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Feline polymorphonuclear neutrophils produce pro-inflammatory cytokines following exposure to *Microsporum canis*

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ABSTRACT

The mechanisms involved in the establishment of the specific immune response against dermatophytes remain unknown. Polymorphonuclear neutrophils (PMNs) are recruited early during the infection process and participate in the elimination of dermatophytes. They could therefore be involved in the induction of the immune response during dermatophytoses by producing specific cytokines. The aim of this work was to assess the *in vitro* cytokine production by feline PMNs exposed to living arthroconidia from the dermatophyte species *Microsporum canis* or stimulated with either a secreted or a structural component of *M. canis*, the latter consisting of heat-killed arthroconidia. The levels of specific cytokines produced by PMNs were determined by capture ELISA and/or quantitative RT-PCR. Results showed that PMNs secrete TNFα, IL-1β and IL-8 following exposure to *M. canis* living arthroconidia and stimulation with both a secreted component and heat-killed arthroconidia. The level of IL-8 mRNA was also increased in PMNs stimulated with *M. canis* living arthroconidia. In conclusion, infective *M. canis* arthroconidia induce the production of pro-inflammatory cytokines by feline PMNs that can be activated either by secreted or structural fungal components. Our results suggest that these granulocytes are involved in the initiation of the immune response against *M. canis*.

1. Introduction

Microsporum canis is a zoonotic dermatophyte responsible for most ringworm in dogs and cats (Weitzman and Summerbell, 1995; Chermette et al., 2008; Mignon and Monod, 2011). Because dermatophytes invade hard keratinized skin structures, considerable attention has focused on the characterization of secreted proteases as putative fungal virulence factors (Monod, 2008; Vermout et al., 2008), but few of them have been demonstrated to be pathogenic factors in *M. canis* (Descamps et al., 2002; Baldo et al., 2010; Bagut et al., 2012). In contrast, little effort has been devoted to the study of the host immune response against *M. canis* specifically and other dermatophytes in general (Almeida, 2008; Mignon et al., 2008). Despite their superficial localization in skin, dermatophytes can induce an adaptive immune response. The cellular response, which is associated with delayed type hypersensitivity (DTH), is known to be correlated with clinical recovery and protection against re-infection

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(Calderon, 1989; Almeida, 2008; Mignon et al., 2008). The first immunological events following the infection are yet to be elucidated, including the role of innate immunity in the set-up of the host-specific immune response. The first epidermal cells encountered by dermatophytes during the infection process are keratinocytes, which can produce a broad spectrum of cytokines upon exposure to these fungi (Nakamura et al., 2002; Shiraki et al., 2006; Tani et al., 2007), including the chemo-attractant for polymorphonuclear neutrophils (PMNs) IL-8 and the pro-inflammatory TNFα (Nakamura et al., 2002). The first leucocytes recruited to the site of infection in dermatophytoses are PMNs (Hay et al., 1988). These cells, along with macrophages, are known to be responsible for the elimination of dermatophytes (Calderon and Hay, 1987; Heddergott et al., 2012). Their potential role in the induction of the specific immune response in dermatophytoses remains unknown but can be reasonably hypothesized. Indeed, in other fungal and microbial infections PMNs can initiate and modulate the adaptive immune response by interacting with dendritic cells and producing specific cytokines (Schaller et al., 2004; Megiovanni et al., 2006; Charmoy et al., 2010). The aim of this study was to evaluate the potential role of feline PMNs during the early stages of *M. canis* infection. To this purpose, PMNs were cultured with various *M. canis* components and the levels of specific cytokines produced by PMNs were assessed.

2. Materials and methods

2.1. Isolation of feline polymorphonuclear neutrophils

Blood from cats was kindly provided by veterinarian practitioners through blood donations taken with the agreement of the cats' owners. Sampled cats were domestic short-haired intact male or female young adults with no history of medical problems. The clinical examination revealed no abnormalities. Cats were negative after testing for infection with Feline Leukemia Virus and Feline Immunodeficiency Virus using the WITNESS® FeLV-FIV test (Prodivet, Eynatten, Belgium). Fungal cultures performed from cat hair were negative for dermatophytes.

Feline PMNs were isolated from heparinized whole blood samples using Polymorphprep™ solution (Axis-Shield, Oslo, Norway). Blood was layered over the density gradient and centrifuged for 30 min at 500 × *g*. Two distinct leukocyte layers (lymphocytes and monocytes in the upper and granulocytes in the lower layer) were obtained. PMNs were harvested, washed and suspended in 24-well cell culture plates (Greiner Bio-One, Frickenhausen, Germany) at a concentration of 1×10^6/ml in RPMI 1640 + GlutaMAX™ medium (Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS) and 1% penicillin-streptomycin (Gibco, Life Technologies). Hemacolor® staining (Merck, Whitehouse station, NJ, USA) was performed to ensure the purity (PMN > 95%) of isolated cells. The latter were vital-stained using the trypan blue dye-exclusion method, and the number of living leukocytes (>98%) was assessed using a Neubauer chamber. Freshly isolated PMNs were used in all experiments.

2.2. Production of M. canis arthroconidia

Arthroconidia were produced from the *M. canis* strain IHEM 21239 by a process previously outlined (Tabart et al., 2007). Briefly, arthroconidia were obtained from 15-day-old cultures on 2% yeast extract/1% peptone agar (VWR Scientific Products, San Dimas, CA, USA) in an atmosphere containing 12% CO_2 at 30 °C. Surface mycelium and conidia were scraped, transferred to PBS and filtered through Miracloth layers (22–25 μm; Calbiochem, La Jolla, CA, USA). Arthroconidia concentration was determined by serial dilutions on Sabouraud's dextrose agar (Sab) medium. Arthroconidia were stored at 4 °C until use. In all experiments, arthroconidia were used within 1 month.

To exclude a possible contamination with an endotoxin (LPS) during arthroconidia production, a PBS solution was prepared using the same procedure except that arthroconidia were omitted. This control PBS solution was further used concomitantly in PMN stimulation experiments (*cf.* below).

2.3. Production of M. canis secreted and structural components

In addition to living arthroconidia, two components were produced to further stimulate feline PMNs: a secreted component and heat-killed arthroconidia representing structural components.

The secreted component was obtained after growing *M. canis* arthroconidia (1×10^5) in 500 ml liquid Sab medium for 5 days at 28 °C under gentle agitation. Culture supernatant was separated from fungal elements by centrifugation, concentrated by ultrafiltration on an Amicon (Millipore, Billerica, MA, USA) 10 kDa membrane and stored at −20 °C until use. Protein concentrations were determined by Bradford's method (Bradford, 1976).

The structural components were obtained by heating *M. canis* arthroconidia (1×10^5) at 95 °C for 10 min. Arthroconidia were cooled down to room temperature and directly used in stimulation experiments. Killing of arthroconidia was confirmed by the absence of growth on Sab medium plates incubated at 27 °C for 21 days.

2.4. Exposure of feline PMNs to M. canis

Feline PMNs (1×10^6/well) were stimulated for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2 (i) with *M. canis* living arthroconidia (1×10^5/well) or with culture medium alone as negative control; (ii) with 10 μg of the secreted component or with liquid Sab medium as negative control; (iii) with 1×10^5/well heat-killed arthroconidia or with PBS as negative control. A positive control consisting in stimulation of PMNs with 1 μg lipopolysaccharide (LPS)/well (purified from *Escherichia coli*; 0111:B4, Sigma–Aldrich) was also performed. All experiments were performed in triplicate using PMNs from three unrelated cats.