Th2 cytokine bias induced by silver nanoparticles in peripheral blood mononuclear cells of common bottlenose dolphins (*Tursiops truncatus*)

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Background Silver nanoparticles (AgNPs) have been widely used in many commercial products due to their excellent antibacterial ability. The AgNPs are released into the environment, gradually accumulate in the ocean, and may affect animals at high trophic level, such as cetaceans and humans, via the food chain. Hence, the negative health impacts caused by AgNPs in cetaceans are of concern. Cytokines play a major role in the modulation of immune system and can be classified into two types, Th1 and Th2. Th1/Th2 balance can be evaluated by the ratios of their polarizing cytokines (i.e., interferon [IFN]-γ/ Interleukin [IL]-4), and animals with imbalanced Th1/Th2 response may become more susceptible to certain kinds of infection. Therefore, the present study evaluated the *in vitro* cytokine responses of cetacean peripheral blood mononuclear cells (cPBMCs) to 20 nm citrate-AgNPs (C-AgNP$_{20}$) by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

Methods Blood samples were collected from 6 captive common bottlenose dolphins (*Tursiops truncatus*). The cPBMCs were isolated and utilized for evaluating the *in vitro* cytokine responses. The cytokines evaluated included IL-2, IL-4, IL-10, IL-12, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α. The geometric means of two housekeeping genes (HKGs), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β2-microglobulin (B2M), of each sample were determined and used to normalize the mRNA expression levels of target genes.

Results The ratio of late apoptotic/necrotic cells of cPBMCs significantly increased with or without concanavalin A (ConA) stimulation after 24 h of 10 μg/ml C-AgNP$_{20}$ treatment. At 4 h of culture, the mRNA expression level of IL-10 was significantly decreased with 1 μg/ml C-AgNP$_{20}$ treatment. At 24 h of culture with 1 μg/ml C-AgNP$_{20}$, the mRNA expression levels of all cytokines were significantly decreased, with the exceptions of IL-4 and IL-10. The IFN-γ/IL-4 ratio was significantly decreased at 24 h of culture with 1 μg/ml C-AgNP$_{20}$ treatment, and the IL-12/IL-4 ratio was significantly decreased at 4 or 24 h of culture with 0.1 or 1 μg/ml C-AgNP$_{20}$ treatment, respectively. Furthermore, the mRNA expression level of TNF-α was significantly decreased by 1 μg/ml C-AgNP$_{20}$ after 24 h of culture.

Discussion The present study demonstrated that the sublethal dose of C-AgNP20 (≤ 1 μg/ml) had an inhibitory effect on the cytokine mRNA expression levels of cPBMCs with the evidence of Th2 cytokine bias and significantly decreased the mRNA expression level of TNF-α. Th2 cytokine bias is associated with enhanced immunity against parasites but decreased immunity to intracellular microorganisms. TNF-α is a contributing factor for the inflammatory response against the infection of intracellular pathogens. In

summary, our data indicate that C-AgNP$_{20}$ suppresses the cellular immune response and thereby increases the susceptibility of cetaceans to infection by intracellular microorganisms.

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2 **peripheral blood mononuclear cells of common bottlenose**

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19 **Abstract**

20 **Background**

21 Silver nanoparticles (AgNPs) have been widely used in many commercial products due to their

22 excellent antibacterial ability. The AgNPs are released into the environment, gradually

23 accumulate in the ocean, and may affect animals at high trophic level, such as cetaceans and

24 humans, via the food chain. Hence, the negative health impacts caused by AgNPs in cetaceans

25 are of concern. Cytokines play a major role in the modulation of immune system and can be

26 classified into two types, Th1 and Th2. Th1/Th2 balance can be evaluated by the ratios of their

27 polarizing cytokines (i.e., interferon [IFN]-γ/ Interleukin [IL]-4), and animals with imbalanced

28 Th1/Th2 response may become more susceptible to certain kinds of infection. Therefore, the

29 present study evaluated the *in vitro* cytokine responses of cetacean peripheral blood mononuclear

30 cells (cPBMCs) to 20 nm citrate-AgNPs (C-AgNP$_{20}$) by quantitative reverse transcriptase

31 polymerase chain reaction (qRT-PCR).

32 **Methods**

33 Blood samples were collected from 6 captive common bottlenose dolphins (*Tursiops truncatus*).

34 The cPBMCs were isolated and utilized for evaluating the *in vitro* cytokine responses. The

35 cytokines evaluated included IL-2, IL-4, IL-10, IL-12, interferon (IFN)-γ, and tumor necrosis

36 factor (TNF)-α. The geometric means of two housekeeping genes (HKGs), glyceraldehyde 3-

37   phosphate dehydrogenase (GAPDH) and β2-microglobulin (B2M), of each sample were

38   determined and used to normalize the mRNA expression levels of target genes.

Results

40   The ratio of late apoptotic/necrotic cells of cPBMCs significantly increased with or without

41   concanavalin A (ConA) stimulation after 24 h of 10 μg/ml C-AgNP$_{20}$ treatment. At 4 h of

42   culture, the mRNA expression level of IL-10 was significantly decreased with 1 μg/ml C-

43   AgNP$_{20}$ treatment. At 24 h of culture with 1 μg/ml C-AgNP$_{20}$, the mRNA expression levels of all

44   cytokines were significantly decreased, with the exceptions of IL-4 and IL-10. The IFN-γ/IL-4

45   ratio was significantly decreased at 24 h of culture with 1 μg/ml C-AgNP$_{20}$ treatment, and the IL-

46   12/IL-4 ratio was significantly decreased at 4 or 24 h of culture with 0.1 or 1 μg/ml C-AgNP$_{20}$

47   treatment, respectively. Furthermore, the mRNA expression level of TNF-α was significantly

48   decreased by 1 μg/ml C-AgNP$_{20}$ after 24 h of culture.

Discussion

50   The present study demonstrated that the sublethal dose of C-AgNP20 ($\leq$ 1 μg/ml) had an

51   inhibitory effect on the cytokine mRNA expression levels of cPBMCs with the evidence of Th2

52   cytokine bias and significantly decreased the mRNA expression level of TNF-α. Th2 cytokine

53   bias is associated with enhanced immunity against parasites but decreased immunity to

54   intracellular microorganisms. TNF-α is a contributing factor for the inflammatory response

55   against the infection of intracellular pathogens. In summary, our data indicate that C-AgNP$_{20}$

56   suppresses the cellular immune response and thereby increases the susceptibility of cetaceans to

57   infection by intracellular microorganisms.

Introduction

59

60   The application of silver nanoparticles (AgNPs) in industry and in consumer products has

61   increased, and the production of AgNPs and the number of AgNP-containing products will

62   increase over time (Massarsky et al. 2014). AgNPs can be released during the production,

63   transport, decay, use, and/or disposal of AgNP-containing products, subsequently draining into

64   the surface water and then accumulating in the marine environment (Farre et al. 2009; Walters et

65   al. 2014). Therefore, the increasing use and growing production of AgNPs, as potential sources

66   of Ag contamination, raise public concerns about the environmental toxicity of Ag (Li et al.

67   2018a). Previous research has demonstrated that AgNPs can precipitate in marine sediments, be

68   ingested by benthic organisms (such as benthic invertebrate species), enter and be transferred

69   from one trophic level to the next via the food chain, and thereby cause negative effects on the

70   animals at different trophic levels, such as algae, invertebrates and fishes (Buffet et al. 2014;

71   Farre et al. 2009; Gambardella et al. 2015; Huang et al. 2016b; Wang et al. 2014). Previous

72   studies have demonstrated that AgNPs are toxic to all tested marine organisms in a dose-

73   dependent manner, indicating that AgNPs may have negative effects on marine organisms at

74   different trophic levels of the marine environment. Immunotoxic effects of AgNPs have been

75   demonstrated in some aquatic animals such as Nile tilapia and mussel (Gagne et al. 2013;

76   Thummabancha et al. 2016). Nevertheless, to date the potential toxicity of AgNPs on marine

77   mammals such as cetaceans has not been sufficiently studied.

78   AgNPs have been demonstrated to cause several negative effects, such as hepatitis,

79   nephritis, neuron cell apoptosis, and alteration of gene expression of the brain, on laboratory

80   mammals (Espinosa-Cristobal et al. 2013; Sardari et al. 2012; Shahare & Yashpal 2013). *In vitro*

81   studies using different cell lines have also indicated that AgNPs can cause damage to DNA, cell

82   membranes, and mitochondria through reactive oxygen species (ROS) dependent/independent

83   pathways and further induce cytotoxicity and genotoxicity (Kim & Ryu 2013). In addition,

84   previous studies conducted in laboratory mammals, including mice and rats, have demonstrated

85   that AgNPs can enter the blood circulation through alimentary tracts and then deposit in multiple

86   organs (Espinosa-Cristobal et al. 2013; Lee et al. 2013; Shahare & Yashpal 2013; van der Zande

87   et al. 2012). Considering the negative effects of AgNPs and the presence of AgNPs in blood

88   circulation, the negative effects of AgNPs on leukocytes should be of concern. Previous studies

89   have demonstrated that AgNPs can cause several negative effects on human polymorphonuclear

90   leukocytes (PMNs) and peripheral blood mononuclear cells (PBMCs). These studies

91   demonstrated that AgNPs could cause morphological alterations, cytotoxicity, atypical cell

92   death, inhibition of de novo protein synthesis, increased production of the CXCL8 chemokine

93   (IL-8), and impaired lysosomal activity in human neutrophils (Poirier et al. 2014; Poirier et al.

94   2016; Soares et al. 2016). Only a few studies have investigated the toxicity of AgNPs on human

95   PBMCs, which have shown that AgNPs can cause cytotoxicity and functional perturbations,

96   including inhibition of proliferative activity and cytokine production (Franco-Molina et al. 2016;

97   Ghosh et al. 2012; Huang et al. 2016a; Orta-Garcia et al. 2015; Paino & Zucolotto 2015; Shin et

98   al. 2007).

99   The environmental contamination level of AgNPs is expected to increase greatly in the near

100   future, and cetaceans, as the top predators in the ocean, will suffer the potentially negative

101   impacts caused by AgNPs. Besides, immunotoxic effects of AgNPs have been demonstrated

102   previously in humans and aquatic animals. Therefore, it is crucial to investigate the immunotoxic

103   effects caused by AgNPs in cetaceans. Generally, *in vivo* experiments are rarely feasible, and the

104   ethical issues concerning the study of immunotoxic effects caused by environmental

105   contaminants in cetaceans are difficult to overcome, so *in vitro* study using blood samples from

106   captive cetaceans would be a logical and crucial approach (Beineke et al. 2010; Desforges et al.

107   2016). Cytokines play a major role in the modulation of the immune system, including

108   lymphocyte proliferation/differentiation, lymphoid development, cell trafficking, and

109   inflammatory response through the interactions between the cytokines themselves and the

110   surface receptors of many different cells (Owen et al. 2013; Tizard 2013a). Previous studies have

111   found that the sequence homology of cytokines among terrestrial and aquatic mammals is low,

112   but conserved molecule regions can still be found on biologically active areas in marine

113   mammals, such as the receptor binding sites of cytokines, suggesting a conserved biological

114   activity of cytokines in both terrestrial and aquatic mammal species (Beineke et al. 2004;

115   Beineke et al. 2010). Therefore, the functions of cytokines on the immune system in cetaceans

116   may be similar to those in mice and humans. Cytokines can be classified into two groups, Th1

117   and Th2, and their secretion pattern is associated with the balance of Th1 and Th2 responses

118   (Kidd 2003; Owen et al. 2013; Tizard 2013b). Th1 response promotes cell-mediated immune

119   response and thus enhances the immunity against intracellular microorganisms, such as

120   *Toxoplasma gondii* and *Brucella* spp., and a variety of viruses. In contrast, Th2 response is

121   associated with enhanced immunity against parasites but decreased immunity to intracellular

122   microorganisms (Owen et al. 2013; Tizard 2013b). The Th1/Th2 balance can be evaluated by the

123   ratios of their polarizing cytokines (i.e., interferon [IFN]-γ/ interleukin [IL]-4), and animals with

124   imbalanced Th1/Th2 response (Th1/Th2 polarization) may become more susceptible to certain

125   kinds of infection (Owen et al. 2013; Raphael et al. 2015; Tizard 2013b).

126   Cytokine profiling is still a relatively new field of immunotoxicology in cetaceans, and thus

127   the enzyme-linked immunosorbent assay (ELISA) kit is not widely used for cytokine profiling in

128   cetaceans (Desforges et al. 2016). Hence, it is more feasible to study the cytokine profiling by

129   molecular biology (i.e., quantitative reverse transcriptase polymerase chain reaction; qRT-PCR).

130 Therefore, the present study evaluates the *in vitro* cytokine responses of cPBMCs to C-AgNP$_{20}$

131 by qRT-PCR. The cytokines measured were as follows: polarizing cytokines of Th1 (IL-12 and

132 IFN-γ) and Th2 (IL-4), and some pro- and anti-inflammatory cytokines (IL-2, IL-10, and tumor

133 necrosis factor [TNF]-α).

134 **Materials and methods**

135 **AgNPs characterization**

136   Considering the extensive use of 20 nm citrate-AgNPs (C-AgNP$_{20}$) in recently reported

137 studies of cetacean and human blood cells (Huang et al. 2016a; Li et al. 2018b; Poirier et al.

138 2014; Poirier et al. 2016), commercial C-AgNP$_{20}$ (Pelco® citrate Biopure™ silver; Ted Pella,

139 CA, USA) was chosen. The C-AgNP$_{20}$ had been extensively washed (without centrifugation) so

140 that the level of trace elements becomes less than 0.000001%. Transmission electron microscopy

141 (TEM) for determining surface area and size/shape distributions, UV-visible spectroscopy for

142 measuring the optical properties, particle hydrodynamic diameter and zeta potential, and

143 dynamic light scattering (DLS) for determining the size distribution were performed according to

144 the manufacturer's instructions and previous studies (Poirier et al. 2014; Poirier et al. 2016). The

145 endotoxin level of C-AgNP$_{20}$ suspension was examined by ToxinSensor™ Single Test Kit

146 (GenScript, NJ, USA), and it was lower than or equal to 0.015 EU/ml. For characterization, the

147 C-AgNP$_{20}$ obtained from the manufacturer were suspended in complete RPMI-1640 medium

148   (RPMI-1640 [Gibco, NY, USA] with 10% fetal bovine serum, 2mM L-glutamine, 50 IU

149   penicillin, and 50 μg streptomycin) at a concentration of 50 μg/ml, and then examined using a

150   JEM-1400 (JEOL, Japan) TEM. The size distribution and zeta potential of the $C-AgNP_{20}$ were

151   determined through Zetasizer Nano-ZS (Malvern Instruments Inc., MA, USA) (Table 1).

152   Measurements were performed by using 100 and 500 μg/ml $C-AgNP_{20}$ in 2 mM citrate buffer

153   (pH 7.4). The $C-AgNP_{20}$ were diluted to 1, 10, and 100 μg/ml with 2 mM citrate buffer and

154   instantly used for subsequent experiments. Two mM citrate buffer was used as a vehicle control

155   (0 μg/ml $C-AgNP_{20}$).

156   **Animals and blood sample collection**

157   All procedures involving animals were conducted in accordance with international

158   guidelines, and the protocol had been reviewed and approved by the Council of Agriculture of

159   Taiwan (Approval number 1051700175). Voluntary blood samples were obtained from six

160   clinically healthy bottlenose dolphins (*Tursiops truncatus*) with confirmation by physical

161   examination, complete blood count, and biochemistry on a monthly basis from 2015 to 2017 at

162   Farglory Ocean Park. Forty millilitres of heparin-anticoagulated whole blood were collected,

163   stored, and shipped at 4 °C within 8 hours for subsequent experiments.

164   **Isolation of cPBMCs**

165   Cetacean peripheral blood leukocytes (cPBLs) were collected by slow spin method with

166   minor modifications (Bossart et al. 2008). The isolated cPBLs were resuspended in RPMI-1640

167   (Gibco, NY, USA) with 10% ethylenediaminetetraacetic acid (EDTA) and subsequently used in

168   the isolation of cPBMCs by density gradient centrifugation method. After centrifugation at 1200

169   ×g for 30 min at 20 °C, the cPBMCs were collected from the cell layer between the RPMI-1640

170   (Gibco) and Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden), washed with RPMI-1640

171   twice, resuspended to a final concentration of 1×10^7 cells/ml in complete RPMI-1640 media,

172   and kept on ice until they were utilized in subsequent experiments. The cell viability of cPBMCs

173   was determined by the trypan blue exclusion method using a hemacytometer, and the cell purity

174   based on the cell size (forward-scattered light; FSC) and inner complexity (side-scattered light;

175   SSC) of cPBMCs were determined by FACScalibur flow cytometry (BD, CA, USA). The

176   cPBMCs with higher than 90% viability and 80% purity were used in this study.

177   **Determination of the sub-lethal dose of C-AgNP$_{20}$ on cPBMCs with/without concanavalin A**

178   **(ConA)**

179   The cytotoxicity of C-AgNP$_{20}$ on cPBMCs was evaluated by the Annexin V-FITC/PI

180   Apoptosis Detection Kit (Strong Biotech, Taipei, Taiwan) according to the manufacturer's

181   instructions. Freshly-isolated cPBMCs were seeded in 96-well plates at a density of 5×10^5

182   cells/well and exposed to C-AgNP$_{20}$ at concentrations of 0, 0.1, 1.0 and 10 μg/ml with or without

183   2 µg/ml ConA (Sigma-Alderich, MO, USA). After 24 h of culture, cells were collected and

184   resuspended in binding buffer for further analysis by FACScalibur flow cytometry (BD). The

185   percentages of early apoptotic (PI – and Annexin +) and late apoptotic/necrotic cells (PI + and

186   Annexin +) were determined. A total of 8,000 events/sample were acquired. The sub-lethal doses

187   of C-AgNP$_{20}$ for cPBMCs were determined and subsequently used in the cytokine expression

188   assay.

189   **qRT-PCR efficiency of each primer sets**

190   The primer sets used in cytokine expression assay are summarized in Table 2. The

191   amplification efficiency (E) of qRT-PCR with each primer set was evaluated by the slope and R^2

192   of standard curves using the equation: $E = 10^{-(1/\text{slope})} - 1$ (Svec et al. 2015). The standard

193   templates for qRT-PCR with target primer sets were prepared by serial dilution of PCR products,

194   which were amplified from cDNA samples of isolated cPBMCs with target primer sets. The PCR

195   product was 500-fold diluted with subsequent six steps of serial 10-fold dilutions, and

196   subsequently used for qRT-PCR. The Cycle threshold (Ct) values of each dilution were

197   evaluated by qRT-PCR with each primer set to generate the standard curves.

198   **Extraction of RNA, synthesis of cDNA and qRT-PCR**

199   Total RNA was extracted from blood samples by RNeasy® Mini Kit (Qiagen, Valencia, CA,

200   USA) according to the manufacturer's instructions. The RNA samples were treated with

201   genomic DNA (gDNA) wipeout solution (Qiagen). Treated samples were then tested by qRT-

202   PCR to confirm the absence of residue gDNA prior to cDNA synthesis. The QuantiTect®

203   Reverse Transcription kit (Qiagen) was used for cDNA synthesis. The reverse transcription was

204   conducted within 4 hours after RNA extraction. The cDNA from each sample was stored at -20

205   ºC for qRT-PCR. The qRT-PCR was performed on Mastercycler® ep realplex (Eppendorf,

206   Hamburg, Germany). Each reaction contained 10 µl of SYBR® Advantage® qPCR Premix

207   (Clontech, California, USA), 7.2 µl of RNase/DNase-free sterile water, 0.4 µl of each 10 mM

208   forward/reverse primers, and 2 µl of DNA template, and the final volume of each reaction was 20

209   µl. Two microliters of RNase/DNase-free sterile water was used as the non-template negative

210   control. The thermocycle conditions were set as follows: initial denaturation at 95 °C for 30 s

211   and 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 20 s, and extension at 72

212   °C for 30 s with fluorescence detection. Furthermore, the melting curve analysis was performed

213   at the end to identify non-specific amplification. All PCR protocols were performed in

214   accordance with the Minimum Information for Publication of Quantitative Real-Time PCR

215   Experiments (MIQE) guidelines (Bustin et al. 2009; Taylor & Mrkusich 2014).

216   **Time kinetics of mRNA expression levels of selected cytokines of cPBMCs**

217   To evaluate the time kinetics of mRNA expression levels of selected cytokines in cPBMCs

218   with ConA, the cytokine gene expression levels of cPBMCs with ConA (0.5 µg/ml) were

219   determined by qRT-PCR (N=4). Freshly-isolated cPBMCs were seeded in 96-well plates at a

220   density of 5×10^5 cells/well and incubated for 0, 4, 8, 12, 16, 20 and 24 h of culture in a

221   humidified atmosphere of 5 % CO_2 at 37 °C. Then the cPBMCs were collected for subsequent

222   mRNA extraction, complementary DNA (cDNA) synthesis, and qRT-PCR. The cPBMCs with 0

223   h incubation were used as control for the calculation of cytokine expression level by $\triangle\triangle CT$

224   method. In addition, the geometric means of two housekeeping genes (HKGs), glyceraldehyde 3-

225   phosphate dehydrogenase (GAPDH) and β2-microglobulin (B2M), of each sample were

226   determined and used to normalize the expression levels of target genes (Hellemans et al. 2007;

227   Vandesompele et al. 2002).

228   **Effects of C-AgNP$_{20}$ on mRNA expression levels of selected cytokines of cPBMCs**

229   The cPBMCs were seeded in 96-well plates at a density of 5×10^5 cells/well and exposed to

230   sub-lethal doses of C-AgNP$_{20}$ with 0.5 μg/ml ConA for 4 and 24 h of culture in a humidified

231   atmosphere of 5 % CO_2 at 37 °C. After incubation, the cPBMCs were collected for subsequent

232   mRNA extraction, cDNA synthesis, and qRT-PCR. PBMCs with 4 and 24 h incubation without

233   C-AgNP$_{20}$ treatment were used as control for the calculation of cytokine expression level by

234     $\triangle\triangle CT$ method. In addition, the geometric means of two HKGs, GAPDH and B2M, of each

235   sample were determined and used to normalize the expression levels of target genes (Hellemans

236   et al. 2007; Vandesompele et al. 2002). The experiment was independently repeated twice in

237   duplicate (N=12).

Statistical analysis

239   In all experiments, the results from duplicates were averaged. To compensate for individual

240   differences, the results at different concentrations of C-AgNP$_{20}$ for each individual were

241   calculated as percentages of the results of the control (exposed to 0 μg/ml C-AgNP$_{20}$). In

242   addition, Th1/Th2 ratios at different concentrations of C-AgNP$_{20}$ were determined by the

243   cytokine mRNA ratios of Th1 (IL-12 or INF-γ) and Th2 (IL-4) polarizing cytokines and then

244   compared to the control. Our data were first checked by Shapiro-Wilk normality test and Brown-

245   Forsythe test, and the results indicated that the assumptions of normality and/or equal variance

246   were violated. Therefore, the Kruskal-Wallis Test (post hoc test: Dunn's multiple comparison

247   test) was subsequently performed on the data. A p value < 0.05 was considered statistically

248   significant, and the analysis was performed in Prism (GraphPad Software, CA, USA). All data

249   were plotted on box-plot graphics. The bar in the middle of the box represented the second

250   quartile (median), and the bottom and top of the box described the first and third quartiles. The

251   whiskers showed that the 75th percentile plus 1.5 times IQR and 25th percentile minus 1.5 times

252   IQR of all data, and any values that greater than these were defined as outliers and plotted as

253   individual points. Asterisks above the boxplots indicated statistically significant differences

254   compared to the control of each experiment.

Results

Characterization of C-AgNP$_{20}$

257   The C-AgNP$_{20}$ in complete RPMI-1640 media were spherical and close to 20 nm in diameter

258   (Fig. 1). The size distributions and zeta potentials of C-AgNP$_{20}$ (100 or 500 µg/ml) in 2 mM

259   citrate buffer are illustrated in Table 1. The size distributions were 30.27 ± 0.18 (100%) and

260   29.64 ± 0.30 (100%) for C-AgNP$_{20}$ at 100 and 500 µg/ml, respectively. The values of the zeta

261   potential were -38.97 ± 1.33 and -44.2 ± 1.35 mV for C-AgNP$_{20}$ at 100 and 500 µg/ml,

262   respectively. Furthermore, the Poly-dispersity Indexes (PDIs) were 0.12 ± 0.00 and 0.11 ± 0.01,

263   indicating that the composition of C-AgNP$_{20}$ in the stock was in a single size mode without

264   aggregates.

Sub-lethal dose of C-AgNP$_{20}$ to the cPBMCs with or without ConA stimulation

266   The treatment of C-AgNP$_{20}$ at 10 µg/ml significantly increased the ratios of late

267   apoptotic/necrotic cells in cPBMCs with or without ConA stimulation. The ratios of early

268   apoptotic and late apoptotic/necrotic cells of cPBMCs with different concentrations of C-AgNP$_{20}$

269   as compared to the control are presented in Fig. 2. After 24 h of 10 µg/ml C-AgNP$_{20}$ treatment,

270   the ratios of late apoptotic/necrotic cells of cPBMCs significantly increased with (median $\pm$

271   interquartile range (IQR): 3.55 ± 3.42; $p=0.0073$) or without ConA stimulation (median $\pm$ IQR:

272   1.78 ± 2.24; $p=0.0103$). In contrast, no statistically significant increases in the ratios of apoptotic

273   and late apoptotic/necrotic cells in cPBMCs were found after 24 h culture with 0.1 and 1.0 μg/ml

274   C-AgNP$_{20}$ treatments. Therefore, 0.1 and 1.0 μg/ml C-AgNP$_{20}$ were defined as the sub-lethal

275   doses for cPBMCs and used in the subsequent mRNA expression levels of selected cytokines in

276   cPBMCs to C-AgNP$_{20}$.

277   **qRT-PCR efficiency of each primer set**

278   Amplification efficiency (E) values for selected HKGs and cytokine genes, including GAPDH,

279   B2M, IL-2, IL-4, IL-10, and IL-12, IFN-γ, and TNF-α, ranged from 91.08 to 101.48% with $R^2 >$

280   0.99. The results are summarized in Table 2.

281   **Time kinetics of mRNA expression levels of selected cytokines of cPBMCs with ConA**

282   **stimulation**

283   The mRNA expression levels of IL-2 and TNF-α were significantly increased at 4 h of culture,

284   gradually decreased from 8 to 20 h of culture, and then mildly but not significantly increased at

285   24 h of culture. The mRNA expression level of IFN-γ was significantly increased at 4 h of

286   culture, gradually decreased at 8 and 12 h of culture, and then increased from 16 to 24 h of

287   culture. In addition, IL-4, IL-10, and IL-12 were significantly increased at 4 h of culture and

288   gradually decreased over time. Therefore, the time points chosen for the following experiments

289   were 4 h and 24 h. All the results are illustrated in Fig. 3.

290   **Effects of C-AgNP$_{20}$ on mRNA expression levels of selected cytokines in cPBMCs**

291   At 4 h of culture, the mRNA expression level of IL-10 was significantly decreased (median ±

292   IQR: 0.7864 ± 0.2355; p=0.0049) at 1 μg/ml C-AgNP$_{20}$, but no significant differences were

293   observed in the mRNA expression levels of other cytokine genes at 0.1 or 1 μg/ml C-AgNP$_{20}$

294   (Figs. 4). Following 24 h of culture with 1 μg/ml C-AgNP$_{20}$, the mRNA expression levels of IL-

295   2, IL-12, IFN-γ, and TNF-α were significantly decreased, but no significant difference was found

296   in those of IL-4 and IL-10. Furthermore, the mRNA expression levels of IL-12 (median ± IQR:

297   0.8337 ± 0.2088; p=0.0339) and IFN-γ (median ± IQR: 0.7894 ± 0.389; p=0.0164) were also

298   significantly decreased at 0.1 μg/ml C-AgNP$_{20}$ (Figs. 4). The Th1/Th2 bias was defined by the

299   ratios of Th1 and Th2 polarizing cytokines. The IFN-γ/IL-4 ratio was significantly decreased

300   following 24 h of culture with 1 μg/ml C-AgNP$_{20}$, and the IL-12/IL-4 ratio was significantly

301   decreased following 4 or 24 h of culture with 0.1 or 1 μg/ml C-AgNP$_{20}$ treatments. Overall, the

302   *in vitro* cytokine responses of cPBMCs with C-AgNP$_{20}$ treatments were biased toward Th2

303   cytokine response (Fig. 5).

304   **Discussion**

305   Our data indicated that the concentration of 10 μg/ml C-AgNP$_{20}$ was lethal dose for cPBMCs

306   after 24 h of culture. Although previous studies of human PBMCs have used a variety of AgNPs

307   (including different sizes and coatings), the lethal dose of AgNPs to human PBMCs is generally

308   higher than 10 μg/ml (Ghosh et al. 2012; Greulich et al. 2011; Huang et al. 2016a; Orta-Garcia et

309   al. 2015; Paino & Zucolotto 2015; Shin et al. 2007). Therefore, our data suggest that cPBMCs

310   may be more vulnerable than human PBMCs to the cytotoxic effects of C-AgNP$_{20}$. However,

311   previous studies have demonstrated that the toxicity and physicochemical characteristics of

312   AgNPs are associated with their surface coating and size (Kim & Ryu 2013), and thus further

313   investigation using the same AgNPs from the same manufacturer is necessary to compare the

314   differences of susceptibility between cetaceans and humans. In addition, the negative effects of

315   AgNPs with different sizes and coatings on the cPBMCs are also worth to be further studied.

316   It has been demonstrated that ConA (a selective T-cell mitogen) induces proliferative

317   activity and gene expression of cytokines in bottlenose dolphins, but no information is available

318   regarding the time course (Hofstetter et al. 2017; Segawa et al. 2013; Sitt et al. 2008). Previous

319   studies on ConA-induced cytokine mRNA expression levels of cPBMCs only presented one or

320   two time points (Segawa et al. 2013; Sitt et al. 2008). Sitt et al. (2008) quantified the ConA-

321   induced cytokine mRNA expression levels of cPBMCs after 48 h of treatment, but the reason for

322   choosing this time point was not explained. Their results showed that the mRNA expression

323   levels of IL-2, IL-4, IL-12, and IFN-γ in cPBMCs are induced after 48 h of ConA stimulation,

324   but those of IL-10 and TNF-α were not increased (Sitt et al. 2008). The other study demonstrated

325   that the mRNA expression level of IL-10 in cPBMCs increased after 6 h of ConA stimulation

326   (Segawa et al. 2013). Therefore, to apply appropriate time points for studying the effects of C-

327    $AgNP_{20}$ on the cytokine mRNA expression levels of cPBMCs, the time kinetics (from 0 to 24 h)

328   of mRNA expression levels of IL-2, IL-4, IL-10, IL-12, IFN-γ and TNF-α in cPBMCs with

329   ConA stimulation were investigated. Our data indicated that the mRNA expression levels of all

330   cytokine genes were significantly increased at 4 h of ConA stimulation and then gradually

331   decreased with time. A longer incubation time was not possible in our study because the cell

332   numbers of isolated cPBMCs were insufficient.

333   Previous studies have investigated the negative effects of AgNPs on cytokines in human

334   PBMCs (Franco-Molina et al. 2016; Greulich et al. 2011; Shin et al. 2007). Uncoated AgNPs

335   (1.5 nm; 1 to 2.5 nm in diameter) significantly inhibited the phytohemagglutinin (PHA)-induced

336   IL-5, IFN-γ, and TNF-α production respectively at concentrations $\geq$ 10 μg/ml, $\geq$ 3 μg/ml, and $\geq$ 3

337   μg/ml in human PBMCs (Shin et al. 2007). It was reported that uncoated AgNPs (100 nm; 90 to

338   190 nm in diameter) at 0.0175 μg/ml can inhibit both PHA and ConA-induced IL-2 production in

339   human PBMCs (Franco-Molina et al. 2016). Furthermore, polyvinylpyrrolidone (PVP)-coated

340   AgNPs (75 ± 20 nm) of 5 to 20 μg/ml significantly increased the generations of IL-6 and IL-8

341   but significantly decreased the release of IL-1ra from human PBMCs, while PVP-coated AgNPs

342   did not affect the productions of IL-2, IL-4 and TNF-α (Greulich et al. 2011). As mentioned

343   above, the effects of AgNPs on cytokine production in human PBMCs remain inconclusive.

344   The mRNA expression levels of IL-4 and IFN-γ were mildly increased and that of IL-12

345   was seemingly unaffected at 4 h of C-AgNP$_{20}$ treatment. IL-4, as a polarizing Th2 cytokine, is

346   mainly produced by T cells (especially the Th2 subset) and mast cells, and it promotes the

347   differentiation of naïve T cells to Th2 cells, stimulates the growth and differentiation of B cells,

348   and induces class switching to IgE, which may promote allergic responses (Owen et al. 2013;

349   Tizard 2013b). IFN-γ, as a polarizing Th1 cytokine and a key mediator of cell-mediated immune

350   response, is produced by Th1 cells, cytotoxic T cells, and NK cells. The major functions of IFN-

351   γ are enhancement of Th1 differentiation, inhibition of Th2 differentiation, and activations of NK

352   cells and macrophages (Owen et al. 2013; Tizard 2013b). IL-12 is also a polarizing Th1 cytokine

353   and is produced by dendritic cells, monocytes, macrophages and B cells. IL-12 induces

354   differentiation of Th1 cells, increases IFN-γ production by T cells and NK cells, and enhances

355   NK and cytotoxic T cell activity (Owen et al. 2013; Tizard 2013b). This mixed pattern of Th1

356   and Th2 cytokines may be indicative of a mixed Th1/Th2 cytokine response of cPBMCs at 4 h of

357   C-AgNP$_{20}$ treatment. However, considering the significant decrease in the IL-12/IL-4 ratio, Th2

358   cytokine response is still predominant in cPBMCs following 4 h of C-AgNP$_{20}$ treatment. The

359   mRNA expression levels of IL-12 and IFN-γ were significantly decreased by 0.1 or 1 μg/ml C-

360   AgNP$_{20}$, and that of IL-4 was seemingly unaffected, in cPBMCs following 24 h of culture. The

361   significantly decreased Th1/Th2 (i.e., IFN-γ/IL-4 and IL-12/IL-4) ratios suggested that the

362   immune response of cPBMCs following 24 h of C-AgNP$_{20}$ treatment is Th2 biased.

363   Furthermore, the mRNA expression level of TNF-α was significantly decreased by 1 μg/ml

364   C-AgNP$_{20}$ after 24 h of culture. TNF-α is a cytokine specifically useful to measure the

365   inflammatory state of an animal and it is primarily produced by macrophages and both Th1 and

366   Th2 cells in response to both acute and chronic conditions (Eberle et al. 2018). Previous studies

367   have demonstrated that TNF-α is a contributing factor in the inflammatory response against

368   infection of intracellular micropathogens such as *Plasmodium* spp., *T. gondii*, *Leishmania major*,

369   and *Trypanosoma* spp. (Korner et al. 2010). Hence, our data indicate that C-AgNP$_{20}$ induced a

370   Th2 biased immune response and suppressed the mRNA expression level of TNF-α in cPBMCs,

371   which may weaken the cellular immune response and further impair the immunity against

372   intracellular organisms and virus. Similar Th2 immune response was observed in other studies

373   that evaluated the expression of cytokines in different cetacean tissues (Jaber et al. 2010). A

374   variety of infections caused by intracellular pathogens in cetaceans have been reported and may

375   be associated with the mass stranding events of cetaceans (Cvetnic et al. 2016; Domingo et al.

376   1990; Domingo et al. 1992; Dubey et al. 2007; Dubey et al. 2008; Mazzariol et al. 2016;

377   Mazzariol et al. 2017). In addition, previous studies suggested that Ag contamination exists in all

378   aspects of the marine ecosystem, and cetaceans may have been negatively affected by Ag

379   contamination (Becker et al. 1995; Caceres-Saez et al. 2013; Chen et al. 2017; Dehn et al. 2006;

380   Kunito et al. 2004; Li et al. 2018a; Mendez-Fernandez et al. 2014; Reed et al. 2015; Rosa et al.

381   2008; Seixas et al. 2009; Woshner et al. 2001). The direct correlation between the infection of

382   intracellular pathogens and the severity of Ag contamination in cetaceans is worth studying.

383   Following 4 h of 1 μg/ml C-AgNP$_{20}$ treatment, the mRNA expression level of IL-10 was

384   significantly decreased and that of IL-2 was mildly increased. In other words, mRNA expression

385   levels of IL-2 and IL-10 were respectively upregulated and downregulated by C-AgNP$_{20}$ in

386   cPBMCs. Subsequently, the mRNA expression level of IL-2 was significantly decreased, and

387   that of IL-10 seemingly unaffected, in cPBMCs following 24 h of treatment of 1 μg/ml C-

388   AgNP$_{20}$. IL-2, which is produced by activated T cells, can stimulate proliferation and

389   differentiation of T and B cells and activates NK cells (Owen et al. 2013; Tizard 2013b).

390   However, a growing body of evidence has indicated that IL-2 is crucial for the development and

391   function of regulatory T cells (Treg cells), which secrete effector cytokines, such as IL-10, to

392   control and modulate the immunity to self, neoplasia, microorganisms, and grafts (Owen et al.

393   2013; Pérol & Piaggio 2016). Considering the roles of IL-2 and IL-10 in immune tolerance, it is

394   speculated that C-AgNP$_{20}$ may play a significant role in peripheral immune tolerance by

395   regulating the balance between IL-2 and IL-10 (Pérol & Piaggio 2016; Veiopoulou et al. 2004).

396   The effect of C-AgNP$_{20}$ on the ConA-induced mRNA expression levels of the selected

397   cytokines in cPBMCs is mainly inhibitory. A previous study found that PVP-AgNPs (10, 25, 40,

398   45, and 110 nm in diameter) could bind to RNA polymerase, disturb the process of RNA

399   transcription, and thus decreased the overall RNA synthesis in mouse erythroid progenitor cells

400   (Wang et al. 2013). Although the down-regulation of mRNA expression levels may be associated

401   with decreased RNA synthesis due to the direct interaction between C-AgNP$_{20}$ and RNA

402   polymerase, it cannot fully explain the unaffected Th2 cytokines (IL-4 and IL-10) of cPBMCs in

403   this study. On the other hand, the ConA-induced proliferative activity of cPBMCs is inhibited by

404   0.1 and 1.0 μg/ml C-AgNP$_{20}$ (Li et al. 2018b), and this phenomenon may be associated with the

405   decreased mRNA expression levels of IL-2, IL-12, IFN-γ, and TNF-α and/or a suppressive effect

406   on DNA/RNA synthesis induced by ConA. Further investigation on the underlying mechanism

407   of AgNPs in cetacean leukocytes is important to ascertain the negative health impact caused by

408   AgNPs on cetaceans, and such investigation would improve the understanding of the potential

409   hazards of AgNPs to environmental condition and human health.

410   Furthermore, although the biodistribution of AgNPs or Ag in cetaceans is still

411   undetermined, previous *in vivo* studies of AgNPs by oral exposure in laboratory rats

412   demonstrated that the Ag concentration in the liver is approximately 10 times higher than that in

413   the blood or plasma (Lee et al. 2013; Loeschner et al. 2011; van der Zande et al. 2012). Based on

414   these animal models, it is presumed that the Ag concentrations in the blood of cetaceans may

415   range from 0.01 to 72.6 µg/ml (Chen et al. 2017; Li et al. 2018a). Although previous studies have

416   indicated that the status of AgNPs in the aquatic environment is complicated and variable (i.e.,

417   the concentrations of AgNPs and other Ag/Ag compounds are still undetermined in

418   cetaceans)(Levard et al. 2012; Massarsky et al. 2014), our data suggest that cetaceans may be

419   negatively affected by AgNPs.

420   **Conclusions**

421   The present study has demonstrated 1) the sublethal dose of C-AgNP$_{20}$ to cPBMCs (≤ 1

422   µg/ml), 2) the time kinetics of mRNA expression levels of selected cytokines in cPBMCs, and 3)

423   the inhibitory effect of C-AgNP$_{20}$ (0.1 and 1 µg/ml) on the mRNA expression levels of selected

424   cytokines of cPBMCs with evidence of Th2 cytokine bias. Taken together, C-AgNP$_{20}$ may

425   suppress the cellular immune response and thus inhibit the immunity against intracellular

426   microorganisms in cetaceans.

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Figure 1

Characterization of C-AgNP$_{20}$.

Representative TEM image of C-AgNP$_{20}$ in complete RPMI-1640.

100 nm
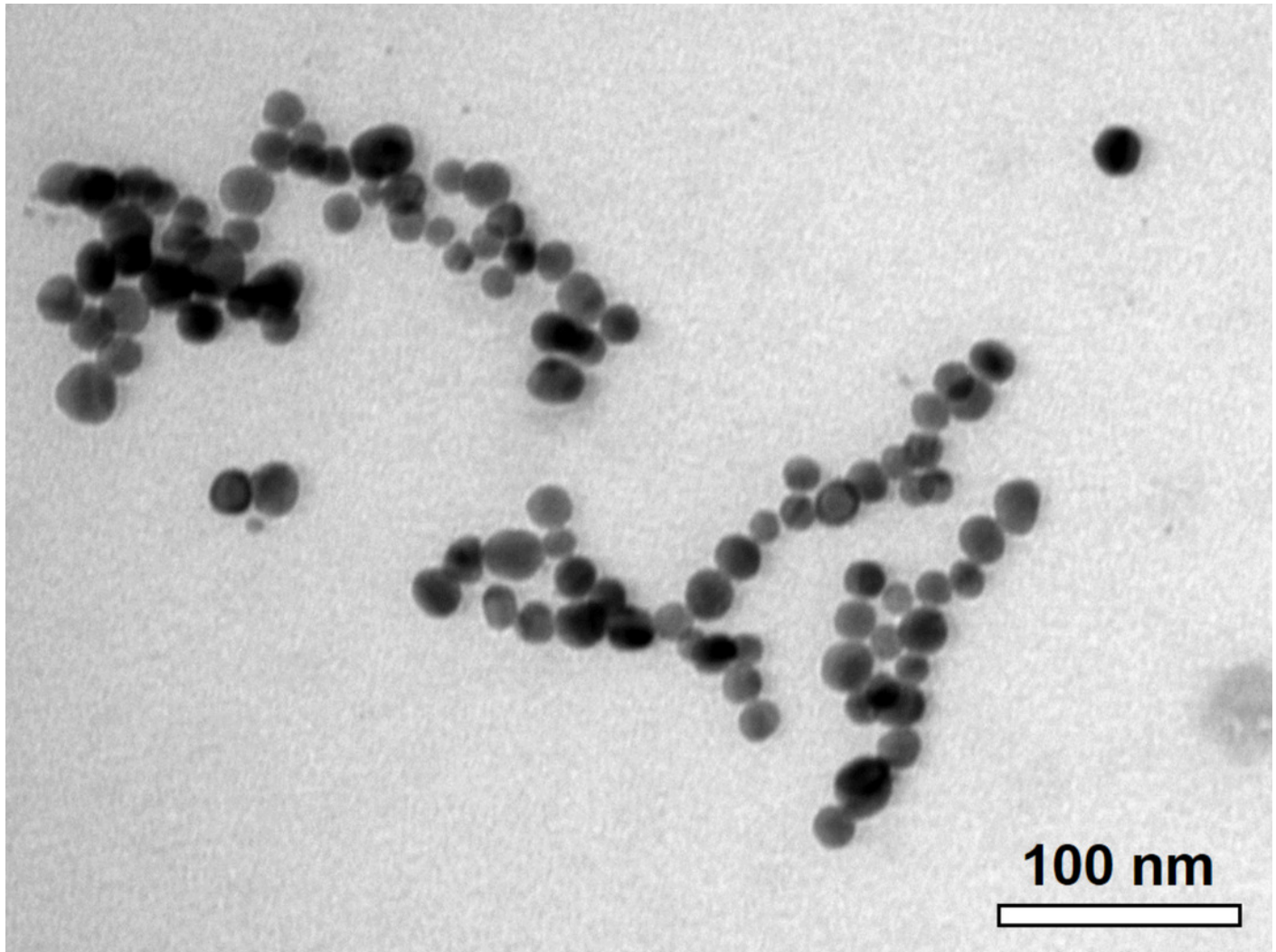
Figure 2

Cytotoxicity of C-AgNP$_{20}$ on cPBMCs after 24 h of culture with or without ConA.

(A) Ratio of apoptotic cPBMCs in percentage between treatment and control without ConA. (B) Ratio of late apoptotic/necrotic cPBMCs in percentage between treatment and control without ConA. (C) Ratio of apoptotic cPBMCs in percentage between treatment and control with ConA. (D) Ratio of late apoptotic/necrotic cPBMCs in percentage between treatment and control with ConA. The bar in the middle of the box represents the median, and the bottom and top of the box describe the first and third quartiles. The whiskers show the 75th percentile plus 1.5 times IQR and 25th percentile minus 1.5 times IQR of all data, and any values that are greater than these are defined as outliers and plotted as individual points. Asterisks indicate statistically significant differences from the control)$p < 0.05$, Kruskal-Wallis Test).

A Early apoptosis
(C-AgNP$_{20}$-treated for 24 h)

B Late apoptosis/necrosis
(C-AgNP$_{20}$-treated for 24 h)

C Early apoptosis
(C-AgNP$_{20}$/Con A-treated for 24 h)

D Late apoptosis/necrosis
(C-AgNP$_{20}$/Con A-treated for 24 h)
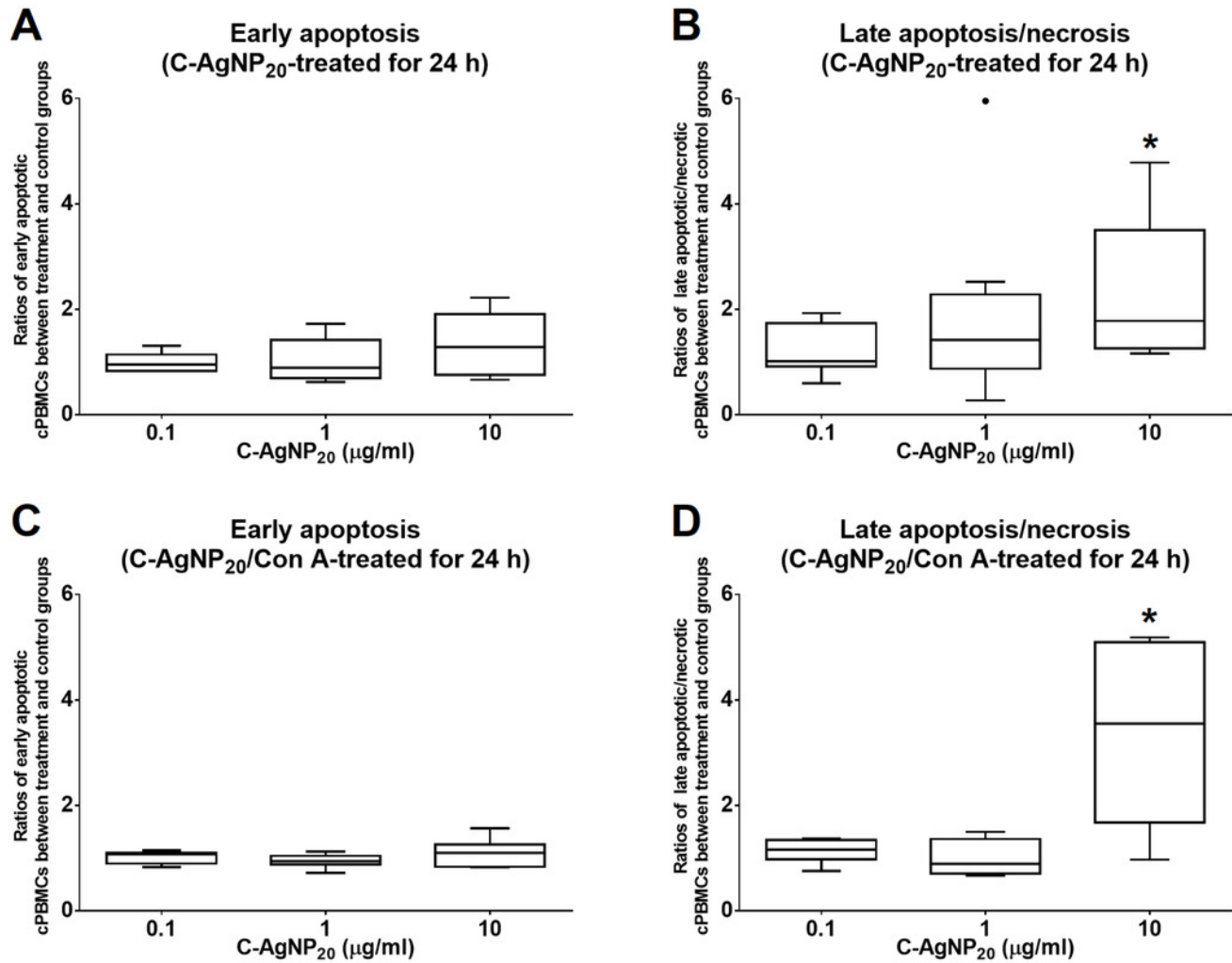
Figure 3

Time kinetics of mRNA expression levels of (A) IL-2, (B) IL-4, (C) IL-10, (D) IL-12, (E) IFN-γ and (F) TNF-α of cPBMCs with ConA.

The bar in the middle of the box represents the median, and the bottom and top of the box describe the first and third quartiles. The whiskers show the 75th percentile plus 1.5 times IQR and 25th percentile minus 1.5 times IQR of all data, and any values that are greater than these are defined as outliers and plotted as individual points. Asterisks indicate statistically significant differences from the control)$p < 0.05$, Kruskal-Wallis Test).
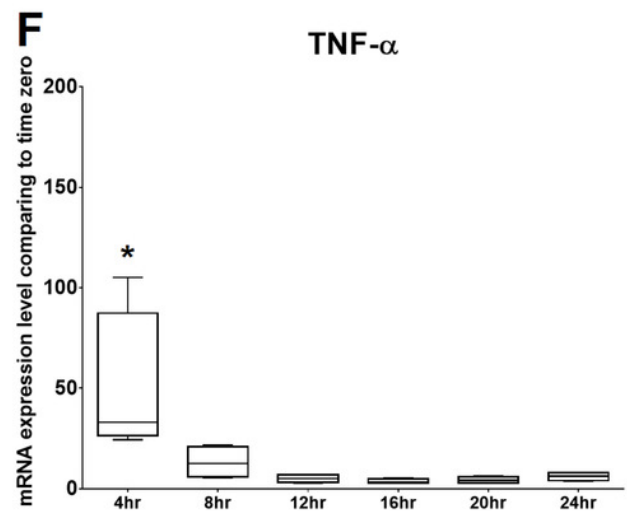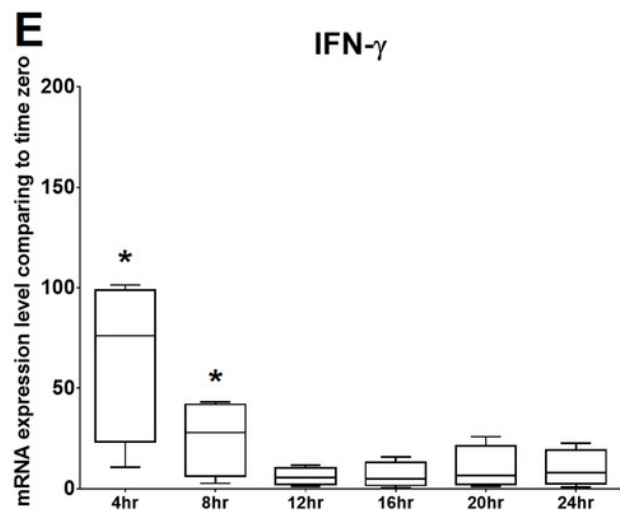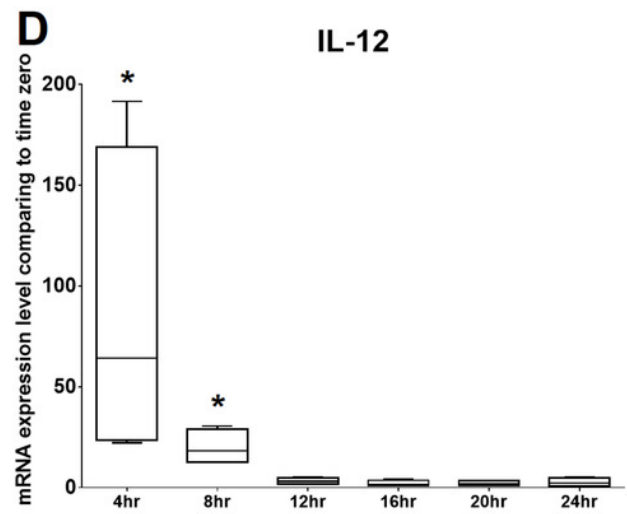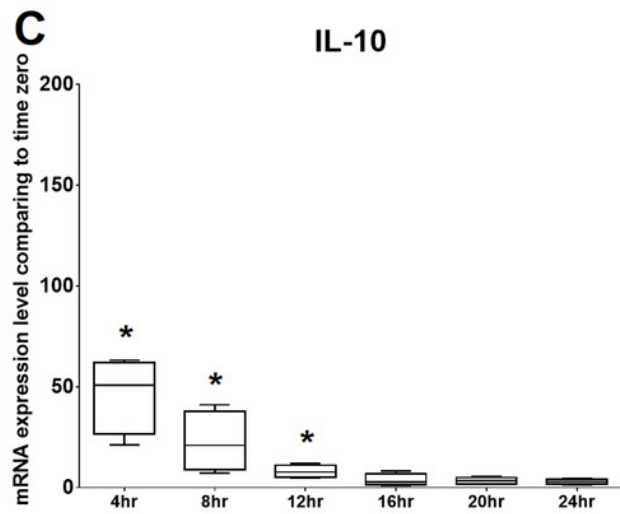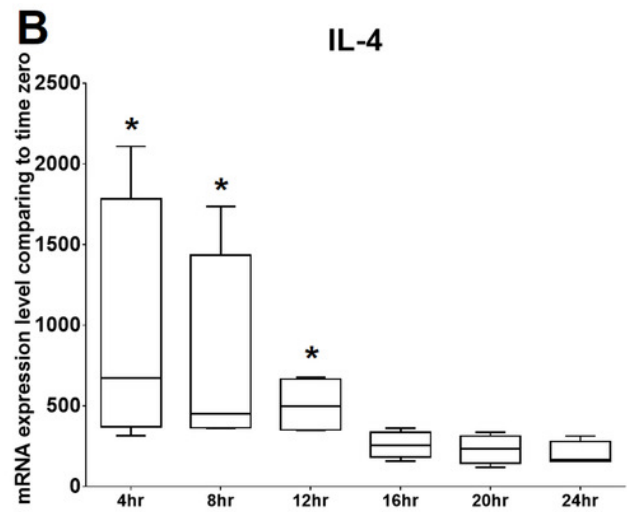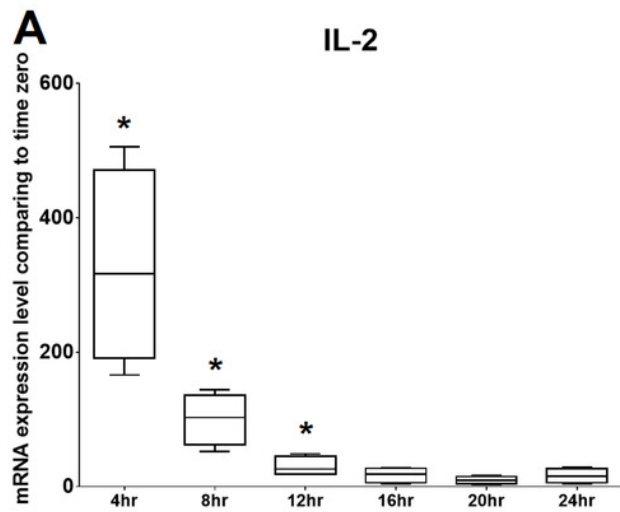
Figure 4

Effects of C-AgNP$_{20}$ on mRNA expression levels of (A) IL-2, (B) IL-4, (C) IL-10, (D) IL-12, (E) IFN-γ and (F) TNF-α of cPBMCs with ConA.

The bar in the middle of the box represents the median, and the bottom and top of the box describe the first and third quartiles. The whiskers show the 75th percentile plus 1.5 times IQR and 25th percentile minus 1.5 times IQR of all data, and any values that are greater than these are defined as outliers and plotted as individual points. Asterisks indicate statistically significant differences from the control)$p < 0.05$, Kruskal-Wallis Test).
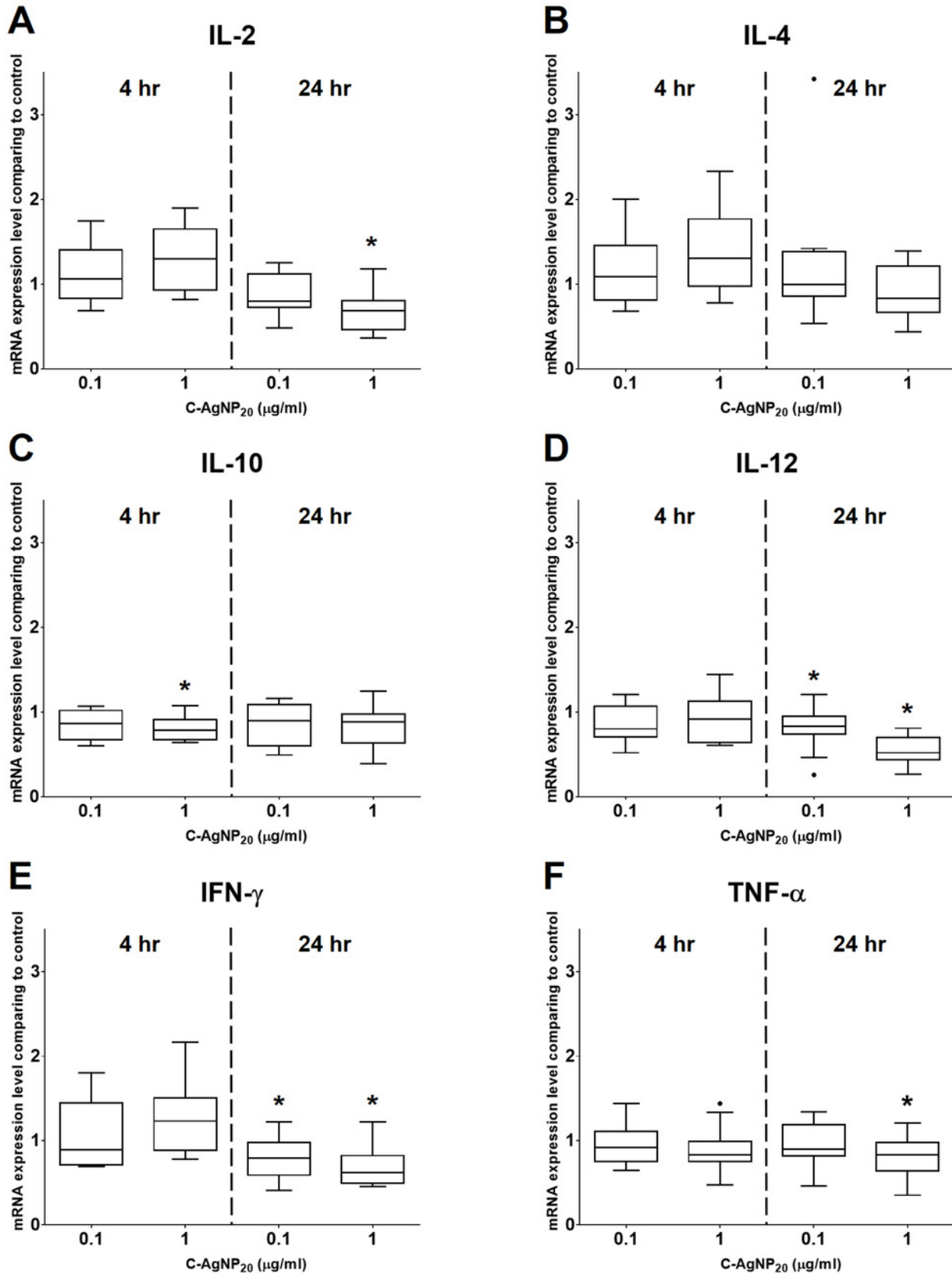
Figure 5

The ratios of Th1 and Th2 polarizing cytokines at 4 and 24 h of culture.

(A) Ratio of IFN-γ and IL-4; (B) Ratio of IL-12 and IL-4. The bar in the middle of the box represents the median, and the bottom and top of the box describe the first and third quartiles. The whiskers show the 75th percentile plus 1.5 times IQR and 25th percentile minus 1.5 times IQR of all data, and any values that are greater than these are defined as outliers and plotted as individual points. Asterisks indicate statistically significant differences from the control ($p < 0.05$, Kruskal-Wallis Test).
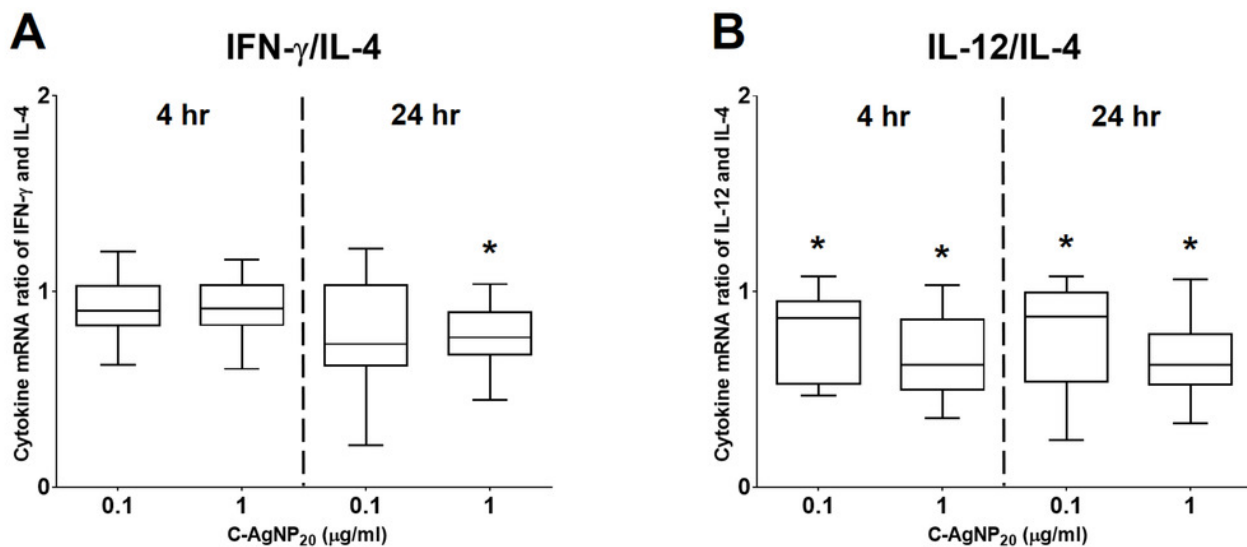
Table 1(on next page)

The size distribution and zeta potential of the 20 nm citrate-AgNPs (C-AgNP$_{20}$).

1   Table 1. The size distribution and zeta potential of the 20 nm citrate-AgNPs (C-AgNP$_{20}$)

Concentration (µg/ml)	100	500
Z-Average (nm)	26.62 ± 0.15	26.54 ± 0.08
Size (nm) (intensity)	30.27 ± 0.18 (100%)	29.64 ± 0.30 (100%)
Zeta potential (mV)	-38.97 ± 1.33	-44.2 ± 1.35
PdI	0.12 ± 0.00	0.11 ± 0.01

Results shown are means $\pm$ SD from three different lectures.

NP were suspended in 2nM citrate buffer and measurements performed at room temperature.

PdI, Poly-dispersity Index.

2

3

Table 2(on next page)

Primer sets used in this study and their efficiencies.

1 Table 2. Primer sets used in this study and their efficiencies

Gene	Accession number	Primer sequence(5'-3')	Efficiency (%)	R^2	Reference
GAPDH	DQ404538.1	CACCTCAAGATCGTCAGCAA GCCGAAGTGGTCATGGAT	100.97	0.9949	Chen et al. 2015
B2M	DQ404542.1	GGTGGAGCAATCAGACCTGT GCGTTGGGAGTGAACTCAG	93.32	0.9984	Chen et al. 2015
IL-2	EU638316	CATGCCCAAGAAGGCTACAGAATTG GTGAATCTTGTTTCAGATCCCTTTAG	91.92	0.999	Sitt et al. 2008
IL-4	EU-638315,	GGAGCTGCCTGTAGAAGACGTCTTTGC CTTCATTCACAGAACAGGTCATGTTTGCC	99.25	0.9982	Sitt et al. 2008
IL-10	AB775207	TGCTGGAGGACTTTAAGGGTTA ATGAAGATGTCAAACTCACTCATG	93.14	0.9986	Segawa et al. 2013
IL-12	EU638319	CAGACCAGAGCGATGAGGTCTTG GGGCTCTTTCTGGTCCTTTAAGATA	91.08	0.9999	Sitt et al. 2008
IFN-γ	EU638318	CAGAGCCAAATAGTCTCCTTCTACTTC CTGGATCTGCAGATCATCTACCGGAATTTG	92.42	0.9976	Sitt et al. 2008
TNF-α	EU638323	GAGGGAAGAGTTCCCAACTGGCTA CTGAGTACTGAGGTTGGCTACAAC	101.48	0.9934	Sitt et al. 2008

2