Modulation of human monocytes by the pathogenic fungus *Candida albicans*

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Table of Contents

LIST OF FIGURES

ABBREVIATIONS

AP	Alternative Pathway
APCs	Antigen Presenting Cells
BMGY	Buffered Glycerol-complex medium
CP	Classical Pathway
C4BP	C4b binding protein
CFHR1	Factor H-related protein 1
DAPI	4'6-diamidino-2-phenylindole
DCs	Dendritic cells
DMEM	Dulbecco's modified eagle medium
DPBS	Dulbecco's phosphate buffered saline
ECL	Enhanced Chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
FHL-1	Factor G-like serum
FITC	Fluorescein Isothiocyanate
H	hour
HRP	horseradish peroxidase
HUVEC	human umbilical vein endothelial cell
iC3b	inactive C3b
IgG	immunoglobulin G
IL-	Interleukin-
IMDM	Isocove's Modified Dulbecco's Medium
IPTG	isopropyl-β-D-thiogalactopyranoside
iTreg	induced T regulatory cell
LB	Luria Broth
LP	Lectin Pathway
LPS	Lipopolysaccharide
LSM	Laser Scanning Microscopy
MAMPs	Microbial-Associated Molecular Patterns
MPO	myeloperoxidase
NHS	normal human serum
nTreg	natural T regulatory cell
OD	optical density
PAMPs	Pathogen-Associated Molecular Patterns
Pra1	pH-regulated antigen protein 1
PRRs	Pattern Recognition Receptors
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute medium

RT	room temperature
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCC	Terminal Complement Complex
TCR	T Cell Receptor
Teff	T effector cell
TGF-β	Tumor Growth Factor – β
Th	T helper cell
TLRs	Toll-like receptors
Treg	T regulatory cell
YPD	Yeast peptone dextrose

Summary

The interaction between human immune cells and pathogens like *Candida albicans* (*C. albicans*) is a constant battle where both cells attack each other. Under normal conditions the human immune system clears infections with microbes. However, as *C. albicans* overcomes the strong human immune defense by employing immune evasion strategies people with an immuno-compromised immune system are at higher risk to become infected with the fungus. As part of the normal flora in the human body *C. albicans* acts as an opportunistic microbe but can also cause superficial and systemic infections. Recently we showed that monocytes release extracellular DNA traps (MoETs) in response to *C. albicans.* The DNA traps share high similarities to neutrophil extracellular traps and also contain citrullinated Histone 3, elastase, myeloperoxidase, and lactoferrin. MoeTs effectively trap and kill *C. albicans*. Furthermore, monocytes secrete cytokines such as IL-1β, IL-6, but also IL-10 in response to *C. albicans*.

C. albicans has the ability to bind human complement regulators onto its surface to evade the complement attack of the innate immune system. One of these regulators is complement factor H which retains its cofactor activity for factor I, cleaving the opsonin C3b into its inactive form iC3b and inhibiting further complement activation. Factor H accelerates the decay of the C3 convertase which results in reduced opsonisation of *C. albicans*, limiting recognition and contact with immune cells and enhancing survival of the pathogen. In this study, a novel function of factor H bound to the surface of *C. albicans* is identified. *C. albicans* surface bound factor H modulates cytokine secretion of human peripheral monocytes by reducing IL-1β and IL-6 and increasing IL-10 secretion. A similar pattern of cytokine modulation is found when factor H is bound to the surface of apoptotic HUVEC cells, which are known to be immunologically silent. Moreover, factor H mediates the same effect when bound to microbial proteins from *Staphylococcus aureus* and *Streptococcus pneumoniae*. Reducing pro-inflammatory cytokine secretion of IL-1β and IL-6 creates a favorable condition for the pathogen to survive. In addition, a parallel increase in anti-inflammatory cytokine IL-10 dampens the inflammatory response, favoring the survival of the fungus. The regulatory effect of factor H is dependent on factor H surface binding and the presence of normal human serum, likely complement C3. However, CR3 knockout in

THP1 cells via CRISPR/Cas9 results in an overall substantially reduced IL-1β but not TGF-β cytokine response to *C. albicans* and therefore represents the dominant recognition receptor of *C. albicans*. CR3 is responsible in signaling cascades that activate the inflamosome and the maturation and secretion of IL-1β. Factor H bound to *C. albicans* does not modulate IL-1β or IL-10 secretion of CR3KO THP-1 cells but enhances TGF-β release. Surface bound factor H generates together with factor I iC3b from C3b. This iC3b alone inhibits IL-1β secretion in LPS-induced monocytes, and induces secretion of IL- l0 and TGF-β. The factor H induced cytokine pattern in monocytes is strong enough to induce naïve $CD4^+$ T cells to differentiate into induced regulatory T cells (iTreg) as shown by increasing intracellular FOXP3 levels in the cells.

Taken together, this study shows a new immune evasion mechanism by *C. albicans*. Recruiting the immune regulator factor H not only serves to reduce complement attack but also to dampen the immune response. Obviously, *C. albicans* exploits factor H function on apoptotic human cells. Elucidating the mechanisms on how *C. albicans* uses complement regulators can provide new strategies to combat fungal infections and to improve human life.

Zusammenfassung

Die Interaktion zwischen humanen Immunzellen wie Monozyten und dem human-pathogenen Pilz *Candida albicans* (*C. albicans*) ist durch einen gegenseitigen Angriff geprägt. Unter normalen Bedingungen kann die humane Immunantwort die meisten mikrobiellen Infektionen erfolgreich bekämpfen. Allerdings haben einige Mikroorganismen wie auch *C.albicans* Strategien entwickelt sich der Immunantwort zu entziehen, weswegen vor allem Patienten mit einem geschwächten Immunsystem ein höheres Risiko haben sich zu infizieren. Als Teil der normalen Flora im Menschen agiert *C. albicans* als opportunistischer Mikroorganismus, kann aber auch unter gegebenen Umständen lebensbedrohliche systemische Infektionen auslösen. Wir haben gezeigt, dass Monozyten in Kontakt mit *C. albicans* DNA Netze (MoETs) auswerfen um die Ausbreitung von *C. albicans* zu hemmen. Die DNA Netze sind sehr ähnlich zu denen von Neutrophilen und enthalten citrullinierte Histone, Elastase, Myeloperoxidase und Lactoferrin um *C. albicans* abzutöten. Außerdem sekretieren Monozyten Zytokine wie IL-1β und IL-6, aber auch IL-10 in Antwort auf *C. albicans*.

C. albicans hat die Fähigkeit humane Komplementregulatoren zu binden um sich vor der Komplementattacke des angeborenen Immunsystems zu schützen. Einer dieser Regulatoren ist Komplement Faktor H, welcher auf *C. albicans* seine Cofaktor-aktivität beibehält und zusammen mit Faktor I das Opsonin C3b auf *C. albicans* zu inaktivem iC3b umformt, welches die Erkennung und Eliminierung von *C. albicans* durch Immunzellen erschwert. In dieser Arbeit wurde eine neue Funktion von Faktor H auf *C. albicans* identifiziert. Gebunden an *C. albicans* reduziert Faktor H die IL-1β und IL-6 Sekretion von Monozyten und verstärkt die IL-10 Freisetzung. Ein ähnliches Zytokinmuster wird beobachtet, wenn Faktor H an apoptotische HUVEC Zellen gebunden ist, die als nicht inflammatorisch beschrieben sind. Auch wenn Faktor H an mikrobielle Proteine von *Staphylococcus aureus* oder *Streptococcus pneumonia* gebunden ist wird eine ähnliche Zytokinmodulation beobachtet. Reduzierte IL-1β und IL-6 Level sind von Vorteil des Erregers, weil es die Inflammation reduziert. Ein gleichzeitiger Anstieg von IL-10, welches die Immunantwort weiter reduziert, erhöht die Überlebenschancen von *C. albicans*. Die Wirkung von gebundenem Faktor H auf Monozyten ist stark genug naive $CD4^+$ T Zellen

vermehrt in induzierte regulatorische T Zellen zu differenzieren. Der regulatorische Effekt von Faktor H benötigt die Oberflächenbindung und vermutlich Konformationsänderung von Faktor H. Ebenso benötigt Faktor H aktives humanes Serum, wahrscheinlich C3, für diese Funktion. Die Deletion von CR3 als Bindungsrezeptor für C3b/iC3b/Faktor H auf THP-1 Zellen via CRISPR/ Cas 9 resultiert in einen substantiellen Erkennungsverlust von *C. albicans* durch Monozyten und zeigt eine zentrale Rolle von CR3 auf. IL-1β und IL-10 Level sind in CR3 KO Zellen nicht durch Faktor H beeinflusst, aber TGF-β.

Zusammenfassend zeigt diese Studie einen neuen Immunevasionsmechanismus von *C. albicans.* Durch die Rekrutierung von Faktor H nutzt *C. albicans* dessen immunmodulatorischen Effekt auf Monozyten aus, so wie Faktor H es vermutlich auf humanen apoptotischen Zellen ausübt. Die Erforschung dieser Evasions-Mechanismen hilft zum einen die Rolle der Regulatoren besser zu verstehen und bietet zum anderen Ansatzpunkte für eine neue Strategie um *C. albicans* Infektionen zu bekämpfen.

1. Introduction

Humans are constantly surrounded by microorganisms. These microorganisms come in contact and interact with the human body either as normal flora causing beneficial effects, but also as pathogens causing harmful effects that can lead into diseases and eventually, death. In response to this constant interaction with microorganisms, the human organism employs the immune system to defend microbial attacks and also to maintain the beneficial effects received from normal flora. This balanced interaction between human and microorganisms has been developed over millions of years. Pathogens are permanently developing strategies to infect and to overcome the human immune system, on the other hand humans train their immune system to counteract infections of pathogens. Understanding the interaction between pathogens and the human immune system is crucial to improve survival and quality of human life. Elaborating the strategies of pathogens to infect and evade the immune system is a prerequisite for the development of new ways for reducing or eliminating infections and increasing the survival rate as well as quality of many human life.

1.1 The human host

1.1.1. The human immune system

The human immune system is composed of several layers of defense including physical barriers, humoral defense, and cellular defense components which altogether work in tandem to protect the body from foreign microorganisms that can cause harm. The first line of defense are the physical barriers provided by the skin, cell surfaces below the skin and their secretions, normal flora that reside and mutually co-exist within the human body, and mechanical barriers such as tight cell junctions which protect the body from dangerous microorganisms. The next line of defenses are the humoral and cellular responses provided by the innate and adaptive immunity which work together in recognizing and clearing any remaining foreign microorganisms that surpass the physical barriers.

1.1.2. Innate and adaptive immunity

Physical barriers are very efficient in protecting against invading microorganisms but in cases of infection the human immune system employs innate and adaptive immune reactions for a highly specific and long lasting immunity. The innate immune system is a spontaneous response to foreign surfaces in the human body. It consists of two arms, mainly a humoral part (such as complement system and anti-microbial peptides) and a cellular part provided by phagocytes (monocytes, neutrophils, and macrophages), basophils, eosinophils, natural killer cells, and dendritic cells. The humoral response of innate immunity (including complement system) is an evolutionary-conserved immune response system which is characterized by series of conserved immune recognition molecules encoded in the human genome. Consequently, since the number of genes in the human genome are limited, this directly limits the number of gene-encoded innate immune recognition molecules. Recognition molecules which are termed pattern recognition receptors (PRRs) detect specific conserved microbial structures, termed microbial associated molecular patterns (MAMPs), which are presented on nearly every type of microorganism [1]. PRRs are composed of lipopolysaccharide-, mannose-, complement-, toll-like- (TLRs), and scavenger receptors. Each receptors recognizes specific ligands and induces inflammatory reaction cascades. Most of these receptors recognize the PAMPs on the microbial surfaces. Mannose and lipopolysaccharides receptors recognizes repeated mannose units and lipopolysaccharides respectively, on the surfaces of infectious agents and their activation triggers endocytosis and phagocytosis. Toll-like receptors are heavily expressed on immune cells such as the phagocytes and lymphocytes. Interaction of TLRs with their specific PAMPs induces NF-κB signaling and the MAP kinase pathway, and induces the inflammasome for the secretion of pro-inflammatory cytokines. However, complement receptors are specific receptors that binds the complement molecules which are activated when the complement system is in contact with microbes, foreign materials, and modified self-cells in the human body. Once activated by a specific microbe, the complement system generates opsonins, anaphylatoxins and chemoattractants which induce the migration of phagocytes to the site of infection, promotes inflammation, and forms lytic pores on the invading microorganisms. After this initial responses, the human immune system continues to a later response by bridging the innate immunity into adaptive

immunity by transferring the information from innate immune response to the adaptive immune response via the antigen presenting cells (APCs). Dendritic cells (DCs) and macrophages are professional APCs that respond to pathogens through pattern recognition receptor (PRRs), which function in the recognition of the invading microorganism [2-3]. Over time APCs can activate the adaptive immune response to the invading pathogens by triggering T cell differentiation [4-5].

The adaptive immunity is characterized by the generation of highly specified T and B lymphocytes equipped with only one clonally-derived antigen-specific receptor. Two broad adaptive immune responses act together: the antibody response and a cell-mediated immune response. Each response is carried out by a specific class of lymphocytes, B cells and T cells, respectively. B cells are activated to secrete antibodies, the immunoglobulins. The antibodies circulate in the bloodstream and permeate the other body fluids, where they bind specifically to the foreign antigen that stimulated their production. The antibody binding neutralizes toxins/foreign materials by, for example, blocking binding sites to receptors on host cells. Antibody binding also marks invading pathogens for clearance mainly by aiding the recognition by phagocytes of the innate immune system for phagocytosis [6].

In cell-mediated adaptive immune responses, activated T cells react directly against a foreign antigen that is presented by APCs of the innate immune system. The T cell reacts to an infected host cell with foreign antigens on its surface, thereby eliminating the infected cell. In other cases, the T cell produces signal molecules that activate macrophages to destroy the phagocytosed microbes [6]. However, until receiving the antigen stimulus from the T cell receptor (TCR), naïve T cells are quiescent and largely metabolically inactive. Following the stimulation, T cells rapidly divide and acquire effector functions, whether becoming T effector cells (Teff) or T regulatory cell (Treg). Upon antigen recognition, CD4+ and CD8+ naïve T cells differentiate into Teff cells that exert their functions as CD4+ T helper cells or as CD8+ cytotoxic T lymphocytes. The $CD4^+$ T helper cells are the activated T cells which react directly against infected host cells and produce signal molecules to activate macrophages. Alternatively, CD4+ T cells can acquire a regulatory T cell phenotype. Tregs expressing the Treg-specific transcription factor Foxp3 develop directly in the thymus as natural Treg (nTreg), but considerable fraction of $Foxp3^+$ Tregs

is also induced from naïve T cells (iTreg) [7]. The nTregs help maintain tolerance against self-antigens and the iTregs are believed to be of functional importance for tolerance to food- and microflora-derived antigens [7-8]. The cytokine environment in which TCR stimulation occurs determines the differentiation of naïve T cells into one or several T cell subsets (Th1, Th2, Th17, Treg), each with its own specific functions to exert/modulate immune reaction (**Table.1**). Additionally, the activation of an adaptive immune response by components of the innate immune system generates an antigen-specific immunological memory carried by the memory T cell, which allows immediate recognition and removal of infectious agents on a second encounter [9].

Table 1. Distinct CD4+ T cell subsets and their functions

T cell subset	Transcription factor	Stimulus cytokine	Secreted cytokine	Function
Th1	T-bet/Stat4	IL-12, IFN-γ	IFN-γ, TNF	Increase of TLRs, induction of cytokine secretion and macrophage activation. Mediates immune response against intracellular pathogens.
Th2	GATA-3/Stat6	IL-4	IL-4, IL-5, IL-9, IL-10, IL-13	Cytokine secretion. Stimulates B cell proliferation and antibody production. Mediates immune response against parasites.
Th17	ROR-γt/Stat3	IL-1, IL-6, IL-23, TGF-β	IL-17, IL-21, IL-22, IL-25, IL-26	B cell recruitment. Immune response against fungal pathogen.
Treg	Foxp3/Stat5	TGF-β, IL-2	IL-10, TGF-β	Supresses T cell proliferation and experimental autoimmune disease. Anti-inflammatory cytokine secretion.

1.1.3. The Complement system

Complement system is first described as heat-labile component of serum in 1896 by Jules Bordet. It was so named for its ability to 'complement' the antibacterial properties of antibody in the heat-stabile fraction of serum [10]. This system is a central part of host innate immunity and consists of more than 50 soluble and membrane-bound proteins, most of which are sequentially activated by proteolytic cleavage. The main attributes of complement functions are the opsonization of the target cell, lysis of the target cell, induction of inflammatory responses through the release of pro-inflammatory molecules, recruitment of effector cells, generation of anaphylactic peptides, cytolytic and antimicrobial compounds, and the induction of effector responses [11]. These effector functions are important to attack and clear infections but also as an efficient and rapid removal system of altered self-cells such as apoptotic and necrotic cells. Complement functions also direct the adaptive responses of the human immune system, co-stimulatory B cell activation and regulation of T cell immunity by the presentation of antigen which has been recognized by the APCs (macrophages and dendritic cells).

1.1.3.1. Activation of the complement system

Complement is activated by three major pathways: the alternative pathway, the classical pathway and the lectin pathway (**Figure.1**). These ways differ in the mechanisms of target recognition and activation, but ultimately converge into generation of complement component molecule C3b and C3 convertase which subsequently creates a loop of C3b generation to increase opsonization on target surfaces. The alternative pathway (AP) is activated by the spontaneous hydrolysis of the central complement component C3 to C3(H_2O). Upon the formation of C3(H_2O), the factor B binding site is exposed. This results in factor B binding to the C3(H_2O) and subsequent cleaving by the protease Factor D into fragments Ba and Bb. Fluid phase C3 pro-convertase (C3(H_2O)Bb) is formed and cleaves C3 into C3b and C3a. C3b exposes for a short time an internal thioester bond that allows stable covalent binding of C3b to hydroxyl groups on proximate carbohydrates and proteins; in this case the nearest cell surfaces [10]. Attached C3b is immediately bound by factor B which is cleaved by factor D resulting in the assembly of the C3 convertase, C3bBb, and the C3 cleaving cascade continues in an amplification loop. This loop is self-

activating and self-amplifying as long as there are no complement regulators present on the opsonized surfaces. The classical pathway is initiated when C1q, in complex with C1r and C1s serine proteases (the C1 complex), binds to the Fc region of antibodies (IgG1 and IgM) which are attached to recognized surfaces (microorganisms, foreign materials, etc). Activation of C1r and C1s in turn cleave C4 and C2 into C4a, C4b and C2a, C2b fragments. C4b and C2b associate to form the classical C3 convertase C4bC2b on pathogenic surfaces and cleaves C3, like the alternative pathway C3 convertase. Upon the formation of the C3 convertase, the cleavage of C3 on the target surfaces is enhanced. The lectin pathway is induced by mannose residues on the target surfaces which is recognized by the mannose-binding lectin (MBL) to be activated. Similar to the classical pathway, the lectin pathway results in cleavage of C4 and C2, but without the presemce of antibody complexes or C1 participation. MBL binds to certain mannose residues on activator surfaces and subsequently interacts with mannan-binding lectin associated serine proteases like MASP and MASP2. The MBL-MASP/-MASP2 complex is similar to Ab-C1q complex of the classical pathway and leads to cleavage of C4. The resulting fragment C4b exposes a binding site for C2 and C4b-bound C2 is subsequently cleaved by MASP2 into C2a and C2b. C2a is then released and C2b remains bound to C4b and forms the classical pathway C3 convertase (C4bC2b). If complement activation progresses, more C3b molecules are deposited on the surface close to the site of generation and surface-bound convertases amplify the cascade [11-12]. C3b as an opsonin is recognized by human cell receptors such as CR1 on neutrophils and CR1g mainly on tissue macrophages [13]. Receptors ligation initiates phagocytosis and subsequently clearance of the opsonized cells. The convertases from the alternative, the classical, and lectin pathways' convertase can interact with deposited C3b on the same surfaces to form C5 convertases (C3bBbC3b and C4bC2bC3b). The C5 convertase cleaves C5 into C5a and C5b which, C5a functions similar as C3a; an anaphylatoxin and chemoattractant, and C5b is deposited on the surface and interacts with more complement molecules. C5a and C3a bind to their receptors (C5aR/C5a receptor like 2, C3aR respectively) which are expressed on leucocytes and several non-immune cells. Upon binding, C5a and C3a stimulate an inflammatory response such as an increase of vascular permeability, extravasation of immune cells (diapedesis), and release of pro-inflammatory mediators. Additionally, C5a and C3a also exert a chemotactic activity for

leucocytes, activated T and B cells, and mast cells, recruiting the immune cells towards the site of complement activation [14]. On the other hand, C5b on target surfaces exposes a binding site for C6 and subsequently binding C7 resulting in the formation of sublytic complex that drills into the target cells' membrane. Further on, binding of C8 to the preformed C5b67 complex recruits additional molecule, C9, forming a lytic pore complex into the target membrane, termed C5b-9 terminal complement complex (TCC) or membrane attack complex (MAC) [15-16]. The TCC forces lysis on the target cells which later are recognized by the phagocytes and removed from the circulation by phagocytosis. However, nucleated cells are resistant to the TCC killing because of the presence of ion pumps and mechanisms that shed TCC. This sublytic attack of TCC increases the calcium concentration in the mitochondrial matrix as a consequence of pore formation, leading to loss of transmembrane potential and triggering of the NLRP3 inflammasome [17].

1.1.3.2. Regulation of the complement system

The human complement system is activated on all surfaces, self and foreign cells. In order to protect host cells from the complement attack, a number of regulators restrict convertase formation. These complement regulator proteins act as membrane-integrated and/or soluble proteins at different activation pathways of the system. Some of these regulator proteins have overlapping functions, to some extent share structural similarities, and function on inhibiting the C3/C5 convertases and the TCC (**Table. 1**).

Factor H and its splice product factor H-like protein (FHL-1) are the major regulators of the alternative complement pathway. These two regulators share the same regulatory functions: binding to the surface bound or soluble C3b to prevent the formation of the C3 convertase C3bBb, accelerating the decay of the existing C3bBb to reduce further C3b generation, and acting as cofactors of factor I-mediated inactivation of C3b, which transforms the active C3b molecule into its inactive form, iC3b [18]. There is also a regulator protein that enhances the activation of the alternative pathway on the surface of apoptotic human cells, named properdin, by stabilizing the alternative pathway convertases [19-20].

The classical pathway and lectin pathway share regulators since they generate the same C3 and C5 convertases. The regulators generally work either on blocking the pathway initiator

molecules, as a decay accelerator of C3 convertase, or as a cofactor of factor I for general complement inhibition. C4BP and C1INH are the central regulators of both the classical and lectin pathways. Like factor H, C4BP acts as a cofactor of factor I and accelerates the C3 convertase from classical and lectin pathways [21]. Another example is C1INH (C1 inhibitor) which blocks serine protease and acts as suicide substrate for C1r, C1s, MASP2, coagulation factors and C3b [22].

The regulators for the terminal pathway work either on the C5 convertase or block the assembly of the TCC. One of the convertase regulators is FHR1, which binds C5b, inhibits C5 convertase activity, and inhibits TCC assembly [23]. Other terminal regulators of the TCC are vitronectin and clusterin, which bind to C7 and C8 respectively to block the assembly of TCC formation [24].

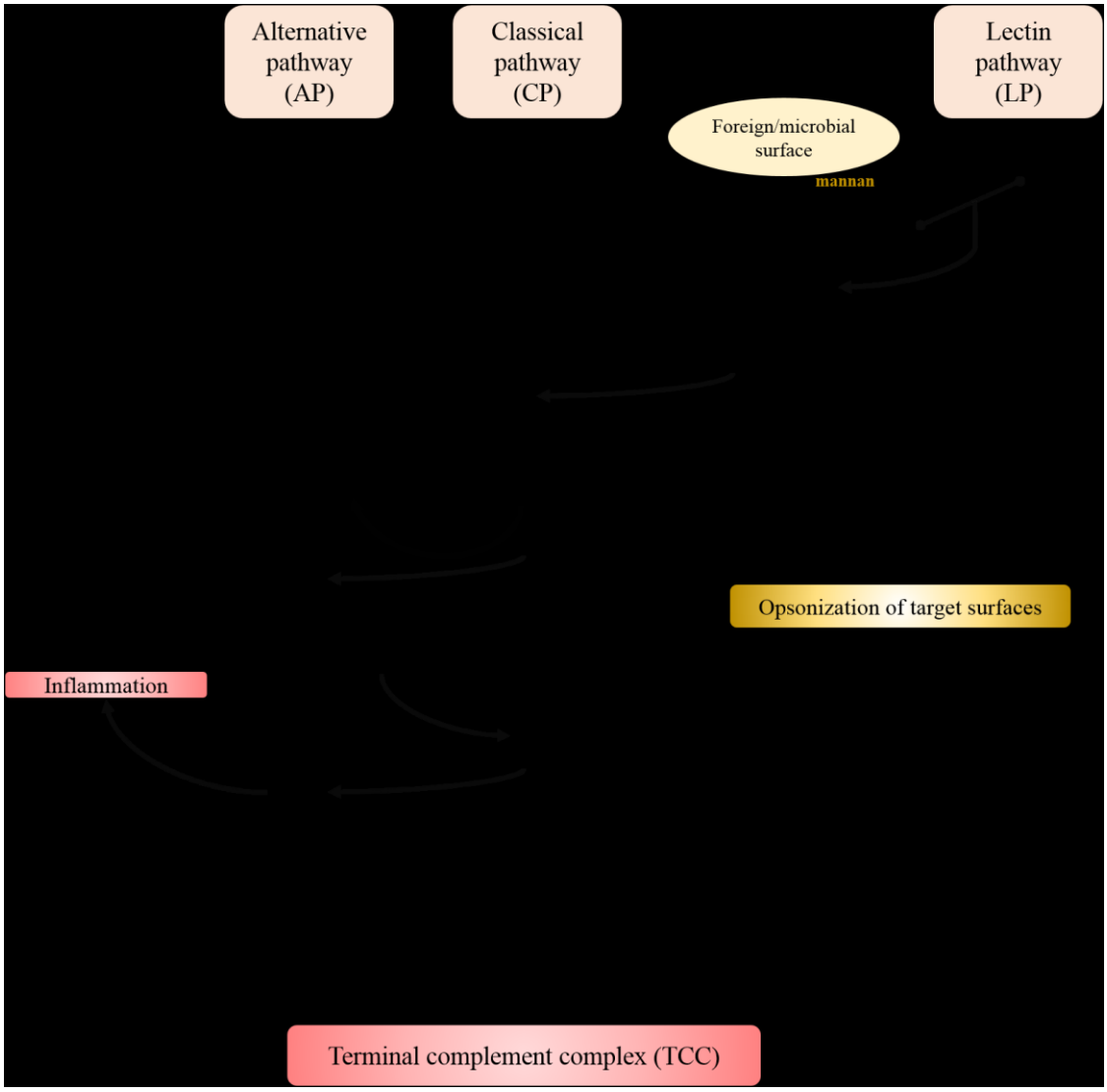


Figure 1. The activation of the complement system via three different pathways.

1.1.3.2.1. Factor H and factor H family proteins

Factor H is composed of 20 homologous domains termed short consensus repeats (SCRs) and circulates in plasma in a high concentration of up to 800 µg/ml [25]. Unlike many other SCR-containing proteins, factor H harbors no other types of domains. Factor H is heavily glycosylated with a high sialic acid content. Deglycosylation of factor H is reported to be without any apparent effect to its function [26]. Several functional sites (**Figure 2**) we located along the 20 SCR domain structure of factor H. The major functional domains are located at the N-terminus (SCRs1-4) and the C-terminus (SCRs18-20). SCRs 1-4 of factor

H bind C3b and act as cofactor for factor I-mediated C3b inactivation. With these SCRs factor H also accelerates the decay of the alternative pathway C3 convertase [27-28]. Factor H binds C3b with two additional binding sites (**Figure 2A**) which have no direct complement regulatory functions and all three sites of factor H interact with distinct C3b domains [29]. The C terminal SCRs18-20 of cator H represent the major cell surface recognition region by interacting with sialic acids, heparin, cell surface exposed glycosaminoglycan (GAGs) and to C3 activation products [30]. As SCRs 18-20 direct factor H to the host cell surfaces, this region is of relevance which is also documented by disease associated mutations in this region [31-32]. Factor H recognizes and binds surface bound C3b and GAGs with SCR 19 and SCR 20 respectively [33-34]. With this dual recognition of GAGs and surface bound C3b by SCRs 18-20, factor H recognizes C3b on the surface of host cells and regulates complement activation. Via this mechanism factor H discriminates between self and foreign cell surfaces, as the latter one lacks the target molecules like GAGs.

The factor H family comprises a group of highly related proteins that includes five complement factor H related proteins (FHR1, FHR2, FHR3, FHR4, FHR5), factor H, and the spliced variant of factor H-like protein 1 (FHL-1). *Factor H* and the five *CFHR* genes are located in a distinct DNA segment on human chromosome 1q32 within the regulation of complement activation (RCA) gene cluster. All *CFHR* genes cluster downstream of the *factor H* gene and [35]. The characteristics of the encoded related proteins are the composition of exclusive SCRs and a high degree of amino acid sequence identities to each other and to factor H. This is shown, for example, for the 3 C-terminal SCRs of FHR1, which share sequence identity from 97% to 100% at the protein level with factor H (**Figure 2B**). This conservation in the recognition SCRs results in very similar ligand binding of FHR1 and factor H. In contrast, FHR1 lacks identity to the N-terminal factor H regulatory domains and thus does not have C3 convertase regulatory activities. Instead, the N-terminal SCRs of FHR1 can form homodimers or heterodimers with FHR2 molecules [36]. The N-terminus also binds to C5b subsequently blocking the C5 convertase formation and TCC assembly [23].

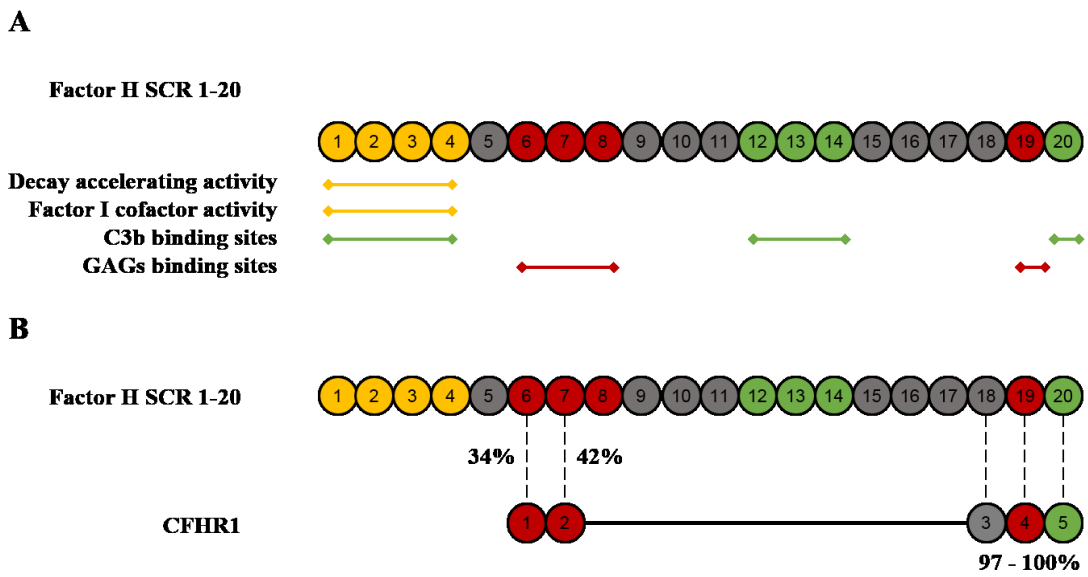


Figure 2. A: Factor H sites and functions aligned to SCRs 1-20. B: FHR1 comparison to factor H. SCRs are aligned according to the amino acid sequence identities (in percentage).

Table 2. Soluble complement regulators and their functions [11]

Regulator	Point of action	Ligand	Function
Factor H	Alternative pathway	C3b and C3d	Cofactor for factor I and acceleration of alternative pathway C3 convertase decay
FHL-1	Alternative pathway	C3b	Cofactor for factor I and acceleration of alternative pathway C3 convertase decay
Properdin	Alternative pathway	C3	Stabilization of alternative pathway convertases
C4BP	Classical and lectin pathway	C4	Cofactor of factor I and acceleration of classical pathway C3 convertase decay
C1INH	Classical and lectin pathway	C1r, C1s, and MASP2	Blocks serine protease and is a suicide substrate for C1r, C1s, MASP2, coagulation factors, and C3b

CFHR1	Terminal pathway (TCC)	C5 convertase and TCC	Inhibition of C5 convertase and TCC assembly
Clusterin	Terminal pathway (TCC)	C7, C8β, C9, and TCC	Transport of cholesterol, HDL, APOA1, and lipids
Vitronectin	Terminal pathway (TCC)	C5b-7 and TCC	Adhesion protein, fibronectin-mediated cell attachment, and Arg-Gly-Asp site coagulation in immune defence against *Streptococcus* spp.

1.1.4. Human immune cells

Human immune cells play a major role in human immune defense against microorganism, as well as clearance of foreign/dead self-cells from the body. They actively respond to invading microorganisms and act together with the innate immune response like complement and antibodies. Termed cellular immunity, immune cells display their protective capabilities by phagocytosis of pathogens, secretion of cytokines that induce inflammation, and secretion of toxic substances to attack extracellular microbes. Immune cells correspond with other cells to become involved and support innate and adaptive immune responses. Specialized immune cells called antigen presenting cells (APCs) stimulate and introduce foreign antigens to antigen-specific cytotoxic T cells. These stimulated T cells induce apoptosis in cells displaying epitopes of the foreign antigen on their surfaces, such as virus-infected cells, cells with intracellular bacteria, and cancer cells displaying tumor antigens.

Leukocytes are a diverse group of immune cells that mediate the human immune response. They circulate through the blood and lymphatic system and are recruited to sites of tissue damage and infection. They are categorized into phagocytes, lymphocytes, and auxiliary cells. Phagocytes are the cells that directly attack foreign particles, including infectious agents, such as microorganisms by phagocytosis and intracellular killing. Neutrophils, monocytes, macrophages, and dendritic cells are the main phagocytes. They search and recognize foreign materials and subsequently eliminate any possible threat by infectious microbes. Phagocytes also induce cytokine secretion to signal other immune cells to migrate to the site of infection and promote inflammation. The lymphocytes include T cells,

B cells, and natural killer cells. Both T cells and B cells are the acting cells of adaptive immunity which react directly to the microbe differently with the help of innate immune cells. T cells are involved in cell immunity and B cells generate antibodies against foreign epitopes. Natural killer cells (NK cells) are part of the innate immune system that recognizes changes on the surfaces of infected cells. Virus infected cells has often a lower expression of antigen presenting major histocompatibility complex class I (MHC class I) enabling these cells to escape cytotoxic T cell killing. However, NK cells recognize this down-regulated antigen presenting molecules on the surface and immediately release cytotoxic (cell-killing) granules which then destroy the altered cells. MHC class I molecules are recognized by NK cell inhibitory receptors and the ligation of these receptors inhibits the activation of NK cells. Basophils, mast cells, and platelets are the main auxiliary cells that help mediate inflammation and recruit other immune cells to the sites of infection. Basophils are a mobile cells that contain granules which on degranulation release histamine, and platelet activating factor. These inflammatory mediators cause increased vascular permeability and smooth muscle contraction to help recruitment of immune cells. Mast cells are similar to basophils, they contain granules which are released when the cell is triggered, causing inflammation on the surrounding tissue since they are immobile and found close to blood vessel in all types of tissues. Platelets are cells that get activated with stimuli that trigger activation mechanism such as thrombin or platelet activating factor (PAF) and release inflammatory mediators to activate coagulation, inflammation, and wound healing. All of these immune cells work accordingly in tandem by interacting and signaling each other to generate inflammatory response in the immune defense.

1.1.4.1. Monocytes

Monocytes are mononuclear phagocytes circulating in blood vessel and have crucial but distinct roles in tissue homeostasis and immunity. This conserved population of leukocytes is present in all vertebrates and represent in humans about 10% of all blood nucleated cells [37]. Monocytes act as immune effector cells, equipped with chemokine receptors and adhesion receptors that mediate migration from bone marrow to blood and to tissues during

infection. They take up cells and foreign materials by phagocytosis and also produce a number of cytokines [38].

Monocytes function primarily as phagocytes and mediators of inflammation. They are a source for replenishing macrophages and dendritic cells in tissues at sites of infections. The strong phagocytic capability is helpful to remove infected and dying cells, but monocytes also play a central role in adaptive immunity by differentiating into macrophages and dendritic cells that subsequently induce CD8+ T cell proliferation and activate CD4+ T cells [39]. Monocytes migrate from the circulation across the endothelium into tissues in response to endothelial cell–bound factors, such as chemokines, that deliver activating and chemoattracting signals. Endothelial cells express chemokine receptor 2 (CCR2) that play a critical role in responding to monocyte chemoattractant protein-1 (MCP-1; as known as CCR ligand 2/CCL2) [40]. CCL2 or MCP-1 is expressed by most nucleated cells in response to pro-inflammatory cytokines or stimulation of innate immune receptors by microbial molecules. CCL2 dimerizes and binds tissue GAGs [41] thereby forming gradients of CCL2 that guide monocytes towards the sites of inflammation. Before entering the sub endothelial space of the tissue, monocytes adhere to the endothelium via integrins. CD11a/CD18 (lymphocyte function-associated antigen 1; LFA-1) and CD11b/CD18 (MAC-1; CR3) integrins on monocytes are responsible for adhesion to the intercellular adhesion molecule I (ICAM-1) of the endothelial cells whereas very late antigen-4 (VLA-4; integrin α4β1) on monocytes interacts with vascular adhesion molecule-1 (VCAM-1) of endothelial cells. These interactions are enhanced upon stimulation of monocytes with pro-inflammatory cytokine IL-1β [42].

Mainly three types of human monocytes are described (**Table 2)**. The $CD14^{hi}CD16^{-}$ monocytes travel along the endothelium of blood vessels and move to sites of inflammation. They also act as precursors of peripheral mononuclear phagocytes. $CD14^{hi}CD16^{+}$ monocytes or the intermediate monocytes $CD16^{+}$ subset which actively produce pro-inflammatory cytokines. $CD14^{low}CD16^{+}$ monocytes (non-classical monocytes) are non-inflamed monocytes which crawl on the luminal side of the endothelium and respond to local danger signals by recruiting neutrophils which trigger focal endothelial necrosis and, subsequently clear the resultant cellular debris [43, 44, 45]. $CD14^{hi}CD16^{-}$ monocytes represent ‘classical monocytes’ which are recruited very rapidly

from the bloodstream to the site of infection as a phagocyte and serve as source for tissue macrophages. In contrast intermediate and non-classical monocytes are also recruited to the sites and act as cytokine and chemokine producer, enhancing and/or regulating the local immune response. Furthermore, we and others describe that monocytes also release extracellular DNA traps, similar to neutrophil, to trap and kill pathogens [46-47].

Monocytes rely on pattern recognition receptors (PRRs) to recognize and interact with pathogen associated molecular patterns (PAMPs) of the invading pathogens. Upon PAMPs engagement, PRRs trigger intracellular signaling cascades culminating in the expression of a variety of pro-inflammatory molecules to promote inflammation and recruitment of other immune cells. One type of PRRs that has been studied extensively is the toll-like receptor (TLR) [48]. Monocytes expresses different types of TLRs (**Table 3**) to recognize bacterial lipoproteins, lipopolysaccharides, heparan sulfate fragments, RNA, and DNA to interact and activate the signaling cascade in monocytes to activate the inflammasome. Complement receptors (such as CR3, CR1, and CR4) on monocytes also play a dominant role in membrane surface recognition, differentiating between self and non-self by recognizing surface bound opsonin C3b, the inactive form C3b (iC3b), and surface bound complement regulators such as factor H and CFHR1 which all induce signaling cascades inside the cell.

Table 3. Different monocyte types and their functions

Monocytes type	Markers	Chemokine receptors	Function
Classical monocytes 75%-85% of total monocytes	$CD14^{hI}$ $CD16^{-}$	$CD192^{hi}$ ($CCR2^{hi}$)	Phagocytic and low pro-inflammatory cytokine production
Intermediate Minor subpopulation of $CD16^{+}$ subset	$CD14^{hi}$ $CD16^{+}$	$CD192^{low}$ ($CCR2^{low}$) $CD195^{+}$ ($CCR5^{+}$)	Pro-inflammatory function active producer of IL-1β and IL-6
Non-classical 10-20% of total monocytes	$CD14^{low}$ $CD16^{hi}$	$CD192^{low}$ ($CCR2^{low}$)	Anti-inflammatory, constitutively produces IL-1RA

Table 4. Monocytes TLRs and their functions

Receptor	Location	Ligand	Ligand location
TLR1	**Cell surface**	**Triacyl lipopeptides**	**Bacterial lipoprotein**
TLR2	**Cell surface**	**Glycolipids, lipoproteins and peptides, beta-glucan, lipoteichoic acid**	**Bacterial peptidoglycans, fungi, Gram positive bacteria**
TLR4	**Cell surface**	**Lipopolysaccharide, heparin sulfate fragments**	**Gram negative bacteria, host cells**
TLR5	**Cell surface**	**Bacterial flagellin**	**Bacteria**
TLR6	**Cell surface**	**Diacyl lipopeptides**	**Mycoplasma**
TLR8	**Cell compartment**	**Small synthetic compound, single strand RNA**	**RNA viruses**
TLR9	**Cell compartment**	**Unmethylated CpG Oligodeoxynucleotide DNA**	**Bacteria, DNA viruses**
TLR13	**Cell compartment**	**Bacterial ribosomal RNA "CGGAAAGACC"**	**Virus, bacteria**

1.1.4.2. Complement receptor 3 (CR3)

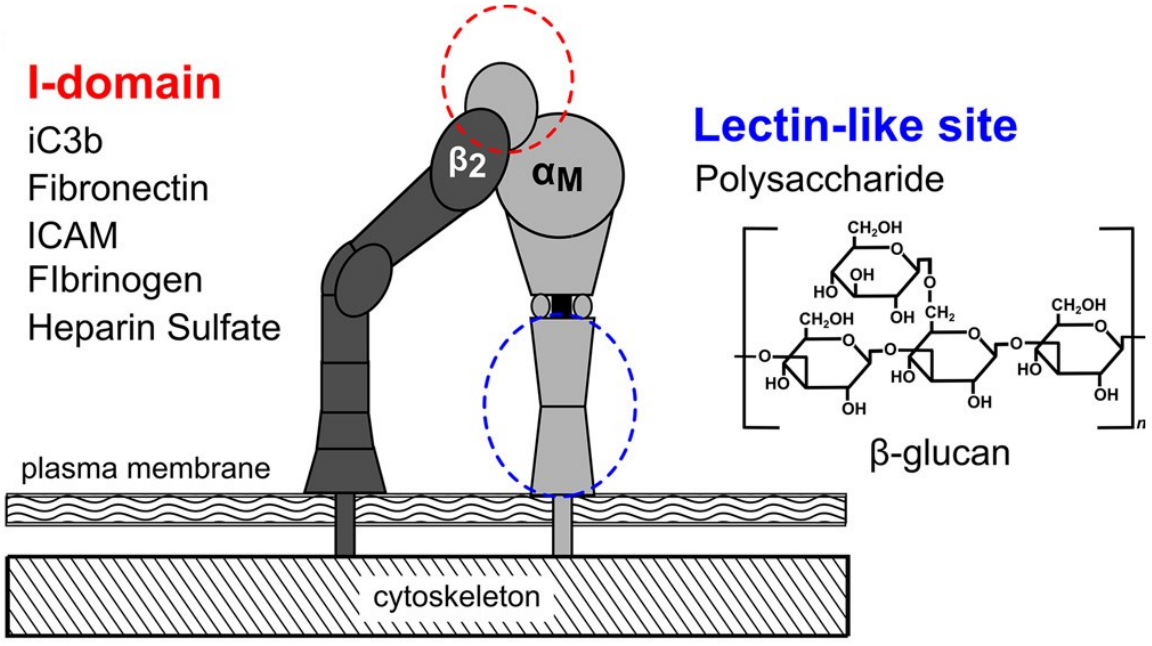
CR3 (αMβ2) is a heterodimeric transmembrane glycoprotein and belongs to the β_2-intergrin family, consisting of CD11b (αM) which is non-covalently associated with CD18 (β2) [50]. Two distinct binding sites in CR3 were identified, an I-domain which is essential for binding and phagocytosis of complement-opsonized particles [50-51] and a lectin domain which is responsible for the non-opsonic binding of the microbes to CR3 (**Figure 3A**).

Recognition via the I-domain is mediated by complement proteins C3b and iC3b on the surface of the microbe, while the lectin domain binds β-glucan, lipopolysaccharides, and surface proteins on microorganisms [52,53,54]. For example, this lectin domain interacts

with the fungal PAMP β-glucan and serves as the dominant receptor for the recognition of fungal cells by human granulocytes. However, soluble β-glucan is not known to directly activate neutrophils through CR3 but rather is thought to initiate a primed state that leads to a faster or an enhanced response when encountering a second stimulus [55]. The soluble β-glucan caused an extension of the extracellular domain of CR3 priming the receptor to an active state to respond for further stimulus (**Figure 3B**).

In addition, CR3 acts as factor H receptor on monocytes [56] and interacts with factor H SCR 7 and SCRs 19-20 as the major binding sites [57]. However, the functional consequences of this interaction has not been completely. Another study reported that binding of factor H to CR3 supported neutrophil adherence and enhanced the release of reactive oxygen species in primed neutrophils [58]. In monocytes, the factor H-CR3 interaction and its intracellular signaling cascade has not been fully explored yet. However, the C3 cleavage product iC3b that is generated by factor I and its cofactor factor H has been reported to interact with CR3 on surfaces and mediates phagocytosis.

A



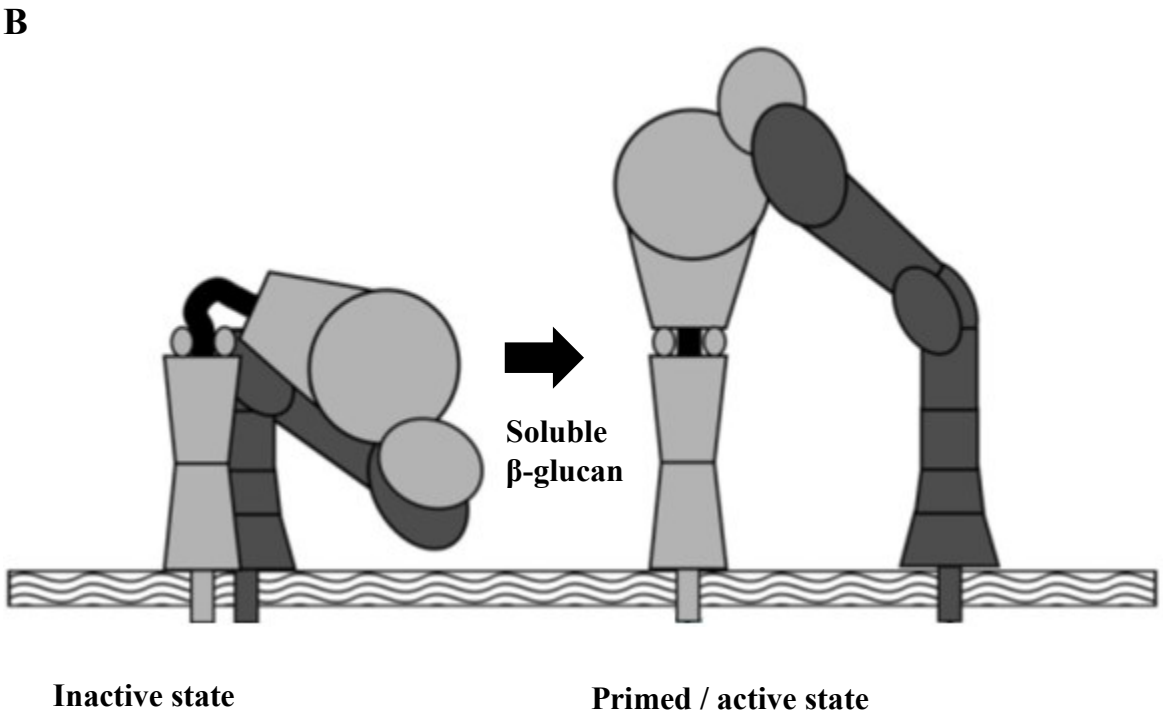


Figure 3. The two domains of CR3 [55]. (A) I-domain and lectin domain in their active state. Each domain can bind to different ligands; I-domain binds to iC3b, fibronectin, ICAM, fibrinogen, and heparin sulfate **(B)** after soluble β-glucan stimulus, CR3 becomes primed in the active state.

1.2 Microbial pathogen

1.2.1. *Candida albicans*

The polymorphic fungus Candida albicans is a member of the normal human micro flora. It is a diploid fungus that lives in the gut, oral-pharyngeal, vulvo-vaginal areas, and on the skin of humans. Normally, *Candida albicans* (*C. albicans*) resides in an individual as a lifelong and harmless commensal. However, under certain circumstances it can cause infections that range from superficial infections of the skin to life-threatening systemic infections. *C. albicans* can cause infection and heavily colonizes the human host when physical barriers are disrupted physical barriers (such as skin and barriers below the skin) and/or in situations of an impaired immune system like immunodeficiency syndrome. In this case, another pathogen infection has weakened the immune system, or

immunosuppressive drugs from chemotherapy or transplantation were used in the patient. Despite applied anti-fungal therapies, both mortality and morbidity mediated by *C. albicans* infections are still high [59, 60, 61] and more than 75% of people with systemic candidiasis infection die. Moreover, resistant *C. albicans* strains are continuously increasing in recent years making *C. albicans* the most frequently isolated fungal pathogen from infected individuals.

C. albicans has a number of traits that enhances the pathogenicity of the fungus, such as the ability to evade the human immune system [62], to switch from yeast to hyphal forms exerting pressure to human cells [63], to secrete proteases that degrade human host components [64], and to express adhesins and invasins [65, 66]. Altogether these traits support both commensalism and pathogenesis of *C. albicans*. In healthy individuals however, the infection with *C. albicans* is cleared by an active immune system and the micro flora of the host body.

1.2.2. *Candida albicans* cell wall

C. albicans interacts with human host cells through various components of the fungus cell surface. It consists of two different layers, the outer layer is composed of mannans and mannoproteins while the inner layer contains chitin, β-1.3 glucan, and Glycophosphatidylinositol (GPI) anchored proteins (**Fig.4**). Both layers are linked by β-1,6 glucan. The attached fungal molecules used for immune evasion are embedded into the fungal cell wall, which comes into contact with the environment and the host immune components [67]. GPI-anchored proteins are attached to β-1,6 glucan while proteins with internal repeats are directly attached to β -1,3 glucan [68]. Cell wall proteins which are non-covalently bound are distributed heterogeneously on the cell surface. These proteins, termed moonlighting proteins, are likely secreted and play another role beside their main identified functions [69]. Pra1 is one of the moonlighting and immune evasion proteins that recruits complement regulators factor H, FHL-1, C4BP, and plasminogen to the surface [70, 71]. Pra1 is also secreted to block the activation and conversion of C3 [72] to prevent opsonization.

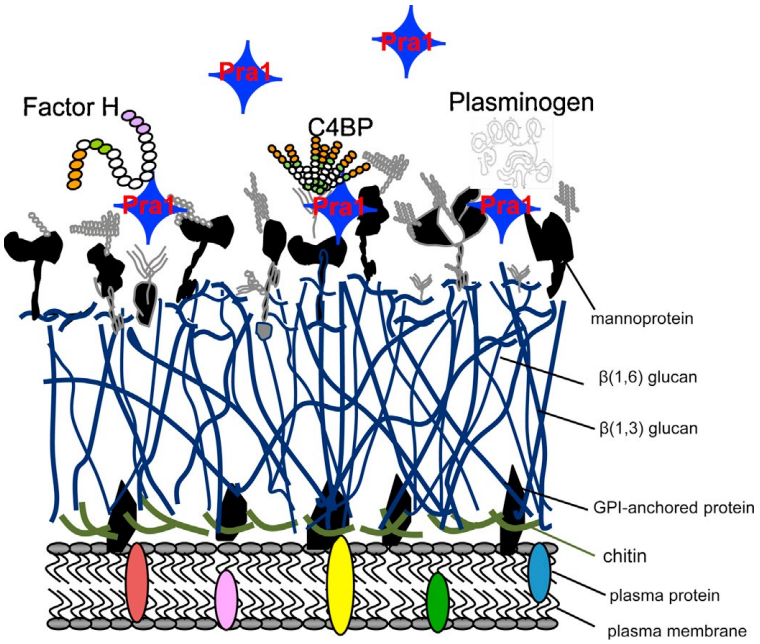


Figure 4. *Candida albicans* cell wall structure [62]. *C albicans* has a thick cell wall which is a highly complex structure composed of glucans (β-1.3 and β-1,6 glucans), chitin, GPI-anchored protein and mannoproteins inserted in network of polysaccharides.

1.2.3. *Candida albicans* induces inflammasome activation

Candida albicans cell wall is the major PAMPs recognized by the host's innate immune cells which occurs via PRRs. The activation of PRRs of the Toll-like receptor (TLRs), RIG-I-like receptors (RLRs), Complement receptor 3 (CR3), or C-type lectin receptors (CLRs) initiates signaling cascades that result in pro-inflammatory gene expression IL-1β and IL-18 [73]. The IL-1β synthesis, processing, and release are tightly controlled and require at least two distinct stimuli (**Fig. 5**). An initial stimulus is received through PRRs, from the recognition of the cell wall of *C. albicans*, results in an accumulation of intracellular stores of pro IL-1β. A second stimulus activates a multi-protein complex containing one or more Nod-like receptors (NLRs), termed inflammasome, which controls the activation of caspase-1 and cleavage of pro-IL-1β and later on the release of active IL-1β [74]. The NLRP3 is the main inflammasome responsible for *C. albicans* infection. Reactive oxygen species (ROS) which is a conserved danger signals, and K+ efflux are required to activate NLRP3 inflammasome known to date [75]. Interestingly, dectin-1/Syk – mediated release of reactive oxygen species and induction of caspases release was shown to be hold a critical role in inflammasome activation in *C. albicans*-infected murine and

human phagocytes [76]. However, secreted aspartic protease (Sap) 2 and Sap6 of *C. albicans* were demonstrated to induce caspase-1-dependent NLRP3 inflammasome activation [77]. The activation of the inflammasome by *C. albicans* culminates into the maturation of pro-IL-1β into mature IL-1β, which is an important pro-inflammatory cytokine that mediates inflammation.

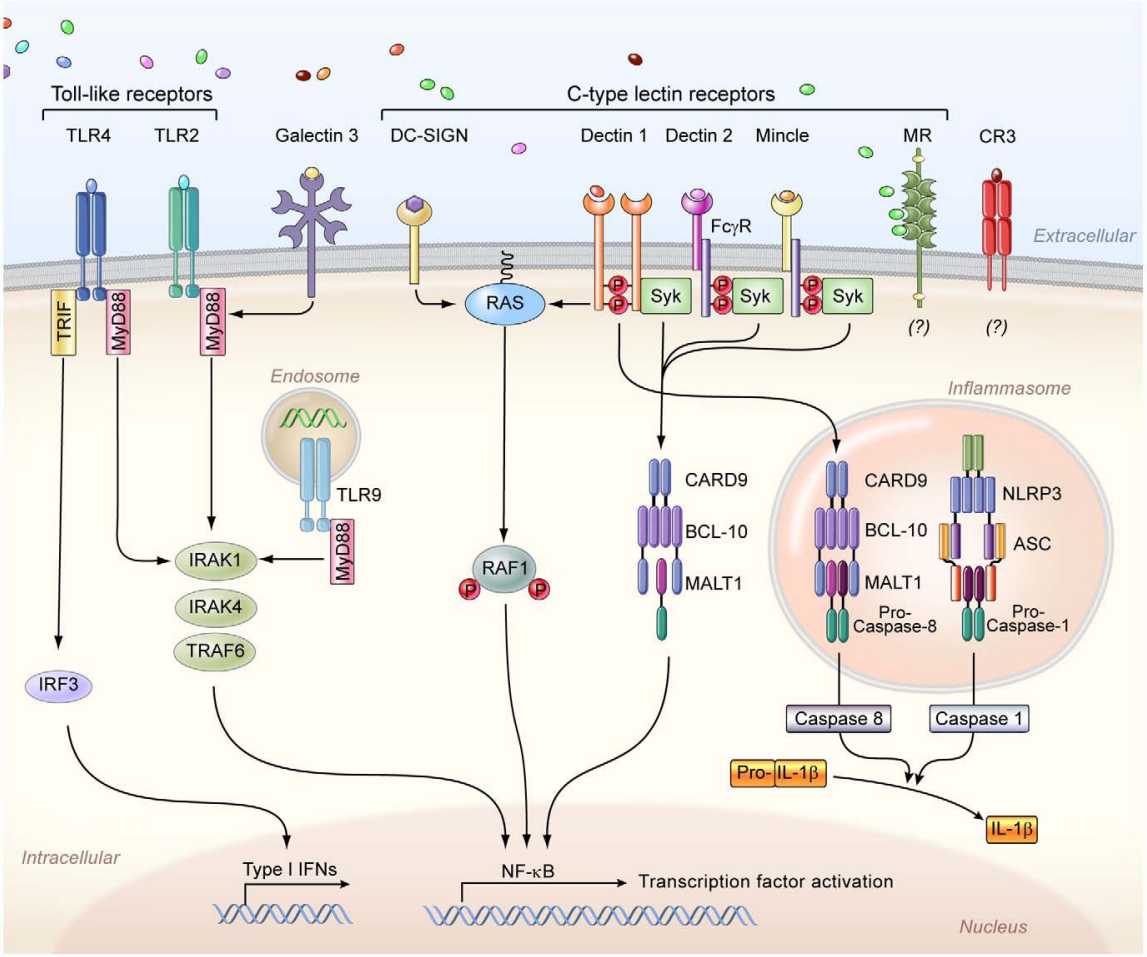


Figure 5. *Candida albicans* recognition induces inflammasome and IL-1β secretion [125]. The IL-1β synthesis, processing, and release are tightly controlled and require at least two distinct stimuli. Activation of the inflammasome is required to activate Caspase 1 which later on cleave Pro IL-1β to mature IL-1β.

1.2.4. *Candida albicans* and complement system

As part of the human microflora, *C. albicans* is permanently in contact with the human immune system. Normaly, fungal overgrowth is suppressed by the human immune system and the other microflora that limit the growth space. Under certain conditions such as environmental triggers (high pH, high CO_2, or 37^0C temperature) which might be harmful to some microbes of the microflora, *C. albicans* changes in morphology, forms filaments, and becomes generally more virulent [78]. The absence of a limiting growth factor like the microflora, or an impaired function of the immune system can lead to *Candida* invasion which ultimately can cause systemic candidiasis and sepsis. During systemic candidiasis, *C. albicans* is exposed to immune components of human blood, the complement system, and antibodies. Upon contact with the blood, *C. albicans* activates all three complement pathways, mainly the alternative pathway that is induced by the C3b deposition on the foreign surface. Similarly, β-1,6 glucans on the surface of *C. albicans* trigger the immune response [79, 80]. Anti- *Candida albicans* antibodies bind to *Candida* and subsequent recognition by C1q activate also the classical pathway. Mannan on *Candida* surface is recognized by mannan binding lectin (MBL) receptor which activates also the lectin pathway [81]. Activation of these complement pathways lead to the opsonization with C3b on the surface of *C. albicans* which enhances recognition of *C. albicans* by immune cells (phagocytes). Also the generation of C3a and C5a that have antimicrobial capabilities and augment cytokine secretion is enhanced [82, 83]. Neutrophils responded to the anaphylatoxic peptides C3a and C5a with a shape change and a respiratory burst [84]. C5a also acts as a chemoattracttant to recruit neutrophils, monocytes, macrophages, and other phagocytes to the site of infection. However, *Candida albicans* is known to recruit complement regulatory proteins to evade the human immune reactions. Complement regulators such as factor H, factor H-like protein 1 (FHL-1), and complement factor H-related protein 1 (CFHR-1) bind to *C. albicans* and retain their activity. With factor H and FHL-1 inhibition of C3b opsonization and CFHR-1 inhibition of C5 convertase, C3a and C5a generation are inhibited, reducing the recruitment of phagocytes which ultimately reduces inflammation. On the other hand, there have been also reports that in without active complement system, factor H, FHL-1, and CFHR-1 increase the attachment of neutrophils to *C. albicans*. Surface bound factor H and CFHR1 also enhance the generation of reactive

oxygen species (ROS) and the release of antimicrobial protease lactoferrin by neutrophils [57].

1.2.5. *Candida albicans* immune evasion

Candida albicans is well known to recruit complement regulators onto its surface and retain their regulatory functions to control and evade a complement attack [11, 62]. Complement regulators factor H, FHL-1, C4BP, and plasminogen are recruited by *C. albicans* surface proteins also called CRASPs (Complement Regulator Acquiring Surface Proteins). The pH-regulated antigen 1 (Pra1) is one of multiple surface proteins of the *C. albicans* that binds complement regulators (factor H, FHL-1, C4BP, plasminogen) to evade immune response on the surface provided by alternative pathway and lectin pathway of the complement activation [70, 71]. Pra1 is also secreted as a complement inhibitor, binding C3 and blocks further cleavage of C3 to C3b and C3a. The reduced opsonization by C3b resulted in the complement cascade inhibition from the C3 level, which also affects the downstream process of C5 cleavage, inhibiting the formation of TCC.

The importance of the recruitment of factor H for *C. albicans* evasion strategies is documented by expression of multiple *C. albicans* surface proteins dedicated to recruit these complement regulators. At present, there are 3 other fungal surface proteins that bind factor H, namely the phosphoglycerate mutase (Gpm1), the high-affinity transporter 1 (Hgtp1), and glycerol-3-phosphate dehydrogenase 2 (Gpd2) [85, 86, 62]. Aside from binding complement regulators, *C. albicans* also secretes proteases Saps 4-6 which interfere and inactivate complement components C3b, C4b, and C5, while also inhibit the TCC formation [64, 87].

1.3 Objective of the study

Previous work showed that *Candida albicans* recruit human complement regulator factor H onto the surface to inhibit complement activation and to reduce opsonization and phagocytosis in the human host. So far, it was unclear whether microbe-bound factor H mediates further regulatory functions like dampening the inflammatory reaction by immune cells. Therefore, the role of complement regulator factor H bound to *C. albicans* was investigated. This study aimed at elucidating whether surface bound factor H modulates the level of secreted inflammatory cytokines of human blood monocytes in the presence of active complement system and affects the immune response to *C. albicans.* In a second step, it is was of interest to follow also the adaptive cellular immune response upon modulated monocytes by factor H. Determining naïve T cell differentiation challenged with supernatants of modulated monocytes was expected to show whether changes of the immune response by monocytes also affect downstream responses and influence adaptive immune responses. As factor H is recruited by a panel of pathogenic microbes, the results will extend the understanding about the immune evasion strategy of many pathogenic microbes, especially of *C. albicans*.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Human sera and cells

Normal human serum (NHS) were obtained from healthy laboratory personnel. Sera were stored at -80^{0}C prior to use. Buffy coats (Friedrich Schiller University Jena, Germany) or fresh blood were collected from healthy volunteers after informed consent according to guidelines from the local ethics committee. Human PBMCs were isolated from buffy coats or fresh blood by density gradient centrifugation with Ficoll-Paque PLUS (GE healthcare, Freiburg). Lymphocytes were removed from the isolated PBMCs using 46% Percoll (GE healthcare, Freiburg) and IMDM (Thermo Fischer scientific, Darmstadt) media density gradient centrifugation. Monocytes were further purified with negative selection using magnetic beads according the manufacturer's protocol provided with pan monocyte isolation kit (Miltenyi Biotec, Bergish Gladbach). Purity of isolated cells was confirmed by identifying CD14 with FITC conjugated anti-human CD14 antibody (Biolegend, London) on the cells using flow cytometry. Human umbilical vein endothelial cells (HUVEC) and THP-1 monocytic cell line were purchased from ATCC (Vanassas, VA, USA). Naïve T cells were isolated similar to the monocytes isolation, the PBMC fraction was incubated with specific fluorescent dye-conjugated antibodies: anti-CD4 (PE) and anti-CD45RA (APC). Cells were washed twice with PBS and then sorted using cell sorter (FACS Aria Fusion special order system, BD) for PE and APC positive.

2.1.2. Chemicals, reagents, kits, and plastic materials

Unless specified otherwise, chemicals and reagents were purchased in the highest quality available from Sigma or Roth. RPMI 1640 medium, IMDM, and Dulbecco's phosphate buffered saline (DPBS) were from Lonza (Verviers, Belgium). Cell detachment enzyme Accutase and human cell apoptosis inducer Staurosporin was purchased from eBioscience (Frankfurt, Germany) and New England Biolabs (Frankfurt, Germany) respectively. Gradient solution Ficoll-pague PLUS and Percoll were purchased from GE Healthcare (Freiburg, Germany). Pan monocyte isolation kit was purchased from Miltenyi Biotec (Bergish Gladbach, Germany). Amaxa human monocyte nucleofector kit was purchased

from Lonza (Cologne, Germany). Polybead®Sulfate 3µm microspheres were manufacture from Polysciences, Inc (Warrington, PA, USA). MaxiSorp microtiter plates were manufactured by Nunc (New York, NY, USA). Nunclon delta surface cell culture plates and Cellstar cell culture flasks for culturing human cells and cell lines were manufactured by Thermo Fisher Scientific (Roskilde, Denmark) and Greiner Bio-one (Frickenhausen, Germany).

2.1.3. Microbial media and supplements

Pichia pastoris yeast cells were cultivated in buffered complex glycerol-complex liquid medium (BMGY, 1% yeast extract, 2% peptone, 100mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base, $4x10^{-5}$% biotin, 1% glycerol) for protein expression purpose. Candida albicans cultivation media were YPD broth (2% glucose, 2% peptone, 1% yeast extract). Solid agar media were prepared as above with additional 1.5% agar. Escherichia coli cells were cultivated in Luria Broth medium (10gr bacto-tryptone, 5gr bacto-yeast extract, 10gr NaCl, pH adjusted to 7.2) or LB agar plates (LB + 1.5% agar). To select colonies containing desired plasmids, LB medium was supplemented with 100µg/ml ampicillin (Invitrogen).

2.1.4. Purified and recombinant proteins

Purified Human C3, C3b, iC3b, C3d, Factor B, Factor H, Factor I were purchased from Complement Technology (Tyler Texas, USA). Recombinant fungal protein Pra1 was expressed from Luo, *et al*., 2011. Recombinant Factor H fragment 18-20 was expressed by Nadine Flach (Department of Infection Biology, HKI, Jena, Germany). Recombinant staphylococcal protein Ssl11 was kindly provided by Anika Westphal (Department of Infection Biology, HKI, Jena, Germany) and recombinant streptococcal protein Pspc was kindly provided by Christian Meinel (Department of Infection Biology, HKI, Jena, Germany).

2.1.5. Antibodies

Primary antibodies used in immunodetection methods were either purchased or provided by colleagues as follows: goat anti-Factor H, goat anti-C3 (Complement Technology),

mouse anti-iC3b (Prof. Thurmann, USA), mouse anti-CD4, mouse anti-CD45RA, mouse anti-CCR7, and alexa 488 conjugated mouse anti-human FOXP3, mouse anti-CD11b (Biolegend, Koblenz, Germany). Corresponding secondary HRP-conjugated antibodies were obtained from Dako (Hamburg, Germany) and corresponding fluorescent dye-coupled antibodies were purchased from Life Technologies (Darmstadt, Germany).

2.2. Methods

2.2.1 Microbiological methods

2.2.1.1. Strains used

Pichia pastoris

Pichia pastoris X33 was used to produce the recombinant his-tagged C. albicans Pra1 described in Luo et al. (2010).

Escherichia coli

DH5α competent E. coli was used for characterization, propagation, and maintenance of the plasmid construct for knocking out CD11b gene.

Candida albicans

The C. albicans strains used in this study were as follows: wild type strain SC5314 and GFP expressing strain.

2.2.1.2. Cultivation and storage of microbial strains

During the various experiments, C. albicans WT and GFP expressing strain were grown in YPD medium at 30^0C overnight with shaking at 180 rpm. Cultures for experiments were maintained on YPD agar plates at 4^0C and colonies were re-streaked on fresh YPD agar plates every 2 weeks. E. coli DH5α was grown in LB at 37^0C overnight with shaking at 180 rpm in cloning experiment. During Pra1 protein expression, Pichia pastoris X33 were grown in BMGY medium according to the protocol.

2.2.2 Protein Biochemistry and Immunological methods

2.2.2.1. Expression and purification of fungal protein Pra1

Recombinant fungal protein Pra1 was expressed and purified in Pichia pastoris using the procedure of Luo et al. (2010). Protein expression was induced by feeding the yeast cultures with 1% pure methanol every 24h for 3 days. Then, culture supernatants were harvested by centrifugation (3000g, 30min), dissolved in 5x binding buffer (10nM Na_2HPO_4, 10mM NaH_2PO_4, 500mM NaCl, 10mM imidazole, pH 7.4), and the expressed proteins were purified from supernatants by metal-chelate affinity chromatography using HisTrap nickel columns (GE healthcare, Qiagen) and the FPLC Äkta purifier system (GE Healthcare). The colums were then washed with 7 column volumes of binding buffer followed by 10 column volumes of 5% elution buffer (binding buffer with 500mM imidazole). Bound proteins were eluted with 10 column volumes of 100% elution buffer. Eluted fractions containing the recombinant protein were combined and concentrated using 10 kDa spin filters (Millipore). Buffer was exchanged to phosphate-buffered saline (PBS) during concentration. The purity of the proteins were assesd by silver staining (section 2.2.2.3) and the concentration was determined using a spectrophotometer from NanoDrop.

2.2.2.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)

The electrophoresis gels were prepared using 4% polyacrylamide stacking gels (acrylamide/bisacrylamide with 37.5:1, 0.5 M Tris-HCl, 10% SDS, 100 mg/ml ammonium persulfate (APS), 0.1% tetramethylethylenediamine (TEMED), pH 6.8 in distilled water) and 8% or 10% polyacrylamide separating gels (acrylamide/bisacrylamide with 37.5:1, 1.5 M Tris-HCl, 10% SDS, 100 mg/ml APS, 0.1% TEMED, pH 8.8 in distilled water). Sample to be analyzed were mixed with 4x reducing buffer (RotiLoad, Roth) and heated at 95^0C for 5 min. Electrophoresis was done at 150mV and pre-stained protein marker (PageRuler, Fermentas) was used as protein size marker.

2.2.2.3. Silver staining

After electrophoresis process, gels were incubated in fixing buffer (30% ethanol, 30% acetate) for 30 min, washed twice with 20% ethanol, and sensitized in 0.02% sodium thiosulfate for 2 min. The gels were rinsed twice for 1 min in distilled water, then stained

with 0.2% silver nitrate for 20 min, and then another twice rinsing in distilled water. Afterwards, gels were developed using developer solution (0.00007% formaldehyde (37%), 3% sodium carbonate, 0.001% sodium thiosulfate) and the reaction was stopped by rinsing with stop solution (2.5% acetic acid, 50% tris/base).

2.2.2.4. Western Blotting (Immunoblotting)

Migrated proteins in SDS PAGE process were transferred into a nitrocellulose membrane (Protran, GE Healthcare) by using the transfer cassette TransBlot turbo (Bio-rad). In the transfer cassette, gels and the membrane are immersed in the transfer buffer (0.045 M Tris, 0.039 nM glycine, 20% methanol, 0.1% SDS) and kept between 2 x sets of 3 Whatmann papers. The blotting was performed with maximum 12V voltage and 1A current for 15 min. Proteins blotted onto the membrane were detected using the enhanced chemiluminescence (ECL) as follows: the blotted membrane was blocked in blocking buffer (1% bovine serum albumin (BSA), 4% milk powder, 0.1% tween 20) for 1h at room temperature or overnight at 4^0C. The blocked membrane was then incubated with appropriate first and secondary antibodies subsequently for 1h at room temperature, washed with wash buffer (0.05% tween in DPBS) and developed with ECL-substrate solution (Applichem) and blot image was captured using Fusion FX imager (Vilber Lourmat).

2.2.2.5. Enzyme-linked immunosorbent assay (ELISA)

In this study ELISA assay was performed to detect interleukins secreted by human primary monocyte cells and THP-1 monocytic cell line. The assay was done using Ready-set-Go® ELISA kit from eBioscience according to the manufacturer's protocol.

2.2.2.6. Flow cytometry

Flow cytometry assays and analyses were performed using the LSR II flow cytometer (BD) and FACSDiva software. Fungal cells and/or human primary cells or cell lines were identified by forward and side scatters from 10000 cells. Primary (dye-conjugated or unconjugated) and corresponding secondary antibodies were used to stain the cells. Data generated from the assays were evaluated using FlowJo software (Tree Star Inc). Incubation and washing steps for the cells were performed in PBS.

2.2.2.7. Confocal laser scanning microscopy

Recruitment of complement regulators on the fungal cell surface by C. albicans were visualized using confocal laser scanning microscopy using LSM 710 (Zeiss) and ZEN software. Factor H (25µg/ml) was incubated with C. albicans for 30 min at 37^0C in DPBS. Cells were then washed and bound factor H was detected with a polyclonal factor H goat antiserum (1:200) followed by secondary Alexa 647-conjugated rabbit anti-goat IgG (1:400). Fungal cell DNA was stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma). Samples (10µl) were spread on microscope slides and dried at 4^0C. A drop of mount fluor (Roti-Mount FluorCare, Roth) was put on the samples as fixing material before the cover slip.

2.2.3. Functional Assays

2.2.3.1. C. albicans uptake by monocytes via phagocytosis

To elucidate the immediate response of monocyte to C. albicans, co-incubation of human primary monocyte and C. albicans cells were performed, and the uptake of the yeast cells by monocytes were quantified overtime. GFP expressing C. albicans cells ($1x10^5$) were incubated with DiD-dyed (30 min, 37^0C) human primary monocyte ($1x10^6$) at 37^0C for different time points ranging from 0 min – 240 min. At each time points, supernatants of the co-incubation were removed and monocytes were detached with accutase enzyme and washed twice with PBS. The washed monocytes were then measured in flow cytometry using forward and side scatters for size determination, DiD staining detection for monocyte and GFP staining for C. albicans. Double positive staining detection of DiD – GFP was measured as phagocytosis event and the amount of the event was compared to the total DiD detection for phagocytosis rate determination.

2.2.3.2. Monocyte inflammatory cytokine response to C. albicans

To determine the inflammatory response of monocyte to C. albicans, human primary monocytes ($6x10^5$) were co-incubated with C. albicans ($6x10^5$) in RPMI 1640 media supplemented with 2 mM l-glutamine (Lonza), 27.5µg/ml gentamycine (Lonza), and 10% normal human serum for 20 hr at 37^0C. Supernatants were collected and assayed for inflammatory cytokines (IL-1β, IL-6, IL-10, TGF-β) level using ELISA method.

2.2.3.3. Acquisition of factor H from serum by Candida albicans

To determine if C. albicans can acquire factor H from human serum, binding assay in combination with laser scanning microscopy and flow cytometry were performed. Yeast cells (1 x 10^6) were washed twice in phosphate-buffered saline (PBS) and then incubated with 10% normal human serum (NHS) or with 25µg/ml purified factor H for 30min at 37^0C. After incubation cells were then washed twice again with PBS.

For flow cytometry, the factor H bound yeast cells were then incubated with antibody goat anti-factor H (1:200 in PBS) for 30 min at RT, with subsequent corresponding secondary antibody conjugated with Alexa 647 dye (1:200) for 30 min at RT. Then the dyed cells were then diluted in PBS for flow cytometry measurement. Forward and side scatters were measured to identify the cells and fluorescent events were measured from 10,000 cells.

For microscopy measurement, the same process as flow cytometry preparation were done and the dyed cells were spread on microscope slides and dried at 4^0C. Fixing material was added before the cover slip and then samples were visualized with the microscope.

2.2.3.4. Binding of factor H by Pra1/Ssl11/PspC microbeads

To enunciate the immune-modulatory effect of factor H on a foreign surface, 25µg/ml Pra1/Ssl11/PspC microbial proteins were immobilized on the surface of 3µm sulfate microspheres (Polysciences) in PBS (30 min, 37^0C), washed twice, and the beads surface were blocked with 1% BSA in PBS (1 hr, 37^0C). The microbial protein microbeads were then incubated with/without 25µg/ml factor H or 25µg/ml fragmented factor H SCR 18-20 for 30 min at 37^0C. The treated microbeads were then incubated with human primary monocyte in a similar way to C. albicans co-incubation and supernatants were assayed with ELISA to determine the monocytes inflammatory response.

2.2.3.5. Recruited factor H cofactor activity assay

Activity of C. albicans surface bound factor H was analyzed by detection of C3b cleavage product, iC3b, on the surface of C. albicans with western blot and fluorescent microscopy methods. Factor H was incubated with C. albicans in PBS for 30 min at 37^0C, washed twice, and then put on ice for 5 min. Afterwards the cells were diluted in 10% NHS with PBS for another 30 min at 37^0C and then washed twice with PBS.

For western blot, the treated cells were lysed using the 4x reducing buffer (RotiLoad, Roth) and heated at 95^0C for 5 min. The mixtures were then ran on SDS PAGE and continued with the western blot protocol, using the primary antibody mouse anti-iC3b.
For Fluorescent microscopy, the treated cells spread on microscope slides and dried at 4^0C. Fixing material was added before the cover slip and then samples were visualized with the microscope.

2.2.3.6. T cell regulation assay

Supernatants from co-incubation of monocytes and C. albicans or factor H/factor H SCR 18-20 bound C. albicans were diluted (10%) in RPMI supplemented with 2 mM l-glutamine (Lonza), 27.5µg/ml gentamycine (Lonza), and 10% fetal calf serum. The diluted supernatants were incubated with the isolated naïve T cells for 6 – 9 days with a media change every 3 days. After incubation, T cells were fixed and permeabilized using the FOXP3 permeabilization buffer from eBioscience according to the manufacturer's protocol. Cells were then internally stained with anti-FOXP3 transcription factor antibody conjugated with Alexa 488 dye. The stained cells were then quantified by flow cytometry.

2.2.3.7. CR3 knockout THP-1 cells cytokine response to C. albicans

This assay was performed as 2.2.3.2. Monocyte cytokine response to C. albicans, with CR3 knockout THP-1 replacing human primary monocytes.

2.2.4. Molecular Biological Methods

2.2.4.1. THP-1 cells CD11b knockout

The generation of CR3 knockout strain was performed using CRISPR/Cas9 method (Zhang lab). Four 20-base guide RNA sequences (oligos) were designed to target the sites in ITGAM gene each adjacent to protospacer adjacent motif (PAM) sequence that contains the canonical trinucleotide NGG. The guide RNA sequences were designed to be inserted into pSpCas9(BB)-2A-GFP (PX458) in the BbsI insertion site under U6 promoter using the golden gate assembly protocol. The RNA guides were designed to target each from the 5' and 3' of the ITGAM gene as follows:

1. 5’ gRNA (sense): 5’-CACCGCTTATGTCATGGGTTCAACT-3’
   5’ gRNA (antisense):5’-AAACAGTTGAACCCATGACATAAGC-3’
2. 5’ gRNA (antisense): 5’-CACCGCGAAGCCCCTTGCGTTCTCT-3’
   5’ gRNA (sense):5’-AAACAGAGAACGCAAGGGGCTTCGC-3’
3. 3’ gRNA (sense): 5’-CACCGACCGCCGCGCTGTACAAGCT-3’
   3’ gRNA (antisense):5’- AAACAGCTTGTACAGCGCGGCGGTC-3‘
4. 3’ gRNA (asense):5’-CACCGTACTGGGGTTCGGCCCCCGG-3’
   3’ gRNA (sense):5’-AAACCCGGGGGCCGAACCCCAGTAC-3’

The insertion of the gRNA sequences to the vector plasmid (pX458) were performed with series of reactions, starting first with a digestion of vector plasmid using a reaction of 1µg of the vector plasmid, 1 unit BbsI (New England Biolabs, NEB), 2 µl of 10x NEBuffer (New England Biolabs), and ddH_2O until 20µl, incubated for 30 min at 37^0C. The cut vector plasmid were run on agarose gel and purified from the gel using QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer’s protocol. The guide RNA oligo pairs were annealed with a reaction of 100 µM forward guide, 100 µM reverse guide, 1 µl 10x T4 ligase buffer, 1 µl T4 PNK, and ddH_2O until 10 µl total volume; run on PCR machine at 37^0C for 30 min, 95^0C for 5 min, and then ramp down to 25^0C at 5^0C/min. Annealed oligos were then diluted with ddH_2O (1:200) before the ligation reaction with cut vector plasmid. The ligation reaction was performed with 50 ng of the digested vector plasmid, 1 µl of the 1:200 annealed oligos, 5 µl of 2x quick ligation buffer (NEB), 1 unit quick ligase enzyme (NEB), and ddH_2O until 10 µl total volume with incubation at room temperature for 10 min. Ligated plasmid were then transformed into E. coli DH5α by heat shock. Then the transformants were plated on a selective LB agar containing ampicillin and grown for 24 hr at 37^0C. Selected colonies were incubated in LB for another 24 hr at 37^0C and plasmids were isolated, cut with BbsI restriction enzyme, and run on agarose gels to determine the availability of the plasmids and oligo insertion. The uncut plasmids were the oligo inserted vector which then were used to be transfected into THP-1 cells using Amaxa® Human Monocyte Nucleofector® Kit according to Maeß, et al., 2014. After 3 days incubation at 37^0C with 5% CO_2, the transfected cells were stained with monoclonal antibody anti-Cd11b (and corresponding secondary antibody conjugated with Alexa-647) and sorted using cell sorter for FITC positive (from the plasmid vector) and Alexa-647

negative. Sorted cells were cultivated further and checked for CD11b availability using western blot; THP-1 cells were lysed before western blot. The CD11b negative cells were then cryo-frozen for storage and cultivated before usage in further experiments.

2.2.5. Maintenance and cultivation of human cell lines

THP-1 cells were maintained in RPMI 1640 media supplemented with 2 mM l-glutamine (Lonza), 27.5µg/ml gentamycine (Lonza), and 10% fetal calf serum (FCS, Gibco). Cells were incubated at 37^0C with 5% CO_2. The cells were passaged every 3-4 days and after 30 passages the cells were discarded and new cyro-stock cells were thawed for new passage line.

2.2.6. Statistical analysis

Standard deviation and significant differences between experiments data were analyzed by either t-test or analysis of variance (ANOVA) using GraphPad Prism 6 software (GraphPad Software Inc.).

3. Results

3.1. The immediate response of monocytes to *Candida albicans*

3.1.1. Monocytes uptake *Candida albicans* via phagocytosis

To characterize the immediate response of human monocytes to *C. albicans*, uptake/phagocytosis of *C. albicans* by monocytes was analysed. Isolated human monocytes were stained with DiD cell dye and then co-incubated with GFP-expressing *C. albicans* at a ratio of 1:1 in growth medium with and without NHS. After 0 – 2 hrs incubation the uptake rate of the pathogen by monocytes was determined every 30 min (with additional time point in 15 min). Using flow cytometry, the phagocytosis rates were determined by comparing the double positive signals (DiD and GFP) representing internalized *C. albicans* to the total DiD signal representing the total number of monocytes. The monocytes phagocytosed *C. albicans* cells in medium and within 90 min about 30% of monocytes had engulfed the fungal cells. When the same assay was followd in NHS, the phagocytosis rate increased to 50% (**Fig.6**). Following in parallel is the survival rate of *C. albicans* shown by the amount of colony forming unit (CFU) on YPD agar plate (2 days, 30^0C) after 90 min of co-incubation with monocytes. The assay revealed a survival rate of *C. albicans* of about 85% in medium but only 40% in 10% NHS. NHS alone already affected *C. albicans* and reduced *C. albicans* survival rate by 65%. Thus *C. albicans* becomes attacked by active components in the serum and opsonization of the fungal cells enhances phagocytosis by monocytes.

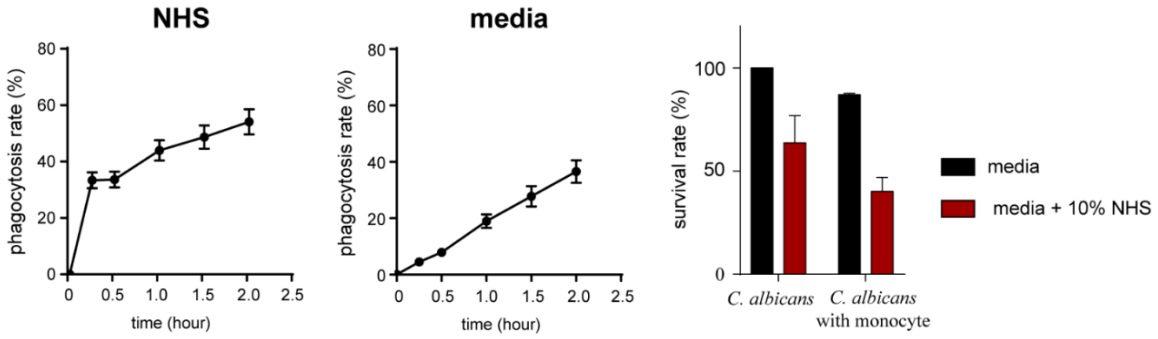


Fig. 6. *Candida albicans* is taken up by monocytes. Phagocytosis of *Candida albicans* by monocyte is enhanced in the presence of NHS as compared to the medium control. After 90 minutes of co-incubation of monocyte and *C.* albicans in 10% NHS, 20% more monocytes have taken up the pathogen as compared to monocytes in medium (without NHS). The survival rate of *C. albicans* was about 85% upon co-incubation with monocytes for 90 min in medium. About 40% of the fungal cells survived after co-incubation in medium with 10% NHS. However, *C. albicans* incubated in active NHS alone is killed by about 35%.

3.1.2. Monocytes release extracellular traps as a response to *C. albicans*

Monocytes immediately phagocytose *C. albicans* cells and also release extracellular DNA traps as seen by real time imaging of monocytes with *C. albicans* (**Fig.7B**). Blood isolated human monocytes were incubated with green fluorescent labelled *C. albicans* in presence of human serum and uptake of *C. albicans* and DNA release was followed in real time (with in-Cell Analyzer) over a time period of 90 - 240 minutes. Monocytes actively uptake *C. albicans* within minutes and continue phagocytosing the pathogen. This uptake was reduced around 70% when the cells were do-incubated in medium alone (**Fig.6**). DNA release in form of traps by monocytes was observed after about 90 minutes of co-incubation. Similar to monocytes, neutrophils released extracellular DNA traps upon 90 minutes of co-incubation with *C. albicans* (**Fig.7A**). Laser scanning microscopy of monocytes with *C. albicans* confirmed DNA trap formation (**Fig.7B**) and further comprehensive characterization of the traps showed the typical signs of extracellular traps [46].

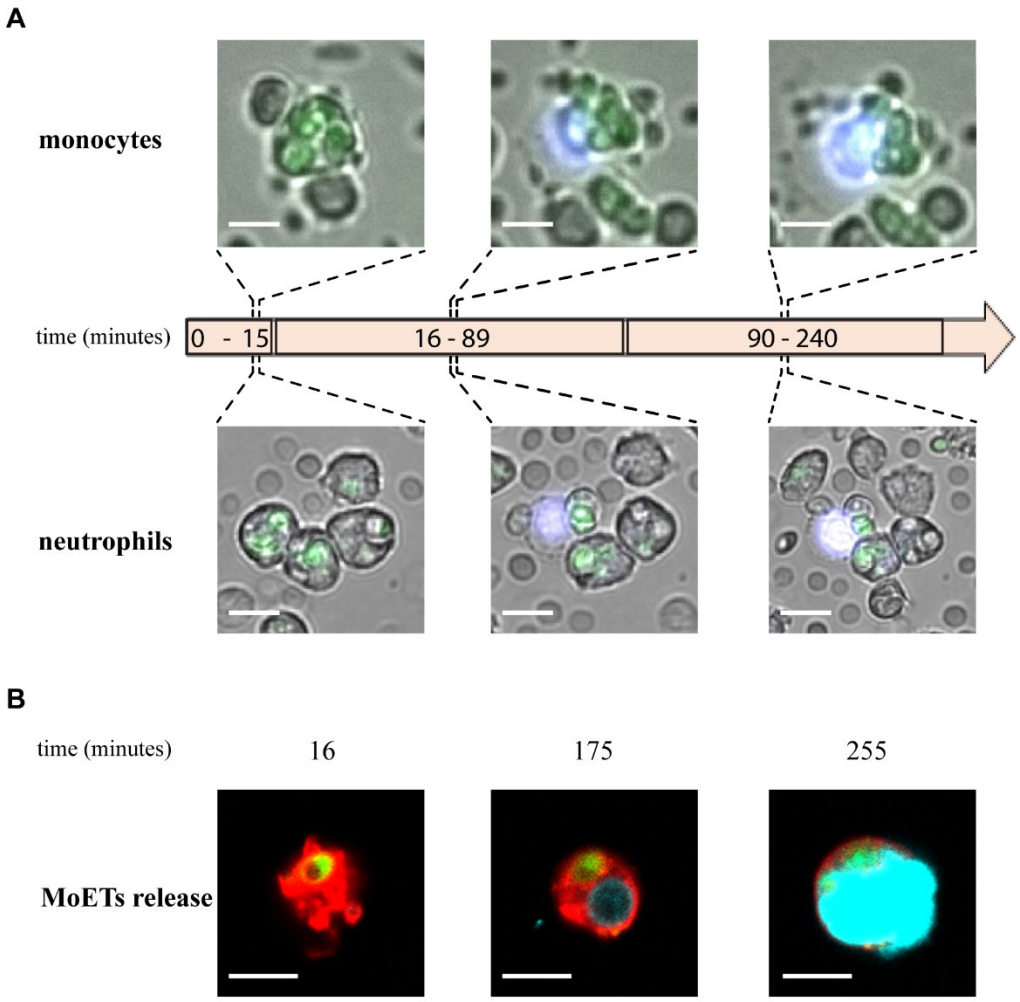


Fig. 7. Monocytes release extracellular DNA traps in response to *C. albicans* similar as neutrophils. (A) Monocytes are shown here to have phagocytosed *C. albicans* in 15 minutes after the start of co-incubation and released their DNA (shown in blue staining – DAPI) starting at minute 90 and last until minute 240. This DNA release is similar to the extracellular DNA traps released by neutrophils as previously reported [88, 89], both from monocytes and neutrophils occuring about 90 minutes after incubation with *C. albicans*. **(B)** Monocytes with internalized *C. albicans* cell decondensed the nuclear DNA after about 16 minutes, continued with DNA decondensation and extracellular DNA was released after 255 minutes. Interaction of monocytes with *C. albicans* was followed using live cell imaging and laser scanning microscopy [46].

3.2. Later response of monocytes to *Candida albicans*

3.2.1. Monocytes secrete inflammatory cytokines in response to *C. albicans*

Having shown the immediate responses of monocytes to *C. albicans*, later responses of monocytes to *C. albicans* were determined by secretion of cytokines. Isolated human monocytes were again incubated with *C. albicans* or human apoptotic cells in NHS supplemented media for 20hr and a set of inflammatory cytokines (IL-1β, IL-6, and IL-10) were evaluated in the supernatants using ELISA. When incubated with *C.* albicans, monocytes generated a significantly higher secretion of IL-1β (3 times higher), IL-6 (10 times higher), and IL-10 (2 times higher) (**Fig.8.A**). Pro-inflammatory cytokines, IL-1β and IL-6, were increased and also the anti-inflammatory cytokine IL-10. In contrast, no significant pro-inflammatory cytokines secretion by monocytes was detected in response to human apoptotic cells while the secretion of the anti-inflammatory cytokine IL-10 also increased (**Fig.8.B**). Thus human monocytes detect *C. albicans* as expected as foreign microorganism, while human apoptotic cells were recognized as 'self'.

3.2.2. *Candida albicans* recruits complement regulator factor H onto its surface

Previous work has shown [70] that *C. albicans* expresses a protein called pH regulated protein 1 / Pra1 that binds to the surface of *C. albicans* and recruits the human complement regulator factor H. To confirm that complement regulator factor H is recruited to the surface of *C. albicans*, purified factor H was bound to *C. albicans* and detected using flow cytometry with polyclonal anti factor-H and antiserum and corresponding fluorescent dye conjugated secondary antibody (**Fig.9.A**). Binding of factor H to *C. albicans* was also evaluated by fluorescent microscopy (**Fig 9.B**). Factor H was bound on the surface of both *C. albicans* yeast form and hyphae form. To show that factor H from normal human serum also bound to *C. albicans*, the fungus was incubated in NHS and binding of factor H was determined as before. *C. albicans* also bound factor H from human serum to the surface (**Fig 9.B**). To establish factor H binding to *C. albicans* in vivo, liver tissue sections from *C. albicans* infected mice were stained for the presence of *C. albicans* using monoclonal antibody to Pra1 together with anti- mouse factor H to show the localization of the binding (**Fig 9.C**). *C. albicans* was detected in the tissue by anti Pra1 staining and factor H was shown to be co-localized with Pra1 (**Fig 9.C**). Having shown that Pra1 and factor H co-

localize in vivo, the binding affinity of Pra1 to factor H was further evaluated using bio-layer interferometry (BLItz) technique. Pra1 was immobilized as a layer on the chip surface and factor H binding affinity to Pra1 (KD = 1.87nM) was determined.

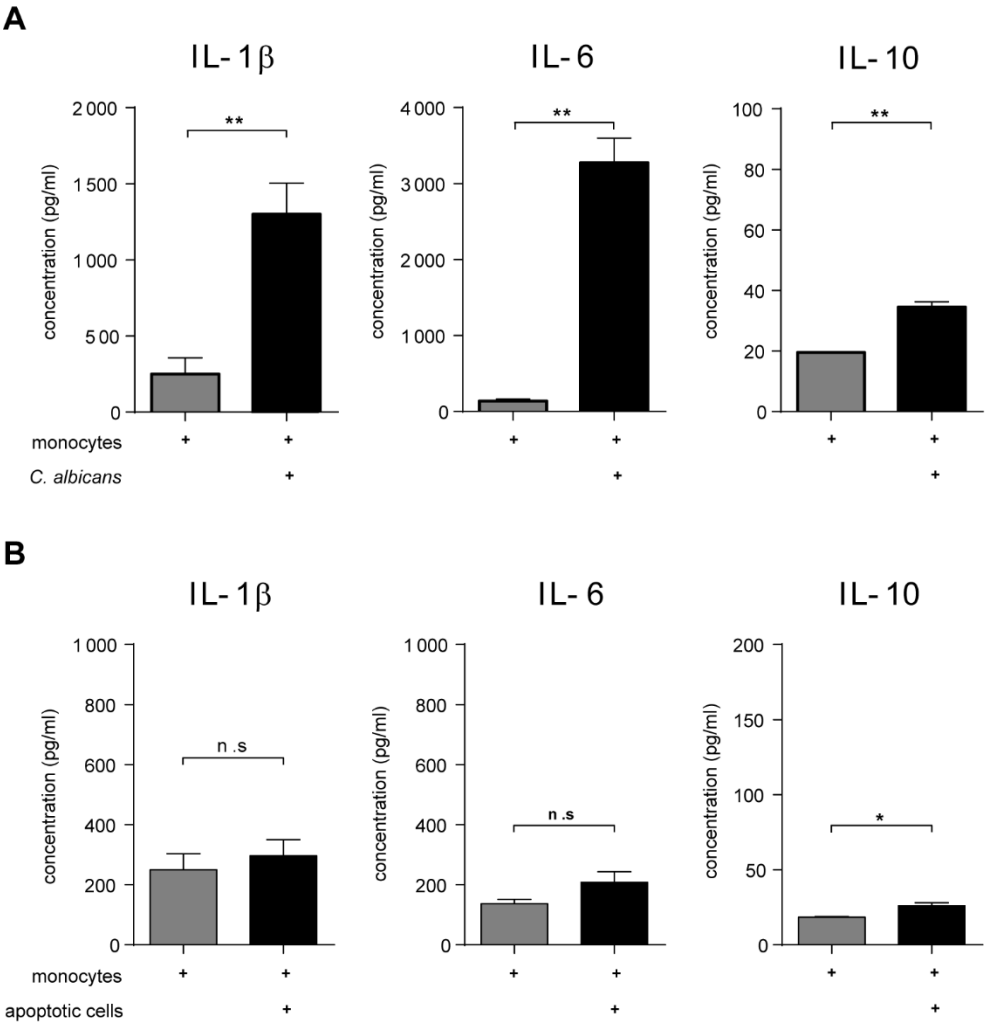


Fig. 8. Monocytes secrete inflammatory cytokines in response to *C. albicans*.
(**A**) Monocytes co-incubated with *C.* albicans for 20 hours release pro-inflammatory cytokines (IL-1β and IL-6) and anti-inflammatory cytokine IL-10 (*p* value of 0.0089 and 0.0092 respectively) (**B**) In contrast to *C. albicans*, human monocytes do not secrete inflammatory cytokines IL-1β and IL-6 when incubated with human apoptotic cells (apoptotic HUVEC cells). The level of anti-inflammatory IL-10 secretion is increasing in both cases (*p* value of 0.0098 and 0.0257 respectively).

3.2.3. Surface bound factor H modulates the cytokine response by monocytes

As a human complement regulator acting on the surface of self-cells, factor H has a regulatory function by increasing the rate of C3 convertase decay that subsequently reduce the amount of C3b opsonization on the surface of the cells. Factor H also has cofactor activity for factor I in cleaving the bound C3b into the inactive form iC3b on the surface of cells.

Recently, an immunomodulatory role of factor H was observed on macrophages [90]. Whether factor H on the surface of *C. albicans* mediate these immune-modulatory response has not been investigated so far. To investigate the modulatory effect of surface recruited factor H on human monocytes, *C. albicans* were incubated with purified factor H and then co-incubated with isolated primary human monocytes for 20hr in NHS supplemented media and the supernatants were analyzed for inflammatory cytokines secretion with ELISA. A fragment of factor H (scr 18-20) which harbors the *C albicans* binding region and C3b binding region was used to simulate binding to *C. albicans* without the regulatory region of factor H. Pro-inflammatory cytokines IL-1β and IL-6 were secreted significantly lower amounts when factor H bound *C. albicans* was co-incubated with monocytes as compared to *C. albicans* with the factor H fragment. In contrast anti-inflammatory cytokine IL-10 was secreted significantly higher (**Fig 10.A.)**, showing that factor H on the surface has a modulatory effect on inflammatory cytokines secretion by monocytes. Factor H binding pushed the immune reaction more into an anti-inflammatory reaction. The same modulatory effects were observed in primary human macrophages upon co-incubation with factor H bound *C. albicans*. The pro-inflammatory cytokines (IL-1β and IL-6) were inhibited and the cytokine IL-10 level increased (**Fig 10.B.**). To investigate whether factor H mediates these immune response also via the original binding site on apoptotic cells, apoptotic HUVEC cells were incubated with factor H, washed and co-incubated with monocytes. Although cytokine production of monocytes to apoptotic self-cells is low (about 80-100 pg/ml IL-1β and about 200-400 pg/ml IL-6) enhanced factor H binding reduced the amount even further (IL-1β by 65% and IL-6 by 50%). IL-10 secretion increased about 60% from 80 pg/ml to about 130 pg/ml. Thus factor H bound to *C. albicans* or to apoptotic cells mediates an immune-modulatory effect (**Fig 10.C.**), indicating that *C. albicans* is recruiting this particular complement regulator inhibit inflammation.

3.2.4. Normal human serum is required for factor H immunomodulatory function

Having shown that surface bound factor H has an immunomodulatory effect in the presence of NHS. We aimed to determine whether NHS is mandatory for the observed factor H modulatory effect. Therefore, recombinant Pra1 was attached onto sulphate microbeads. Loaded microbeads were incubated with monocytes for 20 hr in media with or without NHS. A significant inhibition of pro-inflammatory cytokines IL-1β and Il-6 and significant induction of anti-inflammatory cytokine IL-10 were observed in co-incubation with NHS while no significant changes in cytokine secretion was found in media without NHS (**Fig 11.A.**). Also factor H scr 18-20 had no effect on the cytokine response. These results show that NHS is required for immune modulatory functions of factor H. The finding is in agreement with inhibitory functions observed with factor H bound to *C. albicans* and incubated with human monocytes with or without NHS (**Fig 11.B.**).

3.2.5. Surface bound factor H retains its cofactor activity on *C. albicans*

Factor H is a cofactor of factor I in cleaving the bound opsonin C3b into the inactive form iC3b on the surface of cells. To determine whether this cofactor activity is still retained when factor H is bound to the *C. albicans* surface, *C. albicans* was incubated in active NHS and C3b cleavage to iC3b on the surface was detected using monoclonal antibody anti C3d29 that binds to a region in iC3b but not C3b. Generation of iC3b was evaluated using fluorescent microscopy and western blot. Inactive form of C3b, iC3b, was observed on the surface *C. albicans* after co-incubation with NHS as shown by the fluorescent signals on *C. albicans* by microscopy (**Fig 12.A**). The cleaving of C3b into iC3b was increased over time as shown by increased iC3b bands over time in western blot analysis (**Fig 12.B**). These findings show that factor H retains its cofactor activity for factor I in cleaving C3b into iC3b on the surface of *C. albicans*.

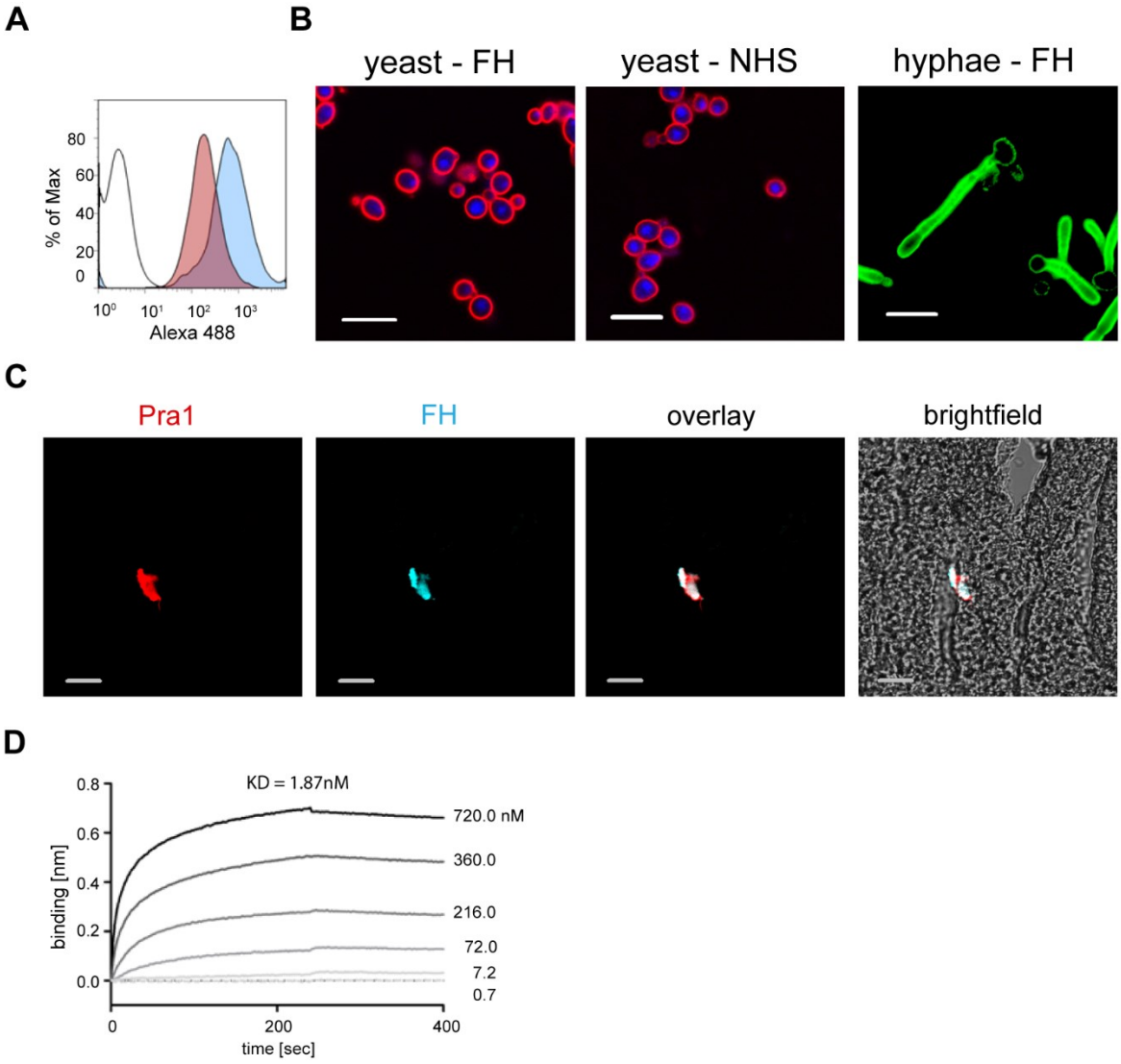


Fig. 9. *C. albicans* recruits factor H to the surface
(A) purified factor H (red) as well as factor H in NHS (blue) bind to *C. albicans* (white). A representative FACS experiment is shown (**B)** Factor H binds to the surface of *C. albicans* cells and hyphae. *C. albicans* yeast and hyphae were incubated for 30 min with human purified factor H or 1in 0% normal human serum and stained with monoclonal antibody anti-factor H combined with red Alexa 647 dye conjugated secondary antibody (in collaboration with Luke Halder). (**C**) Factor H is attached to *C. albicans* in mouse tissue. Murine liver tissue from *C. albicans* infected mice were stained with monoclonal antibody to Pra1 detecting *C. albicans* together with mouse-factor H antibodies. (**D**) The affinity constant (KD) of factor H – Pra1 interaction is 1.87nM, as measured by BLItz analysis.

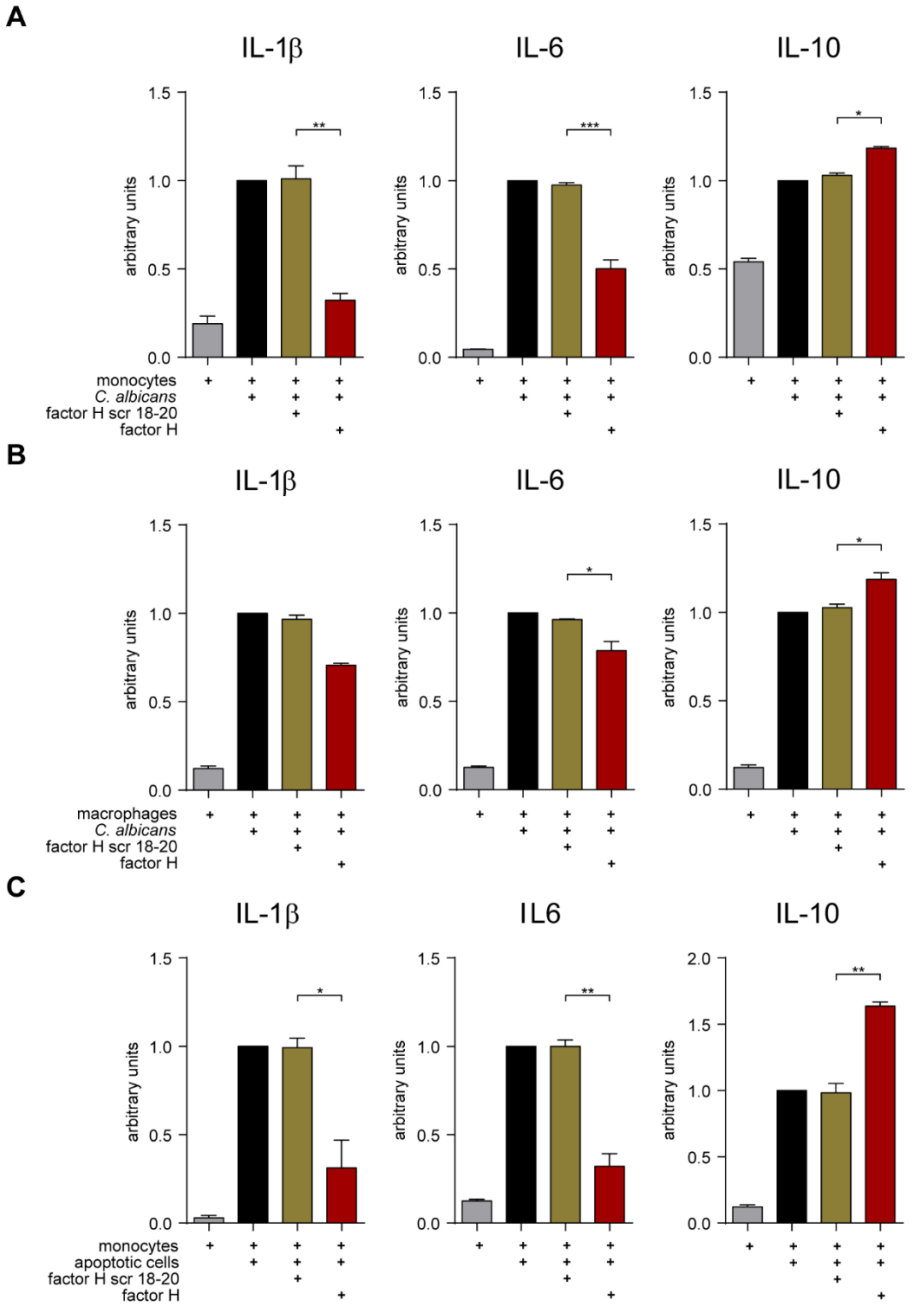


Fig. 10. Surface bound factor H on *C. albicans* modulates the inflammatory cytokine secretion. Monocytes were co-incubated with factor H covered *C. albicans* in medium containing normal human serum (NHS). **(A)** Pro-inflammatory IL-1β and IL-6 secretion is reduced when full length factor H was bound to *C. albicans* compared to the factor H fragment (scr 18-20). IL-1β secretion is reduced by more than 60% (p= 0.0012; arbitrary unit 1 = 1250pg/ml) while IL-6 secretion is reduced by 50% (p=0.008; arbitrary unit 1 = 3000pg/ml). In contrast, anti-inflammatory IL-10 is weakly increased by about 20% (p=0.013; arbitrary unit 1 = 50 pg/ml). **(B)** Similar secretion profile was observed when monocyte derived macrophages were co-incubated with factor H treated *C. albicans*. IL-1β secretion was reduced by about 30% (p=0.0077; arbitrary unit 1 = 2000pg/ml), IL-6 secretion about 20% (p=0.0748; arbitrary unit 1 = 4000pg/ml) and IL-10 was induced by about 18% (p=0.0076; arbitrary unit 1 = 60pg/ml). **(C)** Using factor H covered human apoptotic cells instead of *C. albicans* showed similar effects on IL-1β (p=0.0147; arbitrary unit 1 = 100pg/ml), IL-6 (p=0.0011; arbitrary unit 1 = 300pg/ml), and IL-10 (p=0.0010; arbitrary unit 1 = 130pg/ml) secretion, however, the cytokines level by apoptotic cells is very low.

3.2.6. iC3b modulates the LPS-induced inflammatory response in monocytes

iC3b is generated on the surface of *C. albicans* as a result of C3b cleavage by co-factor activity of factor H for factor I [91]. To investigate whether iC3b has an immune modulatory effect on monocytes, C3b was freshly bound to a cell culture plate by cleaving soluble C3 with a pre-formed C3 convertase (incubation of C3b, factor B, factor P, and factor D in Mg EGTA buffer). Newly formed and bound C3b was then incubated with factor H and factor I to cleave C3b into iC3b. This freshly generated iC3b was subsequently incubated with LPS-induced monocytes and the secreted cytokines in the supernatant were analyzed **(Fig 12.C.)**. Interestingly, iC3b inhibits the secretion of IL-1β but not IL-6, while surface bound C3b did not affect the secretion of pro-inflammatory cytokines IL-1β or IL-6. In addition iC3b increased the secretion of IL-10 and TGF-β. C3b did not affect IL-10 level but seemingly had an effect on TGF-β. In total iC3b reduces about 40% of IL-1β secretion, while increases about 55% of IL-10, and increases 32% of TGF-β secretion.

3.2.7. Supernatants of monocytes co-incubated with *C. albicans* enhances Treg cell differentiation

Having shown that factor H on the *C. albicans* surface is immune modulatory and inhibits inflammatory cytokine responses in monocytes, further regulatory functions were investigated. Having seen that also TGF-β response increased in monocytes, the supernatants of *C. albicans* incubated monocytes were assayed for the effect on T cell differentiation. The supernatant of co-incubated monocytes with factor H treated *C. albicans* were centrifuged to remove any cells and subsequently incubated with isolated naive $CD4^+$ T cells for 5 – 7 days. Differentiation of T cells was followed by staining for intracellular FOXP3 transcription factors as regulatory T cells marker using flow cytometry. Incubation with supernatant from monocytes with *C. albicans* co-incubation resulted in higher number of FOXP3 positive T cells as with supernatants from monocytes alone. Pre-incubation of *C. albicans* with factor H prior to interation with monocytes resulted in two times more FOXP3 positive cells, but not when *C. albicans* was incubated with the factor H fragment 18-20 (**Fig. 13.A**). These data (**Fig 13.B.**) demonstrate that factor H recruited to the surface of *C. albicans* modulates the monocytes in cytokine secretion so that more naïve $CD4^+$ T cells differentiate into a regulatory type.

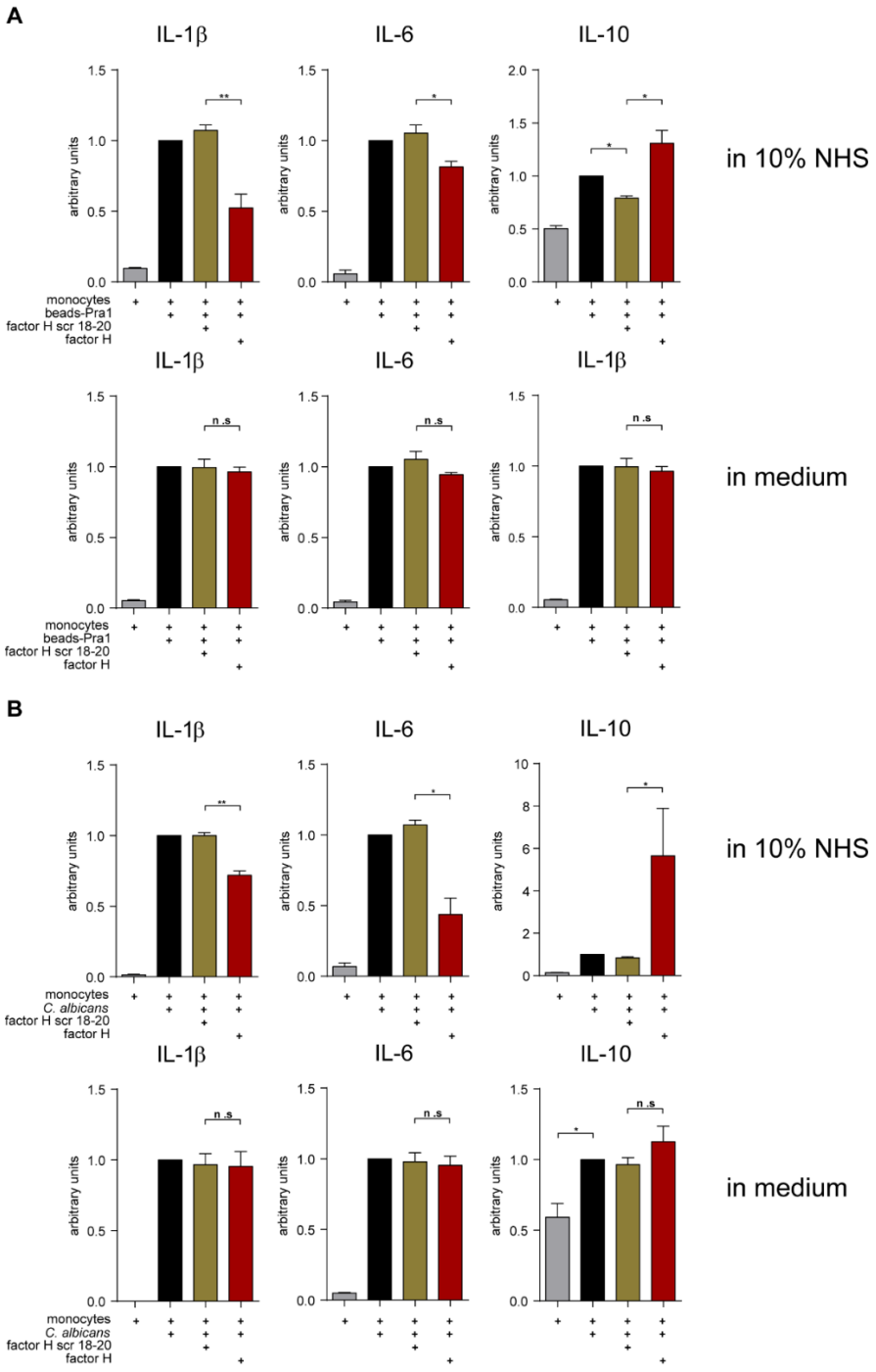


Fig. 11. Factor H attached to *C. albicans* Pra1 covered microbeads modulates the cytokine response of monocytes. Recombinant Pra1 protein was bound onto microbeads and factor H or fragmented factor H SCR 18-20 were bound to Pra1 and incubated with monocytes for 20 hours. **(A, top)** Monocytes pro-inflammatory cytokine (IL-1β and IL-6) secretion was inhibited by bound factor H (p value of 0.006 and 0.0266 respectively) and IL-10 secretion increased (p=0.0132). Factor H fragment 18-20 had no effect. **(A, bottom)** No significant modulation of secreted cytokines occurred when the reaction was in medium without normal human serum (NHS). **(B, top)** Similar cytokine regulation by factor H was found with monocytes co-incubated with factor H treated *C. albicans* in decreased IL-1β and IL-6 secretion (p= 0.0055 and p= 0.0303 respectively) and IL-10 level increased (p= 0.0355). Again the regulatory effect by bound factor H was lost in medium **(B, bottom)**.

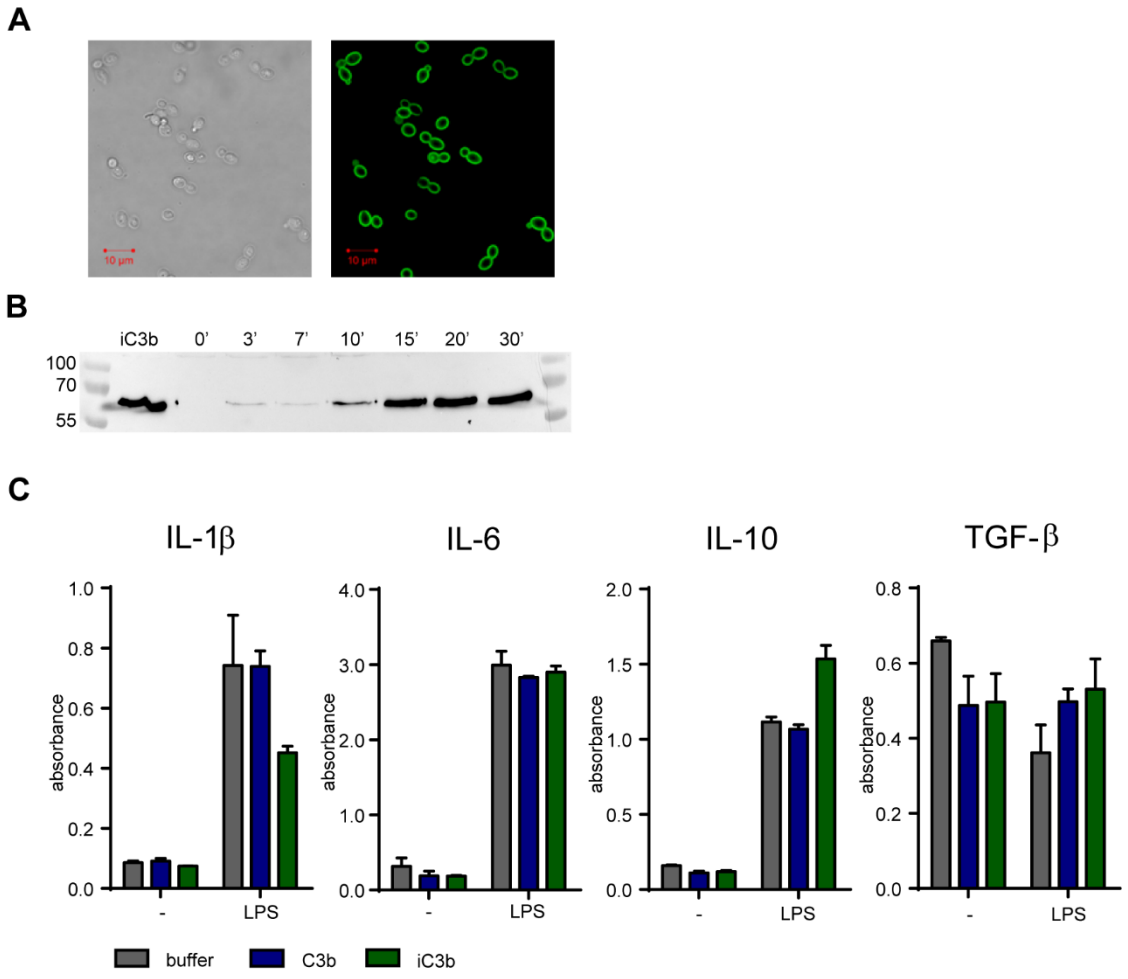


Fig. 12. C3 cleavage product iC3b is generated on the surface of *C. albicans*.
(**A**) Factor H recruited by *C. albicans* retains its cofactor activity for factor I and C3b is cleaved into iC3b in a time dependent manner. Cleavage product iC3b is detected on the surface of *C. albicans* after 20 minutes incubation in 10% normal human serum by laser scanning microscopy using monoclonal antibody anti-iC3b (C3d29,green). (**B**) *C. albicans* bound factor H with factor I generate iC3b rom C3b as detected by western blot analysis (first lane: iC3b band about 61KD). iC3b generation was followed over 30 minutes. (**C**) Surface bound iC3b inhibits the secretion of IL-1β but not IL-6. iC3b also weakly induces IL-10. Surface bound C3b did not modulate the immune response. Monocytes were induced with 2ng/ml LPS in co-incubation with actively generated C3b or iC3b on a cell culture plate.

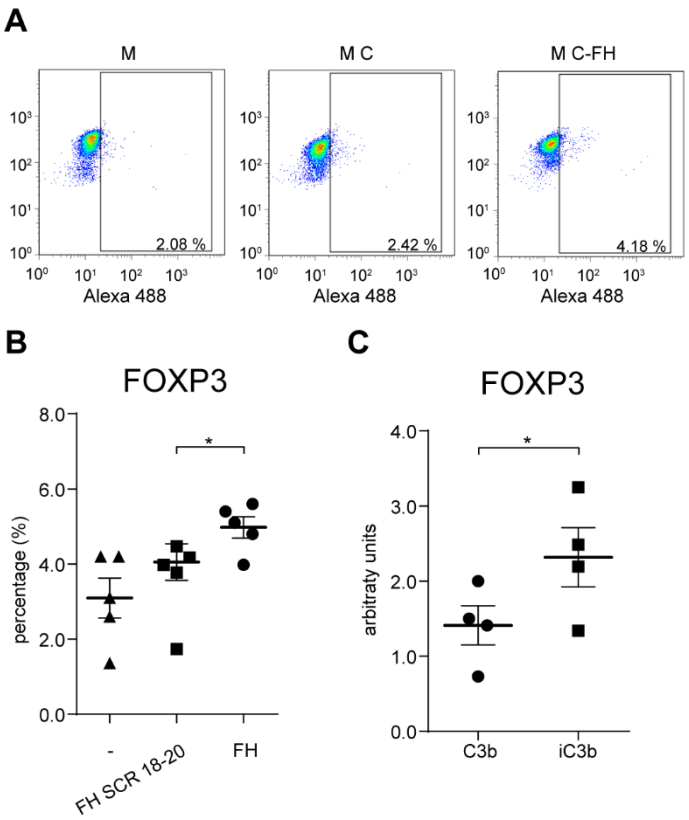


Fig. 13. FOXP3 transcription factor is detected in naïve T-cell after 6 days incubation in supernatant derived from monocytes co-incubated with factor H treated *C. albicans*.
(A) FOXP3 transcription factor expression is induced in naïve T cells when incubated in supernatants from monocytes – *C. albicans* co-incubation. FOXP3 is detected using monoclonal antibody anti-FOXP3 conjugated with alexa 488 dye. Gated naïve T cells with intracellular FOXP3 (1.38%) after 5-7 days are shown, M: monocyte supernatant, MC: supernatant of monocytes co-incubated with *C. albicans*, and MC-FH: monocytes with factor H on *C. albicans*. **(B)** FOXP3 concentration in T cells is increased when cells were incubated in supernatant of monocytes incubated with factor H-treated *C. albicans* (p=0.0237) compared to monocytes with *C. albicans* covered with factor H fragment scr 18-20. **(C)** Higher numbers of FOXP3 positive T cells were found after incubation in the supernatant of iC3b stimulated monocytes incubated with LPS compared to C3b stimulation (p=0.0256).

3.2.8. CD11b knockout in THP-1 monocytic cells using the CRISPR-Cas9 method

C3b, iC3b, and factor H are described to bind to CR3 on neutrophils/macrophages [92, 57, 93] To investigate whether CR3, also called CD11b (integrin αM) and CD18 (integrin β2), is the responsible receptor for factor H modulation of monocytes, a CD11b knockout was performed in monocytes. For this purpose, the monocytic cell line THP-1 cell was used and the ITGAM gene coding for the CD11b chain was disrupted using CRISPR-Cas9 method. A vector plasmid harboring a set of promoter-genes (U6 promoter and cas9) of

Cas9 and the corresponding guide RNAs (gRNAs) for the ITGAM gene were created. Four different guide RNAs were created to ensure disruption of the ITGAM gene. THP-1 cells were transfected via nucleofector method with the gRNA containing vector plasmids. Transfected THP-1 cells were expected to synthesize the Cas9 protein. Together with the transfected gRNAs that lead the Cas9 protein to the ITGAM gene and Cas9 will cut the 5' prime and 3' prime regions of the ITGAM gene. The successful cut will create a point mutation, effectively disrupting the transcription of the gene.

The gRNA sequences were designed using the web application from CRISPR design tool MIT (http://crispr.mit.edu:8079/) by supplying the web tool with human CD11b coding sequences acquired from the NCBI database. The web tool automatically detected the location of multiple PAM (protospacer adjacent motif) sequences and analyzed 20 nucleotides upstream to determine the best possible position for the gRNA. The guide protein Cas9 should cut the section and eliminate off target sites outside the intended gene. The gRNAs were then scored and 4 of the highest scored 5' prime and 3' prime gRNAs, including top strand and bottom strand targets, were selected **(Fig 14.B.)**. These selected gRNAs were generated as oligonucleotides and inserted into the vector plasmid.

The insertion of the gRNAs into the PX458 vector plasmid **(Fig 14.A.)** downstream of the U6 promoter upstream the gRNA scaffold sequence were evaluated by digesting the plasmid with restriction enzyme BbsI. Successfully inserted vector plasmids were identified by cleavage with BbsI since the restriction sequence was disrupted after the insertion of the gRNA oligonucleotides **(Fig 14.C.)**. One representative plasmid from each successful insertion (4 gRNAs → 4 inserted plasmids) were chosen to transfect THP-1 cells using the nucleofection method. The THP-1 cells were transfected with each of the four vector plasmids in 4 different transfection cuvettes according to the protocol previously described by [94]. Transfected cells were grown for 7 days in fresh medium with exchange every third day. To separate transfected from untransfected cells, cells were sorted via anti-CD11b and corresponding secondary antibody conjugated with Alexa 647 dye using the cell sorter. To ensure the availability of the vector plasmid and the absence of CD11b, cells were sorted according to GFP positive and Alexa 647 negative. The sorted cells were grown for another 7 days in medium and presence of CD11b was evaluated using western blot analysis **(Fig 14.D.)** as well as flow cytometry **(Fig 14.E.)**. Clone 4 showed no CD11b

expression in western blot and in flow cytometry. The CD11b knockout THP-1 cells were cryo-frozen to be used for the following experiments.

3.2.9. Factor H immunomodulatory signaling is mediated via CR3

Complement receptor 3 (CR3) binds factor H, C3b, and iC3b [49, 57]. Having shown that surface bound factor H in normal human serum modulates human primary monocytes. This study aimed at investigating whether factor H modulates cytokines secretion via CR3, WT THP-1 cells and CR3KO THP-1 cells generated in this study were used as representatives of human monocytes and co-incubated with factor H bound *C. albicans*. No difference in IL-1β and IL-10 secretion by CR3KO THP-1 cells were observed when incubated with factor H treated *C. albicans* and factor H fragment scr 18-20 covered *C. albicans*. In contrast, WT THP-1 cells secreted less IL-1β and more IL-10 when co-incubated with factor H bound *C. albicans* **(Fig. 15)**. This was similar to human primary monocytes. These results show factor H modulates IL-1β and IL-10 secretion via CR3. In contrast, secretion of TGF-β remained upregulated indicating that factor H does not regulate TGF-β via CR3, but a different pathway.

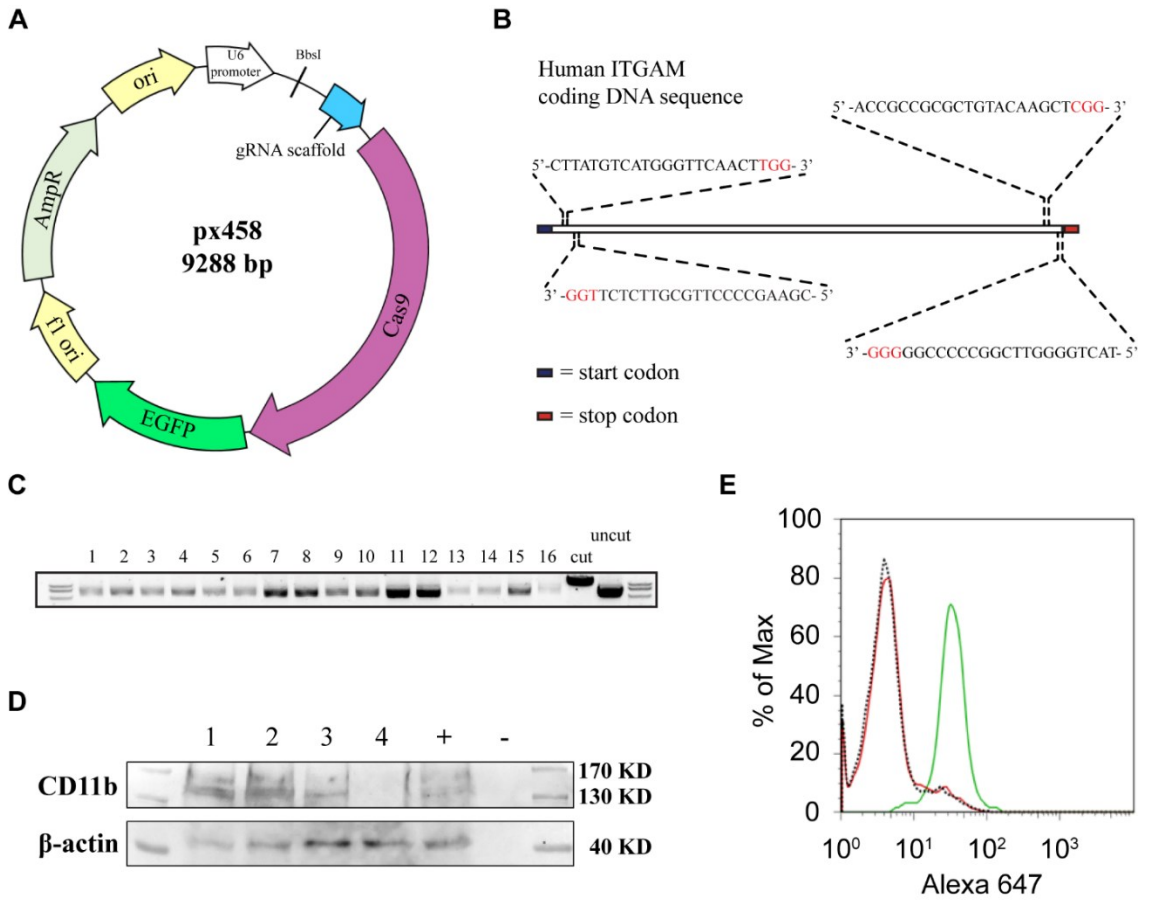


Fig. 14. CD11b knockout in THP-1 monocytic cell line via the CRISPR-Cas9 method. The CRISPR-Cas9 technique was used to knockout the transcription from the gene encoding CD11b in THP-1 cells. A vector plasmid **(A)** containing the gRNA oligo insertion site with downstream scaffold, Cas9 and EGFP genes under control of the U6 promoter were used. **(B)** The selected gRNAs and their recognition sites in the human ITGAM CDS (coding DNA sequence) with the PAM sequence is highlighted in red. **(C)** The evaluation of gRNA oligonucleotides insertions at the BbsI restriction site in the px458 vector plasmid was used, showing that all 16 clones show inserts in their BbsI sites. The plasmids lost their BbsI restriction sites as compared to the control vector px458. **(D)** Clone 4 showed loss of CD11b expression while still retaining the β-actin (housekeeping gene). CD11b expression of transfected THP-1 were analysed using western blot analysis. The numbering (1-4) represent the transfected clone number and '+' represent WT THP-1 cells and '-' represent empty lane as negative control. **(E)** Clone 4 showed no CD11b detection (red curve) compared to the WT THP-1 cell (green curve). Expression of CD11b by clone 4 was followed using flow cytometry using monoclonal CD11b antibody and corresponding secondary antibody conjugated with alexa 647 dye.

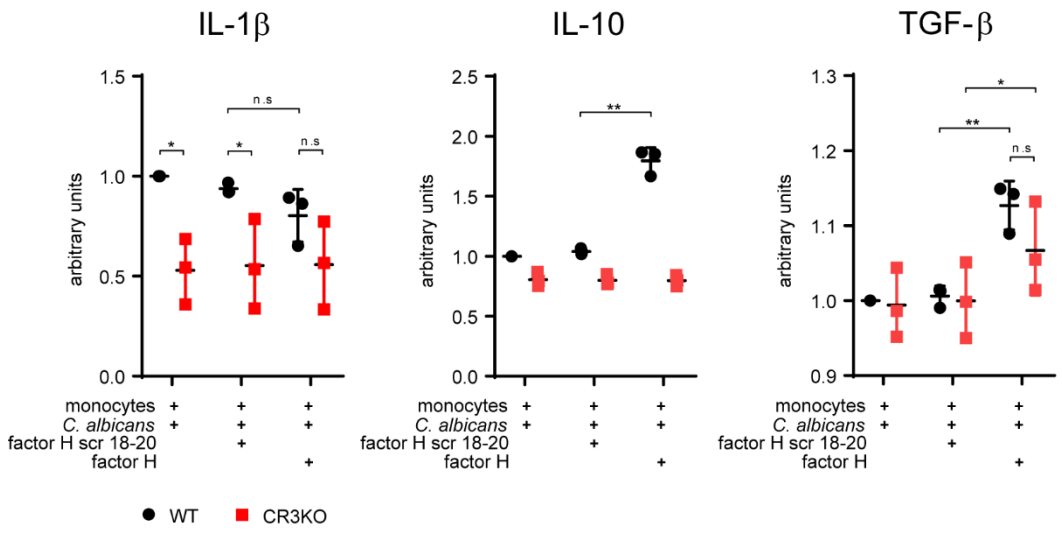


Fig. 15. Factor H immune-modulatory functions are mediated by CR3.
IL-β but not IL-10 or TGF-β secretion is substantially reduced in CR3KO THP-1 cells as compared to WT THP-1 cells in response to *C. albicans*. Factor H attached to *C. albicans* reduced IL-1β (p value of 0.03) but not IL-10 synthesis in CR3KO THP-1 cells, which is increased (p value of 0.006). TGF-β regulation by factor H was unaffected by the CR3 knockout. The increase of TGF-β secretion on WT and CR3KO THP-1 cells by factor H on *C. albicans* were both significant (p value of 0.008 and 0.01 respectively. THP-1 and THP-1 CR3KO cells were incubated with *C. albicans* or factor H / factor H SCR 18-20 covered *C. albicans* and cytokine levels were measured by ELISA. Data represent 3 different experiments. 1 arbitrary unit in IL-1β is a range of 200–350 pg/ml, in IL-10 graph: 50-60 pg/ml, in TGF-β graph: 1200-1500 pg/ml.

4. Discussion

The human body is constantly exposed to microorganisms outside in the environment and also inside the human body. The microorganisms inside the human body that contribute to the human health are considered as microflora and coexist with the host, whereas microorganisms that enter the body and cause infections are considered as pathogens. In cases when the human host immune system is compromised like in HIV infected persons, in transplanted patients or in patients with diabetes, an opportunistic fungal pathogen such as *Candida albicans* can disseminate in the human body and cause damage to the tissues leading to impaired function of the human body or even cause death. Since this pathogen is also a normal microflora in human body, it is considered as a constant potential threat that can attack whenever the human immune system is compromised.

Currently *Candida albicans* in one of the most prevalent cause in fungus infections in humans with a high mortality and morbidity rate. It is an opportunistic fungal pathogen possesses multiple virulence factors and also developed mechanisms to attack and evade human immune system. It has been known that this fungus evade human innate immune response provided by the complement system, although the exact mechanism is not yet well described. One of the known mechanism is the recruitment of human complement regulator on *Candida albicans* surface is helping the fungus to control the complement activation. This study shows that the fungus utilizes the human immune regulator to mask itself from the recognition and also secretes protein that bind and degrade immune effector proteins. Although this fungus is suppressed by the immune system and the other microflora in the body at healthy individuals, a drop in immune system fitness and defects in the immune regulatory system are windows for this fungus to flourish and attack the human host.

The aim of this study is to describe the role of the recruited human complement regulator factor H on the surface of *Candida albicans* to the immune reponse of monocytes.

4.1. The Immediate response of monocytes to *C.albicans*

During the earlier time point of monocytes interaction with *C. albicans* in the complement active environment, phagocytosis events were prevalent and monocytes engulfed *Candida*

albicans which was supported by the complement system. Human monocytes are professional phagocytes that employ receptors on the cell surface to recognize foreign materials and microorganisms that infected human body [95]. The receptors on monocytes such as TLR2, TLR4, dectin 1, and CR3 recognize and interact with β-glucans and mannans on the surface of *Candida albicans* which enhances interaction and attachment of the immune cell to the invading microorganisms, thus increasing phagocytosis. On the other hand, IgG and C3 fragments from complement system (C3b and iC3b) play central roles in the phagocytic activities of monocytes and macrophages [96, 97]. All of the complement pathways are induced by *Candida albicans*. IgG with its Fc portion directly stimulates particle attachment and ingestion/phagocytosis. Moreover, this process is enhanced by opsonization of C3b on the surface and its inactivated form iC3b and the attachment mediated by complement receptors CR1 (CD35) and CR3 (CD11b/CD18).

Candida albicans as fungal pathogen is immediately opsonized with C3 fragments when in contact with human blood, covering its surface with C3b. The complement cascade would be continued on the surface where C3 convertase will be formed from the bound C3b with Factor B (C3bBbC3b) which subsequently triggers more deposits of C3b on the surface of the fungus. This study demonstrates that in the environment with active complement system (10% normal human serum), monocytes uptake *Candida albicans* faster due to the fact of the enhanced attachment and recognition by the surface bound opsonin. The complement activation does not only deposits opsonins on the surface of the fungal pathogen, but also provided antifungal effect as incubation of *C. albicans* in 10% normal human serum reduced the survival of *C. albicans* while co-incubation with monocytes further reduced the survival of *C. albicans*. Similar observation has previously reported on neutrophils killing towards *C. albicans* in the presence of human serum or serum-opsonized *C. albicans* [98, 99]. The uptake of the fungi by neutrophils was greatly enhanced by the presence of human serum in the media or by pre-opsonizing the fungal cell before co-incubation.

Here we observed also a burst release of nucleic acid from the monocytes towards *C. albicans*. Upon contact with *C. albicans* (<240 min) following the phagocytosis response, monocytes decondense their nuclear DNA and release the DNA which form monocyte extracellular traps (MoETs) that cover and fix *C. albicans* cells. These extracellular traps

share many similarities with neutrophil extracellular traps (NETs). For example MoETs contained citH3, elastase, MPO, and lactoferrin [46] described for NETs [100]. NETs effectively kill *C. albicans* with MPO, elastase, and also the release of lactoferrin was previously described [101] and formation of extracellular traps by monocytes with similar properties was confirmed by Halder, *et al*. 2017. Thus monocytes effectively reduce the survival of the fungus by a combination of phagocytosis and MoETs release.

4.2. Advanced response of monocytes to *Candida albicans*

4.2.1. Monocyte secretes inflammatory cytokines in response to *C. albicans*

On inflammation settings, classical monocytes ($CD14^{hi}CD16^{-}$) are recruited rapidly to the site of inflammation from the bloodstream as phagocytes whereas the intermediate monocytes ($CD14^{hi}CD16^{+}$) and the non-classical monocytes (the $CD14^{low}CD16^{+}$) are the cytokines producing cells that recruit neutrophils and other immune cells to the site. This cytokines secretion is considered a specific response of monocytes since the secretion is a process throughout the interaction between monocytes to *C. albicans*, starting from time point 0 until several days. The cytokines that were detected in this study were interleukin-1β, interleukin-6, and interleukin-10.

Co-incubation of monocytes and *C. albicans* revealed an increase in IL-1β and IL-6 secretion showing spontaneous pro-inflammatory response by monocytes to *C. albicans*. The high secretion of IL-1β (400% increase; 1250 pg/ml) and IL-6 (1550% increase; 3300 pg/ml) by monocytes in response to *C. albicans* was presumably because of the detection of the β-glucans on the surface of the fungus by monocytes and also by the contact and interaction enhancement by the deposited opsonins (C3 fragments) from the activated complement system on the fungus surface. The β-glucans are major components of fungal cell walls that trigger IL-1β secretion. Complement receptor 3 (CR3) and Dectin-1 play a crucial role in coordinating β-glucan-induced IL-1β processing as well as death response [102], in addition to the canonical NLRP3 inflammasome and caspase-1 maturation of IL-1β. IL-6 was secreted in very high quantity in the co-incubation of monocytes with *C. albicans* showing a high pro-inflammatory reaction of monocytes to *C. albicans*. This high secretion of IL-6 is acting like a chemokine attracting more leukocyte especially

neutrophils and monocytes, increasing the inflammatory reaction on site also inducing the production of M-CSF receptor on monocytes, skewing the differentiation towards macrophages [103]. Interleukin-1β is a potent inflammatory cytokine that is crucial for host-defense responses to infection and injury [104]. It is an important mediator of the inflammatory response and is involved in cell proliferation, differentiation and apoptosis. IL-1β secretion is also a marker of an activated inflammasome, which catalyzes the proteolytic cleavage of pro IL-1β and pro IL-18 into their mature forms. IL-6 is one of the chemokines that together with IL-1β attract neutrophil to the secretion site. Saturation of IL-6 presence on site can induce a proteolytic processing of IL-6 by receptor of recruited neutrophils, subsequently drives IL-6 *trans-signaling* in the resident tissue cells leading to a switch from neutrophils to monocytes recruitment by suppressing the neutrophil-attracting and enhancing monocyte-attracting chemokines [105].

IL-10 however, is a cytokine with potent anti-inflammatory properties that plays a central role in limiting host immune response to pathogens, thereby preventing damage to the host and maintaining tissue homeostasis. Deficiencies in IL-10 revealed that the majority of intracellular infections are controlled better or cleared faster in the absence of IL-10 [106]. While the absence of IL-10 is often initially beneficial to the host, prolonged IL-10 deficiency can often be detrimental in the long term since the enhanced and prolonged production of inflammatory cytokines can lead to septic shock in cases of infections without the suppressing effect of anti-inflammatory IL-10. Interestingly, IL-10 was upregulated in monocytes when co-incubated with *C. albicans* (90% increase; 38 pg/ml) similar to the IL-10 secretion of monocytes in response to apoptotic cells (50% increase; 30 pg/ml). Presumably, *C. albicans* modulated the IL-10 secretion either by direct interaction with monocytes, or via the complement system similar to as previously reported in macrophages response to apoptotic cells [107].

4.2.2. Surface bound factor H modulates the cytokine response by monocytes

Numerous reports confirm that pathogens recruit human complement regulators on their surfaces to evade a complement attack and essentially evading innate immune responses. *Candida albicans* recruited human complement regulator factor H on its surface as shown in flow cytometry and fluorescent imaging in this study. The binding of factor H was not

only shown *in vitro* but for the first time also *in vivo* in mice. Factor H was recruited onto the surface of *C. albicans*, showing *in vivo* co-localization of fungal surface protein Pra1 and factor H in *C. albicans* infected mice liver tissue. This study shows that surface bound factor H regulated the cytokine secretion of monocytes by inhibiting the secretion of IL-1β and IL-6, and inducing the secretion of IL-10 in a complement active environment. Similar regulatory effects were observed when factor H was bound to apoptotic HUVEC cells. Although the initial response of IL-1β and IL-6 secretion were not as high on monocytes response to apoptotic HUVEC cells compared to *Candida albicans*, the regulation pattern by surface bound factor H is the same. This demonstrates that surface bound factor H on any surfaces is regulating cytokine responses of monocytes. Interestingly, macrophages differentiated from human blood monocytes are also regulated by surface bound factor H the same way. Pra1 was reported to bind factor H and retains its complement regulatory functions [70] but the mechanisms or the function of the recruited regulator to the response of monocytes has not been described yet. The function of factor H in regulating complement activation is mainly degrading the opsonin C3b and blocking further opsonization by accelerating the decay of formed C3 convertase on the surface. The regulatory effect of factor H was only found when factor H was bound onto the surface, not in its soluble form. The additional factor H added to the co-incubation of monocytes with *C. albicans* did not assert any regulatory effect compared to the bound factor H on *C. albicans* surface prior to co-incubation (data not shown).

Microbial pathogens such as *Staphylococcus aureus* and *Streptococcus pneumoniae* are also known to recruit factor H onto their surfaces [108, 109]. Each pathogen has its own surface expressed proteins that bind factor H named staphylococcal superantigen-like protein 11 (SSL11) and PspC, for *Staphylococcus aureus* and *Streptococcus pneumonia* respectively. Here we show that factor H once recruited by these pathogens also modulates the cytokine secretion of monocytes very similar to *C. albicans* (**Fig. 16**). These results show that these pathogens exploit the regulatory functions of factor H on their surfaces, cleaving C3b into iC3b and accelerating the decay of C3 convertase to reduce further opsonization. By reducing the secretion of IL-1β and IL-6, *C. albicans*, *Staphylococcus aureus*, *Streptococcus pneumonia*, and other factor H surface binder pathogens are able to actively impair the inflammatory response of monocytes. The lower level of inflammatory

cytokines creates a favorable condition of lower inflammation reaction and less recruitment of immune cells to attack *C. albicans*. The increased IL-10 secretion also supports *C. albicans* to further dampen the already low inflammatory response and to limit the response of the host immune system to the pathogen.

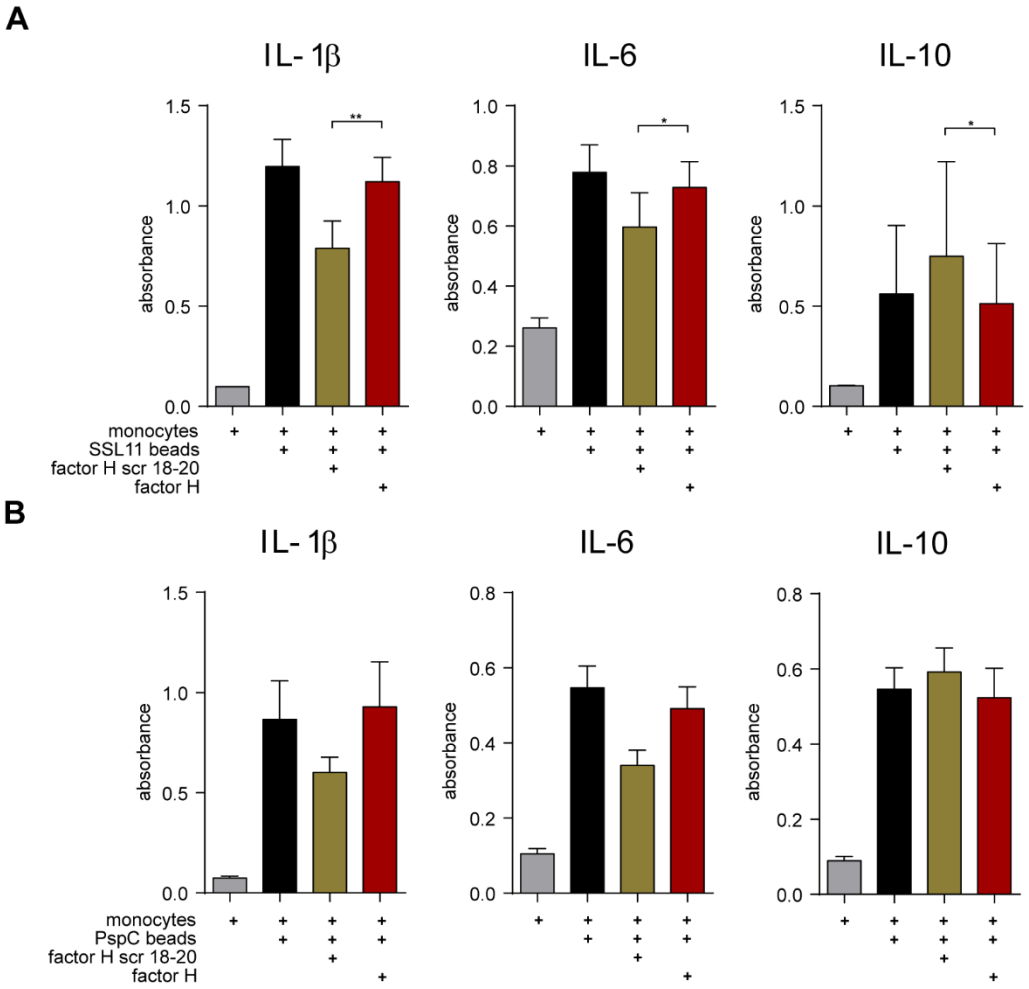


Figure 16. Factor H recruited by *S. aureus* SSL11 and *S. pneumonia* PspC modulates cytokine secretion by monocytes. Each protein recombinant SSL11 and PspC was bound onto microbeads. Factor H or fragmented factor H SCR 18-20 were bound to the recombinant proteins and subsequently incubated with monocytes for 20 hours. **(A)** Monocytes pro-inflammatory cytokine (IL-1β and IL-6) secretion was inhibited by bound factor H on SSL11 microbeads (p value of 0.002 and 0.04 respectively) and IL-10 secretion increased (p=0.02). Factor H fragment 18-20 had no effect. **(B)** Factor H bound to PspC on microbeads had a similar cytokine modulation pattern as SSL11. However, in this case the IL-1β and IL-6 inhibition and and IL-10 changes were statistically not significant.

4.2.3. Normal human serum is required for factor H immune modulatory function

Factor H is a complement regulator of the alternative pathway of complement activation. To assert the role as a complement regulator, an active complement system is required. In this study we investigated the role of normal human serum in correlation with the immunomodulatory function of factor H in modulating the cytokines secretion by monocytes. Sulphate microbeads bound with Pra1 fungal protein was used to mimic foreign surfaces to activate complement. Factor H was subsequently bound to Pra1 microbeads and incubated with monocytes in medium with and without additional normal human serum. The modulation of secreted cytokine by monocytes was dependent on the presence of normal human serum, suggesting that factor H immunomodulatory function requires components in active serum, presumably the complement system. The same principle was observed upon co-incubation of factor H treated *C. albicans* with monocytes, where factor H immunomodulatory functions were only active in normal human serum. This implies that surface bound factor H itself did not regulate the response of monocytes. Interaction between factor H and normal human serum is a prerequisite for factor H to exert its immune modulatory functions.

In further experiments the effect of iC3b which is generated by factor H and factor I, was evaluated to be involved in the immune modulation of monocytes. A question arose whether iC3b itself regulates the cytokine response of monocytes. Freshly generated iC3b modulated LPS-induced monocytes cytokines secretion by inhibiting IL-1β secretion but not IL-6 and iC3b increased the secretion of IL-10 and TGF-β. As comparison, C3b did not modulate IL-1β, IL6, and IL-10 cytokines secretion. However, it seems that to our surprise that C3b has a slight effect on the increase of TGF-β although insignificant. This iC3b modulatory effect seems to contribute partly to surface bound factor H immunomodulatory effect on the monocytes cytokines secretion in the reduction of IL-1β and induction of IL-10 cytokines secretion.

Factor H on *C. albicans* retains its cofactor activity to factor I and the inactive form of C3b, iC3b, was detected on the surface of *C. albicans* after short incubation with normal human serum. Since the surface bound C3b is cleaved into iC3b, further generation of C3b convertase on the surface was halted leading to lower opsonization of the surface and also lower recognition by monocytes. Ultimately, this likely leads to lower recognition and also

a lower response of monocytes towards *C. albicans*. Ross, *et al* reported very early that iC3b interacts with complement receptor 3 (CR3) on phagocytes and they suggested C3 fragments as the major mediating immune clearance factor *in vivo* [110]. Deficiency in factor I prevents the generation of iC3b and predisposes to recurrent bacterial infection as reported previously [111]. However, a more recent studies [107] showed that iC3b on opsonized apoptotic cells mediates anti-inflammatory response on monocytes-derived macrophages by inhibition of pro-inflammatory cytokines (IL-1β and IL-6) and secretion of anti-inflammatory cytokine IL-10 but not TGF-β. The similar regulatory effect was shown in this study, on immunomodulatory functions of factor H on *C. albicans* surface and on apoptotic cells surface, suggesting the same mechanism of modulation.

4.2.4. CR3 mediates Factor H immune modulatory signaling in monocytes

Complement receptor 3 (CR3; CD11b/CD18) is present on all phagocytes strongly interacting with iC3b [92]. By binding to iC3b, CR3 mediates phagocytosis and inflammation. As shown here surface bound factor H, which together with factor I generates iC3b from C3b, modulates the cytokine secretion of monocytes. As iC3b and factor H bind CR3, a CR3 knockout (CD11b knockout) THP-1 monocytic cell line was created and the response of these cells to *C. albicans* was evaluated.

CR3KO THP-1 cell showed a significant lower response of IL-1β secretion compared to THP-1 cells. This is explained by the fact that CR3 is, beside dectin-1, the responsible recognition receptor of β-glucans on the cell wall of the fungus [112, 41]. Recently CR3 was shown to play an essential role for mediating IL-1β secretion of dendritic cells in response to heat killed and to lesser extent, living *C. albicans* [102]. However, the absence of CR3 does not totally abolish the recognition of β-glucans on *C. albicans* due to the recognition by receptors like dectin-1, which is recognizing mannan on the *C. albicans* cell surface. Factor H on *C. albicans* also showed a tendency to inhibit IL-β in THP-1 cells, but not in CR3KO THP-1 cells because of the already low levels of IL-1β in these cells. IL-1β maturation and secretion requires activation of the inflammasome which is triggered by at least two distinct stimuli. CR3 recognition of C3b and iC3b were abolish in the CR3KO THP-1 cells leading to less stimuli and formation and activation of inflammasome, which culminates to low secretion of IL-1β.

Factor H treated *C. albicans* also induced IL-10 secretion in on THP-1 cells but this effect was abolished in CR3KO THP-1 cells, indicating that factor H mediated induction of IL-10 is mediated via CR3. In contrast, factor H on *C. albicans* induced TGF-β in THP-1 and CR3KO THP-1 cells demonstrating that factor H enhancement of this cytokine is not mediated via CR3. In summary CR3 is a central activator of IL-1β in monocytes and factor H acts on IL-10 secretion via CR3, to a minor extend on IL-1β secretion but not on TGF-β via CR3.

4.2.5. Monocytes inflammatory cytokines response to *C. albicans* suppresses adaptive immune response provided by T cells.

Innate immune cells recognize fungal cells by their pattern recognition receptors (PRRs) which leads to secretion of specific cytokines including IL-1β, IL-6, and IL-23 [113, 68, 114]. These cytokines in turn promote skewing of activated $CD4^+$ T cells into the Th17 lineage, which express IL-17, IL-17F, and IL-22. IL-17 and IL-17F signal through common receptor IL17R on , which is crucial for effective anti-*Candida* immunity by inducing innate immunity inflammation through NF-κB and C/EBP [115]. In this study we have identified factor H as a regulator on *C. albicans* which inhibited IL-1β and IL-6 while inducing IL-10 and TGF-β secretion from monocytes. Inhibition of IL-1β and IL-6 likely reduces the promotion of $CD4^+$ Th17 lineage differentiation. As shown in (**Fig.13.A and 13.B**), the supernatants of monocytes incubated with factor H treated *C. albicans* pushed naïve T cells into the direction of induced Treg (iTreg) differentiation as shown by the increasing intracellular FOXP3 detection. Regulatory T cells (Treg) are T cells which suppress the activation, proliferation and effector functions – such as cytokine production – of a wide range of immune cells including $CD4^+$ and $CD8^+$ T cells, NK cells, B cells, and APC. Moreover, transcription factor forkhead box P3 (FOXP3) is one of the intracellular marker for the detection of Treg. Induced regulatory T cells, iTreg, develop from conventional $CD4^+$ T cells outside the thymus: a defining distinction between natural T reg (nTreg) with iTreg cells. IL-1β is known as one powerful cytokine that inhibit the differentiation of $FOXP3^+$ Treg cells [116], inhibition of this cytokine increases the generation of $FOXP3^+$ T cells. On the other hand, induction of IL-10 secretion is known to restrain Th17 cell-mediated inflammation [117]. IL-10 is also described to act with TGF-

β leading to an expansion of $FOXP3^+$ iTregs with enhanced CTLA-4 expression and suppressive capability, comparable to that of natural Tregs [118]. CTLA-4 is an inhibitory protein that exert immune regulatory function, suppressing T cell response by competing with CD28 T cell co-stimulatory molecule in binding to their ligands, CD80 and CD86. Although CTLA-4 regulatory pathway and T reg regulatory function are independent of each other, CTLA-4 was reported to assist T reg regulatory function by blocking the CD28 pathway [119]. Moreover, the induction of TGF-β is possibly mediating suppression of T cell activation, differentiation and proliferation by newly generated Treg cells and known to convert naïve T cells into immune suppressive cells [120, 121, 122]. These induced suppressive cells expressed FOXP3, the critical transcription factor required for the development of $CD4^+$ Treg cells. Induced Tregs display suppressive function similar to natural T reg (nTreg) measured by their ability to inhibit T cell proliferation and Th1/Th17 – mediated autoimmune disease [123, 124]. Therefore, *C. albicans* will benefit at least locally to promote iTreg generation in order to inhibit the Th17 response.

Conclusion

In summary, this study characterizes responses of monocytes towards the fungal pathogen *Candida albicans*. First monocytes phagocytose the *Candida albicans* yeast forms and release monocytic extracellular traps (MoETs) to inhibit dissemination and infection of *C. albicans*. Extracellular traps released by monocytes are very similar to the neutrophil extracellular traps (NETs) and contain cit H3, elastase, MPO, and lactoferrin. These molecules within the extracellular traps act strongly anti-microbial and effectively reducing the survival of *Candida albicans*. Monocytes also recognize *Candida albicans* and secretes cytokines to induce inflammation and to recruit more immune cells to the site of infection. However, *Candida albicans* expresses fungal proteins that recuit the human complement regulator factor H on its surface to modulate complement activation on its surface. This complement regulator recruitment is also known for other microbial pathogen such as *Staphylococcus aureus* and *Streptococcus pneumonia*. The surface recruited factor H retains its regulatory functions as cofactor for factor I, cleaving C3b into iC3b and accelerate the decay of C3 convertase, effectively inhibiting further opsonisation on the

surface. Thus *Candida albicans* exploits the regulatory functions which subsequently decrease the recognition and response of monocytes towards the fungal pathogen. Factor H on *C. albicans* surface but no in fluid phase modulates the cytokine response of monocytes by inhibiting secretion of the central cytokines IL-1β and IL-6 and inducing IL-10 secretion. The inhibition of IL-1β and IL-6 secretion leads to lower inflammation and the induction of IL-10 further dampens inflammation. The factor H regulation is acting partially via complement receptor 3 (CR3), as absence of CR3 diminishes the regulatory effect of surface bound factor H on monocytes IL-1β and IL-10 secretion but has no effect on IL-6 or TGF-β secretion. The cytokines of the modulated monocytes induces naïve T cell differentiation towards induced T regulatory cell (iTreg) shown by the increased FOXP3 detection. This iTreg differentiation will benefit *Candida albicans* in order to inhibit the natural inflammatory reaction of TH17 response towards *Candida* infection.

The finding of this study contribute to further understanding on how *Candida albicans* interact with human innate immune system in general and monocytes in more detailed. The function of the recruited complement regulator factor H was characterized and shown to benefit *Candida albicans* survival by modulating monocytes responses directly and their subsequent effects on T cell differentiation for adaptive immune response. Application of the results presented here can be used to further understand how *Candida albicans* utilizes complement regulator to evade human complement attack.

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DECLARATION OF HONOUR

I hereby declare on my honour that I am familiar with the relevant course of examination for doctoral candidates of the Faculty of Biology and Pharmacy of the Friedrich-Schiller-Universität Jena.

I also declare that I personally composed and wrote the dissertation and that I acknowledged all additional assistance, personal communications, and sources according to the rules of academic work within this dissertation.

I declare that assistance provided by specific individuals during the study and writing of this dissertation has been indicated full.

I declare that I did not enlist any assistance of a doctoral consultant and that no third parties have received either direct or indirect monetary benefits from me for work connected to this submitted dissertation.

I declare that this dissertation has not been submitted as an examination paper for a state or other scientific examination.

I also declare that I did not submit the same, a substantially similar, or a different paper to another postsecondary school.

I am aware that a false declaration will have legal consequences.

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- International conference on Microbial Communication for young scientist (MiCom) 2014. September 2014, presented a poster.
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Publications

- Irmscher, S., Döring, N., Halder, L.D., **Jo, E.A.H**., Kopka, I., Dunker, C., Jacobsen, I. D., Luo, S., Slevogt, H., Lorkowski, S., Beyersdorf, N., Zipfel, P., Skerka, C. (2017). Kallikrein cleaves C3 and activates complement. Journal of Innate Immunity. JIN-2017-8-2/R1. https://doi.org/10.1159/000484257
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