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RESEARCH ARTICLE

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

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ABSTRACT

Candida albicans is a human commensal that causes opportunistic infections. Th17 cells provide resistance against mucosal infection with *C. albicans*; however, the T cell antigens remain little known. Our final goal is to find effective T cell antigens of *C. albicans* that are responsible for immunotherapy against candidiasis. Here, we prepared fractions including cytosol, membrane and cell wall from yeast and mycelial cells. Proteins derived from a membrane fraction of mycelial cells effectively induced differentiation of $CD4^+$ T cells into IL-17A-producing Th17 cells. To confirm the immunological response *in vivo* of 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 adoptively transferred intravenously prevented oral candidiasis by oral infection of *C. albicans*, compared with control anti-CD3-stimulated $CD4^+$ T cells. This was confirmed by the clinical score and the number of neutrophils on the infected tissues. These data suggest that effective T cell antigens against candidiasis could be present in the membrane protein fraction of mycelial cells. The design of novel vaccination strategies against candidiasis will be our next step.

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 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 experience severe thrush. Moreover, deficiency of IL-17 immunity in humans also develops into oropharyngeal candidiasis (Puel *et al.* 2011). *C. albicans*-specific $CD4^+$ T cells that produce cytokines including IL-17 were lost 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*.

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Studies of the pathogenicity of *C. albicans* have focused on the interaction between the organism and host cells. The growth of *C. albicans* is dimorphic depending on environmental factors (Gow *et al.* 2011; Kashem *et al.* 2015). *C. albicans* hyphae adhering to epithelial cells induce clathrin or E-cadherin endocytosis, and 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 under many kinds of stress. However, *C. albicans* cells in patients have some mechanism of survival 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). An example is the *ATO* (ammonia transport outward) gene family that encodes putative acetate and ammonia transporters and is associated with phagosome neutralization (Okai *et al.* 2015). Neutralization of acid in phagolysosome could induce filamentation of *C. albicans* yeast cells in macrophages and damage the host cells. The function of RAB proteins as central regulators involved in phagosome maturation is dysregulated by hyphal formation of *C. albicans* in macrophages (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 little information about *Candida*-derived helper T cell antigens that focus on Th17 differentiation. An epitope isolated as an MHC class II-bound ligand, which is 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 alcohol dehydrogenase (ADH1) protein (one of the mannoproteins) stimulated IL-17A production from a *C. albicans*-specific T cell hybridoma (Trautwein-Weidner *et al.* 2015). Some antigens were reported with a view to vaccination. Cell wall-associated proteins including enolase (ENO1), fructose-bisphosphate aldolase (FBA), glyceraldehyde-3-phosphate dehydrogenase (GAP1), hyphal wall protein-1 (HWP1), methyltetrahydropteroyltriglutamate (Met6), and phosphoglycerate kinase (PGK1) were tested as vaccines combining β-mannan and peptide epitopes against candidiasis (Xin *et al.* 2008). The recombinant N terminus of secreted aspartyl proteinase 2 (Sap2) as a virulence factor was also tested as a mucosal anticandidal vaccine (Sandini *et al.* 2011). These tested proteins are mainly cell surface proteins or are members of the glycolytic pathway.

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

MATERIALS AND METHODS

Fungal strain and growth conditions

Candida albicans SC5314 (Gillum, Tsay and Kirsch 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 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). Cells of each type were separately pooled at −80°C to be crushed physically.

C. albicans expressing green fluorescent protein (GFP) was constructed using the plasmid pGFP-ACT1 (Umeyama *et al.* 2005) linearized with *StuI*, 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). This strain was used for experiments *in vivo* because it is possible to confirm inoculum cells easily.

Preparation of cell fractions

The procedure for *C. albicans* cell fractionation is outlined in Fig. 1B. The harvested yeast cells or mycelia were frozen at −80°C, then crushed immediately with a cold mortar and pestle. The frozen crushed powder was 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 homogenate except for the glass beads was centrifuged for 20 min at 6000 *g*. The supernatant was used to obtain a cytosolic fraction and a membrane fraction. The pellet was used to obtain the cell wall fraction. The supernatant was centrifuged for 60 min at 105 000 g based on the method of Mora-Montes *et al.* (2008). The high-speed supernatant was used as the cytosolic fraction. The high speed-pellet was used as the membrane fraction. To obtain membrane proteins from the membrane fraction, the fraction was treated with 1.5% final concentration of octylglucoside at 4°C for 1 h and then the detergent was removed from the fraction using Pierce detergent removal spin columns (Thermo Fisher Scientific, Waltham, MA, USA). A cell wall fraction was obtained by washing the homogenate five 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 by releasing glycosylphosphatidylinositol-dependent proteins (GPI proteins) from the 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 by releasing mild alkali-sensitive proteins by incubating with 30 mM NaOH at 4°C for 17 h. A heat-kill treatment for whole cells was performed by boiling for 5 min twice. Heat-killed samples were freeze-dried, and used at 10 μg dry weight ml^{-1} for immunological response experiments. Heat-killed mycelial samples 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, Merril and Shifrin 1979).

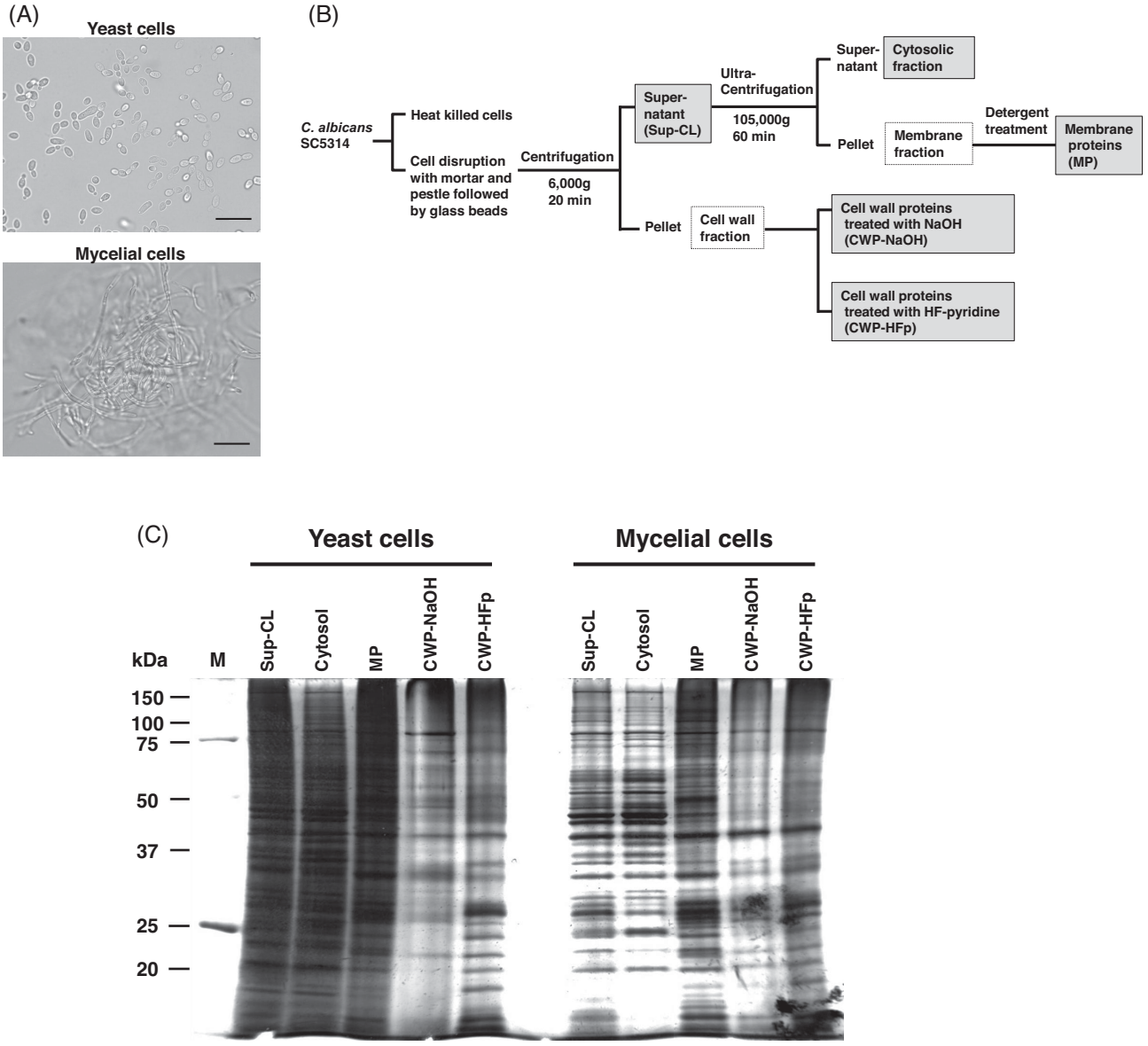

Figure 1. Cell fractionation of yeast and mycelial cells of *C. albicans*. (**A**) Unstained yeast cells and mycelial cells of *C. albicans* SC5314 used for cell fractions viewed at ×1000 magnification. Scale bars indicate 20 μm. (**B**) Preparation of cell fractions. (C) SDS-PAGE of *C. albicans* proteins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

Mice

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

Isolation and culture of dendritic cells

Bone marrow cells were harvested from femurs and tibias of B6 mice and 1 × 10^6 cells per well 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 granulocyte–macrophage colony-stimulating factor (GM-CSF) (PeproTech). On days 3 and 5, each culture was split into two 1 mL wells, and 1 mL fresh medium containing 10 ng mL^{-1} of GM-CSF was added to the original and new wells. On day 7, the cells were harvested and used as bone marrow-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 re-stimulated with 50 ng mL^{-1} phobol myristate acetate (PMA; Sigma-Aldrich) and 500 ng mL^{-1} ionomycin (Sigma-Aldrich) in the presence of 10 μg mL^{-1} brefeldin A (Sigma-Aldrich) at 37°C for 4 h. The cells were stained with anti-CD4 antibody (Ab) (APC, clone RM4-5, BD Biosciences) and permeabilized with 0.1% saponin (Nacalai Tesque). Intracellular cytokines were stained with anti-IL-17A Ab (PE, clone TC11-18H10, BD Biosciences) and anti-IFNγ Ab (fluorescein isothiocyanate, clone XMG1.2, BD Biosciences). All data were obtained using FACSCalibur (BD Biosciences).

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, an immunosuppressed condition and antibiotic 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 live *C. albicans* cells (2×10^8 cells mouse^{-1}) or PBS using a 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 first infection. In anti-CD3 Ab stimulated experiments, CD4$^+$ T cells were isolated from the donor mice given PBS instead of *C. albicans*, and co-cultured with BMDCs and anti-CD3 Ab (clone 145-2C11, BD Biosciences). On day 6, dead CD4$^+$ T cells in the culture were removed using Lympholyte-M (Cedarlane, NC, USA). Donor CD4$^+$ T cells ($1–2 \times 10^6$ cells mouse^{-1}) were adoptively 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 a 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) was 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% fetal bovine serum medium following a 24 h culture at 37°C. Both forms of cell 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 in each fraction were analyzed by SDS-PAGE (Fig. 1C). There was no distinctive difference between proteins from the yeast and mycelial fractions.

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

To determine the effective cell fractions that preferentially led to differentiation into Th17 cells, we examined whether proteins involved in these fractions induce C57BL/6 naive CD4$^+$ T cells isolated from peripheral lymphoid organs to differentiate into Th17 cells. For the yeast cell fractions, none of the fractions led to differentiation into Th17 cells, compared with 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 the mycelial membrane protein (mycelial MP) fraction, compared with heat-killed cells (4.2%) and other fractions (0.7–4.4%). 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 the MP fraction of the mycelial form of *C. albicans*, but not of the 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

As shown in Fig. 2, mycelial MP induced IL-17A production *in vitro*. To test whether mycelial MP could include effective T cell antigens for preventing murine oral candidiasis, we used mice that were adoptively transferred Th17 cells that 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 show only a weak response in producing IL-17A when stimulated by *C. albicans* antigen in uninfected mice, whereas cells from infected mice show a significant response. To increase the proportion of Th17 cells that were adoptively transferred, IL-17A-GFP donor mice were infected orally with 2×10^8 yeast cells per mouse using a feeding needle for mice. Control mice were given 200 μL of PBS per mouse. Ten days later, CD4$^+$ T cells from primary infected IL-17A-GFP mice were

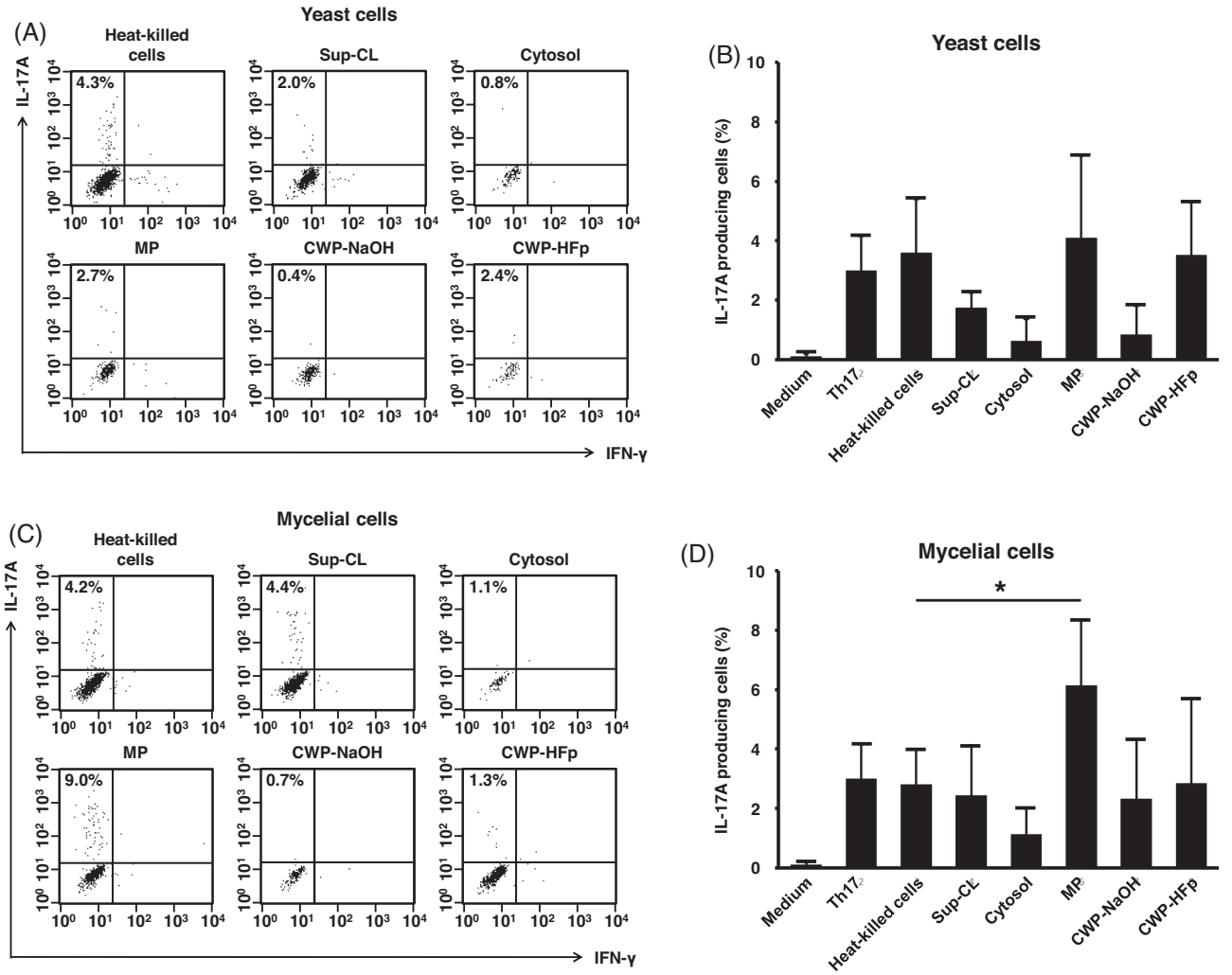

Figure 2. Analysis of *C. albicans* cell fractions involving CD4$^+$ T cell antigens by flow cytometry. (**A** and **C**) Naive CD4$^+$ T cells were magnetically isolated from peripheral lymph nodes and spleen cells. Cytokine production was analyzed by intracellular cytokine staining and flow cytometry. (**B** and **D**) Each bar represents the mean and SD of four independent experiments per group. $^*P < 0.05$ by 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–D are the same in Fig. 1.

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 fungus culture in a stool taken before the oral infection (data not shown). Six days later, the CD4$^+$ T cells from which dead cells had been depleted in culture were adoptively transferred to C57BL/6 recipient mice intravenously. To avoid any influence on adoptively 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} was swabbed on the whole tongue. Assessments of IL-17A-GFP$^+$ expression of adoptively transferred T cells is 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 of 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 are shown in Fig. 3D. Tongues were removed aseptically from each group of oral candidiasis model mice to quantify the fungal burden of the tongue (Fig. 3E). The recovered fungal burden of the tongue was not significantly different among the three groups. The three groups of oral candidiasis model mice showed similar weight loss for 3 days (Fig. 3F). Assessment of IL-17A expression in adoptively transferred CD4$^+$ T cells isolated from the cervical lymph nodes (CLN) and the lymph nodes except CLN of recipient mice is shown in Fig. 3G. When CD4$^+$ T cells from mycelial MP-stimulated mice were adoptively transferred to recipient mice followed by an infection with *C. albicans*, the CD4$^+$ T cells that included Th17 cells gathered more in CLN compared with other lymph nodes of

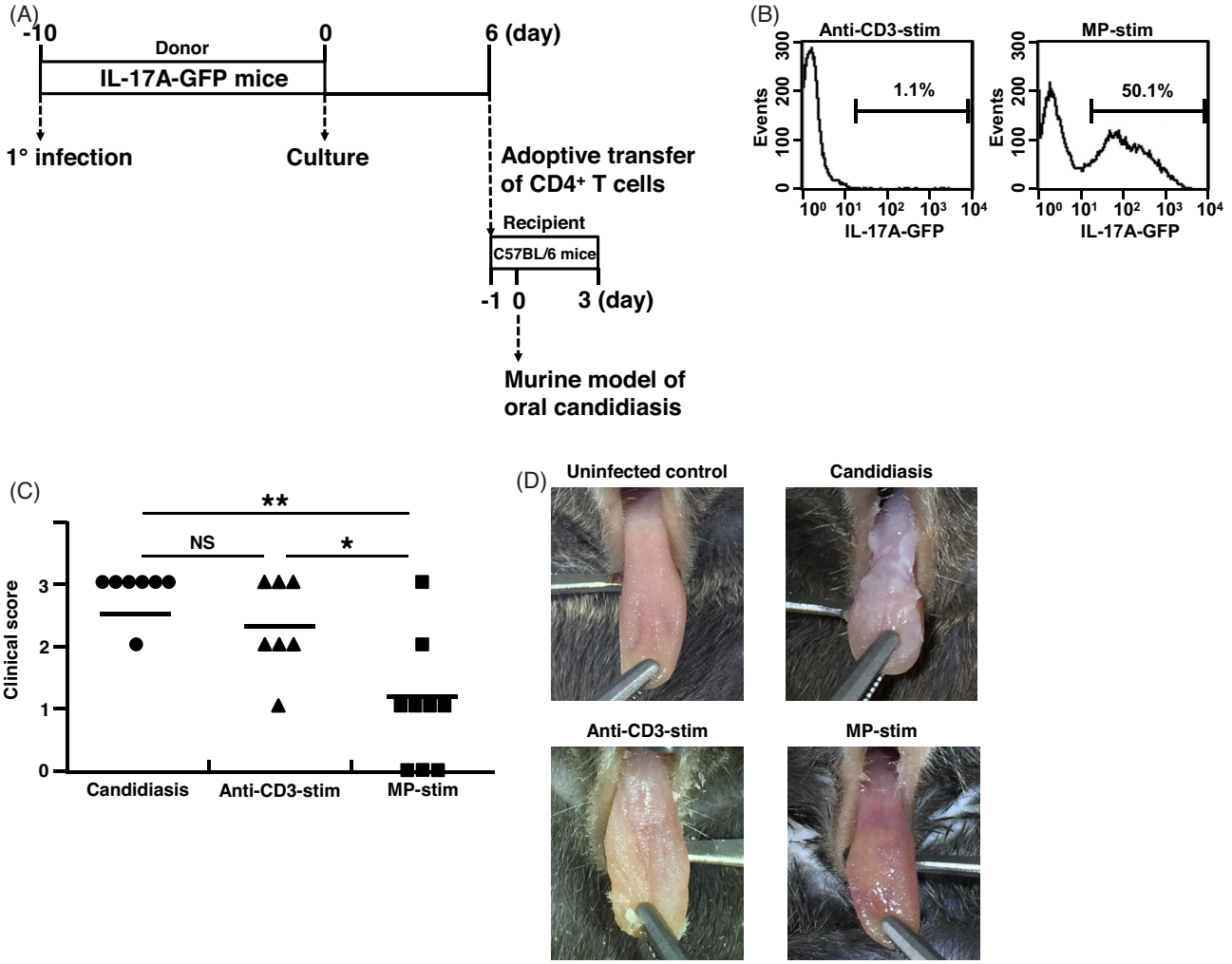

Figure 3. Th17 cells differentiated with mycelial MP prevented oral candidiasis in mice. (**A**) Timeline of infection model. The first (1°) infection in donor mice was by oral inoculation with *C. albicans* or PBS using a feeding needle. $CD4^+$ T cells from lymph nodes and spleen cells of donor mice were cultured with BMDCs and mycelial membrane proteins on day 10 after 1° infection. On day 6, donor $CD4^+$ T cells were adoptively transferred intravenously to recipient mice, followed by determination 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 adoptively transferred $CD4^+$ T cells. Assessment of clinical severity of oral candidiasis by Hise *et al.* (2009) was used. Candidiasis: tongues that developed oral candidiasis as positive control ($n = 7$). Anti-CD3-stim: tongues of recipient mice that were adoptively transferred $CD4^+$ T cells that were stimulated with anti-CD3 Ab ($n = 7$). MP-stim: tongues of recipient mice that were adoptively transferred $CD4^+$ T cells that were stimulated with mycelial MP ($n = 9$). Each data point represents an individual mouse, and the horizontal bar indicates the mean. $^*P < 0.05$, $^{**}P < 0.01$ by a two-tailed Student's *t*-test. Data were evaluated in two independent experiments. (D) Tongues from 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 seven mice. Anti-CD3-stim tongues were from five mice. MP-stim tongues were from eight mice. Error bars represent SD. (F) Weight loss of mice from 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, and nine MP-stim mice were used. Error bars represent SD. (G) IL-17A expression in adoptively transferred $CD4^+$ T cells isolated from the cervical lymph nodes (CLN) (top) 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.

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 the 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 the MP-stimulated group (Fig. 4A). Furthermore, the number of neutrophils infiltrated into the intraepithelial layer in a section of the MP-stimulated group showed a significant reduction compared with the candidiasis ($P < 0.01$) and anti-CD3-stimulated ($P < 0.01$) groups (Fig. 4B). On the other hand, there was also a small reduction in the number of neutrophils in the anti-CD3-stimulated group ($P < 0.05$) compared with the candidiasis group, despite the lack of significance in the difference between them in the clinical score

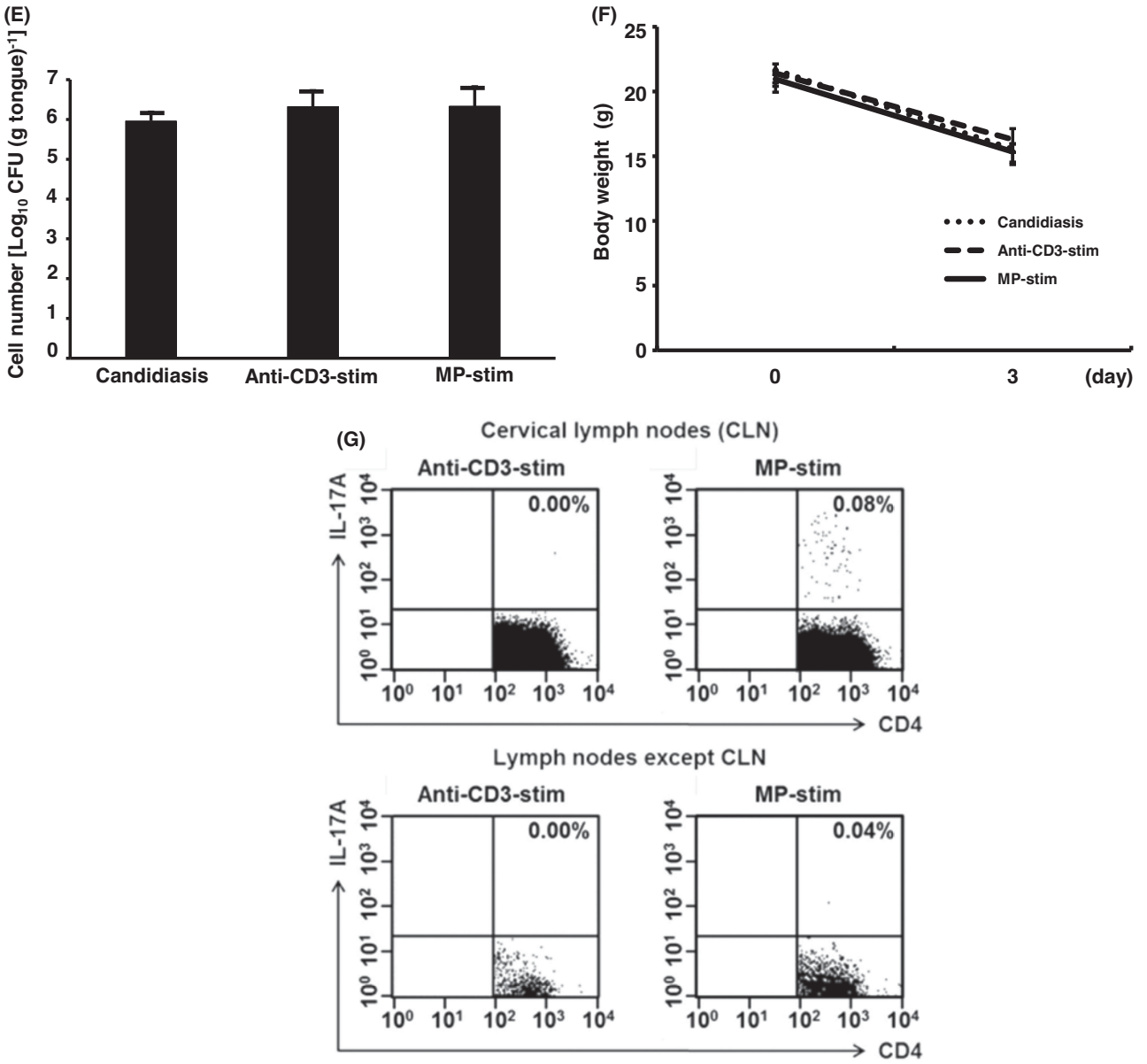

Figure 3 *–continued.*

(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 focused on finding a novel T cell antigen of *C. albicans* to T cell receptor of $CD4^+$ T cells that is effective for Th17 differentiation compared with 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 differential abundance of proteins was found between biofilm and planktonic cells and between yeast cells and mycelia. Moreover, the analysis showed that the differentially abundant cytoplasmic proteins affected several processes and functions. Therefore, differentially abundant proteins would be included among the cell fractions in Fig. 1. However, it has remained unclear what kind of protein effectively responds to $CD4^+$ T cells. Here we recognized that there are different responses to naive helper T cells among the cell fractions. The 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 the mycelial MP could be a protein unique to mycelia or a protein existing in larger amounts 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

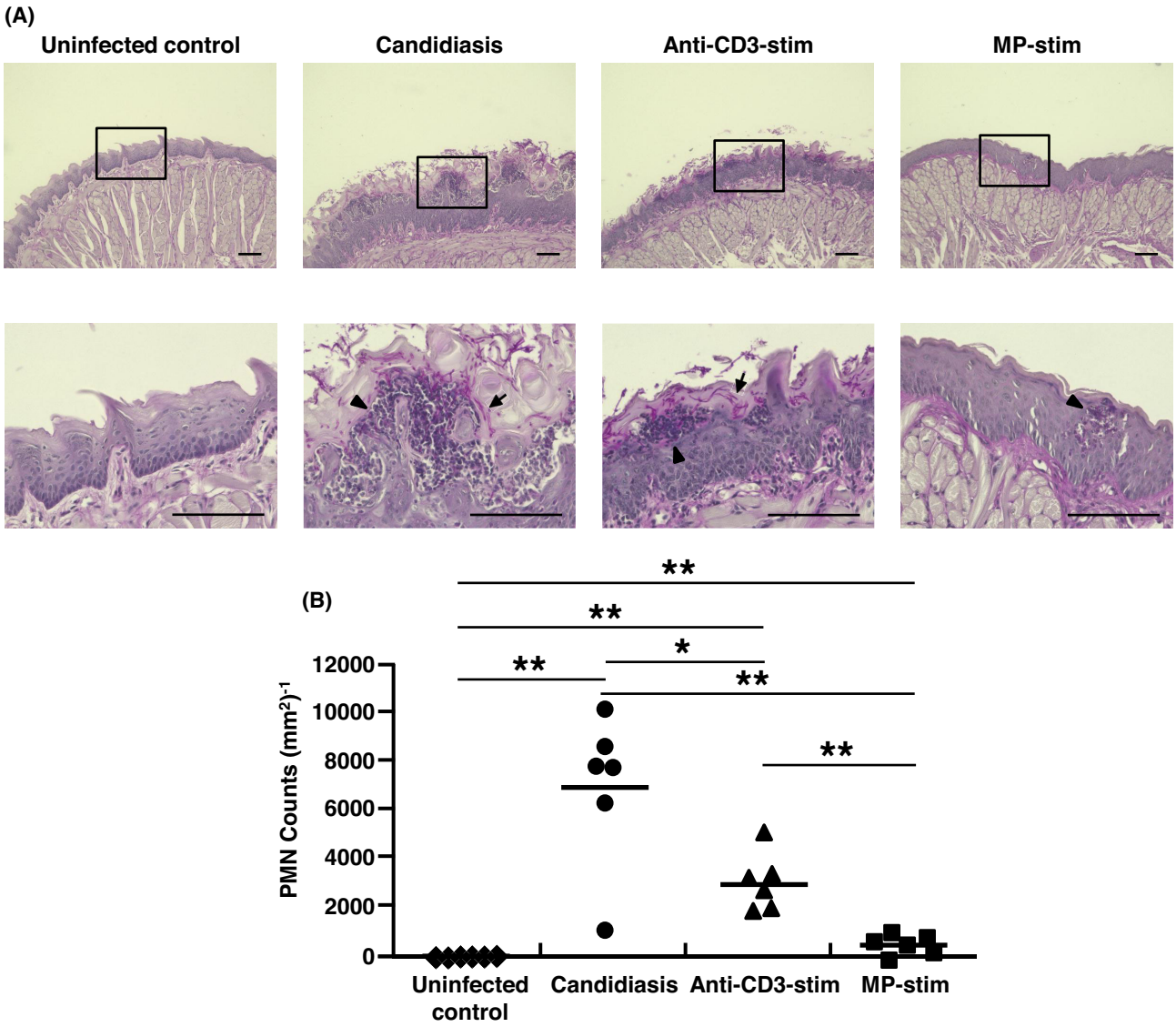

Figure 4. Histological evaluation of *C. albicans* infection. (**A**) PAS-stained histological sections of tongues on day 3 after oral infection with *C. albicans*. Uninfected control group: uninfected healthy tongues ($n = 2$). Candidiasis group: tongues that developed oral candidiasis as positive control ($n = 2$). Anti-CD3-stim group: tongues of recipient mice that were adoptively transferred $CD4^+$ T cells that were stimulated with anti-CD3 Ab ($n = 2$). MP-stim group: tongues of recipient mice that were adoptively transferred $CD4^+$ T cells that were stimulated with mycelial MP ($n = 2$). Arrows and arrowheads indicate *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) Quantification 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.

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.* 2018). In immunological research, 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 immunosuppression drugs and antibiotic administration using swabs of yeast cells on tongues at a 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 comparable to previous reports (Conti *et al.* 2009; Solis and Filler 2012). Clinical severity of recipient mice transferred Th17 cells that were stimulated by mycelial MP in Fig. 3C was reduced, but the CFUs of *C. albicans* on tongues of the 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 can be considered that Th17 cells transferred in the model mice

were activated in mechanisms responding to *C. albicans* yeast cells swabbed on tongues, followed by controlled morpho-type switching of *C. albicans*. Similar weight loss among the three groups shown in Fig. 3F could be governed by skipping feeding for 3 days.

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

In the anti-CD3-stimulated and candidiasis control groups, pathological sections of tongues on which candidiasis occurred showed robust mycelial invasion and neutrophil infiltration into the intraepithelial layer of the tongues (Fig. 4A and B). Candidalysin, discovered by Moyes *et al.* (2016), was 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.* 2018). Therefore, the abundant neutrophil infiltration observed in Fig. 4B would be recruited with candidalysin secreted by robust mycelia. On the other hand, pathological sections of the mycelial MP-stimulated group showed reduced oral candidiasis and infiltration of few neutrophils (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 a mechanism such as 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 induce differentiation of $CD4^+$ T cells to Th17 cells. Moreover, the antigen-specific Th17 cells are able to protect against oral infection with *C. albicans*. On the basis of these findings, we are going to determine the effective T cell antigens from the mycelial membrane proteins of *C. albicans* to design novel vaccination strategies against candidiasis in the next stage of our work.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: ST, TC, JN and YT. Performed the experiments: ST, TC, JN, SI, YN, KA-M, KY and KT. Analyzed the data: ST, TC, JN, YN, KA-M, HK and YT. Wrote the paper: ST, TC, JN and YT. Led the research: YT.

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Conflict of interest. None declared.

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