Complement factor H modulates the activation of human neutrophil granulocytes and the generation of neutrophil extracellular traps

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Abbreviations: CR3, complement receptor type 3 (CD11b/CD18); DHR, dihydrorhodamine; DIC, differential interference contrast; FH, factor H; Fn, fibronectin; HSA, human serum albumin; NET, neutrophil extracellular trap; ROS, reactive oxygen species.

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Abstract

Factor H (FH) is a major inhibitor of the alternative pathway of complement activation in plasma and on certain host surfaces. In addition to being a complement regulator, FH can bind to various cells via specific receptors, including binding to neutrophil granulocytes through complement receptor type 3 (CR3; CD11b/CD18), and modulate their function. The cellular roles of FH are, however, poorly understood. Because neutrophils are important innate immune cells in inflammatory processes and the host defence against pathogens, we aimed at studying the effects of FH on various neutrophil functions, including the generation of extracellular traps. FH co-localized with CD11b on the surface of neutrophils isolated from peripheral blood of healthy individuals, and cell-bound FH retained its cofactor activity and enhanced C3b degradation. Soluble FH supported neutrophil migration and immobilized FH induced cell spreading. In addition, immobilized but not soluble FH enhanced IL-8 release from neutrophils. FH alone did not trigger the cells to produce neutrophil extracellular traps (NETs), but NET formation induced by PMA and by fibronectin plus fungal β-glucan were inhibited by immobilized, but not by soluble, FH. Moreover, in parallel with NET formation, immobilized FH also inhibited the production of reactive oxygen species induced by PMA and by fibronectin plus β-glucan. Altogether, these data indicate that FH has multiple regulatory roles on neutrophil functions. While it can support the recruitment of neutrophils, FH may also exert anti-inflammatory effects

and influence local inflammatory and antimicrobial reactions, and reduce tissue damage by modulating NET formation.

Keywords: complement; CR3; factor H; extracellular DNA; neutrophil extracellular trap; reactive oxygen species

1. Introduction

The complement system is a major humoral component of innate immunity and plays important roles in antimicrobial defense and in maintaining host homeostasis (Ricklin et al., 2010). Complement components and their activation fragments generated upon triggering of the complement cascade also influence the activation and function of various cells through several receptors (Ricklin et al., 2010).

Factor H (FH) is a major inhibitor of the alternative pathway of complement in plasma and on host cellular and non-cellular surfaces (Ferreira et al., 2010; Kopp et al., 2012; Rodriguez de Cordoba et al., 2004). FH is a 155-kDa glycoprotein that is mainly produced in the liver and reaches a median plasma concentration of ~250 μg/ml (Kopp et al., 2012). FH is also produced locally by several types of cells, such as endothelial cells, monocytes and dendritic cells (Brooimans et al., 1990; Li et al., 2011; Whaley, 1980). FH regulates complement activation at the level of the central C3b component by acting as a cofactor in the cleavage of C3b by factor I and by inhibiting formation and accelerating the decay of the alternative pathway C3 convertase enzyme (Kopp et al., 2012; Rodriguez de Cordoba et al., 2004).

In addition to being a complement inhibitor, there is growing evidence for direct regulatory roles of FH on several cell types. FH has been shown to bind to neutrophil granulocytes via

complement receptor type 3 (CR3; CD11b/CD18), and mediate adhesion and cell polarization (Avery and Gordon, 1993; DiScipio et al., 1998; Losse et al., 2010). *Candida albicans*-bound FH facilitated fungal recognition and antifungal responses by neutrophil granulocytes (Losse et al., 2010). FH bound to *Streptococcus pneumoniae* was shown to mediate interaction of pneumococci with human neutrophils and epithelial cells, and facilitate the entry into host cells (Agarwal et al., 2010b). Moreover, FH was shown to facilitate adherence of *Neisseria gonorrhoeae* to CR3-expressing CHO-cells (Agarwal et al., 2010a). FH also binds to monocytes, macrophages, B cells and platelets (Hartung et al., 1984; Iferroudjene et al., 1991; Lambris et al., 1980; Vaziri-Sani et al., 2005). FH promotes the uptake of apoptotic cells by macrophages in a non-inflammatory manner (Mihlan et al., 2009) and has a chemotactic function on monocytes (Nabil et al., 1997). It was also shown that CR3 is involved in FH binding to monocytes and FH can inhibit the C1q-mediated uptake of apoptotic cells (Kang et al., 2012). On B cells, FH was reported to inhibit immunoglobulin secretion and cell differentiation (Tsokos et al., 1985), but the B cell FH receptor could not be identified at the molecular level (Erdei and Sim, 1987). However, these non-canonical, cellular roles of FH are poorly understood.

Neutrophil granulocytes are major inflammatory cells and key players during infections, since they provide the first line of host cellular defense (Mocsai, 2013; Nathan, 2006). They are rapidly recruited to infected tissues and have several killing mechanisms to eliminate pathogens (Kolaczkowska and Kubes, 2013). In addition to phagocytosis and intracellular killing, and the release of antimicrobial factors, neutrophils can trap microorganisms by releasing neutrophil extracellular traps (NETs) (Brinkmann et al., 2004; Nathan, 2006). These web-like structures are formed by activated neutrophils and composed of nuclear chromatin associated with nuclear histones and granular antimicrobial proteins (Brinkmann et al., 2004). Thus, NETs probably do not only function as a trap, but they are also able to play a direct role in killing pathogens

(Brinkmann et al., 2004; Kolaczkowska and Kubes, 2013). NETs are formed in response to a variety of pro-inflammatory stimuli, such as LPS, IL-8 and TNF-α, as well as several microorganisms (Brinkmann et al., 2004; Remijsen et al., 2011). In vitro, phorbol 12-myristate 13-acetate (PMA) is considered the most potent agent to induce NET formation (Brinkmann et al., 2004; Remijsen et al., 2011). In addition to particularly hyphal forms of fungi (Svobodova et al., 2012; Urban et al., 2006), immobilized, purified fungal β-glucan together with fibronectin as an extracellular-matrix component can also stimulate NET formation (Byrd et al., 2013). However, NETs represent not only an effective protection when phagocytosis is not possible due to the large size of microbes (Branzk et al., 2014), but could also be a potential immunostimulatory agent if NET elimination is not completely performed under non-infectious conditions (Farrera and Fadeel, 2013; Leffler et al., 2013; Mocsai, 2013). In addition to the prolonged inflammatory environment and continuous tissue damage, NETs can contribute to the production of autoantibodies (e.g., anti-dsDNA and anti-histones), which may play a role in autoimmune and inflammatory diseases (Leffler et al., 2013; Mantovani et al., 2011; Saffarzadeh and Preissner, 2013; Sur Chowdhury et al., 2014).

The FH receptor CR3 is also a main receptor for recognizing fungal ligands, including β-glucan, on human neutrophils (Losse et al., 2011; Ross et al., 1987; van Bruggen et al., 2009) and it also plays an essential role in immune-complex induced (Behnen et al., 2014) and β-glucan plus fibronectin-induced formation of NETs (Byrd et al., 2013). The present study was designed to investigate the role of FH in modulating the activation and cellular functions of human neutrophils, particularly the generation of NETs.

2. Materials and methods

2.1. Materials

Purified human FH, C3b, factor I, and polyclonal goat anti-human FH antibody were purchased from Merck Ltd. (Budapest, Hungary). Human iC3b was obtained from Complement Technology Inc. (Tyler, Texas). Bovine serum albumin (BSA) was from Applichem (Darmstadt, Germany) and human serum albumin (HSA) was from Sigma-Aldrich Inc. (St. Louis, MO). Horseradish peroxidase (HRP)-conjugated goat anti-human C3 antibody was obtained from MP Biomedicals (Solon, OH). HRP-conjugated rabbit anti-goat immunoglobulins and goat anti-mouse immunoglobulins were from Dako (Hamburg, Germany).

2.2. Cells

Human neutrophil granulocytes were isolated from peripheral blood of healthy individuals. All blood donors gave informed consent. In some cases, neutrophils were isolated from buffy coats obtained from healthy blood donors and provided by the Hungarian National Blood Transfusion Service. The studies were approved by the respective national authorithy (TUKEB ETT, permission number 838/PI/12). Mononuclear cells were removed by Ficoll-Hypaque (Sigma-Aldrich) density gradient centrifugation, then dextran sedimentation using Dextran T-500 (Pharmacia Fine Chemicals, Uppsala, Sweden) was performed. Red blood cells were lysed in hypotonic buffer. Purity of isolated neutrophils was analyzed by flow cytometry using anti-CD16 and anti-CD14 antibodies (BD Biosciences, Heidelberg, Germany) and was over 95%.

2.3. Colocalization assay

FH binding to neutrophils was analyzed by flow cytometry as previously described (Losse et al., 2010). To measure colocalization between FH and CD11b, 10^6 neutrophils were first incubated

with 50 μg/ml FH for 30 min at 22°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 with PBS, Fc receptor blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) was added to reduce nonspecific Ab binding, then a goat anti-human FH antibody (1:500 in PBS containing 1% FBS) was added for 30 min at 4°C, followed by Alexa-488-conjugated rabbit anti-goat Ig (Molecular Probes-Invitrogen, Carlsbad, CA) for 30 min at 4°C. For detection of CR3, CD11b was labelled with biotinylated anti-CD11b (clone M1/70.15; Molecular Probes-Invitrogen) and streptavidin-PE (Sigma-Aldrich). The colocalization was quantified by calculating Pearson's correlation coefficients from at least 100 cells in each sample (Adler and Parmryd, 2010).

2.4. Cellular cofactor assay

2×10^6 neutrophils were incubated with 10 μg/ml FH for 30 min at 22°C in modified Hank's buffer. After washing twice with PBS to remove unbound FH, 3 μg/ml purified C3b and 5 μg/ml factor I were added to the cells in 200 μl final volume in PBS and incubated for 1 h at 37°C. The supernatants were separated on 10% SDS-PAGE gel, transferred to nitrocellulose membrane and analyzed by Western blot using HRP-conjugated anti-C3 antibody to detect cleaved C3b fragments. As a positive control for cofactor activity, FH, C3b and factor I were mixed together in PBS, without cells. The blots were developed by enhanced chemiluminescence (Merck-Millipore).

2.5. Calcium measurement by flow cytometry and microscopy

Neutrophils were washed and incubated for 30 min at 37°C in 5 μg/ml Fluo-4 AM (Molecular Probes-Invitrogen) solution in RPMI-1640 medium (Sigma-Aldrich). After loading with the dye,

168 samples were washed and resuspended in Hank's buffer. 50 μg/ml FH and 2 μg/ml ionomycin
169 (Sigma-Aldrich) as a positive control were used to raise cytoplasmic free calcium level.
170 Fluorescence measurements were performed using a FACS Calibur flow cytometer (BD
171 Biosciences) with an air-cooled argon ion laser (488 nm excitation) and red diode laser (632 nm
172 excitation). Data collection and analysis were done with CellQuest Pro software. Dead cells were
173 excluded by negative gating based on propidium iodide uptake.

174 To investigate the calcium response induced by immobilized FH, neutrophils loaded with
175 Fluo-4-AM were placed into wells of Ibidi microplates (Ibidi, Planegg/Martinsried, München,
176 Germany) at 1.5×10^6/ 200 μl density. Microplates were previously coated overnight at 22°C
177 with 50 μg/ml FH or BSA. Changes in fluorescence intensity of individual cells were monitored
178 for 20 min in Olympus FluoView 500 laser-scanning confocal microscope (excitation: 488 nm)
179 with x60 objective, in time-resolved acquisition mode (1.13 s/frame) immediately after placing
180 them to the microplate. 37°C, 5% CO_2 and humidity were provided by Ibidi gas incubation
181 system for live cell imaging. During data analysis, mean fluorescence intensities obtained from
182 single cell recordings were normalized to differential interference contrast (DIC) intensities to
183 avoid out of focus intensity alteration effects.

184

185 **2.6. Measurement of neutrophil spreading by confocal microscopy**

186 Lab-Tek borosilicate chambered coverglass microplates (NUNC, Rochester, NY) were coated
187 with 50 μg/ml FH or BSA in modified Hank's buffer overnight, then washed three times.
188 Neutrophils (2×10^5 cells) in 200 μl were added and allowed to adhere/spread for 60 min at 37°C
189 in CO_2 thermostat, then fixed with 2% paraformaldehyde for 5 min at 37ºC, followed by washing
190 twice with PBS. For blocking experiments, cells were preincubated with 50 μg/ml anti-CD11b
191 (clone: ICRF44; Biolegend, San Diego, CA) or with control mouse IgG1 mAb (in house) for 20

min at 4°C. The adhered cells were stained with Phalloidin Alexa-488 (Molecular Probes-Invitrogen; 1:100 in 0.1% Triton X-100) for 5 min at 37°C, and then washed four times with PBS. The contact surface of the cells was monitored in Olympus FluoView 500 laser-scanning confocal microscope (excitation: 488 nm). For measuring of the contact zone area we used ImageJ software (*http://rsbweb.nih.gov/ij*) with Analyze Particle tool.

2.7. Cell migration assay

Cell migration assays were performed in serum-free RPMI-1640 medium using Costar 24-transwell plates (Corning Life Sciences, Corning, NY) with 3 μm-pore polycarbonate membranes. 50 μg/ml FH, 50 μg/ml HSA and 1 μM fMLF (both from Sigma-Aldrich) as positive control were added to the lower chamber. Neutrophils were stained with 5 μM Cell tracker green (Invitrogen) for 45 min at 37°C. After washing, 10^6 neutrophils were added to the top chamber for 60 min at 37°C in a CO_2 thermostat, then 25 mM EDTA was added to the lower chamber to release neutrophils adhering to the bottom of the membrane and the bottom of the well. The relative fluorescence intensity of migrated neutrophils was measured using a Fluoroskan Ascent FL (Thermo Scientific, Waltham, MA) microplate reader with excitation and emission filters of 495 nm and 515 nm, respectively.

2.8. ELISA

To determine the FH concentration in the upper chamber of the transwell system, microtiter plates were coated overnight with 1:1000 dilution of polyclonal goat anti-human FH antibody. After washing with PBS containing 0.05% Tween-20. Supernatants diluted 1:1 with PBS were added to the wells and incubated for 1 h at 22°C. After washing, 1:1000 dilution of a mouse anti-FH mAb (A229; Quidel, San Diego, CA) was added for 1 h at 22°C, followed by a secondary

antibody for further 1 h at 22°C. The ELISA was developed using TMB substrate (Kem-En-Tec Diagnostics, Taastrup, Denmark), and the absorbance was measured at 450 nm.

IL-8 in the supernatant of activated neutrophils was determined using a commercial ELISA kit (R&D Systems, McKinley Place, MN).

Lactoferrin was measured using sandwich ELISA. 4 μg/ml anti-lactoferrin mAb (Hytest, Turku, Finland) was immobilized on microtiter plates at 4°C overnight. After blocking with 5% BSA for 1 h, supernatants of activated cells were added for 1 h at 22°C. Lactoferrin was detected using 100 ng/ml HRP-conjugated anti-lactoferrin Ig (antibodies-online, Aachen, Germany), and TMB substrate.

2.9. NET induction by PMA

Wells of 96-well black transparent-bottom plates (Greiner Bio-One, Kremsmünster, Austria) were either left untreated or coated overnight with 50 μg/ml FH in modified Hank's buffer. Neutrophils (10^6 cells) were allowed to adhere to the wells for 30 min at 37°C in CO_2 thermostat. Soluble FH (50 μg/ml) or 100 nM PMA (Sigma-Aldrich) as a positive control was added and after 3 h of incubation in CO_2 thermostat at 37°C, NETs were visualized on adherent neutrophils by addition of 5 μM Sytox Orange nucleic acid stain (Molecular Probes-Invitrogen).

2.10. NET induction by fibronectin and β-glucan

96-well black transparent-bottom plates were coated overnight with 6 μg/ml human fibronectin (Fn, from human plasma; Sigma-Aldrich) in TBS (25 mM Tris [pH 7.2], 150 mM NaCl) and/or with 1 mg/ml β-glucan from *S. cerevisiae* (Sigma-Aldrich) in 50 μl. In some experiments, 50 μg/ml FH or 50 μg/ml iC3b was immobilized. 10^6 cells were pre-treated on ice with 1 nM fMLF for 20 min, then washed and resuspended in serum free RPMI-1640 medium, and 1 mM Mn^{2+}

was added to the cells immediately before plating. After 1 h incubation in CO_2 thermostat at 37°C, NETs were visualized on adherent neutrophils by adding 5 μM Sytox Orange.

In parallel, an adhesion assay was also performed. Neutrophils were stained with 5 μM Cell tracker green CMFDA (Molecular Probes-Invitrogen) for 45 min at 37°C. After washing, 10^6 neutrophils were added to the plates for 1 h. The relative fluorescence intensity of adhered neutrophils was measured using a fluorescence reader with excitation and emission filters of 495 nm and 515 nm, respectively.

2.11. Immunostaining of MPO and citrullinated histone H4

After NET induction, DNA was labelled with 5 μM Sytox Orange, then the neutrophils were fixed with 3% paraformaldehyde for 10 min at 37°C. The cells were permeabilized in 0.1% Triton X-100 (2 min), washed three times in PBS and then FcR blocking reagent (Miltenyi Biotec, Germany) with 5% BSA was added for 30 min at 37°C. For detection of MPO and citrullinated H4, mouse monoclonal anti-MPO (1:500; Hytest Ltd.) and rabbit polyclonal anti-histone H4 (citrulline 3) (1:500; Merck-Millipore) antibodies were used, followed by the corresponding secondary antibodies (Alexa-647-conjugated goat anti-mouse Ig and Alexa-488-conjugated goat anti-rabbit Ig, both from Molecular Probes-Invitrogen) for 30 min at 22°C.

Fluorescence microscopy was carried out on an Olympus FLUOView500 laser-scanning confocal microscope (Hamburg, Germany) equipped with argon ion laser (488 nm) and two He–Ne lasers (with 543 and 632 nm excitation wavelengths, respectively). Typically, fluorescence and DIC images (512x512 pixels) were acquired using a 60x oil-immersion- or 20x objective. Images were processed by ImageJ software (*http://rsbweb.nih.gov/ij*) using the „Image Correlator Plus" colocalization analysis plugin.

2.12. Quantification of NETs

The relative fluorescence intensity of extracellular DNA was measured using a Fluoroskan Ascent FL (Thermo Scientific) fluorescent ELISA microplate reader with excitation and emission filters of 543 nm and 592 nm, respectively. Fluorescence in samples labelled with 5 μM Sytox Orange containing 0.5 mg/ml saponin (Sigma-Aldrich) was taken as maximal signal (100%). Relative fluorescence increase in the examined samples was calculated and referred to as "extracellular DNA (% of max)".

2.13. Detection of reactive oxygen species (ROS)

ROS was measured on PMA- or fibronectin plus β-glucan activated cells in modified Hank's buffer by adding 5 μg/ml dihydrorhodamine (DHR) (Sigma-Aldrich) for the last 15 min of 1 h incubation at 37°C. The fluorescence signal of the oxidized DHR was measured in a fluorescence reader with excitation and emission filters of 485 and 538 nm, respectively.

2.14. Statistical analysis

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

3. Results

3.1. Neutrophil-bound FH retains its cofactor activity and enhances C3b degradation

FH when attached via its C-terminal domains to certain host surfaces, such as endothelial cells, erythrocytes and basement membranes, is thought to play an important role in preventing complement-mediated inflammation and cell damage (Ferreira et al., 2009; Ferreira et al., 2006; Jozsi et al., 2007). Therefore, we tested whether FH when bound to neutrophil granulocytes, can exert complement regulatory activity. First, binding of 50 μg/ml FH was analyzed by flow cytometry and microscopy. FH showed strong specific binding to human neutrophils **(Fig. 1A**), in agreement with previous results (Avery and Gordon, 1993; DiScipio et al., 1998; Losse et al., 2010), and suggesting a receptor-mediated binding different from its loose surface attachment via the host surface glycosaminoglycan/sialic acid binding site. Even though the plasma FH concentration is higher, we used this concentration because in contrast to our in vitro system with neutrophils only, blood contains various cell types in different numbers and with different affinity for FH, and also because our previous data showed saturation of receptors on neutrophils by this amount of FH (Losse et al., 2010). Previous results using monoclonal antibodies suggested that the β_2 integrin CR3 is involved in FH binding to neutrophils (DiScipio et al., 1998; Losse et al., 2010). Here, we confirmed this by confocal microscopy, where the calculated positive Pearson's correlation coefficient (0.3 ± 0.007) indicated colocalization between CD11b and FH **(Fig. 1B)**.

Since FH is the major regulator of the alternative complement pathway, we tested if it is able to facilitate C3b inactivation when bound to CR3 on neutrophils. In the cofactor assay, purified C3b and factor I were incubated with neutrophils, which were either preincubated or not with FH. The cleavage of C3b was analyzed by Western blot. Incubation of C3b with cells alone, in the absence of any added factors I and H, resulted in the cleavage of the C3b α'-chain into

306 fragments with apparent Mw of 68, 46 and 43 kDa **(Fig. 1C, lane 4)**, indicating activity of
307 membrane-anchored complement regulators and/or that of FH, which may already be bound in a
308 small amount on the surface of neutrophils purified from blood (Losse et al., 2010). When
309 neutrophils were preincubated with FH, strongly increased C3b degradation was observed: all of
310 the α'-chains were fragmented **(Fig. 1D, lane 7)**. These results demonstrated that receptor-bound
311 FH could act as a cofactor for factor I, which proteolytically inactivates C3b.
312
313 **3.2. FH supports neutrophil spreading and migration**
314 Because FH was shown to serve as an adhesion ligand for neutrophils and to induce cell
315 polarization (DiScipio et al., 1998), we tested whether FH influences the spreading of
316 neutrophils. Neutrophils were applied to wells coated with FH and BSA, and neutrophil
317 spreading was monitored by confocal microscopy using fluorescent F-actin probe to measure the
318 contact zone area. Under these experimental conditions, significantly increased spreading was
319 observed on immobilized FH compared with BSA **(Fig. 2A and 2B)**. A mAb blocking the ligand
320 binding site on CD11b inhibited spreading on FH, whereas the control mAb had no effect (**Fig.**
321 **2B**).
322 Upon stimulation with immobilized FH, we could observe calcium signal with live cell
323 imaging microscope. The recording was started immediately after the cells were placed into the
324 wells, since as they reached the bottom of the plate an activation stimulus was quickly provided.
325 A small intracellular Ca^{2+} peak occured within a few minutes in all cases after adhesion to the
326 chamber, and was followed by additional intense Ca^{2+} peak and rapid spreading due to the
327 interaction with FH (**Fig. 3A and Supplementary Video 1**). Changes in intracellular Ca^{2+} level
328 were also quantified (**Fig. 3B**). We did not observe similar effect with immobilized BSA
329 (**Supplementary Video 2**). Freshly isolated neutrophils were also incubated with 50 μg/ml FH

for 1-20 min. Soluble FH did not induce Ca^{2+} signals in neutrophils, in contrast to ionomycin, which was used as a positive control **(Fig. 3C)**.

Previous data suggested that FH may also support cell migration, as reported for monocytes and also for neutrophils when exposed to FH-coated *Candida albicans* yeasts (Losse et al., 2010; Nabil et al., 1997). Therefore, we tested whether FH modulates the migratory capacity of neutrophils. The cell migration assays were performed in transwell plates with 3 μm-pore polycarbonate membranes. The measured fluorescence of migrated cells induced by 1 μM fMLF as positive control was set as 100%. Addition of FH to the lower chamber of transwells caused significantly increased neutrophil migration compared with medium control, ~65% of that induced by fMLF **(Fig. 4A)**. We also tested whether FH added to the lower chamber passes to the upper chamber. FH could be detected in all cases in the upper chamber by ELISA, confirming the formation of a FH concentration gradient **(Fig. 4B)**.

3.3. Immobilized FH enhances IL-8 release from neutrophils

Because IL-8 is a known migratory chemokine for neutrophils, we tested if FH is able to induce IL-8 production by neutrophils. Neutrophils were stimulated either with soluble or immobilized FH in FCS-free RPMI-1640 medium for 24 h, then the amount of IL-8 in the supernatants was measured by ELISA. Under these circumstances immobilized FH significantly enhanced IL-8 production, while for soluble FH a similar effect was not observed **(Fig. 5)**. The effect was specific to FH since immobilized fibronectin did not induce enhanced IL-8 release from neutrophils under the same conditions (data not shown).

3.4. NET formation and ROS production induced by PMA and by fibronectin plus fungal β-glucan is inhibited by FH

354 Neutrophils are rapidly recruited in tissues during infections and have a wide repertoire of killing
355 mechanisms to eliminate pathogens, including respiratory burst and NET formation (Brinkmann
356 et al., 2004; Kirchner et al., 2012; Nathan, 2006). Because little is known about how complement
357 modulates NET production, we asked the question whether FH is able to influence the generation
358 of NETs.

359 First, we used a PMA-induced NET formation model (Keshari et al., 2013; Parker et al.,
360 2012), where after 3 h of treatment ~60% of the total DNA was detectable extracellularly (**Fig.**
361 **6A**). Under the same conditions, soluble or immobilized FH alone had no effect on NET
362 production (**Fig. 6B and 6C**). However, when applied together with PMA, immobilized but not
363 soluble FH could significantly decrease the amount of extracellular DNA (**Fig. 6C**). Because FH
364 is known to bind to DNA (Leffler et al., 2010), we tested if FH binding affects the staining of the
365 DNA with Sytox Orange. FH up to 50 μg/ml did not affect the fluorescence signal (data not
366 shown). The formation of NETs was confirmed by confocal microscopic analysis, which showed
367 that the DNA was indeed associated with myeloperoxidase and citrullinated histone H4 (**Figure**
368 **S1**). Because NET generation is usually linked to the production of ROS (Fuchs et al., 2007;
369 Kirchner et al., 2012; Parker et al., 2012), we analyzed the generation of ROS under the same
370 conditions as above. Again, PMA-induced ROS generation by neutrophils was significantly
371 reduced by immobilized but not by soluble FH, and FH alone did not induce ROS under these
372 conditions (**Fig. 6D**).

373 An extracellular matrix-based model was also used, where human fibronectin and β-glucan, a
374 major component of fungal cell wall, together induced NET release, as described by Byrd *et al.*
375 (Byrd et al., 2013). Neutrophils were primed with 1 nM fMLF in the presence of 1 mM Mn^{2+} and
376 showed rapid homotypic cell aggregation upon NET formation (**Fig. 7A**), as described (Byrd et

al., 2013). In this system, FH also inhibited NET formation induced by fibronectin plus fungal β-glucan (**Fig. 7A and 7B**).

As a control, we used iC3b as additional complement ligand of CR3. iC3b is a proteolytically inactivated product of the complement C3 cleavage fragment C3b, which opsonizes pathogens and enhances the cell responses against them. Under our experimental conditions, iC3b did not significantly alter NET generation, thus supporting a specific effect of FH (**Fig. 7B**). We also tested if differences between cell adhesion properties to the applied coats caused the observed effects on NET formation. Neutrophils were loaded with Cell Tracker Green and the percentage of the bound cells was determined compared to the total cell number. As we measured similar cell adherence rates, it can be excluded that the observed differences in NETs are due to altered adhesion, and support the specific inhibitory effect of FH on the release of NETs (**Fig. 7C**).

Similar to the previously observed inhibitory effects of FH on ROS induced by PMA (**Fig. 6**), FH inhibited ROS production in neutrophils stimulated by fibronectin plus β-glucan (**Fig. 7D**). In these experiments, iC3b did not influence ROS production. In addition, we measured lactoferrin production by neutrophils in parallel, which was not modulated significantly by either FH or iC3b under these conditions (**Fig. 7E**).

4. Discussion

FH inhibits the alternative complement pathway in body fluids and also protects self-tissues against complement attack and complement-mediated inflammation. FH can loosely attach to host surfaces, such as endothelial cells, erythrocytes and basement membranes, via glycosaminoglycans and sialic acids, and this binding is enhanced if C3 fragments are also deposited on the surface due to complement activation (Blaum et al., 2015; Ferreira et al., 2009; Jozsi et al., 2007; Kajander et al., 2011). In addition, recruitment of host FH is a common complement/immune evasion strategy of several pathogenic microbes. In some cases, such as for *Neisseria meningitidis*, FH binding is of major importance to avoid complement-mediated lysis; in most cases, however, microbes exploit host complement regulators to evade opsonophagocytosis (Lambris et al., 2008; Ram et al., 1999; Schneider et al., 2006). There is also evidence for FH-mediated adhesion of microbes to host cells, including neutrophils (Losse et al., 2010; Agarwal et al., 2010a; Agarwal et al., 2010b).

In the case of neutrophil granulocytes, the binding of FH was shown to be mediated via the CR3 complement receptor (DiScipio et al., 1998; Losse et al., 2010; Agarwal et al., 2010b). As demonstrated here, despite the specific receptor-ligand interaction, FH retains its cofactor activity (**Fig. 1**); moreover, through the direct effects on neutrophils, it is also able to modulate neutrophil activation and antimicrobial responses.

Previous data provided evidence that FH has a specific receptor on neutrophil granulocytes (Avery and Gordon, 1993; DiScipio et al., 1998). DiScipio *et al.* identified CR3 (CD11b/CD18, $\alpha_M\beta_2$) as the main FH receptor on neutrophils (DiScipio et al., 1998), which was confirmed by our group using specific antibodies against the CD11b (clone ICRF44) and CD18 (clone L130) chains that inhibited FH binding (Losse et al., 2010). In our current study we demonstrated colocalization between CD11b (with mAb clone M1/70.15) and FH by confocal microscopy (**Fig.**

418 **2**). We could not detect similar colocalization with CD18, because depending on the order of
419 labeling only CD18 (clone IB4) or FH could be detected on the cell surface (data not shown),
420 likely due to strong competition between the anti-CD18 mAb and FH for binding. Previously we
421 showed that anti-CD18 almost completely blocked FH binding to neutrophils (Losse et al., 2010).
422 Based on these data, CD18 may have a major role in FH binding. These results, however, do not
423 exclude the existence of additional FH receptors on the cells.

424 FH was described as an adhesion ligand for neutrophils (DiScipio et al., 1998); moreover, as
425 we reported previously, *C. albicans* covered with FH could more efficiently induce migration and
426 become adhered to and phagocytosed by neutrophils than the fungal cells alone (Losse et al.,
427 2010). Similarly, it was shown that FH enhanced the interaction of pneumococci with neutrophils
428 through CR3 (Agarwal et al., 2010b). In addition, FH was described as a chemotactic factor for
429 monocytes (Nabil et al., 1997). Therefore, we studied whether FH can directly, i.e. without a
430 pathogen, affect neutrophil activation, migration and spreading. Soluble FH was indeed able to
431 support neutrophil migration in a transwell assay (**Fig. 4**). While FH is produced in the liver and
432 circulates at relatively high concentration, extrahepatic sources of local FH production are also
433 known. Myeloid cells in tissues and endothelial cells can produce FH upon inflammatory stimuli
434 (Brooimans et al., 1990; Li et al., 2011; Whaley, 1980), which may contribute to generating a
435 local FH gradient and thus promote recruitment of neutrophils.

436 Apparently, the soluble and immobilized forms of FH do not provide the same information to
437 the cells. In our experiments, only immobilized but not soluble FH could trigger calcium
438 response and spreading (**Fig. 2 and 3**), and could induce IL-8 production in neutrophils (**Fig. 5**).
439 IL-8 is a potent proinflammatory chemokine and has a key role in the recruitment and activation
440 of neutrophils (Mantovani et al., 2011). Therefore, presumably an activation process occurs when
441 neutrophils come into contact with FH that is bound to surfaces, which may enhance cell entry to

442 the given area. No effect of soluble FH on neutrophil activation was observed, except for the
443 migration in the case of FH gradient. This is an important observation because of the relatively
444 high average plasma concentration (~250 μg/ml) of FH. Thus, the continuously circulating FH in
445 the body fluids does not stimulate neutrophils; however, when deposited on a pathogen surface
446 (as shown for the fungal pathogen *C. albicans* in vitro) or in the tissues, it may affect the
447 recruitment and activation of these inflammatory cells. This, however, needs to be further studied
448 for in vivo relevance. It should also be noted that integrin receptors can sense differences
449 between soluble and immobilized ligands (Ganpule et al., 1997; Schurpf and Springer, 2011),
450 further supporting the observations that CR3, and probably CR4 (CD11c/CD18), another integrin
451 sharing the β_2 chain but which is expressed at low amount on neutrophils, are specific FH
452 receptors (Losse et al., 2010; Svoboda et al., 2015).
453 Neutrophils are not simply effective and fast killer/effector cells, but depending on the size
454 and nature of the pathogen they deploy different antimicrobial responses. They can selectively
455 release NETs in response to fungal hyphae and pathogens, which are too large to be
456 phagocytosed (Branzk et al., 2014; Byrd et al., 2013; Svobodova et al., 2012). These DNA-based,
457 web-like structures have effective trapping function and are able to prevent pathogen expansion
458 and dissemination. Moreover, neutrophils can eliminate pathogens extracellularly, by releasing
459 antimicrobial peptides, enzymes and reactive oxygen and nitrogen species concentrated to the
460 target area and partly in NETs (Brinkmann et al., 2004; Fuchs et al., 2007; Guimaraes-Costa et
461 al., 2009; Menten-Dedoyart et al., 2012). Extracellular histones exert bactericidal effects (Allam
462 et al., 2014; Brinkmann et al., 2004), but are also toxic to host cells, such as endothelial cells
463 (Allam et al., 2014). Although Byrd *et al.* found that complement does not have an essential role
464 in NET production, since using autologous human serum in their model system did not alter the
465 NETting of the cells when compared to the cells under serum free conditions (Byrd et al., 2013),

there is also evidence for a modulatory role of complement. Pre-activated neutrophils were shown to release NETs upon C5a stimulation (Martinelli et al., 2004; Yousefi et al., 2009). Therefore, we analyzed whether NET release could be modulated by FH. First we used PMA as a general cell activator agent (DeChatelet et al., 1976; Esaguy et al., 1991), for which effective NET inducing ability has been described (Brinkmann et al., 2004; Keshari et al., 2013; Parker et al., 2012). In this model system, the soluble and immobilized forms of FH alone did not induce NETs. Only immobilized FH could modulate NETosis and significantly decreased the PMA-induced NET- and ROS release (**Fig. 6**). Several publications indicated that NETosis is strongly dependent on ROS generation (Fuchs et al., 2007; Keshari et al., 2013; Kirchner et al., 2012), although a ROS-independent process was also described (Byrd et al., 2013; Pilsczek et al., 2010). While the underlying mechanisms of NET release are not yet fully understood, we presume that the decreased NET formation is linked to the decreased ROS production caused by FH in our in vitro NET model.

In addition, an extracellular matrix-based model was used to investigate NET production, where a hyphal infection can be mimicked with immobilized fungal β-glucan (Byrd et al., 2013). A cross-regulatory relationship between β_1 and β_2 integrins has been described, in which the ratio of fibronectin to β-glucan determines the cellular responses. This regulatory mechanism allows superoxid anion production only when neutrophils formed strong contact with fungal hyphae (Lavigne et al., 2006; Lavigne et al., 2007). In our experimental set-up immobilized fibronectin plus β-glucan could effectively induce NET and ROS production. While Byrd *et al.* reported that fibronectin plus β-glucan induced NET formation is a ROS-independent process and they could not detect ROS production upon this stimulus (Byrd et al., 2013), in our experiments there was detectable ROS production. This difference may be due to the different β-glucan preparations and the different cells to surface ratio employed. In addition, instead of ferricytochrome c we used

DHR as a ROS detecting probe, which detects both extracellular and intracellular reactive species with higher response ability and less dependency on the applied buffer (Freitas et al., 2009). In our model we also found that immobilized FH had an inhibitory effect on NET release and ROS production (**Fig. 7**). The NET response to β-glucan plus fibronectin was found to be dependent on CR3 (Byrd et al., 2013), and both FH and iC3b bind also to CR3. iC3b, in contrast to FH, did not inhibit NET release induced by fibronectin plus β-glucan. A direct competition between FH and β-glucan for binding sites on CR3 cannot be excluded; however, FH also strongly inhibited PMA-induced NETosis. While FH reduced ROS production, it did not significantly affect lactoferrin release, thus bactericidal ability of neutrophils in general is not inhibited by FH. It is also possible, however, that some of the released lactoferrin is sequestered by NETs.

Taken together these data provide evidence that FH ensures self protection not only by limiting complement activation, but also by directly mediating cellular responses. On the one hand, FH can promote neutrophil recruitment (**Figs. 4 and 5**) and may enhance antimicrobial responses and phagocytosis (Losse et al., 2010). On the other hand, FH could reduce host damage caused by an inflammatory environment through the inhibition of NET and ROS production. Prolonged presence or enhanced amounts of NETs may be linked to inflammatory and autoimmune diseases, e.g. by providing autoantigens such as dsDNA. Furthermore, extracellular histone may cause cytotoxicity. By inhibiting NET and ROS, FH may limit such adverse reactions. It is tempting to speculate that in FH-associated diseases, such as the kidney disease atypical hemolytic uremic syndrome, hereditary or acquired functional FH deficiency may contribute to local inflammation, NET-mediated complement activation (Leffler et al., 2012; Wang et al., 2015), endothelial damage and thrombus formation, in addition to impairment in complement regulation. The results also raise the possibility that bound FH may be exploited by pathogenic microbes not only for complement evasion, which is a well-documented virulence

514 feature (Lambris et al., 2008), but for NET evasion, too. FH-associated anti-inflammatory effect
515 was demonstrated previously on macrophages, where FH contributed to the non-inflammatory
516 clearance of apoptotic and necrotic cells by inhibiting the pro-inflammatory cytokine production
517 of phagocytosing macrophages (Mihlan et al., 2009).

518 In summary, these data indicate that FH has diverse effects on neutrophil functions. While it
519 can support the recruitment of neutrophils via promoting migration and enhancing IL-8 release,
520 depending on the stimulus context FH could also exert anti-inflammatory effects and influence
521 local inflammatory and antimicrobial reactions as well as tissue damage by modulating NET
522 formation.

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Figure legends

Fig. 1. CR3-bound FH on neutrophils retains its cofactor activity.

(A) Neutrophils were incubated with 50 µg/ml FH in modified Hank's buffer, and bound FH was detected by flow cytometry. One representative histogram out of three independent experiments is shown. **(B)** Representative confocal images show binding of FH to the cell surface of neutrophils (green: FH, red: CD11b, yellow dots represent highly colocalized FH and CD11b). During a colocalization analysis a Pearson's correlation coefficient was calculated from ≥ 100 cells /sample. **(C)** In a cellular cofactor assay, neutrophils preincubated or not with 10 µg/ml FH, were incubated with C3b and factor I. Cell supernatants were separated on 10% SDS-PAGE gel under reducing conditions and analyzed by immunoblotting using anti-C3 antibody to detect C3b fragments. A representative Western blot from three independent experiments is shown. As a positive control, purified FH (10 µg/ml), factor I (5 µg/ml) and C3b (3 µg/ml) have been mixed together in PBS, without cells (lane 2).

Fig. 2. FH supports neutrophil spreading.

(A) FH and BSA were immobilized in 50 µg/ml in chambered microplate wells, then neutrophils were added to each well for 60 min at 37°C. The contact surface of the cells was monitored by confocal microscopy using Phalloidin-Alexa488 as an F-actin probe. Original scale bars, 10 µm. **(B)** FH was immobilized as in (A), and in certain chambers preincubated with 50 µg/ml anti-CD11b (aCD11b) or control mouse IgG1 (mIgG1) antibodies. The contact zone areas were quantified using ImageJ software from 100 cells in each experiment. Error bars represent SEM calculated from three independent experiments performed with neutrophils from different donors. ***$P < 0.001$, one-way ANOVA. ns, not significant.

Fig. 3. Effect of FH on the calcium response of human neutrophils.

(A) Representative confocal images from three independent experiments show neutrophil spreading upon exposure to immobilized FH and the fluorescence intensity of Fluo-4 calcium indicator during this process at different time points. **(B)** Representative single cell calcium response belonging to the above presented images. Mean fluorescence intensities were normalized to DIC intensities to avoid out of focus intensity alteration effects. **(C)** Fluo-4-AM-loaded neutrophils were investigated by flow cytometry for their Ca^{2+} response to 50 µg/ml soluble FH. The maximal response of cells to the Ca^{2+} ionophore ionomycin is shown as a positive control. Data are mean ± SD from three independent measurements.

Fig. 4. FH supports neutrophil migration.

(A) FH was added to the lower well and the cell migration rate was measured by adding Cell Tracker Green loaded neutrophils to the upper well of a transwell system. The cell number was quantified by plate fluorimeter, as described in Materials and methods. Migration induced by 1 µM fMLF was set to 100%. Error bars represent SEM calculated from 16 independent experiments. $*P < 0.05$, one-way ANOVA. **(B)** ELISA was used to determine the amount of FH, added to the lower chamber, in the upper chamber of the transwell system during the experiment described in (A). Data are mean + SEM from seven experiments. $***P < 0.001$, one-way ANOVA.

Fig. 5. FH enhances IL-8 release from neutrophils.

771 Neutrophil supernatants were collected after 24 h stimulation with 50 μg/ml immobilized or
772 soluble FH, and the IL-8 content was determined by a commercial ELISA kit. Data are means ±
773 SEM from five independent experiments. $^{**}P < 0.01$, one-way ANOVA.
774
775 **Fig. 6. PMA-induced NET formation and ROS production is inhibited by FH.**
776 **(A)** Percentage of NET formation upon 100 nM PMA treatment for 0-180 min. The extracellular
777 DNA was quantified by a plate fluorimeter after staining with 5 μM Sytox Orange. Mean ± SEM
778 are shown from three independent measurements. **(B)** NET formation was visualized by staining
779 with 5 μM Sytox Orange. The representative microscopic images illustrate the effects of the
780 indicated treatments. The adherent cell densities are shown in the DIC images, using a 20x
781 objective. Original scale bars, 100 μm. **(C)** Neutrophils were stimulated for 3 h after allowing
782 them to adhere for 30 min. Unstimulated neutrophils in serum-free RPMI medium and
783 neutrophils incubated with 100 nM PMA served as negative and positive controls, respectively.
784 50 μg/ml FH was either immobilized or added in solution in serum-free medium alone, or
785 together with 100 nM PMA. Data are means ± SEM from five independent experiments. $^{*}P <$
786 0.05, one-way ANOVA. **(D)** Under the same conditions, ROS production was measured using 5
787 μg/ml DHR as a fluorescent dye. The ROS level induced by 100 nM PMA was set to 100%. $^{*}P <$
788 0.05, one-way ANOVA.
789
790 **Fig. 7. NET formation and ROS production induced by fibronectin plus β-glucan is**
791 **inhibited by FH.**
792 **(A)** Neutrophils were pretreated with 1 nM fMLF and 1 mM $MnCl_2$, before induction of NET by
793 immobilized fibronectin plus β-glucan. Neutrophils formed aggregates and released NET on this
794 coat after 1 h. FH had an inhibitory effect on this stimulus. The representative microscopic

images show cells stained for NET with 5 μM Sytox Orange. The adherent cell densities and the degree of aggregation are seen in the DIC images, taken using a 20x objective. Scale bars, 100 μm. **(B)** The extracellular DNA was quantified by plate fluorimeter using 5 μM Sytox Orange staining. $^{**}P < 0.01$, one-way ANOVA. **(C)** Cell adhesion was also measured using Cell Tracker Green-loaded cells, treated as in (B). The relative flourescence intensity of 10^6 neutrophils was set to 100% and compared with that measured on the different coats to determine the adhered cell rate. **(D)** Under the same conditions ROS production was assayed using 5 μg/ml DHR. The ROS level induced by 100 nM PMA was set to 100%. $^{*}P < 0.05$, one-way ANOVA. **(E)** Lactoferrin secretion from supernatants after 1 h stimulation was measured by ELISA. Data in **(B)-(E)** are means ± SEM from eight independent experiments.

Video legends

Video 1. Neutrophil spreading and calcium response upon exposure to immobilized factor H.

This video shows neutrophil spreading upon immobilized FH stimulus and the changes in fluorescence intensity of Fluo-4 calcium indicator during this process. The live cell imaging was started immediately after the cells were placed into the coated wells, since as they reach the bottom of the plate an activation stimulus is instantly provided. The focal plane was set during the recording. 20 min real time events were compressed in this video.

Video 2. Neutrophil spreading and calcium response upon exposure to immobilized BSA.

This video shows neutrophil spreading upon immobilized BSA stimulus and the changes in fluorescence intensity of Fluo-4 calcium indicator during this process. The recording was made under the same circumstances as for Video 1.

Fig. 1.

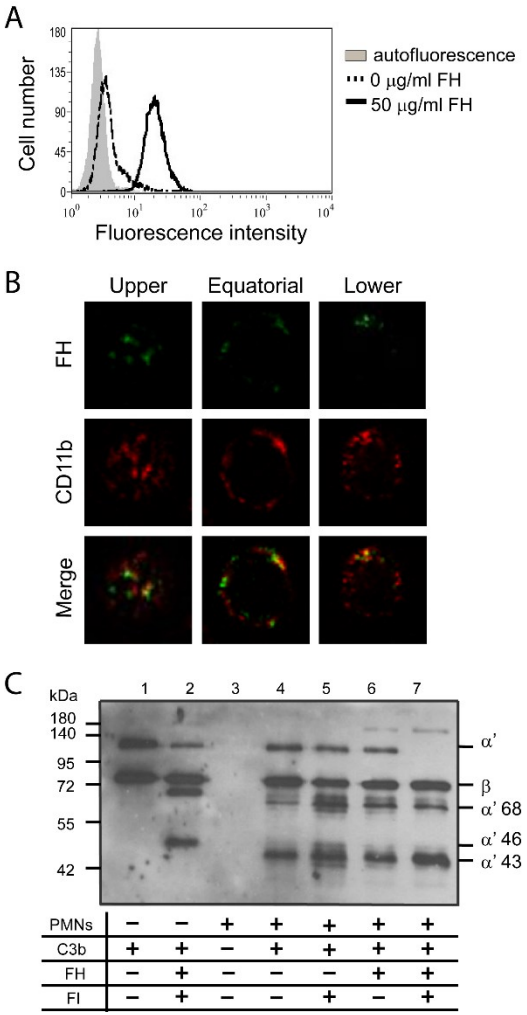

A
Cell number
Fluorescence intensity
autofluorescence
0 μg/ml FH
50 μg/ml FH
B
Upper
Equatorial
Lower
FH
CD11b
Merge
C
kDa
180
140
95
72
55
42
α'
β
α' 68
α' 46
α' 43
PMNs
C3b
FH
FI

Fig. 2.

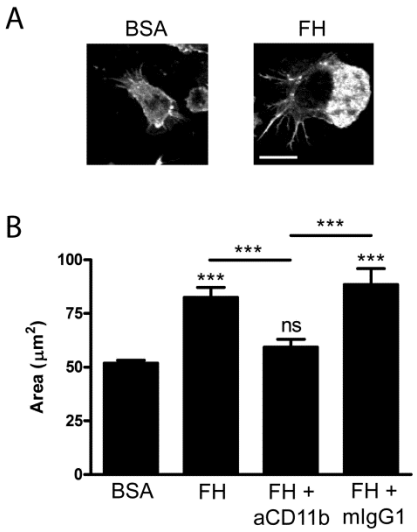

A
BSA
FH
B
Area (μm²)
***
ns
BSA
FH
FH + aCD11b
FH + mIgG1

Fig. 3.

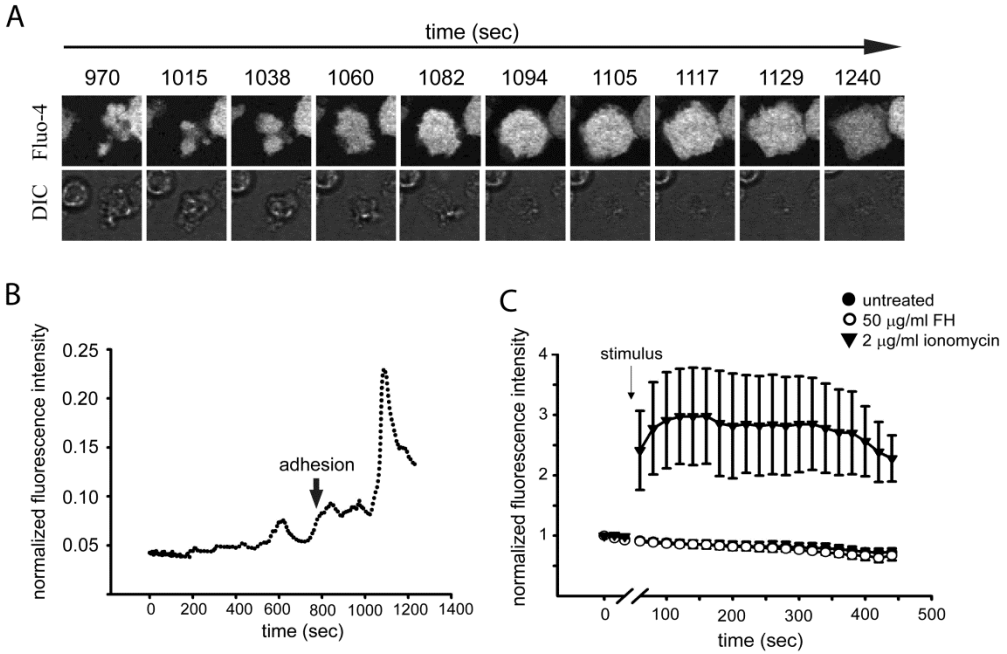


Fig. 4.

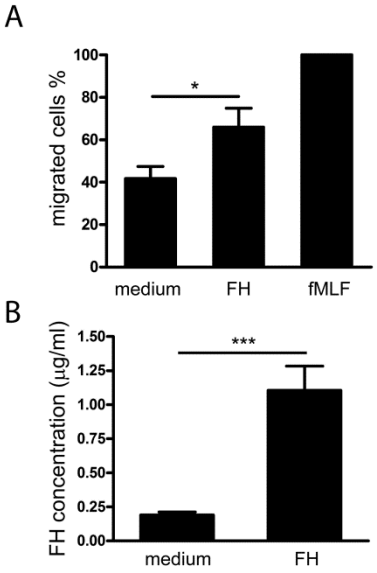


Fig. 5.

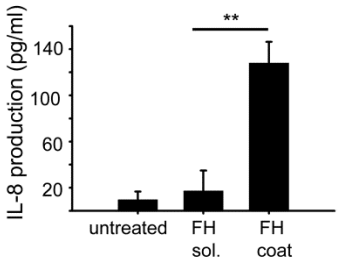


Fig. 6.

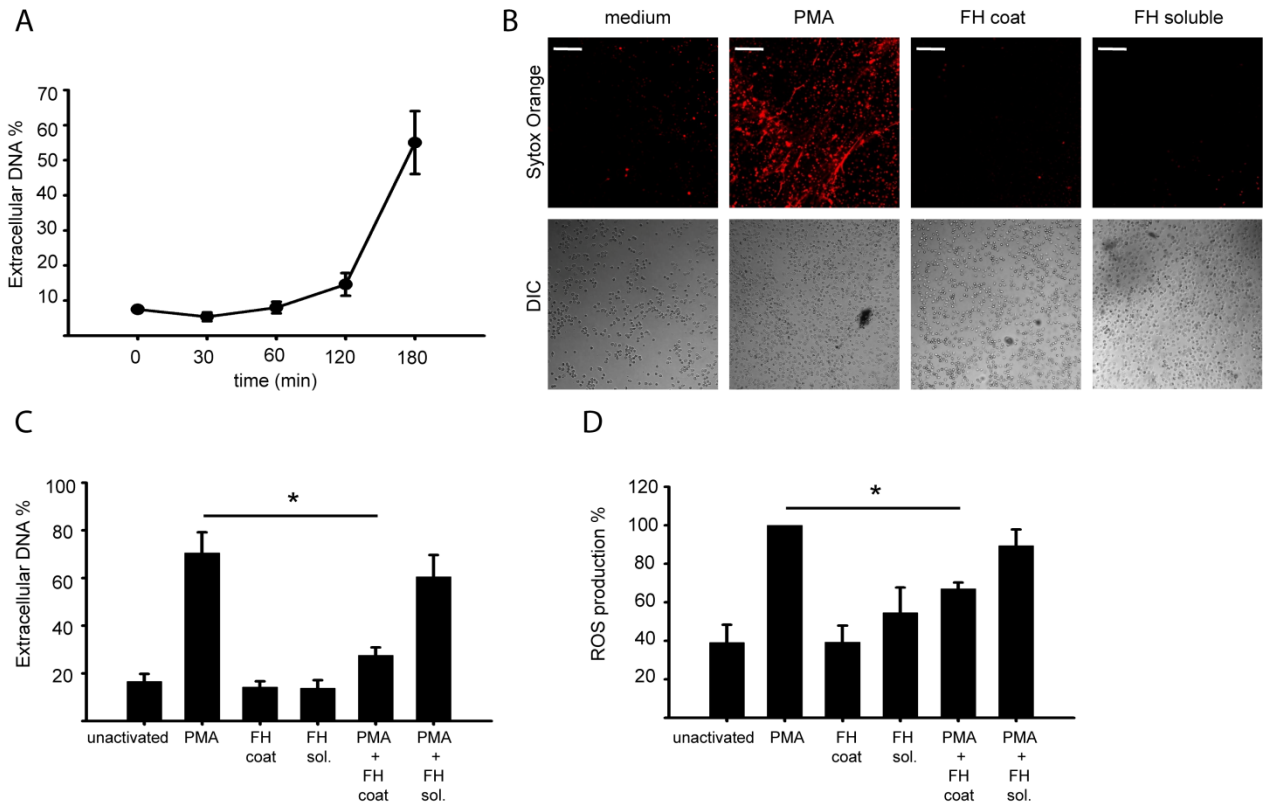
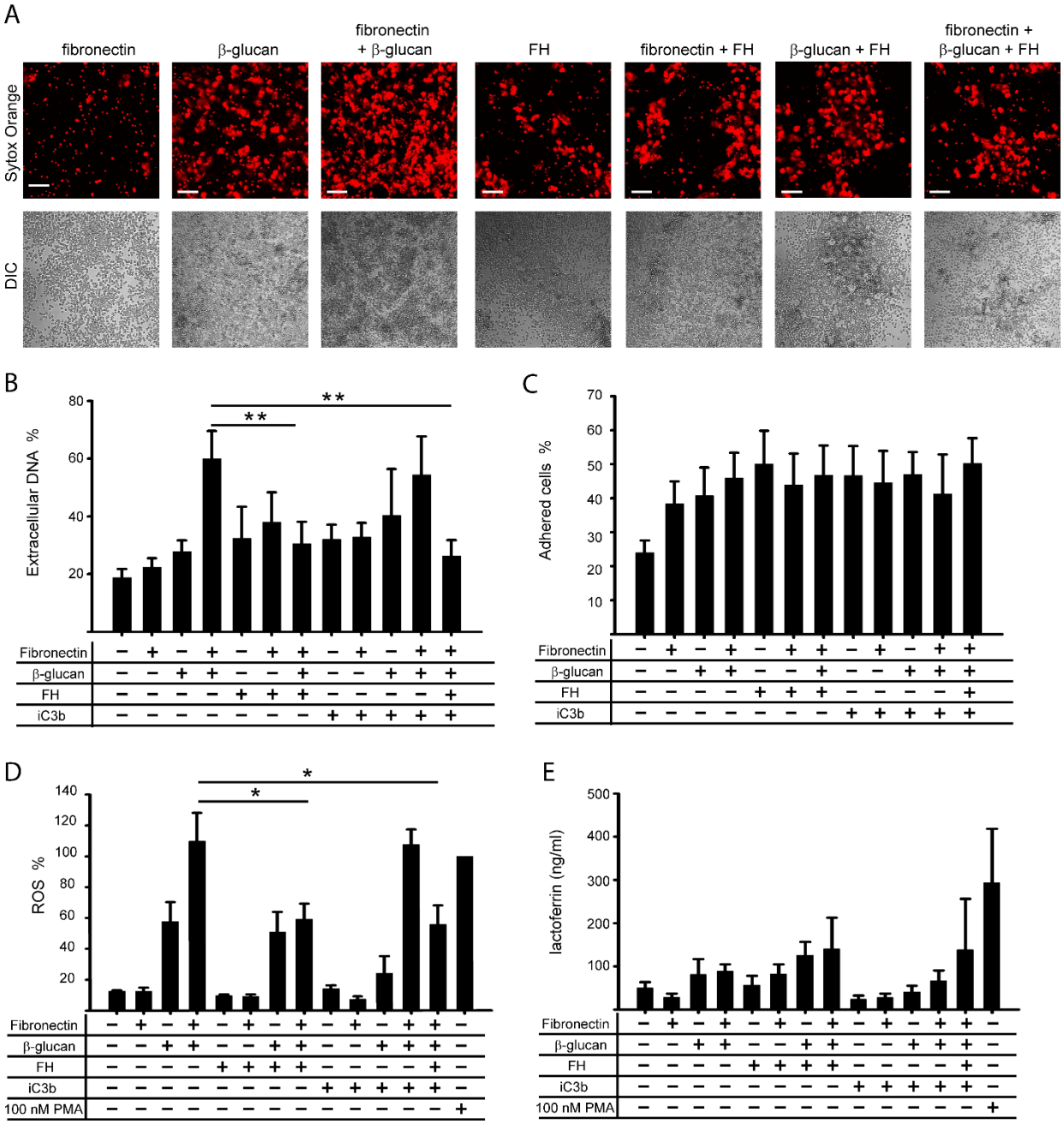

A
Extracellular DNA %
70
60
50
40
30
20
10
0
30
60
120
180
time (min)
B
medium
PMA
FH coat
FH soluble
Sytox Orange
DIC
C
Extracellular DNA %
100
80
60
40
20
*
unactivated
PMA
FH coat
FH sol.
PMA + FH coat
PMA + FH sol.
D
ROS production %
120
100
80
60
40
20
*
unactivated
PMA
FH coat
FH sol.
PMA + FH coat
PMA + FH sol.

Fig. 7.


A
fibronectin
β-glucan
fibronectin + β-glucan
FH
fibronectin + FH
β-glucan + FH
fibronectin + β-glucan + FH
Sytox Orange
DIC
B
Extracellular DNA %
80
60
40
20
**
**
Fibronectin
β-glucan
FH
iC3b
C
Adhered cells %
70
60
50
40
30
20
10
Fibronectin
β-glucan
FH
iC3b
D
ROS %
140
120
100
80
60
40
20
*
*
Fibronectin
β-glucan
FH
iC3b
100 nM PMA
E
lactoferrin (ng/ml)
500
400
300
200
100
Fibronectin
β-glucan
FH
iC3b
100 nM PMA

Complement factor H modulates the activation of human neutrophil granulocytes and the generation of neutrophil extracellular traps

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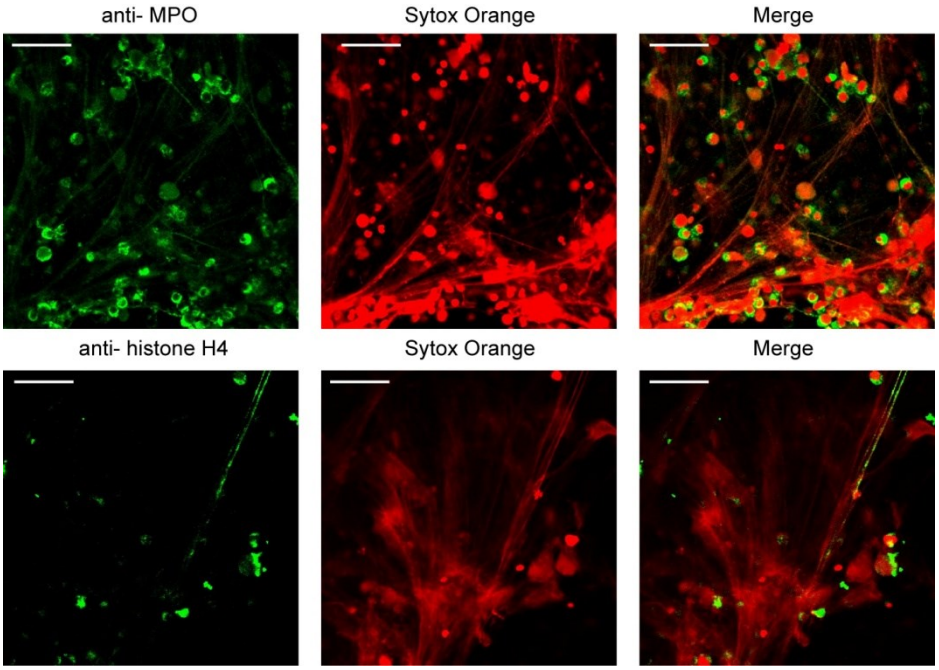

Figure S1. Verification of NETs.
The representative microscopic images show neutrophils stimulated with 100 nM PMA. The decondensed nuclei were counterstained with Sytox Orange (red) after 180 min incubation with PMA. After fixation, staining was performed with primary antibody directed against MPO and Alexa647-conjugated secondary antibody (green, upper panel), or with primary antibody against citrullinated histone H4 and Alexa488-conjugated secondary antibody (green, lower panel). Images were captured with 60x objective. Original scale bars, 50 µm.