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Immunomodulatory Effect Of Host And Fungal Eicosanoids During Host-Pathogen Interactions With Candida Albicans

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IMMUNOMODULATORY EFFECT OF HOST AND FUNGAL EICOSANOIDS DURING HOST-PATHOGEN INTERACTIONS WITH *CANDIDA ALBICANS*

by

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Dedication

To my father, Pranab Kumar Kundu, who has been a constant inspiration and support in every pursuits of my life.

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GENERAL INTRODUCTION

There are approximately 150 species of *Candida* out of which only small numbers are able to establish pathogenesis in humans [1]. Out of the list of all these species, *Candida albicans* is the most commonly isolated species associated with infection. Diseases range from superficial mucosal infections to systemic mycoses. Under normal conditions, *C. albicans* exists as a common member of the oral, gastro-intestinal, and uro-genital microbiota, with carriage rates ranging from 30-70% among healthy individuals [2, 3]. However, exposure rates are likely much higher as the majority of individuals have circulating anti-*Candida* antibodies [4]. Under conditions that permit outgrowth or invasion, *C. albicans* has the potential to cause infection and disease. Therefore, a balanced immune response is required to defend against damaging infections, while tolerating mucosal colonization. However, the mechanism controlling the host-pathogen relationship is relatively unknown.

Previous studies by Noverr *et al.* demonstrated the ability of *C. albicans* to produce immunomodulatory molecules called oxylipins, which are similar in structure and function to host eicosanoids. Eicosanoids are a family of lipid immune signaling compounds that include the prostaglandins and leukotrienes. Specifically, C. albicans produces a compound that cross reacts with mammalian prostaglandin E_2 (PGE_2), called PGEx [2]. Very little is known about the immunomodulatory roles that PGE_2 or PGEx plays during candidiasis. Our hypothesis is that production of oxylipins by both fungi and host are crucial in

modulating the microbiology of the fungus and the host-pathogen interaction in favor of chronic infection or pathogenesis. The goal of this thesis project was to investigate the roles of both host and *Candida* produced eicosanoids during host-pathogen interactions both in vitro and in vivo. This involved investigating interactions with phagocytic and antigen presenting cells and how these cells influence downstream adaptive immune responses during systemic infection. In addition, we investigated effects of host eicosanoids and eicosanoid inhibitors during pathogenesis of mucosal and systemic infections. The results of these studies are clinically significant and may help direct novel pharmacotherapeutic strategies to treat fungal infections.

Virulence Attributes of *Candida albicans*. *C. albicans* is an opportunistic pathogen that normally resides at mucosal surfaces asymptomatically. Therefore, it is not under selective pressure to develop traditional virulence factors found in professional pathogenic microbes. Virulence factors in *C. albicans* are often the factors that allow the organism to persist within the host at mucosal surfaces and systemically. In fact genetic deletion of many Candida "virulence factors" results in avirulence during both mucosal and systemic infection models. It may therefore be more appropriate to define virulence in terms of traits required for survival within the host and evasion from host defenses. Virulence traits in *Candida* includes adhesion to host surfaces, morphogenesis, biofilm formation, antigenic or phenotypic switching, tissue and

non-phagocytic invasion (via proteases and phospholipases), and intracellular survival strategies [5-7].

Morphogenesis

C. albicans is polymorphic and has the ability to grow both as yeast (blastospores), and as hyphae and pseudohyphae (filamentous form). The conversion from yeast to hyphae is induced by environmental conditions such as growth at 37°C, serum, pH > 6.5, D-glucose, CO_2, and nitrogen and carbon starvation (reviewed in [8]). In addition, *C. albicans* produces quorum-sensing compounds that control morphogenesis. At high population densities, farnesol accumulates and represses hyphal formation, whereas tyrosol promotes germination at low population densities when farnesol concentrations are low [9]. In addition, it was discovered that bacterial products influence morphogenesis. Pseudomonas aeruginosa produces a 12 carbon compound called dodecanol, which inhibits morphogenesis, indicating an antagonistic relationship. Another study demonstrated that active serum fractions that promote hyphal formation contain bacterial peptidoglycan (PGN)-like molecules [25]. Analysis of purified bacterial products showed that synthetic muramyl dipeptides (MDPs), subunits of PGN, can strongly promote *C. albicans* hyphal growth. Multiple signal transduction pathways regulate hyphal development, including mitogen activated protein kinase (MAPK) cascades, pH-dependent signaling cascades, and cAMP-dependent protein kinase A (PKA) pathways [10]. These pathways sense and respond to divergent and overlapping environmental signals. Therefore, *C.*

albicans control of morphogenesis can be fine tuned within the various host niches.

The yeast forms are more often associated with asymptomatic colonization whereas the hyphal form is associated with infection and disease. Genes that control hyphal morphogenesis are co-regulated with genes encoding virulence factors such as proteases and adhesions [11]. The ability to switch between yeast and filamentous forms is also required for virulence as mutants that are “locked” in either the yeast form or the hyphal form are avirulent [11, 12]. One study demonstrated that there are distinct roles for both yeast and hyphae in pathogenesis [13]. They engineered a strain that carries one copy of NRG1, a key negative regulator of hyphal development, under the control of a tetracycline-regulatable promoter so the growth forms of the strain can be controlled in vivo. Mice injected with this strain under conditions permitting hyphal formation succumbed to the infection, whereas all of the animals injected under conditions that inhibited this transition survived. Importantly, fungal burdens were almost identical in both sets of animals, indicating that, whereas filament formation appears to be required for the mortality resulting from a deep-seated infection, yeast cells play an important role early in the infectious process by extravasating and disseminating to the target organs. This indicates that hyphal formation is required for mortality via an unknown mechanism. Therefore, this suggests that there are morphotype specific factors and/or immune responses that influence survival.

Biofilm Formation

Another role that morphogenesis plays in the lifestyle of *C. albicans* is in the ability to form biofilms, which are complex aggregations of microbial cells attached to a solid surface, marked by the excretion of a extracellular polysaccharide matrix (ECM) [reviewed in [14-16]. Biofilm formation requires the ability to undergo morphogenesis with germination being induced upon contact with an appropriate surface [17, 18]. Mutants of *C. albicans* that are unable to form hyphae also are unable to form vigorous biofilms. In addition, environmental conditions that induce hyphal formation also facilitate biofilm formation. *C. albicans* readily forms biofilms on abiotic surfaces such as synthetic polymers used in medical devices. In addition, recent studies using experimental models of infection have shown that *C. albicans* forms biofilms on biotic surfaces such as oral and vaginal mucosal tissues in vivo [19, 20].

Biofilm formation occurs in distinct developmental stages: attachment, proliferation, hyphal transformation, maturation, and dispersal [9, 21]. The maturation stage involves a repression of yeast growth, and a proliferation of hyphal growth elevated combined with secretion of ECM. The matrix is composed of carbohydrates, protein, phosphorus, hexosamines, and glucose; however, a large amount of material remains uncharacterized [22]. Further studies by Mukherjee et al. have indicated that the composition of ECM (the protein and the carbohydrate component) varies depending on the biofilm developmental stage [15]. Macromolecular studies have been difficult but one study demonstrated the cell wall component β-glucan could be detected in biofilm

ECM [23]. This is of significance because β-glucan is recognized by dectin-1, a pathogen recognition receptor (PRR), and could influence interactions with the host [24-26].

Studies have shown that *C. albicans* biofilms are highly heterogeneous in terms of structural architecture, which varies greatly by the nature of the substrate surface. For instance biofilms formed on polyvinyl chloride disc have distinct composition compared to that formed on silicone elastomer or polymethylmethacrylate denture strips [15, 27] . Unlike the nature of the substrate resulting in different composition of biofilms, different environmental factors (pH, oxygen availability, fluid shear and redox-gradients) does not seem to be playing role in designing the architecture of the *C. albicans* biofilms. Similar levels of transcript profiles were detected from the biofilms growing under different environmental conditions.

In terms of infection, *Candida* biofilms readily form on indwelling medical devices and represent one of the main portals of entry for systemic and tissue candidiasis [28]. It is estimated that the majority of microbial infections originate from biofilms, with biofilm infection of indwelling medical devices being the single most important cause of nosocomial bloodstream infections (BSI). To underscore this point, it is estimated that 250,000 - 500,000 device-related BSIs occur annually in the US. Among those infections, 10% are caused by *C. albicans*, which has the highest mortality rate (40%) of all BSI causing organisms [29, 30]. Another feature of *C. albicans* biofilms is that they are highly resistant to antifungal agents [31]. The mechanism underlying this increased resistance is

not well understood; however, poor antifungal penetration has been ruled out [32]. Genomic studies have shown that biofilms exhibit unique gene expression patterns compared with planktonic cells, including upregulation of multi-drug resistance pumps [33-35]. The gene pathways that control biofilm formation are still being dissected. There is some overlap with pathways that control morphogenesis; however, a specific biofilm regulator, Bcr1, is required for normal biofilm formation and can still form hyphae. Therefore, biofilms represent a unique lifestyle for *C. albicans*, which requires significant investigation in terms of pathogenesis.

Overview of the Immune Response to *C. albicans Infections*. *C. albicans* causes both superficial mucosal infections and deadly systemic infections which involve the bloodstream and tissues. The type of risk factors and appropriate immune response to infection varies depending on the type of infection. *C. albicans* resides at mucosal surfaces as a commensal organism, where immune responses must be tightly controlled to both prevent overgrowth and infection but also to prevent over-exuberant inflammatory responses. Control of mucosal infections and clearance of systemic infections require both effective innate and adaptive immune responses. The inflammatory response to fungal infections must be followed by resolution of inflammation, which is essential for limiting host damage. Fungal infections tend to be chronic, which indicates that the immune system maintains a very precarious balance between tolerance and inflammation.

Mucosal Infections

C. albicans is frequently isolated from the mucosal regions of vagina, oral cavity and gastrointestinal tract and can be carried asymptomatically in healthy individuals [36, 37]. Changes in the host environment can lead to fungal overgrowth and/or imbalances in host immune responses leading to symptomatic mucosal infection. Risk factors for mucosal infection are tissue specific and include host factors (immunosuppression) and environmental factors (antibiotics and hormones).

Vaginal infection: About 75% of women have at least one incidence of vulvovaginal candidiasis in their lifetime with about 5-10% recurrence rate [38, 39]. Antibiotic treatment, oral contraceptives, hormone replacement therapy, pregnancy, immunosuppressive therapy are the several known predisposing factors leading to this disease [38-40]. In case of vaginitis the resistance and susceptibility is not dependent on adaptive immune responses. This is supported by the fact that women with HIV infection, who are susceptible to oropharyngeal candidiasis (OPC), were no more susceptible to vulvovaginal candidiasis (VVC) than the healthy HIV- population [41]. In addition, experimental models show no role for cell mediated or humoral immunity. In understanding why adaptive immunity is not engaged in vaginal infections, studies have indicated that a strong regulatory immune response is present. Regulatory T cells (Tregs), transforming growth factor β (TGF-β) and plasmacytoid dendritic cells (DCs) were

present at the vaginal mucosa, which can inhibit inflammatory T cell responses [42-44]. Regulatory T cells (Treg) are responsible for dampening inflammation and promoting tolerance at mucosal surfaces via production of IL-10 and TGF-β [45]. In addition, homing receptors on T cells in the draining lymph nodes were down-regulated, indicating an inability to traffic to the vaginal mucosa.

Instead, it is believed that susceptibility is controlled by innate immunity. The vast majority of symptomatic infections in women have increased neutrophil infiltrate that correlated with the observed symptoms of vaginitis. In fact, neutrophil infiltration showed a positive correlation to high vaginal fungal burden. Conversely, those women in these studies who presented as asymptomatic showed no evidence of neutrophil infiltration or inflammation. However, these neutrophil have defective antifungal activity and therefore may contribute to pathology rather than control infection. It has been proposed that a threshold number of C. albicans cells is necessary to stimulate the neutrophil response and symptoms of disease, but that the threshold needed varies depending on the individual [46]. Vaginal epithelial cells also exert a fungistatic activity, which is reduced in women with recurrent vaginitis [47]. In addition, these epithelial cells can sense and respond to *Candida* cells and signal the chemotaxis of neutrophils [48]. Therefore, differences in the sensitivity of vaginal epithelial cells may be involved in determining susceptibility.

Oral infection: There are several types of oral candidiasis including OPC, which is associated with T cell deficiency (HIV and neonates), and denture stomatitis,

which is associated with a contaminated indwelling device but occurs in otherwise healthy individuals. There is a lack of studies investigating denture stomatitis. One study demonstrated that both Th1 and Th2 cytokines are produced in patients [49]. Therefore, disease is not likely due to a Th1 deficiency. Instead the Th1-type immune response is active but seemingly fails to resolve or protect against infection. This could be due to the serial feeding of the tissue with *Candida* as a biofilm on the denture material resulting in the chronic inflammation associated with infection.

OPC involves infections of the palate, tongue and buccal mucosa and can either be pseudomembranous (thrush) or erythematous and is considered an AIDS defining illness [50]. Dissemination of infection generally does not progress from oral infections. While it is clear that CD4+ T cells and adaptive immunity is required for defense against OPC, innate defenses also play a role in controlling fungal growth. Epithelial cells can inhibit up to 80% of *Candida* growth by involving static mechanisms or production of cytokines [51-58]. In addition, saliva contains antimicrobial secretions consisting of calprotectin, β-defensins, and histatins [59, 60]. Along with epithelial cells, neutrophils are described to play role in innate immune responses though it is controversial [8, 61]. Studies have demonstrated reduced numbers of Langherhan cells in patients with frequent oral candidiasis incidences indicating that DCs might have some key role to play towards clearance of *Candida*. [62].

Adaptive immune responses largely involve cell-mediated immunity, while

humoral immunity does not appear to play a role in protection against or susceptibility to OPC [63]. Clinically, OPC is most common in HIV+ patients when CD4+ cell numbers drop below 200 cells/μl [64]. Overall, the prevalence of OPC depends on the status of the local immune mechanisms. Indeed some individuals with <200 CD4+ cells/μl never have OPC while others have recurrent episodes of OPC. Under conditions of CD4+ T cell deficiency it has been proposed that aside from innate defenses, CD8+ T cells help control *C. albicans* and that susceptibility is associated with defects in trafficking of cells to the site of infection [65]. In deciphering how CD4+ T cells control oral C. albicans levels, it was proposed that a Th1 response is required. However, recent studies in experimental models have indicated the role of Th17 cells as well towards inducing protection against oral candidiasis [66]. Interestingly, knockout mice lacking IL-23 or IL-17R (Th17-deficient) were highly susceptible to OPC, whereas mice lacking IL-12 (Th1-deficient) were relatively resistant to infection.

Gastro-intestinal infection: Under normal conditions, humans are colonized with about 10^{14} microorganisms in the GI tract, most of them being bacteria. Fungi are also important part of the microbiota and *Candida* is one of the most commonly isolated genus. Under healthy host conditions, the *Candida* carriage rate inside the GI tract varies somewhere from 30-70% [2, 3]. Increases in GI colonization with *Candida* combined with immunosuppression can lead to spread into the bloodstream, an infection known as candidiasis of endogenous origin [67-70]. Risk factors include antibiotic treatment and parenteral nutrition, which

alter the bacterial microbiota leaving a niche for *Candida* overgrowth. In addition, patients undergoing immunosuppressive treatment and cancer patients are particularly at risk of developing GI candidiasis. One of the major problems associated with GI infection is that it is not clinically detected during the early stages of infection due to inconspicuous symptoms and the inability to differentiate commensal *Candida* from a pathogenic type.

Both innate and adaptive immune responses are important for protecting against gastro-intestinal candidiasis. The key immune cell players involved in initiating potent innate immune responses are NK cells, macrophages and neutrophils. This was verified when gnotobiotic immunodeficient mice with defects in granulocytic cells showed susceptibility to candidiasis. However, *Candida*-specific T cell and antibody responses were mounted, and eventually cleared the infection [71]. Treatment with the inhibitors of nitric oxide synthase (NOS) increased the magnitude of GI candidiasis severity. However, peroxynitrite was the responsible player, as nitric oxide is not candidacidal directly [71]. These studies indicate that these reactive oxygen species released by the phagocytes are important in controlling *Candida*.

Regarding adaptive immunity, studies using congenitally athymic T cell deficient (nu/nu), severe combined immunodeficient (SCID; no T or B cells) mice, or mice specifically lacking α/β or γ/δ T cells have shown that T cells are critical for effective protection against GI candidiasis (6, 9, 31, 104). Mice with T cell deficiencies were more susceptible to GI tract infection, but were not susceptible to sublethal intravenous challenge or dissemination from the GI tract. Various

studies have indicated that during GI candidiasis, Th1 responses are associated with protection whereas the Th2 responses induce non-protective phenotype [72-76]. Antibody mediated protection against GI candidiasis have not been reported and mice lacking B cells are not susceptible to infection [77]. A study indicated that oral immunization had no role in decreasing the colonization of GI tract [78].

More recent studies have investigated the role of DCs in GI infections. Using an experimental murine model, it was demonstrated that Peyer's patch DCs can discriminate between yeast and hyphal forms of *C. albicans* and promote differentiation of inflammatory (Th17/Th1) and tolerogenic DCs (Th2/Treg), respectively [79]. In addition, exacerbation of a Th17 response, characterized by uncontrolled inflammation and dampening of Th1 responses via IL-23 and IL-17, also leads to a worsening of fungal disease in the GI tract [79]. These observations contradict previous studies which demonstrated that yeast promote Th1 responses while hyphae promote Th2 responses from human dendritic cells [80]. It is possible that these differences are due to differences in dendritic cells or the experimental model used.

In the context of *C. albicans* mucosal infections, Tregs are required for effective control of protective Th1 inflammation, which prevents eradication of infection. This not only facilitates generation of memory immunity but also limits pathological inflammatory responses to persistent colonization [81, 82].

Systemic Infections

Systemic candidiasis is an infection associated with high morbidity and mortality. Infections are often misdiagnosed, and delayed treatment combined with drug resistance has resulted in a 40% of crude mortality rate [30, 83, 84]. The total number of *Candida sp*. cases in the U.S. ranges from approximately 10,500 to 42,000 infections per year and is the fourth leading cause of bloodstream infections [85]. At present overall systemic candidiasis rates are on a rise and are associated with enormous amounts of health and economic losses [86]. Risk factors include immune deficiency associated with neutropenic, organ transplant, and cancer patients, use of immunosuppressive drugs, and indwelling medical devices. There are two major pathways by which *C. albicans* may enter the bloodstream: penetration from the GI tract into blood vessels and direct transmission via intravascular catheters [86-88].

Cells of the innate immune response represent the first line of defense against *C. albicans* infections and include effector cells with potent antifungal activity: neutrophils and macrophages [89-91]. While neutrophils generally kill via secreted enzymes and oxidative intermediates, macrophages can phagocytose *C. albicans* and kill via lysosomal and oxidative pathways. In addition, innate immune cells serve in the initial recognition of *C. albicans* via pathogen recognition receptor (PRR) signaling. PRRs, including toll-like receptors (TLRs) and lectins recognize cell wall components of *C. albicans*. Innate cells express PRRs and respond by secreting a variety of immune signaling molecules to direct adaptive responses in a microbe-specific manner.

In particular, macrophages and DCs play important roles in PRR recognition of *C. albicans* and serve as antigen presenting cells, linking innate and adaptive immunity.

Neutrophils are potent antifungal cells and play a key role in the prevention of fungal growth and the invasion of tissues. Defects in neutrophil number and function have been consistently implicated in the pathogenesis of disseminated candidiasis [92, 93]. In particular, defects in oxidative defenses of neutrophils such as myeloperoxidase (MPO) or NADPH oxidase increase susceptibility to systemic infection [94, 95]. *C. albicans* first encounters neutrophils in the bloodstream during systemic infection. Neutrophils inhibit *C. albicans* growth, enhance the fungal response to overcome nitrogen and carbohydrate starvation, and induce the expression of a large number of genes involved in the oxidative stress response [93]. In addition, neutrophils are the primary antifungal cell recruited to the site of tissue infection. Studies have demonstrated that TNF-α, IL-6, and G-CSF play an important role in the recruitment of PMNs at the site of invasive *Candida* infection [96-98]. In the absence of either TNFα or IL-6, the course of experimental disseminated candidiasis is more severe, due to defective PMN recruitment [99]. Treatment of mice with recombinant G-CSF (rG-CSF) leads to a significantly reduced mortality and fungal burden during disseminated candidiasis [100]. This is likely due to the ability of this cytokine to promote proliferation and differentiation of neutrophils from bone marrow. In terms of chronic infections, the role of neutrophils may be primarily as a source of pro-inflammatory cytokines, since these cells have been

shown to produce TNFα, IL-6 and IL-12 upon stimulation with virulent *Candida* strains, but not avirulent, hyphal defective mutants strains[97].

Macrophages also play an essential role during systemic candidiasis. In an experimental model of systemic candidiasis, macrophage depletion resulted in delayed clearance and high kidney fungal burden [101]. Macrophages can kill *Candida* via oxygen-dependent or independent pathway [102]. Oxygen dependent pathways include production of both reactive oxygen and nitrogen intermediates (ROI, RNI). In particular, nitric oxide (NO) has strong antifungal activity; however, *C. albicans* can evade host defense by suppression of NO/superoxide radical production by stimulated macrophages [103-105]. Furthermore, what were thought to be two independent pathways, i.e., nitric oxide and superoxide anion, have now been shown to combine to form a potent macrophage candidacidal molecule, peroxynitrite. Oxygen independent pathways are less well studied. However, upon phagocytosis of *C. albicans*, yeast forms can germinate and lyse macrophages [106]. Therefore, whether the macrophage or the fungus prevails is dependent on several factors, including the type of macrophage and activation state. In contrast to neutrophils, which are important in resistance to early stages of *C. albicans* infections, differentiated macrophages activated by cytokines such as IFN-γ participate in the acquired resistance of hosts with *C. albicans*-specific, cell-mediated immunity.

DCs are one of the most potent antigen presenting cells and has the potential to educate the naïve T cells to further induce adaptive immune responses. DCs arise from the bone marrow progenitor cells called common DC

precursors and migrate via blood to both primary and secondary lymphoid tissues as well as peripheral tissues (mucosal surfaces, skin). Immature DCs are characterized by their low endocytic activity and inability to educate naïve T-cells. Upon phagocytosing antigens or pathogens, they are activated to become mature DCs, which express activation markers CD80 and CD86, co-stimulatory molecules B7.1 and B7.2, as well as high levels of MHC class I and class II molecules. Activated mature DCs then migrate to the regional lymph nodes via the afferent lymphatic vessels where they lose the ability to further uptake antigen. Mature DCs secrete immune signaling compounds and interact with naïve T cells and influence differentiation of different T cell subsets.

There are two main subsets of DCs defined by cellular lineage markers: classical or myeloid type DCs ($CD11c^+$ $CD11b^+$), and plasmacytoid DCs ($CD11c^+$ $B220^+$). Myeloid DCs (mDCs) are the typical DCs displaying dendritic extensions and having antigen-presentation function in steady-state [107]. Also known as lymphoid organ-resident DCs, mDCs are derived from blood-borne progenitors and are found in the thymus, spleen, and lymph nodes, where their actions are restricted to the organ in which they reside. In addition, mDCs are more prevalant in the body and are found in the regions like mucosal linings and skin tissue [63, 108-110]. Plasmacytoid DCs (pDCs) lack dendrites but have a plasmacytoid shape. Unlike mDCs, pDCs are very thinly distributed in the body and they normally reside in lymph nodes, blood and thymus. Signaling via various molecules cause their recruitment at the site of infection. pDCs express lower levels of MHC class II and are much less efficient at inducing T-cell proliferation

compared with mDCs [111]. Mature DCs educate the naive T cells into Th1, Th2, Th17 or Treg phenotype [112, 113]. In terms of *Candida* systemic infection, a Th1 response results in protection and clearance, while a Th2 response is non-protective [114]. Th1 responses require production of phagocyte activating pro-inflammatory cytokines such as IFN-γ, IL-12, IL-6, and TNF-α [74, 115-118]. Tregs or Th3 cells have potent anti-inflammatory activities and aid both controlling inflammation and in resolution of inflammation after infection [119, 120]. Tregs are involved in suppressing inflammation and promoting tolerance via contact dependent and independent mechanisms. In particular, they are associated with secretion of anti-inflammatory cytokines IL-10 and TGF-β [56]. There are several subsets of Tregs: $CD4^+CD25^+$ intrinsic Tregs, and Tregs induced in the periphery. The majority of the time *C. albicans* exists as a commensal, not a pathogen. In this situation, it would benefit the host not to elicit an inflammatory immune response, but instead maintain tolerance via generation of Tregs. However, Tregs could exacerbate disease by dampening protective inflammatory responses in the case of systemic infection [121]. Therefore, dendritic cells and other antigen presenting cells must be able to quickly discriminate between commensal and pathogenic *Candida* growth states and initiate the proper T cell-mediated response.

Th17 cells have a distinct lineage and cytokine profile compared with Th1 and Th2 cells. Naïve T cells differentiate into Th17 cells in the presence of a combination of TGF-β, IL-6, IL-1, and IL-21, while IL-23 is essential for Th17 cell expansion and function. Th17 cells secrete a unique profile of cytokines including

IL-17 (IL-17A), IL-17F, IL-21, IL-22 [66]. Production of these cytokines induces neutrophil activating factors, antimicrobial peptides, and acute response proteins (reviewed in [122]). In terms of systemic candidiasis, the Th17 response is required for protection as mice lacking IL-17 receptor are more susceptible to acute intravenous infection [123, 124]. This was associated with reduced neutrophil recruitment. In addition, vaccination with a hyphal adhesin protein induced a protective Th17 response and Th17 cells enhanced phagocytic killing of *C. albicans* in vitro [125].

DCs have the ability to distinguish and discriminate between the two different *Candida* morphotypes, i.e., hyphae and yeast form. Coordinated signaling via TLRs results in either pro or anti-inflammatory responses by production of different cytokines which will cause changes in downstream signaling molecules to influence adaptive immune responses [126]. Recognition of DCs with yeast form of *Candida* results in stimulation of type 1 cytokines (DC1). This type of interaction involves signaling via TLR4 complexes. Hyphal form of *Candida* signals via TLR2 and cause induction of type 2 cytokines (DC2). Other studies have indicated that interaction with hyphal forms inhibits the maturation followed by activation of DCs [126-128]. Studies by Graaf et al demonstrated that when *C. albicans*-pulsed DCs were injected into the mice, the yeast-pulsed DCs caused a protective response whereas the mice which were injected with hyphae-pulsed DCs lead to a non-protective response [127].

Further, recent studies have demonstrated that *C. albicans* signaling via TLR2 induces production of host PGE_2, which can cause inhibition of innate

responses for antifungal defenses [127]. This indicates that eicosanoids shift the immune response towards that of a non-protective type. Prostaglandins produced by both host and fungal cells also enhance morphogenesis in *C. albicans* suggesting that eicosanoids could participate in modulating the immune responses by both influencing *Candida* and DC biology [77, 129]. Thus, depending on the morphotypes of *Candida* (yeast or hyphal form), the DCs have the ability to modulate the immune responses.

Immunoregulation by Eicosanoids

Host eicosanoid production.

Eicosanoids are one of the most abundant subfamily of oxylipins, which are oxidized fatty acids [47]. Eicosanoids are made up of 20 carbon PUFA metabolites that include the prostaglandins (PGs) and leukotrienes (LTs). Eicosanoids, whose major precursor is arachidonic acid, potent modulators of immune responses [130, 131]. Eicosanoids can be divided into various subfamilies, the main one being prostanoids which are products of a cyclooxygenase (COX). Various subfamilies of the prostanoids are differentiated based on the region of the oxygen substitution in the cyclopentane ring, and also by a number, representing the number of *cis* double bonds in the lipids [132]. For example, a prostaglandin of the E class derived from arachidonic acid will be denoted as prostaglandin E_2 (PGE_2).

The polyunsaturated fatty acids are sequestered in membrane phospholipids and are released upon receiving various endogenous or exogenous signals. Upon the release of precursor fatty acids from the membrane, they are re-esterified or enzymatically converted to eicosanoids. These eicosanoids generally have a very short half-life as they are immediately hydrolysed or undergo spontaneous metabolic inactivation, which could take place by the process of ω-hydroxylation, fatty acid β-oxidation and enzymatic dehydrogenation. As a result of these processes, eicosanoids are generally not stored in the cells. During the initial steps in biosynthesis of eicosanoids, 20 carbon PUFA precursor (generally arachidonic acid) are released from the membrane phospholipids by the action of an enzyme called phospholipase (specifically phospholipase synthases A_2 in mammalian cells) [131]. The initial step of prostaglandin biosynthesis is catalysed by COX (also known as prostaglandin H synthase) which are bifunctional heme-dependent enzymes [133]. In mammals, there are two COX enzymes, COX-1 being constitutive form and COX-2 being inducible form [131]. These enzymes are inhibited by a class of drugs known as NSAIDs (non-steroidal anti-inflammatory drugs), which includes aspirin, indomethacin, etc. The different classes of prostaglandins are then synthesized by specific synthases and hydrolases.

PGE_2 is one of the most well studied prostaglandins and is a pleiotropic signaling molecule. PGE_2 signals via four receptors on host cells: EP1, EP2, EP3, and EP4 [134]. In terms of immune regulation during infection, PGE_2 is associated with anti-inflammatory activities such as inhibition of effector functions

of inflammatory cells. These include inhibition of mediator release from DCs, macrophages, neutrophils, mast cells, basophils and lymphocytes [135]. Studies have shown that PGE_2 can interfere with DC maturation and cytokine secretion, events required for migration to T cell areas and subsequent T cell education and activation [46, 136, 137]. In addition, PGE_2 and other eicosanoids can downregulate macrophage functions including phagocytosis [9, 138-140]. To summarize the different roles of PGE_2 that it plays towards immune cell modulation are to suppress macrophage cytokine and chemokine secretion [141], inhibition of IL-12 and IFN-γ production by NK cells [141, 142], inhibition of T-cell proliferation [71, 143], enhancement of IgE class switching by B-cells [144], up-regulation of antigen-induced mast cell degranulation [145], inhibition of lymphocyte-endothelial cell interactions etc.

Eicosanoid production by host cells can also be influenced by the interaction with fungal pathogens. *C. albicans* induces activation of COX-2 transcription and the production of host PGE_2 synthesis in macrophages, splenocytes, and endothelial cells [146-148]. The ability to control or clear *Candida* infection is dependent on the intensity of the inflammatory response (Th1 vs Th2). Excess of PGE_2 is shown to be associated with prevention of synthesis of TNF-α and IL-12 and aids in the release of Th2 attracting cytokines like IL-10. IL-10 is an important cytokine in regulating immune response and plays key role in maintaining DCs in an immature state [72, 149]. Recent studies have indicated an increased level of PGE_2 production from the vaginal lavage fluids from recurrent vulvovaginal patients [150]. This suggests that fungal

manipulation of host eicosanoid production may be one strategy the organism uses to subvert the immune system.

Candida eicosanoid production

The potential for eicosanoid production in medically important fungi had been overlooked until recently. *C. albicans* causes release of arachidonic acid from host tissues, which could be used for eicosanoids production [149, 151]. *Candida* produces both endogenous oxylipins as well as novel eicosanoid products from exogenous arachidonic acid [77, 148]. A novel eicosanoid product was detected, 3,18-diHETE, which is related to the 3-HETE product detected in the lipomycetaceous fungi. By immunofluorescence, endogenous eicosanoid products were found in hyphae, but not in yeast forms, suggesting that this product may be involved in morphogenesis. The production of this novel eicosanoid could be inhibited by aspirin, which also suppressed the growth of the yeast form and prevented the yeast to hyphal transition of *C. albicans* [152].

A fungal oxylipin was isolated from *Candida* that exhibits cross-reactivity with host PGE_2. This compound, termed PGEx, is bioactive on mammalian cells in vitro similar to PGE_2, indicating the fungal oxylipins can modulate host immune responses [77]. Fungal PGEx inhibited chemokine production, TNF-α production, and splenocyte proliferation while up-regulating IL-10 production. In terms of effects on *C. albicans*, host PGE_2 and fungal PGEx enhance *C. albicans* morphogenesis, and up-regulated hyphal specific genes [77, 129]. *Candida* oxylipin production is also upregulated during biofilm formation [153].

C. albicans does not possess arachidonic acid when grown in vitro. Therefore endogenous PGEx must be structurally distinct from host PGE_2. Because PGEx works similar to PGE_2, it is likely that the ring group which is recognized by EP receptors is similar, but the overall carbon length in shorter. However, supplementation of *C. albicans* cultures with exogenous arachidonic acid significantly increases PGEx production [154]. Therefore, PGEx derived from arachidonic acid was analyzed to determine whether it is authentic PGE_2. Analysis of PGEx using LC-MS/MS determined that it possesses an identical mass and elution time as commercial PGE_2 standard. Further analysis using MS/MS revealed that the fragmentation pattern for the PGEx and PGE_2 standard were identical [155]. This study was indicative of the fact that *C. albicans* can synthesize authentic PGE_2. Hence, *C. albicans* can synthesize both endogenous PGEx and PGE_2 from exogenous archidonic acid. In terms of levels of production, in the presence of arachidonic acid, 99% of PGEx is PGE_2.

Treatment of *C. albicans* with various COX inhibitors decreases fungal prostaglandin production [156]. In vitro studies have also demonstrated that COX inhibitors (aspirin, etodolac, diclofenac, celecoxib, nimesulide, ibuprofen, and meloxicam) inhibit morphogenesis and biofilm formation [157]. In addition aspirin reduces damage to host cells infected with *Candida* by inhibiting extracellular fungal lipases [158]. Unlike mammalian eicosanoid production, little is known about the genes involved in the pathway during prostaglandin formation in fungi. This is due largely to differences between fungal and mammalian enzymes at both the nucleic and amino acid sequence level. It was found that *a*

C. albicans fatty acid desaturase homolog (Ole2) and a multicopper oxidase homolog (Fet3) play roles in prostaglandin production, with mutant strains exhibiting reduced PGE_2 levels compared with parent strains.

CHAPTER 1

Effect of PGEx and PGE_2 on *C. albicans* Interactions with Dendritic Cells in Vitro and In Vivo

ABSTRACT

Candida albicans produces an immunomodulatory oxylipin from arachidonic acid that is structurally identical to host prostaglandin E_2 (PGE_2), called PGEx. In terms of host immune responses, PGE_2 can promote Th2 responses, which are non-protective against fungal infection. Dendritic cells (DCs) are potent antigen presenting cells and reside at mucosal and lymphoid tissues and play an integral role in directing adaptive cell mediated immune responses. We investigated the effect of PGE2 and PGEx on DC maturation and cytokine production at early and late time points in a DC cell line and with murine bone marrow derived DCs. The ability of stimulated DCs to drive adaptive responses against systemic *C. albicans* infection was tested by DC vaccination. In the presence of hyphae, PGE_2 promoted early Th2 cytokine production (2h) as well as increased levels of maturation marker CD86 on the DC cell surface at 18 h. Both PGE_2 and PGEx suppressed Th1 cytokine and production at 18 h. The only notable difference between yeast and hyphae pulsed DCs was in host cell PGE_2 production. Vaccination with yeast-pulsed DCs but not hyphae pulsed DCs lead to protective against systemic infection, with reduced fungal burden in the kidneys. However, treatment with either PGEx or PGE_2 abrogated the ability of yeast pulsed DCs to induce protection. The lack

of protection was associated with inhibition of Th1 cytokines and increased Th2 cytokines in the spleen. Locally, exacerbated Th2 and Th17 cytokines were detected in the kidneys of mice that were not protected against systemic infection. This indicates that host PGE_2 or fungal PGEx can shift adaptive responses in favor of the pathogen and that uncontrolled Th17 responses are detrimental during systemic infection.

INTRODUCTION

Candida albicans poses a significant clinical threat to both immunocompetent and immunocompromised individuals. Chronic colonization at mucosal surfaces with *Candida* is common, with carriage rates among healthy adults ranging from 30-70% [2, 3]. Mechanisms by which Candida persists at mucosal surfaces in the face of an adaptive immune response are relatively unknown. Mucosal persistence can lead to infection, with the severity of Candida-associated diseases ranging from superficial mycoses to systemic candidiasis. Systemic infections are very difficult to treat and account for 40% of crude mortality rate [159].

C. albicans produce immunomodulatory bioactive lipids (oxylipins) that are functionally similar to host eicosanoids, potent regulators of immune responses [156, 160]. *Candida* produces both endogenous oxylipins as well as novel eicosanoid products from exogenous arachidonic acid [156, 161]. A fungal oxylipin was isolated from *Candida* that exhibits cross-reactivity with host PGE_2. This compound, termed PGEx, is bioactive on mammalian cells in vitro similar to PGE_2, indicating the fungal oxylipins can modulate host immune responses [156]. Noverr et. al. reported that host PGE_2 and fungal PGEx enhance *C. albicans* morphogenesis [156, 162]. *Candida* oxylipin production is also upregulated during biofilm formation [157]. Analysis of PGEx using LC-MS/MS determined that it possesses an identical mass and elution time as commercial PGE_2 standard. Further analysis using MS/MS revealed that the fragmentation

pattern for the PGEx and PGE_2 standard were identical [154]. This study was indicative of the fact that *C. albicans* has the potential to synthesize authentic PGE_2. Hence, *C. albicans* can synthesize both endogenous PGEx and PGE_2 from exogenous arachidonic acid. The fact that both the host and yeast can produce similar immunomodulatory signaling molecules suggests that host and pathogen can participate in cross-species communication. Further, oxylipins may enable *Candida* to modulate immune responses in favor of persistence and infection. The role of *Candida* oxylipins in host-pathogen interactions and *Candida* biology is not understood, nor have the biosynthetic pathways been elucidated.

Dendritic cells (DCs) are one of the first types of immune system cells to interact with *C. albicans* at the mucosal surface. The role of dendritic cells is to discriminate between different types of microbes and direct appropriate immune responses, linking innate and adaptive responses. Two types of DCs -myeloid DC (mDC) and plasmacytoid DC (pDC) have been characterized and have distinct origins and functions. Studies have depicted that plasmacytoid DCs ($CD11c^+$ $B220^+$) have been more involved with regulation of immunity and tolerance of induction. On the other hand myeloid DCs ($CD11c^+$ $B220^-$) are involved in the elevation of inflammatory responses [163] . DCs have the ability to distinguish and discriminate between the two different *Candida* morphotypes, i.e., hyphae and yeast form. Coordinated signaling via TLRs results in either pro or anti-inflammatory responses by production of different cytokines which will cause changes in downstream signaling molecules to influence adaptive immune

responses [164]. Recognition of DCs with yeast form of *Candida* results in stimulation of type 1 cytokines (DC1). This type of interaction involves signaling via TLR4 complexes. Hyphal form of *Candida* signals via TLR2 and cause induction of type 2 cytokines (DC2). Studies by Graaf et al demonstrated that when candida-pulsed DCs were injected into the mice, the yeast pulsed DCs caused protective response whereas the mice which were injected with hyphae-pulsed DCs lead to a non-protective response [80]. DCs have the potential to initiate specific T cell responses after interacting with pathogens. In terms of *Candida* infection, a Th1 response results in protection and clearance, while a Th2 response is non-protective, leading to disease [114]. Therefore, depending on the type or nature of fungal morphology, i.e., yeast or hyphal form, the DCs can regulate the T cell immunity caused by *C. albicans* during commensalism or persistent infection, respectively.

PGE_2 is a pleiotropic immune signaling compound and has effects on a variety of cell types, including DCs. Exposure of DCs to PGE_2 leads to inhibition of type I cytokines (TNF-α and IL-12) and promotes release of type II cytokines (IL-10) [36, 37]. Studies have shown that PGE_2 can interfere with DCs maturation and cytokine secretion, events required for migration to T cell areas and subsequent T helper cell education and activation [165-167]. Thus, PGE_2 may play role in promoting a non-protective immune response towards fungal infection. The aim of our studies is to understand the effects of PGE_2 and PGEx on DC maturation and differentiation, and how these effects influence adaptive immune responses during infection.

MATERIALS AND METHODS

Mice. Female retired breeders or 6-8 week old mice of C57BL/6 strain were obtained from Charles River Laboratories (Wilmington, MA). All mice were maintained at an American Association for the Accreditation of Laboratory Animal Care-accredited animal facility at Wayne State University (WSU) and housed in a pathogen-free environment in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals under an animal study proposal approved by the WSU animal investigation committee.

***C. albicans* Strains and Culture Conditions.** The *C. albicans* strain SC5314 used in this study is a prototrophic parental strain [168]. This strain was subcultured from frozen stocks and grown overnight at 30°C in Sabouraud dextrose agar (SDA) (Becton, Dickinson and Company, Sparks, MD). For liquid cultures, isolated colonies from the SDA plates were cultured in Sabouraud dextrose broth (SDB) with constant shaking. To obtain hyphal form, yeast form of *C. albicans* was added to RPMI supplemented with 10% FBS at the concentration of 1 x 10^6 *Candida* per ml of media. Hyphae were generated after 2 h incubation at 37°C.

JAWSII Cell line and Culture conditions. An immature dendritic cell line called JAWSII (ATCC CRL-11904) was used for the purpose. It is a GM-CSF dependent dendritic cell line derived from the bone marrow of C57BL/6 mice

[169-171]. The frozen vial containing the cells were thawed rapidly (approximately 2 minutes) by gentle agitation in a 37°C water bath. After decontaminating the surface of the vial with 70% ethanol, the vial contents were centrifuged at 130xg for 5 to 7 minutes. Following this the pellet was re-suspended with complete RPMI medium (Invitrogen) supplemented with 20% fetal bovine serum and 5 ng/ml murine GM-CSF in a 25 cm^2 tissue culture flask. The cells, upon reaching a confluency of 80-90%, were subcultivated at 1:2 ratio by transferring the floating cells by pipetting. Additionally, the attached cells were removed by rinsing with 0.25% (W/V) Trypsin-0.53mM EDTA solution followed by an incubation period of 5-7 minutes at 37°C incubator. All the cells were pooled and centrifuged at 125 xg for 5 to 10 minutes. After discarding the supernatant, the pellet was resuspended in fresh medium and transferred into a fresh new flask. Following this, the flask was incubated at 37°C incubator in a 5% CO_2 in air atmosphere. Since these are very slowly growing cell line, the medium was renewed at an interval of one time per week.

JAWSII Antigenic Stimulation and Harvest. The JAWS II cells were seeded in six-well plates, each well at the concentration of 5 x 10^6 per well. They were than stimulated with 1μM of PGE_2 (Cayman Chemicals, Ann Arbor). An hour after incubation, the cells were further stimulated with yeast or hyphae in presence and absence of this prostaglandin at a MOI of 1. The control well was stimulated with only PGE_2. After an incubation period of 2 hrs Amphotericin B was added at

a final concentration of 2.5 µg/ml. Further, the cells were trypsinized from the surface using trypsin-EDTA in order to perform RNA extraction from them.

Oligo GEArray DNA Microarray. RNA was extracted from the antigen pulsed JAWSII cells using RNeasy mini kit (Qiagen). A fraction of this RNA sample was run on denaturing agarose gel to verify any possibility of degradation by looking at the 18s and 28s ribosomal bands. A total of 0.1-3 µg RNA was used as a starting material from which cDNA was synthesized using TrueLabeling primer (Superarray Bioscience Corporation). Following this cRNA synthesis, labeling, amplification and purification was performed using manufacturer's provided kit. Further, the biotin-labeled amplified cRNA was hybridized on a prehybridized (the membrane was pre-wet in deionized water followed by replacing it with GEAhyb hybridization solution and rotating the tube in oven for up to 72 hours) array membrane inside a 5 ml disposable screw-top Hybtubes. At least 2 µg of biotin-labeled cRNA was added into the target hybridization mix. The hybridization step was performed overnight inside a roller-bottle hybridization oven at 60°C with continuous but agitation at 5 to 10 rpm. After hybridization steps, a series of washing steps were performed mostly using various concentrations of SSC and SDS solution. After washing, chemiluminescent substrate was added to the hybridization tube and rotated inside hybridization oven allowing the membrane to incubate with the CDP-star for a brief period (2-5min). For image acquisition, a CCD camera was used with an exposure period of 16 minutes and all the images were saved as electronic files in a gray scale in 8 or 16 bit TIFF format.

For data analysis of the arrays, an integrated web-based GEArray expression analysis suite software was used. A two-fold value increase or higher compared to the control was considered as significant increase.

Primer Design. The gene specific primers for mouse IL-4, IL-6, IL-10, IL-12 and GAPDH used for RT qPCR (SABiosciences). These were experimentally verified for their efficiency. The catalog number for IL-4 is PPM03013E, IL-6 is PPM03015A, IL-10 is PPM03017B, IL-12 is PPM03020E and GAPDH is PPM02946E.

qRT-PCR Assay. qRT-PCR assay was performed using a MyiQ thermocycler (Bio-Rad, Hercules, CA, USA). Each well contained a 25-µl reaction mixture that included 12.5µL of 2× SYBR Green PCR Master Mix (Bio-Rad,), 1µl of gene specific 10µM each of forward and reverse primers, 1µL of DNA template at convenient dilution. The following thermocycling pattern was used: 95 °C for 10 min (to activate the hot start DNA polymerase; 95 °C for 15 s, 60 °C for 1 min (40 cycles). A melting-curve analysis was performed at the end of each PCR assay to control that a single PCR-product was amplified. qRT-PCR was carried out on appropriate DNA dilutions in triplicate. For normalization, the mRNA for mouse GAPDH was quantified in parallel for all other samples. For data analysis, difference between the critical threshold Ct values (ΔCt) for each cytokine gene and the housekeeping gene was calculated. The ΔΔCt was obtained from the difference in the ΔCt values between the experimental and

control genes. The fold change in C_T between the cytokine gene expression from the antigen pulsed dendritic cells and the change over unstimulated dendritic cells was determined using the $2^{-\Delta\Delta Ct}$ method [172].

Purification and Culture of Bone Marrow Dendritic Cells. Bone marrow cells were obtained by flushing the femurs of the mice with RPMI supplemented with 10% FBS (Invitrogen). The cells were then incubated with RBC lysis buffer for 2 min followed by adding D-PBS. Following this, the cells were centrifuged at 1000 rpm and resuspended in complete RPMI (cRPMI) containing 5 mM HEPES buffer, 0.3 mg/ml L-glutamine, 5.5 X 10^{-5} mol/L ß-mercaptoethanol, 100 U/ml penicillin, 100 ug/ml streptomycin and 10% FBS.

For obtaining the plasmacytoid DCs (pDCs), the cells were resuspended at the concentration of 10^6 cells/ml in complete medium supplemented with 200 ng/ml recombinant murine Fms-related tyrosine kinase-3 ligand (FLT-3) (Prospec-Tany Tecnogene Ltd, Rehovot, Israel). After incubation of 9 days, the cells were collected by removing the adherent and non-adherent cells by vigorous pipetting with room temperature D-PBS [173].

For generating the myeloid or conventional type of dendritic cells, the bone marrow cells were resuspended in complete RPMI at concentration of 1 X 10^6 cells/ ml and stored in 37°C, 5% CO_2 incubator. On day 1, the non-adherent cells were removed and resuspended at concentration of 5 x 10^5 / ml with RPMI containing 20 ng/ml GM-CSF (Prospec-Tany Technogene Ltd, Rehovot, Israel) and 20 ng/ml IL-4 (Shenandoah Biotechnology). Additional media and 40 ng/ml

of each cytokines were added on day 3. On day 6, the cells were harvested, pelleted and again resuspnded in 30 ml of cRPMI supplemented with 10 ng/ml GM-CSF and 10 ng/ml IL-4. On day 8, the non-adherent cells were harvested and used in experiments [174]

Flow Cytometry. For phenotypic analysis of DC subsets, surface markers were analyzed using flow cytometry. The DC were washed three times in 200 µl cold FACS buffer containing1% BSA / PBS, pH 7.2, 0.1% NaN_3. For purpose of blocking Fcγ receptors, the cells were incubated in FACS buffer containing 5% mice serum for 15 minutes at 4°C. For staining, cell were washed three times and resuspended at a concentration of 1×10^6 cells/ml in cold FACS buffer. Cells were stained for 30 min at 4°C in dark. Cells were stained with following FITC or PE conjugated anti-mouse antibodies according to the manufacturer's instructions: CD86 (maturation marker), CD11b, CD11c, and B220 (ebioscience). Myeloid DCs are $CD11b^+$ $CD11c^+$ and plasmacytoid DCs are $B220^+$ $CD11c^{lo}$ $CD11b^-$. Cells were washed twice with FA buffer and were then fixed with 4% formalin. FITC- and PE-conjugated antibodies used for direct staining obtained from ebiosciences are listed in

Table 1. A minimum of 20,000 events was acquired for each sample with a FACScan flow cytometer (BD) and analysed using Flowjo software.

Isolation of fungal PGEx. *C. albicans* strain SC5314 was grown overnight at 37°C in 10 ml RPMI containing 100 mM arachidonic acid. Culture supernatants

were loaded onto a PGE_2 affinity column (Caymen Chemical), washed, and eluted according to the manufacturers' instructions. The eluates were dried under a stream of nitrogen gas and resuspended in 500 µl PBS. PGE_x concentrations (approximately 20ng) determined using a PGE_2 monoclonal ELISA (Cayman Chemical, Ann Arbor).

Pulsing of DCs and culture of cells. DCs were harvested and washed twice in sterile 1x PBS, counted, and resuspended in a final concentration of $1x10^6$ cells/ml. Cells were plated into 6 well tissue culture treated plates in a volume of $6x10^6$ cells/6ml/well. For pulsing, DC were exposed to unopsonized yeast or hyphae at a DC:fungal cell ratio of 1:2. PGE_2 (Caymen Chemical) or *C. albicans* PGEx was added at a final concentration of 2nM. After 2 h incubation, 2.5 □g/ml amphotericin B was added to prevent fungal overgrowth. After pulsing, cells were left for an additional 18 h in culture. Supernatants were harvested for analysis of immune mediators. Cells were washed and harvested for adoptive transfer experiments.

Enzyme Linked Immunosorbent Assay. Cell culture supernatants (DCs and splenocyotes) were analyzed for the following cytokines by ELISA: IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IFN-γ, TGF-β, TNF-α, IL-23. The detection limits (pg/ml) for the assays were <3.90 for IL-4, < 3.90 for IL-5, <3.90 for IL-6, < 31.25 for IL-10, <15.62 for IL-12, <3.90 for IL-13, < 15.62 for IFN-γ, <62.5 for TGF-β, <7.81 for TNF-α and <7.81 for IL-23 (eBioscience). In addition, DC supernantants were

also tested to analyze the levels of PGE_2 (Cayman chemicals). The absorbance of the plates was measured at 450nm using Multiskan Ex microplate reader (Thermo Electron Corporation) and analyzed with Ascent Software.

Adoptive Immunization and Fungal Challenge. Pulsed and unpulsed BMDCs were trypsinized and washed with 1x sterile PBS pH 7.4 at 1000 rpm. They were counted using a hemocytometer and intraperitoneally injected into the mice at a concentration of 5 X 10^5 cells per mouse in 100 µl of sterile non-pyrogenic PBS (Invitrogen) using a 27-gauge feeding needle attached to a 1-ml syringe. Mice received either a single vaccination at day 0 or a double vaccination at days 0 and 7. Four mice were used for adoptive transfer per each group. 7 d following the last adoptive transfer of BMDCs, the mice were challenged by intravenous tail injection with *C. albicans* at the concentration of 2.5 X 10^5 CFU/mouse in 250 µl of sterile PBS using a 30-gauge feeding needle attached to a 1-ml syringe. Upon assessment of weight analysis for the purpose of determining any incidence of cachexia, there was no significant decrease or change in weights of the mice post systemic challenge with *Candida* for a period of 7 days.

CFU Assay. To determine the amount of fungal growth in the organs of the mice, the kidney and the liver were weighed and homogenized using a tissue tearor. Serial dilutions of tissue homogenates were made in sterile water and

were plated onto sabouraud dextrose agar to determine the CFU per gram per organ in each group of mice.

Splenocyte Harvest and Pulsing. Spleens were excised from the *C. albicans* infected mice and the splenocytes were isolated from them. A 1:1 ratio of both yeast and hyphal forms of *C. albicans* were used at MOI 1:2 to stimulate the splenocytes. After 2 h of incubation, 2.5 µg/ml of amphotericin B was added to prevent *Candida* overgrowth followed by further incubation of 16 h at 37°C, 5% CO_2 incubator. The supernatants were collected and the levels of cytokines were measured by ELISA.

Histological Analysis and Immunohistochemistry. Part of the organs (kidney and liver) were excised and stored immediately in 10% buffered formalin for the purpose of histology. Paraffin-embedded tissues of about 3-4 nm width were stained with periodic acid-Schiff reagent and examined [149]. 5µm sections were cut from paraffin-embedded kidney tissue blocks. Antibodies used along with their respective companies purchased from and the dilution used are listed in Table 2. For heat mediated antigen retrieval, sections were treated with for 20 min with 10mM sodium citrate buffer, 0.05% Tween 20, pH6.0. Incubation with primary antibody was performed overnight at 4°C whereas incubation with the biotin conjugated secondary antibody was performed for 30 min at room temperature. To verify staining specificity, negative control slides or appropriate isotype controls antibodies and at the same dilution as that of respective primary

monoclonal antibodies were used. These isotype controls used were rat IgG, IgM, IgG2b and $IgG_{2a.}$ Samples were analyzed by light microscopy using Olympus BX41 microscope and Qcolor3 software tool.

Statistical Analysis. The Student's *t* test (two-tailed, unequal variance) was used to analyze the significance of differences between two experimental groups. Significance was considered with a $P \leq 0.05$ or less. The data recorded are representative of either two or three independent experiments.

	Name of Antibody	Clone	Dilution
1.	Anti-mouse CD11b	RMMG-1	1:150
2.	Anti-mouse CD86 (B7.2)	GL1	1:160
3.	Anti-mouse CD45R (B220)	RA3-6B2	1:200
4.	Anti-mouse CD11c	N418	1:100

Table 1. List of antibodies and dilutions used for flow cytometry. All antibodies were purchased from eBioscience.

	Name of Antibody	Dilution	Company
1.	IFN-γ (clone RMMG-1)	1:400	Abcam Inc., Cambridge, MA
2.	IL-4 (clone 11B11)	1:100	Abcam Inc., Cambridge, MA
3.	IL-6 (clone)	1:400	Abcam Inc., Cambridge, MA
4.	IL-10 (clone NYRmIL-10)	1:200	Santa Cruz Biotechnology, Inc
5.	IL-12A p35 (clone M-19)	1:200	Santa Cruz Biotechnology, Inc
6.	IL-17 (clone 400210)	1:150	R & D Systems, Inc.
7.	IL-23 (polyclonal)	1:200	Santa Cruz Biotechnology, Inc
8.	Neutrophil (clone NIMP-R14)	1:100	Santa Cruz Biotechnology, Inc

Table 2. List of antibodies and dilutions used for immunohistochemistry.

RESULTS

To determine the effect of prostaglandins on antigenic stimulation of DCs, OligoGEarray was performed to examine early (2h) expression of cytokine genes. Through a simple technique of side-by-side hybridization method, differential expression of the genes between the samples was compared. This oligoarray profiles the expression of 113 genes, including cytokines in the Th1, Th2, Th3 pathways, and other CD4+ T lymphocytes markers and transcriptional factors that regulate the expression of these cytokines. There were also other functional genes involved in immune cell activation, in the Th1 and Th2 type immune responses, antimicrobial humoral responses, etc. We attempted to analyze early cytokine expression in primary bone marrow derived DCs; however, the amounts of cells necessary to obtain high quality RNA precluded using these cells. To obtain enough RNA to perform the oligoarray analysis, we used an immature mouse dendritic cell line (JAWSII), which is a GM-CSF dependent bone marrow derived cell line from C57BL/6. Th2 cytokines IL-4, IL-10, and IL-13 were consistently upregulated in the presence of hyphae and PGE_2. In addition, pro-inflammatory cytokines IL-6 and IL-15 were upregulated in the presence of hyphae and PGE_2 (Table 3). On the other hand, a suppressor of cytokine synthesis gene (SOCS-3) which was used as a control was upregulated in the only PGE_2 treated group. The confirmed the metabolic activity of this compound during the in vitro studies. We were further able to confirm the up-regulation of IL-4, IL-6, and IL-10 genes showed by the oligoarray technique

using qRT-PCR (Figure 1). We observed no up-regulation of IL-12, which indicates that up-regulated genes are not due to errors in RNA concentrations.

	E_2	Y	Y + E_2	H	H + E_2
Th2 Cytokines					
IL-4	0.41	0.37	2.25	1.07	**3.86**
IL-10	0.67	0.64	**4.02**	1.05	**9.18**
IL-13	0.73	0.73	1.08	0.95	**3.03**
Pro-inflammatory Cytokines					
IL-6	1.68	1.09	1.5	1.8	**4.43**
IL-15	0.37	0.31	1.2	0.56	**3.03**
Cytokine Suppressors					
Socs3	**11.11**	2.6	1.1	1.1	1.3

Table 3. Analysis of early cytokine expression in a DC cell line. JAWSII cells were at a concentration of 5 x 10^6 per well and stimulated with PGE^2 for 1 h followed by pulsing with *C. albicans* yeast or hyphae at an MOI of 2. After 2 h, RNA was extracted. Transcript levels were analyzed using a OligoGEarray kit and analyzed using GEArray expression analysis suite software. A two-fold value increase or higher compared to the control is depicted in the table.

	pDC		mDC	
	Untreated	PGEx, PGE_2	Untreated	PGEx, PGE_2
Th1				
IL-12	++++	↓	+	↓
Pro-Inflammatory				
IL-6	Similar	↓	Similar	n/c
Th-17				
IL-23	+	n/c	+++	↓
Th2				
IL-4	Similar		Similar	
IL-10	+	n/c	+/-	↓

Table 4. Relative expression of cytokines after stimulation of dendritic cells with yeast or hyphae in presence or absence of prostaglandins.

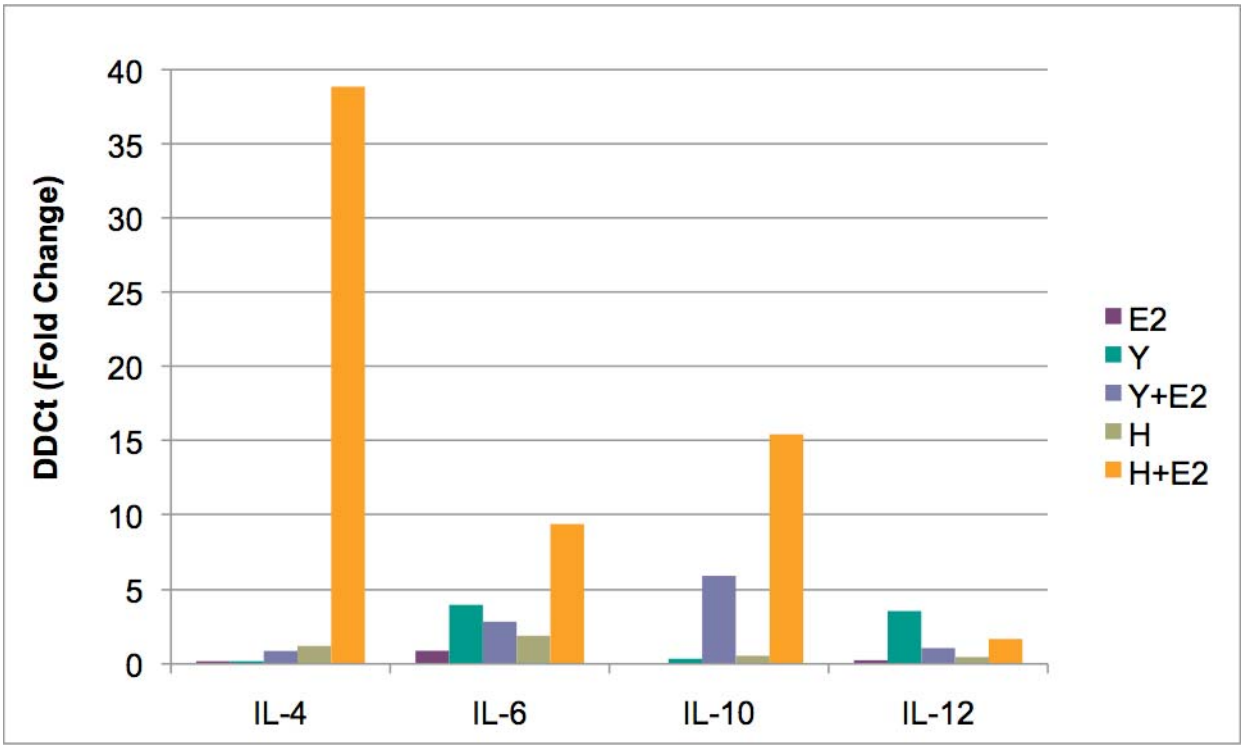


Figure 1. Analysis of early cytokines by quantitative real-time PCR in JAWSII dendritic cell line. JAWSII cells were at a concentration of 5 x 10^6 per well and stimulated with PGE_2 for 1 h followed by pulsing with *C. albicans* yeast or hyphae at an MOI of 2. After 2 h, RNA was extracted and pooled together for each sample group. qRT-PCR was carried out on appropriate DNA dilution in triplicate. For normalization, the mRNA for mouse GAPDH was quantified in parallel for all other samples. For data analysis, difference between the Ct values (ΔCt) for each cytokine gene and the housekeeping gene was calculated. The ΔΔCt was obtained from the difference in the ΔCt values between the experimental and control genes. The fold change in critical threshold (*CT*) between the cytokine gene expression from the antigen pulsed dendritic cells and the change over unstimulated dendritic cells was determined using the $2^{-\Delta\Delta Ct}$ method Data is representative of two independent experiments performed in triplicate.

To confirm the results from studies with the DC cell line extend to primary DC subsets, bone marrow derived plasmacytoid and myeloid type DCs were used in vitro studies. There are several methods of generating DCs from bone marrow in vitro. Culturing cells in the presence of GM-CSF generates myeloid or inflammatory DCs, while FLT3-ligand preferentially expands plasmacytoid or steady state DC populations [175-177]. Both types of DCs were characterized for cell surface marker expression by performing FACS analysis as previously described [178]. GM-CSF differentiated DCs (GM-DCs) were 86.3% $CD11c^{+}$ $B220^{-}$ and 81.32% $CD11b^{+}$ $CD11c^{+}$ indicative of a myeloid DC phenotype (2a and b). On the other hand, Flt-3L differentiated DCs (FL-DCs) were $CD11c^{low}$ (57.7%) compared with GM-DCs (Figure 2c). FL-DCs showed increased expression of B220, with 45.7% were double positive for CD11c and B220, which is indicative of a plasmacytoid DCs phenotype (Figure 2d) [175]. Most of the remaining cells were $CD11c^{+}B220^{-}$. This is to be expected as FLT3-ligand generates a heterogeneous population of DCs similar to steady state splenic DCs. Regardless, FL-DCs are functionally similar to plasmacytoid DCs in experimental models [79, 179]. Therefore, we will refer to GM-DCs as mDCs and FL-DCs as pDCs.

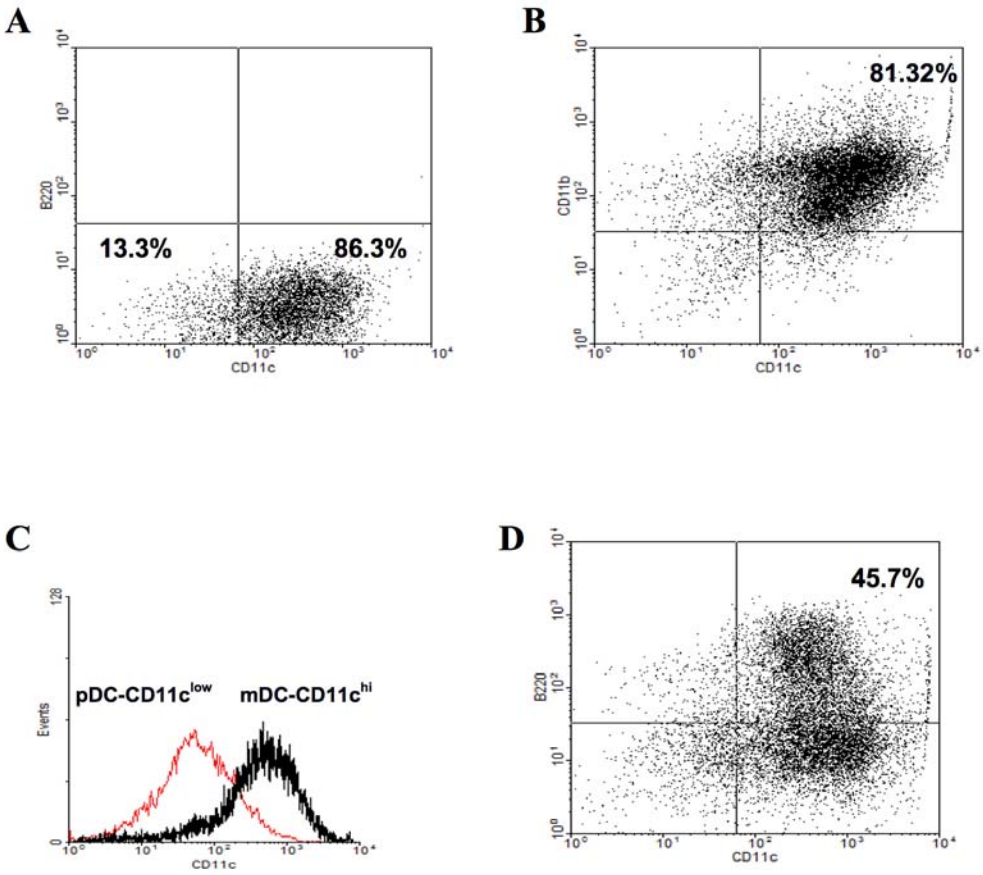


Figure 2. Characterization of bone marrow derived dendritic cells. Murine bone marrow was harvested from C57BL/6 mice and cultured in the presence of GM-CSF (mDCs) or FLT3-ligand (pDCs). A,B) Surface expression of B220, CD11c, and CD11b on mDCs. C) Relative surface expression of CD11c on pDCs (pDC-CD11clow) and mDCs (mDC-CD11chi). D) Surface epression of CD11c and B220 on pDCs. The above data is representative of two independent repeats of FACS analysis.

To investigate the effect of *C. albicans* and prostglandins to induce maturation of mDCs and pDCs in vitro, we analyzed CD86 expression, which is upregulated during antigen presentation by dendritic cells. In unpulsed mDCs 16.3% of cells were $CD11c^{+}CD86^{+}$ and 13.3% of pDCs were $B220^{+}CD86^{+}$ (Figure 3). Upon stimulation of mDCs with *Candida*, CD86 expression increased to 18.9% in the presence of yeast and to 21.42% in the presence of hyphae (Figure 3a). Upregulation of CD86 was more dramatic with pDCs, with increases from 13.3% to 36.44% with yeast and 46.8% for hyphae (Figure 3b). Addition of PGE_2 further increased CD86 expression in both DC types in the presence of both yeast and hyphae. These results demonstrate exposure of mDCs and pDCs to *C. albicans* and PGE_2 induces maturation, with the greatest effects observed in the presence of hyphae and PGE_2.

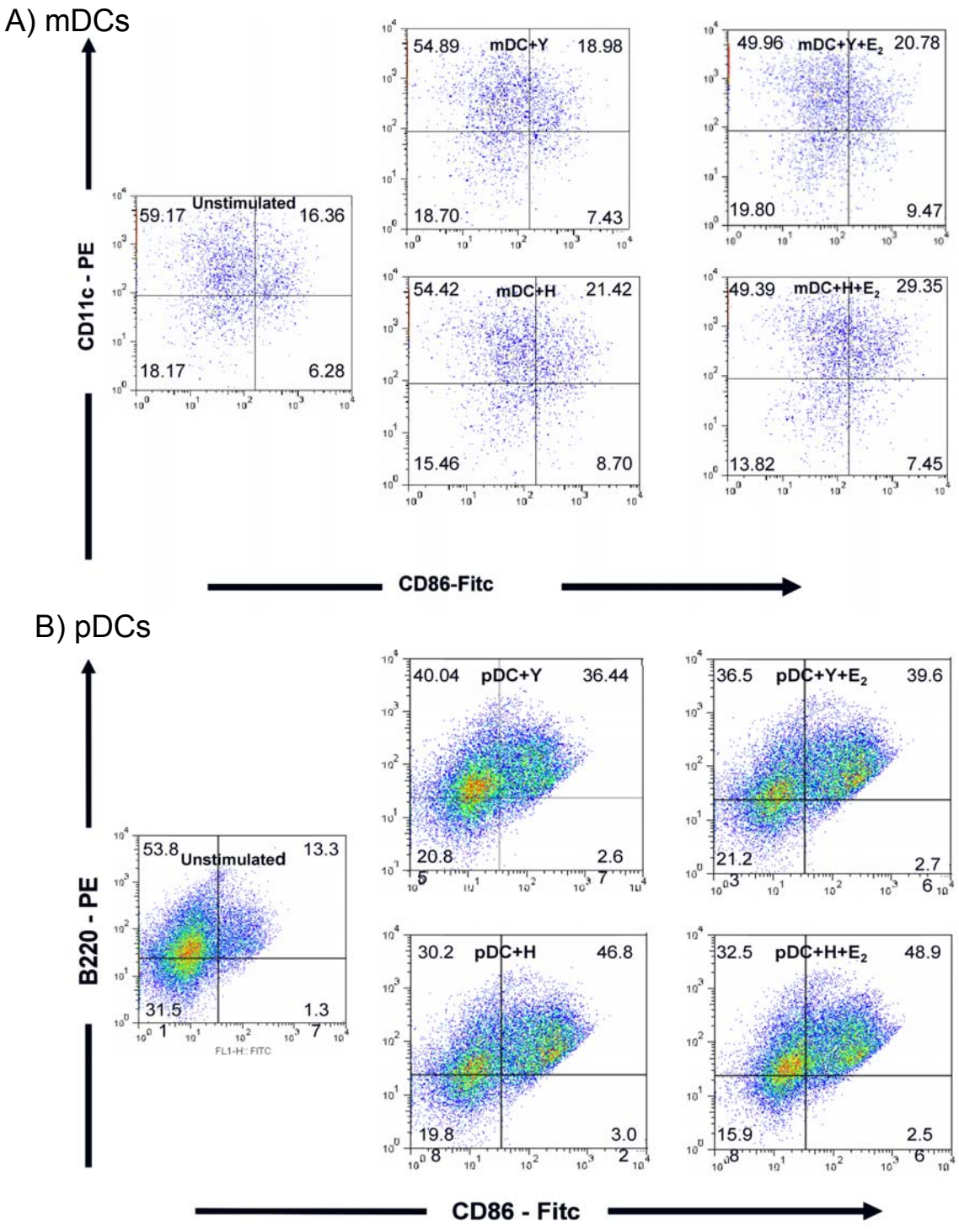


Figure 3. Maturation marker expression on mDCs and pDCs stimulated with *C. albicans* and PGE_2. Murine bone marrow was harvested from C57BL/6 mice and cultured in the presence of GM-CSF (mDCs) or FLT3-ligand (pDCs).

Cells were stimulated with *C .albicans* strain SC5314 yeast (grown in SDB at 30°C) or hyphae (formed in 100% FBS at 37°C for 2h) at MOI of 2 in presence or absence of 2 nM PGE2. After 2 h incubation, *Candida* was killed by adding 2.5 µg/ml of amphotericin B to the culture. Cells were harvested after 18 h and stained with A) CD11c and CD86 for mDCs and B) B220 and CD86 for pDCs. The isotype controls tested negative. The experiment was performed in duplicate with two independent repeats with similar results.

To analyze effects of PGE_2 on early cytokine production in primary DCs, we used qRT-PCR to measure changes in cytokines identified from the oligoarray performed with the JAWSII cell line. Compared with yeast pulsed DCs, hyphal pulsed DCs also tended to have higher IL-4 and lower IL-12 expression, indicative of a shift towards a Th2 phenotype (Figure 4). Similar to what was observed previously with JAWSII cells, treatment of hyphal pulsed DCs with PGE_2 resulted in the highest upregulation of IL-4 and IL-6, with concomitant decreases in IL-12. Similar results were obtained with both mDCs and pDCs. This indicates the PGE_2 treatment can further promote pro-Th2 or pro-Th17 responses from DCs pulsed with hyphae.

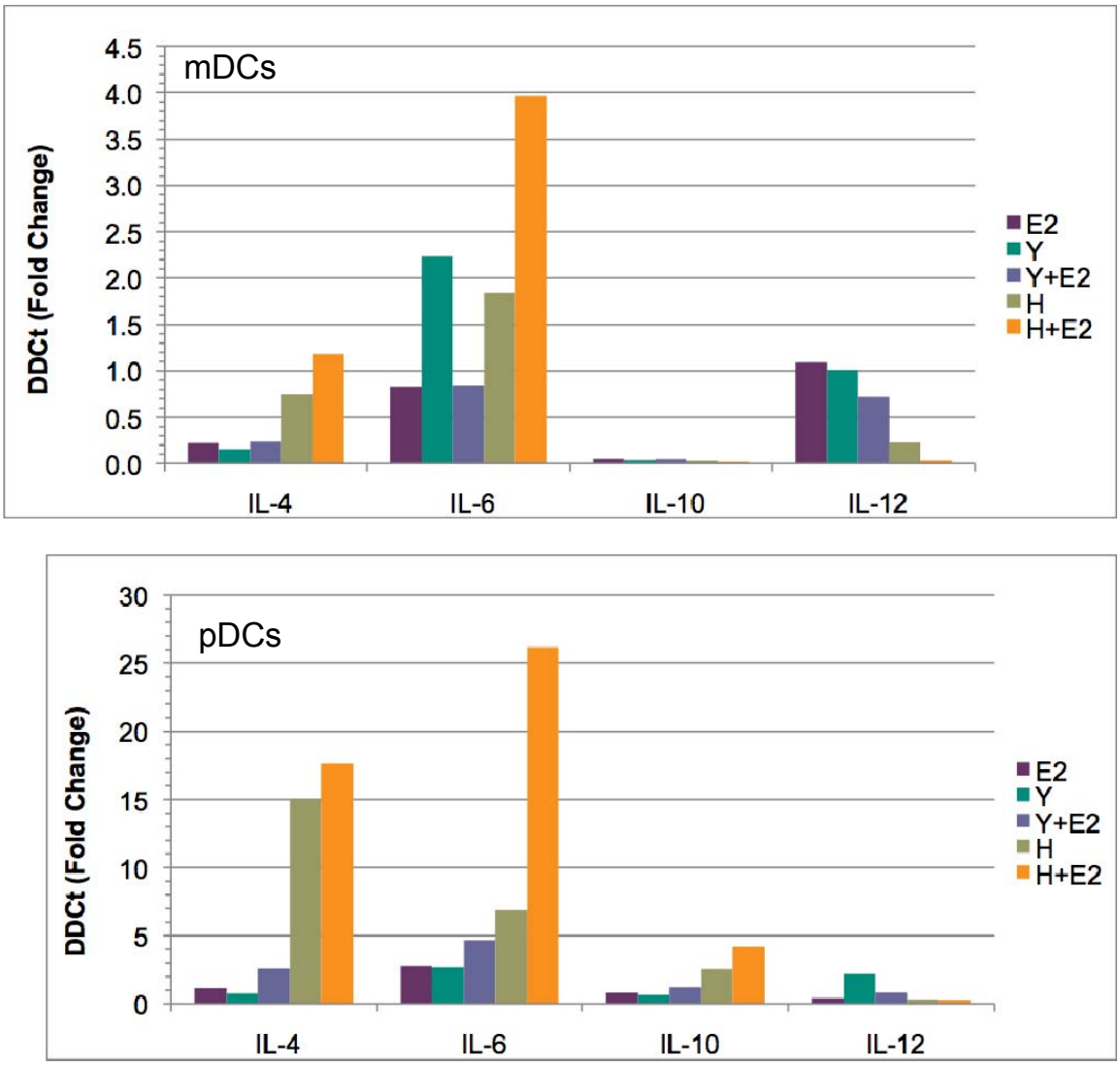


Figure 4. Analysis of early cytokines by quantitative real-time PCR in mDCs and pDCS. Murine bone marrow was harvested from C57BL/6 mice and cultured in the presence of GM-CSF (mDCs) or FLT3-ligand (pDCs). Cells were stimulated with *C .albicans* strain SC5314 yeast or hyphae at MOI of 2 in presence or absence of 2 nM PGE2 or PGEx. After 2 h, RNA was extracted and pooled together for each sample group. qRT-PCR was carried out on appropriate DNA dilution in triplicate. For normalization, the mRNA for mouse GAPDH was quantified in parallel for all other samples. For data analysis, difference between the Ct values (ΔCt) for each cytokine gene and the housekeeping gene was calculated. The ΔΔCt was obtained from the difference in the ΔCt values between the experimental and control genes. The fold change in critical threshold (*CT*) between the cytokine gene expression from the antigen pulsed dendritic cells and the change over unstimulated dendritic cells was determined using the $2^{-\Delta\Delta Ct}$ method. Data is representative of two independent experiments performed in triplicate.

Cytokine analysis of DC culture supernatants at a later time point (18 h) was performed by ELISA to monitor phenotypic differentiation of both mDCs and pDCs after antigenic stimulation (Figure 5). As reported previously, pDCs secreted higher levels of the Th1 cytokine, IL-12, compared to mDCs [79]. Addition of either PGE_2 or PGEx decreased the secretion of this cytokine in both DC subsets. Similarly, a trend towards decreased production of pro-inflammatory cytokine IL-6 was also observed in the presence of prostaglandins. The Th2 cytokine IL-4 showed similar cytokine secretion pattern from both types of dendritic cells, which was slightly increased in presence of prostaglandin. However, IL-10 was inhibited by prostaglandins in mDCs but slightly increased with pDCs. Increased levels of the Th17 cytokine, IL-23, was observed in mDCs as previously reported, which was inhibited in presence of prostaglandins [79]. The comparative summarization of cytokine induction pattern is listed in Table 3. Overall, the data indicate that prostaglandins dampen Th1 cytokines, which could result in generating a non-protective Th2 phenotype.

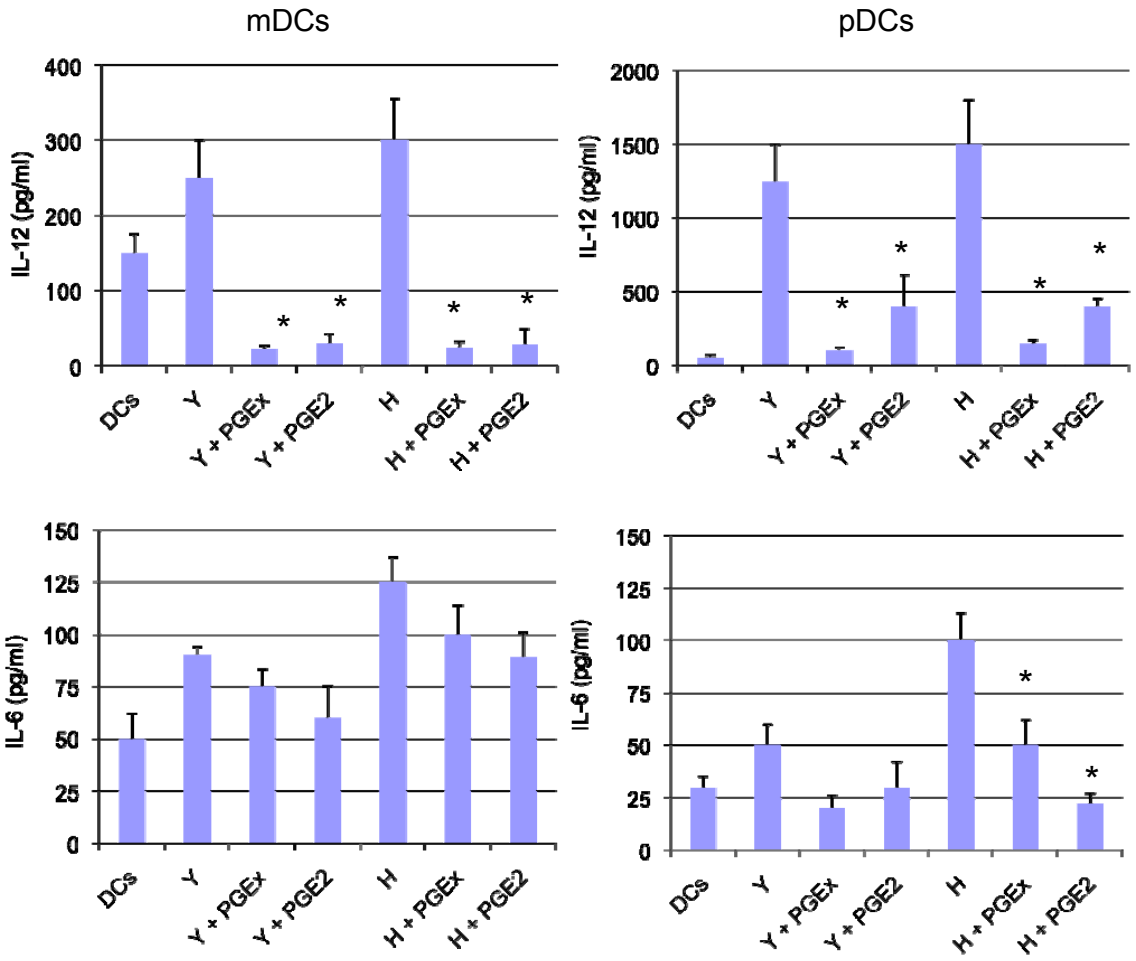
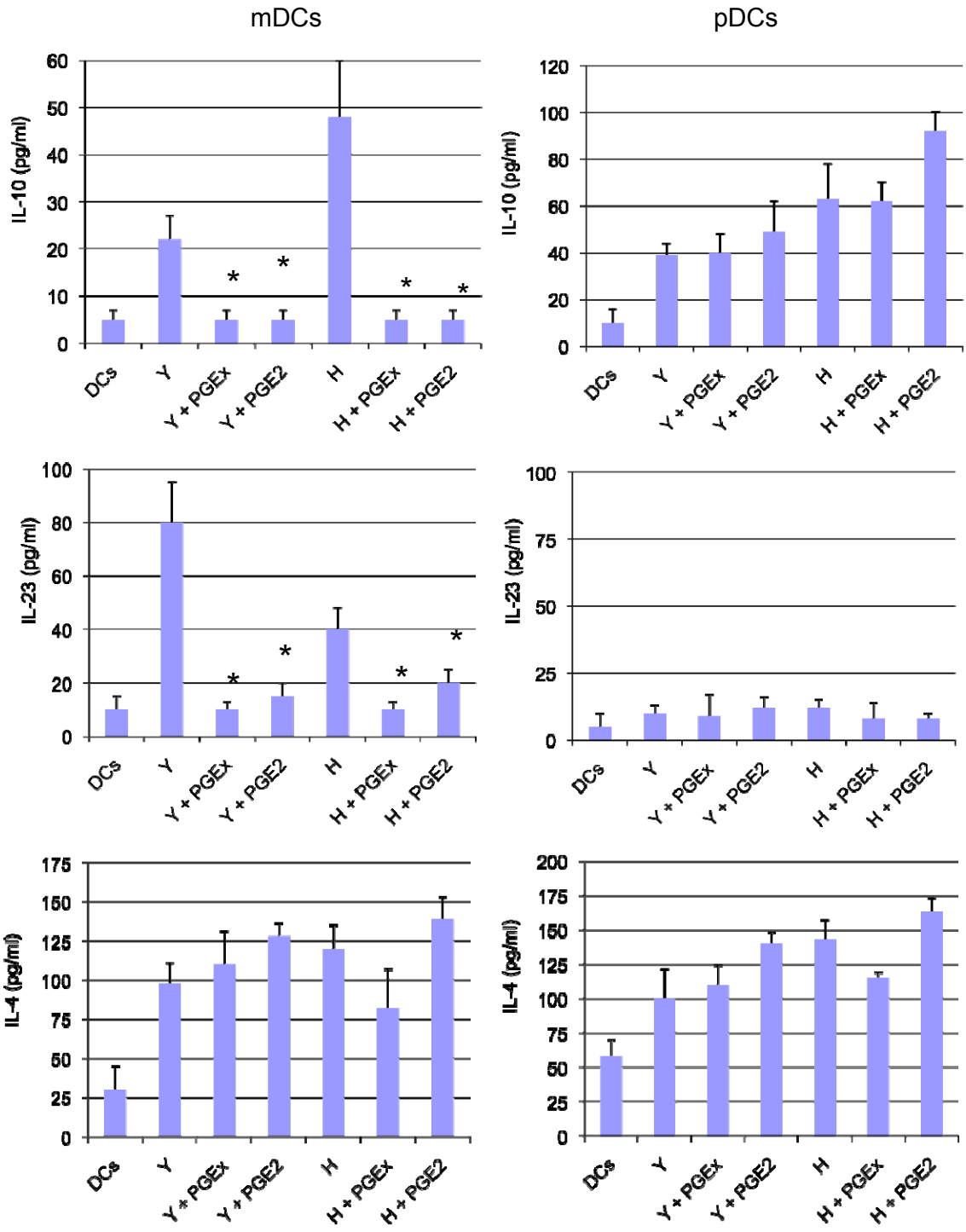


Figure 5. Analysis of late cytokine production by ELISA in mDCs and pDCs. Murine bone marrow was harvested from C57BL/6 mice and cultured in the presence of GM-CSF (mDCs) or FLT3-ligand (pDCs). Cells were stimulated with *C .albicans* strain SC5314 yeast (grown in SDB at 30°C) or hyphae (formed in 100% FBS at 37°C for 2h) at MOI of 2 in presence or absence of 2 nM PGE_2 or PGEx. After 2 h incubation, *Candida* was killed by adding 2.5 µg/ml of amphotericin B to the culture. Cultured supernatants were harvested after 18 h incubation and further used to assay for cytokines by ELISA. Data is representative of three independent experiments performed in triplicates and assayed in duplicate. * $P < 0.05$, prostaglandin treated vs. untreated cells.

Figure 5 continued



	pDC		mDC	
	Untreated	PGEx, PGE_2	Untreated	PGEx, PGE_2
Th1				
IL-12	++++	↓	+	↓
Pro-Inflammatory				
IL-6	Similar	↓	Similar	n/c
Th-17				
IL-23	+	n/c	+++	↓
Th2				
IL-4	Similar	↑	Similar	↑
IL-10	+	n/c	+/-	↓

Table 5. Relative expression of cytokines after stimulation of dendritic cells with yeast or hyphae in presence or absence of prostaglandins.

To determine the effects of *C. albicans* on prostaglandin levels in the DC cultures, we tested the levels of PGE_2 from the culture supernatants. PGE_2 levels were relatively low and did not significantly differ in any of the antigenic groups at 2 h post-pulse (Figure 6). Because *C. albicans* was viable during this period, PGE_2 could be derived from *C. albicans* or the DCs. At 18 h post-chase we observed increased production of PGE_2 from the hyphae pulsed group in both mDCs and pDCs (Figure 6). This PGE_2 is not produced from *Candida* as they were killed after 2h with amphotericin B treatment. These data indicate that hyphae induce significant PGE_2 production from DCs.

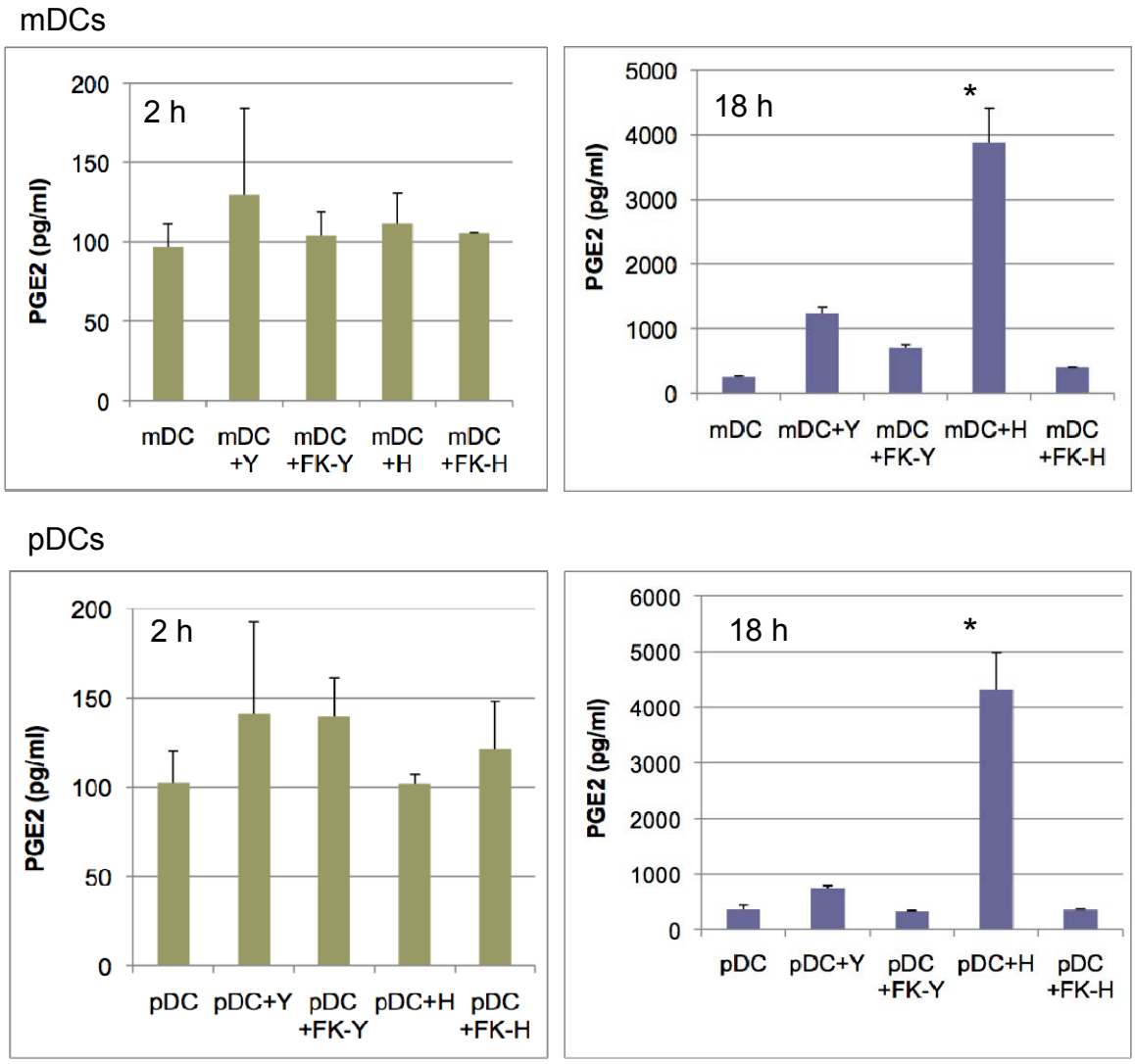


Figure 6. Production of PGE_2 from stimulated mDCs and pDCs. Murine bone marrow was harvested from C57BL/6 mice and cultured in the presence of GM-CSF (mDCs) or FLT3-ligand (pDCs). Cells were stimulated with *C .albicans* strain SC5314 yeast (grown in SDB at 30°C) or hyphae (formed in 100% FBS at 37°C for 2h) at MOI of 2. Supernatant was harvested at 2 or 18 h post-pulse. For the 18 h time point, after 2 h incubation, *Candida* was killed by adding 2.5 µg/ml of amphotericin B to the culture. PGE2 levels were measured using a monoclonal PGE_2 EIA kit. Experiments were performed in triplicate and assayed in duplicate and data is representative of two independent repeats. * $P < 0.05$, prostaglandin pulsed vs. unpulsed cells.

To further investigate the functional activities of the antigen stimulated DCs in vivo, adoptive transfer experiments were performed. In initial experiments, mice received a single adoptive transfer 7 d prior to systemic challenge with *C. albicans* infection (Figure 7). To assess protection against infection, kidney, blood, spleen and liver fungal burden was analyzed, which are the major target organ during systemic infection. In mice vaccinated with antigen pulsed mDCs, no protection was observed after adoptive transfer with antigen pulsed cells (Figure 8a). However, the yeast pulsed pDC-vaccinated mice showed close to a two-fold decrease in kidney fungal burden compared to the other groups (Figure 8b). Interestingly, PGE_2 or PGEx treatment abrogated protective effect of yeast pulsed pDCs during vaccination (Figure 8b). This indicates that mDCs do not impart the same type of protective response as pDCs. Cytokines from restimulated splenic cultures were analyzed to characterize the immune response in vaccinated mice. We observed increased IL-6 production in the mice that received yeast-pulsed pDCs and decreased levels in all other groups (Figure 8). While this indicated that a protective pro-inflammatory response may be elicited in yeast pulsed pDC mice, the splenic response was relatively weak. No significant differences in IL-12, TNF-α, IL-4, IL-10, or TGF-β were observed, with very low levels produced (data not shown).

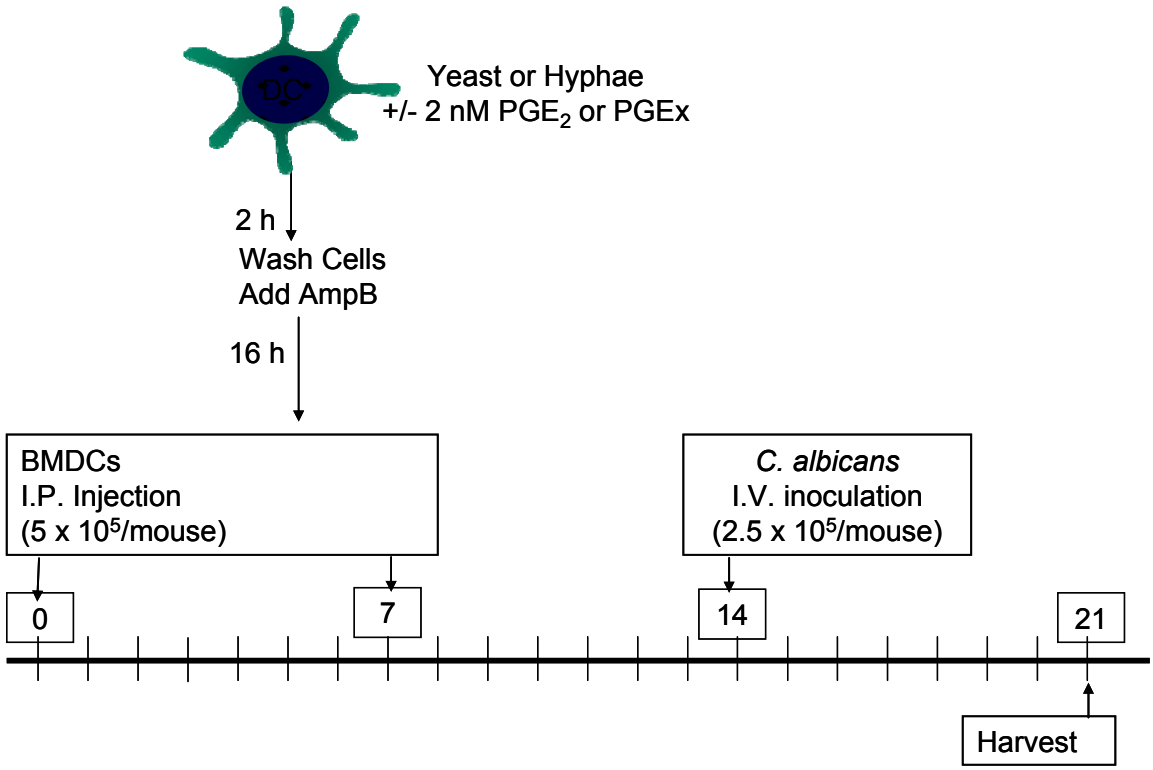


Figure 7. Dendritic cell vaccination or adptive transfer strategy. The antigen pulsed dendritic cells were interperiteonally injected into the mice at a concentration of $5X10^5$/mouse on day 1. On day 7, this adoptive transfer with the pulsed dendritic cells was repeated again. On day 14, the mice were challenges with systemic infection by injecting them with $2.5X10^5$ Candida through tail vein injection. On day 21, the organs (spleen, liver, kidneys, blood) were harvested from accessing protection.

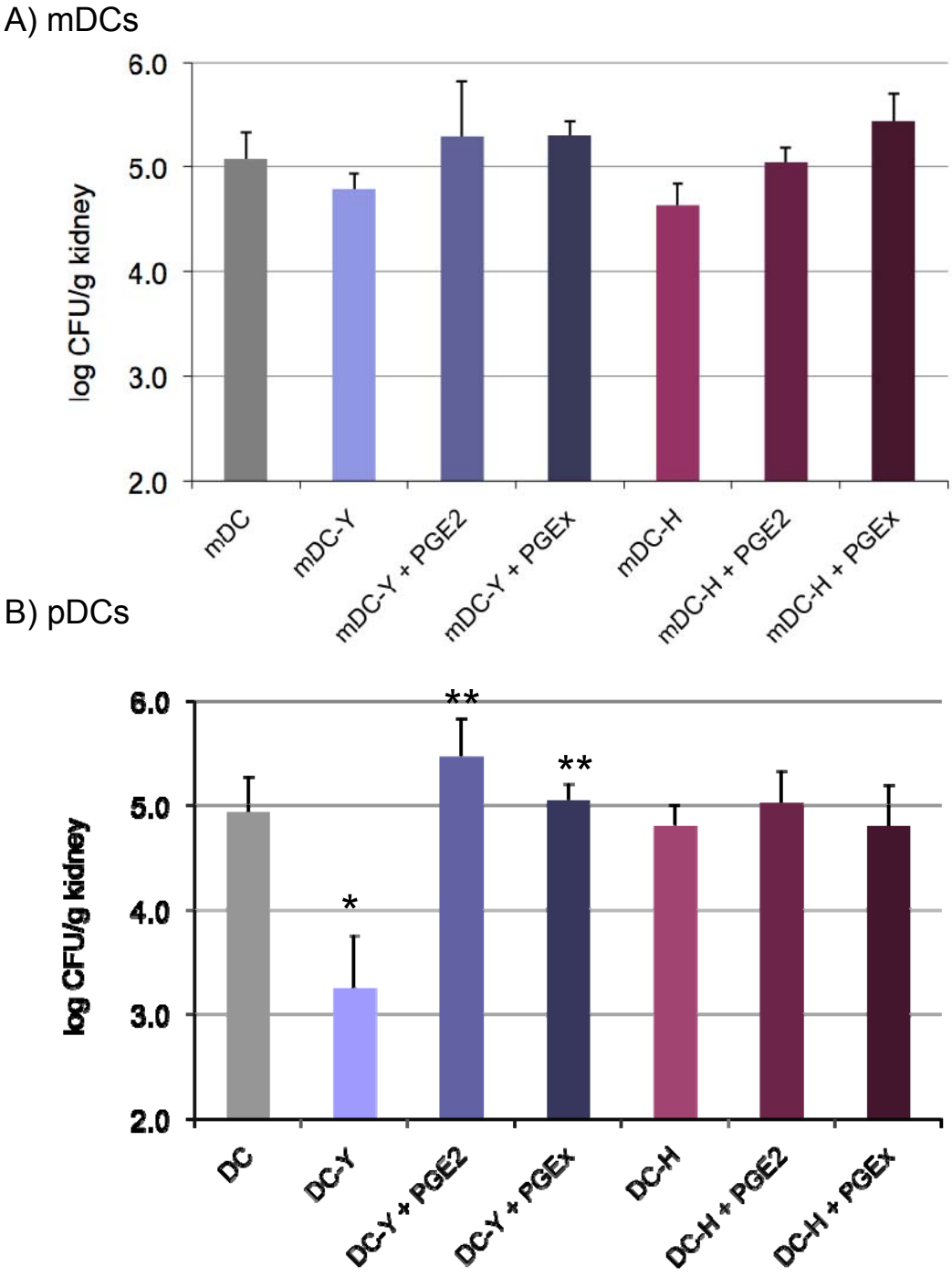


Figure 8. Assessment of protection from *C. albicans* infection following single adoptive transfer with mDCs or pDCs. Antigen pulsed A) mDCs or B) pDCs were harvested after 18h of stimulation. For vaccination, $5x10^5$ cells were

injected via the intraperitonial route in 6-8 week old C57BL/6 mice. After 7 d, mice were inoculated by tail vein injection with *C. albicans* strain SC5314 ($2.5x10^5$ CFU/mouse). After 7 days post-inoculation, the mice were sacrificed, the kidneys excised, homogenized and serial dilution was performed to measure fungal burden. For each group, 4 mice were used. This experiment was repeated four times with similar results. DC = Dendritic cells; Y = Yeast; H = Hyphae. * $P < 0.05$ DC alone vs. experiment groups. ** $P < 0.05$ treated vs. untreated DCs.

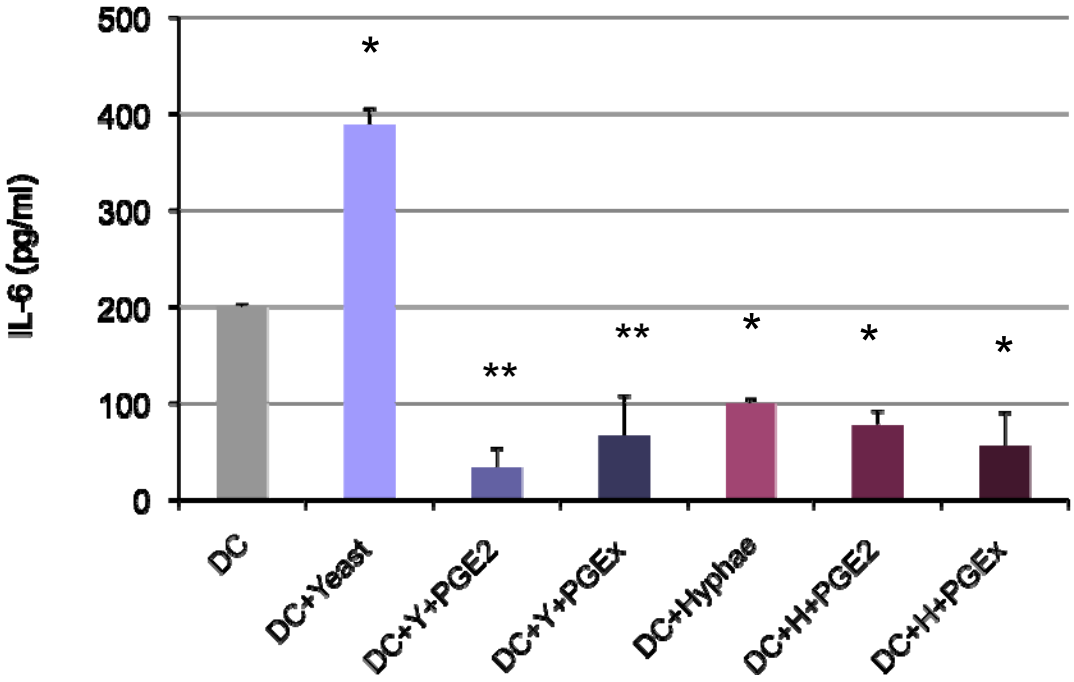


Figure 9. Secretion of cytokines from splenic cultures of mice vaccinated with antigen pulsed pDCs. Spleens were excised from the mice systemically challenged with *C. albicans* following adoptive transfer with pulsed pDCs. Yeast and hyphal forms of *C. albicans* were used in 1:1 ratio to stimulate the splenocytes. After 2h of incubation, 2.5 µg/ml of amphotericin B was added to prevent *Candida* overgrowth followed by further incubation of 16 h. Cytokines in the culture supernatants were analyzed by ELISA. * $P < 0.05$ DC alone vs. experiment groups (pulsed with yeast or hyphae). ** $P < 0.05$ treated (pulsed with PGEx/PGE_2) vs. untreated DCs (pulsed with yeast or hyphae).

To boost the immune response in vaccinated mice, a second adoptive transfer was introduced followed by a systemic challenge. In mice vaccinated with mDCs, yeast-pulsed, but not hyphae-pulsed DCs induced protective responses against systemic infection (Figure 10). While there was a modest decrease in kidney CFUs with yeast-pulsed DCs compared to unpulsed DCs, exposure to PGE_2 or PGE_2 in the DC culture abrogated this effect. Interestingly, mice receiving hyphae-pulsed DCs had increased fungal burden, which was further exacerbated with prostaglandin treatment (Figure 8). This indicated that along with exposure to PGE_2, morphotype specific responses shift the outcome of infection as reported previously [115]. Analysis of splenic Th1 and Th2 cytokines showed that mice receiving prostaglandin treated DCs produced less IL-12 compared with untreated DCs (Figure 11). In addition there was a trend towards increased IL-4 and IL-5 production with hyphae-pulsed DCs, which was further increased with prostaglandin treatment (Figure 11). There were no significant differences with IFN-γ, TNF-α, IL-10, or TGF-β production (Figure 10 and data not shown). Immunohistochemistry analysis showed increases in PMN staining in kidneys of groups that were not protected against systemic infection (Figure 12a). There was also increased IL-4 and decreased IL-12 production in prostaglandin treated or hyphae pulsed groups compared with the mice receiving DCs pulsed with yeast (Figure 12b and d). Overall, however, there was exacerbated Th17 cytokines in all groups of mice (Figure 12f and g).

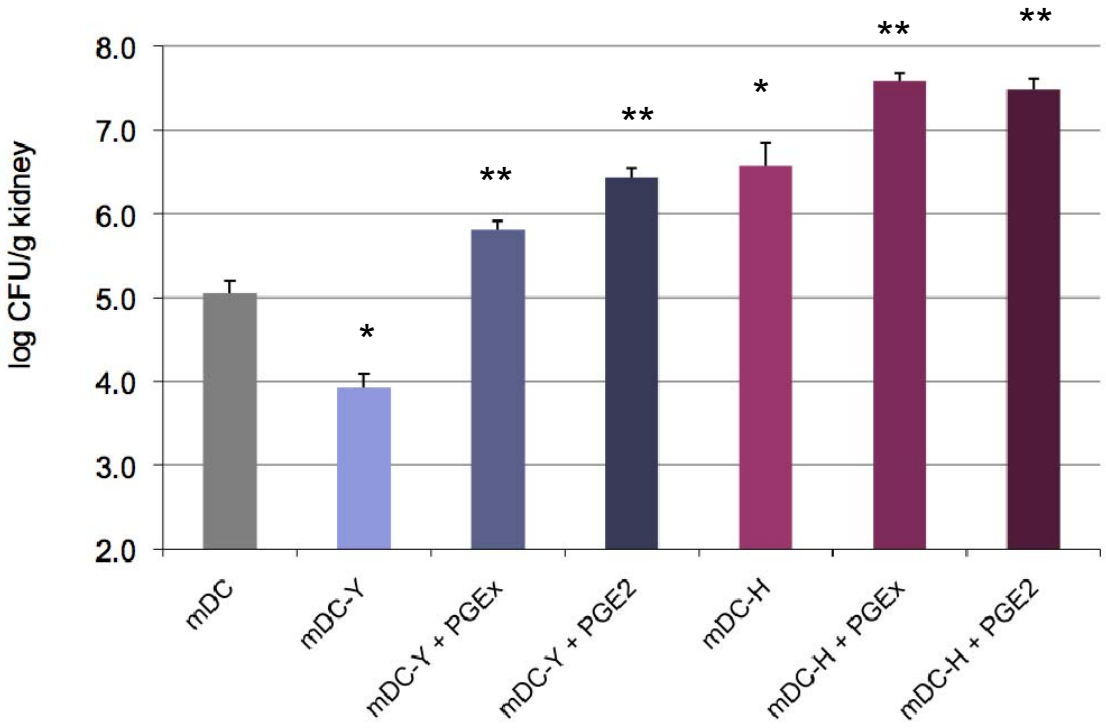


Figure 10. Assessment of protection from *C. albicans* infection following double adoptive transfer with mDCs. Antigen pulsed mDCs were harvested after 18h of stimulation. For vaccination, $5x10^5$ cells were injected via the intraperitonial route in 6-8 week old C57BL/6 mice on day 0 and day 7. Mice were inoculated 7 d after the second vaccination by tail vein injection with *C. albicans* strain SC5314 ($2.5x10^5$ CFU/mouse). After 7 days post-inoculation, the mice were sacrificed, the kidneys excised, homogenized and serial dilution was performed to measure fungal burden. For each group, 4 mice were used. This experiment was repeated four times with similar results. DC = Dendritic cells (plasmacytoid); Y = Yeast cells; H = Hyphae. * $P < 0.05$ DC alone vs. experiment groups (pulsed with yeast or hyphae). ** $P < 0.05$ treated (pulsed with PGEx/PGE_2) vs. untreated DCs (pulsed with yeast or hyphae).

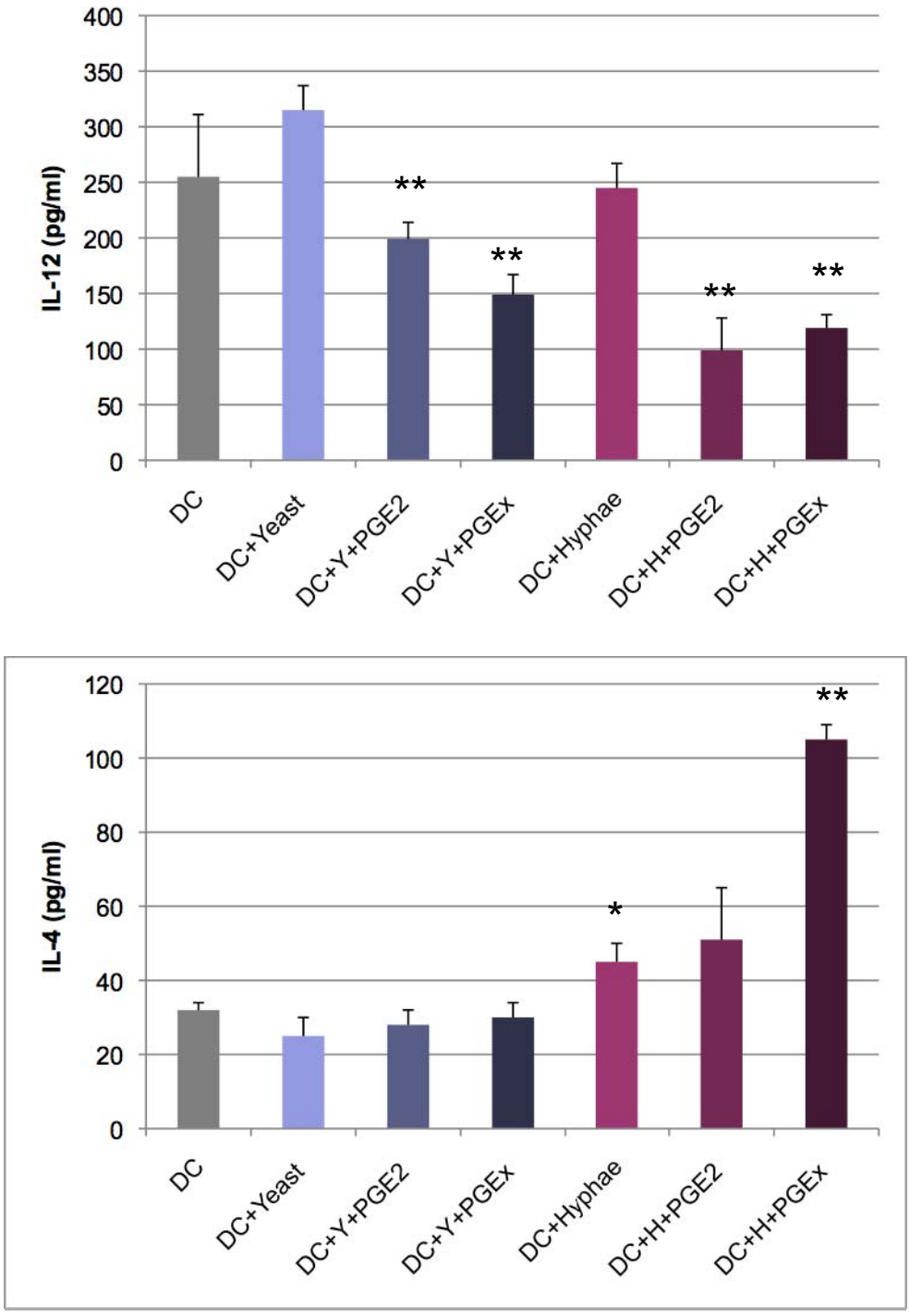
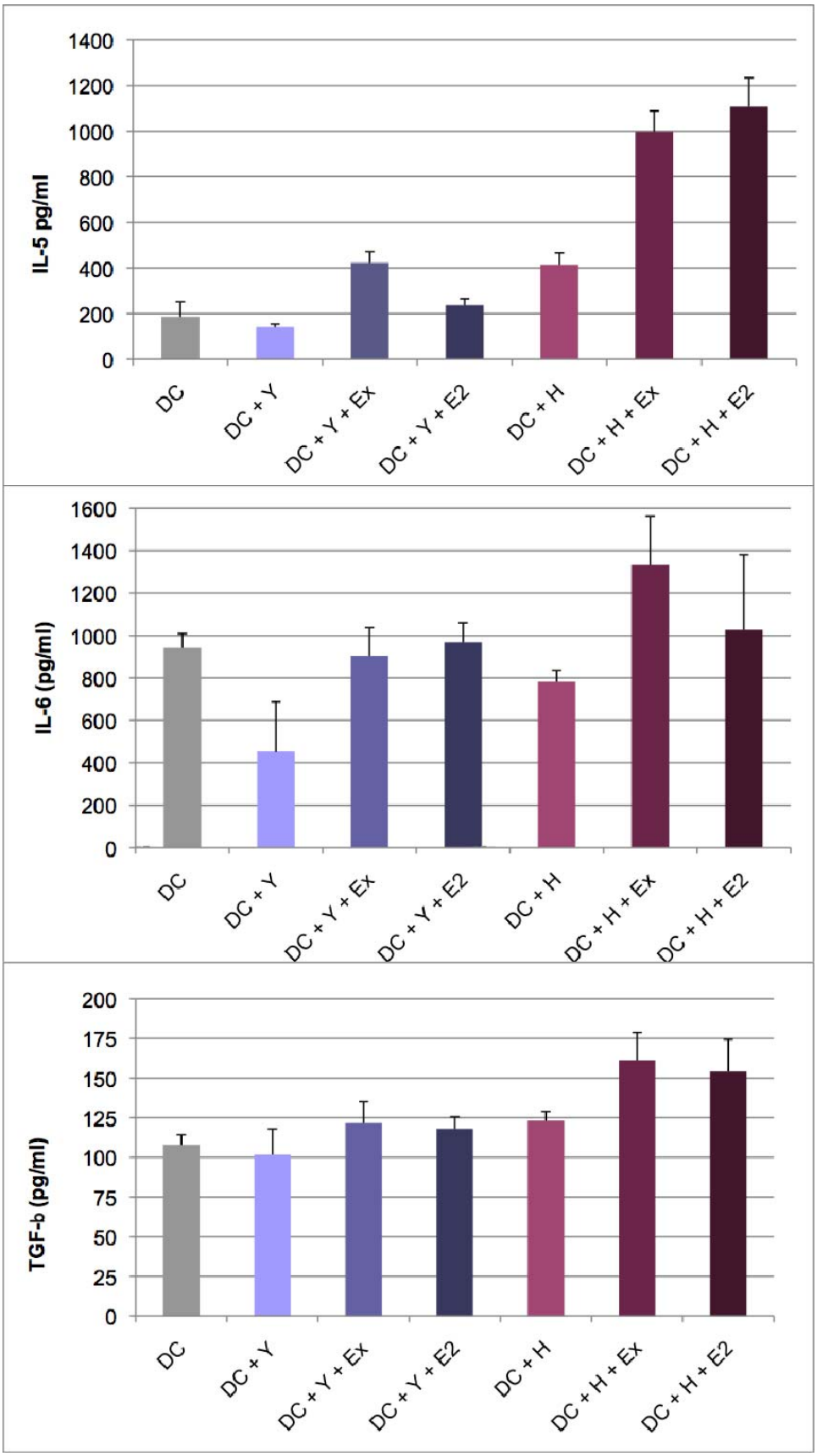


Figure 11. Secretion of cytokines from splenic cultures of mice receiving double vaccination with antigen pulsed mDCs. Spleens were excised from the mice systemically challenged with *C. albicans* following two adoptive transfers with pulsed pDCs. Yeast and hyphal forms of *C. albicans* were used in 1:1 ratio to stimulate the splenocytes. After 2h of incubation, 2.5 µg/ml of amphotericin B was added to prevent *Candida* overgrowth followed by further

incubation of 16 h. Cytokines in the culture supernatants were analyzed by ELISA. * $P < 0.05$ DC alone vs. experiment groups (pulsed with yeast or hyphae). ** $P < 0.05$ treated (pulsed with PGEx/PGE_2) vs. untreated DCs (pulsed with yeast or hyphae).

Figure 10 continued.



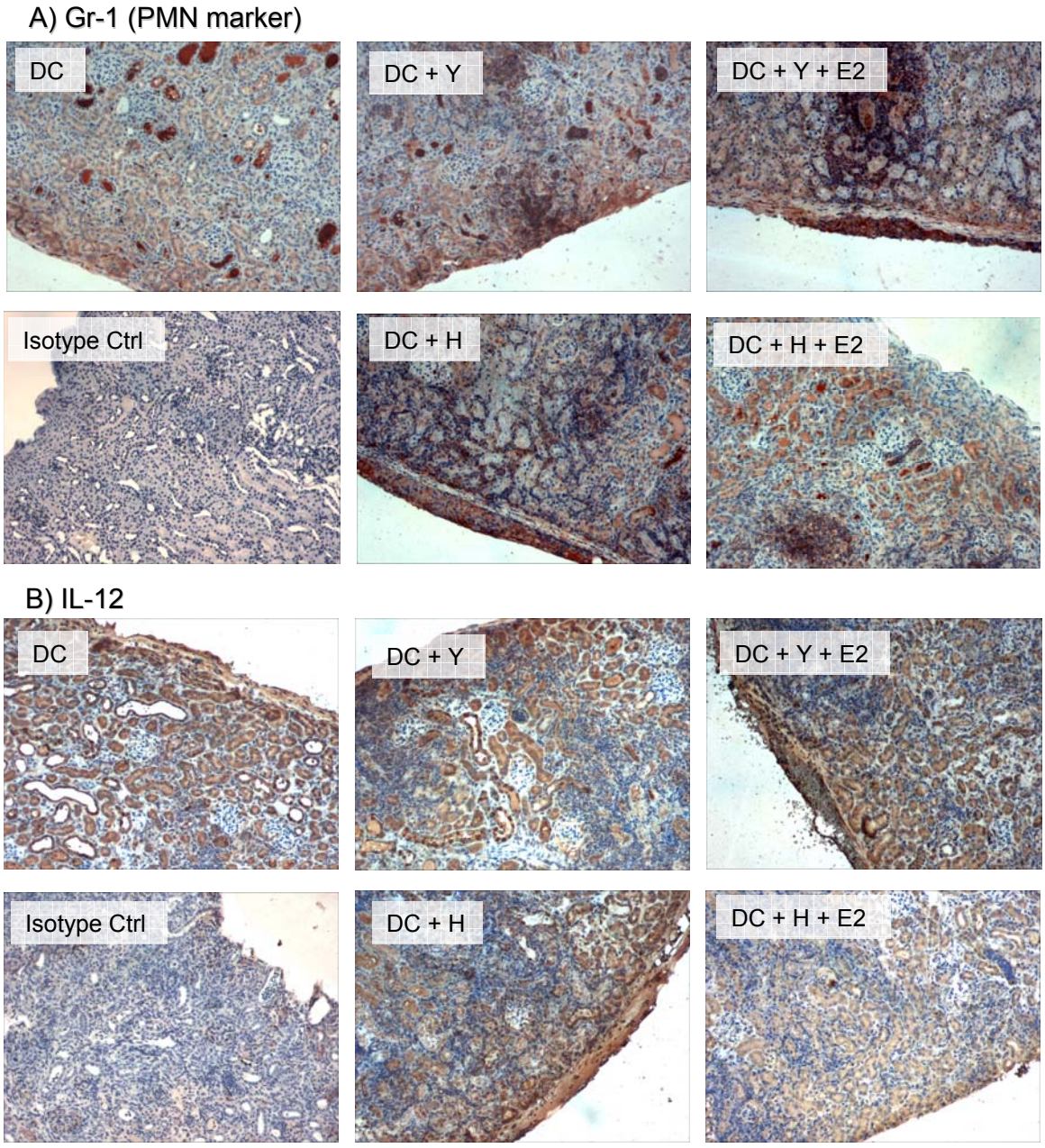
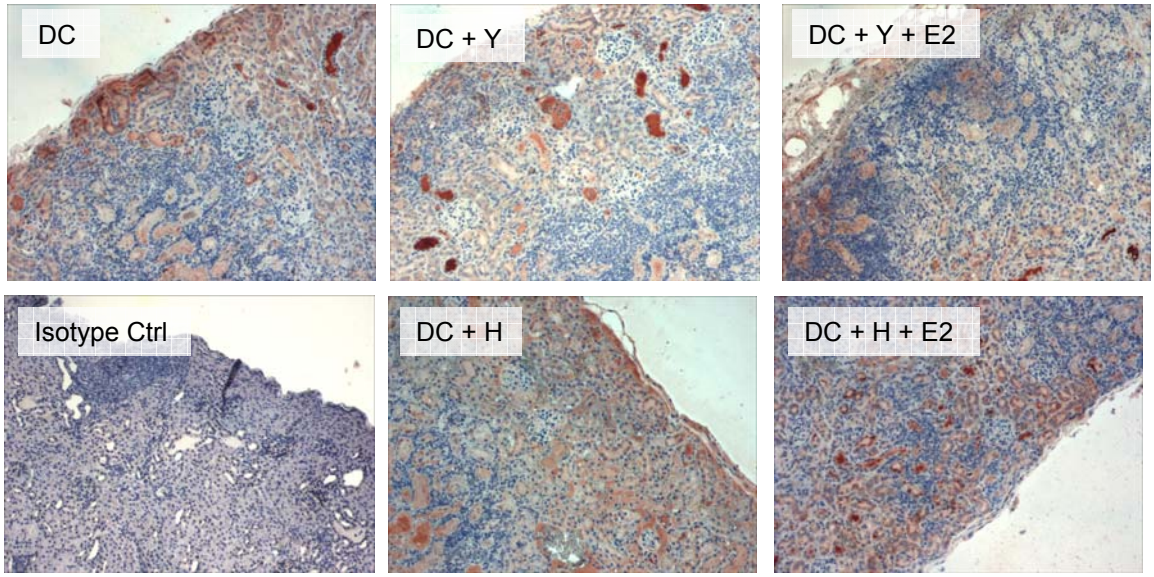


Figure 12. Immunohistochemistry analysis kidneys of mice receiving double vaccination with antigen pulsed mDCs. Kidneys were excised from mice systemically challenged with *C. albicans* following two adoptive transfers with antigen pulsed pDCs. Tissues were fixed in 10% formalin and processed for immunohistochemistry. 5µm sections were cut from paraffin-embedded kidney tissue blocks. Antibodies used along with their respective companies purchased from and the dilution used are listed in

Table **2**. For heat mediated antigen retrieval, sections were treated with for 20 min with 10mM sodium citrate buffer, 0.05% Tween 20, pH6.0. Incubation with primary antibody was performed overnight at 4°C whereas incubation with the biotin conjugated secondary antibody was performed for 30 min at room temperature. To verify staining specificity, negative control slides or appropriate isotype controls antibodies and at the same dilution as that of respective primary monoclonal antibodies were used. These isotype controls used were rat IgG, IgM, IgG2b and $IgG_{2a.}$ Samples were analyzed by light microscopy using Olympus BX41 microscope and Qcolor3 software tool.

Figure 12 Continued

C) IFN-γ



D) IL-4

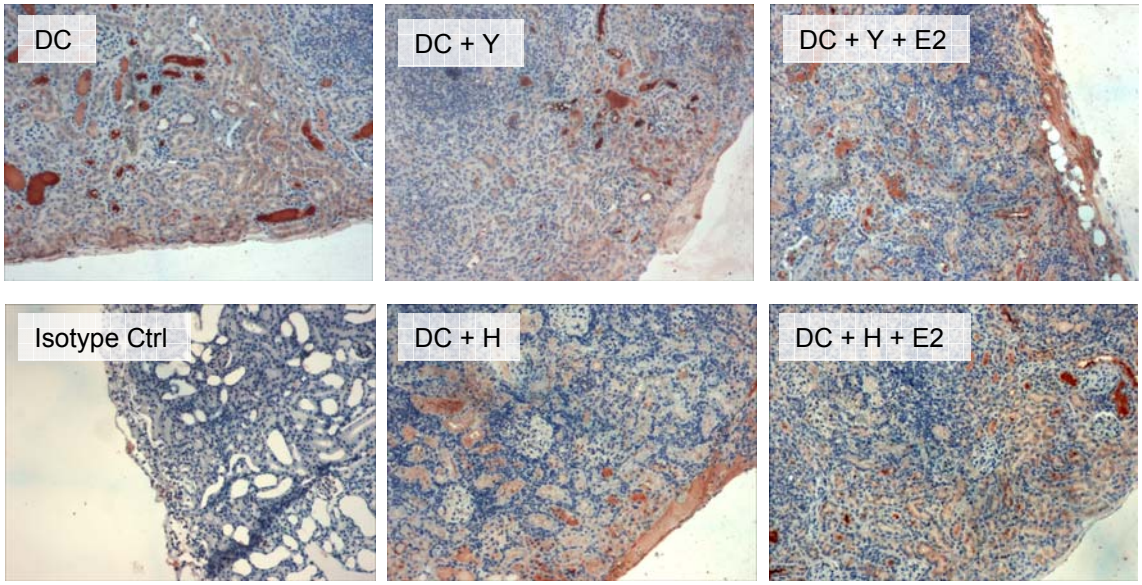


Figure 12 Continued

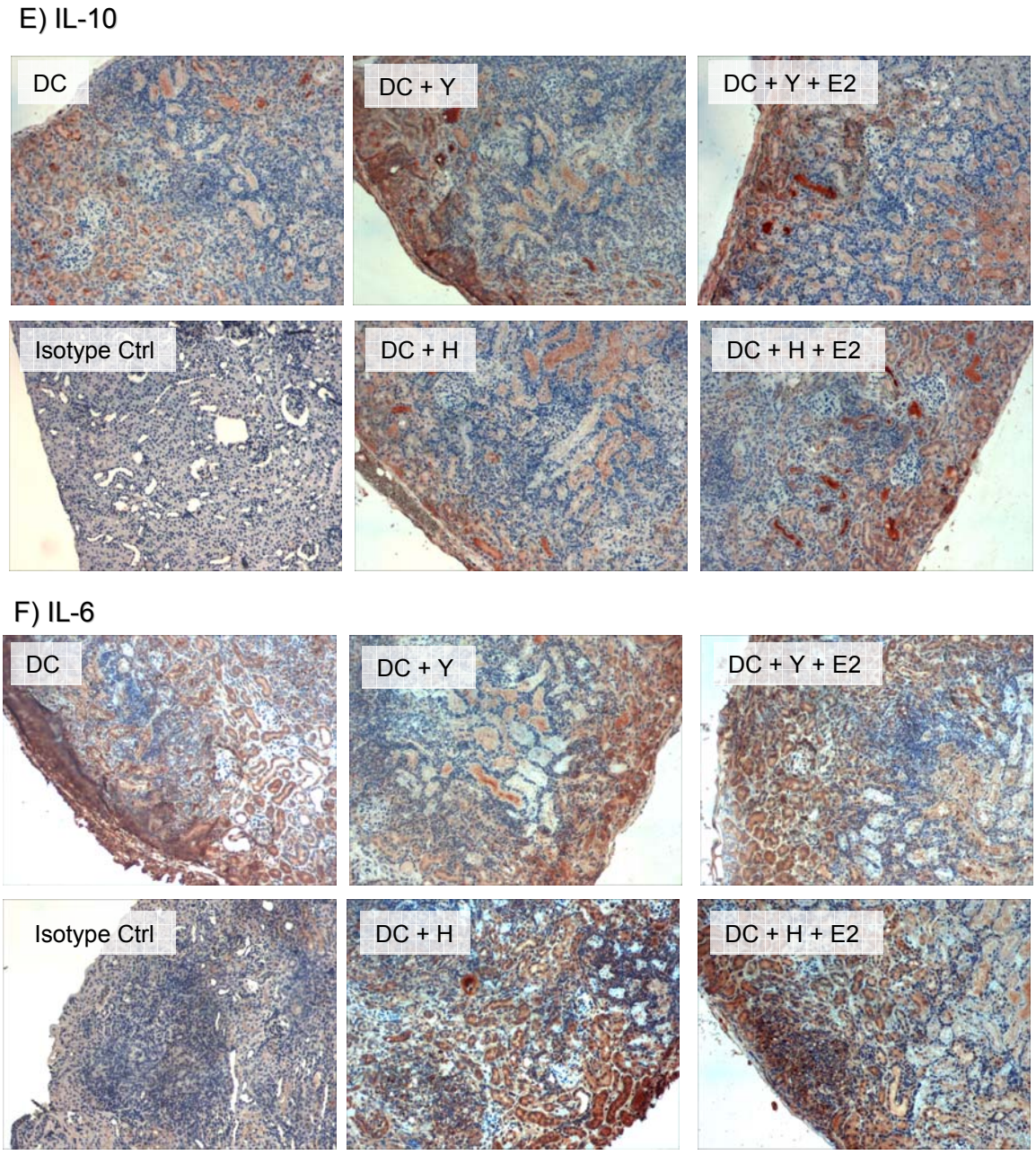
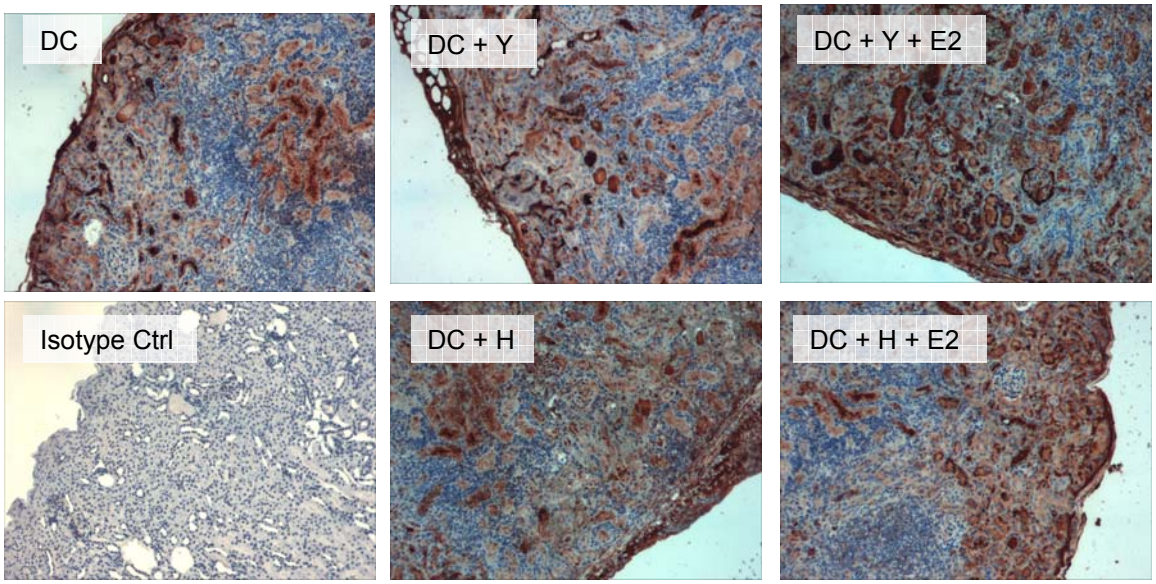
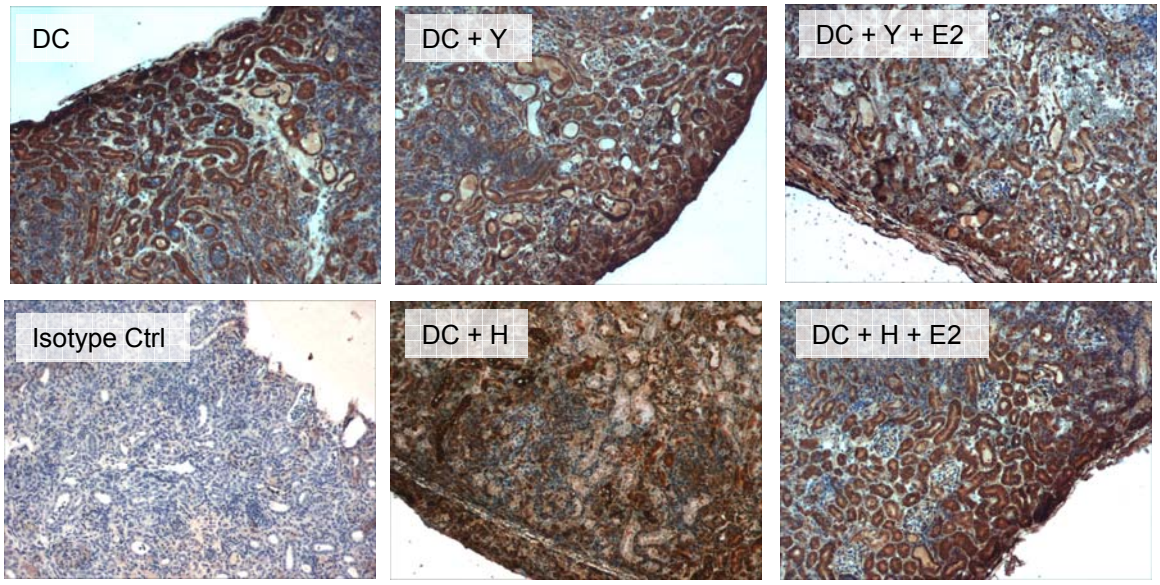


Figure 12 Continued

G) IL-17



H) IL-23



More dramatic results were seen with double adoptive transfers with pDCs in terms of protection. Mice receiving yeast-pulsed pDCs showed a 3 log decrease in fungal CFU in the kidneys at d 7 post-infection (Figure 13). Similar to mDCs, addition of PGE_2 or PGEx abrogated the ability of yeast-pulsed pDCs to induce protection. Hyphae-pulsed pDC exacerbated infection and addition of prostaglandins leads to increased fungal burden. IL-12 levels from restimulated splenocytes correlated with fungal burden, with decreased levels observed in mice that were not protected (Figure 14). IL-4 and IL-5 levels were also increased in mice vaccinated with hyphae pulsed pDCs. Interestingly, IL-6 and TGF-β levels were also increased in non-protected mice. While these cytokines exert both pro- and anti-inflammatory effects, respectively, the combination of the two has been shown to induce development of Th17 cells [180]. However, there was no detectable IL-17 or IL-23 measured from splenocytes (data not shown).

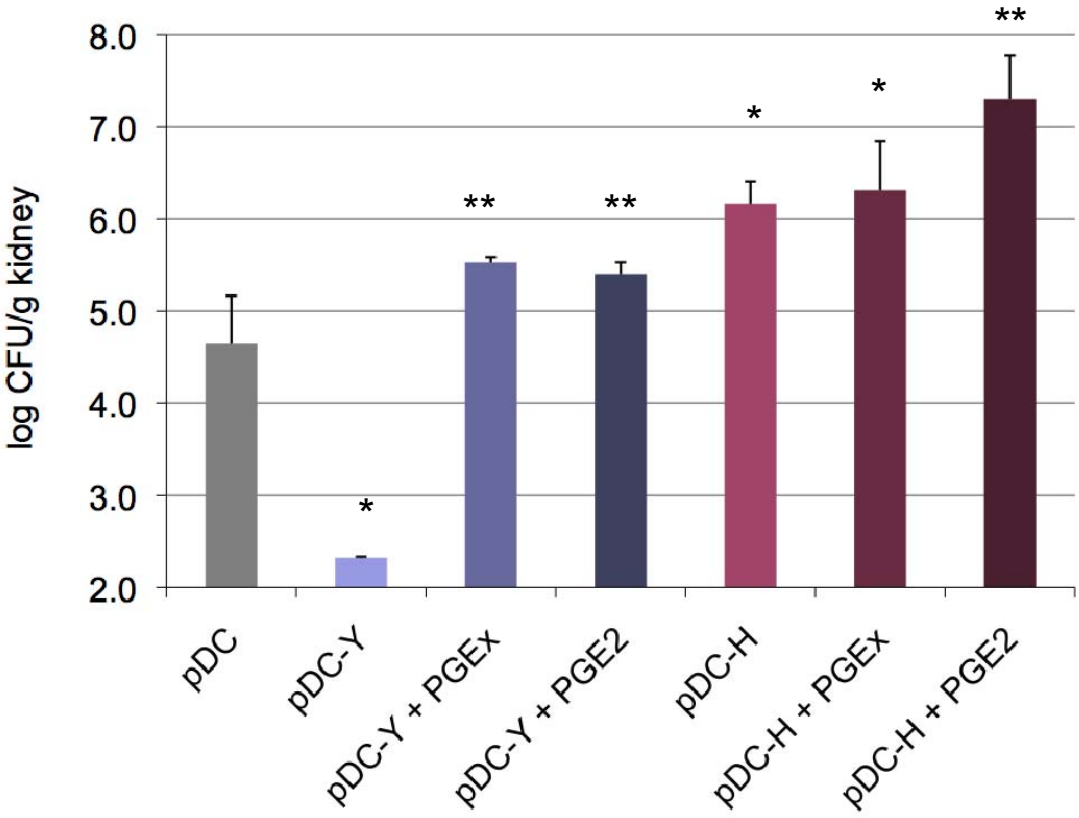


Figure 13. Assessment of protection from *C. albicans* infection following double adoptive transfer with pDCs. Antigen pulsed pDCs were harvested after 18h of stimulation. For vaccination, $5x10^5$ cells were injected via the intraperitonial route in 6-8 week old C57BL/6 mice on day 0 and day 7. Mice were inoculated 7 d after the second vaccination by tail vein injection with *C. albicans* strain SC5314 ($2.5x10^5$ CFU/mouse). After 7 days post-inoculation, the mice were sacrificed, the kidneys excised, homogenized and serial dilution was performed to measure fungal burden. For each group, 4 mice were used. This experiment was repeated four times with similar results. DC = Dendritic cells (plasmacytoid); Y = Yeast cells; H = Hyphae. * $P < 0.05$ DC alone vs. experiment groups (pulsed with yeast or hyphae). ** $P < 0.05$ treated (pulsed with PGEx/PGE_2) vs. untreated DCs (pulsed with yeast or hyphae).

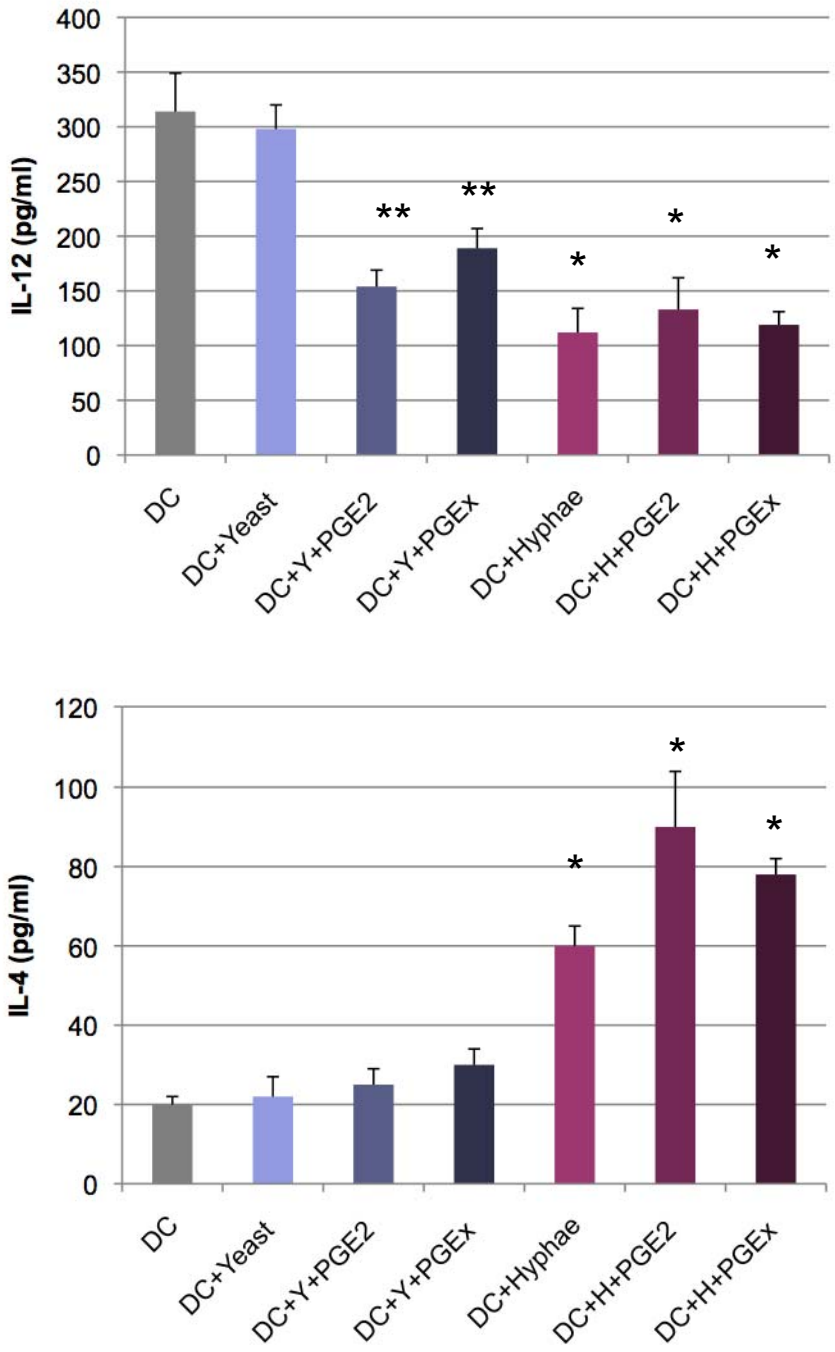
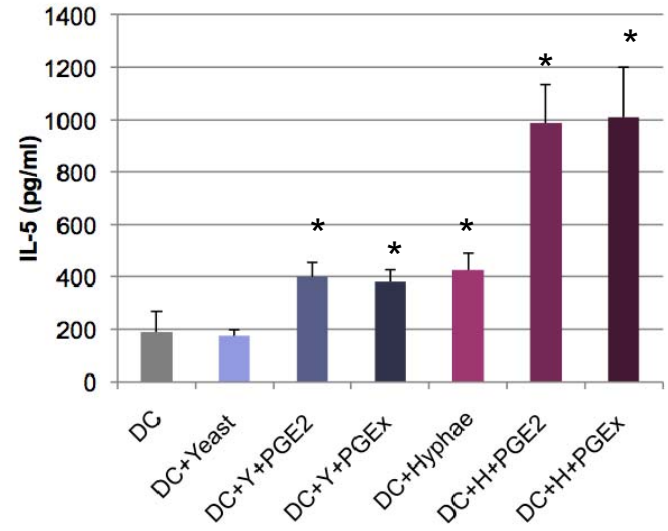
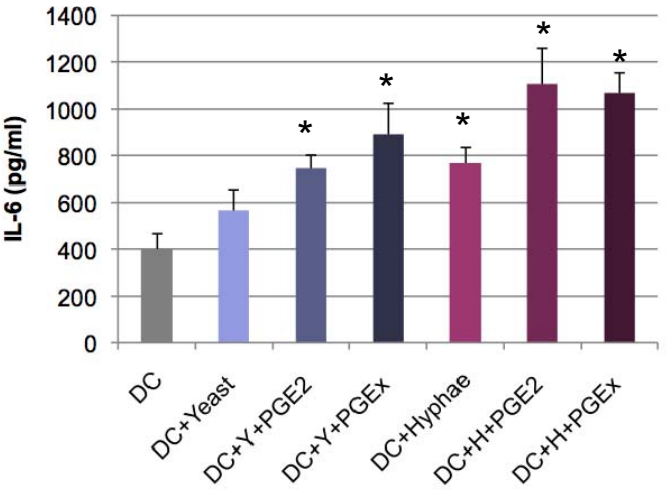


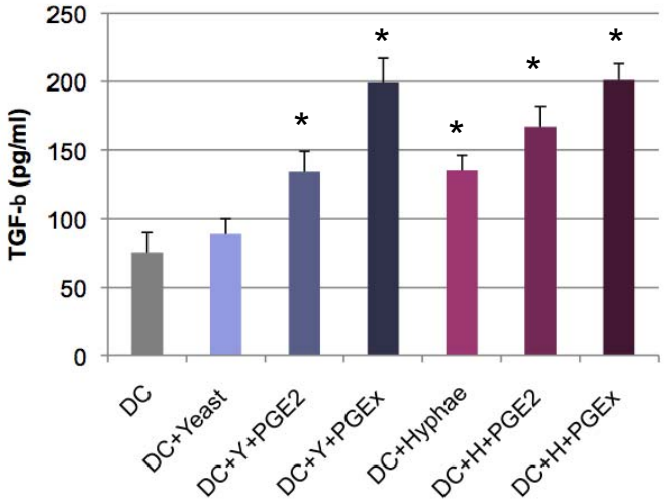
Figure 14. Secretion of cytokines from splenic cultures of mice receiving double vaccination with antigen pulsed pDCs. Spleens were excised from mice systemically challenged with *C. albicans* following two adoptive transfers with antigen pulsed pDCs. Yeast and hyphal forms of *C. albicans* were used in 1:1 ratio to stimulate the splenocytes. After 2h of incubation, 2.5 µg/ml of

amphotericin B was added to prevent *Candida* overgrowth followed by further incubation of 16 h. Cytokines in the culture supernatants were analyzed by ELISA. * $P < 0.05$ DC alone vs. experiment groups (pulsed with yeast or hyphae). ** $P < 0.05$ treated (pulsed with PGEx/PGE_2) vs. untreated DCs (pulsed with yeast or hyphae).

Figure 13 continued.







Overall, protection was associated with increased Th1 and decreased Th2 cytokines in the spleen with pDCs. However, the pattern of cytokines didn't completely match CFUs. We did not find any conclusive results with intracellular cytokines staining from the spleen (data not shown). There are several studies that suggest the cytokine responses associated with protection correlate with local kidney responses as opposed to splenic responses [181, 182]. These studies have depicted drastically increased expression of cytokine genes, various innate immune regulation genes and genes correlated with the initiation of the adaptive immune response. Therefore, kidney but not spleen cytokines were analyzed by immunohistochemistry. Staining revealed increased PMN levels in mice vaccinated with hyphae pulsed DCs, which was even more dramatic in DCs that were treated with PGE_2 (Figure 15a). This indicates a lack of resolution of inflammation. Similar to splenic responses, IL-12 was most prevalent in mice receiving yeast pulsed DCs (Figure 15b). There was dramatic IFN-γ staining in kidneys of mice vaccinated with yeast pulsed DCs, but not in PGE_2 treated or hyphae pulsed DC groups (Figure 15c). Th2 cytokines IL-4 and IL-10 staining correlated with a lack of protection, with the most staining observed with hyphae + PGE_2 pulsed DCs (Figure 15d and e). We also observed increased IL-6 production in non-protected groups, which has previously been associated with a virulent infection (Figure 15f) [181]. Perhaps the most dramatic staining was observed with Th17 cytokines, IL-17 and IL-23. While little to no kidney staining was seen in mice vaccinated with yeast-pulsed DC, heavy staining was seen in non-protected groups (Figure 15g and h). Overall, these data indicate that yeast

pulsed DC induce a Th1 response, while PGE_2 or hyphae shifts the response towards a Th2 and over-exuberant local Th17 response.

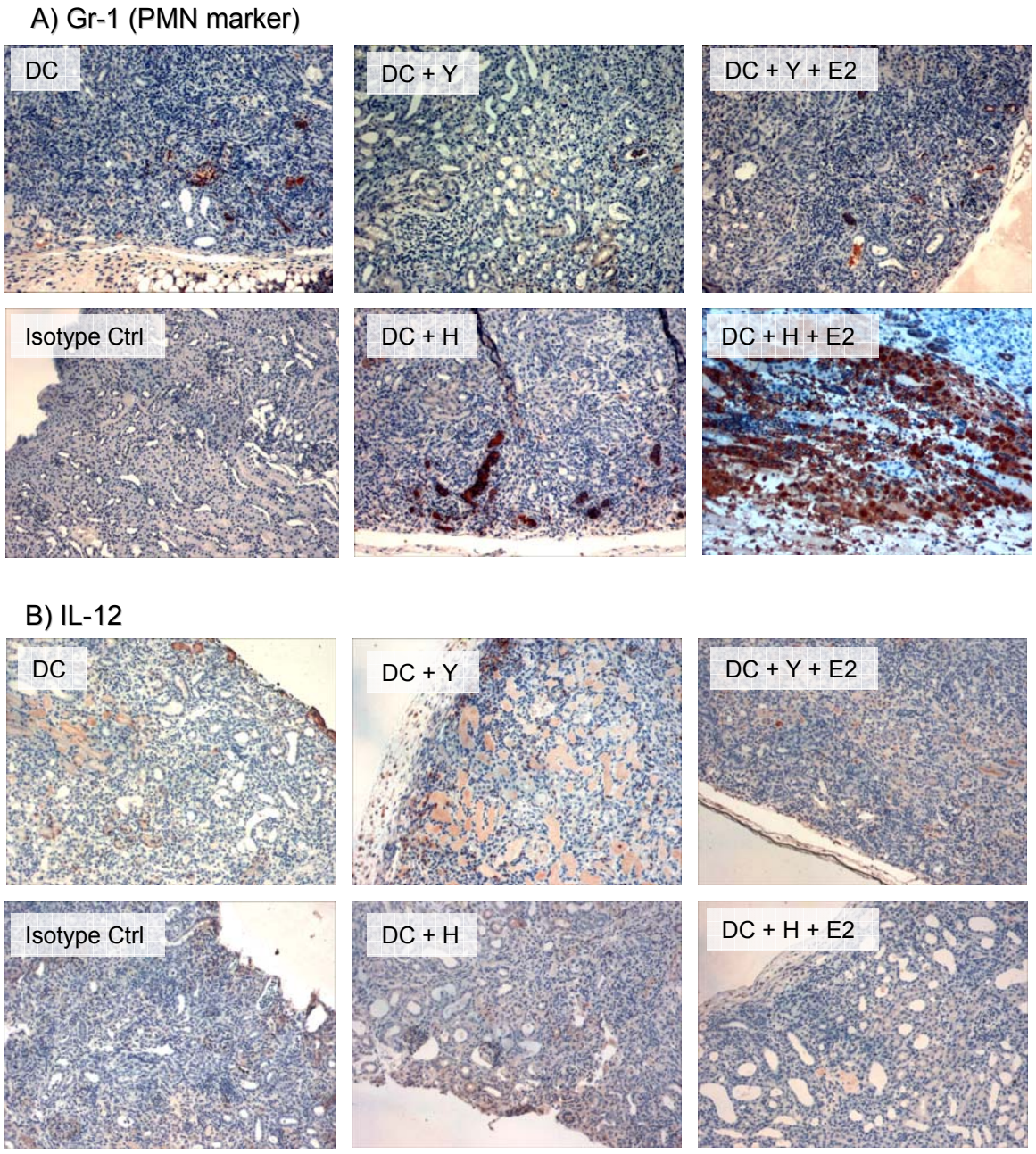
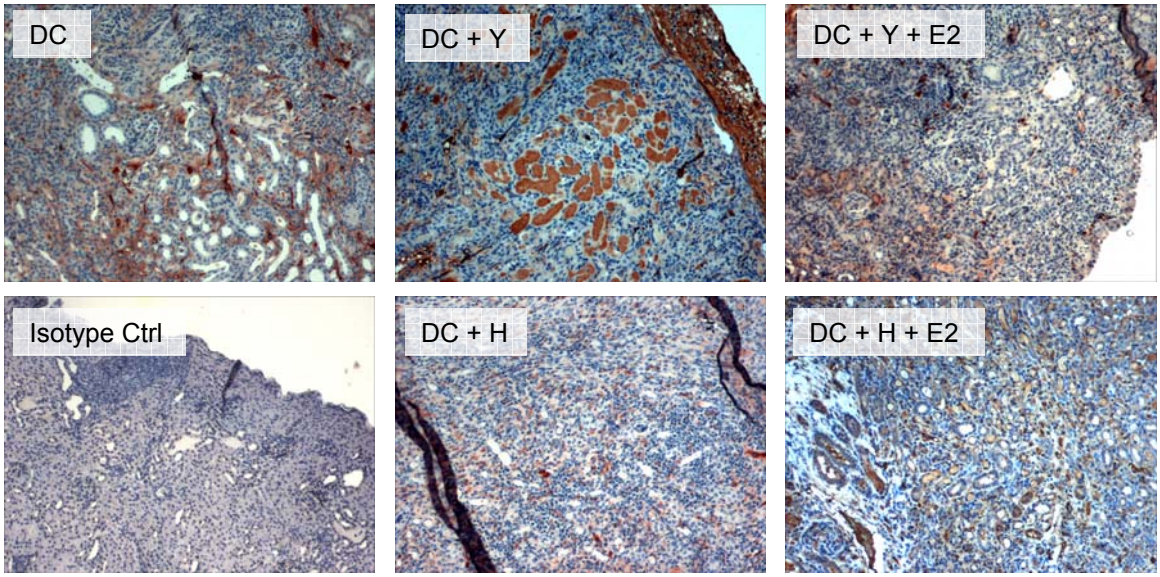


Figure 15. Immunohistochemistry analysis kidneys of mice receiving double vaccination with antigen pulsed pDCs. Kidneys were excised from mice systemically challenged with *C. albicans* following two adoptive transfers with antigen pulsed pDCs. Tissues were fixed in 10% formalin and processed for immunohistochemistry. Staining was visualized by light microscopy.

Figure 15 Continued

C) IFN-γ



D) IL-4

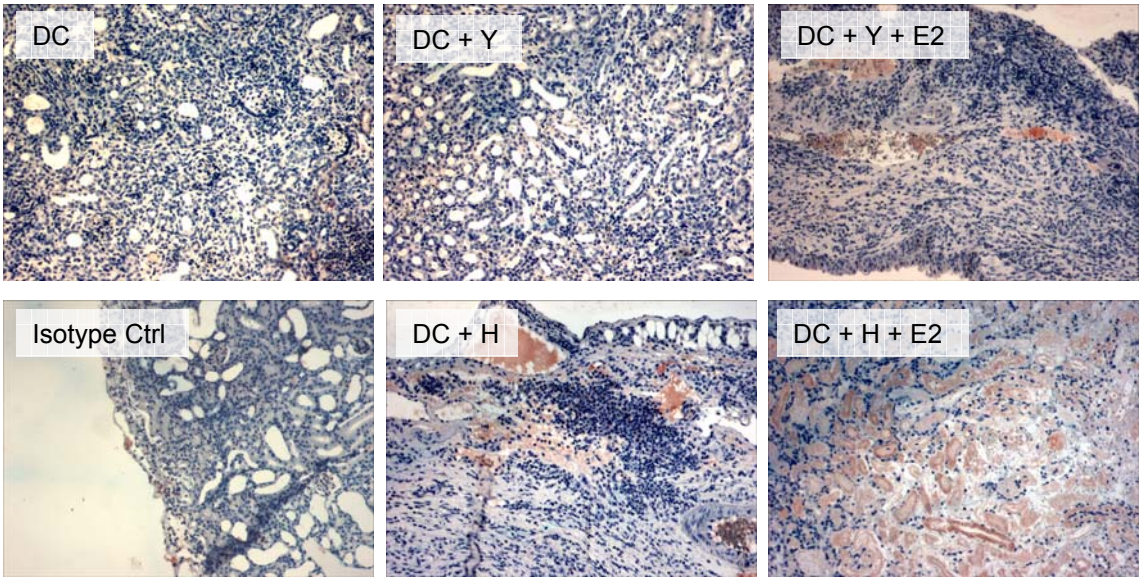
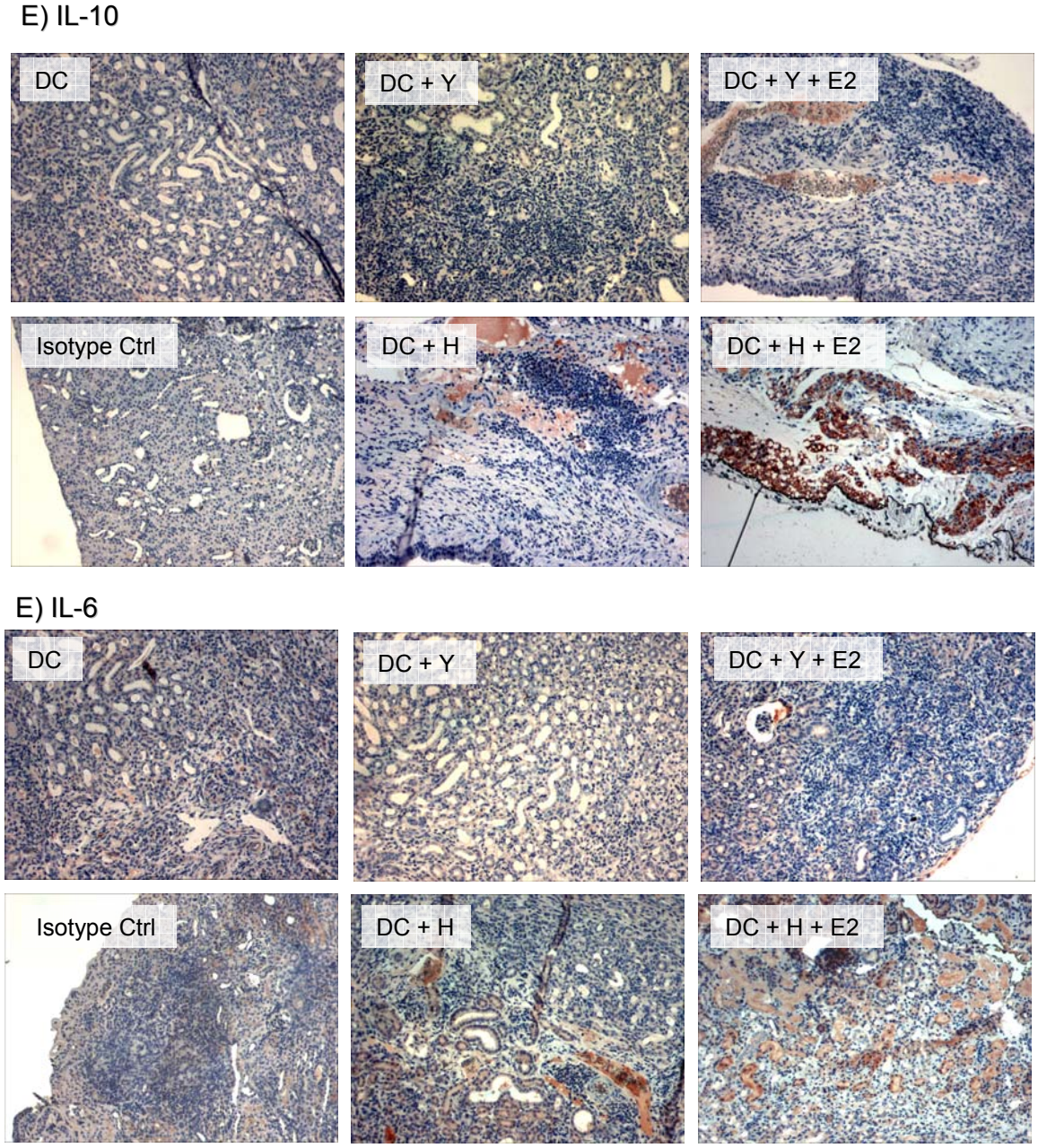


Figure 15 Continued



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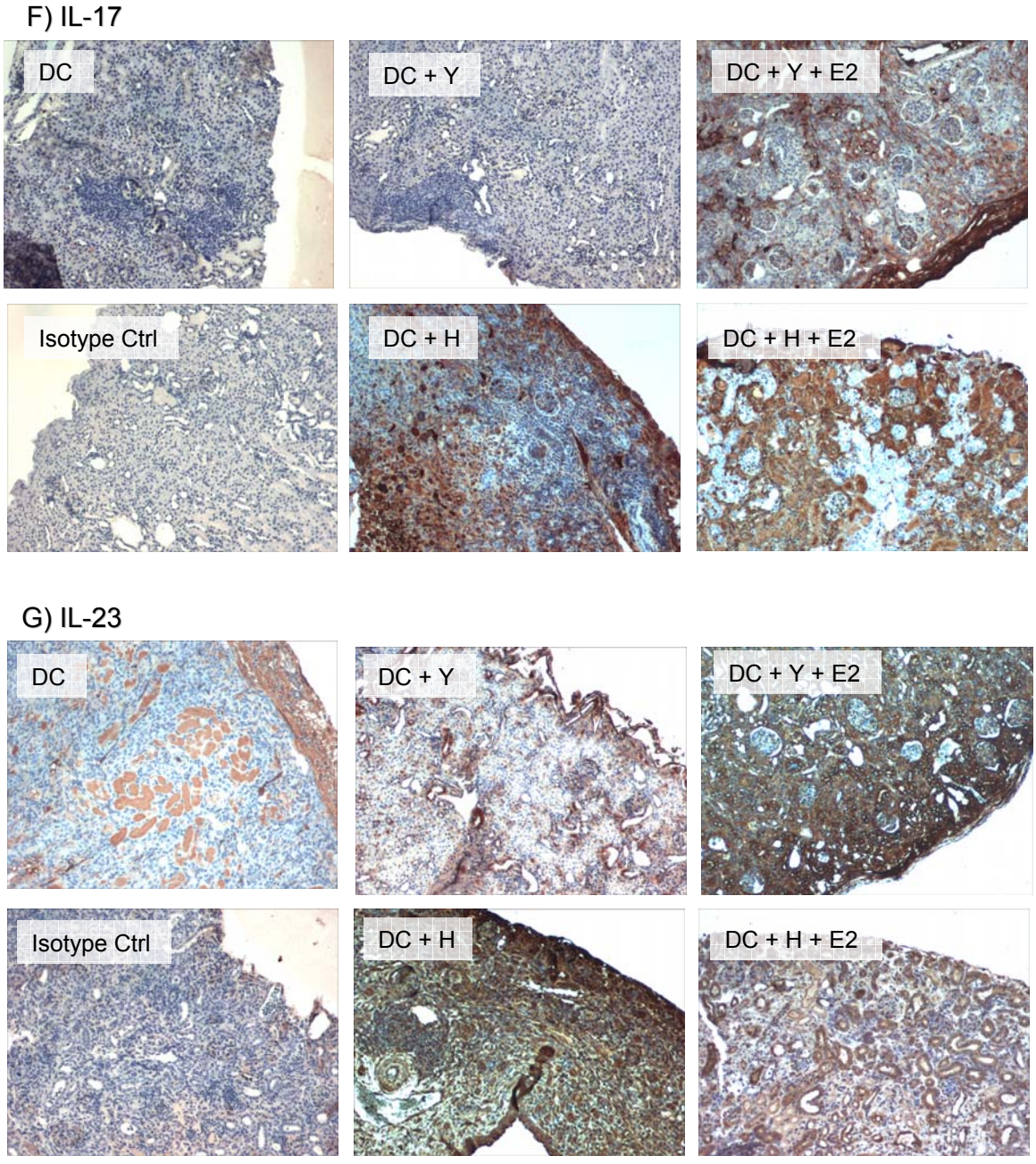

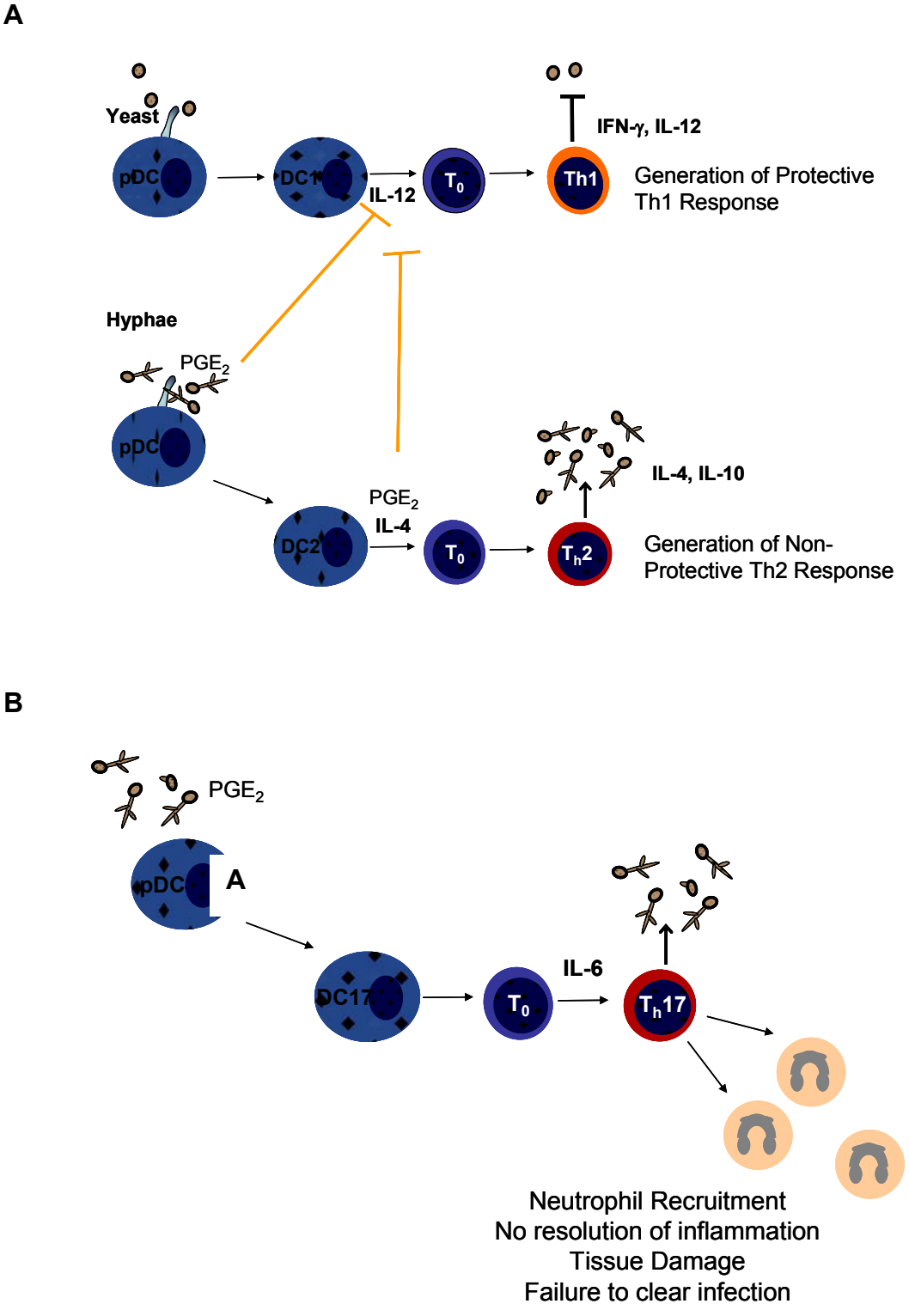

Figure 16. Model for immunomodulation by Candida and PGE2 in (A) Spleen and (B) Kidney.

DISCUSSION

The early cytokine analysis with JAWSII cells revealed upregulation of Th2 cytokines IL-4, IL-10, and IL-13 in the presence of hyphae with PGE_2. This is consistent with the previous data reporting a bias towards non-protective Th2 cytokine responses in macrophages and monocytes in the presence of hyphae, but not yeast [80]. However, the presence of PGE_2 was required for robust Th2 cytokine production from DCs as was reported previously [183]. In addition, pro-inflammatory cytokines IL-6 and IL-15 were upregulated in the hyphae/PGE_2 group. Although pro-inflammatory mediators are required for clearance of *Candida*, this early inflammatory response also correlates with early expression of SOCS-3, a suppressive cytokine, which is involved in promoting a Th2 (DC2) phenotype [184]. We did not see any distinct dichotomy of Th1 versus Th2 response in the yeast versus hyphae treated group perhaps because of the early time point (2h). We also confirmed upregulation of IL-4, IL-6, and IL-10, and down-regulation of IL-12 using qRT-PCR. With this analysis, there were more obvious differences between the yeast and hyphae pulsed DCs, with greatest responses observed with hyphae pulsed DCs treated with PGE_2. Overall, this indicates that PGE_2 promotes a non-protective Th2 type response from DCs (Figure 16).

In analyzing the effects of prostaglandins and antigenic stimulation on DC maturation and activation, we compared cell surface marker expression in

primary bone marrow derived mDCs and pDCs. Overall, the greatest increases in CD86 expression occurred in DCs pulsed with hyphae in the presence of PGE_2. This indicates that DC activation is influence by both *C. albicans* morphotype and PGE_2. Interestingly, there was a more dramatic increase in CD86 with yeast pulsed pDCs (3 fold) compared with mDCs (equivalent to control). This could be one reason why we observed increased protection with yeast pulsed pDC compared to mDCs during systemic *Candida* infection after DC vaccination. This pattern is also reported in other studies where PGE_2 were responsible for upregulation of activation marker in the dendritic cells but in conjunction with a cocktail of other compounds [185]. Analysis of early cytokine production by qRT-PCR confirmed that both mDCs and pDCs behave similarly to the JAWSII cell line. We observed increased IL-4, IL-6 and decreased IL-12 in hyphae pulsed DCs in the presence of PGE_2. This confirms the utility of this cell line and strengthens the argument that hyphae and PGE_2 promote pro-Th2 responses in DCs.

To examine effects of antigenic stimulation and prostaglandins on subsequent responses in pDCs and mDCs, we analyzed cytokine production by ELISA. We observed decreased Th1 cytokine production (IL-12 and IL-6) and slightly increased Th2 cytokine production (IL-4) in the presence of fungal or host PGE_2. Studies have shown PGE_2 to downregulate Th1 and increase Th2 responses, which is supportive of the results from our in vitro cytokine analysis [183, 186, 187]. However, we also observed decreased IL-10 (Th2 and Treg), and IL-23 (Th17). In addition, we did not observe any distinct dichotomy of Th1

versus Th2 response in the yeast versus hyphae treated groups. However, PGE_2 production was significantly increased in the hyphal pulsed DCs compares with yeast pulsed. Because *C. albicans* was killed at the 2 h point, this PGE_2 must be host-derived. Previous studies have shown induction of PGE_2 synthesis via activation of COX-2 transcription by *C. albicans* from various immune cells like splenocytes, macrophages, and endothelial cells [161, 188, 189]. In addition, there are evidences of PGE_2 production from antigen presenting cells during recurrent *Candida* vaginitis [190].

This difference in the pattern of PGE_2 induction could be due to various possibilities like differential PRR signaling or β-glucan contamination or perhaps due to the differences in the concentration of manufacturer's versus PGEx generated in the lab. DCs recognize yeast and hyphal form of *C. albicans* via TL4 and TLR2 receptors [80, 115, 116]. Also, studies have indicated that *C. albicans* signaling via TLR2 pathway leads to the production of PGE_2 from the host and causes persistent type of infection [189]. This discrimination by the DCs to interact via different TLRs could result from the fact that the hyphal cell wall proteins are different from that of the yeast form of *C. albicans*. The presence of important adhesions like Als3 (Agglutinin like sequence) and Hwp1 (Hyphal wall protein), which are antigenic and play role in virulence caused by this fungus has been associated with hyphal forms [191-193]. Prostaglandins produced by both host and fungal cells also enhance morphogenesis in *C. albicans* suggesting that eicosanoids could participate in modulating the immune responses by both influencing *Candida* and DC biology in vivo [156, 162]. Thus,

depending on the morphotypes of *Candida* (yeast or hyphal form), the DCs have the ability to modulate the immune responses. Another factor contributing to the differences in the cytokine responses induced from the bone marrow derived dendritic cells after stimulation with PGE_2 versus PGEx could be because of the β-glucan contamination which is one of the main constituent of Candida cell-wall polysaccharides [194]. Various studies have reported that β-glucan plays active key role towards generating the immune responses by activating the complement proteins and inducing array of inflammatory cytokines like TNF-α and also leukotrienes from the host cells [195]. There is a possibility that PGEx eluted from the anti-PGE_2 column got this β-glucan contamination during washing the anti-PGE_2 column with buffers. Also, there could be differences in the PGE_2 concentration from the manufacturer's PGE_2 with that of the PGEx which was eluted from the anti- PGE_2 column for which the concentration was determined using the monoclonal ELISA kit. All these factors might have possibily contributed to the differences in the cytokine induction pattern after using these compounds for dendritic cell stimulation assay.

To determine the downstream effects of antigenic stimulation in the presence of PGE_2, mice were vaccinated with DCs and challenged systemically. After one round of vaccination, we observed protection only with pDCs pulsed with yeast. This is in agreement with previous work by Zelante et al., which showed that pDCs pulsed with yeast are able to generate protection compared to mDCs [79]. However, after two rounds of vaccination, both yeast pulsed pDCs and mDCs imparted protection compared to the hyphae pulsed group. This

pattern of protective versus non-protective dichotomy with hyphae versus yeast pulsed DCs has been documented in previous studies [80]. This could be due to the fact that the hyphal but not yeast form of *C. albicans* was able to induce high levels of PGE_2 production from the DCs prior to adoptive transfer.

An important characteristic of prostaglandin activity is their potency at very low (nanomolar) concentrations. In relation to this potency, most eicosanoids have very short half-lives, being made de novo (as opposed to stored and released) and acting near the site of their synthesis [134]. Addition of nanomolar quantities of PGE_2 to yeast-pulsed pDCs or mDCs abrogated their ability to induce protection against systemic infection. The concentration of the PGE_2 that was used in our experiment were similar to that detected by the DCs at 18h time point. To analyze the type of immune response generated after vaccination, we measured re-stimulated splenocyte culture cytokine levels. With both pDCs and mDCs, there was decreased IL-12 and increased IL-5 in groups that were not protected after vaccination. This indicates that protection correlates with elaboration of a Th1 response. We also observed increases in IL-4 in mice receiving hyphae pulsed DCs, which was further increased with prostaglandin exposure. Interestingly, we also observed increased IL-6 and TGF-β in non-protected groups. These cytokines are sufficient for the induction of IL-17 and IL-17F expression in TCR-activated naïve CD4+ T cells [196].

The Th17 response also possibly plays a role in generating non-protective response, however, this was more pronounced in the mice kidneys than spleen. Our studies did not depict any recovery of fungal burden from other organs like

mice liver, kidney or blood (data not shown). The findings from the immunohistochemistry studies are also in alignment with the pattern of protection observed in the mice after adoptive transfer. The kidneys from yeast pulsed mice showed the production of protective Th1 cytokines (IFN-γ and IL-12) compared to the other groups, which had more Th2 (IL-4) and pro-inflammatory cytokine (IL-6) expression. The most dramatic staining was seen with Th17 cytokines (IL-17, IL-23) were very strong in the most of the groups except the yeast pulsed pDCs indicating that Th17 pathway is associated with non-protective responses and persistent fungal infection. Although we observed slight protection with double adoptive transfer with mDCs pulsed with yeast, there was dramatic Th17 cytokine staining in the kidneys. It could be that there is a threshold level of *C. albicans* fungal burden that initiates this dramatic response. With pDCs, *C. albicans* was held to 10^2- 10^3 CFU/g tissue, while with mDCs, levels reached 10^4.

Other work on *C. albicans* infections in the GI model have indicated that both IL-23 and Th17 are induced in vaccinated mice and are non-protective, resulting in defective fungal clearance by impairing the anti-fungal activities of PMNs and prolonged inflammation [79]. However, in oral and systemic infection models, IL-17 receptor A (IL-17RA) knockout mice are more susceptible to infection in naïve animals, indicating that the Th17 pathway is necessary during acute infections [108, 123]. Our studies indicate that in later stages of infection in vaccinated mice, the Th17 response cytokines is associated with exacerbated uncontrolled inflammation. Both IL-23 and IL-17 can inhibit neutrophil antifungal

activity resulting in pathological form of inflammation associated with impaired antifungal resistance [79]. Therefore, although neutrophils were recruited to the kidneys, the antifungal activity is likely diminished, as indicated by the high fungal burden. One of the mechanisms by which this could be verified in future studies is by targeting the IL-17 pathway at different points of infection (early, intermediate and late) with antibody-mediated IL-17 depletion in naïve vs. immunized animals. Ours is the first study to report presence of Th17 cytokines in the mice kidneys during systemic candidiasis is associated with a lack of protection.

Our studies using DC vaccination allowed us to investigate downstream effects of host and fungal prostaglandins on *Candida*-DC interactions. Our results indicate that the presence fungal PGE_2 (PGEx) or PGE_2 during the host-pathogen interaction with DCs can drive a non-protective response. This is associated with generation of a non-protective Th2 response and repression of a protective Th1 response. Locally, we also observed exacerbated Th17 responses and uncontrolled fungal growth despite the presence of neutrophils at the site of infection (Figure 16). This study indicates that local host or fungal PGE_2 production could shift responses in favor of the fungus, which is detrimental during systemic infection. Therefore, fungal PGE_2 production may be considered a virulence factor. Understanding the interactions between PGE_2 and its effect on DCs is important towards developing potent therapeutic strategies to treat *Candida* infection. This is especially important given the fact that *Candida* infections are associated with high mortality rate and the antifungal agents like

fluconazole, voriconazole, itraconazole etc. are associated with resistance mechanisms [197-199]. In future, this study will also help towards understanding the prostaglandin pathways and its downstream effects that can be extended towards other pathogenic eukaryotic species.

CHAPTER 2

Effect of PGEx and PGE_2 on *C. albicans* uptake and survival in macrophages

ABSTRACT

Macrophages play key roles in protecting against both mucosal and systemic infections with the opportunistic fungal pathogen *Candida albicans*. However, mechanisms controlling antifungal killing defenses of these phagocytes are not well understood. *C. albicans* produces an immunomodulatory oxylipin called PGEx, that is structurally and functionally similar to host PGE_2. In addition, it can produce authentic PGE_2 from host arachidonic acid. To determine the effects of fungal and host prostaglandins on interactions with macrophages, we studied phagocytosis and intracellular survival, as well as production of reactive oxygen and nitrogen species. Addition of PGE_2 or PGEx inhibited phagocytosis, while promoting intracellular survival of *C. albicans* in RAW264.7 cells. However, increased survival was not accompanied by any reduction in nitric oxide or peroxynitrite. Therefore, the effects of these compounds may involve non-oxidative mechanisms that require further investigation.

INTRODUCTION

C. albicans is an opportunistic fungal pathogen that inhabits mucosal surfaces including the oral cavity, gastrointestinal tract, and vagina [200]. Under conditions that permit the outgrowth of *Candida*, mucosal colonization can lead to infection and dissemination. Diseases range from superficial mucosal infections to life-threatening systemic candidiasis. Risk factors for systemic infection include immunocompromise, antibiotic therapy, and the use of indwelling medical devices. Protective immune responses to candidal infection require both innate and adaptive immunity. There is no evidence of complement or antibody mediated clearance of *C. albicans* infection. Therefore, the first line of defense against infection involves activated innate effector cells, including neutrophils and macrophages.

Macrophages play an essential role during systemic candidiasis. During *C. albicans* systemic infection, mice depleted of macrophages exhibit delayed clearance and high kidney fungal burden [201]. Macrophages have direct antimicrobial activity against such organisms and also promote antigen presentation, polysaccharide sequestration, and production of cytokines and chemokines. Mechanisms implemented by macrophages to combat infection must involve killing *C. albicans*, both yeast and hyphal form. If *C. albicans* survives intracellularly, macrophages could act as a vehicle to disseminate the fungus to immunological privileged niches, which could result in escape from innate and adaptive immune responses generated by the host [202]. There is

also evidence that persistent infection is associated with the intracellular residence of yeast cells in macrophages.

Macrophages can kill *Candida* via oxygen-dependent or independent pathway [203]. Oxygen dependent pathways include production of both reactive oxygen and nitrogen intermediates (ROI, RNI). In particular, nitric oxide (NO) has strong antifungal activity; however, *C. albicans* can evade host defense by suppression of NO/superoxide radical production by stimulated macrophages [103-105]. Furthermore, what were thought to be two independent pathways, i.e., nitric oxide and superoxide anion, have now been shown to combine to form a potent macrophage candidacidal molecule, peroxynitrite. Oxygen independent pathways are less well studied. However, upon phagocytosis of *C. albicans*, yeast forms can germinate and lyse macrophages [106]. Therefore, whether the macrophage or the fungus prevails is dependent on several factors, including the type of macrophage and activation state.

Activation of macrophages is influenced by the microenvironment as well as the nature of the microbe or antigen. Whereas Th1 cytokines such as IFNγ and TNF-α stimulate antifungal activities, Th2 cytokines such as IL-10 are inhibitory [204, 205]. *C. albicans* has also been shown to produce eicosanoids, which are potent immune response regulators derived from arachidonic acid (AA). *C. albicans* produces a compound that cross-reacts with antibodies to host prostaglandin E_2 (PGE_2), called PGEx. In the presence of host AA, PGEx production is significantly upregulated, indicating that *C. albicans* can produce PGE2 from host lipid precursors. Mass spectrometry analysis revealed that

PGEx derived from AA is structurally identical to PGE_2 [154]. Overall, 99.9% of AA-derived PGEx is PGE_2. In terms of macrophages, PGE_2 can inhibit phagocytosis and intracellular killing of bacteria [206, 207]. However, other reports have indicated that PGE_2 is crucial for phagocyte killing of parasites, via induction of nitric oxide [208]. In the present study, we tested the effect of fungal PGE_2 (PGEx) and host PGE_2 on *Candida* phagocytosis and survival in macrophage cell line, RAW264.7 and how these compounds influence oxidative killing mechanisms.

MATERIALS AND METHODS

Cell culture. The macrophage cell line RAW264.7 (originally from ATCC TIB 67) was kind gift from Dr. M. Neely (Wayne State University, Detroit, MI). Cells were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum, 25mM HEPES, 2 mM Glutamine, 100 IU penicillin/ml and 100µg streptomycin/ml (D-10 medium) and incubated at 37°C, 5% CO_2 with 95% humidity. For experiments, cells were seeded in 100 cm^2 T75 flask at the density of 1.5 $X10^5$ cells /ml.

***C. albicans* Strains and Culture Conditions.** The *C. albicans* strain SC5314 used in this study is a prototrophic parental strain [168]. This strain was subcultured from frozen stocks and grown overnight at 30°C in Sabouraud dextrose agar (SDA) (Becton, Dickinson and Company, Sparks, MD). For the liquid cultures, isolated colonies from the SDA plates were inoculated into Sabouraud dextrose broth (SDB) with constant shaking.

Isolation of fungal PGEx. *C. albicans* strain SC5314 was grown overnight at 37°C in 10 ml RPMI containing 100 mM arachidonic acid. Culture supernatants were loaded onto a PGE_2 affinity column (Caymen Chemical), washed, and eluted according to manufacturers instructions. The eluates were dried under a stream of nitrogen gas and resuspended in 500 µl PBS. PGE_x concentrations

(approximately 20ng) determined using a PGE_2 monoclonal ELISA (Cayman Chemical, Ann Arbor).

Phagocytosis assay. Overnight *C. albicans* cultures were washed 2-3 times with 10% DMEM at 4°C by centrifuging at 4000 xg. Sonication was performed at the highest setting for 10 min followed by keeping it on the ice for 30 min to obtain single cell suspensions. Macrophages were infected with *C. albicans* at an MOI of 5 in the presence or absence of PGE_2 or PGEx at a final concentration of 2nM. After 15min or 3h of incubation, the supernatant was removed and the cells were washed three times with 1ml sterile 1x PBS to remove non-adherent organisms. To obtain phagocytosed yeast, macrophages were lysed with sterile water. The recovered yeast cells, from the supernatant fraction as well as after lysis, were plated on SDA media after serial dilution. The formula used for calculations:

% phagocytosis = 1 – [CFU t(initial)/intracellular CFU t(15 min)] X 100

% Intracellular killing = 1 – [intracellular CFU t(15 min)/intracellular CFU t(3 h)] X 100

Fluorescence microscopy. For monitoring phagocytosis in macrophages, RAW264.7 cells seeded on coverslips at a concentration of 10^6 cells/ml. *C. albicans* was stained with 1µl calcufluor at a concentration of 1mg/ml (Fluka, Seelze, Germany) for 10 min at $1x10^6$ yeast cells per 200 µl and added to macrophages at an MOI of 5. To monitor phagocytosis after 15 min incubation,

the supernatant was washed and staining of extracellular non-phagocytosed yeast was quenched with trypan blue. Cells were fixed with 4% paraformaldehyde (in PBS) at 4°C for 10min. To monitor intracellular survival at 3 h, cells were incubated as previously mentioned but Candida was pre-stained with FUN-1 fungal viability stain according the manufacturer's instructions (Invitrogen). FUN-1 distinguishes live (red) versus dead (green/yellow) yeast. For fluorescence microscopy, a Nikon Eclipse E800 and Metamorph software was used.

Growth curve. *C. albicans* was grown overnight in SD broth while shaking at 30°C. The culture was washed, counted, and diluted into 10 ml fresh SD broth at a final concentration of 10^5/ml. PGEx or PGE_2 was added to a final concentration of 2nM and culture were incubated at 30°C with shaking. The absorbance was measured at 600nm at various time points up to 48h. For the blank (control) sample, only SDB without yeast was used. CFU analysis was performed by serial dilution of the cultures at various time points and plating onto SD agar plates.

Nitrite Assay. RAW264.7 cells were plated at a concentration of $5x10^5$ cells/ml in 24-well plates and grown for 24 h with or without L-NMMA (competitive NOS inhibitor) at final concentration of 500μM 24 h prior to infection. Cells were stimulated with LPS (1μg/ml final concentration) and infected with *C. albicans* at MOI 10:1. Cells were incubated at 37°C, 5% CO_2 for 3h. Both PGEx and PGE_2

were used at a final concentration of 2nM. The 24-well plate was centrifuged at 1000 rpm for 10 min and nitrite levels in cell supernatants were assayed using a Griess reagent kit (Molecular Probes) according to the manufacturer's instructions.

Peroxynitrite Assay. RAW264.7 cells were plated at a concentration of $5x10^5$ cells/ml in 24-well plates and grown for 24 h. Cells were washed and stimulated with LPS (1µg/ml final concentration) and infected with *C. albicans* at MOI 10:1 for 3h. Both PGEx and PGE_2 were used at a final concentration of 2nM. Control cells were stimulated with zymosan (200µg/ml final concentration). Cells were incubated at 37°C, 5% CO_2 for 3h. The plate was centrifuged 1000 rpm for 10 min and peroxynitrite levels in cell supernatants were assayed using a peroxynitrite detection kit (Cell Technology Inc.) according to the manufacturer's instructions.

Statistical analysis. The Student's *t* test (two-tailed, unequal variance) was used to analyze the significance of differences between two experimental groups. Significance was considered with a $P \leq 0.05$ or less. The data recorded are representative of either two or three independent experiments.

RESULTS

To study the effect of prostaglandin (PGEx and PGE_2) on macrophage phagocytosis of *C. albicans*, viable intracellular yeast were monitored 15 min after infection. The data showed a decrease in percentage of phagocytosis in the prostaglandin treated groups. Phagocytosis decreased by about 20% in the PGEx treated group, whereas in the PGE_2 treated group, percent phagocytosis was reduced by about 10% (Figure 17).

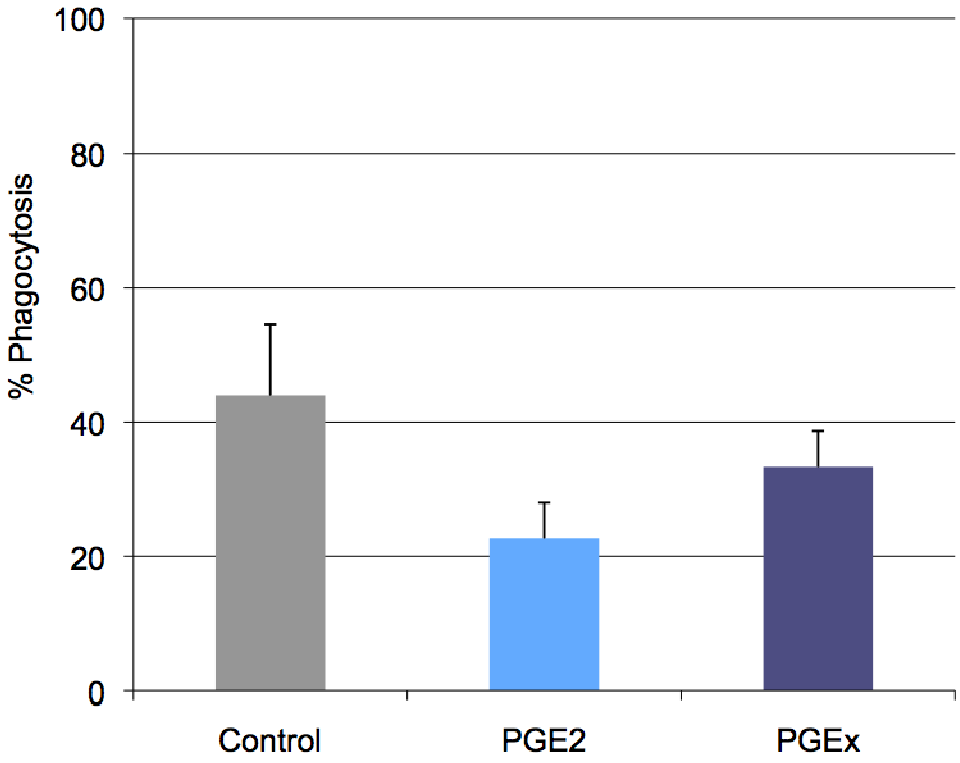

Figure 17. Effect of PGE_2 and PGEx on macrophage phagocytosis of *C. albicans*. *C. albicans* strain SC5314 was grown overnight in SD broth at 30°C. Cultures were washed with D-10 medium followed by sonication to create a single cell suspension. RAW264.7 cells were infected at an MOI of 5 in the presence or absence of PGE_2 or PGEx at a final concentration of 2nM. After 15 min, supernatant was removed and cells were washed three times with sterile 1X PBS. Cells were lysed with sterile water and both supernatants and lysates were diluted and plated onto SD plates to determine CFU. The experiment was repeated 4 times in triplicate.

To determine if prostaglandins influence subsequent intracellular survival viable yeast were monitored 3 h post-infection. We observed a modest increase in intracellular survival in the macrophages treated with PGE_2 (Figure 18). However, there was a significant increase in intracellular survival with macrophages treated with PGEx compared to the control group (Figure 18). To further verify the increase in phagocytosis, we performed fluorescence microscopy. *C. albicans* was labeled with calcofluor white and used to infect macrophages seeded onto cover slips. We observed an increase in the number of intracellular yeast in the presence of PGE_2 or PGEx compared to the control group (Figure 19). Staining of *C. albicans* with FUN-1 also showed increased viable yeast in prostaglandin treated macrophages, particularly with PGEx (Figure 20). To rule out the possibility that prostaglandin was directly contributing to the growth or budding of *C. albicans*, we performed growth curve assays. *C. albicans* was incubated in the presence of PGE_2 or PGEx and growth was monitored over 48 h. We observed no increases in presence of prostaglandin at early or late time points (Figure 21).

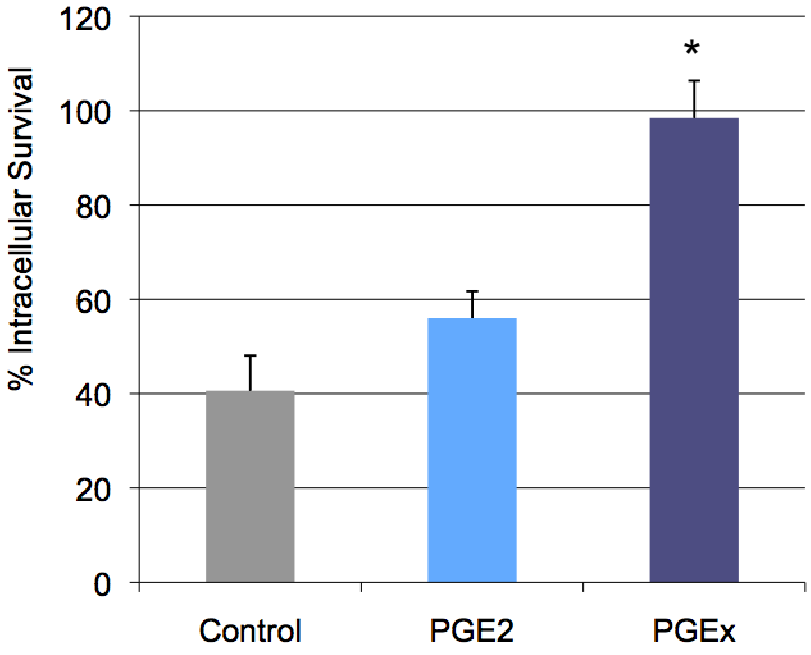

Figure 18. Effect of PGE_2 and PGEx on intracellular survival of C. albicans in macrophages. *C. albicans* strain SC5314 was grown overnight in SD broth at 30°C. Cultures were washed with D-10 medium followed by sonication to create a single cell suspension. RAW264.7 cells were infected at an MOI of 5 in the presence or absence of PGE_2 or PGEx at a final concentration of 2nM. PGEx was obtained by passing the overnight cultured Candia supernatant supplemented with 100mM arachidonic acid through anti-PGE_2 column. PGE_2 was commercial standard prostaglandin obtained from Cayman chemicals, Ann Arbor. After 15 min, supernatant was removed and cells were washed three times with sterile 1X PBS. Cells were lysed with sterile water and both supernatants and lysates were diluted and plated onto SD plates to determine CFU. The experiment was repeated 4 times in triplicate. Experiments were repeated 3 times with 3 replicates each. * $P < 0.05$ control vs. experiment group.

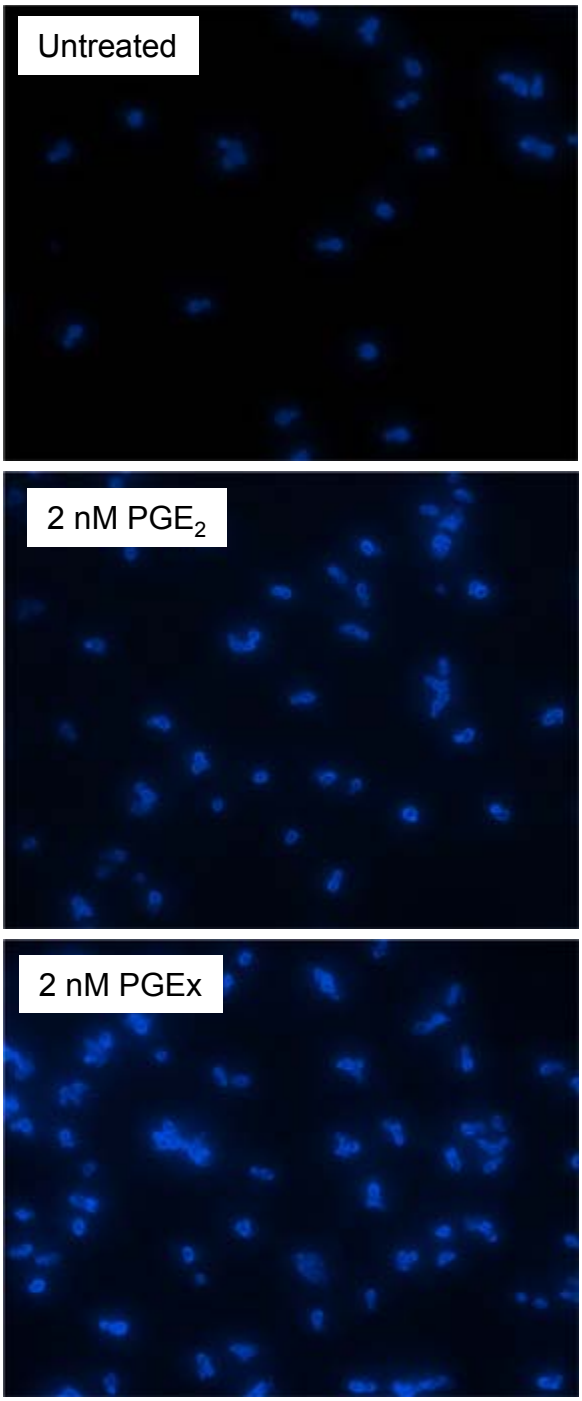

Figure 19. Effect of PGE_2 and PGEx on *C. albicans* phagoctysosis by immunofluorescence microscopy. *C. albicans* strain SC5314 was grown overnight in SD broth at 30°C. Cultures were washed with D-10 medium followed by sonication to create a single cell suspension. Yeast were stained with calcofluor white prior to infection. RAW264.7 cells were seeded onto cover slips and infected at an MOI of 5 in the presence or absence of PGE_2 or PGEx at a final concentration of 2nM. After 3h incubation, the supernatant was washed and

quenched with trypan blue to destain the non-phagocytosed yeast. Cells were fixed with 4% paraformaldehyde and analyzed by fluorescence microscopy using a Nikon Eclipse E800 with Metamorph software. Data is representative of three independent repeats.

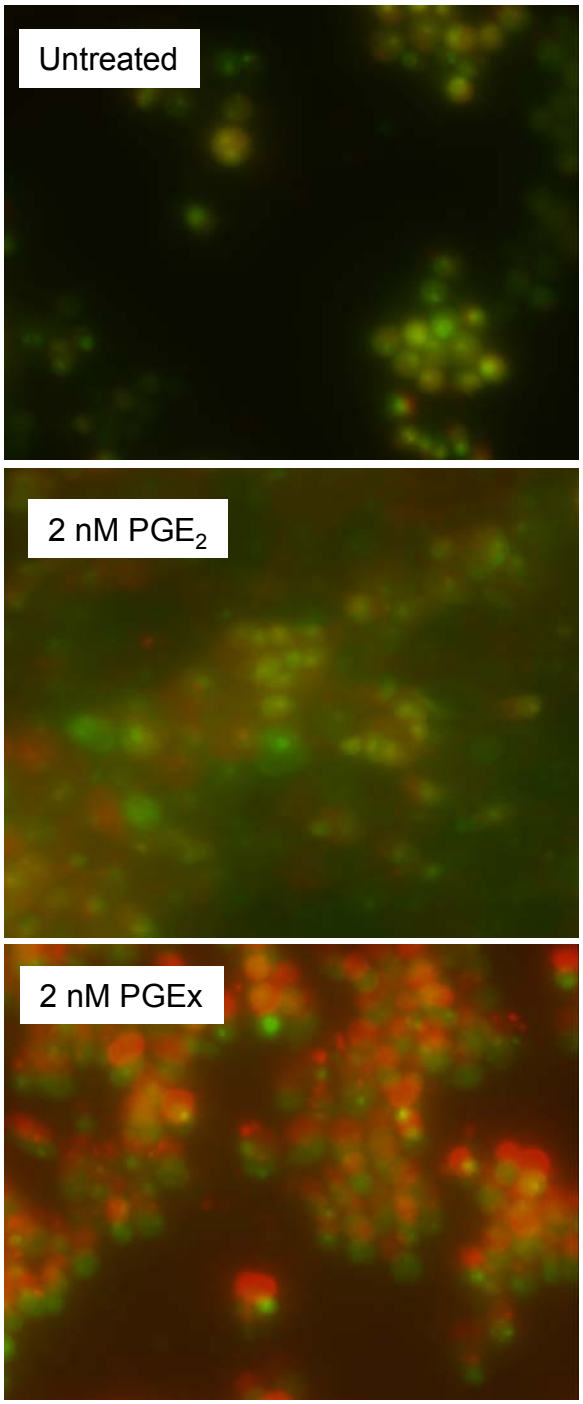

Figure 20. Effect of PGE_2 and PGEx on *C. albicans* intracellular viability. *C. albicans* strain SC5314 was grown overnight in SD broth at 30°C. Cultures were washed with D-10 medium followed by sonication to create a single cell suspension. Yeast were stained with FUN1 prior to infection, which distinguishes live (red) from dead (green-yellow) fungi. RAW264.7 cells were seeded onto cover slips and infected at an MOI of 5 in the presence or absence of PGE_2 or PGEx at a final concentration of 2nM. After 3 h incubation, the cells were

washed and analyzed by fluorescence microscopy using a Nikon Eclipse E800 with Metamorph software. Data is representative of three independent repeats.

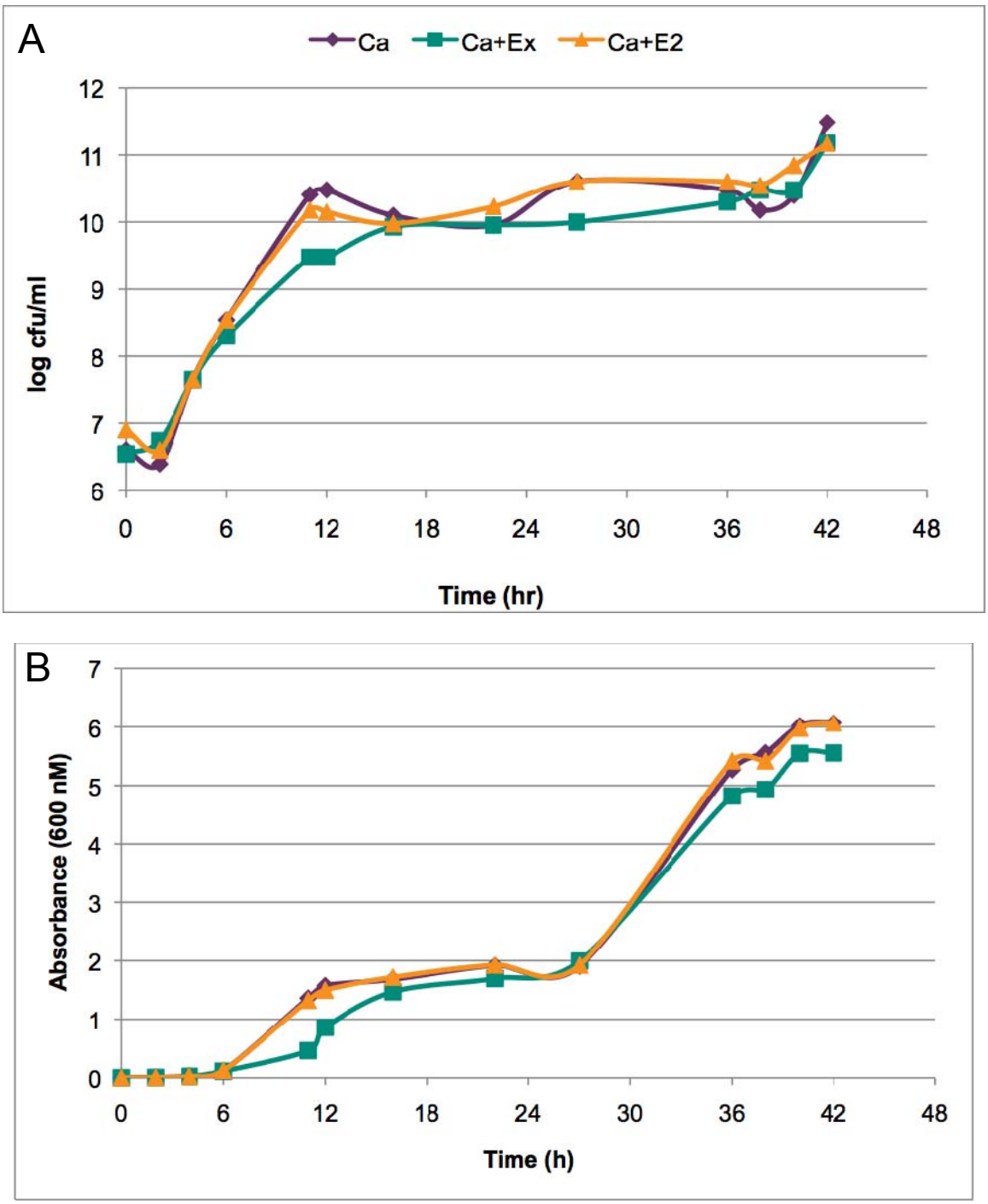

Figure 21. Effect of PGE_2 and PGEx on *C. albicans* growth in vitro. *C. albicans* was grown overnight in SD broth while shaking at 30°C. The culture was washed, counted, and diluted into 10 ml fresh SD broth at a final concentration of 10^5/ml. PGEx was obtained by passing the overnight cultured Candia supernatant supplemented with 100mM arachidonic acid through anti-PGE_2 column. PGE_2 was obtained as commercial standard prostaglandin from

Cayman chemicals, Ann Arbor. PGE_2 and PGEx was added to a final concentration of 2nM and culture were incubated at 30°C with shaking. A) The absorbance was measured at 600nm at various time points up to 48h. For the blank (control) sample, only SDB without yeast was used. B) CFU analysis was performed by serial dilution of the cultures at various time points and plating onto SD agar plates. Data is representative of three indepdendent experiements assayed in duplicate.

While macrophages possess both oxygen dependent and independent antimicrobial defenses, it has been suggested that the oxidative burst is the major antifungal defense. This is due to the fact that yeast germinate with macrophages, which leads to cell lysis. Both reactive oxygen and nitrogen species (ROI/RNI) production contribute to the candidicidal activity of macrophages, with nitric oxide and peroxynitrite exhibiting the strongest antifungal activity [205, 209]. We tested the effect of prostaglandins on inhibiting nitrite and peroxynitrite during infection with *C. albicans*. Macrophages were pre-treated with or without L-NMMA, a competitive iNOS inhibitor, 24 h prior to infection. The results showed a trend in the decrease of nitrite production in each group, which was associated with a slight increase in fungal burden (Figure 22). However, in the PGEx treated macrophages, L-NMMA treatment had no effect on fungal survival. This indicates that the effect of PGEx on increasing intracellular survival is not due to repression of iNOS and nitric oxide production. There were also no significant differences in peroxynitrite production in the presence of PGE2 or PGEx (Figure 23). Therefore, prostaglandins must work to increase intracellular survival by influencing other antifungal pathways.

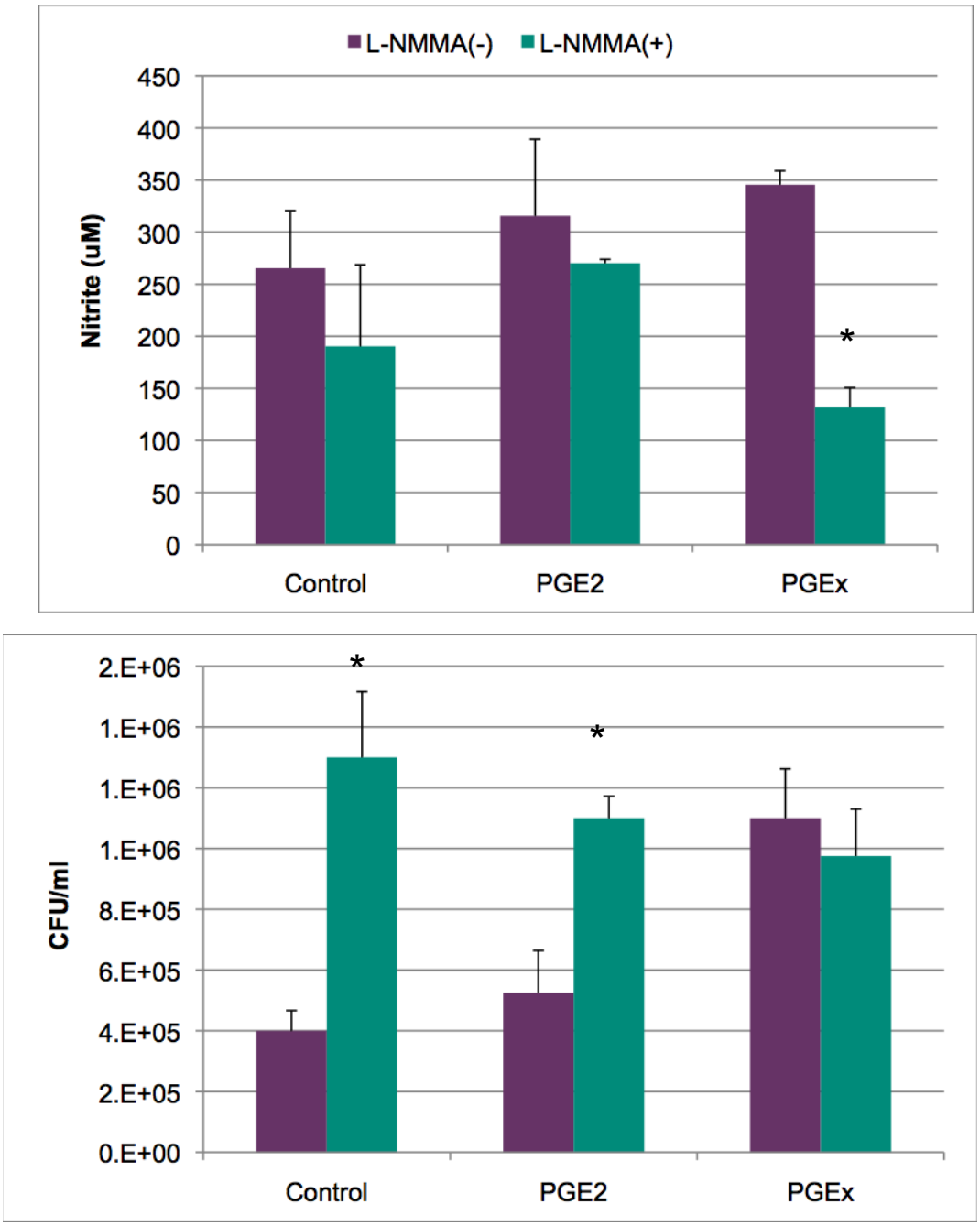

Figure 22. Effect of PGE_2 and PGEx on NO production by macrophages. RAW264.7 cells were seeded overnight in 24-well plate with or without L-NMMA (competitive NOS inhibitor) at final concentration of 500μM 24 h prior to infection. Cells were stimulated with LPS (1μg/ml final concentration) and infected with *C. albicans* at MOI 10:1 for 3h. Both PGEx and PGE_2 were used at a final concentration of 2nM. The 24-well plate was centrifuged at 1000 rpm for 10 min and nitrite levels in cell supernatants were assayed using a Griess reagent kit

(Molecular Probes) according to the manufacturer's instructions. Data is representative of three independent repeats performed in triplicate. * $P < 0.05$ control vs. experimental group.

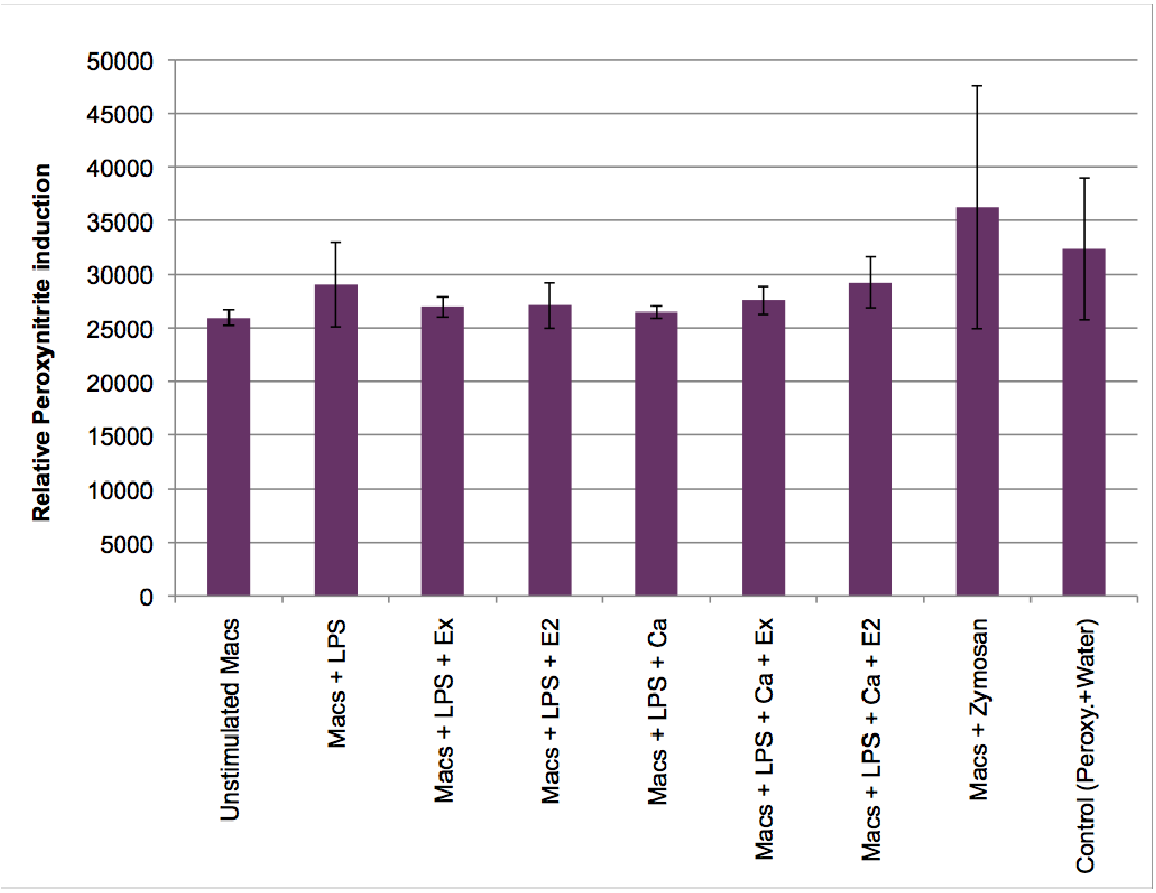

Figure 23. Effect of PGE_2 and PGEx on NO production by macrophages. AW264.7 cells were plated at a concentration of $5x10^5$ cells/ml in 24-well plates and grown for 24 h. Cells were washed and stimulated with LPS (1µg/ml final concentration) and infected with *C. albicans* at MOI 10:1 for 3h. Both PGEx and PGE_2 were used at a final concentration of 2nM. Control cells were stimulated with zymosan (200µg/ml final concentration). Cells were incubated at 37°C, 5% CO_2 for 3h. The plate was centrifuged 1000 rpm for 10 min and peroxynitrite levels in cell supernatants were assayed using a peroxynitrite detection kit according to the manufacturer's instructions. Data is representative of three independent repeats performed in triplicate.

DISCUSSION

Macrophages are potent antifungal effector cells that are required for defense against both mucosal and systemic infections with *C. albicans* [201, 210]. Macrophages have the ability to kill both yeast and hyphal forms of *C. albicans*. However, intracellular killing is restricted to the yeast form, as the hyphal form is not phagocytosed, presumably due to the large size [211]. Intracellular killing is particularly important, as survival within the macrophage could lead to dissemination using the host cell as a vehicle to evade other immune effector responses. Therefore, interactions that govern intracellular survival are of interest in terms of controlling infection. *C. albicans* produces a PGE_2 cross reactive compound called PGEx from endogenous lipid precursors that behaves like host PGE_2. Because PGEx works similar to PGE_2, it is likely that the ring group which is recognized by EP receptors is similar, but the overall carbon length in shorter. In addition, in the presence of arachidonic acid, *Candida* produces copious amounts of authentic PGE_2. Approximately 99.9% of the PGEx derived from arachidonic acid is PGE_2. We therefore tested the effects of commercially available PGE_2 and fungal PGEx (99.9% PGE_2) on influencing macrophage antifungal activity.

Our data shows that PGEx and PGE_2 can decrease phagocytosis and increase intracellular survival of *C. albicans* within macrophages. Interestingly, these results were more dramatic with PGEx, compared with PGE_2. Firstly, the percentage of phagocytosis decreased by about 20% in the PGEx treated

groups, whereas in the PGE_2 treated group, percent phagocytosis came down by about 10% (Figure 1). Secondly, we observed an increase in the intracellular survival of *C. albicans* inside the macrophages by about 10% in the PGE_2 treated group and approximately by 60% in the PGEx treated group compared to the control (Figure 2). This was further verified with our fluorescence microscopy data (Figure 3 and Figure 4). These experiments confirmed that host PGE_2 and fungal PGEx aid in overcoming antifungal defenses of macrophages. The fact that PGEx produced a more potent response may be due to effects of the endogenous PGE_2 cross-reactive compound that is co-isolated with fungal PGE_2. In addition, this may be due to slight variations in the concentration of PGEx. Concentrations of PGEx were determined by ELISA, while the commercially available PGE_2 was determined by analytical methods by the manufacturer.

The effect of PGE_2 and PGEx on increasing intracellular survival did not appear to be due to the direct effect of prostaglandins on C. albicans growth. This is based upon our observation from the growth curve study over a period of 48h (Figure 5). There was no change in the density of *C. albicans* in presence of prostaglandins compared to untreated cells. *C. albicans* can use arachidonic acid as a sole carbon source, which is structurally very similar to PGE_2 [212]. However, levels of this fatty acid that promote growth are much higher than those used in our assays (micromolecular vs. nanomolecular). Therefore, it is unlikely that prostaglandin treatment was used as a nutrient by *C. albicans* during macrophage infection. This data implies that there is perhaps some other

uncharacterized mechanism implemented by prostaglandins that aids in the intracellular survival of *C. albicans* inside RAW264.7 cells.

Preliminary advances have been made in our understanding of how *C. albicans* interacts with RAW264.7 cells in presence of prostaglandins, but large gaps in our knowledge still remains to verify the underlying mechanism. Macrophages employ several candidacidal mechanism to eliminate *C. albicans* [202]. Treatment with PGE_2 or PGEx resulted in increased intracellular survival, which may be due to effects on one specific or combination of various candidacidal mechanisms such as suppression of reactive oxygen, nitrogen intermediates, alteration of trafficking or pH. Previous studies have depicted that hyphal form but not yeast form are susceptible to extracellular antifungal mechanisms employed by macrophages, which likely involves reactive oxidative products [211]**.** Therefore, it is assumed that the yeast form must be eliminated by phagocytosis and intracellular killing methods.

Both reactive oxygen and nitrogen species (ROI/RNI) production contribute to the candidicidal activity of macrophages, with nitric oxide and peroxynitrite exhibiting the strongest antifungal activity [205, 209]. In addition, a complex “cross-regulation” takes place between the COX and NO pathways. It has been reported that PGE_2 can either activate or inhibit NO production depending on the microenvironment NO [213, 214], in part derived from iNOS, negatively regulates the immediate-early induction of COX-2 in response to inflammatory stimuli [215]. Therefore, we examined the effect of host and fungal prostaglandins on RNI production by macrophages. Our data indicate that a nitric

oxide synthase inhibitor slightly reduced NO production from macrophages, which resulted in increased fungal burden in control and PGE_2 treated groups. However, with PGEx treated groups, we observed no increase in fungal burden in the presence of the inhibitor. This may be due to the fact that fungal burden was already maximal with PGE_2 treatment. However, neither PGE_2 nor PGEx alone inhibited nitric oxide or peroxynitrite production; therefore, this is not the mechanism of action of these compounds.

Another main antimicrobial mechanism employed by macrophages is intracellular killing by phagolysosomal fusion, which results in a reduction in pH and activation of degradative enzymes. It has been reported that phagocytosed yeast traffic to late endosomes and fuse with lysosomes [216]. This is followed by germination into hyphae, which distends the host cell membranes, resulting in escape from the macrophage. Germination was found to be dependent on the reduction in pH associated with phagolysosomal fusion. Hyphae, which are not phagocytosed, can penetrate macrophages and also acquire lysosomal markers on the surface of the endosome. This indicates that internalization and phagolysosomal fusion *C. albicans* favors intracellular survival and potentially virulence [216]. Keeping that in mind, it would be worthwhile to study the effects of PGEx and PGE_2 on changes in phagolysososmal fusion and pH during *C. albicans* infection.

In summary, our results suggest that host PGE_2 and fungal PGEx can influence fungal survival during interaction of *C. albicans* with macrophages. In terms of fungal infection, production of prostaglandin by either the host or the

fungus may influence the outcome of the interaction. However, fungal PGEx is more potent than host PGE_2 overall. Intracellularly, this compound may exert a more directed response, inhibiting antifungal effector activity within the target cell. Therefore, development of drugs that specifically inhibit fungal prostaglandin production is warranted.

CHAPTER 3

Role of Eicosanoids in Defense Against Mucosal and Systemic Candidiasis

ABSTRACT

Candida albicans produces both endogenous prostaglandin-like compounds as well as authentic prostaglandins (PGE_2) from host arachidonic acid. Prostaglandin production is highest in the hyphal or biofilm form of *C. albicans*, which are both associated with increased virulence or ability to cause infection. Synthesis of fungal prostaglandins can be inhibited in vitro with eicosanoid inhibitors. In addition, these inhibitors can interfere with *C. albicans* germination and biofilm formation. Therefore, we tested whether eicosanoid inhibitors can affect the outcome of either mucosal infection (vaginitis) or systemic infection (intravenous route) in mice. We compared the effects of a traditional antifungal drug, amphotericin B, with two eicosanoid inhibitors that had been previously shown to inhibit *C. albicans* eicosanoid production in vitro (NDGA and indomethacin). NDGA is a plant polyphenol and inhibits both COX and lipoxygenase (LO), while indomethacin is a non-selective COX inhibitor. In both types of infections, NDGA treatment resulted in decreased fungal burden. This correlated with decreased PGE_2 levels in the host, as well as alterations in cytokine expression. Therefore, eicosanoid inhibitors may useful in combination with conventional antifungal agents in treating fungal infections.

INTRODUCTION

Mucosal and systemic infections caused by *C. albicans* pose a significant clinical threat to the immunocompromised and immunocompetent population [2, 3]. Various persistent forms of infections such as recurrent vaginitis, are associated with increased *C. albicans* colonization and are associated with increased PGE_2 levels locally [217]. Previous studies have demonstrated the production of PGE_2 from both *C. albicans* and host cells, although, the understanding of their role towards pathogenesis and persistence is still at nascent stage. Thus prostaglandins might have some key role to play towards promoting fungal virulence. Therefore, it is important to study the effect of this compound in *C. albicans* colonization, infection, and modulation of immunity.

Prostaglandins are pleiotropic molecules that regulate a variety of physiological processes including modulation of immune response [218]. They can elicit both pro- and anti-inflammatory responses. In terms of innate immunity, PGE_2 can inhibit macrophage phagocytosis and activation and influence dendritic cell maturation and cytokine production. In terms of adaptive immunity, PGE_2 has also shown to be able to inhibit Th1 type of adaptive immune response and promote Th2 type of responses, the later being associated with non-protective immune response to *C. albicans* infection [219-222]. Prostaglandins can be produced by both Candida and the host cells [156, 160, 223]. In mammalian cells, prostaglandins are produced from the arachidonic acid precursor by the action of cyclooxygenase (COX) enzymes, both COX-1 and COX-2. COX-1 is constitutively expressed for steady state production of

eicosanoids, whereas COX-2 is an inducible form associated with immune responses [224]. Previous in vitro studies have demonstrated that *C. albicans* induces PGE_2 production from host cells via the COX-2 pathway [161]. Precursors of *C. albicans* oxylipins can also be used by COX-2 to produce array of other lipid compounds. This emphasizes the presence of transcellular host/fungal eicosanoid pathway [225]. Studies by Douglas et al. have depicted that nonsteroidal anti-inflammatory drugs (NSAIDS), which are cyclooxygenase inhibitors, had inhibitory effects on *C. albicans* germination and biofilm formation, activities associated with infection and virulence [226]. These drugs have the capacity to inhibit the prostaglandin formation from host cells by inhibiting the action of the enzyme(s) COX-1 or COX-2 or both [224]. To this end, in this study we examined the role of host eicosanoids in both mucosal (vaginitis) and systemic infection models through administration of prostaglandin inhibitors.

MATERIALS AND METHODS

Mice. Female retired breeders or 6-8 week old mice of C57BL/6 strain were obtained from Charles River Laboratories (Wilmington, MA). All mice were maintained at an American Association for the Accreditation of Laboratory Animal Care-accredited animal facility at Wayne State University (WSU) and housed in a pathogen-free environment in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals under an animal study proposal approved by the WSU animal investigation committee.

***C. albicans* Strains and Culture Conditions.** The *C. albicans* strain SC5314 used in this study is a prototrophic parental strain [168]. This strain was subcultured from frozen stocks and grown overnight at 30°C in Sabouraud dextrose agar (SDA) (Becton, Dickinson and Company, Sparks, MD). For liquid cultures, isolated colonies from the SDA plates were cultured in Sabouraud dextrose broth (SDB) with constant shaking. To obtain hyphal form, yeast form of *C. albicans* was added to RPMI supplemented with 10% FBS at the concentration of 1 x 10^6 *Candida* per ml of media. Hyphae were generated after 2 h incubation at 37°C.

Vaginal infection model. Three days prior to inoculation, mice were subcutaneously injected with 0.01 mg/mouse of 17β–estradiol valerate (Sigma)

dissolved in sesame oil. This was followed with estrogen administration at every one week interval [43]. The control groups of mice received estrogen treatment and were mock inoculated with PBS. On day 0, mice were inoculated intravaginally with $5x10^6$ *C. albicans* in 30µl PBS using a pipette. On day 2, mice were administered amphotericin B or eicosanoid inhibitors (NDGA, indoemethacin) vaginally at the concentration of 25 mg/kg. After 6 days of drug administration, the mice were euthanized on day 7 and the CFU were recovered after lavaging vaginal tissues twice with sterile PBS (each wash 50µl). The vaginal fungal burden was analyzed by performing serial dilutions of vaginal lavage samples and plating them on SDA plates.

Vaginal Homogenates. For cytokine analysis in the vaginitis model, vaginal tissue was excised and homogenized in 1ml of buffer containing PBS, 0.05% Triton X-100, and protease inhibitor. This volume was further centrifuged at 800g for 5 min to remove the cellular debris and the clear supernatant was collected for cytokine analysis. Previous studies have demonstrated that presence or absence of homogenized tissue does not interfere with the levels of endogenous cytokines following the homogenization process [43].

Systemic infection model. Mice were challenged by intravenous tail injection with *C. albicans* at the concentration of 2.5 X 10^5 CFU/mouse in 250 µl of sterile PBS using a 30-gauge needle attached to a 1-ml syringe. The mice challenged with *C. albicans* were treated with amphotericin B (amphotericin B) or eicosanoid

inhibitors (nordihydroguaretic acid/NDGA), indomethacin) at different concentrations (5, 10, 25 mg/kg) subcutaneously daily for 6 post-infection. On day 7, the kidneys were harvested for CFU analysis and the spleens for cytokine analysis.

CFU Assay. To determine the amount of fungal growth in the organs of the mice, the kidney and the liver were weighed and homogenized using a tissue tearor. Serial dilutions of tissue homogenates were made in sterile water and were plated onto SD agar to determine the CFU per gram per organ in each group of mice.

Splenocyte cultures. For the analysis of cytokines induces in the systemic model, spleens were excised from the *C. albicans* infected mice and the splenocytes were isolated from them. A 1:1 ratio of both yeast and hyphal forms of *C. albicans* were used at MOI 1:2 to stimulate the splenocytes. After 2 h of incubation, 2.5 µg/ml of amphotericin B was added to prevent *Candida* overgrowth followed by further incubation of 16 h at 37°C, 5% CO_2 incubator. The supernatants were collected and the levels of cytokines were measured by ELISA.

Splenocyte Harvest and Pulsing. Spleens were excised from the *C. albicans* infected mice and the splenocytes were isolated from them. A 1:1 ratio of both yeast and hyphal forms of *C. albicans* were used at MOI 1:2 to stimulate the

splenocytes. After 2 h of incubation, 2.5 μg/ml of amphotericin B was added to prevent *Candida* overgrowth followed by further incubation of 16 h at 37°C, 5% CO_2 incubator. The supernatants were collected and the levels of cytokines were measured by ELISA.

Enzyme Linked Immunosorbent Assay. Cell culture supernatants (DCs and splenocyotes) were analyzed for the following cytokines by ELISA: IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IFN-γ, TGF-β, TNF-α, and IL-23. The detection limits (pg/ml) for the assays were <3.90 for IL-4, < 3.90 for IL-5, <3.90 for IL-6, < 31.25 for IL-10, <15.62 for IL-12, <3.90 for IL-13, < 15.62 for IFN-γ, <62.5 for TGF-β, <7.81 for TNF-α and <7.81 for IL-23 (eBioscience). In addition, vaginal lavages and serum samples were also tested to analyze the levels of PGE_2 (Cayman chemicals). The absorbance of the plates was measured at 450nm using Multiskan Ex microplate reader (Thermo Electron Corporation) and analyzed with Ascent Software.

Statistical Analysis. The Student's *t* test (two-tailed, unequal variance) was used to analyze the significance of differences between two experimental groups. Significance was considered with a $P \leq 0.05$ or less. The data recorded are representative of either two or three independent experiments.

RESULTS

To investigate the effects of eicosanoid inhibitors on the course of mucosal candidiasis, we inoculated the estrogenized mice vaginally with *C. albicans.* We compared the effects of a traditional antifungal drug, amphotericin B, with two eicosanoid inhibitors that had been previously shown to inhibit *C. albicans* eicosanoid production in vitro (NDGA and indomethacin) [154]. NDGA is a plant polyphenol and inhibits both COX and lipoxygenase (LO), while indomethacin is a non-selective COX inhibitor. The drugs were administered vaginally daily from day 2 to day 7 at a concentration of 25 mg/kg. This concentration was chosen based on previous studies using topical or oral administration [227-229]. On day 8, the mice were sacrificed and their vaginal fungal burden and induced cytokine levels from the vagina were evaluated. As expected, local amphotericin B treatment reduced vaginal fungal burden by approximately 2 logs (Figure 24). Surprisingly, treatment with NDGA was more effective than amphotericin B at reducing *C. albicans* growth, with approximately 3 logs reduction. However, treatment with indomethacin had no significant effect on fungal burden.

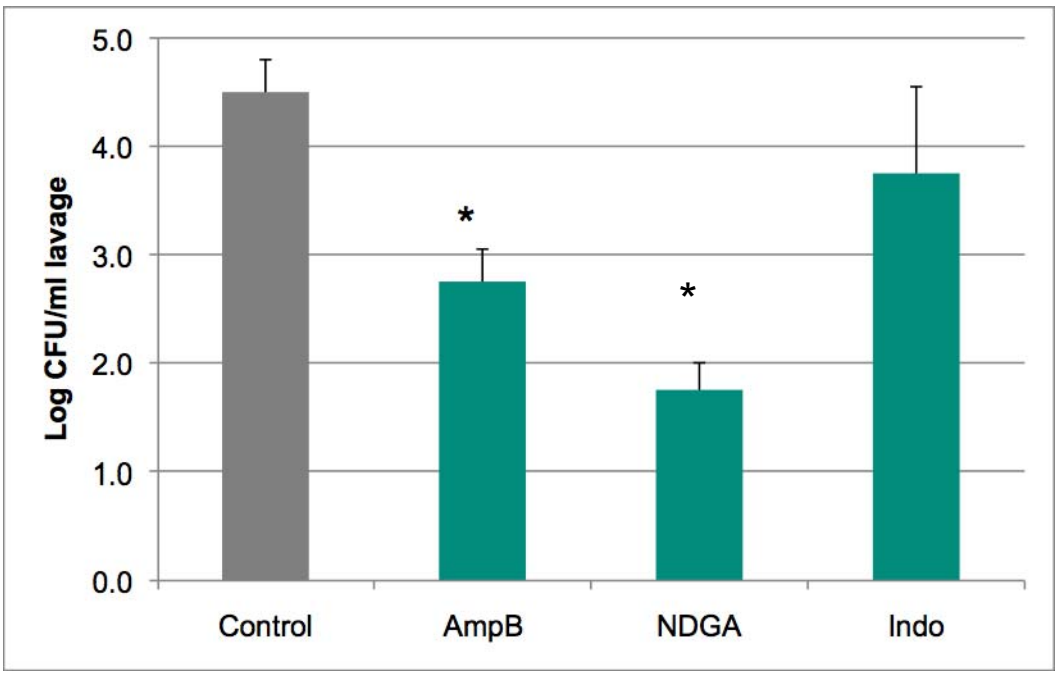

Figure 24. Effect of eicosanoid inhibitors or antifungal drugs on *C. albicans* burden in experimental vaginitis. On day 0, estrogenized mice were inoculated vaginally with $5X10^6$ candida per 10µl PBS. On day 2, eicosanoid inhibitors (NDGA, Indomethacin) or antifungal drugs (amphotericin B) were administered vaginally at the concentration of 25 mg/kg/mice. After 6 days of drug administration, the mice were euthanised on day 7 and the CFU were recovered after lavaging vaginal tissues twice with sterile PBS (each wash 50µl) followed by serial dilution and plating on SDA plates. Results are representative of three independent repeats. * $P < 0.05$ treated vs. untreated group.

To examine effects of drug treatment on vaginal immune mediator production, PGE_2 levels were analyzed in vaginal lavage samples. Compared with uninfected mice, vaginal tissues from mice infected with *C. albicans* showed induction of significant levels of PGE_2 (Figure 25). Treatment with amphotericin B resulted in a reduction in PGE_2 levels, which could be attributable to reduced fungal burden. Treatment with NDGA had the most significant effect on reducing PGE_2 levels, lowering to approximately 100 pg/ml. Interestingly, indomethacin treated mice showed the induction of highest concentration of PGE_2 induction, indicating this drug had no effect on inhibiting eicosanoid production.

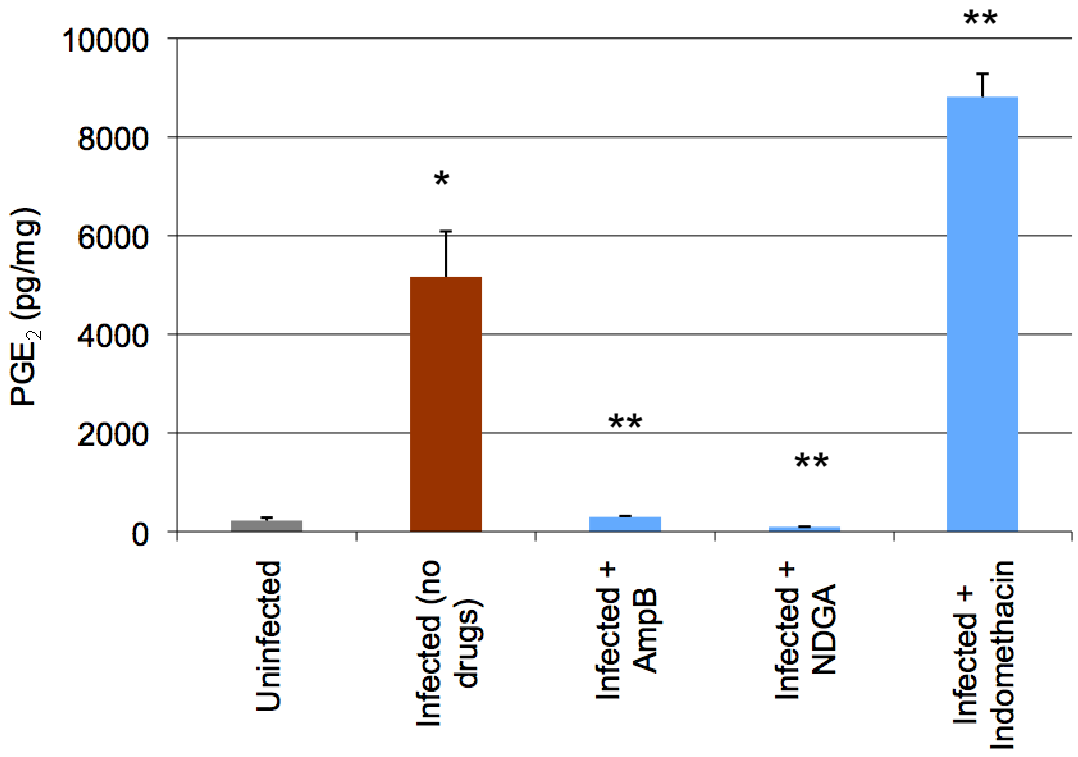

Figure 25. Effect of eicosanoid inhibitors or antifungal drugs on vaginal PGE_2 levels during experimental vaginitis. On day 0, estrogenized mice were inoculated vaginally with $5X10^6$ candida per 10µl PBS. On day 2, eicosanoid inhibitors (NDGA, Indomethacin) or antifungal drugs (amphotericin B) diluted in 10µl of DMSO were administered vaginally at the concentration of 25 mg/kg/mice. After 6 days of drug administration, the mice were euthanised on day 8 and the vaginal tissues were lavaged twice with sterile PBS (each wash 50ul). This homogenized tissue was centrifuged and the supernatant was used to measure PGE_2 levels by ELISA. Results are representative of three independent repeats. * $P < 0.05$ infected vs. uninfected group. ** $P < 0.05$ treated vs. untreated group.

We next analyzed local cytokines responses in vaginal homogenates. We observed a reduction in pro-inflammatory cytokine IFN-γ in infection mice compared with uninfected mice; however, overall levels of this cytokine are relatively low (Figure 26). Treatment with NDGA partially restored IFN-γ production. Infected mice also showed reduced IL-10, which can promote tolerogenic responses at mucosal surfaces. Treatment with NDGA partially restored IL-10 levels. No significant levels of IL-4 or TGF-β were detected in vaginal homogenates.

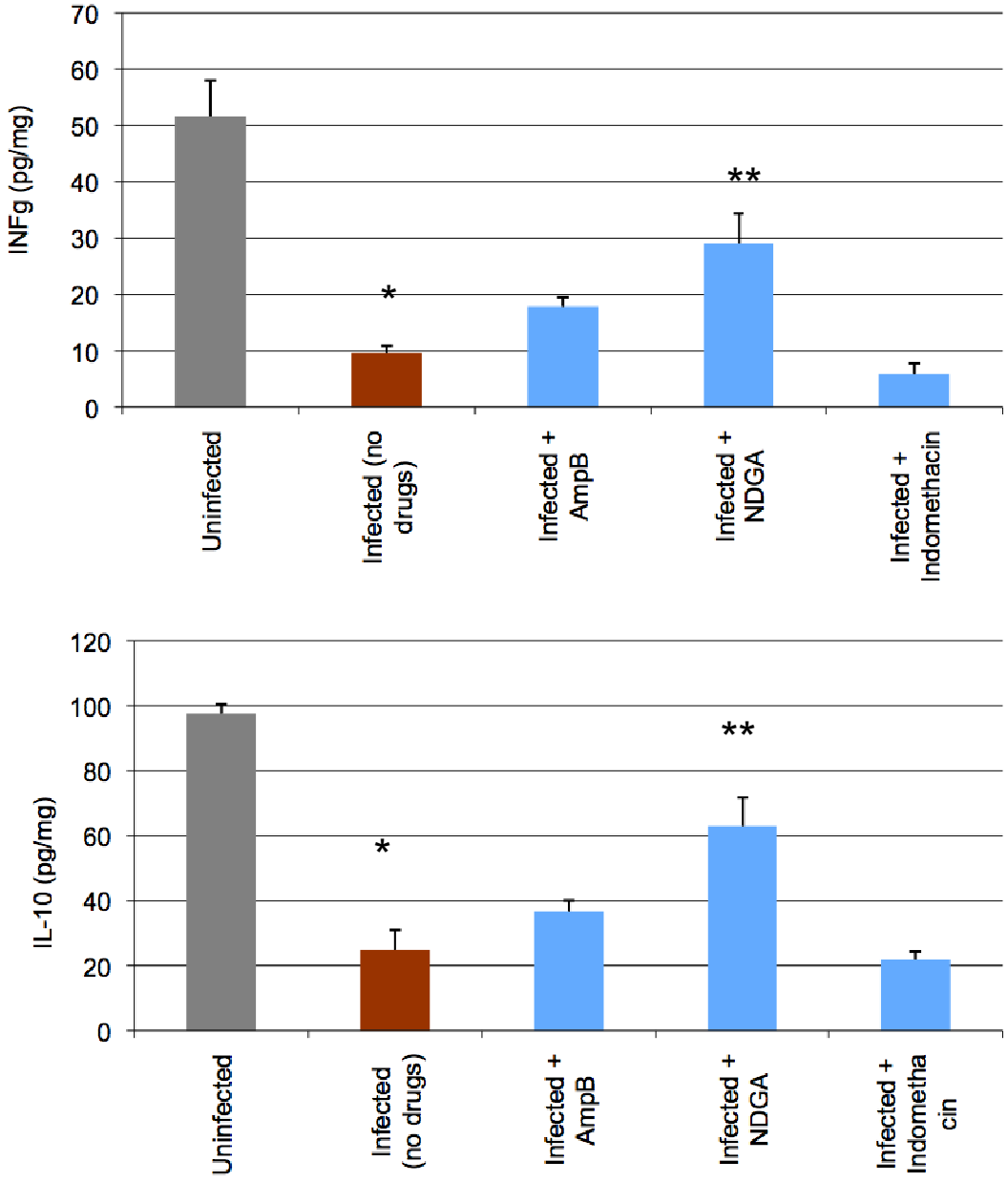

Figure 26. Effect of eicosanoid inhibitors or antifungal drugs on vaginal cytokine levels during experimental vaginitis. On day 0, estrogenized mice were inoculated vaginally with $5X10^6$ candida per 10µl PBS. On day 2, eicosanoid inhibitors (NDGA, Indomethacin) or antifungal drugs (amphotericin B) diluted in 10µl of DMSO were administered vaginally at the concentration of 25 mg/kg/mice. After 6 days of drug administration, the mice were euthanised on day 8 and the vagina was excised and homogenized in 1ml sterile buffer containing PBS, 0.05% Triton X-100, and protease inhibitor. This homogenized

tissue was centrifuged and the supernatant was used to measure cytokine levels by ELISA. Results are representative of three independent repeats. * $P < 0.05$ treated vs. untreated group. ** $P < 0.05$ treated vs. untreated group.

To evaluate effects of eicosanoid inhibitors during systemic infection, mice were infected intravenously and treated with inhibitors by IP injection. After 6 days of daily administration of drugs, the mice were sacrificed on day 7 and kidneys analyzed for fungal burden. After treatment with amphotericin B, lowest fungal burden was observed. Compared with control mice, treatment with amphotericin B significantly reduced fungal burden in the kidney by approximately 4 logs (Figure 27). Treatment with NDGA also reduced fungal burden by approximately 2 logs. However, we observed no effect with indomethacin treatment.

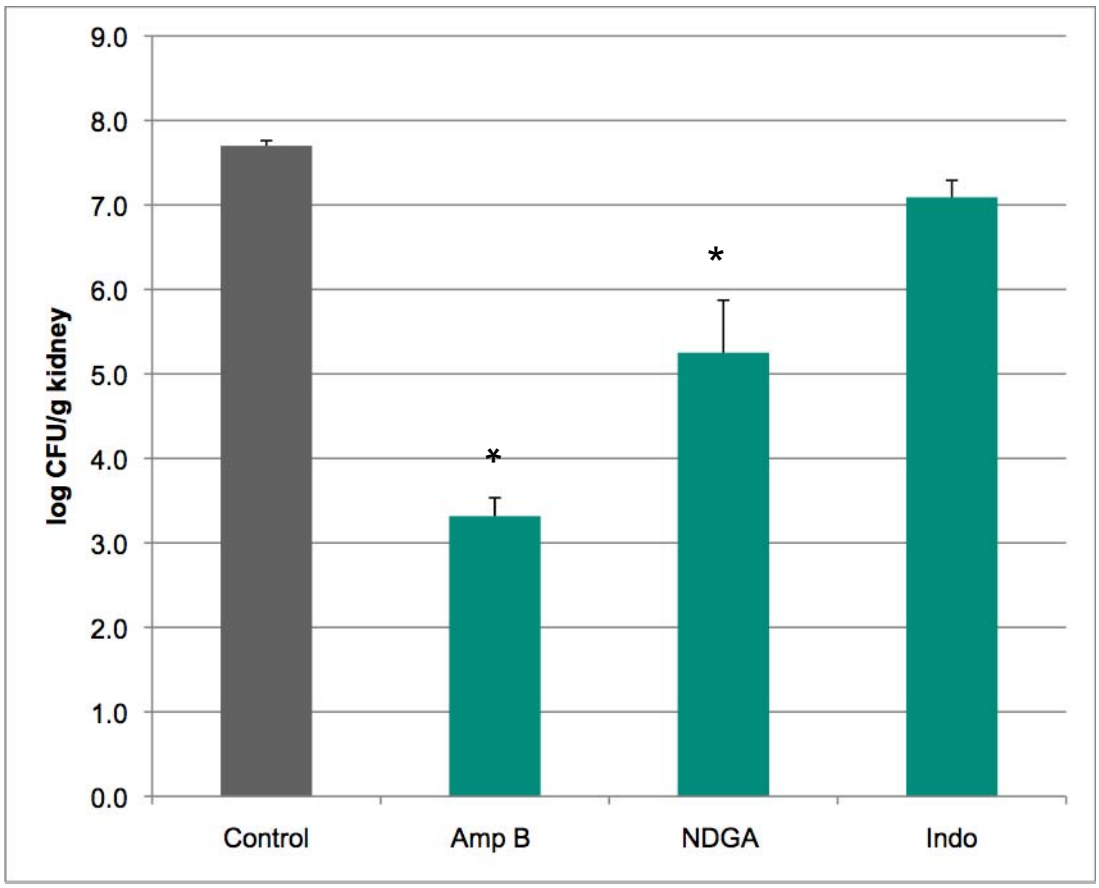

Figure 27. Effect of eicosanoid inhibitors or antifungal drugs on *C. albicans* kidney burden during systemic infection. On day 0, the mice were challenged by intravenous tail injection with *C. albicans* at the concentration of 2.5 x 10^5 CFU/mouse in 250 µl of sterile endotoxin-free PBS. From day 1 to day 6, mice were daily administered drugs via intraperitoneal route at a concentration of 5 mg/kg for NDGA, indomethacin, and aspirin and 0.1 mg/kg for amphotericin B. After 7 days post-inoculation, the mice were sacrificed, the kidneys excised, homogenized and analyzed for fungal burden by serial dilution and plating onto SD plates. Results are representative of three independent repeats. * $P < 0.05$ treated vs. untreated group.

Analysis of PGE_2 levels in serum samples revealed increased levels in mice infected with *C. albicans*, compared with uninfected mice (Figure 28). Treatment with NDGA and amphotericin B resulted in a reduction in PGE_2 levels, while indomethacin had little effect. To determine the effect of eicosanoid inhibitors on immune responses during systemic infection, the spleens were harvested on day 7 following the daily drug treatment and cytokines were analyzed in restimulated splenocyte cultures. Compared with uninfected animals, there was a reduction in IFN-γ levels in infected mice (Figure 29). Treatment with amphotericin B or NDGA restored IFN-γ production in splenocytes. A similar trend was observed with pro-inflammatory cytokine TNF-α. Infected mice showed induction of this cyotkine, which was further increased with amphotericin B treatment. Induction of the Th1 cytokine IL-12 was only observed in amphotericin B treated animals, which correlates with reduced fungal burden. Levels of IL-4 were extremely low, although levels correlated with fungal burden, with lowest levels observed with ampB treatment. Overall this indicates that infection is associated with reduced Th1 promoting cytokines (IFN–γ and IL-12) and a reduction in fungal burden results in increased pro-inflammatory cytokines (IFN-γ and TNF-α).

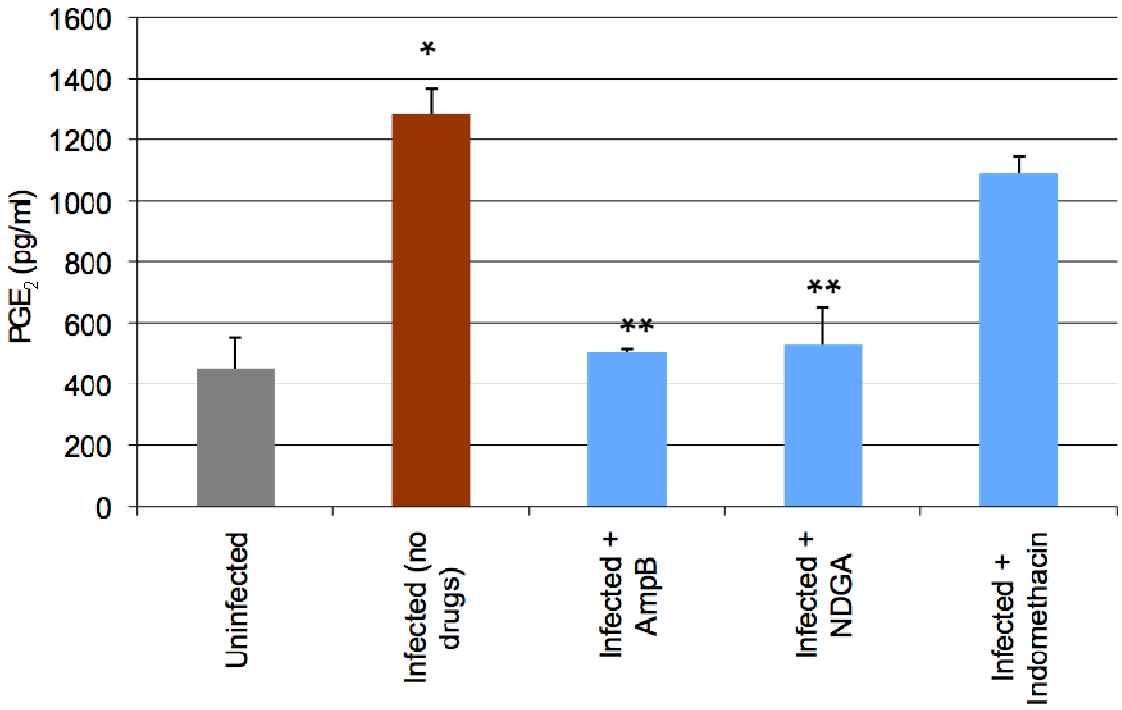

Figure 28. Effect of eicosanoid inhibitors or antifungal drugs on serum PGE_2 levels in mice systemically infected with *C. albicans*. On day 0, the mice were challenged by intravenous tail injection with *C. albicans* at the concentration of 2.5 x 10^5 CFU/mouse in 250 µl of sterile endotoxin-free PBS. From day 1 to day 6, they were daily administered drugs via intraperitoneal route at a concentration of 5 mg/kg for NDGA and indomethacin. For amphotericin B, 0.1 mg/kg/mice dose was used. On day 7, mice were sacrificed, their blood collected in BD yellow vacutainer and spun at 1000xg. The serum was collected to perform ELISA using monoclonal PGE_2 kit. Results are representative of three independent repeats. * $P < 0.05$ treated vs. untreated group. ** $P < 0.05$ treated vs. untreated group.

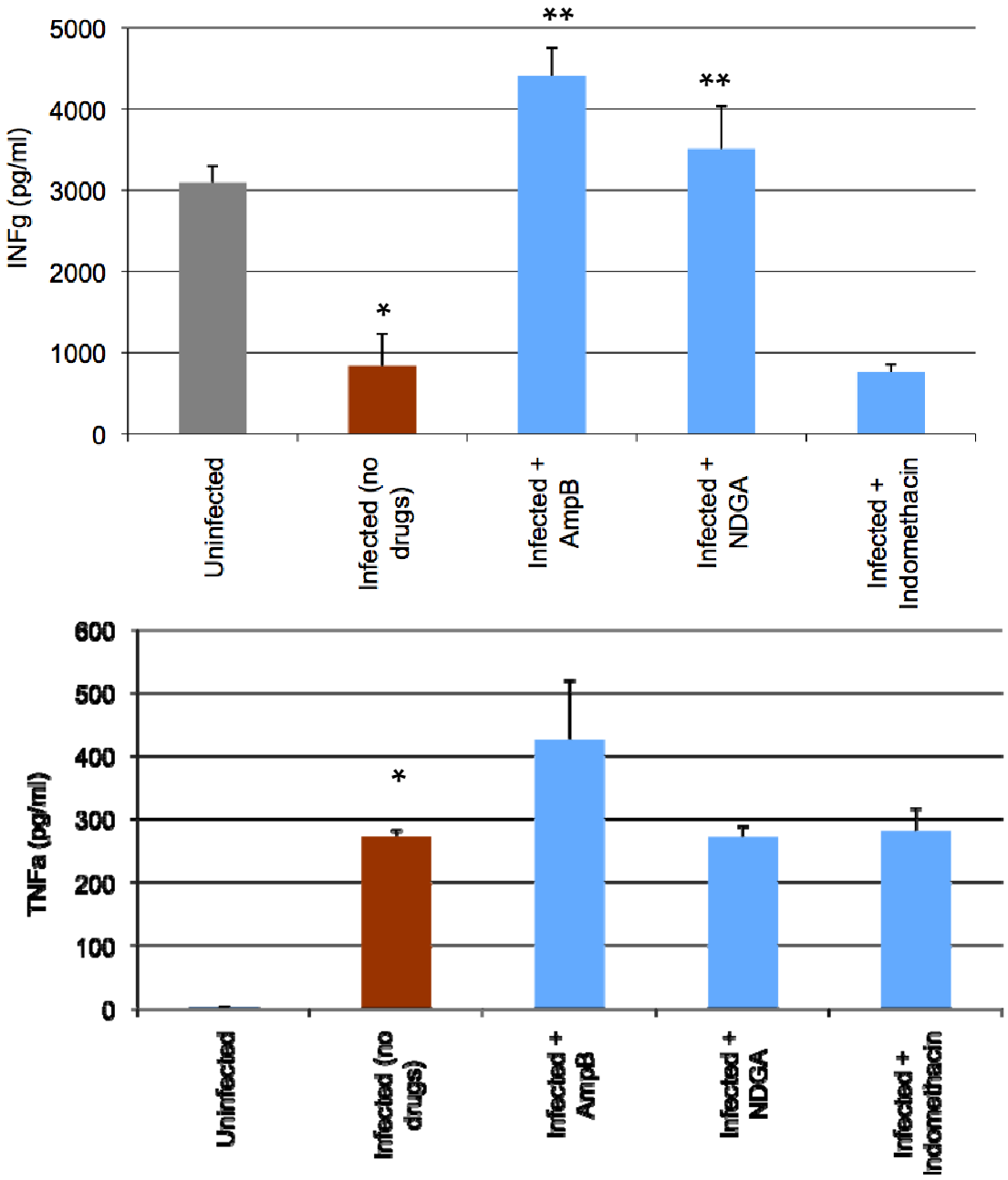

Figure 29. Effect of eicosanoid inhibitors or antifungal drugs on splenic cytokine levels in mice systemically infected with *C. albicans*. On day 0, mice were challenged on day 1 via intravenous tail vein injection with *C. albicans* at the concentration of 2.5 x 10^5 CFU/mouse in 250 µl of sterile endotoxin-free PBS. From day 2 to day 6, they were daily administered drugs via intraperitoneal route at a concentration of 5 mg/kg for NDGA and indomethacin. For amphotericin B, 0.1 mg/kg/mice dose was used. On day 7, mice were sacrificed and their spleens were excised and the splenocytes were isolated. *C. albicans* were used at MOI 1:2 to stimulate the splenocytes. After 2 h of incubation, 2.5

μg/ml of amphotericin B was added to prevent *Candida* overgrowth followed by further incubation of 16 h at 37°C, 5% CO_2 incubator. The supernatants were collected and the levels of cytokines were measured by ELISA. Results are representative of three independent repeats. * $P < 0.05$ treated vs. untreated group.

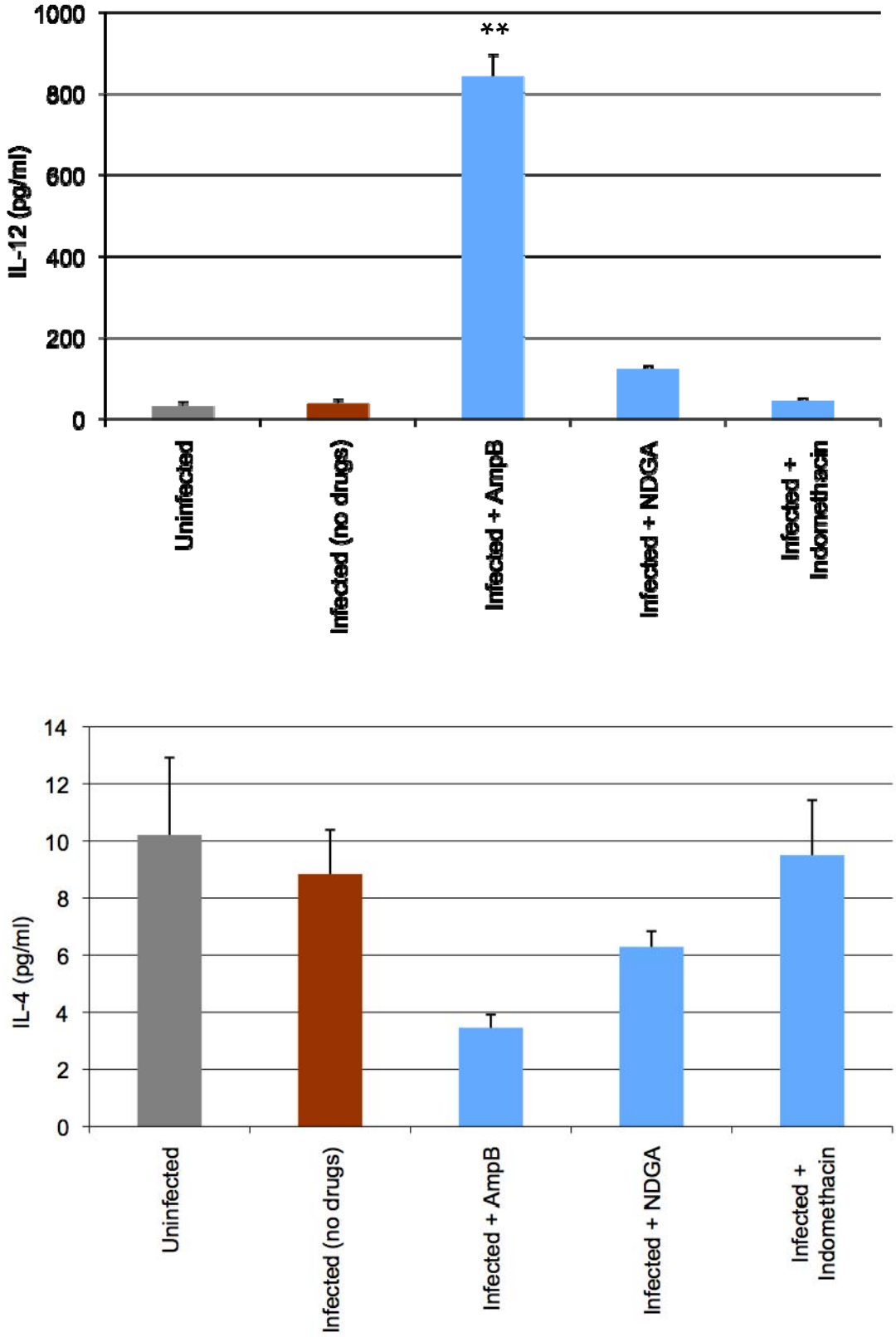

Figure 29 Continued

DISCUSSION

PGE_2 is a pleiotropic molecule that exerts various effects on immune responses depending on the microenvironment, tissue, and cells involved [134, 230]. In addition, PGE_2 and fungal PGEx promote germination and biofilm formation by *C. albicans* [231, 232]. The ability to form hyphae is required for virulence during systemic infection [13]. In addition, hyphal forms and ability to germinate are associated with symptomatic vaginal infections [233, 234]. However, the role of PGE_2 and other eicosanoids towards immunomodulation in mucosal and systemic candidiasis is still largely unknown. In vitro, exposure to eicosanoid inhibitors reduces fungal prostaglandin production, germination, and biofilm formation [154, 157, 226, 231]. At higher doses, these drugs can exert antifungal activity [152, 232, 235]. Therefore, we tested the ability of these drugs to inhibit fungal growth during mucosal and systemic infections and investigate effects on immune responses.

Vulvovaginal candidiasis (VVC) is a very common infection among women. *C. albicans* is the responsible causal organism in up to 80% of vaginitis cases [236]. Studies have shown that unlike systemic infection caused by *C. albicans*, vaginal infection is more associated with local immunity [74, 149, 237]. Recent studies have reported an increased levels of PGE_2 from recurrent VVC patients [150]. *C. albicans* has been shown to directly stimulate PGE_2 production from vaginal epithelial cells [152]. In addition, exogenous PGE_2

administration increases *C. albicans* vaginal infection levels during experimental vaginitis [232]. However, the immunomodulatory effects of prostaglandins during this local immune response are poorly understood [238].

Treatment with the non-specific LO/COX inhibitor NDGA locally during experimental vaginitis dramatically reduced fungal burden. At similar doses, NDGA was more effective than amphotericin B. However, a non-selective COX inhibitor, indomethacin, had no effect, indicating that daily administration of drugs is not having a non-specific effect on fungal colonization levels. At this point it is unclear if the differences in the fungal burden is a consequence of hyphae versus yeast numbers that could result in differences in cfu burden. Previous work by Noverr et al demonstrated the effect of COX-inhibitors towards the inhibition of Candida germination. In both amphotericin B and NDGA treated mice, PGE_2 levels were reduced compared with untreated mice. In the case of NDGA, this is likely due to inhibition of host cell and/or fungal PGE_2 production as previously observed in vitro [154, 239, 240]. Interestingly, PGE_2 production was elevated in the indomethacin treated mice. This was unexpected; however, biphasic effects of indomethacin on prostaglandin production have been reported, with low doses promoting PGE_2 production [241]. It is unknown what the local levels of indomethacin are during vaginal treatment, but this may be one explanation for these observations. Overall, fungal burden correlated with PGE_2 levels. This might be indicative of the fact that high concentrations of PGE_2 are associated with susceptibility towards developing vaginitis.

Analysis of the associated local cytokines from the vaginal tissues showed that infection resulted in reduced pro-inflammatory IFN-γ production. This has also been observed clinically, with decreased IFN-γ associated with recurrent vaginitis [242]. Treatment with either amphotericin B or NDGA resulted in increased IFN- γ, which may be due to reduced fungal burden, or due to reduced PGE_2 levels, which can inhibit IFN- γ [243]. There was also reduced IL-10 production in infected mice, which has been reported previously in experimental vaginitis [244]. IL-10 is an anti-inflammatory cytokine, which can inhibit activation of innate effector cells, which mediate the symptoms of vaginitis. IL-10 levels tend to correlate with fungal burden, as infection induces pro-inflammatory responses [245]. Accordingly, Amphotericin B and NDGA treatment partially rescued IL-10 production. While PGE_2 is a known inducer of IL-10, we hypothesize that the lack of fungal burden and antigenic stimulation is responsible for reduced IL-10 production [246]. Overall, these results emphasized the fact that increased levels of PGE_2 are associated with increased fungal burden and cytokines associated with local immune responses during infection.

Systemic candidiasis is a clinically important infection and is associated with high mortality rates [2, 3]. Both innate and adaptive immunity is required to mount a protective response to infection. Treatment of mice with amphotericin B reduced kidney fungal burden. In addition, treatment with NDGA also reduced C. albicans levels. This was associated with a reduction in serum PGE_2 levels. Similar to the vaginal infection, overall *C. albicans* levels correlated with PGE_2

levels. Studies have shown the role of prostaglandins in generating non-protective responses by dampening anti-fungal defenses [189]. We also observed that treatment with amphotericin B and NDGA was associated with elevated splenic IFN-γ and IL-12, which are required for generation of protective Th1 responses during systemic infection [247]. High fungal burden in untreated mice was associated with slightly increased IL-4 and low IL-12, indicative of a non-protective Th2 response. This association of fungal burden with Th1 vs. Th2 responses has been demonstrated in previous work with mice having disseminated candidiasis [74, 248]. Unlike like NDGA and amphotericin B, indomethacin was not able to reduce the levels of PGE_2 and the balance tilted more towards non-protective response. This may be due to alternative effects of indomethacin on the host immune response. For example, β-glucan, a fungal cell wall component, combined with indomethacin can result in immunocompromise leading to sepsis from the GI tract and mortality in mice [249]. The lethality is strongly related to disruption of the cytokine network. Similar results were observed with killed whole cells of *C. albicans* [250]. Therefore, different eicosanoid inhibitors may have unforeseen effects on global inflammatory responses and must be tested in vivo to determine efficacy against fungal infection.

Although our study points to the fact that PGE_2 is one of the key molecules involved in the process of exacerbating a progressive form of disseminated candidiasis and aids in elevating Th2 responses, the underlying mechanism for this process is not yet clear. From the previous studies showing

the negative role played by the Th2 cytokines during disseminated candidiasis, inhibition of PGE_2 might prove beneficial for the host to clear-up the infection [205, 251]. It is unclear whether NDGA works to inhibit *C. albicans* growth by inhibiting PGE_2 production, or whether direct effects of NDGA on fungal killing resulting in reduced fungal burden causes a reduction in PGE_2. Regardless, the effect of this drug is beneficial and may exert synergistic effects on reducing fungal levels in vivo in combination with a traditional antifungal. Synergism between amphotericin B and NDGA has already been observed in vitro [232]. This is extremely important as resistance to both polyene drugs (amphotericin B drug class) and azoles are on the rise. New therapeutic strategies targeting both PGE_2 and fungal viability may be beneficial for treating fungal infections under these kinds of circumstances.

GENERAL CONCLUSION

Prostaglandins are pleiotropic compounds playing an array of immunomodulatory roles in the mammals. Depending on the type of interaction and the receptors involved, they can have multiple effects on different cells types at a very low (nanomolar) concentrations [134, 230]. Their roles have been widely associated with the effector functions of many immune cells including those of the innate immune system (macrophages, neutrophils), cells that bridge innate and adaptive responses (dendritic cells, epithelial cells), and adaptive immunity (T cells, B cells) [252]. They regulate both acute inflammatory responses as well as chronic inflammation and memory immunity. PGE_2 specifically influences phagocytosis and intracellular killing of microbial pathogens as well as cytokine release from macrophages. PGE_2 also regulates release of effector molecules, migration, maturation and secretion of immunomodulatory compounds from dendritic cells and lymphocytes [165, 167, 253-255].

The fact that *C. albicans* produces both endogenous PGE_2-like compounds as well as authentic PGE_2 from host arachidonic acid suggests that this fungal pathogen may directly influence host responses during colonization and/or infection. Production of eicosanoids by host cells can also be induced upon interaction by fungal pathogens. *C. albicans* has been shown to be able to induce the activation and transcription of host PGE_2 production from various immune cells such as macrophages, endothelial cells, and splenocytes [161,

188, 189]. Therefore both fungal derived and host derived eicosanoids can participate in driving immune responses during fungal infection. Our hypothesis is that Production of oxylipins by both fungi and host modulate the host-pathogen interaction in favor of non-resolving infection or persistence. The goal of this thesis was to determine the immunomodulatory roles of host and fungal eicosanoids during interactions of *C. albicans* with the host. For this purpose we studied the role of prostaglandins in vitro (with dendritic cells and macrophages) as well as in vivo using mucosal and systemic models.

From our studies with the effects of exogenous PGE_2 on dendritic cell-*Candida* interactions, the early cytokine analysis with both JAWSII cells and primary pDCs and mDCs revealed upregulation of Th2 cytokines (IL-4) and down-regulation of Th1 cytokines (IL-12) in the presence of hyphae. This is in agreement with the previous data reporting a bias towards non-protective Th2 cytokine responses in macrophages and monocytes in the presence of hyphae, but not yeast [80, 219, 220, 253-257]. However, the presence of PGE_2 was required for robust Th2 cytokine production from DCs in the presence of hyphae, which is an activity previously reported for PGE_2 [183]. Later cytokine analysis also showed a shift towards a DC2 phenotype in primary DCs stimulated with prostaglandins. The only significant difference in immune mediator production that varied between yeast and hyphae pulsed cells was in host PGE_2 production. Hyphae stimulated significant production at 18 h, the time point used to harvest DCs for adoptive transfer. This indicates that hyphae can prime DCs for PGE_2 release, which could influence nearby immune cells.

This difference in the pattern of PGE_2 induction could be due to differential PRR signaling. DCs recognize yeast and hyphal form of *C. albicans* via TL4 and TLR2 receptors [80, 115, 116]. Also, studies have indicated that *C. albicans* signaling via TLR2 pathway leads to the production of PGE_2 from the host and causes persistent type of infection [189]. Future studies in this area would involve examining DC PGE_2 production in the presence of yeast and hyphae in PRR knockout mice. Overall, these data demonstrate that depending on the morphotypes (yeast or hyphal form), the DCs have the ability to modulate the immune responses in presence of exogenously added prostaglandins.

We observed evidence from early cytokine analysis that effects of prostaglandin exposure are not limited to simply effects on Th1 and Th2 mediators. In addition, we demonstrated that pro-inflammatory cytokines IL-6 and IL-15 were upregulated in the hyphae/PGE_2 group. Early expression of SOCS-3, a suppressor of cytokine, involved in promoting a DC2 phenotype was also upregulated during this time point. [184]. This suggests that PGE_2 exposure can also stimulate pro-inflammatory mediators and that host cells counter-regulate this with SOCS synthesis. In addition, the CD86 expression was greatly increased in primary DCs pulsed with hyphae in the presence of PGE_2, which indicative of maturation and a heightened activation state. Therefore, it was unclear as to how these pulsed DCs would regulate subsequent adaptive responses upon vaccination.

For DC vaccination, two rounds of priming were necessary to induce robust responses. We showed that yeast pulsed pDCs imparts protection

whereas mDCs are less protective in mice with systemic infection. This non-protection was associated with splenic and kidney Th2 responses and at later stages, exacerbated local Th17 responses accompanied by uncontrolled fungal growth at the site of infection. Another major difference between the responses to mDCs and pDCs was evident in the kidney Th17 cytokines. Although we observed slight protection with double adoptive transfer with mDCs pulsed with yeast, there was dramatic IL-17 and IL-23 cytokine staining in the kidneys. The ability of mDCs to promote Th17 responses compared with pDCs has been reported previously [79]. Therefore, mDCs may not be appropriate for fungal vaccination strategies and these results underscore the importance and variability of DC subsets.

Our results are in alignment with other studies indicating that both IL-23 and IL-17 can inhibit neutrophil antifungal activity resulting in pathological form of inflammation associated with impaired antifungal resistance [79]. Therefore, although neutrophils were recruited to the kidneys, the antifungal activity is likely diminished, as indicated by the high fungal burden. To investigate this concept further, it would be necessary to isolate kidney or bloodstream PMNs and examine antifungal activity in vitro. While the Th17 response is required to defend against mucosal and acute systemic infections, ours is the first study to report presence of Th17 cytokines in the mice kidneys of vaccinated mice during systemic candidiasis and its associated lack of protection. This indicates that IL-17 helps initiate protective innate responses during *C. albicans* infections, but if left unchecked, may exacerbate immunopathological inflammation. Future

studies examining the role of Th17 responses during afferent vs. efferent responses are necessary to investigate this possibility.

We examined the effect of PGE_2 and PGEx during the interaction of *C. albicans* with the macrophage cell line, RAW264.7. In this case, we used PGEx derived from arachidonic acid, which is 99.9% PGE_2. Both the fungal derived and commercially available prostaglandins inhibited phagocytosis of *C. albicans*, while increasing intracellular survival in activated macrophages. Interestingly, these effects were more dramatic with PGEx, compared with PGE_2. These experiments confirmed that host PGE_2 and fungal PGEx aid in overcoming antifungal defenses of macrophages. The fact that PGEx produced a more potent response may be due to effects of the endogenous PGE_2 cross-reactive compound that is co-isolated with fungal PGE_2. However, we did not observe such dramatic differences in responses to PGEx vs. PGE_2 with dendritic cells in terms of promoting non-protective responses. To investigate the variable effects of specifically endogenous PGEx on host cells, it would be necessary to isolate the compound in enough quantity to perform experiments. Without addition of exogenous arachidonic acid, very small quantities of PGEx are produced. Therefore, these experiments would be difficult to perform.

We have made some advancement in our understanding on how *C. albicans* interacts with RAW264.7 cells in presence of prostaglandins, but large gaps in our knowledge still remains to verify the underlying mechanism. Both reactive oxygen and nitrogen species (ROI/RNI) production contribute to the candidicidal activity of macrophages, with nitric oxide and peroxynitrite exhibiting

the strongest antifungal activity. Our data indicate that a nitric oxide synthase inhibitor slightly reduced NO production from macrophages, which resulted in increased fungal burden in control and PGE_2 treated groups. However, with PGEx treated groups, we observed no increase in fungal burden in the presence of the inhibitor. This may be due to the fact that fungal burden was already maximal with PGE_2 treatment. However, neither PGE_2 nor PGEx alone inhibited nitric oxide or peroxynitrite production; therefore, this is not the mechanism of action of these compounds.

Future studies should be directed at examining non-oxidative mechanisms that mediate intracellular killing of *C. albicans*. While investigators have demonstrated that *C. albicans* traffics to the phagolysosome, there may be some variation in microenvironment within this compartment that impacts killing [216]. For example, it was recently shown that there is inhibition of the *C. albicans* phagosome fusing with compartments enriched in the lysobisphosphatidic acid [258]. In comparing a wildtype and hyphal deficient mutant, the wildtype strain displayed additional specific survival strategies to prevent its targeting to compartments displaying late endosomal/lysosomal features, such as induction of active recycling out of phagosomes of the lysosomal membrane protein LAMP-1, the lysosomal protease cathepsin D at later time points. Detailed microscopic and cell trafficking experiments are needed to further examine the effects of prostaglandins in the intracellular fate of *C. albicans*.

For synthesis of prostaglandins from arachidonic acid precursors, two cyclooxygenase (COX) enzymes, COX-1 and COX-2, are involved. COX-1 is

expressed constitutively and is responsible for steady-state eicosanoid production whereas COX-2 is the inducible form and responsible for inflammatory eicosanoid production [230]. Previous studies have demonstrated that *C. albicans* induces PGE_2 production via COX-2 in vitro [161]. This enzyme can be inhibited by treatment with various nonsteroidal anti-inflammatory drugs (NSAIDS) including aspirin, indomethacin, naproxen, etc. In addition, certain plant polyphenols inhibit COX activity, including resveratrol and NDGA, which also inhibits lipoxygenase. We evaluated the effects of eicosanoid inhibitors (both an NSAID and plant polyphenol) to determine the effects of host eicosanoids in vivo in mucosal and systemic infection models. It was important to test several infection models as immune responses to *C. albicans* infections are distinct depending on the tissue.

C. albicans vaginitis is a superficial mucosal infection that does not follow the normal paradigm of protective immunity [259]. While cell-mediated immunity is considered the predominant host defense mechanism against other mucosal candidal infections, studies have revealed a lack of a protective role for adaptive immunity. Instead aberrant innate responses to *C. albicans* colonization are responsible, mediated by epithelial cells and neutrophils [260]. In terms of control of the aberrant response, it is believed that immunoregulatory activities of local dendritic cells participate in promoting tolerance to the colonization state. In the experimental vaginitis model, upon treatment with NDGA and indomethacin, our studies demonstrated decreased fungal burden and PGE_2 levels. Treatment with either NDGA resulted in increased IFN-γ, which may be due to reduced

fungal burden, or due to reduced PGE_2 levels, which can inhibit IFN-γ [243]. NDGA treatment also partially restored IL-10 production. IL-10 is an anti-inflammatory cytokine, which can inhibit activation of innate effector cells, which mediate the symptoms of vaginitis. IL-10 levels tend to correlate with reduced fungal burden, as infection induces pro-inflammatory responses [244]. While PGE_2 is a known inducer of IL-10, we hypothesize that the lack of fungal burden and antigenic stimulation is responsible for reduced IL-10 production [246]. Overall, these results emphasized the fact that increased levels of PGE_2 are associated with increased fungal burden and cytokines associated with local immune responses during infection.

Future studies focused on the role of PGE_2 in vaginitis could also be examine the growth state of *C. albicans* in the vaginal tract as PGE_2 also enhances morphogenesis and biofilm formation. Recently, it was reported that *C. albicans* forms biofilms on the vaginal mucosa. However, the role of biofilms in pathogenesis during vaginitis is unknown [261, 262]. In line with this, the role of estrogen in this model should be investigated, which is required for a robust vaginal infection. Estrogen has direct effects on inducing *C. albicans* morphogenesis [263]. PGE_2 stimulates expression and activity of aromatase, the enzyme that is responsible for a key step in the biosynthesis of estrogens [264]. This results in local production of estrogen, which induces PGE_2 formation and establishes a positive feedback cycle. Therefore, effects on both the fungus and host may work together to promote vaginal fungal growth and infection.

We also investigated effects of eicosanoid inhibitors during systemic candidiasis. The ability to clear or control a systemic infection with *Candida* is dependent on the elaboration of a protective adaptive immune response, characterized by induction of Th1 cells and production of pro-inflammatory cytokines. A non-protective response is characterized by expansion of Th2 cells and production of Th2 cytokines [222]. Treatment of mice with NDGA reduced *C. albicans* level in the kidney, which was associated with a reduction in serum PGE_2 levels. We also observed that treatment with NDGA was associated with elevated splenic IFN-γ and IL-12, which are required for generation of protective Th1 responses during systemic infection [247]. Unlike like NDGA, indomethacin was not able to reduce the levels of PGE_2 and the balance tilted more towards non-protective response. While this may be due to alternative effects of indomethacin in protective inflammatory responses, this may also be due to ineffectiveness at directly killing *C. albicans* or inhibiting fungal prostaglandin production during infection. Pharmacologically, this drug may not access *C. albicans* at the site of infection or may lose efficacy in vivo. There is the possibility that using nanotechnology or lipid-based delivery systems may be more effective. Overall, these studies suggested that manipulation of eicosanoid production are effective at reducing fungal burden without suppressing inflammatory response and inhibit fungal growth at same time.

Although the work presented in this dissertation has provided an insight on the immunomodulatory role of prostaglandins during *Candida*-host interactions, many questions still require further investigation. It would be

important to test responses to *C. albicans* mutants lacking the ability to produce eicosanoids would be affected. For this purpose, all the genes responsible for production of eicosanoids in *C. albicans* need to be identified and mutants have to be made to study the downstream response when interacting with the host cells. However, the enzymes and metabolic pathways of fungal eicosanoid production are mostly unknown. This is due largely to differences between fungal and mammalian enzymes at both the nucleic and amino acid sequence level. So far, *OLE2* (fatty acid desaturase) and *FET3* (multicopper oxidase) has been associated with decreased PGE_2 production but they did not completely abrogate it [154]. In addition, a bifunctional LTA4 hydrolase was cloned and characterized from *S. cerevisiae* [265]. This enzyme possessed 42% identity with mammalian leukotriene A4 (LTA4) hydrolase and produced LTB4 from LTA4 in vitro [266]. There exists a homolog of LTA4 hydrolase in *C. albicans*, which can produce LTB4 in vitro. Therefore, future studies could focus on effects of fungal leukotrienes during infection, which may exert completely different effects on the host response compared with prostaglandins.

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Abstract

IMMUNOMODULATORY EFFECT OF HOST AND FUNGAL EICOSANOIDS DURING HOST-PATHOGEN INTERACTIONS WITH *CANDIDA ALBICANS*

by

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Candida albicans, an opportunistic fungal pathogen, poses a significant clinical threat to immunocompromised patients. Diseases associated with this fungus ranges from superficial mucosal infection to life-threatening systemic candidiasis. The mechanisms by which *Candida* persists at mucosal surfaces in the face of an adaptive response are unclear. *Candida* produces immunomodulatory oxylipins that cross-react functionally with host eicosanoids, which are considered to play important role in regulating innate and adaptive immune responses. Our objective was to characterize the role of prostaglandins produced by the host and this fungus during host pathogen interactions, both in vitro with dendritic cells (DCs) and macrophages, and in vivo during pathogenesis. At early time point, there was upregulation of Th2 cytokines IL-4, IL-10, and IL-13 by both plasmacytoid and myeloid DCs in the presence of hyphae and PGE_2. At later time points, fungal or host PGE_2 treatment resulted in decreased Th1 cytokine production (IL-12 and IL-6) and slightly increased Th2

cytokine production (IL-4). DC vaccination experiments demonstrated that yeast pulsed DCs imparted protection, which was abrogated with exposure to host or fungal PGE_2. Non-protective responses were associated with Th2 cytokine production, and at later stages, exacerbated Th17 responses accompanied by uncontrolled fungal growth. Ours is the first study to report presence of Th17 cytokines in the mice kidneys during systemic candidiasis and its associated lack of protection. We also demonstrated that during the interaction of *C. albicans* with the macrophages, PGE_2 and PGEx decreased phagocytosis and increased intracellular survival. We also investigated the effects of eicosanoid inhibitors during mucosal and systemic infection models. During experimental *C. albicans* vaginitis, treatment with inhibitors locally resulted in decreased fungal burden and PGE_2 levels. Similar results were observed during systemic infection, which correlated with restoration of Th1 cytokines. These studies suggested that balanced manipulation with eicosanoid production can work without suppressing inflammatory response and inhibit fungal growth at same time. This could provide newer avenues for developing novel pharmacological intervention strategies to treat *C. albicans* infections.

Autobiographical Statement

EDUCATION

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Wayne State University, Detroit, MI
Masters of Science (with thesis) in Cell and Molecular Biology, July 2005
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2009-2010 Assistant Professor, Natural Sciences Dept, Lawrence Technological University, Southfield, MI. Developed and taught special topic course (Cancer Immunology)

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2002 and 2003 Meritorious scholarship award from Eastern Michigan University .

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