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# Surface Modification of Silver Nanoparticle (AgNP) by Liposomal Encapsulation Mitigates AgNP-Induced Inflammation

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# Surface modification of silver nanoparticle (AgNP) by liposomal encapsulation mitigates AgNP-induced inflammation

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ABSTRACT

Silver nanoparticles (AgNP) are widely used in a variety of consumable products as antibacterial to prevent or treat infection. Unfortunately, evidence exits that AgNP induces inflammation which can worsen with repeated human exposure. However, there is little or no research on how to mitigate these adverse effects due to AgNP induced-toxicity. Here, we investigated if surface modification of AgNP by liposomal encapsulation suppresses AgNP-mediated inflammatory responses in THP1 monocytes and THP1 differentiated macrophages (TDM). AgNP was encapsulated in a dipalmitoyl phosphatidyl choline- (DPPC)/cholesterol-based liposome by extrusion through a 100-nm polycarbonate membrane to form Lipo-AgNP. It was found as expected that AgNP induced significant release of IL-1β, IL-6, IL-8 and TNF-α in THP1 monocytes more than the basal level. Interestingly, release of these cytokines was suppressed by Lipo-AgNP. In TDMs, AgNP and Lipo-AgNP induced IL-8 release ($p < .0001$), but Lipo-AgNP maintained IL-8 release at levels significantly lower than that of AgNP ($p < .01$). However, both AgNP and Lipo-AgNP suppressed IL-1β and TNF-α release in LPS-stimulated THP1 monocytes and LPS-stimulated or unstimulated TDM respectively. We finally showed that Lipo-AgNP inhibits STAT-3 and this may be responsible for regulating the uncontrolled inflammation induced by AgNP likely mediated STAT-3 protein expression in LPS stimulated THP1 monocytes and TDMs, both LPS-stimulated and unstimulated. This data showed that Lipo-AgNP suppressed AgNP induced inflammation, making Lipo-AgNP particularly useful in treatment of bacteria induced inflammatory diseases and inflammatory cancers.

## 1. Introduction

Silver nanoparticles (AgNP) are widely used as an antibacterial agent and are the active agent in > 24% of global nanotechnology driven commercialised product, in dermal applications, medical apparels and in sterilisation products for medical equipment (Vance et al., 2015). While the application of AgNP as an antibacterial agent hold promise in the treatment of bacterial infection, their increased commercialisation translates to increased and repeated human exposure. There are reports of skin irritation and permanent discolouration of the eyes and skin as a result of exposure to AgNP (León-Silva et al., 2016). In support of this, several *in vitro* and *in vivo* studies in recent decades have demonstrated AgNP-induced inflammatory responses. A repeated-dose toxicity assessment carried out on mice that were orally administered with AgNP showed that the nanoparticle, irrespective of size induced significant expression of IL-1, IL-6 and TNF-α, among other pro-inflammatory cytokines in a dose dependent manner (Park et al., 2010). In primary rat brain micro-vessel endothelial cells (rBMEC), Trickler et al. (2010) demonstrated that AgNP induced secretion of interleukin 1β (IL-1β), IL-2 and tumour necrosis-α (TNF-α). Murphy et al. (2016) also reported that exposure of THP1 and primary human monocytes to AgNP resulted in increased mRNA expression of IL-1, IL-6 and TNF-α. In addition to this, increased inflammasome activation was suspected due to increased secretion of pro-IL-1β that was observed upon THP1 exposure to AgNP.

Inflammation is central to the development and progression of various chronic diseases including cancer, sepsis, cardiovascular disease, autoimmune, and neurodegenerative diseases (David et al., 2016; Jones et al., 2003; Mukherjee et al., 2016; Pianta et al., 2017; Seol et al., 2017). Findings in the past decades have shown that IL-1β, IL-6 and TNF-α are the major pro-inflammatory cytokines with roles central to promotion and maintenance of systemic inflammation (Hernandez-Rodriguez et al., 2004; Mori et al., 2011), making AgNP exposure a potential hazard. Cytokines are produced by immune cells such as monocytes and macrophages; and stromal cells such as endothelial cells and fibroblasts for intercellular communication in mediating processes like proliferation, differentiation, growth and immune cell activation (Landskron et al., 2014). The maintenance of the delicate balance between anti- and pro-inflammatory cytokines, however, is what defines the line between normal condition and disease state

for different chronic diseases. For example, IL-1β, IL-6 and TNF-α are the main cytokines driving the damaging inflammatory responses in rheumatoid arthritis (RA), likely through the perturbation of the balance between anti- and pro-inflammatory cytokines in RA joints (Mori et al., 2011). This perturbation may arise due to the amplification of inflammatory response through the activities of IL-1β, IL-6 and TNF-α in inducing expression of other pro-inflammatory cytokines through activation of transcriptional factors such as nuclear factor- κβ (NF-κβ), Signal Transducer and Activator of Transcription-1 (STAT-1) and STAT-3 (Chung et al., 2017; Pugazhenthi et al., 2013).

Taken together, repeated human exposure to AgNP may result in chronic inflammation that may favour autoimmunity or cancer development and the wide application of AgNP increases risk of AgNP induced adverse effects. In this study, we encapsulated AgNP within a dipalmitoyl phosphatidylcholine (DPPC)-based liposome to suppress the nanoparticle-induced inflammation, based on previous studies that have shown that DPPC suppresses AgNP induced inflammation (Murphy et al., 2015; Sweeney et al., 2016). We then showed that the liposomal encapsulation of AgNP suppresses AgNP induced inflammation in both THP1 monocytes and THP1 cells differentiated into macrophages and the exposure to liposomal AgNP correlates with inhibition of STAT-3 expression.

## 2. Experimental

### 2.1. Materials and reagents

Silver nitrate (AgNO$_3$) (CAS Number: 7761-88-8), sodium borohydride (NaBH$_4$) (CAS Number: 16940-66-2), dipalmitoyl phosphatidyl choline (DPPC) (CAS Number: 63-89-8), cholesterol (CAS Number: 57-88-5), Phorbol 12-myristate 13-acetate (PMA) (CAS Number: 16561-29-8) and IL-1β converting enzyme (ICE) inhibitor, Ac-YVAD-CMK, (Cat Number: 178603-78-6), lipopolysaccharide (LPS) from *E. coli* 0111:B4 (Cat Number: LPS25), polymyxin B (CAS Number: 1405-20-5) were all purchased from Sigma Aldrich, Dublin Ireland. Chloroform (CAS Number: 67-66-3) was from Fischer Scientific Dublin, Ireland and Alamar blue (Cat Number: DAL1025) were all from ThermoFischer Scientific, Dublin Ireland. Human IL-1β ELISA kit (Cat Number: HUFI00164), Human TNF-α ELISA kits (Cat Number: HUFI00262) and Human IL-6 ELISA kit (Cat Number: HUFI00180) were all purchased from ELISAGenie, Dublin Ireland. Human IL-8 ELISA MAX™ Deluxe (Cat Number: 431504) was from Biolegend, London, UK.

### 2.2. AgNP synthesis and encapsulation

AgNP of 21 nm size was synthesised through chemical reduction of silver nitrate (AgNO$_3$) by sodium borohydride (NaBH$_4$) and the resulting AgNP was encapsulated in dipalmitoyl-phosphatidyl choline (DPPC) and cholesterol lipid layer to form Lipo-AgNP of 140 nm size as described in our previous study (Yusuf et al., 2018). Briefly, 30 mL of 2 mM solution of NaBH$_4$ was added to an Erlenmeyer flask placed in an ice bath and stirred at 350 rpm for 30 min to equilibrate after which 6 mL of 1 mM AgNO$_3$ was added to the NaBH$_4$ solution one drop per second under constant stirring. The stirring was stopped after all the AgNO$_3$ had been added, and the flask was taken out of the ice bath. To further prevent agglomeration of the AgNP the solution was placed back on the stirrer and stirred until room temperature (RT) was achieved. The resulting golden yellow solution was stable at 4 °C.

For encapsulation, DPPC and cholesterol were dissolved in 5 mL chloroform and the solution was mixed until clear. It was then dried in a vacuum oven at 52 °C overnight (above melting temperature of DPPC). The resultant lipid cake was rehydrated in distilled deionised water (ddH$_2$O) at 60 °C in a shaker. After the lipid was rehydrated, the AgNP solution was added to make a final lipid concentration of 1 mg/mL of DPPC and 0.23 mg/mL of cholesterol to give a 7:3 M

ratio (Briuglia et al., 2015). The solution was then placed in the shaker at 60 °C for another 20 min after which it was vortexed briefly and extruded through a 100 nm Nanosizer polycarbonate membrane extruder (TTScientific, Knoxville, USA). The resulting colloidal mixture was stored at 4 °C before use.

### 2.3. Scanning electron micrograph (SEM) and scanning transmission electron micrograph (STEM) analysis

SEM micrographs for free and encapsulated AgNP were obtained using a Hitachi SU-6600 field emission SEM (Hitachi, Maidenhead, UK) at an accelerating voltage of 25 kV and working distance of 8 mm. For SEM analysis, a 5 μL drop-cast of each sample was made onto a 5 × 5 mm pure silicon wafer substrate (Ted Pella Inc., Redding California, USA) 24 h prior to obtaining the micrographs and allowed to air dry. For STEM analysis, 3 μL of each sample was drop-cast onto a carbon formvar copper grid (Agar Scientific Ltd., Stanstead, UK) 24 h prior obtaining and allowed to air dry.

### 2.4. Dynamic light scattering (DLS) analysis and zeta potential analysis

To measure the hydrodynamic diameter of the nanoparticle in solution, the DLS analysis of both AgNP and Lipo-AgNP was carried out with Malvern Zetasizer Nano ZS (Malvern Panalytical, Malvern, UK). The zeta potential measurement of the nanoparticles was also measured with Malvern Zetasizer Nano ZS instrument set at 25 °C for all the samples. Nanoparticles were loaded into a pre-rinsed folded capillary cell up to the marked portion (usually filled with 1 ml of sample). An applied voltage of 15 and 50 V was used for Lipo-AgNP and uncoated AgNP respectively and a minimum of three different measurements were made for each sample.

### 2.5. Cell culture and THP1 differentiation into monocytic THP1 and THP1 differentiated macrophages

THP1 (ATCC®: TIB-202™) cells were used in this study and were cultured in RPMI-1640 media supplemented with 2 mM ʟ-glutamine and 10% FBS. The cells were incubated at 37 °C, 95% humidity and 5% CO$_2$. For nanoparticle exposure, cells were seeded in a 24-well plate (VWR, Dublin, Ireland) at a density of 3 × 10$^5$ cells/ml of culture media and 1.5 ml of culture media per well. The cells were stimulated with 100 ng/ml (162 nM) PMA for either 24 h or 72 h to induce differentiation into matured monocytic THP1 or THP1 differentiated macrophages (TDM) respectively. The PMA containing media was then replaced with fresh RPMI media and the cells left to incubate for another 24 h (Chanput et al., 2014).

### 2.6. Cell exposure

After this, the culture media containing PMA was removed from the now adhered monocytic THP1 cells or TDMs and another fresh RPMI media containing 30 μg/mL of polymyxin B was replaced for 1 h to inhibit any LPS that might be present in any reagent used. Polymyxin B is known to inhibit LPS induced inflammatory response and the concentration used here was found to inhibit up to 10 μg/mL LPS contamination (Xiao-Xiao et al., 2017). After incubation with polymyxin B, the cells were then treated with different concentrations of uncoated AgNP and Lipo-AgNP containing an equivalent concentration of the uncoated AgNP. As a positive control for inflammatory response, cells primed with 10 μg/mL LPS from *E. coli* 0111:B4 were incorporated on the plate while a negative control of unexposed cells were both incorporated onto the plate. The cells were incubated with treatment for specified period and were stimulated with 5 mM ATP for 1 h prior to collection of the supernatant. In some instances, cells exposed to 20 μM of Ac-YVAD-

CMK, a caspase-1 inhibitor (ICE$_{inh}$), were also incorporated onto the plate. A minimum of three independent experiments were conducted and for each independent experiment, four replicate wells were employed per concentration per plate.

### 2.7. Cytokine release by sandwiched enzyme linked immunosorbent assay (ELISA)

For cytokine release assay, RPMI culture media from THP1 promonocytes, monocytic THP1 and TDMs exposed to AgNP or Lipo-AgNP post priming with LPS and ATP challenge were collected at different time points after which IL-1β, IL-6, IL-8 and TNF-α levels in the culture supernatant were carried out by sandwich ELISA following manufacturer's instructions. Absorbance was measured at 450 nm.

### 2.8. Cell lysate collection

To collect cell lysate for protein expression analysis, culture media was aspirated from the cells after which the cells were rinsed in ice cold $1 \times$ PBS while the plate was kept on ice. The cells were lysed with 100 μl of RIPA buffer containing protease inhibitor cocktail per $3.5 \times 10^5$ cells and the cells were scrapped off and pipetted up and down gently to break up intact cell membranes. The lysate was transferred into a 1.5 ml Eppendorf tube and centrifuged at $13,000 \times g$ for 10 mins at 4 °C. The supernatant was transferred into another 1.5 ml Eppendorf tube and kept on ice to be analysed by ELISA as soon as possible or stored at −80 °C until analysis.

### 2.9. Cell viability

Cell viability of nanoparticle exposed cells (both monocytic THP1 and TDM) to the nanoparticles by Alamar Blue assay (AB), a pre-warmed solution of AB (10% v/v) in serum free media was prepared. The exposure media were removed, and the cells were rinsed with prewarmed sterile $1 \times$ phosphate buffer saline (PBS) after which 1.5 ml of AB solution was added onto the cells. The plates were incubated at 37 °C for 2 h and the resulting florescence of the converted AB dye was measured at 540 nm excitation and 595 nm emission and wavelengths in a Spectra-Max® M2 Multi-Mode Microplate Reader.

### 2.10. Statistical analysis

Statistical analysis of acquired data was carried out with GraphPad Prism version 7. Data was analysed by Two-way analysis of variance (ANOVA) with Sidak or Turkey multiple comparisons test to detect significance in differences. Statistically significant differences in tests were indicated for *p* value < .05.

## 3. Results

### 3.1. Characterisation of nanoparticles

Detail of the nanoparticle characterisation and toxicity profile for AgNP and Lipo-AgNP as well as the drug release profile of Lipo-AgNP in aqueous environment at physiological pH of 7 and acidic pH of 6.5 is present in our recent publication (Yusuf and Casey, 2019). In summary, the DLS analysis of the nanoparticles used in this study are depicted in Table 1 below. DLS analysis indicated that the hydrodynamic radius of AgNP was 20.47 nm while that of the Lipo-AgNP was found to be 139.0 nm. Zeta potential measurements also showed that the encapsulated AgNP (Lipo-AgNP) to be more stable (Zeta of −30.8 mV) than the uncoated AgNP (−25.7 mV). The SEM of both AgNP and Lipo-AgNP confirmed a spherical morphology while the STEM depicted the encapsulation of the AgNP within the liposomes shown as grey area in the micrograph (Fig. 1).

**Table 1**
DLS analysis of both AgNP and Lipo-AgNP.

|              | AgNP              | Lipo-AgNP         |
| ------------ | ----------------- | ----------------- |
| Size (nm)    | 20.47 ± 7.384     | 139.0 ± 22.47     |
| PDI          | 0.186             | 0.127             |
| Zeta (mV)    | −25.7             | −30.8             |

### 3.2. Inflammatory response in monocytic THP1 exposed to AgNP and Lipo-AgNP

IL-1β plays a central role in inflammatory responses throughs its involvement in regulating its own release in addition to that of IL-6 and its crosstalk with TNF-α during inflammation (Di Paolo et al., 2015; Mori et al., 2011). As such, we investigated if exposure of the monocytic THP1 and TDMs to AgNP or Lipo-AgNP would provoke an inflammatory response in these cell types by evaluating the release of IL-1β, IL-6, IL-8 and TNF-α upon exposure to the nanoparticles. Prior to nanoparticle exposure or LPS priming, the cells were exposed to polymyxin B for 1 h to inhibit any LPS contamination that may have occurred in procedures prior to this stage. After this, LPS-primed and unprimed monocytic THP1 and TDMs were exposed to 1 μg/ml and 2 μg/ml of AgNP or Lipo-AgNP for 3 h after which the cells were stimulated with 5 mM ATP for another 1 h when supernatant from the cells was collected for ELISA analysis of IL-1β release. From Fig. 2A, analysis of IL-1β measurement in monocytic THP1 indicated that both 1 μg/ml and 2 μg/ml of AgNP significantly induced IL-1β release compared to untreated and unprimed monocytic THP1 (*p* < .05 and 0.0001 respectively). While Lipo-AgNP maintained no significant induction of IL-1β compared to the unprimed and untreated monocytic THP1 at both 1 μg/ml and 2 μg/ml, Lipo-AgNP significantly suppressed AgNP induced IL-1β release at both concentrations (*p* < .05 and 0.0001 respectively). At 1 μg/ml concentration, both AgNP and Lipo-AgNP were able to significantly suppress LPS induced IL-1β release (*p* < .05) but at 2 μg/ml, the nanoparticles showed no effect on LPS induction of IL-1β release.

Given that IL-1β and TNF-α interact during inflammation to upregulate transcription and expression of pro-inflammatory cytokines, release of TNF-α upon monocytic THP1 exposure to both AgNP and Lipo-AgNP was also investigated following the same procedure used for measuring IL-1β levels. As observed for IL-1β release, Fig. 2B shows that exposure of unprimed monocytic THP1 to 1 μg/ml AgNP resulted in significant induction of TNF-α compared to untreated and unprimed monocytic THP1 (*p* < .05). Coupled with this, exposure of the monocytes to 1 μg/ml Lipo-AgNP did not induce any rise in TNF-α release compared to the untreated and unprimed monocytic THP1. This coupled with the observation that both 1 μg/ml and 2 μg/ml Lipo-AgNP maintained IL-1β levels similar to that of untreated and unprimed indicates that Lipo-AgNP mediate suppression of AgNP-mediated IL-1β and TNF-α release. At 2 μg/ml concentration, both AgNP and Lipo-AgNP induced similar levels of TNF-α as that in untreated and unprimed control monocytes. The contrary was observed for LPS primed monocytic THP1 as exposure to AgNP but not Lipo-AgNP at 1 μg/ml and 2 μg/ml resulted in suppression of LPS mediated induction of TNF-α (*p* < .01 and *p* < .0001 respectively). Also, Lipo-AgNP exposure resulted in significantly higher levels of TNF-α release compared to AgNP exposed monocytic THP1 at 1 μg/ml and 2 μg/ml in the presence of LPS (*p* < .001).

IL-1β and TNF-α are known regulators of IL-6 release (Palmqvist et al., 2008) and based on this we next investigated the effect of AgNP and Lipo-AgNP on IL-6 release. Findings revealed that IL-6 release was not influenced by LPS priming of monocytic THP1 cells even in the cells that were exposed to 1 μg/ml AgNP and Lipo-AgNP alone or
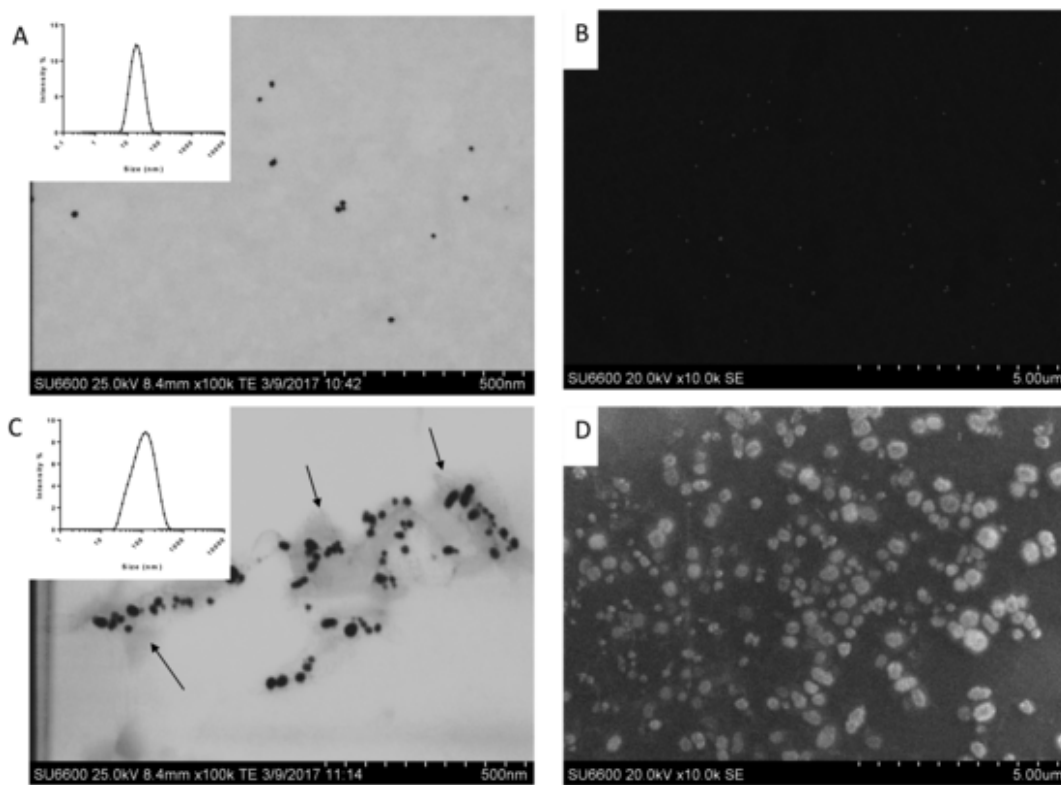
**Fig. 1.** SEM and STEM micrograph of AgNP and Lipo-AgNP: (A) STEM micrograph of AgNP with DLS (inset) (B) SEM of AgNP (C) STEM of Lipo-AgNP with DLS (inset) and (D) STEM of Lipo-AgNP.
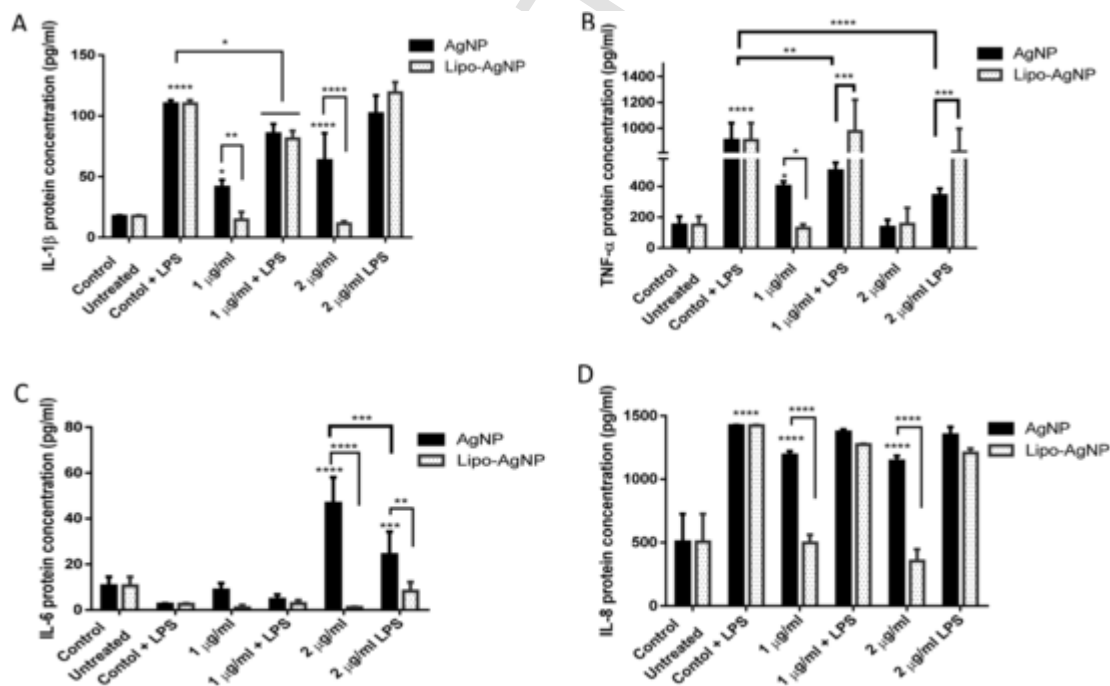


**Fig. 2.** Lipo-AgNP suppress AgNP mediated secretion of IL-1β, IL-6, IL-8 and TNF-α in monocytic THP1 cells. Monocytic THP1 cells that were either primed with 10 μg/mL LPS or unprimed were exposed to 1 μg/ml or 2 μg/ml of AgNP and Lipo-AgNP and incubated for 3 h. The cells were challenged with 5 mM ATP for 1 h before culture supernatant was collected for ELISA analysis of (A) IL-1β (B) TNF-α (C) IL-6 and (D) IL-8 release. Data was presented as mean ± SD of 3 independent experiments. Statistically significant differences within same exposure group were analysed by Two-way ANOVA Tukeys multiple comparison test while differences between different exposure groups were analysed by Sidak multiple comparison test. *$p < .05$, **$p < .01$, ***$p < .001$, **** $p < .0001$.

with LPS (Fig. 2C). Conversely, exposure of the monocytic THP1 to 2 μg/ml AgNP alone or with LPS priming, induced highly significant increase in IL-6 release in comparison to untreated unprimed THP1

monocytes ($p < .0001$ and $p < .001$ respectively). An interesting finding here was the suppression of AgNP induced IL-6 release by Lipo-AgNP both at 1 μg/ml and 2 μg/ml monocytes ($p < .0001$ and $p < .01$ respectively).

It is noteworthy that LPS priming of monocytic THP1 resulted in suppression of IL-6 release although not significantly ($p = .5875$). In the same manner, exposure of LPS-primed monocytic THP1 to 2 μg/ml AgNP resulted in significantly lower release of IL-6 when compared to cells exposed to 2 μg/ml AgNP alone. Taken together, these findings might be indicative of an antagonistic role of LPS on release of IL-6 in the monocytes.

IL-8 is a pro-inflammatory cytokine with tumour promoting roles (Long et al., 2016). IL-8 release upon exposure of unprimed and LPS-primed monocytic THP1 to either AgNP or Lipo-AgNP was investigated. As shown in Figure 2D, there was significant induction of IL-8 release in LPS primed monocytic THP1 (1422.65 pg/ml) compared with unprimed THP1 monocytes (505 pg/ml) ($p < 0.0001$). As observed for IL-1β, exposure of the THP1 monocytes to 1 μg/ml and 2 μg/ml of Lipo-AgNP resulted in similar IL-8 release profile with those that were untreated and unprimed with LPS (498.36 pg/ml and 354.48 pg/ml respectively). Conversely, AgNP at both 1 μg/ml and 2 μg/ml resulted in significant induction in released IL-8 levels (1191.17 pg/ml and 1371.61 pg/ml respectively) ($p < 0.0001$), while Lipo-AgNP significantly suppressed AgNP induced IL-8 release ($p < 0.0001$). However, both AgNP and Lipo-AgNP exposure did not suppress LPS induced release of IL-8 at both concentrations evaluated.

### 3.3. Inflammatory response in TDMs exposed to AgNP and Lipo-AgNP

Following the discovery that Lipo-AgNP was able to suppress AgNP and LPS mediated cytokine release in the monocytic THP1 cells, we proceeded to investigate if this was replicated in TDMs upon their exposure to AgNP and Lipo-AgNP. This was necessary because

monocytes and macrophages are both important components of the innate immune system in processes leading up to inflammation either during injury/infection or disease development such as rheumatoid arthritis (RA) (Ogle et al., 2016; Roberts et al., 2015). As such, release of the same set of cytokines measured in the monocytic THP1 were examined in TDMs under the same conditions.

Analysis of IL-1β release upon LPS priming of TDMs indicated a significant increase or enhancement of IL-1β release by TDMs (188.43 pg/ml) compared to the untreated and unprimed control TDMs (174.45 pg/ml) ($p < 0.05$). Exposure of the unprimed TDMs to 1 μg/ml of AgNP and Lipo-AgNP resulted in significant suppression of IL-1β release (28.41 pg/ml and 41.28 pg/ml respectively) ($p < 0.0001$), although AgNP significantly suppressed the IL-1β release compared to Lipo-AgNP ($p < 0.05$). In a similar manner, 2 μg/ml of AgNP and Lipo-AgNP significantly suppressed IL-1β release in unprimed TDMs (23.73 pg/ml and 48.59 pg/ml respectively) ($p < 0.0001$). Unfortunately, both AgNP and Lipo-AgNP did not suppress LPS mediated release of IL-1β in TDMs (Figure 3A).

The effect of the nanoparticles on TDM response in terms of TNF-α release was next examined. As shown in Figure 3B, the first observation was that TNF-α release in TDMs was more than twice that secreted by monocytic THP1s under all exposure conditions. It was found that LPS priming of TDM induced significantly high level of TNF-a release (3458 pg/ml) compared to unprimed TDM (593.33 pg/ml) ($p < 0.0001$). Exposure of the TDM to 1 μg/ml and 2 μg/ml of Lipo-AgNP resulted in significant suppression of TNF-α release (399.43 pg/ml and 258.33 pg/ml respectively). AgNP exposure on the other hand only resulted in significant suppression of TNF-α release at 2 μg/ml (341.9 pg/ml) ($p < 0.01$). In TDMs that were primed with LPS, exposure to 2 μ
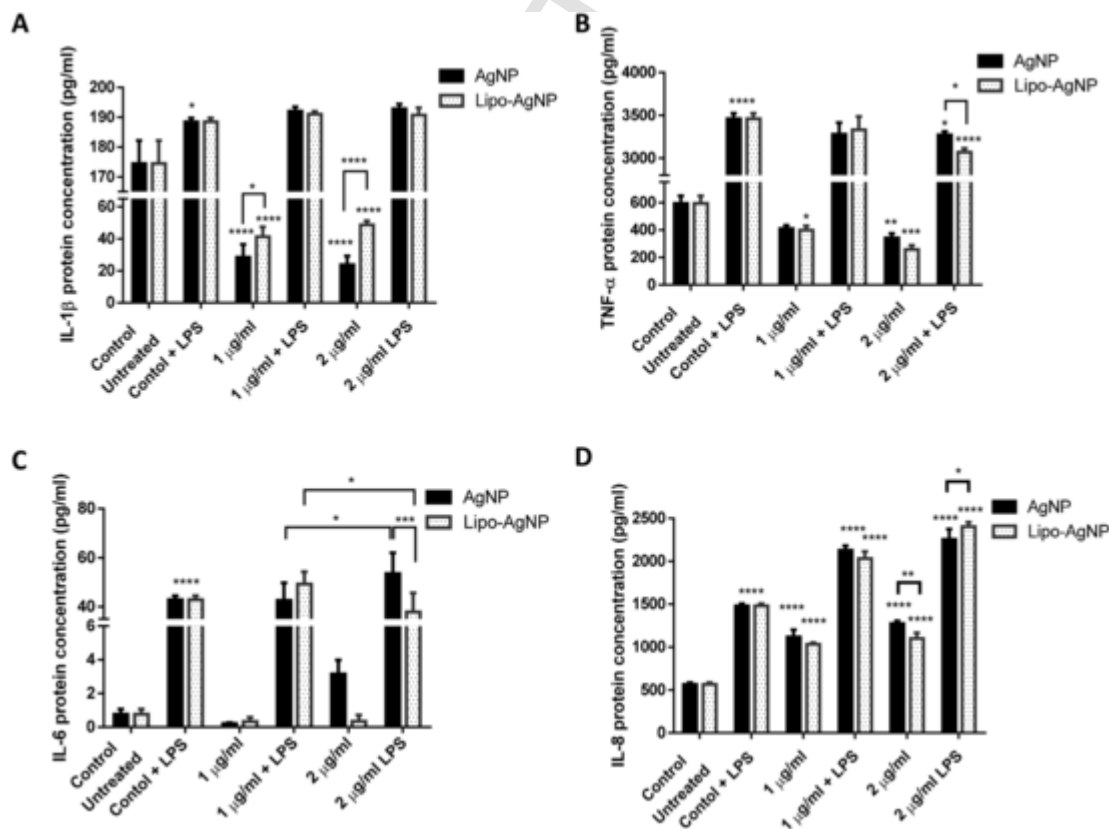


**Figure 3.** Lipo-AgNP suppress AgNP mediated secretion of IL-1β, IL-6 and TNF-α in TDMs. TDMs that were either primed with 10 μg/mL LPS or unprimed were exposed to 1 μg/ml or 2 μg/ml of AgNP and Lipo-AgNP and incubated for 3 h. This was followed by 5 mM ATP challenge for 1 h before culture supernatant was collected for ELISA analysis of (A) IL-1β (B) TNF-α (C) IL-6 and (D) IL-8 release. Data was presented as mean ± SD of 3 independent experiments. Statistically significant differences within same exposure group were analysed by Two-way ANOVA Tukeys multiple comparison test while differences between different exposure groups were analysed by Sidak multiple comparison test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

g/ml AgNP and Lipo-AgNP resulted in significant suppression of LPS induced TNF-α release in the TDMs (3270 pg/ml and 3068 pg/ml respectively) ($p < 0.05$ and $p < 0.0001$ respectively). Furthermore, 2 μg/ml Lipo-AgNP significantly mediated suppression of TNF-α release in comparison with AgNP at the same concentration.

As observed in monocytic THP1, LPS priming of TDMs induced significant release of IL-6 (42.86 pg/ml) when compared with the untreated and unprimed TDMs (0.76 pg/ml) ($p < 0.0001$). In addition, AgNP and Lipo-AgNP at 1 μg/ml and 2 μg/ml did not induce further IL-6 release as the TDMs exposed to the nanoparticles maintained similar IL-6 release as the control. While 1 μg/ml of AgNP and Lipo-AgNP did not affect LPS induce IL-6 secretion in TDMs, exposure of the LPS-primed TDM to 2 μg/ml AgNP resulted in significantly higher release of IL-6 (54.39 pg/ml) compared to 1 μg/ml AgNP (42.59 pg/ml) ($p < 0.05$). The reverse was observed for 2 μg/ml Lipo-AgNP exposure which induced significantly lower IL-6 release compared to 1 μg/ml Lipo-AgNP (37.86 pg/ml to 49.21 pg/ml respectively) ($p < 0.005$) (Figure 3C). In TDMs, observed IL-8 release upon exposure to 1 μg/ml AgNP and Lipo-AgNP (1118.51 pg/ml and 1033.22 pg/ml respectively) was significantly higher compared to control untreated group (566 pg/ml) ($p < 0.0001$). The same was observed for the nanoparticles at 2 μg/ml concentration but AgNP induced significantly higher IL-8 release (1273 pg/ml) compared to Lipo-AgNP (1099.57 pg/ml) ($p < 0.01$) (Figure 3D). LPS priming of the TDMs also resulted in significantly higher release of IL-8 at both 1 μg/ml and 2 μg/ml of both AgNP and Lipo-AgNP ($p < 0.0001$).

### 3.4. Cell viability of monocytic THP1 and TDMs at 4 h post-exposure to AgNP and Lipo-AgNP

We have shown that Lipo-AgNP exhibit immunosuppressive role, even AgNP to some extent. To ensure that the suppressed cytokine release was not due to reduced cell viability or reduction in number of cells that are able to release the cytokine upon exposure to AgNP and Lipo-AgNP, a cell viability assay was carried out. Both LPS primed and unprimed monocytic THP1 and TDMs were exposed to 1 μg/ml and 2 μg/ml of either AgNP or Lipo-AgNP for 3 h after which the cells were stimulated with 5 mM ATP for another 1 h, as done for cytokine release. The cell viability by Alamar blue was then carried out. As shown in Figure 4A and B, exposure conditions for AgNP and Lipo-AgNP did not affect the cell viability of both monocytic THP1 and TDM at 4 h when the cytokine release was evaluated.

### 3.5. Expression of STAT-3 in AgNP and Lipo-AgNP exposed monocytic THP1 and TDMs

Data obtained from this study have shown that AgNP provokes inflammation in both monocytic THP1 and TDMs. We proceeded to investigate the effect of AgNP and Lipo-AgNP exposure on STAT-3 expression. Monocytic THP1 and TDMs that were either primed with LPS or unprimed were exposed to 1 μg/ml of either of AgNP or Lipo-AgNP for 24 h. The lysate collected was subjected to sandwich ELISA analysis and result obtained showed that exposure of LPS primed monocytic THP1 to AgNP was correlated with significant induction of STAT-3 expression compared to LPS primed and untreated control monocytes ($p < 0.05$) (Figure 5A). It was also found that AgNP exposed and LPS-primed monocytic THP1 cells expressed significantly higher STAT-3 protein expression when compared with Lipo-AgNP exposed cells ($p < 0.01$). Contrastingly in TDMs, AgNP exposed cells exhibited significant expression of STAT-3 in both LPS primed and unprimed TDMs when compared to primed and unprimed untreated controls ($p < 0.05$ and $p < 0.001$) as well as LPS primed and unprimed Lipo-AgNP exposed TDMs ($p < 0.05$ and $p < 0.001$) (Figure 5B). However, Lipo-AgNP
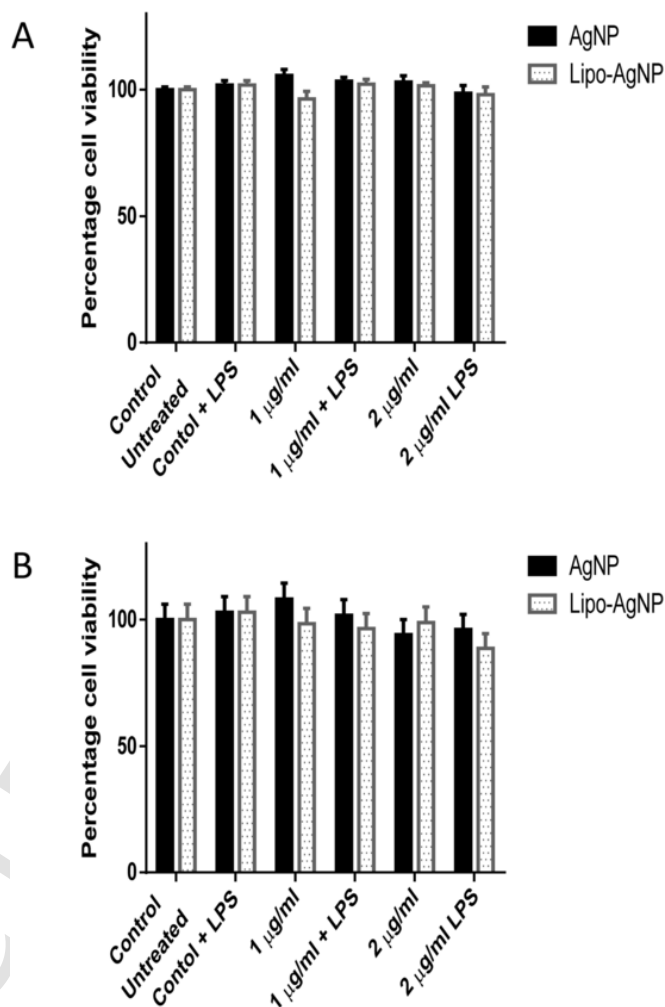
**Figure 4.** Cell viability of monocytic THP1 and TDMs. (A) monocytic THP1 and (B) TDMs that were either primed with 10 μg/mL LPS or unprimed were also exposed to 1 μg/ml and 2 μg/ml AgNP and Lipo-AgNP for 3 h followed by 5 mM ATP challenge for 1 h. The cells were then exposed incubated in 10% Alamar blue containing RPMI media for 2 h. Data is presented as mean ± SD.

exposed TDMs had similar STAT-3 protein expression in THP1 monocytes or TDMs in both LPS primed and unprimed cells.

## 4. Discussion

We have previously shown that Lipo-AgNP at very low dose induced significant caspase 3/7 dependent cell death compared to uncoated AgNP in THP1 cells in a ROS independent manner (Yusuf et al., 2018). Here, the immunomodulatory role of Lipo-AgNP was investigated using THP1 monocytes and TDMs as models for human monocyte and macrophages respectively.

Macrophages unlike monocytes do not readily secrete IL-1β based on our findings and those from other studies (Carta et al., 2011; Madej et al., 2017), this may be attributed to the tissue resident role of macrophages which is less involved promoting inflammation rather than secretion of chemokine required for recruitment of immune cells (Madej et al., 2017). This line of evidence supports our finding that TDMs used in this study secrete significantly higher IL-8 than monocytic THP1. IL-8 is a chemokine that facilitates recruitment of immune cells like neutrophils to site of inflammation and different lines of evidence now exist for its tumour promoting chemokine activities (David et al., 2016; Turner et al., 2014). Our finding here indicated that AgNP also induced higher release of IL-8 compared to Lipo-AgNP, although both nanoparticles induced release of IL-8 higher than the
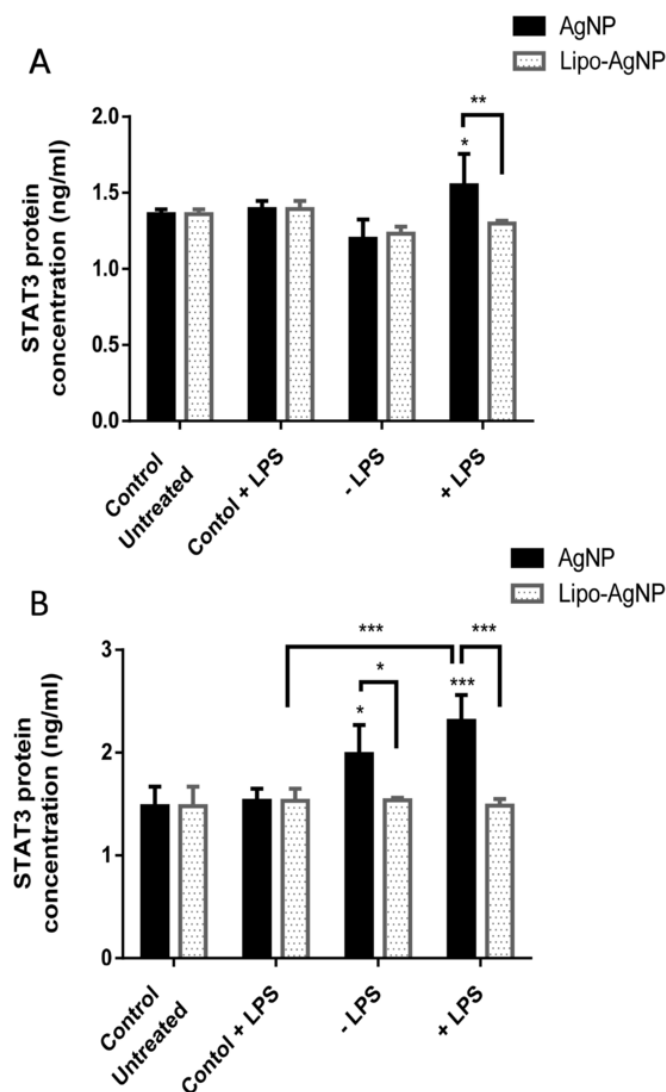
**Figure 5.** AgNP induced STAT-3 expression in monocytic THP1 and TDMs. (A) Monocytic THP1 and (B) TDMs that were unprimed or primed with 10 μg/mL LPS were also exposed with either of 1 μg/ml AgNP or Lipo-AgNP for 24 h. Lysates were collected and analysed by sandwich ELISA for STAT-3 expression. Statistically significant differences within same exposure group were analysed by Two-way ANOVA Tukeys multiple comparison test while differences between different exposure groups were analysed by Sidak multiple comparison test. *p < 0.05, *** $p < 0.001$.

basal levels in TDMs. In addition to this, AgNP induction of IL-8 release in THP1 monocytes was similar to that induced by LPS and this may indicate a possible contribution of AgNP mediated inflammation that may favour tumour development. LPS has been demonstrated to induced cancer development and promote tumour invasion and metastasis (Kurago et al., 2008; Seol et al., 2017), which may be linked to the IL-8 secretion and the chemokine activity. As such, AgNP stimulation of IL-8 release may facilitate IL-8 chemokine activity in the same manner. This may also explain the hormetic effect of AgNP in stimulating THP1 cell proliferation (Yusuf and Casey, 2019)and in HepG2 and A549 cancer cells (Jiao et al., 2014; Sthijns et al., 2017). Interestingly, HepG2 cell proliferation have been shown in another study to be inhibited by gallic acid through inhibition of IL-8 secretion (Lima et al., 2016), highlighting the possible contribution of AgNP-induced IL=8 secretion to cancer cell proliferation. Although, IL-1β release in the AgNP-exposed TDMs was significantly less than in AgNP-exposed THP1 monocytes, LPS priming of the TDM alone or with ATP stimulation resulted in IL-1β release comparable to that exhibited by the

monocytic cell even under caspase-1 inhibition indicating no effect of AgNP or Lipo-AgNP on IL-1β release. Priming of TDMs might have resulted in much more higher transcription rate of NLRP3 in addition to the alternate AIM2 inflammasome utilised by macrophages (Turner et al., 2014), resulting in no net effect of caspase-1 inhibition of IL-1β release upon ATP stimulation.

Inflammation is tightly associated with activities of reactive oxygen species (ROS). Endogenous ROS level can induce cellular responses that activate redox sensitive proteins like NF-κβ causing inflammation due to upregulation of NF-κβ target genes such as IL-1β, IL-6 and TNF-α (Forrester et al., 2018; Mittal et al., 2014; Wang et al., 2007). Likewise, inflammatory response can induce generation of ROS from NADPH oxidase and mitochondrial induced oxidative stress (Yang et al., 2007). Many studies have documented ROS generation to be AgNP main mechanism of action as an antibacterial, even as anticancer (El-Hussein and Hamblin, 2017; Gurunathan et al., 2013; Xu et al., 2012; Yuan et al., 2017), as such we hypothesised Lipo-AgNP will suppress AgNP induced inflammation as it does AgNP ROS generation.

In this study, exposure of the THP1 monocytes to AgNP resulted in significant increase in basal release of IL-1β, IL-6, IL-8 and TNF-α but only IL-8 in TDMs where suppression of IL-1β and TNF-α release was also observed in response to AgNP exposure. In support of this, evidence that AgNP induce inflammation is largely present in the literature. Sweeney et al. (2016) showed that AgNP induced secretion of IL-6 and IL-8 in mouse neuronal cells human alveolar type-I-like epithelial cells. In the same manner, Murphy et al. (2016) demonstrated increased gene expression of IL-1, IL-6 and TNF-α and increased secretion of pro-IL-1β upon exposure of THP-1 and primary human blood monocytes to AgNP. These observations have also been recorded in non-human models. Park et al. (2011) demonstrated that intra-tracheal instillation of silver nanoparticle in mice resulted in a time and dose dependent increase in release of IL-1, IL-2, IL-6 and TNF-α. It was also found that AgNP resulted in upregulation of genes associated with inflammation and tissue damage. Huang et al. (2015) also showed in mouse nerve cells that AgNP induced increased release of IL-1β as well as increased gene expression of CXCL13 chemokine and glutathione synthetase likely due to induced oxidative stress. Thus, this observed inflammation may be linked to ROS generation by AgNP. Interestingly, Lipo-AgNP suppressed both AgNP-mediated inflammation and basal cytokine release by significantly suppressing the release of IL-1β, IL-6, IL-8 and TNF-α in THP1 monocyte. In TDMs, Lipo-AgNP suppressed IL-1β and TNF-α release while maintaining basal level of IL-6 which was aggravated by AgNP. Coupled with the finding from our previous study that Lipo-AgNP suppresses generation of ROS (Yusuf et al., 2018), DPPC which is the major component of the liposome encapsulating AgNP in Lipo-AgNP is known to also possess an immunosuppressive feature (Murphy et al., 2015). Sweeney et al. (2016) had shown that a DPPC containing commercial surfactant prevented release of IL-6 and IL-8 in addition to the near abolishment of ROS generation in human alveolar type-I-like epithelial cells. As such, Lipo-AgNP may have suppressed the inflammatory response by preventing generation of ROS, supporting our finding that AgNP generated ROS induced the observed inflammation. The immunosuppressive activity of Lipo-AgNP opens an application in treatment of RA and other inflammatory diseases. Involvement of bacteria and bacterial-induced inflammation have been reported in the development of RA due to the microbe exacerbation of inflammation and oxidative stress, and sometimes induced autoimmunity (Olsen-Bergem et al., 2016; Pretorius et al., 2017). As such, Lipo-AgNP can serve as a double-edged sword to suppress the induced inflammation at the joint and to exert its antibacterial effect on the pathogens within the RA joint.

As a major PAMP responsible for bacterial induced inflammation during infection, LPS binding to CD14, TLR2 and TLR4 are prominent to initiation of cytokine release. The application of AgNP as antibacterial is fared to drive inflammation into a chronic state that may

favour development of diseases like cancer and autoimmunity. It was discovered that AgNP supressed LPS-induced IL-1β (at 1 µg/ml) and TNF-α release (at both concentration) while Lipo-AgNP (at 1 µg/ml) only suppressed release of IL-1β in THP1 monocytes. While AgNP significantly enhanced LPS induced-IL-6 release, Lipo-AgNP exposure of THP1 monocytes did not induce IL-6 release. In TDMs, only 2 µg/ml Lipo-AgNP mediated suppression of LPS-induced TNF-α. The reason for suppression of LPS induced IL-1β and TNF-α release by either of AgNP or Lipo-AgNP in monocytic THP1 or TDM is not known but it could be that LPS interaction with AgNP modifies the surface chemistry of both AgNP/Lipo-AgNP and LPS resulting in alteration in interaction between LPS and the TLRs. Thus, subsequently modulating expected inflammatory response. However, further studies are required to probe this possibility. Another possible explanation could be that both AgNP and Lipo-AgNP at the right concentrations can inhibit iNOS (inducible nitric oxide synthase) which may have resulted in suppression of LPS-induced IL-1β and TNF-α. In a study by Sarkar et al. (2008), allylpyrocatechol obtained from crude extract of from piper beetle leaf was shown to inhibit iNOS mRNA expression resulting in suppression of LPS-induced secretion of TNF-α, COX-2 and IL-12p40 in RAW 264.7 macrophages. Other compounds such as neocryptotanshinone and naringenin, which are plant extracts were also shown in different studies to inhibit LPS-induced mRNA and protein expression of IL-1β, IL-6 and TNF-α in RAW 264.7 macrophages upon inhibition of iNOS (Kumar and Abraham, 2017; Wu et al., 2015). A study supporting the activity of AgNP in suppression of LPS induced inflammation through suppression of iNOS and nitric oxide was that of Haase et al. (2014), who showed that AgNP suppressed nitric oxide synthesis in human neutrophils and macrophages. Another study also reported that AgNP attenuated the expression of iNOS inhibiting production of nitric oxide in Hep-G2 cells (Zuberek et al., 2017). Although findings of other studies have shown that AgNP can induce expression of iNOS due to increased ROS generation such as in pancreatic cancer and osteoblastic cell lines (Barcinska et al., 2018; Zielinska et al., 2016), this may be as a result of differing cellular responses to AgNP because of the different genetic backgrounds.

We have demonstrated that AgNP induced inflammatory responses, which is suppressed by encapsulation in DPPC based liposome. In particular, AgNP induced release of IL-1β, IL-6 and TNF-α indicating that it may induce persistent inflammation through STAT-3. To investigate this possibility, the effect of AgNP on the expression of STAT-3, a transcriptional factor in the JAK/STAT pathway known to be involved in sustained inflammatory response through its regulation especially by IL-6 family of protein (Wang et al., 2013), was examined. It was found that exposure of LPS-monocytic THP1 to AgNP resulted in significant induction of STAT-3 expression. On the other hand, TDMs exposure to AgNP was found to correlate with significant expression of STAT-3 irrespective of LPS priming. This finding in addition to those existing in the literature suggests a relationship between AgNP exposure and sustenance of inflammatory response mediated by activating STAT-3 expression. For example, IL-1β, IL-6 and TNF-α as found to be induced by AgNP in this study have been previously shown to induce STAT-3 phosphorylation and activation causing prolonged inflammation and joint destruction in RA mice (Mori et al., 2011). IL-6 binding with its receptor, IL-6R, is known to indirectly induce activation
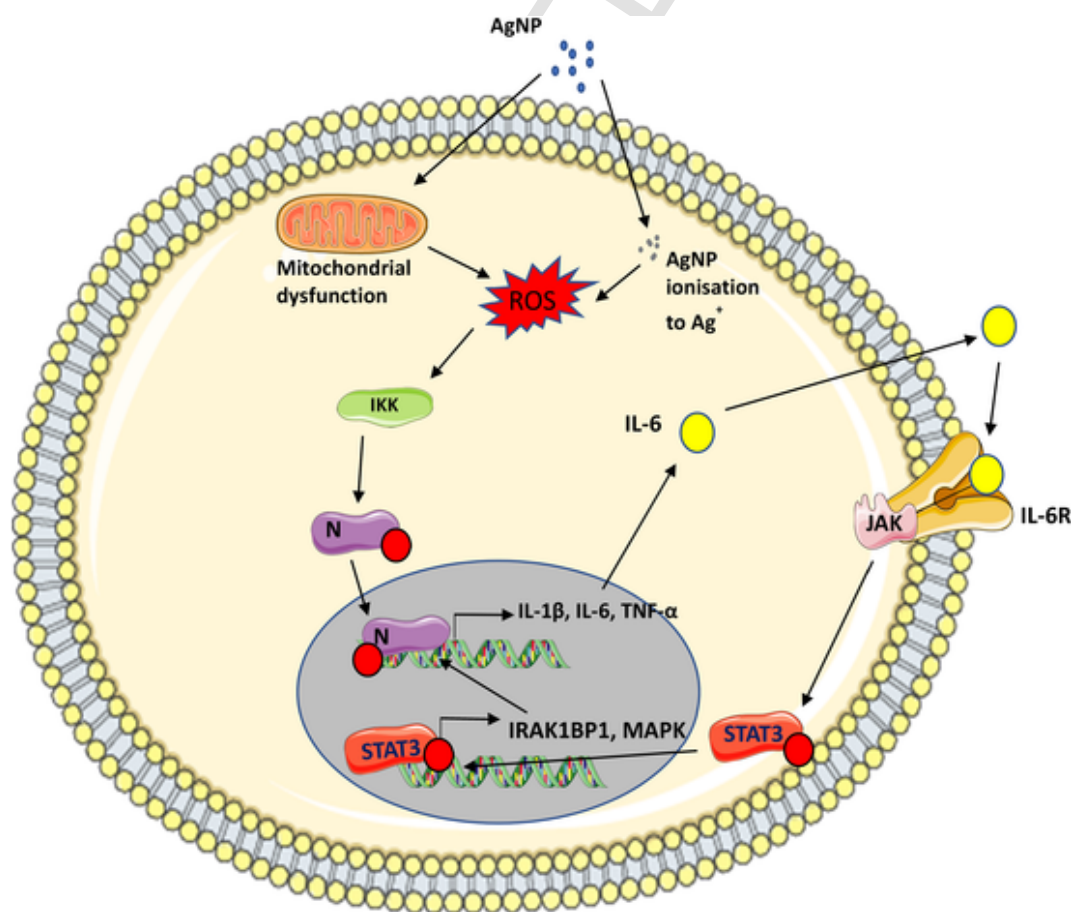


**Figure 6.** Mechanism of AgNP sustenance of perpetual inflammation. Entry of AgNP into the cell can result in generation of ROS through mitochondrial membrane disruption and cytochrome C release or through ionisation of the nanoparticle by the aqueous milieu of the cytoplasm. The generated ROS could activate IKK which in turns activate NF-κβ facilitating its translocation into the nucleus to initiate transcription of pro-inflammatory cytokines like IL-1β, IL-6 and TNF-α. Release of IL-6 and its subsequent interaction with IL-6R can then activate the JAK/STAT-3 pathway leading to phosphorylation and activation of STAT-3 creating a continuous loop of cytokine release.

of STAT-3 through IL-6R interaction with epidermal growth factor receptor (EGFR) or activation of Janus kinase (JAK) leading to STAT-3 activation (Figure 6). This STAT-3 activation subsequently results in prolonged inflammation through continued expression of proteins like the MAP kinase (MAPK) and interleukin-1 receptor associated kinase binding protein-1 (IRAK1BP1) that play crucial role in inflammation and cancer (Wang et al., 2013). The fact that Lipo-AgNP exposure correlates with STAT-3 expression in untreated control THP1 monocytes and TDMs may be indicative of the suppressive role of the encapsulation on AgNP mediated inflammation. Taken together, Lipo-AgNP may be a potential in treatment of RA and the pannus formation in RA joint. Like a double-edged sword, Lipo-AgNP may treat the RA killing the often-colonised bacteria and suppressing the provoked chronic inflammation. In addition, Lipo-AgNP may be useful in treating other bacterial diseases that are characterised with heightened inflammation like ulcerative colitis and Crohn's disease or even cancer like inflammatory breast cancer.

## 5. Conclusion

While AgNP suppression of LPS-induced IL-1β and TNF-α may be indicative of a somewhat favourable contribution of the nanoparticle during LPS triggered inflammation, AgNP provocation of inflammatory response in the absence of LPS indicates otherwise. AgNP is mainly used in preventing bacterial infection which means human exposure to AgNP is highly likely under sterile conditions such as in sterilised items like garments, cosmetics and medical equipment that contain AgNP with intent of preventing rather than treating infection. As such, the increased contact in such conditions may likely drive inflammation that are favourable for chronic diseases like cancer and inflammatory diseases. On the other hand, we have shown that Lipo-AgNP effectively suppressed AgNP mediated inflammatory responses in both monocytic THP1 and TDMs. Based on the evidence presented here and that of our previous study (Yusuf et al., 2018), Lipo-AgNP application will not only translate to lower AgNP concentration required to achieve effective cytotoxicity, but will likely mitigate AgNP-mediated inflammation, preventing vicious cycle of chronic inflammation that favours disease development and progression. This will prove especially useful in treating inflammation induced bacterial disease such as RA and the associated pannus development, ulcerative colitis and Crohn's disease, all of which are characterised with bacterial infection and chronic inflammation since AgNP is an antibacterial coupled with the immunosuppressive properties of Lipo-AgNP.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgement

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