Proceedings

The cytotoxicity of phorbol 12- myristate 13-acetate and lipopolysaccharide on THP-1 cells and optimized differentiation protocol †

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Abstract: In order to evaluate the immunomodulatory potential of novel monofloral Irish honeys, THP-1 monocyte-derived macrophages provided a suitable cell-based model. THP-1 cells can be differentiated to macrophages using phorbol-12-myristate-13-acetate (PMA). Differentiated cells are then challenged with lipopolysaccharide (LPS) to stimulate the inflammatory cascade. Few studies on the cytotoxic concentrations of these two compounds on THP-1 cells are published. Therefore, the viability of treated THP-1 cells was initially evaluated using trypan blue dye exclusion (for PMA), the resazurin assay (for LPS), and propidium iodide (PI) staining (for both). The literature suggests concentrations ranging from 100 ng/ml down to 5 ng/ml PMA are sufficient for THP-1 differentiation. Consequently, further elucidation of the differentiation potential of this sub-cytotoxic PMA concentration range was also evaluated using flow cytometric detection of the macrophage specific CD14 cell surface marker.

Keywords: PMA; LPS; CD14; THP-1; immunomodulatory; differentiation; cytotoxicity

1. Introduction

THP-1 cells are monocytic leukaemia cells isolated from a 1-year-old male [1]. These cells have been used to test the immunomodulatory effects of a range of different compounds, including natural compounds and bioactive foods such as mushroom extracts [2], wild ginger extracts [3], egg yolk antibodies [4], olive extracts [5], buckwheat [6] and honey [7]. Differentiation agents, such as phorbal-12-myristate-13-acetat (PMA), 1,25-dihydroxyvitamin D3 (VD3), and granulocyte macrophage colony stimulating factor (GM-CSF), have been used with varying concentration, exposure time, and rest periods to differentiate THP-1 monocytes to the macrophage M0 phenotype[8]–[10]. These can then be polarized to M1 and M2 macrophage subsets with similar gene expression levels as human monocyte derived macrophages [11].

While a PMA concentration of ~100ng/ml [10] is frequently cited in studies it is difficult to source cytotoxic data for PMA on THP-1 cells in the literature. Choosing the correct cytotoxicity assay is also an important consideration. Some cytotoxicity assays, such as the resazurin, MTT, or neutral red uptake assays, require an untreated cell control to compare the treated samples to, to then calculate the percentage viability. When PMA is added to THP-1 cells it differentiates this monocytic cell type to macrophages which have different metabolic functions[12], [13] and endocytosis rates[14] (important for neutral red uptake assay[15]). These differentiated cells do not proliferate [16] either, unlike the untreated control, consequently a direct comparison of treated THP-1 cells to the untreated control cannot be made.

Once the cytotoxicity of PMA has been determined, the potential to use lower PMA concentrations to mitigate masking of gene expression should be considered. Park *et al.*,[17] and Lund *et al.*,[18] reported differentiation of THP-1 monocytes based on adherence, CD14 mRNA expression, and/or proliferation rates using ~10x lower concentrations than commonly cited in the literature. A review of the literature on THP-1 cells by Chanput *et al*., [10] also highlighted the need to assess cell adherence, surface markers such as CD14 and phagocytosis capacity to ensure the majority of the population is differentiated, although no published value has been found to identify sufficiently differentiated populations of THP-1 cells.

These studies suggest that treating THP-1 cells with PMA at lower concentrations (5-15ng/ml) for 48 hours, including a 24-hour rest period, is sufficient for successful differentiation without masking gene expression levels. The question of whether a longer exposure time (72 hours) makes a difference to the CD14 expression level for lower concentrations of 5 ng/ml and 15 ng/ml PMA versus 100 ng/ml has not been investigated. Hence, it is important to further elucidate if this shorter PMA exposure period (48 h versus 72 h) is sufficient at lower PMA concentrations (i.e., 5ng/ml and 15 ng/ml) to elicit expression of the differentiation-specific, CD14 cell-surface marker.

When THP-1 cells are used for immunomodulatory studies, bacterial lipopolysaccharide (LPS) is often employed to stimulate the inflammatory cascade. LPS can polarize differentiated THP-1 cells after treatment with PMA[12][19]. However, few studies have reported cytotoxicity data on the effects of LPS alone on differentiated THP-1 cells. Studies by Liu *et al.*, 2018 and Huo *et al.*, 2020 reported that a concentration of 10 μg/ml LPS resulted in over 30% cell death. Extremely low seeding densities of differentiated THP-1 cells ($1x10^5$ cells/ml) were used for the MTT[20] and CCK-8[21] cytotoxicity assays in these studies. The current study assessed higher seeding densities of 3.33 $x10^5$ cells/ml - 7.5 $x10^5$ cells/ml. for the resazurin assay in addition to PI staining to confirm the cytotoxic concentrations for LPS and found 10 μg/ml LPS resulted in ~30% cell death.

2. Materials and Methods

Materials

RPMI 1640 media, L-glutamine, penicillin-streptomycin, trypan blue solution, resazurin sodium salt, phosphate buffered saline (without $CaCl_2$ and $MgCl_2$), PMA (P8138) and LPS (L4391) were purchased from Sigma-Aldrich (Merck Life Science Ltd, Ireland). Foetal bovine serum was purchased form Thermo Fisher Scientific (Ireland). Propidium iodide (PI) and CD14 Antibody, anti-human, FITC, REAfinity, Clone REA599, were purchased from Miltenyi Biotec (Miltenyi Biotec, United Kingdom).

Culture of THP-1 cells

THP-1 cells (ECACC 88081201) used were between passage 4 and 15. Cells were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum, 1% l-glutamine, and 1% penicillin-streptomycin in a humidified incubator at 37°C in 5% CO_2. For each experiment cells were seeded 18 hours before addition of PMA to allow cell recovery.

PMA cytotoxicity of monocytic THP-1 cells

PMA cytotoxicity was assessed using the trypan blue dye exclusion assay. THP-1 cells were seeded in 6-well plates at $1x10^6$ cells/ml and exposed to 1000 ng/ml, 500 ng/ml, 100 ng/ml, or 50ng/ml PMA for 72 hours followed by a 24-hour rest period in PMA-free media. All experiments included untreated control cells and cell counts were performed in triplicate for six independent experiments.

Differentiation of THP-1 cells

THP-1 cells were differentiated with 100 ng/ml, 15 ng/ml, or 5 ng/ml PMA for 48 or 72 hours prior to flow cytometry CD14 cell surface marker analysis. Following confirmation of CD14 expression with flow cytometry analysis cells were treated with 15 ng/ml PMA for 72 hours followed by a 24-hour rest period in PMA-free media; referenced as the optimized protocol in the following methods. When PMA media was changed, it was first centrifuged, and the cell pellet resuspended in fresh media and returned to the well, as a small number of cells remain suspended after differentiation regardless of exposure time or concentration.

Flow cytometry analysis of THP-1 cell differentiation

Flow cytometry analysis was performed using a MacsQuant analyzer 10. THP-1 cells were differentiated (100 ng/ml, 15 ng/ml, 5 ng/ml PMA) in 6-well tissue culture plates at a cell density of $1x10^6$ cells/ml for 48 or 72 hours followed by a 24-hour rest period in PMA-free media. When PMA media was changed, it was first centrifuged, and the cell pellet resuspended in fresh media and returned to the well. Untreated cells were included as a comparative control. Cells were washed with PBS, adherent cells scraped, washed again, and incubated with Miltenyi Biotec CD14 Reafinity CD14 Antibody, anti-human, FITC, REAfinity, Clone REA599, for 10 minutes at 4°C as per manufacturers protocol. This Reafinity antibody did not require separate Fc blocking. Following incubation, samples were washed in PBS and resuspended and >50,000 events were recorded. Analysis was carried out with three independent experiments.

LPS Cytotoxicity of differentiated THP-1 cells

Resazurin assay

LPS cytotoxicity and the influence of cell seeding densities was evaluated by the resazurin assay. THP-1 cells were seeded and differentiated as per the optimized PMA protocol with the following seeding densities $5x10^4$ cells/well (96-well plates), and $1.5x10^5$ cells/well (48-well plates). After the 24-hour rest period, LPS was added to wells at concentrations of 10 μg/ml, 1 μg/ml, 0.1 μg/ml, 0.01 μg/ml and the plates incubated for 24 hours before the addition of resazurin to a final concentration of 44 μM for a further 3 hours at 37°C. Relative fluorescence units were measured at an excitation of 528 nm and emission of 590 nm using the Agilent Gen 5 Biotek reader.

PI staining assay

LPS cytotoxicity was assessed with PI staining using the MacsQuant analyzer 10. A cell density of $1x10^6$ cells/ml was seeded in a 6-well tissue culture plate, incubated overnight (18 hours), and treated as per the optimized PMA protocol. Following the 24-hour rest period cells were treated with LPS at concentrations of 10 μg/ml, 1 μg/ml, 0.1 μg/ml, 0.01 μg/ml for 24 hours. Cells were then washed with PBS, adherent cells scraped, and resuspended in PBS for flow cytometry analysis. PI stain was used and >50,000 events were recorded. All data was analysed using FlowJo software (version 10.8.1)

Statistical analysis

All experiments included a minimum of three technical replicates, and three independent experiments. Data is reported as the mean ± standard deviation unless otherwise stated in figures. Statistical analysis included repeated measures ANOVA, one-way ANOVA followed by Dunnett's post hoc test and two-way ANOVA followed by Tukey's post hoc tests. Significance was defined as $p<0.05$. Statistical testing was performed using R studio (version 10.8.1).

3. Results and Discussion

PMA cytotoxicity

In the Trypan Blue dye exclusion assay, the highest PMA concentration tested (1000 ng/ml for 72 hours), resulted in 83.5%.THP-1 cell viability (Figure 1 (left)).

Interestingly cell viability at 100 ng/ml and 50 ng/ml PMA were in accordance with flow cytometry PI staining results, where 100ng/ml PMA treated THP-1 cells at 72 hours resulted in ~7.45% cell death. (Figure 1 (right)). Cells were seeded in 6-well plates at a minimum density of 1 x10^6 cells/ml for both assays. These results cannot be fully compared to the literature as studies detailing PMA cytotoxicity on THP-1 cells could not be identified. However Lund *et al.*[18], briefly mentions a cell viability of 93% for THP-1 cells treated with PMA from concentrations of 5ng/ml to 125ng/ml for 48 hours using trypan blue exclusion dye assay and a viability stain during flow cytometry analysis.

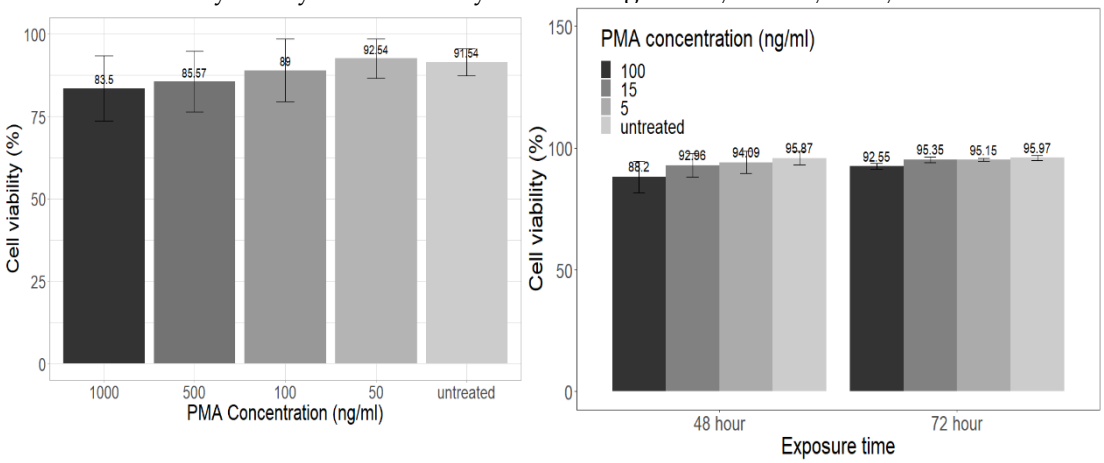

Figure 1 (Right) Effects of PMA on THP-1 cells viability with Trypan Blue exclusion assay. Data shown as the mean ± SD from 6 independent experiments compared with one way ANOVA and Dunnett's post-hoc test**. (Left)** PI stain analysis of PMA differentiated THP-1 cells, where data was subtracted from 100 to identify cell viability and allow for comparison to other cytotoxicity assay results. Data shown as mean ± SD from 3 independent experiments compared with repeated measure ANOVA.

PMA differentiation protocol and CD14 cell surface marker expression

Exposure time

Chanput et al.,[10] highlight that for THP-1 differentiation to occur, PMA exposure should range from 24 to 72 hours at 100ng/ml. Park *et al.*,[17] and Lund *et al.*[18], tested lower concentrations of PMA over a 48-hour exposure. Both studies recommended lower PMA concentrations based on percentage adherence, cell morphology, as well as CD14 expression in the Park *et al*. study. Lund *et al.*,[18] used a 48 hour exposure period based on findings by Schwende *et al.*,[22] who found TNF-α protein expression peaked after LPS exposure with THP-1 cells exposed to PMA for 48 hours. However, it is not stated in the study whether there was a statistically significant difference in TNF-α levels for 48-hour exposure versus 72-hour exposure. CD14, a cell surface protein involved in the LPS receptor complex [23], is used as a marker of macrophage differentiation [24], [25] Assessing CD14 levels after 48- and 72-hour exposure was thus carried out to compare the difference between concentrations of PMA at each exposure time and between concentrations within each exposure time (Figure 2).

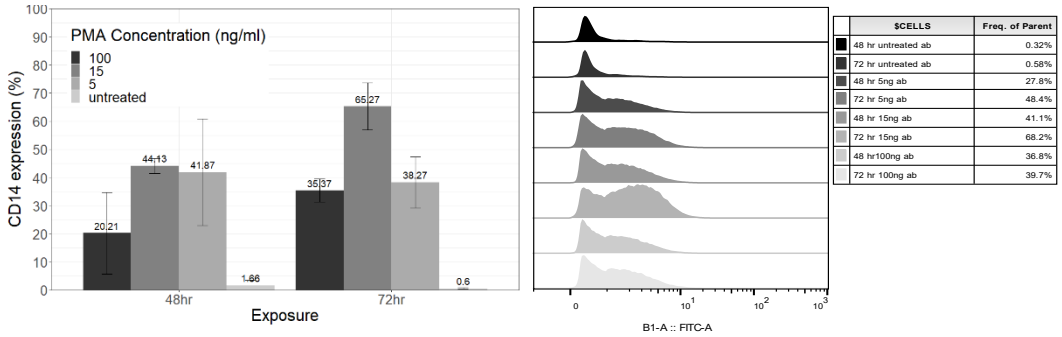

Figure 2 (Left) Bar chart detailing CD14 expression levels (%) for PMA concentrations (ng/ml) grouped by exposure (hours). Data expressed as mean ± SD for three independent experiments compared with repeated measures ANOVA. **(Right)** Stacked histogram of fluorescence representing CD14 cell surface marker antibody levels from PMA treated THP-1 cells for 48-hour and 72-hour exposures, 100 ng/ml -5 ng/ml, ad untreated cells.

There was no statistically significant difference in CD14 levels over exposure time or between exposures with respect to concentration. Overall cells exposed to 15ng/ml of PMA for 72 hours (65.27 % ± 8.3) had the highest CD14 expression followed by cells exposed to 15ng/ml for 48 hours (44.1% ± 2.63) (Figure 2(Left)).

Table 1 Difference in CD14 expression between concentrations given exposure including p-values and significance of three independent experiments. Data comparisons based on mean with repeated measures ANOVA.

Concentrations (ng/ml)	Exposure	Difference (CD14 %)	p-value	Significance (*= p < 0.05)
100 - 15	48hr	-23.92	0.1474	
100 - 5		-21.65	0.2020	
100 - untreated		18.55	0.3049	
15 - 5		2.27	0.9953	
15 - untreated		42.47	0.0109	*
5 - untreated		40.20	0.0148	*
100 - 15	72hr	-29.90	0.0022	*
100 - 5		-2.90	0.9449	
100 - untreated		34.77	0.0008	*
15 - 5		27.00	0.0042	*
15 - untreated		64.67	0.0000	**
5 - untreated		37.67	0.0005	*

Concentration

The highest CD14 levels expressed was observed for 15 ng/ml PMA exposed cells at 48 and 72 hours. Interestingly cells treated with 5 ng/ml PMA had higher CD14 levels than 100 ng/ml for 48 hour and 72-hour exposure (Figure 2). Furthermore, CD14 levels in cells exposed to 5 ng/ml of PMA were similar to that of cells treated with 15 ng/ml exposed for 48 hours. Cells treated with 15 ng/ml or 5ng/ml of PMA for 48 hours were significantly different to untreated cells. (Table 1). The CD14 levels for 15 ng/ml PMA compared to 100ng/ml and 5 ng/ml when exposed for 72 hours were also statistically significant (Table 1). Based on these findings the much lower PMA concentrations at either 48- or 72-hour exposure results in higher CD14 levels thus would be suitable THP-1 differentiation

protocols. However when assessing the differentiation potential of a protocol the proliferation ability, or absence of proliferation[10], [16] can also be considered. In this study it was found that THP-1 cells treated with 5ng/ml PMA for 48 and 72 hours showed continued proliferation (Figure 3).

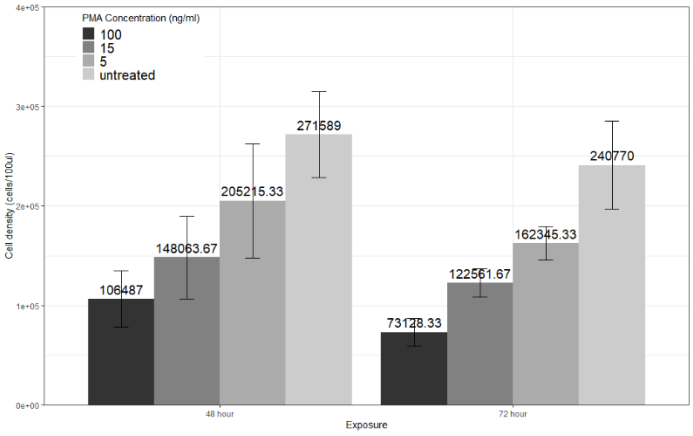

Figure 3 Cell density per 100ul of THP-1 cells treated with PMA grouped by exposure time. Cell seeding density was 1.5 x10^5 cells/100ul. Data expressed as mean ± SD of three independent experiments and compared with repeated measures ANOVA. Data gathered from flow cytometry analysis of CD14 expression.

Based on qualitative observations (not shown) during experiments for all three concentrations of PMA tested, some cells remain in suspension whereas adherence is the trademark of macrophage differentiation [18]. Moreover, the number of cells in suspension appeared to increase as the PMA concentration decreased. Despite the fact that high CD14 levels were observed for 5 ng/ml PMA treated cells, the continued proliferation and the high number of observed suspension cells would suggest a differentiation protocol using 5ng/ml PMA for 48- or 72-hours exposure may not suitably differentiate THP-1 cells. Previous studies have assessed PMA differentiation protocols based on percentage adherence and loss of proliferation but without CD14 expression levels. Lund *et al.*,[18] assessed percentage adherence when THP-1 cells were differentiated with 5ng/ml of PMA for 48 hours with varying rest periods. They found that adherence remained at 80% or higher but decreased as the rest period increased. They did not assess proliferation after differentiation or CD14 levels. Park *et al.*,[17] looked at percentage adherence and found 5ng/ml PMA for 48 hours resulted in over 80% adherence. They also assessed CD14 mRNA levels and found that 2.5ng/ml and 5ng/ml treated cells had levels greater than cells treated with 100ng/ml PMA. They did not assess proliferation after differentiation.

The high percentage adherence for both studies for cells differentiated with 5ng/ml versus the higher number of suspension cells observed in this study could be explained by the calculation method used to determine the percentage. As outlined by Lund *et al.*, non-adherent cells were counted then the number subtracted from the original seeding density and divided by this seeding density. If as found in this study, THP-1 cells treated with 5ng/ml PMA still proliferate than subtracting the number of non-adherent cells from the seeding density may give an inaccurate number as there are more cells present than initial seeding. When looking at percentage adherence, proliferation, and CD14 expression levels together a higher PMA concentration is more suitable.

LPS cytotoxicity

Using the optimized PMA protocol (15 ng/ml for 72 hours), LPS cytotoxicity on differentiated THP-1 cells was determined. Both the resazurin assay and flow cytometric analysis following PI staining reported ~70% cell viability following exposure of PMA-

differentiated THP-1 cells to the highest dose of 10 μg/ml of LPS for 24 hours (Figure 4). Liu *et al.*, [26] reported less than ~70% viability, using the MTT assay, following exposure of cells for a similar time (24 hours) to the same dose of LPS (10 μg/ml), while Huo *et al.*,[21] found that 5 μg/ml LPS resulted in less than ~75% viability, using the CCK-8 assay, with 25 μg/ml LPS resulting in less than 60% viability for 24 hour exposure. Initial experiments with the resazurin assay showed cell viability of over 100% for 10 μg/ml LPS (24 hours) (data not shown). Further work on cell seeding optimization improved the resazurin assay for PMA differentiated THP-1 cells showing a dose dependent decrease in cell viability when using much higher seeding densities ($1x10^5$ cells/ml versus $3.33x10^5$ cells/ml with 96-well plates) (data not shown). Extremely low seeding densities of differentiated THP-1 cells ($1x10^5$ cells/ml) were used for the MTT [20] and CCK-8 [21] cytotoxicity assays by Liu *et al.*, and Huo *et al.*, respectively. The current study assessed higher seeding densities of $3.33x10^5$ cells/ml – $7.5x10^5$ cells/ml. for the resazurin assay in addition to PI staining to confirm the findings from the resazurin assay and found 10 μg/ml LPS resulted in ~30% cell death (Figure 4).

48-well plates were compared to 96-well plates to assess whether large surface areas and higher seeding densities may also affect the cytotoxic effect of LPS. Based on statistical analysis there was no significant differences between 48-well plates and 96-well plates. The much higher seeding density used for the PI staining ($1x10^6$ cells/ml) in a 6-well plate did not show any statistically significant differences to the resazurin assay findings (Figure 4).

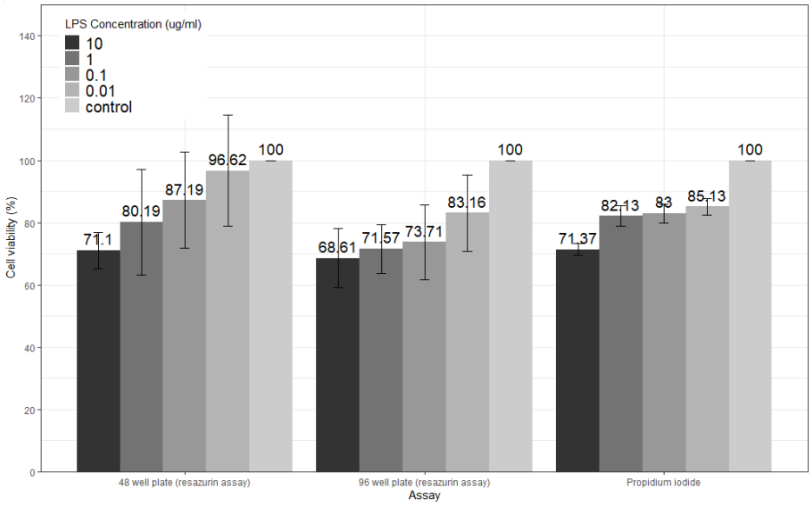

Figure 4 Effects of LPS on PMA differentiated THP-1 cells viability between two cytotoxicity assays, one with two different plate types (resazurin assay) and PI staining where data was subtracted from 100 to identify cell viability and allow for comparison to other cytotoxicity assay results. Data shown as the mean ± SD from 3 independent experiments. Data compared with two-way ANOVA and Tukey's post hoc.

4. Conclusion

Following the results and observations from this study it may be concluded that exposing THP-1 cells to 15 ng/ml PMA for 72 hours is sufficient for differentiation, based on higher CD14 levels compared to 100 ng/ml, with an absence of cell proliferation, and lower levels of suspended cells present. No significant difference was found for concentrations depending on exposure times. Further work to assess the changes in TNF-α levels after exposure to LPS is also required to determine whether 72 hours exposure of PMA differs significantly to 48-hour exposure. The highest concentration of LPS (10μg/ml) resulted in over 70% cell viability. The higher seeding densities for the resazurin assay were more appropriate, $3.3 x10^5$ cells/ml – $7.5 x10^5$ cells/ml versus $1 x10^5$ cells/ml. The type of cytotoxicity assay (PI staining versus resazurin assay) used for LPS exposure showed no significant differences in findings.

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Conflicts of Interest: The authors declare no conflict of interest

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