Protective Role of Naturally Occurring Interleukin-17A-Producing $\gamma~\delta$ T Cells in the Lung at the Early Stage of Systemic Candidiasis in Mice

Dejima, Takashi Division of Host Defense, Medical Institute of Bioregulation, Kyushu University

Shibata, Kensuke Division of Host Defense, Medical Institute of Bioregulation, Kyushu University

Yamada, Hisakata Division of Host Defense, Medical Institute of Bioregulation, Kyushu University

Hara, Hiromitsu Department of Biomolecular Sciences, Faculty of Medicine, Saga University

他

https://hdl.handle.net/2324/25621

出版情報:Infection and Immunity. 79 (11), pp.4503-4510, 2011-11. American Society for Microbiology バージョン: 権利関係:(C) 2011, American Society for Microbiology. **Title:** A protective role of naturally occurring IL-17A-producing $\gamma\delta$ T cells in the lung at the early stage of systemic candidiasis in mice.

A running title: IL-17A-producing $\gamma\delta$ T cells in candidiasis

Authors: Takashi Dejima¹, Kensuke Shibata¹*, Hisakata Yamada¹, Hiromitsu Hara², Yoichiro Iwakura³, Seiji Naito⁴ and Yasunobu Yoshikai¹

Author affiliation:

¹ Division of Host Defense, Medical Institute of Bioregulation, Kyushu University,

Fukuoka, Japan

² Department of Biomolecular Sciences, Faculty of Medicine, Saga University, Saga, Japan

³ Center for Experimental Medicine, Institute of Medical Science, University of Tokyo,

Tokyo, Japan

⁴ Department of Urology, Graduate School of Medical Science, Kyushu University,

Fukuoka, Japan

Key words: Neutrophils, Cytokines, Fungal infection

Corresponding author: Kensuke Shibata Ph.D., Division of Host Defense, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.

Tel: 81-92-642-6962, Fax: 81-92-642-6973, E-mail: k_shibata@bioreg.kyushu-u.ac.jp

Abstract

IL-17A-producing $\gamma\delta$ T cells differentiate in fetal thymus and reside in the peripheral tissues such as lung of naïve adult mice. We here show that "naturally occurring $\gamma\delta$ T cells" play a protective role in the lung at very early stage after systemic infection with Candida albicans. Selective depletion of neutrophils by in vivo administration of anti-Ly6G mAb impaired fungal clearance more prominently in the lung than in the kidney 24 h after intravenous infection with C. albicans. A rapid and transient production of IL-23 was detected in the lung at 12 h, preceding IL-17A production and the influx of neutrophils, which reached to a peak at 24 h after infection. IL-17A KO mice showed reduced infiltration of neutrophils concurrently with impaired fungal clearance in the lung after infection. The major source of IL-17A was $\gamma\delta$ T cell population in the lung, and $C\delta$ KO mice showed little IL-17A production and reduced neutrophil infiltration after infection. Early IL-23 production in TLR2/MyD88-dependent manner and IL-23-triggered Tyk2 signaling were essential for IL-17A production by $\gamma\delta$ T cells. Thus, our study demonstrated a novel role of naturally occurring IL-17A-producing $\gamma\delta T$ cells in the first line of host defense against *C.albicans* infection.

Introduction

Candida albicans is a dimorphic fungus, which causes chronic mucocutaneous candidiasis and, more rarely, multiple organ failures due to systemic disseminations in immuno-compromised hosts (5). Innate immune cells, which recognize components of C. albicans cell walls via pattern recognition receptors (PRRs), are important not only to clear this microorganism by phagocytosis and killing through oxidative and non-oxidative mechanisms, but also to induce acquired immunity by producing pro-inflammatory cytokines (18, 20, 29). TLR2, through activation of MyD88, was involved in protection against C. albicans infection by triggering TNF α , IL-1 β and MIP-2 production from macrophages (30, 50). The antifungal activity of neutrophils and the Th1 response induced by DCs were impaired in IL-1R KO and MyD88 KO mice after systemic infection with C. albicans (4). More recently, it was revealed that α -mannan, which was exposed on the cell walls of C. albicans, bound to one of C-type lectin receptor (CLR), Dectin-2 on DCs and macrophages (39, 40). Dectin-2 KO mice showed impaired Th17 cell differentiation and became susceptible after systemic infection with C. albicans (39). Among innate immune cells, depletion of neutrophils in mice by pretreatment with anti-Gr1 mAb led to death within 4 days after systemic infection with C. albicans (37), indicating the particular importance of neutrophils for

host defense at an early stage when acquired immunity is not completely established, although the detailed mechanism of neutrophil-mediated host defense was not fully understood.

IL-17A is a T cell-derived proinflammatory cytokine which is involved in mobilization and fungicidal activity of neutrophils (15, 19). The protective roles of IL-17A in a murine model of infection with *C. albicans* were recently demonstrated. IL-17R KO mice showed a markedly decreased neutrophil recruitment to infected tissues and an impaired host defense against systemic and oral candidiasis (7, 15). IL-17A KO mice increased susceptibility to systemic infection with *C. albicans* (39). Dectin-2 through activation of caspase-recruiting domain family, member 9 (CARD9) induced Th17 cell differentiation (36, 39). In addition to Th17 cells, other cell types were reported to produce IL-17A, including CD8+ T cells, $\gamma\delta$ T cells and NKT cells, although the involvement of these cells in host defense against *C. albicans* infection was unclear (9).

IL-17A-producing $\gamma\delta$ T cells are known as naturally occurring effectors because they are functionally differentiated within fetal thymus before being exposed to foreign Ags (43). In naive mice, IL-17A-producing $\gamma\delta$ T cells are widely distributed in various organs, but are predominantly found in mucosal tissues such as the gut, peritoneal cavity and lung (21, 43). IL-17A-producing $\gamma\delta$ T cells play important roles in the first line of host defense against *Mycobacterium tuberculosis*, *Escherichia coli* and *Listeria monocytogenes* (13, 22, 42, 49). Various exogenous signals were reported to induce IL-17A production by $\gamma\delta$ T cells. IL-23 and IL-1 β , produced by DCs and macrophages after activation through PRRs, were potent inducers of IL-17A production by $\gamma\delta$ T cells (22, 34, 42, 49). $\gamma\delta$ T cells expressed TLR2 and Dectin-1, which directly recognized *C*. *albicans* and induced IL-17 production and cell proliferation in synergy with IL-23 (23, 26), although the *in vivo* roles of innate receptors on $\gamma\delta$ T cells are still a matter of debate.

In this study, we found a rapid production of IL-17A, which was critical for neutrophil infiltration and host defense in the lung at the very early stage after intravenous infection with *C.albicans*. Resident $\gamma\delta$ T cells in the lung were the major source of early IL-17A production after *C. albicans* infection. IL-23, which was rapidly produced in the TLR2/MyD88-dependent but not in the CARD9-dependent pathway, induced IL-17A production by $\gamma\delta$ T cells, whereas TLR2 expression on $\gamma\delta$ T cells was dispensable for IL-17A production *per se*. These findings provide new insight into the functions of $\gamma\delta$ T cells in the first line of host defense against fungal infection.

Materials and methods

Mice

C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). IL-17A KO, C8 KO TLR2 KO, Tyk2 KO, MyD88 KO, and CARD9 KO mice were generated as previously described (1, 14, 17, 27, 44, 47). These mice were bred in specific pathogen-free conditions in our institute. Six- to eight-week-old male mice were used for the experiments. This study was approved by the Committee of Ethics on Animal Experiment in the Faculty of Medicine, Kyushu University. Experiments were carried out under the control of the Guideline for Animal Experiment.

Microorganisms

C. albicans (No.10261: American Type Culture Collection(ATCC), SC5314) was cultured in a shaking incubator for 24 h at 37°C in Sabouraud broth (Nihon Pharmaceutical, Tokyo, Japan). *C. albicans* was washed extensively with PBS before being resuspended in 50% glycerol-containing PBS. Small aliquots were stored at -80 °C until use. Mice were i.v. inoculated with 1×10^7 CFU of No.10261 or 2×10^5 CFU of SC5314 respectively.

Assessment of fungal growth

At the indicated time after infection, lungs were removed and placed in homogenizers containing 3ml of PBS. Lung homogenates were spread on Sabouraud agar plates (Nihon Pharmaceutical, Tokyo, Japan). After incubation for 24 h at 37°C, colonies were counted.

Lung cell preparation

The lung was minced to yield 1-2 mm pieces and incubated with 1 mg/ml collagenase (GIBCO) and 20 μ g/ml DNase (DN-25, Sigma) in RPMI 1640 containing 10% FCS for 90 min at 37 °C with vigorous vortexing every 15 min. Mononuclear cells were further purified in 33% Percoll by centrifugation at 600×g for 20min.

Measurement of IL-17A, IL-23 and IL-1 β in the lung and kidney

After mononulcear cells from the lung were cultured for 24 h, the supernatant was collected and measured for the production of IL-17A and IL-1β by the DuoSet ELISA Development System (R&D Systems) and the Mouse IL-23 (p19/p40) ELISA Ready-Set-Go (eBioscience), respectively, according to manufacturer's instructions.

Myeloperoxidase (MPO) activity

Mouse MPO ELISA kit (Hycult biotech) was used for the measurement of MPO activity. The protocol was followed according to the manufacturer's instruction. Briefly, after centrifugation of lung homogenates taken from naive or infected mice at $1500 \times g$ at 4°C for 15min, MPO activities in supernatants were analyzed.

Antibodies and flow cytometric analysis

FITC-conjugated anti-CD4 (RM4-5), anti-CD11b (M1/70), anti-CD45.2 (104) mAb, allophycocyanin(APC)-conjugated anti-TCRγδ (GL3), Peridinin chlorophyll protein (PerCP)-Cy5.5-conjugated streptavidin, PE-conjugated anti-CD3ε (145-2C11), anti-Ly6G (1A8) and anti-mIL-17A (TC11-18H10.1) mAb were purchased from BD Biosciences (San Diego, CA). PE-conjugated anti-Gr1 (RB6-8C5) mAb was purchased from Caltag Laboratories (Burlingame, CA). FITC-conjugated anti-TCRβ (H57-597), APC-conjugated F4/80 (BM8), Biotin-conjugated anti-MHC class II (M5/114.15.2), anti-F4/80 (BM8), anti-TCRβ (H57-597) and anti-B220 (RA3-6B2) mAb were purchased from eBioscience (San Diego, CA). Stained cells were run on a FACSCalibur flow cytometer (BD Biosciences). The data were analyzed using CellQuest software (BD Biosciences).

Intracellular cytokine staining

Lung cells were stimulated with or without 10 ng/ml of rIL-23 (R&D systems) for 6 h at 37°C. 10 µg/ml of Brefeldin A (BFA) was added for the last 3 h of incubation. After incubation, cells were stained with various mAbs for 30 min at 4°C. Intracellular staining was performed according to the manufacturer's instruction (BD Biosciences). Briefly, 100 µl of BD Cytofix/Cytoperm solution (BD Biosciences) was added to the cell suspension with mild mixing, then placed for 20 min at 4°C. Fixed cells were washed with 250 µl of BD Perm/Wash solution (BD Biosciences) twice and were stained intracellularly with PE-conjugated anti-mIL-17A mAb for 30 min at 4°C.

In vivo depletion of neutrophils

100 μg of anti-Ly6G mAb (1A8) or the isotype-matched mAb was administered i.p. 1 day before infection.

In vitro mixed cell culture

Cells from the lung of TLR2 KO mice (CD45.2) were mixed in a 1:1 ratio with the lung cells from WT mice (CD45.1). Mixed cells were stimulated with 100 ng/ml of

Pam3CSK4 for 24 h at 37°C. 10 μ g/ml of BFA was added for last 3 h incubation. IL-17A production by $\gamma\delta$ T cells was analyzed by the intracellular staining method described above.

Statistics

Statistical significance was calculated by the Student's *t*-test using Prism software (GraphPad, San Diego, CA). Differences with *p*-values of <0.05 were considered to be statistically significant.

Results

Neutrophils are required for host defense against candidiasis.

To examine in vivo significance of the early infiltration of neutrophils in host defense against systemic infection with C. albicans, we pretreated the mice with neutrophil-specific anti-Ly6G mAb (11), and then infected intravenously with either a virulent or an avirulent strain of C. albicans. Infiltration of neutrophils in the peripheral tissues of mice pretreated with anti-Ly6G mAb was decreased 24hr in systemic infection mouse model of C. albicans (Fig. S1). Neutrophil-depleted mice died within 2 days after infection irrespective of virulence of C. albicans. In contrast, all mice treated with isotype-matched mAb survived beyond 5 days (Fig. 1A). To examine the protective role of early infiltrated neutrophils, fungal clearances in the periphery were analyzed 24hr after infection. We found that fungal clearance of both a virulent and an avirulent strain of C. albicans in the lung but not in the kidney, spleen and liver was significantly impaired 24 hr after infection (Fig. 1B). Histological analysis showed that mycelial form of C. albicans was observed with equal frequency in the kidneys of both neutrophil-depleted and control mice (Fig. 1C), whereas fungal growth of the yeast form of C. albicans was observed in the lungs of neutrophil-depleted mice, but was less frequently detected in control mice (Fig. 1C). These results suggest the importance of neutrophils for host defense in the lung at an early stage after systemic *C. albicans* infection.

A rapid and transient IL-23 production preceded IL-17A production and infiltration of neutrophils in the lung after *C. albicans* infection.

Since IL-17A has been shown to be involved in the mobilization of neutrophils (19), we measured IL-17A production in the lung after infection with C. albicans. Both a virulent and an avirulent strain of C. albicans in the lung was grown after systemic infection, and thereafter both strains cleared completely within a week (Fig. 2A, data not shown). In response to rapid fungal growth in the lung (Fig. 2A), infiltration of neutrophils and IL-17A production were detected as early as 12hr after infection with both a virulent and an avirulent strain and rapidly ceased (Fig. 2B, E). We also examined the kinetics of IL-23 and IL-1 β , which have been shown to promote IL-17A production by Th17, $\gamma\delta$ T, NKT and intestinal innate lymphoid cells (2, 6, 22, 32, 46). Increased productions of IL-23 and IL-1ß preceding IL-17A production and the influx of neutrophils were observed in the lung after infection with both a virulent and an avirulent strain of C. albicans (Fig. 2B-E). These results suggest that IL-17A is involved in neutrophil-mediated host defense in the lung at the early stage after systemic

infection with C. albicans.

IL-17A produced by $\gamma\delta$ T cells plays important roles in neutrophil infiltration and fungal clearance at an early stage of candidiasis.

To directly examine the in vivo significance of early IL-17A production for the infiltration of neutrophils and host defense against C. albicans, IL-17A KO mice were inoculated with C. albicans. Although fungal clearance in the lung was significantly impaired In IL-17AKO mice, reduced but appreciable level of neutrophil infiltration was detected 24hr after infection (Fig. 3A). It was previously shown that IL-17R-mediated signaling is also involved in cytotoxic activation of neutrophils by enhancing their myeloperoxidase (MPO) activity after systemic infection with C. albicans. In agreement with this, MPO activity in the lung of IL-17AKO mice was significantly reduced 24hr after infection (Fig. 3B). In order to identify the cell subsets responsible for early IL-17A production in the lung after C. albicans infection, the lung cells were harvested 24 h after infection, cultured with BFA for 4 h in vitro and examined for intracellular staining for IL-17A. Most of the IL-17A-producing cells were found in $\gamma\delta$ TCR+ cells, but not in CD4+ cells (Fig. 3C). We next examined the involvement of $\gamma\delta$ T cells in IL-17A production and neutrophil infiltration after C.

albicans infection using mice genetically lacking $\gamma\delta$ T cells. IL-17A production was significantly reduced in *C* δ KO mice compared with WT mice at 24 h after intravenous infection with *C. albicans* (Fig. 3D). Neutrophil infiltration and fungal clearance were impaired in *C* δ KO mice at 24 hr after infection (Fig. 3D), suggesting that $\gamma\delta$ TCR+ cells predominantly participate in early IL-17A production, which induce infiltration of neutrophils to clear *C. albicans* after infection.

Tyk2-mediated signaling is critical for IL-23-induced IL-17A production by $\gamma\delta$ T cells.

Tyrosine kinase 2 (Tyk2), a member of the JAK–signal transducer family, is involved in intracellular signaling triggered by IL-23 (28, 41, 44). To determine whether IL-23 was involved in IL-17A production by $\gamma\delta$ T cells after *C. albicans* infection, we inoculated *C. albicans* i.v. into Tyk2 KO mice. IL-17A production in the lung was severely impaired in Tyk2 KO mice, although IL-23 production in Tyk2 KO mice was comparable to that in WT mice (Fig. 4A). These results demonstrated that IL-17A production by $\gamma\delta$ T cells was dependent on the IL-23-Tyk2 pathway after *C. albicans* infection.

The rapid IL-17A production after *C. albicans* infection is TLR2/MyD88 -dependent but CARD9 -independent.

TLR2/MyD88 signaling and Dectin-2/CARD9 signaling are important in the production of proinflammatory cytokines in a murine model of candidiasis and human candidiasis (12, 36, 39, 50, 51). We next examined which signaling is involved in IL-23 production at an early stage after systemic infection with C. albicans. IL-23 production was detected in the lungs of WT mice at 12 h after infection with C. albicans, but such production was completely abolished in MyD88 KO as well as TLR2 KO mice, but not in CARD9 KO mice (Fig. 4B). Consistent with IL-23 production, IL-17A production was detected in the lungs of WT and CARD9 KO mice, but not in MyD88 KO and TLR2 KO mice at 24 h after systemic infection with C. albicans (Fig. 4B). The infiltration of neutrophils and fungal clearance were strikingly diminished in TLR2 KO mice (Fig. 4C). Thus, TLR2/MyD88-mediated signaling is indispensable for early IL-23 and IL-17A production, which were important for neutrophil-mediated fungal clearance in the lung at 24 h after systemic C. albicans infection.

Exogenous IL-23, induced through TLR2, is involved in IL-17A production by $\gamma\delta$ T cells.

A significant fraction of y8 T cells are reported to express TLR2 involved in IL-17A production (23). To further determine whether TLR2 expression on $\gamma\delta$ T cells is required for IL-17A production, we set up the following experiment. Lung cells from TLR2 KO mice were stimulated in vitro with a TLR2 ligand, Pam₃CSK₄, in the presence of lung cells from WT mice, then IL-17A production by TLR2-deficient γδ T cells was analyzed by flow cytometry. The percentage of IL-17A-producing γδ T cells from TLR2 KO mice increased significantly in the presence of TLR2-sufficient lung cells (Fig. 5A, B). Therefore, it was revealed that TLR2-mediated signaling in $\gamma\delta$ T cells was not required for C. albicans-induced IL-17A production. The above results suggest that IL-17A production by $\gamma\delta$ T cells requires IL-23 provided by C. albicans-stimulated lung cells. Therefore, we next examined the ability of exogenous IL-23 to stimulate naive $\gamma\delta$ T cells and found that in vitro culture with IL-23 induced production of IL-17A by resident $\gamma\delta$ T cells (Fig. 5C).

Discussion

Local infiltration of neutrophils is one of the earliest events induced by microbial infection and is critical for host defense against various pathogens including *C. albicans*. In the present study, we found the involvement of $\gamma\delta$ T cells in the infiltration of neutrophils in the lung at an early stage following systemic infection with *C. albicans*, through IL-17A production. IL-23, which was produced in the lungs as early as 12 h after systemic infection with *C. albicans* in a TLR2/MyD88-dependent but not in a CARD9-dependent manner, stimulated preexisting $\gamma\delta$ T cells in the lungs to produce IL-17A. Thus, naturally occurring IL-17A-producing $\gamma\delta$ T cells play important roles in the first line of host defense by controlling neutrophil infiltration at an early stage after systemic infection with *C. albicans*.

We found in this study that neutrophil-depleted mice impaired fungal clearance in the lung 24 h after infection. We also found that CARD9 KO mice could survive beyond 3 days, suggesting that CARD9 was dispensable in neutrophil-mediated host defense at an early stage (data not shown). In contrast, CARD9, which was activated through Dectin-2, was important for host defense by inducing Th17 cell differentiation after systemic *C. albicans* infection (39). Indeed, Dectin-2 KO mice began to die around 12 days after *C. albicans* infection (39). These results indicate a different mechanism to

protect against *C. albicans* at an early stage and late stage after infection. Interestingly, we found that the yeast form of *C. albicans* was observed in the lung one day after systemic infection whereas another group and our present study showed that the mycelial form of *C. albicans* was observed in the kidney (39). Interestingly, it was shown that dendritic cells internalize yeast or mycelial form of *C. albicans* with different PRRs and subsequently induced different helper T cell subsets *in vivo* (10, 38). As such, it was speculated that PRRs involved in the recognition are different from the yeast and mycelial form of *C. albicans* and consequently the mechanism responsible for controlling fungal growth differ at the early stage from those at the late stage after systemic infection with *C. albicans*. However, to prove this hypothesis, further studies to elucidate the interaction between *C. albicans* and immune cells should be performed.

In this study, we demonstrated that TLR2 was indispensable for IL-17A production by $\gamma\delta$ T cells at an early stage after systemic infection with *C. albicans*. Although $\gamma\delta$ T cells expressed TLR2 involved in IL-17A production and proliferation (23, 33), we could not observe such evidence resulting from direct recognition by $\gamma\delta$ T cells. IL-17A production by $\gamma\delta$ T cells was induced in response to IL-23, but TLR2 expression on $\gamma\delta$ T cells was dispensable for the effector function. Consistent with this, IL-17A production was not induced by purified $\gamma\delta$ T cells after stimulation with TLR2 ligands, Pam3CSK4

alone (33, 34). We also found that the number of $\gamma\delta$ T cells in the lung did not increase within 24 h after systemic infection with C. albicans (data not shown), suggesting that TLR2 induced IL-17A production, but not proliferation of $\gamma\delta$ T cells in the lung at this stage. Treg cells express TLR2, which is involved in maintaining suppressive function and survival (45). TLR2-dependent IL-10 production by C. albicans-infected splenocytes was induced after stimulation with heat-killed C. albicans (31). In fact, we found that IL-10 production in the lung was observed at 24 h after systemic infection with C. albicans (data not shown). Although the involvements of Treg cells for host defense in the lung after systemic C. albicans infection were unclear, IL-17A production by $\gamma\delta$ T cells was induced in the presence of IL-10 after infection. Taken together, TLR2, but not other PRRs, is important for protection in the lung at an early stage after systemic C. albicans infection by regulating IL-17A production by γδ T cells. Our data also suggest the involvement of factors other than IL-17A for neutrophil-mediated host defense against C. albicans, because IL-17-independent neutrophil infiltration was observed in the lung 24hr after infection. It was previously shown that neutrophil infiltration after systemic infection with C. albicans was partly induced via Dectin1-mediated signaling which augments productions of IL-6 and the chemokines granulocyte colony-stimulating factors (G-CSF) MCP-1 and MIP-1 α , and

granulocyte-monocyte colony-stimulating factors (GM-CSF) (48). Among these chemoattractants, increased expression of *G-CSF* in the lung was observed independently of IL-17A in intratracheal infection mouse model with *M. bovis* BCG (49). Indeed, G-CSF-deficient mice had reduced number of neutrophils in the blood 7d after systemic infection with *C. albicans* (3). Moreover, candidacidal activity was slightly impaired in G-CSF-deficient neutrophils. These results suggest that G-CSF may act synergistically with IL-17A for infiltrations of neutrophils and fungal clearance in the lung after systemic infection with *C. albicans*.

IL-23 is an inflammatory cytokine that induces IL-17A production not only by $\gamma\delta$ T cells, but also by Th17, NKT and intestinal innate lymphoid cells (2, 6, 22, 32). Consistent with previous findings, IL-17A production by $\gamma\delta$ T cells was induced in response to IL-23 at an early stage after *C. albicans* infection. Intriguingly, other T cell subsets did not produce IL-17A in the lung, indicating that resident IL-17A-producing $\gamma\delta$ T cells are a unique subset able to respond to IL-23 immediately after systemic infection with *C. albicans*. Supporting the rapid response, it was shown that more than 50% of $\gamma\delta$ T cells in mucosal tissues such as PEC, LPL constitutively expressed IL-23R in naive mice (35). Similar to $\gamma\delta$ T cells, Th17 cells express IL-23R which induces IL-17A production and cell proliferation (2, 24). In addition to the protective roles of

IL-23, IL-23 potentially induces autoimmune disorders such as EAE, inflammatory bowel disease (IBD) and rheumatoid arthritis (RA) in which Th17 cells as well as IL-17A-producing $\gamma\delta$ T cells are involved (8, 16, 25). In this regard, since responsiveness to IL-23 should be tightly regulated, it is of interest to elucidate the mechanism to maintain IL-23R expression on IL-17A-producing $\gamma\delta$ T cells involved in host defense against systemic *C. albicans* infection.

In conclusion, we demonstrated the involvement of IL-17A-producing $\gamma\delta$ T cells at an early stage of host defense in the lung against fungal infection. Although Th17 cells are functionally differentiated in the periphery after systemic infection with *C. albicans* (39), IL-17A-producing $\gamma\delta$ T cells, which arise within the thymus, are abundantly present in the peripheral tissues of naive mice independent of pathogens (43). This unique developmental pathway and tissue localization of naturally occurring IL-17A-producing $\gamma\delta$ T cells may be important for protection in a first line of host defense against pathogens including *C. albicans*.

Acknowledgements

We thank Kiyomi Akasaki, Akiko Yano, Mihoko Ookubo and Miki Kijima for secretarial assistance and lab members for their helpful discussions. We thank Hiroji Chibana for providing virulent strain of *C. albicans*, SC5314. This work was supported by a Grant-in-Aid for Scientific Research from the Japan Society for Promotion of Science (K.S. and Y.Y.), by The Mochida Memorial Foundation for Medical and Pharmaceutical Research (K.S.), and by the program of Founding Research Centers for Emerging and Reemerging Infectious Diseases launched as a project commissioned by the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan (Y.Y.).

Disclosures

The authors have no financial conflict of interest.

References

- Adachi, O., T. Kawai, K. Takeda, M. Matsumoto, H. Tsutsui, M. Sakagami, K. Nakanishi, and S. Akira. 1998. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. Immunity 9:143-150.
- Aggarwal, S., N. Ghilardi, M. H. Xie, F. J. de Sauvage, and A. L. Gurney. 2003. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. J Biol Chem 278:1910-1914.
- Basu, S., G. Hodgson, H. H. Zhang, M. Katz, C. Quilici, and A. R. Dunn. 2000. "Emergency" granulopoiesis in G-CSF-deficient mice in response to Candida albicans infection. Blood 95:3725-3733.
- Bellocchio, S., C. Montagnoli, S. Bozza, R. Gaziano, G. Rossi, S. S. Mambula, A. Vecchi, A. Mantovani, S. M. Levitz, and L. Romani. 2004. The contribution of the Toll-like/IL-1 receptor superfamily to innate and adaptive immunity to fungal pathogens in vivo. J Immunol 172:3059-3069.
- Bodey, G. P., M. Mardani, H. A. Hanna, M. Boktour, J. Abbas, E. Girgawy, R. Y. Hachem, D. P. Kontoyiannis, and Raad, II. 2002. The epidemiology of Candida glabrata and Candida albicans fungemia in immunocompromised patients with cancer. Am J Med 112:380-385.
- 6. Buonocore, S., P. P. Ahern, H. H. Uhlig, Ivanov, II, D. R. Littman, K. J. F. Powrie. 2010. Innate lymphoid Maloy, and cells drive interleukin-23-dependent innate intestinal pathology. Nature **464**:1371-1375.
- 7. Conti, H. R., F. Shen, N. Nayyar, E. Stocum, J. N. Sun, M. J. Lindemann, A. W. Ho, J. H. Hai, J. J. Yu, J. W. Jung, S. G. Filler, P. Masso-Welch, M. Edgerton, and S. L. Gaffen. 2009. Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. J Exp Med 206:299-311.
- Cua, D. J., J. Sherlock, Y. Chen, C. A. Murphy, B. Joyce, B. Seymour, L. Lucian, W. To, S. Kwan, T. Churakova, S. Zurawski, M. Wiekowski, S. A. Lira, D. Gorman, R. A. Kastelein, and J. D. Sedgwick. 2003. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. Nature 421:744-748.
- 9. Cua, D. J., and C. M. Tato. 2010. Innate IL-17-producing cells: the

sentinels of the immune system. Nat Rev Immunol 10:479-489.

- d'Ostiani, C. F., G. Del Sero, A. Bacci, C. Montagnoli, A. Spreca, A. Mencacci, P. Ricciardi-Castagnoli, and L. Romani. 2000. Dendritic cells discriminate between yeasts and hyphae of the fungus Candida albicans. Implications for initiation of T helper cell immunity in vitro and in vivo. J Exp Med 191:1661-1674.
- Daley, J. M., A. A. Thomay, M. D. Connolly, J. S. Reichner, and J. E. Albina. 2008. Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. J Leukoc Biol 83:64-70.
- Glocker, E. O., A. Hennigs, M. Nabavi, A. A. Schaffer, C. Woellner, U. Salzer, D. Pfeifer, H. Veelken, K. Warnatz, F. Tahami, S. Jamal, A. Manguiat, N. Rezaei, A. A. Amirzargar, A. Plebani, N. Hannesschlager, O. Gross, J. Ruland, and B. Grimbacher. 2009. A homozygous CARD9 mutation in a family with susceptibility to fungal infections. N Engl J Med 361:1727-1735.
- Hamada, S., M. Umemura, T. Shiono, K. Tanaka, A. Yahagi, M. D. Begum, K. Oshiro, Y. Okamoto, H. Watanabe, K. Kawakami, C. Roark, W. K. Born, R. O'Brien, K. Ikuta, H. Ishikawa, S. Nakae, Y. Iwakura, T. Ohta, and G. Matsuzaki. 2008. IL-17A produced by gammadelta T cells plays a critical role in innate immunity against listeria monocytogenes infection in the liver. J Immunol 181:3456-3463.
- Hara, H., C. Ishihara, A. Takeuchi, T. Imanishi, L. Xue, S. W. Morris, M. Inui, T. Takai, A. Shibuya, S. Saijo, Y. Iwakura, N. Ohno, H. Koseki, H. Yoshida, J. M. Penninger, and T. Saito. 2007. The adaptor protein CARD9 is essential for the activation of myeloid cells through ITAM-associated and Toll-like receptors. Nat Immunol 8:619-629.
- Huang, W., L. Na, P. L. Fidel, and P. Schwarzenberger. 2004. Requirement of interleukin-17A for systemic anti-Candida albicans host defense in mice. J Infect Dis 190:624-631.
- Hue, S., P. Ahern, S. Buonocore, M. C. Kullberg, D. J. Cua, B. S. McKenzie, F. Powrie, and K. J. Maloy. 2006. Interleukin-23 drives innate and T cell-mediated intestinal inflammation. J Exp Med 203:2473-2483.
- 17. Itohara, S., P. Mombaerts, J. Lafaille, J. Iacomini, A. Nelson, A. R. Clarke, M. L. Hooper, A. Farr, and S. Tonegawa. 1993. T cell receptor delta gene mutant mice: independent generation of alpha beta T cells

and programmed rearrangements of gamma delta TCR genes. Cell **72**:337-348.

- 18. Janeway, C. A., Jr., and R. Medzhitov. 2002. Innate immune recognition. Annu Rev Immunol 20:197-216.
- 19. Kolls, J. K., and A. Linden. 2004. Interleukin-17 family members and inflammation. Immunity **21**:467-476.
- 20. Lee, W. L., R. E. Harrison, and S. Grinstein. 2003. Phagocytosis by neutrophils. Microbes Infect 5:1299-1306.
- 21. Lochner, M., L. Peduto, M. Cherrier, S. Sawa, F. Langa, R. Varona, D. Riethmacher, M. Si-Tahar, J. P. Di Santo, and G. Eberl. 2008. In vivo equilibrium of proinflammatory IL-17+ and regulatory IL-10+ Foxp3+ RORgamma t+ T cells. J Exp Med 205:1381-1393.
- 22. Lockhart, E., A. M. Green, and J. L. Flynn. 2006. IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection. J Immunol 177:4662-4669.
- 23. Martin, B., K. Hirota, D. J. Cua, B. Stockinger, and M. Veldhoen. 2009. Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals. Immunity 31:321-330.
- McGeachy, M. J., Y. Chen, C. M. Tato, A. Laurence, B. Joyce-Shaikh, W. M. Blumenschein, T. K. McClanahan, J. J. O'Shea, and D. J. Cua. 2009. The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells in vivo. Nat Immunol 10:314-324.
- 25. Melis, L., B. Vandooren, E. Kruithof, P. Jacques, M. De Vos, H. Mielants, G. Verbruggen, F. De Keyser, and D. Elewaut. 2010. Systemic levels of IL-23 are strongly associated with disease activity in rheumatoid arthritis but not spondyloarthritis. Ann Rheum Dis 69:618-623.
- 26. Mokuno, Y., T. Matsuguchi, M. Takano, H. Nishimura, J. Washizu, T. Ogawa, O. Takeuchi, S. Akira, Y. Nimura, and Y. Yoshikai. 2000. Expression of toll-like receptor 2 on gamma delta T cells bearing invariant V gamma 6/V delta 1 induced by Escherichia coli infection in mice. J Immunol 165:931-940.
- 27. Nakae, S., Y. Komiyama, A. Nambu, K. Sudo, M. Iwase, I. Homma, K. Sekikawa, M. Asano, and Y. Iwakura. 2002. Antigen-specific T cell

sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses. Immunity **17**:375-387.

- 28. Nakamura, R., K. Shibata, H. Yamada, K. Shimoda, K. Nakayama, and Y. Yoshikai. 2008. Tyk2-signaling plays an important role in host defense against Escherichia coli through IL-23-induced IL-17 production by gammadelta T cells. J Immunol 181:2071-2075.
- Netea, M. G., and L. Marodi. 2010. Innate immune mechanisms for recognition and uptake of Candida species. Trends Immunol 31:346-353.
- 30. Netea, M. G., R. J. Stuyt, S. H. Kim, J. W. Van der Meer, B. J. Kullberg, and C. A. Dinarello. 2002. The role of endogenous interleukin (IL)-18, IL-12, IL-1beta, and tumor necrosis factor-alpha in the production of interferon-gamma induced by Candida albicans in human whole-blood cultures. J Infect Dis 185:963-970.
- 31. Netea, M. G., R. Sutmuller, C. Hermann, C. A. Van der Graaf, J. W. Van der Meer, J. H. van Krieken, T. Hartung, G. Adema, and B. J. Kullberg. 2004. Toll-like receptor 2 suppresses immunity against Candida albicans through induction of IL-10 and regulatory T cells. J Immunol 172:3712-3718.
- 32. Rachitskaya, A. V., A. M. Hansen, R. Horai, Z. Li, R. Villasmil, D. Luger, R. B. Nussenblatt, and R. R. Caspi. 2008. Cutting edge: NKT cells constitutively express IL-23 receptor and RORgammat and rapidly produce IL-17 upon receptor ligation in an IL-6-independent fashion. J Immunol 180:5167-5171.
- 33. Reynolds, J. M., B. P. Pappu, J. Peng, G. J. Martinez, Y. Zhang, Y. Chung, L. Ma, X. O. Yang, R. I. Nurieva, Q. Tian, and C. Dong. 2010. Toll-like receptor 2 signaling in CD4(+) T lymphocytes promotes T helper 17 responses and regulates the pathogenesis of autoimmune disease. Immunity 32:692-702.
- 34. Ribot, J. C., M. Chaves-Ferreira, F. d'Orey, M. Wencker, N. Goncalves-Sousa, J. Decalf, J. P. Simas, A. C. Hayday, and B. Silva-Santos. 2010. Cutting edge: adaptive versus innate receptor signals selectively control the pool sizes of murine IFN-gamma- or IL-17-producing gammadelta T cells upon infection. J Immunol 185:6421-6425.
- 35. Riol-Blanco, L., V. Lazarevic, A. Awasthi, M. Mitsdoerffer, B. S. Wilson,

A. Croxford, A. Waisman, V. K. Kuchroo, L. H. Glimcher, and M. Oukka. 2010. IL-23 receptor regulates unconventional IL-17-producing T cells that control bacterial infections. J Immunol 184:1710-1720.

- 36. Robinson, M. J., F. Osorio, M. Rosas, R. P. Freitas, E. Schweighoffer, O. Gross, J. S. Verbeek, J. Ruland, V. Tybulewicz, G. D. Brown, L. F. Moita, P. R. Taylor, and C. Reis e Sousa. 2009. Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection. J Exp Med 206:2037-2051.
- 37. Romani, L., A. Mencacci, E. Cenci, G. Del Sero, F. Bistoni, and P. Puccetti. 1997. An immunoregulatory role for neutrophils in CD4+ T helper subset selection in mice with candidiasis. J Immunol 158:2356-2362.
- 38. Romani, L., C. Montagnoli, S. Bozza, K. Perruccio, A. Spreca, P. Allavena, S. Verbeek, R. A. Calderone, F. Bistoni, and P. Puccetti. 2004. The exploitation of distinct recognition receptors in dendritic cells determines the full range of host immune relationships with Candida albicans. Int Immunol 16:149-161.
- 39. Saijo, S., S. Ikeda, K. Yamabe, S. Kakuta, H. Ishigame, A. Akitsu, N. Fujikado, T. Kusaka, S. Kubo, S. H. Chung, R. Komatsu, N. Miura, Y. Adachi, N. Ohno, K. Shibuya, N. Yamamoto, K. Kawakami, S. Yamasaki, T. Saito, S. Akira, and Y. Iwakura. 2010. Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against Candida albicans. Immunity 32:681-691.
- Sato, K., X. L. Yang, T. Yudate, J. S. Chung, J. Wu, K. Luby-Phelps, R.
 P. Kimberly, D. Underhill, P. D. Cruz, Jr., and K. Ariizumi. 2006. Dectin-2 is a pattern recognition receptor for fungi that couples with the Fc receptor gamma chain to induce innate immune responses. J Biol Chem 281:38854-38866.
- 41. Shaw, M. H., V. Boyartchuk, S. Wong, M. Karaghiosoff, J. Ragimbeau, S. Pellegrini, M. Muller, W. F. Dietrich, and G. S. Yap. 2003. A natural mutation in the Tyk2 pseudokinase domain underlies altered susceptibility of B10.Q/J mice to infection and autoimmunity. Proc Natl Acad Sci U S A 100:11594-11599.
- 42. Shibata, K., H. Yamada, H. Hara, K. Kishihara, and Y. Yoshikai. 2007.

Resident Vdelta1+ gammadelta T cells control early infiltration of neutrophils after Escherichia coli infection via IL-17 production. J Immunol **178**:4466-4472.

- Shibata, K., H. Yamada, R. Nakamura, X. Sun, M. Itsumi, and Y. Yoshikai. 2008. Identification of CD25+ gamma delta T cells as fetal thymus-derived naturally occurring IL-17 producers. J Immunol 181:5940-5947.
- 44. Shimoda, K., K. Kato, K. Aoki, T. Matsuda, A. Miyamoto, M. Shibamori, M. Yamashita, A. Numata, K. Takase, S. Kobayashi, S. Shibata, Y. Asano, H. Gondo, K. Sekiguchi, K. Nakayama, T. Nakayama, T. Okamura, S. Okamura, and Y. Niho. 2000. Tyk2 plays a restricted role in IFN alpha signaling, although it is required for IL-12-mediated T cell function. Immunity 13:561-571.
- Sutmuller, R. P., M. H. den Brok, M. Kramer, E. J. Bennink, L. W. Toonen, B. J. Kullberg, L. A. Joosten, S. Akira, M. G. Netea, and G. J. Adema. 2006. Toll-like receptor 2 controls expansion and function of regulatory T cells. J Clin Invest 116:485-494.
- 46. Sutton, C. E., S. J. Lalor, C. M. Sweeney, C. F. Brereton, E. C. Lavelle, and K. H. Mills. 2009. Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. Immunity 31:331-341.
- 47. Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda, and S. Akira. 1999. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity 11:443-451.
- Taylor, P. R., S. V. Tsoni, J. A. Willment, K. M. Dennehy, M. Rosas, H. Findon, K. Haynes, C. Steele, M. Botto, S. Gordon, and G. D. Brown. 2007. Dectin-1 is required for beta-glucan recognition and control of fungal infection. Nat Immunol 8:31-38.
- Umemura, M., A. Yahagi, S. Hamada, M. D. Begum, H. Watanabe, K. Kawakami, T. Suda, K. Sudo, S. Nakae, Y. Iwakura, and G. Matsuzaki.
 2007. IL-17-mediated regulation of innate and acquired immune response against pulmonary Mycobacterium bovis bacille Calmette-Guerin infection. J Immunol 178:3786-3796.
- 50. Villamon, E., D. Gozalbo, P. Roig, J. E. O'Connor, D. Fradelizi, and M.
 L. Gil. 2004. Toll-like receptor-2 is essential in murine defenses

against Candida albicans infections. Microbes Infect 6:1-7.

51. Woehrle, T., W. Du, A. Goetz, H. Y. Hsu, T. O. Joos, M. Weiss, U. Bauer, U. B. Brueckner, and E. Marion Schneider. 2008. Pathogen specific cytokine release reveals an effect of TLR2 Arg753Gln during Candida sepsis in humans. Cytokine 41:322-329.

Figure legends

Figure 1. Susceptibility of mice depleted of neutrophils against systemic *C.albicans* infection.

Mice were pretreated with 100µg of anti-Ly6G mAb (1A8) or isotype-matched mAb (isotype-matched) 1 day before i.v. infection with 1×10^7 CFU of No.10261 or 2×10^5 CFU of SC5314. (A) Survival rates of mice (n=10 in each group) receiving anti-Ly6G mAb or isotype-matched control mAb were monitored after i.v. infection with *C. albicans*. P-value was analyzed by Log-rank test. (B) Fungal clearance in the lung, kidney, spleen and liver was analyzed at 24 h after infection. Data are shown as the mean \pm SD of five mice for each group. **p*<0.05, ***p*<0.01, ****p*<0.001. (C) The lung and kidney, which was taken from mice 24 h after infection with 1×10^7 CFU of No.10261, were stained with Periodic acid-Sciff (PAS) (original magnification, \times 400). Data are representative of three independent experiments.

Figure 2. Kinetics of fungal clearance, neutrophil infiltrations and cytokine productions in the lung after systemic infection with *C. albicans*.

After mice were injected intravenously with 1×10^7 CFU of No.10261 or 2×10^5 CFU of SC5314 respectively, (A) fungal clearance, (B) neutrophil infiltration, (C, D, E)

increased IL-23, IL-1 β and IL-17A productions in the lung were analyzed. Data are shown as the mean \pm SD of five mice at each time point.

Data are representative of three independent experiments.

Figure 3. Protective role of IL-17A-producing $\gamma\delta$ T cells after systemic infection with *C*. *albicans*.

(A) IL-17A KO mice impaired neutrophil infiltration and fungal clearance in the lung after infection.

WT or IL-17A KO mice were i.v. infected with 1×10^7 CFU of *C. albicans*.

After 24 h, neutrophil infiltration (left) and fungal clearance (right) in the lung of infected mice were analyzed. Data indicate the mean \pm SD of five mice. **p*<0.05, ***p*<0.01.

(B) MPO activity in the lung of WT or IL-17A KO mice after infection.

MPO activities of lung homogenates taken from WT and IL-17A KO mice 24 hr after infection were shown. Data indicates the mean \pm SD of five mice. **p*<0.05.

(C) Identification of IL-17A-producing cells in the lung after infection.

After i.v. infection with 1×10^7 CFU of *C. albicans*, lung cells were cultured in the presence of BFA for 4h. After incubation, IL-17A+ cells were analyzed by intracellular

staining. Data were shown after gating on MHC class II-negative CD3-positive cells. The number in the upper right quadrant indicates the percentage of IL-17A+ cells in $\gamma\delta$ TCR+ cells (left) or CD4+ cells (right).

(D) Cδ KO mice significantly reduced IL-17A production and impaired neutrophil infiltration and fungal clearance after infection.

WT or C δ KO mice were i.v. infected with 1×10^7 CFU of *C.albicans*.

After 24 h, IL-17A production (left), neutrophil infiltration (middle) and fungal clearance (right) in the lung of infected mice were analyzed. Data are shown as the mean \pm SD of three to five mice for each group. *p<0.05, **p<0.01.

Data are representative of three independent experiments.

Figure 4. IL-17A production by $\gamma\delta$ T cells is induced by IL-23 in a TLR2/MyD88-dependent, but CARD9-dependent manner.

(A) Tyk2 signaling was indispensable for IL-17A production by $\gamma\delta$ T cells.

After i.v challenge with 1×10^7 CFU of *C. albicans* to WT and Tyk2 KO mice, IL-23 and IL-17A productions in the lung were analyzed by ELISA. Data indicate the mean \pm SD of three mice. ***p*<0.01. N.S. indicates statistically not significant between groups.

(B) IL-23 production was dependent on a TLR2/MyD88-mediated, but not on a

CARD9-mediated pathway.

After i.v. challenge with 1×10^7 CFU of *C.albicans* to WT, MyD88 KO, TLR2 KO and CARD9 KO mice, IL-23 and IL-17A productions in the lung were analyzed by ELISA. Data indicate the mean ±SD of three to five mice. **p<0.01. N.S. indicates statistically not significant between groups.

(C) TLR2 signaling was important for neutrophil-mediated host defense in the lung after systemic infection with *C. albicans*.

WT or TLR2 KO mice were i.v. infected with 1×10^7 CFU of *C. albicans*. After 24 h, neutrophil infiltration (left) and fungal clearance (right) in the lung of infected mice were analyzed. Data are shown as the mean \pm SD of three mice for each group. **p*<0.05. Data are representative of three independent experiments.

Figure 5. Exogenous IL-23 induces IL-17A production by $\gamma\delta$ T cells.

(A, B) TLR2 expression on $\gamma\delta$ T cells was not required for IL-17A production.

TLR2-deficient lung cells, mixed with or without TLR2-suffcient lung cells at a 1:1 ratio, were cultured in the presence of 100 ng/ml of Pam₃CSK₄ for 24h. BFA was added for the last 3 h of incubation. (A) After incubation, IL-17A+ cells were analyzed intracellularly. Data were shown after gating on $\gamma\delta$ TCR+ cells. Numbers in the

quadrants indicate IL-17A+ cells within TLR2-deficient (CD45.2+) $\gamma\delta$ T cells. (B) % of IL-17A-producing cells in TLR2-deficient (CD45.2+) $\gamma\delta$ T cells were shown. Data indicates the mean ± SD of six individual wells. *p<0.05.

(C) Exogenous IL-23-induced IL-17A production by resident $\gamma\delta$ T cells in the lung.

Lung cells of naive mice were stimulated with PBS (left) or 10 ng/ml of IL-23 (right) for 6 hr. BFA was added for the last 3 h of incubation. After stimulation, cells were analyzed for intracellular IL-17A. Data were shown after gating on MHC class II-negative CD3-positive cells. The number in the right quadrant indicates the percentage of IL-17+ cells in $\gamma\delta$ TCR+ cells.

Data are representative of three independent experiments.