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Original Article

Extracellular vesicles derived from *Malassezia furfur* stimulate IL-6 production in keratinocytes as demonstrated in *in vitro* and *in vivo* models

Yu-Jing Zhang[a], Yang Han[a], Yu-Zhe Sun[a], Hang-Hang Jiang[a], Min Liu[b], Rui-Qun Qi[a], Xing-Hua Gao[a,*]

[a] NHC/Ministry of Education/Liaoning Province Key Laboratory of Immunodermatology, National Joint Engineering Research Center for Diagnosis and Treatment of Skin Diseases, The First Hospital of China Medical University, Shenyang, Liaoning Province, 110001, PR China
[b] Institute of Respiratory Disease, The First Hospital of China Medical University, Shenyang, Liaoning Province, 110001, PR China

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ABSTRACT

Background: Malassezia is one of the commensal microorganisms colonized on human skin and has been shown to be related to several inflammatory cutaneous disorders. Previous studies indicated that *Malassezia. sympodialis* (*M. sympodialis*) can produce extracellular vesicles, however, the immunoregulatory function of *Malassezia* extracellular vesicles on keratinocytes has not been studied.
Objective: To investigate the extracellular vesicular production capability of *Malassezia. furfur* (*M. furfur*) and examine their immunoregulatory effects both *in vitro* and *in vivo*.
Methods: Extracellular vesicles derived from *M. furfur* were isolated by sequential ultracentrifugation procedure. Their structure and diameter were determined by negative stain TEM and NTA, respectively. Confocal microscopy was used to visualize the internalization of these nanoparticles into HaCaT cells and mice epidermal keratinocytes. The expressions of inflammatory cytokines were screened using PCR Array assay and validated *in vitro* by qPCR and ELISA assays. *In vivo* cytokine production was measured by the IHC method. The role of NF-κB in such process was evaluated in HaCaT cells by western blot assay.
Results: Our results showed that *M. furfur* produced ovoid-shaped nanoparticles, which could be then internalized into HaCaT cells, as well as mice epidermal keratinocytes. IL-6 expression was significantly enhanced in response to extracellular vesicular stimulation both *in vitro* and *in vivo*, in which process the activation of NF-κB was involved.
Conclusion: M. furfur has the ability to release extracellular vesicles, which can be internalized into keratinocytes and promote the production of IL-6 with the involvement of NF-κB dependent pathway. Such findings reveal some important new insights into *Malassezia* pathogenesis and therapy.

1. Introduction

Malassezia is a commensal resident fungus which can colonize on human skin, particularly in areas where sebum secretion is abundant [1]. In some cases, *M. furfur* infection can result in several superficial dermatoses such as pityriasis versicolor, dandruff, seborrheic dermatitis, *Malassezia* folliculitis, and even systemic sepsis [2]. Previous studies showed that direct contact between the yeasts and keratinocytes is an essential requirement in *Malassezia* induced epidermal inflammation [3,4]. However, current results revealed virulent factors of *Malassezia* including lipase, phospholipase [5–7], bioactive indoles [8], and other irritating *Malassezia* metabolites, which are extracellular macromolecules, are involved in the disease development.

As extracellular substance containers [9], extracellular vesicles, nanoparticles with lipid bi-layered membrane structures, are recently brought into focus due to the important roles they play in cellular nutrient absorption, pathophysiological processes and cell-to-cell communication [10]. In addition to

Abbreviations: M. furfur, Malassezia furfur; *M. sympodialis, Malassezia sympodialis*; TEM, transmission electronic microscopy; NTA, nanoparticle tracking analysis; Dil, dialkylcarbocyanine iodide; FITC, fluorescein isothiocyanate; DAPI, 4′,6-diamidino-2-phenylindole; LDH, lactate dehydrogenase; IHC, immunohistochemistry; IL, interleukin.

* Corresponding author at: NHC/Ministry of Education/Liaoning Province Key Laboratory of Immunodermatology, The First Hospital of China Medical University, No.155 Nanjing Bei Street, Heping District, Shenyang, Liaoning Province, 110001, PR China.
E-mail address: gaobarry@hotmail.com (X.-H. Gao).

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mammalian cells, the ability to produce extracellular vesicles is also discovered in bacteria, yeast and parasitic cells [11–13]. Fungal extracellular vesicular trans-cell wall transportation was first described in *Cryptococcus neoformans* [12]. Following this initial report, the existence of extracellular vesicles was subsequently identified in *Paracoccidioides brasiliensis* [14], *Histoplasma capsulatum*, *Sporothrix schenckii*, and *Candida albicans* [15]. In the *Malassezia* genus, extracellular vesicular secretion was first observed in *Malassezia sympodialis* (*M. sympodialis*), followed by investigations of small RNA and allergen components in *M. sympodialis* extracellular vesicles [16,17]. In a nanoparticle-host interaction study, Gehrmann et al. demonstrated that extracellular vesicles from *M. sympodialis* could induce the release of IL-4 and TNF-α in PBMCs obtained from atopic eczema patients. Such effects might contribute to the inflammation observed in atopic eczema patients [18].

Given that *Malassezia* colonizes on the surface layer of the skin, a more effective approach to study the immunoregulatory functions of *Malassezia* extracellular vesicles would be achieved by investigating the direct interactions between extracellular vesicles and keratinocytes, which play critical roles in mediating innate immune responses [19]. Interestingly, such an approach has rarely been reported in the literature. Our goal in this study was to investigate the immunoregulatory effects of *Malassezia* extracellular vesicles on keratinocytes along with an examination of some of the possible molecular mechanisms involved. We found that extracellular vesicles derived from *M. furfur* can induce the production of IL-6 as determined in both *in vitro* and *in vivo* assays. Such effect was associated with the activation of nuclear factor-kappa B (NF-κB) signaling pathway.

2. Materials and methods

2.1. Yeast strain and culture conditions

M. furfur (ATCC 14521) was recovered and cultured on a modified Dixon agar (mDixon) plate at 30 °C for 3 d to prepare for the isolation of extracellular vesicles. The mDixon agar plate consists of malt extract 36 g, desiccated oxbile 20 g, Tween-40 10 ml, peptone 6 g, glycerol 2 ml, oleic acid 2 ml, agar 15 g, DI water 1000 ml, pH adjusted to 6 with HCl, and autoclaved at 121 °C for 20 min.

2.2. Cell line and treatments

HaCaT cells (GENECHEM, Shanghai, China), an immortalized human keratinocyte cell line [20], were cultured in high glucose DMEM medium (BI, CT, USA) supplemented with 10% fetal bovine serum (BI) and 1% penicillin/streptomycin (BI) at 37 °C in 5% CO_2 atmosphere within a cell incubator. See the Supplementary methods for the information of cell treatment.

2.3. Yeast viability

Malassezia yeast viability was measured with use of the FUN1 cell stain kit (Thermo Fisher, MA, USA) according to the protocol handbook. See the Supplementary methods for additional information.

2.4. M. furfur *extracellular vesicle isolation and quantification*

The method used to isolate extracellular vesicles from *M. furfur* complied with an established ultracentrifugation protocol, in particular for yeast extracellular vesicle isolation, as described previously by Rodrigues et al. [21], with a slight modification. See the Supplementary methods for additional information.

2.5. Negative-stain transmission electron microscopy (TEM)

Pellets obtained following ultracentrifugation were resuspended and fixed in 2.5% glutaraldehyde, mounted on copper-mesh formvar grids and negatively stained with phosphotungstic acid (pH = 6.5). Samples were observed with use of Hitachi H-7650 transmission electron microscopy (Hitachi, Tokyo, Japan) operating at 80 kV.

2.6. Nanoparticle tracking analysis (NTA)

The size and diameter of extracellular vesicles were determined by NTA with ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany) and corresponding software ZetaView 8.04.02. See the Supplementary methods for additional information.

2.7. Cell viability

Lactate dehydrogenase (LDH) is a cytoplasmic enzyme contained within intact cell membranes. Leakage of LDH into the extracellular space indicates cell membrane damage. To evaluate whether isolated extracellular vesicles would induce cell lysis in HaCaT cells, LDH levels within supernatants of extracellular vesicle-HaCaT cell co-incubations were measured. See the Supplementary methods for additional information.

2.8. Internalization of extracellular vesicles by HaCaT cells

lipophilic dialkylcarbocyanine iodide (Dil) stained extracellular vesicles, as described previously [22], were co-incubated with HaCat cells, whose cell membrane and nuclei were labeled with fluorescein and DAPI, respectively, for 1, 6 or 12 h. The internalization was visualized with use of laser confocal microscopy. See the Supplementary methods for additional information.

2.9. RT^2 Profiler PCR Array analysis

Inflammation and Toll-like Signaling Pathway plates (#PAHS-018Z, Qiagen, Hilden, Germany) were used to perform the RT^2 Profiler PCR Array. See the Supplementary methods for additional information.

2.10. RNA extraction and quantitative real-time PCR (qPCR) analysis

Transcriptional levels of *IL1B*, *IL6*, *CXCL8*, *RELA*, *FOS* and *JUN* were measured by qPCR analysis with the oligonucleotide sequences of PCR primers listed in Table 1. See the Supplementary methods for additional Information.

2.11. Protein preparation and western blot analysis

The translational levels of NF-κB p65 and phosphorylated p65 were detected by western blot using rabbit-derived primary antibodies. See the Supplementary methods for additional information.

2.12. ELISA assay

Concentrations of IL-1β, IL-6 and IL-8 in cell culture supernatants were quantified using human IL-1β, IL-6, and IL-8 ELISA kits (Novus Biologicals, CO, USA), respectively. See the Supplementary methods for additional information.

2.13. Inhibition of NF-κB activation

NF-κB specific inhibitor Helenalin (Abcam, ab146197), dissolved in DMSO at a storage concentration of 100 mM, was added

Table 1
Oligonucleotide sequences of qPCR primers and amplicon lengths of interested genes and housekeeping gene.

Gene symbols	Protein names	Primers	Oligonucleotide sequences	Amplicon length
IL1B	IL-1β	Forward	AAAAGCTTGGTGATGTCTGG	177
		Reverse	TTTCAACACGCAGGACAGG	
IL6	IL-6	Forward	GAGGAGACTTGCCTGGTGAA	104
		Reverse	GCTCTGGCTTGTTCCTCACTAC	
CXCL8	IL-8	Forward	CCACCGGAAGGAACCATCTC	156
		Reverse	GGAGTATGTCTTTATGCAC	
RELA	NF-κB p65	Forward	GGATGGCTTCTATGAGGCTGA	132
		Reverse	GGGTTGTTGTTGGTCTGGATG	
FOS	c-Fos	Forward	GGCAAGGTGGAACAGTTATCTC	143
		Reverse	TCTTCTAGTTGGTCTGTCTCCG	
JUN	c-Jun	Forward	AAACGACCTTCTATGACGATGC	110
		Reverse	CAGGTTCAGGGTCATGCTCT	
GAPDH	GAPDH	Forward	AAGAGCACAAGAGGAAGAGAGAGAC	103
		Reverse	GTCTACATGGCAACTGTGAGGAG	

to medium 2 h prior to extracellular vesicle stimulation at a final concentration of 10 μM. Negative control groups were treated likewise using equal volume of solvent.

2.14. Extracellular vesicular transdermal permeation and immunohistochemical stain (IHC) assay

Female BALB/c mice were purchased from Liaoning Changsheng Biotechnology Co. Ltd. All animals, 6–8 weeks of age, were raised under pathogen-free conditions at 25 °C and 50–60% humidity. Vesicular transdermal permeation and IL-6 synthesis of mice epidermal keratinocytes were measured with use of laser confocal microscopy and IHC assays, respectively. See the Supplementary methods for additional information.

2.15. Statistical analysis

Experimental data were expressed as means ± SD. One-way analyses of variance (one-way ANOVA) and Tukey's test were used for comparisons involving multiple groups. Student's t-tests were used for comparisons between two samples if they have the equal variances otherwise *t*-tests with Welch's correction were performed. Results were displayed using GraphPad Prism 7, Photoshop CC 2017 and RStudio. Statistically significant differences were indicated by asterisks (*), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3. Results

3.1. M. furfur *produces extracellular vesicles*

Extracellular vesicles have been successfully enriched and observed in several fungal genuses [12,15,23], and results from recent studies have suggested that vesicular secretion occurs in *M. sympodialis* [16–18]. Consequently, we designed experiments to search for extracellular vesicles in another subspecies of the *Malassezia* genus, *M. furfur*. Prior to vesicular extraction, yeast cell metabolic viability was measured with use of the FUN1 stain. With this method, metabolically active yeast cells are clearly marked with orange-red fluorescent cylindrical intravacuolar structures (CIVS), while dead cells exhibit a diffuse green-yellow fluorescence. Our results demonstrated that almost all yeast cells were viable compared to that of the heat-killed group (Fig. 1a). Therefore, the possibility of vesicles being derived from dead cells would be very unlikely. Negative-stained transmission electronic microscopy (TEM) analysis of vesicle preparations identified the presence of membrane compartments with variable dimensions and electronic densities (Fig. 1b). As dimension measurements resulting from TEM can be limited and problematic, a nanoparticle tracking analysis (NTA) system [24] was also employed to supplement these TEM data. Our NTA results revealed that dimensions ranged between 40–400 nm with a mean diameter of 112 nm (Fig. 1c and d and Supplementary Video). These data confirmed that *M. furfur* produces extracellular vesicles with heterogenous characteristics.

3.2. Internalization of M. furfur *extracellular vesicles by HaCaT cells*

To investigate the immunoregulation function of *M. furfur* extracellular vesicles on keratinocytes, we first assessed the potential for recognition between vesicles and keratinocytes. Enriched vesicles were quantified as based upon sterol concentrations and stained with red fluorescence compound dialkylcarbocyanine iodide (Dil), which labels the lipid component of the vesicles. Dil stained vesicles were incubated with HaCaT cells in a time-based sequence. With increasing incubation times, the accumulated internalized vesicles exhibited a perinuclear distribution pattern. After 12 h of incubation, several lighter and enlarged merged vesicle dots could be visualized when assessed with laser scanning confocal microscopy (Fig. 2a). No red fluorescent signals were observed in the negative control group (data not shown).

Within the supernatants of our LDH analysis experiments, no significant differences were present among HaCaT cells exposed to vesicles of different sterol concentrations (Fig. 2b). These results suggest that under our test conditions, vesicles were not able to damage the HaCaT cells.

3.3. M. furfur *extracellular vesicles enhance the production of IL-6 in HaCaT cells*

Based upon results from our preliminary experiments on exposure duration and vesicular concentrations (Supplementary Fig. 1a), a 1 h duration and 500 ng/ml vesicular sterol concentration were selected. Under such conditions, the genome-DNA free RNA samples extracted from HaCaT cells stimulated with PBS or extracellular vesicles were subjected to PCR Array assay. Gene expression levels relative to *GAPDH* ($2^{-\Delta Ct}$) were graphed as a heatmap (Fig. 3a and Supplementary Table 1), in which top 10 genes with the greatest differential expression were illustrated (Fig. 3b). IL-1β, IL-6 and IL-8, play important roles in the mediation of inflammatory cutaneous disorders [25], were focused in this study and we designed experiments to validate the PCR Array results *in vitro*. After vesicular exposure, transcription levels of IL-1β and IL-6 were significantly induced as evidenced by qPCR assay, however the IL-8 mRNA level showed an opposite trend against our

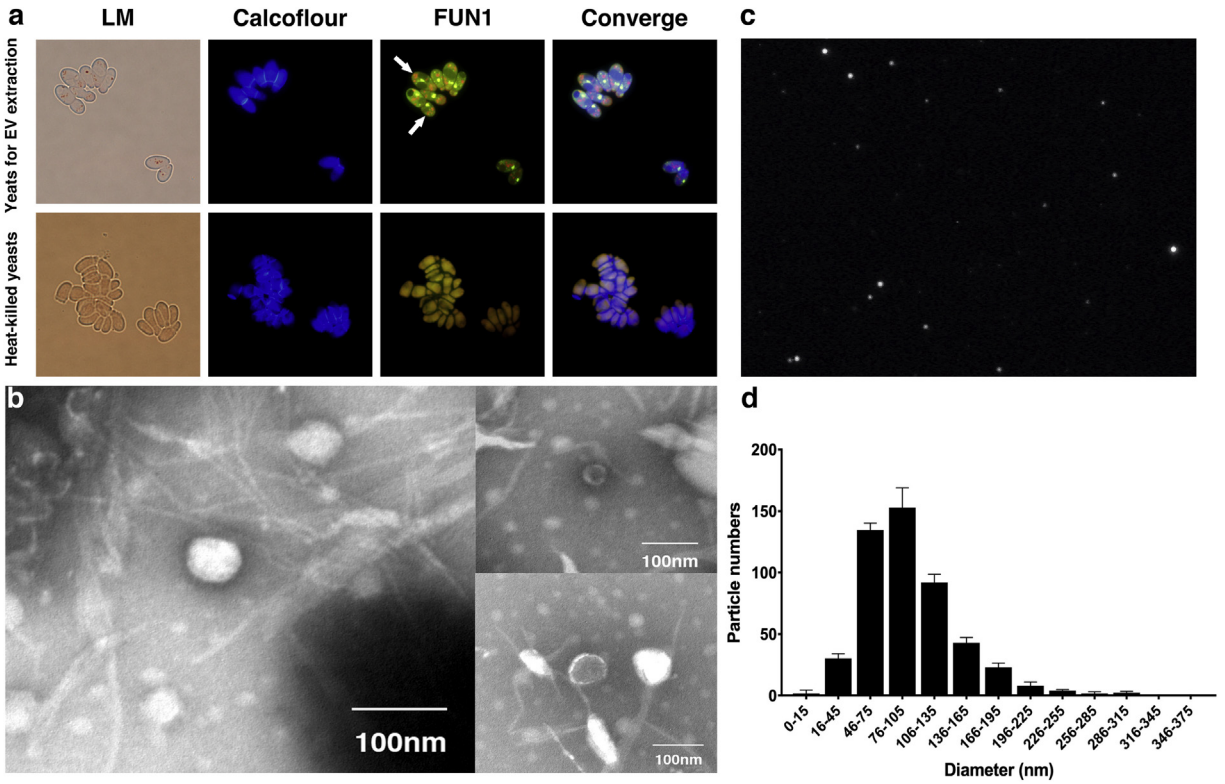

Fig. 1. *M. furfur* produces extracellular vesicles. (a) FUN1 stain. *M. furfur* prepared for vesicle isolation were stained with FUN1 and Calcofluor after incubation and viewed with use of fluorescence microscopy using a filter set at excitation values in the fluorescein and DAPI ranges, respectively. Yeasts killed at 80 °C for 30 min were used as negative controls (second row). Cylindrical Intra Vacuolar Structures (CIVS, orange-red inclusions as indicated by white arrows) were observed in almost all the yeasts as compared to the diffuse yellow-green stain indicating dead cells. (b) TEM. *M. furfur* extracellular vesicles isolated by ultracentrifugation and visualized with use of TEM. (c and d) NTA. The diameter distribution of fungal extracellular vesicles as analyzed by NTA showed a range from 40 to 400 nm with a mean diameter of 112 nm. Abbreviations: *M. furfur*, *Malassezia furfur*; DAPI, 4′,6-diamidino-2-phenylindole; TEM, transmission electronic microscopy; NTA, nanoparticle tracking analysis.

screening study (Fig. 3c). In further support of these findings, the supernatant of HaCaT cells exposed to extracellular vesicles was collected and subjected to ELISA assay for detection of these cytokines. Our results showed that as compared with PBS group, the secretion of IL-6 displayed a time dependent trend to increase, with the maximal enhancement occurring at 12 h (Fig. 3e). Similar findings were not observed for the secretion of IL-1β and IL-8 (Fig. 3d and f).

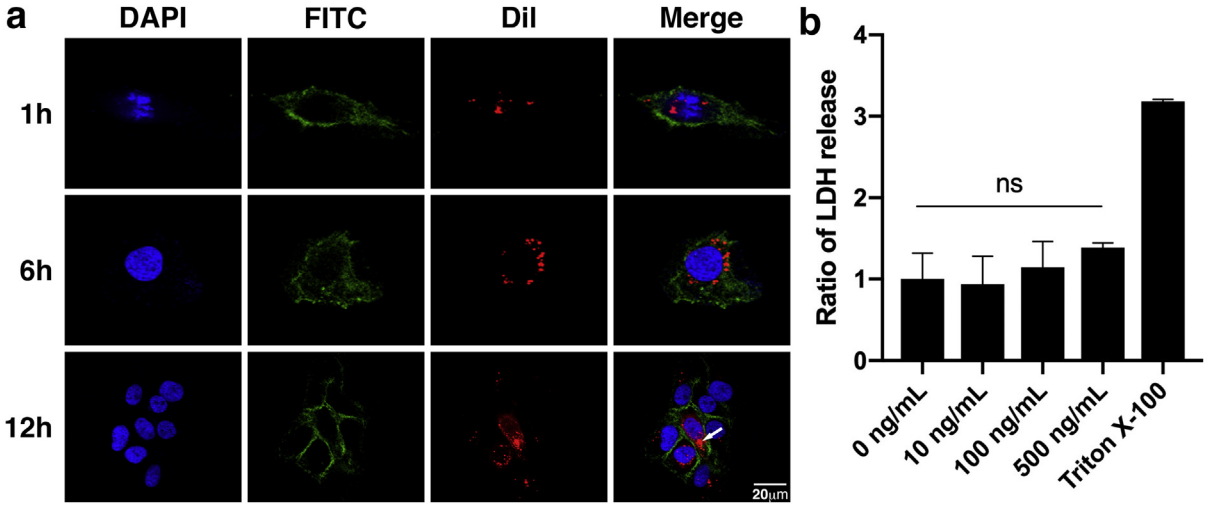

Fig. 2. Internalization of *M. furfur* extracellular vesicles by HaCaT cells. (a) Confocal microscopy. DiI stained extracellular vesicles were incubated with HaCaT cells for 1, 6 or 12 h, followed by cell membranes (CD44) and nuclei being labeled with FITC and DAPI, respectively. The perinuclear accumulation of extracellular vesicles can be observed as an increasing incubation period. The white arrow indicates merged vesicles as presented in a lightened and enlarged scale. (b) LDH assay. The sterol concentration of extracellular vesicles was adjusted to 0, 10, 100 or 500 ng/ml with serum free DMEM. After co-incubation, supernatants were collected and the LDH levels were measured within the different groups. No significant differences were obtained among the groups in response to exposure of the various vesicular concentrations tested. Supernatants from non-treated HaCaT cells (0 ng/ml) and Trition X-100 (10%) treated cells were considered as negative and positive controls, respectively. DMEM medium without HaCaT cells was considered as a blank control. Abbreviations: DiI, dialkylcarbocyanine iodide; FITC, fluorescein isothiocyanate; DAPI, 4′,6-diamidino-2-phenylindole; LDH, lactate dehydrogenase.

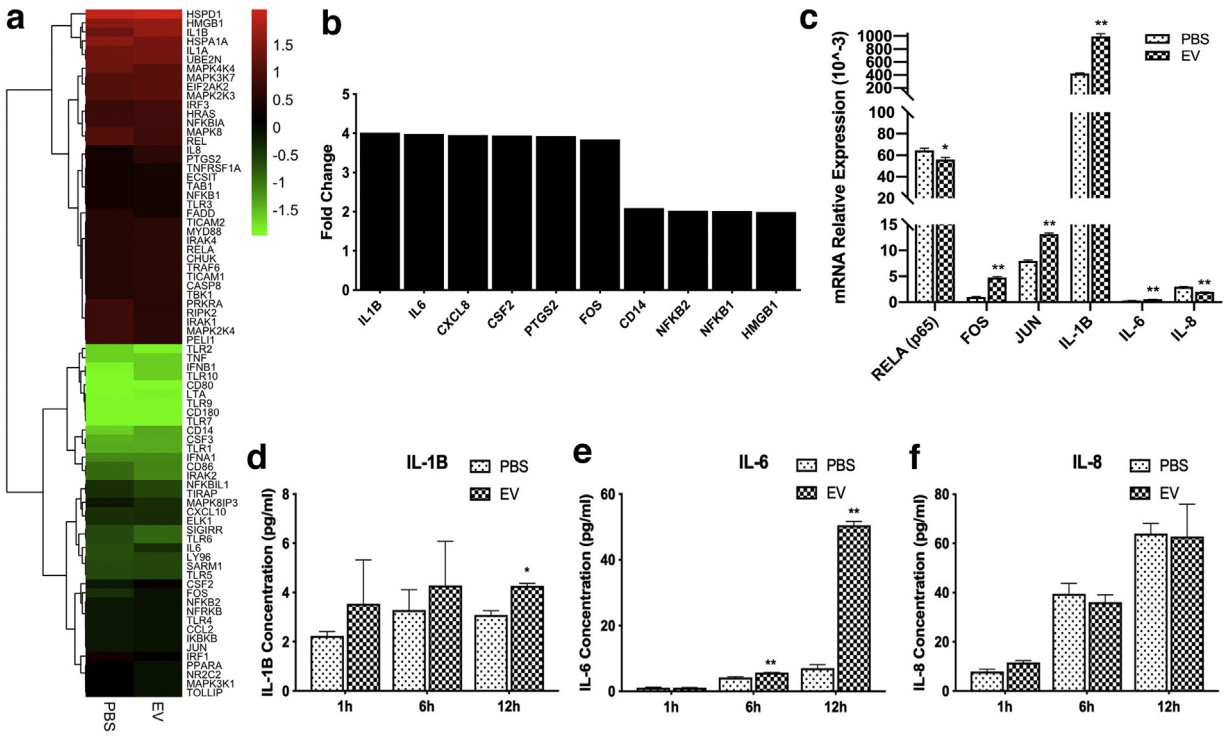

Fig. 3. RT^2 Profiler PCR Array screening of gene expressions and *in vitro* validation by qPCR and ELISA assays. (a) Gene expression levels relative to *GAPDH* ($2^{-\Delta Ct}$) are scaled and plotted as a heatmap. Up-regulated and down-regulated genes are colored in red and green, respectively. (b) Top 10 genes with the greatest differential expression in PCR Array screening are illustrated as fold changes ($2^{-\Delta\Delta Ct}$). (c) Validation of the results of PCR Array as determined with use of qPCR method. Within this assay, after 1 h of vesicular exposure, in which the expression trends of *IL-1B*, *IL-6*, *FOS* and *JUN* were in accordance with that of the PCR Array, whereas the expression of *RELA* and *IL-8* exhibited an opposite trend. (d to f) Validation of the PCR Array results as determined by ELISA assay. With use of this ELISA assay the secretion of IL-6 displayed a time dependent trend to increase, with the maximal enhancement occurring at 12 h. Similar trends were not observed for the secretion of IL-1β and IL-8. Statistically significant values are presented as asterisks (*), $^{*}P < 0.05$, $^{**}P < 0.01$. All data represent that of the results from 3 independent experiments. Abbreviations: IL-1β, interleukin 1β; IL-6, interleukin 6; IL-8, interleukin 8; EV, extracellular vesicle.

3.4. M. furfur *extracellular vesicles promote IL-6 synthesis in epidermal keratinocytes of mice*

Given the results obtained in HaCaT cells as demonstrated *in vitro*, in this experiment we investigated whether *M. furfur* extracellular vesicle could induce similar biological effects in epidermal keratinocytes in an *in vivo* model, as well as the role of the epidermal barrier in this process. To accomplish this goal, the permeation capacity of extracellular vesicles into epidermis was first evaluated. Prior to the experiment, an epidermal barrier destruction model was prepared on the half of dorsal skin of mice with use of the tape stripping method [26]. Subsequently, Dil stained extracellular vesicles were topically applied to this dorsal area with both epidermal conditions. Skin samples were collected at 1 h and 8 h time points following application of extracellular vesicles. With use of confocal microscopy, we observed that Dil stained extracellular vesicles had extended throughout the entire epidermal layer within 1 h following exposure in the tape stripping epidermis (Fig. 4a–c) and penetrated below the basement membrane after 8 h of exposure (Fig. 4g–i). In contrast, extracellular vesicles applied to the intact epidermis showed a delayed trend with regard to this penetration (Fig. 4d–f and j–l). Nonetheless, it can be confirmed that in both conditions, extracellular vesicles can penetrate into the epidermis and be absorbed by epidermal keratinocytes. Moreover, we observed a follicular accumulation of extracellular vesicles under fluorescence microscopy regardless of the barrier status.

Having confirmed this prerequisite, experiments were then performed to assess IL-6 expression *in vivo* after exposure to extracellular vesicles (Dil free). Extracellular vesicles with various sterol quantities were topically applied onto the dorsal skin under different conditions of epidermal barrier status. Skin samples were prepared for detection of IL-6 expression by IHC assay as well as for measurement of epidermal thickness. As the results indicated, under both conditions, no statistically significant differences were obtained in epidermal IL-6 expression among groups stimulated with a small quantity of extracellular vesicles (0, 10 or 100 ng), although there was trend for increased expression in the intact epidermis with increasing vesicular quantities. With an exposure quantity of 500 ng, a substantial enhancement of IL-6 expression was observed in both conditions as compared with that of groups exposed to PBS or those treated with lower concentrations of vesicles (Fig. 5a and b). Results obtained from epidermis thickness analysis were in accordance with that observed with IL-6 expression in the intact epidermis. However, in the tape stripping epidermis group no significant differences in epidermal thickness were observed among skin samples stimulated with the varying concentrations of extracellular vesicles (Fig. 5c).

3.5. NF-κB *activation is involved in the production of IL-6 stimulated by* M. furfur *extracellular vesicles in HaCaT cells*

NF-κB plays a central role in the inflammatory response to infection or tissue injury [27]. To investigate its role in the production of IL-6 as promoted by *M. furfur* extracellular vesicles, the expression level of p65, an important subunit of NF-κB, along with its activated form, phosphorylated p65, was examined based on a time sequence after vesicular stimulation by western blot. The results revealed that the expression of phosphorylated p65 started to increase at 30 min after vesicular exposure compared to the

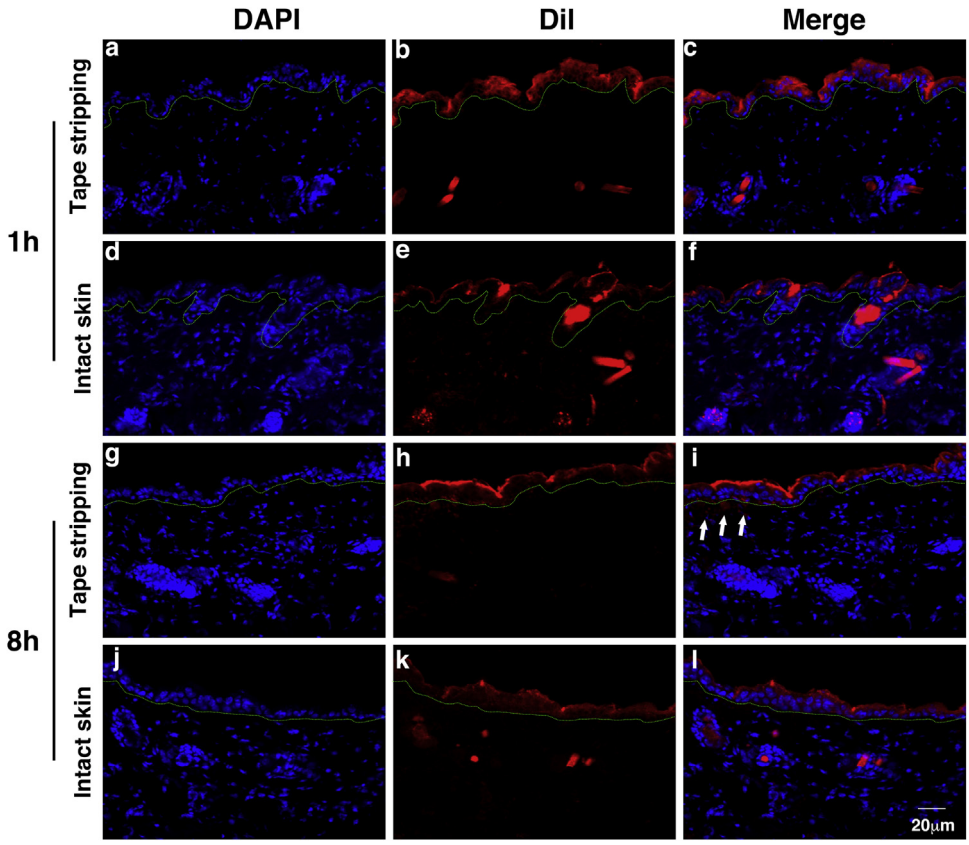

Fig. 4. Extracellular vesicular permeation into epidermis of mice. Dil stained extracellular vesicles were topically applied on the dorsal skin area of BALB/c mice with or without tape stripping treatment. After 1 or 8 h of exposure, skin sections were stained with DAPI and visualized under fluorescence microscopy. At 1 h post-exposure, extracellular vesicles had penetrated into the entire layer of epidermis (a–c) in the tape stripping group, whereas most of vesicles remained at the stratum corneum layer in the intact skin group (d–f). After 8 h of exposure, extracellular vesicles (indicated by white arrows) can be observed within the dermal layer below the basement membrane (indicated by green dotted lines) in the tape stripping group (g–i), and in the intact skin group, the entire epidermal layer permeation was observed (j–l). Hair follicular accumulation of extracellular vesicles can be observed in both conditions. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; Dil, dialkylcarbocyanine iodide.

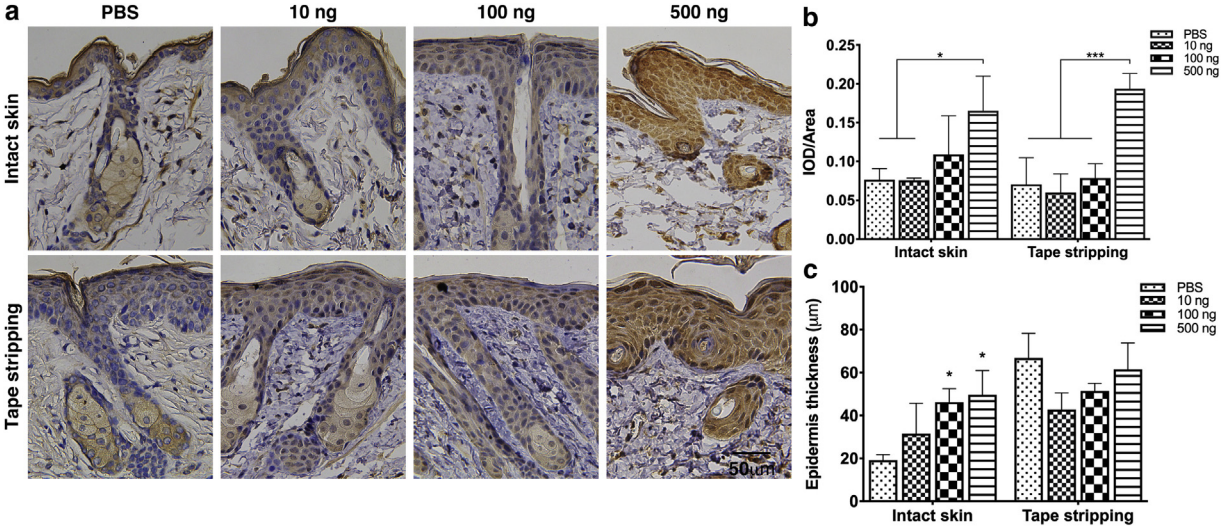

Fig. 5. *M. furfur* extracellular vesicles promote IL-6 synthesis in epidermal keratinocytes *in vivo*. (a and b) Expression of IL-6 was determined in skin samples from the intact skin and the tape stripping groups at 48 h after topical application of extracellular vesicles with various sterol quantities. As compared with that of the PBS group, there was an increasing trend for IL-6 expression in both conditions, with maximal expressional differences observed in groups stimulated with vesicles of 500 ng sterol quantity. (c) The epidermal thickness of vesicle groups in response to 100 or 500 ng sterol quantity was significantly thicker than that of PBS group in the intact skin group, however no such differences were observed in the tape stripping group. Statistically significant values are presented as asterisks (*), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Data represent that of the results from independent IHC assays performed with use of 4 BALB/c mice per group.

control group. In contrast, p65 expression showed no time-dependent changes in response to this stimulation (Supplementary Fig. 2a). To collaborate these findings, Helenalin, an NF-κB specific inhibitor, was administrated prior to vesicular stimulation and its inhibitory effects were assessed (Supplementary Fig. 2b). As performed in our previous experimental conditions, the supernatants from HaCaT cells were collected at 12 h following stimulation with extracellular vesicles and subjected to ELISA assay. Following pretreatment with Helenalin, IL-6 production was significantly reduced despite with exposure to extracellular vesicles (Supplementary Fig. 2c). These data suggested that the activation of NF-κB is critically involved in the IL-6 production in HaCaT cells induced by *M. furfur* extracellular vesicles.

4. Discussion

The host response to fungal invasion represents a complicated process due to the variety of fungal antigens or components that may be present. As the carrier of these substances, fungal extracellular vesicles play an important role in this host-fungi crosstalk [28]. In this report, our results provide the first evidence demonstrating that *M. furfur* produces extracellular vesicles and their resultant pro-inflammatory effects on keratinocytes as shown in both *in vitro* and *in vivo* models.

The isolation method for these vesicles followed the standard protocol as described by Rodrigues et al. [21]. In addition, prior to ultracentrifugation, we employed a process to evaluate yeast cell viability as achieved with use of the FUN1 stain to guarantee that only a tiny proportion were dead cells. The vesicles we harvested were similar in diameter and distribution as that reported for *M. sympodialis* [16,17]. Results from our TEM analysis revealed an ovoid-shaped membrane structure of the vesicles with distinct electronic densities, implying a diversity of contents in the package. Recent studies on the components of vesicles as derived from *M. sympodialis*, have provided valuable background information regarding our observations. Rayner et al. confirmed the presence of small RNA sequence reads with well-defined start and stop positions in extracellular vesicles from *M. sympodialis* [16]. Quantitative proteomics as performed at this same research center, identified 110 enriched proteins, including hydrolase and enzymes with catalytic activity [17].

Two possible mechanisms have been proposed regarding the means through which mammalian exosomes are incorporated into host cells and their contents delivered to the cytoplasm [29]. Feng and colleagues suggested that a phagocytotic capability of target cells is essential for the internalization pattern [30]. While cells with phagocytic ability incorporate exosomes mainly by active phagocytosis, most exosomes attach to the cell membrane through means other than that involving internalization in non-phagocytic cells. Our results, indicating an absence of Dil on the host cell membrane suggest that *M. furfur* extracellular vesicles are delivered into HaCaT cells by phagocytosis. These findings support the results of Johansson's study on the interaction between extracellular vesicles from *M. sympodialis* and primary human keratinocytes [17]. In that study, they demonstrated that vesicle internalization by keratinocytes was dependent on an active process, as internalization only occurred at an incubation temperature of 37 °C, but not at 4 °C.

Studies involving nanoparticle application to the skin, as a means of facilitating local or systemic drug administration, have become a popular area of research [31]. However, limited information exists on extracellular vesicular transdermal permeation [32]. Here, we designed experiments to evaluate whether extracellular vesicles derived from *M. furfur* could penetrate the epidermis as well as the influence of epidermal barrier status on this process. Our results showed extracellular vesicles penetrated into the entire epidermal layer and were absorbed by epidermal keratinocytes in both epidermal barrier conditions. However, we did observe some notable differences between the intact *versus* the tape stripping groups. Specifically, in the intact skin group, where most of the vesicles remained at the stratum corneum layer at 1 h after exposure, a delay was observed, whereas an entire epidermal penetration was present by this time in the tape stripping group. The stratum corneum layer, composed of flattened, anuclear corneocytes and extracellular lipid matrix [33], is considered as the rate limiting barrier for substance permeating across the skin [31,34]. And there are evidences showing that transdermal permeation is selective with regard to particle sizes [31]. Considering the wide range of the extracellular vesicular sizes, with diameters from 40 to 400 nm, we postulated that in addition to serving as a rate limiting barrier, the stratum corneum may also function as a selective mesh that would be capable of expelling large sized vesicles. However, in some diseased conditions of the skin this barrier may be disrupted and thus alter the degree of permeation as related to vesicular size. Further works will be required to verify this speculation.

We also observed another interesting phenomenon as obtained with the confocal microscopy analysis. Notably, we detected high affinity of the *M. furfur* extracellular vesicles to hair follicles, regardless of epidermal barrier status. This was not observed under conditions where we applied vesicular free Dil solution to the skin (data not shown). Such findings are like that observed with a topical application of liposomal formulation, a bilayer structure similar to extracellular vesicles, on the skin of hamster ears [35]. Although the mechanisms for these effects remain unclear, these findings can provide some novel insights into the pathogenesis of *Malassezia* related follicular diseases.

Studies on fungal extracellular vesicles have revealed that merging of these nanoparticles with host cells can influence physiological processes and modify the production of several immunoregulatory cytokines [18,36,37]. However, to the best of our knowledge, no studies have been directed toward investigating the immunoregulatory effects of *Malassezia* extracellular vesicles on keratinocytes. To this end, we designed *in vitro* and *in vivo* experiments to investigate such a problem. We found that IL-6, as indicated by our ELISA assay, were significantly upregulated, while no such trend was obvious for IL-1β or IL-8. These findings were also observed in the *in vivo* mouse model, as obtained in both healthy and disrupted epidermal conditions. Based on the results of confocal microscopy and IHC assays, we speculated that the epidermal barrier does play a rate-limiting role with regard to vesicular transdermal permeation but has no significant effect on the prevention of the vesicular fractions with IL-6 promoting effect from entering into the epidermis. IL-6, a soluble mediator with a pleiotropic effect on inflammation, immune responses and hematopoiesis [38], is believed to be involved in many cutaneous disorders. For example, it has been reported that IL-6 aids keratinocyte proliferation and contributes to the development of psoriasis [39]. In our current results, we found a vesicular concentration dependent increase in epidermal thickness within the intact skin group, which is well consistent with the trend of IL-6. In contrast, within the tape stripping group there were no significant differences among the groups in response to the various vesicular concentrations, possibly due to the interference of epidermal trauma. Furthermore, we found that the NF-κB activation is involved in extracellular vesicle induction IL-6 production, suggesting a possible therapeutic target of NF-κB in reducing the inflammatory responses in *M. furfur* infection.

In summary, we demonstrated the existence of *M. furfur* extracellular vesicles, with their morphological and dimensional features identified. Based on the observations of our *in vitro* and *in vivo* experiments, it seems reasonable to conclude that *M. furfur*

extracellular vesicles are immunologically active and have the potential to play a role in the pathogenesis of *Malassezia* related cutaneous disorders. More specific fractions of these vesicles and their contents remain to be elucidated.

Conflict of interests

The authors have no conflict of interests to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jdermsci.2019.03.001.

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