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Title: 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 was 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.

Liège, Monday 08th October 2012

Ref: original article VETMIC-D-12-7272

Dear Editor in chief,

Would you please find herewith our manuscript entitled: « Feline polymorphonuclear neutrophils produce pro-inflammatory cytokines following exposure to *Microsporum canis* » which is a revised form of the above referred original manuscript.

We would like to thank you for your consideration of our work as well as for your comments and the opportunity you gave us to submit an amended version of our manuscript. We would like also to thank the reviewers for their critical remarks.

Our specific answers to comments are as follow:

Editor's comments: As an Editor it is not only my duty to assure the scientific quality of manuscripts, but also to make sure that the limited printing space available is used as efficient as possible. In this respect I have to ask you to reduce the length of your Introduction to one page. I have asked this from numerous authors and do not wish to make an exception for your manuscript.

The length of the introduction was reduced as requested.

Reviewer 1:

My main concerns relates to:

- some minor typo-errors occurring in the manuscript (i.e., line 258 "Trichomonas vaginalis") that should be corrected during revision.

Line 254: "*Trichomona vaginalis*" was replaced by "*Trichomonas vaginalis*".

- the discussion section, which would benefit from some modification. The content of lines 214-215 is over repeated in this part, so I would ask to the Authors to adjust it throughout the text. The concepts of lines 215-219 should be for other parts of the work.

The content of the lines 214-219 was deleted to avoid repetition in the work.

Reviewer 2:

Material and Methods: The authors should specify whether sampled cats were healthy, as I assume. More specifically, clinical pictures of cats and results of their haematological analysis should be reported.

The cats were healthy as revealed by a clinical examination performed by a veterinary practitioner. No clinical picture was taken because of the total absence of obvious lesions. However, a fungal culture was performed and was negative for dermatophytes in all cases. Concerning haematological analyses, a blood smear confirmed that feline PMNs had no morphological abnormalities and represented 40-75% of total white blood cells. Cats were negative after testing for infection with Feline Leukemia Virus and Feline Immunodeficiency Virus.

The text was modified as suggested (lines 71-77).

Discussion:

Page 12, lines 240-243: the sentences "This is not related to the transcriptional inability of PMNs. These cells are not terminally differentiated, though that was a long-held belief. Indeed, several studies have reported the regulation of gene expression in PMNs stimulated with pathogens (Fradin et al., 2007; Ma et al., 2011)" needs to be clarify. The authors should better discuss why high levels of TNF<alpha> were detected by Elisa and not by qRT-PCR.

Lines 226-233: the sentences have been clarified.

Lines 226-241: the difference between the results obtained by ELISA and qRT-PCR for TNFα detection has been better discussed.

Page 12, lines 260-264: the sentences "Interestingly, in addition to containing strong activators of feline PMNs, the secreted component induces DTH in immune guinea pigs having spontaneously recovered from an experimental infection with (data not shown). This indicates." are not supported by any experimental procedures. Please specify whether the data were obtained by the authors in the present study or if the results belong to an other study.

The data were obtained by Mignon and coworkers in other studies which are not yet published. However, these data were obtained using experimental procedures which are identical to those described in another article published by Mignon and coworkers (Mignon et al., 1999).

The “data not shown” was therefore replaced by “Mignon, personal communication” (line 257).

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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 was 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*.

Key words: dermatophytes, *Microsporum canis*, feline ringworm, polymorphonuclear neutrophils, pro-inflammatory cytokines

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 (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, 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.

Material and Methods

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% foetal 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.

Production of *Microporum 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).

Production of *Microsporum 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.

Exposure of feline PMNs to *Microsporum 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.

Quantification of cytokine secretion by feline PMNs

After *M. canis* exposure, PMN culture supernatants were collected by centrifugation for 5 min at 500 *g*, filtered (0.2 µm) and stored at -80°C after being shock-frozen in liquid nitrogen. The amount of TNFα, IL-1β, IL-8 and IFNγ secreted in culture supernatants was measured by capture ELISA using a DuoSet® kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. The concentration of each cytokine was determined using a standard curve generated by 2-fold serial dilutions of the recombinant cytokine. Absorbance values were measured at 450 nm using a Multiskan RC spectrophotometer (ThermoLabsystems, Altrincham, Cheshire, UK).

Quantification of cytokine mRNA level in feline PMNs

PMNs total RNA was isolated using a High Pure RNA Isolation kit (Roche Applied Sciences, Lewes, East Sussex, UK) following the manufacturer's instructions. Template cDNA was synthesized from RNA by reverse transcription, using iScript™ cDNA Synthesis kit (Bio-rad, Hercules, CA, USA). Reverse transcriptase was omitted in control reactions. Oligonucleotides were selected using the Primer-BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHomeAd). The sequences of oligonucleotide primers for feline 18S rRNA (internal control), TNFα, IL-1α, IL-8 and IL-18 were synthesized by Eurogentec (Liège, Belgium) (Table 1). The qPCR reactions were assembled using the iQ™ SYBR® Green Supermix (Bio-rad) and subjected to the following protocol in a MiniOpticon System (Bio-rad): 10 min at 95°C and 40 cycles of 45 s at 95°C, 45 s at 60°C and 45 s at 72°C. The melting curve was performed from 45°C to 95°C in 1°C/15 s increments. The number of copies of each mRNA was evaluated using a standard curve generated by serial dilutions of cDNA standard samples (from 10 to 1×10^9) using the Thermal cycler software (Bio-rad). Each transcript level was normalized to that of 18S rRNA from the corresponding sample.

Statistical analysis

The amount of secreted cytokines and the level of cytokine mRNA in PMNs exposed to *M. canis* components were compared with those determined in corresponding negative control PMNs.

Significant differences between two data sets were assessed using a general linear model (GLM procedure of SAS; SAS Institute Inc., Cary, NC, U.S.A.) with significance defined as $P < 0.05$.

Results

PMNs produce TNFα, IL-1β and IL-8 following exposure to *Microsporum canis* living arthroconidia

The amount of TNFα, IL-1β, IL-8 and IFNγ secreted by PMNs co-cultured with living arthroconidia was evaluated by capture ELISA. Stimulated PMNs produced significantly more TNFα (2.5-fold), IL-1β (8-fold) and IL-8 (25-fold) in culture supernatant than non-stimulated PMNs, whereas IFNγ secretion was not affected by stimulation (Fig. 1). The cytokine response evaluated by ELISA was actually induced by arthroconidia and not by a possible contamination with an endotoxin (LPS). Indeed, the cytokine production of PMNs stimulated with the control PBS solution did not differ from that of non-stimulated PMNs (data not shown).

Cytokine mRNA levels in PMNs exposed to *M. canis* living arthroconidia were evaluated by qRT-PCR and compared with those in non-stimulated PMNs (Fig. 2). In response to arthroconidia stimulation, the level of IL-8 mRNA expressed by PMNs increased significantly (35-fold). A slight increase of TNFα mRNA level was also observed; nevertheless, this result was not significant. A slight and non-significant decrease of IL-18 mRNA level was even observed in stimulated PMNs. The level of IL-1α mRNA expression in stimulated and non-stimulated PMNs was similar.

A secreted and a structural component from *Microsporum canis* are potent stimulators of PMNs

To investigate which *M. canis* components were responsible for pro-inflammatory cytokine production by PMNs stimulated with living arthroconidia, cells were exposed to either secreted, represented by the secreted component (or liquid Sab medium as specific negative control) or structural, represented by heat-killed arthroconidia (or PBS as specific

189 negative control) fungal components. A positive control consisting of stimulation of PMNs
190 with LPS was also performed.

191 Figure 3 shows the concentrations of TNFα, IL-1β and IL-8 in culture supernatants of
192 PMNs exposed to the secreted component and heat-killed arthroconidia. As feline PMNs
193 stimulated with *M. canis* living arthroconidia did not produce IFNγ (Fig. 1), the presence of
194 this cytokine was not further tested. As expected, LPS induced a significant increase of TNFα
195 (4.5-fold), IL-1β (4.8-fold) and IL-8 (10-fold) production in PMNs culture supernatant. A
196 significant increase of TNFα (5-fold), IL-1β (11-fold) and IL-8 (2-fold) production was
197 observed upon stimulation with heat-killed arthroconidia. These structural components
198 induced a similar (TNFα) and even a higher (IL-1β) cytokine production than LPS. Feline
199 PMNs stimulated with the secreted component produced significantly more TNFα (3.3-fold),
200 IL-1β (3-fold) and IL-8 (2-fold) than PMNs stimulated with liquid Sab medium.

201 Cytokine mRNA levels in PMNs exposed to the secreted component and heat-killed
202 arthroconidia are shown in Figure 4. A significant increase of TNFα (4-fold) and IL-8 (14-
203 fold) mRNA levels was observed in PMNs stimulated with LPS. Unexpectedly, LPS did not
204 induce a significant increase of IL-1α and IL-18 mRNA expression in PMNs. Cytokine
205 mRNA levels were similar in PMNs stimulated with heat-killed arthroconidia and PBS. A
206 non-significant increase of IL-1α, IL-8 and IL-18 mRNA expression in PMNs stimulated with
207 the secreted component was observed.

208

209 **Discussion**

210 Results obtained by ELISA show that, in response to *M. canis* living arthroconidia
211 stimulation, feline PMNs secrete TNFα, IL-1β and IL-8, which are three pro-inflammatory
212 cytokines. It has been suggested that PMNs play a role in protection by producing pro-
213 inflammatory cytokines in other fungal infections. In an *in vitro* model of oral candidosis, the

addition of human PMNs to epithelium induces the production of IL-1α, IL-1β, TNFα and IL-8 (Schaller et al., 2004). In response to *Paracoccidioides brasiliensis*, a fungus responsible for systemic mycoses, PMNs produce IL-8 (Acorci-Valério et al., 2010). This cytokine is a potent chemotactic factor for PMNs, promoting degranulation in these cells and enhancing their antifungal activity (Djeu et al., 1990). In our experimental conditions no IFNγ secretion from feline PMNs stimulated with *M. canis* arthroconidia was observed. This is not surprising because the main IFNγ-producing cells are T lymphocytes (Rengarajan et al., 2000). The pro-inflammatory cytokines produced by feline PMNs exposed to the infective spores of *M. canis*, namely arthroconidia, indicate that these cells may recruit and activate other immune cells like dendritic cells, macrophages and PMNs themselves. Further studies are needed to understand more precisely how PMNs can interact with other immune cells and to verify their role in the set-up of the specific immune response against *M. canis*.

Results obtained by qRT-PCR show that in response to *M. canis* living arthroconidia stimulation, the mRNA levels of cytokines do not vary in PMNs, except for an upregulation of IL-8. The considerable release of TNFα detected by ELISA is not correlated to the level of mRNA expression. This result is surprising but similar observations have been reported in other studies (Sawant and McMurray, 2007; Saegusa et al., 2009). This is not related to the inability of PMNs to modulate their gene expression in response to environmental changes. Indeed, in our study, the TNFα mRNA expression increases significantly in PMNs stimulated with LPS (Fig. 4). Additionally, other studies have reported the regulation of gene expression in PMNs stimulated with pathogens (Fradin et al., 2007; Ma et al., 2011). The discrepancy between ELISA and qRT-PCR results concerning TNFα detection could be related to either the short half-life of PMNs in culture or most probably, the few RNA they contain due to their high content of dense chromatin (Dockrell et al., 2007). More specifically, the amount of isolated mRNA could be insufficient to detect a low but potentially significant increase in

TNFα transcript despite the high sensitivity of the commercial kits. According to our results, ELISA rather than qRT-PCR seems to be more appropriate for quantification of PMN feline cytokines.

We have demonstrated that feline PMNs produce pro-inflammatory cytokines upon exposure to *M. canis* living arthroconidia. To further investigate which fungal components could be responsible for this activation, a secreted and a structural component consisting of heat-killed arthroconidia were produced and used for stimulation experiments. They were shown to induce the release of TNFα, IL-1β and IL-8, suggesting that both secreted and structural *M. canis* components are involved in the activation of feline PMNs. Regarding structural components, Saegusa et al. (2009) reported similar results with heat-killed *Candida albicans* and *Saccharomyces cerevisiae* that induce TNFα, IL-1β and IL-8 production by human PMNs. Additionally, zymosan, a glucan prepared from a yeast cell wall and LPS, a component of the outer membrane of Gram-negative bacteria, are commonly used to stimulate the production of cytokines by PMNs (Bellocchio et al., 2004). The role of specific secreted components in the activation of PMNs has also been demonstrated for other pathogenic microbes, namely, *C. albicans* (Losse et al., 2011) and *Trichomonas vaginalis* (Nam et al., 2012). Interestingly, in addition to containing strong activators of feline PMNs, the secreted component induces DTH in immune guinea pigs having spontaneously recovered from an experimental infection with *M. canis* (Mignon, personal communication). This indicates that the secreted component should contain Th1 antigens. Consequently, the characterization and the selection of appropriate protective antigens from the secreted component appear to be of major importance in the development of an effective vaccine against *M. canis.*

In our experiments, feline PMNs and *M. canis* components are in close contact. PMNs are able to capture PAMPs (pathogen associated molecular patterns) of the fungus via their PRRs (pattern recognition receptors). Among PRRs expressed by PMNs, TLRs (toll like

receptors) are well characterized in humans and mice but not in cats. In human PMNs, the TLR-2 and TLR-4 are involved in the recognition of fungal PAMPs and therefore contribute to the establishment of a protective immune response (Bellocchio et al., 2004; Acorci-Valério et al., 2010). These TLRs could also be involved in the recognition of *M. canis* PAMPs and consequently induce TNFα, IL-1β and IL-8 secretion by feline PMNs. The characterization of these receptors would be very helpful in furthering our understanding of the mechanisms involved in the first steps of the establishment of immunity against *M. canis.*

Conflict of interest statement

None.

Acknowledgements

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Figures

Figure 1

Microsporum canis living arthroconidia induce pro-inflammatory cytokines secretion by feline polymorphonuclear neutrophils (PMNs).

Feline PMNs were cultured in the absence (control) or the presence of *M. canis* arthroconidia for 24 h at 37°C in a humidified atmosphere containing 5% CO_2. The amount of TNFα, IL-1β, IL-8 and IFNγ secreted in culture supernatant was evaluated by capture ELISA. In response to *M. canis* arthroconidia stimulation, PMNs produce significantly more ($^{***}P < 0.001$) TNFα, IL-1β and IL-8 in culture supernatant while IFNγ secretion is not affected by stimulation.

Data are representative of 3 independent experiments (mean ± SEM).

Figure 2

Microsporum canis living arthroconidia induce an increase of IL-8 mRNA expression in feline polymorphonuclear neutrophils (PMNs).

Feline PMNs were cultured in the absence (control) or the presence of *M. canis* arthroconidia for 24 h at 37°C in a humidified atmosphere containing 5% CO_2. The levels of TNFα, IL-1α, IL-8 and IL-18 mRNA were quantified by qRT-PCR and the number of mRNA copies was evaluated using a standard curve. In response to *M. canis* arthroconidia stimulation, only IL-8 mRNA expression increases significantly in PMNs ($^{*}P < 0.05$).

Data are representative of 3 independent experiments (mean ± SEM).

Figure 3

Feline polymorphonuclear neutrophils (PMNs) secrete pro-inflammatory cytokines in response to a secreted component (SC) and heat-killed arthroconidia (HKA) from *Microsporum canis*.

PMNs were stimulated for 24 h with either lipopolysaccharide (LPS, positive control) or *M. canis* HKA (or PBS as specific negative control) or *M. canis* SC (or liquid Sab medium as specific negative control) and the amount of TNFα, IL-1β and IL-8 secreted in culture supernatant was evaluated by capture ELISA. A significant increase in TNFα, IL-1β and IL-8 secretion by PMNs was observed in culture supernatant of PMNs stimulated with LPS, SC and HKA, indicating that both secreted and structural components from *M. canis* are able to stimulate PMNs.

Data are representative of 3 independent experiments (mean ± SEM).

$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

Figure 4

The cytokine mRNA expression in feline polymorphonuclear neutrophils (PMNs) does not vary in response to a secreted component (SC) and heat-killed arthroconidia (HKA) from *Microsporum canis*.

PMNs were stimulated for 24 h with either lipopolysaccharide (LPS, positive control) or *M. canis* HKA (or PBS as specific negative control) or *M. canis* SC (or liquid Sab medium as specific negative control). The levels of TNFα, IL-1α, IL-8 and IL-18 mRNA were quantified by qRT-PCR and the number of mRNA copies was evaluated using a standard curve. No significant modification in the cytokine mRNA expression by PMNs was observed except an increase of TNFα and IL-8 mRNA level upon LPS stimulation (**$^{*}P < 0.05$**).

Data are representative of 3 independent experiments (mean ± SEM).

Table

Table 1. Sequences of oligonucleotide primers

Target gene	Forward sense (5'→3')	Reverse sense (5'→3')
18S rRNA	CGGCTACCACATCCAAGGA	GCTGGAATTACCGCGGCT
TNFα	CTTCTCGAACTCCGAGTGACAAG	CCACTGGAGTTGCCCTTCA
IL-1α	CAAATCAGTTGCCCATCCAAA	TGTGCCTGGACCCCAAGCAA
IL-8	ACACCAGACCCACACACTGCA	TCTGAAAGTCAGTGACAGAGGGTAG
IL-18	GGAGATCAACCTGTGTTTGAGGAT	GATGGTTACTGCCAGACCTCTAGTG

Figure
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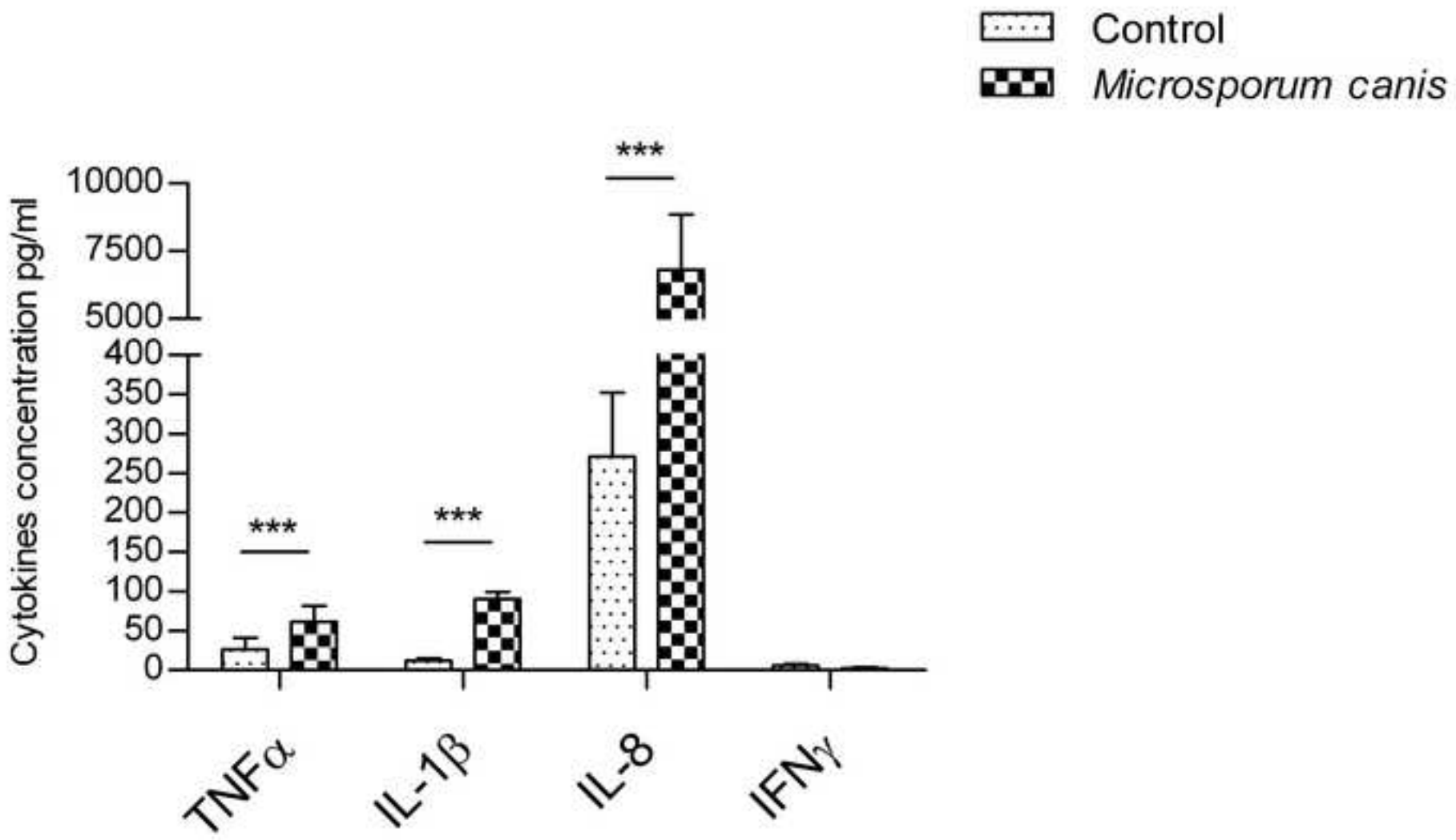

Figure 2

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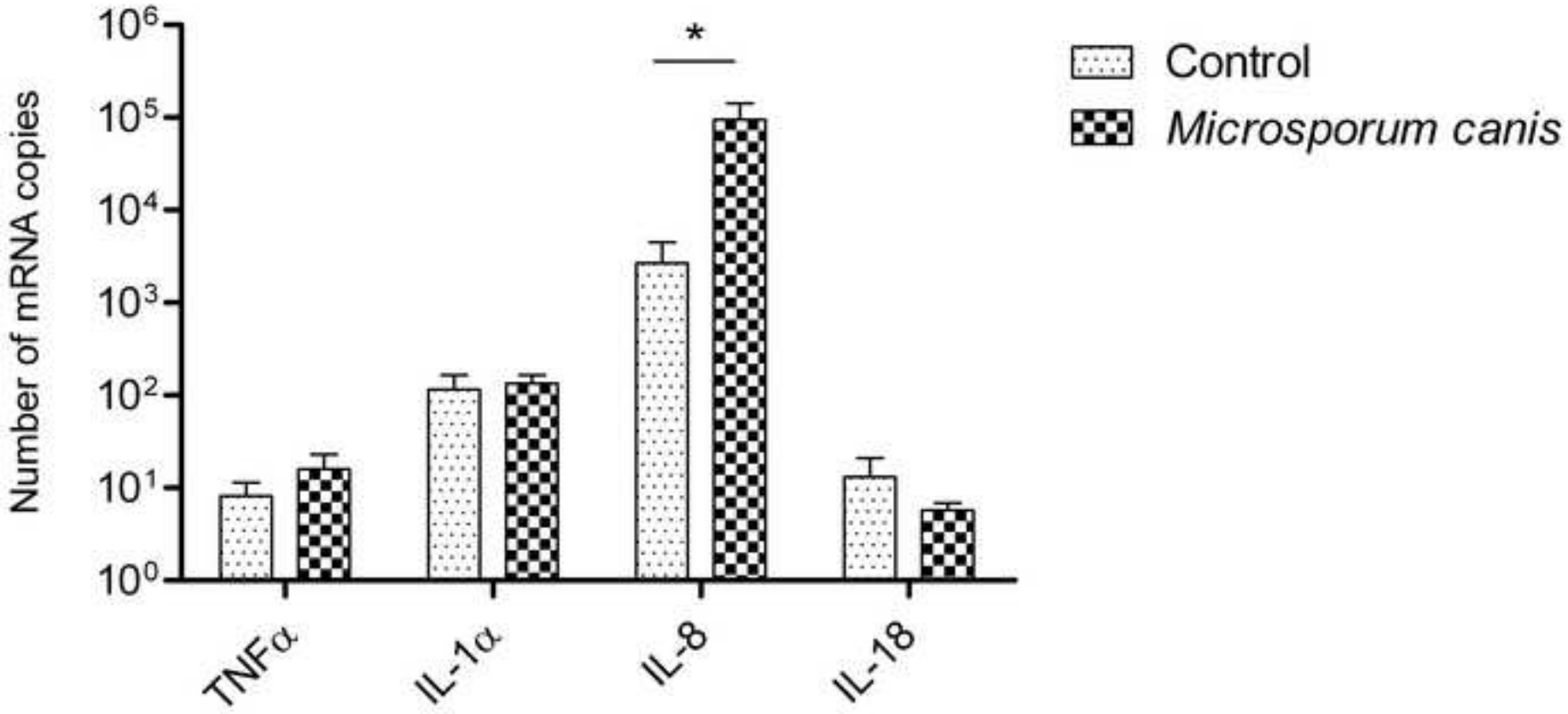

Figure 3

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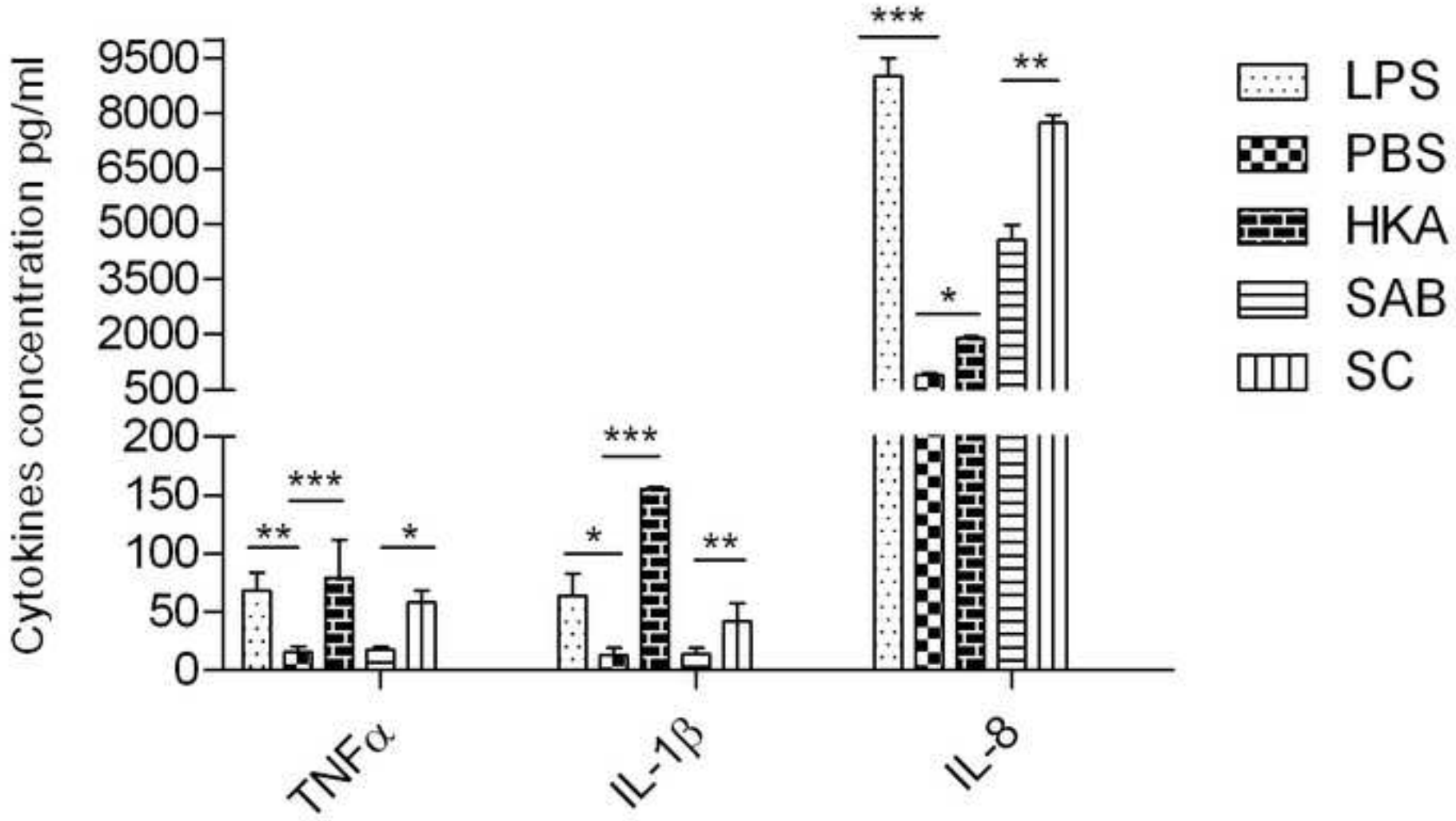

Figure 4

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