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Inhibition of Alternative and Terminal Complement Pathway Components Modulate the Immune Response Against Bacteria and Fungi in Whole Blood

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Keywords: *Aspergillus fumigatus* | blood | C3aR | C5aR1 | C5aR2 | complement system | *Escherichia coli* | inflammation

ABSTRACT

Complement activation plays a critical role in the inflammatory response to *Escherichia coli* and *Aspergillus fumigatus* conidia. However, the specific contributions of complement components, including anaphylatoxin receptors, remain unclear. Using an ex vivo lepirudin whole blood model, we examined the activation of all three complement pathways (C4c, C3bc, and sC5b-9) induced by these microbes. We also assessed granulocyte and monocyte receptor expression of CD11b, CD64, C3aR, C5aR1, and C5aR2, along with phagocytosis, leukocyte activation (MPO), and cytokine release. Additionally, we investigated selective inhibition of complement components FD, C3, C5, and C5aR1. Both microbes increased complement activation products (C3bc and sC5-9), CD11b and CD64 expression, MPO release, and proinflammatory cytokines (IL-1β, IL-6, IL-8, and TNF), while decreasing C3aR, C5aR1, and C5aR2 expression. Complement inhibition reduced CD11b and CD64 expression and partially restored C3aR and C5aR1 levels, with minimal effects on C5aR2. FD, C3, and C5 inhibition reduced downstream complement markers, with FD and C3 inhibition also reducing phagocytosis, and only C3 inhibition reducing MPO release. The cytokine response varied by microbe: *E. coli* triggered higher proinflammatory cytokines, and FD and C3 inhibition generally reduced cytokine release, while C5 inhibition was less effective. Interestingly, *A. fumigatus*-induced cytokines significantly increased with C5aR1 inhibition, highlighting immune response differences related to C5aR1 signalling in bacterial versus fungal infections. In conclusion, regulation of inflammation through FD, C3, C5, and C5aR1 underscores the immunoregulatory role of the complement system in anti-microbial immune responses.

Abbreviations: C, complement component; C3aR, complement C3a receptor; C5aR, complement C5a receptor; CCL, C-C motif chemokine ligand; CD, cluster of differentiation; CXCL, C-X-C motif chemokine ligand; F(ab), antigen-binding fragment; FD, Factor D; HI, heat-inactivated; HRP, horseradish peroxidase; Ig, immunoglobulin; IL, interleukin; INF, interferon; mAb, monoclonal antibody; MCP, monocyte chemoattractant protein; MFI, median fluorescence intensity; MIP, macrophage inflammatory protein; MPO, myeloperoxidase; pAb, polyclonal antibody; PRM, pattern recognition molecule; sC5b-9, soluble C5b-9 complex; SD, standard deviation; T120, incubation control 120 min (incubation, no stimulation); T120stim, Stimulation control 120 min (incubation, stimulation); T30, Incubation control 30 min (incubation, no stimulation); T30stim, stimulation control 30 min (incubation, stimulation); Tb, baseline control (no incubation, no stimulation); TNF, tumour necrosis factor.

Ida Mariegaard, Beatrice Fageräng, and Laura Pérez-Alós should be regarded as shared second authors. Peter Garred and Anne Rosbjerg should be regarded as shared last authors.

1 | Introduction

The complement system is a key player in humoral innate immunity and is comprised of a multitude of soluble and membrane-bound proteins [1]. Its main immunological functions include the recognition and removal of pathogens and damaged host cells and the attraction and activation of immune cells [1, 2]. Initial activation of the complement system upon pathogen exposure is triggered by the recognition of the microbes by different pattern recognition molecules (PRMs) belonging to the classic and lectin pathways [1, 3]. The cascade is enforced by the alternative pathway amplification loop, which can generate a many-fold increase in complement component C3 processing depending on the rate-limiting protein factor D (FD) [2, 4]. C3 cleavage creates the opsonin C3b and smaller fragment C3a [2]. Further sequential protein cleavage in the complement cascade generates another small immunologically active protein C5a, and together, C3a and C5a are termed anaphylatoxins [5]. Anaphylatoxins regulate the immune response by attracting granulocytes and monocytes to the site of complement activation and activating immune cells in general, leading to changes in cell surface marker expression and cell physiology [2]. However, despite similar structural properties of anaphylatoxins C3a and C5a [6], immune cells react differently to exposure to C5a compared to C3a [2], as conclusively demonstrated for neutrophils in terms of changes in cell physiology and cell surface marker expression [7]. C5a signals through complement C5a receptor 1 (C5aR1) and complement C5a receptor 2 (C5aR2), and C3a activates complement C3a receptor (C3aR) [2]. Signalling through anaphylatoxin receptors has been described to modulate the inflammatory response [2, 8–10]. Especially, the activation of C5aR1 has been associated with a strong pro-inflammatory response [8]. However, an anti-inflammatory effect of C5aR1 signalling in a mouse sepsis model was recently described as well [10]. Similarly, C5aR2 is associated with a pro-inflammatory response during experimental sepsis but has also been described as a regulator of C5aR1 expression [8, 11]. In contrast to C5aR1, activation of C3aR has been described as associated with an anti-inflammatory response; however, during chronic inflammation, C3aR signalling has been described as pro-inflammatory [2, 8]. In addition, C5aR1, and partly C5aR2, have been extensively reviewed for their role as potential anti-inflammatory targets, whereas much less is known about the function of C3aR [8, 9].

The involvement of the complement system in regulating the inflammatory response and disease-related aberrations in complement function has led to the establishment of multiple complement-targeting drugs [12, 13]. For example, targeting C5 via eculizumab has been successful in improving the disease severity of multiple complement-mediated diseases, such as atypical hemolytic uremic syndrome [14] and paroxysmal nocturnal hemoglobinuria [15]. In addition, dual inhibition of the complement system and CD14 has shown promising results for dampening inflammation in microbe-stimulated whole blood, as demonstrated for *Escherichia coli* [16], *Staphylococcus aureus* [17], and *Aspergillus fumigatus* [18]. However, targeting the complement system must be considered carefully, as complement inhibition may have clinical implications on anti-microbial defensive mechanisms. Supporting this notion, susceptibility to certain infections in patients with primary or acquired complement deficiencies, such as FD, C3, and C5, has been described [19]. While inhibition of C5 has mainly been connected to meningococcal infections [20], it appears that C5-targeted treatment can also be associated with systemic fungal infections [21]. Hence, a functional complement system, including key components, such as C3 and C5, appears to be essential in particular for immune-mediated protection regarding certain fungal pathogens [22, 23]. In these years, complement-targeting drugs are in vast development [24]; hence, it is important to consider these potential risks.

Here, we investigated and compared the inflammatory response to the bacterium *E. coli* and the fungus *A. fumigatus* in human whole blood and investigated the contribution of key complement components from the alternative pathway and the terminal pathway (FD, C3, C5, and C5aR1) by focusing on granulocyte and monocyte surface expression of activation markers (CD11b and CD64) and anaphylatoxin receptors (C3aR, C5aR1, and C5aR2). In addition, we investigated the role of complement activation in MPO secretion and cytokine release to understand the implications of complement-targeted therapies in infection by bacteria and fungi.

2 | Methods

2.1 | Preparation of Heat-Inactivated *Aspergillus fumigatus* Conidia

The *A. fumigatus* strain 6871 was obtained by kind donation from Luigina Romani (Institute of Infectious Diseases, University of Perugia, Italy). The strain was clinically isolated from a case of invasive aspergillosis that caused patient death. *A. fumigatus* cultivation was conducted on Sabouraud glucose agar with chloramphenicol (Merck, Darmstadt, Germany, cat. no.: 89579), which was prepared according to the manufacturer's instructions. Agar-containing Petri dishes (VWR, Radnor, PA, USA, cat. no.: 391-0468P) were inoculated with *A. fumigatus* and incubated at 37°C for approximately a week. *A. fumigatus* conidia were harvested subsequently with 10 mL of PBS (Region H Apoteket, Copenhagen, Denmark, cat. no.: 864485), with added 0.01% Tween 20 (Merck, cat. no.: 8221840050) (PBS/0.01% Tween 20) per Petri dish. The final cell suspension was purified with a 40 μm filter (VWR, cat. no.: 732-2757P), to remove residual fungal hyphae. Afterwards, the cell suspension was centrifuged (1400×g, 10 min, at room temperature [RT]) and the supernatant was removed. Following, the cell pellet was washed three times with 10 mL of PBS/0.01% Tween 20. Thereafter, the pelleted cells were resuspended in PBS/0.01% Tween 20 and filtered with a 10 μm filter (pluriSelect, Leipzig, Germany, cat. no.: 43-10010-50), to remove aggregated and germinated conidia. The cell concentration was then microscopically determined and adjusted to 1×10^9 cells/mL. Following, purified *A. fumigatus* conidia were heat-inactivated (HI) at 121°C/2 bar for 20 min by autoclaving. Finally, HI *A. fumigatus* conidia were aliquoted and stored at 4°C.

2.2 | Preparation of Heat-Inactivated *Escherichia coli*

HI *E. coli* (American Type Culture Collection, Manassas, VA, USA, cat. no.: 33572, strain LE392) were kindly supplied by Tom Eirik Mollnes (Department of Immunology, Oslo University

Hospital and University of Oslo, Oslo, Norway; Research Laboratory, Nordland Hospital, Bodø, Norway). In brief, *E. coli* were cultured with LB medium (ATCC Medium 1065), washed with sterile PBS, heat-inactivated at 60°C for 1 h, and then frozen for long-term storage. For usage, frozen bacteria are thawed and repeatedly washed with sterile PBS. Afterwards, bacteria are resuspended in sterile PBS, adjusted to 1×10^9 cells/mL, and stored at 4°C.

2.3 | FITC-Conjugation of HI *Aspergillus fumigatus* Conidia and HI *Escherichia coli*

HI *A. fumigatus* conidia and HI *E. coli* were added to 15 mL sterile falcon tubes (Sarstedt, Nümbrecht, Germany, cat. no.: 62.554.502), containing 5 mL of 1 M sodium bicarbonate buffer (Merck, cat. no.: S5761; in-house adjusted to pH 9.0). Following this, HI *A. fumigatus* conidia and HI *E. coli* were labelled with an in-assay concentration of 8.26 and 4.13 μg/mL of FITC (Merck, cat. no.: F7250) respectively, which was prediluted in sterile DMSO (Merck, cat. no.: D8418) and added to the respective solution. Tubes were agitated for 30 min at RT in the dark. Continuing, the cells were pelleted by centrifugation (HI *A. fumigatus* conidia: 1000 × *g*, 6 min, at RT; HI *E. coli*: 3000 × *g*, 10 min, at RT), and the supernatant was removed. Cells were washed three times as described before, using sterile PBS/0.01% Tween 20 (for HI *A. fumigatus* conidia) or sterile PBS (for HI *E. coli*). The respective previously mentioned buffer was used to ultimately resuspend pelleted cells. The final FITC-labelled HI *A. fumigatus* conidia suspension was counted via a haemocytometer. The concentration of FITC-labelled HI *E. coli* bacteria was determined via spectrometry (Optical Density (OD), 600 nm), by comparing cell suspension absorption to a known standard. Cell suspensions were aliquoted and stored at 4°C.

2.4 | Lepirudin Whole Blood Model

A previously documented lepirudin whole blood model [25] was utilised to determine the effects of selective complement inhibition in human blood responding to a bacterial or fungal challenge. Blood was drawn into blood vials (Greiner Bio-One, Kremsmünster, Austria, cat. no.: 454241) containing lepirudin (Refludan) (Celgene, Windsor, UK) at the final concentration of approximately 50 μg/mL. Blood was immediately stored on wet ice after sampling. Within 1 h of blood sampling, 390 μL of blood was added to sterile 1.8 mL tubes (Thermo Fisher Scientific, Waltham, MA, USA, cat. no.: 368632) within an ice-water bath, containing 45 μL of one of the following (in-assay concentrations, residual volumes filled with DPBS (Merck, cat. no.: D8662)): DPBS (added to baseline (Tb), incubation (T30), and stimulation control (T30stim)), 0.2 μM IgG1k isotype monoclonal antibody (mAb)-based $F(ab')_2$ fragment (BioLegend, San Diego, CA, USA, cat. no.: 401408; mock antibody control), 0.2 μM FD inhibitory mAb 31A9-based $F(ab')_2$ fragment (Genentech, South San Francisco, CA, USA), 10 μM C3 inhibitor compstatin-analog CP40 ([26]; kindly provided by John D. Lambris), 0.67 μM C5 inhibitor eculizumab (Soliris; Alexion Pharmaceuticals, Boston, MA, USA), 0.2 μM C5aR1 inhibitory mAb 18–41-6-based $F(ab')_2$ fragment ([27]; produced in-house). Prior processing of mouse mAb inhibitors into $F(ab')_2$ fragments was conducted via a kit (Thermo Fisher Scientific, cat. no.: 44980). Samples were incubated for 5 min within the ice-water bath. Following, samples were stimulated by adding either 15 μL of DPBS (added to baseline control (Tb) and incubation controls (T30 and T120)), or a final concentration of 1×10^7 HI *E. coli* (FITC-labelled) or 1×10^7 HI *A. fumigatus* conidia (FITC-labelled). Afterwards, samples, except Tb, were transferred to a blood tube roller (Boule Medical, Spånga, Sweden, Mixer 820) at 37°C and incubated for 30 min (T30 samples; flow cytometric analysis, plasma analysis of complement activation and MPO expression) or 120 min (T120 samples; plasma analysis of cytokine expression), respectively. A baseline control (Tb; no stimulation, no incubation) and two incubation controls (T30/T120; no stimulation, incubation) were included. After incubation, samples were transferred back to the ice-water bath, and 20 μL of blood (Tb, T30 samples) was transferred to a respective well of a microplate (Corning, cat. no.: 3799) on ice (six times per sample for all FACS staining conditions), containing 20 mM EDTA (Merck, cat. no.: 03690, pH adjusted in-house to 7.4) and 10.3 mM sodium citrate (Becton Dickinson, Franklin Lakes, NJ, USA, cat. no.: 367714) at a final concentration. The treated blood samples (Tb, T30 samples, T120 samples) were stopped afterward with an in-assay concentration of 20 mM EDTA and centrifuged (3000 × *g*, 15 min, at 4°C) to obtain plasma. Purified plasma was aliquoted and stored at −80°C. The blood-containing microtiter plate was rested at RT for 10 min before treatment with 250 μL of red blood cell (RBC) lysis buffer (Thermo Fisher Scientific, cat. no.: HYL250) per well for 10 min at RT in the dark. Following, the sample-containing microtiter plate was centrifuged (500 × *g*, 5 min, at 4°C). After centrifugation, the microtiter plate was decanted and pressed on paper towels to remove residual supernatant. Subsequently, the microtiter plate was washed three consecutive times (500 × *g*, 5 min, at 4°C) with 200 μL of DPBS (Merck, cat. no.: D8537), with added 0.5% bovine serum albumin (BSA) (Merck, cat. no.: A8327) (DPBS/BSA). Samples were first blocked by addition of 40 μL of diluted human IgG isotype control (Thermo Fisher Scientific, cat.: 02-7102) at an in-assay concentration of 200 μg/mL (after application of staining antibodies) and incubated for 15 min on ice in the dark, followed by addition of 40 μL of the respective stain master mix, containing 0.5 μL CD14 BB700 (Becton Dickinson, cat. no.: 566465), 0.25 μL CD15 BV421 (BioLegend, cat. no.: 323040), 1 μL CD45 SBV790 (Bio-Rad Laboratories, cat. no.: MCA87SBV790), and either no additional PE-conjugated antibody (PE Fluorescence Minus One (FMO) control; master mix 1), 5 μL CD11b PE (BioLegend, cat. no.: 301306; master mix 2), 5 μL C5aR1 PE (BioLegend, cat. no.: 344304; master mix 3), 5 μL C3aR PE (BioLegend, cat. no.: 345804; master mix 4), 5 μL C5aR2 PE (BioLegend, cat. no.: 342404; master mix 5), or 5 μL CD64 PE (BioLegend, cat. no.: 399504; master mix 6). Samples were stained for 15 min on ice in the dark. Following, samples were washed four consecutive times with 200 μL of DPBS/BSA as described before. Finally, samples were resuspended in 200 μL DPBS/BSA, kept on ice in the dark, and analysed via a BD FACSCelesta flow cytometer (Becton Dickinson, standard configuration BVYG) by recording non-gated events for 60 s per sample on medium speed. Compensation was conducted with beads (Thermo Fisher Scientific, cat. no.:

01-3333-42), using one of the following antibodies for staining per compensation control: CD11b PE (BioLegend, cat. no.: 301306), CD14 BB700 (Becton Dickinson, cat. no.: 566465), CD15 BV421 (BioLegend, cat. no.: 323040), CD45 SBV790 (Bio-Rad Laboratories, cat. no.: MCA87SBV790), and CD66b FITC (BioLegend, cat. no.: 305104; used as a surrogate to compensate for any FITC-related fluorescence spillover).

2.5 | Analysis of Soluble Activation Products

2.5.1 | C4c, C3bc, and sC5b-9

384-well ELISA plates (Thermo Fischer Scientific, cat. no.: 464718) were coated by adding 25 μL of 2 μg/mL C3b-binding mAb BH6 ([28]; used for C3bc assay, produced in-house), 2 μg/mL C4c-binding mAb 99-72-18 ([29]; used for C4c assay, produced in-house), or 2 μg/mL C9-binding mAb aE11 ([30]; used for sC5b-9 assay, produced in-house) in PBS (Region H Apoteket, cat. no.: 864485) to the respective plates/wells. Plates were incubated overnight at 4°C. On the experiment day, plates were washed three times with PBS with 0.05% Tween 20 added (PBS/0.05% Tween 20). Samples and standards were added to the respective plates and wells after diluting them 1:2000 (C3bc assay), 1:40 (C4c assay), and 1:5 (sC5b-9 assay) in sample buffer (PBS, with added 0.05% Tween 20, 20 mM EDTA (Merck, cat. no.: EDS-500G), 0.5% bovine serum (Thermo Fisher Scientific, cat. no.: 16170086), and 1 μg/mL mouse polyclonal IgG isotype control (Thermo Fisher Scientific, cat. no.: 10400C)). All assays were conducted using a zymosan-activated serum standard (ELISA standard). Plates were incubated in the following manner: no plate agitation, 60 min, at 4°C (C3bc assay); plate agitation, 60 min, at RT (C4c assay); plate agitation, 90 min, at RT (sC5b-9 assay). Afterwards, plates were washed three times with PBS/0.05% Tween 20 before 25 μL of the following detection antibody was added to each respective plate/well: 1 μg/mL rabbit anti-human C3c polyclonal (pAb) (C3bc assay; Agilent, Santa Clara, CA, USA, cat. no.: A0062), 0.4 μg/mL rabbit anti-human C4c pAb (C4c assay; Agilent, cat. no.: Q0369), 2 μg/mL biotinylated mouse anti-human C6 sC5b-9 mAb 9C4 (sC5b-9 assay; [31]; produced in-house). All assay plates were incubated under agitation for 90 min at RT. Following, all assay plates were washed three times with PBS/0.05% Tween 20. Thereafter, 25 μL of the respective secondary antibody/reagent was added to each assay plate at the dilution of 1:2000 in PBS/0.05% Tween 20: swine anti-rabbit-horseradish peroxidase (HRP) conjugated pAb (C3bc/C4c assay; Agilent, cat. no.: P0399), or Streptavidin-HRP conjugated (sC5b-9 assay, Cytiva, Marlborough, MA, USA, cat. no.: RPN1231V). Assay plates were incubated under agitation for 60 min at RT. Subsequently, plates were washed three times with PBS/0.05% Tween. Finally, 25 μL of TMB One (Kementec, Taastrup, Denmark, cat. no.: 4380A) was added to each plate/well and assay plates were developed for 15 min at RT. The reaction was stopped by the addition of 25 μL of 0.3 M sulfuric acid (Kementec, cat. no.: 4695A). Assay plates were analysed at 450 nm and 630 nm OD (corrected signal: 450–630 nm) with a plate reader (Synergy HT absorbance reader; BioTek Instruments, Winooski, VT, United States). An unstimulated lepirudin sample was used as an internal control between plates. The plates were handled by the Biomek NX^{P} Automated Workstation (Beckman Coulter, Brea, CA, United States).

2.5.2 | MPO

MPO expression was determined with the "Human Myeloperoxidase DuoSet ELISA" kit (R&D systems, Minneapolis, MN, cat. no.: DY3174), according to the supplier's recommendations. All additional required reagents were obtained from the manufacturer (R&D systems, cat. no.: DY008B). An unstimulated lepirudin plasma sample was used as an internal control between plates. Samples and ELISA standard were added to the respective plates and wells, after diluting them 1:100 in diluent reagent. Assay plates were developed for 4 min at RT. Assay plates were analysed at 450 nm and 540 nm OD (corrected signal: 450–540 nm) using the Synergy HT absorbance reader.

2.5.3 | Cytokines

Cytokine expression was analysed with the "Bio-Plex Pro Human Cytokine 27-plex Assay" kit (Bio-Rad Laboratories, cat. no.: M500KCAF0Y) according to the manufacturer's instructions, as previously reported [32]. Sample analysis was conducted with a Luminex 200 System (Luminex, Austin, TX, USA).

2.6 | Data Analysis

FACS data was processed via "FlowJo" (version 10.10.0), by using the indicated set of gates (Figure S1). The following formula was utilised to assess phagocytosis: Phagocytic Index = percentage of FITC-positive cells × FITC median fluorescence intensity (MFI) of the FITC-positive cell population. Estimated complement activation and MPO levels were interpolated by regression analysis using a four-parameter logistic curve fitting via "GraphPad Prism" (version 10.1.2 (324)). Complement activation results were given in Complement Arbitrary Units per mL (CAU/mL), whereas MPO and cytokine results were given in pg/mL. Statistical analyses were conducted by one-way ANOVA, utilising matched data, assumed Gaussian data distribution, and non-assumed sphericity as options ("RM one-way ANOVA, with the Geisser-Greenhouse correction") and Fisher's LSD multiple comparisons, with individual variances computed for each comparison, using the individual stimulation control as a point for comparison.

3 | Results

3.1 | Analysis of Complement Activation Products C4c, C3bc, and sC5b-9

Whole blood stimulation with *E. coli* and *A. fumigatus* conidia led to a substantial increase in observed C3bc and sC5b-9 levels in plasma, while C4c levels seemed unaffected (Figure 1). Inhibition of C3; however, resulted in an increase in C4c (only significant for *E. coli*, Figure 1A). Observed levels of C3bc were strongly receptive

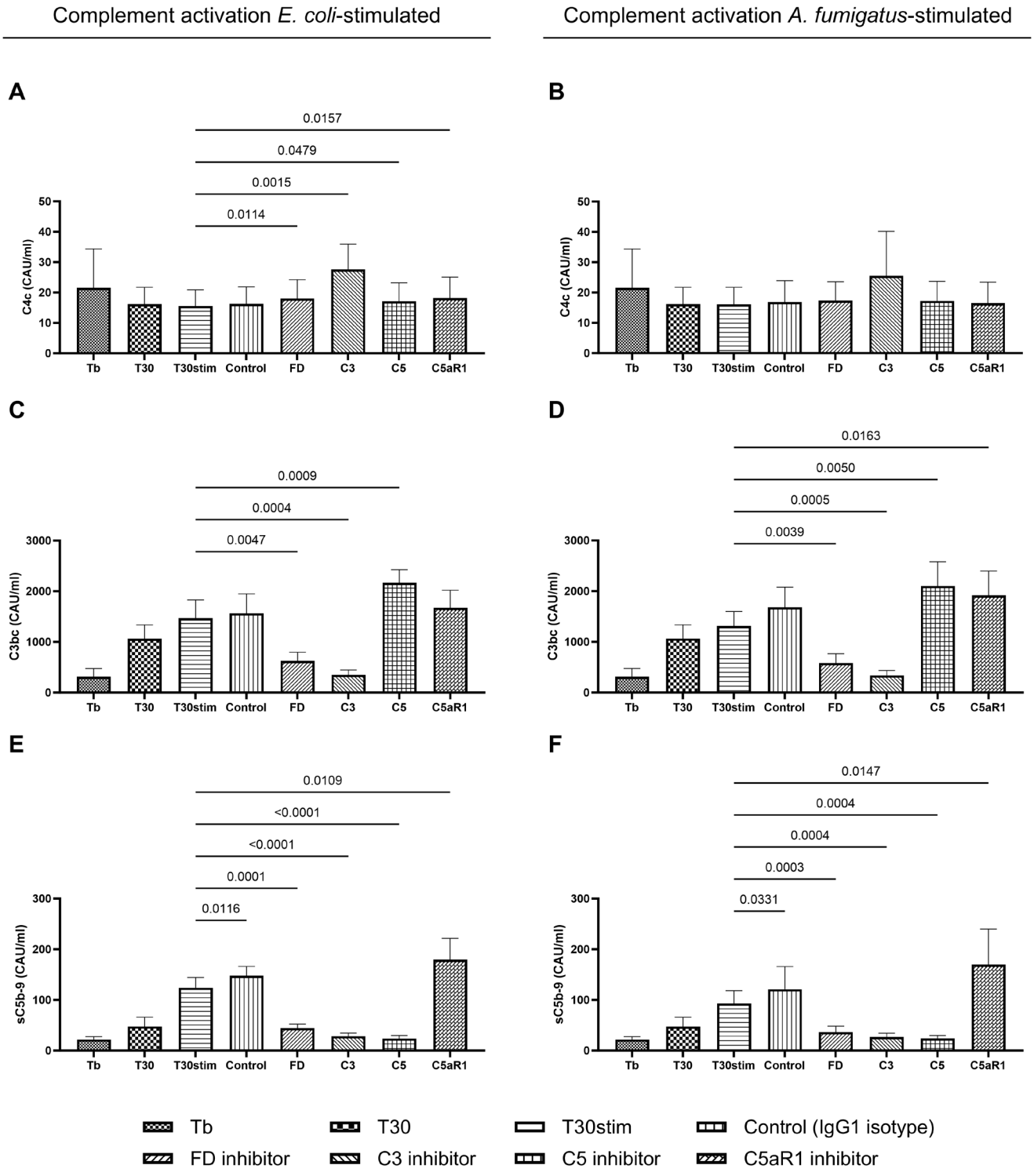

FIGURE 1 | Complement activation in *E. coli* and *A. fumigatus*–stimulated whole blood. Analysis of complement activation in whole blood, as assessed by activation products C4c (A, B), C3bc (C, D), and sC5b-9 (E, F), utilising either *E. coli* (A, C, E) or *A. fumigatus* conidia (B, D, F) as a stimulating agent. Multiple controls, including a baseline control (Tb; no incubation, no stimulation), an incubation control (T30; incubation, no stimulation), and a stimulation control (T30stim; incubation, stimulation) are included to assess the model-dependent complement activation progression. Individually targeted components of the complement system include FD, C3, C5, and C5aR1, with an IgG1 control for reference. The presented data are based on six individual repeats featuring a different healthy donor per repeat. Data are presented as mean with one-sided SD for better visibility.

to complement targeting, leading to a profound and significant reduction in C3bc after inhibition of FD and C3, while inhibition of C5 led to a significant C3bc increase in whole blood stimulated with either *E. coli* or *A. fumigatus* conidia (Figure 1C,D). In addition, inhibition of C5aR1 significantly increased C3bc levels in *A. fumigatus* conidia-stimulated whole blood. Similarly, recorded levels of sC5b-9 depended on the type of complement inhibition, with inhibition of FD, C3, and C5 profoundly and significantly

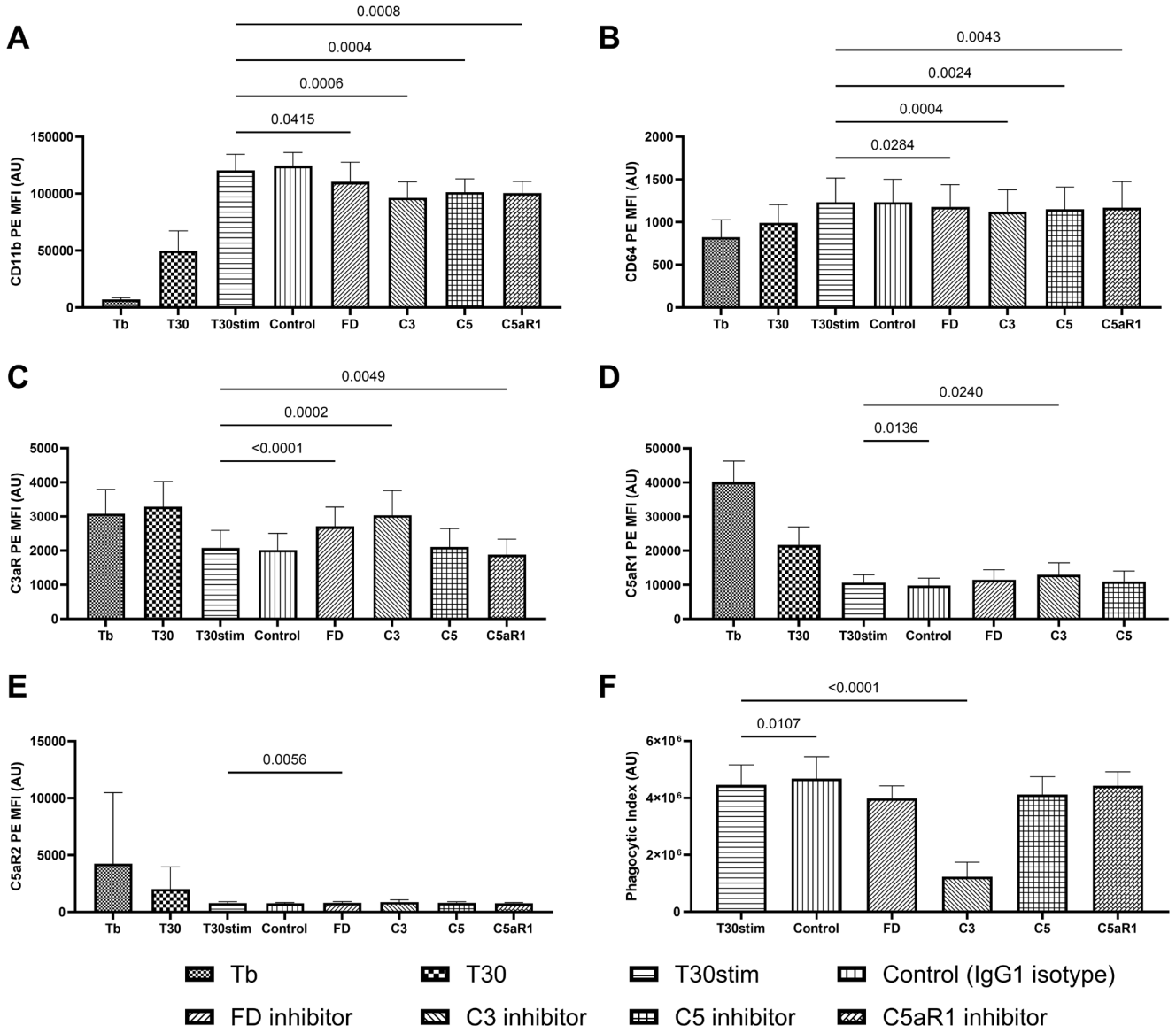

FIGURE 2 | Granulocyte receptors and phagocytosis in *E. coli*-stimulated whole blood. Investigation of whole blood activation markers (CD11b and CD64), as well as anaphylatoxin receptor expression (C3aR, C5aR1, and C5aR2) on granulocytes, as well as phagocytosis (Phagocytic Index), after whole blood stimulation with FITC-labelled *E. coli* (A–F). Several controls, including a baseline control (Tb; no incubation, no stimulation), an incubation control (T30; incubation, no stimulation), and a stimulation control (T30stim; incubation, stimulation) are included to assess the model-dependent marker progression. Individually targeted components of the complement system include FD, C3, C5, and C5aR1, with an IgG1 control for reference. The presented data are based on six individual repeats featuring a different healthy donor per repeat. Data are presented as mean with one-sided SD for better visibility.

reducing measured sC5b-9 levels regardless of the microbe, while inhibition of C5aR1 led to a significant increase in observed sC5b-9 readout (Figure 1E,F). Investigation of the C5aR1 inhibitory mAb 18-41-6-based $F(ab')_2$ fragments revealed a small remaining residue of non-processed antibodies, likely increasing complement activation through binding to cellular C5aR1.

3.2 | Analysis of Granulocytic and Monocytic Blood Activation Markers, Anaphylatoxin Receptor Expression, and Phagocytosis

Exposure of whole blood to *E. coli* and *A. fumigatus* conidia resulted in an upregulation of investigated blood activation markers CD11b and CD64 (Figures 2A,B–5A,B), as well as a general down-regulation of anaphylatoxin receptors C3aR, C5aR1, and C5aR2 (Figures 2C–E–5C–E). The exposure also resulted in the interaction of granulocytes and monocytes with *E. coli* and *A. fumigatus* conidia shown by the Phagocytotic Index (Figures 2F–5F).

Inhibition of FD, C3, and C5, and C5aR1 led to a significant reduction of CD11b expression on granulocytes stimulated with *E. coli* or *A. fumigatus* conidia (Figures 2A and 3A). Reduction of CD11b on monocytes by complement inhibitors was less pronounced, with a significant reduction of CD11b expression observed for whole blood inhibition with FD, C3, and C5, but not C5aR1 (Figures 4A and 5A).

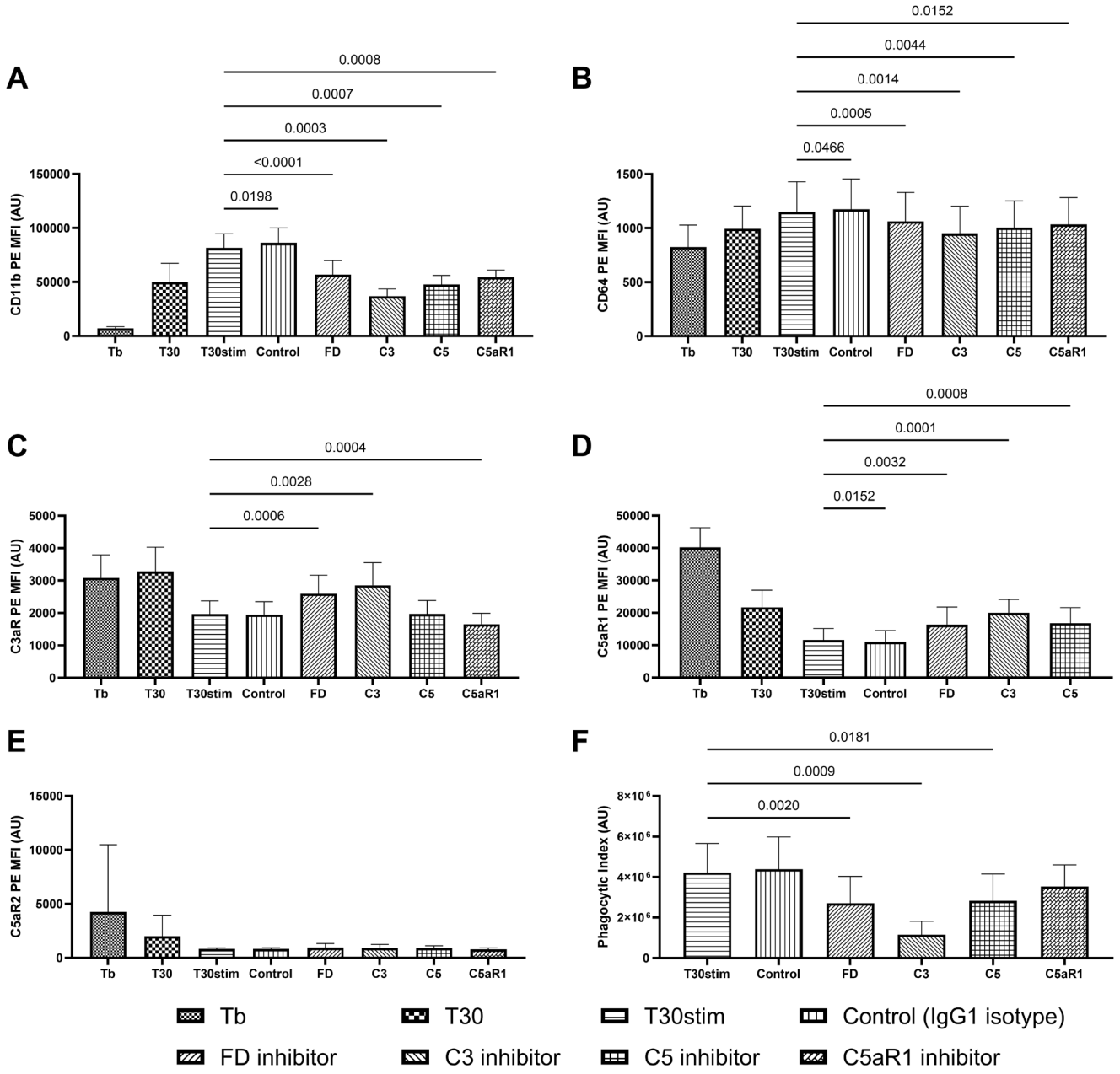

FIGURE 3 | Granulocyte receptors and phagocytosis in *A. fumigatus* conidia-stimulated whole blood. Investigation of whole blood activation markers (CD11b and CD64), as well as anaphylatoxin receptor expression (C3aR, C5aR1, and C5aR2) on granulocytes, as well as phagocytosis (Phagocytic Index), after whole blood stimulation with FITC-labelled *A. fumigatus* conidia (A–F). Several controls, including a baseline control (Tb; no incubation, no stimulation), an incubation control (T30; incubation, no stimulation), and a stimulation control (T30stim; incubation, stimulation) are included to assess the model-dependent marker progression. Individually targeted components of the complement system include FD, C3, C5, and C5aR1, with an IgG1 control for reference. The presented data are based on six individual repeats featuring a different healthy donor per repeat. Data are presented as mean with one-sided SD for better visibility.

The reduction of granulocytic CD64 upregulation by complement inhibitors followed a similar trend compared to the CD11b profile, with a significant reduction of CD64 expression observed when whole blood was exposed to FD, C3, C5, or C5aR1 inhibition, while no relevant significant effects were observed for monocytic CD64 expression (Figures 2B–5B).

Complement inhibition in microbe-stimulated whole blood affected the expression of the anaphylatoxin receptors differently depending on the inhibitor. Complement inhibition at the level of FD and C3 strongly and significantly improved the expression of C3aR, for which the expression seemed to be largely unaffected by incubation conditions (comparison between Tb and T30), for both granulocytes and monocytes (Figures 2C–5C). Interestingly, inhibition of C5aR1 further reduced C3aR expression regardless of the investigated immune cell and stimulation condition. In *E. coli*-mediated stimulation, C3 inhibition significantly increased granulocytic C5aR1

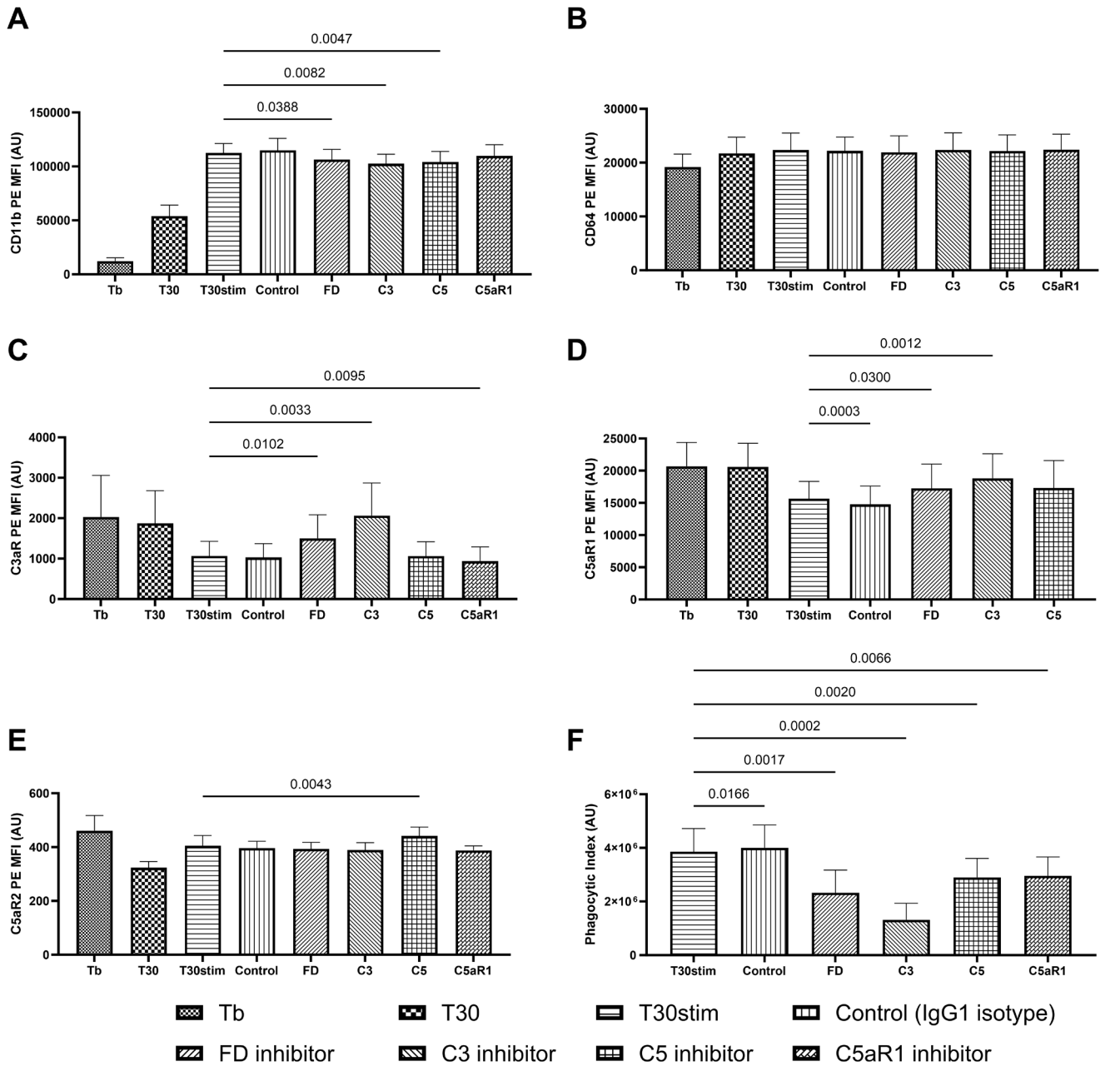

FIGURE 4 | Monocyte receptors and phagocytosis in *E. coli*-stimulated whole blood. Investigation of whole blood activation markers (CD11b and CD64), as well as anaphylatoxin receptor expression (C3aR, C5aR1, and C5aR2) on monocytes, as well as phagocytosis (Phagocytic Index), after whole blood stimulation with FITC-labelled *E. coli* (A–F). Several controls, including a baseline control (Tb; no incubation, no stimulation), an incubation control (T30; incubation, no stimulation), and a stimulation control (T30stim; incubation, stimulation) are included to assess the model-dependent marker progression. Individually targeted components of the complement system include FD, C3, C5, and C5aR1, with an IgG1 control for reference. The presented data are based on six individual repeats featuring a different healthy donor per repeat. Data are presented as mean with one-sided SD for better visibility.

expression compared to the stimulated blood without inhibitors, which also applied to *A. fumigatus* conidia, through inhibition of FD, C3, and C5 (Figures 2D and 3D). Monocytic C5aR1 expression after complement inhibition essentially led to similar outcomes, with the addition that not only C3 but also FD inhibition significantly increased the C5aR1 expression during *E. coli* and *A. fumigatus* conidia stimulation (Figures 4D and 5D). A greater dynamic range of granulocytic C5aR1 expression (comparison between Tb, T30, T30stim) compared to monocytic C5aR1 expression was observed, which seems to also be more sensitive to incubation conditions. An analysis of C5aR1 expression after C5aR1 inhibition was omitted due to the competitive binding of the C5aR1 inhibitor and detection antibody to the receptor. Complement inhibition of whole blood had limited effects on granulocytic C5aR2 expression, with only inhibition of FD resulting in a significant (although marginal) improvement of C5aR2 expression in the case of *E. coli*-based stimulation, but not for

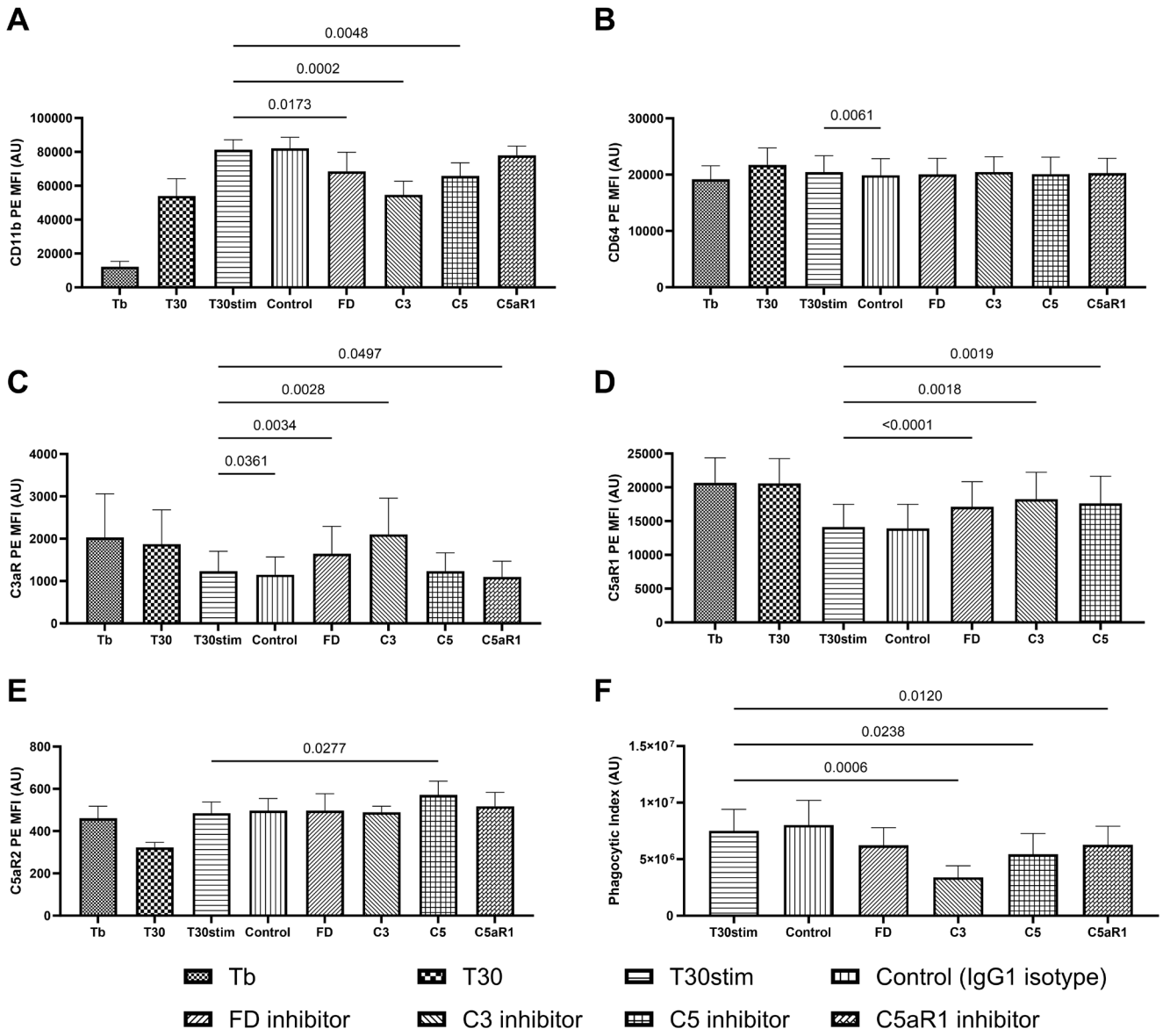

FIGURE 5 | Monocyte receptors and phagocytosis in *A. fumigatus* conidia-stimulated whole blood. Investigation of whole blood activation markers (CD11b and CD64), as well as anaphylatoxin receptor expression (C3aR, C5aR1, and C5aR2) on monocytes, as well as phagocytosis (Phagocytic Index), after whole blood stimulation with FITC-labelled *A. fumigatus* conidia (A–F). Several controls, including a baseline control (Tb; no incubation, no stimulation), an incubation control (T30; incubation, no stimulation), and a stimulation control (T30stim; incubation, stimulation) are included to assess the model-dependent marker progression. Individually targeted components of the complement system include FD, C3, C5, and C5aR1, with an IgG1 control for reference. The presented data are based on six individual repeats featuring a different healthy donor per repeat. Data are presented as mean with one-sided SD for better visibility.

A. fumigatus conidia (Figures 2E and 3E). Interestingly, a significant improvement of monocytic C5aR2 expression was observed after C5 inhibition regardless of the stimulation condition (Figures 4E and 5E).

Finally, C3 inhibition resulted in a profound and significant reduction of phagocytosis of *E. coli* and *A. fumigatus* conidia by both granulocytes and monocytes (Figures 2F and 5F). In addition, inhibition of FD and C5 significantly reduced granulocytic phagocytosis of *A. fumigatus* conidia. For monocytes, a significant reduction in phagocytosis was further observed for the inhibition of C5 and C5aR1 after exposure to *E. coli* or *A. fumigatus* conidia, with targeting of FD further significantly reducing phagocytosis of *E. coli*.

3.3 | Analysis of Soluble Leukocyte Activation Markers: MPO And Cytokines

In addition to complement activation products, we measured the release of MPO in whole blood stimulated with *E. coli* and *A. fumigatus* conidia. Both microbes facilitated a substantial increase in released MPO compared to controls (Tb, T30) (Figure 6) and in both cases, inhibition of C3 significantly reduced measured MPO levels. Inhibition of FD, C5, or C5aR1 did not significantly affect the MPO release.

Finally, the release of cytokines was assessed (Figures 7–9). The following selected cytokines displayed a profound increase after whole blood stimulation with *E. coli*: TNF, IL-1β,

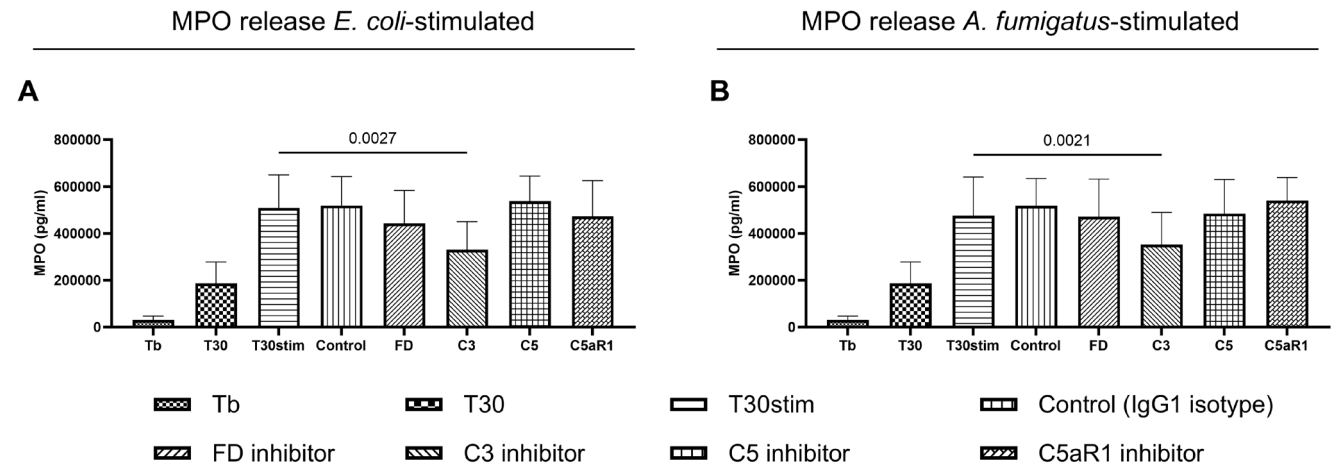

FIGURE 6 | MPO release in *E. coli* and *A. fumigatus*-stimulated whole blood. Assessment of MPO release in whole blood stimulated by either *E. coli* (A) or *A. fumigatus* conidia (B). Multiple controls, including a baseline control (Tb; no incubation, no stimulation), an incubation control (T30; incubation, no stimulation), and a stimulation control (T30stim; incubation, stimulation) are included to assess the model-dependent MPO release. Individually targeted components of the complement system include FD, C3, C5, and C5aR1, with an IgG1 control for reference. The presented data are based on six individual repeats featuring a different healthy donor per repeat. Data are presented as mean with one-sided SD for better visibility.

IL-6, IFNγ, IL-1RA, IL-4, IL-17A, IL-8/CXCL8, MCP-1/CCL2, MIP-1α/CCL3, and MIP-1β/CCL4. Whole blood stimulation with *A. fumigatus* conidia led to similar outcomes; however, the expression of inflammatory cytokines such as TNF, IL-1β, IL-6, and IFNγ was generally lower compared to *E. coli*-mediated whole blood stimulation. For whole blood stimulated with *E. coli*, the expression of the following cytokines was influenced by complement inhibition: TNF (FD: significant decrease), IFNγ (C5: significant increase), IL-1RA (C5: significant increase), IL-17A (FD: significant decrease), IL-8/CXCL8 (FD, C3, and C5: significant decrease), and MIP-1β/CCL4 (C5: significant increase). For *A. fumigatus*-stimulated whole blood, complement inhibition influenced the expression of cytokines as follows: TNF (FD: significant decrease; C5aR1: significant increase), IL-1β (FD and C5: significant decrease; C5aR1: significant increase), IL-6 (FD: significant decrease), IFNγ (FD: significant decrease; C5aR1: significant increase), IL-1RA (C3: significant decrease; C5aR1: significant increase), IL-4 (FD: significant decrease; C5aR1: significant increase), IL-17A (FD: significant decrease; C5aR1: significant increase), IL-8/CXCL8 (FD, C3, and C5: significant decrease), MCP-1/CCL2 (FD, C3, C5, and C5aR1: significant decrease), MIP-1α/CCL3 (FD: significant decrease; C5aR1: significant increase), and MIP-1β/CCL4 (C5aR1: significant increase).

4 | Discussion

This study aims to investigate the degree and pattern of inflammatory response and understand the implications of inhibiting selective complement system components in infections with bacterial and fungal pathogens, more specifically, *E. coli* and *A. fumigatus*. The immune reaction to these microbes was assessed in lepirudin whole blood exposed to heat-inactivated FITC-labelled *E. coli* and *A. fumigatus* conidia. In detail, the effect of inhibiting FD, C3, C5, and C5aR1 was investigated by analysing complement activation products (C4c, C3bc, and sC5b-9), the expression of leukocyte activation markers (CD11b and CD64) and anaphylatoxin receptors (C3aR, C5aR1, and C5aR2) on granulocytes and monocytes. Moreover, phagocytosis was measured, as well as leukocyte activation (MPO) and cytokine release.

Whole blood exposure to FITC-labelled *E. coli* and *A. fumigatus* conidia resulted in profound changes in investigated read-out parameters, with a marked increase in C3bc and sC5b-9 documenting a robust complement activation. CD11b increased markedly, CD64 to a lesser extent, whereas a general reduction of anaphylatoxin receptor expression was observed. Granulocytic and monocytic phagocytosis increased as well as the release of MPO and pro-inflammatory cytokines. This supports our previous demonstration of complement activation progression of whole blood stimulated with *E. coli* and *A. fumigatus* conidia, with *A. fumigatus* conidia giving rise to a relatively lower level of immune cell activation marker (CD11b) and cytokine expression [18]. Similarly, we observed a lower CD11b increase after whole blood stimulation with *A. fumigatus* conidia, while complement inhibition also seemed to more potently reduce *A. fumigatus* conidia-dependent CD11b upregulation on granulocytes and monocytes, supporting previous findings.

Expectedly, inhibition of FD and C3 resulted in a significant reduction of C3bc and sC5b-9, while inhibition of C5 significantly reduced sC5b-9 levels. Interestingly, inhibition of C3 resulted in higher levels of C4c, while C5 inhibition significantly increased measured C3bc levels regardless of the stimulation agent. Both observations may be caused by an ongoing complement activation, resulting in the accumulation of processed complement components upstream of the point of inhibition, which has previously been proposed in a study of the complement dynamics of *A. fumigatus* [33]. However, since our results concern complement activation products of the fluid phase, a different scenario may be present. Currently, there is no documentation of possible feedback loops due to complement inhibition, which could explain these findings.

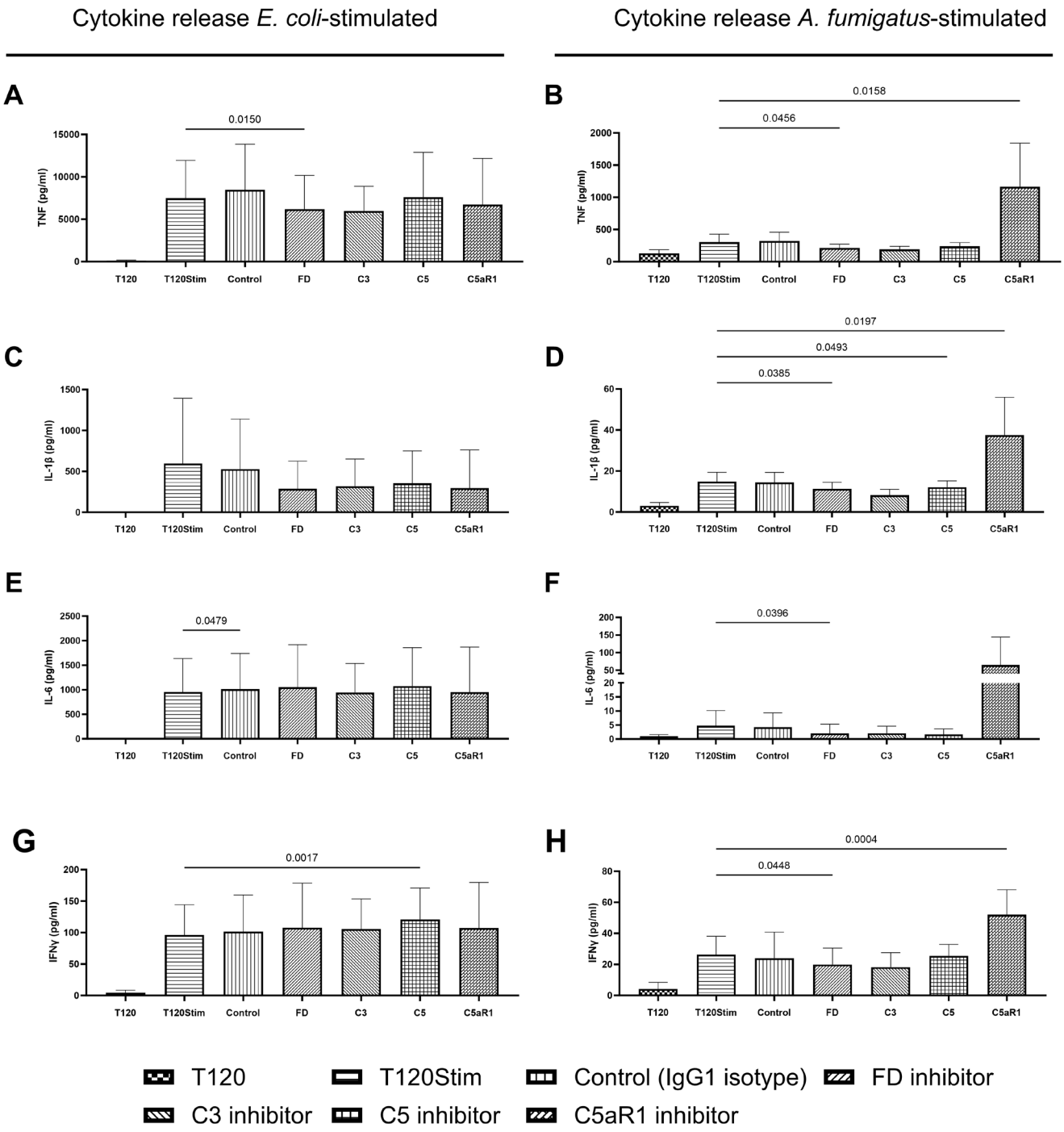

FIGURE 7 | Analysis of cytokine release in whole blood. Whole blood was stimulated with either *E. coli* (A, C, E, and G) or *A. fumigatus* conidia (B, D, F, and H), featuring TNF, IL-1β, IL-6, and IFNγ. Multiple controls, including an incubation control (T120; incubation, no stimulation), and a stimulation control (T120stim; incubation, stimulation) are included to assess the model-dependent cytokine release. Individually targeted components of the complement system include FD, C3, C5, and C5aR1, with an IgG1 control for reference. The presented data are based on six individual repeats featuring a different healthy donor per repeat. Data are presented as mean with one-sided SD for better visibility.

A general mechanism behind both observations may be possible, but is speculative with current complement knowledge, requiring further investigation.

Inhibition of C5aR1 significantly increased C3bc (in the case of whole blood stimulation with *A. fumigatus* conidia) and sC5b-9 levels. We confirmed subsequently that a small fraction of full-size mAb after the $F(ab')_2$ conversion of the C5aR1 mAb inhibitor remains and therefore may induce Fc-mediated activation of complement on the surface of blood cells.

Inhibition of FD, C3, C5, and C5aR1 significantly reduced the expression of leukocyte activation markers CD11b and CD64. This is likely attributed to reduced anaphylatoxin production or signalling, since it has been demonstrated that C5aR1 stimulation increases CD11b expression [25]. CD11b is part of the

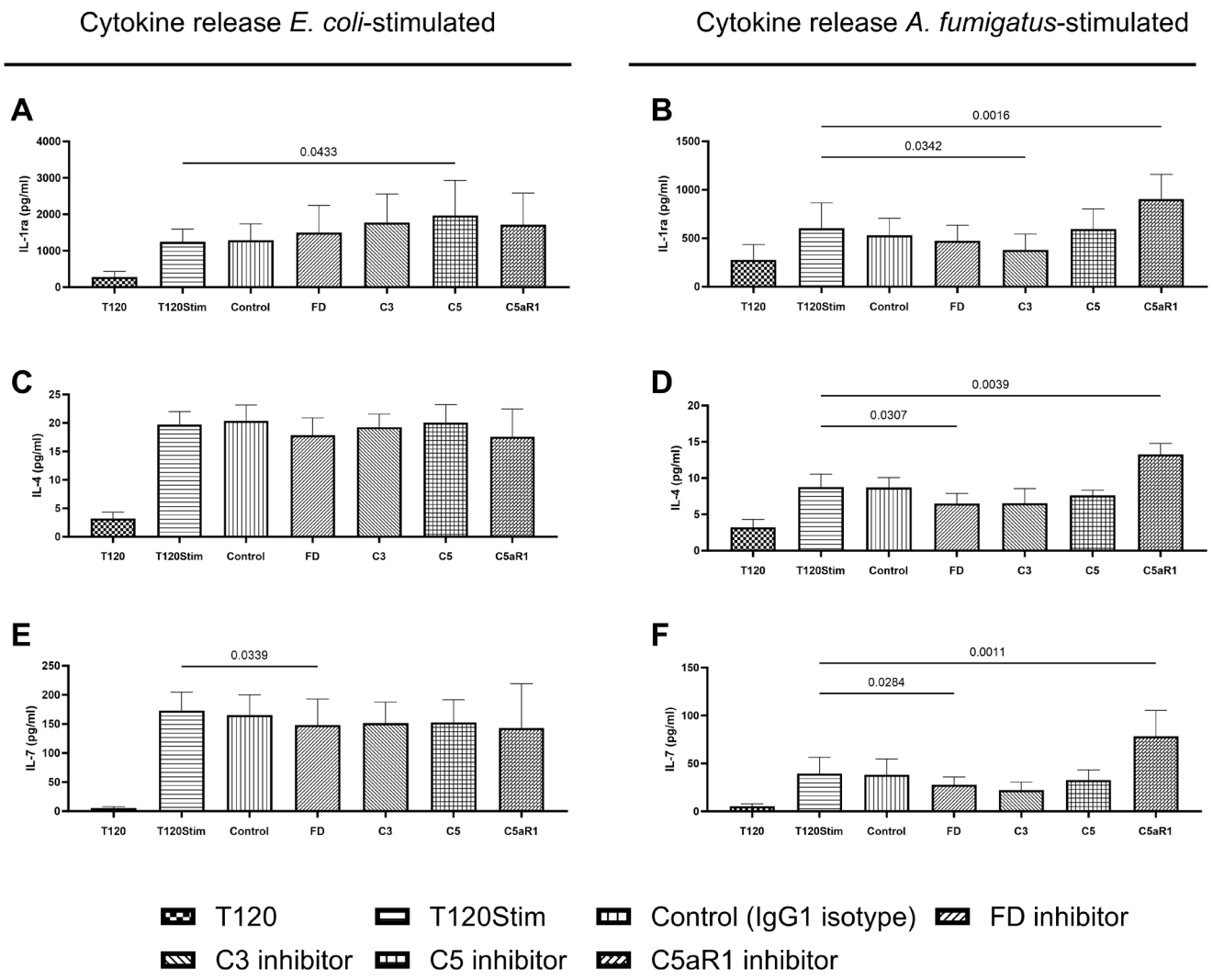

FIGURE 8 | Analysis of cytokine release in whole blood. Whole blood was stimulated with either *E. coli* (A, C and E) or *A. fumigatus* conidia (B, D and F), featuring IL-1RA, IL-4, and IL-17A. Multiple controls, including an incubation control (T120; incubation, no stimulation), and a stimulation control (T120stim; incubation, stimulation) are included to assess the model-dependent cytokine release. Individually targeted components of the complement system include FD, C3, C5, and C5aR1, with an IgG1 control for reference. The presented data are based on six individual repeats featuring a different healthy donor per repeat. Data are presented as mean with one-sided SD for better visibility.

complement receptor 3 (CR3), which recognises iC3b-marked surfaces and induces phagocytosis, therefore playing a crucial role in protection against microbial pathogens and also contributes to homeostasis by removal of apoptotic cells [34, 35]. CD64 has been reported to be a valuable tool to assess the general inflammation state of blood, as the marker displays long-term stability at ambient temperature, and its upregulation is dependent on an inflammatory response [36]. However, CD64 upregulation has previously been thought to be pathogen-dependent, as it has been demonstrated to be a strong biomarker in bacterial sepsis but not fungal or viral infections [37]. Nonetheless, both *E. coli* and *A. fumigatus* conidia caused an increase of neutrophilic CD64 in whole blood. The complement inhibitor-dependent reduction of CD64 followed the trend of CD11b, displaying significant CD64 expression reduction for all assayed complement inhibitors. Monocytic CD64 did not show the same responsiveness to stimulation and complement inhibition compared to neutrophilic CD64. Notably, inhibition of FD generally reduced blood activation marker progression, emphasising its key role in the alternative pathway as a rate-limiting component [4].

Generally, inhibition of the complement components partly reversed the downregulation of anaphylatoxin receptors, which was likely through the reduction of C3a and C5a, which under normal circumstances would activate the receptors leading to subsequent internalisation [38]. Both inhibition of FD and C3 had a rescuing effect on C3aR expression, leading to a nearly preserved expression during C3 inhibition. Similarly, inhibition of FD and C3 overall elevated the expression of C5aR1 for both granulocytes and monocytes regardless of the microbial agent. In addition, C5 inhibition resulted in a significant improvement of C5aR1 expression for both granulocytes and monocytes in whole blood stimulated with *A. fumigatus* conidia, but not *E. coli*. Expression of C5aR1 after C5aR1 inhibition has been excluded from statistical analysis, due to the validated competitive binding of C5aR1 inhibitor and C5aR1 detection antibody. In general, complement inhibition seemed to also have a larger

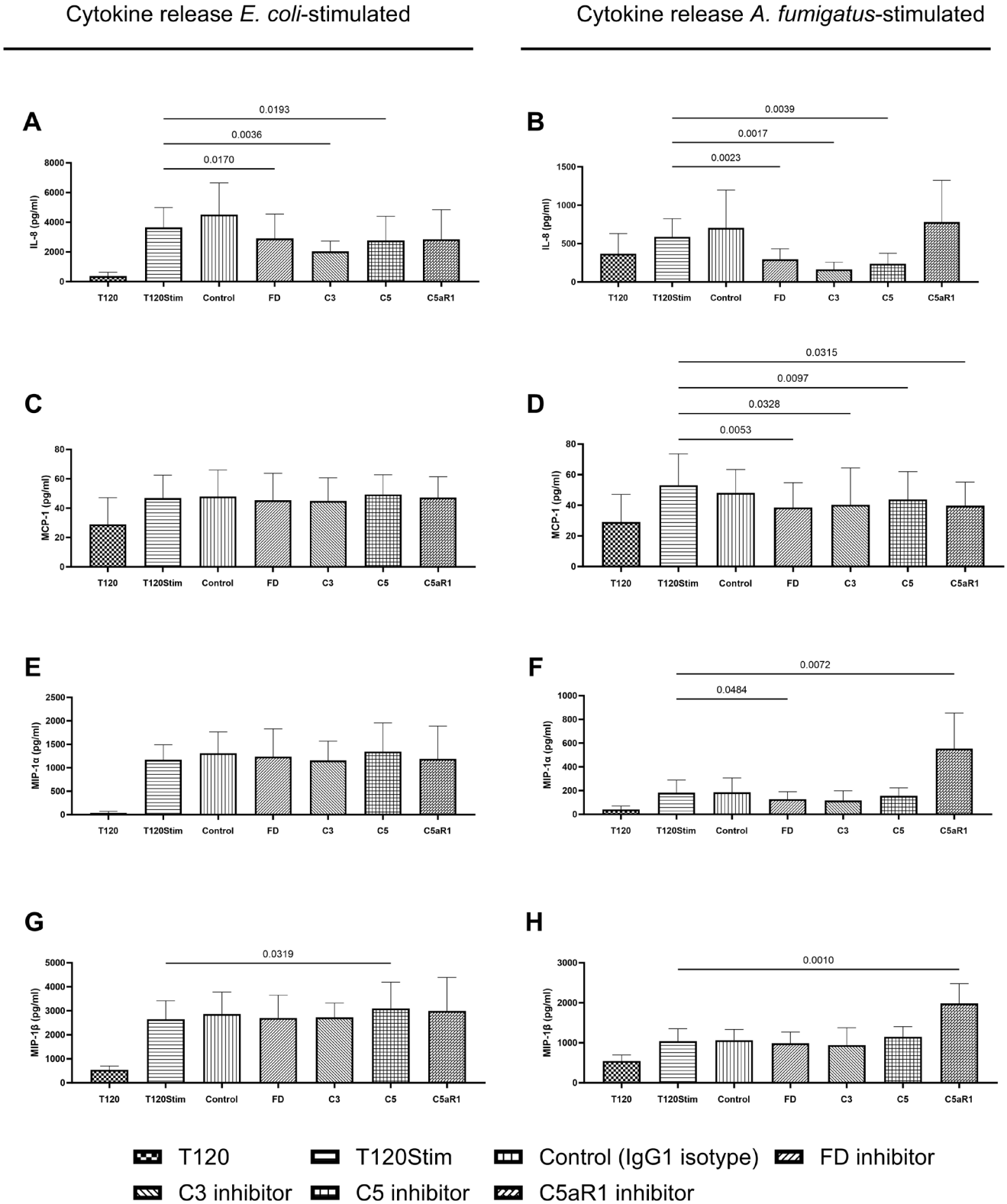

FIGURE 9 | Analysis of cytokine release in whole blood. Whole blood was stimulated with either *E. coli* (A, C, E, and G) or *A. fumigatus* conidia (B, D, F, and H), featuring IL-8/CXCL8, MCP-1/CCL2, MIP-1α/CCL3, and MIP-1β/CCL4. Multiple controls, including an incubation control (T120; incubation, no stimulation), and a stimulation control (T120stim; incubation, stimulation) are included to assess the model-dependent cytokine release. Individually targeted components of the complement system include FD, C3, C5, and C5aR1, with an IgG1 control for reference. The presented data are based on six individual repeats featuring a different healthy donor per repeat. Data are presented as mean with one-sided SD for better visibility.

preserving effect on C5aR1 expression on immune cells stimulated with *A. fumigatus* conidia compared to *E. coli*, emphasising different immunostimulatory properties of these microbes. Monocytic C5aR2 expression was significantly improved due to C5 inhibition regardless of stimulation agent, while granulocytic C5aR2 was largely unaffected by complement inhibition. It has been previously described that C5aR1 is expressed at a high level on granulocytes and monocytes, while C3aR expression has been demonstrated, and C5aR2 expression is considered much lower than C5aR1 expression [39]. In addition, the magnitude of anaphylatoxin receptor expression changes was cell-dependent, with granulocytes displaying a greater reduction of C5aR1 in response to bacterial and fungal agents compared to monocytes. The sensitivity of neutrophilic C5aR1 has been described before as a potential marker of infection [9].

Inhibition of C3 potently reduced the phagocytosis of FITC-labelled *E. coli* and *A. fumigatus* conidia (Phagocytic Index), emphasizing the key role of C3 in pathogen opsonization as previously described [2, 23, 40]. In addition, the inhibition of FD also dampened the phagocytosis, which underlines the important contribution of the alternative pathway to generate sufficient C3b [2]. Interestingly, inhibition of the C5a/C5aR1 axis reduced phagocytosis of *E. coli* and *A. fumigatus* by monocytes, whereas the inhibitory effect on neutrophilic phagocytosis only applied to *A. fumigatus*. This supports recent findings demonstrating that C5a-mediated signalling likely is very important for phagocytosis and killing of fungal pathogens [22, 41].

Only inhibition of C3 led to a significant reduction of MPO release in whole blood stimulated with either *E. coli* or *A. fumigatus* conidia. MPO is released intracellularly and extracellularly by phagocytes, such as neutrophils, after phagocytosis of pathogens [42]. We therefore propose that inhibition of C3 impairs the interaction of immune cells with the pathogens (as demonstrated via the significant reduction of phagocytosis (Phagocytic Index)), resulting in a reduced release of MPO.

Cytokine profiles differed substantially between *E. coli*- and *A. fumigatus*-stimulated blood, as stimulation with *E. coli* led to a much higher production of inflammatory cytokines, such as IL-1β, IL-6, and TNF, which was previously described [18]. This result may be partly explained by structural differences. Bacterial lipopolysaccharides (LPS) have been demonstrated to be inducers of IL-1β, IL-6, and TNF production [43]. Conversely, *A. fumigatus* conidia are immunologically protected via an external protein layer (rodlet-like proteins), masking more immunogenic structures underneath [44]. In addition, a high prevalence of *E. coli*-associated LPS-targeting antibodies has been described in serum for healthy blood donors [45]. Moreover, *Aspergillus*-targeting antibodies have also been described in healthy donors, with a further increase during *Aspergillus*-mediated infection [46]. Species-specific antibodies may influence the immune response for *E. coli*- and *A. fumigatus*-stimulated whole blood.

Complement inhibition led to various outcomes, depending on the target. Interestingly, inhibition of FD resulted in a significant decrease of many cytokines in the case of whole blood stimulation for both *E. coli* and *A. fumigatus* conidia (TNF, IL-17A, and IL-8/CXCL8) or *A. fumigatus* conidia alone (IL-1β, IL-6, IFNγ, IL-4, MCP-1/CCL2, and MIP-1α/CCL3), often matching expression profiles seen by C3 inhibition. Despite visibly reducing the release of many cytokines, the statistical significance of C3 inhibition remained limited. In detail, C3 inhibition led to a significant reduction of IL-1RA (*E. coli*-stimulated whole blood), MCP-1/CCL2 (*A. fumigatus*-stimulated whole blood), and IL-8/CXCL8 (*E. coli*−/*A. fumigatus*-stimulated whole blood). Inhibition of C5 only selectively decreased cytokine expression profiles (such as IL-1β and MCP-1/CCL2 in case of whole blood stimulation with *A. fumigatus* conidia, and IL-8/CXCL8 in case of whole blood stimulation with *E. coli*/*A. fumigatus* conidia) but also led to the significant increase of certain cytokines (IL-1RA in case of whole blood stimulation with *A. fumigatus* conidia, MIP-1β/CCL4 if whole blood was stimulated with *E. coli*). Inhibition of C5aR1 led to considerable differences between *E. coli*- and *A. fumigatus*-stimulated whole blood. While significant increases in the release of many cytokines were observed for whole blood stimulated with *A. fumigatus* conidia (TNF, IL-1β, IFNγ, IL-1RA, IL-4, IL-17A, MIP-1α/CCL3, and MIP-1β/CCL4), no effect was observed for *E. coli*-stimulated whole blood. However, the release of MCP-1/CCL2 was significantly reduced if C5aR1 was targeted prior to whole blood stimulation with *A. fumigatus* conidia.

Despite the statistical significance/insignificance of relative cytokine profiles, the inhibition of both FD and C3 led to profound reductions in released cytokines. This has been described before in the case of the single inhibition of C3 and combined inhibition of C3 and CD14 for *A. fumigatus* conidia-stimulated whole blood [18]. Similarly, the combined inhibition of C3 and CD14 has been demonstrated to effectively attenuate an *E. coli*-mediated cytokine response in whole blood [47]. Blockade of FD, which is the key rate-limiting component of the alternative pathway, has been proposed as an ideal therapeutic target due to its very low concentration, highly specific functionality, and specific substrate (in comparison to C3) [4], which is supported by our observations. The C5-mediated cytokine release in *A. fumigatus*-stimulated whole blood was not as dramatic as C3-mediated release, again supporting previous observations [18].

Notably, C5aR1 inhibition in whole blood stimulated with *A. fumigatus* conidia resulted in a substantial increase in the release of multiple cytokines. This may be caused by the previously stated observation of incomplete $F(ab')_2$ processing of the C5aR1 inhibitory mAb leading to complement activation. However, as this effect is seen solely in the case of whole blood stimulation with *A. fumigatus* conidia and certain cytokines display comparable release levels between *E. coli*- and *A. fumigatus* conidia-stimulated whole blood (such as IFNγ and IL-4), a biological causation cannot be ruled out. Especially striking are the opposite trends of IL-1β release after C5aR1 inhibition in *E. coli*- and *A. fumigatus* conidia-stimulated whole blood, with an observed non-significant decrease and significant IL-1β release, respectively. However, as antibodies are normally not able to pass the cellular membrane [48], we are only able to inhibit extracellular C5aR1 activation, but not intracellular. Since the discovery of the complosome, the relevance has been recently demonstrated by the C5aR1 inhibitor JPE1375, which attenuated mitochondrial C5aR1 signalling in cholesterol crystal-stimulated monocytes, reducing IL-1β secretion [49]. Furthermore, it was recently demonstrated that C5aR1-deficient mice respond to pathogen challenge (polymicrobial faeces) with a substantial increase in pro-inflammatory IFNγ

release, which seems to be further influenced by the pathogen load [10]. We observed a similar effect regarding IFNγ release if C5aR1 was targeted prior to *A. fumigatus*-mediated stimulation of whole blood. In congruence with the previously mentioned study by Sommerfeld et al., we hypothesize that C5aR1 signalling may also possess anti-inflammatory properties and therefore presents a regulatory mechanism, which might further be influenced by the pathogen type.

The inactivation method for both *E. coli* and *A. fumigatus* conidia (heat treatment) should further be considered when interpreting these results. While live and heat-inactivated *E. coli* have been demonstrated to induce a comparable inflammatory response [50], heat-treatment of *A. fumigatus* conidia may influence immunogenicity [51]. Inactivation of *E. coli* and *A. fumigatus* conidia, however, improves the whole blood assay by removing the need for sample fixation, which would greatly impair the detection of weakly expressed markers, such as C5aR2. In addition, it provides greater control over in-assay concentrations of *E. coli* and *A. fumigatus* conidia. In a clinical setting, dead bacterial or fungal cells might contribute to the adverse inflammatory response to the same extent as live cells, which therefore reduces the significance of the CFU count to determine the magnitude of the inflammatory response. We have previously demonstrated the effect of certain concentrations of heat-inactivated *E. coli* and *A. fumigatus* conidia on the inflammatory response [18]. It should also be noted that the presented whole blood model requires substantial concentrations of bacterial and fungal cells to obtain a measurable and meaningful inflammatory response within the used time frame. As demonstrated for bacterial sepsis, much less microbial burden in patients is observed [52], which should be additionally considered for interpreting the magnitude of the immune response.

In summary, we investigated the effect of selective complement inhibition on whole blood stimulated with either FITC-labelled *E. coli* or *A. fumigatus* conidia. Inhibition of C3, C5, FD, and C5aR1 resulted in a general reduction of blood activation markers (CD11b and CD64), while especially FD and C3 inhibition partly recovered the downregulation of expression of C3aR and C5aR1 (but not C5aR2) as well as substantially reduced phagocytosis of *E. coli* or *A. fumigatus* conidia. Interestingly, inhibition of C5 seemed to overall interrupt phagocytosis of *A. fumigatus* conidia more potently compared to *E. coli*. Inhibition of C3 was able to significantly reduce the measured release of MPO in both *E. coli*- and *A. fumigatus*-stimulated whole blood. Especially targeting of FD resulted in a significant reduction of the release of many cytokines with similar effects compared to C3 inhibition, validating FD as a key element of complement amplification. Inhibition of C5aR1 resulted in a profound upregulation of cytokine release in case of whole blood stimulation with *A. fumigatus* conidia, but not *E. coli*, which we attribute to potential pathogen-dependent immunoregulatory patterns.

Author Contributions

L.C., T.E.M., P.G. and A.R. conceptualised the presented study. Project supervision was carried out by T.E.M., P.G. and A.R. Study-related experiments were designed and conducted by L.C., I.M., B.F. and L.P.-A; T.E.M. and V.H. contributed by supplying HI *Escherichia coli* and *Aspergillus fumigatus* conidia, respectively. Data were analysed by L.C., I.M., B.F., L.P.-A. and A.R. L.C. made the first draft, and all authors critically revised the draft before submission.

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Ethics Statement

Human whole blood was received from anonymous donors via the Blood Bank at Copenhagen University Hospital—Rigshospitalet in Copenhagen. Informed written consent from individual blood donors was not required or obtained. For this study, in accordance with local and national guidelines, ethical approval was not required.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All study-associated data may be obtained from the corresponding authors upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.