Heat-Killed *Malassezia Pachydermatis* Suspension Modulates the Activity of Macrophages Challenged with *Encephalitozoon Cuniculi*

Jéssica Feliciana Codeceira
Universadidade Paulista - UNIP
Anuska Marcelino Alvares-Saraiva
Universidade Paulista - Unip
Elizabeth Cristina Perez Hurtado
Universidade Paulista - Unip
Diva Denelle Spadacci-Morena
Instituto Buntantan
Selene Dall´Acqua Coutinho
Universidade Paulista - Unip
Maria Anete Lallo (anetelallo@hotmail.com)
Universidade Paulista - UNIP https://orcid.org/0000-0003-3578-3087

Research article

Keywords: anti-inflammatory cytokines, macrophages, Malassezia suspension, microsporidia, phagocytosis

Posted Date: November 17th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-105125/v1

Abstract

Background

Encephalitozoon cuniculi, are obligate intracellular fungi that determine emerging diseases in humans and other animals, with economic relevance. Studies have shown that *Malassezia* are able to modulate the production of pro and anti-inflammatory cytokines by keratinocytes and human monocytes and has an immunomodulatory effect. In the present work, we evaluate the effects of heat-killed *M. pachydermatis* suspension on the modulation of the response of macrophages challenged with the microsporidia *E. cuniculi*.

Methods

Heat-killed *M. pachydermatis* suspension was used to treat macrophage for 1 hour, subsequently, macrophages were infected with spores of *E. cuniculi* (2:1) and evaluated at 15, 30, 60 minutes and 48 hours. To determine the phagocytic capacity (PC) and index (PI), cultures were stained with Calcofluor and the spores, internalized or not, were counted.

Results

Microbicidal and phagocytic activity was evaluated by transmission electron microscopy (TEM). The untreated macrophages and had PC, PI, and larger amounts of phagocytosed spores than those treated, but by TEM it was observed that in macrophages treated the microbicidal activity was faster, since in the phagocytic vacuoles there were few spores in different degrees of degeneration and few amounts of amorphous material. Macrophages treated with heat-killed *M. pachydermatis* suspension showed lower PI and PC, with the incipient presence of *E. cuniculi* in phagosomes inside these macrophages. Additionally, these same treated macrophages had a mixed pattern of cytokine release with a Th1, Th2 and Th17 profile, with emphasis on IL-10, IL-4, IL-17, IL-6 and IFN-g secretion, particularly greater production of anti-inflammatory cytokines.

Conclusions

Our results showed that the treatment with heat-killed *M. pachydermatis* suspension determined an increase in the release of cytokines and reduced the phagocytic activity of macrophages challenged with *E. cuniculi*

Background

Microsporidia constitute a broad group of fungi belonging to the Phylum Microsporidia [1]. These unicellular beings have mandatory intracellular development. There are currently more than 1200 species classified and distributed in more than 140 genera, being described in invertebrate and vertebrate hosts [1, 2]. Microsporidia are considered emerging infectious agents that affect hosts from the main biomes in the world, from terrestrial to aquatic, thus reaching sectors of food production, from which they are also

consumed by human beings [3, 4]. The host after contracting the disease, which most often originates in the intestinal or respiratory epithelium, can spread to other parts of the body [5, 6]. This spread can occur due to the pathogen's ability to infect macrophages, which often, instead of eliminating the pathogens, can become “Trojan horses”, in which they serve as reservoirs, transporting spores and spreading the disease [7].

Pathogens can manipulate the immune cell response in their favor to evade death and persist in the host [7, 8]. Innate immunity involving mainly monocytes and macrophages is considered an important defense mechanism against pathogenic fungi involved in systemic mycoses. These phagocytes play a key role in host defense by ingestion and inactivation of invading organisms, cytokine production, and interactions with other cells participating in adaptive immunity such as T and B lymphocytes. Interaction of macrophages with intracellular pathogens that express one or more PAMPs (pathogen-associated molecular patterns), provides a signal which stimulates macrophage activation through toll-like receptors 4 [8]. These stimuli generally induce the activation and secretion of inflammatory cytokines. These cytokines may modulate monocyte and macrophage functions by stimulating or inhibiting the oxidative and non-oxidative microbicidal activities of these cells in an autocrine and paracrine fashion [8]. The imbalance in pro- and anti-inflammatory cytokine production by these cells during infection with pathogenic fungi may determine the disease progression or microorganism death. It has been shown that the lipid-rich layer that lines the yeasts of *Malassezia* sp., modulate the production of cytokines avoiding the induction of a nonspecific inflammatory response in the host, thus allowing the commensal relationship with the host's skin [9].

In order to evaluate the immunomodulatory potential of this yeast, *Malassezia* sp. killed by heat was administered to mice, before the challenge with pathogenic bacteria (*Salmonella typhimurium*) or with tumor cells [10, 11]. In these studies, previous treatment with killed *Malassezia* determined resistance to infection and tumor development, indicating that this pathogen, similarly to what is observed with *Propionibacterium acnes*, has an immunomodulator effect and may constitute an adjuvant for vaccines [12].

As there are no treatments against microsporidia, finding therapeutic forms that stimulate immunity and imply the host's response may implement infection control measures. In the present work, we evaluate the effects of heat-killed *M. pachydermatis* suspension on the modulation of the response of macrophages challenged with the microsporidia *Encephalitozoon cuniculi*.

Methods

Heat-killed *M. pachydermatis* suspension

M. pachydermatis (standard strain CBS-1696 isolated from dog) was maintained in Sabouraud medium incubated at 32ºC for 5 to 7 days at the Universidade Paulista - Unip, Molecular Biology Laboratory. The fungus extract was made with a suspension containing 5 yeasts for each macrophage (5:1), diluted in PBS and boiled at 120ºC for 30 minutes and then filtered with a 0.20 µm filter to remove debris.

E. cuniculi spores

Spores of *E. cuniculi* (genotype I) (from Waterborne Inc., New Orleans, LA, USA) that were used in this experiment were previously cultivated in a rabbit kidney cell lineage (RK-13, ATCC CCL-37) in RPMI medium supplemented with 10% of fetal calf serum (FCS- Sigma-Aldrich, St. Louis, MO, USA), pyruvate, nonessential amino acids (Sigma-Aldrich, St. Louis, MO, USA), and gentamicin at 37 °C in a humidified atmosphere with 5% CO_2. The spores were purified by centrifugation and cellular debris was excluded by 50% Percoll.

Macrophage cultures

RAW 264.7 macrophages were maintained in RPMI (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% FCS (R10 at 37 °C in a humidified atmosphere with 5% CO_2. The maintenance of the cultures to obtain the macrophages was made with periodic changes of the medium R10, respecting the necessity of the culture. The medium was changed every 2 days, until 70 to 80% of confluence was reached.

Macrophages treatment and infection

Macrophages were platted in 24-well plates with $3x10^5$ cells/well in 300 µl of R10 and incubated overnight at 37 °C in a 5% CO_2 atmosphere. Heat-killed *M. pachydermatis* suspension were added for 1 hour to the macrophages. After removal of supernatants, the cultures were infected with *E. cuniculi* spores at a 2:1 concentration (2 *E. cuniculi* spores for each macrophage) for 15, 30, 60 minutes and 48 hours. The experiments were done in triplicate. After the established periods, the supernatants were removed and stored at -80ºC for subsequent measurement of cytokines. To investigate to visualize the spores inside the phagocytic cells, 10 µl of Calcofluor (Sigma-Aldrich, St. Louis, USA) was added per cell cultures. Phagocytic capacity and index were calculated according to the formula: PC = number of phagocytes containing at least one ingested spore/100 phagocytes and PI = total number of phagocytic spores/100 phagocytes containing spores.

Quantification of Cytokines

Cytokines were measured in culture supernatants using CBA Mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences, CA, EUA), according to the manufacturer's instructions. A mixture of 25 µl of each sample, 25 µl of mixed beads containing specific capture antibodies, and 25 µl of PE-conjugated detection antibody was incubated for 2 h at room temperature in the dark. Subsequently, the samples were centrifuged, washed, and resuspended for analysis by two-color flow cytometry using C6 Accuri (BD Biosciences, Mountain View, CA, USA). All the analyses were done using the software FCAP Array 3.0.

Transmission Electron Microscopy (TEM)

Macrophages was adjusted to $1×10^7$ cells, transferred to 25 cm^2 bottles and incubated overnight in R10 medium at 37 °C with 5% CO_2 atmosphere. Then, heat-killed *M. pachydermatis* suspension were added

for 1 hour to the macrophages, respecting the 5:1 ratio. After removal of the extract, the cultures were infected with *E. cuniculi* spores at a 2:1 concentration for 15, 60 minutes and 48 hours. The cultures were collected and fixed using 2% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.2) at 4 °C for 10 h. They were then post-fixed in 1% OsO_4 buffer for 2h. Semi-thin sections stained with toluidine blue were made for visualization by light microscope, and ultrathin sections were made for TEM analysis (LEO EM 906E).

Statistical Analysis

Prism 5 (GraphPad Software Inc, La Jolla, CA, EUA) using a one-way analysis of variance (ANOVA) followed by Bonferroni test post hoc analysis was used to compare samples with untreated macrophage cells. All results are presented as mean ± SEM. A p value of 0.05 or less was considered significant.

Results

In order to observe the modulation of phagocytic activity of macrophages by heat-killed *M. pachydermatis* suspension, phagocyted spores were counted under the fluorescence microscope to obtain phagocytic capacity (PC) and phagocytic index (PI) analysis at times 15, 30, 60 minutes and 48 hours after infection. Untreated macrophages had higher PC, PI and phagocyted spore amounts than those treated at 1 hour and 48 hours (Fig. 1).

By TEM, in the untreated macrophages it was observed that in 15 and 30 minutes of infection there was a small amount of phagocytic spores. At 60 minutes, these untreated macrophages exhibited many phagocytic vacuoles with intact and degenerating spores (Fig. 2A, B, C). It was also frequently observed the presence of amorphous material, suggesting of lytic activity, with exocytosis of this material. These same findings were observed at 48 hours, but with few intact spores and much amorphous material within phagosomal vacuoles (Fig. 2D, E, F). In this study, we also observed the fusion of vacuoles homotypic phagosomes containing a single *E. cuniculi* spore forming of megasomes (Fig. 2F). No multiplication stages of *E. cuniculi* were visualized inside macrophages.

In treated macrophages after 60 minutes and 48 hours of infection, the phagocytosis of spores occurred in cell cultures characterized by projections of the cellular membrane of macrophages near or around the spores (Fig. 3). Few intact spores were seen outside or inside of macrophages. The presence of phagocytic vacuoles with spores in degeneration phases or amorphous material was incipient (Fig. 3). Neither stages of multiplication of *E. cuniculi* within macrophages were visualized (Fig. 3).

Infection of macrophages with *E. cuniculi* spores determined the production of IL-6 after 15 minutes, a cytokine already detected in other experiments carried out with this pathogen (Fig. 4). However, macrophages treated with heat-killed *M. pachydermatis* suspension showed the most significant changes in cytokines levels (Fig. 5). The production of IFN-γ, IL-6, IL-17, IL-4 and IL-10 cytokines increased in macrophages treated with heat-killed *M. pachydermatis* suspension when compared to *E. cuniculi* infected macrophages (Infected) or treated plus infected macrophages (Infected + Treated) (Fig. 5). These

results suggest that the heat-killed *M. pachydermatis* suspension increases the release of Th1, Th2 and Th17 cytokines, while the pathogen *E. cuniculi* favored the decline in the release of these cytokines.

Discussion

Herein, we have demonstrated the immunomodulatory effect of heat-killed *M. pachydermatis* suspension in macrophages activity. Macrophages treated with heat-killed *M. pachydermatis* suspension showed lower index and phagocytic capacity, with the incipient presence of *E. cuniculi* in phagosomes inside these macrophages at the time of observation. Additionally, these same treated macrophages had a mixed pattern of cytokine release with a Th1, Th2 and Th17 profile, with emphasis on IL-10, IL-4, IL-17, IL-6 and IFN-γ secretion, particularly greater production of anti-inflammatory cytokines.

It has been reported that several bacterial species have a high biological activity that inhibits tumor or bacteria growth when administered *in vivo* [10, 11]. Most of the studies have been performed by using *Propionibacterium* and *Lactobacillus* [12, 13]. Even though these bacteria possess the ability to activate macrophages, either *in vivo* or *in vitro*, the precise mechanisms concerning the antitumor or bactericidal effect of activated macrophages have not been defined [10, 11]. Mice treated with killed *Malassezia* sp. showed resistance to *Salmonella typhimurium* infection [10]. Although the survival rate of mice treated with *Malassezia* sp. was slightly lower than that of mice treated with *P. acnes*, the clearance of bacteria from the blood and the inhibition of bacterial grow thin the liver and spleen occurred at almost the same levels in mice treated with *Malassezia* sp. [10] and *P. acnes* [12]. In contrast, our results showed less phagocytic activity associated with a profile of anti-inflammatory macrophages.

Heat-killed *P. acnes* potentiated a type I hypersensitivity inflammation (Th2 response), including late phase reaction, when it was injected simultaneously with antigen implantation (coagulated ovalbumin). When the animals were treated with *P. acnes* previously to antigen inoculation a complete change to a typical Th1 immune response was obtained, suggesting that different immunization protocols induced either a Th1 or a Th2 cytokine pattern [14, 15]. The hypothesis that significant reduction in pro-inflammatory cytokine production by human peripheral blood mononuclear cells (PBMCs) in response to *Malassezia* species (formalized and viable) [16] was related to the unusually high quantity of lipid associated with the yeast cell wall. It has been demonstrated that removing the lipid layer surrounding the *Malassezia* yeast causes increased pro-inflammatory cytokine production and reduced IL-10, suggesting that the lipid layer confers the evasion of the inflammatory response by keratinocytes [17]. The *M. restricta* extract increased the mRNA and protein expression of Th1-attracting CXC chemokine ligand (CXCL)10 and STAT1 activity and phosphorylation in keratinocytes, which was suppressed by a Janus kinase inhibitor [17]. However, our results showed a more anti-inflammatory profile with the treatment of macrophages with heat-killed *M. pachydermatis* suspension. Corroborating these results, in previous studies, we demonstrated in macrophages infected with live *M. pachydermatis* intense proliferation of the fungus with the death of macrophages and release of high levels of IL-4 [18].

Untreated macrophages demonstrated high index and phagocytic capacity and intense phagocytosis by TEM, with the presence of many intact or degenerating spores inside phagosomes. A peak IL-6 release was demonstrated after 15 minutes of challenge and, in the other periods, cytokine levels were similar to the heat-killed *M. pachydermatis* suspension treated and challenged macrophages but lower than in the only treated macrophages with heat-killed *M. pachydermatis* suspension. These results suggest that the challenge with the *E. cuniculi* microsporidia reduced the release of cytokines, despite the stimulus caused by the heat-killed *M. pachydermatis* suspension.

In a previous study, we demonstrated that diabetes mellitus (DM) increased the susceptibility of C57BL/6 mice to encephalitozoonosis and DM mice *infected with E. cuniculi* showed higher levels of IL-6 than DM-*uninfected* mice, suggesting that DM may also modulate a pro-inflammatory state of the organism [19]. IL-6 is a pleiotropic cytokine that mediates several biological functions, including regulation of the immune system by anti-inflammatory and pro-inflammatory production [20]. We speculate that the cytokine IL-6 released by macrophages shortly after the challenge with *E. cuniculi* may be involved in the pronounced phagocytic activity observed in this group and not seen in the groups treated with heat-killed *M. pachydermatis* suspension.

E. cuniculi replication is possible in peritoneal macrophages [21]. However, when macrophage is activated by spores opsonized by specific antibodies, lysosomes and fusion occurs and the spores were easily destroyed [22, 23]. Herein, we identified inside macrophages treated or not with heat-killed *M. pachydermatis* suspension phagocytic vacuoles with spores in degenerate and intact stages, thus reinforcing that endocytosis/phagocytosis was evidently the preferred entry form for *E. cuniculi* spores in macrophages, corroborating the literature data. Additionally, phagocytosis by treated macrophages was less than that observed in untreated macrophages, which suggests impairment of the phagocytic activity of these macrophages, despite the presence of the pathogen.

Conclusions

In summary, the treatment of macrophages with heat-killed *M. pachydermatis* suspension determined the release of cytokines with anti-inflammatory profile and reduced the phagocytic activity after challenged with *E. cuniculi*.

Declarations

Funding

Not applicable.

Ethics declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no other competing interests.

References

1. Han B, Weiss LM. Microsporidia: obligate intracellular pathogens within the Fungal kingdom. Microbiol Spectr. 2017; 5(2): 10.1128/microbiolspec.FUNK-0018-2016.
2. Anane S, Attouchi H. Microsporidiosis: epidemiology, clinical data and therapy. Gastroenterol Clin Biol. 2010;34:450–64.
3. Stentiford GD, Becnel JJ, Weiss LM, et al. Microsporidia - Emergent Pathogens in the Global Food Chain. Trends Parasitol. 2016;32(4):336–48.
4. Heyworth MF. Genetic aspects and environmental sources of microsporidia that infect the human gastrointestinal tract. Trans R Soc Trop Med Hyg. 2017;111(1):18–21. doi:10.1093/trstmh/trx001.
5. Field AS, Milner Da Jr. Intestinal microsporidiosis. Clin Lab Med. 2015;35(2):445–59.
6. Han B, Takvorian PM, Weiss LM. Invasion of host cells by microsporidia. Front Microbiol. 2020;11:172. https://doi.org/10.3389/fmicb.2020.00172.
7. Han Y, Gao H, Xu J, Luo J, Han B, Bao J, Pan G, Li T, Zhou Z. Innate and adaptive immune responses against microsporidia infection in mammals. Front Microbiol. 2020;11:1468. doi:10.3389/fmicb.2020.01468.
8. Thakur A, Mikkelsen H, Jungersen G. Intracellular pathogens: host immunity and microbial persistence strategies. J Immunol Res. 2019. doi:10.1155/2019/1356540.
9. Thomas DS, Ingham E, Bojar RA, et al. *In vitro* modulation of human keratinocyte pro-and anti-inflammatory cytokine production by the capsule of *Malassezia* species. FEMS Immunol Med Microbiol. 2008;54:203–14.
10. Takahashi M, Ushijima T, Ozaki Y. Studies on the biological activity of *Pityrosporum.* I. Activation of complement and generation of macrophage chemotactic factors in normal human serum by *Pityrosporum*. J Med Mycol. 1984;24:241–4.
11. Takahashi M, Ushijima T, Ozaki Y. Biological activity of *Pityrosporum.* II Antitumor and immune stimulating effect of *Pityrosporum* in mice. J Nat Cancer Inst. 1986;77:1093–7.
12. Ananias RZ, Rodrigues EG, Braga EG, Squaiella CC, Mussalem JS, Longhini ALF, et al. Modulatory effect of killed-*Propionibacterium acnes* and its purified soluble polysaccharide on peritonial exudate cells from C57Bl/6 mice: major NKT cell recruitment and increased cytotoxicity. Scand J Immunol. 2007;65:538–48. doi:10.1111/j.1365-3083.2007.01939.x.

13. Criscuolo E, Caputo V, Diotti RA, Sautto GA, Kirchenbaum GA, Clementi N. Alternative methods of vaccine delivery: an overview of edible and intradermal vaccines. J Immunol Res. 2019. doi:10.1155/2019/8303648.
14. Braga EG, Ananias RZ, Mussalem JS, et al. Treatment with *Propionibacterium acnes* modulates the late phase reaction of immediate hypersensitivity in mice. Immunol Lett. 2003;88:163–9.
15. Kalis C, Gumenscheimer M, Freudenberg N, Tchaptchet S, Fejer G, Heit A, et al. Requirement for TLR9 in the immunomodulatory activity of *Propionibacterium acnes*. J Immuno 2005; 174:4295–300. doi:10.4049/ jimmunol.174.7.4295.
16. Kesavan S, Walters CE, Holland KT, Ingham E. The effects of *Malassezia* on pro-inflammatory cytokine production by human peripheral blood mononuclear cells *in* v*itro*. Med Mycol. 1998;36:97–106.
17. Hau CS, Kanda N, Makimura K, Watanabe S. Antimycotics suppress the Malassezia extract-induced production of CXC chemokine ligand 10 in human keratinocytes. J Dermatol. 2014;41(2):124–34. doi:10.1111/1346-8138.12380.
18. Moreira RTF, Lallo MA, Alvares-Saraiva AM, Hurtado ECP, Konno FT, Spadacci-Morena D, Coutinho SD. Dichotomous response of *Malassezia*-infected macrophages to *Malassezia pachydermatis* and *Malassezia furfur*. Med Mycol. 2019;57(5):628–35. https://doi.org/10.1093/mmy/myy104.
19. Francisco Neto A, Dell'Armelina Rocha PR, Perez EC, Xavier JG, Peres GB, Spadacci-Morena DD, Alvares-Saraiva AM, Lallo MA. Diabetes mellitus increases the susceptibility to encephalitozoonosis in mice. PLoS One. 2017;12:e0186954.
20. Schett G. Physiological effects of modulating the interleukin-6 axis. Rheumatology. 2018;57:43–50.
21. Weidner E. Interactions between *Encephalitozoon cuniculi* and macrophages. Zentralblatt fur bakteriol Parasit. 1975;49:1–9.
22. Niederkorn JY, Shadduck JA. Role of antibody and complement in the control of *Encephalitozoon cuniculi* infection by rabbit macrophages. Infect Immu. 1980;17:995–1002.
23. Schmidt EC, Shadduck JA. Mechanisms of resistance to the intracellular protozoan *Encephalitozoon cuniculi* in mice. J Immun. 1984;133:2712–9.

Figures

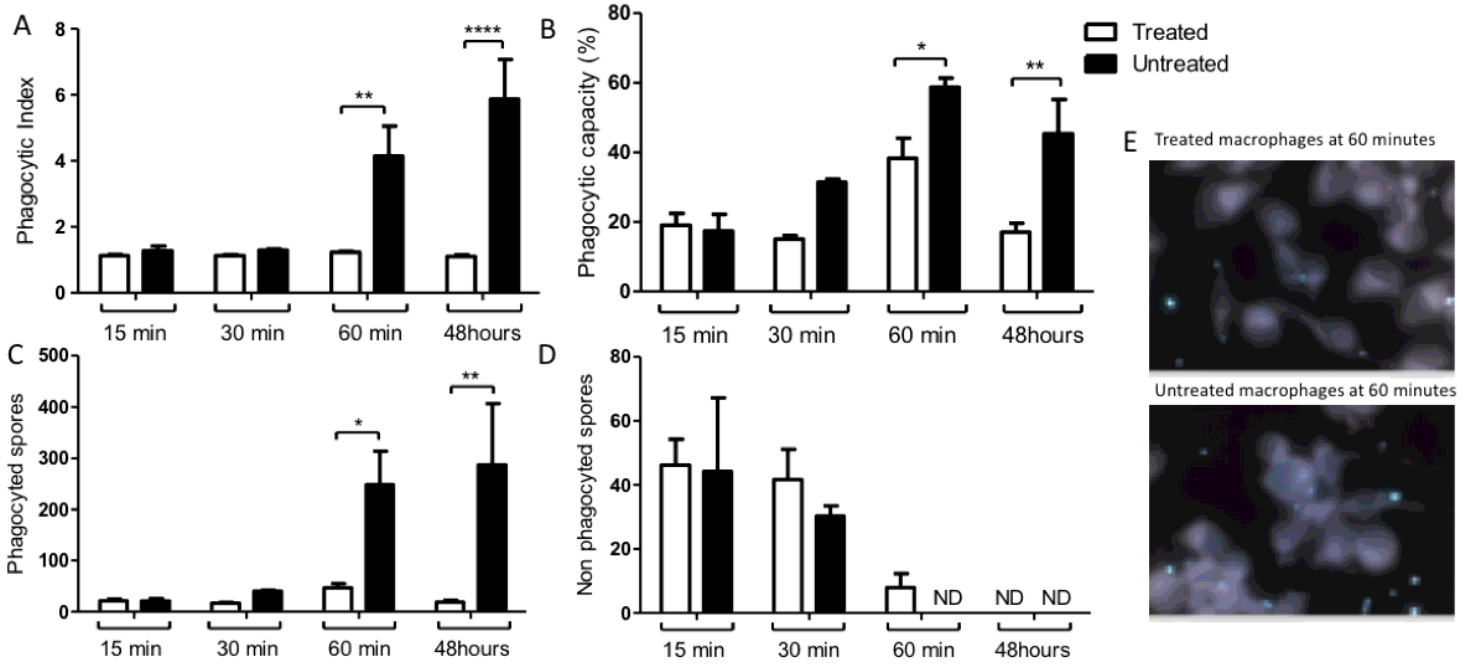

Figure 1

Phagocytic index and capacity of macrophages treated or untreated with heat-killed Malassezia pachydermatis suspension and infected with E. cuniculi at 15, 30, 60 minutes and 48 hours. A) Phagocytic index. B) Phagocytic capacity. C) Number of E. cuniculi spores phagocytized by macrophages. D) Number of E. cuniculi spores not phagocytized by macrophages. E) Photomicrography of macrophages treated or untreated with heat-killed M. pachydermatis suspension and infected with E. cuniculi under calcofluor stain. Data are represented as mean ± SEM (*P < 0.05, ** P < 0.01, **** P < 0.0001, One-way ANOVA with Tukey post-test)

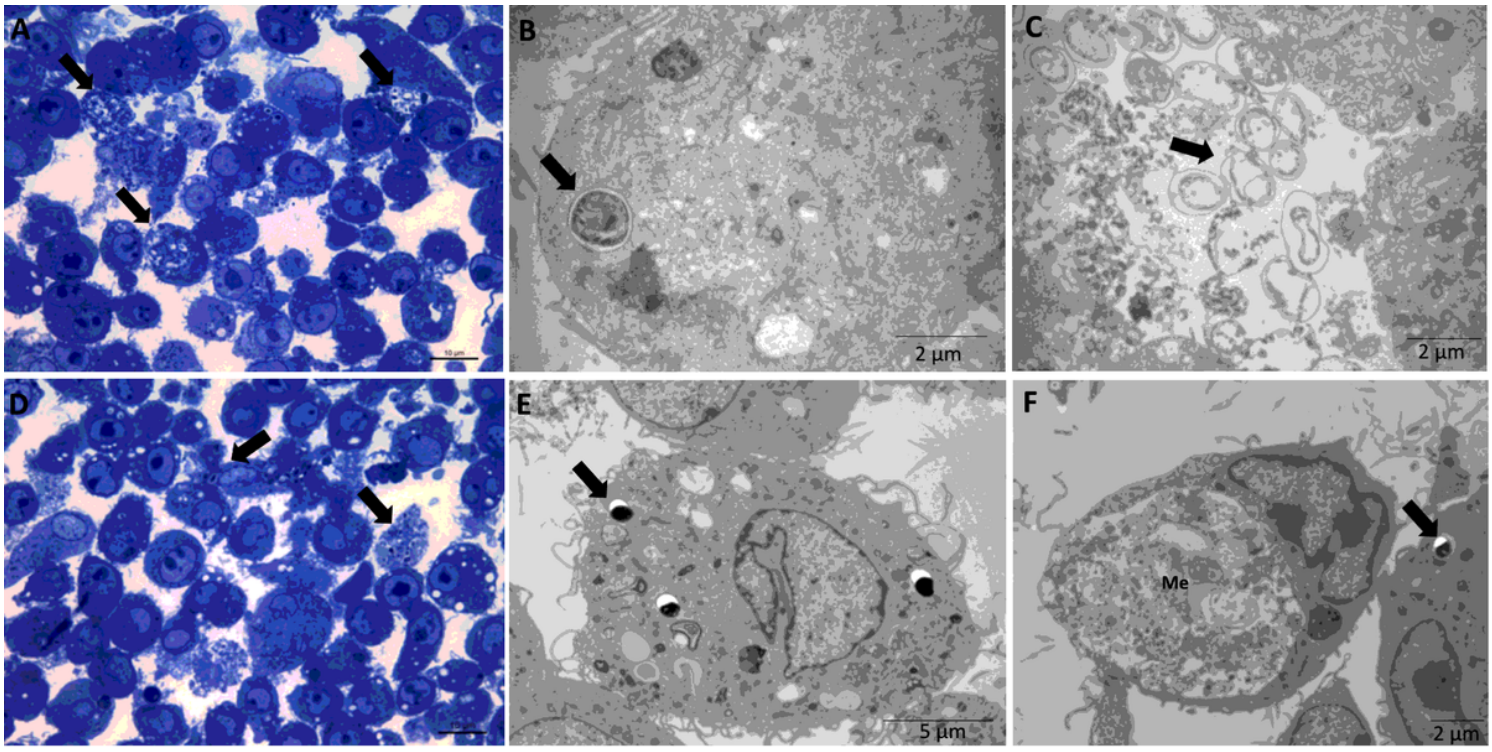

Figure 2

Phagocytic activity of macrophages untreated with heat-killed M. pachydermatis suspension and infected with E. cuniculi after 60 minutes or 48 hours. (A) Photomicrography of macrophages with spores inside by Toluidine Blue stain at 60 minutes. (B) Intact spore inside macrophage by ultramicrography at 60 minutes. (C) Many preserved spores outside macrophages, by ultramicrography at 60 minutes. (D) Photomicrography of macrophages with spores inside by Toluidine Blue stain, at 48 hours. (E) Homotypic phagosomes vacuoles in macrophages, with intact (arrow) ou degenerating spores by ultramicrography at 48 hours. (F) Megasome (me) with amorphous and electron dense material and E. cuniculi spores (arrow) inside. Ultramicrography of macrophages at 48 hours.

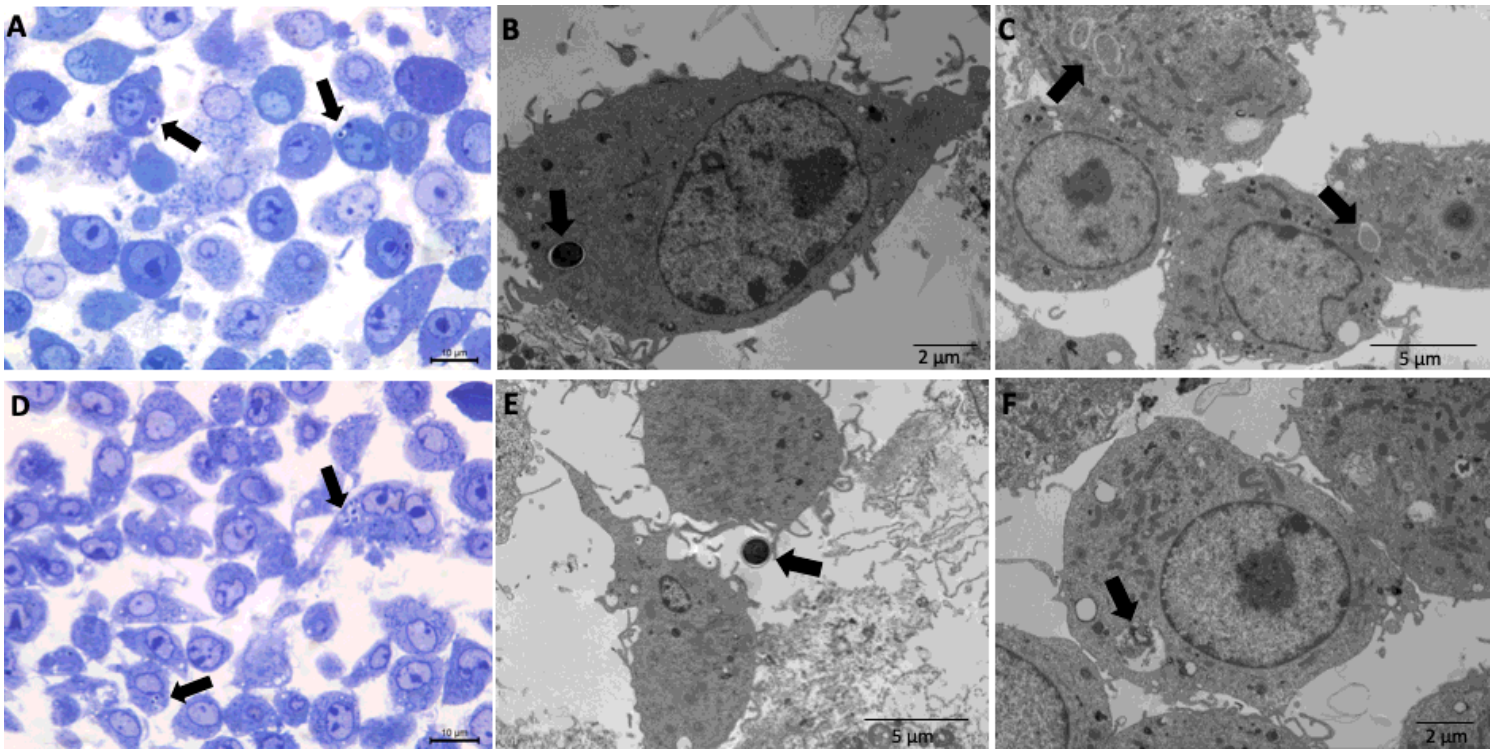

Figure 3

Phagocytic activity of macrophages treated with heat-killed M. pachydermatis suspension and infected with E. cuniculi after 60 minutes or 48 hours. (A) Photomicrography of macrophages with spores inside by Toluidine Blue stain at 60 minutes. (B) Intact spore inside macrophage by ultramicrography at 60 minutes. (C) Homotypic phagosomes vacuoles in macrophages, with degenerating spores (arrow) by ultramicrography at 60 minutes. (D) Photomicrography of macrophages with spores inside by Toluidine Blue stain at 48 hours. (E) Projections of the cellular membrane of macrophages involving the spores (arrow) of E. cuniculi by ultramicrography at 48 hours. (F) Phagosomes vacuoles with amorphous material inside in the macrophages (arrow) by ultramicrography at 48 hours.

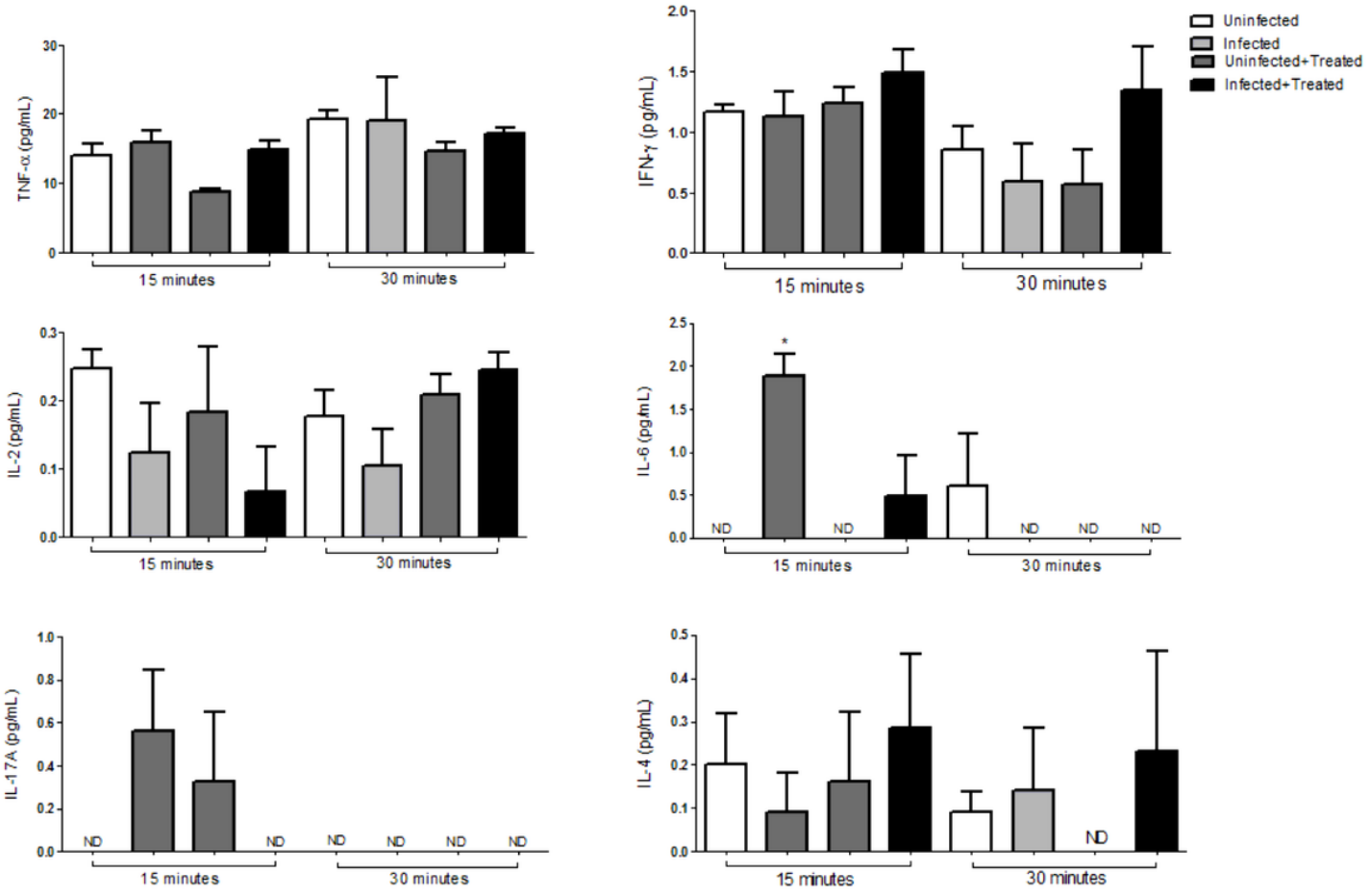

Figure 4

Nitric oxide and hydrogen peroxide production by macrophages treated or untreated with heat-killed Malassezia pachydermatis suspension and infected with E. cuniculi at 15, 30, 60 minutes and 48 hours. The data are presented as mean ± SEM (*P < 0.05, ** P < 0.01, *** P <0.001, One-way ANOVA with Tukey post-test)

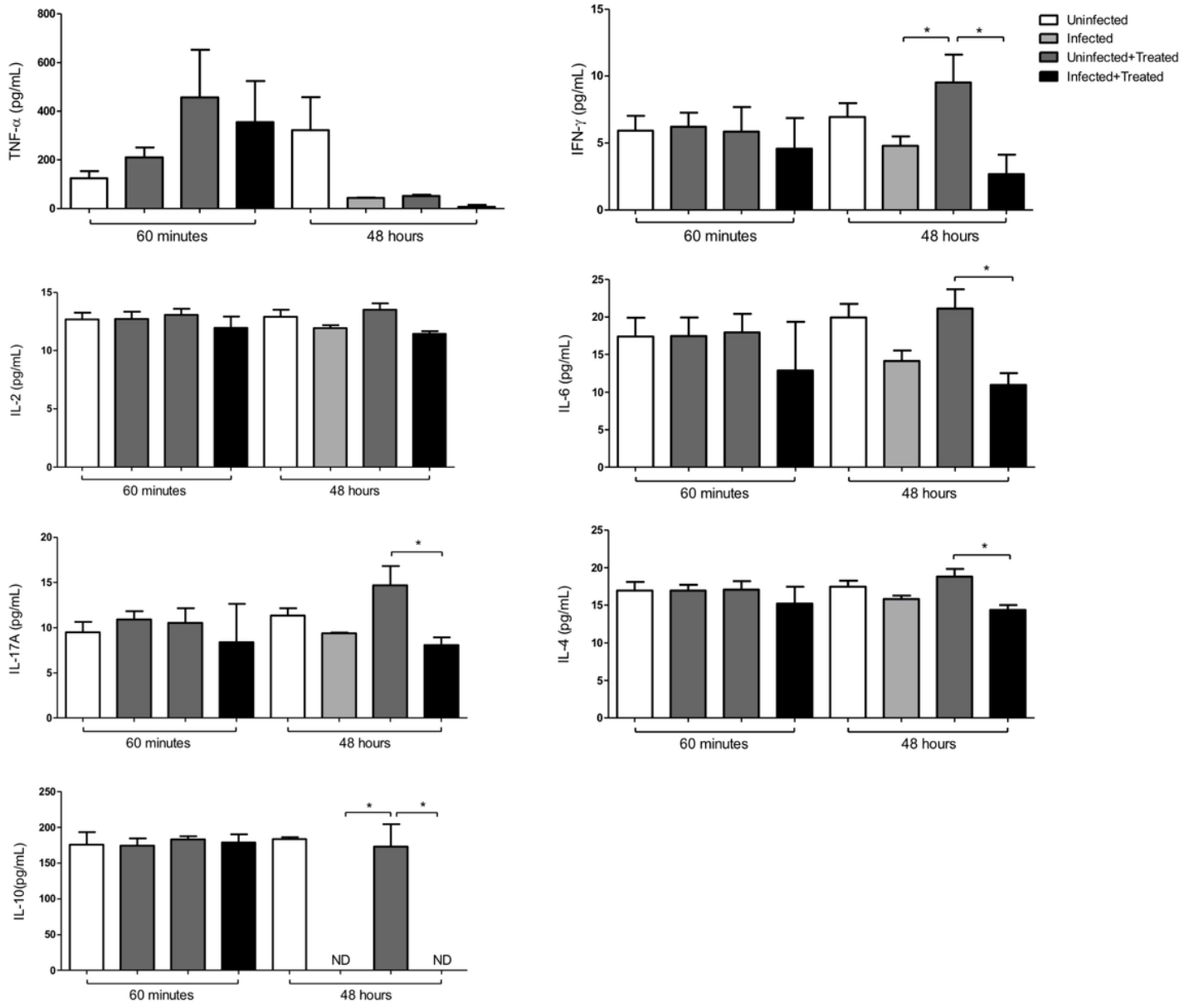

Figure 5

Cytokines levels in the supernatants of macrophages treated or untreated with heat-killed Malassezia pachydermatis suspension and infected with E. cuniculi at 15, 30, 60 minutes and 48 hours. Data are represented as mean ± SEM (** P <0.01, *** P <0.001and ND = not detected, One-way ANOVA with multiple comparisons and Tukey post-test).