ORIGINAL PAPER

# Cytokine Levels in the *In Vitro* Response of T Cells to Planktonic and Biofilm *Corynebacterium amycolatum*

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### Abstract

Unravelling of the interplay between the immune system and non-diphtheria corynebacteria would contribute to understanding their increasing role as medically important microorganisms. We aimed at the analysis of pro- (TNF, IL-1β, IL-6, IL-8, and IL-12p70) and anti-inflammatory (IL-10) cytokines produced by Jurkat T cells in response to planktonic and biofilm *Corynebacterium amycolatum*. Two reference strains: *C. amycolatum* ATCC 700207 (R-CA), *Staphylococcus aureus* ATCC 25923 (R-SA), and ten clinical strains of *C. amycolatum* (C-CA) were used in the study. Jurkat T cells were stimulated *in vitro* by the planktonic-conditioned medium (PCM) and biofilm-conditioned medium (BCM) derived from the relevant cultures of the strains tested. The cytokine concentrations were determined in the cell culture supernatants using the flow cytometry. The levels of the cytokines analyzed were lower after stimulation with the BCM when compared to the PCM derived from the cultures of C-CA; statistical significance ($p < 0.05$) was observed for IL-1β, IL-12 p70, and IL-10. Similarly, planktonic R-CA and R-SA stimulated a higher cytokine production than their biofilm counterparts. The highest levels of pro-inflammatory IL-8, IL-1β, and IL-12p70 were observed after stimulation with planktonic R-SA whereas the strongest stimulation of anti-inflammatory IL-10 was noted for the BCM derived from the mixed culture of both reference species. Our results are indicative of weaker immunostimulatory properties of the biofilm *C. amycolatum* compared to its planktonic form. It may play a role in the persistence of biofilm-related infections. The extent of the cytokine response can be dependent on the inherent virulence of the infecting microorganism.

K e y   w o r d s: *Corynebacterium amycolatum*, biofilm, planktonic cells, cytokines, Jurkat T cells

## Introduction

Within recent years there has been a considerable increase in the number of literature data reporting non-diphtherial corynebacteria (also known as diphtheroids) as the causative agents of opportunistic and nosocomial infections in humans (De Miguel-Martinez et al. 1996; Yoon et al. 2011; Bernard 2012b; Nhan et al. 2012; Qin et al. 2017; Santos et al. 2017; Kang et al. 2018). These bacteria are members of the microbiota of the skin and mucous membranes (Qin et al. 2017; Santos et al. 2017; Kang et al. 2018). The potential association between coryneforms and clinical infections can be attributed to immunosuppression, severe underlying medical disorders or invasive procedures (Nhan et al. 2012; Cacopardo et al. 2013; Kimura et al. 2017; Qin et al. 2017). In order to shed more light on the role

of diptheroids as medically relevant microorganisms, their inherent low virulence should be confronted to the increasingly reported multidrug resistance (Yoon et al. 2011; Bernard 2012b; Kimura et al. 2017; Qin et al. 2017), and the ability to adhere to biotic and abiotic surfaces and/or to form biofilms (Kwaszewska et al. 2006; Souza et al. 2015a; Souza et al. 2015b; Qin et al. 2017; Kang et al. 2018).

The biofilm-producing bacteria are protected both against antibiotics (Fux et al. 2005; Lebeaux et al. 2014), and the host innate and adaptive immune responses (Souza et al. 2015b). Biofilm infections are associated with simultaneous activation of both arms of the host immune response. Neither of them, however, can eliminate the biofilm pathogen, but instead, in synergy, causes collateral surrounding tissue damage due to the release of phagocytic enzymes, oxidative radicals

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and/or due to formation of immune complexes (antigen-antibody) (Costerton et al. 1999; Jensen et al. 2010). Moreover, during a biofilm-related infection, planktonic bacteria released from the biofilm can spread into the bloodstream or in the vicinity the source of the infection. These strategies favor microbial persistence and chronicization of the infectious process which significantly complicates the therapy of biofilm-associated infections (Costerton et al. 1999; Lebeaux et al. 2014).

Although studies focused on the investigation and comparison of immune mechanisms stimulated in response to planktonic and biofilm types of bacterial growth have already been undertaken for medically important bacteria such as *Staphylococcus aureus* (Leid et al. 2002; Secor et al. 2011; Brady et al. 2018), *Enterococcus faecalis* (Mathew et al. 2010; Daw et al. 2012; Jarzembowski et al. 2018) or *Pseudomonas aeruginosa* (Jensen et al. 2010), little is known about mechanisms involved in the stimulation of immune responses by opportunistic *Corynebacterium* spp. Another issue worth a thorough investigation is an interplay between the immune system and polymicrobial biofilms what have been increasingly reported in chronic infections such as diabetic foot ulcers or surgical site infections (Dowd et al. 2008; Wolcott et al. 2009).

Hence, the aim of the study was the investigation of the impact of *Corynebacterium amycolatum* soluble products of the planktonic-conditioned medium (PCM) and biofilm-conditioned medium (BCM) on human Jurkat T cells. The production of cytokines demonstrating pro-inflammatory (TNF, IL-1β, IL-6, IL-8, IL-12p70) and anti-inflammatory properties (IL-10) was analyzed. The research was conducted with the use of clinical strains of *C. amycolatum*. Additionally, two reference strains represented by *C. amycolatum* ATCC 700207 and *S. aureus* ATCC 25923 were used to analyze the potential immunostimulatory effect of their mono- and mixed cultures.

## Experimental

### Materials and Methods

**Bacterial strains.** Two reference strains: *C. amycolatum* ATCC 700207, *S. aureus* ATCC 25923, and ten clinical strains of *C. amycolatum* isolated from patients with bacteremia were used in the study. The preliminary characterization of the isolated clinical strains to the species level was based on the analysis of their biochemical profile (APICoryne; bioMérieux, France). The phenotypic identification was followed by the analysis of the sequences of the specific fragment of the 16S rRNA gene (Genomed, Poland) (Drancourt et al. 2000; Bernard et al. 2002a; Fernández-Natal et al. 2008).

**Cell culture.** Human T lymphocytes cell line (Jurkat, Clone E6-1 ATCC®TIB-152) was used in the study. Cells were cultured in RPMI 1640 culture medium (Sigma Aldrich, USA) containing 10% heat-inactivated fetal calf serum (FCS) (Merck, USA), supplemented with 1% solution of antibiotics and antimycotic (Antibiotic, Solution Stabilized, Sigma Aldrich, USA). All cultures were kept in a humidified 5% $CO_2$ incubator at 37°C.

**Planktonic and biofilm *C. amycolatum* culture and preparation of the planktonic-conditioned media (PCM) and the biofilm-conditioned media (BCM).** The strains were initially assessed for the biofilm production using the assay with crystal violet that was carried out as previously described (Alves et al. 2016) with some modifications. The assay was performed in the 96-well microtiter plates (NUNC, Thermo Fisher Scientific Inc, Denmark). Overnight cultures of bacterial isolates (ca. McFarland 4 turbidity) were diluted 1:100 in RPMI 1640 medium containing 10% FCS. Aliquots (200 μl) of each diluted bacterial culture were inoculated into five consecutive wells of the microtiter plate. The RPMI 1640 broth (200 μl) was used as a negative control. Biofilms were grown statically for 24, 48, 72, and 96 h at 37°C in aerobic conditions. Following incubation, the wells were carefully washed twice with 0.9% NaCl, and dried for 1 h at 50°C. Biofilms in the wells were stained with 0.1% crystal violet (CV; 200 μl) for 15 min to determine total biofilm biomass. After staining, the wells were washed by flushing the plate three times with 200 ml of distilled water to remove the unbound CV and air-dried. The biofilm-bound dye was extracted with 200 μl of 70% (v/v) ethanol. The optical density (OD) was determined at 570 nm using the microplate reader. Based on the mean OD values for each strain at the time points mentioned above, the optimal biofilm incubation period was determined for the harvesting of BCM for the stimulation of the Jurkat T cells.

After confirmation of the ability to produce biofilm of a given strain, its planktonic and biofilm culture was performed in order to obtain PCMs and BCMs used subsequently to stimulate the Jurkat T cell lines. This was done for each of ten *C. amycolatum* strains.

Planktonic and biofilm cultures of the reference strains: *C. amycolatum* ATCC 700207, and *S. aureus* ATCC 25923 were also performed in monoculture and the mixed cultures. The "mixed cultures" were achieved in the proportion of 1:1 of two bacterial species when their suspensions of the 0.5 McFarland density ($1.5 \times 10^8$ CFU/ml) were mixed and grown together. It provided the same number of bacterial cells (of both species) in the final volume at the beginning of the mixed culture growth.

Planktonic cultures of the strains tested were grown in glass tubes (with the inoculum of McFarland 0.5 turbidity, approximately corresponding to $1.5 \times 10^8$ CFU/ml)

in RPMI 1640 medium supplemented with 10% FCS, and gently agitated for 48 hours at 37°C. Biofilms were grown from the bacterial inoculum (McFarland 0.5 turbidity) of each strain in RPMI 1640 medium supplemented with 10% FCS in 24-well plates (volume 1000 μl, Nunc, USA) for 48 hours at 37°C. Both types of bacterial cultures resulted in the different number of bacterial cells following the incubation. For *C. amycolatum*, the planktonic and biofilm cultures resulted in the cell density of approximately $9.0 \times 10^8$ CFU/ml (McFarland 3.0 turbidity) and $1.2 \times 10^9$ CFU/ml (McFarland 4.0 turbidity), respectively. For *S. aureus*, the planktonic and biofilm cultures resulted in the cell density of approximately $6 \times 10^8$ CFU/ml (McFarland 2.0 turbidity) and $9 \times 10^8$ CFU/ml (McFarland 3.0 turbidity), respectively. For the mixture of *S. aureus* and *C. amycolatum*, the planktonic (PCMmix) and biofilm (BCMmix) cultures resulted in the cell density of approximately $6 \times 10^8$ CFU/ml (McFarland 2.0 turbidity) and $9 \times 10^8$ CFU/ml (McFarland 3.0 turbidity), respectively. For each of ten clinical *C. amycolatum* strains, and the reference *C. amycolatum* ATCC 700207 and *S. aureus* ATCC 25923 strains, the supernatants were preserved after 48-hour culture of the planktonic and biofilm bacteria. The supernatants were filtered with the use of the MILIPORE filters (the pore size: 0.22 μm) (Merck, USA). The supernatants were prepared directly before their inoculation into to the human cell culture (1:10 dilution, a final volume of the cells culture medium – 1000 μl).

The whole protein content was measured in the supernatants of all bacterial strains investigated before inoculation of the medium for growth of Jurkat T cells. For this purpose, the absorbance was measured in the BioPhotometer (Eppendorf BioPhotometer, Germany) at a wavelength of 280 nm. The absorbance values ranged from 0.018 to 0.720 (planktonic cultures) and from 0.022 to 0.776 (biofilm cultures). The protein content expressed by the optical density (OD) was higher in the biofilm culture of each investigated isolate when compared to its planktonic culture. These supernatants were subsequently used to stimulate cytokine production in Jurkat T cells.

***In vitro* stimulation of Jurkat T cells with PCM, BCM, PCMmix, BCMmix.** For the experiments, Jurkat T cells (at the density of $1 \times 10^5$ cells/well) were seeded independently three times in the wells of 24-well culture plates (Nunc, USA) and incubated with the bacterial conditioned media (PCM, BCM, PCMmix or BCMmix) for 24 h at 37°C and 5% $CO_2$ at a ratio of 1:10 (total volume – 1000 μl). After incubation, the supernatants of the cell culture were collected and analyzed for cytokine concentrations.

**Detection of cytokines by flow cytometry.** The level of cytokines in the supernatants of Jurkat T cell culture after their exposure to the PCM, BCM, PCM-mix, and BCMmix was determined using the BD Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit (Becton Dickinson, USA), according to the manufacturer's protocol. The method was based on the cytokine binding to the specific antibody (conjugated to phycoerythrin). The resulting complex emitted a fluorescent signal in the proportion to the concentration of the cytokine that was recorded with the flow cytometer. The fluorescence intensity allowed distinguishing between different cytokines (TNF, IL-1β, IL-6, IL-8, IL-12p70, and IL-10) and the mean fluorescence intensity represented the concentration of cytokines. The results were acquired and analyzed on a FACSCalibur (BD Bioscience, USA) flow cytometer using the Cellquest software (BD Bioscience, USA).

**Statistical analysis.** Statistical analysis was conducted with the use of Statistica 9.0PL software (Statsoft, Poland). Distribution of variables was checked with the Kolmogorov-Smirnow test with Lilliefors correction and with W Shapiro-Wilk test. Due to the non-Gaussian distribution of variables we used nonparametric tests for further analyses. The assessment of differences between groups was done with the Kruskal-Wallis ANOVA test and paired posthoc test.

Differences were considered statistically significant with $p < 0.05$. Results are shown as median value (Me), mean value (x), minimal (min) and maximal values (max).

All experiments were performed in triplicate in three independent experiments.

## Results

The range of OD values for *C. amycolatum* strains tested for the ability to produce biofilm were as follows: at 24 h – from 0.089 to 0.101 (mean: 0.097), at 48 h – from 0.113 to 0.385 (mean: 0.201), at 72 h from 0.101 to 0.437 (mean: 0.142), at 96 h from 0.088 to 0.199 (mean: 0.139). Since the highest mean OD was recorded at the 48 h-time point, this period of incubation was used in further research.

Stimulation of the Jurkat T cells line with ten clinical strains of *C. amycolatum* revealed significant differences in the effects exerted by to the PCM and BCM derived from the planktonic and biofilm cultures, respectively.

The level of two cytokines, namely IL-1β and IL-12p70, produced by Jurkat T cells was significantly ($p = 0.0027$ and $p = 0.0222$, respectively) lower after stimulation with the BCM when compared to the PCM (Fig. 1A, Fig. 1B). The level of the remaining pro-inflammatory cytokines, TNF, IL-6, and IL-8, was also lower but the differences did not achieve statistical significance (. 1C-E).
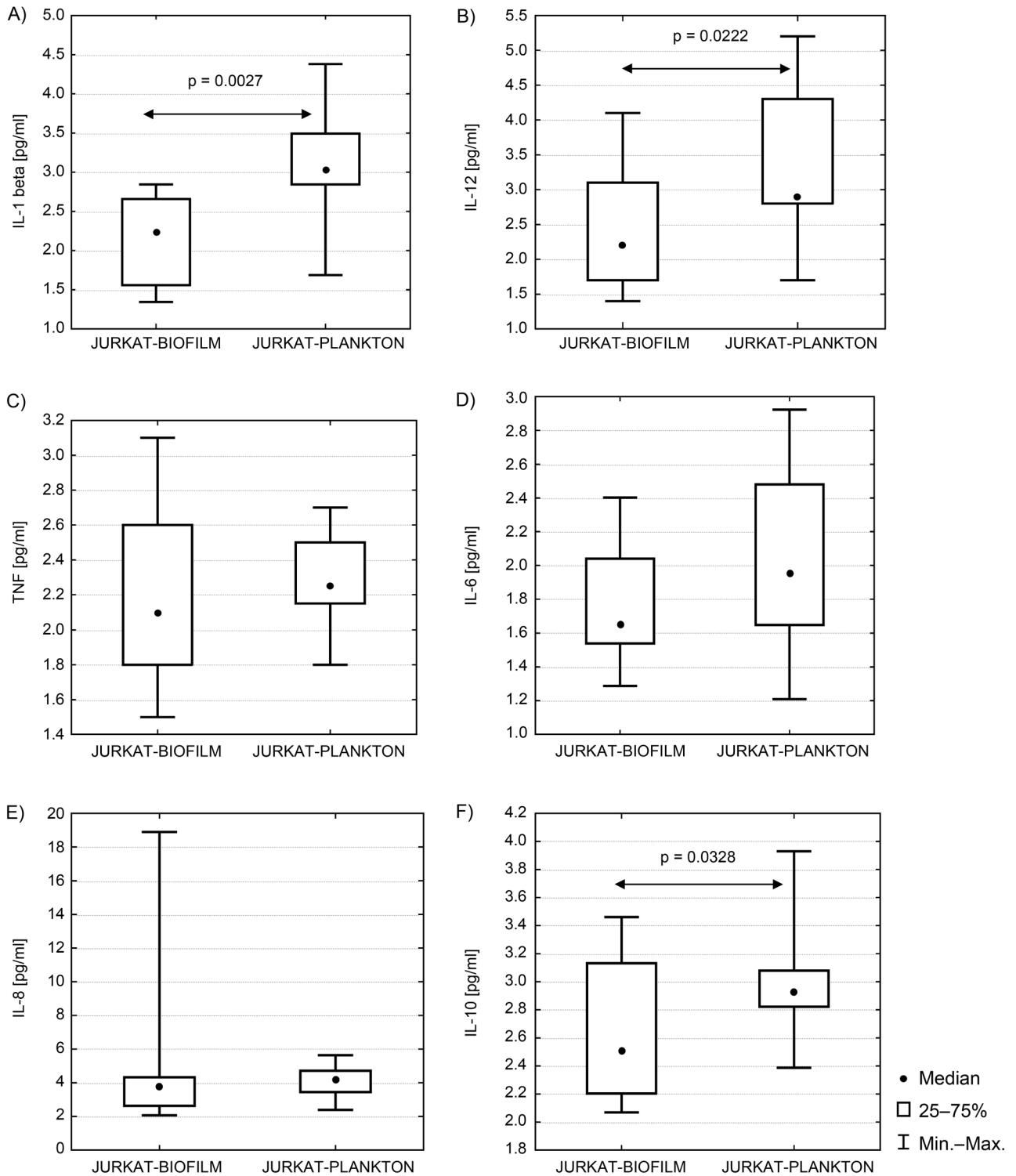
Fig. 1. Concentrations of cytokines produced by Jurkat T cells in response to the to the PCM and BCM of planktonic and biofilm cultures of clinical *C. amycolatum* strains.

We observed a statistically significant ($p=0.0328$) lower concentration of anti-inflammatory IL-10 produced by Jurkat T cells in response to the supernatant of the biofilm culture of *C. amycolatum* when compared to the planktonic culture (Fig. 1F).

A similar tendency was observed when the planktonic and biofilm types of growth of *C. amycolatum*

ATCC 700207 and *S. aureus* ATCC 25923 monocultures were compared. Namely, the supernatants of planktonic forms of both reference strains caused a greater stimulation of the cytokine production including the anti-inflammatory IL-10 than their biofilm counterparts. The only exception was the similar level of IL-6 observed after stimulation with the
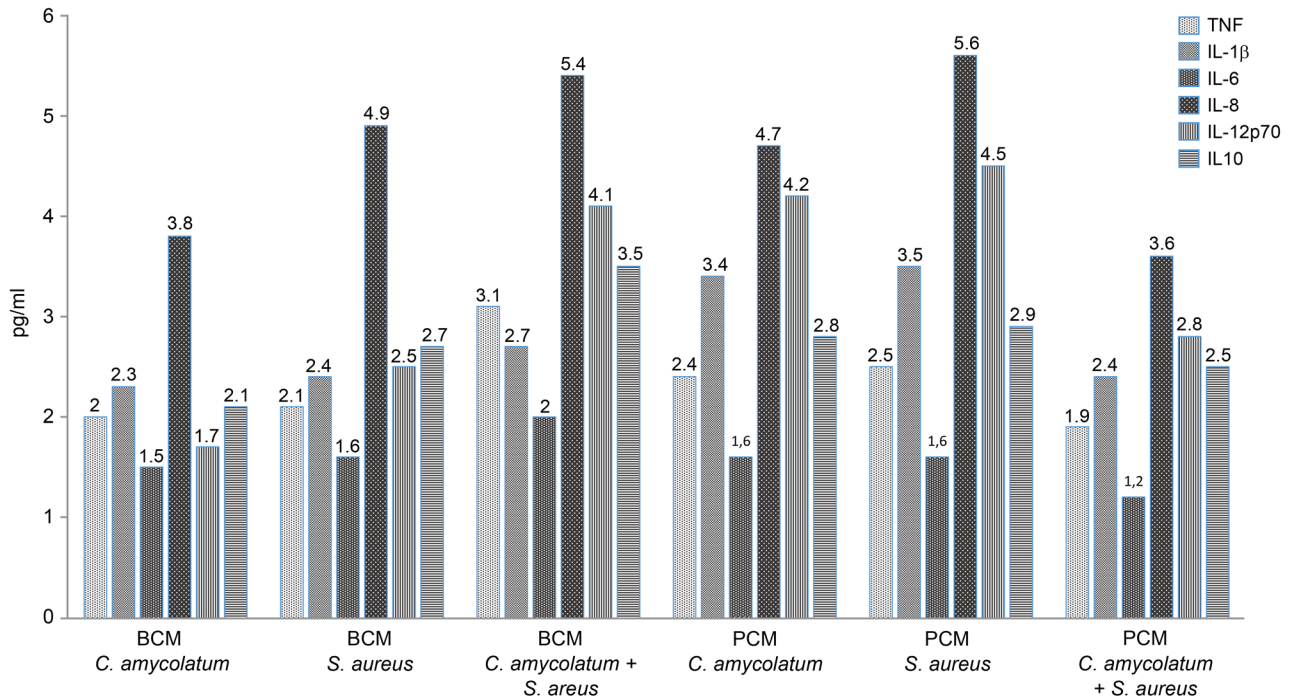
Fig. 2. Levels of cytokines (pg/ml) produced by Jurkat T cells in response to the PCM and BCM derived from mono- and mixed cultures of the reference *C. amycolatum* and *S. aureus* strains.

PCM and BCM derived from the culture of *S. aureus* ATCC 25923 (Fig. 2).

Stimulation of Jurkat T cells by the PCM and BCM from the mono- and mixed cultures of *C. amycolatum* ATCC 700207 and *S. aureus* ATCC 25923 strains revealed that the BCM derived from the mixed culture of the two species (BCMmix) stimulated the production of pro-inflammatory IL-6, TNF, and anti-inflammatory IL-10 to the highest levels (Fig. 2). When the two reference species were considered separately, it was observed that the supernatant of planktonic and biofilm *S. aureus* ATCC 25923 cultures had a stronger stimulatory effect than the monoculture of *C. amycolatum* ATCC 700207, with the greatest increase in the production of pro-inflammatory IL-8, IL-12 p70, and IL-1β being observed for planktonic *S. aureus* (Fig. 2).

## Discussion

Many potentially pathogenic bacteria grow in a planktonic state that can be attributed to acute infections and in biofilms that are associated with chronic infections. Bacteria growing within the biofilm communities secrete an extracellular matrix, form complex structures, demonstrate diverse metabolic activity and are phenotypically distinct from their planktonic counterparts. Therefore, it can be expected that the host immune response may contribute to different outcomes associated with these two disease types

(Secor et al. 2011; Brady et al. 2018). To the best of our knowledge, the immune response manifested by the cytokine release after stimulation of immune cells with the planktonic and biofilm diphtheroids has not been studied yet. In our study, the planktonic and biofilm cultures of *C. amycolatum* were conducted under conditions analogous to those used for the stimulation of Jurkat T cells. The same composition of the medium used for the planktonic and biofilm cultures and the stimulation of Jurkat T cells minimized the risk of an additional, non-specific action of the bacterial medium on the culture of eukaryotic cells.

The assessment of Jurkat T cell inflammatory responses in our study revealed a statistically significant ($p < 0.05$) increase in the levels of pro-inflammatory cytokines such as IL-1β and IL-12 after stimulation with the PCM compared to the BCM.

These results are in line with the previously published results regarding other potentially pathogenic bacteria. Secor et al. (2011) revealed that *S. aureus* biofilm and planktonic-conditioned medium induced distinct responses in human keratinocytes *in vitro*. The authors observed that in spite of higher production of cytokines induced by the BCM after four hours of exposure, the BCM induced cytokine levels were lower when compared to the cytokine production induced by the PCM after 24 hours. After 24 hours of exposure, the supernatant of *S. aureus* biofilm induced sustained low level of cytokine production when compared to the near exponential increases of cytokines in keratinocytes

treated with the supernatant of planktonic culture. Daw et al. (2012) observed that macrophages infected with the biofilm cells of *Enterococcus faecalis* secreted lower levels of pro-inflammatory (IL-6, MCP-1, and TNF-α) cytokines. According to Mathew et al. (2010), the biofilm cells of *Enterococcus faecalis* in contact with macrophages showed higher potential for surface adherence, the intracellular survival, and produced IL-6 and TNF-α in lower concentrations when compared to planktonic cells.

An unexpected result obtained in the present study was the level of anti-inflammatory IL-10, which was significantly ($p < 0.05$) decreased after stimulation with the BCM compared to the PCM derived from *C. amycolatum* culture. As mentioned previously, there is a relative decrease in inflammation intensity in patients with the biofilm infections possibly contributing to the persistence of these infections. Hence, biofilm infections are rather associated with an increase in IL-10, which has been reported to play a role in shaping the inflammatory milieu typical of the biofilm infection (Heim et al. 2015; Gutierrez-Murgas et al. 2016).

On the other hand, according to what has been published by Saraiva and O'Garra (2010), induction of IL-10 often occurs together with pro-inflammatory cytokines, although pathways that induce IL-10 may negatively regulate these pro-inflammatory cytokines. Gutierrez-Murgas et al. (2016) reported a murine model of a catheter-associated *Staphylococcus epidermidis* biofilm infection in the central nervous system. Cytokine analysis of the tissue surrounding the catheters revealed higher levels of IL-10 in the infected group compared to the healthy mice. On the other hand, the authors observed increased levels of pro-inflammatory cytokines including IL-1β, IL-6, CXCL2, and CXCL-1 in the homogenates of adjacent catheter-associated tissue and reported the lack of pro-inflammatory IL-12p70; thus, highlighting the role of anti-inflammatory pathways involved in response to *S. epidermidis* catheter infection. We observed that *C. amycolatum* biofilm led to the general weaker stimulation of the cytokine responses including those with pro- and anti-inflammatory activities when compared to the planktonic cells. Our results can be indicative of a weak immune stimulation by the diphtheroid species used in the study. It can result from an inherent low-virulence of this microorganism and we cannot exclude that the cytokine response can be pathogen-specific.

Another part of this study was to investigate the immunomodulatory effect of *C. amycolatum* co-cultured with *S. aureus*. According to what has been already published (Dowd et al. 2008), coaggregation of bacteria together into a functional equivalent pathogroups provides the functional equivalence of well-known pathogens, such as *Staphylococcus aureus*, giving the biofilm community the factors necessary to maintain chronic biofilm infections.

Stimulation of the Jurkat T cell line by the PCM and BCM derived from the mono- and mixed cultures of the reference *C. amycolatum* ATCC 700207 and *S. aureus* ATCC 25923 strains revealed that the BCM derived from the mixed culture of the two species induced the highest levels of both anti-inflammatory IL-10, and pro-inflammatory IL-6 and TNF. It may support the above-mentioned hypothesis on the greater ability of co-aggregated microorganisms to maintain chronic infections what was reflected here by a stronger immunomodulatory effect. When the two reference species were considered separately, it was observed that *S. aureus* had a stronger stimulatory effect on the immune cells than *C. amycolatum* with the greatest increase in the production of pro-inflammatory IL-8, IL-12 p70, and IL-1β observed for planktonic *S. aureus*. This may indicate that the cytokine production can be pathogen specific. On the other hand, co-existence of different bacterial species in the biofilm community can be considered an important strategy involved in the interaction with the host and facilitating the microbial persistence.

It should also be noted that the interpretation of the results obtained is burdened with some limitations. The conditions used in *in vitro* experiments and the use of selected immune cells do not reflect all pathologic processes occurring *in vivo*, and the complex interplay between other cells of the immune system and their cytokine/chemokine products secreted in response to infection. As an example, a recently discovered population of myeloid-derived suppressor cells (MDSCs) can be given. These cells have been reported to be the main source of IL-10 during *S. aureus* orthopedic implant biofilm infection. MDSCs negatively regulate inflammatory mechanisms through their suppressive action, production of IL-10, an ability to limit the monocyte/macrophage recruitment in chronic inflammation, tumors or bacterial biofilm infections (Heim et al. 2015).

Hence, the unravelling of the interplay between the immune system and the coryneform bacteria as well as other bacteria that occurs within the biofilm communities requires further studies, which would shed more light on the increasing role of these medically important microorganisms. The results presented here can be considered as a starting point in this investigation.

**Conflict of interest**

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