The effects of Malassezia on pro-inflammatory cytokine production by human peripheral blood mononuclear cells \textit{in vitro}

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\textit{Malassezia} spp., the causative agents of pityriasis versicolor, are members of the normal human cutaneous microflora. Utilizing a combination of both enzyme-linked immunosorbent assay (ELISA) and bioassay, we have investigated the ability of both formalin-preserved and viable \textit{Malassezia} (serovars A, B and C) to modulate pro-inflammatory cytokine (IL-6, IL-1\(\beta\) and TNF-\(\alpha\)) release by human peripheral blood mononuclear cells (PBMNC) \textit{in vitro}, over a 48-h co-incubation period. The results demonstrated that formalin-preserved \textit{Malassezia} (serovars A, B and C) at mid-exponential phase were generally able to induce a pro-inflammatory cytokine response at a yeast cell to PBMNC ratio of 1:1. In addition, the results consistently demonstrated that at a yeast cell to PBMNC ratio of 20:1, formalin-preserved \textit{Malassezia}, irrespective of serovar, growth phase or PBMNC donor, were capable of significantly (\(P<0.05\)) decreasing the release of both immunochemical IL-6 and IL-1\(\beta\) plus bioactive IL-1\(\beta\) and TNF-\(\alpha\) below that of unstimulated culture medium control values. This was apparent following 24- and 48-h co-incubation times, where maximal cytokine production was detected after 24 h. Similar results were obtained for the effect of viable \textit{Malassezia} on pro-inflammatory cytokine release by PBMNC. Our results suggest that a possible inhibitory component, present perhaps within the cell wall of \textit{Malassezia}, was responsible for this depressive effect on pro-inflammatory cytokine production.

\textbf{Keywords} \textit{Malassezia}, peripheral blood mononuclear cell, pro-inflammatory cytokine

\section*{Introduction}

\textit{Malassezia} spp. are dimorphic, lipid-dependent yeasts which form an integral part of the human cutaneous microflora, existing at high population densities (up to \(10^8\) cfu cm\(^{-2}\)) notably in the seborrhoeic regions of the upper trunk [1]. The genus \textit{Malassezia} has recently been revised and enlarged using morphology, ultrastructure, physiology and molecular biology to include seven species, the three former taxa \textit{M. furfur}, \textit{M. pachydermatis} and \textit{M. sympodialis}, and four new taxa \textit{M. globosa}, \textit{M. obtusa}, \textit{M. restricta} and \textit{M. slooffiae} [2].

\textit{M. sympodialis}, \textit{M. globosa} and \textit{M. restricta} correspond to serovars A, B and C, respectively, in the classification of Cunningham et al. [3].

\textit{Malassezia} spp. are aetiological agents of the chronic, superficial, mycotic skin disorder pityriasis versicolor in which the mycelial phase predominates [4]. Hyphae invade the stratum corneum both between and inside corneocytes [5], resulting in an alteration in skin pigmentation together with a lack of any visible erythema associated with lesions. Yeast phase \textit{Malassezia} has also been implicated in the pathogenesis of other superficial dermatoses, the most important ones being seborrhoeic dermatitis (severe and difficult to treat when associated with AIDS) [6,7], folliculitis [8] and atopic dermatitis [9]. However, the mechanisms by which this commensal microorganism causes dermatoses are not yet clear. In
addition, Malassezia have more recently been identified as causative agents of life-threatening, iatrogenic, catheter-related sepsis both in immunocompromised individuals and low-birth-weight neonates receiving parenteral lipid alimentation [10–12].

The relationship between Malassezia and host defence mechanisms remains unclear. While there has been extensive research into specific immune responses to Malassezia [13–18], there have been few investigations into the interactions of Malassezia with non-specific defence mechanisms. The most recent investigations failed to demonstrate any significant difference in humoral and cell-mediated immune responses specific to Malassezia serovars A, B and C in patients suffering from Malassezia-associated dermatoses when compared to controls [13,14]. However, conflicting data report both higher Malassezia-specific immunoglobulins [15] and IgG [18] in patient groups compared to controls, together with data indicating that patients with pityriasis versicolor exhibit impaired cell-mediated immune responses to Malassezia [16].

The interaction between Malassezia and human mononuclear phagocytes has not been studied in detail previously. There is one report indicating that Malassezia serovar B (M. globosa) may depress IL-1β production by human peripheral blood mononuclear cells in vitro [19]. In order to determine whether this was also the case with other serovars of Malassezia, we have investigated the effects of both formalin-preserved and viable Malassezia serovars A, B and C on pro-inflammatory cytokine production by human peripheral blood mononuclear cells in vitro, together with an investigation into the possible adsorption of pro-inflammatory cytokines by non-specific receptors of Malassezia.

**Materials and methods**

**Yeast**

The six strains of Malassezia used in this study (serovar A/M. sympodialis; strain 13 and E10; serovar B/M. globosa; strain 21´3 and E8 and serovar C/M. restricta; strain 42´2 and E2) were subcultured on Malassezia agar [20] on the basis of differential colonial morphology. Strains 13, 21´3 and 42´2 are type strains (originally isolated from clinically normal skin) which have been used in the genetic classification of the genus Malassezia by Guého et al. [2], while strains E10, E8 and E2 were freshly isolated from healthy volunteers. All six strains were differentiated into serovars A, B and C by indirect immunofluorescence [3] and grown aerobically in modified milk (MM) medium [20] at 34°C. Each strain was harvested at both mid-exponential and late stationary phase of growth. Culture purity was determined prior to and following batch preparations. In general, serovars A and B usually attained late stationary phase following 60 h of incubation, while serovar C usually took more than 90 h to reach late stationary phase. Cells of all six strains were washed three times in phosphate buffered saline (PBS; pH 7.4) and resuspended in PBS plus 1% (v/v) formaldehyde and stored at room temperature.

**Peripheral blood mononuclear cells**

Human peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood obtained by venepuncture from various healthy donors (four in total). Heparinized whole blood was diluted 1:1 with RPMI-1640 (Life Technologies) supplemented with 60 μg ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin, 20 mm N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid (HEPES; Life Technologies) and 5% (v/v) heat-inactivated fetal calf serum and the mononuclear cell fraction was obtained by density centrifugation on Lymphoprep® (Nycomed). PBMC were resuspended at 10⁶ cells ml⁻¹ in culture medium (RPMI-1640 supplemented with penicillin, streptomycin, 20 mm HEPES, 10% (v/v) heat-inactivated fetal calf serum and 2 mm l-glutamine) and viability determined by trypan blue dye exclusion.

**Co-incubation of PBMC with formalized Malassezia**

PBMC were seeded into sterile, 96-U-well plates (Beckton Dickinson Labware) at a concentration of 10⁶ PBMC per well. Three different yeast cell to PBMC ratios for all three serovars were tested (1:1; 10:1 and 20:1). Yeast cell preparations were washed three times in sterile PBS to ensure complete removal of formaldehyde and Malassezia suspensions (prepared in RPMI-1640 culture medium) were added to PBMC in triplicate to give a final culture volume of 200 μl per well. Lipopolysaccharide (LPS from Escherichia coli; Sigma; 20 μg ml⁻¹) served as a positive control and RPMI-1640 culture medium (with supplements) served as a negative control. Cell-free supernatants were harvested at 0 h, 24 h and 48 h of co-culture at 37°C in an atmosphere of 5% (v/v) CO₂ in air, stored at −20°C and assayed for IL-6, IL-1β and TNF-α. PBMC growth and viability were assessed at 0 h, 24 h and 48 h by adaptation of the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay [21]. Replicate wells were prepared for the purpose of mononuclear cell viability measurements. Briefly, 10 μl of MTT (5 mg ml⁻¹ in PBS;
pH 7-4) was added to these wells and incubated for a further 4 h. One hundred microlitres of sodium dodecyl sulphate (SDS; 10% [wt/vol] in 0·01 m HCl) was added to each well and the plates were incubated in a moist box overnight at 37 °C. A170 values were then determined (MR7000 plate reader; Dynatech).

Co-incubation of PBMNC with viable Malassezia

Three strains of Malassezia (serovar A/M. sympodialis; strain E10; serovar B/M. globosa; strain E8 and serovar C/M. restricta; strain E2) were grown aerobically in modified milk medium [20] at 34 °C and harvested during late stationary phase. Culture purity was determined prior to and following batch preparations. Each strain was washed three times in PBS and resuspended in RPMI-1640 culture medium (with supplements). PBMNC were seeded at a concentration of 10⁶ cells well⁻¹. One yeast cell to PBMNC ratio was tested (20:1) for all three serovars. Cell-free supernatants were harvested following 0 h and 24 h co-incubation in an atmosphere of 5% (v/v) CO₂ in air and stored at −20 °C until assayed for pro-inflammatory cytokine content. Replicate wells were prepared for mononuclear cell viability determinations. Briefly, 10 μl of culture was removed from each replicate well and mononuclear cell viability was determined by total counting (haemocytometer) and trypsin blue dye exclusion.

Cytokine assays

Immunoochemical IL-6 and IL-1β were measured by utilizing an ‘in-house’ ELISA and a commercial ELISA kit (Endogen, USA), respectively. The IL-6 ELISA was carried out by coating Maxisorp 96-well plates (Nunc, Roskilde, Denmark) with rabbit antihuman IL-6 polyclonal antibodies (prepared ‘in-house’). One hundred microlitre aliquots of mononuclear cell supernatants were added to respective wells and, following this, a secondary mouse antihuman IL-6 monoclonal antibody was added (prepared ‘in-house’). The antibody–cytokine–antibody complex was detected by a reaction of biotinylated antimouse antibodies and a horseradish–peroxidase–avidin conjugate (Vector Laboratories, Peterborough, UK). The colour reaction was detected by the addition of orthophenylendiamine (OPD; Sigma, Dorset, UK) and the reaction stopped with 2·5 m H₂SO₄. The ELISA was standardized by using a recombinant IL-6 reference standard (National Institute for Biological Standards and Controls; NIBSC). Bioactive IL-1β was measured using an adaptation of a murine thymocyte proliferation assay [22] in which thymocytes from freshly sacrificed C3H Hej mice proliferate in response to bioactive IL-1 in the presence of saturating levels of IL-2. Briefly, thymus tissue was aseptically homogenized to produce a single-cell thymocyte suspension which was adjusted to 4 × 10⁶ cells ml⁻¹ in RPMI-1640 supplemented with 60 μg ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin, 10% (v/v) heat-inactivated fetal calf serum, 50 mM 2-mercaptoethanol, 2 mM l-glutamine and interleukin-2 (200 U ml⁻¹) (Boehringer Mannheim). Thymocytes were seeded into 96-well plates at a concentration of 2 × 10⁶ thymocytes per well, which also contained 50 μl of test sample (each sample doubly diluted, ranging from 1/2 to 1/128). Plates were incubated at 37 °C for 3 days in a 5% (v/v) CO₂ in air atmosphere. Thymocyte proliferation was determined by the MTT assay.

Bioactive TNF-α was measured by cytolysis of a target mouse lung fibroblast cell line (L929). Briefly, L929 cells were grown for 48 h in Dulbecco’s modified Eagles’ medium (DMEM; Life Technologies), 10% (v/v) heat-inactivated fetal calf serum, 2 mM l-glutamine, 7-5% (w/v) NaHCO₃, 60 μg ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin. The cells were harvested by trypsin-EDTA treatment and plated in 50 μl volumes (2 × 10⁵ cells ml⁻¹) into sterile, flat-bottom, 96-well microtitre plates (Beckton Dickinson Labware) for 3 h in an atmosphere of 5% (v/v) CO₂ in air. Twofold dilutions of PBMNC supernatants (50 μl) were added to L929 monolayers within wells and plates were incubated at 37 °C for 3 days in a 5% (v/v) CO₂ in air atmosphere. L929 cell viability was determined by the MTT assay.

Both bioassays were standardized using recombinant IL-1β and TNF-α reference standards (NIBSC). All samples incorporated into the bioassays were also incubated with appropriate neutralizing polyclonal antibodies to confirm true positivity (antihuman IL-1β and TNF-α neutralizing antibodies; British Biotechnology). The dilution of each test sample and standard giving 50% of the maximum response was determined by probit analysis. Cytokine values were expressed in pg ml⁻¹ and later converted to units of specific activity (nanogram of cytokine per optical density at 570 nm).

Adsorption of interleukin-6 by Malassezia (serovars A, B and C)

Formalized, intact cells of three type strains (serovars A/M. sympodialis; strain 13; serovar B/M. globosa; strain 21·3 and serovar C/M. restricta; strain 42·2) (late stationary phase) were resuspended at 4 × 10⁶ cells ml⁻¹ in RPMI-1640 supplemented with penicillin, strep-
Fig. 1 Specific activities of bioactive IL-1β released by PBMNC from donors I (a, b) and II (c–f) co-incubated for 48 h with formalized midexponential and late stationary phase serovar A; strain 13 (a, b), serovar B/strain 21·3 (c, d) and serovar C/strain 42·2 (e, f). Positive [LPS (+)] and negative [CM (−)] controls are shown. Significant increases/decreases (MSD $P<0.05$ [**]) in comparison with the negative control are indicated. For a and b, MSD values are ($T=0$ h) 1·00; ($T=24$ h) 0·5; and ($T=48$ h) 0·5. For c and d, MSD values are ($T=0$ h) 0·09; ($T=24$ h) 1·00 and ($T=48$ h) 1·60. For e and f, MSD values are ($T=0$ h) 0·4; ($T=24$ h) 1·7 and ($T=48$ h) 1·60. 1 MAL: 1 PBMNC (a) is equivalent to one Malassezia yeast cell per human peripheral blood mononuclear cell.

tomycin, 20 mM HEPES, 10% (v/v) heat-inactivated fetal calf serum and 2 mM L-glutamine, and added to wells of 96-U-well plates in 50 μl volumes. Interleukin-6 (NIBSC) was added in 50 μl volumes (1 in 2 dilution) at three concentrations of 2000 pg ml$^{-1}$, 1000 pg ml$^{-1}$ and 200 pg ml$^{-1}$. The plate was rotated for 1 h (802/TW suspension mixer, Luckham Ltd, UK) and incubated at 37 °C for 24 h in an atmosphere of 5% (v/v) CO$_2$ in air. Cell-free supernatants were harvested after 24 h and stored at −70 °C until assayed for IL-6 by ELISA.

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Cytokine production in response to Malassezia

Fig. 2 Specific activities of immunochemical IL-6 released by PBMNC from donors I (a, b) and II (c–f) co-incubated for 48 h with formalized midexponential and late stationary phase serovar A/strain 13 (a, b), serovar B/strain 21-3 (c, d) and serovar C/strain 42-2 (e, f). Positive [LPS (+)] and negative [CM (−)] controls are shown. Significant increases/decreases (MSD $P<0.05$ [**]) in comparison with the negative control are indicated. For a and b, MSD values are $(T=0\text{ h})$ 0·2; $(T=24\text{ h})$ 1·5; and $(T=48\text{ h})$ 1·5. For c and d, MSD values are $(T=0\text{ h})$ 0·2; $(T=24\text{ h})$ 1·1; and $(T=48\text{ h})$ 1·3. For e and f, MSD values are $(T=0\text{ h})$ 0·22; $(T=24\text{ h})$ 1·9; and $(T=48\text{ h})$ 1·9. 1 MAL: 1 PBMNC (a) is equivalent to one Malassezia yeast cell per human peripheral blood mononuclear cell.

Statistical analysis

Values of cytokine specific activities were analysed by two-way analysis of variance. Minimum significant differences (MSD $P<0.05$) between means ($n=3$) were calculated by the $T$-method [23].

Results

IL-1β production by PBMNC co-incubated with Malassezia (serovars A, B and C)

The levels of IL-1β released by PBMNC from two donors in the presence of formalized Malassezia...
Fig. 3 Specific activities of bioactive TNF-α released by PBMC from donors I (a, b) and II (c–f) co-incubated for 48 h with formalized midexponential and late stationary phase serovar A/strain 13 (a, b), serovar B/strain 21·3 (c, d) and serovar C/strain 42·2 (e, f). Positive [LPS (+)] and negative [CM (−)] controls are shown. Significant increases/decreases (MSD \( P < 0.05 \) \( ** \)) in comparison with the negative control are indicated. For a and b, MSD values are \( (T = 0 \text{ h}) 0·3; (T = 24 \text{ h}) 0·6; \) and \( (T = 48 \text{ h}) 0·678 \). For c and d, MSD values are \( (T = 0 \text{ h}) 1·1; (T = 24 \text{ h}) 0·5; \) and \( (T = 48 \text{ h}) 1·20 \). For e and f, MSD values are \( (T = 0 \text{ h}) 1·3; (T = 24 \text{ h}) 1·3; \) and \( (T = 48 \text{ h}) 1·20 \). 1 MAL: 1 PBMNC (a) is equivalent to one Malassezia yeast cell per human peripheral blood mononuclear cell.

(serovars A, B and C), over a 48-h co-culture period, were determined by bioassay. Figure 1 shows the effect of midexponential and late stationary phase yeast cells of serovar A/M. sympodialis (strain 13), serovar B/M. globosa (strain 21·3) and serovar C/M. restricta (strain 42·2) on IL-1β production by PBMC incubated with three different yeast cell to PBMC ratios. The levels of bioactive IL-1β released by PBMC into supernatants in response to Malassezia ranged between 0 and 450 pg ml\(^{-1}\), with maximal cytokine production

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following a 24-h co-incubation. Growth and viability of PBMC, as assessed by the MTT assay, did not vary significantly and PBMC remained viable throughout the 48-h co-culture period.

Exponential phase cells of serovar A (*M. sympodialis*) significantly induced IL-1\(\beta\) production \((P<0.05)\) after 24 h and 48 h, when compared with respective negative control values, at a yeast cell to PBMC ratio of 1:1 (Fig. 1a). This was not apparent for late stationary phase cells at the same ratio (Fig. 1b). Cells of both growth phases (serovar A/*M. sympodialis*) produced low levels of IL-1\(\beta\) (24 and 48 h), which were not significantly different from constitutive negative control values at ratios of 10:1 and 20:1. Exponential phase cells of serovar B (*M. globosa*) significantly induced IL-1\(\beta\) production \((P<0.05)\) at a ratio of 1:1 (24 h). This was not apparent for stationary phase cells at this ratio (Fig. 1d). Exponential phase cells of serovar B (*M. globosa*), at a ratio of 10:1, produced levels of IL-1\(\beta\) not significantly different compared with the negative control. However, at a ratio of 20:1, levels of IL-1\(\beta\) were significantly lower than constitutive negative control values (Fig. 1c). Stationary phase cells of serovar B (*M. globosa*) also significantly depressed IL-1\(\beta\) production at all three yeast cell to PBMC ratios (Fig. 1d). A similar pattern of results was observed with cells of serovar C (*M. restricta*) (both growth phases) (Fig. 1e, f). The positive control (LPS; 20 ng well\(^{-1}\)) significantly increased IL-1\(\beta\) production by PBMC in all determinations. The weaker IL-1\(\beta\) response of donor I (Fig. 1a, b) compared with donor II (Fig. 1c–f) reflects the variation in PBMC responsiveness between blood donors in vitro.

**IL-6 production by PBMC co-incubated with Malassezia (serovars A, B and C)**

PBMC co-incubated for 48 h with formalized *Malassezia* (serovars A, B and C) at three different yeast cell to PBMC ratios showed a similar dose-dependent depression of immunochemical IL-6 release by PBMC (Fig. 2). The levels of immunochemical IL-6 in supernatants in response to *Malassezia* ranged between 0 and 750 pg ml\(^{-1}\). All three serovars, at a yeast cell to PBMC ratio of 1:1 (exponential phase) induced IL-6 production to levels which were significantly greater than the negative control \((P<0.05)\). No cytokine induction was apparent in response to late stationary phase cells of all three serovars at this ratio. At a ratio of 10:1 (all three serovars at both growth phases), levels of immunochemical IL-6 were either not significantly different or were depressed compared with negative control values. However, following a 24-h co-incubation, a yeast cell to PBMC ratio of 20:1 significantly depressed levels of immunochemical IL-6, in all 6 determinations (Fig. 2a–f) to below those values of the negative control \((P<0.05)\).

**TNF-\(\alpha\) production by PBMC co-incubated with Malassezia (serovars A, B and C)**

Figure 3 shows the effect of *Malassezia* (serovars A, B and C) (midexponential and late stationary phase) on bioactive TNF-\(\alpha\) production by PBMC. The detected levels of bioactive TNF-\(\alpha\) released by PBMC in response to *Malassezia* ranged between 0 and 436 pg ml\(^{-1}\), with maximal cytokine production detected following 24-h co-incubation. Again, the depression of pro-inflammatory TNF-\(\alpha\), induced by intact whole *Malassezia* yeast cells, was generally found to be concentration-dependent, while late stationary phase cells appeared to reduce the levels of TNF-\(\alpha\) more than midexponential phase cells. As with IL-1\(\beta\) (Fig. 1), the weaker TNF-\(\alpha\) response of donor I (Fig. 3a, b) compared with donor II (Fig. 3c–f) reflects variation in PBMC responsiveness between blood donors in vitro.

**The effects of different strains of Malassezia (serovars A, B and C) on pro-inflammatory cytokine production by PBMC from various donors**

The effects of one further strain of all three serovars (formalized) at late stationary phase (serovar A/strain E10; serovar B/strain E8 and serovar C/strain E2) was tested on pro-inflammatory cytokine production by PBMC from three different donors. Only one yeast cell to PBMC ratio was evaluated (20:1). The MTT assay results indicated that PBMC remained viable for the 24-h co-incubation period.

Levels of immunochemical IL-1\(\beta\) and IL-6 plus bioactive IL-1\(\beta\) and TNF-\(\alpha\) released by PBMC in response to intact, whole *Malassezia* yeast cells were generally significantly lower \((P<0.05)\) when compared with constitutive negative control values, seen with all three donors (data not presented). Levels of immunochemical IL-1\(\beta\) showed good correlation with bioactive values (Pearson’s product moment correlation coefficient \(r = 0.768\)).

**The effects of viable strains of Malassezia (serovars A, B and C) on pro-inflammatory cytokine production by PBMC from various donors**

In order to determine whether viable *Malassezia* yeast cells exhibited similar effects to formalized cells on
pro-inflammatory cytokine production, the same three strains of each serovar (E10, E8 and E2) were tested using the same three PBMC donors. Again, only one yeast cell to PBMC ratio was evaluated (20:1). Figure 4 shows the results for immunochemical IL-6 released by PBMC from all three donors, in response to viable Malassezia. Levels of IL-6 in response to viable Malassezia ranged from 0 and 330 pg ml\(^{-1}\) and were significantly lower (\(P<0.05\)) when compared with negative control values. PBMC remained viable for the 24-h co-incubation period as determined by total counts and trypan blue dye exclusion. Similar results were gained for TNF-\(\alpha\) and IL-1\(\beta\).

**Adsorption of cytokines by Malassezia (serovars A, B and C)**

Table 1 shows the results of the investigation into the possible adsorption of IL-6 by non-specific receptors of Malassezia serovars A (strain 13), B (strain 21.3) and C (strain 42.2). This experiment was carried out to confirm whether adsorption of pro-inflammatory cytokines by Malassezia was one possible explanation for the significant reduction of IL-1\(\beta\), IL-6 and TNF-\(\alpha\) in response to the yeast cells. The results clearly show that there was no significant difference between tests and controls. Similar results were obtained for TNF-\(\alpha\) determined by bioassay.

**Discussion**

Walters et al. first reported a significant reductive effect of Malassezia serovar B (M. globosa) on IL-1\(\beta\) production by PBMC in vitro [19]. However, parameters such as different yeast cell to PBMC ratios, different serovars/strains at different phases of growth, other pro-inflammatory cytokines or the use of viable yeast cells were not investigated. Therefore it was important to study this phenomenon in greater detail.

The results presented here for formalin-preserved Malassezia cells demonstrate that at a yeast cell to PBMC ratio of 1:1, exponential phase cells of serovars A and B (synonymous with M. sympodialis and M. globosa, respectively) were able to induce the production of IL-1\(\beta\), IL-6 and TNF-\(\alpha\) in vitro. Exponential phase Malassezia cells of serovar C (M. restricta) were only able to induce IL-6 and TNF-\(\alpha\) production at this ratio. However, late stationary phase Malassezia serovars A, B and C, at the same ratio, usually had no effect on pro-inflammatory cytokine production, perhaps suggestive of a biochemical difference in the Malassezia yeast cell wall/surface components between the two phases of growth. At a yeast cell to PBMC ratio of 20:1, whole, intact cells of Malassezia serovars A, B and C, harvested during midexponential or late stationary phase were able to significantly reduce the production of IL-1\(\beta\), IL-6 and TNF-\(\alpha\) by PBMC in vitro. This was sometimes apparent at a yeast cell to PBMC ratio of 10:1, and was not due to an adverse effect of the yeast cells on PBMC viability.

The effect of viable Malassezia cells on pro-inflammatory cytokine production by human mononuclear cells was also tested. Since preliminary studies using formalin-preserved cells revealed that late stationary phase cells appeared to be more immunosuppressive than exponential phase cells, viable
Malassezia were only evaluated at one growth phase (late stationary) and at one yeast cell to PBMC ratio (20:1). The results consistently revealed that viable Malassezia cells were also capable of significantly reducing levels of IL-1β IL-6 and TNF-α, thereby validating results gained with formalin-preserved cells. Extensive experiments using viable Malassezia cells were not carried out because of technical difficulties associated with the use of live microorganisms in tissue culture experiments.

The possibility that the Malassezia yeast cells adsorbed the pro-inflammatory cytokines produced by the PBMC in vitro was tested using recombinant IL-6 and TNF-α. The results showed that there was no reduction in the levels of these cytokines following incubation with formalin-preserved Malassezia yeast cells. A previous study has reported similar results for IL-1β [19].

Pro-inflammatory cytokine production in response to another dimorphic yeast, Candida albicans, by human natural killer cells [24], PBMC [25] and mononuclear phagocytes [26] has been studied previously and shown to be stimulated, but only at a yeast to cell ratio of 1:1 or below. In 1991, Jeremias et al. investigated the effect of both viable and heat-killed strains of C. albicans on TNF-α and IL-1β production by PBMC in vitro [25]. Their studies revealed production of both cytokines in proportion to the concentration of yeast cells. However, the ratios of yeast cells to PBMC evaluated were 1:1 or below, without comparison to control cultures. Interestingly, it was also reported that viable cells were found to preferentially induce IL-1β production while heat-killed cells induced significantly higher TNF-α levels than viable cells, suggesting that TNF-α and IL-1β production were stimulated by different yeast cell moieties. To our knowledge, no further in vitro experiments using C. albicans at a yeast-to-cell ratio greater than 1:1 have been reported.

The most consistent observation in this study was that both formalized and viable Malassezia yeast cells, irrespective of serovar, strain or PBMC donor, at a yeast cell to PBMC ratio of 20:1, significantly decreased the in vitro production of IL-1β, IL-6 and TNF-α by PBMC, compared to unstimulated negative control values. It is highly unlikely that extracellular enzymes/toxins or secondary fungal metabolites such as azelaic acid [27] were responsible for the observed effect due to the fact that both formalin-preserved and viable Malassezia had the same reductive effect on pro-inflammatory cytokine production. A recent investigation revealed that purified capsular polysaccharide (glucuronoxylomannan [GXM]) of the opportunistic yeast Cryptococcus neoformans suppressed TNF-α release by LPS-treated monocytes, in a dose-dependent manner [28]. In addition, GXM was shown to inhibit IL-1β production by monocytes treated with a stimulatory capsular variant of C. neoformans. It was suggested that reduction of pro-inflammatory cytokine release in response to C. neoformans could contribute to the in vivo progression of cryptococcosis.

It is logical to postulate that cell wall/surface components of Malassezia play an important role in the reduction of pro-inflammatory cytokine release by human mononuclear cells in vitro. Unfortunately, little is known about the cell wall/surface composition of this microorganism. Malassezia yeast cell walls contain an unusually high lipid content (≈15–20%) [29], which has been implicated in the high osmotolerance of the microorganism [30]. This is very different from the cell wall lipid content of other yeasts such as C. albicans (1–2%) [31] and Saccharomyces cerevisiae (1–2%), respectively [32]. A recent ultrastructural investigation identified an outer lamellar layer, unique to Malassezia, which seems to be equivalent to a capsule, thought to contain lipids and believed to participate in the attachment of Malassezia yeast cells to corneocytes in pityriasis versicolor [30]. Whether this has any bearing on the Malassezia–PBMC interaction also remains to be determined.

During infection of the skin, pro-inflammatory cytokines are believed to play a critical role in the onset of inflammation [33]. It would be reasonable to speculate that if Malassezia behave in their in vivo microenvironment as they do in vitro, high numbers of Malassezia yeast cells, even if released into the dermal compartment, would give rise to minimal inflammation, a key clinical feature of pityriasis versicolor. Histological sections of lesional skin in pityriasis versicolor have revealed that the cellular infiltrate is minimal when compared to the fungal load [34] and Malassezia are located in all layers of the stratum corneum. The capacity of Malassezia to reduce pro-inflammatory cytokine production by PBMC is therefore consistent with the pathology. It is possible that in individuals with Malassezia-associated inflammatory dermatoses, such as seborrhoeic dermatitis, the physiology of the normal commensal yeast is altered, perhaps due to changes in the availability of nutrients on the skin surface, rendering the yeast abnormally capable of inducing an inflammatory response.

References


