Candidiasis shows tongues that developed oral candidiasis as positive control (n = 7). Anti-CD3-stim shows tongues of recipient

mice adoptive-transferred $CD4^+$ T cells which were stimulated with anti-CD3 Ab (n = 7). MP-stim shows tongues of recipient mice adoptive-transferred $CD4^+$ T cells which were stimulated with mycelial MP (n = 9). Each dot represents an individual mouse, and the horizontal bar indicates the mean. *, $P < 0.05$ by a two-tailed Student's *t*-test. Data were evaluated in two independent experiments. (D) Tongues shown are on day 3 after oral infection with *C. albicans*. A representative result is shown. (E) Quantitative fungal burden of tongues on day 3 after oral infection with *C. albicans*. Candidiasis tongues as positive control were from 7 mice. Anti-CD3-stim tongues were from 5 mice. MP-stim tongues were from 8 mice. Error bars represent SD. (F) Weight loss of mice that performed oral candidiasis experiments. Mice were weighed on day 0 and day 3 after oral infection with *C. albicans*. Eleven candidiasis mice were used as positive control. Seven anti-CD3-stim mice were used. Nine MP-stim mice were used. Error bars represent SD. (G) IL-17A expression in adoptive-transferred $CD4^+$ T cells isolated from the cervical lymph nodes (CLN) (above) and the lymph nodes except CLN (bottom) of recipient mice was detected by flow cytometry. Independent experiments were repeated twice. A representative result is shown.

Figure 4 Histological evaluation of *C. albicans* infection. (A) PAS-stained histologic sections of tongues on day 3 after oral infection with *C. albicans.* Uninfected control group shows uninfected healthy tongues (n = 2). Candidiasis group shows tongues that developed oral candidiasis as positive control (n = 2). Anti-CD3-stim group shows tongues of recipient mice that adoptive-transferred $CD4^+$ T cells which were stimulated with anti-CD3 Ab (n = 2). MP-stim group shows tongues of recipient mice that adoptive-transferred $CD4^+$ T cells which were stimulated with mycelial MP (n = 2). Arrows and arrowheads point to *C. albicans* and PMNs, respectively. Sections of tongue in each group were viewed at 100× to 400× magnification. All bars are 100 μm. A representative result is shown. (B) Quantitation of histological evaluation of PMNs. Six sections per mouse from two mice per group were pathologically evaluated for the number of PMNs in microscopic field, and expressed as the number per mm^2. Experiments were independently repeated twice. *$P < 0.05$, **$P < 0.01$ by a two-tailed Student's *t*-test.

Yeast cells



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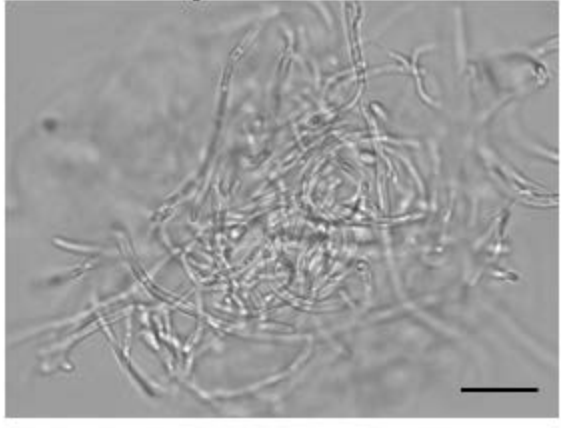
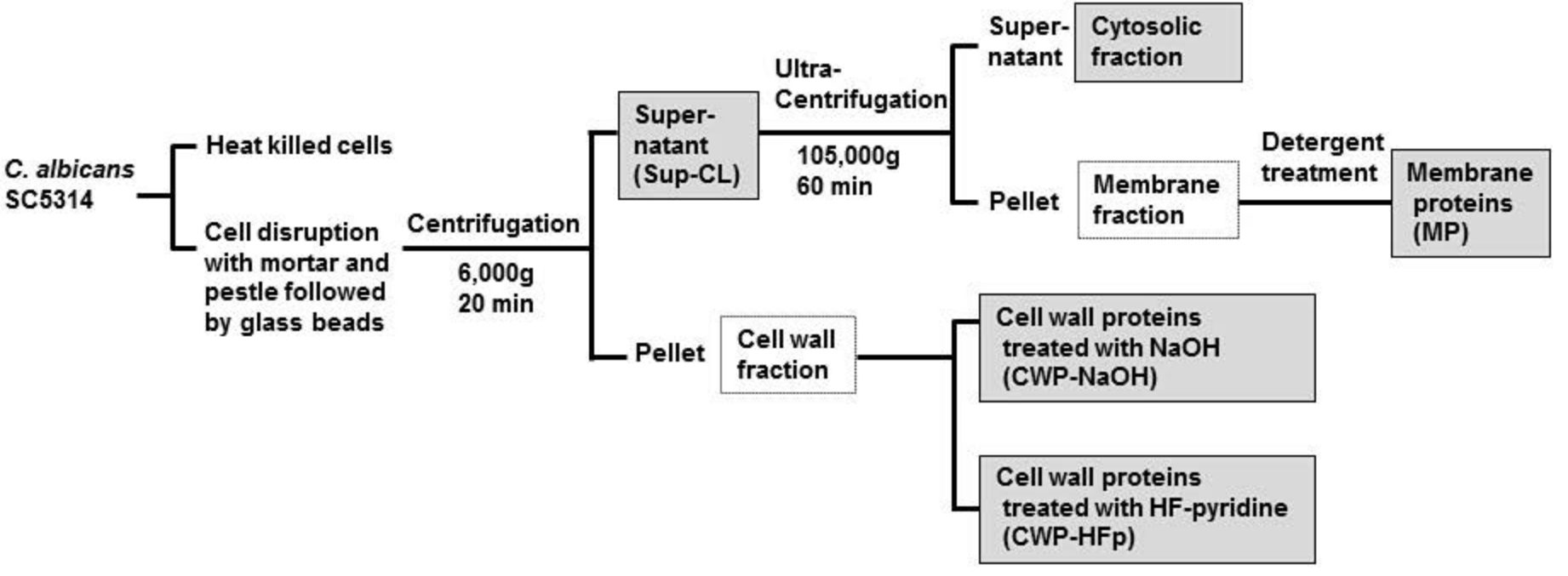
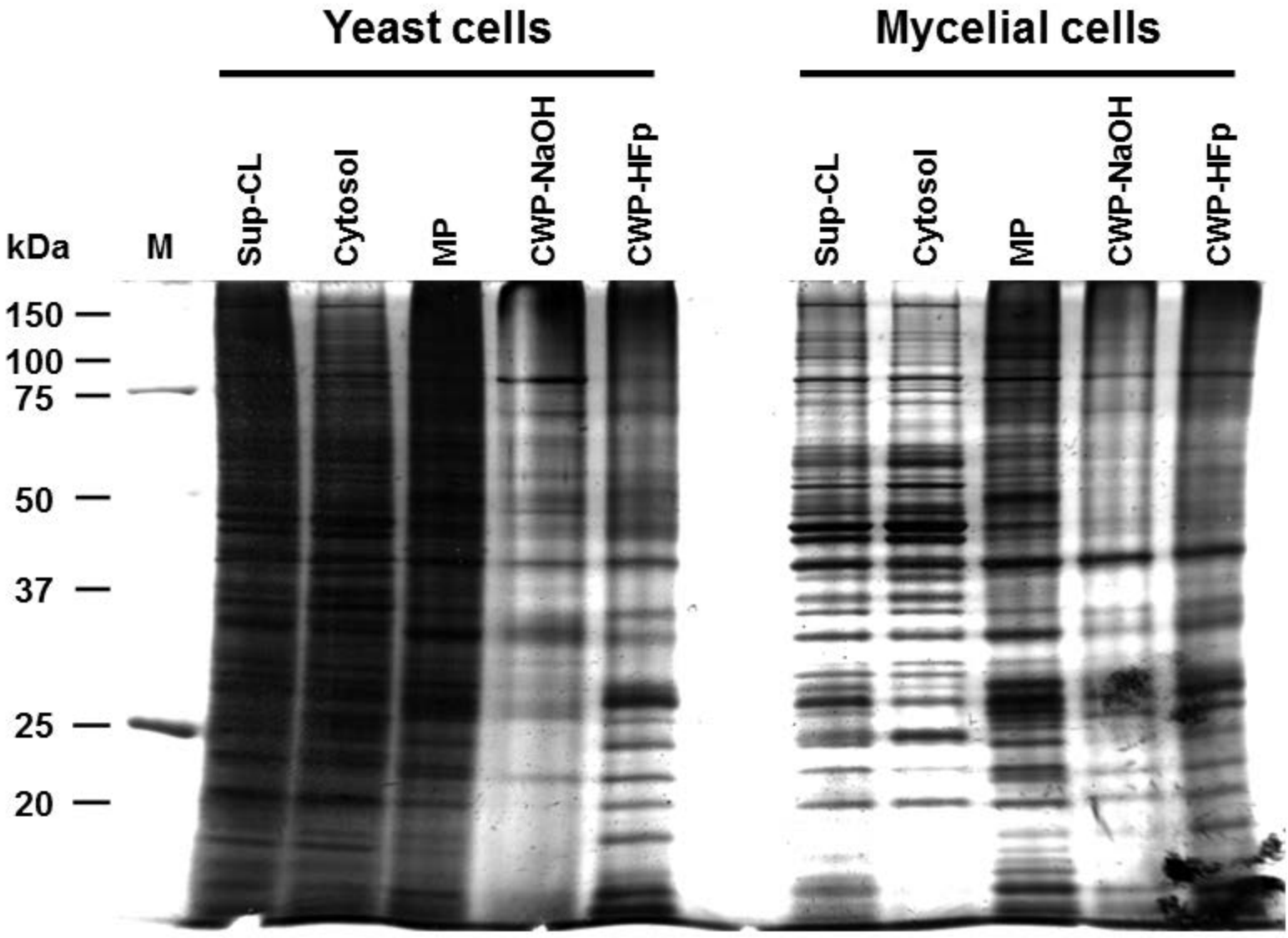


Tasaki.S Fig1A

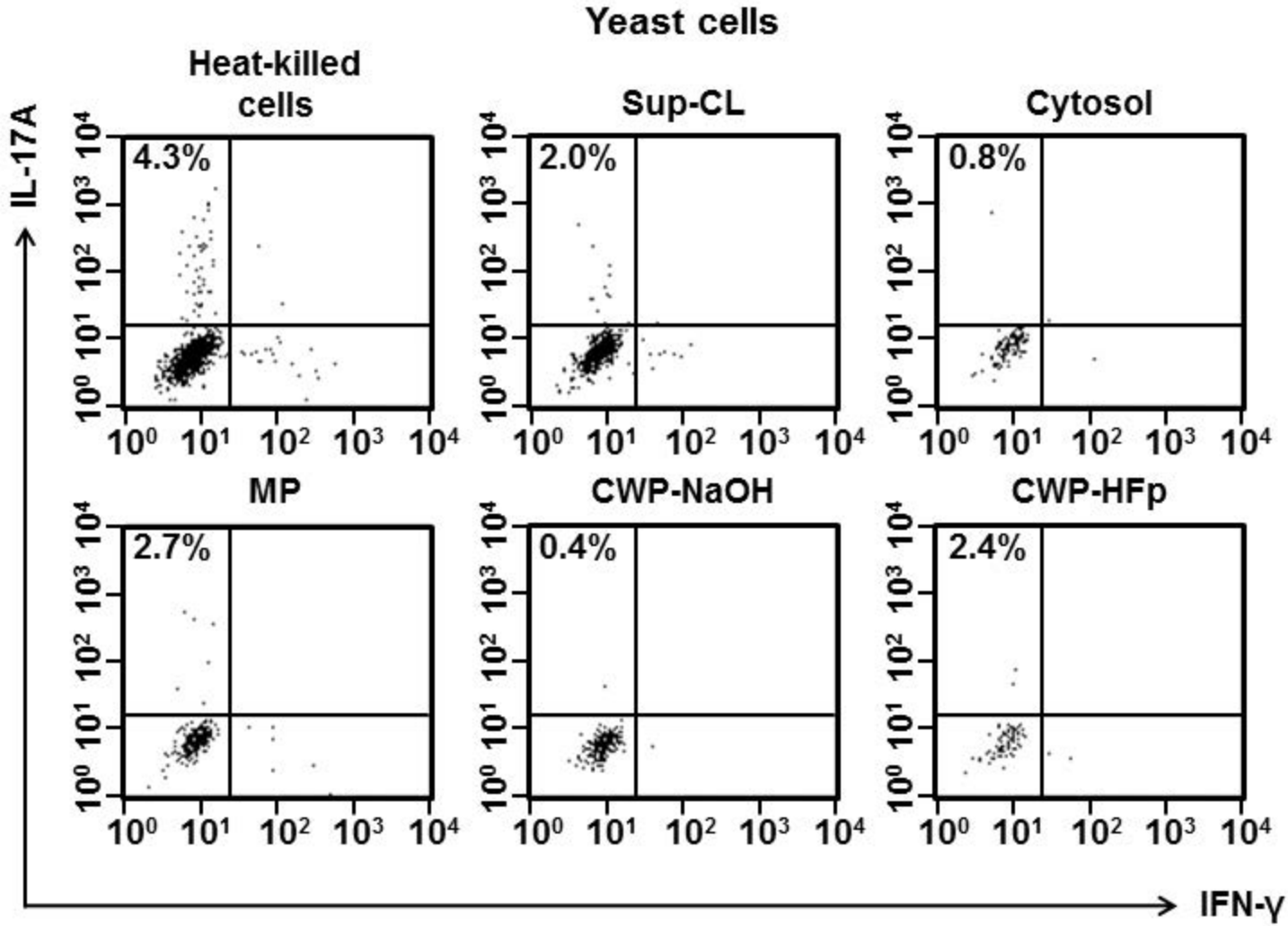
Tasaki.S Fig1B





Tasaki.S Fig1C

Tasaki.S Fig2A



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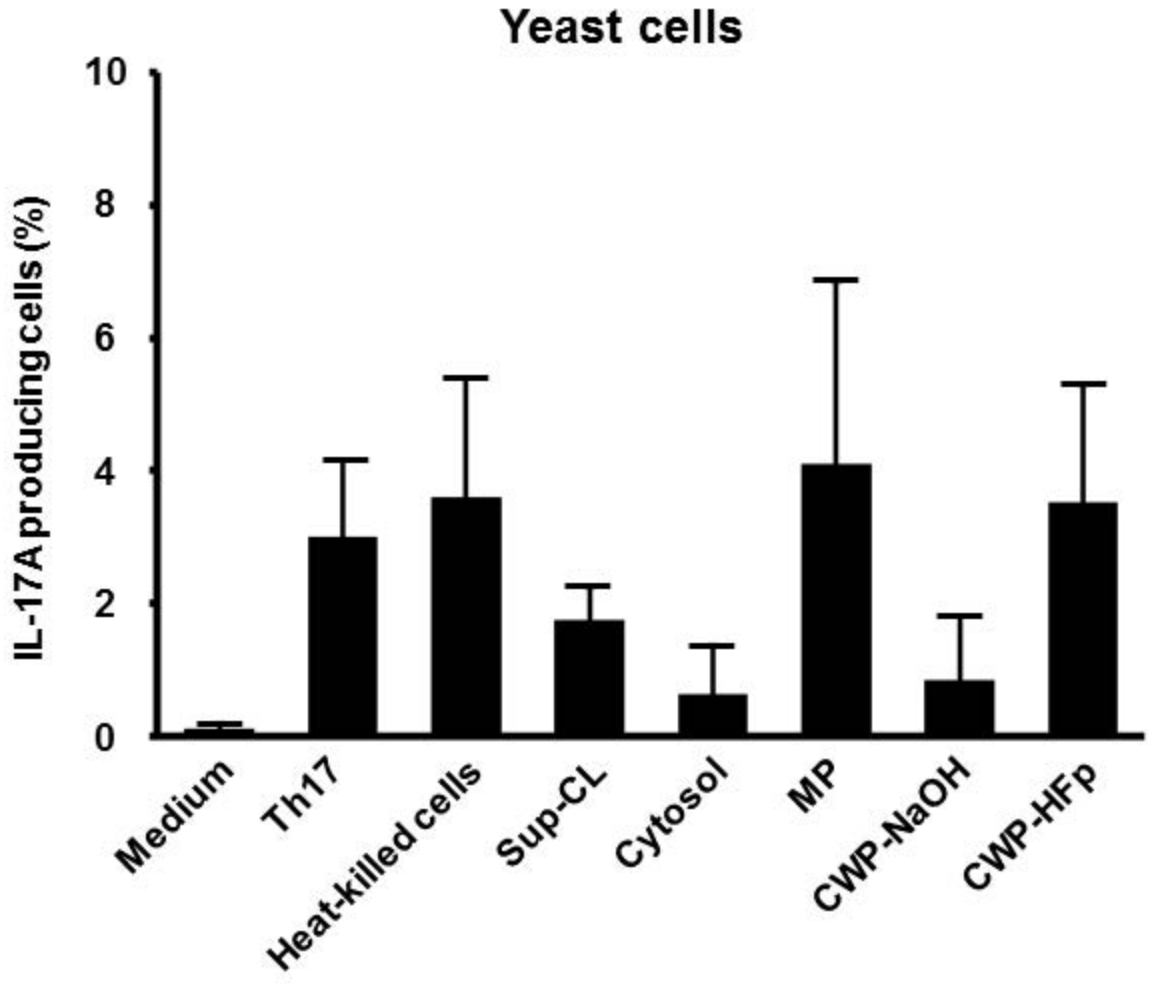

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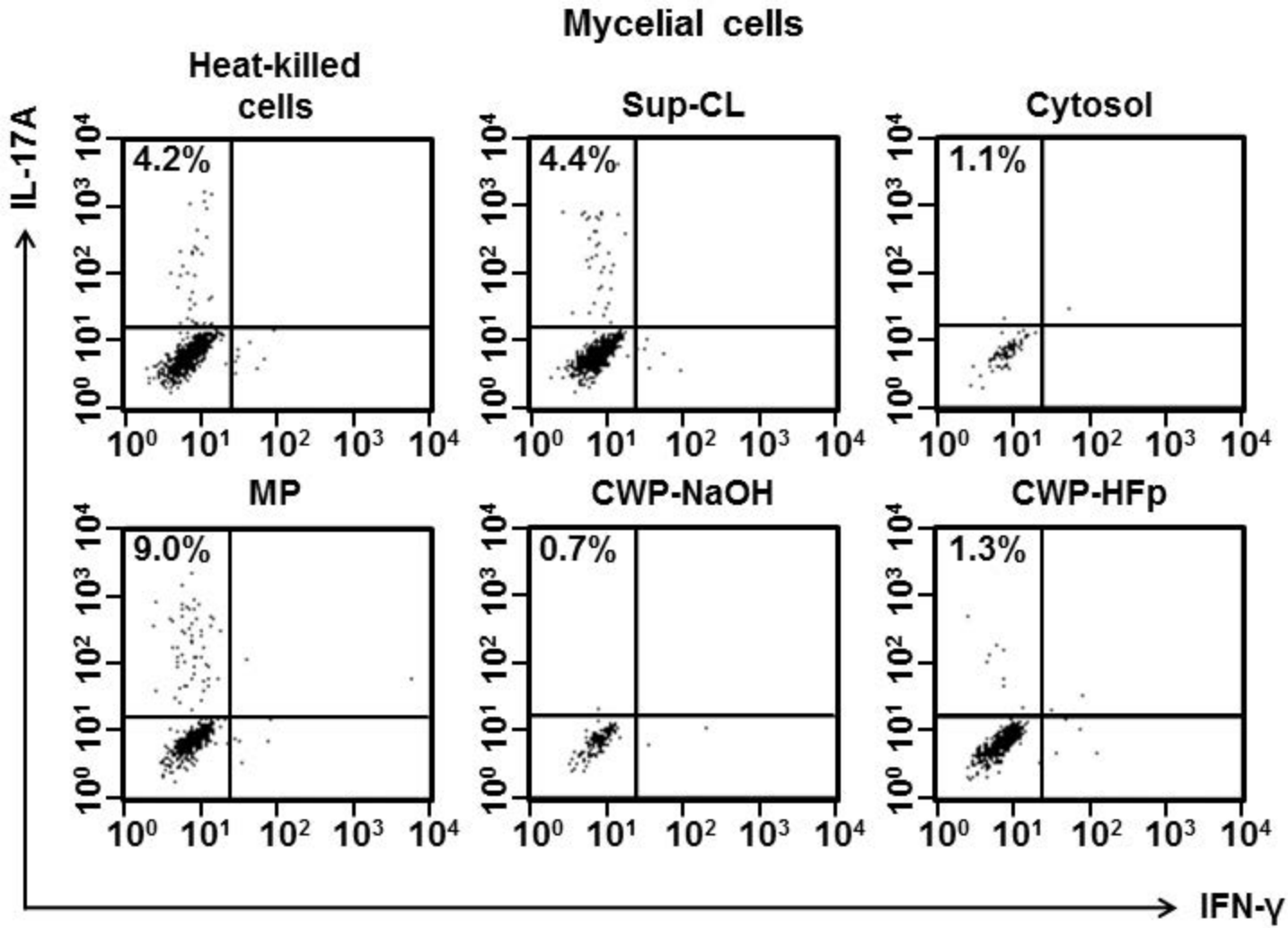

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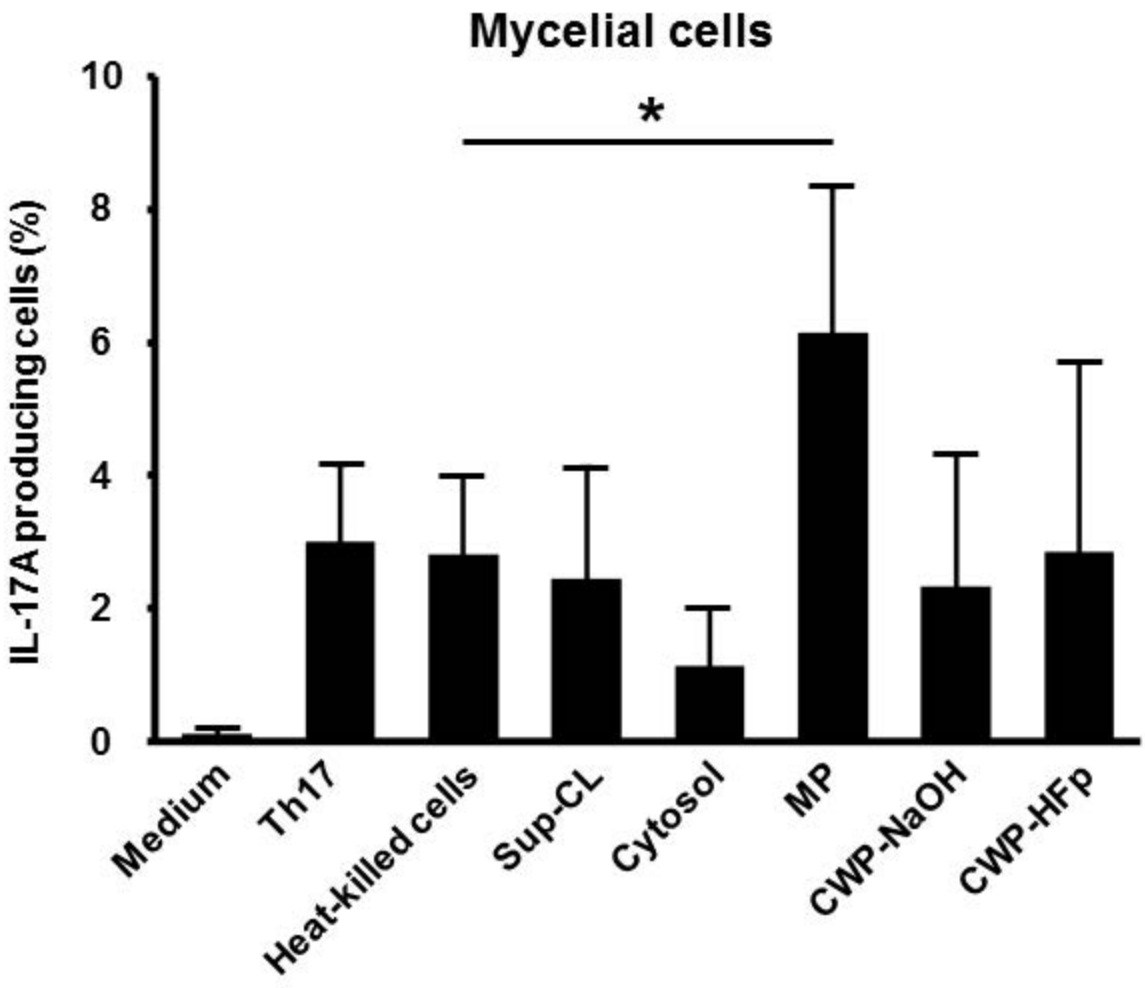

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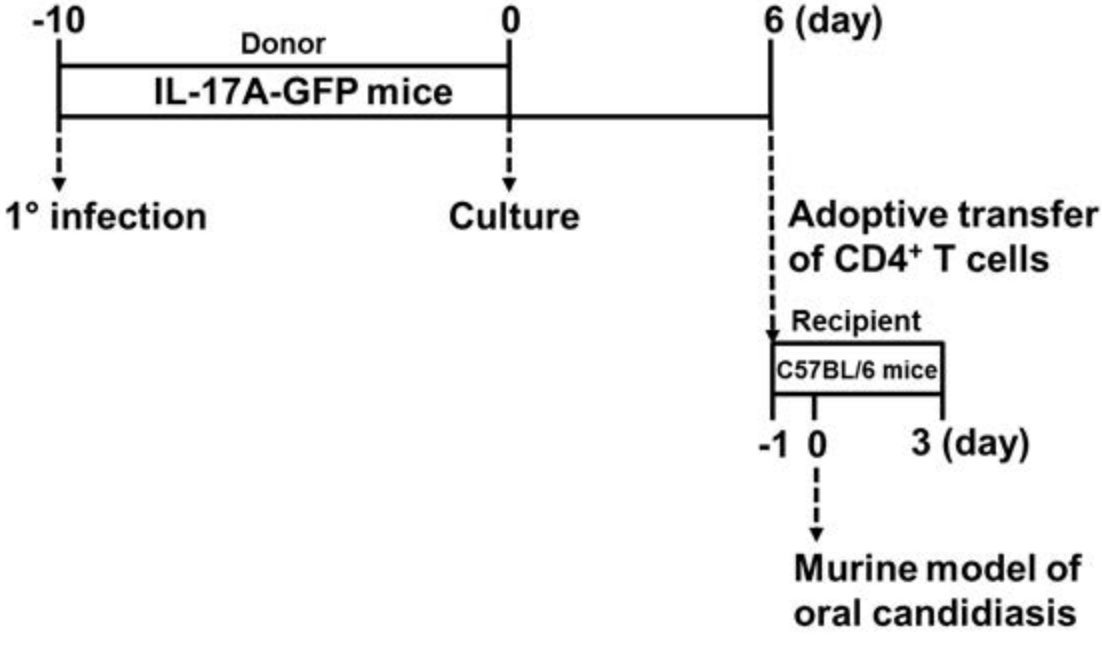

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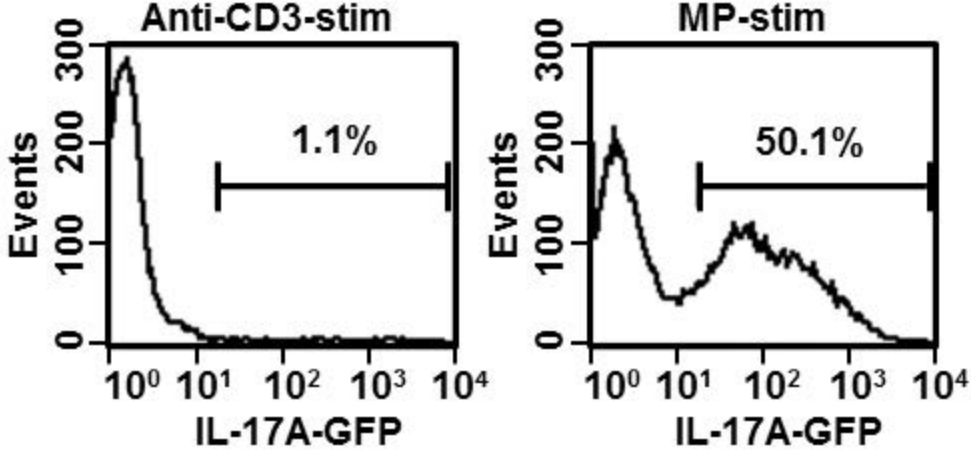

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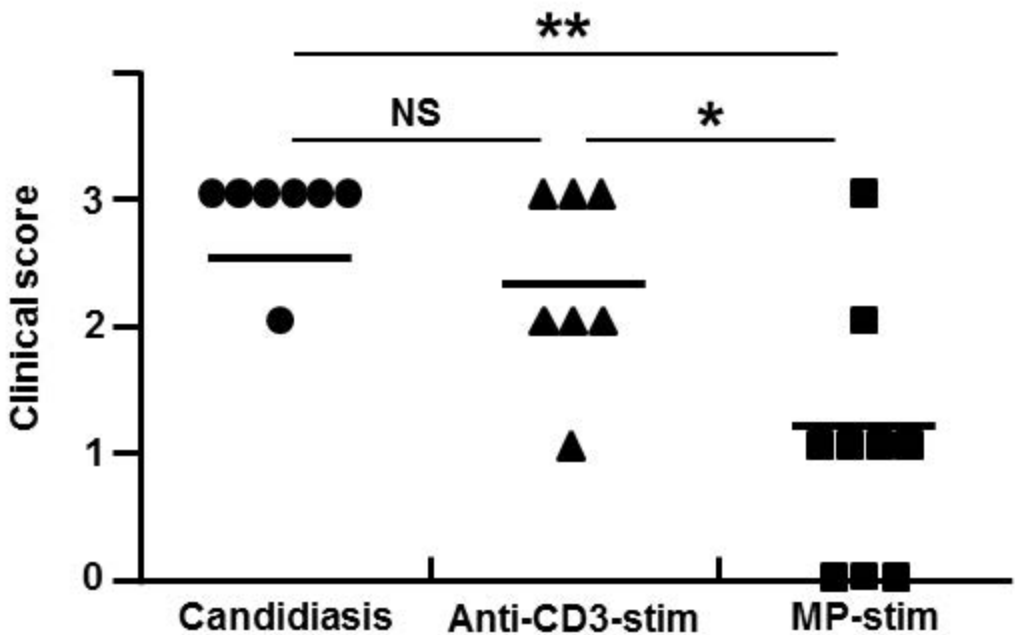

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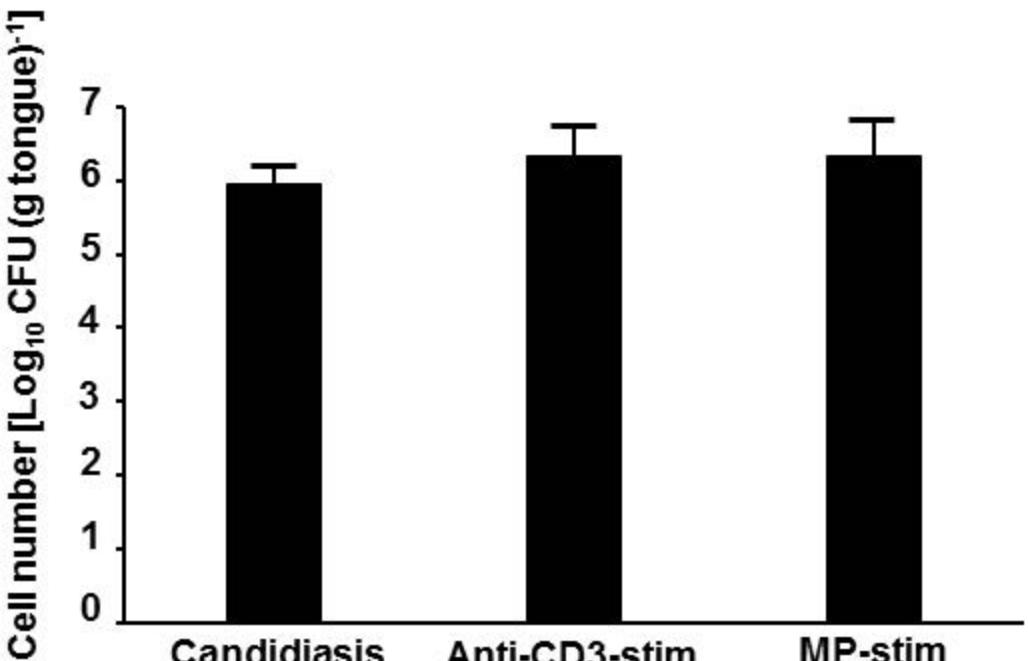

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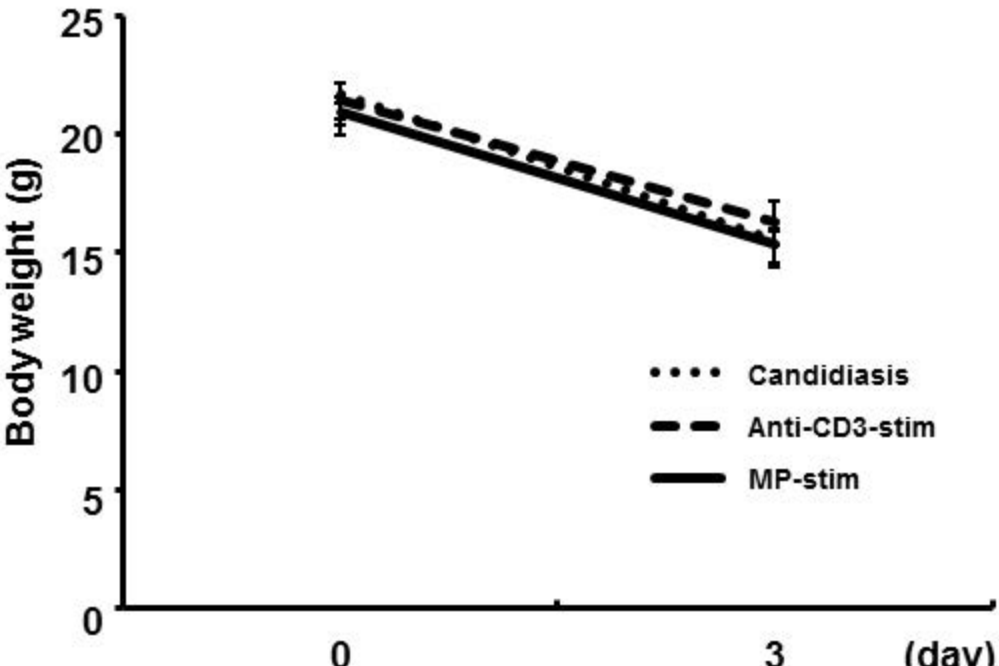

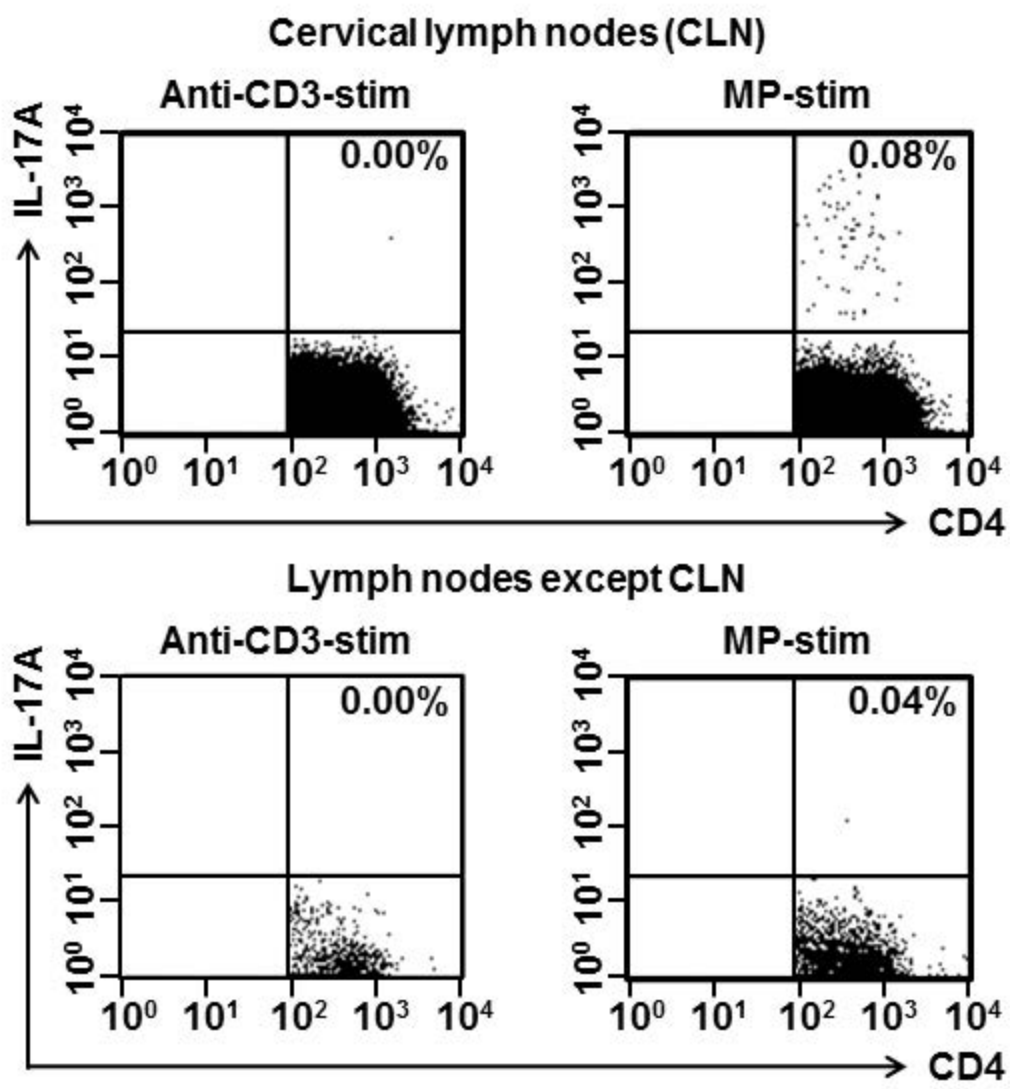

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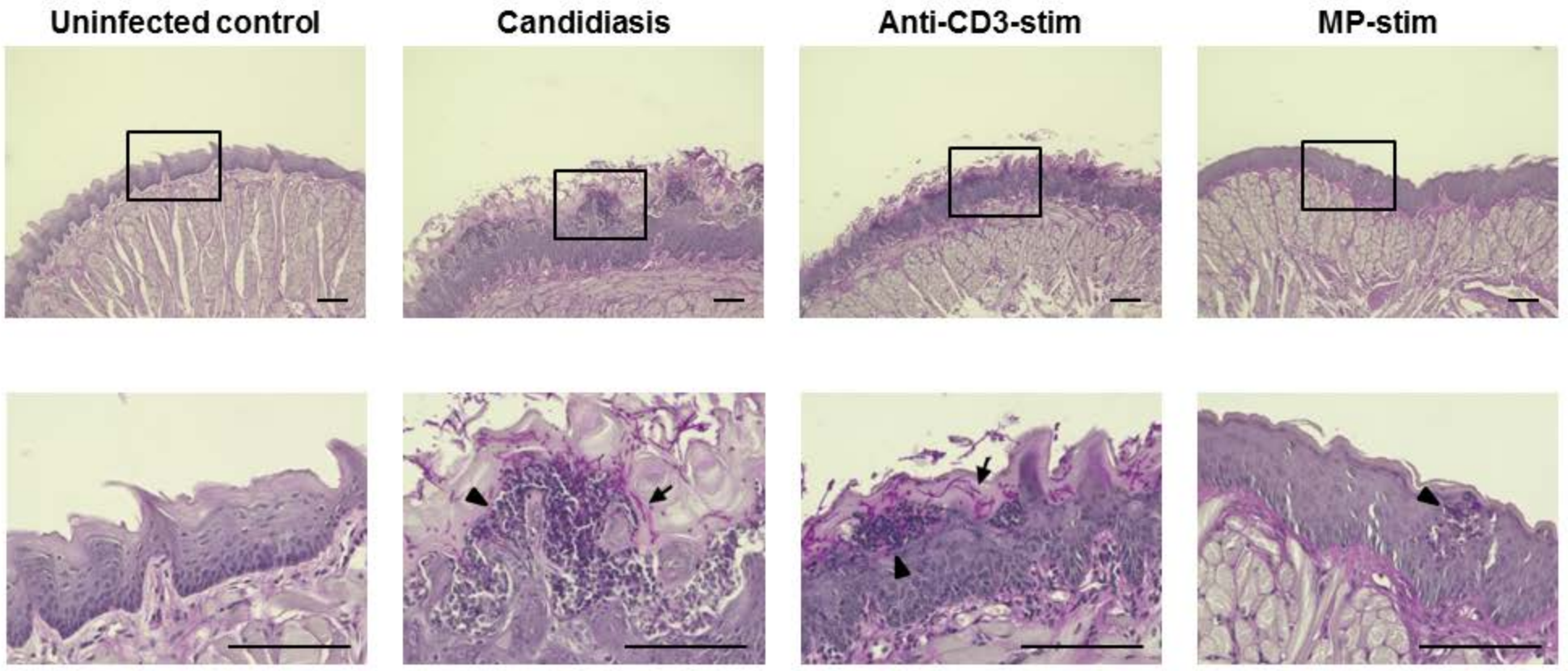
Tasaki.S Fig4A



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