



Title	Differences in interleukin-1 β release-inducing activity of <i>Candida albicans</i> toward dendritic cells and macrophages.
Author(s)	Hasebe, A; Saeki, A; Yoshida, Y et al.
Citation	Archives of oral biology, 93, 115-125 https://doi.org/10.1016/j.archoralbio.2018.06.004
Issue Date	2018-09
Doc URL	https://hdl.handle.net/2115/75324
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Type	journal article
File Information	A0B Revise2.pdf



Differences in interleukin-1 β release-inducing activity of *Candida albicans* toward dendritic cells and macrophages

Akira Hasebe ^a, Ayumi Saeki ^a, Yasuhiro Yoshida ^b and Ken-ichiro Shibata ^{a,*}

Departments of Oral Molecular Microbiology ^a and Biomaterials and Bioengineering ^b,
Faculty of Dental Medicine and Graduate School of Dental Medicine, Hokkaido
University, Kita 13, Nishi 7, Kita-ku, Sapporo 060-8586, Japan.

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* Corresponding author: Ken-ichiro Shibata, Department of Oral Molecular
Microbiology, Faculty of Dental Medicine and Graduate School of Dental Medicine,
Hokkaido University, Kita 13, Nishi 7, Kita-ku, Sapporo 060-8586, Japan.

Tel.: +81 11 706 4240, Fax: +81 11 706 4901, E-mail: shibaken@den.hokudai.ac.jp

Abstract

Objective: The purpose of this study is to elucidate differences in the mechanism of the IL-1 β release-inducing activity of *Candida albicans* toward dendritic cells and macrophages because IL-1 β is one of the proinflammatory cytokines which is crucial in host defense against candidiasis.

Design: Two *C. albicans* strains were used in this study. One strain is uridine-auxotrophic (CAI4) that needs uridine to grow and form hyphae, and another is a strain without any specific auxotrophy (pACT1-GFP), which forms hyphae naturally by culturing with serum components. Murine macrophage and dendritic cell lines were primed with LPS and then stimulated with *C. albicans* CAI4 or pACT1-GFP.

Results: Both strains of *C. albicans* induced IL-1 β release from dendritic cells, and *C. albicans* pACT1-GFP induced IL-1 β release but CAI4 induced little amounts in macrophages. These differences were suggested to be due to the difference in the amount of extracellular ATP released in the cell culture supernatants induced by *C. albicans* CAI4 or pACT1-GFP. For induction of IL-1 β release from both macrophages and dendritic cells by *C. albicans*, direct contacts of the microbes with cells were required. In addition, macrophages required morphological change of *C. albicans* from yeast to hyphae for induction of IL-1 β release, whereas dendritic cells did not require it.

Dead *C. albicans* could induce IL-1 β release from dendritic cells, but could not from macrophages.

Conclusions: There are different mechanisms by which *C. albicans* induces IL-1 β release from dendritic cells and macrophages.

Keywords:

interleukin-1 β ; *Candida albicans*; dendritic cells; macrophages

Highlights

- ▶ IL-1 β was induced in DCs and macrophages with *C. albicans* direct contact.
- ▶ The induction in DCs was irrespective of the morphology of *C. albicans*.
- ▶ The induction in macrophages needed direct contact with live *C. albicans*.
- ▶ The induction in macrophages needed form change of *C. albicans* from yeast to hyphae.

1. Introduction

Candidiasis, one of the opportunistic infections caused by *Candida albicans*, is a serious problem for immunocompromised patients because it can be systemic and fatal (Kullberg & Arendrup, 2015). *C. albicans* is a commensal resident of the human oral cavity in approximately 50% of individuals (Kullberg & Arendrup, 2015; Reichart, Samaranayake, & Philipsen, 2000), and one of its representative characteristics is the ability to perform a dimorphic switch from the spherical yeast growth form to filamentous hyphal cells in response to various environmental cues (Han, Cannon, & Villas-Bôas, 2011). This dimorphic switch ability is associated with its pathogenicity (Calderone & Fonzi, 2001), and the continuous morphological changes result in the expression of many fungal pathogen-associated molecular patterns (PAMPs). Various pattern recognition receptors (PRRs) on host cells recognize these PAMPs and trigger signaling pathways and cellular responses, including the production of cytokines (Becker, Ifrim, Quintin, Netea, & van de Veerdonk, 2015).

Interleukin (IL)-1 β is an important cytokine that induces the differentiation of T helper (Th) 17 cells, which are essential for effective host defense against *C. albicans* by producing IL-17 (Feller, Khammissa, Chandran, Altini, & Lemmer, 2014). IL-1 β is synthesized as a precursor form (pro-IL-1 β) in the cytoplasm by various stimuli via

PRRs. Mature IL-1 β is produced by cleavage of pro-IL-1 β by caspase-1, and caspase-1 activation is induced by the activation of inflammasomes. Inflammasomes are multiprotein complexes comprising a nucleotide-binding domain-like receptor (NLR), the adaptor protein apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC), and procaspase-1. NLRP3 is a representative cytosolic NLR, and the NLRP3 inflammasome is activated when stimulated by bacteria, viruses, particulates, (Koizumi et al., 2012; Lupfer & Kanneganti, 2013) and fungi such as *C. albicans* (Hise et al., 2009).

Many types of cells, including macrophages and dendritic cells (DCs), produce IL-1 β , and both cell types are crucial for host defense against *C. albicans*. During the early stages of candidiasis, the role of macrophages varies over the course of infection and appears to be the most important (Ngo et al., 2014). DCs are also indispensable in the antifungal response, because they are responsible for sensing the fungal PAMPs via their PRRs, secreting cytokines and chemokines into the environment, capturing fungal particles by phagocytosis, and presenting antigens to T cells to induce an adaptive immune response (Ramirez-Ortiz & Means, 2012). In the present study, we attempted to elucidate difference in the mechanism of the IL-1 β release-inducing activity of *C. albicans* toward DCs and macrophages.

2. Materials and Methods

2.1 *C. albicans* culture

Both *C. albicans* pACT1-GFP and CAI4 were kindly provided by Professor Alistair J. P. Brown (University of Aberdeen, UK). *C. albicans* pACT1-GFP has no specific auxotrophy, and *C. albicans* CAI4, the parental strain of *C. albicans* pACT1-GFP, is a uridine auxotroph. *C. albicans* CAI4 is derived from *C. albicans* SC5314, which was isolated from human. Dextrose minimal broth (SD broth), based upon yeast nitrogen base without amino acids (0.67% w/v) (Difco/Becton Dickinson, Sparks, MD, USA) supplemented with dextrose (2%, w/v) (Wako, Tokyo, Japan) was prepared to grow *C. albicans* pACT1-GFP (Sherman, 2002). *C. albicans* CAI4 was grown in SD broth supplemented with 25 mg/ml of uridine (MP Biomedicals, Irvine, CA, USA) because of its auxotrophy (Cormack et al., 1997). The organisms were cultured under aerobic conditions at 37 °C, harvested, and washed three times with sterilized phosphate-buffered saline (PBS). The forms of *Candida* cells harvested from the broth were confirmed as yeast under an optical or a phase-contrast microscope. *C. albicans* was stained with trypan blue and the density was adjusted to 1.5×10^8 cells/ml. Aliquots were made and stored at -80 °C.

2.2 *Antibodies and reagents*

Goat anti mouse IL-1 β antibodies (Abs) were purchased from R&D Systems (Minneapolis, MN, USA), and horseradish peroxidase-conjugated anti-goat IgG was purchased from Jackson Immuno Research (West Grove, PA, USA). Fc blocker (anti-mouse CD16/32) was purchased from BioLegend (San Diego, CA, USA).

Phycoerythrin (PE)-conjugated anti-TLR4 mAb (clone UT41) and its isotype control mAb (clone P3.6.2.8.1) were purchased from eBioscience (San Diego, CA, USA). Anti- β actin (clone AC-15) was purchased from Sigma-Aldrich (St Louis, MO, USA).

Anti-caspase-1 p20 (clone Casper-1) and anti-NLRP3 (clone Cryo-2) were purchased from Adipogen (San Diego, CA, USA). Polyclonal rabbit anti-ASC Abs were purchased from Novus Biochemicals (Littleton, CO, USA).

A caspase-1 inhibitor, z-YVAD-fmk, was purchased from Abcam (Cambridge, UK).

Escherichia coli lipopolysaccharide (LPS) from O111:B4 was purchased from Sigma-Aldrich. The chemiluminescent substrate was purchased from Millipore (Bedford, MA, USA).

All other reagents were purchased from commercial sources and were of analytical or reagent grade.

2.3 *Cell culture*

XS106, a murine DC line, was kindly provided by Professor Akira Takashima

(University of Texas Southwestern, Dallas, TX, USA) (Mohan, Hopkins, & Mabbott, 2005). J774.1 (JCRB0018), a murine macrophage cell line, was obtained from the Japanese Collection of Research Bioresources (Osaka, Japan). XS106 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich) containing 10% (v/v) fetal bovine serum (FBS) (Invitrogen/Thermo Fisher Scientific, Carlsbad, CA, USA), 2 mM L-glutamine, 1 mM sodium pyruvate (Sigma-Aldrich), 10 mM HEPES buffer (Sigma-Aldrich), 100 U/ml penicillin G, 100 µg/ml streptomycin (Sigma-Aldrich), 50 µM 2-mercaptoethanol (Sigma-Aldrich), 1% (v/v) non-essential amino acids (Sigma-Aldrich), 0.5 ng/ml murine recombinant granulocyte macrophage colony-stimulating factor (Pepro Tech, Rocky Hill, NJ, USA), and 5% (v/v) culture supernatant derived from NS47 fibroblast cells (Mohan et al., 2005). J774.1 cells were cultured in RPMI 1640 medium (Sigma-Aldrich) containing 10% (v/v) FBS (Invitrogen/Thermo Fisher Scientific), 100 U/ml penicillin G, and 100 µg/ml streptomycin (Sigma-Aldrich). Cells were grown at 37 °C in a humidified atmosphere of 5% CO₂. Twenty four-well plates and 0.4-µm pore size Transwell® inserts were purchased from Corning (Lowell, MA, USA).

2.4 *Measurement of IL-1 β*

XS106 or J774.1 cells were added to a 24-well plate at 6×10^5 cells per well in

600 µl of their appropriate media and incubated at 37 °C for 2 h in a 5% CO₂ atmosphere. The cultures were centrifuged at 400 × g for 5 min, and the cells were washed with RPMI 1640 medium containing 1% (v/v) FBS. After washing, the cells were resuspended in 300 µl of the same medium containing 100 ng/ml of LPS for priming. After incubation for 4 h, the cells were stimulated with *C. albicans* at 37 °C for 16 h in a 5% CO₂ atmosphere. Concentrations of chemicals used and an effector-to-target (E/T) ratio (ratio of *Candida* number to cell number) are indicated in the figures. The E/T ratio was 1 unless otherwise stated. The amounts of IL-1β in the cell culture supernatants were measured using an OptEIA™ Set Mouse IL-1β enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences, San Jose, CA, USA). The ELISAs were repeated at least twice and the results are expressed as the mean ± SD of duplicate or triplicate assays of a representative experiment. Mature IL-1β in the cell culture supernatant was detected using western blotting using the antibodies and the chemiluminescent substrate described above.

2.5 Cell death assay

Cells were stimulated as described and the culture supernatants were harvested every 3 h up to 18 h. The lactate dehydrogenase (LDH) content in the supernatants was measured using a CytoTox 96 kit (Promega, Madison, WI, USA) according to the

manufacturer's instructions. Cytotoxicity was calculated against the maximum release of LDH, which was obtained by cell lysis with 0.09% (v/v) Triton X-100. The percentage of cytotoxicity was calculated as LDH released in [tested sample (A490)/maximum LDH release (A490)] $\times$ 100.

2.6 Determination of TLR4 expression levels

XS106 or J774.1 cells were collected and adjusted to 1×10^6 cells/ml in PBS containing 1% (w/v) bovine serum albumin (BSA, PBS/1% BSA). After incubating for 20 min, they were incubated with Fc blocker (anti mouse CD16/32, BioLegend) for 20 min and then incubated anti TLR4 mAbs, or their appropriate isotype control mAbs. The cells were washed with PBS/1% BSA after each step. Data for 30,000 cells falling within an appropriate forward light scatter and side light scatter were analyzed using a FACS Calibur instrument (BD Biosciences). Data were analyzed using the FlowJo software (Tree Star, Ashland, OR, USA). Relative mean fluorescence intensities (MFIs) were calculated as [(MFI of cells incubated with anti TLR4 antibody)/(MFI of cells incubated with isotype control antibody)].

2.7 Quantitative real-time RT-PCR

XS106 or J774.1 cells were primed with LPS and stimulated with live *C. albicans* pACT1-GFP or CAI4, as described. Their total RNAs were then extracted using a

ReliaPrep™ RNA Miniprep Systems (Promega) and cDNAs were synthesized using the ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo Co., Ltd, Osaka, Japan). For real-time PCR analysis, PCR amplification was performed in the presence of a TaqMan probe using a StepOne Real-Time PCR system (Applied Biosystems, Carlsbad, CA). Specific primers for mouse IL-1 β and GAPDH were purchased from Applied Biosystems. The cycling threshold (C_T) value was defined as the number of PCR cycles in which the fluorescence signal exceeds the detection threshold value. The normalized amount of a target mRNA (N_t) was calculated from the C_T values obtained for both the target and *Gapdh* mRNA using the equation $N_t = 2^{C_t(\text{GAPDH}) - C_t(\text{target})}$. Relative mRNA expression was obtained by setting the N_t in non-stimulated samples as 1 in each experiment. The results are expressed as the mean $\pm$ SD of triplicate assays of a representative experiment.

2.8 *Measurement of extracellular ATP*

XS106 and J774.1 cells were prepared and stimulated as described. The extracellular ATP in the cell culture supernatant was quantified using an ATP Bioluminescence Assay Kit CLS II (Roche) according to the manufacturer's instructions. Luminescence was measured using a Wallac 1420 ARVOsx (PerkinElmer, Inc., Waltham, MA, USA) and the ATP concentration was determined using a standard

curve.

2.9 Induction of morphological changes of C. albicans

To induce the morphological change to the hyphal form, *C. albicans* pACT1-GFP was incubated in PBS containing 20% FBS and *C. albicans* CAI4 was incubated in PBS containing 20% FBS supplemented with uridine (25 mg/ml) at 37 °C for 16 h. Dead *C. albicans* was prepared by amphotericin B treatment at 37 °C for 16 h, and then the organisms were washed three times with sterilized PBS.

2.10 Statistical analysis

For multiple observations, Student's t-test was used for the statistical analysis. Statistical significance was set at a P-value of less than 0.05. All experiments were repeated two or more times and similar results were obtained. Representative data are shown.

3. Results

3.1 *IL-1 β release from XS106 and J774.1 cells in response to C. albicans*

C. albicans pACT1-GFP induced IL-1 β release from both XS106 and J774.1 cells in an E/T ratio-dependent manner (Fig. 1A). *C. albicans* CAI4 also induced IL-1 β release from XS106 cells in an E/T ratio-dependent manner, but induced IL-1 β at a lower level in J774.1 cells (Fig. 1A). To confirm whether the IL-1 β detected was the mature form, the culture supernatant was subjected to western blotting analysis. The result showed that mature forms of IL-1 β (17.5 kDa) were released in the supernatants stimulated with *C. albicans* CAI4 or pACT1-GFP (Fig. 1B). These data implied that there are differences in abilities of these two strains of *C. albicans* to induce IL-1 β release from XS106 and J774.1 cells.

3.2 *Time course of IL-1 β release and cell death in response to C. albicans*

Pyroptosis, a caspase-1-dependent cell death mechanism, occurs during IL-1 β release via inflammasome activation (Bergsbaken, Fink, & Cookson, 2009). Therefore, a time course of IL-1 β release and the percentage of dead cells were examined. In XS106 cells, both the amount of IL-1 β and the percentage of dead cells increased in a time-dependent manner in response to *C. albicans* CAI4 or pACT1-GFP (Fig. 2A and B). In J774.1 cells, *C. albicans* pACT1-GFP induced IL-1 β release and cell death in a

time-dependent manner, whereas *C. albicans* CAI4 induced both smaller amounts of IL-1 β and a lower percentage of dead cells compared with those induced by *C. albicans* pACT1-GFP (Fig. 2C and D). These findings suggested that pattern of IL-1 β release in the culture supernatants is related to cell death.

3.3 *Involvement of caspase-1 in the induction of IL-1 β*

Mature IL-1 β secretion is induced after pro-IL-1 β processing by caspase-1, which is activated by inflammasomes (Lamkanfi & Dixit, 2014). Therefore, the effect of the caspase-1 inhibitor, z-YVAD-fmk, on IL-1 β induction was examined. As expected, z-YVAD-fmk attenuated the IL-1 β -inducing activities of *C. albicans* CAI4 or pACT1-GFP in a dose-dependent manner (Fig. 3). This suggested that caspase-1 is involved in the IL-1 β release induced by *C. albicans* CAI4 or pACT1-GFP.

3.4 *Cell surface expression level of TLR4, and mRNA expression level of *Il1B**

Cell surface expression level of TLR4 was determined, because TLR4 is indispensable for recognition of *E. coli* LPS, (Akira et al. 2006) which was used for priming. It was found that both XS106 and J774.1 cells expressed TLR4 on their cell surfaces without stimulation, although the expression level in J774.1 cells was higher than that in XS016 cells (Fig. 4A). Thus, the expression levels of *Il1B* mRNA in these cells were examined when they were primed with LPS and/or stimulated with *C.*

albicans. There were no significant differences of *II1B* mRNA expression levels in both XS106 and J774.1 cells primed with LPS alone or subsequently stimulated with *C. albicans* (Fig. 4B). These results suggested that the induction of *II1B* mRNA transcription is dependent on LPS but not on *C. albicans* in both XS106 and J774.1 cells.

3.5 *Levels of inflammasome-related molecules*

The levels of molecules related to inflammasome activation were determined using cell lysates of XS106 or J774.1 cells. The NLRP3 level increased after priming with LPS and/or stimulation with *C. albicans* CAI4 or pACT1-GFP, whereas there were no significant differences in the levels of ASC and pro-caspase-1 under any stimulation (Fig. 5). Interestingly, no mature caspase-1 was detected under these conditions. Pro-IL-1 β levels increased by priming with LPS following with or without stimulation with *C. albicans* CAI4 or pACT1-GFP (Fig. 5), suggesting that the induction of pro-IL-1 β was mainly caused by stimulation with LPS. These results demonstrated that there are no differences in the patterns of expression of inflammasome-related molecules between XS106 and J774.1 cells.

3.6 *ATP release by XS106 cells and J774.1 cells*

ATP released from damaged cells is capable of activating the NLRP3

inflammasome (Iyer et al., 2009; Riteau et al., 2012); therefore, XS106 and J774.1 cells were primed with LPS and/or stimulated with *C. albicans* CAI4 or pACT1-GFP. It was found that stimulation with *C. albicans* CAI4 or pACT1-GFP induced ATP release by XS106 cells (Fig. 6). In addition, there was no significant difference in the amount of ATP in response to *C. albicans* CAI4 or pACT1-GFP (Fig. 6). By contrast, *C. albicans* pACT1-GFP induced ATP release by J774.1 cells, but *C. albicans* CAI4 did not (Fig. 6). This result suggested that the difference in the amount of IL-1 β induced in XS106 and J774.1 cells was caused by differences in their extracellular ATP concentrations.

3.7 Morphological change of *C. albicans* under co-culture with cells

C. albicans can switch from the spherical yeast growth form to filamentous hyphal cells under certain conditions, which is crucial for its pathogenicity (Calderone & Fonzi, 2001). *C. albicans* pACT1-GFP constitutively expresses GFP and has no specific auxotrophy (Barelle et al., 2004). By contrast, *C. albicans* CAI4 is uridine auxotrophic and is a parental strain of pACT1-GFP, constructed by deletion of the second copy of *URA3* (*ura3:: λ imm434/ura3:: λ imm434*) (Fonzi & Irwin, 1993).

Therefore, the addition of uridine is necessary for the growth of *C. albicans* CAI4.

XS106 cells (Fig. 7) or J774.1 cells (Fig. 8) were prepared in 24-well plates and observed using a phase-contrast microscope up to 14 h. When *C. albicans* CAI4 was

cultured with XS106 cells, it maintained its spherical yeast form (Fig. 7B). However, the addition of uridine to the culture medium induced *C. albicans* CAI4 to change from yeast to the filamentous hyphal form in a time-dependent manner (Fig. 7C). *C. albicans* pACT1-GFP showed the morphological change from yeast to hyphae without the addition of uridine (Fig. 7D). Similar results were obtained using J774.1 cells (Fig. 8A–D). These findings demonstrated that the morphological change of *C. albicans* CAI4 from yeast to hyphae requires uridine and that no supplements are required for the morphological change of *C. albicans* pACT1-GFP to filamentous hyphae cells under these conditions. These results also implied that the morphological change of *C. albicans* to hyphal cells is critical for J774.1 cells to induce IL-1 β release, whereas no morphological change is required for XS106 cells to induce IL-1 β release.

3.8 Relationship between the morphology of *C. albicans* and the induction of IL-1 β

To examine the effects of the morphological change of *C. albicans* on the induction of IL-1 β , XS106 or J774.1 cells were primed with LPS and then cultured with live *C. albicans* CAI4 or pACT1-GFP, with or without uridine. The results showed that the addition of uridine had no effects on the induction of IL-1 β release by *C. albicans* CAI4 and pACT1-GFP toward XS106 cells (Fig. 9A). By contrast, the addition of uridine enabled *C. albicans* CAI4 to induce IL-1 β release from J774.1 cells (Fig. 9B).

There were no effects of uridine addition on the IL-1 β release-inducing activity of *C. albicans* pACT-1-GFP (Fig 9B). These results suggested that the morphological change of *C. albicans* from the yeast to hyphal form is critical for the induction of IL-1 β by J774.1 cells, but not by XS106 cells.

3.9 Requirement of direct contact of *C. albicans* with XS106 or J774.1 cells to induce IL-1 β release

The induction of IL-1 β release by *C. albicans* toward J774.1 cells was found to be dependent on the morphological change of *C. albicans*; therefore, we attempted to confirm whether *C. albicans* metabolites are involved in the induction of IL-1 β release, because metabolites produced during the transformation might be crucial for the activity. XS106 or J774.1 cells were primed with LPS and then incubated with live *C. albicans* using transwell culture plates to avoid direct contact between *C. albicans* with the cells. XS106 or J774.1 cells were cultured in the lower chamber and whole cells of *C. albicans* CAI4 or pACT1-GFP were added to the transwell insert (upper chamber). As controls, whole cells of *C. albicans* CAI4 or pACT1-GFP were incubated together with XS106 or J774.1 cells in the same wells for direct contact. Before harvesting the supernatants to measure the amount of IL-1 β released, morphological changes to the hyphal form of *C. albicans* pACT1-GFP and CAI4 under the addition of uridine to the

culture medium were confirmed under phase-contrast microscopy (data not shown). IL-1 β was released into the supernatant from the direct contact culture system, but not from the transwell culture system (Fig. 10). This result suggested that direct contact of *C. albicans* with XS106 or J774.1 cells is crucial for the induction of IL-1 β release, and the activity is not caused by soluble *C. albicans* metabolites.

3.10 *Involvement of viable C. albicans in the induction of IL-1 β release*

The results in section 3.9 showed that direct contact of *C. albicans* with XS106 or J774.1 cells was critical for inducing IL-1 β release; therefore, we next determined whether the viability of *C. albicans* affects the activity. Both the yeast and hyphal forms of *C. albicans* CAI4 or pACT1-GFP were prepared and then killed by treatment with amphotericin B. The dead cells of the two strains were washed with PBS and used to stimulate XS106 or J774.1 cells. Both strains of *C. albicans* induced IL-1 β release from XS106 cells, irrespective of their viability, although the activities of dead cells of both strains of *C. albicans* were significantly weaker than those of their live counterparts (Fig. 11A). In contrast, the dead cells of both strains of *C. albicans* could not induce IL-1 β release from J774.1 cells (Fig. 11B).

These results demonstrated that J774.1 cells required *C. albicans* to be alive for induction of IL-1 β release. On the other hand, both live and dead *C. albicans* induced

the release from XS106 cells, although the induction by dead *C. albicans* was weaker than that by live *C. albicans*.

4. Discussion

Although little is known about the differential recognition of *C. albicans* forms by host cells, there have been a number of studies on the relationship between their forms and the ability to induce cytokines. For example, in DCs, *C. albicans* hyphae induce IL-4 production, but not IL-2 production; whereas the yeast form induces IL-12 production (D'Ostiani et al., 2000). IL-8 secretion by oral epithelial cells requires the transformation of *C. albicans* into hyphae (Dongari-Bagtzoglou & Kashleva, 2003). Attempts have been made to address these differences in cytokine induction by differences in cell surface β -glucans between yeast and hyphae, because β -glucans were found to be rich on yeast bud scars but not on hyphal surfaces (Gantner, Simmons, & Underhill, 2005). However, Wheeler *et al.* reported that there was no difference in β -glucan exposure between yeast and hyphal cells in any of the stages of infection measured (Wheeler, Kombe, Agarwala, & Fink, 2008). Recently, Lowman *et al.* suggested that differences in the structural features of β -glucan between yeast and hyphae is important in innate immune recognition (Lowman et al., 2014). Many studies are in progress to elucidate how the immune system interacts with the various morphological forms of fungi.

In agreement with our results, Joly *et al.* reported that *C. albicans* mutants

incapable of forming hyphae were defective in their ability to induce IL-1 β secretion from macrophages (Joly et al., 2009). In addition, Cheng *et al.* reported that germ-tube formation of *C. albicans* is critical for the stimulation of IL-1 β production by macrophages (Cheng et al., 2011). However, Wellington *et al.* reported that no morphogenesis is required for IL-1 β production by macrophages (Wellington, Koselny, & Krysan, 2012). These discrepancies in the manner of IL-1 β induction in macrophages are speculated to reflect differences between the *C. albicans* strains used in these studies. In addition, the difference between the mammalian cells used in these studies might also have contributed to the observed discrepancies, because IL-1 β production is reported to be regulated differentially in monocytes and macrophages (M. Netea et al., 2009).

The NLRP3 inflammasome is considered a key contributor for a large number of danger signals and is the main platform for IL-1 β processing. One of the most efficient NLRP3 activators is extracellular ATP, which acts mainly through a purinergic receptor, P₂X₇ (Ferrari et al., 1997). Inhibitors of the P₂X₇ receptor, such as oxidized ATP, can abrogate ATP-induced IL-1 β release from immune cells (Ferrari et al., 1997), and mouse macrophages lacking the P₂X₇ receptor do not release IL-1 β in response to ATP (Solle et al., 2001). Therefore, ATP-mediated activation of the P₂X₇ receptor has

been hypothesized to play a pivotal role in IL-1 β production (Pelegrin & Surprenant, 2007; Sanz et al., 2009). In the present study, differences in extracellular ATP concentrations were observed in supernatants from J774.1 cells stimulated with *C. albicans* CAI4 or pACT1-GFP (Fig. 6), which suggested that ATP is one of the key components in the IL-1 β inducing activity. Further studies are needed to determine how ATP is released extracellularly from immune cells in response to *C. albicans* infection.

IL-1 β is a potent inflammatory cytokine that is mainly produced by monocytes, macrophages, and DCs (Dinarello, 2011). IL-1 β plays important roles in multiple autoinflammatory syndromes and in the host defense against infectious diseases. In infectious diseases, IL-1 β upregulates T cell activation and initially regulates the development of Th17 cells (van de Veerdonk, Netea, Dinarello, & Joosten, 2011; Weaver, Harrington, Mangan, Gavrieli, & Murphy, 2006) which are crucial for anti-*Candida* host defense (Laan et al., 1999). IL-1 β is also required for the production of IL-17 and IL-22 by natural killer T (NKT) cells (Moreira-Teixeira et al., 2011) and natural killer (NK) cells (Hughes et al., 2010), respectively. For the maturation of IL-1 β , cleavage of pro-IL-1 β by caspase-1 is required, and caspase-1 activation is induced by the activation of inflammasomes, which are multiprotein complexes comprising NLR, ASC, and procaspase-1. Recently, many studies have reported the involvement of NLR

family members in host defense mechanisms against *Candida*, although the mechanisms remain unclear. The pathological roles of NLRP3 and NLRC4 inflammasomes in *C. albicans* infection have been determined. Similar to studies on other oral pathogens (Belibasakis & Johansson, 2012), the NLRP3 inflammasome has been shown to play key pathological roles in *C. albicans* infection. NLRP3 inflammasomes were suggested to be critical in the host defense against *C. albicans* infection by controlling caspase-1-mediated cleavage of pro-IL-1 β (Hise et al., 2009). Hyphae formation of *C. albicans* has been reported to trigger activation of NLRP3 inflammasomes (Joly et al., 2009). Many studies suggested the importance of NLRP3 inflammasomes in *C. albicans* recognition by the immune system. In addition, the importance of NLRC4 inflammasomes in *Candida* infection was also reported. Tomalka *et al.* suggested that NLRC4 plays an important role in host defense against mucosal *Candida* infection (Tomalka et al., 2011). Interestingly, a negative role of NLRC4 was also reported, in which NLRC4 inhibits the NLRP3 inflammasome via production of an IL-1 receptor antagonist during vaginal candidiasis in the presence of IL-22 (Borghi et al., 2015). NLRP10 has also been suggested to be important in the fight against *C. albicans* infection, although the absence of NLRP10 did not affect the activation of NLRP3 for IL-1 β secretion (Joly et al., 2012).

Several cell surface PRRs might be involved in the recognition of fungi. TLR2 has been reported to be involved in the recognition of fungal components (Gil & Gozalbo, 2009; M. G. Netea et al., 2006), such as zymosan (Ozinsky et al., 2000), although disagreement persists regarding the precise fungal components involved (van de Veerdonk, Kullberg, van der Meer, Gow, & Netea, 2008). TLR4 might also participate in antifungal defense, because the absence of TLR4 facilitated infection of the host by fungal pathogens (Bellocchio et al., 2004; Ding et al., 2005; Mambula, Sau, Henneke, Golenbock, & Levitz, 2002). However, recognition of fungal components by PRRs remain unclear (van de Veerdonk et al., 2008). Other PRRs that are essential for fungal component recognition are C-type lectin receptors (CLRs). The CLRs involved in the recognition of fungal components are dectin-1, 2, 3; the macrophage mannose receptor (MR); and DC-specific ICAM3-grabbing nonintegrin (DC- SIGN) (van de Veerdonk et al., 2008). Dectin-1 recognizes β -glucan to control fungal infection (Taylor et al., 2007). Dectin-2 recognizes β -glucan (Brown et al., 2002) and α -mannans (Saijo et al., 2010). Recently, the heterodimer of Dectin-2 and Dectin-3 was shown to bind α -mannans more effectively than their homodimers (Zhu et al., 2013). The roles of MR in *Candida* infection are also important, because it is involved in the induction of many cytokines, such as IL-1 β , IL-6, granulocyte-macrophage colony-stimulating factor, and

IL-17 (van de Veerdonk et al., 2009; Yamamoto, Klein, & Friedman, 1997). DC-SIGN mediates the uptake of fungal particles (Cambi et al., 2003) and its murine homolog, SIGNR1, regulates TLR2 signaling (Ohtani et al., 2012). Several PRRs that might be involved in the recognition of fungal components; however, we only examined the cell surface expression levels of TLR2 and TLR4 in this study. Studies are in progress to determine the roles of NLRs and PRRs in response to *C. albicans*.

Conflicts of interest

There is no conflict of interest related to this study.

Source of funding

This work was supported by the Grants-in-Aid for Scientific Research (C26462775) and (17K1160607) provided by the Japan Society for the Promotion of Science.

Acknowledgements

The authors thank the Support section for Education and Research and Department of Biochemistry and Molecular Biology in the Faculty of Dental Medicine and Graduate School of Dental Medicine, Hokkaido University.

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Figure Legends

Figure 1. Interleukin (IL)-1 β release from XS106 and J774.1 cells in response to *C. albicans*. (A) XS106 and J774.1 cells were primed with lipopolysaccharide (LPS), and then stimulated with live *C. albicans* at various effector-to-target (E/T) ratios. (B) Determination of the mature form IL-1 β in the cell culture supernatants by western blotting analysis.

*Indicates a statistically significant difference ($p < 0.05$).

Figure 2. Time course of the interleukin (IL)-1 β release (A, C) and cytotoxicity (B, D) induced using live *C. albicans*. The cells were primed with lipopolysaccharide (LPS), and then stimulated with live *C. albicans*.

*Indicates a statistically significant difference in activity between *C. albicans* CAI4 and pACT1-GFP ($p < 0.05$).

Figure 3. Effect of z-YVAD-fmk on the interleukin (IL)-1 β release-inducing activity of live *C. albicans*. XS106 and J774.1 cells were incubated with z-YVAD-fmk at various concentrations for 1 h before priming with lipopolysaccharide (LPS). NS represents no stimulation.

*Indicates a statistically significant difference ($p < 0.05$).

Figure 4. (A) TLR4 expression on XS106 and J774.1 cells. The experiment was repeated twice and similar results were obtained. (B) Relative quantity of *Il1B* mRNA in XS106 and J774.1 cells.

Figure 5. Western blotting analysis of nucleotide-binding domain-like receptor protein 3 (NLRP3), apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC), caspase-1, interleukin (IL)-1 β , and β -actin in the cell lysates of XS106 cells and J774.1 cells. Density of each protein band was analyzed by the Image J software.

Figure 6. Release of ATP by XS106 and J774.1 cells in response to *C. albicans*.

*Indicates a statistically significant difference ($p < 0.05$).

Figure 7. Effects of uridine on the morphogenesis of *C. albicans* incubated with XS106 cells at different time intervals. *C. albicans* cells in the yeast form were added to XS106 cell culture and then images were captured every 1 h up to 6 h and 14 h. Large circles or ovals are XS106 cells, and small circles or filamentous forms are *C. albicans*.

Representative images captured at 0 h, 6 h, and 14 h are shown. Images were captured at a magnification of $\times 100$ under a Nikon TS-100 phase-contrast microscope.

Figure 8. Effects of uridine on the morphogenesis of *C. albicans* incubated with J774.1 cells at different time intervals. *C. albicans* cells in the yeast form were added to J774.1

cell culture and then images were captured every 1 h up to 6 h and 14 h. Large circles or ovals are J774.1 cells, and small circles or filamentous forms are *C. albicans*.

Representative images captured at 0 h, 6 h, and 14 h are shown. Images were captured at a magnification of $\times 100$ under a Nikon TS-100 phase-contrast microscope.

Figure 9. Effects of uridine on the interleukin (IL)-1 β release-inducing activity of *C. albicans*. XS106 cells (A) and J774.1 cells (B) were primed with lipopolysaccharide (LPS), and then stimulated with live *C. albicans* in the presence or absence of uridine. NS represents no stimulation with *C. albicans*.

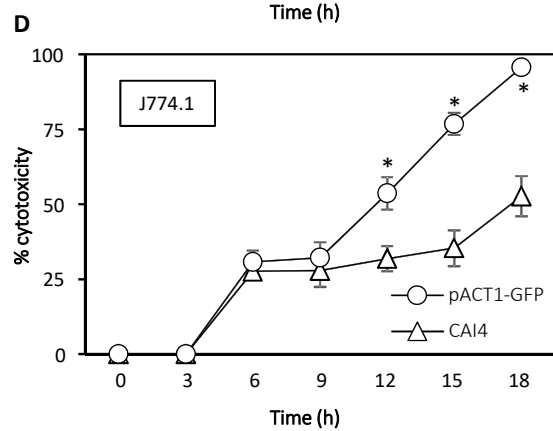
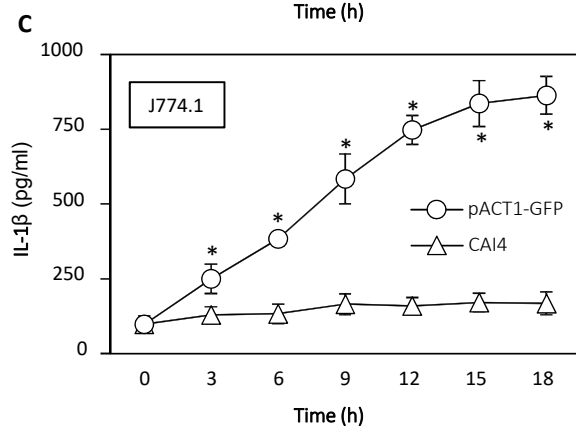
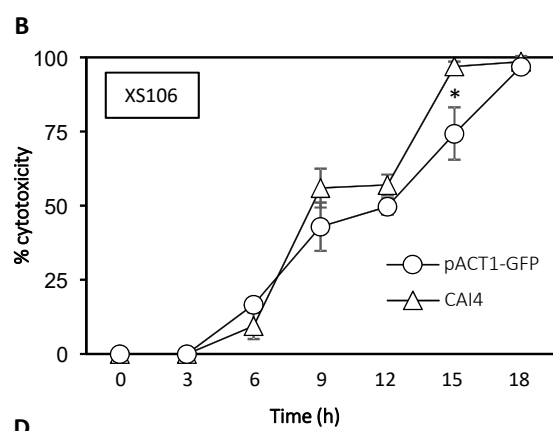
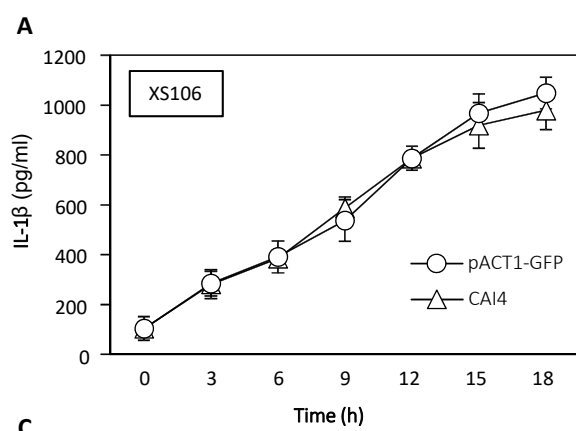
*Indicates a statistically significant difference ($p < 0.05$).

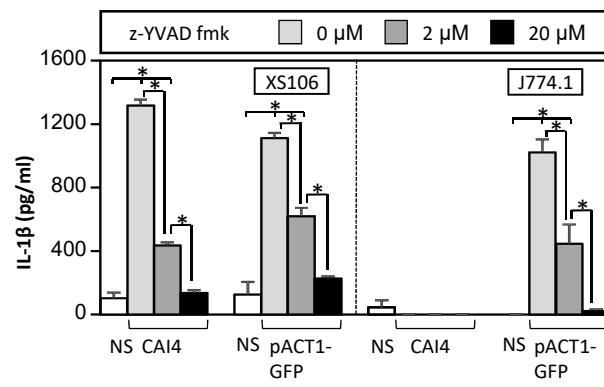
Figure 10. Effects of contact of *C. albicans* with XS106 or J774.1 cells on the induction of interleukin (IL)-1 β release. XS106 and J774.1 cells were primed with lipopolysaccharide (LPS), and then stimulated directly or indirectly (Transwell) with live *C. albicans*. NS represents no stimulation with *C. albicans*.

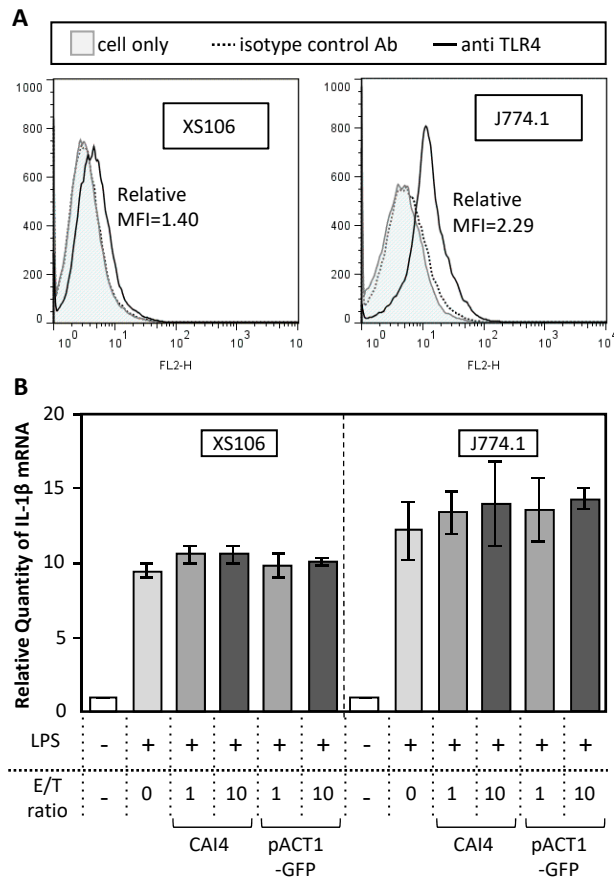
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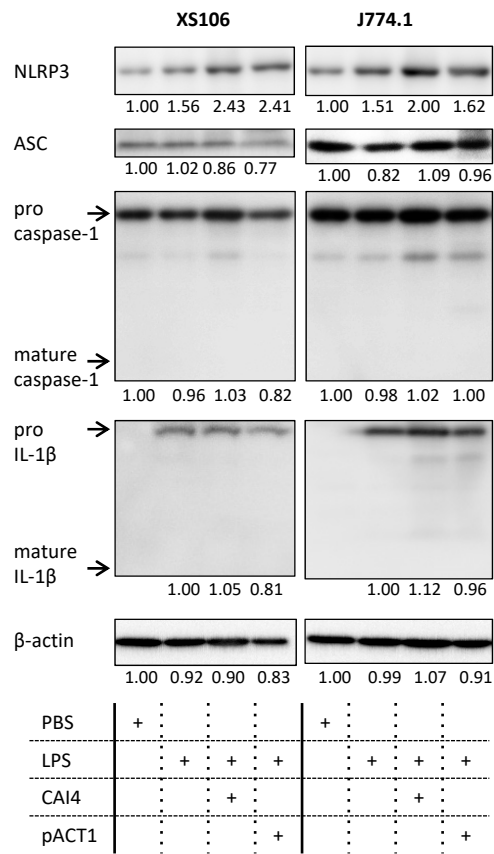
Figure 11. Effects of *C. albicans* viability on the induction of interleukin (IL)-1 β release. XS106 cells (A) and J774.1 cells (B) were primed with lipopolysaccharide (LPS), and then stimulated with live or dead *C. albicans* cells in the presence or absence of uridine.

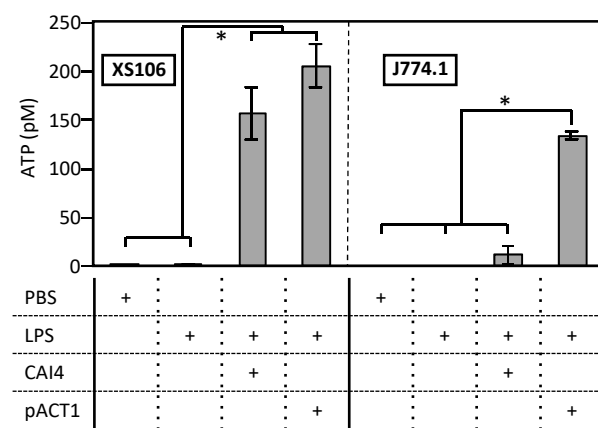
*Indicates a statistically significant difference ($p < 0.05$).



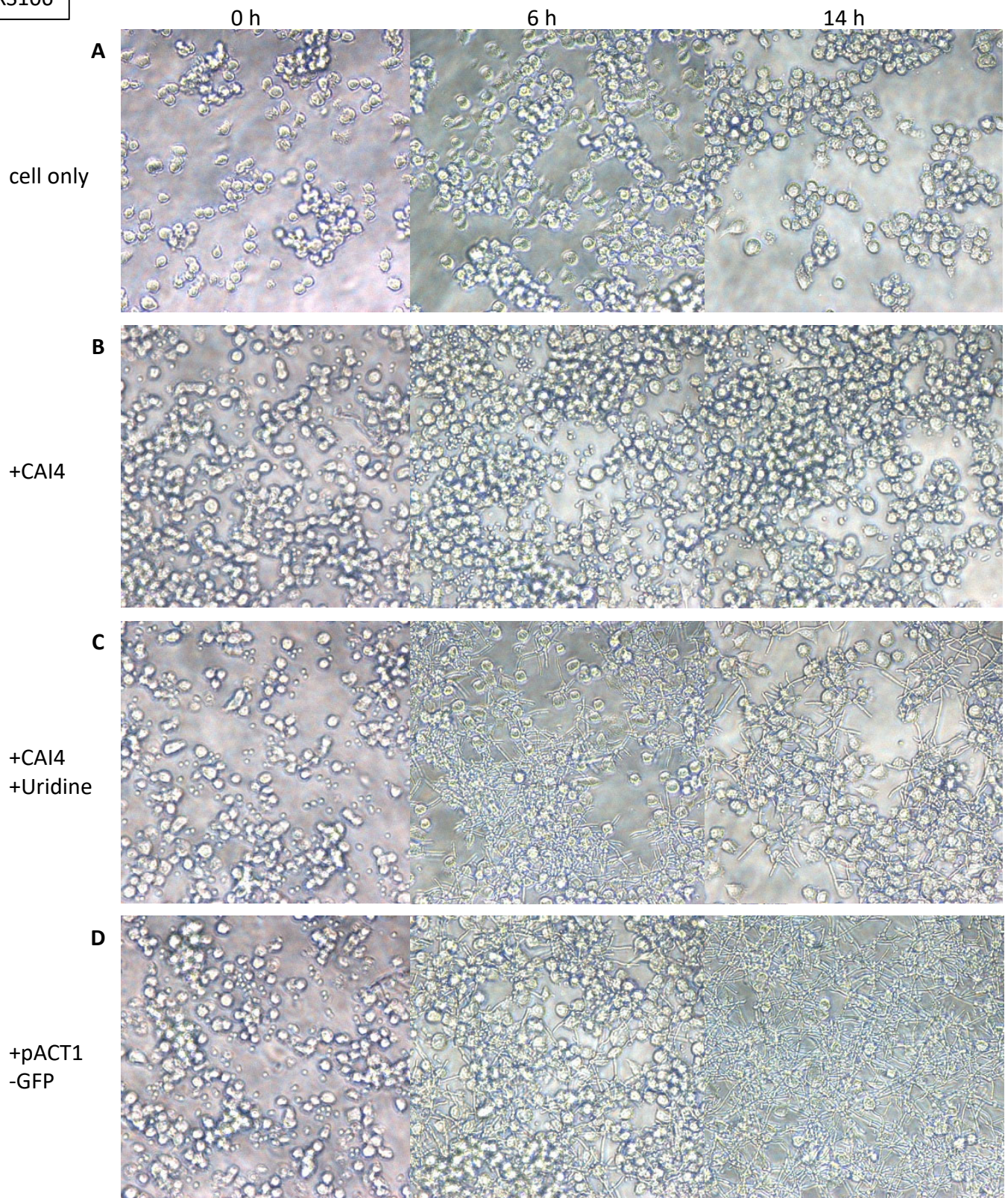








XS106



J774.1

