Effect of *Candida glabrata* β-glucans on immunosuppressive properties of dendritic cells

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ผลของเบต้ากลูแคนส์ของแคนดิดากลาบราตาต่อคุณสมบัติการกดภูมิคุ้มกันของเดนไดรติกเซลล์

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เบต้ากลูแคนส์เป็นคาร์โบไฮเดรตประกอบด้วยโครงสร้างที่ตำแหน่ง 1, 3 และ 1, 6 อยู่ในผนังเซลล์ของแคนดิดากลาบราตา ทำหน้าที่เป็นตัวกระตุ้นเชิงชีวภาพต่อกลไกการตอบสนองของระบบภูมิคุ้มกัน เดนไดรติกเซลล์ทำหน้าที่นำเสนอแอนติเจนอย่างมีประสิทธิภาพโดยมีบทบาทสำคัญทั้งระบบภูมิคุ้มกันแบบไม่จำเพาะและจำเพาะ วัตถุประสงค์ของงานวิจัยเพื่อศึกษาผลการตอบสนองทางภูมิคุ้มกันวิทยาของเดนไดรติกเซลล์ต่อเบต้ากลูแคนส์ของแคนดิดากลาบราตา เดนไดรติกเซลล์ที่กลายมาจากเซลล์ไขกระดูกและถูกกระตุ้นด้วยเบต้ากลูแคนส์ที่ความเข้มข้นแตกต่างกัน พบการแสดงออกที่มากขึ้นอย่างมีนัยสำคัญทางสถิติที่ระดับ 0.05 ของสัญลักษณ์ซีดี-80, -86, -40 และเอ็มเอชซีทู รวมไปถึงพบการตอบสนองของเดนไดรติกเซลล์มีผลต่อการผลิตอินเตอร์ลิวคิน 10 ให้เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติที่ระดับ 0.05 อีกทั้งเดนไดรติกเซลล์ที่ถูกกระตุ้นด้วยเบต้ากลูแคนส์มีส่วนส่งเสริมการผลิตอินเตอร์ลิวคิน 10 ที่ผลิตจากที-เซลล์ อินเตอร์ลิวคิน 10 เป็นไซโตไคน์ที่ทำหน้าที่ส่งสัญญาณยับยั้งกระบวนการอักเสบและส่งเสริมกระบวนการกดภูมิคุ้มกัน นอกจากนั้นผลวิจัยเบื้องต้นพบว่าเบต้ากลูแคนส์ของแคนดิดากลาบราตามีผลต่อการลดระดับปริมาณของแอนติบอดีที่ต่อต้านดีเอ็นเอของตัวเอง (anti-(double-stranded)-DNA antibodies) ในหนูที่เป็นสัญลักษณ์ของโรคลูปัส (*Fcgr2b*$^{-/-}$ lupus-prone mice) ผลงานวิจัยนี้แสดงให้เห็นว่าเบต้ากลูแคนส์ของแคนดิดากลาบราตาสามารถเหนี่ยวนำสภาวะการกดภูมิคุ้มกันในเดนไดรติกเซลล์และที-เซลล์ รวมไปถึงอาจจะเป็นประโยชน์ต่อการบรรเทาอาการของโรคลูปัสในหนูทดลอง

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Phawida Tummamunkong : Effect of *Candida glabrata* β-glucans on immunosuppressive properties of dendritic cells. Advisor: Assoc. Prof. PATCHAREE RITPRAJAK, D.D.S., Ph.D.

β-glucan is a polysaccharide and consists of the backbone of β-(1, 3)-glucan and/or β-(1, 6)-glucan structures. β-glucans are extracted from the cell wall of *Candida* sp, including *Candida glabrata*. Interestingly, the biological activity of *Candida* β-glucan has been represented as a stimulator in the mechanism of immune responses. Dendritic cells are powerful antigen-presenting cells that play a significant role in both innate and adaptive immunity. In the present study, we evaluated the effect of *C. glabrata* β-glucans on dendritic cell (DC) immunologic responses. Firstly, bone marrow-derived DCs (BMDCs) were induced with *C. glabrata* β-glucans in a dose-dependent manner. The expression of CD80, CD86, CD40, and MHCII in BMDC markedly increased after stimulation. *C. glabrata* β-glucan-stimulated BMDCs significantly enhanced the production of immunosuppressive cytokine, interleukin-10 (IL-10). Secondly, BMDC after stimulation could promote a high level of IL-10 secretion by T cells. IL-10 plays a critical role in limiting the inflammatory immune response and its ability to mediate the immunosuppressive response. Furthermore, the level of serum anti-(double-stranded)-DNA antibodies promisingly decreased in *Fcgr2b*$^{-/-}$ lupus-prone mice after *C. glabrata* β-glucans immunization in an *in vivo* experiment. Our results imply that *C. glabrata* β-glucans have the induction of immunosuppressive responses in dendritic cells and T cells and may be beneficial for the amelioration of lupus disease.

Field of Study:	Medical Microbiology (Interdisciplinary Program)	Student's Signature .....
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Phawida Tummamunkong

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CHAPTER I

INTRODUCTION

β-glucans are in the form of complex polysaccharides that are covalently linked to other polysaccharides, proteins, and a side-chain-branched (1,3;1,6)-β-glucan [1]. Different sizes and branching patterns of β-glucans lead to various immune responses. Understanding the mechanism behind the induction of β-glucans in immunomodulation is helpful for clinical treatment. Several studies revealed that β-glucans directly restored immune suppression associated with tumor-educated dendritic cells that promoted helper and cytotoxic T lymphocyte priming and improved antitumor responses [2]. Anti-tumor monoclonal antibodies combined with soluble yeast glucans were used as an adjuvant to recruit granulocytes (tumor killer cells) triggered by CR3 recognition of tumor cell-bound cells [3]. Hence, β-glucans have essential biological functions that are useful for the development of clinical applications.

β-glucans can be isolated from various sources (cereals, mushrooms, lichens, algae, and microorganisms) with different characteristics such as glycosidic links, degree of branching, molecular weight, and solubility [4]. In this study, we focus on β-glucans in the *Candida* cell wall. Mannans, chitins, and β-glucans are the major components of fungal cell walls [5]. β-glucans of *Candida* species have been identified as an immunomodulator of anti-*Candida* immunity and have mainly induced the expression

of immunostimulatory molecules and the production of pro-inflammatory cytokines [6, 7]. The interaction of *Candida* β-glucans acts between innate and adaptive immune responses. Innate immune activation is triggered by *Candida* β-glucans through macrophages, dendritic cells, and neutrophils, as well as epithelial cells [8]. β-glucans mainly induce inflammatory responses through recognition of pattern recognition receptors (PRRs) such as C-type lectin receptors and Toll-like receptors (TLRs) to initiate adaptive immunity [9, 10]. Adaptive immune responses are orchestrated and rely on antigen-presenting cells (APC), such as macrophages, dendritic cells, and B cells. Naïve T lymphocytes recognize antigens via major histocompatibility complex (MHC) class I or MHC class II molecules on APCs, especially dendritic cells [11]. Dendritic cells play a crucial role in the presentation of antigens that help drive naïve T cells to specific effector T cells. Furthermore, yeast β-glucans can induce autophagy as a crucial mechanism for DC maturation and influence T cell differentiation [12].

The members of the *Candida* species reveal the most prevalent opportunistic fungal pathogens. *Candida glabrata* is a member of the non-*Candida Albicans Candida* (NCAC) species which is defined as the human pathogen [13]. *C. glabrata* in systemically infected immunocompetent mice was promoted to rapid induction of immunoreactive proteins for pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-a), interleukin-12 (IL-12), and gamma interferon (IFN-γ) and lack of induction of anti-inflammatory cytokine (IL-10). Unlike *C. albicans*-infected mice, IL-10 was rapidly

induced but delayed (i.e., TNF-a) or absent (i.e., IL-12 and IFN-γ) induction of immunoreactive pro-inflammatory cytokines [14]. Whereas our preliminary result shows that *C. glabrata* β-glucan isolates strongly induced IL-10 production from bone marrow-derived dendritic cells (BMDC) *in vitro.* Subsequently, *C. glabrata* β-glucan was administered to mice, resulting in enhanced IL-10 production under PPARγ expression, promoting attenuation of colitis and elimination of *C. glabrata* [15].

Dendritic cells (DC) serve as unique sentinels of the immune system, which are the most potent antigen-presenting cells, driving both T-cell immunity and tolerance [16]. Apart from antigen presentation, dendritic cell-based immunotherapy is the clinical application that uses the beneficial impacts of both types of DC (immunogenic and tolerogenic DCs) [17]. Immunogenic DCs are used by cancer patients to initiate antitumor immune responses [18]. Tolerogenic DCs (tolDCs) have potently maintained tolerance to self-antigen. These DCs play an important role in inducing peripheral tolerance through specific mechanisms such as regulatory T cell expansion, suppression of effector T cells, and secretion of immunosuppressive cytokines (e.g., IL-10, TGF-β) [19]. Standard immunosuppressive therapies often do not specify the target of the disease and are accompanied by severe side effects. In contrast, tolDCs have an attractive therapeutic approach to induce, enhance, or restore tolerance to specific antigens [20]. For example, estriol-generated tolDCs improved inhibitory costimulatory markers (PD-L1, PD-L2, B7-H3, and B7-H4) and immunoregulatory expression of IL-10 and TGF-b

mRNA to protect against inflammatory autoimmune diseases [21]. DC treated with dexamethasone generated from allergic patients to natural rubber latex (NRL) plays an important role in modulation of allergen-specific T cell responses and IgE production to support their potential use in allergen-specific immunotherapy [22].

Here, we investigated the effect of DC and T cell responses through induction of *C. glabrata* β-glucans. Dendritic cells were derived from bone marrow cells of mice and stimulated with microparticulate β-glucans *in vitro*. We used the *ex vivo* coculture system for the observation of T cell responses. Preliminary results show that *C. glabrata* β-glucans primed-DCs lead to immunosuppressive responses by IL-10 secretion. IL-10 is an anti-inflammatory cytokine and powerfully influences immunosuppression. The immunosuppression state in DC contributes to the generation of immune non-responsiveness or tolerance to antigens. These data have the potential to manage immune-mediated diseases, especially allergies, transplantations, autoimmunity, or chronic inflammatory diseases. Our research provides a benefit for the development of immunotherapy in immune-mediated diseases.

CHAPTER II

HYPOTHESIS AND OBJECTIVE

Research questions

1. How do dendritic cells respond to *C. glabrata* β-glucans *in vitro*?

2. How do T cells respond to *C. glabrata* β-glucans-stimulated dendritic cells *in vitro*?

3. Can *C. glabrata* β-glucans ameliorate lupus disease *in Fcgr2b*$^{-/-}$ lupus-prone mice?

Hypothesis

C. glabrata β-glucans can promote immunosuppressive effect in dendritic cells and ameliorate lupus disease in *Fcgr2b*$^{-/-}$ lupus-prone mice.

Research objectives

1. To determine dendritic cell response to *C. glabrata* β-glucans.

2. To determine T cell response to dendritic cells after *C. glabrata* β-glucans stimulation.

3. To determine the amelioration of lupus disease in response to *C. glabrata* β-glucans *in Fcgr2b*$^{-/-}$ lupus-prone mice.

CHAPTER III

LITERATURE REVIEW

β-glucans

β-glucans are natural polysaccharides with other polysaccharides, proteins, and side chains. Its form mostly occurs in the cell wall of some plants, fungi, bacteria, mushrooms, yeast, and seaweeds [23]. β-glucans are well known for their property in stimulating the host immune system and increasing the resistance to various viral, bacterial, protozoan, and fungal diseases [24]. For clinical application, β-glucans have been considered an immunomodulator that improves antimicrobial and anticancer activities [25]. Yeast-derived β-Glucan in cancer therapy has been involved in trained immunity, where innate cells take on memory phenotypes. Additionally, it has the ability to regulate the tumor microenvironment (TME) by bridging the innate and adaptive immune responses and by modulating the phenotype of immune-suppressive cells to be immune-stimulatory [26]. In contrast, β-Glucans partially represented biological activity with anti-inflammatory effects. Yeast β-Glucan could decrease pro-inflammatory modulators of TNF-α, IL-6, IL-1β, CCL2, and SAA3 and increase the anti-inflammatory factors of *Azgp1* at protein and/or mRNA levels in a model of diabetic mice [27]. *Candida albican* β-Glucan strongly induced the anti-inflammatory cytokine, IL-1Ra which specifically induced an Akt/PI3 K-dependent anti-inflammatory IL-1Ra response [28]. Both cultured macrophages alone and cocultured macrophages and lymphocytes *in vitro* exposure to 1,3-β-glucan promote the secretion of anti-

inflammatory cytokines, resulting in a decrease in the expression of T helper 1 cytokines and an increase in T helper 2 [29]. Furthermore, dietary oat β-glucan is a good prognostic for use in Crohn's disease dietotherapy by reducing the gene expression of pro-inflammatory cytokines and their protein concentration in an animal model [30].

Sources of β-glucans

Up to now, β-glucans are divided into five groups as shown in Figure 1 [4]. Depending on advances in research, β-glucans have been isolated from cereal sources and have a specific combination of β-(1⟶4) and β-(1⟶3) linkages into linear long-chain polysaccharides of high-molecular-weight linear long chain polysaccharides [31]. Another β-glucans have been isolated from sources of microorganisms, as well as from some mushrooms, lichens, and seaweed/algae [4]. In our proposed study, we isolated β-glucans from *Candida* species. *Candida* β-glucans have been isolated from their cell wall [32]. β-glucan extracted from the different sources leads to various characteristics such as glycosidic linkages, degree of branching, molecular weight, and solubility [4].

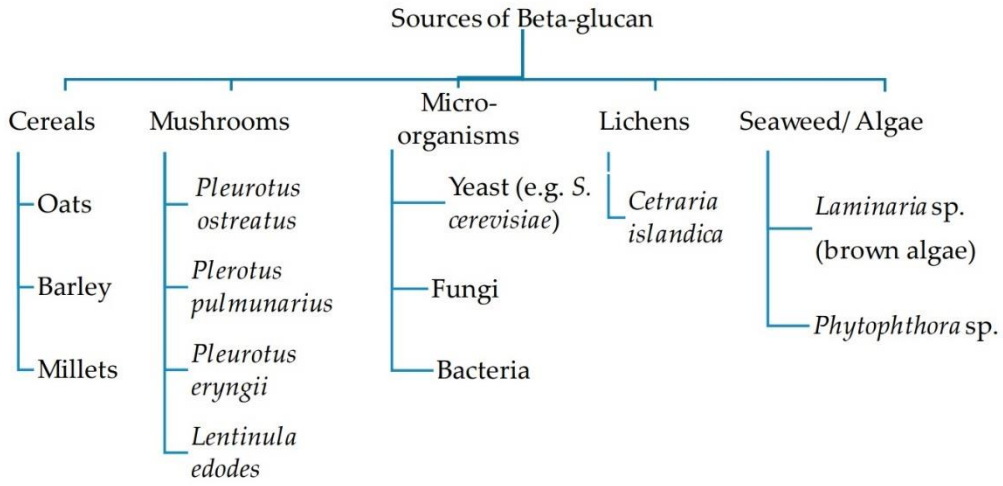


Figure 1 Sources of β-glucans.

Candida β-glucans

Candida species reveal the most prevalent opportunistic fungal pathogens. In healthy humans, *Candida* species are commensal fungi in nature and commonly colonize mucous membranes and the skin [33]. The *Candida* cell wall consists of proteins, lipids, β-glucans, mannans, and chitins. The outer layer of the cell wall is made up of mannans and the inner layers are mainly composed of β-glucan and chitin skeleton [34]. Mannans and β-glucans have significant biological activities in stimulating the host immune system [35]. Immune responses to *Candida* species orchestrated both innate and adaptive immunity. β-glucans are the main component identified as pathogen-associated molecular patterns (PAMPs) and recognized by pattern recognition receptors (PRRs) [36, 37]. C-type lectin receptors (CLRs) such as dectin-1, dectin-2 and dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), mannose receptor (MR), Mincle, Galectin-3, toll-like receptors (TLRs)

(such as TLR2 and TLR4) and complement receptor 3 (CR3) have all been associated with the recognition of fungal cell wall components as shown in Figure 2 [36, 38]. The most well-known β-glucan receptor is dectin-1. For example, *C. krusei* β-glucans partially induced host immune defenses via dectin-1 receptor and promoted the production of IL-10 by T cells [39]. Dectin-1 triggers cytokine production through Syk-dependent production of the T-helper 2 type anti-inflammatory cytokine (interleukin-10) and Toll-like receptor-Myd88–dependent stimulation of monocyte-derived proinflammatory cytokines (tumor necrosis factor) after *C. albicans* infection [6]. Dectin-1 modulated the limitation of systemic infections and the generation of differential adaptation of the *C. albicans* strain *in vivo* [40]. In addition, mice lacking the dectin-1 receptor are more susceptible to systemic infection by *C. glabrata* [41].

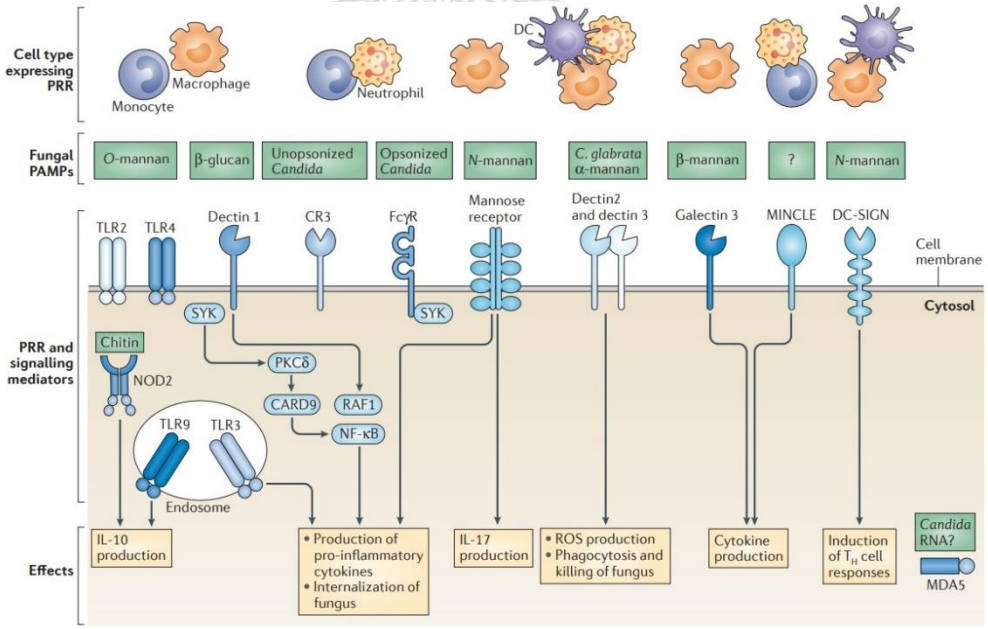


Figure 2 Recognition of *Candida* species by various PRRs on innate immune cells.

Defense against fungal infection is provided through the interplay between innate and adaptive immunity. Dendritic cells (DC) are critical for the initiation of adaptive T -cell mediated immune defense [42]. The recognition of *Candida* β-glucans by β-glucans receptors expressed on the surface of DC leads to T cell differentiation. Naïve $CD4^+$ T cells differentiate into effector T helper cells by the presentation of DC [11]. Intracellular bacterial infection mediates immune protective responses by inducing T helper 1, whereas T helper 2 cells are stimulated by helminth infection [43]. T helper 17 cells are markedly characterized for the protective immune responses of fungal invasion with a minor fraction belonging to the Th1 or Th2 subsets [44, 45]. Th17 expresses chemokine receptors (CCR4 and CCR6) [46] and mediates cytokine production (IL-17A, IL-17F, and IL-22) to generate defenses against fungal infection [38].

C. glabrata and _C. albicans_ β-glucans

β-glucans are in the inner layer of the *Candida* cell wall and consist of carbohydrate backbones with many different of side-chain-branched β-1,3 and 1,6-glucan as shown in Figure 3 [34]. *C. albicans* β-glucans are the major components, accounting for 50-60% by weight of the cell wall [47]. The cell wall composition of *C. glabrata* contains approximately 50% more protein and the relative level of total glucan is lower than the walls of *C. albicans* and *Saccharomyces cerevisiae* [48]. Furthermore, *C. glabrata* blastoconidia (1 to 4 μm) has been considered smaller than *C. albicans* blastoconidia (4 to 6 μm) [49]. *C. albicans* is a dimorphic yeast, and host invasion can occur through two mechanisms, including the induction of host

endocytosis and the formation of hyphae [50]. The individual characteristics of *C. albicans* led to a stronger inflammatory response than *C. glabrata* infection [51].

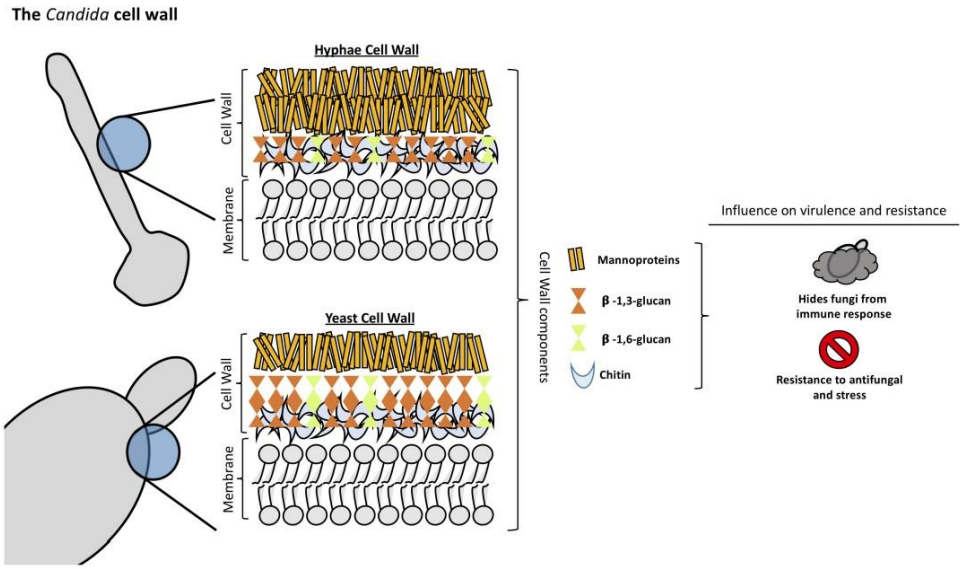


Figure 3 Structural organization and composition of *C. albicans* cell wall.

C. glabrata is a non-dimorphic yeast and penetrates the host bloodstream by accidental breach of natural barriers [52]. *C. glabrata* is markedly recognized and ingested by macrophages more than *C. albicans* [53]. However, *C. glabrata* is a commensal microorganism like *C. albicans,* and thus there are similar common host defense mechanisms that effectively control *C. glabrata* [49].

Dendritic cells

Dendritic cells (DCs) are unique sentinel leukocytes that respond early to antigens or danger signals and initiate innate and adaptive immunity [16]. Their function has an efficient ability to activate naïve T lymphocytes by expression of activation markers and secretion of cytokines and chemokines. DCs generally express CD11c and

MHC class II molecules, but once activated, they differentiate into mature DCs and their expression levels of surface MHC–peptide complexes and of co-stimulatory molecules obviously increase and potentially activate T cells [54]. There are four main subsets of DCs including conventional DCs (cDCs), monocyte-derived DCs (MoDCs), plasmacytoid DCs (pDCs), and Langerhans cells (LCs) [42]. Based on their functional responses, DCs have been defined as 'gatekeepers of the immune system', acting to orchestrate the maintenance of immune homeostasis in both immune activation and immune tolerance [55, 56]. Immunogenic DCs play a critical role in activating innate immunity through pattern recognition receptors (such as Toll-like receptor, C-type lectin receptor) and contribute to the priming of T cell differentiation [55].

On the other hand, tolerogenic DCs have been driven and maintained immune suppression by inducing immune silence to encounter antigens. They participate in both central and peripheral tolerance [57]. Therefore, tolerogenic DCs have been defined as the target of immunosuppressants for the regulation of hypersensitivity immune responses. For instance, quercetin which is a potent immunosuppressive agent, strongly generated an inhibitory effect on LPS-induced DCs by decreasing the production of proinflammatory cytokines/chemokines and the expression levels of MHC class II and costimulatory molecules. These reactions can be used for the treatment of chronic inflammation, autoimmunity, and transplantation by abolishing DC function [58]. Probiotic bacteria and steroids can generate immunosuppressive effects in human intestinal DCs for the treatment of acute ulcerative colitis [59].

Strategies in the clinical treatment of autoimmune disease, hypersensitivity, chronic inflammatory diseases, and transplantation focus on the inhibition of immune response by enhancing immune tolerance.

Role of dendritic cells and T cells in response to *C. glabrata* β-glucans

In this study, we investigated DCs and T cell response to *C. glabrata* β-glucans. DC functions, as previously described, play an important role in the protective reaction and antigen presentation. The expression of DC activation markers and cytokine production are the main signals in response to antigens. Our preliminary results show that murine bone marrow-derived DCs (BMDCs) can express the differential activation marker and secrete pro-inflammatory and anti-inflammatory cytokines. Interestingly, *C. glabrata* β-glucans-induced BMDCs can promote a high level of IL-10 as an anti-inflammatory cytokine. Previous evidence supports the notion that IL-10 mediates immunosuppression. IL-10 and transforming growth factor (TGF)-β1 can generate a local immunosuppressive environment in HPV-associated cervical neoplasia [60]. Blocking IL-10 resulted in alleviation of MDSC-mediated immunosuppression and improved survival in *in vivo* tumor progression [61]. IL-10 production in our preliminary *in vitro* assay may support the generation of tolerogenic properties in DCs. Recently, induced tolerogenic DCs are one type of dendritic cells. Their maturation can promote T cell tolerance due to exogenous signals such as microbial components, pharmacological and dietary agents, or certain physiological agents, and also by other specific metabolites, cytokines, and growth factors [62]. *C. glabrata* β-glucans in our

study are used as a stimulator to modulate immune responses in BMDC. Normally, understanding the immunosuppressive reaction in clinical management has traditionally been focused on lymphocytes. In this study, we designed the *in vitro* coculture assay of DCs and T cells for the observation of T cell responses. The tolerogenic characteristics of DC and/or T cells after induction of *C. glabrata* β-glucans are powerful tools to alleviate allergies, transplantation, autoimmunity, or chronic inflammatory diseases and develop new strategies for clinical therapy.

The differentiation of dendritic cell (DC) maturation can promote the development of T-cell polarization. DC-mediated T-cell polarization is determined by three signals. First, the T cell receptor (TCR) recognizes the pathogen-derived peptides which are presented by MHC class II molecules on the cell surface of DCs. Second, the binding of CD28 and CD80/CD86 which are expressed by DCs, is the co-stimulatory signal. Third, the polarizing signal mainly mediates through cytokines and chemokines [63, 64]. Naïve T lymphocytes differentiate into different types and functions of T helper cells as shown in Figure 4 [65]. Type 1 T helper cells (Th1) require interferon-γ (IFN-γ) and interleukin-12 (IL-12). Transcription factor T-bet has been defined as the master regulator in Th1 cell differentiation. IFN-γ mainly induces the expression of T-bet, whereas IL-12 mediates Th1 cell lineage differentiation [66]. Type 2 T helper cells (Th2) produce IL-4, IL-5, and IL-13 which are significant for antibody-mediated immunity and defense against parasite infection [67]. Furthermore, Th2 cells are associated with

allergy and asthma [68]. Type 17 T helper cells (Th17) play an important role in controlling extracellular pathogen infection. Th17 cells play critical roles in inflammatory and autoimmune diseases. Th17 cells efficiently induce the lineage-specific transcription factor retinoic-acid-receptor-related orphan nuclear receptor γt (RORγt) as well as IL-17A secretion [69]. The maintenance of immune homeostasis is associated with immunosuppressive function or immune tolerance and prevents autoimmunity. Regulatory T cells (Treg) are determined by stable expression of the lineage-specific transcription factor Foxp3 and high amounts of the interleukin (IL-2) receptor α-chain (CD25) [70]. Treg cells commonly secrete IL-10, TGF-β and IL-2 for the generation of human $CD4^+CD25^+Foxp3^+$ T cells [71].

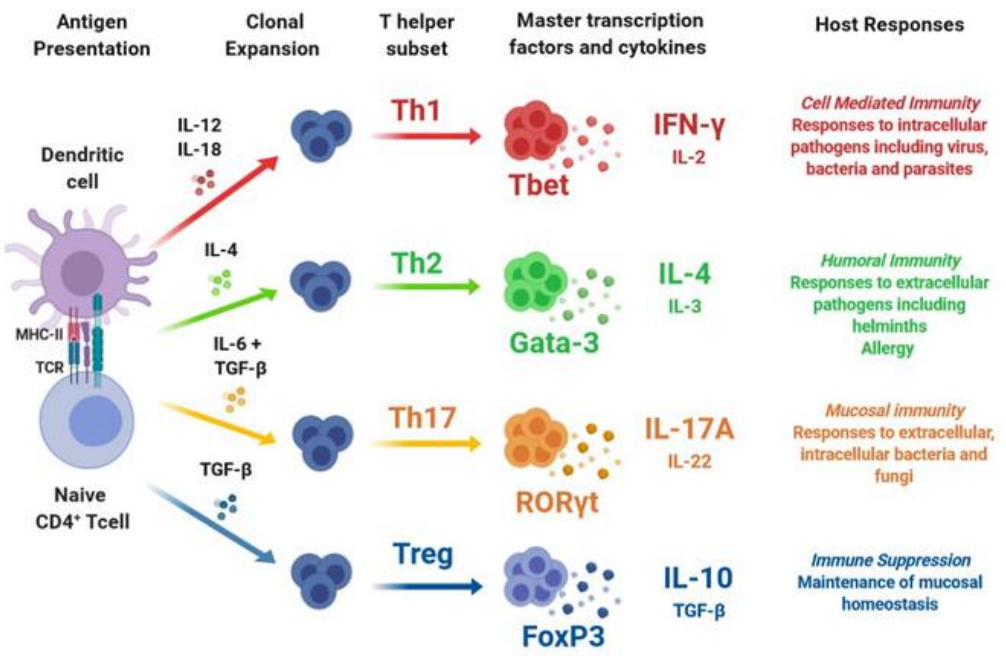


Figure 4 Overview of T helper subsets.

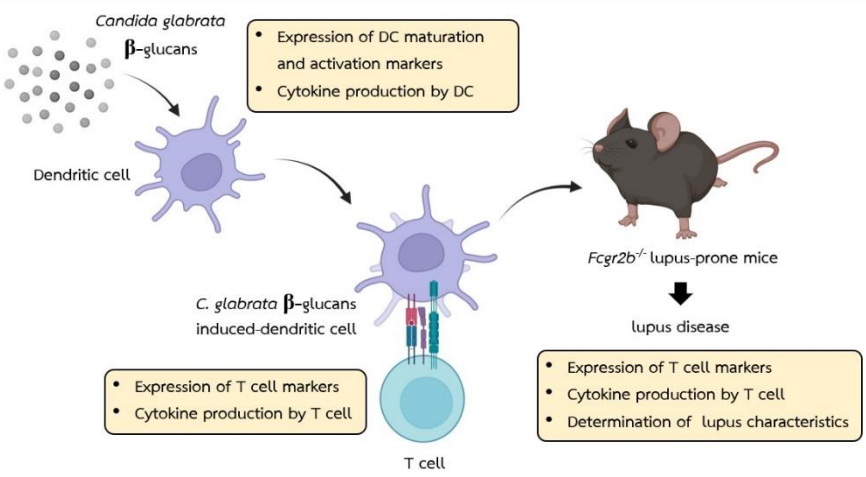
Lupus disease and *Fcgr2b* knockout lupus-prone mice

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease and is associated with multisystemic inflammatory responses [72, 73]. Autoimmunity is involved in the loss of self-tolerance and is driven by the defective mechanism of elimination of immune complexes and apoptotic waste, leading to chronic inflammation [74, 75]. Conventional lupus characteristics include anti-dsDNA, creatinine, proteinuria, urine sediments, and complement levels [76]. In this study, we used the model of mice prone to Fc gamma receptor 2b knockout (*Fcgr2b*) to determine the improvement of lupus disease in response to *C. glabrata* β-glucans. The advantages of this model are convenience and suitable for screening the amelioration of lupus disease after immunization with *C. glabrata* β-glucans. Fcgr2b is the only inhibitory Fc receptor and is important in the balance between promoting and regulating immune responses. The main function of Fcgr2b is the inhibition of activating signal transduction during immune activation [77]. SLE is associated with the dysfunction of the Fcgr2b. Therefore, *Fcgr2b*$^{-/-}$ lupus-prone mice are used as the representative lupus model. *Fcgr2b*-deficient mice from a C57Bl/6 background spontaneously develop autoantibodies and autoimmune glomerulonephritis which are defined as lupus-like disease, but do not occur from a BAlB/c background [78]. However, our study still needs to be performed in other models of autoimmunity disease to provide more advanced and insight information.

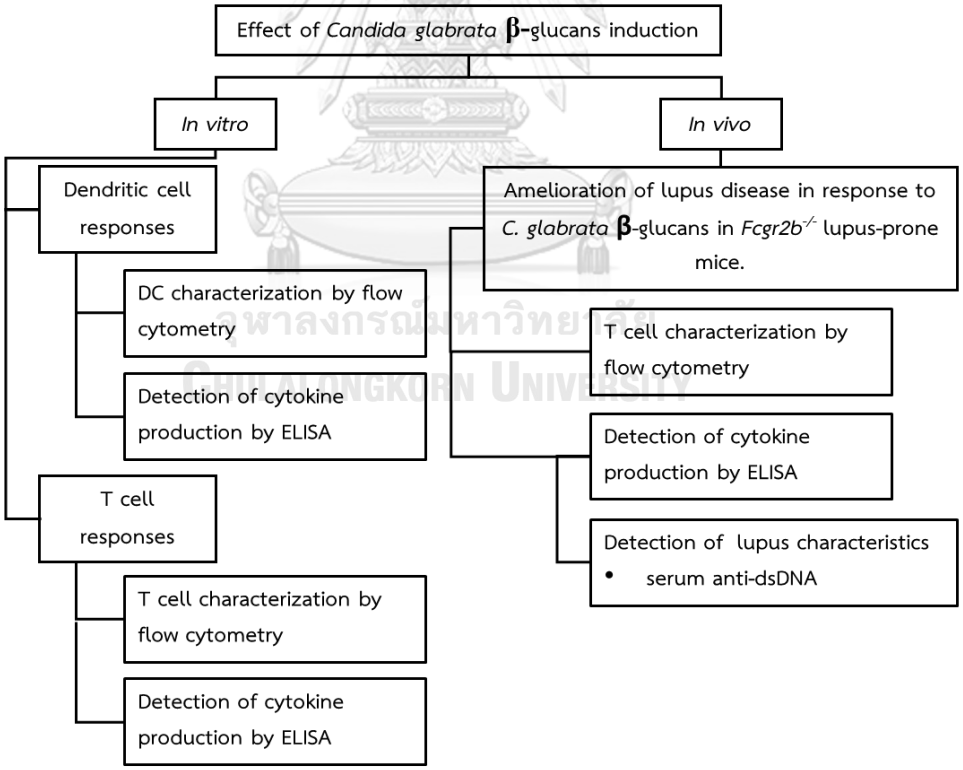
CHAPTER IV

METHODOLOGY

Conceptual framework



Experimental design



CHAHTER V

MATERIALS AND METHODS

Animals

Wild-type mice (6- to 8-week-old female C57BL/6 mice) were purchased from Siam Nomura International. Fcgr2b-deficient mice (*Fcgr2b*$^{-/-}$) on a C57BL/6 background were received from Assoc. Prof. Asada Leelahavanichkul and provided by Dr. Silvia Bolland (NIAID, NIH, Maryland, USA). The mice were housed at the Chulalongkorn University Laboratory Animal Centre. All animal procedures were reviewed and approved by the Institute Animal Care and Use Committee of Chulalongkorn University Laboratory Animal Centre (IACUC) (Animal Protocol 19-33-010).

Preparation of microparticulate β-glucans (mp- βG)

Candida glabrata (ATCC2001) and *C. albicans* (ATCC 24433) were grown in YPD medium at 30 ° C for 8 h with shaking at 180 rpm. All yeast cultures were diluted to OD_{600} of 0.1 and cultured at 30°C for 12-14 h with shaking at 150 rpm. Micro-particulate β-glucans (mp-βG) were extracted following the protocol provided by [39]. Briefly, mp-βG was extracted with 0.75 N sodium hydroxide, 2 N phosphoric acid, and 1% phosphoric acid in absolute ethanol. The sediment was harvested, and the pH was adjusted to 7.0. The sediment was then washed with sterile water for 15 minutes in boiling and repeated three times. The mp-βG pellets were collected, lyophilized, and stored at -20 °C until use. All procedures were performed using endotoxin-free water and containers. For mp-βG preparation, *C. glabrata* mp-βG should be prepared at a

low concentration (approximately 5 mg/ml). 50 mg of lyophilized mp-βG were resuspended in 10 ml of endotoxin-free water by stirring at room temperature overnight. Next, the pellets were harvested by centrifugation, washed with endotoxin-free water three times, and adjusted pH to 7.0. The mp-βG was depyrogenated with 1-2 ml of 250 mM sodium hydroxide for 50 mg of particulate glucan and then dispersed with 250 mM phosphoric acid (final pH 6.5-7.2) for neutralization. The pellets were washed with endotoxin-free water five times and suspended in endotoxin-free water. The mp-βG suspension was autoclaved at 121°C, 15 psi for 20 min. After cooling down, the suspension was stored at 4 ° C until use and available for 3 months. The concentration (5 mg/ml of *C. glabrata* mp-βG and 10 mg/ml of *C. albicans* mp-βG) was calculated by weight/volume. *C. glabrata* mp-βG was used in all experiments. *C. albicans* mp-βG was used in the *in vitro* dendritic cell and T cell coculture assay.

Generation of bone marrow-derived dendritic cells (BMDCs)

Bone marrow (BM) cells were collected from the femurs and tibias of six-week-old female mice. BM cells were flushed out using a 25-gauge needle (NIPPO), suspended in culture medium, and filtrated through a cell strainer. BM cells were washed with culture medium twice. BM cells (2×10^6 cells) were counted and cultured in a 24-well plate in 1 ml of RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM Glutamax, 100 U / ml penicillin, and 100 mg/ml streptomycin. Bone marrow cells were derived using recombinant mouse FLT3L (Biolegend) and recombinant murine GM-CSF (Peprotech). BM cells were cultured with 200 ng/ml of

rm FLT3L on day 0, 100 ng / ml of rm FLT3L on day 3, 100 ng / ml of rm FLT3L, and 20 ng / ml of rm GM-CSF on day 6, and 20 ng / ml of rm GM-CSF on day 8. Cells were incubated under a humidified atmosphere of 5% CO_2 at 37 ° C for 9 days. Half of the culture media was replaced every 3 days.

Stimulation of BMDCs with *Candida glabrata* β-glucans

BMDCs were stimulated with 12.5, 25, and 50 μg/ml of *C. glabrata* β-glucans. Unstimulated BMDCs were used for negative control. BMDCs were treated with 1 μM Dexamethasone on day 7 of cell culture and stimulated with 0.5 μg/ml LPS on day 9 as a positive control. There were seven experimental groups (N = 5). After 24 and 48 h of stimulation, cells were harvested for evaluation of the expression of DC maturation markers using flow cytometry analysis. Culture supernatant was collected for cytokine measurement using an enzyme-linked immunosorbent assay (ELISA).

Flow cytometry analysis

Fluorescent-conjugated antibodies against markers were used as follows: mouse CD11c (N418), mouse CD40 (3/23), mouse CD80 (16-10A1), mouse CD86 (CL-1), and mouse I-A/I-E (MHC class II) (M5/114.152) for DC maturation. Mouse CD3 (2C11), mouse CD4 (RM4-5), mouse CD25 (PC61), mouse IL-10 (JES5-16E3), mouse IFN-γ (XMG1.2), mouse IL-17A (eBio17B7), and mouse/human FoxP3 (FJK-16s) for T cell population assay. Before staining with specific antibodies, cells were incubated with Fc block (purified anti-mouse CD16/32) to reduce nonspecific binding. All antibodies were obtained from BioLegend (San Diego, CA, USA), except for the anti-FoxP3

antibody, which was obtained from eBioscience (San Diego, CA, USA). Stained cells were fixed with 4% paraformaldehyde and suspended in FACs buffer (0.1% sodium azide and 1% heat-inactivated fetal bovine serum in PBS buffer). Cells were analyzed by flow cytometry (CytoFLEX, Beckman Coulter, San Diego, CA, USA) and data was determined using Kaluza Flow Analysis Software (Beckman Coulter).

Cytokine measurement

The pro-inflammatory cytokine (tumor necrosis factor (TNF)-α, IL-1β, interferon (IFN)-γ, IL-6, IL-23, and IL-12) and anti-inflammatory cytokine (IL-10) were detected in the culture supernatant. Cytokine in the culture supernatant of BMDC and DC: T coculture assay were measured by sandwich ELISA following the manufacturer's instructions. All ELISA kits were purchased from Biolegend, except the IL-23 ELISA kit, which was obtained from eBioscience. The absorbance was measured at 450 nm using a microplate reader (EPOCH2C, BioTek).

***In vitro* dendritic cell and T cell coculture assay**

For BMDC, cells were stimulated with 25 μg/ml of *C. glabrata* and *C. albicans* β-glucans. Unstimulated BMDCs were used for negative control. There were three experimental groups (N = 3). After 24 h of stimulation, cells were washed twice with RPMI medium. $CD3^+$ T cells in the spleen were isolated from wild-type mice and purified using the Pan T Cell Isolation Kit II (Miltenyi Biotec). Purified $CD3^+$ T cells (1×10^6 cells per well) were cocultured with stimulated BMDCs (1×10^5 cells) at a DC: T cell ratio of 1:10 in the presence of 30 ng/ml of soluble anti-CD3 antibody (145-2C11,

Biolegend) in a 48-plate well plate following the protocol provided by [39]. After 48 h of culture, cells were evaluated for the expression of CD3, CD4, CD25, FoxP3, and IL-10 using flow cytometry analysis. Culture supernatant was collected for cytokine measurement using ELISA.

***In vivo* study in *Fcgr2b*$^{-/-}$ lupus-prone mice**

For the lupus mice model, *Fcgr2b*$^{-/-}$ lupus-prone mice developed anti-dsDNA antibodies at approximately six months of age and were used as the representative model of lupus disease. In this study, lupus characteristics were determined by serum anti-dsDNA. Serum anti-dsDNA was detected by the coated Calf-DNA (Invitrogen, Carlsbad, CA, USA). Before immunization, anti-dsDNA antibodies were measured in the serum of *Fcgr2b*$^{-/-}$ lupus-prone mice to represent the lupus characteristics. *Fcgr2b*$^{-/-}$ lupus-prone female mice were divided into two groups (4 mice per group) including *C. glabrata* β-glucan immunization and PBS control. Apoptotic bodies were isolated from *Fcgr2b*$^{-/-}$ lupus-prone female mice and used as the autoantigen in this model. Apoptotic bodies were prepared and received from Dr Phuriwat Khiewkamrop. *Fcgr2b*$^{-/-}$ lupus-prone female mice were immunized with *C. glabrata* β-glucan and apoptotic bodies following the timetable as shown in Table 1. On day 0, 50 μg of apoptotic bodies were subcutaneously injected with 2 mg of *C. glabrata* β-glucan or PBS in 200 μl at the neck of mice. The mice were then injected with 25 μg of apoptotic bodies and 2 mg of *C. glabrata* β-glucan or PBS on days 7, 21, and 42. At sacrifice, lymph nodes, spleen, kidney, urine, and blood were harvested after 2 weeks of the last

injection. Lymph nodes and spleen were digested and cultured for an *ex vivo* restimulation assay. The kidneys were soaked in 10% neutral buffered formalin for histology. Blood was used for the detection of serum anti-dsDNA.

Table 1 The timeline of *C. glabrata* β-glucan and apoptotic bodies immunization.

Day of injection	**Age of mice**	**Immunization**
Day 0	5-month-old	50 ug apoptotic body + 2 mg *C. glabrata* β-glucan or PBS
Day 7	5-month-old	25 ug apoptotic body + 2 mg *C. glabrata* β-glucan or PBS
Day 21	5-month-old	25 ug apoptotic body + 2 mg *C. glabrata* β-glucan or PBS
Day 24	5-month-old	Collecting blood
Day 42	6-month-old	25 ug apoptotic body + 2 mg *C. glabrata* β-glucan or PBS
Day 45	6-month-old	Collecting blood
Day 54	6-month-old	At sacrifice, lymph nodes, spleen, and blood were collected.

Ex vivo re-stimulation assay

To investigate the immunologic responses of T cells, spleen and lymph nodes were collected from mice after 7 days of the last injection and digested. There were 2 experimental groups (4 mice per group, N = 4). The spleen was minced through a cell strainer. Cells were washed with culture medium twice by centrifuging at 1,500 rpm for 5 minutes. The cell suspension was lysed with 1 ml of RBC lysis buffer (Biolegend) for 1 minute. Then, 9 ml of culture medium was added to stop the reaction. Splenocytes were washed and suspended in culture medium. Lymph nodes were suspended in 5 ml of culture medium and digested using a sterile glass bead in a 50-ml tube. 300 units/ml of Collagenase IV (Gibco) and 10 units/ml of DNase I (Thermo Fisher Scientific) were added to lymph nodes. lymph nodes were incubated at 37 ° C with shaking for 30-45 minutes. The cell suspension was filtrated through a 0.45 μM membrane filter. Lymph node cells were washed and suspended in culture medium.

Splenocytes and lymph node cells (2×10^6 cells per well) were counted and cultured in a 48-well plate. Cells were re-stimulated with 20 μg/ml of apoptotic bodies. After 24 and 48 hours of stimulation, the culture supernatant was collected for cytokine measurement using ELISA. Cells were collected and divided into 2 sets for intracellular staining. Both sets were incubated with 50 ng/ml of phorbol 12-myristate 13-acetate, PMA (Sigma), and 1 μM of ionomycin (Sigma) for 1 hour. Then, cells were incubated with monensin (Biolegend) or Befeldin A (Sigma) for 3 hours later. After 4 hours of blocking cell transporter processes, cells were stained and evaluated for the

expression of CD3, CD4, CD25, FoxP3, IL-10, IFN-γ, and IL-17A using flow cytometry analysis.

Assessment of cell proliferation

Spleen and lymph nodes were collected from mice after 7 days of the last injection and digested. Splenocytes and lymph node cells (2×10^5 cells per well in 200 µl) were cultured in a 96-well plate. Cells were re-stimulated with 20 µg/ml of apoptotic bodies for 72 hours. Cell proliferation was detected using CellTiter 96® AQueous One Solution Cell Proliferation Assay, MTS (Promega), and following the manufacturer's instructions. Briefly, cells were incubated with 20 µl of CellTiter 96® AQueous One Solution Reagent (Promega) and incubated at 37° C for 4 hours. The absorbance was measured at 490 nm using a microplate reader (EPOCH2C, BioTek). The percentage of cell proliferation was calculated by normalization with the negative control.

Statistical analysis

All data were presented as mean ± standard deviation and analyzed using One-way ANOVA with Tukey's HSD post-hoc test in SPSS28 software (IBM, New York, NY, USA). Values of $p < 0.05$ were determined to be statistically significant.

CHAPTER VI

RESULT

Effect of *C. glabrata* β-glucans on dendritic cell viability and CD11c expression.

To evaluate the proportions of DC maturation, the BMDCs were stimulated with 12.5, 25, and 50 μg/ml of *C. glabrata* β-glucans. Cells were characterized using flow cytometry. Live BMDCs gate was placed based on side scatter (SSC) and forward scatter (FSC) as shown in the A1 gate (Figure 5A). Our results reveal that the percentage of live BMDCs did not alter in *C. glabrata* β-glucans stimulated-BMDCs when compared to unstimulated BMDCs. Whereas 25 and 50 μg/ml of *C. glabrata* β-glucans-stimulated BMDCs at only 24 h after stimulation slightly reduce the percentage of live BMDCs. Moreover, the percentage of live BMDCs in *C. glabrata* β-glucans-stimulated BMDCs significantly decreased at 24 and 48 h when compared to 1 μM dexamethasone and LPS-stimulated-BMDCs (Figure 5B). BMDCs were derived with FLT3L and GM-CSF in the DC culture system. GM-CSF derived-BMDCs present a heterogeneous group of cells that comprises conventional DCs and monocyte-derived macrophages. The expressions of maturation genes in GM-DCs were great after LPS induction, while GM-Macs represented the great expression of interferon-stimulated genes. Flt3L is a cytokine and growth factor for the differentiation of dendritic cells. Flt3L-dependent differentiation of common DC precursors and pre-DCs was safe and used for the DC culture method [79]. Subsequently, the electronic gate of the DC population was identified by gating the $CD11c^{+}$ marker (A2 gate) as shown in Figure 5A. All doses of *C.*

glabrata β-glucans significantly reduced both the percentage and the geometric mean fluorescence intensity (MFI) of CD11c at 48 h when compared to unstimulated BMDCs but did not occur at 24 h (Figures 5C and 5D). BMDCs after dexamethasone treatment were used for positive control in this experiment. CD11c expression was markedly reduced after LPS stimulation in dexamethasone treated-BMDCs when compared to unstimulated BMDCs. Accordingly, dexamethasone has been defined as an anti-inflammatory drug and is used for the tolerance study model [80, 81]. Our results show that CD11c expression of BMDCs after 1 μM dexamethasone treatment significantly decreased when compared to LPS stimulated-BMDCs. Furthermore, CD11c expressions in *C. glabrata* β-glucans stimulated-BMDCs were significantly enhanced when compared to 1 μM dexamethasone and LPS stimulated-BMDCs (Figures 5C and 5D).

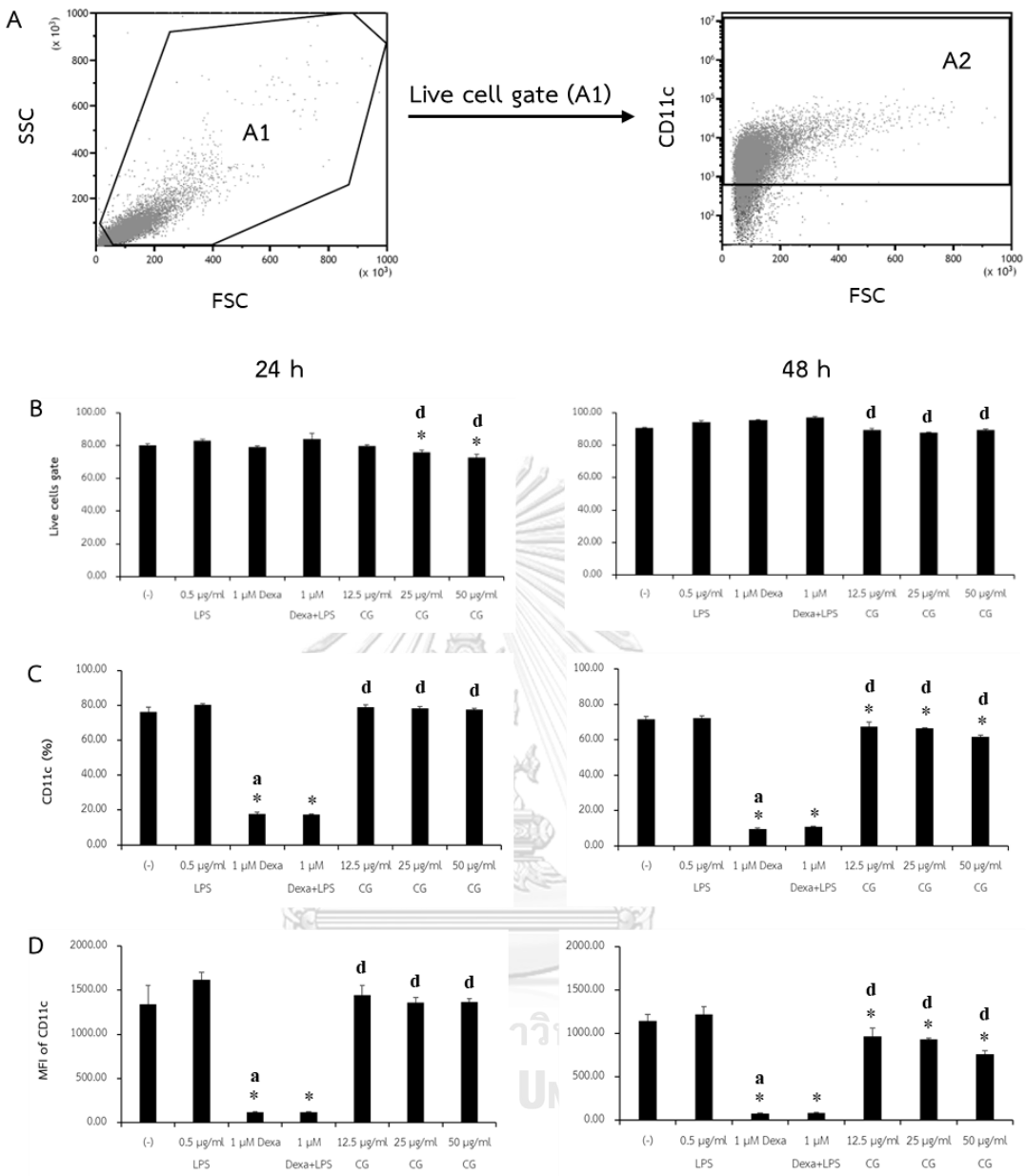


Figure 5 Evaluation of CD11c^{+} cell proportion, CD11c expression, and live BMDC in response to *C. glabrata* β-glucans.

(A) Dot plot analysis of live BMDCs was shown using side scatter (SSC) and forward scatter (FSC). The A1 gate was placed to select the live BMDCs. The CD11c^{+} cells were evaluated in the A1 gate and gated based on the dot plot analysis of CD11c

and FSC as shown in the A2 gate. (B) The live BMDC gate, (C) $CD11c^+$ cell proportion and (D) CD11c expression were analyzed and gated in the A1 gate. * $p < 0.05$ when compared with unstimulated BMDCs, $^a p < 0.05$ when compared with LPS stimulated-BMDCs, $^d p < 0.05$ when compared with 1 μM dexamethasone and LPS stimulated-BMDCs: N= 5.

Effect of *C. glabrata* β-glucans on dendritic cell maturation.

C. glabrata β-glucans stimulated-BMDCs significantly increased the expression of CD80, CD86, CD40, and MHCII when compared to unstimulated BMDC at both time points. Although dexamethasone-only and dexamethasone-LPS-treated BMDCs still negatively regulated the maturation markers. The electronic gate of the DC population was identified by placing the live cell gate (A1 gate) as shown in Figure 5A. The CD80, CD86, CD40, and MHCII expression of BMDCs after 1 μM dexamethasone treatment significantly decreased when compared to LPS stimulated-BMDCs. The percentages of each DC subpopulation were consistent with their geometric MFI levels (Figures 6 and 7). The percentage and geometric MFI of all markers in *C. glabrata* β-glucans stimulated-BMDCs were significantly increased compared to 1 μM dexamethasone and LPS stimulated-BMDCs (Figures 6 and 7).

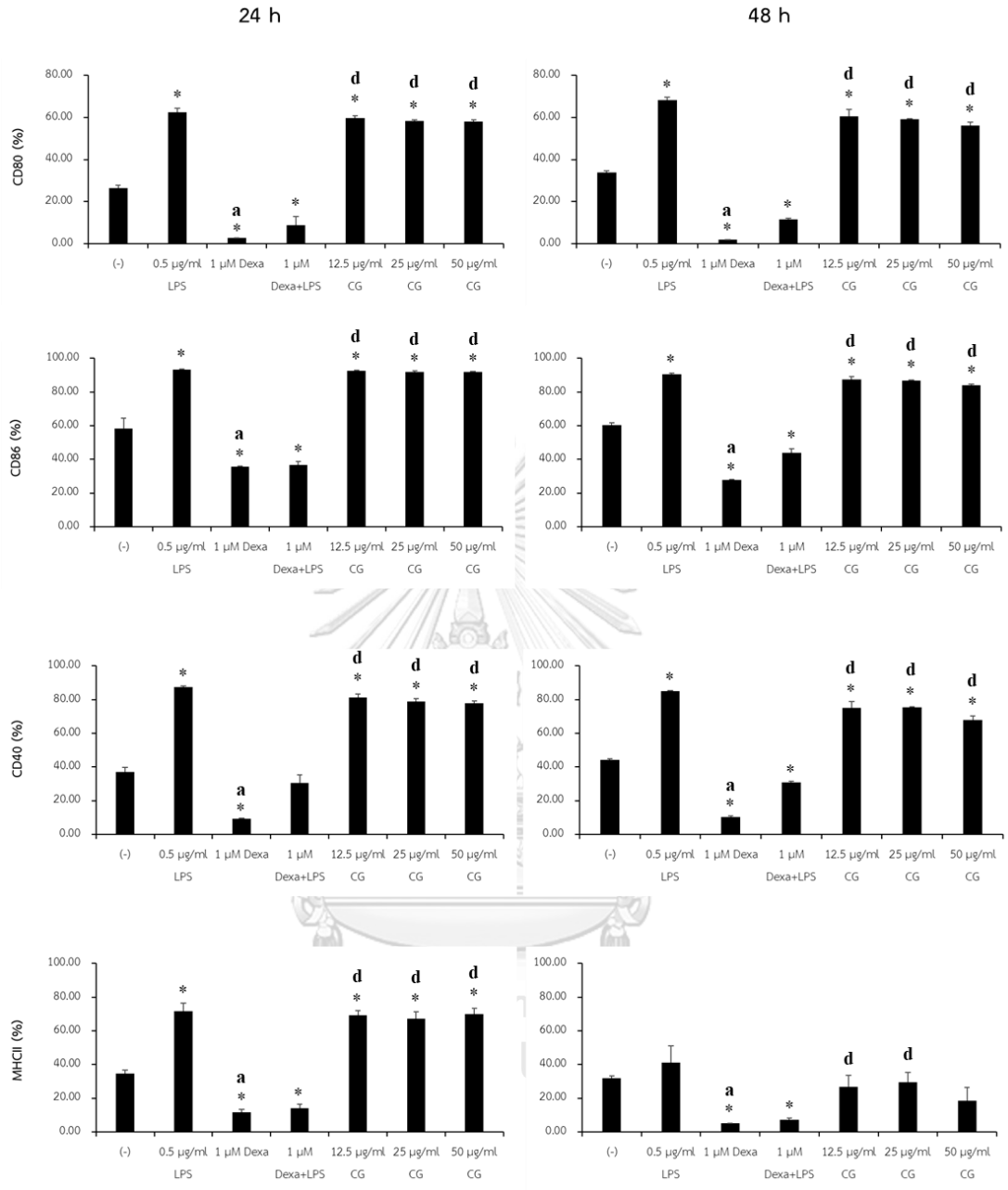


Figure 6 The percentage of differential phenotypic BMDC maturation after stimulation with *C. glabrata* β-glucans.

The cell proportions of $CD80^+$, $CD86^+$, $CD40^+$, and $MHCII^+$ were analyzed and gated in a live BMDC gate (A1 gate). * $p < 0.05$ when compared with unstimulated BMDCs, [a] p

< 0.05 when compared with LPS stimulated-BMDCs, [d] $p < 0.05$ when compared with 1 μM dexamethasone and LPS stimulated-BMDCs: N= 5.

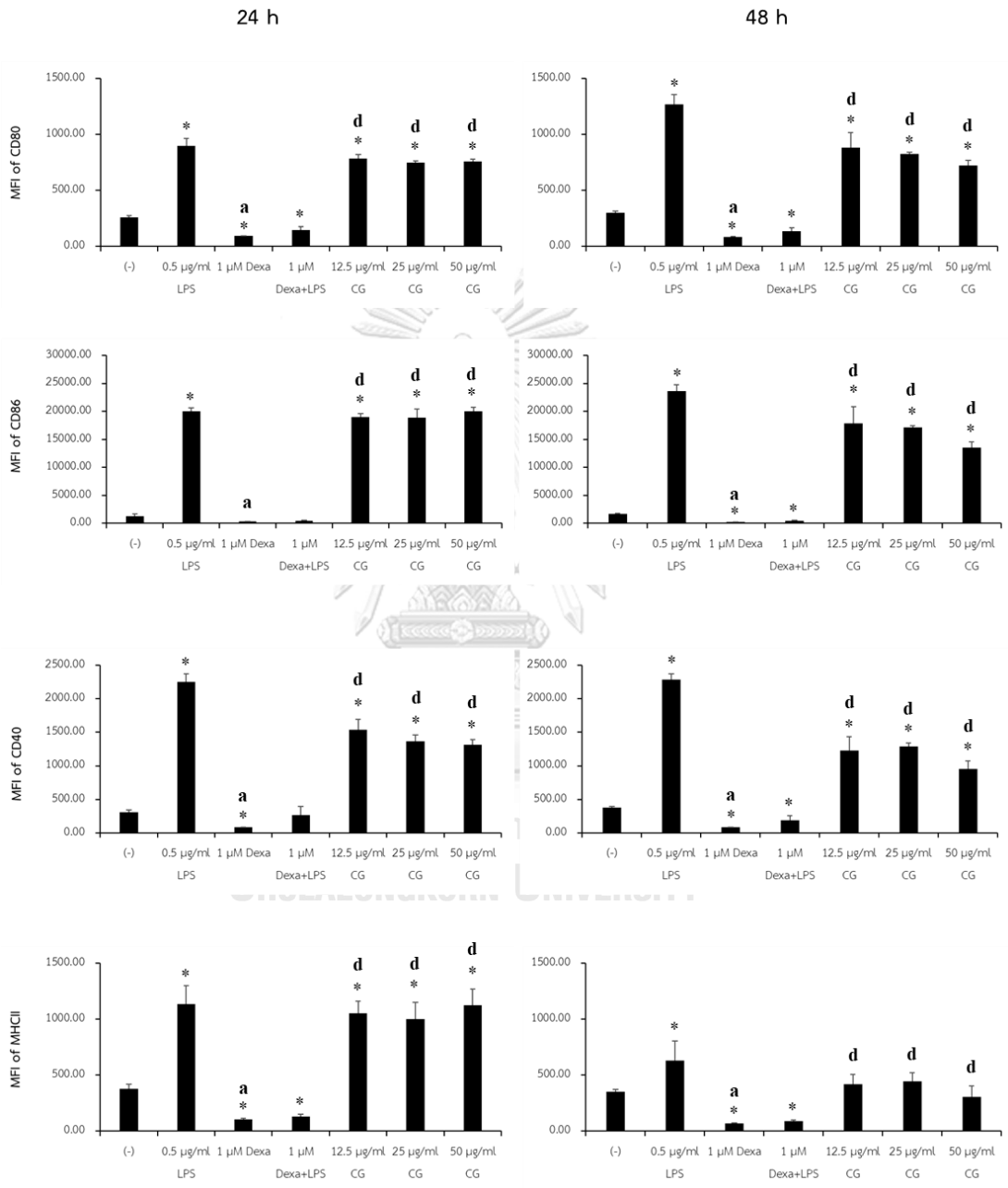
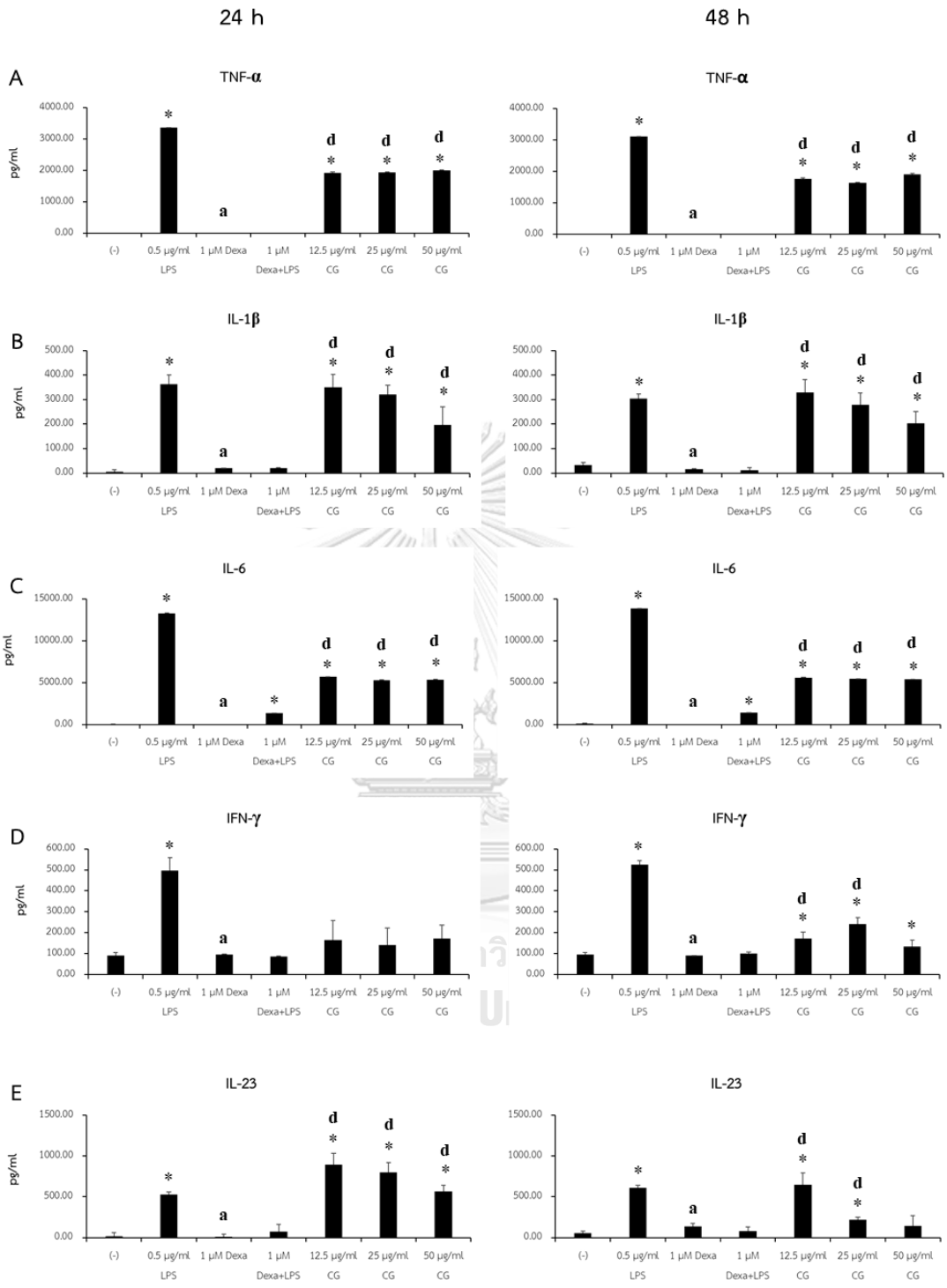


Figure 7 The geometric mean fluorescence intensity (MFI) of differential phenotypic BMDC maturation after stimulation with *C. glabrata* β-glucans.

The expressions of CD80, CD86, CD40, and MHCII were analyzed and gated in a live BMDC gate (A1 gate). * $p < 0.05$ when compared with unstimulated BMDCs, [a] $p < 0.05$ when compared with LPS stimulated-BMDCs, [d] $p < 0.05$ when compared with 1 μM dexamethasone and LPS stimulated-BMDCs: N= 5.

BMDC stimulated with *C. glabrata* β-glucans had a differential impact on the production of pro-inflammatory and anti-inflammatory cytokines.

The levels of pro-inflammatory cytokines (TNFα, IL-1β, IFN-γ, IL-6, IL-23, IL-12) and an anti-inflammatory cytokine (IL-10) in the culture supernatant were quantitated by ELISA. Pro-inflammatory cytokine levels after three doses of *C. glabrata* β-glucans stimulation at both time points were significantly enhanced compared to unstimulated BMDC, but lower than after LPS stimulation (Figure 8). The levels of pro-inflammatory cytokines in dexamethasone treated-BMDCs significantly decreased when compared to LPS stimulated-BMDCs. Surprisingly, all doses of *C. glabrata* β-glucan augmented the massive production of the anti-inflammatory cytokine, IL-10 (Figure 8G). *C. glabrata* β-glucans stimulation significantly enhanced the production of pro- and anti-inflammatory cytokines when compared to 1 μM dexamethasone and LPS stimulated-BMDCs but did not alter the IL-12 production. Next, we observed the alteration of T cells in response to BMDCs after *C. glabrata* β-glucan induction. We expected that *C. glabrata* β-glucans-stimulated BMDCs may impact T polarization by enhancing IL-10 production.


24 h
48 h
A
TNF-α
B
IL-1β
C
IL-6
D
IFN-γ
E
IL-23
pg/ml
(-)
0.5 μg/ml LPS
1 μM Dexa
1 μM Dexa+LPS
12.5 μg/ml CG
25 μg/ml CG
50 μg/ml CG

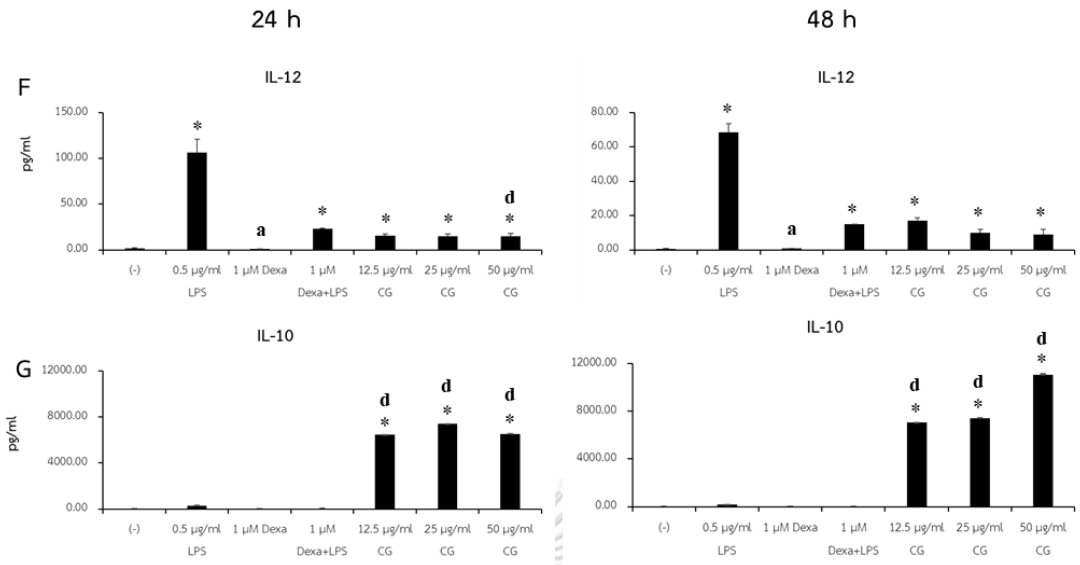


Figure 8 Measurement of cytokines in *C. glabrata* β-glucans stimulated BMDCs. The levels of pro-inflammatory cytokines (TNF-α, IL-1β, IFN-γ, IL-6, IL-23, IL-12) and an anti-inflammatory cytokine (IL-10) in the culture supernatant were quantitated by ELISA. * $p < 0.05$ when compared with unstimulated BMDCs, ^{a} $p < 0.05$ when compared with LPS stimulated-BMDCs, ^{d} $p < 0.05$ when compared with 1 μM dexamethasone and LPS stimulated-BMDCs: N= 5.

Expression of T cells in response to *C. glabrata* β-glucans stimulated BMDCs.

Having demonstrated the effect of BMDC maturation and cytokine production in response to *C. glabrata* β-glucans (Figures 5 to 8), we subsequently observed the orchestration of T cell responses by *C. glabrata* β-glucans induction. For this experiment, we performed an *in vitro* DC-T coculture experiment in the presence of a low concentration (30 ng/ml) of a soluble anti-CD3 monoclonal antibody. The binding of soluble CD3 and T cells leads to signal transduction into T cells [82]. The BMDCs were stimulated with 25 μg/ml of *C. glabrata* and *C. albicans* β-glucans for 24 h and then co-cultured *in vitro* with T cells in the presence of anti-CD3 antibody. DCs alone and T cells alone were also incubated with the soluble anti-CD3 antibody, which was used for the negative control. Live cells were gated based on side scatter (SSC) and forward scatter (FSC) as shown in the A1 gate (Figure 9A). The percentage of live cells did not alter in T cell responses after coculture with *Candida* β-glucans stimulated-BMDCs when compared to the negative control (Figure 10A). The percentage of $CD3^+CD4^+$ cells was evaluated in the A1 gate and gated based on the dot plot analysis of CD3 and CD4 as shown in the $CD3^+CD4^+$ gate (Figure 9A). The expressions of CD3 and CD4 were defined as T helper cells [83]. The percentage of CD25, FoxP3, IL-10, IFN-γ and IL-17A were gated based on the dot plot analysis in the A1 gate (Figure 9B). The expression of $CD4^+CD25^+$ as an IL-2 receptor and T lymphocyte activation marker significantly increased in T cells after coculture with *C. glabrata* β-glucans stimulated-BMDCs when compared to unstimulated BMDCs and *C. albicans* β-glucans stimulated-

BMDCs (Figure 10B). Whereas *C. albicans* and *C. glabrata* β-glucans stimulated-BMDCs did not impact the expression of $CD4^+FoxP3^+$, $CD4^+IL\text{-}10^+$, $CD4^+IFN\text{-}\gamma^+$ and $CD4^+IL\text{-}17A^+$ in T cells (Figure 10C, 10D, 10E and 10F).

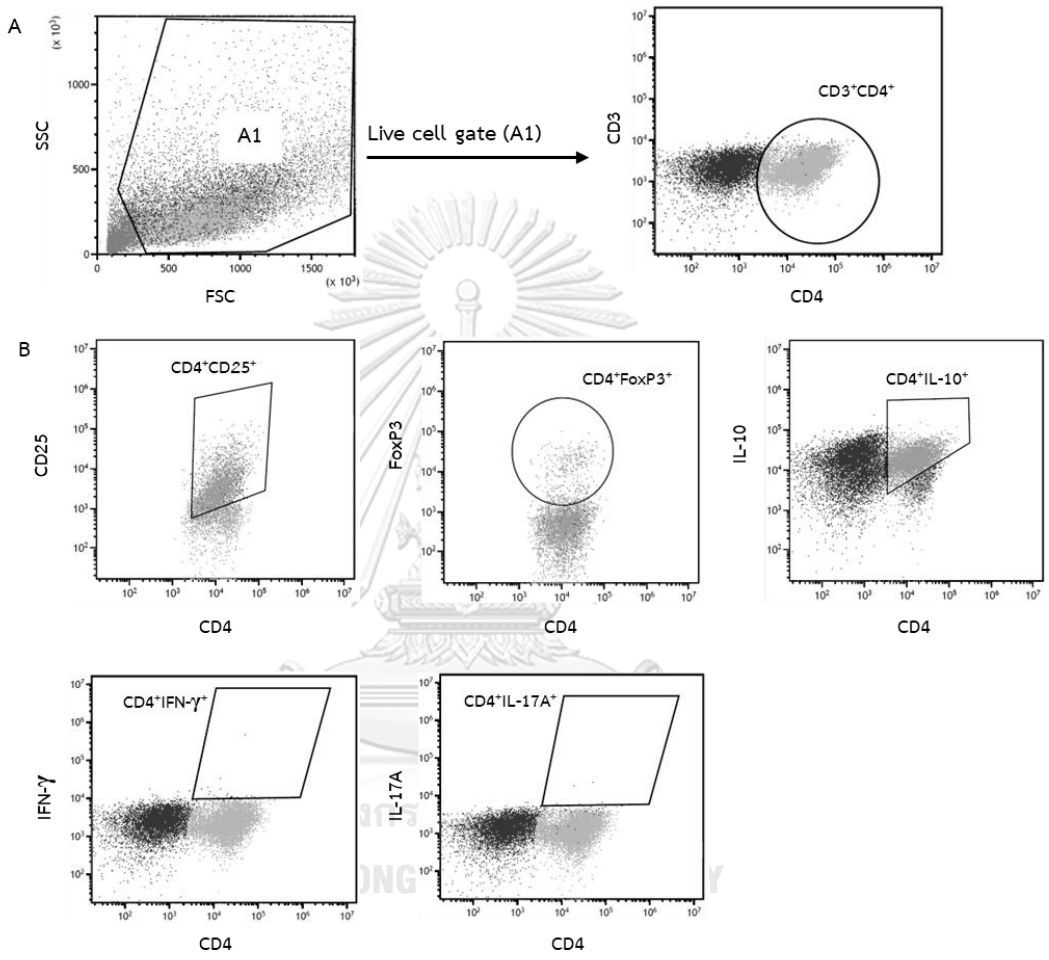


Figure 9 Dot plot analysis of the expressions of T cell markers in response to *C. glabrata* β-glucans stimulated BMDCs.

(A) The live cell gate was shown using side scatter (SSC) and forward scatter (FSC). The A1 gate was placed to select the live cells. The $CD3^+CD4^+$cells were evaluated in the A1 gate and gated based on the dot plot analysis of CD3 and CD4 as shown in the

$CD3^+CD4^+$ gate. (B) The percentage of CD25, FoxP3, IL-10, IFN-γ and IL-17A were analyzed and gated in the A1 gate.

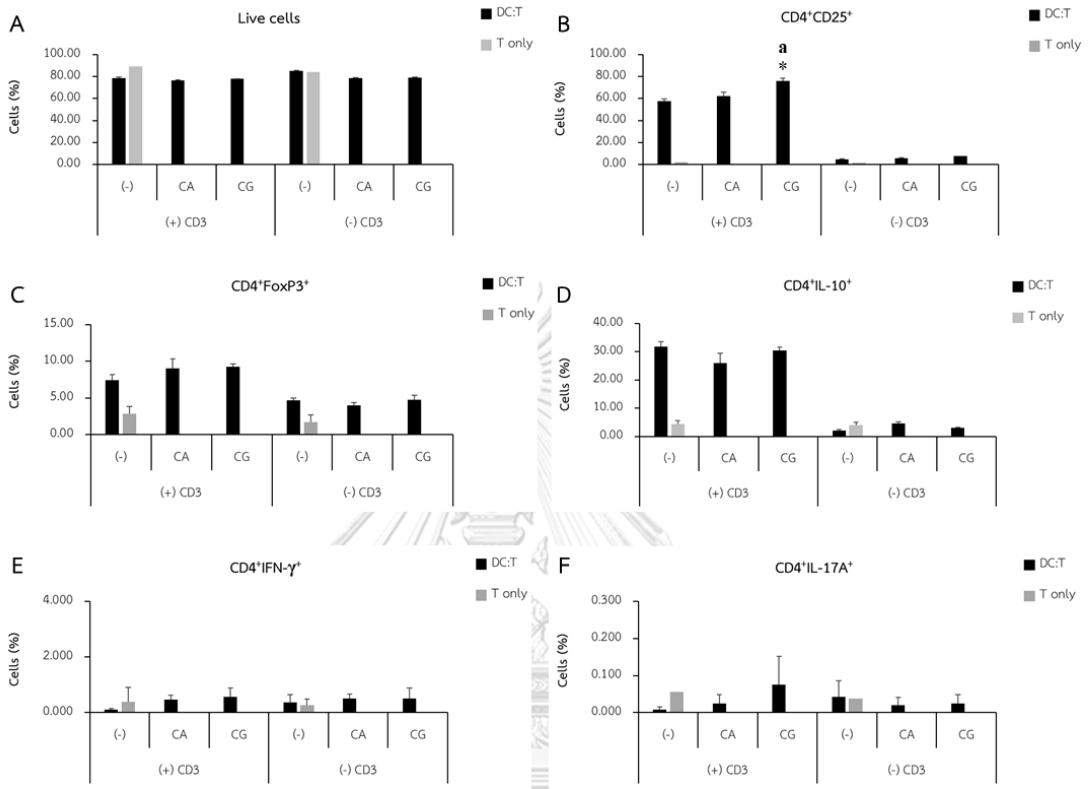


Figure 10 Effect of *C. glabrata* β-glucans stimulated BMDCs on the expression of T cells.

The percentages of T-cell markers were analyzed from dot plot analysis as shown in Figure 8. * $p < 0.05$ when compared with unstimulated-BMDCs, [a] $p < 0.05$ when compared with *C. albicans* β-glucans: N= 3.

BMDC stimulated with *C. glabrata* β-glucans had a differential impact on the cytokine production in T cells.

We subsequently observed the cytokine production of T cell response to *C. glabrata* β-glucans-stimulated BMDCs. T cells significantly augmented IL-2 production after *C. glabrata* and *C. albicans* β-glucans -stimulated BMDCs induction. Moreover, IL-2 production in DC: T coculture of *C. glabrata* β-glucans was markedly enhanced when compared to *C. albicans* β-glucans -stimulated BMDCs induction (Figure 11A). IL-2 was discovered as a T cell growth factor and essential for the proliferation of T cells and the generation of effector and memory cells [84]. In our results, we found that IL-10 and IFN-γ were predominantly secreted from T cells after 48 h of coculture with BMDC pulsed with *C. glabrata* β-glucans (Figures 11B and 11C). The level of IL-10 in the DC: T coculture of *C. glabrata* β-glucan induction was approximately 1.75-fold higher than its non-induction (Figure 11B). Also, the level of IFN-γ in the DC: T coculture of *C. glabrata* β-glucan induction was approximately 1.1-fold higher than its non-induction (Figure 10C). Whereas IL-17A production in T cells did not alter after *C. glabrata* and *C. albicans* β-glucans-stimulated BMDCs induction when compared to the negative control (Figure 11D).

T helper 1 mainly produces IFN-γ, whereas T helper 17 mainly produces IL-17A. T-cell activation plays an important role in adaptive immune responses. IL-10 is secreted from regulatory T cells and promotes the function of anti-inflammation [83,

85]. We found that *C. glabrata* β-glucans-induced BMDCs can promote the high secretion of IFN-γ and IL-10. The levels of IFN-γ and IL-10 were approximately 1.4- and 1.8-fold higher than T cells cocultured with *C. albicans* β-glucans stimulated BMDCs, respectively (Figure 12).

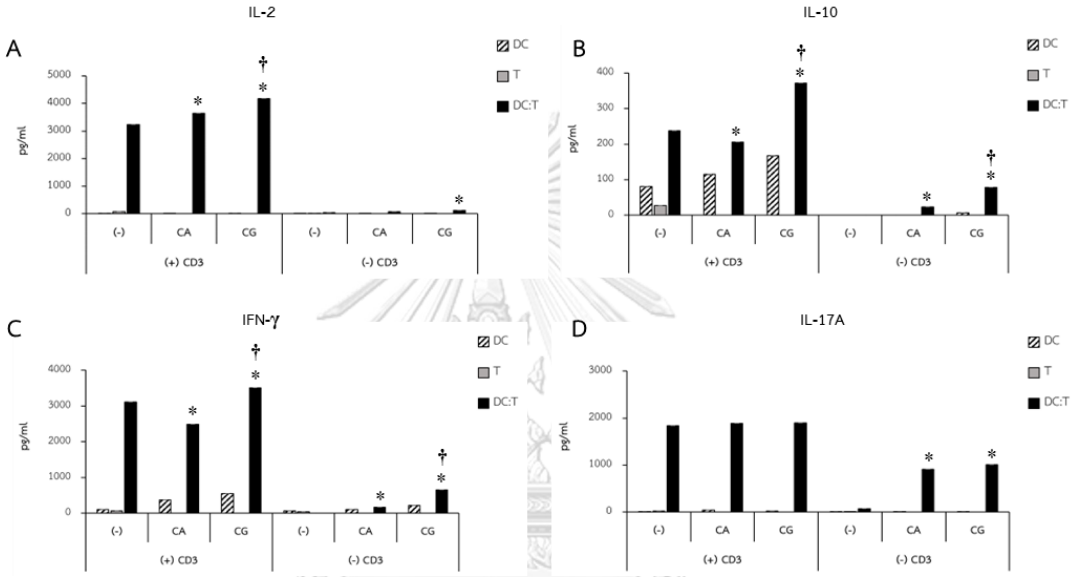


Figure 11 Effect of *C. glabrata* β-glucans stimulated BMDCs on the cytokine production of T cells.

The levels of IL-2, IL-10 IFN-γ, and IL-17A in the culture supernatant were quantitated by ELISA. * $p < 0.05$ when compared with unstimulated BMDCs, † $p < 0.05$ when compared with *C. albicans* β-glucans: N= 3.

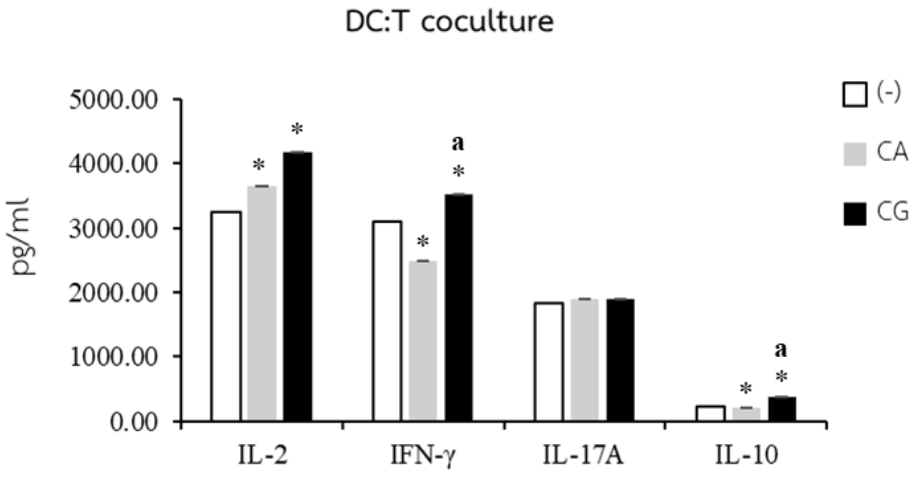


Figure 12 Comparison between the secretion of pro- and anti-inflammatory cytokines from T cells in response to *Candida* β-glucans.

* $p < 0.05$ when compared with unstimulated BMDCs (white bar), [a] $p < 0.05$ when compared with T cells cocultured with *C. albicans* β-glucans stimulated BMDCs (black bar): N= 3.

Effect of *C. glabrata* β-glucans on the development of anti-dsDNA antibodies in *Fcgr2b*$^{-/-}$ lupus-prone mice.

Fcgr2b$^{-/-}$ lupus-prone mice at six months of age were used as the representative model of lupus disease. In this study, we measured the level of serum anti-dsDNA antibodies for screening the lupus characteristic. Having investigated the induction of *C. glabrata* β-glucans on massive IL-10 secretion in dendritic cells *in vitro*, they may generate an immunosuppressive state in *Fcgr2b*$^{-/-}$ lupus-prone mice leading to ameliorate lupus disease. There are two experimental groups (*C. glabrata* β-glucan

immunization and PBS control). Mice were administrated with *C. glabrata* β-glucans as adjuvant and apoptotic bodies as the autoantigens. PBS and autoantigens were injected into mice and used for the negative control. Our result demonstrated that anti-dsDNA antibodies at days 45 and 54 after *C. glabrata* β-glucans immunization predominantly decreased in lupus-prone mice when compared with PBS control (Figure 13).

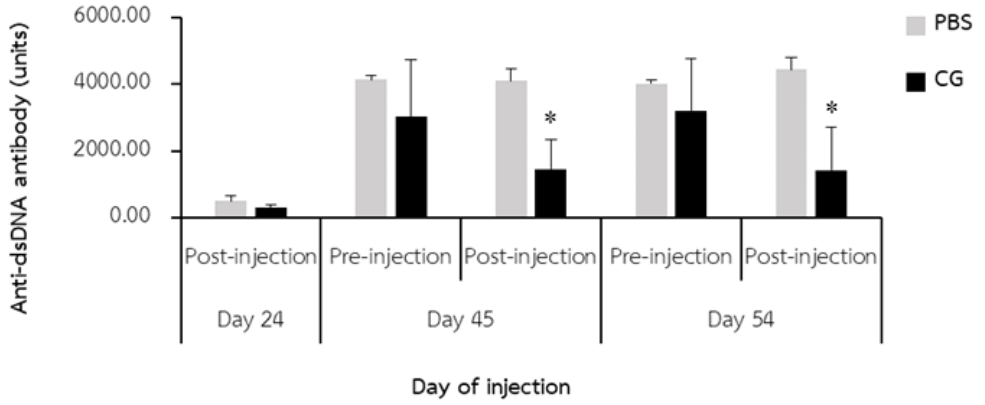


Figure 13 The development of anti-dsDNA antibodies in *Fcgr2b*$^{-/-}$ lupus-prone mice after *C. glabrata* β-glucans immunization.

* $p < 0.05$ when compared with PBS control: N= 4.

Effect of *C. glabrata* β-glucans on cell proliferation in splenocytes and lymph node cells of *Fcgr2b*$^{-/-}$ lupus-prone mice.

We used the *ex vivo* re-stimulation assay for determining the cell proliferation of splenocytes and lymph node cells. The percentage of cell proliferation was calculated and analyzed using the colorimetric method (CellTiter 96® AQueous One Solution Cell Proliferation Assay, MTS). We found that the percentage of cell

proliferation in lymph node cells did not alter after being re-stimulated with autoantigens, whereas the percentage of cell proliferation in splenocytes significantly increased after *C. glabrata* β-glucans immunization in *Fcgr2b*$^{-/-}$ lupus-prone mice (Figure 14).

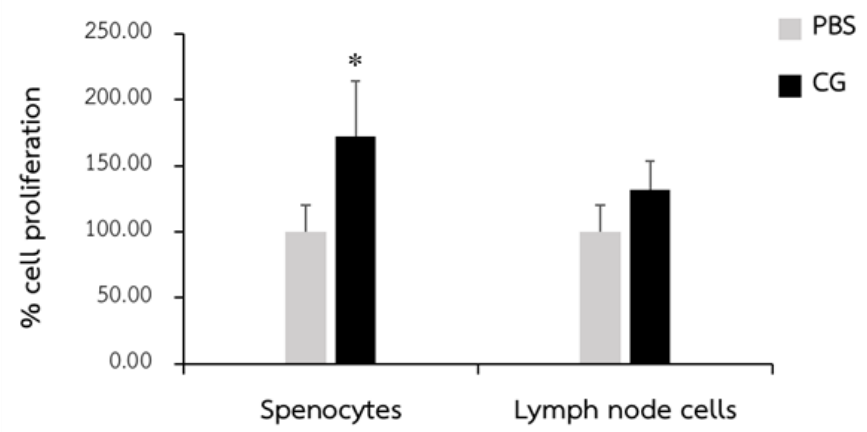


Figure 14 Effect of *C. glabrata* β-glucans on cell proliferation in splenocytes and lymph node cells of *Fcgr2b*$^{-/-}$ lupus-prone mice.

The percentage of cell proliferation was evaluated using an MTS assay. * $p < 0.05$ when compared with PBS control: N= 4.

Expression of T cells from splenocytes and lymph node cells in *Fcgr2b*$^{-/-}$ lupus-prone mice in response to *C. glabrata* β-glucans immunization.

Our investigation of dendritic cell responses to *C. glabrata* β-glucans demonstrated that *C. glabrata* β-glucans may impact the immunosuppressive state in dendritic cells via IL-10 secretion. In this experiment, we performed an *in vivo* experiment in *Fcgr2b* knockout lupus-prone mice to determine the improvement of lupus disease in response to *C. glabrata* β-glucans. Spleen and lymph nodes were collected from immunized mice. We used the re-stimulation assay for the evaluation of T-cell responses. Splenocytes and lymph node cells were analyzed using flow cytometric analysis. Live cells were gated based on side scatter (SSC) and forward scatter (FSC) as shown in the A1 gate (Figure 15A). The percentage of $CD3^+CD4^+$ cells was evaluated in the A1 gate and gated based on the dot plot analysis of CD3 and CD4 as shown in the $CD3^+CD4^+$ gate (Figure 15A). The percentage of FoxP3, IL-10, and IL-4 were gated based on the dot plot analysis in the A1 gate (Figure 15B). Our results reveal that *C. glabrata* β-glucans immunization in *Fcgr2b* knockout lupus-prone mice did not alter the expression of FoxP3, IL-10 and IL-4 in both splenocytes and lymph node cells when compared to PBS control (Figures 16).

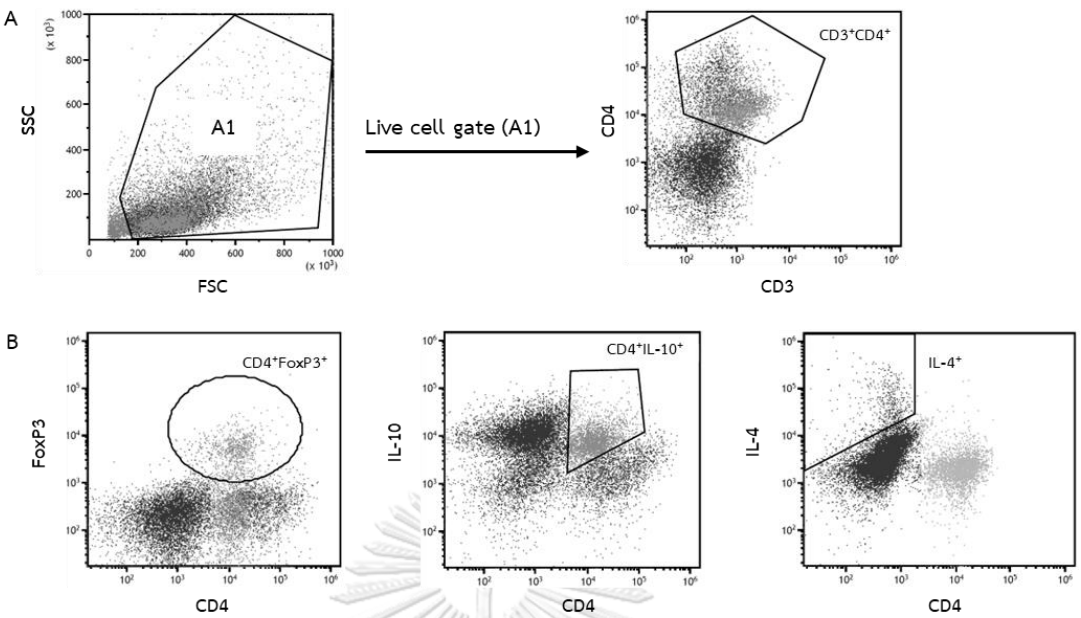


Figure 15 Dot plot analysis of CD3, CD4, FoxP3, IL-10 and IL-4 expressions in response to *C. glabrata* β-glucans immunization.

(A) The live cell gate was shown using side scatter (SSC) and forward scatter (FSC). The A1 gate was placed to select the live cells. The $CD3^+CD4^+$cells were evaluated in the A1 gate and gated based on the dot plot analysis of CD3 and CD4 as shown in the $CD3^+CD4^+$ gate. (B) The $CD4^+FoxP3^+$, $CD4^+$IL-10, and IL-4^+ expressions were analyzed and gated in the A1 gate.

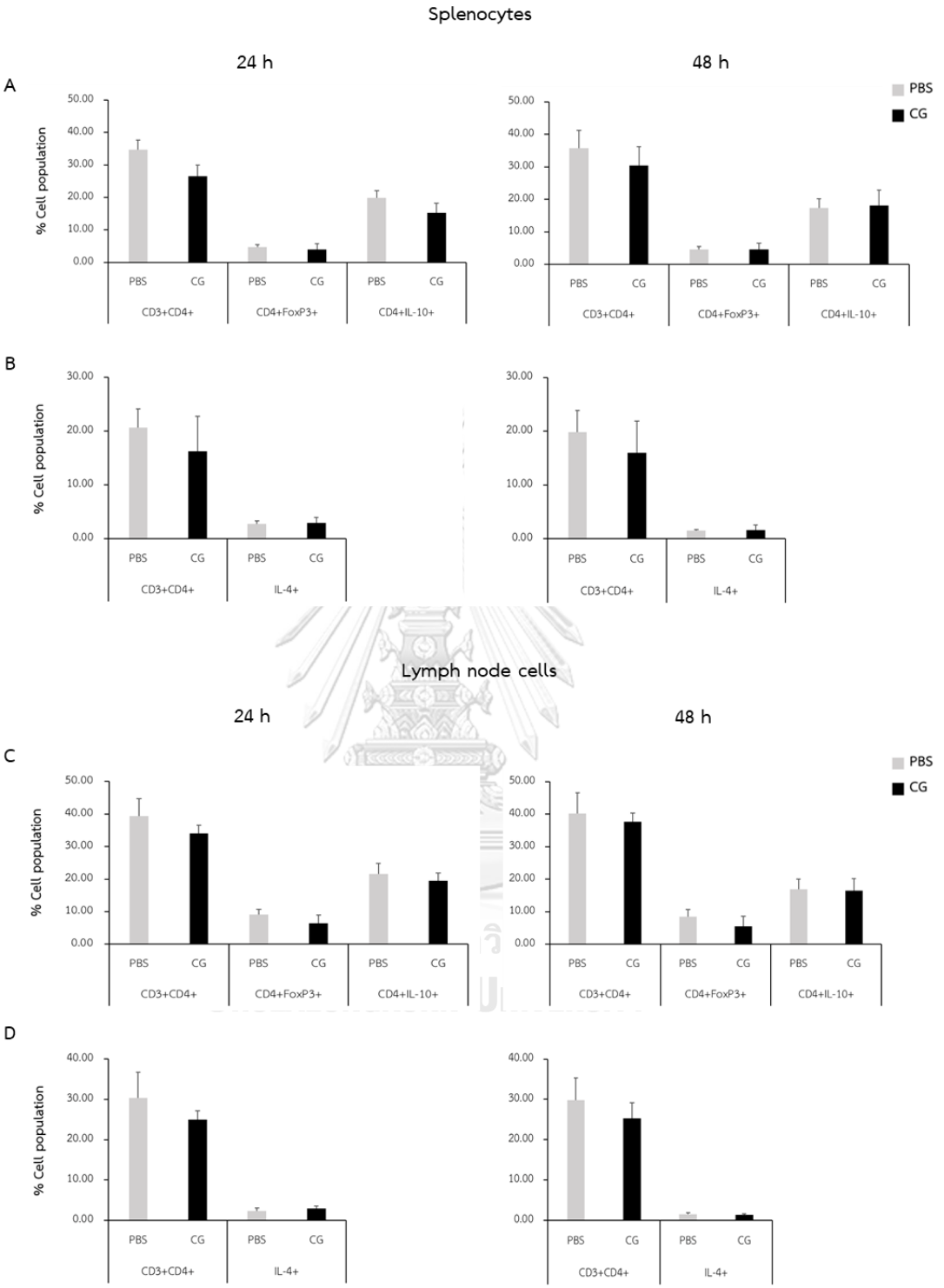


Figure 16 Effect of *C. glabrata* β-glucans on the expression of $CD4^+FoxP3^+$, $CD4^+IL\text{-}10^+$ and $IL\text{-}4^+$ from splenocytes and lymph node cells in *Fcgr2b*$^{-/-}$ lupus-prone mice.

The percentages of T-cell markers were analyzed from dot plot analysis as shown in figure 15. * $p < 0.05$ when compared with PBS control: N= 4.

Cytokine production in T cells from splenocytes of *Fcgr2b*$^{-/-}$ lupus-prone mice.

C. glabrata β-glucans immunization in the lupus mice model had a differential impact on cytokine secretion. We used the re-stimulation assay for the evaluation of T-cell responses. Splenocytes and lymph node cells were re-stimulated with apoptotic bodies and cultured for 24 and 48 h. IFN-γ, IL-4 and IL-17A were mainly secreted from T helper 1, T helper 2, and T helper 17, respectively. Regulatory T cells can promote IL-10 production [83, 85]. We found that IFN-γ production in splenocytes significantly increased in the culture supernatant of *C. glabrata* β-glucans immunization at 24 and 48h. The levels of IFN-γ at 24 and 48h were approximately 18.92- and 27.40-fold higher than PBS control, respectively. Whereas IL-10, IL-17A and IL-4 production did not alter in splenocytes of *C. glabrata* β-glucans immunization (Figure 17A). In lymph node cells of *C. glabrata* β-glucans immunization, IL-17A production significantly increased at both time points but did not occur the alteration of IL-10, IFN-γ, and IL-4 production. The levels of IL-17A at 24 and 48h were approximately 10.12- and 7.42-fold higher than PBS control, respectively. In comparison between immune activation and suppression, we found that the level of IL-17A at 24 h in lymph node cells was approximately 5.04-fold higher than the IL-10 level but did not occur in splenocytes (Figure 17B).

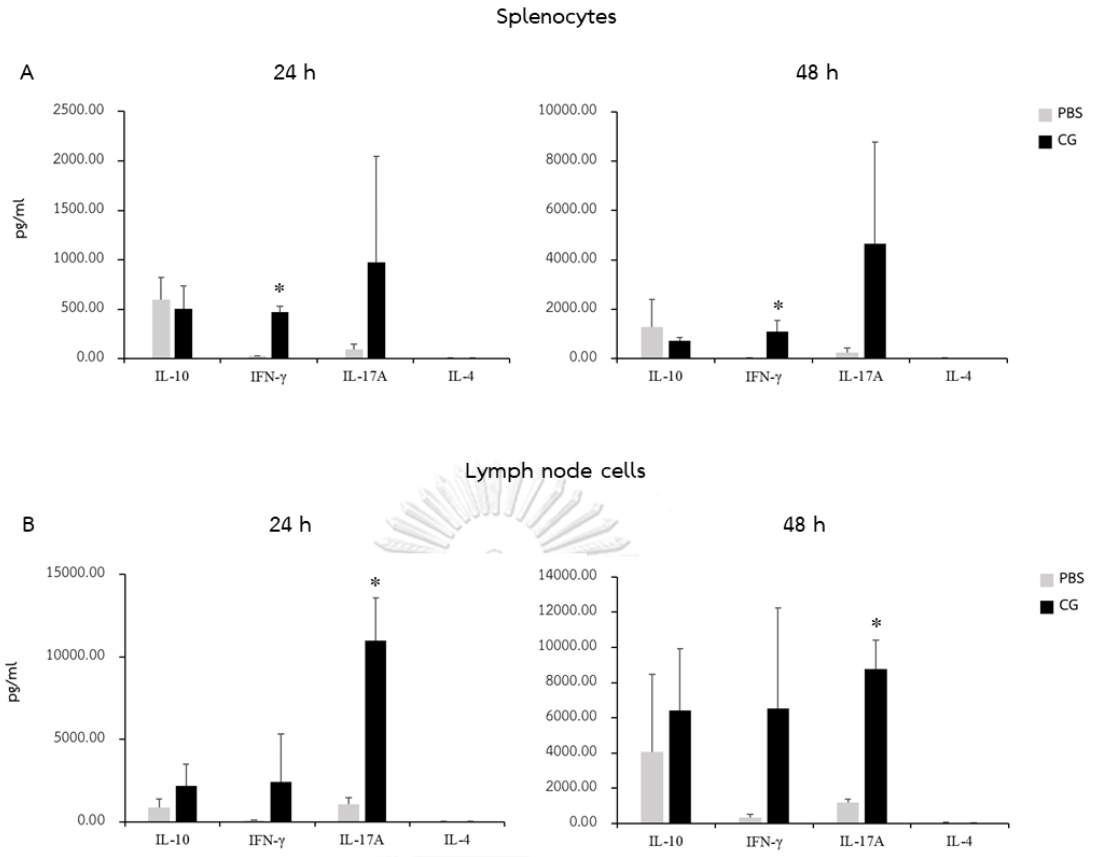


Figure 17 Effect of *C. glabrata* β-glucans on the cytokine production of T cells from splenocytes and lymph node cells of *Fcgr2b*$^{-/-}$ lupus-prone mice.

The levels of IL-10, IFN-γ, IL-17A and IL-4 in the culture supernatant were quantitated by ELISA. * $p < 0.05$ when compared with PBS control: N= 4.

CHAPTER VII

DISCUSSION

C. glabrata β-glucans had a differential impact on the dendritic cell maturation and cytokine production.

β-glucans have essential biological functions that are represented as a stimulator in the mechanism of immune responses. Our results reveal that *C. glabrata* β-glucans had a differential impact on the phenotype of bone marrow-derived dendritic cells (BMDC) as shown in figures 4-6. We used dexamethasone treatment for the tolerance study model [80, 81]. Our results show that CD11c expression was markedly reduced in BMDC treated with dexamethasone alone and dexamethasone-LPS (Figure 5). In addition, dexamethasone resulted in the inhibition of DC differentiation and no change in the DC viability [86]. The immature DCs used for tolerance induction maintain a steady immature state with predominant production of interleukin (IL) -10 [87]. In our investigation, the expressions of CD80, CD86, CD40, and MHCII were identified by gating live BMDCs in both dot plots and histogram analysis. We found that *C. glabrata* β-glucans stimulated-BMDCs significantly increased the expression of CD80, CD86, CD40, and MHCII compared to unstimulated BMDC. Although dexamethasone-only and dexamethasone-LPS-treated BMDCs still negatively regulated the maturation markers. The percentage and geometric MFI of all markers in *C. glabrata* β-glucans stimulated-BMDCs were slightly reduced compared to LPS stimulated-BMDCs (Figures 6 and 7). Parallel to defective antigen-specific T-cell

stimulation, the expression of MHCII and co-stimulatory molecules (CD80 and CD86) in DCs after treatment with nanoparticles containing OVA and dexamethasone [88]. Our finding implies that *C. glabrata* β-glucans may affect DC function.

Both pro-inflammatory and anti-inflammatory cytokines in dendritic cells had a differential response to *C. glabrata* β-glucans, especially IL-10 production (Figure 8). Previously, dendritic cells matured through the CD40-CD40L interaction leading to high amounts of IL-10 production [89]. IL-10 production by pulmonary dendritic cells mediated tolerance induced by respiratory exposure to the antigen [90]. The effect of massive IL-10 secretion by dendritic cells may drive the differentiation of naïve T cells into effector regulatory T cells. Dendritic cells producing IL-10 and IL-27 can enhance IL-10 production of T cells, leading to the generation of oral tolerance [91]. Resident CD141 (BDCA3)$^{+}$ dendritic cells in human skin are capable of IL-10 production to induce regulatory T cells that suppress skin inflammation [92]. Selective probiotic bacteria prime dendritic cells to drive the development of regulatory T cells, producing increased levels of IL-10 [93]. Therefore, enhanced IL-10 production in *C. glabrata* β-glucan-induced BMDCs may impact T polarization, especially regulatory T cells. Consequently, we observed the alteration of T cells in response to BMDCs after *C. glabrata* β-glucan induction.

C. glabrata β-glucans-stimulated BMDCs had a differential impact on the cytokine production in T cells.

We used the *in vitro* DC-T coculture experiment to investigate T-cell alteration in response to *C. glabrata* β-glucan-pulsed BMDCs. *C. albicans* β-glucans were used as a positive stimulator. *C. albicans* β-glucans have been represented as a stimulator for host immune responses [94]. In this experiment, *C. albicans* β-glucan-induced BMDCs did not influence T-cell responses (Figures 10 and 11). Our results reveal that *C. glabrata* β-glucan-pulsed BMDCs did not alter the expression of $CD4^+FoxP3^+$, $CD4^+IL\text{-}10^+$, $CD4^+$ IFN-$\gamma^{+,}$ and $CD4^+IL\text{-}17A^+$ in T cells (Figure 10). Whereas both pro- and anti-inflammatory cytokine was predominantly secreted from T cells after 48 h of coculture with BMDC pulsed with *C. glabrata* β-glucans (Figure 11). In comparison between immune activation and suppression, we found that the level of IFN-γ secretion significantly augmented when compared to IL-10 secretion in T cells after coculture with *C. glabrata* β-glucans stimulated-BMDCs (Figure 12). This result implies that IL-10 producing-BMDCs after *C. glabrata* β-glucans stimulation did not impact anti-inflammatory responses in T cells and mainly promoted inflammatory responses. We found that the level of IL-10 in the coculture system was less when compared to the IL-10 level in BMDCs. Because the number of BMDCs in the coculture system (10^5 cells) was 10-fold lower than the BMDCs (10^6 cells) in the DC culture system. Several shreds of evidence have shown that β-glucans from *C. glabrata* cell wall impact host immune responses through C-type lectin receptors [41, 95]. Survived mice after *C. glabrata*

infection was associated with rapid induction of mRNAs and corresponding immunoreactive proteins for the proinflammatory cytokines (TNF-α, IL-12, and IFN-γ) and the lack of induction of protein for the anti-inflammatory cytokine (IL-10) [14]. Collectively, *C. glabrata* β-glucan acts as an inducer in the generation of an immunosuppressive state in dendritic cells through IL-10 secretion. These DCs could not modulate the differentiation and function of T cells in immunosuppressive responses. Next, we used the *in vivo* study for determining the alteration of lupus disease in response to C. *glabrata* β-glucans in the lupus mice model. We expected that IL-10-producing dendritic cells through *C. glabrata* β-glucan induction may generate an immunosuppressive state in the lupus mice model and improve lupus disease after *C. glabrata* β-glucans immunization.

The alteration of lupus disease in response to *C. glabrata* β-glucans in *Fcgr2b*$^{-/-}$ lupus-prone mice.

In our investigation in an *in vitro* experiment, we found that *C. glabrata* β-glucans could trigger dendritic cells leading to the massive secretion of the anti-inflammatory cytokine, IL-10. IL-10 has a significant role in generating the immunosuppressive state [96]. Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease [72] and is associated with the dysfunction of Fcgr2b as an inhibitory Fc receptor [97]. In this study, *Fcgr2b*$^{-/-}$ lupus-prone mice were used as the representative model of lupus disease. *Fcgr2b*$^{-/-}$ lupus-prone mice developed increased anti-dsDNA antibodies at six months of age, indicating lupus

disease [98]. The alteration of anti-dsDNA antibodies is detected for screening the lupus characteristic. We found that *C. glabrata* β-glucans immunization at days 45 and 54 significantly decreased the level of anti-dsDNA antibodies in *Fcgr2b*$^{-/-}$ lupus-prone mice when compared with PBS control (Figure 13). This finding implies that the *C. glabrata* β-glucans may play a role in promising alleviation of lupus disease. To date, the management of SLE has moved from conventional treatments to biological treatments to decrease the risk of progressive irreversible damage accrual and increased mortality in patients with this chronic disease [99]. Immunomodulation and immunosuppressive state are being considered for SLE therapeutic approaches [100, 101]. In our results, the augmented IL-10 secretion from *C. glabrata* β-glucans stimulated-dendritic cells may be used and adapted for the biological therapies in SLE.

Splenocytes and lymph node cells were collected and re-stimulated with autoantigens in an *ex vivo* experiment. We found that the percentage of cell proliferation in lymph node cells did not alter but altered in splenocytes after re-stimulation, suggesting that *C. glabrata* β-glucans may generate immunosuppressive environments in lymph nodes (Figure 14). Otherwise, the expression of FoxP3, IL-10 and IL-4 did not alter in both splenocytes and lymph node cells after re-stimulation (Figure 16). Moreover, the level of IL-10 and IL-4 secretion did not alter in splenocytes and lymph node cells after re-stimulation (Figure 17). We found that augmented IL-17A production (5.04-fold) in lymph node cells was higher than IL-10 production

suggesting that *C. glabrata* β-glucans may impact immune activation in lymph nodes. The previous discoveries that T cell activation and oligoclonal T cell responses occurred in the regional lymph nodes (LN) of nephritic mice during acute lupus glomerulonephritis (GN) [102]. Basophils and the T helper type 2 environment could promote the production of autoantibodies resulting in the development of lupus nephritis [103]. IL-10–producing $CCR6^{+}$T cells were highly abundant in lymph nodes of SLE patients and could play a prominent pathogenic role in SLE [104]. Collectively, our findings implied that *C. glabrata* β-glucan immunization in lupus-prone mice probably mediated the alteration of immune responses in lymph nodes.

In conclusion, our investigation implies that β-glucan isolates in the cell wall of *C. glabrata* could promote the massive production of anti-inflammatory or immunosuppressive cytokine, interleukin-10 (IL-10), which might lead to generating the immunosuppressive state in dendritic cells. Moreover, we found that the capacity of *C. glabrata* β-glucan in an *in vivo* experiment promisingly controls the levels of autoantibodies in *Fcgr2b*$^{-/-}$ lupus-prone mice which may be beneficial for the amelioration of lupus disease. However, this study still needs to provide more advanced information in *in vivo* experiments on the alteration of lupus characteristics and the characterization of dendritic cells and T cells after *C. glabrata* β-glucan immunization in *Fcgr2b*$^{-/-}$ lupus-prone mice.

APPENDIX A

MATERIALS AND EQULPMENT

1.	24-wells plate (Cell Culture plate)	USA
2.	48-wells plate (Cell Culture plate)	USA
3.	96-wells plate (Cell Culture plate)	USA
4.	96 wells flat bottom plate	USA
5.	Biological Safety Cabinet (BSC) Class II	Singapore
6.	Centrifuge 4-16KS	Germany
7.	Centrifuge Allegra X-30R	USA
8.	CO_2 Incubator for cell culture	USA
9.	Water bath	Germany
10.	Automated Cell Counter (Invitrogen)	USA
11.	Inverted microscope IX70	Japan
12.	Incubator shaker (Daihan)	Korea
13.	Orbital shaker incubator	Latvia
14.	Pipette tip 10, 200, 1000 µL	USA
15.	Serological pipette 5, 10, 25 ml	USA
16.	Micropipette 2, 10, 200, 1000 µL	USA
17.	Serological pipette controller	USA
18.	1.5 ml microcentrifuge tube	USA
19.	15- and 50-ml conical tube	USA

20.	Microplate washer 405LS	USA
21.	Microplate reader Epoch2C	USA
22.	Flow cytometry (CytoFLEX)	USA
23.	Autoclave HVA-110	Japan
24.	Hot air oven (Memmert)	Germany

APPENDIX B

BIOLOGICAL/CHEMICAL AGENTS AND REAGENTS

1. Absolute Ethanol Grade AR	New Zealand
2. Sodium hydroxide (QREC)	New Zealand
3. Phosphoric acid (QREC)	New Zealand
4. Sodium azide (QREC)	New Zealand
5. Sodium chloride (QREC)	New Zealand
6. Di-Sodium hydrogen phosphate dihydrate (QREC)	New Zealand
7. Potassium dihydrogen phosphate (QREC)	New Zealand
8. Potassium Chloride (QREC)	New Zealand
9. Sodium bicarbonate (QREC)	New Zealand
10. Sodium carbonate (QREC)	New Zealand
11. Tri-potassium Citrate Monohydrate (QREC)	New Zealand
12. Citric acid Monohydrate (QREC)	New Zealand
13. Sodium Hydroxide (QREC)	New Zealand
14. Endotoxin-free water	Thailand
15. RPMI 1640 Medium (Gibco)	USA
16. Fetal bovine serum (Gibco)	USA
17. Glutamax (Gibco)	USA
18. Penicillin-Streptomycin (Gibco)	USA
19. 2-mercaptoethanol (Invitrogen)	USA

20. Dulbecco's Phosphate-Buffered Saline, DPBS (Gibco)	USA
21. Recombinant mouse FLT3L (Biolegend)	USA
22. Recombinant murine GM-CSF (Peprotech)	USA
23. Bovine Serum Albumin, BSA (Capricorn)	Germany
24. Dexamethasone (Sigma)	USA
25. Lipopolysaccharides, LPS (Sigma)	USA
26. Collagenase IV (Invitrogen)	USA
27. DNase I (ThermoFisher)	USA
28. All antibodies for flow cytometry (Biolegend)	USA
29. anti-FoxP3 antibody (eBioscience)	USA
30. All antibodies for ELISA (Biolegend)	USA
31. IL-23 ELISA kit (eBioscience)	USA
32. DMSO (Sigma)	USA
33. 30% Hydrogen peroxide (QREC)	New Zealand
34. TMB (3,3,5,5-tetramethylbensidine)	USA
35. Paraformaldehyde	USA
36. Phorbol 12-myristate 13-acetate, PMA (Sigma)	USA
37. Ionomycin (Sigma)	USA
38. Brefeldin A (Sigma)	USA
39. Monensin (Biolegend)	USA
40. Coated Calf-DNA (Invitrogen)	USA

41. Pan T Cell Isolation Kit II (Miltenyi Biotec) USA

42. Cell Proliferation Assay Kit, MTS (Promega) USA

APPENDIX C

REAGENTS PREPARATION

1. Complete RPMI1640 for BMDCs culture

RPMI 1640	45 ml
200 mM Glutamax	50 µl
100% heat inactivated FBS	5 ml
100x Penicillin/Streptomycin	500 µl

2. Complete RPMI1640 for splenocytes and lymph node cells culture

RPMI 1640	44 ml
200 mM Glutamax	500 µl
100% heat inactivated FBS	5 ml
100x Penicillin/Streptomycin	500 µl
1000x 2-mercaptoethanol	50 µl

3. 70% Ethanol

100% Ethanol	70 ml
Sterile water	30 ml

4. 1x Dulbecco's Phosphate-Buffered Saline, DPBS

Dulbecco's Phosphate-Buffered Saline, DPBS	9.55 g
Endotoxin-free water	1000 ml

5. 1x Phosphate-buffered saline

Sodium chloride, NaCl	8 g
Di-Sodium hydrogen phosphate dihydrate, Na_2HPO_4	1.16 g
Potassium dihydrogen phosphate, KH_2PO_4	0.2 g
Potassium Chloride, KCL	0.2 g
Distilled water	1000 ml

6. FACs buffer

1x Phosphate-buffered saline	495 ml
100% heat inactivated FBS	5 ml
Sodium azide (NaN_3)	0.5 g

7. Coating buffer for ELISA

Sodium bicarbonate, NaHCO3	8.4 g
Sodium carbonate, Na_2CO_3	3.56 g
Distilled water	1000 ml

8. 0.2M TMB buffer

Tri-potassium Citrate Monohydrate	33.25 g
Citric acid Monohydrate (MW=210.4 g/mol)	19.69 g
Distilled water	500 ml

9. TMB solution

0.2M TMB buffer	10 ml
DMSO	250 µl
30% Hydrogen peroxide	2.5 µl
TMB (3,3,5,5-tetramethylbensidine)	2.5 mg

10. 10N Sodium hydroxide

Sodium hydroxide, NaOH	40 g
Distilled water	100 ml

11. 4% Paraformaldehyde

Paraformaldehyde	8 g
10N Sodium hydroxide	20 µl
1x Phosphate-buffered saline	200 ml

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