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Neutrophil swarming toward *Cryptococcus neoformans* is mediated by complement and leukotriene B4

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ABSTRACT

Swarming behavior of neutrophils has been noticed in both sterile injury and infection models and the mechanisms are being unveiled. So far, no *in vitro* model has been established to study neutrophil swarming to microbes. In the current study, using live-cell imaging, we observed *in vitro* neutrophil swarming toward *Cryptococcus neoformans*, a fungal pathogen causing human meningoencephalitis. Complement C3 and CD11b expression are essential for neutrophils to form cell swarms surrounding *C. neoformans*. Leukotriene B4 (LTB4) was quickly released by neutrophils during their interactions with *C. neoformans*. Blockade of LTB4 synthesis inhibited the swarming response to *C. neoformans*. Importantly, blockade of LTB4 synthesis also significantly reduced neutrophil recruitment in the lung vasculature of mice infected intravenously with *C. neoformans*, demonstrating a critical role of LTB4 in intravascular neutrophil swarming during infection. Together, this is the first report of neutrophil dynamics of swarming toward a microorganism *in vitro*, mediated by complement and LTB4.

1. Introduction

As the most abundant innate immune cells, neutrophils not only play a fundamental role in defense against invading pathogens, but also serve as critical mediators of sterile inflammation in acute and chronic diseases [1]. To exert their functions, neutrophils must be recruited to sites of infection and inflammation. Decades ago, neutrophils have been observed to aggregate in the presence of chemotactic factors and mechanic stirring [2–4]. Recently, with the development of imaging techniques, much progress has been made on neutrophil dynamics during their migration to sites of infection and inflammation [5].

With the use of intravital microscopy, neutrophils have been recently shown to rapidly migrate toward sites of sterile tissue injury [6,7] or infections [8–12]. As the dynamic of neutrophil accumulation is similar to swarming behavior of some insects, the behavior of neutrophils is commonly referred to as “neutrophil swarming” [5]. It has been recently shown that the lipid leukotriene B4 (LTB4), secreted by neutrophils, plays a central role in neutrophil activation and migration to formyl peptides [13]. More recently, *in vivo* imaging has revealed that both LTB4 and integrins are required for neutrophil swarming in the extravascular space of a damaged tissue [8]. However, the role of LTB4 in intravascular neutrophil swarming has not been determined *in vivo* [5].

Although neutrophil swarming has been recently described *in vivo* using both tissue injury models and infection models [8,9,11], this important behavior of neutrophils has been poorly addressed *in vitro*. Questions remain as to whether neutrophils swarm to pathogens in an *in vitro* system and how it happens.

Using live-cell imaging, we have recently established an *in vitro* model to study the dynamic interactions of neutrophils with *Cryptococcus neoformans*, a human pathogenic fungus causing fatal meningoencephalitis [14]. Given the complexity of *in vivo* environment, *in vitro* models provide an alternative approach to dissect mechanisms underlying host-pathogen interactions, with the advantage of being able to precisely control experimental conditions. In the current study, we visualized the swarming of neutrophils toward *C. neoformans in vitro* and examined the underlying mechanisms.

Abbreviations: C3, complement component 3; C5, complement component 5; C5a, complement component 5a; C5aR, complement component 5a receptor; CXCL1, chemokine (C-X-C motif) ligand 1; CXCL2, chemokine (C-X-C motif) ligand 2; rC5a, recombinant complement 5a; LTB4, Leukotriene B4; TRITC, Tetramethylrhodamine.

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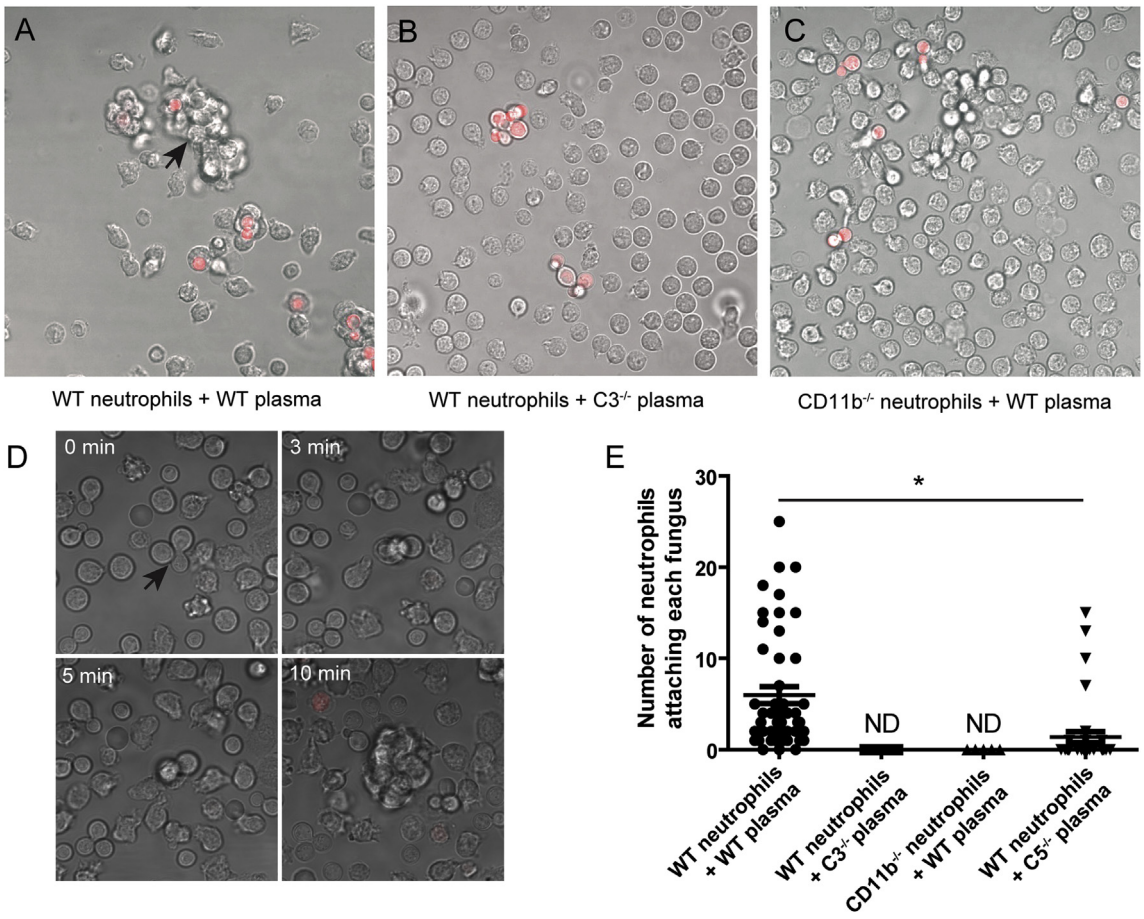

Fig. 1. Essential role of complement and CD11b in neutrophil swarming to *C. neoformans*. (A and B) Live-cell imaging showing that neutrophil swarming to *C. neoformans* (red, labeled with TRITC) occurred in the presence of wild-type mouse plasma (A, arrow, see also Video S1), but not $C3^{-/-}$ mouse plasma (B). (C) No swarming to *C. neoformans* (red) was observed for $CD11b^{-/-}$ neutrophils in the presence of wild-type mouse plasma (see also Video S2). (D) Series of images showing that neutrophils swarm to *C. neoformans* (arrow) when co-cultured in the presence of $C5^{-/-}$ mouse plasma. Red: dead cells stained by sytox orange (see also Video S3). (E) Quantification of the number of neutrophils attaching each fungus 30 min after coincubation using live-cell imaging. Data are presented as means ± SEM. Data are representative of results from 2 independent experiments. *, $p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2. Materials and methods

2.1. Animals and C. neoformans

C57BL/6 mice were purchased from National Cancer Institute (Frederick, MD, USA). $C3^{-/-}$ (JAX003640), $CD11b^{-/-}$ (JAX003991), and $C5^{-/-}$ (JAX000461) mice were purchased from the Jackson laboratory (Bar Harbor, ME, USA) and bred in the animal facility of the University of Maryland. For all experiments, eight- to ten-week-old mice were used. The animal use protocol was approved by the Institutional Animal Care and Animal Use Committee (IACUC) of the University of Maryland.

Encapsulated *Cryptococcus neoformans* H99 strain (serotype A, ATCC no. 208821) was used throughout the experiments. A single fungal colony was inoculated into Sabouraud's dextrose broth and cultured to exponential phase at 32 °C with gentle rotation (180 rpm) for 24 h. The yeast cells were washed twice with PBS and counted before use.

2.2. Neutrophil isolation and culture

Neutrophils were isolated from tibia and femur of adult mice using 69% and 78% Percoll (GE, Pittsburg, PA, USA) as previously described [15] and resuspended in Hanks' balanced saline solution (HBSS) without calcium and magnesium. Neutrophils (5×10^5) were incubated with *C. neoformans* (5×10^4) in 96-well plates containing 200 μL RPMI 1640 in the presence of 40% fresh mouse plasma at 37 °C. In some experiments, neutrophils were incubated with recombinant C5a (rC5a, 250 ng/ml, R&D Systems, Minneapolis, MN, USA) in the presence or absence of Zileuton (100 μM, 5-lipoxygenase inhibitor, Cayman chemicals, Ann Arbor, MI, USA). The supernatants and cells were collected for ELISA and qPCR, respectively.

2.3. Live cell imaging of neutrophil swarming

Live cell imaging was performed in a 35-mm glass-bottom dish (Thickness no. 1.5; MatTek, Ashland, MA, USA) containing 200 μl RPMI-1640 supplemented with 40% fresh mouse plasma. Neutrophils and yeast cells were added at an effect to target ratio of 10:1 (5×10^5:5×10^4). In some experiments, sytox orange (5 μM, Thermofisher scientific, Waltham, MA, USA) were added to detect cell viability. Neutrophil swarming was visualized using Zeiss LSM 510 system coupled with a CO_2 module and a temperature control module (PECON, Germany) connected to a transparent chamber to maintain 5% CO_2 and 37 °C.

2.4. Animal infections

Mice were intravenously infected with 5×10^6 *C. neoformans*. In