Serum opsonin ficolin-A enhances host-fungal interactions and modulates cytokine expression from human monocyte-derived macrophages and neutrophils following *Aspergillus fumigatus* challenge

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Abstract

Invasive aspergillosis is a devastating invasive fungal disease associated with a high mortality rate in the immunocompromised; such as leukaemia patients, transplant patients and those with HIV/AIDS. The rodent serum orthologue of human L-ficolin, ficolin-A, can bind to and opsonize *A. fumigatus*, the pathogen that causes invasive aspergillosis, and may participate in fungal defence. Using human monocyte-derived macrophages and neutrophils isolated from healthy donors, we investigated conidial association and fungal viability by flow cytometry and microscopy. Additionally, cytokine production was measured via cytometric bead arrays. Ficolin-A opsonization was observed to significantly enhance association of conidia, while also inhibiting hyphal growth and contributing to increased fungal killing following incubation with monocyte-derived macrophages and neutrophils. Additionally, ficolin-A opsonization was capable of manifesting a decrease in IL-8, IL-1β, IL-6, IL-10 and TNF-α production from MDM and IL-1β, IL-6 and TNF-α from neutrophils 24 h post-infection. In conclusion, rodent ficolin-A is functionally comparable to human L-ficolin and is capable of modulating the innate immune response to *A. fumigatus*, down-regulating cytokine production and could play an important role in airway immunity.

Key words; aspergillosis; macrophage; neutrophil; cytokines; innate immunity

Introduction

Aspergillus fumigatus (*A. fumigatus*) is the most common invasive mold pathogen and is the primary causative species of the devastating disease, invasive pulmonary aspergillosis (IA) in immunocompromised hosts. IA is an ever increasing challenge in the developed world and is associated with a mortality rate of around 30-40% if treated and 100% if left untreated [1]. Those most at risk of disease include leukaemia sufferers, solid and haematopoietic transplant patients, neutropenic and diabetic patients or those undergoing corticosteroid therapies [2-4]. *A. fumigatus* infects via the propagation of its conidia (spores) in to the air, which are then inhaled by the host. Due to the small size of the conidia, those that evade mucocilliary clearance can invade the depths of the alveolar space where they can germinate into filamental (hyphal) structures. This invasive stage can lead to thrombosis, tissue necrosis and dissemination to other organs such as the skin and brain, leading to death [5,6].

Defence is initiated by the innate immune system and predominantly comprises of type II epithelial cells, alveolar macrophages and neutrophils. Macrophages are traditionally acknowledged to be essential in the phagocytosis and removal of conidia, whereas neutrophils are necessary for protection against the invasive hyphal stage whereby they produce fungistatic neutrophil extracellular traps (NETs) and degranulate, releasing antimicrobial compounds [7-10]. Serum opsonins such as ficolins, proteins similar to the collectins mannose-binding lectin (MBL) and the surfactant proteins (SP)-A and –D, can assist and enhance the functions of these host cells.

Ficolins are novel opsonins composed of an N-terminal collagen-like domain and a C-terminal fibrinogen-like (FBG) domain with lectin activity predominantly for the acetylated carbohydrate, *N*-acetylglucosamine (GlcNAc). However, ficolins do not bind exclusively to acetylated carbohydrates and can recognise pathogen specific structures such as (1,3)-β-D-

glucan, lipotechoic acid and LPS, in addition to interacting with acute phase proteins such as C-reactive protein (CRP) and pentraxin-3 (PTX3) [11]. Humans possess three types of ficolin; the membrane-bound M-ficolin and the serum ficolins L-ficolin and H-ficolin. Only orthologues of human L-ficolin and M-ficolin can be found in rodents and are termed ficolin-A and ficolin-B, respectively.

Others have demonstrated that ficolin-A can recognise *A. fumigatus* conidia and we have recently demonstrated that ficolin-A is capable of recognizing *A. fumigatus* conidia, increasing the quantity of conidia associating with the A549 type II epithelial cell line and magnifying the production of pro-inflammatory IL-8 [12,13]. However, the protective roles of ficolins and whether opsonization can enhance phagocytosis by professional phagocytes during *Aspergillus* defence is poorly characterised. Therefore, we utilized ficolin-A and investigated if ficolin-A opsonization of *A. fumigatus* could enhance conidial associations, contribute to killing or modulate inflammatory cytokine production following incubation with human macrophages and neutrophils.

Materials and Methods

Fungal pathogens

A clinical isolate of *A. fumigatus* was used as described previously [14]. Resting conidia were obtained after *A. fumigatus* was subcultured on Sabouraud dextrose agar (Oxoid, Basingstoke, UK) at 37 °C for 7 days, and conidia were harvested using sterile physiological saline (Oxoid, Basingstoke, UK). Resting live conidia were used immediately or fixed in 4% PBS–formaldehyde for 10 min at room temperature (RT), washed, and resuspended in PBS. Fixed *A. fumigatus* conidia were stored at 4 °C for up to 1 month until further use. To fluorescently label *A. fumigatus* conidia, up to 10^9 particles were incubated for 24 h at 4°C with 0.5 mg mL^{-1} fluorescein isothiocyanate (FITC; Sigma) in FITC labelling buffer (500 mM $NaHCO_3$, 500 mM Na_2CO_3, 0.9% (w/v) NaCl), washed repeatedly with PBS and centrifuged at 775 g for 4 min until the supernatant was clear in colour.

Ethical approval

Ethical approval for blood donation by healthy participants was obtained from the Faculty of Health Research Ethics Committee (Ref. Mechanisms of airway diseases – 2008042). Blood was acquired through venepuncture of healthy participants who gave informed consent at the time of collection. All donors were not on medication at the time of collection. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Cells and reagents

All experiments were conducted using human monocyte-derived macrophages (MDM) or peripheral blood neutrophils. MDM and neutrophils were isolated from healthy donor blood via a 68% percoll gradient modified from Walsh et al (1999) [15]. Monocytes were selected for by adherence to tissue culture plastic ware for 1h and differentiated in RPMI-1640 supplemented with 10% autologous human serum and 50 I.U ml^{-1} penicillin and 50 µg ml^{-1} streptomycin over 5-9 days. Granulocytes were removed from a separate Percoll layer and were stained with Kimura to determine the percentage of neutrophils in the population. Neutrophils were briefly maintained in RPMI-1640 supplemented with 10% heat-inactivated foetal calf serum and 50 I.U mL^{-1} penicillin and 50 µg ml^{-1} streptomycin (All purchased from Invitrogen, Paisley, UK). Granulocyte preparations containing greater than 90% neutrophils and exhibiting >98% viability (as determined by trypan blue staining) were placed in culture. Recombinant ficolin-A was produced from CHO-DXB11 cells containing ficolin-A cDNA and purified by affinity chromatography on *N*-acetylglucosamine (GlcNAc)-Sepharose columns as previously described [16]. Ficolin-A protein was eluted with 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 300 mM GlcNAc (Sigma-Aldrich, United Kingdom). Finally, GlcNAc was removed by dialysis and ficolin-A presence confirmed by SDS-PAGE. This was kindly provided by the laboratory of Prof. Russell Wallis (University of Leicester, UK). Experiments were all performed in serum-free conditions and data are representative of a minimum of three independent experiments.

Association assays

MDM or human neutrophils were seeded in 24-well plates (Thermo Scientific, Loughborough, UK) in supplemented RPMI-1640 at 37 °C in a 5% CO_2 atmosphere. FITC-labelled fixed *A. fumigatus* conidia (FL1-A) were opsonized with 5 µg mL^{-1} ficolin-A for 1 h at 37°C. Ficolin-A-opsonized *A. fumigatus* conidia (5 x 10^5) were incubated for 2 h with

adherent MDM or neutrophils in suspension (ratio of conidia to cells of 5:1) at pH 5.7 or pH 7.4 at 37 °C in a 5% CO_2 atmosphere. Following incubation, non-adherent conidia and cells were removed and the adherent cells were washed with warm supplemented RPMI 1640. Adherent cells were subsequently removed by the use of trypsin-EDTA and gentle trituration prior to washing. Cells were fixed in 4% PBS–formaldehyde for 10 min at RT and analyzed by flow cytometry using a BD Accuri C6 flow cytometer with BD CFlow software. The percentage of MDMs and neutrophils associated with *A. fumigatus* conidia was determined by gating on the cell populations based upon their size and granularity (forward scatter channel/side scatter channel [FSC/SSC]). Additionally, the cells staining positive for conidia-derived FITC fluorescence were gated to quantify conidial association with cells (FL1-A [Exλ 488 nm, Emλ 530/33 nm]).

Fungal viability assays

Fungal viability was measured using a LIVE/DEAD® viability kit (Invitrogen, Paisley, UK). Assays were conducted as outlined in the protocol. In brief, live resting conidia were opsonised with 5 μg mL^{-1} prior to incubation with MDM or neutrophils for 6 h. Following cell challenge, conidia were stained with 15 μM FUN-1 cell stain and incubated in the absence of light for 30 min at 30 °C. FUN-1 stain is metabolised inside the fungus from yellow-green to orange-red fluorescence if the fungi are metabolically active and possess intact membranes. Dead fungi display diffuse bright green staining. Following incubation, supernatants were aspirated and the cells were washed with warm supplemented RPMI-1640. Adherent cells were subsequently removed by the use of trypsin/EDTA and gentle trituration. Cells and supernatants were fixed in 4% PBS/formaldehyde for 10 min at RT. Fungal viability was quantitated by flow cytometry using a BD Accuri C6 flow cytometer with BD CFlow® Software and measuring FL1-A (Exλ 488 nm, Emλ 530/33 nm). Microscopic

analysis was conducted after 24 h and growth was observed using an Axiovert 40 CFL microscope (Zeiss) at 10x objective.

Cytokine determination

Cytokine production from supernatants was quantitated using a BD cytometric bead array (CBA) Human Inflammatory Cytokines kit (BD Biosciences, Oxford, UK). Assays were conducted as outlined in the protocol. In brief, live un-opsonized or ficolin-A opsonized conidia (ratio of conidia to cells of 5:1) were added to MDM or neutrophils for 24 h prior to collection of the supernatant and storage at -80°C. Capture beads for the measurement of IL-8, IL-1β, IL-6, IL-10 and TNF-α were mixed together prior to their addition to the supernatant sample and standard tubes. Following the addition of capture beads to the samples, Human Inflammatory Cytokine PE Detection Reagent was added to all tubes and incubated for 3 h in the absence of light. Following incubation, samples were washed in wash buffer for 5 min at 200 g prior to aspiration of the supernatant, re-suspension in wash buffer and flow cytometry (Exλ 488 nm, Emλ 585/40nm) and (Exλ 633 nm, Emλ 780/30 nm) on a BD Accuri C6 flow cytometer with BD CFlow® Software, collecting 1500 events as outlined in the protocol.

Statistical analysis

Results were expressed as mean ± SD. Descriptive and two-tailed Students *t*-test analyses were performed using GraphPad prism software (version 5). One-way ANOVA's were performed using SigmaStat software (version 3.5). A value of $p<0.05$ was considered statistically significant.

Results

Ficolin-A opsonization enhances association of A. fumigatus with human monocyte-derived macrophages and contributes to enhancing fungal killing.

Macrophages are the resident lung phagocytes responsible for the removal of *A. fumigatus* conidia. Therefore, we investigated whether ficolin-A opsonization of *A. fumigatus* could enhance association with macrophages and contribute to fungal killing. Experiments were conducted both at pH 5.7 conditions as well as pH 7.4, as we had previously observed acidic conditions to be optimal for ficolin binding [12].

MDM were gated based on size and granularity (FSC-A/SSC-A; Fig. 1A) and association was determined by the detection of FITC positive cells (Fig. 1B and C). We observed that the percentage of the MDM population staining positive for conidial association was not altered (Fig. 1D). However, we observed an increase in fluorescence within the gated cell population suggesting that the number of FITC-labelled conidia associated with the MDM was significantly enhanced following ficolin-A opsonization in acidic conditions (based upon the median fluorescence intensity) (Fig. 1E; $p=0.00728$).

Next, we investigated the importance of ficolin-A opsonization to potentiate the ability of MDM to kill *A. fumigatus* conidia. Both the free and cell-associated *A. fumigatus* were gated based on size and granularity (Fig. 1F). Fungal viability assays demonstrated a significant reduction in fungal viability (demonstrated by an increase in green fluorescence) following opsonization by ficolin-A in acidic conditions (pH 5.7), as observed by flow cytometry (Fig. 1G; $p=2.2 \times 10^{-5}$).

Ficolin-A opsonization enhances association of A. fumigatus with human neutrophils and contributes to reducing fungal viability

Neutropenic patients are at significant risk for invasive aspergillosis, highlighting the importance of neutrophils in *Aspergillus* defence. In particular they are integral in the destruction of the invasive hyphal stage. Therefore, we also investigated whether ficolin-A opsonization of *A. fumigatus* could enhance phagocytosis and contribute to the reduction of fungal viability by human neutrophils that had been isolated from healthy donor blood.

Neutrophils were gated based on size and granularity (FSC-A/SSC-A; Fig. 2A) and association was determined by the detection of FITC positive cells (Fig. 2B and C). As for the macrophages, flow cytometry analysis indicated no difference in the percentage of the neutrophil population associated with conidia (Fig. 2D). Again however, a significant increase the fluorescence indicated that the number of FITC-labelled conidia associated with the neutrophils (based upon the median fluorescence intensity) was observed following ficolin-A opsonization in acidic conditions (Fig. 2E; $p=0.03215$).

As before, we were interested in investigating fungal viability following neutrophil challenge with un-opsonized or ficolin-A opsonized conidia. Light microscopy indicated that in the absence of ficolin-A in pH 5.7 or pH 7.4 conditions, hyphal growth was very dense (Fig. 2F, G). Conversely, following challenge with ficolin-A opsonized conidia in pH 5.7 conditions, hyphal growth was significantly reduced and branching much less dense (Fig. 2H). A minimal reduction in hyphal growth was also observed following ficolin-A opsonization in pH 7.4 conditions (Fig. 2I).

Additionally, the free and cell-associated *A. fumigatus* were gated based on size and granularity (Fig. 2J), fungal viability assays demonstrated a significant decrease in fungal viability following opsonization by ficolin-A in pH 5.7 conditions, as observed by flow cytometry (Fig. 2K; p=0.00262).

Ficolin-A opsonization modulates the secretion of inflammatory cytokines from MDM and neutrophils in response to A.fumigatus

Previous observations demonstrated the ability of ficolin-A opsonization to elicit an increase in IL-8 production from the A549 cell line following live conidial challenge [12]. Whether ficolin-A could modulate cytokine production from other cell types involved in *Aspergillus* defence was poorly understood. Therefore, we utilised cytometric bead arrays and measured the concentration of IL-8, IL-1β, IL-6, IL-10 and TNF-α produced by MDM and neutrophils following challenge with un-opsonized or ficolin-A opsonized live *A. fumigatus* conidia.

Indeed, challenge of MDM with ficolin-A opsonized conidia led to a significant reduction in production of all of the cytokines tested (Fig. 3A-E). Ficolin-A itself in the absence of conidia was capable of stimulating an increase in IL-6, IL-10 and TNF-α production (Fig. 3C-E). Additionally, following challenge of neutrophils with ficolin-A opsonized conidia we observed a significant reduction in the production of IL-1β, IL-6 and TNF-α (Fig. 4B, C, E). As with the MDM, unbound ficolin-A was capable of stimulating a significant increase in the production of IL-8, IL-1β, and TNF-α from neutrophils (Fig. 4A, B, E).

Discussion

Our study focused on whether opsonization of *A. fumigatus* by ficolin-A could significantly enhance *Aspergillus*-phagocyte interactions and modulate cytokine production. This led to a few new observations. Firstly, ficolin-A opsonization enhanced the quantity of *A. fumigatus* conidia associated with MDM and neutrophils. Next, ficolin-A opsonization significantly inhibited hyphal growth and contributed to the reduction of fungal viability in the presence of phagocytes. Finally, ficolin-A opsonization manifested a reduction in inflammatory cytokine production from MDM and neutrophils. This led us to postulate that ficolin-A could play an important role in airway immunity via efficient recognition and removal of *A. fumigatus* and modulation of cytokine production.

We recently demonstrated that in an acidic pH representative of infection/inflammation, the affinity of ficolin-A binding to *A. fumigatus* conidia was greatly increased and this increase in recognition subsequently led to enhanced conidia-epithelium interactions [12]. However, the interactions of ficolin-A opsonized *A. fumigatus* with professional phagocytes were unknown.

Following inhalation of *A. fumigatus*, macrophages are essential at ameliorating the early stages of infection whereby conidia are phagocytosed and destroyed in acidic phagolysosomes [17,18]. Conversely, neutrophils are traditionally recognised to be recruited to the site of infection during the later stages (in response to IL-8) where they assist the inhibition of fungal invasion by degranulation and the production of fungistatic NETs following adherence to the hyphal cell wall, albeit recently they have also been implicated in the early phagocytosis of conidia [10,19-21].

Initial observations indicated that ficolin-A was capable of enhancing conidial associations with the murine macrophage cell line Raw 264.7 (data not shown) which prompted the use of primary cells. Additionally, ficolin-A was also capable of enhancing the association of

conidia by both human MDM and neutrophils but only in acidic conditions. We have recently described that the binding of ficolin-A to *A. fumigatus* occurs with greatest affinity in acidic conditions, therefore functional enhancement in these conditions comes as no surprise [12]. Potentiation of cell-microbe interactions is not uncommon amongst serum opsonins, as the human orthologue of ficolin-A, L-ficolin, in addition to the functionally and structurally similar molecules MBL, SP-A and SP-D, have all been observed to enhance phagocytosis of pathogenic microorganisms by macrophages and polymorphonuclear cells [22,23,12,24-27].

Although phagocytosis is important, conidial killing is even more crucial to sterilize infected tissues. Therefore we utilised fungal viability assays and investigated the contribution of ficolin-A to killing. In our study, we demonstrated that ficolin-A opsonization enhanced the ability of macrophages and neutrophils to significantly inhibit hyphal growth and reduce fungal viability. The most striking observation was how significantly ficolin-A opsonization reduced hyphal density following incubation with neutrophils. Conversely, the presence of ficolin-A almost completely inhibited germination when incubated with MDM.

Recent *in vitro* and patient evidence has highlighted that L-ficolin can also enhance opsonophagocytic killing by human MDM in addition to neutrophils and is present in the fungal infected lung [28]. Moreover, L-ficolin can interact with the acute phase protein, PTX3 to enhance recognition and complement deposition [29], indicating a role in antifungal immunity. However, to date, no *in vivo* studies have been conducted confirming the importance of ficolin-A in opsonophagocytosis and defence against *A. fumigatus*. However, there is a body of evidence describing the integral role of the related opsonins, SP-D and MBL, in airway immunity *in vivo* [25,27,30]. Deficiencies in human L-ficolin have also been observed to exacerbate susceptibility to recurrent respiratory infections, suggesting that its rodent orthologue, ficolin-A, could also potentially play a significant role in airway immunity

in a transgenic mouse model [31]. This is something we are currently investigating in our laboratory.

As indicated earlier, we have previously observed that ficolin-A opsonization leads to an increase in IL-8 secretion from A549 cells, a cytokine that is crucial for the recruitment of neutrophils during *Aspergillus* infection. Cytokines are key regulators of inflammation and play an essential role in the defence against fungal challenge. Conidial challenge can induce the production of a wide array of cytokines and chemokines including IL-1β, IL-2, IL-5, IL-6, IL-8, IL-10, IL-13, IL-17A, IL-22, IFN-γ, TNF-α, GM-CSF and MCP-1 [20,12,32-37]. In this study we highlighted that MDM challenged with ficolin-A opsonized conidia produced significantly less IL-8, IL-1β, IL-6, IL-10 and TNF-α, whereas only IL-1β, IL-6 and TNF-α production was lower from neutrophils. Ficolins have been described to have the potential to be both pro- or anti-inflammatory, dependent upon the cell type involved [38,12,39,40]. We have previously observed that L-ficolin opsonization can modulate an anti-inflammatory response from MDM and neutrophils, while additionally, others have reported that ficolin-A can dampen LPS-induced inflammatory responses on mast cells [39,28]. In addition, we again show that unbound ficolin-A can increase cytokine production. The mechanisms by which these effects are achieved are currently unknown but preliminary studies in our lab have indicated the potential importance of the MAPK signalling cascades and TLRs.

In conclusion, we demonstrate that ficolin-A plays an important role in potentiating the functions of macrophages and neutrophils against *A. fumigatus* challenge *in vitro* and is functionally comparable to human L-ficolin. Additionally, we highlight that ficolin-A may be important in the down-regulation of cytokine production post-infection but relevant ficolin-A *in vivo* studies are lacking.

Acknowledgements

We are grateful to Dr. Orla Jupp (University of East Anglia), for processing blood and providing us with monocytes and neutrophils. We would also like to thank Prof. Russell Wallis and Dr. Umakhanth Venkatraman Girija for supplying us with recombinant rat ficolin-A. This work was supported by a Faculty of Health, University of East Anglia, UK PhD studentship (FMH 04.4.66 C4).

Conflict of interest

The authors have no conflicts of interest.

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Figure legends

Fig. 1. Association and killing of ficolin-A opsonized *A. fumigatus* conidia with MDM. FITC-labelled *A. fumigatus* conidia (5 x 10^5) were opsonized with 5 µg mL^{-1} ficolin-A prior to incubation with MDM (conidia:MDM ratio of 5:1) in pH 5.7 and pH 7.4 conditions for 2 h. To measure killing, live unlabelled conidia were incubated with MDM for 24 h prior to LIVE/DEAD staining. **A** Gating strategy used to distinguish macrophages. **B** MDM in the absence of FITC-labelled conidia. **C** MDM in the presence of FITC-labelled conidia. **D** The percentage of MDM associated with conidia in pH 5.7 and pH 7.4 conditions in the presence or absence of ficolin-A. **E** The relative number of associated FITC-labelled conidia (based upon the median fluorescence intensity; FL1-A) either un-opsonized (-ficolin-A) or following opsonization by ficolin-A (+ficolin-A). **F** Gating strategy used to measure fungal viability. **G** Fungal death after incubation with MDMs based upon the median fluorescence intensity of FL1-A. Heat killed conidia were used as a positive control for fungal killing. Results are representative of the average of all data points gained from three independent experiments. Error bars represent SD and significance was determined via two-tailed Students *t*-test. An asterisk indicates a significant difference; $p<0.05$.

Fig. 2. Association and killing of ficolin-A opsonized *A. fumigatus* conidia with neutrophils. FITC-labelled *A. fumigatus* conidia (5 x 10^5) were opsonized with 5 µg mL^{-1} ficolin-A prior to incubation with neutrophils (conidia:neutrophil ratio of 5:1) in pH 5.7 and pH 7.4 conditions for 2 h. To measure killing, live unlabelled conidia were incubated with neutrophils for 24 h prior to LIVE/DEAD staining. **A** Gating strategy used to distinguish neutrophils. **B** Neutrophils in the absence of FITC-labelled conidia. **C** Neutrophils in the presence of FITC-labelled conidia. **D** The percentage of neutrophils associated with conidia in pH 5.7 and pH 7.4 conditions in the presence or absence of ficolin-A. **E** The relative

number of associated FITC-labelled conidia (based upon the median fluorescence intensity; FL1-A) either un-opsonized (-ficolin-A) or following opsonization by ficolin-A (+ficolin-A). **F** Hyphal germination following incubation of un-opsonized conidia in pH 5.7 or. **G** in pH 7.4. **H** Hyphal germination following incubation of ficolin-A opsonized conidia in pH 5.7 or. **I** in pH 7.4. **J** Gating strategy used to measure fungal viability. **K** Fungal death after incubation with neutrophils based upon the median fluorescence intensity of FL1-A. Heta killed conidia were used as a positive control for fungal killing. Results are representative of the average of all data points gained from three independent experiments. Error bars represent SD and significance was determined via two-tailed Students *t*-test. An asterisk indicates a significant difference; $p<0.05$.

Fig. 3. Inflammatory cytokine release from MDM following challenge with un-opsonized or ficolin-A opsonized conidia. Supernatants were collected after 8 h and 24 h time points during challenge of MDM with live *A. fumigatus* conidia (5×10^5) either un-opsonized or ficolin-A opsonized (5 μg mL^{-1}) prior to the conduction of cytometric bead arrays (conidia:MDM ratio of 5:1). **A** Quantity of IL-8 production. **B** Quantity of IL-1β production. **C** Quantity of IL-6 production. **D** Quantity of IL-10 production. **E** Quantity of TNF-α production. Following *A. fumigatus* challenge. **MDM** is representative of MDM alone. **+ ficolin-A** represents MDM in the presence of ficolin-A alone. **+ AF** and **+AF +ficolin-A** are representative of un-opsonized *A. fumigatus* or ficolin-A opsonized conidia, respectively. Results are representative of the average of all the data points gained from three independent experiments. Error bars represent the SD. Significance was determined via one-way ANOVA and pair-wise comparisons were conducted using the Student-Newman-Keuls method. An asterisk indicates a significant difference: $p<0.05$.

Fig. 4. Inflammatory cytokine release from neutrophils following challenge with un-opsonized or ficolin-A opsonized conidia. Supernatants were collected after 8 h and 24 h time points during challenge of neutrophils with live *A. fumigatus* conidia (5×10^5) either un-opsonized or ficolin-A opsonized (5 μg mL^{-1}) prior to the conduction of cytometric bead arrays (conidia:neutrophil ratio of 5:1). **A** Quantity of IL-8 production. **B** Quantity of IL-1β production. **C** Quantity of IL-6 production. **D** Quantity of IL-10 production. **E** Quantity of TNF-α production. Following *A. fumigatus* challenge. **Neutrophils** is representative of neutrophils alone. **+ ficolin-A** represents neutrophils in the presence of ficolin-A alone. **+ AF** and **+AF +ficolin-A** are representative of un-opsonized *A. fumigatus* or ficolin-A opsonized conidia, respectively. Results are representative of the average of all the data points gained from three independent experiments. Error bars represent the SD. Significance was determined via one-way ANOVA and pair-wise comparisons were conducted using the Student-Newman-Keuls method. An asterisk indicates a significant difference: $p<0.05$.