Biofilm of *Klebsiella Pneumoniae* Minimize Phagocytosis and Cytokine Expression by Macrophage Cell Line

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Abstract

Infectious bacteria in biofilm mode is involved in many persistent infections. Owing to its importance in clinical settings, many *in vitro* and *in vivo* studies are being conducted to study the structural and functional properties of biofilms, their drug resistant mechanism and survival mechanism of planktonic and biofilm cells. In this regard, there is not sufficient information on the interaction between *Klebsiella* biofilm and macrophages. In this study, we have made an attempt to unravel the interaction between *Klebsiella* biofilm and macrophages in terms of phagocytic response and cytokine expression. *In vitro* phagocytosis assays was performed for heat inactivated and live biofilms of *K. pneumoniae*, together with the expression analysis of TLR2, iNOS, inflammatory cytokines such as IL- β 1, IFN- γ , IL-6, IL-12, IL-4, TNF- α and anti-inflammatory cytokines, IL-10. A phagocytic rate of an average of 15% was observed against both heat inactivated and live biofilms, when LPS+IFN- γ activated macrophages were used. This was significantly higher than non-activated macrophages when tested against heat inactivated and live biofilms (average 8%). Heat-inactivated and live biofilms induced similar phagocytic response and up-regulation of pro-inflammatory genes in macrophages, indirectly conveying that macrophage response is to some extent dependent on the biofilm matrix.

1. Introduction

K. pneumoniae is a Gram-negative, encapsulated opportunistic pathogen that colonizes almost every part of the human body with the most preferred sites being the respiratory, gastrointestinal and urinary tracts (Moore et al. 2002). *K. pneumoniae* causes both hospital and community-acquired infections (Chung 2016). Pneumonia, meningitis, urinary tract infections and catheter-related bloodstream infections are some of the potential illness caused by this bacterium (Szita 2013). The major risk factors associated with *K. pneumoniae* infection include central venous catheterization, urinary catheterization, mechanical ventilation, prolonged stay in an intensive-care unit, low birth weight in preterm infants and individuals with impaired immunity (Aiola et al. 2012).

Klebsiella spp are characterized by the presence of capsular polysaccharides (CPS), type 1 and 3 fimbriae that act as major virulence factors and contribute in the adhesion and colonization of host tissues. Also, these virulence factors are also essential for biofilm formation on indwelling medical devices (Chung 2016).

To overcome the infections caused by planktonic and biofilm forms of *K. pneumoniae*, both humoral and cell-mediated immune defenses are involved (Paczosa and Mecsas 2016). The host immune responses to planktonic *K. pneumoniae* have been extensively investigated (Blanchette and Orihuela 2012; Thurlow et al. 2011a), however, information related to host immune responses to *K. pneumoniae* biofilms remain largely unexplored. The role of immune defenses and the immune evasion mechanism by *Klebsiella* sp have been studied using both *in vitro* and *in vivo* models (Lawlor et al. 2006). The first line defense mechanism includes ciliary lining, the flow of urine, peristalsis, mucus, bile and digestive enzymes (Kaper et al. 2004). Once it overcomes these mechanical barriers, humoral and cellular innate defenses function

to eliminate the pathogen. Humoral defenses consist of, i). the complement system, which forms membrane attack complex and causes cell lysis, in addition to their role as an opsonin, ii). defensins, which are bactericidal and disrupt the cell membrane, and iii). transferrin that sequester iron, which facilitates bacterial growth. However, studies have shown that most *Klebsiella* strains appear to evade or resist the complement-mediated membrane attack formation and opsonophagocytosis under both *in vitro* and *in vivo* conditions (Paczosa and Mecsas 2016).

Besides, there have also been studies to show the importance of IL-8, CXCL1, and leukotriene B4 against infection (Paczosa and Mecsas 2016; Xiong et al. 2015). Similarly, studies on the role of TLR during *Klebsiella* infection have shown that mice defective in TLR4 signaling showed increased bacterial load and consequent mortality, suggesting the importance of bacterial recognition in immune activation (Thurlow et al. 2011a). Upon bacterial recognition by pattern recognition receptors such as TLR4, there is an initiation of pulmonary innate immunity against *Klebsiella* by the release of cytokines such as TNF- α . Another important cytokine required for defense against *K. pneumoniae* is IL-12, which in turn is essential for the induction of IFN- γ . IFN- γ is a critical mediator in *Klebsiella* clearance and prevention of dissemination from the lungs and improved survival rates in case of localized pulmonary infection (Blanchette and Orihuela 2012). On the contrary, the detrimental role of anti-inflammatory IL-10 in persistent *K. pneumoniae* infection has also been well proved in studies (Yoshida et al. 2001). Most of these studies have looked at planktonic cells and the consequence of *K. pneumoniae* biofilm interaction with immune cells is not clear. The establishment of biofilm on the tissues of the susceptible host facilitates the expression of virulent traits and causes infections of the upper and lower respiratory tract, otitis media, tonsillitis, cystic fibrosis, urinary tract infections, and chronic bacterial prostatitis (Mahmood and Mahmood 2015). Several studies in the last decade had proven the increased antibiotic resistance of *Klebsiella* in the biofilm state when compared to planktonic cells (Anderl et al. 2000; Wei et al. 2018). Biofilms have also been hypothesized to contribute to reduced bacterial clearance by the innate defense mechanisms of the host. This includes limited penetration of leucocytes and their antimicrobial products into the biofilm, reduced phagocytic activity, and modified gene expression patterns and genetic shifts. Previously, our lab has explored the macrophage response toward *K. pneumoniae* planktonic cells. (Lalitha et al. 2017). Further, as a first report, we have attempted to analyse the interactions between macrophage cells and *K. pneumoniae* biofilms *in vitro* in terms of phagocytic activity, TLR, iNOS, proinflammatory and anti-inflammatory cytokine expression during phagocytosis in order to understand the consequence of such interactions for persistent *in vivo* infections. This is based on our hypothesis that biofilms of *K. pneumoniae* can modulate macrophage responses during infection.

2. Materials And Methods

2.1 Strain

The studied strain *Klebsiella pneumoniae* NDM-05-506 was procured from Microbial Culture Collection Centre, Pune, India. The strain was found to have metallo- β -lactamase, that hydrolyse almost all β -lactam

antibiotics (Yong et al. 2009). The strain was further proven to be a proficient biofilm producer by our lab (Lalitha et al. 2017).

2.2 Cell lines

Raw264.7 macrophage cell line was procured from National Centre for Cell Science, Pune, India. Cells were maintained in DMEM with 10% FBS and 1X antibiotics in 5% CO₂ with 95% moisture at 37°C.

2.3 Preparation of biofilm for interaction study

To study the interactions of macrophage and *Klebsiella* biofilm, the bacteria were cultured overnight in nutrient broth at 37°C. The culture was then centrifuged at 5,000 X g for 10 min. The cell pellets were washed with PBS and re-suspended in nutrient broth. The bacterial cells were plated at a density of 10⁶ cells/mL on acid washed coverslips (22 × 22 mm²), placed in 6 well plates containing nutrient broth and incubated for 72 h at 37°C to form a biofilm. After incubation, the non-adherent cells were removed by washing thrice with sterile 1X PBS. The biofilm formed on coverslip was then transferred to 6 well plates. For phagocytosis assay, both live and heat inactivated biofilms were used. The heat inactivation was used to analyse the role of biofilm exopolysaccharide (EPS) alone in macrophage activation and immune response against *Klebsiella* biofilm. Rather than extracting the EPS, the heat method was chosen to inactivate the bacterial cells enclosed in the EPS matrix without disrupting the matrix. So where the biofilm was heat treated, they will be henceforth called as heat inactivated biofilm and biofilm not given any heat treatment will be henceforth called as live biofilm.

For heat inactivation, two different conditions were used: heat treatment at 56°C for 30 min in a water bath and 56°C for 1 h using a bacteriological incubator. After heat treatment, both live and heat inactivated biofilms were estimated by Crystal violet assay (CV)

and also microscopic assessment was made using biofilms stained with ConA-FITC (30 µg/mL) and propidium iodide (1 µg/mL).

2.4 Phagocytosis assay

To study the phagocytic response of macrophage towards the biofilm, Raw264.7 macrophage cell line was used. Cells were harvested and placed in T25 flask in DMEM media with 10% FBS for 4 days in CO₂ incubator with 95% humidity to get confluency. The cells were then trypsinized with trypsin-EDTA solution from T25 flask. Trypsinized macrophage cells were neutralized and stored in DMEM media with 10% FBS without antibiotics.

The cells were plated at a density of 10⁵ cells/mL with complete DMEM medium without any antibiotics and then added to the biofilm that was formed and stained with FITC on a coverslip in 6 well plate (as mentioned in the above section). The interaction study at a ratio of 1:10 (macrophage:bacteria) was performed for both heat inactivated and live biofilm by including the following groups: i). Non-activated macrophages added to live biofilm, ii). non-activated macrophages added to heat inactivated biofilm for 6 h [to know the interactions between non-activated macrophages and biofilm alone], iii). LPS+IFN-γ.

activated macrophages added to live biofilm, and iv). LPS+IFN- γ activated macrophages added to heat inactivated biofilm.

The macrophages were activated by 3 $\mu\text{g/mL}$ LPS (*Pseudomonas aeruginosa* originated, L9143, Sigma) and 100 pmol IFN- γ for 6 h. [In our previous study with planktonic cells, 2 h incubation was used to activate macrophages by LPS+IFN- γ . Since we presumed that biofilm interactions need more time, we extended the time to 6 h (Lalitha et al. 2017)].

After incubation, the coverslips were washed with 1X PBS and treated with 0.005% trypan blue to quench ConA-FITC from biofilm cells (Lowe et al. 2013) that were not engulfed by macrophages. After washing, macrophage cells were stained with Giemsa stain for 15 min and then observed under a fluorescent microscope using a green filter for FITC stained biofilm, while stained macrophage was observed under bright field. The phagocytosis rate was calculated as follows:

Phagocytosis rate (%) = $\frac{\text{Macrophage with an internalised biofilm} - \text{Macrophages without internalized biofilm}}{\text{Total number of macrophages}} \times 100$

2.5 TLR2, iNOS and cytokine expression

To analyse the expression pattern of TLR2, iNOS and cytokines in Raw 264.7 macrophages during phagocytosis of heat inactivated and live biofilm, the following experimental groups were included.

Group 1: Non-activated macrophages alone, Group 2: LPS+IFN- γ activated macrophages, Group 3: Non-activated macrophages presented to live biofilm, Group 4: LPS+IFN- γ activated macrophages presented to live biofilm, Group 5: Non-activated macrophages presented to heat inactivated biofilm, Group 6: LPS+IFN- γ activated macrophages presented to heat inactivated biofilm.

After six hours, the cells were washed with DEPC treated 1X PBS. Total RNA was then extracted from macrophages using the TRIzol method and converted into cDNA by PrimeScript™ RT-PCR Kit (Takara). The following genes were amplified, TLR2, iNOS, proinflammatory cytokines such as IL- β 1, IFN- γ , IL-6, IL-12, IL-4 and TNF- α , anti-inflammatory cytokines IL-10 and GAPDH. These genes were amplified by initial denaturation at 95°C for 5 min followed by 40 cycles at 95°C for 30 seconds. The different primers, annealing conditions (30 seconds) are mentioned in table 1 and the final extension was at 72°C for 30 seconds. The primer pairs were confirmed to amplify the genomic DNA fragments of macrophages from the control group (macrophages alone). All the groups were tested in triplicate on a real time PCR system (Eppendorf, Germany) using DyNAmo Flask SYBR Green qPCR kit (Thermo Scientific) and analysed with the $2^{-\Delta\Delta C_t}$ method and normalized with GAPDH and control.

2.6 Statistical analysis

All experiments were performed in duplicates and the data analysis was executed in GraphPad Prism 6. Sidak's test following one-way ANOVA were performed to test statistical significance for multiple comparisons. Where $P < 0.05$ was considered to indicate a statistically significant difference. All graphs were prepared with GraphPad Prism 6 and were expressed as the mean $\pm$ SD of triplicates.

3. Results

3.1 Heat treated *K. pneumoniae* biofilms

Figure 1, shows a visual comparison of heat-inactivated biofilms using a water bath and incubator. Dead cells were observed in both conditions. However, the number of biofilm cells were observed to be less in water bath treated when compared to incubator treated samples. The picture also reveals that the biofilm matrix was not disrupted during heat inactivation at 56°C for 1 h using a bacteriological incubator. The untreated biofilm was observed to have dense clusters of live cells. The CV assay (Figure 2) shows the presence of more than 80% of biofilm formation in incubated treated cells at the same time, the cells in biofilms are mostly dead which are visualised in florescent micrographs when compared with untreated biofilms. Based on these observations, biofilms inactivated using the incubator was selected for all further analyses.

3.2 The phagocytic response of macrophage towards *K. pneumoniae* biofilms

To understand the role of macrophages in phagocytosis of *K. pneumoniae* biofilm, we co-incubated both these cells under different treatment conditions as mentioned earlier. LPS+IFN- γ was used to stimulate the macrophages, and heat-inactivated or live biofilms were used for interaction. As can be seen from the results (Figure 3), the phagocytic rate of non-activated macrophage against heat inactivated biofilm was around $7\pm 1.8\%$ as compared to that against live biofilm ($9.4\pm 2.0\%$). This shows that there is certainly a basal response by macrophages against *K. pneumoniae* biofilms. However, when the macrophage were activated with LPS+IFN- γ before exposure to heat inactivated or live biofilms of *K. pneumoniae*, there was a significant increase in the phagocytic rate. In the case of heat inactivated biofilm, the phagocytic rate was $15\pm 2.8\%$ when LPS+IFN- γ activated macrophages were used and in the case of live biofilm a phagocytic rate of $16\pm 2.2\%$ was observed when LPS +IFN- γ activated macrophages were used. It should be noted here that the presence of LPS+IFN- γ during macrophage interaction with either heat-inactivated or live biofilms consistently produced an increase in phagocytic response though it was statistically insignificant.

3.3 Cytokine responses of RAW 264.7 macrophage to *K. pneumoniae* biofilms

Another aspect of macrophage-microbial interaction is the production of cytokines by macrophages as a response to both recognition and phagocytic-killing of the microbe. To test whether this is true in the case of macrophage interaction with *K. pneumoniae* biofilms as well, we allowed LPS+IFN- γ -activated macrophage to interact with either heat-inactivated or live biofilms of *K. pneumoniae* and analysed both pro- and anti-inflammatory cytokine gene expression together with TLR2 and iNOS genes. As can be observed from the results, there was a general increase in all the pro-inflammatory cytokines (IL- β 1, TNF-

α , IL-6, IL-12, IFN- γ), TLR2 and iNOS when LPS was used for macrophage activation (group 2), as is well known. However, the use of LPS+IFN- γ for activation resulted in a significant increase in the anti-inflammatory IL-4 cytokine gene while there was a strong inhibition in the expression of IL-10 cytokine, when compared to unstimulated macrophages (group 1). Thus, it is clear that macrophage activation by LPS+IFN- γ is a well-recognized prerequisite for setting up of pro-inflammatory immune responses. Using this as a background data, we next introduced both LPS-activated or inactivated macrophages to either heat inactivated or live biofilms. When comparing the results of both unactivated macrophage with either heat inactivated (group 3) or live biofilm (group 5) with that of group 2 cells, it is clear that there is no significant increase in any of the genes analysed, suggesting that LPS+IFN- γ activation is very essential for inducing proinflammatory cytokine responses in macrophages. However, when group 3 responses are compared with that of group 5 cells there appears to be some change in gene expression. This is seen especially for TLR2 and IL-12 that showed a significant increase of 5-fold and 1.4-fold, respectively, for group 5 cells when compared to group 3 cells (group 3 IL-12 expression was in the negative). On the other hand group 5 macrophages showed a moderate increase in gene expression when compared to group 3 cells, while IL-10, as expected, remained unchanged (Figure 4).

When macrophages were activated with LPS+IFN- γ and then exposed to heat inactivated (group 6) or live (group 4) biofilms there was a significant increase in all the genes analysed, except for IL-10 that showed no change. When comparing group 4 cells with group 3, it was observed that TNF- α showed the highest increase of 6-fold, followed by IL-6 and IL-4 (5-fold each), iNOS (4-fold), IL- β 1 (3-fold) and IFN- γ (2-fold). A similar but more or less a consistent enhancement was observed across all the genes analysed in the case of group 6 cells when compared to group 5 macrophages, with an increase ranging from 1.7 fold to 3-fold. These groups of cells showed a consistent increase in gene expression when compared to group 4 macrophages. Perhaps heat treatment of biofilm modifies certain surface properties producing these differences. Nevertheless, the highest response was observed for TLR2 in the case of group 4 macrophages wherein it showed an increase of 9-fold over its control (group 3) macrophage. Taken together these results suggest that (i) LPS+IFN- γ activation of macrophage is essential for a better phagocytic response towards *K. pneumoniae* biofilm and, (ii) activated macrophages can produce a stronger cytokine response when exposed to live biofilm, indicating better recognition of live biofilm cells by macrophages. (iii) Both heat-inactivated and live biofilms induced similar phagocytic response, up regulation of pro-inflammatory genes in macrophages, indirectly conveying that macrophage response is to some extent dependent on biofilm matrix.

4. Discussion

K. pneumoniae is a nosocomial pathogen whose resistance to antibiotics has become a problem worldwide and this is owed partly to its ability to form biofilms (Riquelme et al. 2018). Such biofilm formation is seen quite commonly with MDR strains and extensively drug resistant *K. pneumoniae* (Vuotto et al. 2017). Together with capsule and fimbriae, biofilm formation has become a major virulence mechanism (Lawlor et al. 2005) leading to immune evasion and persistent infection (Sahly et al. 2008). Although there are studies to show the immune clearance of *K. pneumoniae* strains by the cells of the

immune system (Riquelme et al. 2018), the basis for such recognition and interaction is still not fully understood, and moreover, most studies have looked at only the planktonic or heat-inactivated cells. Thus, there is a general lack of information on the *K. pneumoniae* biofilm-immune cell interaction and this formed the basis of this study.

To understand the importance of biofilm in immune evasion, we used both heat-inactivated and live biofilms as targets and our results show that unactivated RAW 264.7 macrophages indeed show a basal phagocytic response towards biofilms, irrespective of the nature of the biofilm. In general, the biofilm matrix is comprised of polysaccharides, proteins, extracellular DNA and the composition of matrix varies with the strain of bacteria concerned (Allison 2003). We were able to obtain a higher phagocytic rate when macrophages were activated with LPS+IFN- γ , before being exposed to biofilm. Even in this case, the activated macrophages failed to distinguish between heat-inactivated and live biofilms and on average the phagocytic rate was around 15% for either heat-inactivated or live biofilms. This suggests that RAW 264.7 macrophages can phagocytose biofilm strains of *K. pneumoniae*. However, macrophage activation is needed to enhance this response, which suggests that the priming step is important for the activation of the innate immune response (Inagawa et al. 2016). Nevertheless, the rate of phagocytosis was an average of 15% for both biofilms and this could be due to a variety of factors such as incubation time, the role of biofilm in suppressing phagocytosis etc. (Riquelme et al. 2018). Similarly previous *in vitro* experiments demonstrated minimal *S. aureus* phagocytosis (Thurlow et al. 2011b). In the case of *S. epidermidis* biofilm, impaired phagocytosis and reduced activation of J774A.1 macrophages were noticed (Schommer et al. 2011). In our previous study we have shown the ability of the activated RAW 264.7 macrophages to phagocytose heat-inactivated planktonic *K. pneumoniae* clinical strain (Lalitha et al. 2017) by 32% and this difference we believe is due to the use of biofilm as a target here. Another criterion that could be considered for such an interaction is the exo-polysaccharide and thus it remains to be seen if macrophage activation by exo-polysaccharide of *K. pneumoniae* could be important in altering macrophage responses.

Having shown the ability of activated macrophages to phagocytose biofilms, we attempted to understand the ability of the biofilm to modulate cytokine, TLR2 and iNOS gene expression in the macrophages. It is already proven that the resistant strains of *K. pneumoniae* have the ability to not only resist phagocytic killing but also alter the polarisation state of the macrophages (Tsuchimoto et al. 2015), which could be important in determining the successful establishment of infection by *K. pneumoniae*. A pattern of cytokine secreted is one of the major mechanisms that determine macrophage polarisation (Martinez and Gordon 2014) and microbes especially resistant ones are well known to skew the innate immune responses to a more anti-inflammatory type (Regueiro et al. 2011). Our study shows that when activated macrophages were exposed to heat-inactivated or live biofilms, there was a significant increase in pro-inflammatory cytokine genes together with the expected increase in TLR2 and iNOS. Interestingly, anti-inflammatory IL-10 showed no up regulation in any of the treatment groups. Surprisingly, both heat-inactivated and live biofilms induced similar up regulation of pro-inflammatory genes in macrophages, suggesting a minor role of exo-polysaccharide in modulating macrophage cytokine responses. However, we would like to emphasize that this needs a more detailed study using isolated exo-polysaccharide. At

this juncture, we would like to point out that in the case of group 3 macrophages (unactivated + exposed to live biofilm) and group 5 macrophages (unactivated + exposed to heat-inactivated biofilm) the gene expression was lower than group 2 (activated macrophage alone) macrophages, suggesting the possibility of (i) suppression of macrophage immune responses by the biofilm, and (ii) the essentiality of macrophage priming by LPS + IFN- γ , at least *in vitro*. Nevertheless, the results of gene expression analyses clearly show that during RAW 264.7 macrophage interaction with *K. pneumoniae* biofilm, there is a modulation of the macrophage responses towards a pro-inflammatory one and this could be important in increasing the clearance efficacy of innate immune cells. Nevertheless, this modulation was not similar for LPS+IFN- γ activated Raw264.7 macrophages and planktonic *K. pneumoniae* cells. The cytokine expression was found to have a significant increase in, IL-4 (8-fold), IL-12 (5-fold), TNF- α (7-fold), IFN- γ (17-fold)(Lalitha et al. 2017), whereas, in case of biofilm, the cytokine modulation was very minimal ranging between 1-3 fold increase in cytokine expression. Taken together, the *in vitro* results suggest that *K. pneumoniae* in biofilm mode elicits a minimal phagocytic response and cytokine expression by macrophages.

The lack of analysing the protein expression level using either ELISA or flow cytometry are limitation of the present study. The cytokine mRNA expression results opened up for further detailed transcriptomics approaches to understand the molecular mechanism of host pathogen interactions.

The *in vitro* model was developed to co-culture *Klebsiella* biofilm and macrophage cells to better understand the phagocytic response of macrophage, especially towards biofilm cells. In comparison with our previous results of macrophage and planktonic cell, response, the present study suggests that *K. pneumoniae* biofilm elicits a minimal phagocytic response and cytokine expression by macrophages. Also, these results show that for a better *K. pneumoniae* biofilm recognition and clearance by macrophages, their activation state is essential in determining both the phagocytic rate and polarisation state, at least under *in vitro* conditions. Perhaps this could be important in terms of devising strategies for treating extremely resistant biofilm producing strains of *K. pneumoniae*.

Declarations

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Authors Contribution

All authors contributed equally to this work.

Ethics Declaration

The study does not involve any ethical subjects.

Consent for publication

Not applicable

Conflict of Interest

There is no conflict of interest.

Availability of Data and Materials

The data of this article are included within the article. For any further information on data or materials can be requested from the corresponding authors.

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Figures

Figure 1

Heat-inactivated *K. pneumoniae* biofilm. (A) Heat treatment at 56°C for 30 min in water bath; (B) 56°C for 1 h using bacteriological incubator ; (C) Untreated biofilm (Scale bar = 144 μm).

Figure 2

Estimation of heat-inactivated *K. pneumoniae* biofilm by CV assay. Live *K. pneumoniae* biofilm was inactivated by incubation at 56°C for 1 h using bacteriological incubator and 56°C for 30 min in water bath. Statistics performed by one way ANOVA followed by Holm-Sidak comparison tests.

Interaction between Macrophage Raw264.7 and *Klebsiella pneumoniae* biofilm

Figure 3

Interaction between Macrophage (Mφ) Raw264.7 and *K. pneumoniae* biofilm Non- activated Mφ exposed to the live biofilm (A) and heat inactivated biofilm (B); Phagocytosis assay in the presence of LPS and IFN-γ (co-treated) to the live biofilm (A) and Heat inactivated biofilm (B). Increased phagocytic response was observed in both the cases of heat inactivated and live biofilm in the presence of LPS. Where arrow represents internalized bacterial biofilm by macrophages (Scale bar = 10 μm). Statistics performed by two way ANOVA followed by Sidak's multiple comparison test

Figure 4

Cytokine gene expression assay. Evaluation of cytokines in RAW 264.7 Macrophage cell lines (Mφ) in various conditions. Heat inactivated or live biofilms of *K. pneumoniae* exposed to activated Mφ showed increased level of cytokines (TLR2, iNOS, IL-6, IL-β1, IFN-γ, IL-4, IL-12, TNF-α) except IL-10. One way ANOVA followed by Sidak's multiple comparison tests were performed for comparing the mean cytokine expression between the groups. * represents significant fold increase (p < 0.05).

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