Mechanism and effect of *Candida albicans* recognition by murine B cells

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Abstract

Candida albicans is a polymorphic fungus that colonizes mucosal tissue, and is thus a frequent member of the human microbiome. While it is no threat to healthy individuals, *C. albicans* can become pathogenic in immunocompromised hosts, being one of the most common human fungal pathogens, and one of the leading causes of nosocomial infections. For many years, and especially due to the fact that patients with B cell or immunoglobulin deficiencies do not show any increased propensity to *C. albicans* infections, B cells and antibodies were considered not to play any role in defensive responses against this pathogen. However, several studies have contradicted this view so that nowadays, even though not considered as major players like neutrophils or T_H17 cells, B cells are seen as helpers in mounting efficient anti-*C. albicans* protective responses.

To study the interplay between *C. albicans* and B cells, mouse splenic B cells were isolated and cultured in the presence of heat-killed *C. albicans* yeast, hyphae, or the *Saccharomyces cerevisiae* cell wall preparation zymosan. Cultures in unsupplemented medium or medium supplemented to mimic either T cell-independent or T-cell dependent B cell activation were analysed. The obtained results allowed to conclude that zymosan and heat-killed *C. albicans* hyphae, but not yeast, are able to increase B cell activation in a MyD88-dependent manner, and mostly via TLR2. This conclusion was drawn from the observed increase in AID induction and IgG1, IL-10 and IL-6 production. Dectin-1 and specific BCR recognition were not required for this effect. Furthermore, it was possible to observe that *C. albicans*-induced TLR signalling is able to cooperate with BCR signalling to increase B cell proliferation.

The results of this study lead to the hypothesis that in a physiological context, *C. albicans*-induced TLR activation of B cells might not only cooperate with BCR signalling to enhance the production of specific anti-*C. albicans* antibodies, but also enhance protective T_H17 responses through IL-6 production. The importance of pursuing such a hypothesis, and others related to the role of B cells in *C. albicans* infections, resides in the fact that despite the increasing effort made in recent years to diagnose and fight fungal infections, they still display a high mortality rate, making potential protective approaches urgently needed.

Zusammenfassung

Candida albicans ist ein polymorpher Pilz, welcher Schleimhäute besiedelt und somit ein häufiges Mitglied des menschlichen Mikrobioms ist. *C. albicans* stellt zwar keine Bedrohung für gesunde Individuen dar, kann jedoch in immunsuppremierten Wirten pathogen werden. Er gilt als einer der verbreitetsten fungalen Krankheitserreger im Menschen und einer der Hauptverursacher von nosokomialen Infektionen. Viele Jahre lang, und vor allem aufgrund dessen, dass Patienten mit einer B-Zell- oder Immunglobulin-Defizienz keine erhöhte Tendenz zu *C. albicans*-Infektionen aufweisen, wurde angenommen, dass B-Zellen und Antikörper in der Abwehr dieses Pathogens keine Rolle spielen. Allerdings wurde dieser Ansicht durch mehrere Studien widersprochen, sodass B-Zellen, wenngleich sie nicht als Hauptspieler gelten, wie beispielsweise Neutrophile oder T_H17-Zellen, mittlerweile als Helfer bei der Entwicklung von wirkungsvollen anti-*C. albicans* Abwehrreaktionen angesehen werden.

Um die Interaktion zwischen *C. albicans* und B-Zellen zu untersuchen, wurden B-Zellen aus der Milz von Mäusen isoliert. Die Kultivierung dieser Zellen wurde in Gegenwart von durch Hitze getöteten, *C. albicans* Hefen, Hyphen, oder der *Saccharomyces cerevisiae*-Zellwand-Präparation Zymosan durchgeführt. Es wurden Kulturen in unbehandeltem Medium oder in Medium, das entweder eine T-Zell-unabhängige oder eine T-Zell-abhängige B-Zell-Aktivierung nachahmt, analysiert. Die erhaltenen Ergebnisse ließen darauf schließen, dass sowohl Zymosan, als auch durch Hitze getötete *C. albicans* Hyphen, jedoch nicht Hefen, die B-Zell-Aktivierung erhöhen. Dies geschieht in Abhängigkeit von MyD88 und vor allem via TLR2. Diese Schlussfolgerung basiert darauf, dass ein Anstieg in der AID-Induktion und der Produktion von IgG1, IL-10 und IL-6 gemessen wurde. Eine Beteiligung von Dectin-1 und der spezifischen Erkennung durch den BCR an diesen Effekten konnte nicht nachgewiesen werden. Weiterhin konnte gezeigt werden, dass *C. albicans*-induzierte TLR-Signale in der Lage sind, zusammen mit BCR-Signalen die Proliferation der B-Zellen zu steigern.

Die Ergebnisse dieser Studie führten zu der Hypothese, dass *C. albicans*-induzierte TLR-Aktivierung der B-Zellen nicht nur mit BCR-Signalen kooperierten könnte, um die Produktion von spezifischen Antikörpern gegen *C. albicans* zu erhöhen, sondern auch die schützende T_H17 Antwort durch IL-6-Produktion verstärken könnte. Trotz der in den letzten Jahren unternommenen Anstrengungen zur Diagnose und Bekämpfung von Pilzinfektionen ist die Sterblichkeitsrate in solchen Fällen hoch ist und potenzielle Schutzmaßnahmen werden dringend benötigt. Daher ist es wichtig, dass solche Hypothesen, wie auch andere, welche die Rolle der B-Zellen in *C. albicans*-Infektionen untersuchen, weiter erforscht werden.

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1. Introduction

1.1 The immune system

The human body is challenged by multiple infectious agents and harmful substances on a daily basis while being equipped with physical and chemical barriers to contain these threats. However, upon disruption of these barriers, it is up to the immune system to fight and clear the invading pathogens. To identify infectious agents, immune cells express on their surface pathogen recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) present in several pathogens [1-6]. After recognition, the immune system is responsible for containing, and if possible, resolving the infection. To do so, the immune system is divided into innate and adaptive immunity, the first being a fast and broad response and the latter a slower but highly specific one.

1.1.1 The innate immune system

As mentioned above, the innate immune system delivers a fast first response in case of pathogenic infection. Major cellular players of the innate immune system are macrophages, dendritic cells (DCs), granulocytes (especially neutrophils), and natural killer cells, which possess a vast array of effector functions such as phagocytosis, cytokine production and cytotoxic capacity [1]. The fact that innate cells express several PRRs and exist in relatively abundant numbers in the circulation makes them able to target the invader in a matter of hours. The term PRR comprises different types of germline-encoded receptors, most being classified in different families depending on their domain homology [7]. The five PRR families are Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs) and AIM2-like receptors. PRRs recognize conserved molecular motifs associated to pathogens – PAMPs, each PRR recognizing a defined type of molecule. Several types of molecules can serve as PAMPs, such as carbohydrates and peptides present in bacteria, viral nucleic acids and glucans and chitin from fungi [8]. As such, PRRs can recognize and initiate an immune response against Gram-positive and –negative bacteria, viruses, fungi and protozoa [8]. Important to note is the fact that PRRs are also expressed by cells of the adaptive immune system [9, 10]. Besides PAMPs, PRRs also recognize damage-associated molecular patterns (DAMPs) released by damaged/stressed host cells [11].

The innate immune system also includes a humoral component, the complement system. The complement system is composed of several proteins that react one to another, forming proteolytic cascades that lead to opsonization of pathogens (to increase phagocytosis), recruitment of other components of the immune system and pathogen membrane lysis [1]. Three different pathways can lead to complement activation, the classical pathway, the lectin pathway and the alternative pathway, each being activated by different molecules present on the surface of pathogens [1, 12].

1.1.2 The adaptive immune system

In opposition to the innate immunity, the adaptive immunity comprises a slow but highly specific response. Two cell types originated from common lymphoid progenitors constitute the adaptive immune system, T lymphocytes and B lymphocytes, also called T and B cells. In the absence of infection, T and B cells are generally inactive – naïve cells. However, upon encountering their specific antigen, these cells become activated, proliferate and differentiate giving rise to specialized effector cells [1].

Although both cell types present an antigen-specific receptor on their surface, their receptor and effector functions are very different. T cells are usually activated upon T cell receptor (TCR) recognition of their cognate antigen, which is presented by major histocompatibility complex (MHC) molecules [1]. After activation, they can be categorized according to their effector function in helper ($CD4^+$) and cytotoxic ($CD8^+$) T cells [13]. While cytotoxic T cells kill infected or abnormal cells by releasing cytotoxic molecules, helper T cells provide help to effector cell types (including B cells) by expressing stimulatory molecules and cytokines. Different subtypes of helper T (T_H) cells have been identified based on their cytokine secretion profile and transcription factor production, with T_H1 cells producing interferon gamma (IFNγ), interleukin 2 (IL-2) and tumor necrosis factor alpha (TNF-α), T_H2 cells producing IL-4, IL-5 and IL-13 and T_H17 cells producing IL-17 [14]. A special type of $CD4^+$ T cells are regulatory T cells, which have the ability to control immune responses by influencing several immune cell types [15].

B cells are mainly activated by recognition of their cognate antigen through their B cell receptor (BCR) [1]. Upon activation, they undergo several processes that influence the effector function and improve the affinity of the produced antibodies. Ultimately, B cells can differentiate into antibody secreting cells, thus becoming plasma cells [16]. Moreover, B

cells possess antigen-presenting cell (APC) capacity, presenting antigens through MHC class II molecules [1]. Some activated B and T cells can also mature to long-living memory B and T cells, thus providing the basis for a fast and highly specific response when re-encountering a known pathogen [17, 18].

Due to the topic of this dissertation, a more in depth introduction to B cell biology is provided in the next chapters. The importance of the innate and adaptive immunity in the context of *Candida albicans* infection will also be addressed.

1.2. B cells

As mentioned before, B cells are part of the adaptive system and have the production of antibodies as their hallmark. However, before becoming antibody secreting cells, B cells need to undergo several development, activation and differentiation steps. Only a B cell that expresses a rearranged functional BCR and several other important proteins can leave the bone marrow, be activated by its cognate antigen, undergo processes to increase immunoglobulin affinity and finally differentiate into a plasma or memory cell [19]. The BCR is a Y-shaped protein, also known as immunoglobulin (Ig), composed of two identical heavy and two identical light polypeptide chains linked by disulphide bridges [20, 21]. Both heavy and light chains possess a constant and a variable region, where the latter is responsible for antigen binding. Although possessing no functional difference, two types of light chain can be found in immunoglobulins, a kappa (κ) light chain (which occurs more frequently), and lambda (λ) light chain [22, 23]. An antibody is the secreted form of the BCR. In mammals, five distinct immunoglobulin isotypes can be distinguished by their constant region, IgM, IgD, IgG, IgA and IgE, each possessing a distinct functional activity [24]. Finally, it is also important to note that B cell functions go beyond antibody secretion, as they also act as antigen-presenting cells and cytokine producers [25].

1.2.1 B cell development

B cells arise from common lymphoid progenitors in the bone marrow, where they undergo several stages of development [26, 27]. Throughout these different stages ,the precursor cells migrate within the bone marrow until reaching the sinus of the marrow cavity [28]. Once cells are committed to the B cell lineage, they express the specific transcription factors E2A

and early B cell factor (EBF), thus becoming pro-B cells [29]. During this stage, B cells initiate the functional rearrangement of their immunoglobulin gene segments, allowing the generation of a broad B cell repertoire. The first rearrangements lead to the formation of the immunoglobulin heavy chain [26]. In this case, the diversity (D) gene segment and the joining (J) gene segment undergo rearrangement, a process followed by the rearrangement of an upstream region, the variable gene segment (V) [30, 31]. An effective VDJ rearrangement results in the formation of a μ chain [32]. The expression of the μ chain along with a surrogate light chain and signal transducing proteins allows the formation of a pre-BCR, its surface expression marking the transition into the pre-B cell stage [33, 34]. During this stage, cells undergo proliferation followed by VJ rearrangement of the light chain [26]. To avoid the expression of more than one specific immunoglobulin, light chain rearrangement occurs first in the κ chain, occurring only in the λ chain in case of an unsuccessful κ chain rearrangement [35]. Once a successful light chain is formed, it pairs with the μ chain to form the BCR [36]. At this point cells become immature B cells and can leave the bone marrow, though still expressing only immunoglobulins of the IgM isotype [19].

After leaving the bone marrow, B cells migrate to secondary lymphoid organs, like the spleen or lymph nodes, where they can encounter their cognate antigen, be activated and further differentiate [19, 37]. However, before being considered mature, B cells go through two transient transitional stages: T1, before they acquire recirculation capacity and T2, when they acquire the ability to recirculate but still express markers of immaturity [38, 39].

1.2.2 B cell lineage subsets

B cells can be divided into 3 different main subsets: B1 and B2 cells, with B2 cells being further divided into follicular (FO) and marginal zone (MZ) B cells [40-42].

B1 cells arise early in embryonic development, originating from B1 progenitors derived from hematopoietic stem cells in the fetal liver [43]. These cells reside predominantly in the peritoneal and pleural cavities, presenting self-renewing capacity to maintain their population [44, 45]. A small contribution of bone marrow precursors to the B1 cell population in adulthood has also been verified [41, 45, 46]. B1 cells were first distinguished from other B cells through the expression of CD5 on their surface (B1a), although there are also cells with B1 characteristics that do not express this marker (B1b)

[41]. Regarding their effector function, B1 cells present a restricted immunoglobulin repertoire, predominantly producing IgM antibodies against antigens that do not require T cell help, these being mainly polysaccharides or phospholipids present in commensal bacteria [42, 47, 48]. The fact that these natural IgM antibodies recognize conserved microbial structures allows them to function as a first barrier against pathogen infection [49, 50]. Moreover, since these antibodies are polyspecific, they also recognize self-antigens, being important for tissue homeostasis [51, 52]. B1 cells can also produce polyreactive IgA antibodies, contributing to mucosal immunity [53].

B2 cells originate from progenitors in the bone marrow and pass through several development stages until they become mature B cells. Most mature B2 cells migrate to B cell follicles in the secondary lymph nodes where they become FO B cells. Alternatively, mature B cells can migrate to the outer white pulp of the spleen, residing between the marginal sinus and the red pulp where they become MZ B cells [40, 54]. These two types of mature B cells can be distinguished by the expression of different surface markers, like CD21, which is highly expressed in MZ but not in FO B cells [55]. Moreover, due to their different localization, MZ and FO B cells present different effector functions. By localizing inside follicles in proximity to T cell zones, FO B cells mount good T cell-dependent (TD) responses [40]. Consequently, they are involved in the germinal center formation, where they undergo affinity maturation and can differentiate into plasma and memory cells, or in extrafollicular responses [56, 57]. On the other hand, MZ B cells, located at the interface between the circulation and a lymphocyte-rich zone, strongly respond to T cell-independent (TI) blood-borne pathogens, mounting fast but less specific responses [47, 58]. These cells can also differentiate into plasma cells, although secreting only IgM immunoglobulins [55]. Moreover, they present some memory-like properties such as a pre-activated phenotype, self-renewal capacity and the ability to live as long as the host [40].

1.2.3 B cell activation

Mature B cells become activated upon recognition of antigens. Whether that happens via the recognition of their cognate antigen by their BCR with T cell help, i.e. via T cell-dependent activation [59], or via recognition of conserved or repetitive antigens by their TLRs or BCR cross-linking, i.e. via T-cell independent activation [9], depends on the cell type, location and milieu.

1.2.3.1 T-cell independent activation

Antigens that activate B cells without depending on T cell help are called T cell-independent antigens. These are usually week antigens that do not trigger T cell responses but can be recognized by B cells in two different ways: 1) through TLR recognition, a so-called T cell-independent type 1 antigen response (TI-1) and 2) through BCR cross-linking, leading to a T cell-independent type 2 antigen response (TI-2). Since TLRs are a family of transmembrane proteins that recognize distinct microbial molecular motifs, TI-1 antigens are mostly PAMPs [9]. A well-studied example is LPS, a component present on the outer membrane of gram-negative bacteria, known to be recognized by TLR4 and to induce proliferation [60]. On the other hand, TI-2 antigens are molecules bearing repetitive structures that have the ability to cross-link BCRs. An example of such structures are bacterial polysaccharides [61]. TI responses are mostly observed in B1 and MZ B cells, leading to the production of IgM against invading pathogens, and limiting their growth until a full specific response is mounted [47]. Several studies have also showed TI B cell responses leading to class switched antibodies. Examples include the presence of IgA against commensal bacteria in the intestinal mucosa [53] and IgG responses elicited by live virus infections [62]. Of note is the fact that all TI responses are largely influenced by the cytokine milieu at the site where B cell/antigen interaction takes place [9].

1.2.3.2 T-cell dependent activation

Conversely, antigens that activate B cells depending on T cell help are called T cell-dependent antigens. While migrating or recirculating between secondary lymphoid organs, a B cell can recognize its cognate antigen via its BCR, internalizing and processing it to be presented on its surface through MHC class II [63]. Consequently, antigen-presenting B cells get trapped in the T cell zone (near the T/B cell zone border) of secondary lymphoid tissue where they can be in contact with activated T cells that recognize the expressed antigen [64, 65]. At this point, cells proliferate and generate long-lived B/T cell interactions [66]. From there, T cell-dependent activation of B cells can lead to two different fates, the differentiation into short-lived plasma cells in extrafollicular foci or the formation of a germinal center reaction to form long-lived plasma and memory cells [57, 67]. Crucial for both B and T cell growth and differentiation is also the interaction between the CD40 receptor expressed on the surface of B cells with its ligand, CD40L (CD154), expressed on the surface of activated T cells [68]. Both the expression of CD40L and the production of cytokines and chemokines

involved in T cell help are triggered by the binding of the T cell receptor (TCR) to its antigen presented by MHC class II molecules. Consequences of T cell help to B cells include increased survival, proliferation, differentiation, hypermutation, class switching, adhesion and attraction [59].

1.2.4 Antibody responses

Regarding the production of antibodies, three different types of antibody-secreting cells with different differentiation and secreted antibody profiles can be distinguished: 1) B1 cells that secrete natural antibodies [69], 2) short-lived plasma cells formed in extrafollicular responses [16, 57], and 3) long-lived plasma cells originated from germinal center reactions [16, 70]. These three types of cells differentiate at distinct time points and locations, generating antibodies with different degrees of affinity. Since the generation of natural antibodies was already described above, this section focuses on extrafollicular and germinal center reactions.

1.2.4.1 Extrafollicular reaction

As the name indicates, extrafollicular reactions occur in foci outside B cell follicles. This type of reaction is elicited by certain antigens and is not dependent on B cell types or forms of activation [57]. In fact, both FO and MZ B cells can undergo extrafollicular differentiation, in a T cell-dependent or -independent fashion, although MZ B cells seem to be faster and more efficiently recruited into this type of response [47, 57, 71]. In T cell-dependent antibody responses, early high antibody affinity also appears to favour extrafollicular reactions [72]. In this case, after being activated, B cells proliferate and differentiate into plasmablasts, migrating to the red pulp in the spleen or to the medullary chords in lymph nodes to form the so-called extrafollicular foci [73] (Figure 1). There, plasmablasts associate with $CD11c^{high}$ DCs for survival and further differentiation into plasma cells [74]. In this phase, no CD4 T cell-help is needed, although it has been shown that CD40 ligation can extend plasmablast growth and differentiation [75, 76]. Most plasma cells generated in extrafollicular responses are short-lived, surviving only for approximately 3 days [57, 77]. However, it has been shown that this type of response is also able to generate long-lived plasma cells [78]. Commitment of B cells to the extrafollicular pathway is also associated with the expression of Blimp-1, the key transcription factor for plasma cell

differentiation, and the downregulation of Bcl-6, a transcriptional repressor that suppresses the program of plasma cell formation [79].

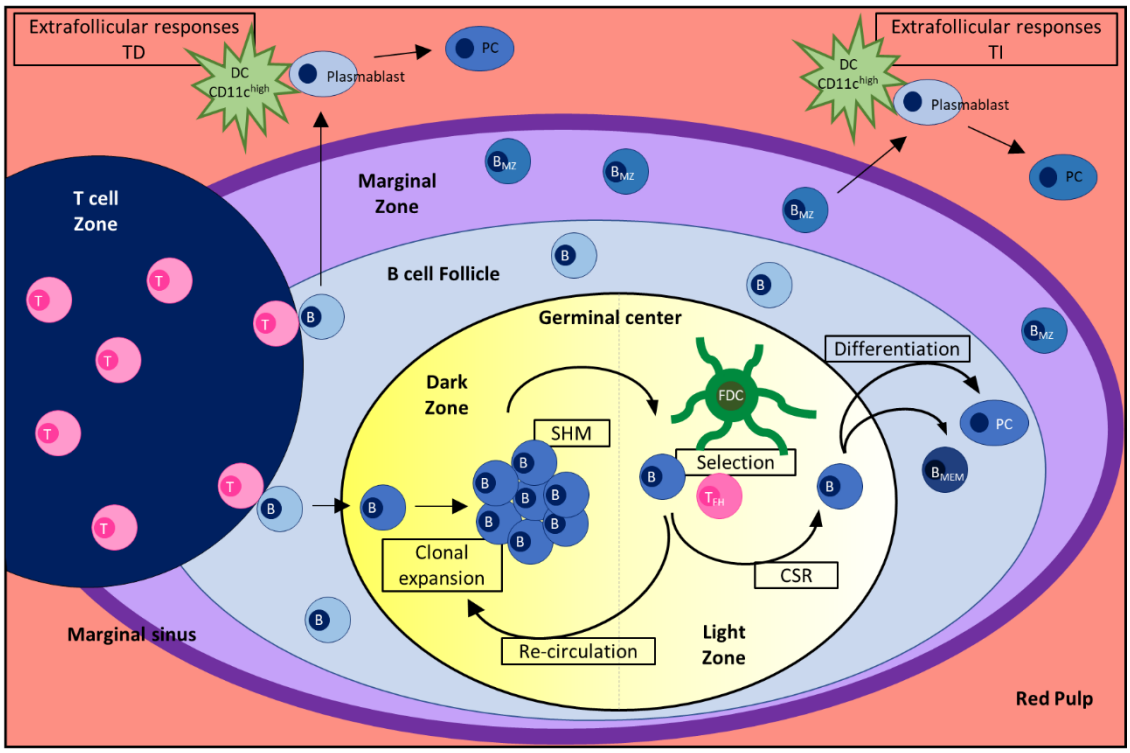


Figure 1. **B cell activation in the spleen.** Activation of both marginal zone and follicular B cells can occur independently or dependently of T cell help. Marginal zone B cells are mostly activated in a T cell-independent fashion, which can result in plasmablast differentiation and subsequent migration and formation of extrafollicular foci. There, B cells associate with CD11c^{high} DCs which allows for further differentiation, mostly to short-lived plasma cells. On the other hand, after being activated by their cognate antigen, B cells committed to a follicular fate get trapped close to the T/B cell border where they receive stimuli from T cells. The most common fate of these cells is the formation of germinal centers, which are divided into two different microenvironments. In the dark zone, B cells undergo clonal expansion and somatic hypermutation, whereas after migration to the light zone, cells are subjected to selection and can undergo class switch recombination to modify their effector functions. Subsequently, B cells can either differentiate into memory or plasma cells and leave the germinal center, or re-circulate to the dark zone to accumulate more advantageous mutations. Similar to what occurs with marginal zone B cells, follicular B cells can also form extrafollicular foci, where they differentiate into short-lived plasma cells. However, in this case plasmablast differentiation and migration is usually dependent on T cell help.

TD – T cell-dependent, TI – T cell-independent, CSR – class switch recombination, SHM – somatic hypermutation, MZ – marginal zone, DC – dendritic cell, FDC – follicular dendritic cell, MEM – memory cell, PC – plasma cell.

1.2.4.2 Germinal center reaction

Germinal center reactions are mostly triggered by T cell-dependent B cell activation (Figure 1). Interacting B and T cells commit to the GC program still outside the follicles, upregulating several GC-associated proteins and most decisively, the transcription factor Bcl-6 [80]. Only after commitment do cells migrate to the follicles, where as soon as 4 days after contact with the cognate antigen GC precursors can form early GCs [67]. At this point, B cells grow and differentiate into blasts, settling in the existing network of follicular dendritic cells (FDCs) in the centre of the follicle [81, 82]. IgM^+ IgD^+ B cells are displaced by the B cell blasts to form the mantle zone around the GC [70]. From days 5 to 7 the blasts continue their proliferation (clonal expansion) until a fully established GC with two defined microenvironments, the dark and the light zone, is formed [56, 83] (Figure 1). It is, however, important to note that different antigens trigger different GC kinetics and organization.

The dark zone of the GC is highly populated by B cell blasts, also containing FDC. In this zone B cells proliferate and accumulate until they circulate to the light zone [67, 84]. Most importantly, while in this zone B cell undergo somatic hypermutation (SHM), the process during which mutated high affinity BCRs can be generated [85] (Figure 2). SHM occurs thanks to the action of the protein activation-induced cytidine deaminase (AID), which deaminates deoxycytidine residues into deoxyuridine (C:G into U:G), causing lesions in the rearranged immunoglobulin variable regions [86, 87]. These lesions are subsequently recognized and processed, introducing mutations that can increase antibody affinity. The type of DNA repair pathway used by the cell to resolve a particular lesion dictates the type of mutation introduced [87].

On the other hand, the light zone of the GC is populated not only by B cells and FDC but also by follicular helper T cells (T_{FH}) and macrophages [80]. The presence of these cells is important for the process of positive selection occurring at this point [88]. Having reached the light zone, B cells move to the network of FDCs and macrophages where high amounts of antigen are available. Since B cells expressing higher affinity BCRs can better recognize their cognate antigen, these cells are also more successful in forming MHC complexes and in competing for T_{FH} help, thus being positively selected to further differentiate [67, 84]. Cells not able to efficiently capture their antigen die by apoptosis [67, 88]. At this stage, B cells may undergo class switch recombination (CSR), the process that allows to change the effector function (isotype) of the already formed high affinity antibodies (Figure 2). CSR is

also dependent on AID activity. In this case, AID deaminates doxycytidines in the switch regions present in the heavy chain, causing DNA double-strand breaks [89]. These breaks are subsequently ligated, excising the intervening DNA and approximating the already mutated VDJ segment to a different downstream constant region. Since different constant regions code for different immunoglobulin isotypes, this process leads to switch from IgM to IgG, IgE or IgA isotypes. After being positively selected and undergoing CSR, B cells can either recirculate to the dark zone to acquire more favourable mutations [90], or differentiate into plasmablasts or memory B cell precursors [56]. Of note is the fact that B cells only reside in the light zone for some hours [56].

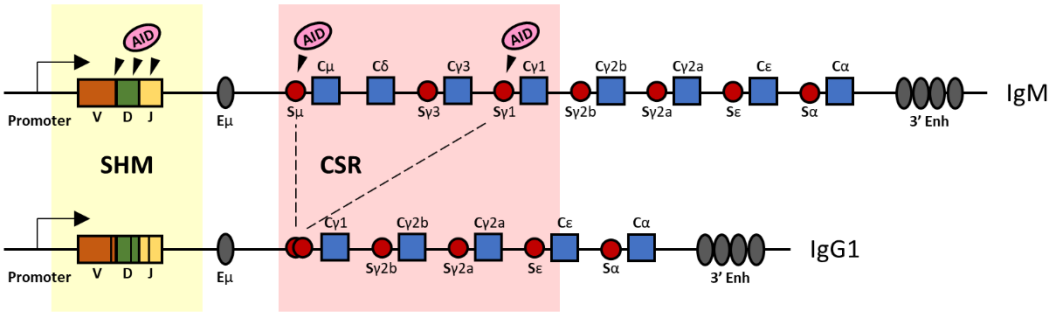


Figure 2. **Somatic hypermutation (SHM) and class switch recombination (CSR).** In germinal centers, B cells improve the effector function of the produced immunoglobulins by undergoing two processes of immunoglobulin diversification. SHM occurs in the dark zone of the GC and is characterized by the introduction of mutations, usually substitutions, that confer a greater diversification to the immunoglobulin variable region. On the other hand, CSR occurs in the light zone of the GC. In this process, double-strand breaks induced in the switch regions (S) upstream of the different constant regions (C) lead to the excision of the intervening DNA and approximation of the VDJ segment to a new constant region. This results in isotype switching, altering immunoglobulin effector function. AID is a key enzyme in both processes since it deaminates deoxycytidine into deoxyuracil inducing DNA lesions. It is the subsequent pathway of resolution of these DNA lesions that dictates the outcome of the diversification process.

V – variable gene segment, D – diversity gene segment, J – joining gene segment, E/Ehn – enhancer, S – switch region, C – constant region.

1.2.5 B cell signalling pathways

As described in the previous chapters, BCR signalling is indispensable throughout the different stages of development and differentiation of B cells. However, other signalling pathways also play critical roles in B cell activation (Figure 3). In the case of T cell-dependent activation, the activation of the CD40 receptor is crucial for processes like GC formation, selection and CSR [91]. Also necessary for GC formation and CSR is the presence of cytokines, here represented by IL-4 [92]. T cell-independent type 2 activation

relies on TLR signalling, which can also synergize with the BCR to promote CSR before T cell help is available [93].

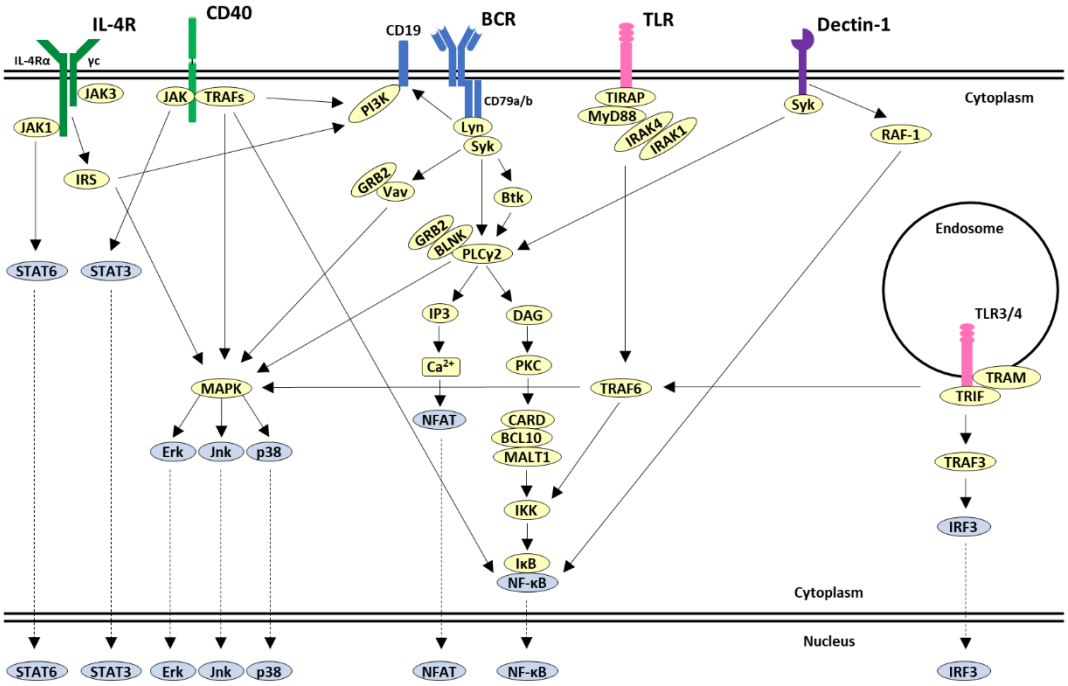


Figure 3. **B cell signalling pathways.** B cells express several types of receptors on their surface, most importantly the B cell receptor (BCR), but also co-stimulatory receptors, cytokine receptors and receptors specialized in the recognition of pathogen-associated molecular patterns. To this latter category belong several toll-like receptors, and the c-type receptor dectin-1. The B cell receptor is indispensable for B cell survival, with its signalling also being responsible for proliferation and cellular activation. To that end, B cell signalling triggers the activation of several proteins converging into the activation of transcription factors such as NF-κB, NFAT and the MAP kinases Jnk, Erk and p38. Crucial for some B cell intrinsic functions, such as class switch recombination, is the presence of the co-stimulatory receptor CD40, which is activated by the T cell surface protein CD154, and the presence of cytokine receptors, here represented by the IL-4 receptor. The effector functions of these receptors are largely dependent on the JAK-STAT pathway and MAPK cascades, CD40 signalling also triggering NF-κB activation. TLR signalling, dependent on adaptor proteins such as MyD88 or TRIF, has been shown to synergize with BCR signalling by activation of the same transcription factors. Moreover, TLR activation induces the expression of type I IFN genes through the activation of IRF3. Dectin-1 is also able to induce NF-κB activation, both through a Syk-dependent and Syk-independent pathway. Activation of NF-κB and other transcription factors facilitates their nuclear localization, leading to the transcription of several different genes.

1.2.5.1 BCR signalling

The BCR is composed by a transmembrane immunoglobulin noncovalently bound to two signalling transducing molecules, CD79a (Igα) and CD79b (Igβ) [94]. Upon binding of a cognate antigen to the BCR, Scr family protein kinases, especially Lyn, are recruited and phosphorylate the ITAM motives present on the cytoplasmic tails of CD79 molecules

[95]. This phosphorylation results in the recruitment of the signalosome (Figure 3), composed by other kinases as Syk and Btk, Vav proteins, adaptor proteins like Grb2 and BLNK and the signalling enzymes PLCγ2 and PI3K [96, 97]. As Syk becomes responsible for the phosphorylation of the CD79 proteins, Lyn provides signal amplification by continuous recruitment of kinases and formation of a complex with the co-receptor CD19 and other molecules [98]. The formation of this complex allows the lowering of the threshold of B cell activation [99]. The continuous transduction of signal upon antigen recognition triggers BCR aggregation in clusters in plasma membrane domains termed lipid rafts [100]. Once the signalosome is formed, PLCγ2 is dually phosphorylated by the kinases Syk and Btk, producing the second messengers diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3) [97]. While DAG is an activator of protein kinase C (PKC), IP3 leads to calcium influx from the endoplasmic reticulum [101].

Upon initial BCR activation and signal amplification, multiple downstream effectors are activated. As an example, calcium influx activates the transcription factor NFAT [102]. Another example is the activation of the mitogen-activated protein kinase (MAPK) pathway, where MAPKs like Erk, Jnk and p38 regulate transcription factors such as Elk1, c-Myc, c-Jun and ATF2 [101, 103]. Last but not least, stimulation via PKC results in the phosphorylation and subsequent proteasomal degradation of IκB (Figure 3), facilitating NF-κB translocation to the nucleus and inducing gene transcription [101, 104]. By activating all these different pathways, BCR signalling can provide survival, proliferation and activation signals to the cell.

1.2.5.2 CD40 signalling

The CD40 receptor is a member of the tumor necrosis factor receptor (TNFR) family of surface molecules, being expressed in a variety of cells like epithelial, endothelial cells and APCs [105]. In B cells, the engagement of CD40 by its ligand, CD40L or CD154, expressed by $CD4^+$ T cells, leads to the association of the CD40 cytoplasmic domain with a set of proteins that belong to the family of TNFR-associated factors (TRAFs) [91, 106]. The association with different TRAFs (1, 2, 3, 5 and 6) leads to the activation of different signalling pathways among which are the canonical and non-canonical NF-κB pathways, the MAPKs Erk, Jnk and p38, and the JAK-STAT pathway (Figure 3) [91]. The activation of these pathways ultimately results in the transcriptional activation of several genes, among which are anti-apoptotic and cell cycle promoting genes [107, 108]. Of note is the fact that

most pathways activated upon CD40 receptor engagement are also activated by BCR antigen recognition, allowing efficient synergy of signals initiated by these receptors.

1.2.5.3 IL-4R signalling

IL-4 signalling can be initiated by the binding of IL-4 to two different receptor complexes (Figure 3). Both complexes encompass the IL-4Rα chain (CD124), a transmembrane protein ubiquitously expressed in fairly low amounts, that heterodimerizes with the common gamma chain (γc) to form the type I receptor, or with the IL-13Rα1 chain to form the type II receptor [109]. The binding of IL-4 leads to the recruitment and activation of Janus-family kinases (JAKs), with JAK1 binding to the IL-4Rα chain, JAK3 to the γc chain and JAK2 to the IL-13Rα1 chain [109-111]. Also triggered by ligand binding is the tyrosine phosphorylation of the IL-4Rα chain itself [112], as well as the phosphorylation of IRS1/2 and STAT6 by JAKS [113, 114]. While IRS initiates a variety of signalling pathways such as the PI3K and MAPK pathways enhancing survival and proliferation [109], STAT6 translocates to the nucleus where it has an important role in gene regulation [115]. As an example, STAT6 activation is responsible for enhancing the expression of germline immunoglobulin ε and γ1, showing a synergy with CD40 signalling in promoting CSR [116].

The combination of anti-CD40 antibodies or recombinant CD40 ligand with IL-4 is often used in *in vitro* B cell cultures, since it promotes increased B cell proliferation, survival and an activated phenotype, as well as CSR from IgM to IgG1 or IgE [117, 118].

1.2.5.4 TLR signalling

Until now, thirteen different TLRs have been reported (TLR1 to TLR13), each presenting specificity to one type of PAMPs [2]. While TLRs 1, 2, 4, 5, 6 and 10 are present at the cell surface, the remaining TLRs are found in endosomes. An exception being TLR4, which can be found both on the cell surface or intracellularly after being endocytosed [119]. TLR1, 6 and 10 form heterodimers with TLR2 [120-122]. Regarding TLR expression in B cells, several studies show that different human B cell subsets express different TLRs, with naïve B cells presenting lower expression levels than plasma or memory B cells [123-127]. In the case of murine B cells, a study from 2007 showed that except for TLR5, TLRs 1 to 9 are expressed in splenic and mucosal B cell subsets [128]. Interestingly, due to the insertion of a stop codon, TLR10 is a non-functional pseudogene in mice [129].

TLR signalling has been mainly studied in cells of the innate immune system, proving to be similar among different TLRs and relying on TIR domain-containing adaptor proteins [130]. In most TLRs, the recognition of PAMPs activates a signalling cascade that involves the adaptor protein Myeloid differentiation primary response 88 (MyD88) [131] (Figure 3). As an exception, TLR3 and endosomal TLR4 signal through a different adaptor protein (TRIF) [132, 133]. In the MyD88-dependent pathway, MyD88 is recruited by another TIR domain-containing protein, TIRAP, which allows the assembly of signalling complexes at the TLR location [134]. Once engaged with the TLR, MyD88 forms a complex with the proteins IRAK4 and IRAK1, the Myddosome [135]. This complex can in turn trigger the activation and autopolyubiquitination of TRAF6, leading to the activation of two distinct pathways, the IKK complex/NF-κB pathway and MAPK cascades [136]. In B cells, the triggered translocation of NF-κB to the nucleus can influence several processes, such as cell proliferation and survival [137, 138], class switching [93, 139], plasma cell differentiation [140, 141], pro-inflammatory cytokine production [142-144] and induction of important B cell proteins such as AID [93]. The activation of MAPK members triggers the activation of transcription factors which are important for regulation of inflammatory responses [145]. As mentioned before, the TRIF-dependent pathway is used by TLR3 and endosomal TLR4. However, while TLR3 can directly interact with TRIF, TLR4 needs the bridging adaptor protein TRAM [119]. Differently from MyD88, TRIF triggers not only the activation of TRAF6 but also TRAF3 [130, 146]. In this case, TRAF3 recruits IKK related kinases to induce IRF3 phosphorylation and subsequent translocation to the nucleus, where it induces the expression of type I IFN genes. Other IRFs have also been shown to be involved in TLR signalling [147-150].

1.2.5.5 Dectin-1 signalling

Dectin-1 is a PRR member of the C-type lectin receptor (CLR) family [151]. Since it is the most prominent receptor for β-glucans, a major constituent of the fungal cell wall, it plays an important role in the immune response against fungal pathogens [152, 153]. While dectin-1 is most prominently expressed in cells of the innate immunity, its expression on human B cells has been described [154]. Whether dectin-1 can also be expressed in mouse B cells remains inconclusive due to contradictory reports [155, 156].

Upon ligand recognition, dectin-1 signalling is initiated by the phosphorylation of the ITAM motif present in its intracellular tail. This results in recruitment and activation of

Syk, which phosphorylates PLCγ2 leading to subsequent activation of the CARD9-Bcl10-Malt1 complex (Figure 3) [157-159]. Stimulation of PLCγ2 phosphorylation thus leads to the activation of the transcription factors NF-κB, NFAT and IRF1/5 as well as MAPK/Erk signalling. [158, 160-163]. NF-κB can also be activated via Raf-1, in a Syk-independent way [164].

A consequence of dectin-1 signalling is the inflammasome activation-mediated production of IL-1β [165]. Two different mechanisms for IL-1β processing have been shown to be triggered by dectin-1 activation, one dependent on the assembly of the canonical NLRP3 inflammasome and caspase-1 [165], and a second via a noncanonical caspase-8 inflammasome [166]. While most studies concerning the involvement of dectin-1 in IL-1β production have been made using cells from the innate immune system, a recent study shows that also in human B cells dectin-1 activation can lead to IL-1β modulation via the NLRP3 inflammasome [167].

1.2.6 B cell effector functions

Although the main function of a B cell is to produce antibodies with high affinity and a correct isotype for a determined function, B cells are also professional antigen-presenting cells which produce cytokines. Therefore, while several B cell functions are mediated by the binding of antibodies to its targets, there are a variety of antibody-independent functions which are mediated by B cell-produced cytokines or that rely on their APC capacity.

1.2.6.1 Antibody-mediated functions

After being secreted by B cells, differently diversified/class switched antibodies mediate different functions, being involved in immune responses against bacterial, fungal, viral and parasitic pathogens [168]. Important is the fact that antibodies do not only trigger responses via effector cells, but also by direct binding to the pathogen.

One of these direct interactions is neutralization, which occurs when neutralizing antibodies bind to the pathogen, inhibiting or limiting the infection of susceptible cells [168]. This process can occur during different stages of infection (before, during or after attachment), causing diverse effects such as aggregation, immobilization, inhibition of attachment, inhibition of growth, or even death of the pathogen [168-174]. Antibodies can also neutralize pathogen-secreted toxins [175, 176].

Through pathogen-binding, some antibodies are also able to activate the classical complement pathway, leading to cell lysis or internalization by phagocytes [177-180]. Antibody binding to its specific antigen exposes a binding site for the complement molecule C1q (part of the C1 protein complex), which binds to Fc regions of immunoglobulin molecules, activating the complement cascade [180]. Consequently, complement activation can result in the formation of a membrane attack complex which creates transmembrane pores, therefore leading to cell lysis [168, 181]. Another result of antibody-mediated complement activation is the processing of C3b, which deposits on antigen/antibody complexes on the surface of pathogens, opsonizing them for phagocyte internalization [168, 182]. Of interest is the fact that C1q can only bind to IgM or IgG immunoglobulins, IgM being the most potent complement activator, followed by IgG3, IgG1 and IgG2 [180].

Not depending on their variable region but on their Fc part, antibody immune complexes (ICs) can as well bind to Fc receptors. However, the outcome of this engagement varies depending on the Fc receptor-bearing cell, the form of the IC, the cytokine milieu and the presence of complement [168]. Two examples are the phagocytosis mediated by antibody-coated pathogens [183], and antibody-dependent cellular toxicity (ADCC). The latter occurs when antibodies form a bridge between an infected cell (or pathogen) and an Fc-bearing effector cell, causing cell lysis or apoptosis [168, 184, 185]. Moreover, Fc-Fc receptor binding can lead to host immune modulation, affecting different processes such as the generation, secretion and repression of pro- and anti-inflammatory substances, regulation of B cell activity and survival, and TLR signalling [186-191]. Of note is the fact that the same antibody can be involved in more than one type of response [177].

1.2.6.2 Antigen presentation

Although B cells are professional APCs, internalizing some protein antigens and presenting them in MHC molecules to T cells, they differ from other APCs by being specific for their cognate antigen [63]. While this specialization was first regarded as a disadvantage - few B cells with specificity to a given antigen - two distinct aspects give B cells the ability to efficiently present antigens: their BCR and their location.

APCs internalize antigen via three different mechanisms, phagocytosis, fluid-phase pinocytosis and receptor-mediated endocytosis [63]. Receptor-mediated endocytosis occurs through BCR recognition of the cognate antigen, allowing B cells to concentrate small amounts of antigen that can, as a result, be efficiently presented to T cells [192].

Interestingly, the BCR affinity to a given antigen has been shown to be proportional to the capacity of B cells to present that antigen to CD4 T cells [193]. In addition, BCR signalling leads to changes that stimulate the traffic of antigen [194, 195] and the synthesis of MHC molecules [196], steps which are also necessary for efficient antigen presentation.

Regarding their location, B cells find themselves in a privileged niche for B-T cell interactions - the secondary lymphoid organs. As such, while B cells rely on T cell help, they can also participate in T cell responses by presenting antigen. However, it is important to note that this APC function is dependent on the type and form of the antigen being presented [63, 197]. Advocating for the importance of T cell help in this context is the fact that both BCR crosslinking and CD40/CD154 engagement are necessary to activate the APC function of B cells. While BCR crosslinking provides growth, proliferation and survival signals, and increases CD86 expression [198], CD40/CD154 engagement contributes to CD86 stabilization, CD80 induction and enhancement of antigen processing [199-201]. Conversely, it has been shown that the lack of APC function in B cells might lead in some cases to impaired T cell helper function and IL-4 production [202]. Also important to mention is the fact that B cells appear to be able to initiate T cell responses even in the absence of other APCs [203, 204], a function that is only possible due to the progressive interaction that occurs between B and T cells [63].

Studies using experimental animal models have also shown that antigen presentation by resting B cells is able to induce T cell tolerance [205-207].

1.2.6.3 Cytokine-mediated functions

Even though the fact that B cells produce a vast array of cytokines has been known for a long time [208-210], the field of cytokine-mediated B cell effector functions is still understudied, with many observations made in single experimental models [25]. However, marked progress has been made recently. Three mains areas of the immune system where B cell-secreted cytokines show an important influence can be highlighted: $CD4^+$ T cell responses, tissue development and repair, and dampening of inflammatory immune responses.

The effect of B cell-produced cytokines on $CD4^+$ T cell responses is mediated by a variety of cytokines. TNF, IFNγ and IL-6 have been shown to promote T_H1 cell differentiation in different contexts, while IL-6 has also been shown to support T_{FH} and T_H17 cell responses [25, 211-215]. Also involved in T_H1 cell differentiation, although indirectly,

is the secretion of GM-CSF, which is suggested to increase IL-12 production by DCs [216]. B cells have also been suggested to affect T_H2 cell responses in a context of infection, in this case via IL-2, which seems to be important for effector and memory T cell formation [217], and lymphotoxin α1β2 (LTα1β2), which is indirectly involved in relocalization of T_H2 cells against helminths [218]. Moreover, B cell-secreted cytokines can indirectly support $CD4^+$ T cell responses by influencing cells from the innate immunity, with several studies showing stimulation of macrophage activation via IFNγ [219], and suggesting that IL-17-producing plasma cells can promote the accumulation of IL-10-producing neutrophils [220, 221].

Regarding tissue development, it is in the lymphoid tissue where the effects of B cell-secreted cytokines can best be seen [25]. These effects are mostly mediated by LTα1β2 and include development and localization of FDCs, T cells and the marginal zone in the spleen [222-224], lymph node remodelling during immune responses [225], maturation of isolated lymphoid follicles in the gut [226] and tertiary lymphoid tissue formation [227]. LTα1β2 appears to be also involved in tissue repair, with studies showing its effects in prostate regeneration and tumor regrowth [228, 229]. Another B cell-produced cytokine involved in the development of lymphoid tissue is TNF, shown to be required for marginal zone formation in the spleen and FDC development in lymph nodes and Peyer's patches [209, 222, 230].

Through release of IL-10 and IL-35 B cells can also dampen inflammatory immune responses [25]. This effect has been shown both *in vitro* and *in vivo* in contexts of autoimmunity and infection such as chronic intestinal inflammatory condition or *Salmonella* infection [231-238]. Depending on the context, the effects of these B cell-secreted cytokines can be protective or deleterious [236, 239].

1.3. *Candida albicans*

Candida albicans is a commensal fungus commonly found in gastrointestinal, oral and vaginal mucosal tissues of healthy individuals [240]. In fact, it is estimated that *Candida* species can be found in approximately 70% of the human population [241, 242]. However, this benign commensal can also cause severe infections in immunocompromised hosts, being one of the most common fungal pathogens of humans and one of the leading causes of nosocomial infections [243]. Despite increasing research efforts in recent years, compared

to other infections, fungal infections are still understudied and often misdiagnosed, leading to a high mortality rate [244].

1.3.1 Colonization versus invasion

In an immunocompetent host, *C. albicans* colonizes mucosal surfaces without triggering any major response, since it is controlled by the present microbial flora, the epithelial barriers and the innate immune system [245]. However, in case of a disruption in these control mechanisms, *C. albicans* can become invasive and trigger an array of immune responses, therefore becoming pathogenic [246, 247]. One characteristic that plays a crucial role in commensal-to-pathogen differentiation is polymorphism, as *C. albicans* is able to switch between yeast, pseudo-hyphae or true hyphae forms [248]. While yeast cells present a simple ellipsoid shape, hyphae cells form long filaments that can penetrate through the physical barriers of the host. Moreover, this yeast-to-hyphae shift leads to the exposure of cell wall components, as well as to the expression of hyphae-associated proteins, both factors known to increase *C. albicans* virulence [249]. Once in hyphal form, *C. albicans* has the ability to damage the epithelial barrier through a process that occurs in three subsequent stages: (1) adhesion, mediated by cell surface adhesins, some predominantly expressed in hyphae, (2) invasion, which can occur via host-mediated induced endocytosis or hyphae-mediated active penetration, and (3) damage, triggered by hyphae intraepithelial invasion and growth, and potentiated by virulence factors [245, 250]. Once inside the host, it is the job of the immune system to fight the invasion.

1.3.2 Anti-*Candida albicans* immune responses

The immune response against *C. albicans* starts by the recognition of conserved PAMPs present on its cell wall by PRRs expressed by immune cells [247]. The cell wall of *C. albicans* can be generally differentiated into two lawyers, the outer layer, mainly composed of N- and O-linked mannans, and the inner layer, composed of β-glucans and chitin [251]. The outer layer also contains mannoproteins [252]. Several groups of PRRs are involved in the recognition of these cell wall components: Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs).

Table 1 lists the PRRs currently known to be involved in *C. albicans* recognition, as well as their PAMPS, localization and attributed functions.

Table 1. **Pattern recognition receptors involved in *Candida albicans* recognition** (table adapted from Naglik 2014 [240] and Netea 2015 [247]).

Receptor family	Receptor	Recognizes	Expressed in	Functions	Refs
Toll-like receptors (TLRs)	TLR2	Phospholipo-mannan	Most immune cells	Pro-inflammatory cytokines	[253]
	TLR4	O-mannan		Pro-inflammatory cytokines	[253]
	TLR3	RNA		IFN-γ secretion	[254]
	TLR7	RNA		Pro-inflammatory cytokines	[255, 256]
	TLR9	DNA, chitin		Anti-inflammatory cytokines	[257]
C-type lectin receptors (CLRs)	Dectin-1	β-glucan	Monocytes, macrophages and neutrophils	Pro-inflammatory cytokines, TLR-signal amplification, NET formation	[151, 152, 258-262]
	Dectin-2/3	α-mannan	DCs, macrophages and neutrophils	T_H17 modulation, ROS production, pro-inflammatory cytokines	[263, 264]
	Mannose receptor	N-mannan	Macrophages	Pro-inflammatory cytokines – IL-17	[253, 265]
	DC-SIGN	N-mannan	DCs	T_H activation and differentiation	[266]
	MINCLE	α-mannan	Monocytes and neutrophils	TNF secretion	[267]
	Galectin-3	β-mannan	Macrophages	TNF secretion	[268]
	MBL	Mannan	Soluble	Complement activation	[269]
NOD-like receptors (NLRs)	NOD2	Chitin	Macrophages and neutrophils	IL-10 production	[257]
RIG-I-like receptors (RLRs)	MDA5 (IFIH1)	RNA?	Macrophages	IFN-β secretion	[270]
Complement receptors	CR3	β-glucan	Neutrophils	Phagocytosis and killing of unopsonized *Candida*	[271]
Fc receptors	FcγR	IgG	Neutrophils	Killing of opsonized *Candida*	[272]

The PRR identified as possessing a major role in recognition of *C. albicans* is dectin-1, as it recognizes a major constituent of the fungal cell wall - β-1,3 glucan [153]. However, several studies indicate that this type of glucan is masked by the outer layer of mannans, needing to be exposed for dectin-1 recognition [273]. Whether this exposure occurs only in budding yeast or also upon transition to the hyphal form seems to still be controversial [240, 247, 274, 275]. This example is only one of several on how yeast and hyphae are differentially recognized by the immune system [245, 276, 277].

As mentioned in a previous section, dectin-1 induces intracellular signals that lead to the activation of NF-κB and other transcription factors [158, 160]. A vast array of functions has been attributed to the binding of β-glucans to dectin-1, such as the stimulation of phagocytosis, secretion of pro-inflammatory cytokines, production of reactive oxygen species (ROS), mast cell activation and the prevention of excess release of neutrophil extracellular traps (NETs), protecting the host from immune response-associated tissue damage [258, 262, 278-280]. Moreover, dectin-1-induced secretion of cytokines was shown to influence DC maturation, and T_H1 and T_H17 differentiation [164, 281].

Further confirming the importance of dectin-1 in *C. albicans* immune defence is the fact that both mice and humans with impaired dectin-1 expression show increased susceptibility to *C. albicans* [279, 282]. When systemically infected, dectin-1 knockout mice displayed a higher mortality rate, which was proven to occur due to impaired cytokine production and poor neutrophil-mediated killing [282]. However, it is also of note, that a report from 2013 showed the requirement for dectin-1 to control systemic *C. albicans* infections in mice to be fungal strain-specific [283]. Patients carrying a mutation resulting in impaired dectin-1 expression and function are more susceptible to mucocutaneous candidiasis, which is also proven to be associated with a defect in cytokine production (such as TNFα, IL-6 and IL-17) [279]. Nevertheless, these patients presented no increased susceptibility to systemic candidiasis, probably due to normal phagocytosis and killing of *C. albicans*. Finally, dectin-1 activation has also been reported to amplify immune responses triggered by TLR2 and TLR4 engagement in monocytes and macrophages [260, 261, 284].

Several TLRs have been shown to be involved in the recognition of *C. albicans* cell wall components (see Table 1). Moreover, studies in MyD88 knockout mice, where TLR signalling is impaired, showed increased susceptibility of these mice to *C. albicans* infection [285], consolidating the importance of TLRs in response to this pathogen. In humans, while most of the available data point to an important role of TLRs in defence against *C. albicans*, the specific contribution of individual TLRs has been difficult to pinpoint [240, 286]. The two most studied TLRs in this context are TLR4 and TLR2, since a study from 2002 demonstrated their importance in the host defence against *C. albicans* [287]. In this study TLR4 knockout mice were more susceptible to *C. albicans* infection, which was shown to occur due to a decrease in chemokine secretion and neutrophil activation [287]. This increased susceptibility has since been both corroborated and contradicted [285, 288]. One

possible explanation for this divergence was published in 2010, indicating that TLR4 recognition is variable among *C. albicans* strains [289]. Another protective function attributed to TLR4 was the production of pro-inflammatory cytokines such as IFN-γ and TNF-α [285, 290-292]. Regarding TLR2, its main attributed function in response to *C. albicans* infection is immune suppression, since it has been shown to mediate the production of regulatory cytokines such as TNF-α and IL-10, and to promote T_{reg} survival [285, 287, 291, 293]. Results of *in vivo* models of *C. albicans* infection of TLR2 knockout mice are as well contradictory. While it was shown that TLR2 knockout mice were more resistant to disseminated candidiasis due to better chemotaxis and enhanced killing capacity of its macrophages [293], another study described a higher susceptibility of these mice due to a decrease in neutrophil recruitment caused by impaired chemokine production [294]. One can speculate that the discrepancy between these results might be a consequence of different infection models, since a third study has reported a comparable susceptibility to *C. albicans* primary infection between TLR2 knock and control mice, but a decrease survival in case of re-infection [285].

From the moment *C. albicans* overcomes the epithelial barrier and is first recognized, it is the job of the immune system to trigger a chain of effector mechanisms that lead to the clearance of the pathogen (Figure 4). The first response comes from the epithelial cells themselves, which produce not only cytokines to recruit phagocytic immune cells, but also β-defensins with anti-*Candida* activity [295, 296]. Also providing a fast response are the tissue-resident macrophages, which are able to phagocytose invading *C. albicans* cells and to produce cytokines involved in neutrophil recruitment [253, 297]. Neutrophils, recognized as the most potent *C. albicans* killers, are the key players in several anti-*Candida* mechanisms. These mechanisms include phagocytosis, production of ROS and anti-microbial factors, and the release of neutrophil extracellular traps (NETs), which can trap *C. albicans* cells inhibiting their growth via antimicrobial proteins [298-302]. Moreover, studies showing that neutropenia is a major risk factor for candidiasis have confirmed the importance of neutrophils in this context [303, 304]. Other innate immune cells such as monocytes and natural killer cells have also been reported to contribute to the clearance of *C. albicans* upon infection [297, 305, 306].

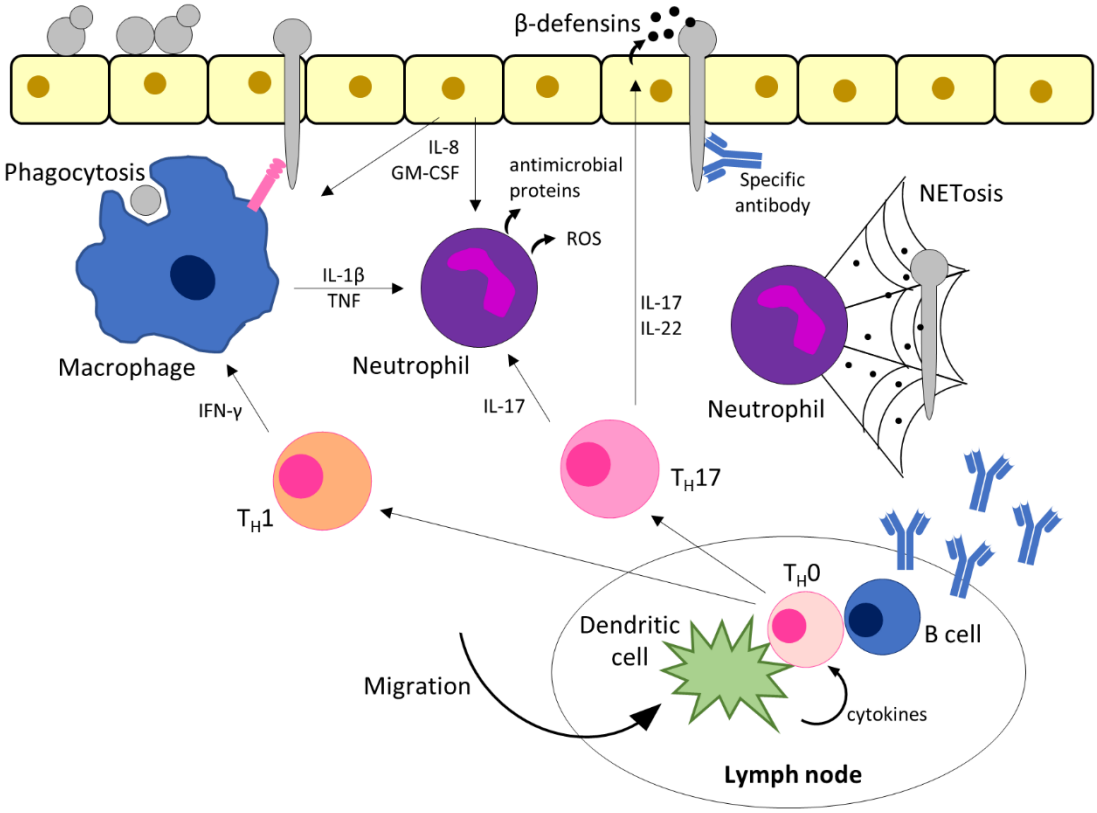


Figure 4. **Immune response upon *C. albicans* invasion.** The first line of defence against *C. albicans* is the epithelial barrier. More than forming a physical barrier, epithelial cells mount a first response by secreting defensins with anti-*Candida* activity, and cytokines that lead to the recruitment of phagocytic cells. Once in the tissue, *C. albicans* encounters tissue-resident macrophages, which provide a fast response through their phagocytic capacity and secretion of pro-inflammatory cytokines. The secretion of pro-inflammatory cytokines by both epithelial cells and macrophages leads to the recruitment of neutrophils, cells with a strong *Candida*-killing capacity. Not only are neutrophils capable of phagocytosis, they also secrete antimicrobial proteins and reactive oxygen species (ROS) which are deleterious for *C. albicans*. Moreover, neutrophils can release neutrophil extracellular traps (NETs) that capture and inhibit *C. albicans* growth. Upon fungal recognition, dendritic cells (DCs) migrate to secondary lymphoid organs where they contribute to adaptive immune responses. There, they act as antigen-presenting cells leading to the activation of T cells, also influencing T helper (T_H) cell differentiation through the cytokine milieu they provide. In the context of *C. albicans* invasion, T_H17 and to a smaller extent T_H1 responses play an important role. While T_H17 cells secrete IL-17 and IL-22 that participate in neutrophil recruitment and β–defensin production by epithelial cells, T_H1 cells produce IFN-γ, a potent phagocytic cell activator cytokine. Upon activation, B cells produce anti-*C. albicans* antibodies which can either opsonize the fungal cells, targeting them for phagocytosis by neutrophils, or directly influence the cell wall, mediating processes that lead to decreased pathogenicity. Examples of such effects are the inhibition of growth and adhesion.

The activation of the adaptive immune system upon *C. albicans* recognition is mediated by DCs, which migrate to secondary lymphoid organs to shape T helper responses via antigen presentation and cytokine secretion. The type of DC involved is of special

importance, since the nature of the triggered T cell response is determined by the cytokine milieu T cells encounter [240, 307]. T_H17 responses are of special interest for *C. albicans* immunity, as IL-17 secretion has been shown to be a key event in protective responses [308-310]. T_H17 cells produce not only IL-17 but also IL-22, both proven to trigger neutrophil recruitment and activation, and to induce secretion of β-defensin in epithelial cells [308, 311]. Also relevant are T_H1 responses, since these cells produce IFNγ, a potent phagocytic cell activator [312-314]. Although performing less prominent roles, other immune cell types and humoral components such as B cells, ILCs and the complement system, have been shown to be involved in the host response to *C. albicans* [240, 247].

1.3.3 B cells in *Candida albicans* immunity

For several years, clinical data and studies on patients with B cells or immunoglobulin deficiencies pointed to no major role of B cells in *C. albicans* infection [315, 316]. However, many studies are now changing this view by proposing that antibodies are involved in multiple protective mechanisms [317-332]. Among these studies, many have shown monoclonal antibodies which recognize cell wall components of *C. albicans* and whose binding leads to a decrease in pathogenic capacity. Examples of functions mediated by these antibodies are inhibition of growth, hyphae formation, adhesion, biofilm formation and metabolic processes [323, 326, 327, 329, 332-334]. The exact mechanisms through which these effects are achieved have not in all cases been identified, but are probably related to the disruption of the fungal cell wall [335]. Antibodies can also exert protective functions through Fc receptor-dependent ADCC or by priming other components of the immune system with anti-*C. albicans* action [336, 337]. By recognizing shared cell wall components or by influencing other immune cells, certain antibodies provide cross-protection against more than one fungal pathogen [326, 338]. However, it is important to keep in mind that the masking of cell wall components and polymorphism of *C. albicans* poses an additional difficulty in the production of high affinity antibodies. Even though antibodies against different cell wall glycans and glycoproteins have been identified, and mannoproteins recognized as the major component eliciting antibody responses [335, 339], a recent study has shown that the glucan epitopes are those which contribute the most to these responses [340].

In a clinical setting, anti-*C. albicans* antibodies can be very valuable in two distinct scenarios, vaccination and diagnosis. Regarding vaccination, even though many efforts have been made in the recent years, few vaccines directed to *C. albicans* have successfully completed Phase I clinical trials [240]. One of such vaccines was the monoclonal antibody MycoGrab/efungmab, targeting the heat shock protein 90 (Hsp90) [341]. Although it progressed through to Phase III clinical evaluation against invasive systemic candidiasis, due to claimed production difficulties it was never commercialized [317]. Two vaccines currently in clinical evaluation against recurrent vulvovaginal candidiasis are PEV7, which is constituted by the *C. albicans* protein Sap2 and an influenza virosome [342], and NDV-3, which contains the N-terminal region of the hyphal protein Als3 and is formulated using alum adjuvant [337, 338, 343]. Since the diagnosis of *C. albicans* infections is often difficult and in many case still dependent on a blood culture test, new and non-invasive diagnostic tools are necessary. While the detection of (1-3)-β-D-glucan, mannan and/or anti-mannan antibodies is currently used as a tool to diagnose fungal infections [344, 345], the specificity and/or sensitivity of these methods, although high, still does not allow for an unequivocal positive diagnosis [346]. Therefore, the identification of novel *Candida albicans*-associated proteins whose presence could confirm the existence of an infection – for example, a specific antibody - would be advantageous. To that end, a recent study analysed the serologic profiles of systemic candidemia patients, identifying a set of 19 *C. albicans*-reactive IgGs with possible diagnosis value [340]. However, probably due to the fact that *C. albicans* is a commensal and a basal level of antibodies is present in most individuals, no unambiguous anti-*C. albicans* antibody pattern for a consistent diagnosis was found. Even though it can now be affirmed that B cells/antibodies play a role in the defence against *C. albicans*, many details of their involvement are still undiscovered.

1.4. Aim of the Project

Although many advances in the understanding the role of antibodies in the context of *Candida albicans* infection have recently been made, not much is known about the mechanisms through which B cells interact with this pathogen. Therefore, the first aim of this project was to investigate the impact of *C. albicans* on B cells, as well as to uncover the mechanism through which this pathogen is recognized. For that purpose, mouse splenic B cells were cultured *ex vivo* in the presence of both heat-killed *C. albicans* (HKCA) yeast and hyphae, and of the fungal cell wall preparation zymosan. The outcome of such interaction was measured using flow cytometry and ELISA techniques, which allow to determine B cell-related functions such as class switch recombination, and antibody and cytokine production. The use of a reporter mouse made it possible to assess AID activation, a measure of B cell activation due to its importance in processes crucial for high affinity antibody production. To identify the receptors responsible to recognize *C. albicans*, B cells from mice presenting impaired signalling in different receptors were analysed. Examples of the mice used were dectin-1 knockout, where the receptor dectin-1 was not functional, MyD88 knockout, where the absence of the protein MyD88 abrogates TLR signalling and B1-8f, whose BCR is only capable of recognizing an irrelevant antigen.

The second aim of this project was to go deeper into *C. albicans*-triggered B cell functions. To this end, and not forgetting the role of B cells as cytokine producers, the cytokine secretion profiles of B cells co-cultured with HKCA or zymosan were screened using a BioPlex assay and the prominent candidates further analysed by ELISA.

This study hopes to open the door to a more in-depth analysis of how exactly B cells recognize *C. albicans* in a physiological context.

2. Materials and Methods

2.1 Materials

2.1.1 Buffers, media and solutions

Table 2. List of used buffers, media and solutions, and respective composition.

Buffer/medium	Application	Composition
1x PBS		137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.76mM KH_2PO_4, in dH_2O. pH7.4
cRPMI medium	Culture of mouse B cells	RPMI1640, 10% FBS, 100U/mL penicillin/100µg/µL streptomycin, 100mM HEPES, 50µM β-mercaptoethanol
YPD medium	*Candida albicans* growth	1% yeast extract, 1% peptone, 2% glucose, in dH_2O For solid medium: add 2% agar
YNB medium	*Candida albicans* growth	0.67% Yeast nitrogen base, 1% glucose, in dH_2O
YNBNP medium	*Candida albicans* growth	0.67% Yeast nitrogen base, 0.2% glucose, 5mM N-acetylglucosamine, in 25mM potassium phosphate buffer pH7.0
Carbonate-Bicarbonate buffer pH9.5 – ELISA coating buffer	Immunoglobulin ELISA	0.2M Na_2CO_3, 0.2M $NaHCO_3$, in dH_2O. pH adjusted to 9.5
ELISA washing buffer	Immunoglobulin ELISA	1x PBS, 0.1% Tween-20
ELISA buffer	Immunoglobulin ELISA	1x PBS, 0.1% Tween-20, 1% Milk Powder
0.05M Phosphate-Citrate buffer pH5.0 - ELISA substrate buffer	Immunoglobulin ELISA	0.2M Na_2HPO_4 x $7H_2O$, 0.1M Citric acid, dH_2O- pH adjusted to 5.0
ELISA substrate solution	Immunoglobulin ELISA	1 tablet OPD (o-phenylenediamine dihydrochloride, 10mg, Sigma) in 25 mL ELISA substrate buffer, add 10µL 30% H_2O_2
Wash buffer	ELISA Ready-Set-Go! kit	1x PBS, 0.05% Tween-20

2.1.2 Cell culture stimulants

Table 3. List of used cell culture stimulants

Stimulant	Company	Catalog No.
LPS	Sigma	L4391
α-CD40	eBioscience	16-0402-86
IL-4	eBioscience	14-8041-80
Zymosan	Wako	269-01493

2.1.3 Antibodies

Table 4. List of used antibodies.

Antibody	Conjugated/Purified	Application	Company	Catalog No.
α-mouse B220	FITC	Flow cytometry	BD Biosciences	553088
α-mouse B220	PE	Flow cytometry	BD Biosciences	553090
α-mouse B220	APC	Flow cytometry	BioLegend	103212
α-mouse B220	BV785	Flow cytometry	BioLegend	103246
α-mouse CD3ε	PE	Flow cytometry	BD Biosciences	553064
α-mouse CD21	FITC	Flow cytometry	BD Biosciences	561769
α-mouse CD23	PE	Flow cytometry	BD Biosciences	561773
α-mouse CD95	PE	Flow cytometry	BD Biosciences	55458
α-mouse dectin-1	PE-Cy7	Flow cytometry	eBioscience	25-5859-80
α-mouse dectin-1	FITC	Flow cytometry	Thermo Fischer Scientific	MA5-16480
α-mouse IgG1	FITC	Flow cytometry	BD Biosciences	553443
α-mouse IgG1	PE	Flow cytometry	BD Biosciences	550083
α-mouse IgG1	Biotin	ELISA	BD Biosciences	553441
α-mouse IgG1	Purified	ELISA	BD Biosciences	553445
Mouse IgG1, κ	Purified	ELISA	BD Biosciences	557273
α-mouse IgM	APC	Flow cytometry	BioLegend	406509
PNA	FITC	Flow cytometry	Vector	FL-1071

Antibodies used in IL-6 and IL10 ELISA were provided in ELISA Ready-Set-Go! kits from eBioscience (IL-6 cat. no. 88-7064-88, IL-10 cat. no. 88-7105-88).

2.1.4 Mice

- The AIDCreRosa26YFP line was generated by crossing AIDCre and Rosa26YFP mice. Rosa26YFP mice were obtained from Dr. Helen Morrison from the Leibniz Institute on Aging, Fritz Lipmann Institute. AIDCre mice were purchased from The Jackson Laboratories.
- Dectin-1 knockout mice were obtained from Dr. med. Hortense Slevogt from the Host Septomics, Jena University Hospital.
- B1-8f transgenic mice were obtained from Prof. Dr. Klaus Rajewsky from the Max Delbrück Center for Molecular Medicine.
- MyD88 knockout mice were obtained from Prof. Dr. med. Thomas Kamradt from the Institute of Immunology, Jena University Hospital.

These mouse lines were bred and housed under specific pathogen-free conditions in the “Serviceeinheit Experimentelle Biomedizin”, Friedrich Schiller University Jena. Mice

were bred heterozygously to obtain littermate knockouts and wildtype controls. All used mouse lines were on a C57BL7/6 background.

- TLR2 knockout, TLR4 knockout and C57BL7/6 control mice were obtained from Prof. Dr. Marcus Fulde from the Institute of Microbiology and Epizootics, Freie Universität Berlin. Mice were housed by the research group Microbial Immunology, Leibniz Institute for Natural Product Research and Infection Biology, Hans Knöll Institute.

2.2 Methods

2.2.1 Preparation of heat-killed *Candida albicans*

The *Candida albicans* strain SC5314 was obtained from Nicole Engert, Microbial Immunology, Leibniz Institute for Natural Product Research and Infection Biology, Hans Knöll Institute. A single colony of *C. albicans* was inoculated overnight in YPD at 30°C, 180rpm to obtain a starting culture. To differentiate between yeast and hyphae, cells from the initial culture were inoculated at 10^6 cells/mL in YNB or YNBNP for 24 hours. For yeast cells, the inoculation was done in YNB at 30°C, 180rpm. For hyphae cells, the inoculation was done in YNBNP at 37°C, 180rpm. Cultures were then washed and set to a density of 10^8 yeast cells/mL in PBS. The same dilution factor was used for hyphal cells. Diluted cells were distributed into 1.5mL tubes and heat-killed by incubation at 80°C for 15min, 800rpm. Cells were stored at -20°C. To ensure cell death, cells were thawed and plated in YPD agar plates at 37°C. To prepare 100mg/mL working stocks, cells were centrifuged, weighed and resuspended in the appropriate volume of sterile PBS. To reduce cell clumping, hyphae cells were sonicated prior to usage.

2.2.2 B cell isolation

Primary B cells were isolated from spleens of 8- to 16-week-old mice. When comparing genotypes, littermate mice of the same sex were used. The same isolation and culture procedures were used for all analysed mouse lines. In between steps, cells were washed in PBS 2% FBS by centrifuging for 8min at 500g (before splenic cell suspensions) or 10min at 300g (splenic cell or B cell suspensions), 4°C. Spleens were harvested and pressed through a 70µM strainer in PBS 2% FBS to obtain homogeneous cell suspensions.

Erythrocytes were removed by incubation with Red Blood Cell Lysing Buffer (Sigma) for 5 to 10 minutes. Erythrocyte debris was filtered using a 40µL strainer, and the strainer abundantly washed. For B cell isolation by negative selection, cell suspensions were incubated for 15min with 10µL anti-CD43 beads (Miltenyi Biotec) per 10^7 cells, and the unbound beads washed out. Cell suspensions were then resuspended at a density of 10^8 cells per 500µL of PBS 2% FBS, and passed through MACS columns in a magnetic separator (Miltenyi Biotec). Columns were rinsed with PBS 2% FBS. The unlabeled fraction, which was not retained in the column, contained the purified B cells. Cells were diluted in Trypan Blue and counted using a hemocytometer.

The purity of the isolated cells was analysed by flow cytometry. To that end, cell aliquots from before and after MACS were stained with α-B220-FITC and α-CD3-PE for 30min at 4°C. After incubation, cells were washed for 5min at 3000rpm, 4°C, and resuspended in PBS 2% FBS for flow cytometry analysis. For dead cell exclusion, cells were stained with DAPI (Sigma) prior to analysis. In all experiments, flow cytometry was performed using a LSR Fortessa cytometer (BD Biosciences), and the results analysed using FlowJo software (FlowJo, LLC).

2.2.3 B cell culture and stimulation

Isolated B cells were seeded at a density of $5x10^5$ cells/mL in cRPMI in 48-well plates. cRPMI was either further unsupplemented, or supplemented with 10µL/mL LPS and 20ng/mL IL-4, or 1µg/mL α-CD40 and 20ng/mL IL-4. In some experiments cRPMI was instead supplemented with 1 or 10µg/mL α-IgM. After 3 to 4 hours of incubation at 37°C, zymosan, HKCA yeast or HKCA hyphae was added to the cells for a final concentration of 30µg/mL zymosan or 500µg/mL HKCA. Cells were kept in culture in a final volume of 1mL/well for 5 days at 37°C. At day 3 of culture, 500µL of cell suspension was recovered and replaced by 500µL of fresh medium containing the same stimulation conditions. The remaining cells were recovered at day 5 of culture. After recovery, cells were centrifuged at 1200rpm for 5min, 4°C, and the supernatants collected and stored at -20°C and -80°C for posterior analysis. Cells were washed with PBS 2% FBS and prepared for flow cytometry analysis.

2.2.4 Flow cytometry analysis of culture B cells

B cells recovered on days 3 and 5 of culture were analysed by flow cytometry to determine the percentage of IgG1-positive cells. Cells were stained with anti-B220-PE, anti-IgG1-FITC and anti-IgM-APC in PBS 2% FBS for 30min at 4°C in the dark. Staining was stopped by the addition of PBS 2% FBS and cells washed by centrifugation at 1200rpm for 5min, 4°C. Cells were subsequently resuspended in PBS 2% FBS, and DAPI (Sigma) was added to each sample prior to analysis. In experiments involving AIDCre-Rosa26YFP mice, cells were stained with anti-B220-APC and anti-IgG1-PE, and the percentage of YFP-positive cells analysed in addition. Flow cytometry was performed using a LSR Fortessa cytometer with a coupled High Throughput Sampler (BD Biosciences) to allow automated sample acquisition from 96-well microtiter plates. The results were analysed using FlowJo software (FlowJo, LLC).

2.2.5 ELISA analysis of immunoglobulin secretion in B cell culture supernatants

B cell culture supernatants recovered on days 3 and 5 of culture were analysed by ELISA to determine the concentration of IgG1. To that end, Nunc MaxiSorp ELISA 96-well plates were coated with 2µg/mL anti-IgG1 in ELISA coating buffer (50µL/well), and incubated overnight at 4°C. On the following day, the coating solution was discarded and the plates were washed three times with 200µL/well ELISA washing buffer. Unspecific binding was prevented by blocking with 200µL/well ELISA buffer for at least 30min at room temperature. ELISA buffer was discarded and the wells washed once. To prepare a standard curve, 8 different purified IgG1 concentrations were prepared by 1:2 serial dilutions in ELISA buffer. The standard curve started with 0.1µg/mL purified IgG1 and was prepared in duplicate. Supernatants were analysed undiluted and diluted in ELISA buffer, supernatants collected at day 3 of culture diluted 1:5 and 1:10, and supernatants collected at day 5 of culture diluted 1:10 and 1:100. Standard dilutions and supernatants were added to the plate and incubated for 1 hour at room temperature (50µL/well). After sample incubation, plates were washed three times. For IgG1 detection, plates were incubated with anti-IgG1-biotin (1:500 in ELISA buffer, 50µL/well) for 1 hour at room temperature, followed by three times washing, and incubation with streptavidin-HRP (BioLegend) (1:1000 in ELISA buffer, 5µL/well) for another 1 hour at room temperature. Before addition of the substrate solution, plates were again washed three times. 100µL of ELISA substrate solution was added to each

well, and the plates incubated in the dark at room temperature. After 20min, the reaction was stopped with 3N HCl (25µL/well) and the absorbance measured at 492nm using a plate reader. Results were analysed using Microsoft Excel.

2.2.6 B cell proliferation

Proliferation of freshly isolated B cells was measured by following CFSE dilution via cell division using flow cytometry. For this purpose, the Vybrant CFDA SE Cell Tracer Kit (Thermo Fischer Scientific) was used, as the non-fluorescent CFDA SE is highly cell permeable, being converted to CFSE once in the cytoplasm. After isolation, B cells were set to a density of $5x10^6$ cells/mL in pre-warmed PBS and stained with 1µM CFDA SE for 10min at 37°C. After staining, the stain was quenched by addition of 5 times cold cRPMI. Cells were then re-pelleted, resuspended in pre-warmed cRPMI and incubated for additional 20min at 37°C to ensure complete modification of the dye. Following incubation, cells were re-pelleted and set in the appropriate conditions for culture and stimulation. CFSE dilution was analysed by flow cytometry daily between days 1 and 4 of culture. In addition, cells were stained with anti-B220-PE and DAPI (Sigma) for live/death exclusion as previously described. Flow cytometry was performed using a LSR Fortessa cytometer with a coupled High Throughput Sampler (BD Biosciences) to allow automated sample acquisition from 96-well microtiter plates. The results were analysed using FlowJo software (FlowJo, LLC). The number of divisions during the analysed time period was calculated by $Log_2(\frac{MFI_{d1}}{MFI_{d4}})$ using Microsoft Excel. MFI, mean fluorescent intensity.

2.2.7 Dectin-1 RT-PCR

The presence of dectin-1 mRNA in B cells was analysed by RT-PCR. RNA from mouse splenic cells, isolated B cells and the murine macrophage cell line RAW 264.7 was isolated using the Quick-RNA Miniprep kit (Zymo Research) accordingly to manufacturer's instructions. RNA was stored at -80°C. RNA purification was confirmed by electrophoresis, by running the isolated RNA in a 1% agarose gel. cDNA synthesis was performed using the First Strand cDNA Synthesis Kit for RT-PCR (Roche) accordingly to manufacturer's instructions. cDNA was stored at -20°C. To detect dectin-1, the following pair of intron spanning primers was designed: Dectin-1_fwd 5'-ACCACAAGCCCACAGAATCAT-3'

and Dectin-1_rev 5'- GACTTGAAACGAGTTGGGGAAG-3', amplifying a product of 347 base pairs. Tables 5 and 6 show the used PCR reaction mix and PCR conditions, respectively. The primer pair β-Act1-fw 5'-ACCTTCAACACCCCAGCCATGTACG-3' and β-Act2-re 5'-CTAATCCACATCTGCTGGAAGATGG-3' was used to detect β-actin as a loading control. These primers generated a product of 698 base pairs.

Table 5. PCR reaction mix for dectin-1 RT-PCR

	Per sample
dH_2O	11.2µL
PCR Buffer (10x)	2µL
MgCl2 (25 mM)	1.6µL
dNTP´s (2 mM)	2µL
Dectin-1_fwd (10 µM)	0.5µL
Dectin-1_rev (10 µM)	0.5µL
Taq Polymerase	0.2µL
cDNA	2µL
Total	20µL

Table 6. PCR conditions for dectin-1 RT-PCR

	Temperature	**Time**	**Cycles**
Denaturation	94°C	5 min	
Denaturation	94°C	60 sec	35 X
Annealing	65°C	50 sec	
Elongation	72°C	60 sec	
Elongation	72°C	5 min	
	4°C	∞	

2.2.8 Dectin-1 expression analysis by flow cytometry

The expression of surface dectin-1 in mouse B cells was analysed by flow cytometry using two different anti-dectin-1 antibody clones. Splenic B cells were stained with anti-B220-BV785 and anti-dectin-1-PE-Cy7 (clone bg1fpj) or dectin-1-FITC (clone 2A11) in PBS 2% FBS for 30min at 4°C in the dark. Staining was stopped by the addition of PBS 2% FBS and cells washed by centrifugation at 3000rpm for 5min, 4°C. Cells were then resuspended in PBS 2% FBS, and DAPI (Sigma) was added to each sample prior to analysis. Flow cytometry was performed using a LSR Fortessa cytometer (BD Biosciences) and the results analysed using FlowJo software (FlowJo, LLC).

2.2.9 B cell subset analysis by flow cytometry

Splenic B cells were analysed to determine percentages of follicular versus marginal zone B cells, and of germinal center B cells. To label follicular and marginal zone B cells, isolated cells were stained with anti-B220-APC, anti-CD21-FITC and anti-CD23-PE. To label germinal center B cells, isolated cells were stained with anti-B220-APC, anti-CD95-PE and PNA-FITC. Staining was performed in PBS 2% FBS for 30min at 4°C in the dark, and stopped by further addition of PBS 2% FBS and centrifugation at 3000rpm for 5min, 4°C. Cells were then resuspended in PBS 2% FBS, and DAPI (Sigma) was added to each sample prior to analysis. Flow cytometry was performed using a LSR Fortessa cytometer (BD Biosciences) and the results analysed using FlowJo software (FlowJo, LLC).

2.2.10 Cytokine screening using LEGENDplex™

B cell culture supernatants were analysed for the presence of the cytokines TNF-α, IFN-γ, IL-2, IL-5, IL-4, IL-6, IL-10 and IL-13 using the bead-based immunoassay LEGENDplex™ (BioLegend). The assay was conducted accordingly to manufacturer's instructions. Flow cytometry was performed using a LSR Fortessa cytometer (BD Biosciences) and the results analysed using the LEGENDplex™ Data Analysis Software (BioLegend) and Microsoft Excel.

2.2.11 ELISA analysis of IL-10 and IL-6 presence in B cell culture supernatants

B cell culture supernatants recovered at day 5 of culture were analysed for the presence of IL-10 and IL-6 by ELISA using Ready-Set-Go! ELISA kits (eBioscience). The assay was conducted accordingly to manufacturer's instructions. Results were analysed using Microsoft Excel.

2.2.12 Statistical analysis

Statistical significance was determined using the two-tailed unpaired Student's t-test. Statistical analysis was performed using Microsoft Excel. p values under 0.05 were considered significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

3. Results

3.1. How do B cells respond to *Candida albicans*? – Experimental setup

The first aim of this study was to investigate how B cells respond to the presence of both the yeast and hyphal forms of *Candida albicans*. For that purpose, conditions under which B cells could be exposed to both isolated forms of *C. albicans* needed to be established. Co-cultures using live *C. albicans* were not possible, since these resulted in non-viable B cells. Therefore, a protocol for growth and heat-killing of both yeast-only and hyphae-only cells was established and heat-killed *C. albicans* (HKCA) stocks were generated (see Materials and Methods). To study the effect of *C. albicans* on B cell activation, mouse splenic B cells were isolated using MACS and cultured in the presence or absence of HKCA yeast or hyphae. As control, B cells were also cultured in the presence of zymosan, a cell wall preparation from *Saccharomyces cerevisiae*, which is widely used as a model for fungus-induced immune stimulation [261, 347]. Considering that optimal culture conditions for fungal recognition by B cells were not established, three different culture media were tested: 1) unsuplemented medium (unstimulated), 2) medium supplemented with LPS and IL-4, mimicking a T cell-independent activation of B cells, and 3) medium supplemented with α-CD40 and IL-4, mimicking a T cell-dependent activation of B cells. After 3 and 5 days of culture, B cells were analysed by flow cytometry and the culture supernatants stored to be further analysed by ELISA. Figure 5 shows the general workflow used for the experiments. In all experiments, the purity of isolated cells was confirmed by flow cytometry, being generally above 95% (Figure 6).

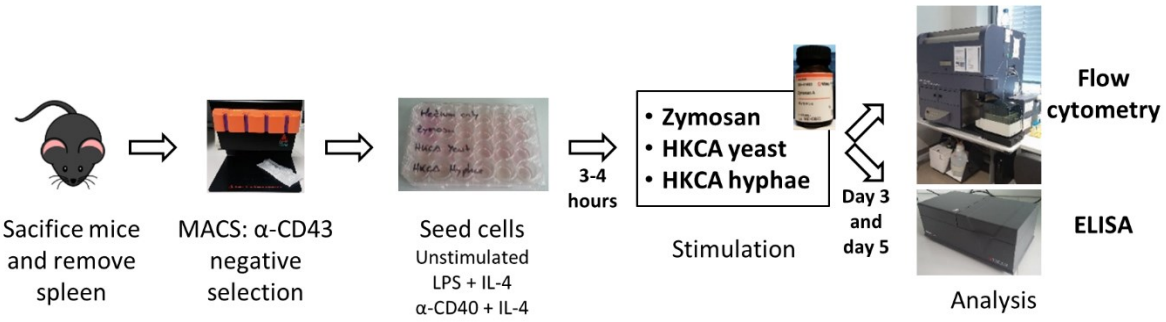


***Figure 5*. Workflow of B cell isolation, culture and analysis.** Splenic mouse B cells were isolated by MACS and cultured (5x10^5 cells/mL) for 5 days, either unstimulated or stimulated with LPS+IL-4 or anti-CD40+IL-4 in the presence of Zymosan, HKCA yeast or HKCA hyphae. On day 3 and 5 of culture, the cells and supernatants were recovered and analysed by flow cytometry and ELISA, respectively.

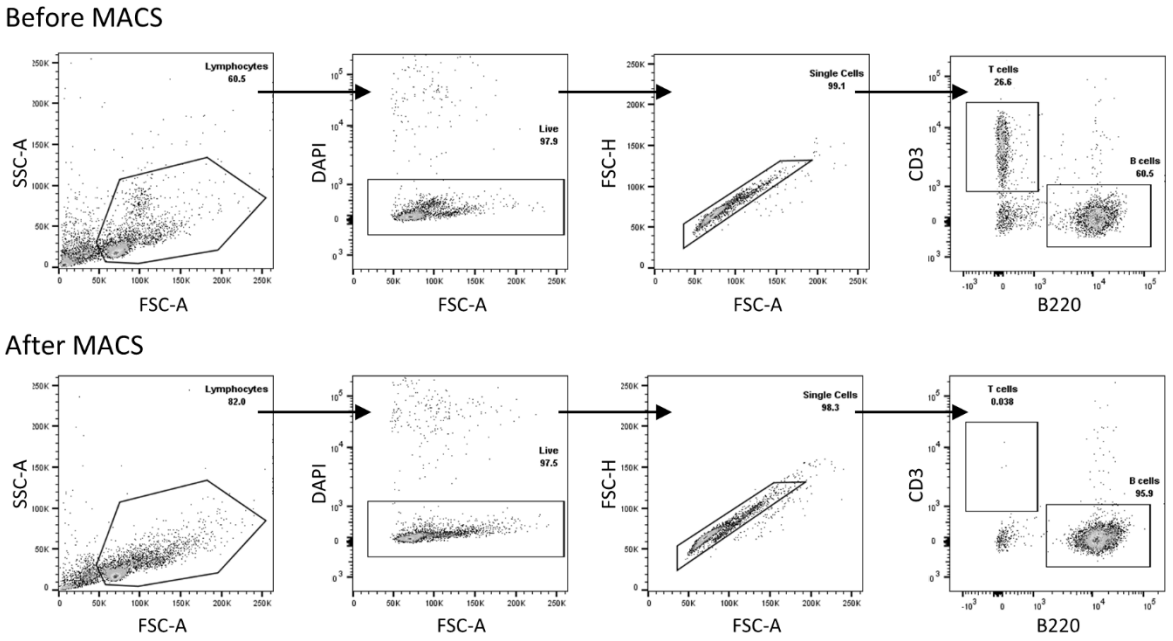


***Figure 6*. Gating strategy to confirm purity of isolated B cells.** Splenic mouse B cells were isolated by MACS with anti-CD43 beads (negative selection). Splenic cells and cells obtained after B cell isolation were analysed by flow cytometry. Cells were gated to exclude debris, dead cells and doublets. Dead cells were excluded by positive DAPI staining. B cells ($B220^+$) and T cells ($CD3^+$) were gated within the single cell gate. Representative plots are shown.

3.2. *Candida albicans* hyphae increase IgG1 production in B cells

The first parameter analysed to assess B cell activation upon culture with HKCA and zymosan was class switch recombination. The presence of IL-4 in the culture medium leads to *in vitro* IgG1 class switching, making it possible to measure the percentage of IgG1-positive cells in the culture by flow cytometry (gating strategy shown in Figure 7). Figure 8A shows that only cells harvested on day 3 and cultured in unsupplemented medium display a slight increase in class switching upon contact with zymosan and HKCA hyphae. On the other hand, HKCA yeast lead to a decrease in the percentage of IgG1-positive cells in supplemented medium. Therefore, the data show that, in general, the presence of HKCA hyphae or zymosan do not increase class switch recombination in B cells.

Considering that class switching does occur (Figure 8A), it would be possible that HKCA could modulate IgG1 secretion. As such, the second analysed parameter was the concentration of IgG1 in the culture supernatants, which was determined by ELISA. Figure 8B shows that both zymosan and HKCA hyphae trigger an increase in IgG1 production when compared to medium only. This effect is significant for both T cell-dependent and unstimulated conditions, and also seen as a trend in T cell-independent stimulation

conditions. In opposition, the presence of HKCA yeast seems to cause a decrease in IgG1 production, especially in T cell-independent stimulation conditions. The low class switching and IgG1 production observed in cells cultured in unsupplemented media is likely linked to the reduced cell numbers obtained after 3 and 5 days of culture (data not shown). This leads to the conclusion that additional survival/proliferation signals are indispensable to maintain B cells in culture, even in the presence of zymosan or HKCA.

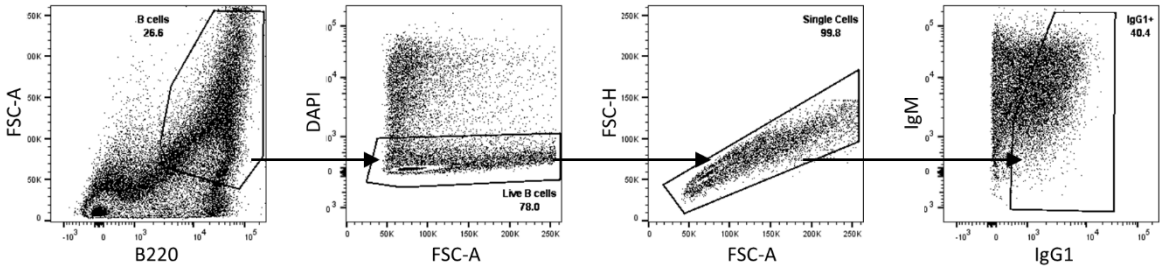


***Figure 7*. Gating strategy for IgG1 class switch recombination analysis.** Splenic mouse B cells were isolated by MACS and cultured ($5x10^5$ cells/mL) for 5 days, either unstimulated or stimulated with LPS+IL-4 or anti-CD40+IL-4 in the presence of Zymosan, HKCA yeast or HKCA hyphae. On day 3 and 5 of culture, the cells were analysed by flow cytometry to determine the percentage of IgG1-positive cells. B cells were first gated to exclude debris and other cells or particles ($B220^+$). Dead cells were excluded by positive DAPI staining. Cells were subsequently gated for single cells and the percentage of $IgG1^+$ cells was obtained within this gate. Representative plots show cells stimulated with anti-CD40+IL-4 in the presence of HKCA hyphae recovered after 5 days in culture.

To determine if the enhanced response induced by zymosan and HKCA was caused by an increase in proliferation, B cells were labelled with carboxyfluorescein succinimidyl ester (CFSE) and its dilution via cell division was measured by flow cytometry for 4 days. The resulting histograms, depicted in Figure 9, show that in the case of cells cultured in unsupplemented medium, the presence of zymosan or HKCA hyphae (and not yeast) leads to a significant increase in proliferation. However, likely due to substantial proliferation of cells in basal stimulation conditions, that increase is not as pronounced in cells cultured either in the presence of LPS+IL-4 or α-CD40+IL-4. It can be thus concluded that proliferation is not the cause for the observed increase in IgG1 production.

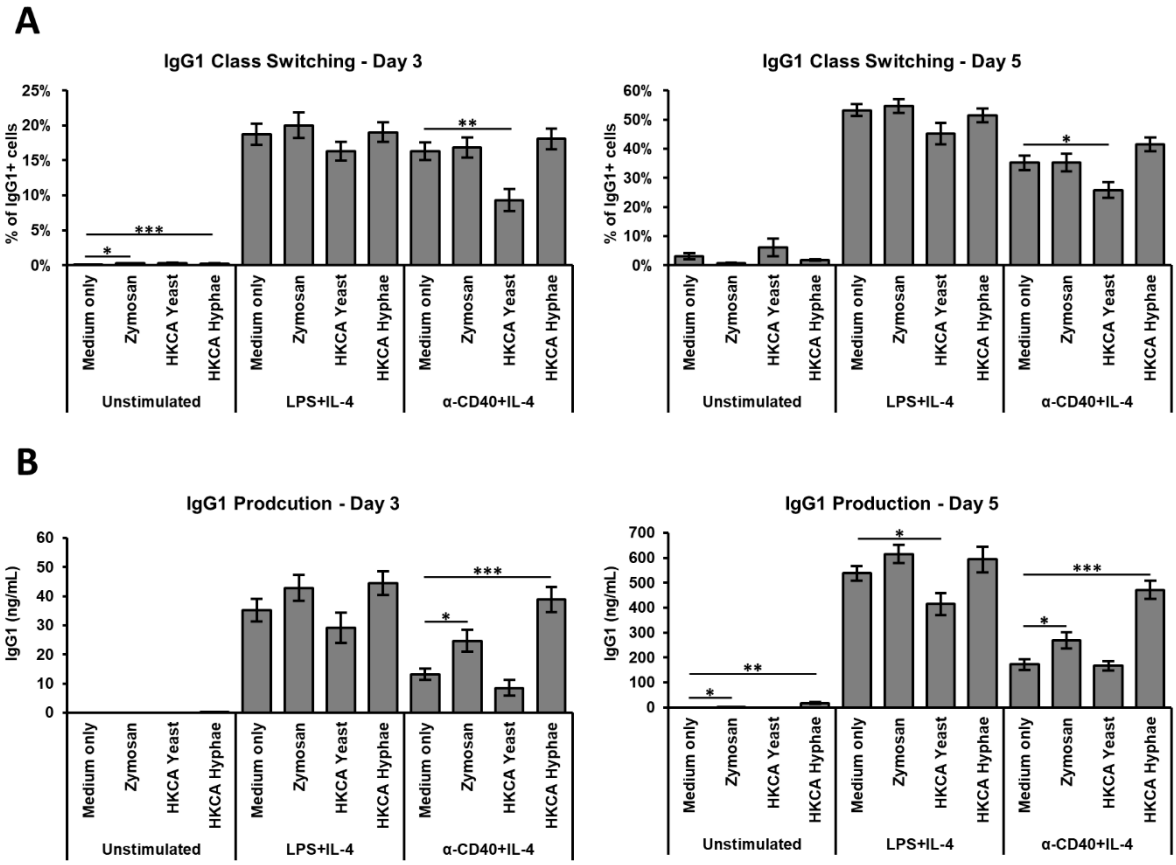
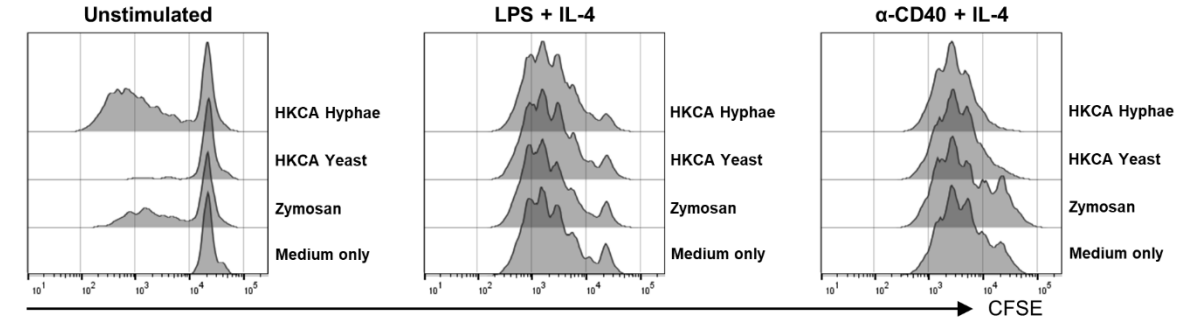


***Figure 8*. B cell stimulation with Zymosan and HKCA hyphae leads to an increase in antibody production.** Splenic mouse B cells were isolated by MACS and cultured ($5x10^5$ cells/mL) for 5 days, either unstimulated or stimulated with LPS+IL-4 or anti-CD40+IL-4 in the presence of Zymosan, HKCA yeast or HKCA hyphae. (**A**) **IgG1 class switching**. Percentage of IgG1-positive cells within live B cells was measured by flow cytometry after 3 and 5 days of culture. (**B**) **IgG1 production**. Concentration of secreted IgG1 in cell culture supernatants after 3 and 5 days of culture was measured by ELISA. Data represent mean ± SEM of 10 mice, with triplicate measurements performed for each mouse. *p<0.05, **p<0.005, ***p<0.001

With proliferation ruled out, the next step was to determine if the increased IgG1 production was a result of increased B cell activation. A good readout for this question is the presence of AID, a key enzyme for class switch recombination, whose expression in B cells is expected to correlate with B cell activation. AIDCre-Rosa26YFP are mice where exon 1 of AID is substituted by Cre recombinase, while also carrying at the Rosa26 locus a loxP-flanked cassette which contains a stop sequence and is linked to a YFP gene (Figure 10A) [348, 349]. As such, triggering AID expression leads to the expression of Cre recombinase which excises the loxP-flanked cassette, enabling YFP expression (Figure 10A). To analyse AID activation, B cells from AIDCre-Rosa26YFP$^{cre/+\ fl/+}$ and AIDCre-Rosa26YFP$^{cre/+\ +/+}$ mice were cultured in the presence of HKCA yeast, hyphae and zymosan, and the percentage of YPF-positive cells was measured by flow cytometry. These experiments were performed

in cooperation with Sally Böde. As confirmed in Figure 10B, only cells containing the flanked cassette (fl/+) are able to express YFP. Analysis of the results show that in unstimulating conditions the presence of zymosan and HKCA hyphae lead to an increase in the percentage of AID-positive cells (Figure 10B). The same effect was observed in cells stimulated with α-CD40 and IL-4, although the difference caused by the presence of zymosan was not statistically significant. Interestingly, in this stimulating condition the presence of HKCA yeast seems to decrease the percentage of AID-positive B cells in culture. In cells stimulated with LPS and IL-4 neither the presence of zymosan or HKCA lead to a significant difference. These results follow the same pattern observed in Figure 8B, indicating that the increase in IgG1 production in the presence of zymosan and HKCA hyphae occurs due to an increase in B cell activation.



	Cell divisions	SEM	
Medium only	0.61	0.08	***
Zymosan	1.73	0.04	
HKCA Yeast	1.09	0.48	***
HKCA Hyphae	2.28	0.11	

	Cell divisions	SEM
Medium only	2.86	0.16
Zymosan	3.10	0.14
HKCA Yeast	3.03	0.15
HKCA Hyphae	3.17	0.13

	Cell divisions	SEM	
Medium only	2.72	0.06	*
Zymosan	2.36	0.21	
HKCA Yeast	2.85	0.14	
HKCA Hyphae	3.19	0.09	

Figure 9. **Increase in IgG1 production in the presence of zymosan and HKCA hyphae is not due to proliferation.** Splenic mouse B cells were isolated by MACS and cultured (5x10^5 cells/mL) for 4 days, either unstimulated or stimulated with LPS+IL-4 or anti-CD40+IL-4 in the presence of Zymosan, HKCA yeast or HKCA hyphae. Cells were stained with CFSE before culture and B cell proliferation was measured by flow cytometry based on the CFSE dilution. Representative plots from day 4 are shown. Tables show number of cell divisions in 4 days. Data represent mean ± SEM of 3 mice, , with triplicate measurements performed for each mouse. *p<0.05, **p<0.005, ***p<0.001

Taken together, these results lead to the conclusion that *C. albicans* hyphae, not yeast, enhance the B cell response through cell activation and not via proliferation, as seen by an increased antibody production and AID activation. The finding that the same effect is observed with the fungal cell wall preparation zymosan indicates a scenario in which B cells can recognise exposed fungal cell wall components directly.

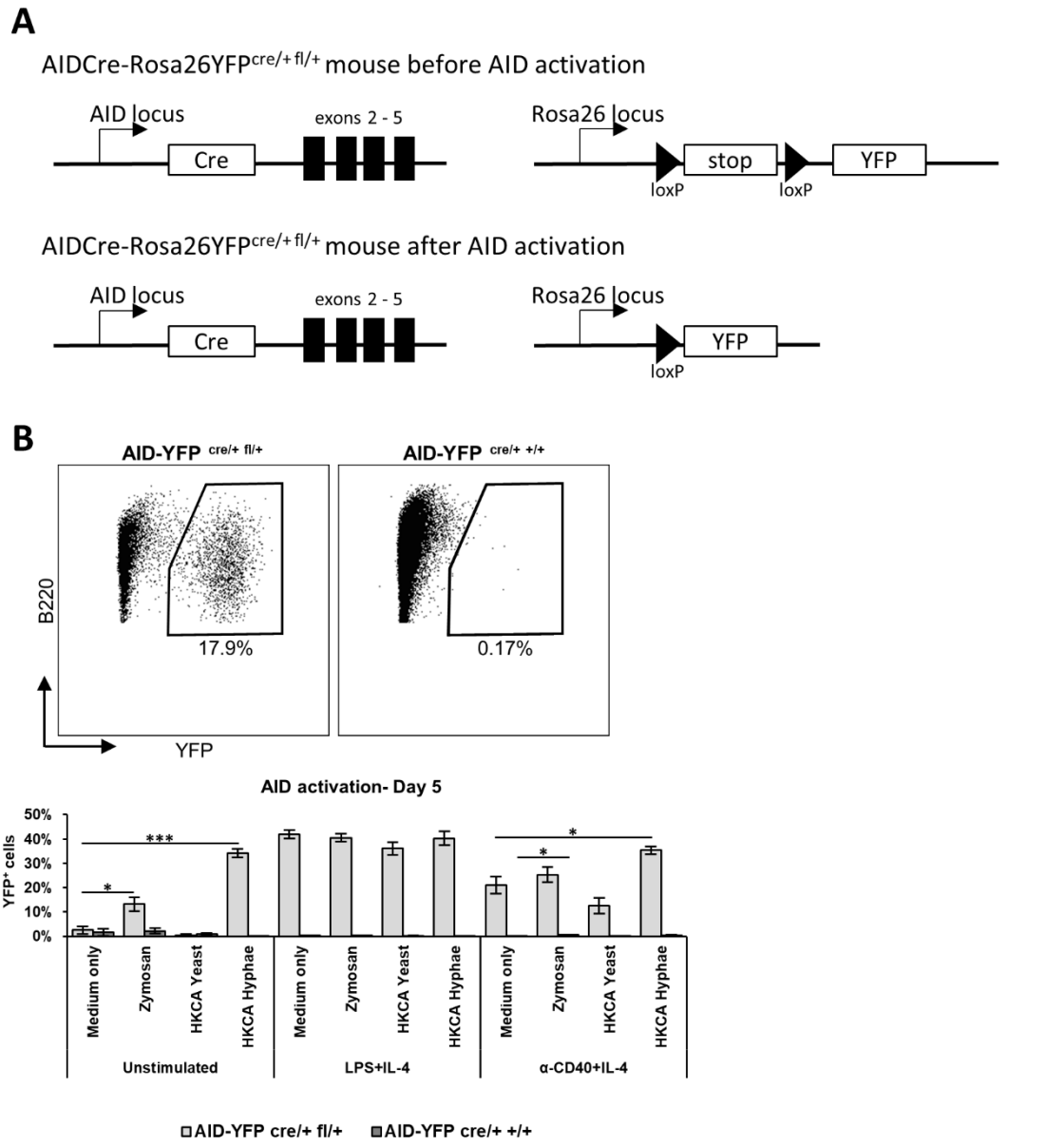


***Figure 10*. B cell stimulation with HKCA hyphae leads to an increased AID activation.** (**A**) Schematic representation of the modified loci in AIDCre-Rosa26YFP mice before and after AID activation. (**B**) Splenic B cells from AIDCre-Rosa26YFP$^{cre/+\ fl/+}$ and AIDCre-Rosa26YFP$^{cre/+\ +/+}$ mice were isolated by MACS and cultured (5x10^5 cells/mL) for 5 days, either unstimulated or stimulated with LPS+IL-4 or anti-CD40+IL-4 in the presence of Zymosan, HKCA yeast or HKCA hyphae. The percentage of YFP-positive cells within single live B cells was measured by flow cytometry after 5 days of culture. Representative plots show B cells stimulated with anti-CD40+IL-4 5 days after culture. Data represent mean ± SEM of 3 mice per genotype, with triplicate measurements performed for each mouse. *p<0.05, **p<0.005, ***p<0.001

3.3. The enhanced IgG1 production triggered by HKCA is not dependent on Dectin-1

The next step in this study was to investigate which receptors were responsible for recognising fungi and trigger the observed increase in IgG1 production. Since one of the most abundant components of the *C. albicans* cell wall is β-glucan, the first candidate receptor to be studied was dectin-1. Dectin-1 is a C-type lectin receptor that recognizes β-glucans, and is required for the control of *C. albicans* infections in mice [282]. Moreover, zymosan, mainly constituted of β-glucans, is also known to be recognized by dectin-1 in innate immune cells [261]. Therefore, a mouse in which the first three exons of dectin-1 were replaced with an neomycin resistance cassette (Dectin-$1^{-/-}$) was used to investigate whether the observed enhanced B cell response was dependent on *C. albicans* recognition via dectin-1 [282].

Since in the literature the expression of dectin-1 in mouse B cells is a controversial topic [155, 350, 351], the expression of dectin-1 in splenic B cells was first assessed by RT-PCR (Figure 11A) and flow cytometry (Figure 11B). RT-PCR results show that dectin-1 mRNA, although at low levels, can indeed be expressed in mouse B cells. Surface expression of dectin-1 was analysed by flow cytometry using two distinct anti-dectin-1 antibody clones, 2A11 and bg1fpj, both previously used in other studies [259, 352]. While a distinct dectin-1 positive population could be seen in splenic non-B cells of Dectin-$1^{+/+}$ mice, which was not present in non-B cells of Dectin-$1^{-/-}$ mice (Figure 11B), a positive smear was observed in B cells from mice of both genotypes. As the knockout of dectin-1 can be confirmed by the absence of dectin-1 mRNA (Figure 11A), the positive signal observed in B cells is probably the result of unspecific binding of the anti-dectin-1 antibodies. Consequently, despite confirming the existence of dectin-1 mRNA in mouse B cells, it was not possible to confirm the expression of dectin-1 on their surface.

To assess the influence of dectin-1 in B cell activation, B cells from Dectin-$1^{-/-}$ and Dectin-$1^{+/+}$ mice were isolated and cultured in the presence of zymosan, HKCA yeast and HKCA hyphae. As a readout, class switching to IgG1 and IgG1 production were measured as above. Both Figures 12A and 12B show no differences between Dectin-$1^{-/-}$ and Dectin-$1^{+/+}$ mice for class switching or IgG1 production upon addition of HKCA hyphae and zymosan to the culture. Therefore, it can be concluded that dectin-1 is not necessary for the observed enhancement of the B cell response induced by zymosan or HKCA hyphae.

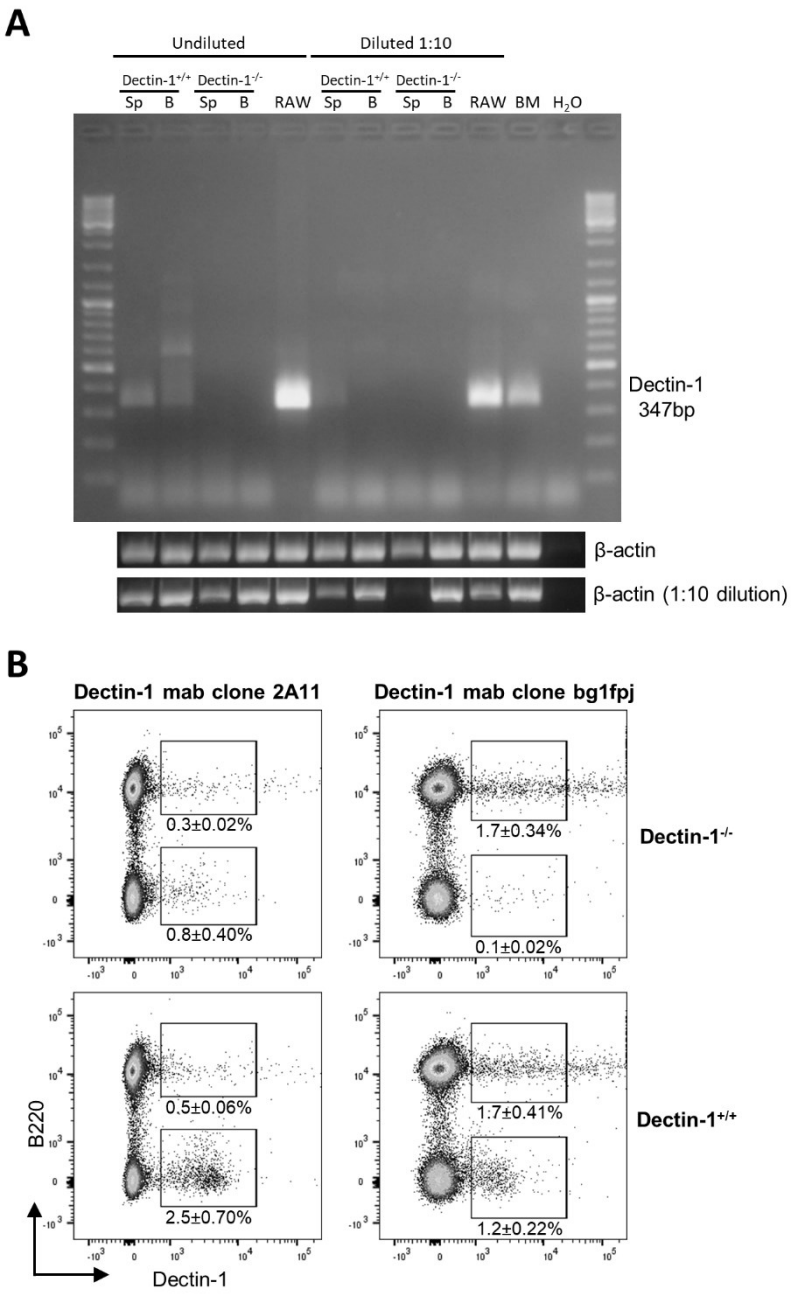


***Figure 11*. Mouse splenic B cells express dectin-1 mRNA.** (**A**) The presence of dectin-1 mRNA was analysed by RT-PCR in splenic and B cells from Dectin-$1^{-/-}$ and Dectin-$1^{+/+}$ mice, the macrophage cell line RAW 264.7 and bone marrow-derived macrophages. β-actin was used as a loading control. For a more accurate visualisation of the loading control, all samples were further diluted 1:10. Bone marrow-derived macrophages cDNA was kindly provided by René Winkler. (**B**) Splenic cells from Dectin-$1^{-/-}$ and Dectin-$1^{+/+}$ mice were stained with anti-dectin-1 antibody and analysed by flow cytometry. Two different antibody clones were tested, bg1fpj (PE-Cy7) and 2A11 (FITC). The populations of interest were gated after exclusion of debris, dead cells and doublets. The dectin-1 gating strategy was chosen based on the population of dectin-1-positive cells (non-B cells) stained with the anti-dectin-1 antibody clone 2A11. Representative dot plots are shown. Data represent mean ± SD of 3 mice per genotype.

Sp – splenic cells, B – B cells, RAW – RAW 264.7 macrophages, BM – bone marrow-derived macrophages

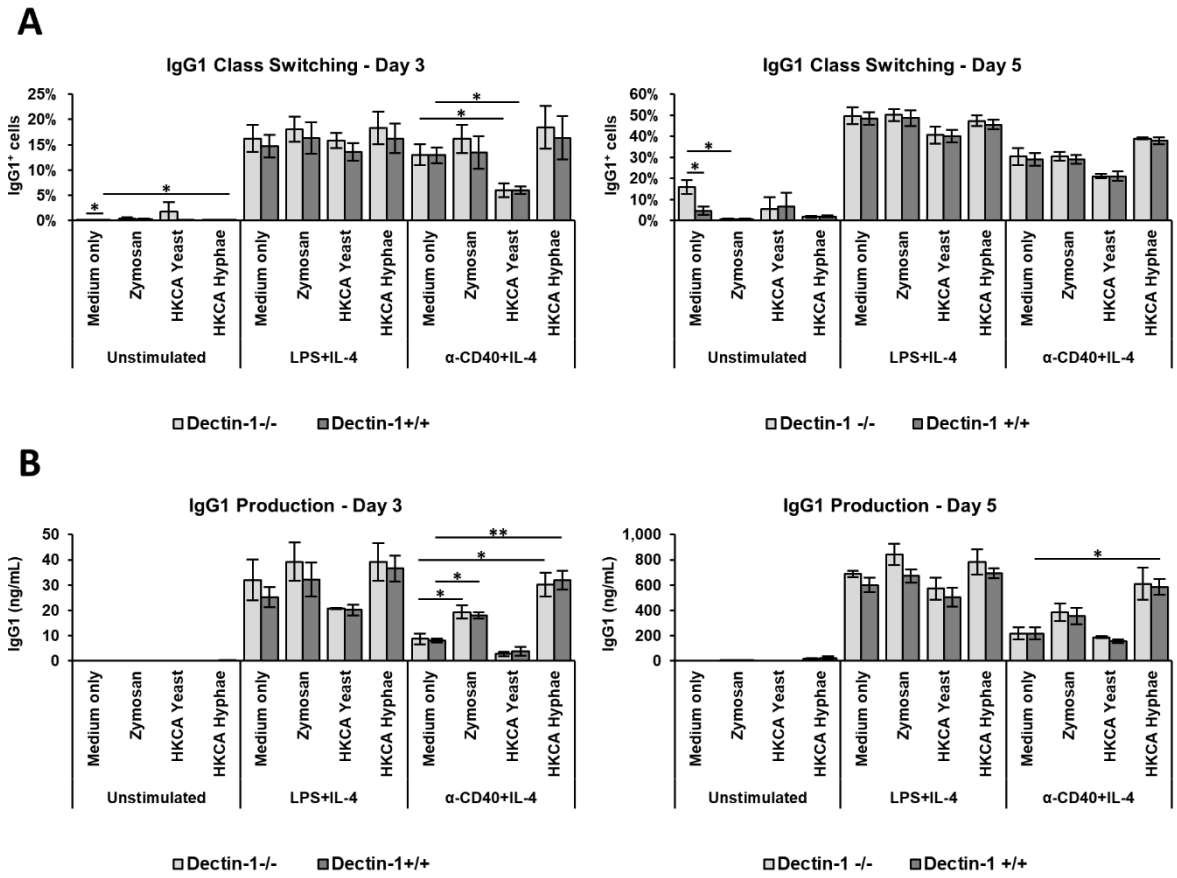


***Figure 12*. Increased antibody production upon stimulation with Zymosan or HKCA hyphae is not dependent on dectin-1.** Splenic B cells from Dectin-$1^{-/-}$ and Dectin-$1^{+/+}$ mice were isolated by MACS and cultured ($5x10^5$ cells/mL) for 5 days, either unstimulated or stimulated with LPS+IL-4 or anti-CD40+IL-4 in the presence of Zymosan, HKCA yeast or HKCA hyphae. (**A**) **IgG1 class switching**. Percentage of IgG1-positive cells within live B cells was measured by flow cytometry after 3 and 5 days of culture. (**B**) **IgG1 production**. Concentration of secreted IgG1 in cell culture supernatants after 3 and 5 days of culture was measured by ELISA. Data represent mean ± SEM of 3 mice per genotype, with triplicate measurements performed for each mouse. *p<0.05, **p<0.005, ***p<0.001

3.4. The enhanced IgG1 production triggered by HKCA is not dependent on recognition by a specific B cell receptor

A second receptor which might recognize *C. albicans* in B cells is their B cell receptor. Differently from PRRs, the BCR does not recognize molecular patterns, being instead activated by its own specific cognate antigen. BCR activation leads in turn to NF-κB activation and AID expression, which is needed for class switch recombination. Therefore, BCR recognition of HKCA hyphae and zymosan would be in line with the observed increase in IgG1 production. Since BCR deletion is lethal for B cells, to test this hypothesis a mouse bearing a transgenic BCR that only recognises an irrelevant antigen (4-hydroxy-3-

nitrophenylacetyl) – the B1-8f mouse (B1-8f$^{tg/tg}$) - was used [353]. To confirm that B1-8f mice do not show B cell population abnormalities in the spleen, the percentage of follicular, marginal zone and germinal center B cells of B1-8f$^{tg/tg}$ and B1-8f$^{+/+}$ mice were analysed by flow cytometry. As can be seen in Figure 13, there are no significant differences between splenic B cell populations from mice of both genotypes.

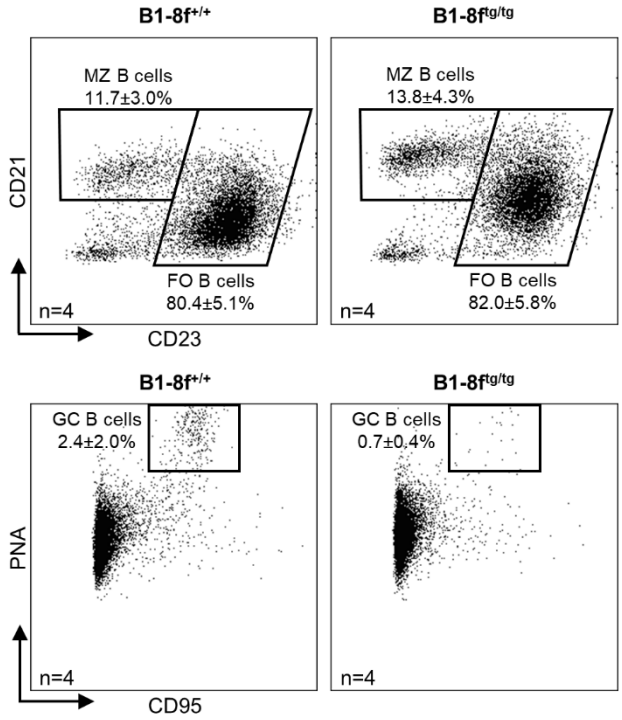


***Figure 13*. B1-8f mice do not display alterations in the major splenic B cell populations.** Splenic B cells from B1-8f$^{tg/tg}$ and B1-8f$^{+/+}$ mice were isolated by MACS and different B cell subsets were analysed by flow cytometry. Populations of interest were gated among B cells (B220^{+}) after debris, dead cells and doublets exclusion. MZ, marginal zone B cells (CD21^{hi}CD23^{-}). FO, follicular B cells (CD21^{-}CD23^{+}). GC, germinal center cells (PNA^{hi}CD95^{+}). Representative dot plots are shown. Data represent mean ± SD of 4 mice per genotype.

B cells from B1-8f$^{tg/tg}$ and B1-8f$^{+/+}$ mice were thus isolated and cultured in the presence of zymosan, HKCA yeast and HKCA hyphae, and as a readout, class switching to IgG1 and IgG1 production were measured as above. Curiously, when observing Figure 14A it is of note that when cultured in T cell-independent stimulation conditions, B cells from B1-8f$^{tg/tg}$ mice present a higher basal class switching to IgG1 than cells from B1-8f$^{+/+}$ mice. This difference was not seen in other culture conditions. Regarding IgG1 production, Figure 14B shows no difference between B1-8f$^{tg/tg}$ and B1-8f$^{+/+}$ mice, with exception of unstimulated cells in presence of HKCA hyphae and cells cultured in T cell-independent

stimulation conditions in presence of zymosan. Taken together, these results show that the incapability of recognizing *C. albicans*-specific antigens does not affect the ability of HKCA hyphae and zymosan to increase IgG1 production, leading to the conclusion that the enhancement of the B cell response displayed upon contact with zymosan or HKCA hyphae is not dependent on antigen-specific sensing by the BCR.

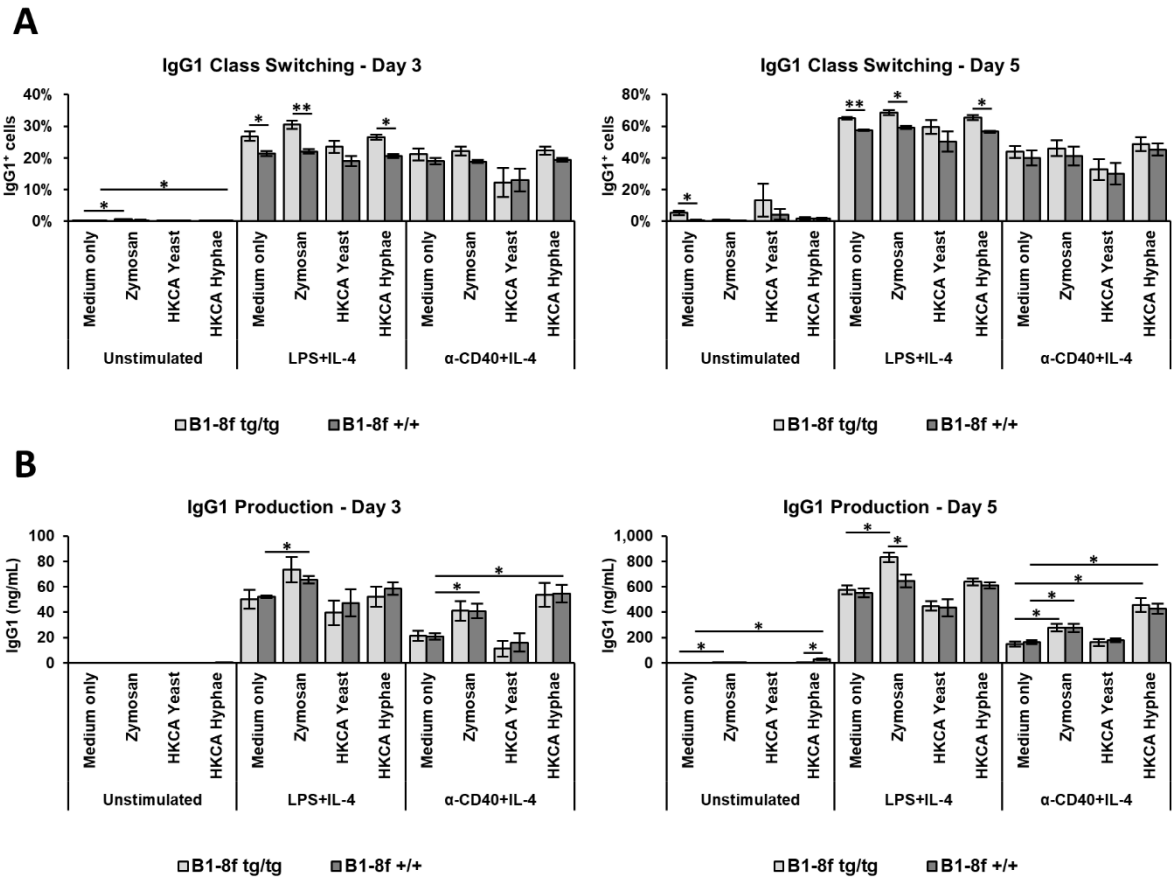


Figure 14. **Increased antibody production upon stimulation with Zymosan or HKCA hyphae is not dependent on specific B cell receptor recognition.** Splenic B cells from B1-8f$^{tg/tg}$ and B1-8f$^{+/+}$ mice were isolated by MACS and cultured (5x10^5 cells/mL) for 5 days, either unstimulated or stimulated with LPS+IL-4 or anti-CD40+IL-4 in the presence of Zymosan, HKCA yeast or HKCA hyphae. (**A**) **IgG1 class switching**. Percentage of IgG1-positive cells within live B cells was measured by flow cytometry after 3 and 5 days of culture. (**B**) **IgG1 production**. Concentration of secreted IgG1 in cell culture supernatants after 3 and 5 days of culture was measured by ELISA. Data represent mean ± SEM of 3 mice per genotype, with triplicate measurements performed for each mouse. *p<0.05, **p<0.005, ***p<0.001

3.5. MyD88 signalling is mostly responsible for the enhanced IgG1 production triggered by HKCA hyphae and zymosan

Another major inducer of B cell activation that may also sense *C. albicans* is the TLR. TLRs are widely expressed in both human and mouse immune cells and can recognize not only fungi but also bacteria, protozoa and virus. Regarding B cells, one particular study has comprehensively demonstrated that multiple TLRs are expressed in different mouse B cell subsets, being able to be activated to trigger proliferation and antibody secretion [128]. TLRs are also known to synergize with the BCR, enhancing B cell activation [93]. So far 12 TLRs have been described in mouse (TLR 1-13, except 10), most of them depending on the adaptor protein MyD88 for signal transduction [119]. Therefore, a mouse where the c-terminal cytoplasmic domain of MyD88 gene was replaced with a neomycin cassette ($MyD88^{-/-}$) [354] was used to test if TLRs are the PRRs that lead to an enhancement in IgG1 production upon recognition of HKCA hyphae. Splenic B cells from $MyD88^{-/-}$ and $MyD88^{+/+}$ mice were thus cultured in the presence of zymosan, HKCA yeast and HKCA hyphae, and IgG1 class switching and production were measured as before. These experiments were performed in collaboration with Melissa Wich. Since LPS is recognized by TLR4, the absence of MyD88 abrogates the transduction of survival/proliferation signals given by LPS, thus explaining the reduced class switching and antibody production in $MyD88^{-/-}$ B cells cultured in the presence of LPS+IL-4 (Figure 15). Regarding class switch recombination of cells either unstimulated or stimulated with α-CD40+IL-4, no differences were registered between $MyD88^{+/+}$ and $MyD88^{-/-}$ mice (Figure 15A). However, in the case of IgG1 production, the observed increase occurring in B cells cultured in T cell-dependent activation conditions upon addition of zymosan or HKCA hyphae was impaired in $MyD88^{-/-}$ mice (Figure 15B). While the effect caused by the presence of zymosan was totally abrogated, the increase in IgG1 production caused by the presence of HKCA hyphae was only strongly reduced. In conclusion, the data show that the enhancement of the B cell response upon culture in presence of zymosan and HKCA hyphae is largely dependent on MyD88 signalling, pointing towards a scenario where B cells recognise *C. albicans* via TLRs.

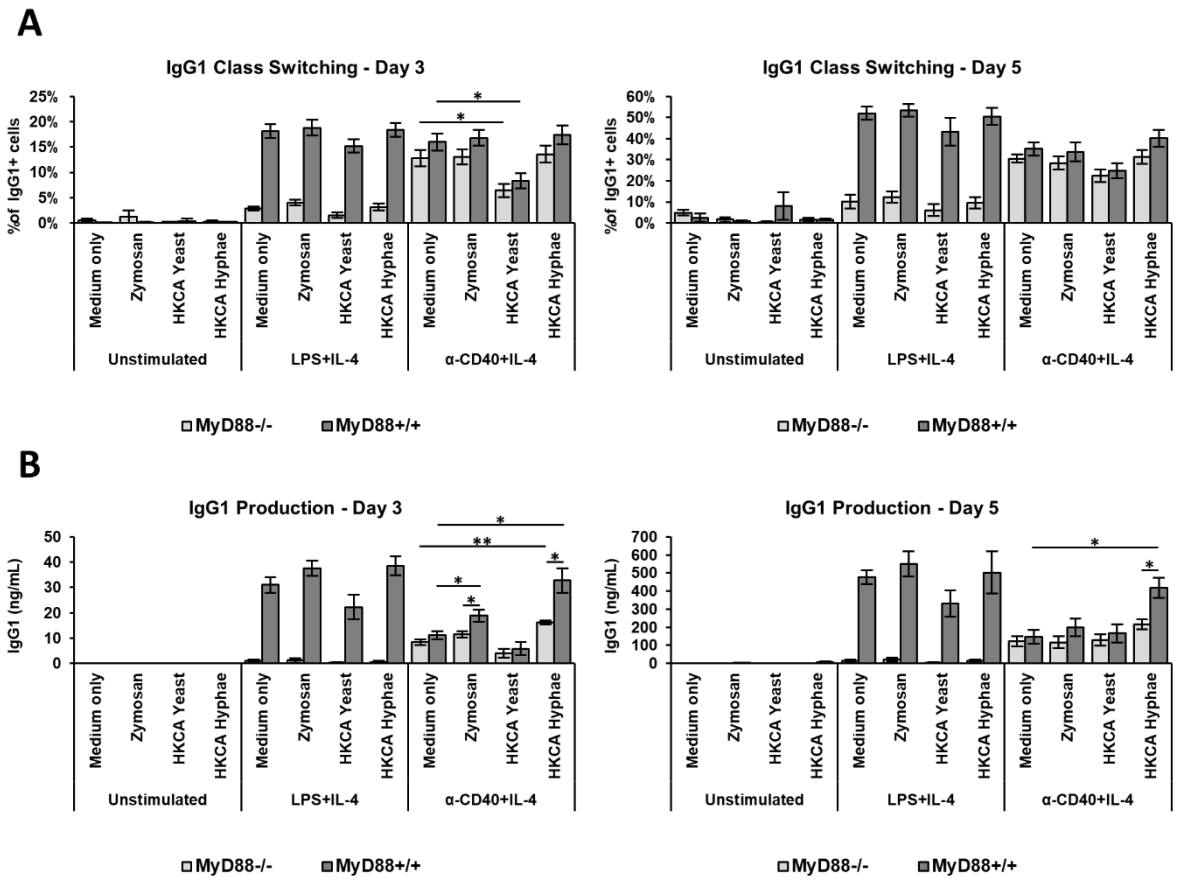


***Figure 15*. Increased antibody production upon stimulation with Zymosan or HKCA hyphae is mostly dependent on MyD88.** Splenic B cells from MyD88$^{-/-}$ and MyD88$^{+/+}$ mice were isolated by MACS and cultured (5x10^5 cells/mL) for 5 days, either unstimulated or stimulated with LPS+IL-4 or anti-CD40+IL-4 in the presence of Zymosan, HKCA yeast or HKCA hyphae. (**A**) **IgG1 class switching**. Percentage of IgG1-positive cells within live B cells was measured by flow cytometry after 3 and 5 days of culture. (**B**) **IgG1 production**. Concentration of secreted IgG1 in cell culture supernatants after 3 and 5 days of culture was measured by ELISA. Data represent mean ± SEM of 4 mice per genotype, with triplicate measurements performed for each mouse. Since cells lacking MyD88 cannot transduce signals triggered by LPS, in cells stimulated with LPS+IL-4 the difference between MyD88$^{-/-}$ and MyD88$^{+/+}$ cells is statistically significant for all samples. *p<0.05, **p<0.005, ***p<0.001

3.6. TLR2 recognition of HKCA hyphae and zymosan results in enhanced IgG1 production

Several TLRs are known to be in involved in *C. albicans* recognition in innate immune cells [355]. Two of these are TLR2 and TLR4, respectively known to mostly recognize mannans and phospholipomannans present in the cell wall [292]. Therefore, to further confirm TLR involvement in fungal cell wall recognition by B cells, mice without TLR2 or TLR4 expression were analysed. The TLR2 knockout mouse ($TLR2^{-/-}$) was created by insertion of a neo cassette that disrupted the sequence encoding the extracellular region and part of the transmembrane domain of TLR2 [356]. The TLR4 knockout mouse ($TLR4^{-/-}$) resulted from a spontaneous mutation where a 74723bp deletion completely removed the Tlr4 coding sequence [357, 358]. As before, B cells from TLR knockout mice and wildtype controls were isolated and cultured in the presence of zymosan and HKCA yeast and hyphae, and IgG1 class switching and production were measured to assess B cell responses. Since LPS is recognized by TLR4, the absence of TLR4 signalling leads to a low survival/proliferation of cells cultured in LPS+IL-4. Figure 16A shows that the absence of TLR2 or TLR4 does not alter the percentage of IgG1 class switched cells in any of the studied conditions. However, as seen in Figure 16B, the absence of TLR2 reduces the increase in IgG1 production caused by zymosan or HKCA hyphae in cells cultured in T cell-dependent activation conditions. The effects of the absence of TLR4 are less easy to interpret. Though not statistically significant, there appears to be a trend also pointing to a reduced increase in IgG1 production in $TLR4^{-/-}$ cells cultured in T cell-dependent activation conditions.

In summary, the data show that B cells can recognise zymosan and HKCA hyphae but not yeast via TLR2 and maybe to a lower extent via TLR4, leading to an increase in antibody production. These data do not exclude a minor involvement of other TLRs or even other receptors, since the absence of TLR2, TLR4 and MyD88 signalling does not completely abrogate the increase in IgG1 production in presence of HKCA hyphae.

After showing that *C. albicans* can trigger TLR stimulation in B cells, the question remains as to the outcome of this increased stimulation. Are these activated B cells more prone to produce cytokines and with it influence the immune response? And can this TLR stimulation cooperate with other signals?

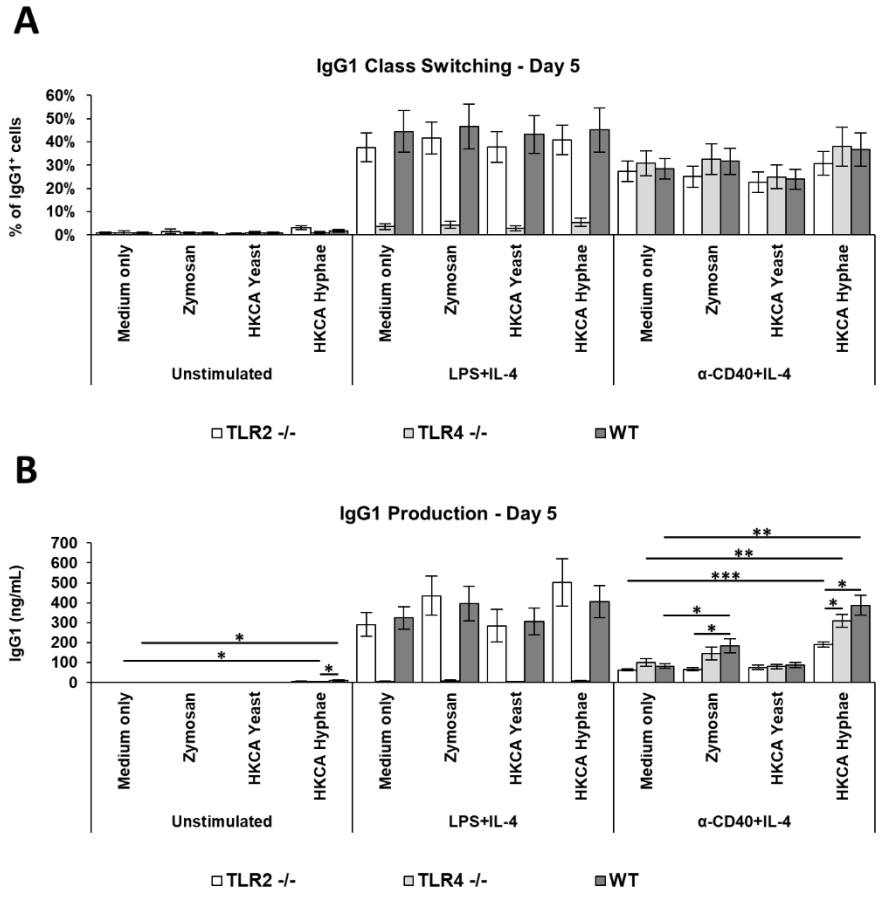


***Figure 16*. Increased antibody production upon stimulation with Zymosan or HKCA hyphae is mediated via TLR2 recognition.** Splenic B cells from TLR2$^{-/-}$, TLR4$^{-/-}$ and wildtype (WT) control mice were isolated by MACS and cultured (5x10^5 cells/mL) for 5 days, either unstimulated or stimulated with LPS+IL-4 or anti-CD40+IL-4 in the presence of Zymosan, HKCA yeast or HKCA hyphae. (**A**) **IgG1 class switching**. Percentage of IgG1-positive cells within live B cells was measured by flow cytometry after 5 days of culture. (**B**) **IgG1 production**. Concentration of secreted IgG1 in cell culture supernatants after 5 days of culture was measured by ELISA. Data represent mean ± SEM of 4 mice per genotype, with triplicate measurements performed for each mouse. Since cells lacking TLR4 expression cannot recognise LPS, in cells stimulated with LPS+IL-4 the difference between TLR4$^{-/-}$ and WT cells is statistically significant for all samples. *p<0.05, **p<0.005, ***p<0.001

3.7. TLR recognition of HKCA cooperates with BCR signalling to increase proliferation

Synergy between TLR and BCR activation has been proposed by several different studies [93, 359, 360]. To investigate if that also occurs for TLR activation triggered by *C. albicans*, B cells from wildtype mice were isolated and cultured in medium supplemented with anti-IgM in the absence or presence of zymosan, HKCA yeast and HKCA hyphae. Figure 17 shows that in fact, the presence of HKCA hyphae, and to a lesser extent Zymosan, leads to an increase in the α-IgM-triggered proliferation. Consequently, this data indicates

that in an *in vivo* scenario it would be beneficial for *Candida*-specific B cells to be further activated by TLR recognition.

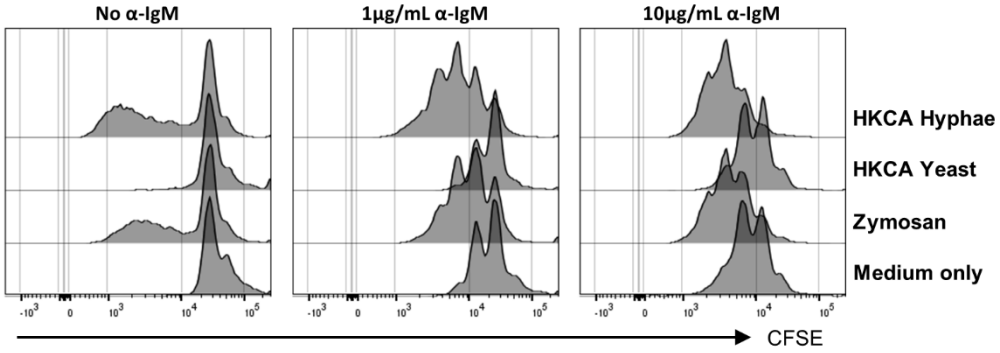


***Figure 17*. TLR recognition of zymosan and HKCA hyphae synergizes with BCR activation enhancing B cell proliferation.** Splenic mouse B cells were isolated by MACS and cultured ($5x10^5$ cells/mL) for 3 days, either unstimulated or stimulated with 1 or 10µg/mL anti-IgM in the presence of Zymosan, HKCA yeast or HKCA hyphae. Cells were stained with CFSE before culture and B cell proliferation was measured by flow cytometry based on the CFSE dilution. Representative plots from day 3 are shown.

3.8. HKCA hyphae and zymosan recognition by B cells leads to IL-6 production

One feature of activated B cells, in particular the ones activated via TLR, is the ability to produce cytokines. Thus, using a bead-based immunoassay that can quantify multiple soluble analytes at the same time, it was possible to screen cytokine production by B cells in different culture conditions. For this purpose, wildtype mouse B cells were cultured in both T cell-independent and T cell-dependent activation conditions in the absence or presence of zymosan, HKCA yeast and HKCA hyphae. After 5 days of culture, supernatants were recovered for analysis. Among the analysed cytokines - TNF-α, IFN-γ, IL-2, IL-5, IL-4, IL-6, IL-10 and IL-13 - only IL-10 and IL-6 production increased in the presence of zymosan or HKCA hyphae (Figure 18). Interestingly, while IL-10 production was only detected on cells cultured in the presence of LPS and IL-4, the increase in IL-6 production was only visible in cells cultured in the presence of α-CD40 and IL-4. No increase in cytokine production was registered in the presence of HKCA yeast.

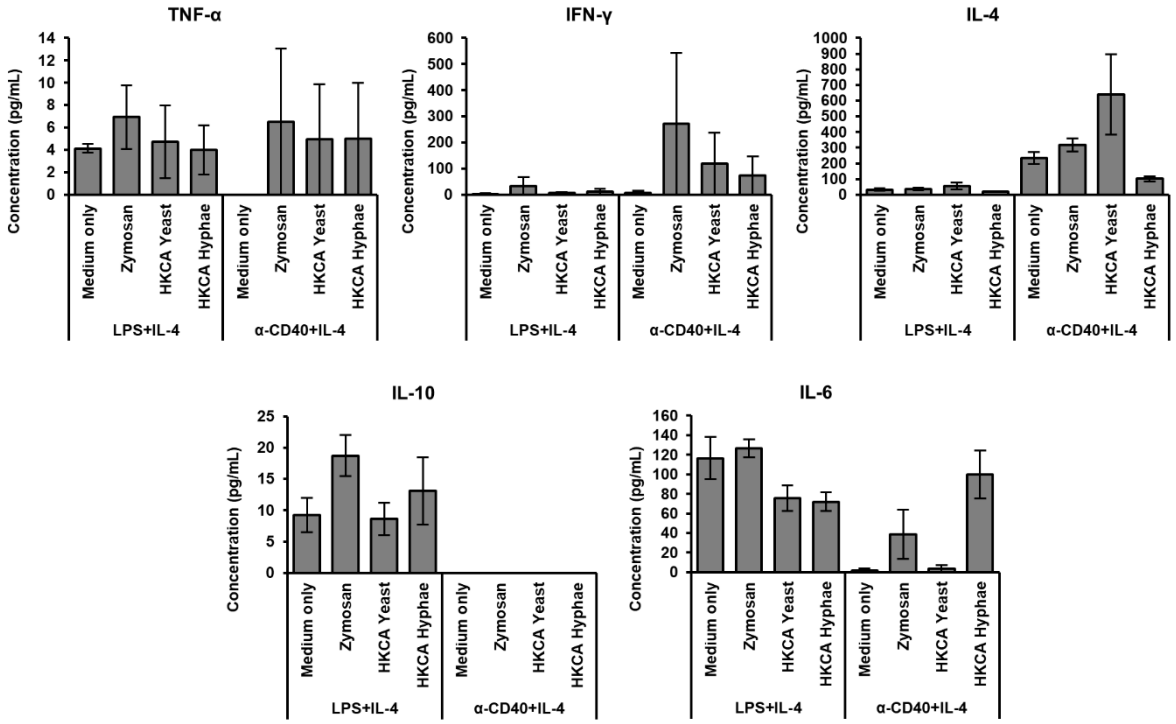


***Figure 18*. Cytokine production screening.** Splenic B cells from wildtype mice were isolated by MACS and cultured (5x10^5 cells/mL) for 5 days, either stimulated with LPS+IL-4 or anti-CD40+IL-4 in the presence of Zymosan, HKCA yeast or HKCA hyphae. Supernatants recovered after 5 days of culture were screened for differences in secreted cytokines using the bead-based immunoassay LEGENDplex™. Among the tested cytokines, TNF-α, IFN-γ, IL-2, IL-5, IL-4, IL-6, IL-10 and IL-13, no IL-2, 5 or 13 was detected. Data represent mean ± SEM of 3 mice, with duplicate measurements performed for each mouse.

To further investigate the relation between IL-10 and IL-6 production and recognition of *C. albicans* by B cells, supernatants from both MyD88$^{-/-}$ and MyD88$^{+/+}$ B cell cultures were analysed by ELISA. The obtained results show that culture conditions mimicking T cell-dependent B cell activation could only trigger a very low production of IL-10 and that the presence of zymosan or HKCA hyphae did not lead to a significant increase in IL-10 concentration (Figure 19A). However, in conditions mimicking T cell-independent B cell activation, where a basal IL-10 production is already seen, the presence of HKCA hyphae lead to an almost two-fold increase in IL-10 secretion. Strikingly, while supernatants from cells cultured in unsupplemented medium have no detectable IL-10, the presence of HKCA hyphae triggers a high production. Additionally, the fact that the B cells from MyD88$^{-/-}$ mice do not show this high IL-10 production leads to the conclusion that it occurs in a TLR-dependent fashion.

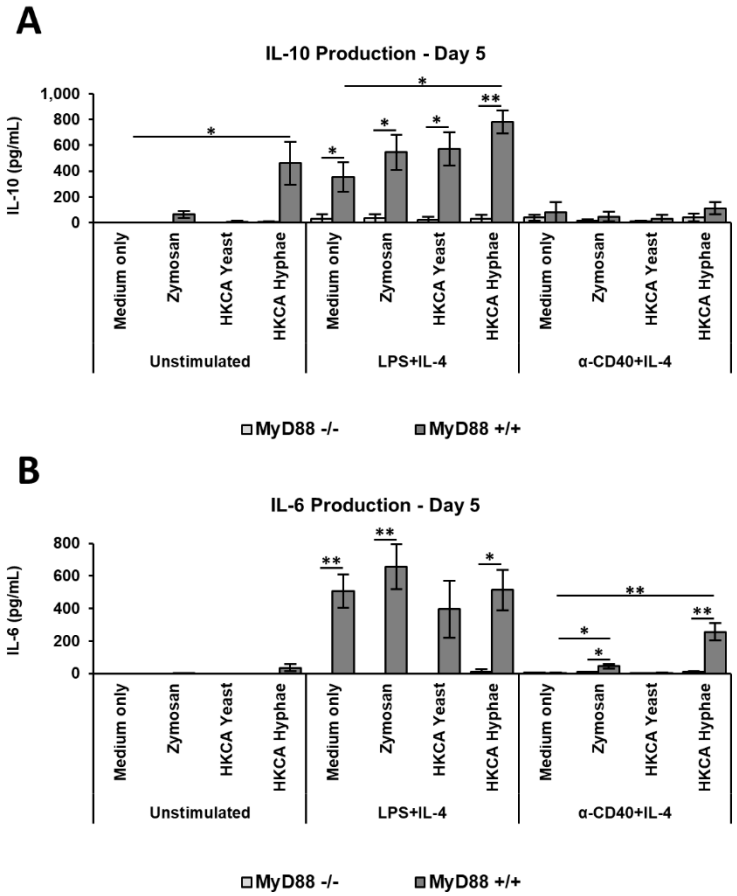


***Figure 19*. Increased IL-10 and IL-6 production upon stimulation with Zymosan or HKCA hyphae is dependent on MyD88 activation.** Splenic B cells from MyD88$^{-/-}$ and MyD88$^{+/+}$ mice were isolated by MACS and cultured (5x10^5 cells/mL) for 5 days, either unstimulated or stimulated with LPS+IL-4 or anti-CD40+IL-4 in the presence of Zymosan, HKCA yeast or HKCA hyphae. Concentration of secreted IL-10 and IL-6 in cell culture supernatants after 5 days of culture was measured by ELISA. Data represent mean ± SEM of 4 mice per genotype, with triplicate measurements performed for each mouse. *p<0.05, **p<0.005, ***p<0.001

Regarding IL-6, culture conditions mimicking T cell-dependent B cell activation did not lead to significant IL-6 production (Figure 19B). However, the addition of zymosan or HKCA hyphae increases IL-6 secretion, in line with the increased B cell response observed before. This effect is also MyD88-dependent, since B cells deficient in MyD88 do not upregulate IL-6 production in the presence of zymosan or HKCA hyphae. Similarly to what was seen for IL-10, B cells cultured in unsupplemented medium do not secrete IL-6, producing it only in the presence of HKCA hyphae (not statistically significant). In the case of B cells cultured in T cell-independent activation conditions, while the basal activation already leads to high IL-6 secretion, the presence of zymosan or HKCA hyphae does not significantly increase the amount of secreted IL-6. HCKA yeast do not increase IL-6 or IL-10 production in any of the studied conditions.

As a whole, these results show that *C. albicans*-induced B cell activation leads to an increased secretion of cytokines, which can contribute to the shaping of an immune response.

To conclude, the presented data indicates a scenario where B cells recognize *C. albicans* hyphae via their TLRs, especially TLR2, leading to an increased B cell response as seen by increased antibody production, AID upregulation and cytokine secretion, as well as increased proliferation upon additional BCR stimulation.

4. Discussion

This study shows that the co-culture of B cells with heat-killed *C. albicans* hyphae and the yeast cell wall preparation zymosan results in an increase in B cell activation, which leads to enhanced IgG1 and IL-6 secretion. Heat-killed *C. albicans* yeast cells did not trigger the same effect. Moreover, it was possible to show that *C. albicans* hyphae and zymosan recognition by B cells occurs mainly in an MyD88-dependent manner, being significantly mediated through TLR2. The contribution of dectin-1 or specific B cell receptor-mediated recognition to this effect was ruled out. Hyphae and zymosan-induced TLR signalling is also able to cooperate with BCR activation to enhance B cell proliferation.

The relevance of this study relies on the fact that recent data have highlighted the importance of B cells and antibodies in defence against *C. albicans*, with some monoclonal antibody vaccines reaching clinical trial phase [337, 341-343]. Several studies have shown that antibodies can not only opsonize fungal cells, but also directly influence the cell wall, resulting in deleterious outcomes for *C. albicans* like inhibition of growth, adhesion or biofilm formation, and disruption of metabolic processes [323, 326, 329, 332-334, 361]. Since the protective antibodies are rare among many non-protective ones, their effect is conditioned by their titer and/or isotype. Protective effects of antibodies also depend on the infection type and location, especially in the case of cell-mediated effects, as not all cell types are involved in responding to the different forms of infection [317]. While the importance of antibodies on anti-*C. albicans* responses has recently drawn attention of several researchers, fewer studies focus on the physical interaction between *C. albicans* (or its components) and B cells [156, 165, 215, 362]. An in-depth understanding of B cell activation mechanisms in the context of *Candida albicans* invasion would allow for an effective usage of B cells/antibodies in the defence against this pathogen.

4.1. B cell activation by fungal cell wall components

As the primary objective of this study was to investigate the interaction between B cells and *C. albicans*, mouse B cells were co-cultured with the *Saccharomyces cerevisiae* cell wall preparation zymosan, HKCA yeast or HKCA hyphae in different B cell-activating culture conditions. These different conditions not only allow to assess the strength of *C.*

albicans-triggered signals, but also to mimic (as accurately as possible) the different *in vivo* B cell activation conditions/locations.

The first condition consisted in medium without addition of further stimulants (unstimulated). This allows the assessment of the influence of *C. albicans* not only in B cell activation, but also in survival and proliferation since B cells need external survival/proliferation signals to survive *ex vivo*. Moreover, it could show an ability of *C. albicans* to trigger a T cell-independent B cell activation. Similarly to unstimulated B cells, most B cells co-cultured with HKCA yeast did not survive. Co-culture with zymosan or HKCA hyphae, even though resulting in some B cell proliferation (Figure 9), still presented a low number of surviving cells. These results lead to the conclusion that zymosan and HKCA hyphae do activate B cells but cannot provide enough survival and/or proliferation signals to alone maintain them in culture.

The second tested culture condition also aimed to mimic a T cell-independent B cell activation (TI), which *in vivo* occurs mostly outside of B cell follicles. To that end, cells were cultured in medium supplemented with LPS and IL-4, as LPS is known to activate mouse B cells in culture by providing survival signals and strongly inducing proliferation via TLR engagement [89, 363]. Moreover, since LPS is a component of the outer membrane of Gram-negative bacteria, in the presence of *C. albicans,* this culture condition also resembles the co-stimulation by bacterial and fungal components that occurs in the gut. This setting is of special interest when considering that the gut is the major reservoir of *C. albicans*, and contains a high amount of B cells [364, 365].

The third tested condition was the supplementation of medium with α-CD40 and IL-4. Since the CD40 receptor is activated through binding of a ligand present on T cells, and this binding is important for survival, proliferation and CSR, the activation of CD40 by an α-CD40 antibody intends to mimic a T cell-dependent B cell activation (TD) [68, 89]. *In vivo*, B-T cell interaction occurs next to B cell follicles, leading to germinal center formation, a process that allows for specific antibody generation (see Figure 1). Although GC formation does not occur *in vitro*, CSR can be triggered by α-CD40 in the presence of certain cytokines. The most frequently used cytokine in CSR studies is IL-4, its addition allowing class switching to IgG1. The addition of other cytokines or cytokine combinations triggers class switching to different isotypes.

Two common assays to study B cell responses are the analysis of CSR and the quantification of secreted antibodies. Measuring the percentage of IgG1-positive B cells, it was observed that the presence of zymosan or HKCA have little to no influence on CSR (Figure 8A). An exception is the fact that in conditions mimicking TD activation, HKCA yeast leads to a decrease in the percentage of IgG1 switched cells. While no difference was observed in class switching to IgG1, the same was not true regarding the production of IgG1 antibodies. In T cell-dependently activated B cells, the presence of both zymosan and HKCA hyphae lead to an increase in antibody secretion (Figure 8B), indicating that B cells do respond to fungal cell wall components. In the case of TI culture conditions, while the differences between co-cultured B cells and controls are small and not statistically significant, they show a trend that points to the same result as obtained in TD conditions. HKCA yeast cells do not have the same influence in antibody secretion. Contrary to hyphae, they appear to lead to a decrease in IgG1 production, although only significant in cells stimulated with LPS and IL-4 for 5 days. It is important to note that since this primary *in vitro* B cell culture does not mimic a germinal center reaction, the results do not indicate the production of specific anti-fungal antibodies, but a more innate-like response where an increased production of antibodies is an indicator of an increased B cell activation.

Regarding unstimulated cells, despite the aforementioned lack of survival of B cells cultured without further stimulation, it is undeniable that the presence of zymosan or HKCA hyphae leads not only to some proliferation (Figure 9), but also to the activation of the few remaining live B cells (Figure 8B). The contrast between the significant increase in the percentage of IgG1-positive cells in the presence of zymosan or HKCA hyphae at day 3 and the variable results observed at day 5 (Figure 8A) is probably equally related to the low numbers of surviving B cells in culture.

The analysis of B cell proliferation in TI or TD B cell activating conditions showed that both conditions lead to B cell proliferation, with the addition of zymosan or HKCA not significantly influencing the number of cell divisions occurring during the culture period (Figure 9). The only exception was observed in B cells co-cultured with HKCA hyphae in the presence of α-CD40+IL-4, which divided on average 1.17 times more than B cells cultured alone. However, in a biological context this increase leads only to a small increase in cell number, thus not being responsible for the observed augmented antibody production. This observation adds to the conclusion that B cells are directly activated by zymosan and HKCA hyphae, and reach an overall higher activation state than just proliferating more.

This hypothesis was further confirmed by the analysis of AID, a key enzyme for SHM and CSR expressed during B cell activation [86, 366], as T cell dependently-activated B cells showed increased AID activation in the presence of zymosan or HKCA hyphae (Figure 10B). Actually, both TI and TD B cell activation (via LPS or α-CD40) have been shown to lead to AID induction, this occurring via NF-κB activation (see Figure 3) [367, 368]. However, similarly to what was observed for antibody secretion, in case of LPS + IL-4 stimulated B cells, the addition of zymosan or HKCA hyphae did not influence AID activation (Figures 8B and 10B). A conceivable explanation for this result relies on the fact that LPS itself is known to be a potent immune stimulator. It is possible that the stimulation provided by LPS is strong enough to saturate the B cell signalling machinery, preventing a further enhancement of the response. The requirement for α-CD40 co-stimulation is ruled out, as the cells cultured in non-activating conditions (unstimulated) also show the capacity to respond to the presence of zymosan or HKCA hyphae (Figures 8, 9 and 10). The involvement of NF-κB activation in the HKCA hyphae or zymosan-induced increase in B cell activation is currently being investigated in our laboratory by Sally Böde. To that end, B cells from NF-κB eGFP reporter mice are isolated, cultured and stimulated in the same conditions described in this study, and the GFP signal measured through time by flow cytometry.

The fact that the same effect, although with different strengths, is observed in the presence of both zymosan, a yeast fungal cell wall preparation, and hyphae from *C. albicans*, implies that B cells can recognize fungal cell wall components. Moreover, since *C. albicans* is killed and extensively washed before contact with B cells, and no other cell type is added to the culture, it indicates that the interaction is direct and not dependent on secreted factors. The different or even absent recognition of *C. albicans* yeast might result from a different composition or exposure of its cell wall components, since it has been shown that *C. albicans* yeast and hyphae present differences in both cell wall composition and exposure [245, 275, 369]. Several cell types have been shown to differentially recognize yeast and hyphae [262, 274, 291], with some reports showing preferential hyphal-induced activation in cells like macrophages or epithelial cells [370-373], and a recent one showing higher PBMC activation triggered by yeast cells [374]. Also interesting is the fact that some *C.* albicans-induced epithelial cell responses appear to be independent of the classical PRRs [372].

The difference in response strength triggered by zymosan or HKCA hyphae may also rely on differences in component composition and/or exposure. Although zymosan's main

constituents are glucans and mannans, thus being known as a dectin-1/TLR2 agonist, it also contains proteins, chitins and glycolipids [261, 347]. It is also important to consider that the process of killing *C. albicans* by heat alters cell wall exposure when compared to the living fungus [375]. It was however a necessary procedure, since co-culture of B cells with living *C. albicans* led to B cell death, and fungal killing by UV or thimerosal was respectively unreliable for hyphal killing or toxic for B cells even upon extensive washing (data not shown). Despite this alteration in cell wall exposure, a recent study using different strains of *C. albicans* showed that both live and heat-killed hyphae cells induce similar cytokine responses in human PBMCs, although an increased response is triggered by heat-killed yeast cells [374]. The same study controlled the effect of sonication, here necessary to allow hyphal clump dissociation and posterior pipetting, proving that sonication does not lead to any difference in response when compared to non-sonicated cells. Many other studies rely on heat-killing of *C. albicans* to assess its influence on the immune system [156, 274, 287, 291, 374, 375].

After concluding that B cells are able to directly recognize fungal cell wall components, namely the ones exposed in zymosan or HKCA hyphae, the question arising is through which receptor B cells recognize these components.

4.2. Dectin-1 does not play a role in B cell activation by *Candida albicans*

Among a variety of receptors expressed by B cells the decision to study dectin-1 as the possible PRR responsible for zymosan and *C. albicans* hyphae recognition was supported by two distinct lines of thought. First, the fact that dectin-1 is one of the most investigated PRRs in *C. albicans* recognition, possessing an important role both in macrophage and neutrophil responses against this pathogen [260-262, 375]. And second, a study by Seo *et al.* [156], which not only showed that mouse B cells express dectin-1, but also claimed that its activation was able to reinforce LPS-driven IgG1 production. Until this study, dectin-1 expression was never detected in mouse B cells [155], although it was shown to be present in human B cells [154]. The observed results are thus in line with the conclusion that B cells can be directly activated by fungal cell wall components, pointing towards a scenario where dectin-1 would be the PRR sensing zymosan and HKCA hyphae. Further corroborating this hypothesis is a previous publication which shows that the dectin-1 agonist curdlan directly influences B cell activation [165].

Due to the discrepancies between published studies, the expression of dectin-1 in mouse B cells was again assessed both by RT-PCR and flow cytometry. While the presence of dectin-1 mRNA could be confirmed, unspecific binding of anti-mouse dectin-1 antibodies did not allow for a surface expression assessment (Figure 11). The unspecific binding of the tested antibody clones was stressed by the fact that only the B cells but not the splenic non-B cells from dectin-1 knockout mice showed a positive signal for dectin-1 staining (Figure 11B). The absence of dectin-1 surface expression in neutrophils and monocytes of these knockout mice had been previously confirmed by flow cytometry [282].

When analysed, however, B cells from dectin-1 knockout mice showed the same enhanced antibody response to the presence of both zymosan and HKCA hyphae as seen in wildtype mice (Figure 12). This observation excludes dectin-1 from being the PRR enhancing B cell activation. Although these results do not support the observations made by Seo *et al*. [156], due to the different experimental procedures used, they can also not contradict them. Seo *et al*. studied the influence of dectin-1 agonists in LPS-activated B cells, showing that both HKCA and depleted zymosan (zymosan treated with hot alkali to remove TLR-stimulating properties [261]) could enhance IgG1 production. The first and most notorious difference between this and our study is the absence of IL-4, as LPS alone (without IL-4) is a weak inducer of IgG1 production [376]. Additionally, *Seo et al*. used an LPS concentration 10 times lower than the one used in the experiments of this study. As such, a possible explanation for the discrepancy in the observed results might be a scenario where in weak B cell activating conditions (low LPS amounts and absence of IL-4), dectin-1 is able to boost antibody production by cooperating with LPS-induced TLR4 signalling. On the other hand, and as aforementioned, in strong LPS stimulating conditions HKCA does not seem to increase IgG1 secretion. A second important difference between these two studies is the HKCA used. While in this study both HKCA yeast and hyphae were differentially prepared using the *C. albicans* strain SC5314, in the publication from *Seo et al*, the HKCA was commercially acquired and no discrimination between yeast or hyphae or *C. albicans* strain is mentioned. Information obtained on the website of the HKCA vendor states that the used strain is ATCC 10231 in the yeast form. This difference may also be important, as several studies have stressed out differences in recognition between yeast, hyphae and different *C. albicans* and mouse strains [262, 274, 275, 283, 289, 291].

Once extracted, and despite their differences, glucans from both yeast and hyphae can be recognized by dectin-1 [275]. However, in intact cells, it has been published that

dectin-1 is not able to recognize *C. albicans* hyphae due to the masking of the β-glucan content by outer cell wall components [274]. More recent publications have contradicted this, although it is still accepted that hyphae recognition by dectin-1 might not be as efficient as for yeast [240, 247, 273, 283, 377]. These data might provide an alternative explanation to why dectin-1 from mouse B cells does not seem to play any role in the HKCA hyphae-induced antibody production. A second possibility is a low surface expression of dectin-1 in mouse B cells. That would explain why previous publications did not observe dectin-1 expression in these cells [155], and also the absence of difference in zymosan-induced enhancement of B cell activation between wildtype and dectin-1 knockout mice.

Despite the many differences, there is one point in which our study and the one from Seo *et al.* both agree, HKCA alone is able to trigger B cell proliferation (although in Seo *et al.* this observation is made in a total splenic cell culture and not isolated B cells). On the other hand, depleted zymosan was not able to induce B cell proliferation (in isolated B cells). Comparing the effects of zymosan and depleted zymosan in the two studies, the observed results point to a scenario where zymosan induces B cell proliferation through TLR engagement. A follow up article from Seo *et al.* [351] shows that dectin-1 engagement selectively induces IgG1 class switching in LPS-activated mouse B cells, although no *C. albicans* was used in this study.

The results obtained in our study do not exclude a more prominent role of dectin-1-mediated pathogen recognition in human B cells. In fact, a recent study shows that upon β-glucan recognition by dectin-1, human B cells upregulate the secretion of the pro-inflammatory cytokines TNF-α, IL-6 and IL-8, and that the medium from β-glucan-stimulated B cells is able to elicit neutrophil chemotaxis [378]. Interestingly, β-glucan did not induce B cell proliferation or IgM secretion.

4.3. *Candida albicans*-induced enhancement of B cell activation is not dependent on BCR specific recognition

Other than PRRs, B cells express a specific B cell receptor which is indispensable for B cell survival and known to influence antibody production upon recognition of cognate antigens. As *C. albicans* is a common commensal of humans, anti-*Candida* antibodies can be frequently found in healthy individuals, implying the presence of B cells bearing BCRs

that recognize *C. albicans* antigens [340, 379]. In mice, however, *C. albicans* is not part of the microbiota, which may decrease the probability of *Candida*-specific B cells in non-infected mice [380]. Nevertheless, the presence of B cells with a BCR which recognizes a conserved molecular pattern in both *C. albicans* and zymosan is possible, hypothesising that the BCR could be involved in the observed increase in B cell activation. This hypothesis was, however, excluded by the analysis of B cells from B1-8f mice (Figure 14). Since B1-8f mice possess a transgenic BCR only able to recognize the antigen 4-hydroxy-3-nitrophenylacetyl (NP) [353], the fact that their B cells still showed an increased activation upon co-culture with zymosan or *C. albicans* hyphae proved other receptor present in B cells to be responsible for fungal cell wall recognition. This result reiterates the observed increase in antibody production as an innate-like response and mostly an indicator of general rather than antigen-specific B cell activation. Importantly, this study does not exclude the occurrence of *Candida*-specific B cells in *in vivo* mouse models of colonization or invasion, but shows an alternative, and possibly parallel, innate-like activation.

4.4. B cells recognize *Candida albicans* via toll-like receptors, especially via TLR2

Excluded dectin-1 and the BCR, TLRs pose as the most likely receptors to recognize fungal cell wall components and to influence B cell activation. Three different aspects support this hypothesis: 1) TLR activation is a key event in T cell-independent B cell activation, as TLR signalling synergizes with BCR signalling to trigger AID expression and CSR [93]; 2) TLR agonists have been shown to synergize with CD40L to induce activation, proliferation and plasma cell differentiation in mouse B cells [381, 382]; 3) Several TLRs have been shown to recognise *C. albicans* [253-257]. Analysing B cells from mice deficient in the adaptor protein MyD88, required for signalling in most TLRs, it was possible to confirm that the increase in B cell activation triggered by cell wall components was highly dependent on TLR signalling (Figure 15). The absence of MyD88 abrogated the increase in IgG1 production triggered by zymosan in B cells cultured in the presence of α-CD40+IL-4, and significantly impaired the same effect triggered by HKCA hyphae. Due to the absence of TLR4 signalling transduction in these mice, the activation of B cells by LPS is impaired, leading to cell death by lack of survival and proliferation signals [383].

As zymosan is known to be recognized by dectin-1 and TLR2 [261], and no alteration in its effect was visible in the absence of dectin-1 (Figure 12), it was already expected that an impairment in TLR signal transduction would abrogate the enhancement in B cell activation. The fact that *C. albicans* can be recognized by several receptors (see Table 1) might explain why HKCA hyphae are able to slightly increase IgG1 secretion even in the absence of MyD88/TLR signalling. On this topic, it is also important to consider that not all TLRs rely on the MyD88 for signal transduction. Instead of MyD88, endosomal TLR4 and TLR3 recruit the adaptor protein TRIF, potentially being able to recognize *C. albicans* and transduce signal even in the absence of MyD88. Apart from TLRs, other receptors known to recognize *C. albicans* have been shown to be expressed in B cells [154, 384-388]. Interestingly, some of these receptors, such as galactin-3 or NOD2, are only expressed and/or modulated upon B cell activation [387, 388].

Once TLR signalling was identified as the major responsible trigger for the *C. albicans*-induced increase in B cell activation, it was important to pinpoint the specific TLR or TLRs involved in this effect. Strong candidates were TLR2 and TLR4, the most prominent TLRs in *C. albicans* responses of innate immune cells [253]. While TLR2 has been shown to recognize *C. albicans* phospholipomannan, triggering the production of different cytokines, TLR4 recognizes the O-linked mannan present in *C. albicans*, inducing the secretion of pro-inflammatory cytokines [285, 287, 290-293, 389]. Also important for this study is the fact that both TLRs have been shown to respond to both live and heat-killed *C. albicans* [287, 390].

The analysis of B cells from TLR2 knockout mice cultured in TD conditions showed that in the absence of TLR2, the increase in IgG1 production triggered by HKCA hyphae was significantly smaller than in the control (Figure 16). These results are thus in line with what was observed in MyD88 knockout mice, proving that B cells recognize *C. albicans* hyphae (and probably cell wall components) via TLR2. As expected, in the absence of TLR2 signalling zymosan was unable to increase IgG1 production.

Corroborating the presented results are the *in vitro* studies by Jain *et al*. [381] and Boeglin *et al.* [382], which claim that TLR2 can synergize with CD40 signalling to favour B cell activation. In both studies the stimulation of mouse B cells with α-CD40 and a TLR2 agonist (not derived from fungi) resulted in increased AID induction and antibody production. Jain *et al*. shows that further CD40 stimulation of TLR2-stimulated resting B

cells augments proliferation and activation, accompanied by enhanced expression of several activation markers such as CD40, CD86, CD80, TLR2, MHC and IgM, and increased Erk and Akt phosphorylation. Furthermore, other B cell functions such as antigen uptake, T cell help and differentiation of marginal zone precursors, also appear to be enhanced in these stimulating conditions. Boeglin *et al.* focus on the fact that TLR2 and CD40 synergy increases antibody-secreting cell differentiation, which is shown not only by increased antibody secretion but also by enhanced Blimp-1 and AID expression.

As in wildtype mice, B cells from TLR2 knockouts cultured in TI conditions do not show a statistically significant increase in IgG1 production in the presence of either zymosan or HKCA hyphae (Figure 16). Since LPS is recognized by TLR4, and both TLRs share the same signalling pathway [119], these results support the aforementioned scenario where LPS might saturate TLR signalling, preventing TLR2-induced increase in B cell activation. This hypothesis can be further supported by evidence showing that some LPS-induced B functions are dose-dependent, reaching a plateau after a certain LPS concentration [391-395].

The curious fact that, even though *C. albicans* is able to trigger B cell activation and AID expression, no enhanced CSR is observed, might be explained by a study from Pone *et al*. [93]. In this publication, the ability of TLR2 agonists to influence CSR is shown to be dependent on BCR crosslinking, which is not present in any of the tested culture conditions. However, these data might indicate that in an *in vivo* setting, B cells which have their BCR crosslinked through the recognition of their cognate antigen, could have their CSR capacity enhanced by the presence of *C. albicans* hyphae.

TLR4 appears not to be important for fungal cell wall-dependent enhancement of B cell activation, since compared to wildtype, B cells from TLR4 knockout mice show no significant change in IgG1 production when in co-culture with zymosan or HKCA hyphae (Figure 16). While this result was expected for zymosan stimulation, since it is a preparation known to induce TLR2 and not TLR4 signalling [396], it was more intriguing for *C. albicans* hyphae. It might, however, be explained by studies advocating that TLR4 has reduced capacity for hyphae recognition, most probably due to the reduced cell wall mannan expression in comparison with *C. albicans* yeast [291, 374]. Variable TLR4 recognition of different *C. albicans* strains has also been observed [289].

In conclusion, even though the involvement of other PRRs is likely, the presented data show that TLR2 is the major contributor to the observed *C. albicans*-triggered increase in B cell activation. Further supporting this conclusion are studies on the interplay between B cells and different bacteria/bacterial surface proteins, which show a TLR/TLR2 dependence for pathogen-mediated B cell activation [212, 397-399]. Observed effects of this activation are increased proliferation, formation of antibody-secreting cells (IgM and IgG), cytokine secretion (IFNγ, IL6 and IL-10), and upregulation of co-stimulatory molecules such as MHC class II, CD80 and CD86. Some studies further describe the involvement of protein tyrosine kinases, NF-κB and Erk signalling in these TLR-mediated B cell effector functions [398, 399]. Interestingly, along with the major involvement of TLR2, Alugupalli *et al*. [397] also reported a minor role for TLR4 in *Borrelia hermsii*-induced B cell responses, concluding that multiple TLRs might redundantly contribute to B cell activation by pathogens.

4.5. *Candida albicans*-induced TLR activation cooperates with B cell receptor signalling

Several studies have reported a synergistic effect during TLR and BCR activation, leading to increased AID induction and class switch recombination [93, 360, 400]. Since no appreciable CSR is induced in the absence of cytokines, B cell proliferation was chosen as a parameter to evaluate cooperation between TLR and BCR signalling upon *C. albicans* recognition by B cells.

As observed before, B cells cultured without anti-IgM stimulation remained mostly non-proliferative, although the presence of HKCA hyphae or zymosan was able to trigger proliferation in some cells (Figures 9 and 17). The presence of HKCA yeast did not induce any substantial proliferation. On the contrary, stimulation with anti-IgM led to B cell proliferation, which was increased by the presence of both zymosan and HKCA hyphae (Figure 17). Corroborating previous results, HKCA hyphae seems to have a slightly stronger stimulation capacity than zymosan. Comparing the two different anti-IgM concentrations, stimulation with a 10µg/mL of anti-IgM triggers a higher effect than 1µg/mL. Preliminary data suggest that the increase in B cell proliferation triggered by the combined stimulation with anti-IgM and HKCA hyphae or zymosan is not as pronounced in the absence of TLR2.

Taken together, these data show that in B cells, *C. albicans*-mediated TLR engagement can cooperate with BCR signalling, increasing B cell proliferation.

The fact that anti-*C. albicans* antibodies are found in both healthy colonized individuals and infected patients [340, 379], associated with the importance of TLR signalling for optimal antibody responses in a context of infection [139, 401-403], indicates that the dual TLR2/BCR cooperation observed upon *C. albicans* recognition should also occur *in vivo*. However, to answer this question, factors such as B cell subsets, location and type of infection need to be carefully addressed. A first insight into this topic is currently being addressed in our laboratory by Sally Böde, who aims to trace B cell responses upon *C. albicans* colonization using the aforementioned AIDCre-Rosa26YFP mice. For TLR/BCR cooperation to occur, B cells need not only to be in contact and recognize the pathogen via their TLRs, but also to be able to recognize their cognate antigen via their BCR [93]. Two different scenarios for this dual engagement have been reviewed by Pone *et al*. [139]: 1) Simultaneous TLR engagement and BCR crosslinking leading to the generation of moderate affinity antibodies (CSR can occur but no SHM); 2) Recognition of PAMPs/antigens by both TLR and BCR engagement prime B cells to undergo a GC reaction (CSR and SHM occur). This latter scenario involves T cell help and leads to the generation of high affinity antibodies. Regarding cell subsets, while the first scenario is more frequent in MZ B cells, the second occurs mostly in recirculating follicular B cells [139, 403]. Moreover, evidence shows that while during an initial absence of T cell help, TLR/BCR dual engagement can lead to early CSR and production of low affinity antibodies, in a later phase, these switched cells can be primed to enter a GC reaction and increase the affinity of their antibodies [139, 404]. In this case, the GC reaction is potentiated by the known TLR-mediated upregulation of the costimulatory molecules MHC class II, CD40, CD80 and CD86, which favours T cell help [405, 406]. Finally, it is necessary to keep in mind that different types of infection lead to different types of antibody response [337]. Further *in vivo* studies can determine the impact of *C. albicans* recognition by B cells via TLR2 in the production of anti-fungal antibodies.

4.6. *Candida albicans* recognition by B cells leads to IL-6 (and IL-10) production

Another hallmark of B cell activation is cytokine production. Stimulated B cells have been shown to produce cytokines such as TNF, IL-6, IL-10 and IFN-γ, influencing other components of the immune system in an antibody-independent manner [25]. As such, and since *C. albicans* and phospholipomannan-induced TLR2 activation have also been shown to trigger cytokine responses in different cells types [260, 292, 389, 407], it is reasonable to suspect that a similar effect can be observed in B cells. To investigate this hypothesis, culture supernatants were screened for cytokine presence, leading to the identification of IL-6 and IL-10 production as being influenced by zymosan and HKCA hyphae (Figure 18).

A more in-depth analysis of IL-10 production by ELISA showed that contrary to what has been described so far, a significant difference is observed in cells cultured in LPS+IL-4 supplemented medium (Figure 19A). In this case, the presence of HKCA hyphae results in an enhanced IL-10 secretion. Of note is the fact that LPS is per se a known IL-10 inducing factor for B cells [391]. The same effect is visible in cells only stimulated with HKCA hyphae, meaning that either the few cells able to survive in this culture condition secrete high amounts of IL-10, or that IL-10 secretion is an early response. Surprisingly, no substantial IL-10 production is observed in cells cultured in α-CD40+IL-4 supplemented medium, which seems to indicate that this culture condition impairs IL-10 secretion. Supporting this hypothesis, a study on human B cells has shown that IL-4 downregulates IL-10 production in a dose-dependent manner [208, 408]. The fact that despite the presence of IL-4, IL-10 production is still detectable in B cells cultured with LPS, might be related to LPS being a stronger IL-10 inducer than α-CD40 [409].

Although LPS stimulation is abrogated in MyD88 deficient mice, impeding a clear assessment of TLR involvement, the fact that unstimulated B cells from these mice do not respond to HKCA hyphae like their wildtype littermates, indicates that the increase in IL-10 production is a TLR-dependent effect. These results give strength to the observed trend that HKCA hyphae can also increase B cell activation in the absence of T cell help.

C. albicans has been shown to trigger IL-10 production via TLR2 signalling in DCs and macrophages, a fact that contributed to TLR2 activation being regarded as anti-inflammatory [285, 291, 293]. A study describing TLR2 knockout mice as more resistant to disseminated candidiasis also contributed to this vision [293]. However, despite IL-10

production, TLR2 signalling has also been implicated in the production of pro-inflammatory cytokines such as TNF and IL-6 [287, 291, 292, 407]. These observations combined with a study where TLR2 knockout mice show increased susceptibility to *C. albicans* infection due to a decreased neutrophil recruitment [294], make the designation of anti-inflammatory receptor attributed to TLR2 arguable.

In B cells, IL-10 has been shown to contribute to plasma cell differentiation and antibody secretion in both an autocrine and paracrine way [408, 410]. As such, an increase in IL-10 secretion leads to an increase in the number of plasma cells, which can increase even further the amount of IL-10 produced. Such effects can be deleterious for the host in a context of infection, since an excess of IL-10 production by plasma cells/plasmablasts does not only limit inflammatory T cell responses, but has also been shown to inhibit neutrophil migration [411-413]. Altogether these data point to a scenario where invading pathogens can exploit an overactivation of B cells to decrease the triggered immune response. Corroborating this hypothesis are studies which found plasmacytosis during severe viral infections, and studies claiming that in a model of systemic *Salmonella typhimurium* infection, plasma cells express IL-10 in a MyD88-dependent manner, impairing inflammatory T cell, neutrophil and NK cell activity [235, 237, 414, 415].

In conclusion, even though the presented data appear to support the ability of *C. albicans* to induce IL-10 secretion via TLR-recognition in B cells, due to the discrepancies in B cell activation conditions in which IL-10 and other signals of B cell activation occur, further investigation is necessary. Contributing to this discrepancy is probably the presence of IL-4. If proven true, *C. albicans* would be able to exploit B cell activation and differentiation into plasma cells to, through increased secretion of IL-10, dampen the host immune responses.

Regarding IL-6 production, results obtained by ELISA confirmed that IL-6 secretion by B cells cultured in T cell-dependent activation conditions was enhanced in the presence of both zymosan or HKCA hyphae (Figure 19B). Furthermore, and similar to what was observed for IgG1 production and AID induction, this effect was proven to be MyD88-dependent. Once again, although the response caused by zymosan or HKCA hyphae in LPS+IL-4 activated B cells is not statistically significant, it does display a clear trend. These results further support the scenario where *C. albicans* enhances B cell activation mostly via TLR2, being in agreement with three important aspects: 1) IL-6 has since long been known

to be expressed in activated B cells and to possess an important role in immunoglobulin production [416-418]. 2) Both *in vitro* TI and TD B cell activation conditions (LPS, CD40 ligand and IL-4) stimulate IL-6 production [208, 419, 420]. 3) TLR2 stimulation is able to trigger IL-6 secretion in different cells types [215, 292, 407, 421-423].

The importance of IL-6 in the immune defence against pathogens goes beyond its role in plasma cell differentiation. In this context, IL-6 is produced by a variety of cell types, mainly upon TLR-mediated recognition of PAMPs, and its secretion influences mostly T cells but also other cells such as neutrophils and monocytes [424, 425]. While previous reports simply stated that IL-6 blocks neutrophil accumulation at sites of infection or inflammation [426-428], subsequent studies show that in fact, IL-6 exerts a regulatory effect on the transition between distinct phases of the immune response [429-431]. In a first phase, upon pathogen recognition epithelial and endothelial cells recruit neutrophils to the site of infection. However, this neutrophil accumulation leads to an increase in IL-6 signalling in tissue resident cells, triggering a change in the chemokine responses that favours the recruitment of monocytes and T cells instead of neutrophils [432]. IL-6 signalling further regulates this transition by increasing the expression of the adhesion molecule CD62 in T cells [433], and by promoting pro-apoptotic signals in neutrophils while supplying anti-apoptotic signals in T cells [430, 434, 435]. As such, it can be said that IL-6 acts in the transition from innate to adaptive immunity.

Despite its important role in regulating immune cell recruitment, in the context of defence against invading pathogens, IL-6 is better known for its role in shaping T cell responses. Upon sensing of PAMPs, antigen-presenting cells such as macrophages, dendritic cells and B cells have been shown to upregulate their IL-6 production, contributing to the cytokine milieu that dictates specific T cell differentiation [212, 214, 436, 437]. Consequently, and along with TGF-β, the presence of IL-6 is able to trigger the differentiation of naïve T cells into T_H17 cells, repressing TGF-β-induced differentiation of regulatory T cells, and thus skewing the T cell response into a pro-inflammatory type [438, 439]. In combination with IL-21, IL-6 also influences T_{FH} differentiation, further helping the production of high affinity antibodies [440, 441]. Moreover, IL-6 is also involved in $CD8^+$ T cell responses [442].

Regarding mechanisms of defence against *C. albicans*, both neutrophils and T_H17 cells are known to be major players, making IL-6 an indispensable cytokine for an effective

response [247, 443, 444]. In fact, mice lacking IL-6 have been shown to be more susceptible in a systemic infection model of *C. albicans* [445]. As such, and since B cells are a major source of IL-6 that can stimulate T cells in secondary lymphoid organs, and B cell-specific IL-6 knockout exhibit impaired T_H17 responses, the role of B cells as IL-6 producers may certainly be of relevance in a context of anti-fungal defence [212, 214, 446]. Supporting the importance of B cell-derived IL-6 in a context of *C. albicans* invasion, a recent study from Li *et al*. [215] shows that anti-*C. albicans* T_H17 responses can be induced by IL-6-secreting human B cells. Although not as prominently, *C. albicans*-activated B cells also seem to induce T_H1 differentiation. Apart from IL-6, the observed B cell-induced T helper cell stimulation is also dependent on the MHC class II and the co-stimulatory molecules CD80 and CD86. Of note is the fact that HKCA protein extracts and not intact cells were used for *in vitro* B and T cell stimulation. Finally, Li *et al*. show decreased proliferation and IL-17 production in response to *C. albicans* in T cells from patients undergoing B cell depleting treatment, thus concluding that IL-6 from B cells is important for anti-fungal T_H17 responses.

Barr *et al*. [212] reached a similar conclusion regarding the importance of B cells in anti-bacterial responses. In this study, B cells respond to *Salmonella enterica* by producing IL-6 in an MyD88-dependent manner, which influences IL-17 secretion by T cells. Interestingly, this study distinguished between two phases of B cell contribution to T cell differentiation. The first early phase is almost exclusively dependent on MyD88 signalling, and elicits T_H1 and T_H17 responses by B cell-secreted cytokines. In a second phase, B cell antigen recognition and uptake by the BCR and subsequent presentation on MCH class II molecules allows for generation of memory T cell responses.

Considering the results from these two studies, and our observation that mouse B cells produce IL-6 in response to *C. albicans* hyphae in a TLR-dependent manner, it would be interesting to evaluate the involvement of TLR-mediated *C. albicans* recognition in B cell induction of anti-*C. albicans* T_H17 responses. If proven important, modulation of TLR activation in B cells might be beneficial for immunocompromised patients, as it could, for example, improve T_H17-mediated neutrophil recruitment in individuals where other mechanisms of neutrophil recruitment are impaired. Experiments addressing the impact of HKCA-activated mouse B cells/IL-6 production in T cell polarization are currently being conducted in our laboratory by Melissa Wich.

Even though the observed induction of both IL-10 (anti-inflammatory) and IL-6 (pro-inflammatory) in B cells seems counterproductive, it has also been observed by others in a context of TLR-dependent recognition of bacterial components [212, 399]. As discussed above, the production of IL-10 might be triggered by the pathogen as a way of counteracting the inflammatory milieu created by IL-6 production, leading to impaired T cell and neutrophil responses [237, 413]. However, it is important to remember that IL-10 also contributes to plasma cell differentiation and antibody production, and most importantly, it protects the host from damage during the acute phase of an inflammatory response [408, 410, 447, 448]. As such, and as reviewed before [449], while the absence of IL-10 is beneficial in early stages of infection, prolonged IL-10 deficiency can lead to conditions as severe as septic shock. In line with this argument, IL-10 knockout mice have been shown to display an early resistance to acute systemic candidiasis [450]. Additional information about the spatial and temporal elements of the observed cytokine responses is thus crucial to further interpret the obtained results.

4.7. Conclusion

The present study proposes a model in which mouse B cells recognize *C. albicans* hyphae in an MyD88-dependent manner, mostly via TLR2, which leads to an increased B cell activation characterized by augmented AID induction and antibody and cytokine production (Figure 20). The same effect, although more modest, can also be triggered by the fungal cell wall preparation zymosan, indicating that the TLR2 recognition occurs most probably via cell wall components. Intriguingly, *C. albicans* yeast did not trigger any B cell response. Moreover, *C. albicans*-inducted TLR signalling is able to cooperate with BCR activation to increase B cell proliferation.

Even though there are many differences between mouse and human immunology [451], with the exception of TLR4, which is not widely expressed in human B cells, the B cell receptors, pathways and functions addressed here are known to occur in both species [119, 127, 139, 215]. Most importantly, the Master's project from Melissa Wich conducted in parallel with this study shows that zymosan and HKCA hyphae but not yeast also activate B cells isolated from human blood. These results thus strengthen the believe in a parallelism between the obtained results using a mouse model and what can be observed in humans.

Since specific anti-*C. albicans* antibodies, protective and not, can be found in *C. albicans*-infected patients and also in colonized healthy individuals, it is no novelty that B cells can recognize *C. albicans* antigens [340, 379]. In that direction, a vaccine exploiting B cell responses for treatment of recurrent vulvovaginal candidiasis is currently in Phase II clinical development [337, 452]. However, details on B cells and *C. albicans* interplay have been scarcely addressed. This study thus aimed to overcome that bridge, unveiling some mechanisms through which B cells might be involved in not only antibody-dependent, but also cytokine-dependent anti-*C. albicans* responses. Better understanding of such mechanisms helps in the search for new methods to increase the efficiency of anti-*C. albicans* responses in immunocompromised hosts, where fungal infections can even become life-threatening.

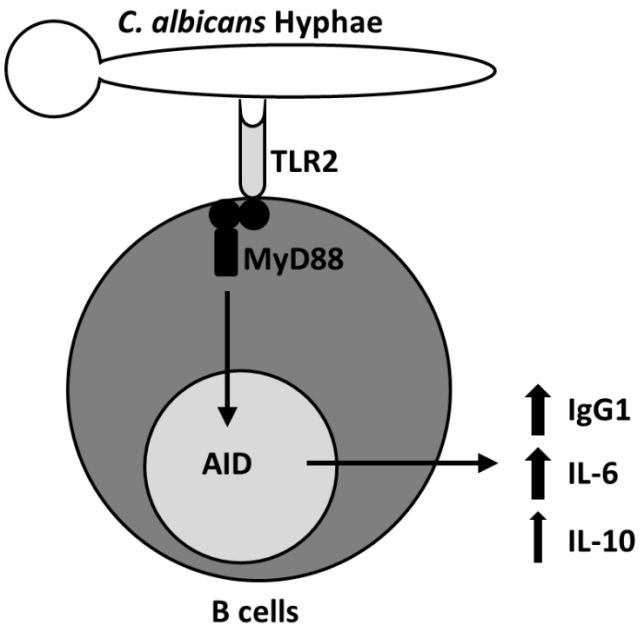


***Figure 20*. Proposed model of *Candida albicans* hyphae recognition by murine B cells.** B cell recognise *C. albicans* hyphae via TLR, mostly via TLR2, and therefore in a MyD88-dependent fashion, leading to an increase in AID activation and IgG1, IL-6 and IL-10 production.

5. Outlook

Despite the accumulated evidences showing that *C. albicans* recognition by TLR leads to B cell activation, further experiments would add information to the proposed model. Since production of AID, antibodies and cytokines is associated with NF-κB activation, which is known to be also induced by TLR signalling, increased NF-κB activation upon co-culture of B cells with HKCA would provide a link among all observed effects [139]. One possibility to address this question is the aforementioned isolation and culture of B cells from NF-κB reporter mice. Further phenotypic characterization of the analysed B cells would also add to the collected information, as B cell activation accompanied by antibody production is usually associated with an increased expression of co-stimulatory molecules, such as MHC class II, CD80 and CD86, and plasma cell differentiation. Finally, and since TLR2 usually heterodimerizes with TLR1 or TLR6, it would be interesting to investigate the roles of TLR1 and TLR6 in *C. albicans* recognition by B cells [119].

To gather more information about the influence that *C. albicans*-mediated B cell activation exerts in a physiological context, *in vivo* experiments are also necessary. A good model to analyse B cell responses to *C. albicans in vivo* is to subject AIDCre-Rosa26YFP mice to *C. albicans* colonization and subsequent dissemination. That fact that cells in which AID is induced (activated cells) become yellow, allows for tracking of activated B cells within the mouse. With this tool, information regarding activated subsets and preferable localization of these cells in the different contexts of colonization versus dissemination can be collected. Of note is the fact that this route of infection (starting from colonization) is advantageous over the commonly used intravenous administration, as it mimics the route of infection that most commonly occur in humans.

According to the literature [19, 139], and supported by the observed results, TLR signalling in B cells can act in two distinct contexts: 1) Antibody production, which can occur in a T cell-independent way, with or without BCR involvement, and lead to the generation of low to medium affinity antibodies, or in a T cell-dependent way, involving germinal center reactions and resulting in the generation of high affinity antibodies; 2) Cytokine production, which triggers a change in the cytokine milieu influencing other cell types, especially T cells.

To address the involvement of *C. albicans* recognition by B cell TLRs in antibody production *in vivo*, the antibody response of mice with a B cell-specific TLR signalling impairment could be studied upon the same aforementioned *C. albicans* colonization and dissemination model. Followed by measurement of specific and non-specific antibody titers and analysis of B cell subsets, this model could give information about which type of antibody activation is affected by lack of *C. albicans*-triggered TLR signalling. This B cell-specific model is especially advantageous as DCs, necessary for appropriate antibody responses, also express TLRs, complicating the distinction between direct and indirect TLR effects on antibody production. It is, however, important to keep in mind that a clear-cut distinction between T cell-dependent and T cell-independent B cell responses is unlikely, as in a context of microbial infection PAMPs eliciting TLR recognition are in close contact with protein antigens.

Regarding cytokine production, it has been previously shown that IL-6 secreted by human B cells upon *C. albicans* recognition leads to the differentiation of T_H17 cells [215]. Still uncharacterized is the involvement of TLR signalling in this process. To address that question, B cells from MyD88 knockout mice can be co-cultured with wildtype T cells in the presence of HKCA hyphae, the analysis of T cells revealing if the absence of TLR signalling can in fact impair T_H17 differentiation. Confirmation of this hypothesis *in vivo* would be advantageous, once again requiring B cell-specific MyD88 knockout mice and the measurement of IL-6 and T_H17 responses.

To conclude, as a long-term objective this project aims to contribute to the knowledge that allows for novel anti-*C. albicans* therapies. The presented hypotheses proven true, the modulation of B cell TLRs might trigger a boost in both protective antibody and T_H17 responses, presenting an optional therapy for patients suffering from *Candida* infections.

6. List of Abbreviations

ADCC	Antibody-dependent cellular toxicity
AID	Activation-induced cytidine deaminase
APC	Antigen-presenting cell
BCR	B cell receptor
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CFSE	Carboxyfluorescein succinimidyl ester
CR	Complement receptor
CSR	Class switch recombination
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific ICAM-grabbing non-integrin
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
Erk	Extracellular signal–regulated kinase
FBS	Fetal bovine serum
FDC	Follicular dendritic cell
FO	Follicular
GC	Germinal center
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HKCA	Heat-killed *Candida albicans*
HRP	Horseradish peroxidase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
IRF	Interferon regulatory factor
JAK	Janus kinase
Jnk	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
LTα1β2	Lymphotoxin α1β2
MACS	Magnetic-activated cell sorting
MAPK	Mitogen-activated protein kinase
MBL	Mannose-binding lectin
MDA5	Melanoma Differentiation-Associated protein 5
MHC	Major histocompatibility complex
MINCLE	Macrophage inducible Ca2+-dependent lectin receptor

mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response 88
MZ	Marginal zone
NET	Neutrophil extracellular trap
NFAT	Nuclear factor of activated T-cell
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	Nod-like receptor
NOD2	Nucleotide-binding oligomerization domain containing 2
NP	4-hydroxy-3-nitrophenylacetyl
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PNA	Peanut agglutinin
PRR	Pathogen recognition receptor
RAG	Recombination-activating gene
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SHM	Somatic hypermutation
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TD	T cell-dependent
TD	T cell-dependent
T_{FH}	T follicular helper cell
TGF-β	Transforming growth factor β
T_H	T helper cell
TI	T cell-independent
TI	T cell-independent
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRIF	TIR-domain-containing adapter-inducing interferon-β
WT	Wildtype
YFP	Yellow fluorescent protein
DAMP	Damage-associated molecular pattern

7. References

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8. Declaration of Authorship

I hereby declare that I am familiar with the relevant course of examination for doctoral candidates at the Faculty of Biological Science of the Friedrich Schiller University Jena. In accordance, I declare that the submitted dissertation was composed entirely by me and is based on my own work. Any other aids and sources have been treated with due acknowledgement. In any moment was I assisted by a doctoral consultant, nor did I provide any monetary compensation to third parties for work connected to this dissertation. This dissertation has never been submitted for examination elsewhere.

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Place, date Signature

9. Curriculum Vitae

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Publications

Manuscript in preparation	Marta Ferreira-Gomes, Melissa Wich, Sally Böde, Ilse D. Jacobsen and Berit Jungnickel "*Candida albicans* hyphae stimulate antibody and IL-6 production in B cells via TLR2/MyD88 signalling"

List of Scientific Presentations

Oral Presentations

April 2018	16th B Cell Forum of the Study Group Biology of B Lymphocytes of the German Society for Immunology, Masserberg, Germany "*Candida albicans* hyphae stimulate antibody and IL-6 production in B cells via TLR2/MyD88 signalling"

Poster Presentations

March 2018	14th Spring School on Immunology, organized by the German Society for Immunology, Ettal, Germany Marta Ferreira-Gomes, Melissa Wich, Sally Böde, Ilse D. Jacobsen and Berit Jungnickel "*Candida albicans* hyphae stimulate antibody and IL-6 production in B cells via TLR2/MyD88 signalling"
March 2016	Annual Conference of the Association for General and Applied Microbiology (VAAM), Jena, Germany Marta Ferreira-Gomes, Stephanie Greim, Ilse D. Jacobsen and Berit Jungnickel "B cells and antibodies in protective immunity to *Candida albicans* infection"

February 2016 — 14th B Cell Forum of the Study Group Biology of B Lymphocytes of the German Society for Immunology, Zeist, The Netherlands
Marta Ferreira-Gomes, Stephanie Greim, Ilse D. Jacobsen and Berit Jungnickel
"B cells and antibodies in protective immunity to *Candida albicans* infection"

September 2015 — 49. Wissenschaftliche Tagung der DMykG and 1st International Symposium of the CRC/Transregio FungiNet, Jena, Germany
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4th European Congress of Immunology – Vienna, Austria
Marta Ferreira-Gomes, Betty Hebecker, Ilse D. Jacobsen and Berit Jungnickel
"B cells and antibodies in protective immunity to *Candida albicans* infection"

April 2015 — 5th International Student Conference on Microbial Communication - MiCom, organized by the Jena School for Microbial Communication (JSMC), Jena, Germany
Marta Ferreira-Gomes, Betty Hebecker, Ilse D. Jacobsen and Berit Jungnickel
"B cells and antibodies in protective immunity to *Candida albicans* infection"

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