Secreted aspartic protease 2 of *Candida albicans* inactivates factor H and the macrophage factor H-receptors CR3 (CD11b/CD18) and CR4 (CD11c/CD18)

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Abstract

The opportunistic pathogenic yeast *Candida albicans* employs several mechanisms to interfere with the human complement system. This includes the acquisition of host complement regulators, the release of molecules that scavenge complement proteins or block cellular receptors, and the secretion of proteases that inactivate complement components. Secreted aspartic protease 2 (Sap2) was previously shown to cleave C3b, C4b and C5. *C. albicans* also recruits the complement inhibitor factor H (FH), but yeast-bound FH can enhance the antifungal activity of human neutrophils via binding to complement receptor type 3 (CR3). In this study, we characterized FH binding to human monocyte-derived macrophages. Inhibition studies with antibodies and siRNA targeting CR3 (CD11b/CD18) and CR4 (CD11c/CD18), as well as analysis of colocalization of FH with these integrins indicated that both function as FH receptors on macrophages. Preincubation of *C. albicans* yeast cells with FH induced increased production of IL-1β and IL-6 in macrophages. Furthermore, FH enhanced zymosan-induced production of these cytokines. *C. albicans* Sap2 cleaved FH, diminishing its complement regulatory activity, and Sap2-treatment resulted in less detectable CR3 and CR4 on macrophages. These data show that FH enhances the activation of human macrophages when bound on *C. albicans*. However, the fungus can inactivate both FH and its receptors on macrophages by secreting Sap2, which may represent an additional means for *C. albicans* to evade the host innate immune system.

Keywords: *Candida albicans*, complement, CR3, factor H, fungal pathogen, immune evasion, protease, Sap2

Abbreviations: CCP, complement control protein domain; CR3, complement receptor type 3 (CD11b/CD18); CR4, complement receptor type 4 (CD11c/CD18); FH, factor H; MDM, monocyte-derived macrophages; Sap2, secreted aspartic protease 2

1. Introduction

Candida albicans is an opportunistic fungal pathogen in humans that is part of the mucosal microflora in the majority of the human population. It can cause a range of superficial and invasive life-threatening infections in individuals with a compromised immune system (Arendrup, 2010). *C. albicans* possesses several virulence factors that assist its success to persist and cause infection in the human host. One of the virulence traits of *C. albicans* is the release of proteolytic enzymes, such as secreted aspartyl proteases (Sap), which cleave fluid-phase and extracellular matrix proteins and thus cause tissue damage and facilitate infection (Schaller et al., 2005). Sap2 is a secreted enzyme essential for *C. albicans* growth in a protein-rich environment that cleaves peptide bonds between hydrophobic amino acids (Hube and Naglik, 2001). In the human host, Sap2 cleaves various proteins of the extracellular matrix, antimicrobial peptides, and the complement components C3b, C4b and C5, and contributes to fungal virulence (Schaller et al., 2003; Meiller et al., 2009; Gropp et al., 2009).

The complement system is a crucial humoral component of innate immunity, which serves the immediate protection against intruding microorganisms (Ricklin et al., 2010). Beside its role in pathogen elimination mediated directly by formation of the lytic terminal complement complex C5b-9 or indirectly by opsonization (mainly with C3b and C4b complement fragments), the complement system participates in the disposal of immune complexes and apoptotic cells, and the modulation of activation of the cellular components of the immune system (Ricklin et al., 2010).

Because of its destructive potential for the host, the complement system encompasses several regulator molecules. Factor H (FH) is the major soluble regulator of the alternative pathway, which acts in body fluids as well as at cellular surfaces by assisting the degradation of C3b by factor I (termed cofactor activity) and by preventing the formation and accelerating the decay of the alternative pathway C3-convertase. FH consists of 20 complement control

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protein (CCP) domains, of which the N-terminal CCPs 1-4 are responsible for both the cofactor- and decay accelerating activity of FH, and the C-terminal CCPs 19-20 mediate host surface binding (Kopp et al., 2012).

Several pathogenic and non-pathogenic microbes, including various fungi, were shown to sequester host complement inhibitor molecules, such as FH, to escape from the complement system (Lambris et al., 2008). Due to the thick fungal cell wall, complement cannot harm pathogenic fungi directly by lysis. However, the activation of complement can lead to opsonization of the fungal surface with C3b and C4b, and result in the generation of chemotactic and pro-inflammatory activation fragments, such as C3a and C5a (Speth et al., 2008; Cheng et al., 2012), which together facilitate the elimination of the pathogen by attracting neutrophils and macrophages and enhancing opsonophagocytosis. In order to evade these complement-mediated defence processes, some fungi bind complement regulators, such as FH and C4b-binding protein, through a variety of surface-bound and secreted proteins (Poltermann et al., 2007; Vogl et al., 2008; Luo et al., 2009) and thus restrict complement activation in their environment. In addition, FH was reported to influence cellular adhesion and/or antifungal responses. Due to its binding via various domains, FH can bridge different pathogens, such as *Streptococcus pneumoniae*, *Neisseria gonorrhoeae* and *C. albicans*, with the CR3 complement receptor and enhance cellular invasion or uptake of these pathogens and the immune response of the host cells (Agarwal et al., 2010a; Agarwal et al., 2010b; Losse et al., 2010). FH, when bound to the surface of *C. albicans,* was shown to mediate fungal recognition by neutrophils via primarily CR3, and increase the production of lactoferrin and reactive oxygen species by these host cells (Losse et al., 2010).

FH binding to various immune cells has been described; in some cases, however, neither the nature of the FH receptor, nor the relevance of this interaction is known, such as in the case of B lymphocytes (Lambris and Ross 1982; Erdei and Sim, 1987). FH has been

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identified as a ligand of L-selectin on leukocytes (Malhotra et al., 1999). FH binds to neutrophils via complement receptor 3 (CR3; CD11b/CD18) (Avery and Gordon 1993; DiScipio et al., 1998) and this interaction is mediated through the CCP7 and CCPs 19-20 of FH (Losse et al., 2010). It is also to be noted that neutrophils express a unique, fucosylated form of CR3, which may influence ligand interactions of CR3 (van Gisbergen et al., 2005). Purified FH has been shown to be chemotactic for human monocytes at a nanomolar concentration (Nabil et al., 1997) and the coincubation of human monocytes with FH stimulated the secretion of reactive oxygen species and IL-1β in a dose-dependent manner (Schopf et al., 1982; Iferroudjene et al., 1991). In addition, macrophages stimulated with FH exhibited an elevated production of tromboxane and prostaglandine E (Hartung et al., 1984). FH has recently been shown to bind in part to CR3 on monocytes and down-modulate the C1q-mediated uptake of apoptotic cells (Kang et al., 2012), and it was also shown to enhance the phagocytosis of apoptotic particles and decrease the release of IL-8 and TNF-α by macrophages (Mihlan et al., 2009). Nevertheless, the nature of the FH receptor on mononuclear phagocytes and the role of pathogen-bound FH in the response of macrophages are poorly characterized.

The tissue-resident mononuclear phagocytes and the recruited neutrophils represent the first and major defence against *C. albicans*. The fungus is recognized by the phagocytes either directly through pattern recognition receptors or indirectly, after opsonization, via opsonic receptors. The CR3 and the related CR4 (CD11c/CD18) receptors are major opsonic receptors, which bind the C3b cleavage product iC3b. CR3 can also bind directly to fungal surface moieties, such as beta-glucan and pH-regulated antigen 1, which interactions are important in the antifungal innate cellular response (van Bruggen et al., 2009; Soloviev et al., 2007, 2011; Losse et al., 2011).

Macrophages express more CR4 in comparison with neutrophils, however, the role of FH-CR3/CR4 interaction in the context of antifungal response of macrophages has not been studied yet. Therefore, the aim of this study was to reveal the role of CR3 and CR4 in FH binding on human macrophages and to study whether Sap2 can facilitate the evasion of the FH-CR3/CR4 mediated activation of macrophages.

2. Materials and Methods

2.1. Ethical approval

The studies were performed with approval of the Research Ethics Committee of the Medical Faculty of Friedrich Schiller University, Jena (permission number 2268-04/08) and by the respective Hungarian authorities (permission number ETT TUKEB 838/PI/12.). Informed consent for the use of blood samples was obtained according to the Declaration of Helsinki.

2.2. Materials

Purified human FH and the goat FH-specific polyclonal antibody were purchased from Merck (Schwalbach, Gemany). The anti-CD11b (clone ICRF44), anti-CD11c (clone B-ly6), anti-CD18 (clone L130) monoclonal antibodies and isotype controls were purchased from BD Biosciences (Heidelberg, Germany). HRP-conjugated rabbit anti-goat IgG, FITC-conjugated rabbit anti-goat IgG and F(ab')$_2$ fragments of goat anti-mouse IgG were obtained from Dako (Hamburg, Germany).

2.3. Human cell culture

THP-1 macrophages were differentiated from THP-1 monocytic cells (DSMZ, Braunschweig, Germany) by incubation with 10 nM PMA in RPMI 1640 medium (LONZA, Wuppertal, Germany) containing 10% FCS (PAA, Cölbe, Germany) for 24 h.

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (GE Healthcare, Freiburg, Germany) density gradient separation and erythrocytes were lysed using a hypotonic salt solution. Monocytes were obtained from PBMC by positive selection with CD14 magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany), following the manufacturer's instructions. Briefly, PBMC were resuspended in MACS buffer (DPBS with 0.5% BSA and 2 mM EDTA) containing CD14 microbeads. After incubation for 30 min at

4°C, cells were washed and resuspended in MACS buffer and loaded onto MiniMACS LS Separation Columns (Miltenyi Biotech). After washing out of non-labeled cells with MACS buffer, monocytes were eluted and the purity of CD14-positive monocytes was determined by flow cytometry.

Monocyte derived macrophages (MDM) were obtained by culturing isolated monocytes for 7 days in X-VIVO 15 medium supplemented with 10% heat-inactivated FCS (PAA), 2 mM ultraglutamine (Lonza) and 50 µg/ml gentamicin sulfate (Lonza) and by addition of 500 IU/ml GM-CSF (Immunotools, Friesoythe, Germany) every 48 h. MDM were resuspended at 2×10^6 cells/ml in serum-free X-VIVO 15 medium and cultivated at 37°C in a humidified atmosphere containing 5% CO_2.

2.4. FH binding assay

THP-1 macrophages or monocyte-derived macrophages (5×10^5 cells) were incubated with 12.5–100 µg/ml FH in DPBS with Ca^{2+} and Mg^{2+} (Lonza) for 30 min at 20°C. After washing, goat anti-FH pAb (1:500) followed by FITC-labelled anti-goat Ig antibody were added for 20 min at 4°C. 10,000 cells were measured using a BD LSRII flow cytometer (BD Biosciences, Heidelberg, Germany). Dead cells were excluded from the analysis based on propidium iodide staining. Data were analyzed using the FACSDiva (BD Biosciences) and FlowJo softwares (Tree Star, USA). For blocking experiments, cells were pre-incubated with 20 µg/ml anti-CD11b, anti-CD11c, anti-CD18, or control mAb for 20 min at 4°C and, after washing, incubated with 25 µg/ml FH. For receptor expression measurements, cells were incubated with 10 µg/ml mouse anti-CD11b, anti CD11c, anti-CD18, or control mAb for 20 min at 4°C followed by FITC-labelled anti-mouse antibody. 10,000 cells were measured by flow cytometry as described above.

2.5. Microscopic measurement of molecule colocalization

Colocalization between FH and CD11b or between FH and CD11c was detected in human monocyte-derived macrophages. 10^6 cells were incubated with 50 µg/ml FH for 30 min at 20°C in modified Hank's buffer (142 mM NaCl, 1 mM Na_2SO_4, 5 mM KCl, 1 mM NaH_2PO_4, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 5 mM glucose, 10 mM HEPES; pH 7.4). After washing, goat FH-antiserum (1:500 in modified Hank's buffer + 1% FBS) was added for 30 min at 4°C, followed by the corresponding secondary antibody for 30 min at 4°C. For detection of receptors, CD11b was labelled using biotinylated anti-CD11b and streptavidin-PE, while CD11c was visualized using anti-CD11c conjugated with Alexa 647 dye. The colocalization was quantified by calculating Pearson's correlation coefficients from at least 100-150 cells in each sample as previously described (Adler and Parmryd, 2010). This coefficient has a value between -1 and +1; a value > 0.2–0.3 indicates a strong correlation between the two studied molecules.

2.6. RNA silencing of MDMs

Monocytes were isolated from buffy coat obtained from healthy donors and provided by the Hungarian National Blood Transfusion Service by magnetic separation of the CD14+ monocytes (Miltenyi) from PBMC as previously described (Sándor et al., 2009, 2013). Cells were cultivated in CellGro serum-free medium supplemented with 100 ng/mL rHu GM-CSF. RNA silencing was performed according to the method of Prechtel (Prechtel et al., 2007). We used commercially available predesigned Qiagen AllStar Negative control siRNA and Qiagen Genome Wide predesigned siRNA for CD11b (Hs_ITGAM_5) and CD11c (Hs_ITGAX_6) silencing. Cells were transfected on day 3 and day 5 of differentiation with 20 µg siRNA to generate CD11b, CD11c or negative control silenced MDMs at day 6. CD11b and CD11c expression were analyzed on day 6 by cytofluorimetry and subsequent experiments were

carried out the same day.

2.7. Candida *cell culture and inactivation*

C. albicans yeast cells of the strain SC5314 were routinely maintained on yeast peptone dextrose agar (20 g peptone, 10 g yeast extract, 20 g glucose, 15 g agar per liter). For experiments, cells were grown in yeast peptone dextrose medium at 30°C with shaking for 16 h and washed twice in Dulbecco's phosphate buffered saline (DPBS; Lonza, Wuppertal, Germany) before use. For some experiments *Candida* cells were inactivated by incubation in DPBS (LONZA, Germany) containing 0.05% thimerosal (Sigma-Aldrich, Taufkirchen, Germany) for 1.5 h at 20°C. After extensive washing in DPBS, yeast cells were resuspended in DPBS at a concentration of 1×10^8/ml and the loss of viability was confirmed by plating on yeast peptone dextrose agar.

2.8. Cell stimulation and cytokine measurement

Inactivated or live yeast cells of *C. albicans* SC5314 strain (1×10^7) were preincubated with 50 μg/ml FH, HSA and 20% NHS. MDMs were stimulated with *C. albicans* cells at a MOI of 4, 50 μg/ml FH or 100 μg/ml Zymosan (Sigma-Aldrich). Culture supernatants were collected 20 h later and the measurement of IL-6 and IL-1β was performed using commercial sandwich ELISA kits from ImmunoTools and eBioscience (Frankfurt, Germany), respectively, according to the manufacturer's instructions.

2.9. Sap2 cleavage experiments

The culture supernatants containing specifically the *C. albicans* protease Sap2 were obtained from strain SAP2ex4 as described previously (Staib et al., 2008). Briefly, *C. albicans* strain SAP2ex4 is a derivative of wild-type strain SC5314 containing in a *sap2* knock-out

background a copy of the *SAP2* gene under control of the doxycycline-inducible promoter. After preculture in SD medium (20 g glucose and 6.7 g YNB with ammonium sulfate [MP Biomedicals] per liter) at 30°C, the yeast cells were diluted 1:100 in YCB-BSA medium (23.4 g yeast carbon base, 4 g of bovine serum albumin per litre, pH 4.0) containing 50 μg /ml of doxycyclin (supernatant w/ Sap2) and grown for two days at 30°C. For control, culture medium alone (supernatant w/o Sap2) was used.

To analyze the effect on cell surface receptors, 1×10^6 THP-1 macrophages were incubated with 60 μl supernatants of *C. albicans* (diluted 1:2 in DPBS) or DPBS alone for different time periods and washed with DPBS containing 1% FCS (PAA) and 0.1% NaN_3 (Carl Roth). Cells were then incubated with 10 μg/ml monoclonal antibodies against CD11b, CD11c and CD18 and the isotype control (BD Biosciences, Germany) for 20 min at 4°C followed by the corresponding FITC-labelled antibody (1:100, Dako) for 20 min at 4°C. 10,000 cells were measured by flow cytometry as described above.

Cleavage of FH was analyzed by adding 30 μl of the Sap2-containing supernatant to 200 nM FH (30 μg/ml) in 60 μl DPBS (pH 5.0) for different time periods. The mixture was separated by SDS-PAGE, transferred to a nitrocellulose membrane and detected using a goat polyclonal anti-FH antibody (Calbiochem) and a HRP-labelled secondary antibody (Dako). The bands were detected by enhanced chemiluminescence.

2.10. Cofactor assay

The functional activity of Sap2-pretreated FH was analyzed in a cofactor assay. FH (0.25 or 1 μg) preincubated in DPBS alone or in DPBS containig *C. albicans* supernatant with or without Sap2 induction was added to a mixture of C3b (0.5 μg) and FI (0.025 μg) in 30 μl DPBS and incubated for 30 min at 37°C. Samples were collected, separated by SDS-PAGE

and analyzed by Western blot. The C3b cleavage products were detected using a polyclonal goat anti-C3 antibody (1:2500, Sigma-Aldrich) and a HRP-labeled anti-goat Ig (1:200, Dako).

2.11. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA). A *p* value < 0.05 was considered statistically significant.

3. Results

3.1. FH binds to human macrophages via CR3 and CR4

First, FH binding to THP-1 cells and human monocyte-derived macrophages was analyzed by flow cytometry. FH showed dose-dependent binding to both THP-1 cells and blood-derived primary macrophages (**Fig. 1**), similar to FH binding to neutrophils (Losse et al., 2010). The binding of FH to blood-derived macrophages showed higher variability compared with its binding to the cell line, which is likely due to the biological variability of the macrophages derived from different blood donors. This was further supported by the measurement of the expression of the CR3 and CR4 on the used cells (**Fig. 2A**).

To analyze which of the two related integrins CR3 and CR4 mediate binding of FH to macrophages, three approaches were used. First, PMA-activated THP-1 cells and human monocyte-derived macrophages were pre-incubated with mAbs binding to CD11b, CD11c and CD18 respectively, followed by incubation of the cells with FH. Binding of FH was measured by flow cytometry. All three CR3/CR4-specific mAbs reduced FH binding to macrophages, suggesting that both CR3 and CR4 participate in binding this complement regulator (**Fig. 2B**).

Next, FH binding was measured after treatment of the macrophages with siRNA targeting CD11b or CD11c, which resulted in at least 50% reduction in the detectable cell surface CD11b and CD11c, respectively (**Fig. 2C**). FH showed ~20% less binding to macrophages after silencing of CD11b (**Fig. 2D**). Silencing of CD11c showed no effect on FH binding, likely because of the primary role of CR3 and/or the CD18 chain in FH binding.

In addition, we measured the colocalization of bound FH and the CR3 and CR4 complement receptors on the cells by CLSM (**Fig. 3**). The calculated Pearson's correlation coefficients for FH and CD11b (0.362 ± 0.126) as well as for FH and CD11c (0.215 ± 0.106) indicated that bound FH and the alpha-chain of both integrins are in proximity on the cell

membrane. Co-localization of FH and CD18 could not be measured because the anti-CD18 mAb very efficiently inhibited FH binding to the cells and even displaced bound FH.

3.2. FH enhances the production of the pro-inflammatory cytokines IL-1β and IL-6 in macrophages

We have previously shown that *C. albicans*-bound FH by binding to CR3 on human neutrophils enhanced the release of the pro-inflammatory IL-8 (Losse et al., 2010). Additionally, IL-1 release by monocytes was described upon addition of FH (Iferroudjene et al., 1991). Therefore, we measured the release of IL-1β and IL-6 in cocultures of human MDM with *C. albicans* yeast cells preincubated with FH, NHS or with HSA. Approximately 10% and 25% enhancement in the production of IL-1β and IL-6, respectively, was observed when live fungal cells with FH were co-cultured with MDM compared with HSA-treated controls (**Fig. 4A and 4D**). However, FH strongly and significantly enhanced production of both cytokines by MDM when fungal cells were inactivated with thimerosal before preincubating them with FH (**Fig. 4B and 4E**). Similarly, macrophages incubated with zymosan and FH produced more IL-1β and IL-6 than macrophages stimulated with zymosan alone (**Fig. 4C and 4F**). Taken together, these results suggested that FH can increase the production of pro-inflammatory cytokines by macrophages upon exposure to fungal cells, while the live fungi can counteract this.

3.3. Sap2 cleaves FH

Because fungal proteases are known to inactivate various host factors and some of the Saps were shown to degrade the host complement proteins C3b, C4b and C5, we studied whether this mechanism may also be used by *C. albicans* to inactivate FH. Because Sap2 is a protease with broad specificity, we measured its capacity to degrade human FH. Supernatant of *C.*

albicans cells producing Sap2 upon induction was used at acidic pH known to be optimal for the activity of this enzyme (Staib 1965; Hube et al., 1994) and which may occur at sites of fungal infection such as the vagina and/or during certain infection stages (De Bernardis et al., 1995; Staib et al., 2000). This Sap2-rich supernatant when co-incubated with FH efficiently cleaved this human complement regulator as visualized by Western blot (**Fig. 5A**). We also tested the cofactor activity of FH upon Sap2 treatment. As expected, cleavage of FH by Sap2 resulted in the complete loss of its capacity to facilitate factor I-mediated cleavage and inactivation of C3b (**Fig. 5B**). *C. albicans* culture supernatant without Sap2 induction did not result in FH cleavage (**Fig. 5A, lane 1**) and the such treated FH maintained its cofactor activity (**Fig. 5B, lane 9**). Sap5 was not induced under these conditions and did not result in FH cleavage, measured as control (not shown).

3.4. Sap2 inactivates CR3 and CR4 on macrophages

We also studied whether Sap2 is able to inactivate the cell surface FH-binding proteins CR3 and CR4. To this end, THP-1 cells were incubated with supernatants of *C. albicans* culture without or with induction of Sap2 production, and the expression of the CD11b, CD11c and CD18 molecules on the cells was determined by flow cytometry. Incubation with the supernatant of Sap2-expressing yeasts resulted in significant and time dependent decrease of the detectable CD11b and CD18 on THP-1 cells, while the control culture supernatants, without Sap2 induction, did not alter the expression of the CR3 and CR4 chains (**Fig. 6**). The time-dependent decrease in detectable CR3 and CR4 suggests proteolytic cleavage by Sap2 rather than simply binding of the protease to these integrins. The viability of the cells was not significantly altered upon incubation with the yeast culture supernatant for the tested time period (not shown).

4. Discussion

The host immune system applies several mechanisms to control fungal infections, involving particularly humoral and cellular elements of innate immunity. Complement has a major role in innate host defence against infections by opsonization and lysis of microbes. However, microorganisms acquired various means to evade host innate responses, including the action of the host complement system (reviewed in Lambris et al., 2008).

FH is a major host complement inhibitor that can be found in human plasma at a high concentration (~250 µg/ml) and is also secreted by various immune cells locally (Kopp A et al., 2012). Thus, FH is in direct contact with *C. albicans* during commensalism and/or infection and can be exploited by the fungus to inhibit complement activation on its surface (Meri et al., 2002). In addition, FH may enhance the antifungal response of immune cells by bridging the pathogen and the host cell, as *C. albicans*-bound FH was shown to enhance neutrophil responses (Losse et al., 2010).

FH binding to immune cells, such as B cells, monocytes, macrophages and neutrophils, was described; however, its receptors and cellular functions are poorly characterized (Erdei and Sim 1987; Malhotra et al., 1999). FH binding to neutrophils and the role of CR3 as a FH receptor is established (Avery and Gordon 1993; DiScipio et al., 1998; Losse et al., 2010). Similarly, a recent study showed that FH binds in part via CR3 to monocytes (Kang et al., 2012). Our previous study on human neutrophils suggested that CR4 (CD11c/CD18) may also be involved in binding FH; however, neutrophils express much less CR4 in comparison with CR3. Therefore, we studied the human monocytic cell line THP-1 and also monocyte-derived macrophages that do express significant amounts of CR4 (**Fig. 2**). While dose-dependent FH binding to macrophages was observed, no saturation was reached at the tested physiological FH concentrations (**Fig. 1**). Under similar conditions, saturable FH

binding to neutrophils was observed previously (Losse et al., 2010), likely reflecting the difference in the number of expressed CR3 and CR4 and/or the difference in carbohydrate composition of CR3 (van Gisbergen et al., 2005). Various approaches, using blocking mAbs to the integrin chains, colocalization measurements and application of siRNA to silence CD11b and CD11c suggested that indeed both receptors are involved in binding FH on macrophages (**Figs. 2-3**). Silencing of CD11b resulted in ~50% detectable CD11b and only ~20% decrease in FH binding and that of CD11c had no effect on FH binding to the cells, which can be explained by the quick upregulation of membrane CR3 from intracellular stores at 37°C, as observed previously (Sándor et al., 2013). The data also raise the possibility that CD18 is instrumental in FH binding, because its inhibition by mAbs strongly and almost completely inhibited FH binding to MDM (Fig. 2) and, as previously shown, to neutrophils (Losse et al., 2010). We assume that the colocalization measurement was not successful due to a strong competition between anti-CD18 and FH for binding to the cells, implying that they bind in close proximity. These results, however, do not exclude that other FH receptors exist on macrophages.

Because it was reported that FH can stimulate the release of IL-1β by human monocytes (Iferroudjene et al., 1991) and we found that FH enhanced *C. albicans*-induced IL-8 release by neutrophils, we analyzed the role of pathogen-bound FH in the production of proinflammatory IL-1β and IL-6 by MDM. We found slight but significant increase of both cytokines when live yeasts with FH were added to the cell culture and a more pronounced increase when thimerosal-inactivated yeasts were used (**Fig. 4**). In addition, we found that FH can enhance zymosan-induced IL-1β and IL-6 production in MDM, i.e. without yeast cells but using yeast extract, whereas FH alone had no effect. Thus, FH enhanced pathogen-induced proinflammatory cytokine release, in contrast to the previously described anti-inflammatory effect when macrophages phagocytosed apoptotic particles (Mihlan et al., 2009). This

suggests that FH can positively or negatively modulate the cytokine response of macrophages, depending on the nature of the phagocytosed particle and the additional stimuli mediated by specific receptors.

CR3 is a multifunctional receptor with a number of host and non-host ligands, including FH and the major opsonin iC3b, as well as fungal beta-glucan and Pra1 (Ross and Lambris, 1982; van Bruggen et al., 2009; Soloviev et al., 2007). Thus, CR3 mediates direct interaction of phagocytic cells with the fungal cell wall and is integrally involved in the elicitation of cellular immune response. Defects in the function of this integrin may result in susceptibility to infections (Ross et al., 1985; Andrews and Sullivan, 2003). Under experimental conditions, live *C. albicans* cells elicit less cellular responses than do thimerosal-inactivated cells, as shown previously for neutrophils (Losse et al., 2011) and here for macrophages (**Fig. 4**). This may be in part due to secreted Pra1, which can bind to and block CR3 and thus reduce neutrophil responses, such as increased myeloperoxidase, lactoferrin and cytokine production (Losse et al., 2011). Inactivation by yeast-derived Sap2 represents an additional means by which *C. albicans* can block this receptor on host cells (**Fig. 6**). While Sap2 could theoretically bind to all these receptors without necessarily cleaving them, this is very unlikely, because Sap2 is a promiscuous protease that cleaves a large number of substrates. Furthermore, the time-dependent decrease in detectable CR3 and CR4 on the cells indicates proteolytic cleavage rather than simply binding of Sap2 to these integrins. In either case, however, Sap2 impairs the availability of CR3 and CR4 on the cells.

We also found that Sap2 efficiently cleaves FH which then loses its ability to act as a cofactor for the inactivation of complement C3b (**Fig. 5**). This suggests that Sap2 does not only inactivate CR3 but also one of its ligands, shown here and previously to be involved in antifungal defence (Losse et al., 2010). The FH-related protein FHR-1 was similarly cleaved by Sap2 (data not shown). FHR-1 lacks domains homologous to the N-terminal complement

regulatory domains of FH and has no FH-like cofactor- and decay accelerating activity (Timmann et al., 1991). Instead, FHR-1 was described as an inhibitor of C5 and thus the terminal complement pathway (Heinen et al., 2009), although other groups could not confirm this effect (Strobel et al., 2011; Goicoechea de Jorge et al., 2013). Nevertheless, the C-terminal domains of FHR-1 and FH share high sequence similarity (Józsi et al., 2015), which explains why FHR-1 can bind to CR3 (Losse 2010). FHR-1 was shown also to enhance antifungal activity of neutrophils when bound on *C. albicans* (Losse et al., 2010).

Because FH is an efficient complement inhibitor utilized by many microbes to evade complement attack, one can argue that the mechanism described here would be of disadvantage for the pathogen. One should consider that Sap2 is a promiscuous protease that cleaves a number of host factors, including complement components that are involved in the propagation of the complement cascade, such as C3b, C4b and C5 (Gropp et al., 2009). Similar to our results, other groups have recently shown cleavage of FH by microbes (Miller et al., 2014; Riva et al., 2015). Thus, it is likely that even if the host complement inhibitor FH is inactivated by this protease, complement activity will be impaired by the action of Sap2 at other points in the cascade. Furthermore, the local pH will regulate activity of the protease that might be relevant at sites of infection where often the pH is reduced (e.g., vagina).

In conclusion, FH is shown to bind to CR3 and CR4 on human macrophages and enhance proinflammatory cytokine response in the presence of yeasts. Sap2 of *C. albicans* was found to inactivate FH, FHR-1, CR3 and CR4, which mechanism could represent an additional means by which *C. albicans* evades the host innate immune system.

Acknowledgements

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Figure legends

Figure 1. Binding of FH to THP-1 cells and to human monocyte-derived macrophages.

(A) THP-1 cells and (B) human monocyte-derived macrophages (MDM) were incubated with the indicated amounts of purified FH. FH binding was detected by flow cytometry using polyclonal anti-FH antibody. Data are expressed as fold mean fluorescence relative to the control without FH and show mean $\pm$ SD from three experiments. (C) Representative histograms showing binding of 25 μg/ml FH, used in receptor blocking experiments, to MDM.

Figure 2. Role of CR3 (CD11b/CD18) and CR4 (CD11c/CD18) in binding FH to macrophages.

(A) Expression of the CR3 and CR4 receptor chains on THP-1 cells and human MDM was measured by flow cytometry using specific mAbs. Data are expressed as fold mean fluorescence relative to the control without FH and show mean + SD from three experiments.

(B) Human MDM were preincubated with mAbs specific to CD11b, CD11c and CD18, or with isotype control. Binding of 25 μg/ml FH was measured as described for Fig. 1. Normalized data represent mean + SD from three experiments. *, $p < 0.05$ and ***, $p < 0.001$, one-way ANOVA.

(C) Human MDM were pretreated with control, CD11b- and CD11c-specific siRNA as described in Methods. Cell surface expression of the CD11b and CD11c integrin chains was measured as described in (A).

(D) In parallel, FH binding was measured after siRNA treatment. Normalized data represent mean + SD from three experiments.

Figure 3. Microscopy analysis of colocalization of FH with CR3 and CR4 on MDM

Representative confocal images from three experiments show binding of FH to cell surface of macrophages (green: FH, red: CD11b or CD11c, yellow dots represent highly colocalized FH and integrin α-chains). To assess colocalization, a Pearson's correlation coefficient was calculated between FH and CD11b (A) or between FH and CD11c (B) from $\geq$ 100 ROI/image.

Figure 4. Effect of yeast-bound FH on the cytokine response of macrophages.

Live *C. albicans* yeasts were preincubated with 50 µg/ml FH and then coincubated with human MDM. The release of (A) IL-1β and (D) IL-6 was measured by ELISA from the supernatants after 20h. (B) IL-1β and (E) IL-6 release by MDM were measured upon coincubation with thimerosal-inactivated yeast cells pre-incubated with FH or with 20% NHS. (C) IL-1β and (F) IL-6 release by MDM upon incubation with FH, zymosan or FH plus zymosan. Human serum albumin (HSA) was used as control protein. Normalized data represent mean + SD of at least three experiments. *, $p < 0.05$, one-way ANOVA.

**Figure 5. Sap2 of *C. albicans* inactivates FH.**

(A) Purified FH was incubated for the indicated time (t) with *C. albicans* culture supernatants, either with or without induction of Sap2 expression. FH was then analyzed by Western blot using polyclonal anti-FH. (B) Cofactor activity of FH treated or not with Sap2 was analyzed after incubation with C3b and factor I (FI) by Western blot using polyclonal anti-C3.

Figure 6. Sap2 of *C. albicans* inactivates CR3 and CR4.

THP-1 cells were incubated with *C. albicans* culture supernatants, with or without the induction of Sap2 expression. The expression of CR3 and CR4 was measured after the

indicated time using mAbs specific to the receptor chains. Data represent mean + SD of three

experiments. ***, $p < 0.001$, one-way ANOVA.

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Figure 1

A

B

C
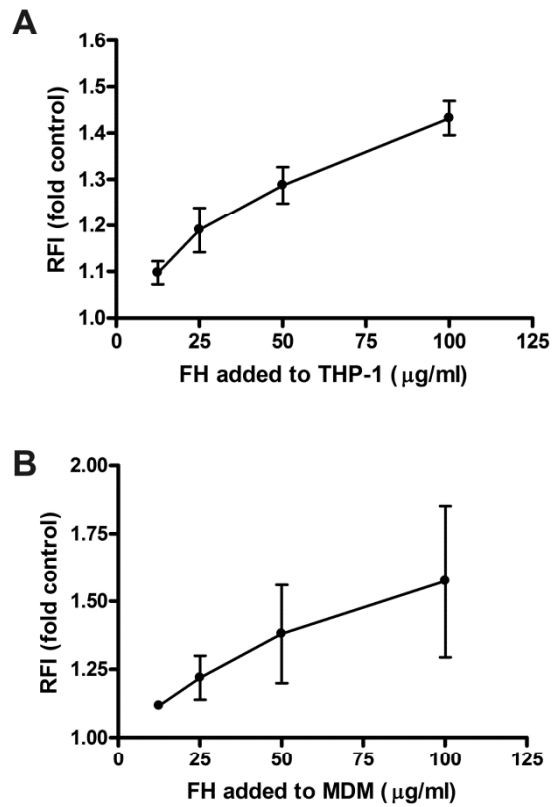
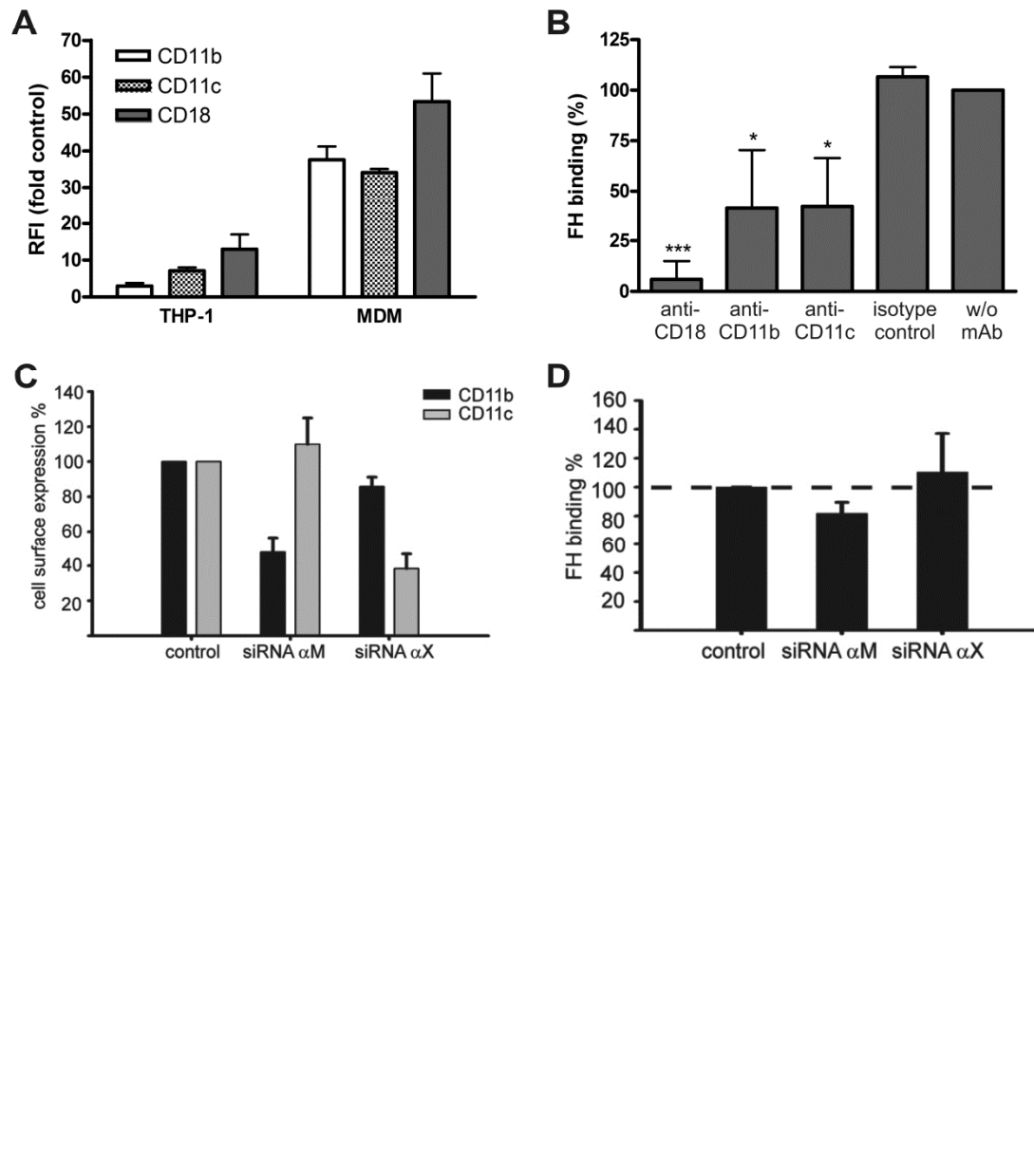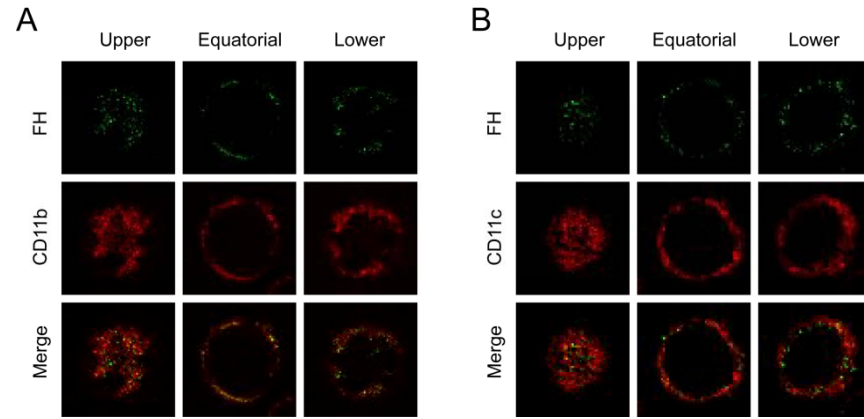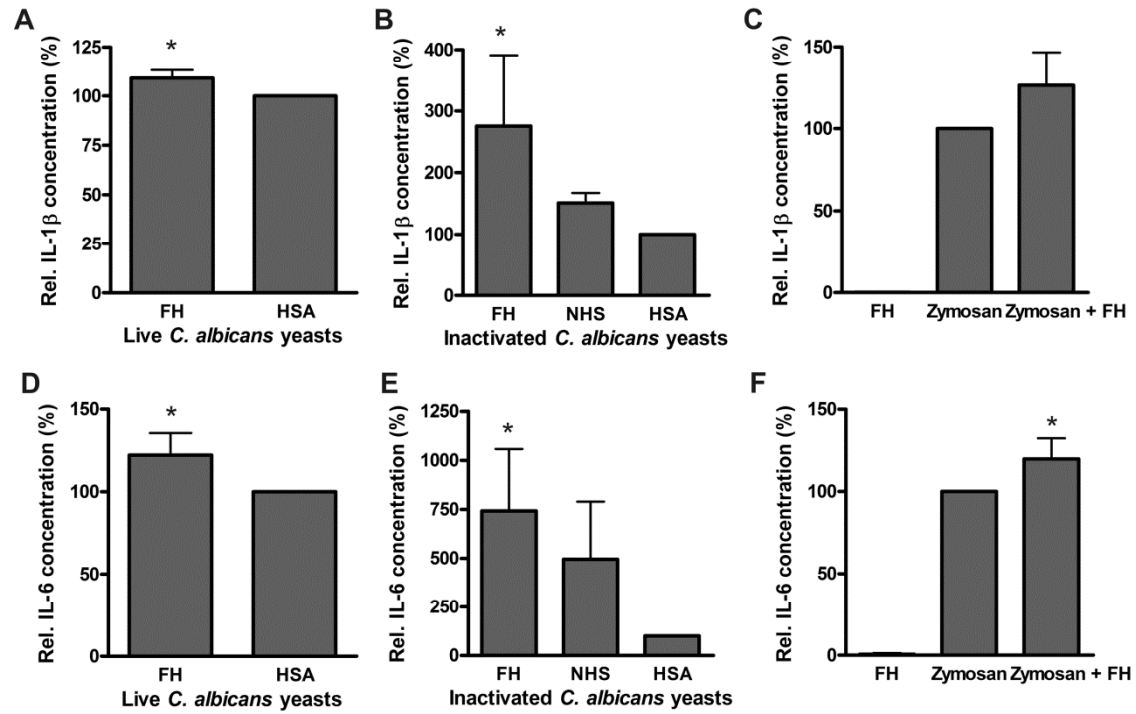Svoboda et al. 2015
Figure 2

A

B

C

D

Figure 3

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Figure 4

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Figure 5
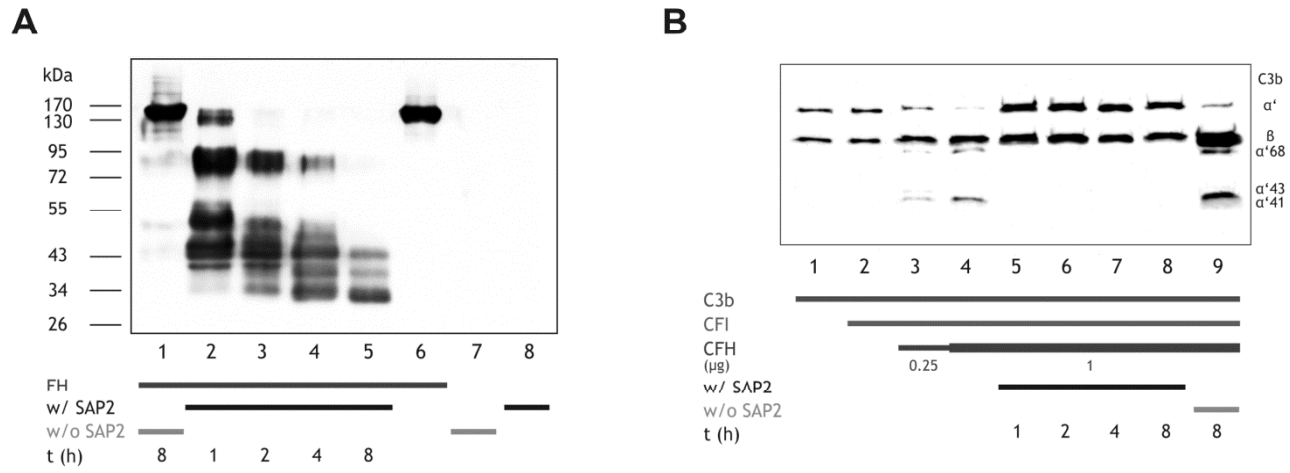
A

kDa								
170								
130								
95								
72								
55								
43								
34								
26								

1 2 3 4 5 6 7 8

FH
w/ SAP2
w/o SAP2
t (h)   8 1 2 4 8

B

C3b
α'
β
α'68
α'43
α'41

1 2 3 4 5 6 7 8 9

C3b
CFI
CFH (µg)   0.25   1
w/ SAP2
w/o SAP2
t (h)   1 2 4 8 8

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Figure 6

Legend:
- DPBS (3h)
- Sap2 ctrl. (3h)
- Sap2 (1h)
- Sap2 (2h)
- Sap2 (3h)

% MFI

CD11b   CD11c   CD18