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DIFFERENTIAL DENDRITIC CELL RESPONSES TO *CANDIDA* SPECIES

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บทคัดย่อ

แมนแนนในชั้นนอกสุดของผนังเซลล์ของเชื้อราอาจเป็นโมเลกุลแรกที่ปฏิสัมพันธ์กับเดนไดรติกเซลล์ในร่างกาย และมีผลทำให้เกิดการกระตุ้นการตอบสนองทางภูมิคุ้มกันซึ่งกำหนดการดำเนินของโรค อย่างไรก็ตามปัจจุบันยังไม่ทราบแน่ชัดว่าความแตกต่างของโครงสร้างของแมนแนนในเชื้อรากลุ่มแคนดิดามีผลกระทบต่อการตอบสนองของเดนไดรติกเซลล์และทีเซลล์อย่างไร ดังนั้นวิทยานิพนธ์นี้จึงทำการศึกษาเปรียบเทียบผลของแมนแนนที่สกัดจากผนังเซลล์ของเชื้อรา *Candida albicans*, *Candida tropicalis*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis* และ *Candida dubliniensis* ต่อการตอบสนองของเดนไดรติกเซลล์ที่พัฒนามาจากไขกระดูกของหนูเมาส์ พบว่าแมนแนนของ *C. albicans* และ *C. tropicalis* ไม่สามารถกระตุ้นเดนไดรติกเซลล์ได้ แต่สามารถกระตุ้นการหลั่งของไซโตไคน์ชนิด IFN-g และมีผลต่อการตอบสนองของทีเซลล์ชนิด Th1 แมนแนนของ *C. glabrata* กระตุ้นเดนไดรติกเซลล์ได้ดี แต่ไม่สามารถกระตุ้นการหลั่งไซโตไคน์ของเดนไดรติกเซลล์ ในทางตรงข้าม แมนแนนของ *C. krusei* กระตุ้นเดนไดรติกเซลล์ได้อย่างมีประสิทธิภาพ และยังกระตุ้นให้เซลล์หลั่งไซโตไคน์ที่เหนี่ยวนำการอักเสบในปริมาณมาก ซึ่งการตอบสนองนี้อาจมีผลทำให้เกิดภาวะการอักเสบที่มากเกินไป ในขณะเดียวกัน แมนแนนของ *C. dubliniensis* กระตุ้นการตอบสนองของเดนไดรติกเซลล์ได้ปานกลาง ซึ่งอาจเกี่ยวข้องกับความสามารถของ *C. dubliniensis* ที่ไม่ทำให้เกิดพยาธิสภาพที่รุนแรง แมนแนนจากผนังเซลล์ของ *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis* and *C. dubliniensis* ไม่มีผลต่อความมีชีวิตของเดนไดรติกเซลล์ เป็นที่น่าสนใจว่าแมนแนนของ *C. krusei* กระตุ้นให้เดนไดรติกเซลล์หลั่งไซโคไคน์ที่เหนี่ยวนำการอักเสบในปริมาณมากบ่งบอกถึงสภาวะที่เดนไดติกเซลล์ถูกกระตุ้นอย่างรุนแรง และด้วยสภาวะนี้เดนไดรติกเซลล์จึงเกิดการตายแบบอะพอพโทซิส การตายแบบอะพอพโทซิสของเดนไดรติกเซลล์นั้นถูกเหนี่ยวนำผ่านวิถีสัญญาณ TLR2 และ MyD88 ถึงแม้ว่าเดนไดรติกเซลล์จะตายจากการกระตุ้นโดยแมนแนนของ *C. krusei* แต่เดนไดรติกเซลล์นี้ยังสามารถกระตุ้นการตอบสนองของทีเซลล์ได้ และพบว่าการกระตุ้นวิถีสัญญาณ MyD88 โดยมีผลต่อการควบคุมทิศทางการตอบสนองของทีเซลล์ชนิด Th1/Th17 ซึ่งการควบคุมดังกล่าวน่าจะเป็นผลมาจากการเปลี่ยนแปลงของไซโตไคน์ชนิด IL-12 และ IL-6

โครงสร้างและองค์ประกอบของแมนแนนในผนังเซลล์ของเชื้อราแคนดิดามีความสำคัญอย่างยิ่งในควบคุมการตอบสนองของเดนไดรติกเซลล์ ซึ่งมีผลต่อการกระตุ้นภูมิคุ้มกันที่ป้องกันการติดเชื้อ หรือช่วยให้เชื้อราหลบหลีกภูมิคุ้มกันได้ ดังนั้นแมนแนนของเชื้อรากลุ่มแคนดิดาอาจมีความเกี่ยวข้องกับพยาธาสภาพทางภูมิคุ้มกันและความรุนแรงของการติดเชื้อแคนดิดาทางระบบและทางผิวหนังเยื่อเมือก

สาขาวิชา ชีววิทยาช่องปาก ลายมือชื่อนิสิต .....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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THU NGUYEN NGOC YEN: DIFFERENTIAL DENDRITIC CELL RESPONSES TO *CANDIDA* SPECIES. ADVISOR: ASST. PROF. PATCHAREE RITPRAJAK, D.D.S., Ph.D., CO-ADVISOR: ASSOC. PROF. ORANART MATANGKASOMBUT, D.D.S., Ph.D., 86 pp.

Abstract

Mannan in the outermost layer of *Candida* cell wall may be the first molecules that interact with host dendritic cells (DCs) and activate immune responses that determine disease outcomes. However, little is known how different mannan structures of common *Candida* species affect DC activation and T cell responses. Thus, this work compared the effects of mannans of *Candida albicans*, *Candida tropicalis*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis* and *Candida dubliniensis* on bone marrow-derived DC (BMDC) responses in mice. *C. albicans* and *C. tropicalis* mannan could not activate DC maturation, however, they induced IFN-gamma production which consequently provoked Th1 responses. *C. glabrata* mannan potentially induced DC maturation, but it failed to mediate the cytokine production. In contrast, *C. parapsilosis* mannan caused strong DC activation and high production of several pro-inflammatory cytokines which possibly promote hyperinflammation. Meanwhile, *C. dubliniensis* mannan induced moderate BMDC responses, which may correlate with its lower pathogenicity. Cell wall mannan of *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis* and *C. dubliniensis* had no effect on DC viability. Interestingly, *C. krusei* mannan triggered the massive production of pro-inflammatory cytokines in DCs, and this high activation state of DCs consequently leaded to the cellular apoptosis. The induction of DC apoptosis upon *C. krusei* mannan was mediated via TLR2- and MyD88 pathway. Although DCs underwent apoptosis upon *C. krusei* mannan stimulation, they were still capable of initiating T cells responses. In addition, the activation of MyD88 in DCs by *C. krusei* mannan controlled Th1/Th17 switching by virtue of the polarizing cytokines IL-12 and IL-6. The structure and composition of *Candida* cell wall mannan crucially promotes either host protective immunity or fungal immune evasion through differential activation of DCs. Therefore, mannan of each *Candida* species may be involved in the immunopathogenesis and disease severity of systemic and mucocutaneous candidiasis.

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Field of Study: Oral Biology
Academic Year: 2017

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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Chapter 1

INTRODUCTION

Background and rationale

Commensal *Candidia* species in human microbiota recently have been contributed to the high prevalence of fungal infection worldwide due to the raising of population at risk, including immunocompromised patient (AIDS, cancer, transplantation, neonatant and older population), advanced medication (intravenous catheter placement, aggressive surgery) and wide usage of broad-spectrum antimicrobial agents. Over the past two decades, there is a shift in epidemiology of *Candida* infection all over the world. The incidence of infection caused by *Candida albicans* is decreasing, while the prevalence of infection caused by non-*albicans Candida* species (NACs) is increasing and even becomes predominant in some areas. This emerging trend of fungal infection becomes a serious threat for individuals and public health because invasive infections caused by NACs are high mortality and more difficult to eliminate due to their resistance to common antifungal agents. In addition, knowledge about the host immune responses against *Candida* species is limited because most of studies in the past focus on *Candida albicans.*

Host defense against fungal infection requires the initiation of both innate and adaptive immunity. Among immune cells, dendritic cells (DCs) play an important role in linking innate and adaptive immunity, as well as controlling immune homeostasis. Various receptors on DC surface are responsible for recognizing the fungal cell wall and initiating the signaling lead to protective immune responses. Mannan is the outer-most polysaccharide layer in *Candida* fungal cell walls, considered as an important antigen for host cell to interact with. The dissimilar mannan structures in various *Candida* species may result in the distinct immune response due to the

differences in DC activation. However, little is known about the effects of interaction between DCs and mannan of NACs. Understanding how DCs regulated immune responses against *Candida* species, especially NACs, will help to generate more effective immunotherapies.

Research questions

1. How do dendritic cells differently response against cell wall mannan of *Candida albicans* and non-*albicans Candida* species?
2. How do the distinct *Candida* mannan-stimulated DCs influence T cell responses

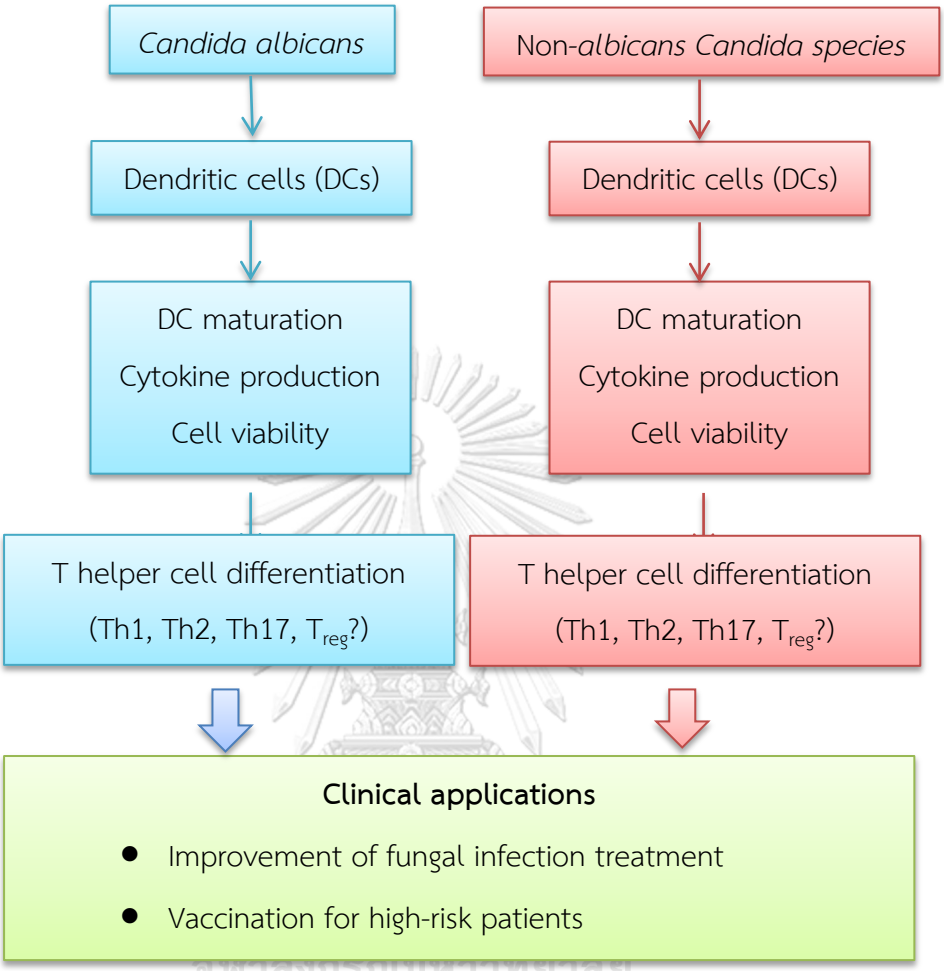
Hypothesis

Cell wall mannans of *Candida albicans* and non-*albicans Candida* differently affect DC responses, and consequently lead to the distinct T cell responses.

Objectives

1. To determine the impact of cell wall mannans of *Candida albicans* and systemic candidiasis-related non-*albicans Candida* on DC and T cell responses *in vitro* and *in vivo*.
2. To investigate the effect of cell wall mannans of *Candida albicans* and oral candidiasis-related non-*albicans Candida* on DC responses *in vitro*.

Conceptual framework



Benefits of study

This study provided the fundamental knowledge for the development of the new and effective clinical therapeutics that contribute to the prevention and treatment of *Candida* infection.

Chapter 2

REVIEW OF RELATED LITERATURE

Candida species infection

The increasing incidence of fungal infections has been reported over the last decades. *Candida* species are among the majority of human fungal pathogens that cause infections in both mucocutaneous and systemic forms. Notably, oral candidiasis and vulvovaginal candidiasis are the most common fungal infections of the oral and vaginal mucosa, respectively (1). Invasive Candidiasis is considered to be the most important global opportunistic mycosis, and is ranked as the third most common cause of hospital-acquired sepsis from 75 countries (2).

Although *Candida* species is the commensal fungi that frequently resides on skin and mucous membranes of gastrointestinal tract and reproductive tract, it can overgrow and lead to illnesses ranging from non-life-threatening to invasive processes that may contribute to morbidity and mortality under some specific condition, such as the disorder of locally mucosal microbiota or individual's immunocompromised state. A number of predisposing factors for the significant increase in candidiasis incidence have been implicated such as the rising of Acquired Immunodeficiency Syndromes (AIDS), the increased population of patients treated with cancer chemotherapy or immunosuppressive therapy in transplantation and the use of broad spectrum antibiotics in hospital as well as in community. The new advanced medical practices, such as intravascular catheters and central venous catheters, total parenteral nutrition and invasive surgeries also can contribute to the risks for candidemia in patients (3).

Invasive *Candida* infections are usually related to high morbidity and mortality. In the United States, although *Candida* species are ranked as the fourth most common cause of nosocomial bloodstream infection (BSI), the crude mortality percent caused by them was at the highest rate (4). Even a lot of effort was spent on

researches, improvements of diagnosis and treatment process to fight against *Candida* infection worldwide, the burden of this disease does not seem to be decreased. *Candida* species are also a cause of oral candidiasis, which is a common problem in the elderly and immunocompromised individuals, whose defense mechanisms are impaired locally or systemically. Local and systemic predisposing factors leading to pathogenic condition include ill-fitting prosthodontic appliances, xerostomia, malnutrition, and hormonal and endocrine disorders (5). Recently, *Candida* has developed anti-fungal resistance resulting from the widespread use of broad-spectrum antibiotics and prolonged antibiotic therapy (6, 7). Therefore, the new strategy for anti-fungal treatment must be established.

An emerging threat from non-*albican Candida* infections

Among the genus *Candida, Candida albicans* historically reported to be the predominant species responsible for *Candida* infections. However, the epidemiology of candidiasis worldwide has been changing recently due to the rising proportion of infections caused by non-*albicans Candida* species (NACs) during the past two decades. A study analyzing candidemia information from North American medical centers in 4 years (2004-2008) found that the incidence of candidemia caused by NACs was higher than caused by *Candida albicans*. In general, this change in epidemiology could be a result of severe immunosuppression or illness, prematurity, exposure to broad-spectrum antibiotics and older patients. Among NACs, the majority species identified included *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, *Candida krusei* and *Candida dubliniensis*. In addition, patients with *Candida parapsilosis* and *Candida krusei* infection had the lowest and highest crude mortality, respectively (8). In European countries, *Candida glabrata* and *Candida parapsilosis* were also reported with the highest prevalence among the NACs infection (9). In Asia, a similar trend of change in epidemiology of *Candida* infection has been recorded. In China, from 2009 to 2011, *Candida albicans* and *Candida parapsilosis* were the most prevalent species identified from patient with candidemia. However, the incidence of *Candida parapsilosis* infection increased

while the incidence of *Candida albicans* infection decreased over the time (10). Among the *Candida* species isolated from oral cavities of oral candidiasis patients in Japan, *Candida albicans* and *Candida glabrata* presented at 95%. Interestingly, the prevalence of the latter increased while the prevalence of the former decreased significantly (11). In India, *Candida tropicalis*, *Candida parapsilosis* and *Candida glabrata* are the most frequent isolated species from blood samples of patient in hospital, with the highest incidence appear at neonatology department (12). Another recent study in India also confirmed that NACs became predominant pathogens isolated from various clinical specimens (13). The underlying reasons of this shift of epidemiology have not been understood well. However, some factors include administration of corticosteroids, central venous catheter placement, and pre-existing candiduria have been reported to be associated independently with candidemia caused by NACs (14). Previous exposure to fluconazole also contributed to the increasing risk of blood stream infection due to NACs (15).

It is interesting that infections caused by NACs distribute in particular geographical areas. For examples, *C. glabrata* predominantly causes invasive candidiasis in USA and Northern or Central Europe, while *C. tropicalis* is more popular in some parts of Asia and Latin America (16). NACs quickly became threats in both systemic or blood stream infection and mucocutaneous infection such as oral candidiasis.

Systemic candidiasis-related NACs

C. tropicalis, *C. glabrata* and *C. krusei* are among NACs that are predominantly isolated from systemic candidiasis patients and related with high mortality. *C. tropicalis* usually found in patients carrying solid tumors or haematologic diseases. Aging and neoplastic patients were infected with *C. glabrata* more frequently than the other species (12). *C. krusei* systemic infection is strongly associated with prior fluconazole prophylaxis, haematological malignancies, bone marrow transplants and neutropenia (17). In the emerging image of increasing prevalence of NACs in *Candida* infection worldwide, the threat are even more serious as NACs were demonstrated high resistance to antifungal agents (18). A study about

the antifungal susceptibility of NACs in China showed that a part of isolated *C. tropicalis* and *C. glabrata* were fluconazole resistant (19). It has been reported that *C. krusei* is multidrug-resistant because it is completely resistant with fluconazole and showed reduced susceptibility to other antifungal agents such as flucytosine and amphotericin B (20).

Oral candidiasis-related NACs

C. parapsilosis and *C. dubliniensis* are two common NACs isolated from normal flora of healthy individuals and from oral candidiasis lesion (21-23). *C. parapsilosis* is a complex comprising three distinct species, *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis*, which are common colonizers of the oral cavity. *C. parapsilosis* has been recognized as a major human pathogen since it has high prevalence worldwide (24). *Candida parapsilosis* is a frequent pathogen isolated in catheter-related infections as it usually colonizes on skin (25). This species also commonly showed resistance to antifungal drug during clinical treatment (26). In young patients with cancer, *C. parapsilosis* appears as the second most abundant species, after *C. albicans*, causing oral candidiasis (27). *Candida dubliniensis* is closely related to *C. albicans*, and has been found at high prevalence in the oral cavities of HIV-infected patients and healthy individuals (21, 28). In addition, a study in diabetes mellitus patients, who use insulin, reported that *C. dubliniensis* was the second most common species in the oral environment (29). Additionally, besides oral candidiasis, *C. albicans*, *C. parapsilosis* and *C. dubliniensis* have recently been isolated from dental caries and periodontitis (30, 31).

The treatment responses were significantly different between patients infected with *Candida albicans* and NACs. Higher mortality was found associated with NACs blood stream infection in comparision with *Candida albicans* infection. These emerging trends of *Candida* infection caused by NACs rise a necessity to understand clearly their pathogenesis and how the host immunity response against these species in order to develop more effective stratergies for disease prevention and treatment.

Candida cell wall

The cell wall is an important structure for fungus because it provides a protective barrier, maintains the cell stability and contributes to the fungal adhesion to host cells. On the other hand, the cell wall contains components that have immunogenic properties and may play a major role in the interaction with host immune cells. Fungal cell walls of *Candida* species are mainly constructed by three main layers of carbohydrates, including mannans (polymers of mannose), β-glucans (polymers of D-glucose linked by β-glycosidic bonds) and chitins (polymers of N-acetyl-D-glucosamine) (Fig. 2.1). These structures are considered as major unique PAMPs of fungi and make fungi become distinguishable to the host mammalian cells. For this reasons, the cell wall of *Candida* species has been the focus of attention.

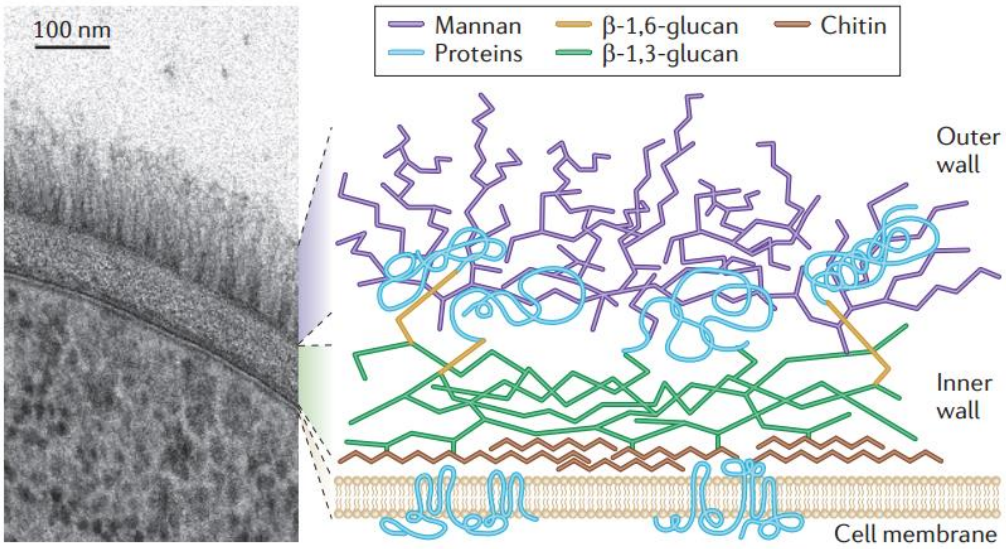


Figure 2.1 Cell wall structure of *Candida* (32)

Candida cell wall are mainly constructed by three main layers of carbohydrates, including mannans, β-glucans and chitins. Mannan is the outer most layer of the cell wall.

Candida cell wall mannan

Structures of *Candida* mannan

Mannan, the outermost layer of the cell wall, plays an essential role in host interaction, including the initiation and regulation of the anti-*Candida* host immune responses. *Candida* mannans are composed of α- and β-linked mannose units, arranged into three structures include linear O-linked mannan, highly branched N-linked mannan and phospholipomannan (33) (Fig. 2.2).

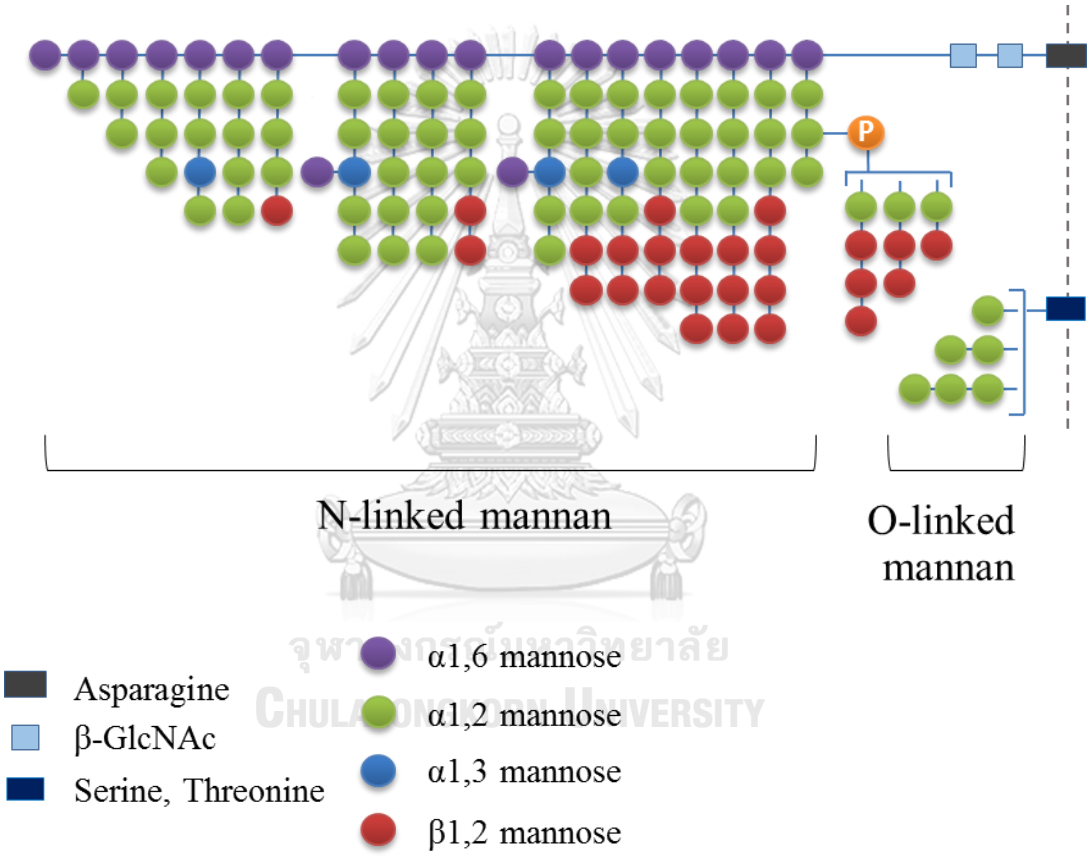


Figure 2.2 Structure of *Candida albicans* mannan (34)

C. albicans mannan consists of a short chain O-linked mannan oligosaccharide, and a branch of N-linked mannan polysaccharide moieties. This N-linked mannan contains a long chain of α-1,6-linked mannose backbone connected with an oligomannose side chain mainly containing α-1,2-, α-1,3-, and β-1,2-linked mannose residues with a few phosphate groups (35, 36)

Although cell walls of NACs are poorly investigated, different cell wall mannans of some *Candida* species have been identified (35) (Fig. 2.3). *C. tropicalis* shares mannan moieties with some serotypes of *C. albicans* (37). On the contrary, cell wall mannan of *C. glabrata* is small branches containing low α-mannan content and one or two units of β-1,2-linked mannose residues (38). *C. krusei* mannan is lightly branch that contains a long chain of α-1,2-linked mannose backbone with one or two α-1,6-linked mannose residues located in the middle of the chain, and short side chain of α-1,2-linked mannose residues (39, 40). These evidences suggest that different *Candida* species has distinct structures of cell wall mannan.

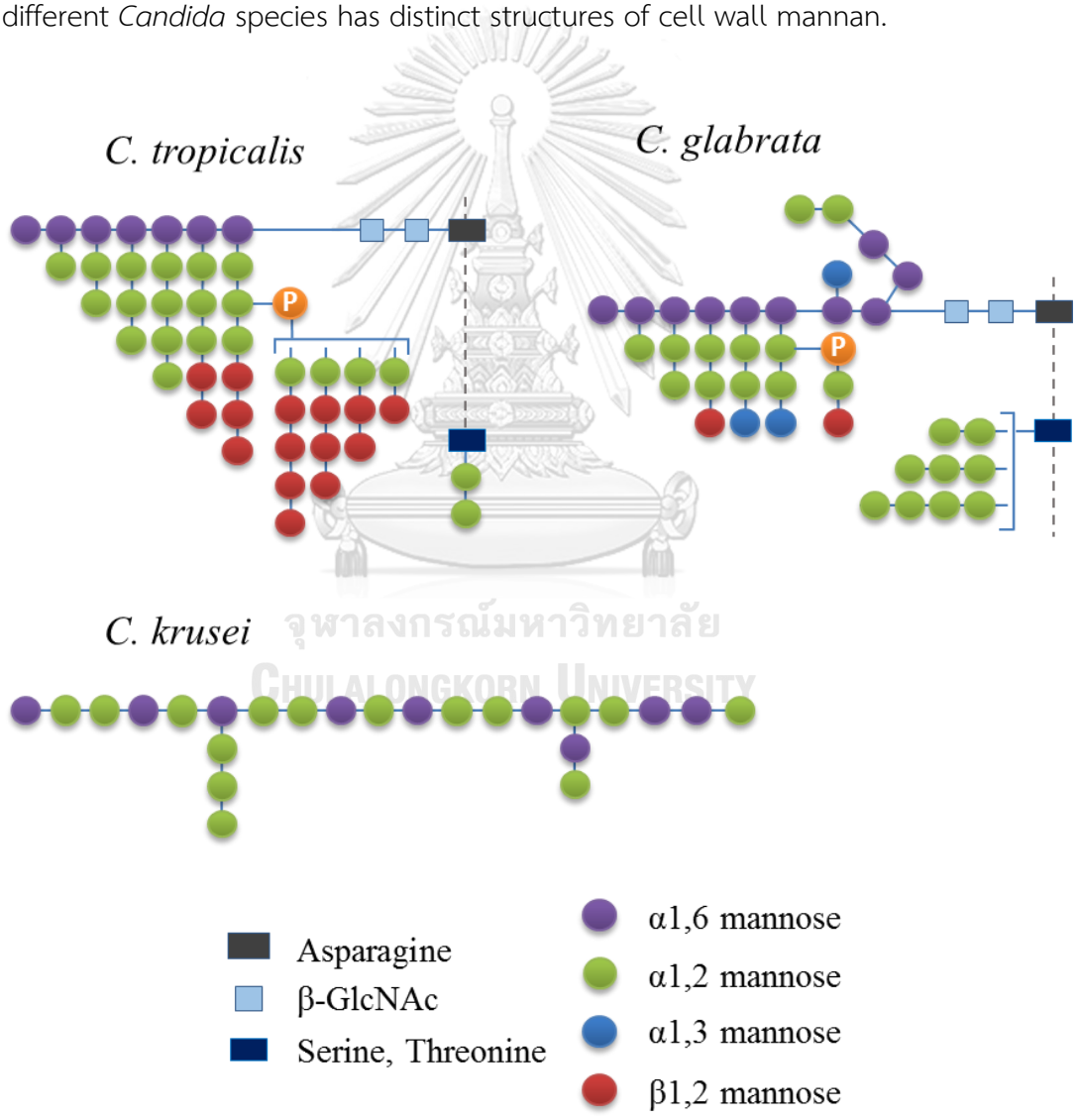


Figure 2.3 Mannan structures of some common NACs (37-39)

Role of mannan in host immune response

It has been reported that patients with systemic *Candida* infection have high circulating levels of mannan in blood. In addition, mannan is detected in early stage and associated with the disease severity (41). Therefore, mannan may initially interact with the host innate immune system. Mannan has been known for its ability to induce protective immune response. Host defense against *Candida albicans* requires the recognition of α-mannan by dectin-2, which initiates Th17 response (42). Furthermore, α-mannan on the surface of *Candida albicans* hyphae can be recognized by a heterodimer of dectin-3 and dectin-2, results in the activation of NF-κB (43). Mannan of *Candida parapsilosis* has showed its importance in IL-1β, TNFα and IL-6 stimulation, which trigger the inflammatory responses (44). On the contrary, mannan may act as a virulent factor for fungal invasion. *Candida albicans* mannan had been identified as a potent inhibitor of cell-mediated immunity in Candidiasis since 1978 (45). A study using *mnn10* mutant of *Candida albicans* found that N-linked mannan, which is the main structure of mannan, was critical for the pathogenesis of *Candida albicans* (46). In addition, dendritic cells reduced ability of producing inflammatory cytokines by interacting with β-1,2-mannosides in N-linked mannan of *Candida albicans* (47). These evidences suggest an important role of mannan in the initiation and regulation of host immune response. However, little is known about immune response against NACs mannans. Therefore, more studies should be generated to investigate the role of NACs mannan in immune response to strengthen the knowledge for fighting with these emerging threats.

Host defense against *Candida* infection

DCs in innate and adaptive immunity

Host defense against infections caused by fungi generally depends on the activation of innate immunity, followed by a stimulation of specific cellular immune responses mediated by T-cells or humoral immunity mediated by B-cells. In order to initiate an immune response, antigen presenting cell play a critical role due to their

abilities to capture, process and present antigen to T cell. Dendritic cells (DCs), which are considered as potent antigen-presenting cells, take the central position of the immune system due to their capacity to link innate and adaptive immunity. DCs bridge innate and adaptive immunity by shaping T cell responses following PRR-dependent cytokine production. Only DCs are able to prime naive T cells to generate life-long memory against pathogens. Their major functions are antigen presentation and activation of T cell responses as well as maintaining immune tolerance. For T cell initiation, DCs start an immune response by catching antigen and presenting it in the complex with peptide–major histocompatibility complex (MHC) molecule to antigen-inexperienced T cells which called naïve T cells in lymphoid tissues (48, 49).

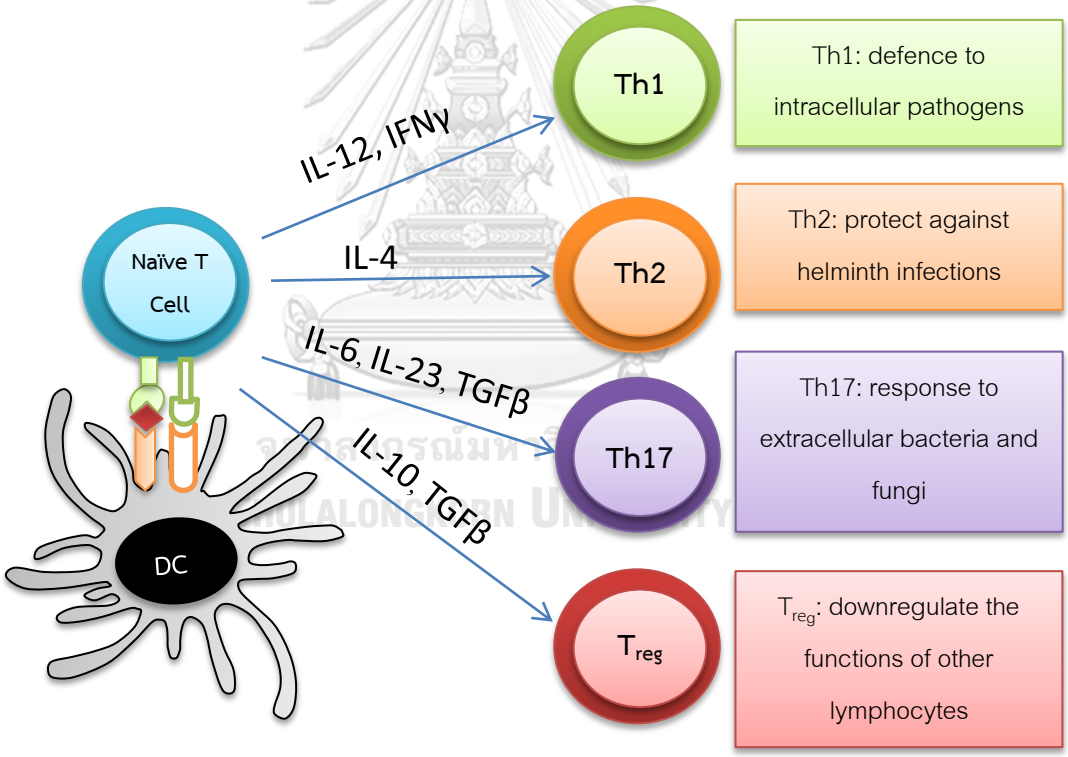


Figure 2.4 Mature dendritic cells secrete different types of cytokines to orchestrate the differentiation of helper T cell subsets (50).

After DCs in the peripheral tissues are activated by the invading pathogen, they become maturate and migrate into regional lymph nodes to present antigen and activate naïve T cell. Meanwhile, the mature DCs secrete cytokines to promote and dictate the

differentiation of effector T cell subsets including: T helper (Th) 1, Th2, Th17 and T_{reg}. Each T cell subsets has distinct function and role in immune response.

DCs additionally have an imperative part in controlling humoral immunity by interacting directly with B cells or indirectly through the helper T cells populations to promote B cell activation and antibody production. DCs can also interact with several types of innate immune cells such as natural killer (NK) cells, mononuclear phagocytes and mast cells for initiating an acute response against infection. Differentiated DCs, which have not experienced any pathogenic antigen, are considered as immature DCs. They are broadly resident in peripheral tissue and have high ability of phagocytosis but low capacity of antigen presentation and low expression of costimulatory molecules. After encountering pathogens, DCs become mature or activated, and undergo a maturation program resulting in up-regulation of MHC molecules, CD40 and costimulatory molecules for example: CD80, CD86, as well as the acquisition of CCR7, which permits migration of the DCs into the draining lymph node. In the interaction with T cells in draining lymph node, mature DCs can polarize the differentiation of naïve $CD4^+$ T cells and $CD8^+$ T cells T into appropriated antigen-specific effector T cells with unique functions and cytokine profiles according to different types of pathogenic antigens. After the activation, CD4 T cells will be differentiated into T helper 1 (Th1), Th2, Th17, or regulatory T cells (T_{Reg}) with different profies of cytokine production. In order to prime T cell differentiation, DCs secrete cytokines such as interferons (IFNs) and interleukin (IL)-12, critical for generation of Th1 differentiation, or IL-4, critical for Th2 polarization, or IL-6 and IL-23 and TGF-β for Th17 generation, or IL-10, which are important for regulatory T cell differentiation (51-53). Among antigen-specific T cell subsets primed by DCs, Th1 cells play a critical role in protection against intracellular microbes, Th2 cells are important to fight against helminths, Th17 cells help to resist extracellular bacilli, or T follicular helper (T_{FH}) cells help B cells to differentiate into antibody-secreting cells. On the other hand, naïve $CD8^+$ T cells can differentiate to effector CTLs which play central role in killing cell infected with virus or cancer cells (49). By contrast, self-

antigen presentation by immature DCs to T cells results in a situation called immune tolerance characterized either by T cell anergy or the raising differentiation of regulatory T cells (FoxP3$^+$ T cell) which can inhibit other lymphocyte functions (54). Thus, the ability to control the fate of the immune response makes DCs both central to balancing immunity and a prime target for vaccine development against the fungi.

DCs subset characteristics

Basing on the function and the expression of surface markers, DCs are divided into two main subsets: conventional (c)DCs and plasmacytoid (p)DCs. pDCs is a small group of DCs that lowly express major histocompatibility complex class II (MHC-II), costimulatory molecules and CD11c in the steady state. They have ability to produce large amount of IFN-α in response to virus via the sensing of Toll-like receptors (TLRs) 7 and 9. On the other hand, cDCs represent all other DCs in both lymphoid and nonlymphoid tissues, characterized by high expression of CD11c and MHC-II on their surface. cDCs have high abilities to capture damaged tissue, environmental- and seft-antigens, as well as superior capacities of antigen processing, migration into lymnodes, presentation phagocytosed antigens to T cells and priming naïve T cell activation and polarization. Because of these characteristics, most studies focused on the role of cDCs when they investigated the host- fungal pathogen interaction (55, 56).

Furthermore, cDC population consists of several distinct subsets. These subsets were characterized by their differences in developmental specificities, functions and phenotypes. In nonlymphoid tissue, mouse cDCs have two major subsets included CD103$^+$CD11b$^-$ and CD11b$^+$ cDC. CD103$^+$CD11b$^-$ cDCs (or CD103$^+$ cDCs) reside predominantly at the interface with the environment and express abundant of receptors for sensing pathogenic antigens and tissue damages. They also have high ability of migration into draining lymph nodes to present antigen to T cells. CD103$^+$ cDCs have been reported that they are more potent for activating CD8$^+$ T cell immune responses than other cDC subsets not only because of their enhanced capacity of cross-presentation but also their increased CD8$^+$ T cell-generation

potential by secreting IL-12 and IL-15 (56). On the other hand, dermal $CD11b^+$ cDCs show predominant role in MHC-II presentation and drive the generation of antigen-specific $CD4^+$ T cells (57). cDCs characterized as nonlymphoid tissue DCs but found in peripheral lymph nodes are called tissue-migratory cDCs. Lymphoid tissue-resident cDCs have different characteristics. They consist mainly of two subpopulations, $CD8^+$ and $CD11b^+$ cDCs. Lymphoid tissue $CD8^+$ cDCs are similar to nonlyphoid tissue $CD103^+$ cDCs in origin, phenotype, transcriptional profile and functions. Thymic $CD11b^+$ DCs are best identified by the expression of CD172a, more mature phenotype, and produce more amount of the $CD4^+$ T cell attractant chemokines in comparison with their $CD8^+$ counterparts (58).

Because of limitation in collecting sample from human, studies about human DCs usually isolated DCs from blood and skin. In human skin, dermal cDCs are divided into 2 subsets: $CD1a^+CD14^-$ DCs and $CD1a^-CD14^+$ DCs. In human blood circulation, people found 2 major subsets that expressed non-overlapping markers: CD1c and CD141. When activated with TLR3 ligand, $CD141^+$ DCs can secrete high amount of IL-12 and type I IFN and cross-present antigen to CD8 T cells . However, $CD1c^+$ cDCs are predominant in human blood and also can produce IL-12 and cross prime $CD8^+$ T cell differentiation (59). $CD1c^+$ and $CD141^+$ cDCs whose phenotypes are resemble blood cDCs are also found in human spleen and lymph nodes and considered as lymphoid tissue-resident DCs (60).

Epidermal Langerhans cell (LC) is a special cDCs subset that locates in the epidermis of skin. Different from other cDCs, LC express lower MHC-II and CD11c, but extremely high CD207 (C-type lectin langerin). LCs could have tolerogenic function in steady state by induce T_{Reg} or protective effect in inflamed condition by limiting T_{Reg} (61).

DCs and fungi interaction

Different pathogenic microorganisms will give rise to at least partly different immune responses. This can be seen already at the DC level, by studying how the different DCs respond to various pathogens and antigens. For recognition, DCs

express a large variety of pattern recognition receptors (PRRs) that bind to pathogen-associated molecular patterns (PAMPs) in fungi. Activation of PRRs induces downstream intracellular signaling pathways that initiate the maturation of DCs by high expressing MHC, CD40, costimulatory molecules and secreting cytokines that drive T cell differentiation.

As *Candida albicans* has been the most common pathogen causing fungal infection, the immune responses against this species were broadly investigated. Among them, responses of DCs in the interaction with *Candida albicans* were also reported in some papers. *Candida albicans* can growth in two forms, included unicellular yeast and filamentous form as hyphae. DCs could uptake both forms, however, they gave different responses. The difference has been demonstrated in mice that the yeast form of the *Candida albicans* induced IL-12 production and priming of Th1 cells, hyphae inhibited IL-12 and Th1 generation while produced a lot of IL-4, an important cytokine for Th2 differentiation (62, 63). However, very few studies have been performed to investigate the immune response of DCs against NACs, especially the recently increasing prevalence infection-caused species (*Candida parapsilosis*, *Candida tropicalis, Candida glabrata, Candida krusei* and *Candida dubliniensis*). Because of the powerful function of DCs in regulating immune response against infectious disease, it is necessary to understand more about the interaction between DCs and NACs.

Among the PRRs that can recognize these PAMPs, Toll-like receptors (TLRs) and C-type lectin receptors are best investigated. The major TLRs playing a role in recognizing fungal structures such as fungal DNA, zymozan, O-linked mannans and phospholipomannan are TLR9, TLR2 and TLR4 (64). These TLRs require adaptor myeloid differentiation primary response protein 88 (MyD88) for transferring the signals, following by the induction of proinflammatory cytokines and resulting in driving Th1 differentiation for antifungal responses (55). It is unknown clearly about the involvement of TLRs in the induction of fungal-specific Th17 cells. On the other hand, mice with deficiencies in CLRs showed high susceptibility to fungal infections suggested that CLRs play an important role in fungal sensing and activation of

antifungal immune response. Among the CLR family members, Dectin 1, Dectin 2, macrophage-inducible C-type lectin (Mincle), DC-specific ICAM3-grabbing non-integrin (DC-SIGN), macrophage mannose receptor (MR) were confirmed for their abilities of recognizing fungal carbohydrates and initiating protective innate and adaptive immune response against fungi (65). Dectin 1 mainly binds to β-glucans on fungal cell wall and send signals into two independent downstream signaling pathways includes spleen tyrosine kinase (SYK) and RAF pathway, leading to NF-κB activation, resulting in inducing production of pro- and anti-inflammatory cytokines and chemokines that drive the differentiation of Th1 and Th17 cells (66, 67). Dectin 2 and Mincle recognize high-mannose structures and pair with adaptor FcRγ to induce cytokine releasing through SYK-CARD9 pathway. In addition, Dectin 2 activated by α-mannan induced the polarization of Th17 (42, 68). Besides, N-linked mannans are recognized by the mannose receptor and DC-SIGN on DCs and these ligations directly involved in the producing of IL-6, an important cytokines for Th17 generation (69, 70) (Fig. 2.5).

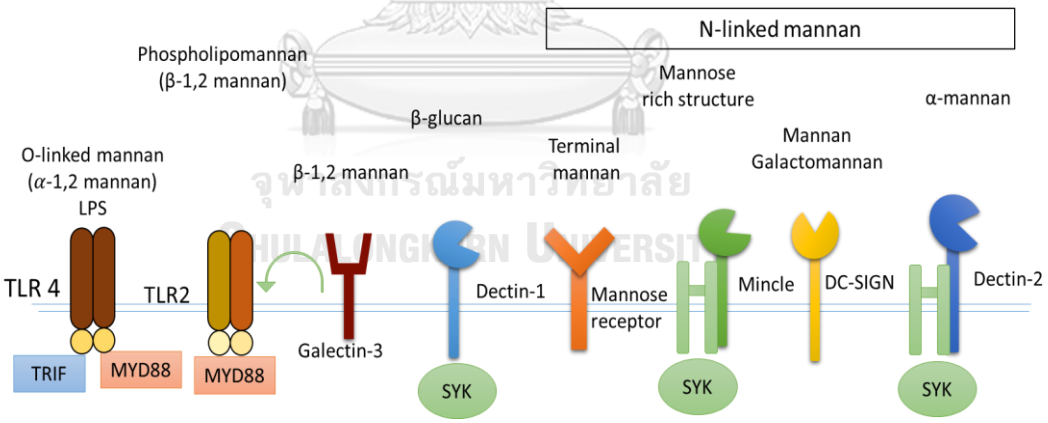


Figure 2.5 Pattern recognition receptors sensing *C. albicans* (35)

CLRs and TLRs are the major receptor groups on DCs that responsible for recognition structures of *Candida* cell wall components. For examples, TLR2 can recognize phospholipomannan, mainly composed of β-1,2 mannan, and activate MyD88 pathway. Dectin-2 can recognize α-mannan and activate DC response via SYK.

Studies investigated the role of mannosylation of *Candida albicans* mannan in the interaction with immune host cells gave evidences that different structures of mannan may affect to recognition and response ability of the host. For instance, deletion of genes encode glycosidase that processes N-mannan core resulted in the decreased production of pro-inflammatory cytokines by human monocytes (71). β-mannans has been known for their inhibitory effect on pro-inflammatory cytokines production (IL-6, IL-12 and TNF-α) (47).

The unique mannan structure of each *Candida* spp. (Fig. 2.2 and 2.3) might lead to specific responses of DCs when they were recognized by multiple host PRRs. During the process of a fungal recognition, a variety of PRRs may be stimulated by PAMPs in different affinity between the fungi and host or in different combinations among the PRRs depending on the fungal morphologies, species and on the host cell types. Therefore, the final immune response will depend not only on the relative degree of stimulation of the individual receptors but also on the level of receptor cooperation and the cellular localization (64).

Taken together, this study arms to investigate DC responses against common NACs in comparison with *Candida albicans*, focusing on their cell wall mannans. In the context of high resistance to antifungal agents of NACs, new knowledge on the mechanisms of host defense against *Candida* infections can lead to the development of novel immunotherapeutic approaches include vaccination, therapeutic antibodies, recombinant cytokines and adoptive transfer of primed immune cells to combat these emerging pathogens.

Chapter 3

RESEARCH METHODOLOGY

Cultivation of *Candida* yeasts

Six *Candida* species selected for our study are as follow: *C. albicans* (ATCC 24433), *C. krusei* (ATCC 6258), *C. glabrata* (ATCC 2001), *C. tropicalis* (ATCC 750), *C. parapsilosis* (ATCC 90018) and *C. dubliniensis* (NCPF 3949 or MYA-646). The strains chosen for *C.albicans*, *C. krusei*, *C. glabrata*, *C. tropicalis* and *C.parapsilosis* are reference strains for quality control and antifungal drug susceptibility testing, while the strain for *C. dubliniensis* is the only available standard strain (Microbiologics Inc., Minnesota, USA). The fungi were grown on YPD agar (2% yeast extract, 1% peptone, 2% dextrose, and 2% agar) (HiMedia, Mumbai, India and Ajax Finechem, NSW, New Zealand) at 30°C for 2 days. The colonies were subcultured in YPD broth at 30°C with 200 rpm shaking for 8 h to reach the log phase. In some experiments, the yeast cultured were then diluted to OD600 of 0.05, and cultured at 30°C for 14 h with 145 rpm shaking.

Preparation of heat-inactivated yeasts

Yeast cells were harvested, washed twice, and diluted to a concentration of $1x10^8$ cells/ml in sterile phosphate buffered saline (PBS; GIBCO, ThermoFisher Scientific, NY, USA). The cells were then inactivated by heating at $100^{\circ}C$ for 10 minutes. The heat-inactivated (HI) yeast cells were collected by centrifugation and resuspended in complete RPMI at a concentration of $1x10^8$ cells/ml.

Isolation and purification of mannan

Mannan extraction was performed as previously described (72), with minor modifications. Briefly, one hundred grams of wet *Candida* yeast cell pellets were

resuspended in 250 ml of citrate buffer, pH 7.0, 0.02 M for *C. albicans*, *C. krusei*, *C. glabrata*, *C. tropicalis*, and 0.1 M for *C. parapsilosis* and *C. dubliniensis*. We used 0.1 M citrate buffer for the mannan extraction of *C. parapsilosis* and *C. dubliniensis* since the lower concentrations (0.05 and 0.02 M) could not extract the mannan from these organisms. To rule out the possibility that the different buffer concentrations used could affect our results, we compared the *C. albicans* mannan that was extracted with 0.1 M and 0.02 M citrate buffer, and found that there was no difference in the amount of mannan or BMDC responses (data not shown). Next, the yeast suspension was autoclaved at 121°C for 90 min, and the supernatant were separated and collected. Thereafter, the remaining yeast cells were resuspended in 375 ml of the same buffer, and re-autoclaved at 121°C for 90 min. The supernatant from the second round of autoclaving was collected and combined with that from the first round. Equal volume of Fehling's solution was then added to the total supernatant, and the mixture was stirred overnight at 4°C. The sediment was collected and dissolved in 6-8 ml of 3N hydrochloric acid (HCl), and the solution was added drop-wise into 100 ml of methanol-acetic acid (ratio 8: 1, v/v). The mannan precipitate was collected by centrifugation and washed with HCl and methanol-acetic acid repeatedly until the precipitate becomes colorless. Mannan was then dissolved in sterile water. Mannans from *C. albicans, C. krusei*, *C. glabrata* and *C. tropicalis*, were further dialyzed in sterile water. Since the dialysis method caused a tremendous loss of *C. parapsilosis* and *C. dubliniensis* mannan, we used Biogel P-4 (Bio-Rad, CA, USA) column as the alternative method for changing the buffer. The concentration of mannan was determined using phenol-sulfuric acid method (73). Mannan was kept in lyophilized form. Endotoxin free water was used for yeast culture and mannan extraction, and endotoxin was eliminated from all glassware by dry heat method.

Scanning Electron Microscopy

To observe the morphology and microscopic structures of live and HI *Candida* cells, the samples were prepared for scanning electron microscopic analysis as described previously (74). Briefly, *Candida* cells were fixed in 2% glutaraldehyde in

0.1 M phosphate buffer, pH 7.2 for 18 h at 4°C. Then, the fixed yeast cells were washed with PBS and deposited on 0.02 μM membrane filter. Post-fixation procedure was performed using 2% osmium tetroxide for 2 h at room temperature. The specimens were dehydrated in a series of ethanol gradients, critical point-dried in CO_2 (K850, Quarum Technologies, UK), coated with gold (JFC-1200, Jeol, USA), and investigated by a scanning electron microscope (Quanta250, FEI, USA) with 20,000x and 50,000x magnifications.

Animals

Five to eight-week-old female BALB/c mice were purchased from National Laboratory Animal Center, Mahidol University) and were housed at Chulalongkorn University Lab Animal Center. All animal experiments were performed in accordance with the protocol approved by the Institutional Animal Use and Care Committee of Chulalongkorn University Lab Animal Center (Protocol number 1573005).

Generation and stimulation of bone marrow-derived dendritic cells (BMDCs)

BMDCs were generated as previously described (75). Briefly, bone marrow cells were flushed out from femur and tibia of mice. The cells were seeded in 24-well plates at 1×10^6 cells in 1 ml per well, and were cultured in DC medium containing RPMI 1640 (GIBCO, ThermoFisher Scientific, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO, ThermoFisher Scientific, NY, USA), 0.2 mM Glutamax (GIBCO, ThermoFisher Scientific, NY, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (HyClone, UT, USA), 10 ng/ml recombinant murine GM-CSF and 10 ng/ml recombinant murine IL-4 (Peprotech, NJ, USA). The cell culture plates were incubated at 37°C with 5% CO2 for 7 days. The half of culture media was replaced at day 2, 4 and 6.

On day 7, BM-DCs were stimulated with HI *Candida* yeasts or soluble mannan extracted from *Candida* cell wall at the concentration of 12.5, 25 and 50 μg/ml for 24-48 h. After stimulation, cultured media were collected for cytokine measurement and the cells were harvested for investigating the expression of DC maturation

markers. Unstimulated BMDCs were used as the negative control, and lipopolysaccharide (LPS) stimulated BM-DCs were used as the positive control. LPS was purchased from Sigma-Aldrich, MO, USA.

Cell viability assay

To determine the cell viability, BMDCs were cultured in 96-well plate at $1x10^5$ cells/well in 200 µl DC medium. BMDCs were stimulated with HI *Candida* yeasts or soluble mannan for 48 h. After stimulation, 20 µl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Life technologies, ThermoFisher Scientific, OR, USA) were added into each well, and the culture plates were incubated in the dark, at 37oC with 5% CO2 for 90 min. Dimethyl sulfoxide (DMSO; AMRESCO, OH, USA) was used to dissolve the purple formazan crystals produced by live cells. The optical density (OD) was measured at 570 nm using a microplate reader (Synergy H1, BioTek, VT, USA). The percent of cell viability was calculated by normalization with the negative control.

Treatment with inhibitors and TLR antagonists

For SYK inhibition, BMDCs were pre-incubated with 1 µM SYK inhibitor (R406; InvivoGen, CA, USA) for 30 min prior to stimulation. DMSO 0.1% was use as the negative control of SYK inhibitor (76).

For MYD88 inhibition, BMDCs were pre-incubated with 100 µM MYD88 inhibitor peptide (NBP2-29328; Novus Biologicals, CO, USA) or control peptide for 24 h prior to stimulation (77).

For TLR blockade, BMDCs were pre-incubated with anti-mouse/human TLR2 mAb (Biolegend, CA, USA) or anti-mouse TLR4 mAb (Biolegend) for 2 h prior to stimulation. Rat IgG was used as the isotype control.

***In vivo* immunization and *ex vivo* re-stimulation assay**

Twenty-five of eight-week-old female BALB/c mice were divided in to 5 groups, with 5 animals per group. One group is negative control. The other 4 groups

are experimental group for investigating the effect of C. *albicans*, C. *krusei*, C. glabrata and C. tropicalis mannans in vivo. Mice were subcutaneously injection in the scruff of the neck with the the mixture of *Candida* mannan (50 µg mannan per 1 g of body weight) and OVA (30 µg per animal; Sigma Aldrich) in 200 µl PBS at day 0 and day 7. On day 14, mice were sacrificed using CO_2 chamber. The cervical, axillary and brachial LNs were excised, digested with collagenase type IV (Sigma Aldrich) at 37^{o}C for 30 min, and subsequently incubated with DNase I (Sigma Aldrich) at room temperature for 10 min. The cells were washed, and resuspended in RPMI 1640 supplemented with 10% heat-inactivated FBS, 0.2 mM Glutamax, 100 U/ml penicillin and 100 mg/ml streptomycin, and 55 µM 2ME (GIBCO). For the negative control, mice were immunized with OVA in PBS.

For *ex vivo* re-stimulation assay, LN cells (4 x 10^6 cells) were cultured in 24-well plates in 1 ml media at the presence or absence of 250 µg/ml OVA. The culture supernatants were collected at 48 h and 72 h after OVA stimulation.

In vitro OT-II T cell stimulation

Splenocytes from OT-II mice were provided by Dr. Jin Mo Park (Cutaneous Biology Research Center, Massachusetts General Hospital/Harvard Medical School, Boston, MA), and T cells were enriched by immunomagnetic beads (Pan T Cell Isolation Kit II, mouse; Miltenyi Biotec, CA, USA). BMDCs were stimulated with *Candida* mannan for 30 h and pulsed with 500 µg/ml whole OVA protein overnight, and washed twice with the media. Then, the mannan-stimulated OVA-pulsed BMDCs were co-cultured with OT-II T cells at T:DC ratio of 10:1. The supernatants were collected at 48 h and 72 h for cytokine determination by ELISA.

Measurement of cytokines

Supernatants collected from BMDCs, LN cell and OT-II T cell cultures at indicated time points were quantitated by standard sandwich Enzyme-Linked Immunosorbent Assay (ELISA) using commercially available paired antibody sets for IL-1β, IL-4, IL-6, IL-10, IL-12, IL-17, IL-23, IFN-γ and TNF-α. ELISA sets were purchased

from Biolegend, CA, USA and eBioscience, San Diego, CA. The procedures were performed according to the manufacturer's instruction.

Flow cytometric analysis

All cells were incubated with Fc Block (anti-CD16/CD32; Biolegend) before labeling with the specific antibody. To determine BMDC maturation, the cells were labeled with fluorochrome-conjugated mAb against murine CD11c, CD40, CD80, CD86 and I-A/I-E (Biolegend). For apoptosis assay, BMDC were labeled with annexin V and 7-AAD (Biolegend). To assess immune cell population, LN cells were labeled with fluorochrome-conjugated mAb against murine B220, CD3**ε**, CD4, CD8a, CD44, CD62L, IFN-**γ**, IL-4 (Biolegend), IL-17A and FoxP3 (eBioscience, CA, USA). For intracellular staining, the cells were incubated with 50 ng/ml PMA (Sigma, MO, USA), 1 μM ionomycin (Sigma Aldrich), and 2 μg/ml Brefeldin A (PanReac AppliChem, Darmstadt, Germany) for 4 h prior to staining. The isotype matched antibodies were used as the control. All stained cells were acquired on a flow cytometry (CytoFLEX, Beckman Coulter, CA, USA) and the data were analyzed on by CellQuest Pro software (BD Bioscience) or Kaluza Flow Analysis Software (Beckman Coulter).

Statistical Analysis

All data values were expressed as mean ± SD, and the sample size was indicated in each figure legend. The statistical analysis was performed using one-way ANOVA with post-hoc Turkey HSD test for the comparison of 3-5 groups, and using Student's T test for the comparison between 2 groups. Values of $p<0.05$ were considered significant.

Chapter 4

RESULT AND DISCUSSION

Part I

Objective 1: To determine the impact of cell wall mannans of *Candida albicans* and systemic candidiasis-related non-*albicans Candida* on DC and T cell responses *in vitro* and *in vivo*.

Results

C. krusei mannan induced DC maturation and triggered the massive production of pro-inflammatory cytokines

DC maturation is essential for T cell activation. As a result, it is important to link innate and adaptive immunity in response to pathogens (48). To evaluate whether cell wall mannans extracted from four distinct *Candida* species differentially affected the phenotypic maturation of DCs, BMDCs were cultured and then stimulated at day 7 with the various concentrations of mannans (12.5, 25 and 50 μg/ml) for 24 h. BMDC were subsequently characterized by the flow cytometric analysis of the maturation markers, including CD40, CD80, CD86 and MHC class II (Fig. 4.1, 4.2 and 4.3). DC population were first identified by gating on a DC marker, CD11c (Fig. 4.1 A), and geometric mean fluorescent intensity (MFI) of expression of the maturation markers were assessed using a histogram analysis (Fig. 4.1 B and 4.3 A). Unstimulated BMDCs were used as the negative control. BMDCs stimulated with *C. albicans* and *C. tropicalis* mannan did not undergo maturation when compared to the negative control, while those stimulated with *C. krusei* and *C. glabrata* mannan were potentially activated. *C. krusei* mannan upregulated the expression of CD40, CD86 and MHC class II on BMDCs, and further induced the highest level of CD40, especially at the highest mannan concentration. Although, *C. glabrata* mannan obviously induced CD40, CD80, CD86 and MHC Class II expression on BMDCs, those expression were slightly different from BMDCs stimulated with *C. krusei* mannan.

To determine the number of DCs that underwent maturation, the dot plot analysis was performed to evaluated the percentage of $CD11c^{+}CD40^{+}$, $CD11c^{+}CD80^{+}$, $CD11c^{+}CD86^{+}$ and $CD11c^{+}$MHC class II^{+} cells (Fig. 4.2 and 4.3 B). The results of the percentage of each DC subpopulations were consistent to their geometric MFI level (Fig. 4.3 A and 4.3 B).

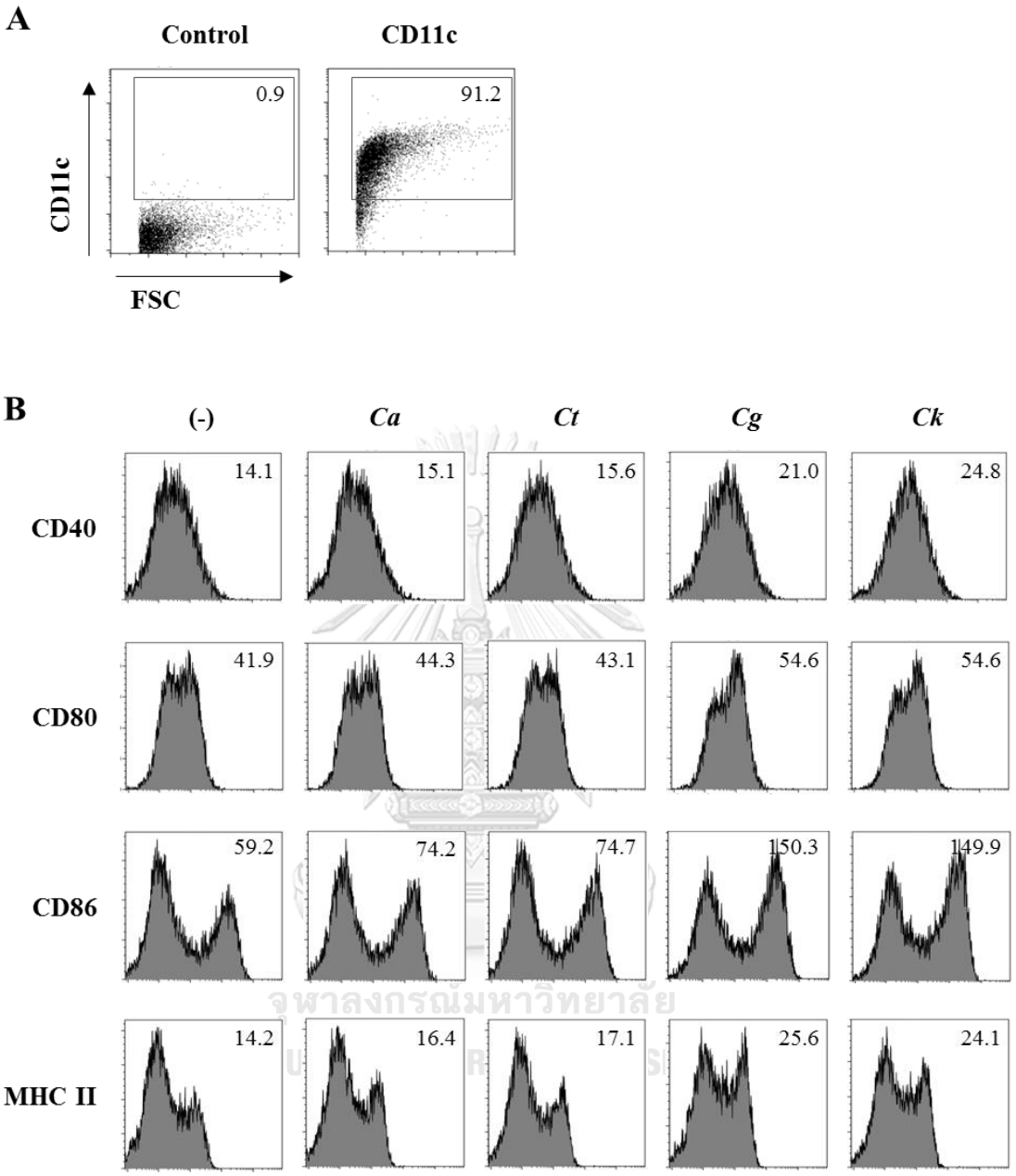


Figure 4.1 Flow cytometric analysis of the expression level of DC maturation markers

BMDCs were stimulated with *Candida* mannans, and the expression level of CD40, CD80, CD86 and MHC class II on $CD11c^+$ cells was assessed using flow cytometry. The dot plot analysis of BMDC was first shown using side scatter (SSC) and forward scatter (FSC), and the live cells were gated. **(A)** DCs were identified by gating on $CD11c^+$ population. The left panel is isotype control staining, and the right panel is CD11c staining. The number indicated the percentage of $CD11c^+$ cells. **(B)** Histogram analysis of CD40, CD80, CD86 and MHC class II expression on $CD11c^+$ cells. The number indicated the geometric MFI; (-), unstimulated BMDCs; *Ca*, *C. albicans*; *Ct*, *C. tropicalis*; *Cg*, *C. glabrata*; *Ck*, *C. krusei*.

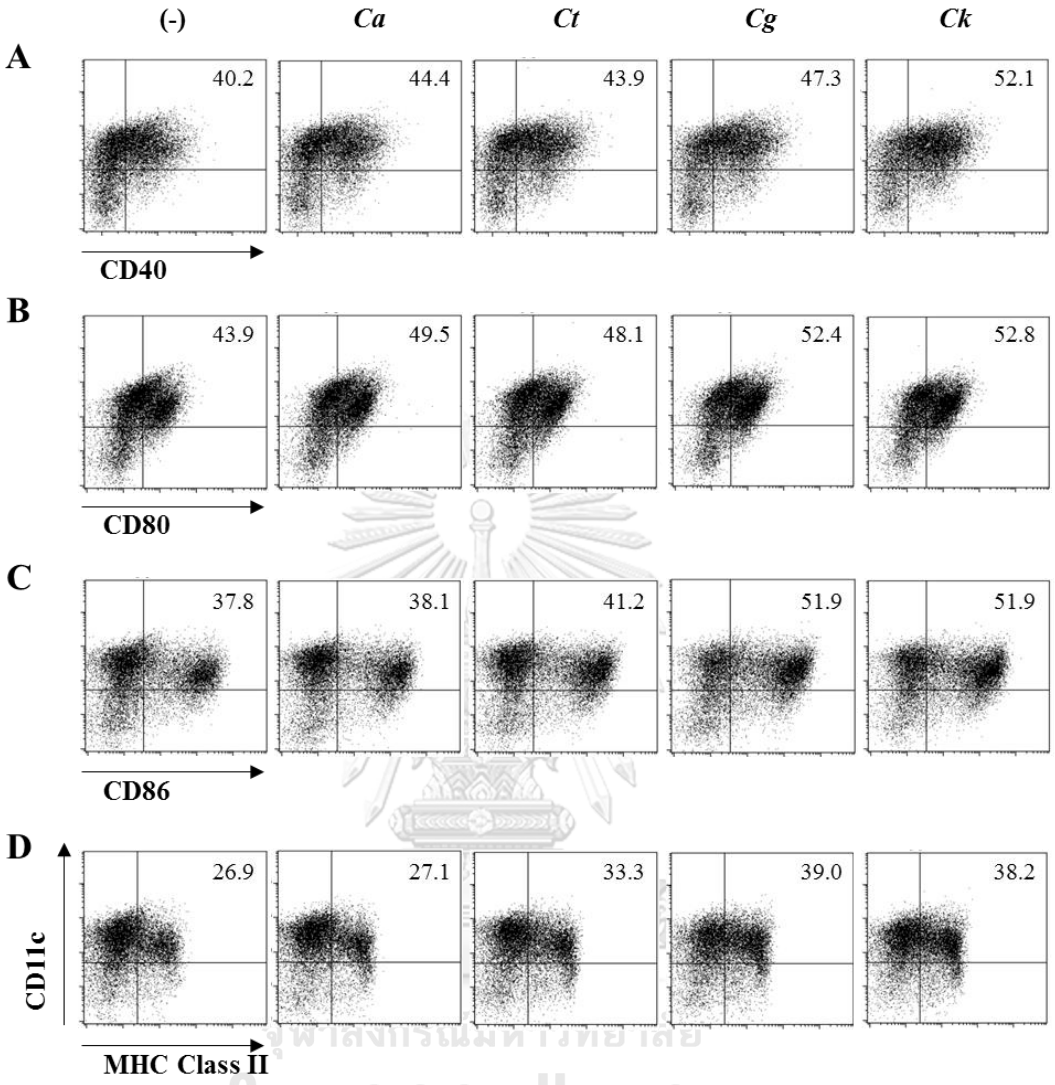


Figure 4.2 Flow cytometric analysis of the percentage of maturated $CD11c^+$ cells

BMDCs were stimulated with *Candida* mannans, and the percentage of $CD40^+$, $CD80^+$, $CD86^+$ and MHC class II^+ cells within $CD11c^+$ population were determined using flow cytometry. The live cells were gated based on SSC and FSC, and $CD11c^+CD40^+$, $CD11c^+CD80^+$, $CD11c^+CD86^+$ and $CD11c^+$MHC class II^+ cells were analyzed as shown in the dot plot. The number indicated the percentage of double positive cells. (-), unstimulated BMDCs; *Ca*, *C. albicans*; *Ct*, *C. tropicalis*; *Cg*, *C. glabrata*; *Ck*, *C. krusei*.

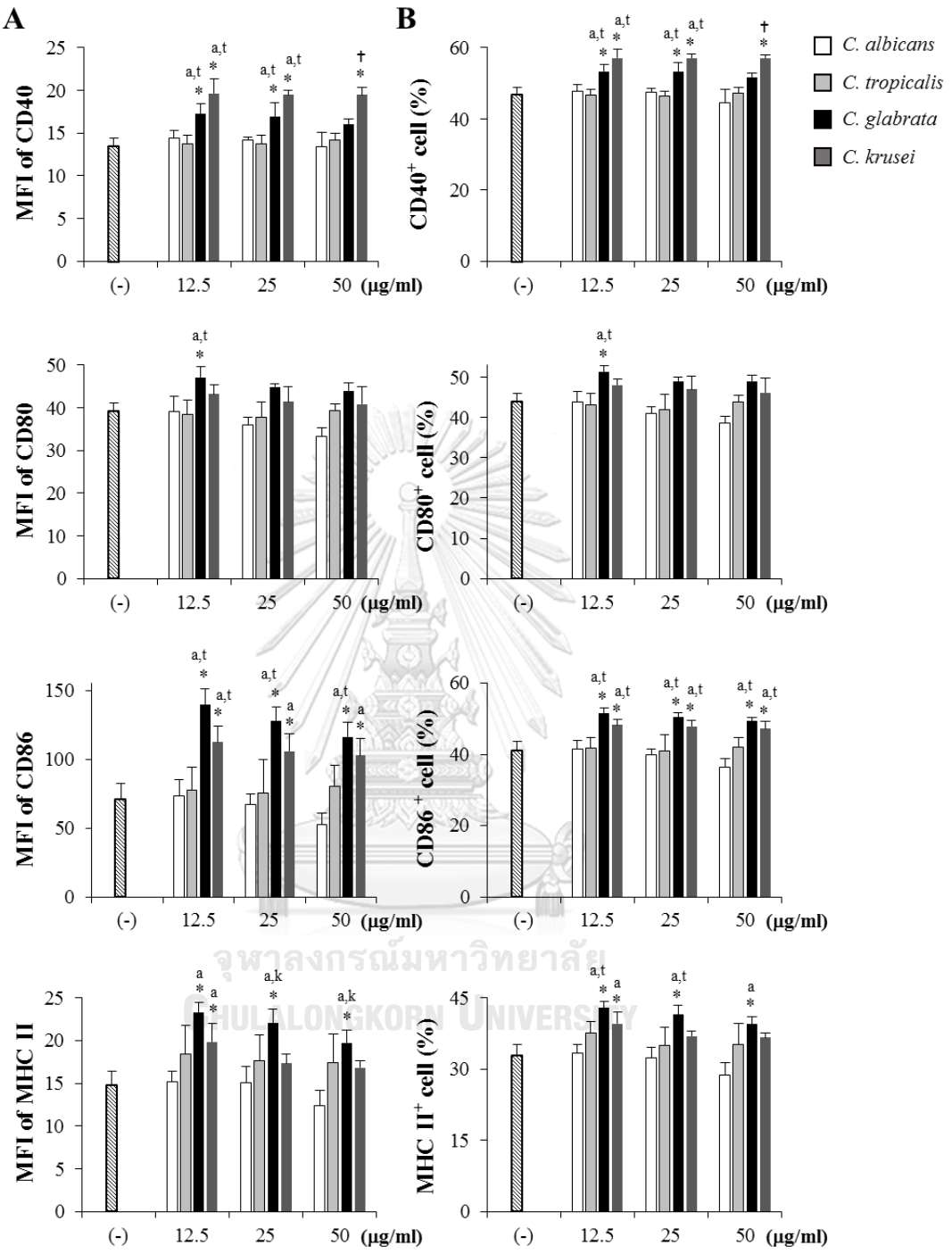


Figure 4.3 Differential phenotypic maturation of BMDCs in response to *Candida* mannans

BMDCs were stimulated with *Candida* mannans at the indicated concentrations. Twenty-four hours later, the expression of CD11c, CD40, CD80, CD86 and MHC class II were assessed using flow cytometry as shown in Fig. 4.1 and 4.2. Live BMDCs were defined

based on side scatter (SSC) and forward scatter (FSC), and an electronic gate was placed on the $CD11c^+$ cells to determine DC population. **(A)** Subsequently, the geometric mean fluorescence intensity (MFI) were evaluated using histogram analysis, and **(B)** the percentage of $CD40^+$, $CD80^+$, $CD86^+$ and MHC class II^+ cells within $CD11c^+$ population were determined. * $p<0.05$ when compared to the unstimulated BMDCs, ^{a} $p<0.05$ when compared to *C. albicans*, ^{t} $p<0.05$ when compared to *C. tropicalis*, ^{g} $p<0.05$ when compared to *C. glabrata,* ^{k} $p<0.05$ when compared to *C. krusei,* † $p<0.05$ when compared to all groups; n = 5; Data are representatives of two independent experiments. (-), unstimulated BMDCs; *Ca*, *C. albicans*; *Ct*, *C. tropicalis*; *Cg*, *C. glabrata*; *Ck*, *C. krusei*.

Next, we quantitated the production of pro-inflammatory cytokines, TNF-α, IL-1β, IL-6, IL-23, IFN-γ, IL-12, and anti-inflammatory cytokines, IL-4 and IL-10 by DCs, in response to *Candida* mannans (Fig. 4.4). BMDCs were stimulated with the various concentrations of *Candida* mannans (12.5, 25 and 50 μg/ml) for 24-48 h. Supernatants of cell culture were collected and used for ELISA to quantify amount of cytokines secreted by DCs. *C. albicans* and *C. tropicalis* mannan induced the significant level of IFN-γ in BMDCs when compared to the negative control. However, in parallel to the DC maturation, *C. albicans* and *C. tropicalis* mannans could not stimulate BMDCs to produce the other cytokines. *C. glabrata* mannan significantly upregulated IFN-γ, and slightly promoted IL-6 and IL-23 production, however, *C. glabrata* mannan failed to stimulate BMDC to produce the other cytokines regardless of its high capability of DC activation. Of note, *C. krusei* mannan at all concentrations augmented the massive production of TNF-α, IL-6, IL-23, IFN-γ and IL-12. All *Candida* mannans could not promote the production of the anti-inflammatory cytokines, IL-4 and IL-10.

Our findings suggest that the cell wall mannan from the distinct *Candida* species differentially impact on DC maturations and functions, and *C. krusei* mannan, in particular, elicit the robust inflammatory responses of DCs.

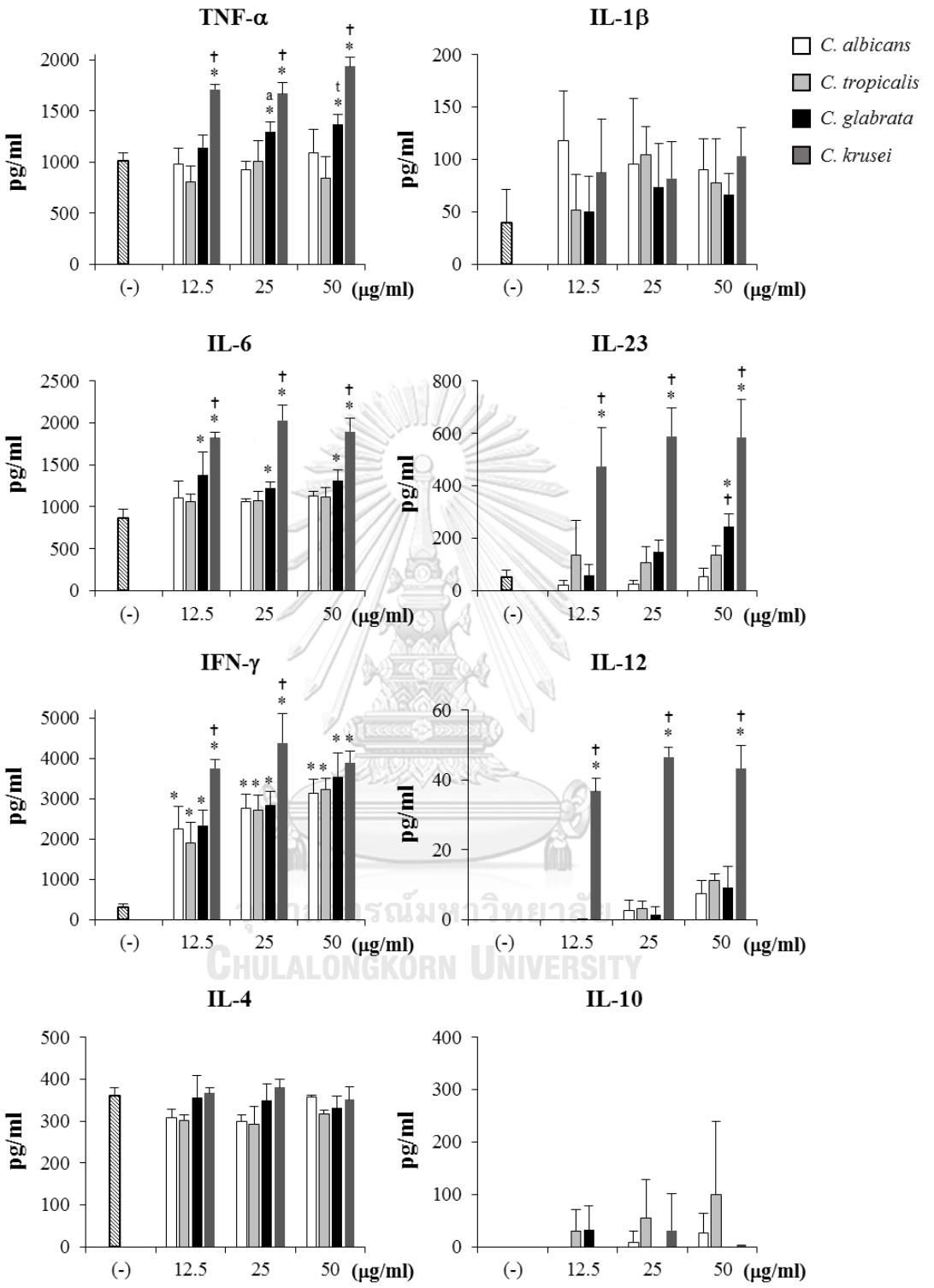


Figure 4.4 *C. krusei* mannan induced massive pro-inflammatory cytokine production

BMDCs were stimulated with *Candida* mannans at the indicated concentrations and the cytokine production in the culture supernatant was measured by ELISA. The level of

TNF-α, IL-6, IL-10 and IL-23 was detected at 24 h after stimulation, and the level of IL-1β, IFN-γ, IL-12 and IL-4 was detected at 48 h after stimulation. * $p<0.05$ when compared to the unstimulated BMDCs, ^{a} $p<0.05$ when compared to *C. albicans*, ^{t} $p<0.05$ when compared to *C. tropicalis*, † $p<0.05$ when compared to all groups; n = 5; Data are representatives of two independent experiments. (-), unstimulated BMDCs; *Ca*, *C. albicans*; *Ct*, *C. tropicalis*; *Cg*, *C. glabrata*; *Ck*, *C. krusei*.

C. krusei mannan reduced BMDC viability by the induction of cellular apoptosis

The signal transduction via PRRs leaded to the transcriptional expression of the inflammatory cytokines and triggered cellular apoptosis (78, 79). On the basis of the vigorous pro-inflammatory cytokine production of BMDCs upon *C. krusei* mannan stimulation, we thus hypothesized that *C. krusei* mannan may mediate cell death. BMDCs were incubated with *Candida* mannans at various concentrations and the cell viability was determined by MTT assay. The percentage of cell viability was expressed as a percentage relative to the unstimulated BMDC OD. We found that *C. krusei* mannan at all concentrations, but not the other *Candida* mannans, obviously decreased BMDC viability (Fig. 4.5 A). To specify the viability of DC in BMDC culture, we observed the alterations of $CD11c^{+}$ population in BMDCs stimulated with *Candida* mannans, using flow cytometric analysis. $CD11c^{+}$ cells were gated, and the live cells were identified based on forward scatter (FSC) and side scatter (SSC) (Fig. 4.5 B). The percentage of the live cells in $CD11c^{+}$ population was notably reduced when BMDCs were incubated with *C. krusei* mannan, while those live cells were not affected by the mannans from the other three *Candida* species (Fig. 4.5 C and Fig. 4.5 D).

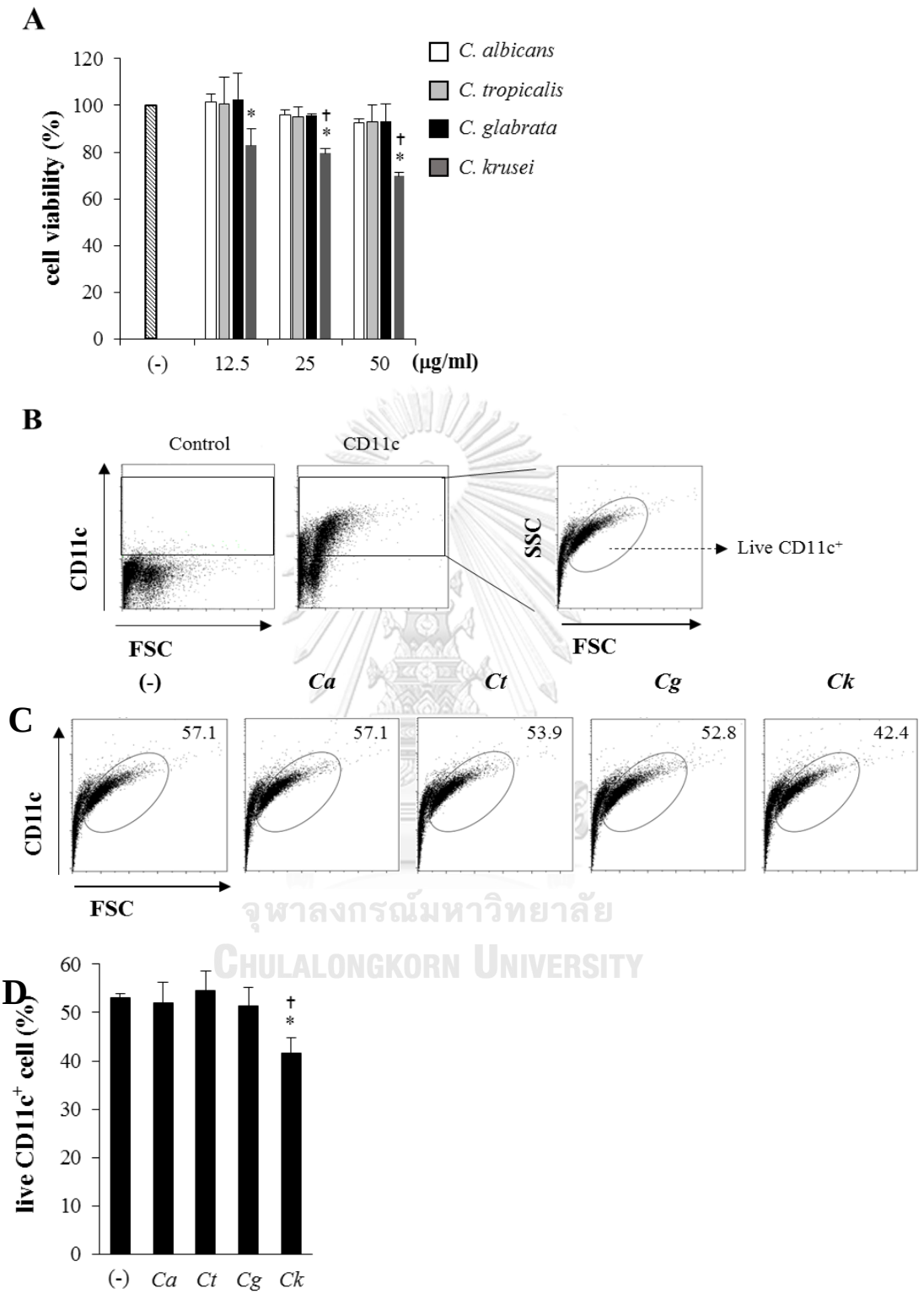


Figure 4.5 *C. krusei* mannan reduced DC viability

(A) BMDCs were incubated with various concentrations of *Candida* mannans for 48 h, and the cell viability was determined by MTT assay. The percentage of cell viability was expressed as a percentage relative to the unstimulated BMDCs. **(B)** BMDCs were incubated with 25 μg /ml of *Candida* mannans for 72 h, and the percentage of live $CD11c^+$ cells were determined. First, $CD11c^+$ population was gated and the live cells were identified based on SSC and FSC. **(C)** Showed the live DC in dot plot analysis. The number indicated the percentage of live $CD11c^+$ cells. **(D)** Bar graph showed percent of live DC after stimulation with *Candida* mannans. * $p<0.05$ when compared to the unstimulated BMDCs, † $p<0.05$ when compared to all groups; n = 5; Data are representatives of two independent experiments. (-), unstimulated BMDCs; *Ca*, *C. albicans*; *Ct*, *C. tropicalis*; *Cg*, *C. glabrata*; *Ck*, *C. krusei*.

Next, to determine whether *C. krusei* mannan mediated DC apoptosis, we used flow cytometry to analyze apoptotic cells. BMDCs were stimulated with 25 μg/ml of *Candida* mannan for 48 h. Unstimulated and mannan stimulated BMDCs were stained with CD11c, Annexin V and 7AAD. In the $CD11c^+$ population, cells that were single positive for Annexin V or double positive for Annexin V and 7AAD (Fig. 4.6 A) were identified as apoptotic DCs. Consistently, only *C. krusei* mannan markedly induced DCs to undergo apoptosis (Fig. 4.6 B). Altogether, our data imply that *C. krusei* mannan affects the viability of DCs probably via a process of activation-induced cellular apoptosis.

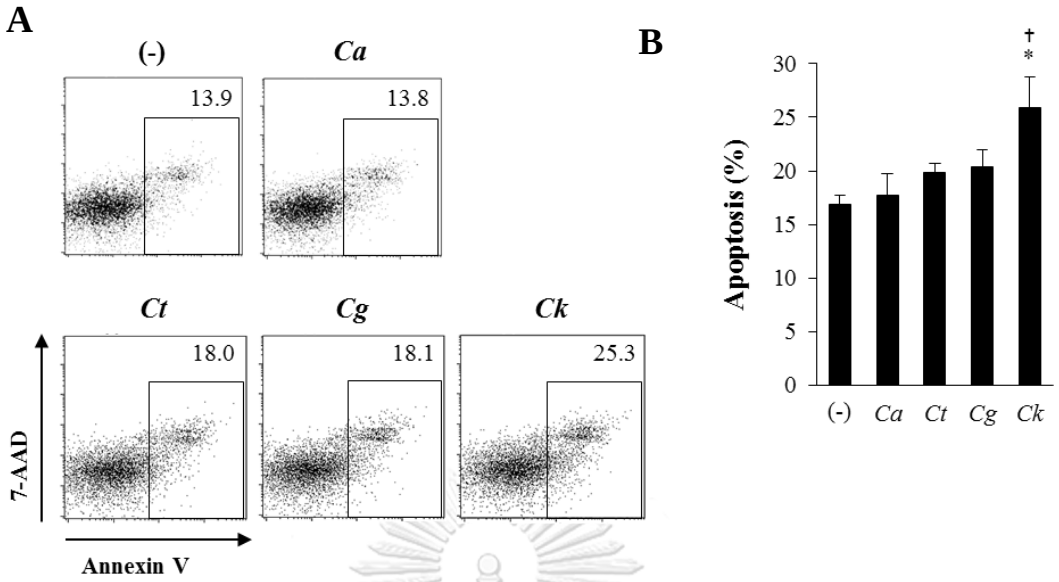


Figure 4.6 *C. krusei* mannan mediated DC apoptosis

BMDCs were incubated with 25 μg/ml of *Candida* mannans for 48 h, then the cells were stained with CD11c, Annexin V and 7AAD. **(A)** $CD11c^+$ cells were gated and the Annexin V^+, and Annexin V^+7AAD^+ were identified as apoptotic fraction. The number in the dot plot indicated the percentage of apoptotic DCs. **(B)** the percentage of apoptotic $CD11c^+$ cells was shown as the bar graph. * $p<0.05$ when compared to the unstimulated BMDCs, † $p<0.05$ when compared to all groups; n = 5; Data are representatives of two independent experiments. (-), unstimulated BMDCs; *Ca*, *C. albicans*; *Ct*, *C. tropicalis*; *Cg*, *C. glabrata*; *Ck*, *C. krusei*.

C. krusei mannan mediated DC apoptosis via MyD88-dependent pathway

C-type lectin receptors (CLRs) such as dectin-2, dectin-3 and Mincle are the main receptors recognizing *Candida* mannans, however, some structures of the mannans engage to TLR2 and TLR4 (65, 80). According to evidences showing that mannan structures of *Candida* species are diverse (35, 37), we therefore questioned which pathway is responsible for *C. krusei* mannan-mediated DC apoptosis. To elucidate the signal transduction underlying the apoptosis induction of *C. krusei* mannan, the downstream signaling of Syk-coupled CLRs and TLR-MyD88 in BMDCs

was interfered with the specific inhibitors. BMDCs were pre-treated with Syk (1 μM) or MyD88 (100 μM) inhibitors, and subsequently were stimulated with 25μg/ml *C. krusei* mannan (Fig. 4.7 A and 4.7 B). The cell viability was determined using MTT assay. *C. albicans* β-glucan was used as the positive control for SYK activation since it mediated signal transduction via dectin-1-Syk (81), and we found that *C. albicans* β-glucan did not affect BMDC cell viability (Fig. 4.7 A). The inhibition of Syk in unstimulated and β-glucan stimulated BMDCs showed the significant decrease in cell viability, while Syk inhibitor did not abrogate *C. krusei* mannan-induced cell death (Fig. 4.7 A). In parallel experiment, lipopolysaccharide (LPS) was used as the positive control for MyD88 activation. LPS stimulation showed the marked reduction of BMDC viability as similar as *C. krusei* mannan stimulation (Fig. 4.7 B). Interestingly, the inhibition of MyD88 rescued the viability of BMDCs that stimulated with LPS and *C. krusei* mannan (Fig. 4.7 B). To confirm the involvement of MyD88 pathway in *C. krusei* mannan-stimulated DC apoptosis, BMDCs were pre-treated with MyD88 inhibitor and then incubated with LPS or *C. krusei* mannan for 48 h. Percent of apoptotic DCs was determined as previous experiment using flow cytometry analysis. The result of apoptosis staining demonstrated that MyD88 inhibitor, to a great extent, interfered with LPS- and *C. krusei* mannan-mediated apoptosis of $CD11c^{+}$ population (Fig. 4.7 C and 4.7 D). These results suggest the intriguing molecular mechanism of MyD88-dependent signaling pathway in DC apoptosis in response to *C. krusei* mannan stimulation.

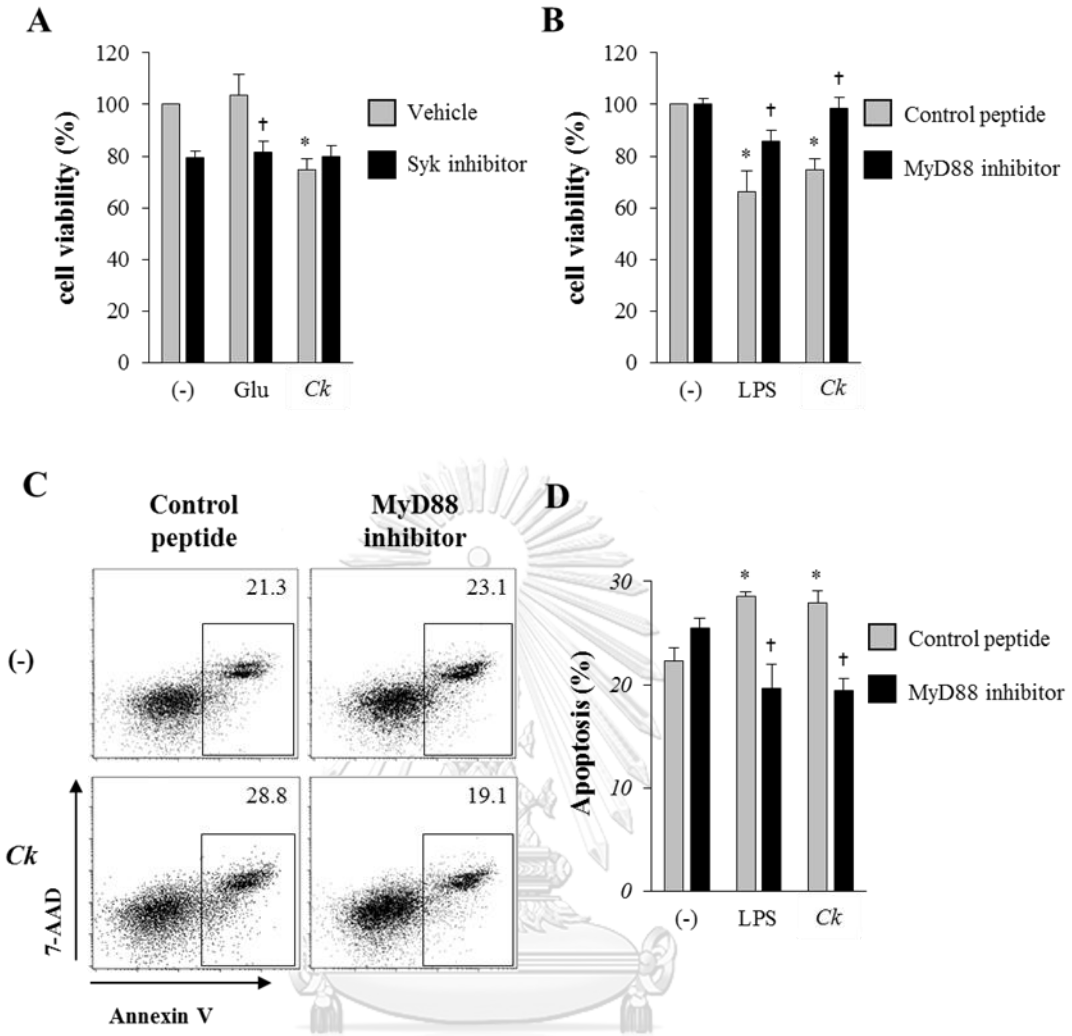


Figure 4.7 Mediation of DC apoptosis by *C. krusei* mannan was dependent on MyD88 signaling pathway

(A) BMDCs were pre-treated with vehicle control or Syk inhibitor, and the cells were incubated with 25 μg /ml of *C. krusei* mannans for 48 h. **(B)** BMDCs were pre-treated with control peptide or MyD88 inhibitor, and the cells were incubated with 25 μg/ml of *C. krusei* mannans for 48 h. The BMDC viability was determined by MTT assay. The percentage of cell viability was expressed as a percentage relative to the unstimulated BMDCs. **(C)** and **(D)** BMDCs were pre-treated with control peptide or MyD88 inhibitor, and the cells were incubated with 25 μg /ml of *C. krusei* mannans for 48h. Then, the cells were stained with CD11c, Annexin V and 7AAD. $CD11c^+$ cells were gated and the Annexin V^+, and Annexin V^+7AAD^+ were identified as apoptotic fraction. The number in the dot plot indicated the percentage of apoptotic DCs. (D) The percentage of apoptotic

$CD11c^+$ cells was shown as the bar graph. n = 3; * $p<0.05$ when compared to the unstimulated BMDCs, † $p<0.05$ when compared to the vehicle control or the control peptide treated BMDCs; n = 5; Data are representatives of three independent experiments. (-), unstimulated BMDCs; *Ca*, *C. albicans*; *Ct*, *C. tropicalis*; *Cg*, *C. glabrata*; *Ck*, *C. krusei*.

DC apoptosis-mediated by *C. krusei* mannan was associated with TLR2 activation

As mention above, TLR2 and TLR4 are partly involved in the recognition of the mannosyl residuals of *C. albicans* cell wall (65, 82, 83). To investigate the functional relevance of TLR-triggered DC apoptosis upon *C. krusei* mannan stimulation, the activation of TLR2 and TLR4 were inhibited by the blocking antibodies. As the positive control, TLR2 ligation with Pam_3CSK_4 significantly enhanced DC apoptosis, and this effect of Pam_3CSK_4–induced cell death was eradicated by the treatment with anti-TLR2 blocking Abs. Notably, the blockade with anti-TLR2 mAbs merely diminished the effect of *C. krusei* mannan on DC apoptosis (Fig. 4.8 A and 4.8 C), and these findings were concordant with the results of MyD88 inhibition (Fig. 4.7 D). In parallel experiment, BMDCs also significantly underwent apoptosis upon LPS stimulation when compared to the negative control (Fig. 4.7 D, 4.8 B and 4.8 D). In contrast to the results of TLR2 blockade (Fig. 4.8 A and 4.8 C) and MyD88 inhibition (Fig. 4.7 D), the blockade of TLR4 aggravated the cell death (Fig. 4.8 B and 4.8 D). Besides, the induction of DC apoptosis by *C. krusei* mannan was not impeded by anti-TLR4 blocking Abs (Fig. 4.8 B and 4.8 D). Therefore, our data indicate that the activation through TLR2, but not through TLR4, possibly play a role in the mechanism of DC apoptosis induction by *C. krusei* mannan.

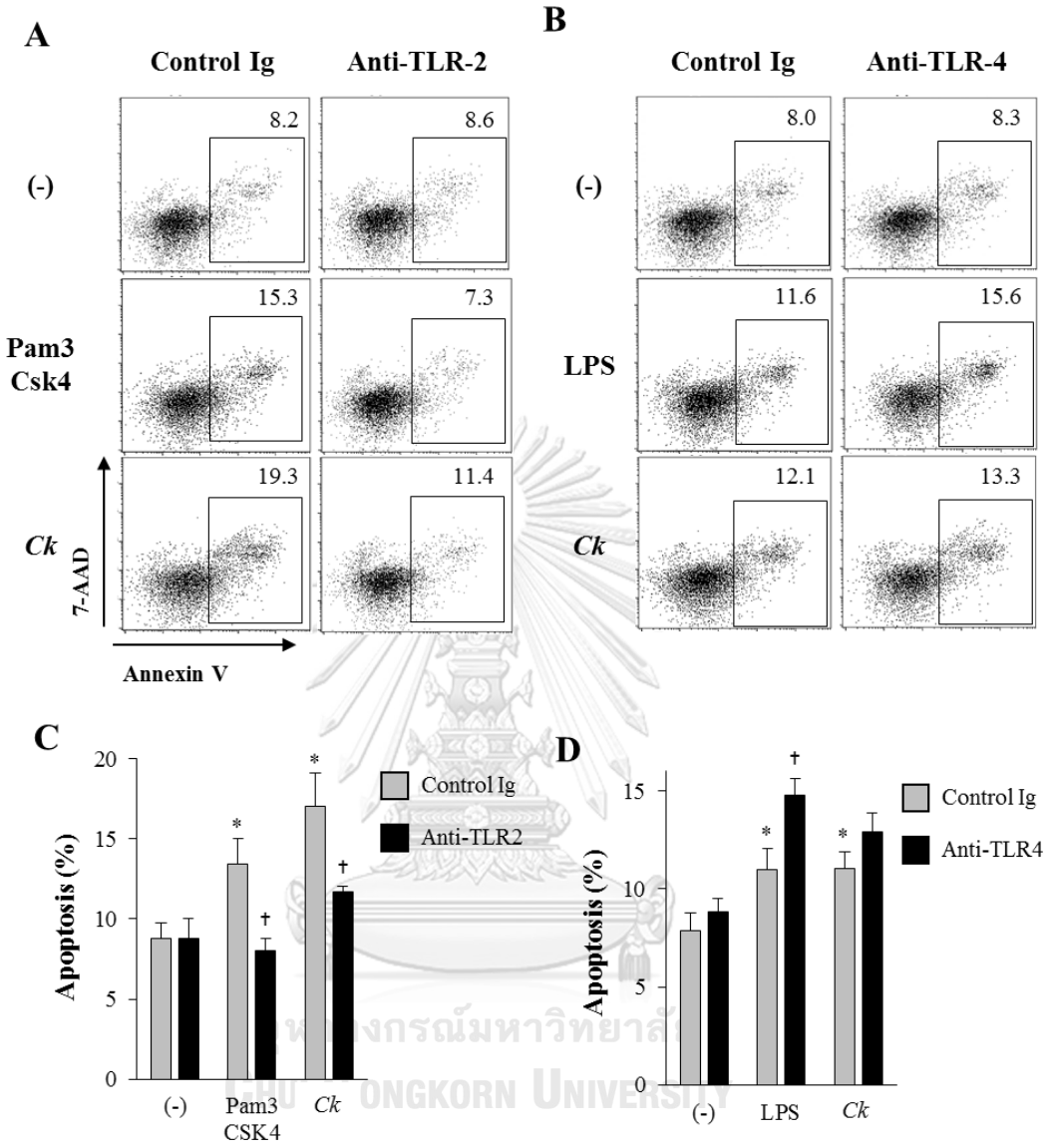


Figure 4.8 *C. krusei* mannan mediated DC apoptosis via the activation of TLR2

BMDCs were pre-treated with control rat IgG or **(A)** and **(C)** anti-mouse TLR2 mAbs, or **(B)** and (D) anti-mouse TLR4 mAbs, and the cells were incubated with 25 μg/ml of *C. krusei* mannans for 48 h. Then, the cells were stained with CD11c, Annexin V and 7AAD. $CD11c^{+}$ cells were gated and the Annexin V^{+}, and Annexin $V^{+}7AAD^{+}$ were identified as apoptotic fraction as shown in (A) and (B). The number in the dot plot indicated the percentage of apoptotic $CD11c^{+}$ DCs. **(C)** and **(D)** The percentage of apoptotic $CD11c^{+}$ cells was shown as the bar graph. n = 5; * $p<0.05$ when compared to the unstimulated BMDCs, † $p<0.05$

when compared to the control rat IgG treated BMDCs; n = 3; Data are representatives of three independent experiments. (-), unstimulated BMDCs; *Ca*, *C. albicans*; *Ct*, *C. tropicalis*; *Cg*, *C. glabrata*; *Ck*, *C. krusei*.

C. krusei mannan-stimulated DCs orchestrated antigen-specific Th17 responses

Since *C. krusei* mannan impaired DC viability, we next questioned how *C. krusei* mannan-stimulated DCs influence antigen-specific T responses. As the large number of DCs resides and distributes in the skin (84), the mice were immunized twice with the mixture of 30 μg ovalbumin and 1 mg of each *Candida* mannan (OVA-*Candida* mannan) via a subcutaneous route in other that DCs could be directly exposed to the stimuli. Seven days after the last immunization, the immune cell population including: T cells ($CD3^+B220^-$), B cells ($CD3^-B220^+$), CD4 T cells ($CD3^+CD4^+$), CD8 T cells ($CD4^+CD8^+$), memory T cells ($CD4^+CD62L^+CD44^{lo}$, $CD4^+CD62L^-CD44^+$ and $CD4^+CD62L^+CD44^{hi}$), and Th subsets (Th1 ($CD4^+IFN\text{-}\gamma^+$), Th2 ($CD4^+IL\text{-}4^+$), Th17 ($CD4^+IL\text{-}17^+$ and T_{reg} ($CD4^+FoxP3^+$) in $CD3^+$ population) in regional lymph nodes (RLNs) was determined by flow cytometric analysis (Fig. 4.9 and 4.10). The total numbers of RLN cells were significantly increased when the mice were immunized with OVA-*C. albicans* mannan and OVA-*C. krusei* mannan. However, all immunized mice did not exhibite the altered proportion of the immune cell population (Fig. 4.10). Several evidences have revealed that *Candida* cell wall- and mannan-stimulated DCs dictated the fate of CD4 T cell polarization (42, 55), we thus further examined T cell responses *ex vivo*. The antigen-primed LN cells of each *Candida* mannan-immunized group of mice were collected and re-stimulated with 250 μg/ml OVA for 48 h, and then Th cytokines, IFN-γ, IL17, IL-4 and IL-10 were determined by ELISA (Fig. 4.11). After *ex vivo* antigen re-exposure, OVA-*C. albicans* mannans and OVA-*C. tropicalis* mannan immunized LN cells remarkably secreted IFN-γ, IL-4 and IL-10 when compared to the negative control, OVA-*C. glabrata* mannan and OVA-*C. krusei* mannan group. Meanwhile, immunization with OVA-*C. glabrata* mannan could not

activate the cytokine production. Noteworthy, the augmented IL-17 production was particularly observed in OVA-*C. krusei* mannan immunized LN cells.

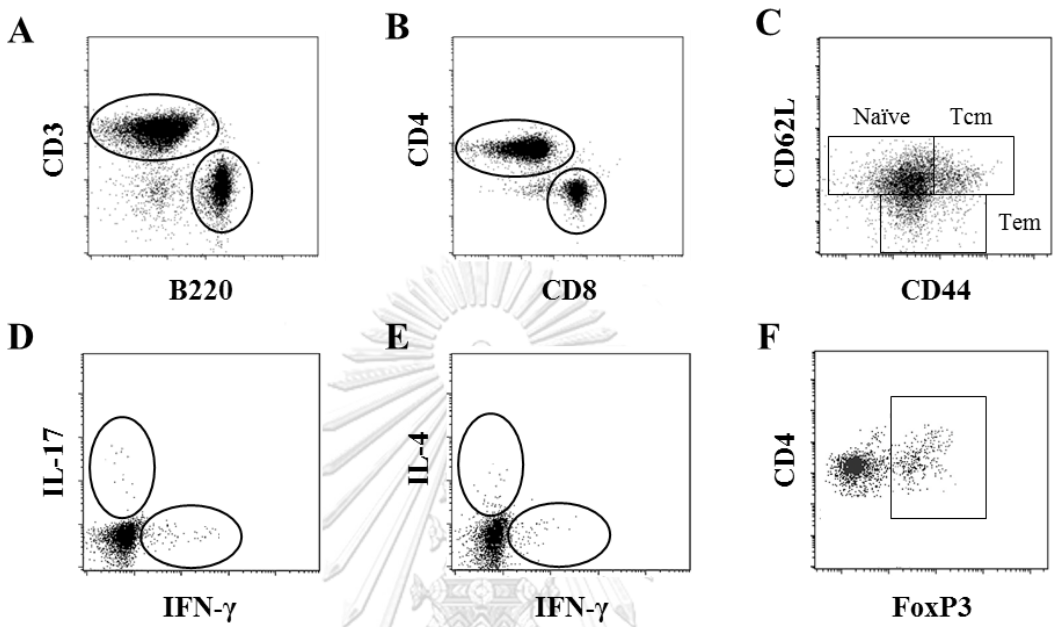


Figure 4.9 Flow cytometric analysis of immune cell population

Mice were subcutaneously immunized with the mixture of OVA and *Candida* mannans at day 0 and day 7. At day 14, the immune cell population in RLN cells were investigated using flow cytometric analysis. **(A)** Show the dot plot analysis of $CD3^+$ and $B220^+$ cells. **(B)** $CD3^+$ cells were gated, and $CD4^+$ and $CD8^+$ cells were subsequently identified. To determine the naïve and memory phenotype, $CD3^+CD4^+$ cells were first gated, and **(C)** $CD62L^+CD44^{lo}$ (naïve), $CD62L^-CD44^+$ (effector memory T cells or Tem), and $CD62L^+CD44^{hi}$ (central memory T cells or Tcm) were then identified. To determine T helper cell subpopulation, $CD3^+CD4^+$ cells were gated, and **(D)** $IL\text{-}17^+$ and $IFN\text{-}\gamma^+$ or **(E)** $IL\text{-}4^+$ and $IFN\text{-}\gamma^+$ or **(F)** $CD4^+FoxP3^+$ T cells were then identified.

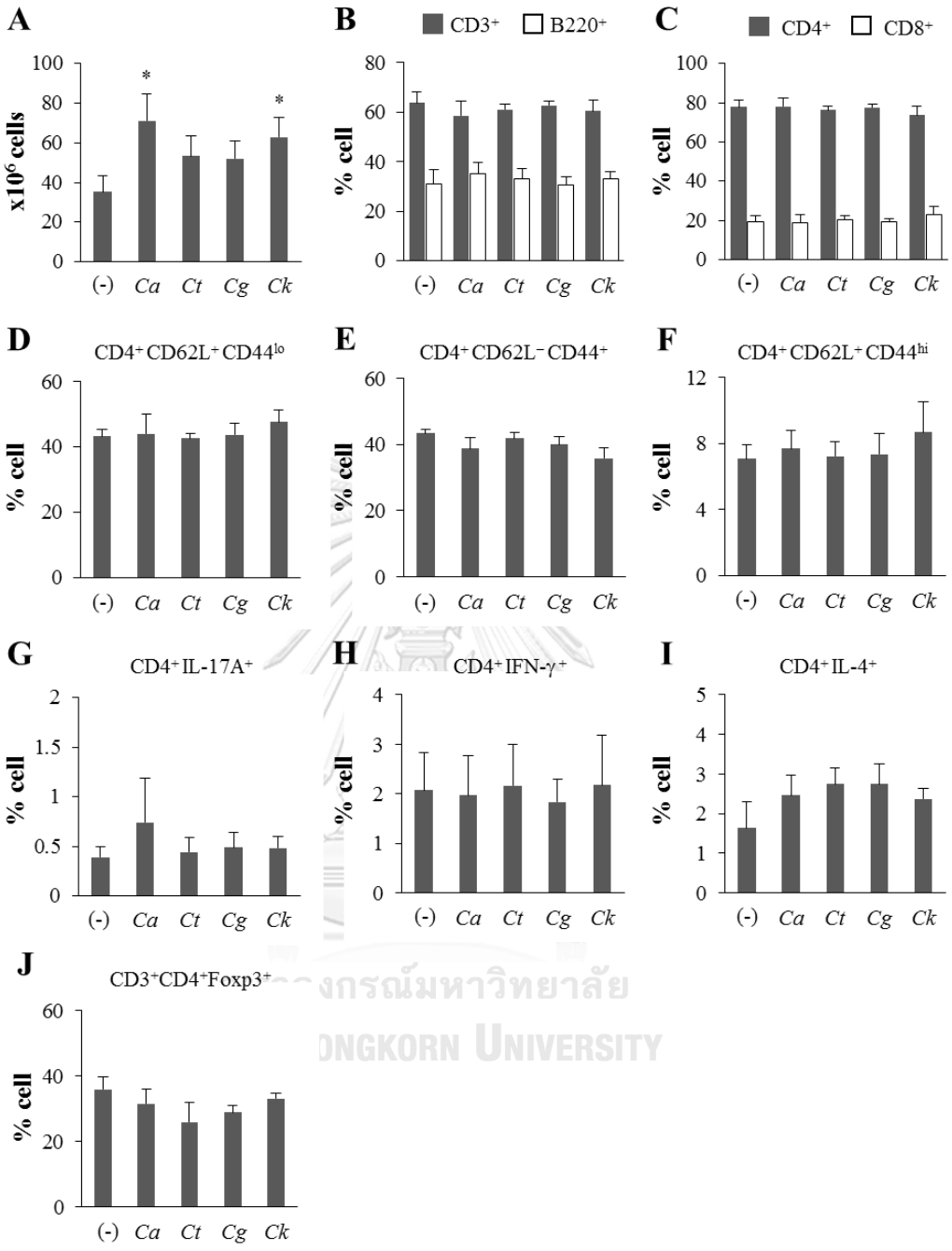


Figure 4.10 Alteration of cell number and immune cell population in the RLNs

Mice were subcutaneously immunized with the mixture of OVA and *Candida* mannans at day 0 and day 7. At day 14, the immune cell population in RLN cells were investigated using flow cytometric analysis as described in Fig. 4.9. **(A)** Number of total LN cells. The percentage of **(B)** T cells ($CD3^+$) and B cells ($B220^+$), **(C)** CD4 T cells and CD8 T cells, **(D)** naïve $CD4^+$ T cells, **(E)** $CD3^+CD4^+$ Tem, **(F)** $CD3^+CD4^+$ Tcm , **(G)** IL-17^+ T cell, **(H)** IFN-γ^+ T

cells, **(I)** IL-4^{+} T cells**, (J)** CD4^{+}FoxP3^{+} T cells. *p<0.05 when compared to the negative control group; n = 5; Data are representatives of two independent experiments. (-), OVA alone as a negative control, *Ca*, OVA-*C. albicans*; *Ct*, OVA-*C. tropicalis*; *Cg*, OVA-*C. glabrata*; *Ck*, OVA-*C. krusei*.

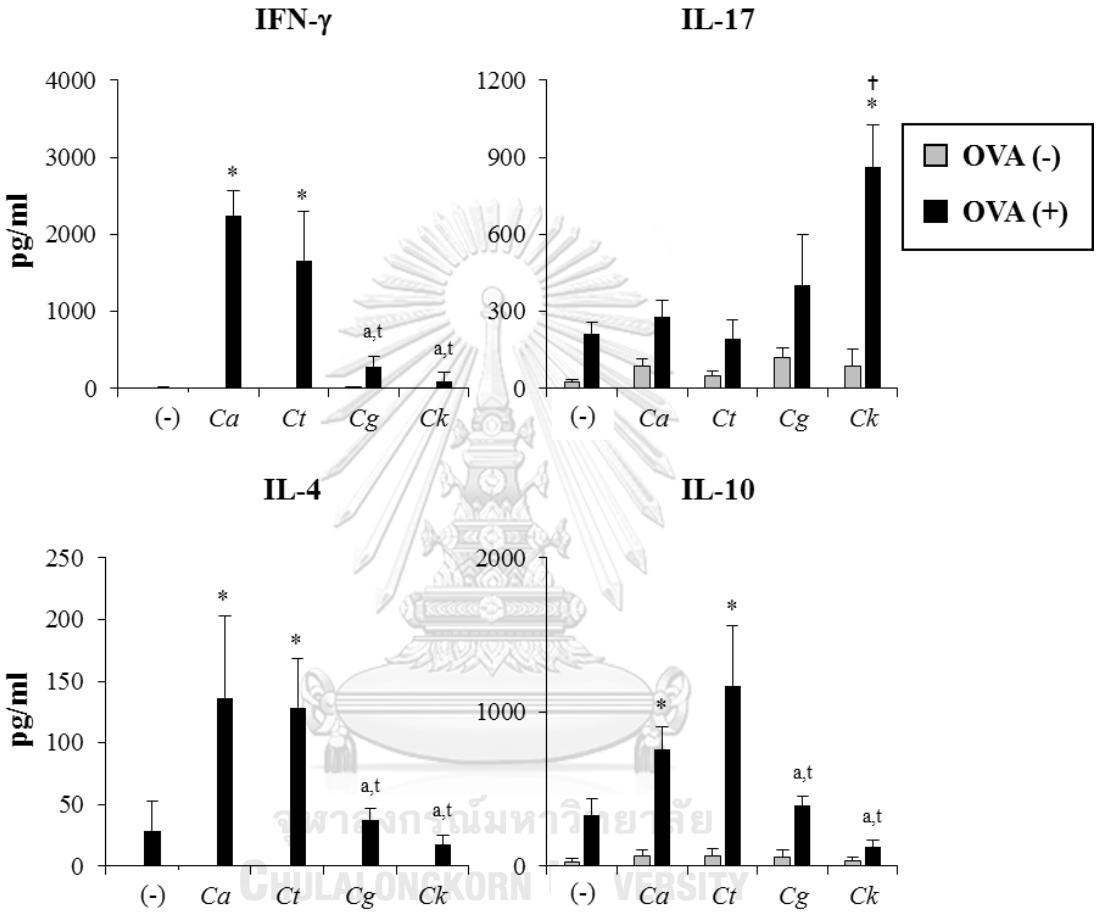


Figure 4.11 *In vivo* immunization of *C. krusei* mannan governed Th17 response in an antigen-specific manner

Mice were subcutaneously immunized with the mixture of OVA and *Candida* mannans at day 0 and day 7. At day 14, the RLN cells were re-simulated with (+) or without (-) OVA, and the level of IFN-γ, IL-17, IL-4 and IL-10 in the culture supernatant were quantitated by ELISA. * p<0.05 when compared to the negative control group, ^{a} p<0.05 when compared to *C. albicans*, ^{t} p<0.05 when compared to *C. tropicalis*, † p<0.05 when compared to all groups; n = 5; Data are representatives of two independent experiments. (-), OVA alone as a negative control; *Ca*, OVA-*C. albicans*; *Ct*, OVA-*C. tropicalis*; *Cg*, OVA-*C. glabrata*; *Ck*, OVA-*C. krusei*.

The *in vivo* mannan immunization may not only activate DCs but also possibly affect the response of other immune cells. Therefore, we investigated the direct impact of *C. krusei* mannan-stimulated DCs on CD4 T cell responses by performing the *in vitro* co-culture system. BMDCs were primed with *Candida* mannans and were subsequently pulsed with OVA, then, the BMDCs were co-cultured with the T cells isolated from OT-II TCR transgenic mice. The secretion of IFN-γ, IL-17, IL-4 and IL-10 in the culture supernatant was readout, representing T helper cell responses. However, IL-4 and IL-10 were not detectable in this culture system. Concurring with the *ex vivo* re-stimulation assay, *C. abicans* and *C. tropicalis* mannan-stimulated DCs profoundly augmented IFN-γ production from OT-II T cells, while *C. krusei* mannan-stimulated DCs preferably induced IL-17 production (Fig. 4.12 A and 4.12 B). Collectively, our results reveal that although DCs activated with *C. krusei* mannan apparently loss their viability by undergoing apoptosis, they are, in particular, capable of orchestrating the Th17 response in an antigen-specific manner.

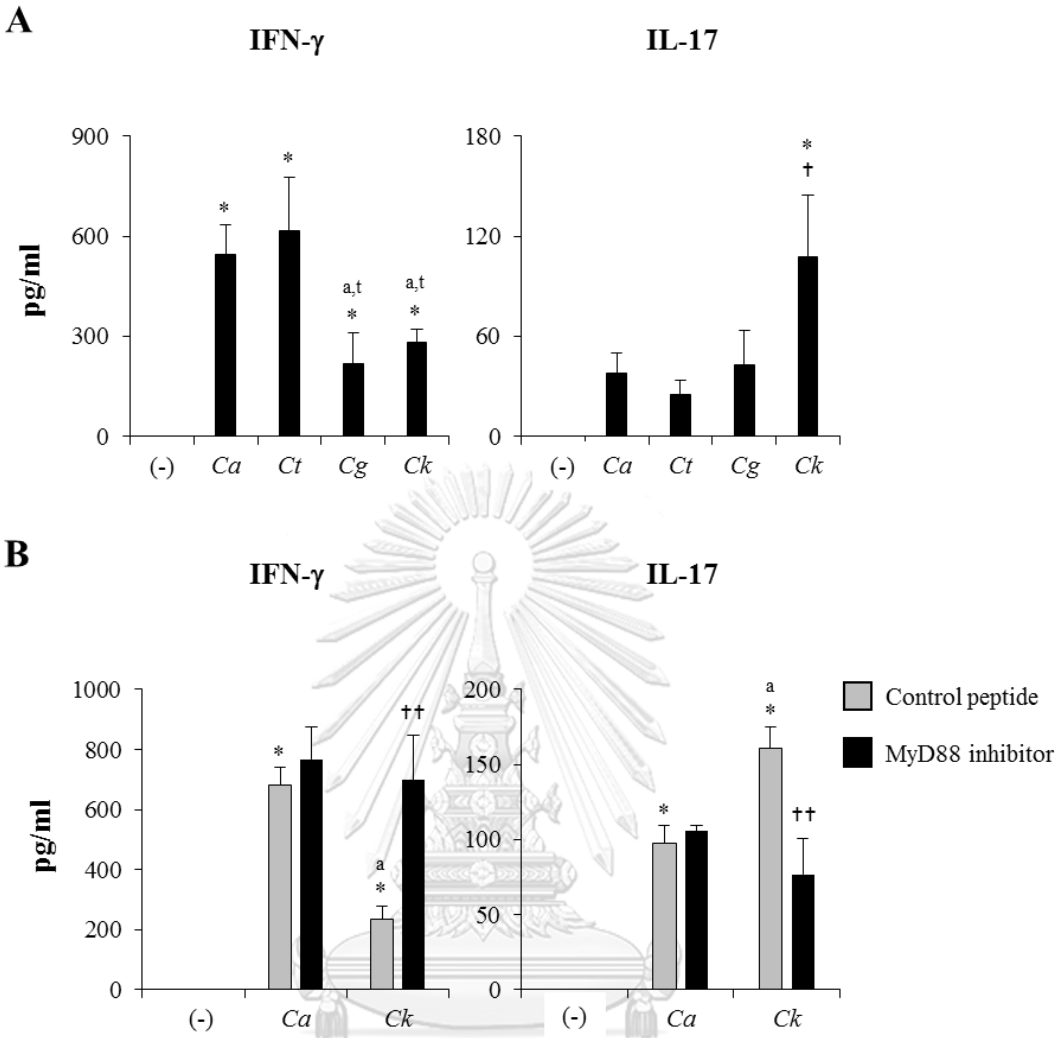


Figure 4.12 *C. krusei* mannan-stimulated DCs induced IL-17 production from TCR transgenic T cells via the MyD88 dependent pathway

(A) BMDCs were stimulated with 25 μg/ml of *Candida* mannans, and **(B)** BMDCs were pre-treated with control peptide or MyD88 inhibitor, and the cells were stimulated with 25 μg/ml of *C. albicans* or *C. krusei* mannans. Subsequently unstimulated and mannan-stimulated BMDCs were pulsed with OVA, and the BMDCs were then co-cultured with OT-II T cells for 48 h. The level of IFN-γ and IL-17 in the culture supernatant was determined by ELISA. * $p<0.05$ when compared to the negative control group, ^{a} $p<0.05$ when compared to *C. albicans*, ^{t} $p<0.05$ when compared to *C. tropicalis*, † $p<0.05$ when compared to all groups, †† $p<0.05$ when compared to the control peptide treated BMDCs; (A) n = 5 and (B) n = 4; Data are representatives of two independent experiments. (-), T cell incubated with OVA as a negative control; *Ca*, *C. albicans*; *Ct*, *C. tropicalis*; *Cg*, *C. glabrata*; *Ck*, *C. krusei*.

Th17 induction by *C. krusei* mannan-stimulated DC was obligated to MyD88 signaling pathway

We have demonstrated the involvement of the MyD88-dependent pathway in the DC response to *C. krusei* mannan, we thus questioned whether MyD88 in *C. krusei* mannan-stimulated DCs was also required for Th17 skewing. BMDCs were pre-treated with MyD88 inhibitor and then were primed with *C. albicans or C. krusei* mannans. Subsequently those BMDCs were pulsed with OVA, and were co-cultured with OT-II T cells. The inhibition of MyD88 in DCs stimulated with mannan from *C. krusei*, but not *C. albicans*, converted the production of IL-17 to IFN-γ in the activated OT-II T cells (Fig. 4.12 B). We wondered how MyD88 involves in the production of cytokines in DC, which contribute to the balance between Th1 and Th17. Therefore, we investigated the IL-12, IL-6 and IL-23 production of BMDCs pre-treated with MyD88 inhibitor, followed by *C. krusei* mannan stimulation. Consistently, the MyD88 inhibition in BMDCs stimulated with *C. krusei* mannan substantially enhanced IL-12 production, which is require for Th1 induction (85), but reduced IL-6 production, which is the key cytokine for Th17 induction (85, 86) (Fig. 4.13 A).

We further verified the implication of TLR2 and TLR-4 on the cytokine production of *C. krusei* mannan-stimulated DCs by using the functional blocking Abs. We found that TLR2 blockade significantly suppressed the production of IL-6, but not IL-12 and IL-23, in BMDCs stimulated with Pam_3CSK_4 and *C. krusei* mannan (Fig. 4.13 B). Meanwhile, the blockade of TLR4 diminished IL-12, IL-6 and IL-23 in LPS-stimulated BMDCs. However, TLR4 blockade did not provide the effect on all cytokine production in *C. krusei* mannan-stimulated DCs (Fig. 4.13 C).

These findings indicate that the MyD88 pathway, and partly the TLR2 activation, regulates the cytokine responses of DCs upon *C. krusei* mannan stimulation, and these possibly provide the shift toward Th1 and Th17 immunity (Fig 4.14).

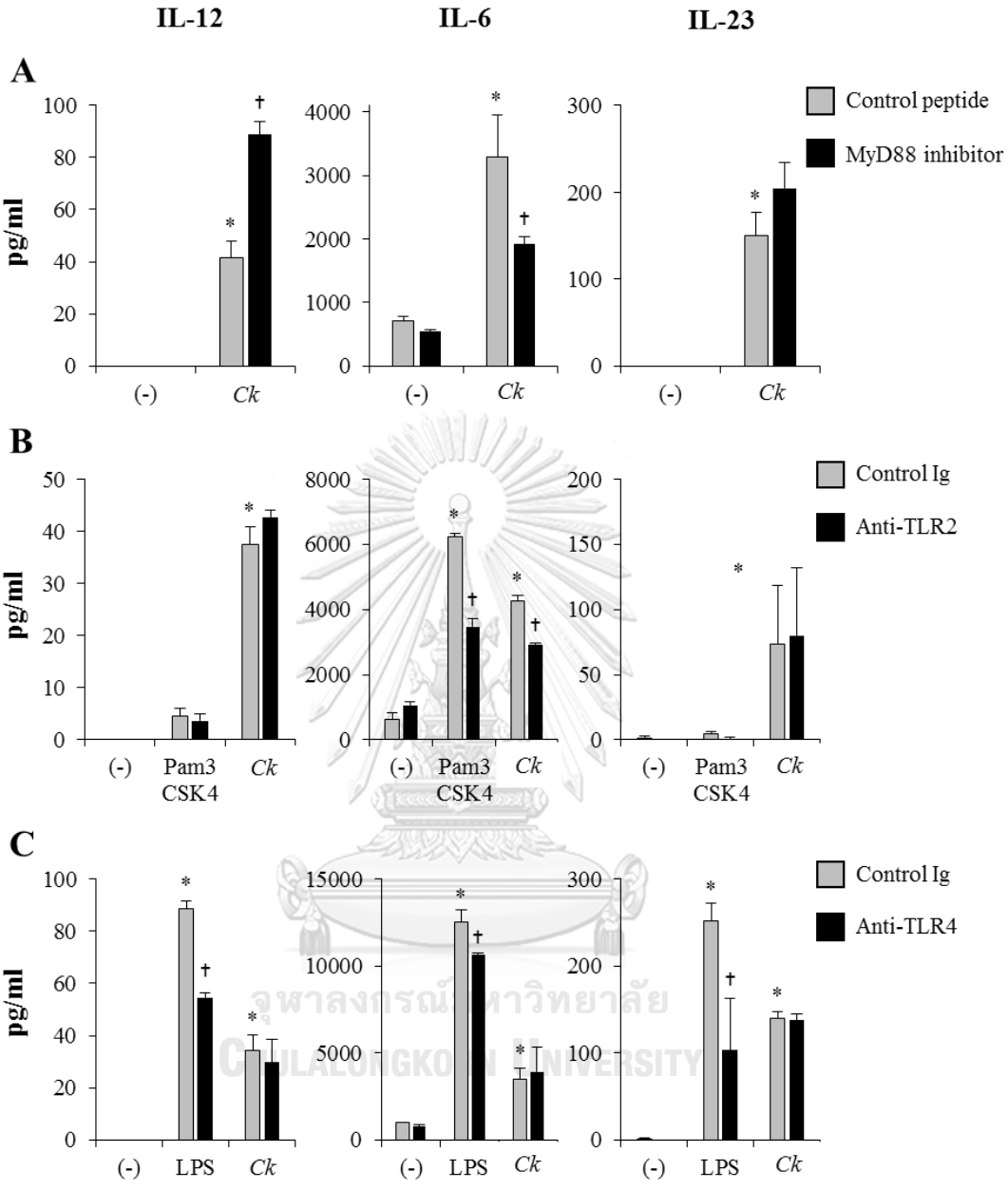


Figure 4.13 Production of Th1 and Th7 polarizing cytokine from *C. krusei* mannan-stimulated DC was dependent on MyD88 and TLR2 pathway

BMDCs were pre-treated with **(A)** control peptide or MyD88 inhibitor, **(B)** control IgG or anti-mouse TLR2 mAbs, and **(C)** control IgG or anti-mouse TLR4 mAbs. Then the cells were stimulated with 25 μg/ml of *C. krusei* mannans, and the level of IL-12, IL-6 and IL-23 in the culture supernatant were determined by ELISA. n = 3; * $p<0.05$ when compared to the unstimulated BMDCs, † $p<0.05$ when compared to the control peptide

or control rat IgG treated BMDCs; n = 3; Data are representatives of three independent experiments. (-), unstimulated BMDCs; *Ca*, *C. albicans*; *Ct*, *C. tropicalis*; *Cg*, *C. glabrata*; *Ck*, *C. krusei*.

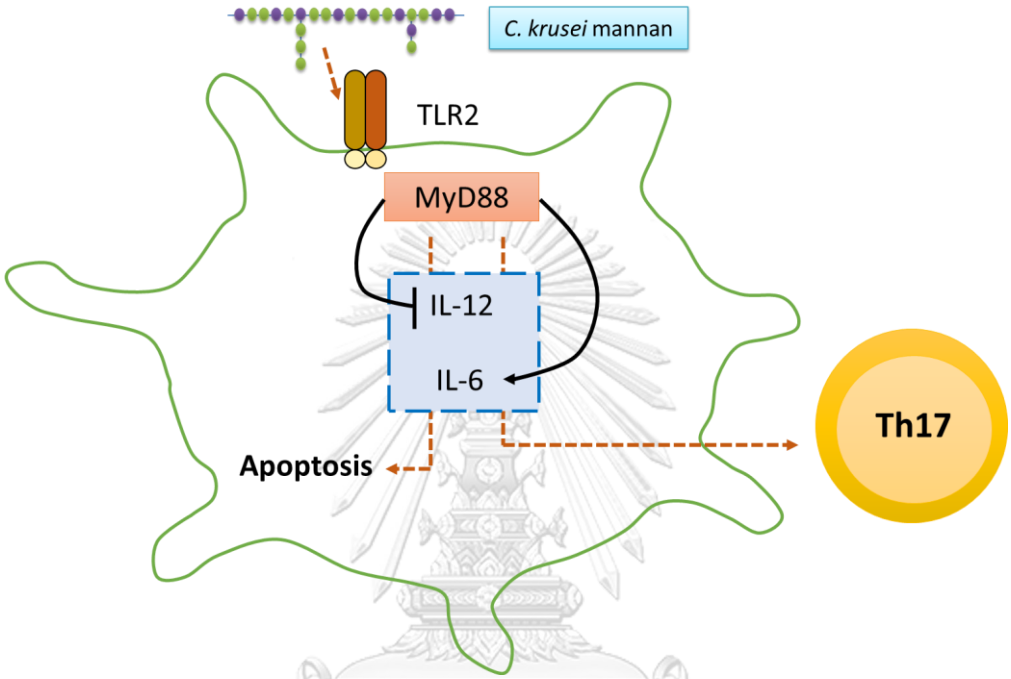


Figure 4.14 *C. krusei* mannan mediated DC apoptosis and induce Th17 response via MyD88 and TLR2 signaling pathway

Discussion

Dendritic cells are potent and versatile APCs that sense numerous pathogens via PRRs which transduce intracellular signal to initiate the protective immunity as well as the immune tolerance (56). Here, we investigate the molecular mechanism underlying the induction of DC and T cell immunity in response to *C. krusei* mannan stimulation.

Our results showed the quantitative differences in DC responses against the cell wall mannan of distinct *Candida* species (Fig. 4.3 and 4.4), which possibly were due to their differences in structure and proportion of mannose residues (35, 37, 38). *C. albicans* mannan consists of a short chain O-linked mannan oligosaccharide, and a branch of N-linked mannan polysaccharide moieties. This N-linked mannan contains a long chain of α-1,6-linked mannose backbone connected with an oligomannose side chain mainly containing α-1,2-, α-1,3-, and β-1,2-linked mannose residues with a few phosphate groups(35, 36). *C. tropicalis* shares mannan moieties with some serotypes of *C. albicans*, and is antigenically identical to *C. albicans* serotype A (37, 87, 88). Concurring with the mannan structure and content of these two *Candida*, we observed the similar effects of *C. albicans* and *C. tropicalis* mannan on DC (Fig. 4.3 and 4.4), as well as T cell responses (Fig. 4.11 and 4.12). On the contrary, cell wall mannan of *C. glabrata* is small branches containing low α-mannan content and one or two units of β-1,2-linked mannose residues (35, 38). Meanwhile, *C. krusei* mannan is lightly branch that contains a long chain of α-1,2-linked mannose backbone with one or two α-1,6-linked mannose residues located in the middle of the chain, and short side chain of α-1,2-linked mannose residues (39, 40). Since the mannans of *C. glabrata* and *C. krusei* differed from those of *C. albicans* and *C. tropicalis*, they produced the different effect on DC maturation and cytokine production (Fig. 4.3 and 4.4).

C. krusei mannan induced DC maturation and the massive cytokine production, which verified the state of high activation of DCs, and probably leaded to the mediation of cellular apoptosis (78, 79). Concordantly, *C. albicans* and *C. tropicalis* mannan had no effect on DC maturation and less effect on the cytokine

production, therefore, they could not mediate DC apoptosis. Furthermore, *C. glabrata* mannan was the potent stimulus that induced DC maturation, but it failed to promote the cytokine production, and as a result, this may contribute to the inability of *C. glabrata* mannan to mediate DC apoptosis (Fig. 4.5 and 4.6). Besides, *C. albicans*, *C. tropicalis* and *C. glabrata* mannan particularly induced the production of IFN-γ that may act as an autocrine and enable the survival of BMDCs (89-91).

Ample evidences have revealed the central role of CLRs and Syk in the recognition of *Candida* mannans, and the protection against *C. albicans* infection (80, 92). Our results demonstrated that the inhibition of Syk reduced the cell viability of unstimulated and β-glucan stimulated BMDCs (Fig. 4.7), which are consistent to the previous studies (93-95). The signal transduction via Syk is thus required for the cell survival, as the activation of Syk leaded to STAT3 phosphorylation which mediating cell growth and differentiation (95). For this reason, the mannan of *C. albicans*, *C. tropicalis* and *C. glabrata* possibly ligated to the receptors that activated Syk (68, 80) and consequently resulted in the prevention of apoptosis in BMDCs (Fig. 4.5 and 4.6). On the contrary, we found that *C. krusei* mannan mediated BMDC apoptosis via the activation of TLR-2 and MyD88 (Fig. 4.7 and 4.8), which probably transduce the downstream signal through a pathway involving Fas-associated death domain protein (FADD) and caspase 8 (96). Of note, although DCs underwent apoptosis upon *C. krusei* mannan, these DCs retained their immunogenic functions that initiated T cell responses (Fig. 4.12).

A previous study reported that the ligation of *C. albicans* mannan to dectin-2, or mannose receptor promoted IL-6 and IL-23 production in APCs, and hence mediated Th17 response (42, 83). However, our *C. albicans* and *C. tropicalis* mannan could induce only IFN-γ production in BMDCs (Fig 4.4), and consequently skewed Th1 responses (Fig. 4.11 and 4.12). These different results could be due to the differences in mannosyl composition. The previous study cultured yeasts in a specific condition with carbon-limiting, low pH, and low temperature, where the yeasts could not synthesized β-linked mannose (42). In contrast, we grew the yeasts in a normal condition, which possibly allowed them to synthesize the entire α- and β-linked mannose components. It is likely that the presence of β-linked mannose in *C.*

albicans mannan reduced the inflammatory cytokine production in DCs (47). In addition, the intracellular signal through DC-SIGN, a CLR that also recognizes N-linked mannan of *C. albicans* (70), appeared to inhibit dectin-1-dependent Th17 generation, instead of favoring Th1 response, upon *Mycobacterium tuberculosis* infection(97). Therefore, our observation could support that the cell wall mannan may play a role in *C. albicans* immune evasion by a shift in Th1/Th17 balance (46, 98), leading to the debilitated Th17 protective immunity.

The results of cytokine production by *Candida* mannan-stimulated BMDCs *in vitro* (Fig. 4.4) may partly explained the outcomes of the *in vivo* experiment (Fig 4.11). *C. albicans* and *C. tropicalis* mannan-stimulated BMDCs induced IFN-γ production, which is important cytokines for polarizing Th1 differentiation. These 2 mannans were failed to induce other cytokines. On the other hand, *C. krusei* mannan-stimulated BMDCs massively produced pro-inflammatory cytokines, especially high concentration of IL-6 and IL-23, which are essential for Th17 differentiation (50). This phenomenon of DCs might result in the increasing of IL-17 production by antigen-specific T cell response, primed by *C. krusei* mannan *in vivo*.

TLR2 and TLR4 also serve as an immune sensor recognizing the specific mannose residues of *Candida* species (65), and are essential for host defense against *C. albicans* infection (99, 100). TLR4 specifically interacts with O-linked mannan (82), which contains α-1,2-linked mannose residues (34), while TLR2 directly engages to phospholipomannan (101), which contains phospholipid and β-1,2-linked mannose residues. In addition, the recognition of β-linked mannose by galectin-3 is associated with TLR2 activation (36). Intriguingly, the structure and composition of *C. krusei* mannan differed from those identified carbohydrate ligands of TLR2 and TLR4, but it is likely that *C. krusei* mannan was recognized by TLR2. Of note, the distinct structure of *C. krusei* mannan, which contains the interlaced α-1,2- and α-1,6-linked mannose residues (39, 40), possibly facilitated the specificity of the ligand-receptor binding. Furthermore, a previous study demonstrated that the partial acetolysis gave rise to the mannooctose of α-1,2- and α-1,6-linked mannose residues and this *C. krusei*-specific structure might be responsible for the TLR2/MyD88 activation.

Signal transduction via TLR2 and MyD88 in DCs has been shown to be involved in Th17 polarization (102, 103). Consistently, our findings demonstrated that the activation of MyD88 in DCs by *C. krusei* mannan controlled Th1/Th17 switching by virtue of the polarizing cytokines IL-12 and IL-6 (Fig. 4.12 and 4.13). Though, the certain role of TLR2 reconcile with MyD88 in *C. krusei* mannan-mediated Th17 response is still unclear, it may, at least in part, trigger IL-6 which is the key cytokine for Th17 generation (104, 105).

In conclusion, the structure and composition of *Candida* cell wall mannan crucially influence the concomitant regulation of host protective immunity and fungal immune evasion through differential activation of DCs. Therefore, the comprehension of *Candida* recognition by PRRs and the induction of adaptive immunity may bring important contributions to the development of new clinical therapeutic applications.

Part II

Objective 2: To investigate the effect of cell wall mannans of *Candida albicans* and oral candidiasis-related non-*albicans Candida* on DC responses *in vitro*.

Results

Effect of *Candida* yeast cell walls and soluble mannan on DC viability

To determine the effect of yeast cell walls on DC response without the influence from any secretory molecules from live cells, the yeast cells were inactivated by heat. We examined the morphology and microscopic structures of yeast cells by a scanning electron microscope, and we found that the heat inactivation method did not disrupt the cell wall integrity (Fig. 4.15 A and Fig 4.15 B).

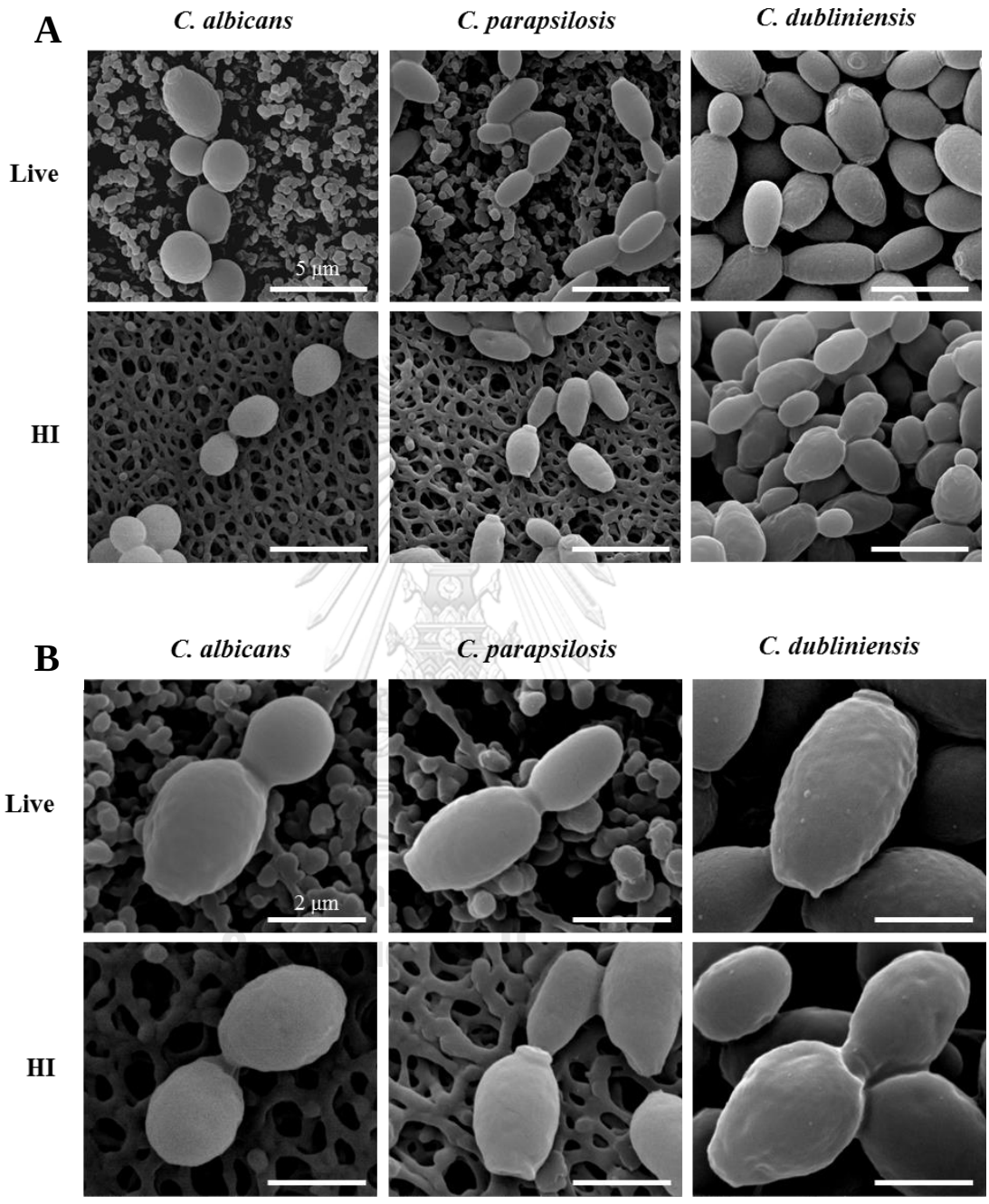


Figure 4.15 Morphology and cell wall integrity of live and HI *Candida* species

Morphology and cell wall integrity of live and HI *C. albicans*, *C. parapsilosis* and *C. dubliniensis* were observed using a scanning electron microscope with (A) 20,000x and (B) 50,000x magnification. Scale bars as indicated.

By using the heat-inactivated (HI) *Candida* yeasts, we expected that cell wall is the first component interacting with DCs. To evaluate the cytotoxicity of HI *Candida* yeasts and soluble mannans in BMDCs, MTT assay was performed. Heat-inactivated *C. albicans, C. parapsilosis,* and *C. dubliniensis* significantly reduced DC viability in a DC/yeast ratio dependent manner, and all *Candida* yeasts showed similar levels of cytotoxicity on BMDCs (Fig. 4.16).

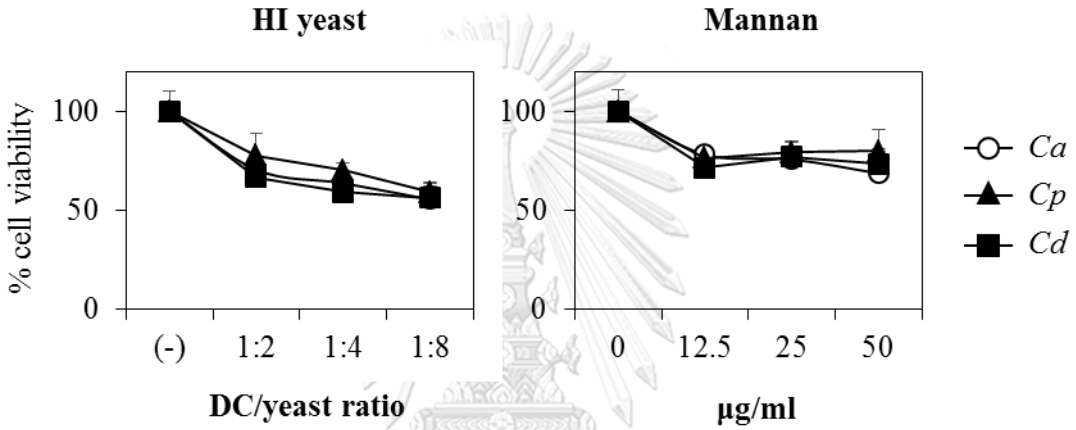


Figure 4.16 Cell viability of BMDCs stimulated with HI *Candida* yeasts and soluble mannans

BMDCs were incubated with HI *Candida* yeast cells and soluble mannans for 48 h. Then, the cell viability was determined by MTT assay. The various DC/yeast ratios and mannan concentrations were performed as indicated. Unstimulated BMDCs were used as the negative control (-). n = 5; Data are representatives of two independent experiments. *Ca*, *C. albicans*; *Cp*, *C. parapsilosis*; *Cd*, *C. dubliniensis*. The statistical analysis of significant differences is shown in Tables 4.1 and 4.2.

Table 4.1 Cell viability (mean ± SD %) of BMDCs stimulated with HI *Candida* yeasts

Candida species	DC/Yeast ratio		
	1:2	**1:4**	**1:8**
C. albicans	70.47 ± 6.63*	63.67 ± 4.65*	55.52 ± 8.23*,[1]
C. palapsilosis	77.76 ± 10.78*	70.19 ± 3.86*	59.12 ± 5.33*,[1]
C. dubliniensis	66.72 ± 3.66*	59.25 ± 4.22*	56.26 ± 6.21*,[1]

*$p<0.01$ comparison with the negative control (100.00 ± 10.19 %).

[1] $p<0.05$ comparison with ratio of 1:2.

Table 4.2 Cell viability (mean ± SD %) of BMDCs stimulated with *Candida* mannans

Candida species	Mannan concentration (μg/ml)		
	12.5	**25**	**50**
C. albicans	78.16 ± 3.57*	75.70 ± 8.60*	68.61 ± 10.80*
C. palapsilosis	76.26 ± 5.12*	79.35 ± 3.09*	79.83 ± 8.75*
C. dubliniensis	71.42 ± 7.24*	76.83 ± 8.27*	73.39 ± 7.87*

*$p<0.01$ comparison with the negative control (100.00 ± 10.47 %)

There was no difference among *Candida* species at the same mannan concentration.

Induction of DC activation by *Candida* yeast cell walls and soluble mannan

To examine the effect of yeast cell wall on DC responses and to avoid the undesirable consequences from cell death, we selected the DC/yeast ratio of 1:2 with DC viability above 65%, which was similar to the effect of mannan at all concentrations, for all subsequent experiments (Fig. 4.16, Table 4.1 and 4.2). The DC purity was first confirmed by flow cytometric evaluations of a DC marker, CD11c molecules. The percentage of $CD11c^+$ cells was analyzed by electronic gating on live cells that expressed CD11c (Fig. 4.17 A and Fig. 4.18 A). Consistently with the MTT results, the percentage of $CD11c^+$ BMDCs stimulated with HI *Candida* yeasts and soluble mannan were comparable (Fig. 4.18 A).

Next, DC maturation upon HI *Candida* yeast and soluble mannan stimulation was observed by an assessment of the expression of activation markers, CD40, CD80, CD86 and MHC class II, on $CD11c^+$ cells (Fig. 4.18 B-E). BMDCs stimulated with HI *C. parapsilosis* exhibited a significantly lower level of expression of CD86 and MHC class II, but not CD40 and CD80, when compared to BMDCs stimulated with HI *C. albicans* and *C. dubliniensis*. There was no difference in the expression of all activation markers on BMDCs when compared HI *C. albicans* to HI *C. dubliniensis*. On the contrary, when BMDCs were stimulated with soluble mannan at the highest concentration, *C. parapsilosis* mannan produced the strongest CD40 expression when compared to *C. dubliniensis* and *C. albicans*. However, comparable levels of CD80, CD86 and MHC class II expression were observed for *C. parapsilosis* and *C. dubliniensis* mannan, and these were notably higher than in BMDCs stimulated with *C. albicans* mannan. Our findings suggest that the cell wall and soluble mannan of each *Candida* species differentially induced DC activation. Furthermore, the immunogenicity of the whole yeast cell wall, albeit with the outermost immobilized mannan layer, was distinct from the soluble mannan.

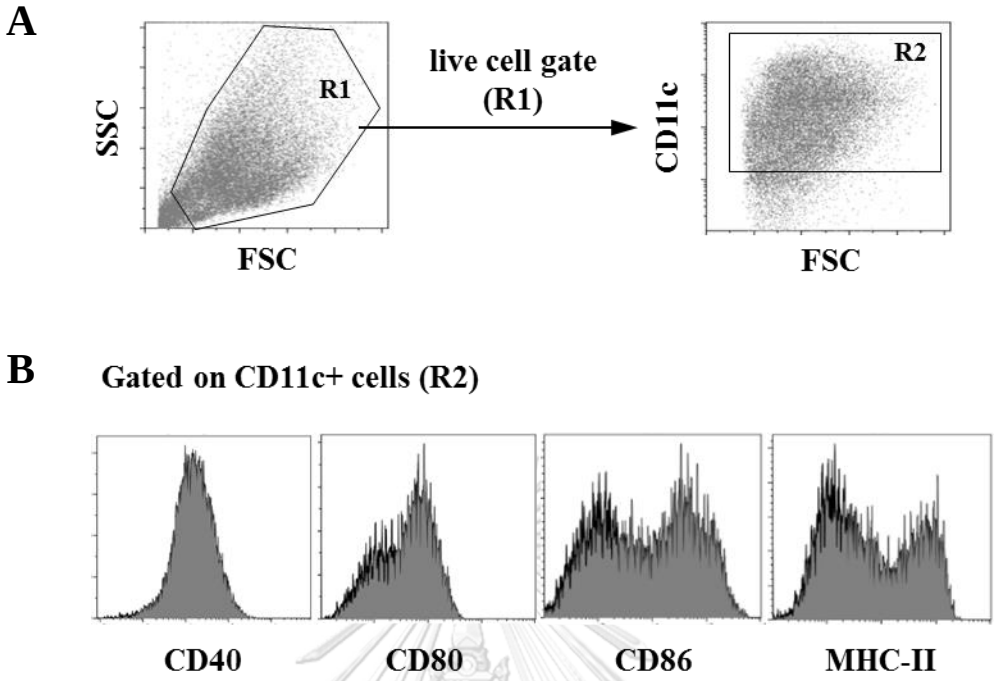


Figure 4.17 Diagram of flow cytometric analysis

After BMDC stimulation, the percent of $CD11c^+$ cells and the expression level of CD40, CD80, CD86 and MHC class II was assessed using flow cytometry. (A) The dot plot analysis of BMDC was first shown using side scatter (SSC) and forward scatter (FSC), and the R1 gate was placed to select the live BMDCs. In R1 gate, the $CD11c^+$ cells were identified as R2 gate based on the dot plot analysis of CD11c and FSC. (B) The expression levels of CD40, CD80, CD86 and MHC class II of $CD11c^+$ population (R2 gate) were subsequently analyzed as shown in the histograms.

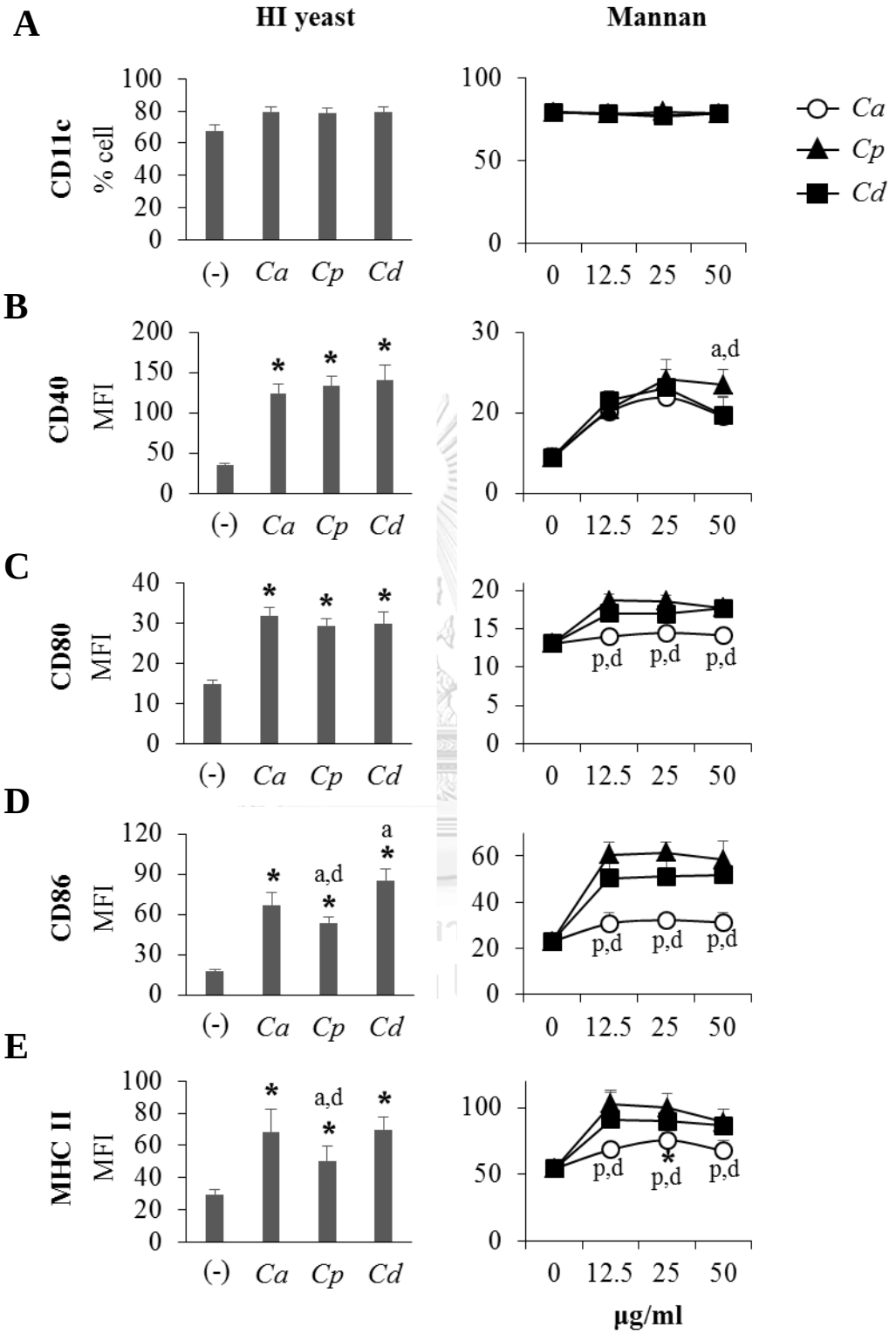


Figure 4.18 Induction of BMDC maturation by HI *Candida* yeasts and soluble mannan

BMDCs were stimulated with HI *Candida* yeast cells at the DC/yeast ratio of 1:2 and soluble mannan at the indicated concentrations. Forty eight hours later, the expression

of CD11c, CD40, CD80, CD86 and MHC class II were assessed using flow cytometry as shown in Figure 4.17. (A) Percentage of $CD11c^+$ population in BMDCs was analyzed, and the expression levels of (B) CD40, (C) CD80, (D) CD86, and (E) MHC class II were determined using geometric mean fluorescence intensity (MFI). * $p<0.05$ when compared to the negative control, ^{a} $p<0.05$ when compared to *C. albicans*, ^{d} $p<0.05$ when compared to *C. dubliniensis*, ^{p} $p<0.05$ when compared to *C. parapsilosis*; n = 5; Data are representatives of two independent experiments. *Ca*, *C. albicans*; *Cp*, *C. parapsilosis*; *Cd*, *C. dubliniensis*. All concentrations of *C. parapsilosis* and *C. dubliniensis* mannan induced significantly higher levels of CD40, CD80, CD56 and MHC class II expression at $p<0.05$ when compared to the negative control.

Functional properties of DCs activated with *Candida* yeast cell walls and soluble mannan

The pro-inflammatory cytokines, IL-1β and TNF-α, are primary cytokines detected in oral candidiasis lesion (80, 106, 107), therefore, we compared the production of these two cytokines in BMDCs in response to HI *Candida* and the mannans (Fig. 4.19). Heat-inactivated *C. dubliniensis* induced the highest level of IL-1β (Fig. 4.19 A), while mannans from all *Candida* could not promote this cytokine production. Besides, all HI *Candida* upregulated the expression of TNF-α to comparable levels, but *C. parapsilosis* mannan induced the highest level of TNF-α (Fig. 4.19 B). In addition, at the highest concentration of mannan, BMDCs stimulated with *C. albicans* mannan significantly produced more TNF-α than those stimulated with *C. dubliniensis* mannan.

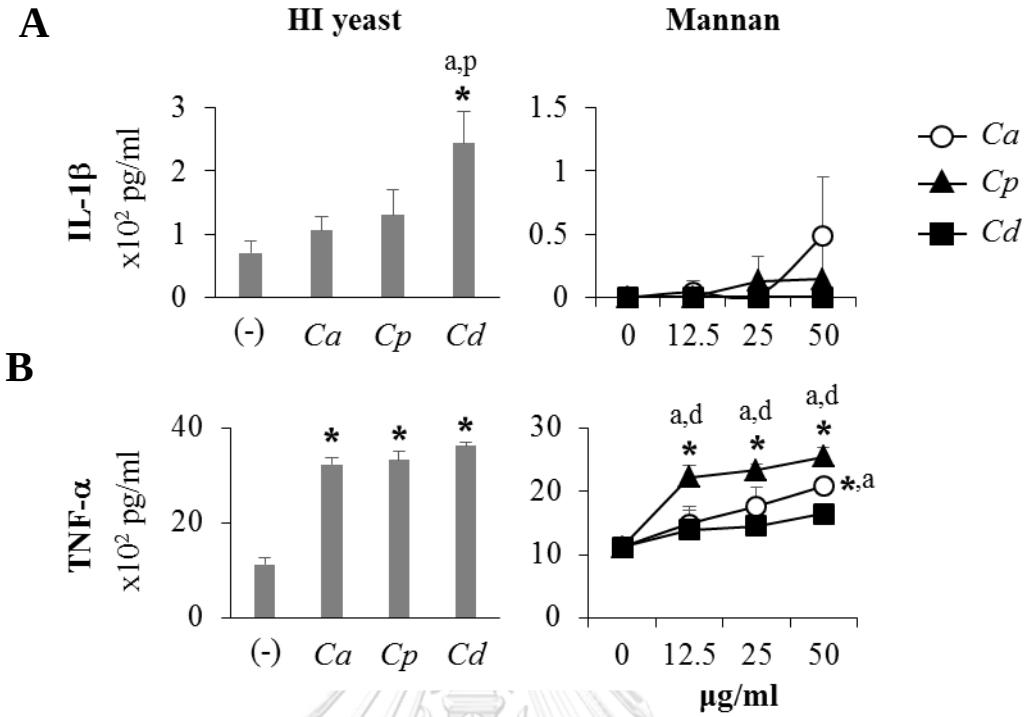


Figure 4.19 Level of pro-inflammatory cytokines, IL-1β and TNF-α, produced by BMDCs stimulated with HI *Candida* yeasts and soluble mannans

BMDCs were stimulated with HI *Candida* yeast cells and soluble mannan for 24 h. Subsequently, (A) IL-1β and (B) TNF-α were measured by ELISA. * $p<0.05$ when compared to the negative control, [a] $p<0.05$ when compared to *C. albicans*, [d] $p<0.05$ when compared to *C. dubliniensis*, [p] $p<0.05$ when compared to *C. parapsilosis*; n = 5; Data are representatives of two independent experiments. *Ca*, *C. albicans*; *Cp*, *C. parapsilosis*; *Cd*, *C. dubliniensis*.

Since the cytokine production from DCs in response to stimuli provides the foundation to direct the type of effector T lymphocytes (64), we thus evaluated BMDC secretion of T helper cell polarizing cytokines: IFN-γ and IL-12 (Th1), IL-6 and IL-23 (Th17), IL-4 (Th2) and IL-10 (T_{reg}), when activated with HI yeast cells or soluble mannan (Fig. 4.20). In comparison to HI *Candida* yeast cells, the soluble mannan was a weaker stimulus as it induced lower levels of all cytokines (Fig. 6a-f). When compared among HI *Candida* species, HI *C. dubliniensis* induced the highest

production of IFN-γ, IL-12, IL-6, IL-23, and IL-10 (Fig. 4.20 A-D and 4.20 F). In contrast, the soluble mannan from *C. dubliniensis* induced a weak production of these five cytokines, while *C. parapsilosis* mannan induced the highest level of cytokine production (Fig. 4.20 A-D and 4.20 F). Interestingly, neither HI *Candida* yeast cells nor soluble mannan could promote IL-4 production (Fig. 4.20 E). On the other hand, all HI *Candida* yeast cells, but not the soluble mannan, significantly induced the production of IL-10 compared to the negative control. Consistently with our observation of differential induction of DC activation markers, the whole yeast cell wall and soluble mannan differed in the capability for pro-inflammatory cytokine induction. Altogether, the cell wall and soluble mannan of the NACs triggered a stronger inflammatory response of DC when compared to those of *C. albicans*.

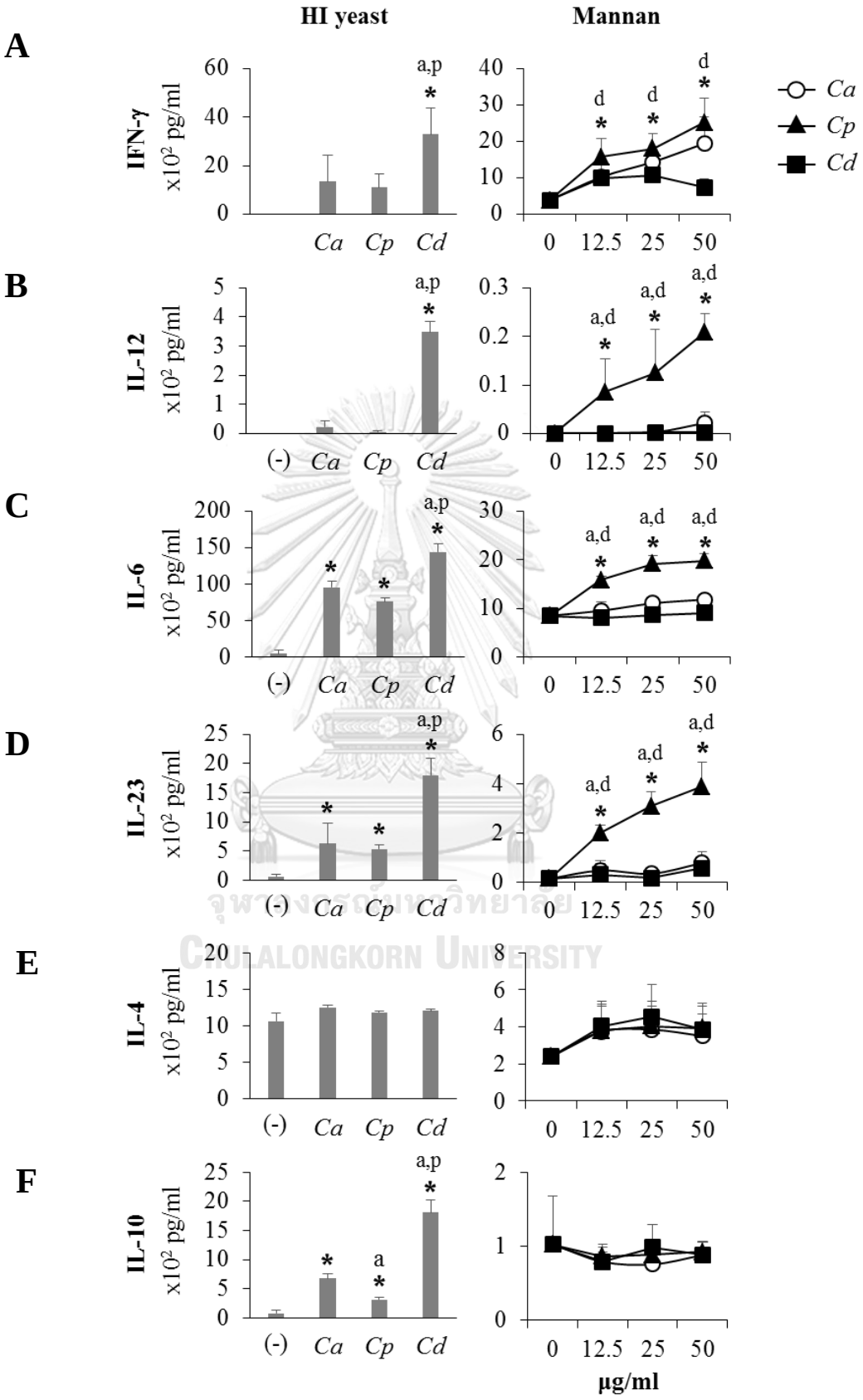


Figure 4.20 Production of T helper cell polarizing cytokine from BMDCs stimulated

with HI *Candida* yeasts and soluble mannan

BMDCs were stimulated with HI *Candida* yeast cells and soluble mannan for 24 or 48 h (please see detail in materials and methods), and then (A) IFN-γ, (B) IL-12, (C) IL-6, (D) IL-23, (E) IL-4 and (F) IL-10 were measured by ELISA. * $p<0.05$ when compared to the negative control, [a] $p<0.05$ when compared to *C. albicans*, [d] $p<0.05$ when compared to *C. dubliniensis*, [p] $p<0.05$ when compared to *C. parapsilosis*; n = 5; Data are representatives of two independent experiments. *Ca*, *C. albicans*; *Cp*, *C. parapsilosis*; *Cd*, *C. dubliniensis*.

Discussion

Oral mucosal DCs play a major role in the induction of immunity and tolerance, and the outcomes of the immune response depend on the functional versatility of these DCs, which in turn depends on the various types of pathogens and stimuli. Here, we compared the responses of DCs to the cell wall and the soluble mannan of *C. albicans*, *C. parapsilosis* and *C. dubliniensis,* which are important opportunistic fungi that can cause oral candidiasis. Although mannan locates at the outermost layer of *Candida* cell wall and should be the first component that interacts with host cells, we found that DC responses against whole cell wall and soluble mannan were distinct (Fig. 4.16-4.18). *Candida* cell wall comprises mannan, glucan, and chitin, which are located at the outermost, middle and innermost layer of the cell wall, respectively (1). Thus, the results observed when BMDCs were stimulated with HI yeast cells could be due to the combination of interactions with all components of the cell wall. Furthermore, heat treatment of *Candida* yeast cells possibly led to an increased exposure of β-glucan on the cell wall surface, and consequently increased the signaling via dectin-1 receptor (82, 108).

Our study using the soluble mannan extracted from *Candida* cell wall provided a more direct evidence for mannan-mediated DC responses. *Candida* mannan engages with diverse receptors, such as dectin-2, mannose receptor (MR), DC-SIGN, galectin-3, complement receptor 3 (CR3), TLR-2, TLR-4 and TLR-6 (1), depending on its structure (33, 42, 47). Cell wall mannan of *Candida* species not only differs in the structure, but also the proportions of the various structures (35, 109, 110). These could result in the differential DC activation and cytokine production in response to the HI yeasts and the soluble mannan (Fig. 4.18, 4.19 and 4.20). Evidences showed that α-mannan of *C. albicans* binds to dectin-2, while β-mannan is recognized by galectin-3 and TLR-2 (1, 42, 111). It should be noted that *C. albicans* mannan contains large branches with high content of α-mannan, therefore, the signal transduction via dectin-2 may be dominant (42, 110). In contrast, *C. dubliniensis* contains high level of β-mannan, which possibly engages to galectin-3 (36, 112) that activates the TLR-2 signaling pathway. *C. parapsilosis* mannan contains short

branches with low mannosyl content (110, 113). It is still ambiguous which receptor is specific for *C. parapsilosis* mannan. Nevertheless, a previous study suggests that the recognitions of *C. parapsilosis* and *C. albicans* cell wall by human peripheral blood mononuclear cells were distinct (113) (Fig. 4.18, 4.19 and 4.20).

Studies in mouse model of oral candidiasis infected with *C. albicans* showed the induction of IFN-γ, IL-12, IL-6 and IL-23 in oral tissues (114, 115). We also found the production of these cytokines from BMDCs stimulated with HI *C. albicans* (Fig. 4.20). On the other hand, while a previous study demonstrated that soluble mannan extracted from *C. albicans* induced the production of IL-6 and IL-23 (42), we found that *C. albicans* mannan mainly induced IFN-γ production in a dose-dependent fashion (Fig. 4.20). These different cytokine induction results could be due to the differences in mannosyl composition of the extracted mannan. While our mannan possibly contained both α- and β-mannosyl linkages, the mannan in the previous study may be composed of only α-mannosyl linkages, because yeast cells were cultured in a carbon-limiting, low pH, and low temperature condition where β-mannosyl linkages could not be synthesized (42).

Several reports indicated that β-glucan was the key component provoking protective immunity and mannan played more role in *Candida* immune evasion (1, 116). Our observation that *C. albicans* mannan mediated IFN-γ production may, at least in part, help to explain this phenomenon (Fig. 4.20). Our results suggest that mannan-induced IFN-γ from DCs may skew T cell polarization towards Th1, resulting in the down-modulation of Th17 protective immunity.

To date, there is little evidence on the role of oral immunity on the pathogenesis of *C. parapsilosis* and *C. dubliniensis*. A recent study in mouse model of *C. parapsilosis* infection has demonstrated that the level of proinflammatory cytokines was correlated to disease severity (117). The transcriptome profile in murine macrophage also has demonstrated a significant upregulation of pro-inflammatory cytokine genes, such as TNF-α and IL-1β, in response to *C. parapsilosis* (118). In addition, the same study further showed that *C. parapsilosis* promoted high TNF-α production in human monocyte-derived macrophage model (118). The recent study using *C. parapsilosis och1* mutant that lacks N-linked mannan in the cell wall

implicated the role of *C. parapsilosis* mannan as a virulence factor. Consistently, we found that BMDCs stimulated with soluble mannan of *C. parapsilosis* was highly activated and produced high levels of proinflammatory cytokines, but a low level of the anti-inflammatory cytokine IL-10 (Fig. 4.18 and 4.20). In a newborn mouse infection model, the *och1* mutant exhibited less pathogenicity comparing to the wild type *C. parapsilosis* (119). Although the involvement of the high pro-inflammatory cytokine production induced by *C. parapsilosis* mannan still needs further investigation, the aforementioned evidences support the important role of the cell wall mannan in *C. parapsilosis* immunopathogenicity. On the contrary, BMDCs stimulated with *C. dubliniensis* mannan produced only IFN-γ, while HI *C. dubliniensis*-stimulated BMDCs produced high levels of both pro- and anti-inflammatory cytokines (Fig. 4.19 and 4.20). Although the consequence of this result is still unclear, it is possible that HI *C. dubliniensis* induced IL-10, which plays a major role in immune regulation (44). In addition, *C. dubliniensis* mannan have the lowest potential to induce TNF-α in BMDCs. Altogether, these findings may support previous studies that suggested lower pathogenicity of *C. dubliniensis* (120, 121).

Many *Candida* species are dimorphic fungi, which can undergo phenotypic switching between the yeast and filamentous forms. Our current work investigated cell wall mannan of *Candida* yeast cells since this form initially colonizes host tissues and blood stream. Furthermore, *C. parapsilosis* lacks hyphae formation (24), thus we focused our attention on the comparison of yeast forms only. Nevertheless, it has been demonstrated that cell wall mannan of *Candida* yeast and hyphae differs in composition and structure (34, 122), and this difference possibly leads to distinct immune responses against the yeast and hyphae form (80). Thus, further study on DC responses to both yeast and hyphae forms of different *Candida* species are required.

Our results in this study are based on the responses of murine BMDCs, which could be directly applied in understanding mouse models of *Candida* infection. Nevertheless, murine BMDCs may partly differ from human monocyte-derived DCs (MoDCs). Thus, there is a limitation in applying these results to human infection. Yet, murine BMDCs are widely used as the platform for studying DC immunobiology as well as vaccinology and immunotherapy. In addition, *in vitro* generated BMDCs have

been well proved to be potent APCs that can activate and polarize T cells (75). Moreover, a large number of cells were required for our experiments, and this makes the use of human monocytes impossible for this study. Therefore, future work on human DCs is still required in order to translate our findings to the human system.

In summary, cell wall mannan may function as a virulence factor promoting the immunopathogenicity and immune evasion of oral *Candida* species through differential activation of DCs. A better understanding on the pathogenesis and the induction of protective immunity against various *Candida* species is a necessary foundation for the development of clinical diagnostic tools and therapeutic applications.

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APPENDIX

VITA

Miss Thu Nguyen Ngoc Yen was born in January 22, 1988 in An Giang, Vietnam. She graduated the degree of Doctor of Dental Surgery (D.D.S) with second-class honors from Faculty of Odonto-Stomatology, University of Medicine and Pharmacy at Ho Chi Minh City, Vietnam in academic year 2012. She started her study in doctoral degree of oral biology program in 2013 at Faculty of Dentistry, Chulalongkorn University. All of her research work was performed at the Oral Biology Research Center, Faculty of Dentistry, Chulalongkorn University.