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Research Paper

Direct antimicrobial effects of chemokines on *Cryptococcus spp*, with special emphasis on a 'CXC' chemokine

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ABSTRACT

Cryptococcus species are ingenious human pathogens that are widespread globally. They continue to cause over 200,000 deaths per year. Presently due to the rise in resistance and therapy failure, it is necessary to shift the focus to an alternate therapeutic strategy against this pathogen. One promising approach is to emphasize the host defense system in order to develop more precise and customized treatment strategies. In this regard, research has revealed that interferon-γ-inducible CXCL10 chemokine, amongst other chemokines spanning both CXC and CC categories, has a direct killing effect *in vitro* against *Cryptococcus neoformans* and *Cryptococcus gattii*, with a significantly greater microbicidal effect against the former. Moreover, when CXCL10 is used in combination with CCL5, there is a significant reduction in the survival of *C. gattii* at normal-serum level concentration, indicating a previously unreported synergistic effect of these two chemokines. Confocal and STED microscopic studies have demonstrated that CXCL10 has both cell wall/membrane and intracellular targets against this fungus. These findings present new possibilities for developing chemokine-derived small molecule antifungals and may represent a step forward in creating precision medicine tailored to each patient.

Introduction

Drug discovery for diseases with complex etiology poses significant challenges. Each disease has its own phenotypical manifestation and a labyrinth of interacting partners spanning both the host and the pathogen, influencing specific aspects of the host's immune responses. Cryptococcosis is a disease whose susceptibility [1–3] has been broadly categorized as a defective adaptive immune response [4] which can act as a trigger for the reemergence of a dormant cryptococcal infection [5]. Unfortunately, current treatments for this disease are limited by cytotoxic effects of the drugs on the host, limited efficacy, or the development of resistance upon long-term usage [6]. Frequent treatment failure due to the emergence of resistant strains, commonly seen in other microbial infections, has now also become a menace associated with fungal meningitis. This reiterate an urgent need for both early diagnosis and improved therapeutics including immune-replacement or immune-modulation therapies against this disease [7].

T-cell responses are key to controlling cryptococcal infections [8]. Protective immune response of $CD4^+$ T cells [9], specifically protective chemokines [10] like **T**umor **N**ecrosis **F**actor Alpha (TNFα), **I**nterferon **G**amma (IFNγ, **I**nter**l**eukin 8 (IL-8), CXCL10 (also known as Interferon Gamma **I**nducible **P**rotein-10 or IP-10), **M**acrophage **I**nflammatory **P**rotein-1 alpha (MIP-1α), MIP-1β, RANTES, and **M**onocyte **C**hemoattractant **P**rotein-1 (MCP-1), are known to be major mediators of leukocyte recruitment [11] into sites of *Cryptococcus neoformans, Candida albicans* and *Toxoplasma gondii* infection as has been evidenced from mouse models [12,13]. A variety of chemokines have a broad spectrum of microbicidal activity against human pathogens including gram-negative bacteria (e.g. *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa,* etc.), gram-positive bacteria (e.g. *Staphylococcus aureus, Enterococcus faecalis, Streptococcus pyogenes*), parasites (e.g. *Leishmania mexicana* and *Plasmodium falciparum*) and fungi (e.g. *Candida albicans* and *Cryptococcus spp.*) [14–17]. Conversely, several bacteria have been reported to possess mechanisms for specifically blocking the antimicrobial activities of chemokines [18]. Till date, a plethora of literature surveys mostly points to the classical leukocyte chemotactic activity of chemokines which directs and or activates other killer immune cells, as an immune response of the host that may kill the pathogen eventually. Whether this "microbicidal effect" is also direct, is as yet mostly unexplored, except in few articles on bacteria [19], where it was shown that few of them also interfere with the infectious agents directly.

Chemokines are small (8–12 kDa) proteins produced by a variety of cell types like epithelial, endothelial, fibroblast, lymphocytes, macrophages, monocytes, neutrophils, smooth mast cells and are known

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for their role in modulation of infectivity, angiogenesis, autoimmunity, tumor metastasis. The steady-state kinetics of chemokines or their role in homeostasis, together with their inflammatory role with or without immune modulation is a vast and complicated field, which continues to generate a lot of interest. The most commonly used nomenclature for these molecules mostly based on human chemokines, broadly divide into 2 major subfamilies- CC and CXC depending on whether the 2N-terminal cysteines have an amino acid between them (CXC) or not (CC). The other names currently used are XC, or CX3C which are variants of the 2 broad families mentioned above [11,20,21].

Currently, there is no antimicrobial chemokine in clinical use. Thus, the discovery and use of chemokine-based novel antimicrobials, either in the form of peptides, or as synthetic **i**nnate **d**efense **r**egulator (IDR) peptides having both antimicrobial and immunomodulatory effects may have an immense potential towards the development of "precision medicine". Given this backdrop, the identification of chemokine(s) with specific and direct killing effects in a certain diseased environment can be crucial in the development of novel antimicrobials and improved immunotherapeutics.

This study reports the first evidence of significant direct killing activity of a chemokine on *Cryptococcus spp.* This is particularly relevant as the more virulent, but less common *Cryptococcus gattii* [22], which usually infects immunocompetent hosts, also shows susceptibility against CXCL10 and a significant synergistic effect was observed when it was used together with CCL5 chemokine. Confocal and STED imaging validated that the targets of this chemokine are both extracellular and intracellular in both species. Further research on the determination of these antimicrobial cellular targets of chemokines, together with the development of chemokine-derived small molecule drugs can eventually lead to better alternate treatment plans and disease−management strategies.

Materials and methods

Materials

Strains used

One representative strain *C. neoformans var. grubii*, of molecular type VN I or H99 (wild type) and *C. gattii* of molecular type VG II or R265 were a kind gift from Dr. Maurizio Del Poeta, Stony Brook University, Long Island, New York, US.

Media

Yeast **P**eptone **D**extrose (YPD) SIGMA cat # Y1375 with or without agar for fungal growth. **D**ulbecco's **M**odified **E**agle **M**edia (DMEM) with phenol indicator AL007A HIMEDIA.

Chemokines and drug(s)

Human recombinant chemokines are from Peprotech, USA. Fluconazole (FLC) cat # 22239 and/ or amphotericin B (Amp B) 54713 (SRL), drugs−of−choice in case of cryptococcosis, were used as positive controls. Alamar blue from BIORAD cat #BUF012A was used for Alamar Blue assays to determine the killing effect of chemokines.

Antibodies

Primary monoclonal antibody against CXCL10 in rabbit from Invitrogen Cat #JA10-82 was used. Secondary antibody anti-rabbit IgG (H+L), F(ab")2 Fragment (Alexa Fluor® 488 Conjugate) Cat # 4412 and DAPI (a kind gift from Dr. Rajnish K Singh, MBU, BHU) from Cell signaling Technology, were used for immune imaging purposes.

All other reagents, including common laboratory reagents, are either from SRL, SIGMA, or HIMEDIA.

Methodology

Alamar blue assay

Active metabolism of fungal vegetative cells with or without chemokine treatment was quantified using the oxidation−reduction indicator dye Alamar blue in response to the chemical reduction of the treatment medium [23]. From log phase starter culture of *C. neoformans* and *C. gattii* grown overnight, a fresh subculture was started on shaking −incubator at 30 °C. After the culture reached exponential phase (OD ~0.6/ml), a stock solution was made with 1×10^6 cells/ml in respective media (DMEM with phenol indicator, pH 7.4 and 50 mM Hepes). In a 96-well culture plate, 100 μl of this culture with or without drug/ chemokine were added into each well. Vehicle control had 0.03% Human Serum Albumin (HSA) or water in which the chemokines or drugs were dissolved, respectively. The plates were placed on a shaking- incubator at 30 °C, 120 rpm. After 4 h (hrs), Alamar blue was added at a 1:10 dilution to each well including blanks lacking the pathogen. The sample plate was then protected from light and incubated for an additional 16 h at 37 °C on a shaker. The reduction of Alamar blue as an index of fungal cell number and proliferation was then estimated by measuring sample well absorbance at 570 nm and 600 nm in a BioRad iMark Micro plate Reader. Calculations regarding the amount of reduced Alamar Blue % were made according to its spec sheet protocol.

Measurement of colony forming units (CFU)/ml

For CFU determinations, three replicate wells from each treatment group were harvested 4 h post treatment, and several dilutions were prepared according to predetermined values specific for each treatment (dilution values ranged from no dilution to 1:100,000, which typically resulted in countable colonies per plate). Sample dilutions were plated onto YPD agar plates and incubated for 24−48 h at 30 °C before the colonies were enumerated.

Immunofluorescence

Fungal cells were sub cultured from a fresh secondary culture as described before in triplicate wells. Cells harvested 4 h post-treatment in Alamar Blue assays, were pooled from 3 or more wells and spun down at 15,000 rpm for 5 min at room temperature. The pellet was washed in **T**ris **B**uffered **S**aline (TBS) thrice, 1 droplet typically of 5 μl on Poly-lysine treated coverslips air-dried, fixed in 4% paraformaldehyde for 5 min, and permeabilized with 0.1% Triton X- 100 in TBS. After 10 min Triton X-100 was aspirated, rinsed twice with TBS-Triton X-100 (TBS-T), and blocked in 5% blocking buffer for 30 min at room temperature. Primary antibody was added at a dilution ranging from of 1:1000-2000 and incubated overnight at 4 °C. The coverslips were washed thrice with TBS-T and incubated in secondary antibody with Alexa fluor 488 at a dilution of 1:2000-3000 for 2 h at 4 °C. Coverslips were washed and mounted on slides in antifade with or without DAPI mounting media and visualized first under Leica DMi8 inverted fluorescence microscope before processing for confocal imaging [24−26].

Super-resolution confocal and stimulated emission depletion (STED) microscopy

The laser Scanning Super Resolution Microscope of Leica Microsystem, model SP8 STED, was used for imaging purposes [27]. Confocal imaging was done with blue (405 nm) and green (488 nm). For confocal +DIC imaging green, with a range of 495−559 nm, PMT detectors (range 411−462 nm and 496−580 nm) for blue and green respectively, with a scan speed of 200 Hz, Pin hole 1 AU, Line average 4 were used Visualization was with 63X (oil) magnification and XYZ scan mode. STED/ Super Resolution imaging +DIC were done with channel Green (488) only, with HyD detector with a range of 495 −570 nm, Pin hole 1AU, scan mode XYZ STED laser 592 nm, and magnification 100X (oil). Using the SP8 STED 3X: the lateral resolution

Table 1
The + sign is for direct killing. +++ sign denotes ≥ 80% killing, ++ ≥ 60% < 80%, + shows ≥20% < 60% while – means < 20% or no significant direct killing by these chemokines.

¿	Chemokines	*C. neoformans*	*C. gattii*
1	**CXCL7**	**+**	–
2	**CXCL9**	**++**	**+**
3	**CXCL10**	**+++**	**++**
4	**CXCL11**	–	–
5	**CXCL20**	–	–
6	**CXCL21**	**+**	–
7	**CXCL27**	**+**	–
8	**CCL5**	**+++**	**+**
9	**CCL7**	**+**	–
10	**CCL9**	**+**	–
11	**CCL20**	**+**	–
12	**CCL21**	**++**	–
13	**CCL27**	**+**	–
14	**CCL28**	**++**	–

that can be achieved is ~ 50 nm. Only bright photostable samples in the confocal mode were processed for STED. Excellent performance up to 50 nm deep into the sample could be achieved by single color STED imaging with Alexafluor 488 labeled secondary antibody where 100x oil STED WHITE is the lens of choice for standard fixed samples and structures close to the coverslip.

Statistical analyses

Statistical analyses and graphing were performed using MS Excel and /or GraphPad Prism 6.0 software. Experimental groups were analyzed using an unpaired, two-tailed student *t*-test. Significant differences were determined to have a P value of ≤ 0.001.

Results and discussion

Determination of direct killing by CXC and CC chemokines on C. neoformans *and* C. gattii

Fourteen chemokines were selected, seven from each CXC and CC category, based on their reported antifungal properties. Alamar blue assays were performed to determine and compare the direct killing effects of these chemokines against *Cryptococcus spp. in vitro*. The results have been presented in Table 1.

(Table 1) Antifungal effects of chemokines against *Cryptococcus spp*. Compares the antimicrobial effects of selected chemokines against *Cryptococcus spp*. CXCL10 and CCL5 are the most potent in showing a direct killing effect on both *C. neoformans* and *C. gattii in vitro*.

Quantifying the direct killing effects of CXCL10 and CCL 5 on C. neoformans

The potential direct killing effects by chemokines CXCL10 and CCL5 against *C. neoformans* were investigated and quantified by Alamar Blue assay as shown in Fig. 1A. The chemokines were used at their physiologically relevant concentration, usually observed during infections [28]. Treatment with CXCL10 and /or CCL5 resulted in a significant reduction of ≥ 80% in cryptococcal survival, similar to the effects of the antifungals Fluconazole and Amphotericin B (drug-of-choice for cryptococcosis). CXCL7 and CCL9 were less effective and showed ≥ 60% fungal survival in comparison to the no-drug controls. In order to confirm whether the reduced absorbance measured by the assay either indicated the absence of a viable cell population due to death or a dormant but live cell population, **CFU**/ml of *C. neoformans* were measured as shown Fig. 1B. The results revealed that the presence of CXCL10 or CCL5 significantly reduced CFU/ml than the initial inoculum compared to the untreated or less effective chemokine-treated controls, indicating that the cryptocccal population was killed rather than remaining dormant.

Determination of half maximal inhibitory concentration or IC_{50} and synergistic effects of CXCL10 and CCL5 against C. neoformans *and* C. gattii

The microbicidal effect of CXCL10 and CCL5 against *C. neoformans* was found to be effective in achieving complete pathogen killing as depicted in Fig. 2A at concentration of < 4 μM and < 0.5 μM respectively. The half maximal inhibitory concentration (IC_{50}) values for CXCL10 and CCL5 were determined to be 1.694 ± 0.01384 μM and 0.1695 ± 0.003258 μM respectively. Notably, CXCL10 and CCL7 could achieve ≥60% killing in *C. neoformans* at lower concentrations than when used individually, as shown in Fig. 2B. *C. gattii*, which exhibited little or no susceptibility to any of the tested chemokines except for CXCL10, was killed significantly more by CXCL10 and CCL5 when used in combination, even at a low concentration typically observed in the serum of an uninfected host.

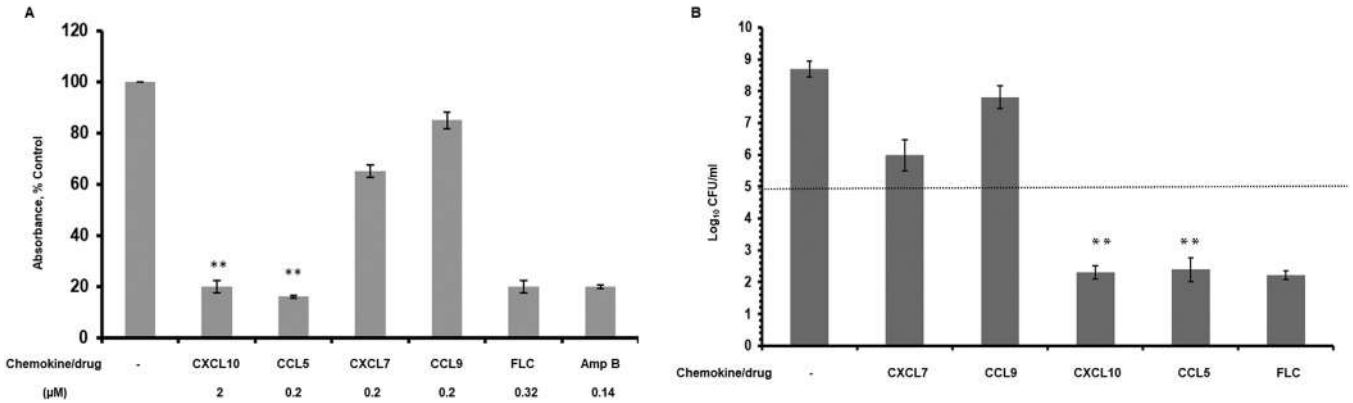

Fig. 1. *Quantification of the direct killing against C. neoformans by chemokines*. A. CXC chemokines CXCL10, CXCL7 and CC chemokines CCL5, CCL9 were added into wells in triplicate at the indicated concentration to a final volume of 100 μl of DMEM plus 50 mM Hepes with phenol indicator containing 10^5 Cryptococcal cells. Media with cells and without any chemokines or drugs served as controls. Alamar blue data are expressed as a percentage of untreated control and represented mean values, standard errors of the means (SEM) for three independent experiments. P value ** < 0.001 compared with untreated control. B. Chemokine-treated *C. neoformans* cells were pooled from 3 wells of a 96-well plate, washed in PBS, and plated on YPD plates in serial dilution. The dotted line represents the initial inoculum. CFU data are expressed as CFU/ml (log_{10} scale) and a representative data set is shown from three separate experiments, P value ** < 0.001 compared with untreated control.

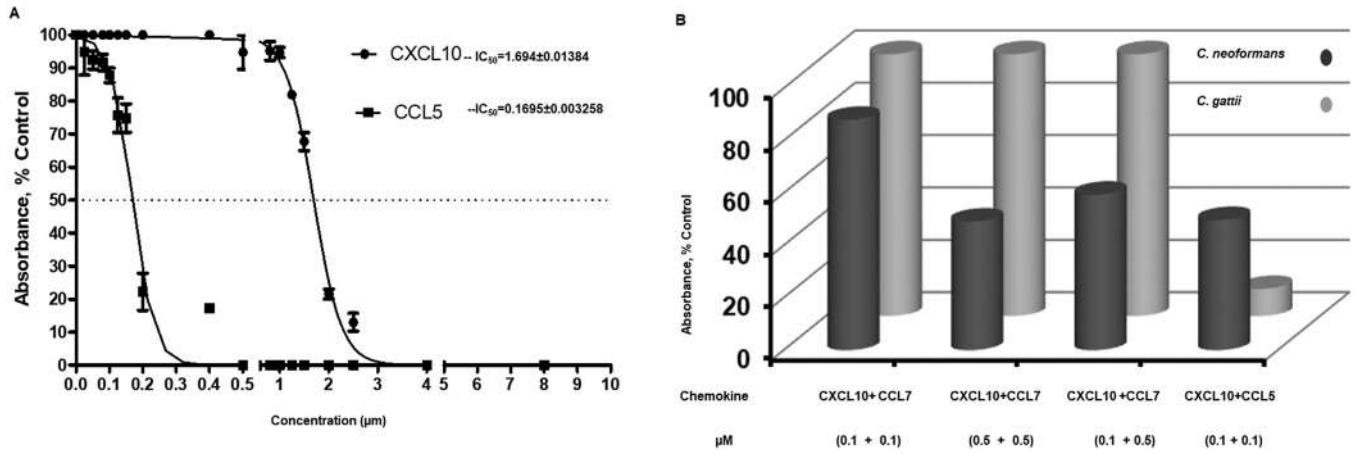

Fig. 2. *Concentration curve and synergistic effects of antimicrobial chemokines.* A. 10^6/ml of log phase secondary culture of *C. neoformans* cells were treated with CXCL10 and CCL5 in a concentration ranging from 0 to 8 μM. Alamar blue reduction is expressed as a percentage of the untreated control and data points represent mean values ± SEM; $n = 4$ independent experiments, with IC_{50} values of the CXCL10 and CCL5 at 1.694 ± 0.01384 and 0.1695 ± 0.003258 μM, respectively. B. CXCL10, CCL5 and CCL7 (a chemokine with less or no killing effect in *C. neoformans* and *C. gattii*) in different combinations were used together at varying concentrations and direct killing effects were quantified by Alamar Blue assay.

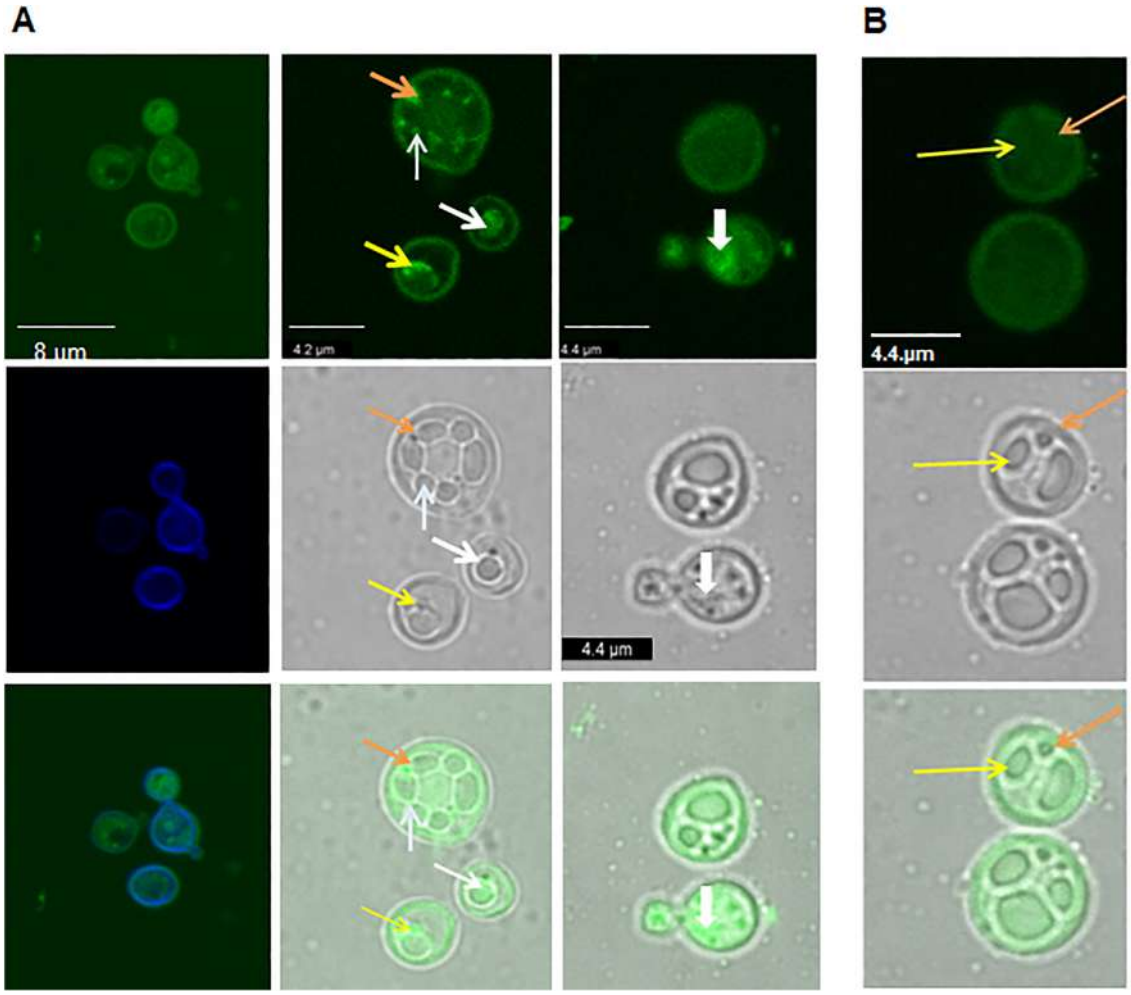

Fig. 3. *Confocal STED Microscopy on CXCL10 treated Cryptococcus spp.* (A) Single or dual color confocal and STED images of chemokine-treated *C. neoformans* with Alexafluor 488 ligated secondary antibody against a CXCL10 monoclonal primary antibody. The left panels shows single (green) upper, middle denoting DAPI, while the overlay confocal images in the lowermost box, demonstrates chemokine binding on the cell membrane/wall. Scale bar ~ 8 μM. The other 2 panels are single-color STED images with the uppermost square showing the extracellular and intracellular targets in green. The middle is the DIC imaging and the lowermost overlap. Colored arrows points to the different intracellular structures having CXCL10 binding targets. The filled arrow denotes the concentration of fluorescence at the budding site, Scale bar ~4 μm. (B). Confocal STED images of *C. gattii* showing cell wall/membrane and intracellular targets of CXCL10.

Confocal and STED microscopy

Confocal imaging and Stimulated Emission Depletion Microscopy (STED) are powerful tools for non-invasive visualization of specific components or processes in cells or organisms. STED imaging can produce super-resolved images (< 200 nm) in a very short period of time making it useful for investigating whether a chemokine binds to specific targets in cryptococcal cells, both extracellular and intracellular targets. In order to explore this, CXCL10-treated *C. neoformans* and *C. gattii* were imaged using confocal and STED microscopy as illustrated in Fig. 3A and B. Confocal images confirmed CXCL10 binding to cellular targets, while STED imaging revealed the precise location of the chemokine binding targets in Cryptococcal cells treated with CXCL10 and stained with monoclonal antibodies against the chemokine. The results showed that CXCL10 has multiple binding targets both inside and on cell wall/membrane. A concentration of fluorescence was also observed in or around the budding site. The CXCL10 chemokine binds to the cell wall/membrane of the fungus and, after internalization, binds to various intracellular targets, as denoted by arrows in the figure. Thus, STED imaging provided a means to visualize the ultrastructures inside the cell with which CXCL10 had bound, providing insights into probable targets of this chemokine.

Frequent development of drug and treatment resistance underscores the pressing need for innovative therapeutic approaches to combat a broad range of human diseases, particularly Multidrug Resistant (MDR) bacteria and fungi, which poses serious global threat. The widespread use of antifungals has driven pathogens like *Cryptococcus* spp. to develop several means which includes, but are not limited to 'titan cells' [29] and 'cross-linked capsule' [26] to escape the host immune response effectively, leading to the emergence of resistance. The candidacy of a molecule for pharmacological development and applications is strengthened if it can potentially circumvent the host immunomodulatory action in addition to being microbicidal. Host-derived molecules are likely to be more adept at avoiding the host defense system and immune rejection, making them potential and successful candidate for exogenous application. The microbicidal activity of chemokines, along with previously unknown synergistic effects of CXCL10 and CCL5 reported here, can be further explored to develop alternative therapies such as chemokine –derived small molecule(s) alone, or in combination, against cryptococcosis. In general, small molecules, most antimicrobial chemokines, exhibit activity against multiple organisms, suggesting further possibilities for exploring chemokine-based treatment strategies for other microbes as well.

Conclusion

This study highlights the direct microbicidal effect of chemokines against the human pathogen *Cryptococcus spp*. While killer cells activated by immune responses may eventually reduce pathogen burden, certain immune cells like chemokines have the potential to target both the cell wall/membrane and intracellular structures of the pathogen, leading to its direct killing. The significant finding is the effectiveness of CXCL10, a 'CXC' chemokine, in killing *Cryptococcus spp*. Thus chemokine-based therapy provides a promising alternative that can be tailored to individual patients' immune responses especially as drug resistance continues to be a major challenge. This research contributes to advancing the development of personalized and effective treatments against microbial infections.

Declaration of Competing Interest

None.

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