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Three-dimensional spheroid cultures of A549 and HepG2 cells exhibit different lipopolysaccharide (LPS) receptor expression and LPS-induced cytokine response compared with monolayer cultures

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Lipopolysaccharide (LPS) is a potent modulator of pathogen-induced host inflammatory responses. Lipopolysaccharide signaling to host cells is correlated with the expression of well-characterized LPS receptors. We have developed three-dimensional (3-D) cell cultures (spheroids) that are more representative of *in vivo* conditions than traditional monolayer cultures and may provide novel *in vitro* models to study the inflammatory response. In this work, we have compared F-actin organization, LPS-induced pro-inflammatory cytokine response and LPS receptor expression between spheroid and monolayer cultures from A549 lung epithelial cells and HepG2 hepatocytes. Significant junctional F-actin was seen at the cell–cell contact points throughout the spheroids, while monolayer cells showed stress fibers of actin and more prominent F-actin localized at the cell base. A time course of cytokine release in response to LPS showed that A549 spheroids secreted persistently higher levels of interleukin (IL)-6 and IL-8 compared with monolayer cultures. Unlike monolayer cultures, HepG2 spheroids responded to LPS by releasing a significant level of IL-8. We identified a significant increase in the expression of CD14 and MD2 in these spheroids compared with monolayers, which may explain the enhanced cytokine response to LPS. Thus, we suggest that 3-D spheroid cell cultures are more typical of *in vivo* cell responses to LPS during the development of inflammation and would be a better *in vitro* model in inflammation studies.

Keywords: 3-D culture, LPS, LPS receptor, pro-inflammatory cytokine, F-actin

Introduction

Lipopolysaccharide (LPS), also known as endotoxin, is the main component of the outer membrane of Gram-negative bacteria. It plays a critical role in inducing host activation of cytokine release and the inflammatory response.[1] Excessive inflammation may occasionally result in organ damage and severe sepsis, which is a serious clinical problem world-wide.[2] Despite extensive experimental and clinical studies over the last decades, there is still no effective specific therapy to treat septic patients.[2]

Lipopolysaccharide-induced cell signaling is mediated by the collaboration of plasma LPS-binding protein (LBP) and LPS receptors including CD14, MD2 and Toll-like receptor 4 (TLR4).[1,3,4] Plasma LBP binds LPS, which facilitates the transfer of LPS to cell surface-bound CD14. CD14, in turn, shuttles LPS to the transmembrane receptor TLR4 associated with MD2. Activation of TLR4 by LPS then triggers the intracellular mitogen-activated protein kinase (MAPK) and nuclear factor (NF)-κB signaling cascade, and induces cell responses to LPS.[1] The pro-inflammatory effects of LPS have been correlated with the expression of LPS receptors both *in vivo* and *in vitro*.[5–7] Furthermore, both

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CD14 and MD2 also exist in a soluble form and soluble CD14 (sCD14) can bind and transfer LPS to the cell-surface receptor complex in cells lacking membrane-bound CD14.[1,8] However, it has been indicated that membrane CD14, rather than sCD14, plays a greater role in LPS-induced responses in uroepithelial and Kupffer cells.[9,10]

In LPS-induced pathophysiological conditions, unrestrained inflammation may cause cardiovascular derangements and dysfunction of multiple organs, particularly in the lung, liver, kidney, heart and small intestine. The lung and liver are two major infection targets and sources of inflammatory mediators.[11–13] Lung epithelium and hepatocytes can respond to microbial components by regulation of tissue-specific functionality and cytokine production.[14–16] Cells derived from lung and liver cultured in two-dimensional (2-D) monolayers have been widely utilized to investigate the biological mechanisms involved in the pathogen–host interaction.[17,18] However, epithelial cells or hepatocytes grown as monolayer cultures undergo dedifferentiation, decrease in tight junctions and cell adhesion, loss of apical polarity and tissue function due to the disassociation of these cells from their native three-dimensional (3-D) tissue structure.[19–21]

Compared with monolayer cultures, the recently developed rotatory 3-D cultures (spheroids) exhibit a longer period of maintenance, enhanced cell–cell adhesion, diverse gene expression profile and improved functionality. Thus, the 3-D culture model may more closely resemble the *in vivo* condition and has been proposed as a better *in vitro* model in biomedical research.[20,22,23] So far, transformed and primary cells derived from the liver have been used to generate spheroids for hepatic function and toxicity studies.[24,25] Recently, 3-D cultures of airway and lung epithelial cells have also been explored and they display relatively *in vivo*-like characteristics in cell morphology, epithelial regeneration and functional performance.[20,21,26] However, the application of a 3-D culture model of hepatocytes and lung epithelial cells in inflammation is limited and the biological basis of such a model in response to pro-inflammatory stimuli is poorly understood.

The present study aimed to determine the cell–cell contacts by characterizing the filamentous-actin (F-actin) and E-cadherin distribution in spheroid and monolayer cultures from A549 lung epithelial cells and HepG2 hepatocytes. The time course of cell responses to LPS in production of pro-inflammatory cytokine interleukin (IL)-6 and IL-8 was compared between 3-D and 2-D cultures. In addition, the expression of molecules involved in the recognition and signaling to LPS, CD14, MD2 and TLR4 were examined in all the cultures. To the best of our knowledge, this is the first report providing evidence of up-regulation of LPS receptor expression and cytokine responses to LPS in 3-D cultures and suggests that such cell cultures may be a more accurate model to study LPS-induced pathogenesis in non-leukocyte cells.

Materials and Methods

Cell lines

The human tumorigenic lung epithelial cell line A549 (American Type Culture Collection, Teddington, UK) was cultured in RPMI 1640 (Sigma-Aldrich, Gillingham, UK) supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 200 nM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Paisley, UK). The human Caucasian hepatoma cell line HepG2 (European Collection of Cell Cultures, Salisbury, UK) was kept in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich) with the similar supplements as used in the A549 cell cultures. The cells were routinely subcultured using 0.05% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich) at a split ratio of 1 : 5. Subcultured cells were allowed to recover for at least 2 d before performing experiments to form 3-D cultures using gyrotatory shaking.

Spheroid culture

A549 and HepG2 spheroids were generated by a gyrotatory-mediated method as described previously.[27] Briefly, monolayer cultures were detached using 0.05% trypsin/0.02% EDTA solution and 1×10^6 cells in single-cell suspensions were added to each well of 6-well plates. The plates were placed on a gyrotatory shaker (New Brunswick, St Albans, UK) at 83 rpm for the first 24 h, and rotated at 77 rpm thereafter.

Scanning electron microscopy

Spheroids were collected and washed with phosphate-buffered saline (PBS), followed by routine fixation with 4% glutaraldehyde (TAAB Laboratories, Aldermaston, UK). After washing, the spheroids were dehydrated using a graded ethanol series (20, 30, 50, 70, 80 and 100%) and then transferred to mixtures of ethanol and hexamethyldisilazane (HMDS; Sigma-Aldrich) in a series of ratios at 2 : 1, 1 : 1 and 1 : 2, respectively. Following rinse in HMDS, the cells were sputter coated with gold and examined using a XL30 Environmental Scanning Electron Microscope (ESEM; Philips-FEI, Eindhoven, The Netherlands).

Fluorescence staining and confocal microscopy

Fluorescence staining of F-actin in A549 and HepG2 cell cultures was performed as described previously.[27] Briefly, the monolayer cells grown on a slide or cell aggregates of the spheroid culture were fixed in 90% ethanol for 20 min. Samples were washed in PBS, permeabilised for 30 min with 0.1% Triton X-100 (Sigma-Aldrich) and blocked for 30 min with 1% bovine serum albumine (BSA) (Sigma-Aldrich). F-Actin was stained for 20 min with 10 U/ml Phalloidin-Alexa 488 conjugate (Invitrogen) and mounted in VectaShield containing 4′-6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) that was used to stain nuclei in the cells. For immunofluorescence labeling of E-cadherin, monolayer or spheroid cultures were fixed using 2% paraformaldehyde for 15 min, followed by permeabilization in 0.1% Triton X-100 for 10 min. The cells were blocked with 1% BSA for 30 min and incubated with rabbit polyclonal antibody against E-cadherin (Abcam, Cambridge, UK) overnight at 4°C. The samples were stained with a fluorescein (FITC)-conjugated swine anti-rabbit immunoglobulin (Dako, Cambridge, UK) and then counterstained with DAPI. Confocal images of stained monolayer cells and spheroids were captured by an UltraView spinning disk confocal microscope and visualised using the Volocity software (Perkin Elmer, Waltham, MA, USA).

Immunoassay

The cell monolayers and spheroids grown in 6-well plates were incubated with fresh cell culture media containing 1.0 μg/ml of LPS (from *Escherichia coli* O111 : B4; Sigma-Aldrich) for up to 72 h. Cell culture supernatants were harvested at different time points and stored at −80°C until analysis for IL-6 and IL-8 concentrations by a sandwich enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (R&D Systems, Abingdon, UK). The cytokine concentrations were calibrated by total cellular protein amount measured by a protein assay reagent (Bio-Rad Laboratories, Hemel Hempstead, UK).

Reverse transcription and real time PCR analysis

Cellular RNA was isolated using TRIzol reagent (Invitrogen) and digested with RQ1 RNase-free DNase (Promega, Southampton, UK). RNA was reverse-transcribed using the Superscript II pre-amplification system (Invitrogen). The following primers, designed by the Invitrogen OligoPerfect™ Online Software (<http://tools.invitrogen.com>), were used to amplify the target genes: IL-6, forward 5′-TAC CCC CAG GAG AAG ATT CC, reverse 5′-TTT TCT GCC AGT GCC TCT TT; IL-8, forward 5′-CGA TGT CAG TGC ATA AAG ACA, reverse 5′-TGA ATT CTC AGC CCT CTT CAA AAA; CD14, forward 5′-GCC GCT GTG TAG GAA AGA AG, reverse 5′-AGG TTC GGA GAA GTT GCA GA; MD2, forward 5′-AAT CTT CCA AAG CGC AAA GA, reverse 5′-GGG CTC CCA GAA ATA GCT TC; TLR4, forward 5′-TGA GCA GTC GTG CTG GTA TC, reverse 5′-CAG GGC TTT TCT GAG TCG TC; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5′-ACA GTC AGC CGC ATC TTC TT, reverse 5′-GAC AAG CTT CCC GTT CTC AG. cDNA of target genes was amplified using a SYBR® GreenER™ qPCR SuperMix Kit (Invitrogen) on the iCycler Thermal Cycler (Bio-Rad Laboratories).

Flow cytometry

Monolayer and spheroid cultures were dissociated by 10–20 min of treatment with Accutase (Sigma-Aldrich) that leaves most of the cell surface molecules intact.[27] The single-cell suspension containing 1×10^5 cells was blocked with 1% BSA in PBS. To assess membrane-bound CD14 expression, cells were stained with a phycoerythrin (PE)-labeled mouse monoclonal anti-CD14 IgG2a (Dako). An isotypic murine IgG2a (eBioscience, Hatfield, UK) was used as negative control for CD14 detection. Similarly, cell surface TLR4 expression was examined using a PE-mouse monoclonal anti-TLR4 IgG2a (eBioscience). The expression of MD2 was investigated using an indirect method by first incubating the cells with a rabbit polyclonal anti-MD2 (Abcam) and subsequent staining with a FITC-conjugated swine anti-rabbit Immunoglobulin (Dako). For the negative control in MD2 tests, the primary antibody was omitted. The data were collected on a FACSVantage flow cytometer (Becton Dickinson, Oxford, UK) and histograms were generated using the software WinMDI v.2.9. Relative cell surface LPS receptor expression levels were presented using the geometric mean fluorescence intensity (GMFI) ratio and calculated by the formula:

$$\text{GMFI ratio} = \frac{\left(\begin{array}{c}\text{Experimental GMFI} \\ -\text{negative control GMFI}\end{array}\right)}{\text{negative control GMFI}} \times 100\%$$

Statistical analysis

Results were presented as mean ± SEM. Data were analyzed using Student's two-tailed *t*-test and differences were considered significant at $P < 0.05$.

RESULTS

3-D culture formation and junctional marker distribution in A549 and HepG2 spheroids

Using the gyrotatory technique, A549 or HepG2 cells in suspensions started to fuse after 2 h of plating. During the initial culture time, smaller and irregular aggregates continued to join together to form bigger aggregates. After 3 d for A549 (Fig. 1A) and 5 d for HepG2 cells (Fig. 1B), sphere-like 3-D aggregates (spheroids) of 50–100 μm in diameter were formed. The cells in 3-D cultures are in close contact with each other. It has been shown that the substance exchange between cells inside spheroids and the external environment is hampered when the size of 3-D culture exceeds 250 μm in diameter.[28] Therefore, 3-d A549 spheroids and 5-d HepG2 spheroids were used in the experiments. For comparison, subconfluent A549 and HepG2 monolayer cultures with the same passage number as spheroids were used.

Fluorescence staining showed cortical F-actin distribution (green, pentagons) in cells within both A549 and HepG2 spheroids (Fig. 1C,D). Confocal microscopy further demonstrated that junctional F-actin (arrows) was significantly organized at the points of cell-cell contact throughout the spheroids (Fig. 1E,F), indicating that it stabilizes and strengthens intercellular adhesive interactions.[27,29] In contrast, A549 and HepG2 monolayer cells showed a different F-actin distribution pattern. The average height of a confluent monolayer culture of A549 or HepG2 cells was 10–13 μm examined by confocal microscopy scanning. Stress fibers of F-actin in the cytoplasm were found at the middle section of monolayers (5–7 μm from the top; Fig. 1G,H). Figure 1I,J presents cell base sections (10–13 μm for the top) of identical areas as Figure 1G,H, respectively. The results show that more prominent F-actin formation in monolayer cells localized at the cell base where cells attached to the surface of the tissue culture plates, instead of the cell–cell contact points.

Confocal microscopy further indicated that 3-D cultures of A549 and HepG2 cells had brighter and more continuous E-cadherin labeling on the cell surface and at the cell–cell contact sites (Fig. 1K,L) compared with 2-D cultures, which exhibited scattered E-cadherin staining throughout the cytoplasm and only limited E-cadherin expressed at the junctions (Fig. 1M,N). There was no significant E-cadherin staining in control cells when the primary antibody was omitted (data not shown).

Enhanced cell response to LPS in cytokine production from spheroids

To compare the LPS-induced inflammatory response between 2-D and 3-D cultures, the monolayers and

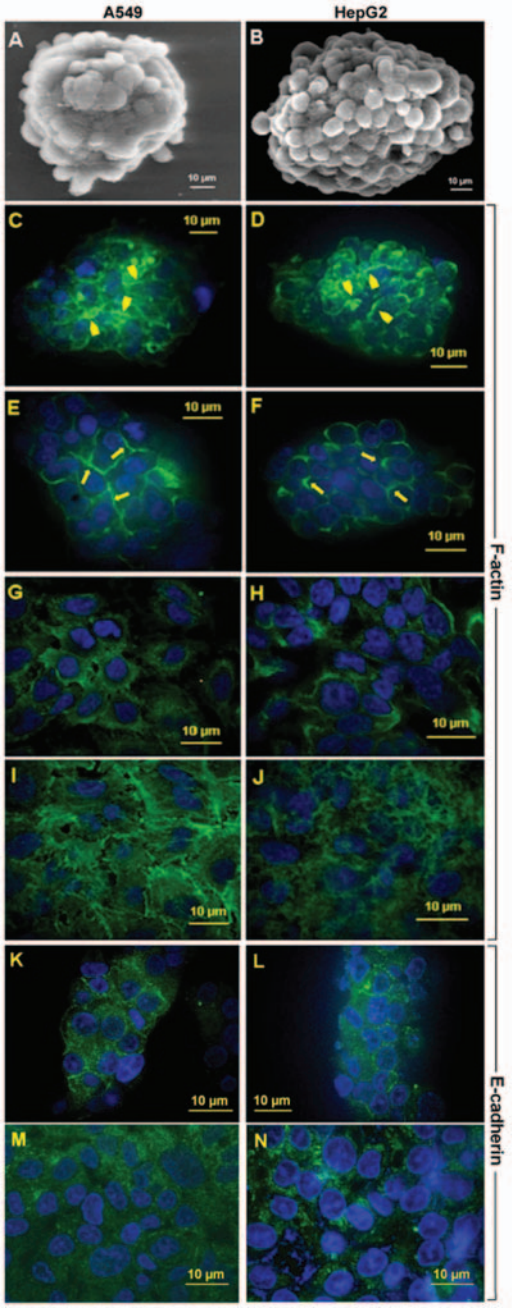

Fig. 1. Formation of A549 and HepG2 spheroids and comparison of junction markers (F-actin and E-cadherin) between spheroid and monolayer cultures. Scanning electron microscopy showed that A549 cells in single-cell suspensions formed 3-D spheroids after 3 d (A) and HepG2 spheroids formed after 5 d (B). F-actin staining (green) in A549 (C) and HepG2 (D) spheroids showed cortical distribution (pentagons) in spheroid cells. Confocal micrographs showed significant junctional F-actin (arrows) at the points of cell–cell contact throughout A549 (E) and HepG2 (F) spheroids. Cells grown in A549 (G) and HepG2 (H) monolayers formed F-actin stress fibers observed at the middle section of the monolayers by confocal microscopy. Large amount of F-actin cytoskeleton distributed at the cell base area contacting the dish surface (I, A549; J, HepG2). Immunofluorescence staining and confocal microscopy showed different distribution pattern of E-cadherin (green) between spheroid (K and L) and monolayer (M and N) cultures (K and M, A549; L and N, HepG2). The nuclei staining with DAPI (blue) was used to show individual cells (C-N).

spheroids were stimulated with LPS at a concentration of 1.0 μg/ml for up to 72 h. It was found that LPS treatment did not significantly affect cell viability and cell viability was always >90% during the periods of experiments (data not shown). As shown in Figure 2A, a significant LPS-induced IL-6 secretion was found in both A549 monolayers and spheroids compared with untreated cells during 3–72 h ($P<0.05$). The A549 spheroids treated with LPS released a markedly enhanced level of IL-6 in comparison with LPS-treated monolayer counterparts between 24 h and 72 h (24 h, $P=0.002$; 48 h, $P=0.042$; 72 h, $P<0.001$). Moreover, IL-6 secretion level in LPS-stimulated spheroids remained persistent between 6–72 h. In contrast, A549 monolayers exposed to LPS presented a time-dependent decrease in IL-6 release between 6–72 h, indicating that the cells grown in monolayers gradually lose cytokine responsiveness to LPS.

Similarly, IL-8 secretion from both types of A549 cultures was induced by LPS during 6–72 h (Fig. 2B) and an up-regulation of LPS-induced IL-8 secretion between 24 h and 72 h was observed in the spheroids relative to monolayers (24 h, $P=0.014$; 48 h, $P=0.14$; 72 h, $P=0.025$). We found a significantly higher level of IL-8 secretion compared with IL-6 in A549 cells, which is consistent with previous reports.[30,31] In addition, the secretion of IL-8 in untreated control cells gradually increased over time in contrast to IL-6 that showed

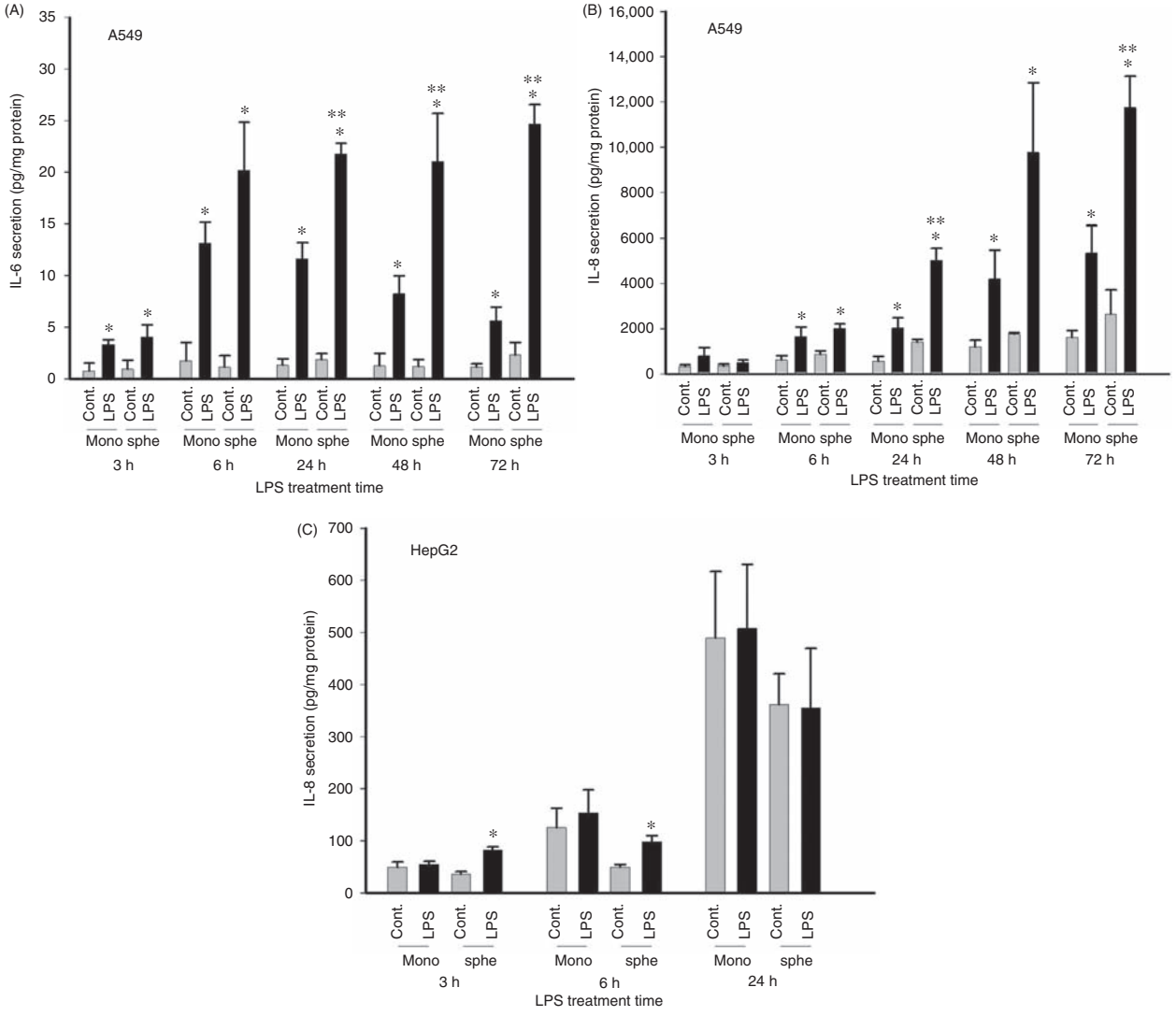

Fig. 2. Comparison of LPS-induced pro-inflammatory cytokine secretion between monolayers and spheroids from A549 and HepG2 cells. Monolayer and spheroid cultures were treated with 1.0 μg/ml of LPS for up to 72 h. Cell culture supernatants were collected at indicated time points and concentrations of IL-6 secreted from A549 cells (A) and IL-8 from A549 (B) and HepG2 (C) cells were determined by ELISA. Data are from three to four independent experiments with duplicate samples. *$P<0.05$ versus untreated cells of similar culture type at the same time point; **$P<0.05$ versus LPS-treated monolayer control at the same time point. Mono, monolayer; Sphe, spheroid.

relatively consistent levels during the period of experiments. The difference in secretion kinetics between IL-6 and IL-8 shown here is in agreement with one recent study from Mochizuki and co-workers[31] and may be caused by different mechanisms involved in cytokine regulation.

HepG2 cells had only trace amount of IL-6 secretion and LPS did not modulate IL-6 release from either monolayer or spheroid cultures. In addition, LPS failed to induce IL-8 release from HepG2 monolayers during 3–24 h treatment (Fig. 2C). Although the basal IL-8 secretion level in untreated HepG2 spheroids was not significantly different from monolayers over time, LPS stimulation did raise the IL-8 level in HepG2 spheroids compared with the untreated spheroid control after 3–6 h (3 h, $P = 0.005$; 6 h, $P = 0.022$). There was no significant IL-8 induction by LPS in HepG2 spheroids thereafter.

We then tested if the enhanced LPS-induced cytokine release in spheroids is due to regulation at mRNA level. Figure 3A shows that IL-6 mRNA in A549 spheroids was increased 12-fold ($P = 0.011$ versus untreated control) after 3 h of LPS stimulation, while the induction in monolayers was only 5.4-fold ($P = 0.013$ versus untreated). Lipopolysaccharide brought about a 52.3-fold increase in IL-8 mRNA expression in A549 spheroids but a 27.1-fold induction in A549 monolayers (both $P < 0.0001$ versus untreated; Fig. 3B). Figure 3C shows that LPS treatment led to a 2.5-fold induction of IL-8 mRNA expression in HepG2 spheroids compared with untreated control ($P = 0.023$) and there was no change in IL-8 mRNA level between LPS-treated and untreated HepG2 monolayers, which coincides with the finding of IL-8 antigen secretion.

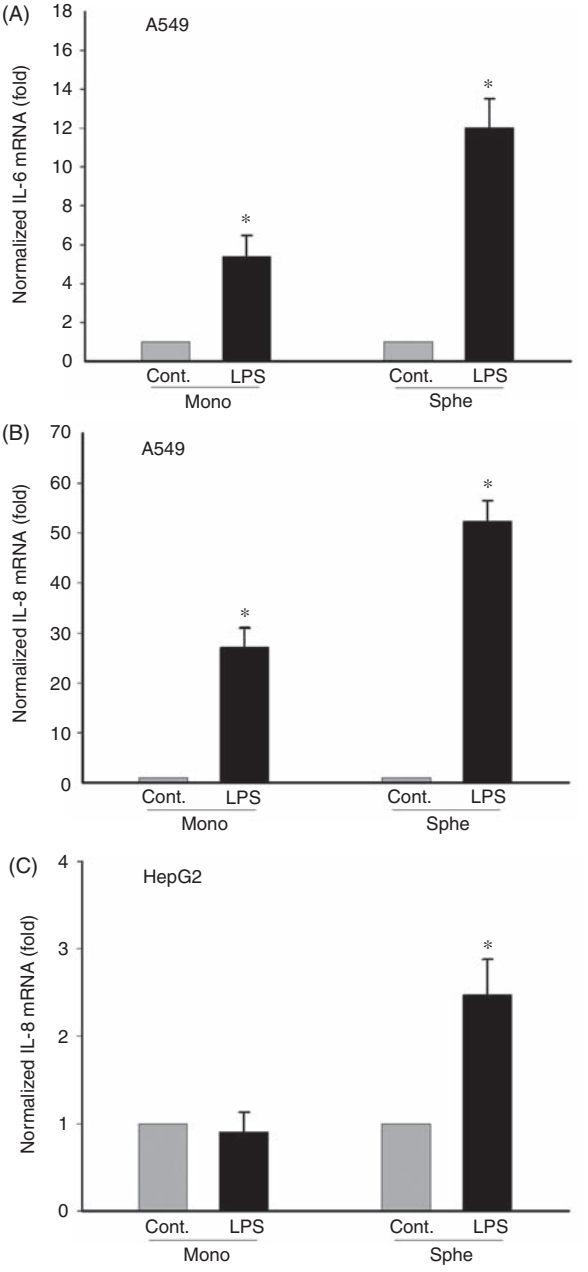

Fig. 3. Lipopolysaccharide-induced cytokine mRNA levels in monolayer and spheroid cultures. After 3 h of treatment with 1.0 μg/ml LPS, cellular RNA was isolated, reverse-transcribed, and cDNA amplified by real-time PCR. Cytokine mRNA expression was normalized by GAPDH mRNA as an internal control. Quantitative data showed relative mRNA levels of IL-6 in A549 cells (A) and IL-8 in A549 (B) and HepG2 cells (C) from three separate experiments. *$P < 0.05$ versus untreated cells of similar culture type.

Variable up-regulation of LPS receptor expression in spheroids

We compared the levels of LPS receptor expression in both 3-D and 2-D cultures. The results indicated that CD14 and MD2 transcript levels in A549 spheroids were markedly higher than monolayers (CD14, $P = 0.014$; MD2, $P = 0.018$; Fig. 4A), although the increase in TLR4 mRNA expression had no statistical significance. In HepG2 cells, only CD14 mRNA level showed increased expression in spheroids relative to monolayers ($P = 0.005$), while mRNA specific to MD2 or TLR4 had no difference between the two types of culture (Fig. 4B).

Flow cytometric histograms showed increased surface CD14 and MD2 proteins in cells of A549 spheroids (Fig. 5A,C), which can be confirmed by the statistical analysis of GMFI ratio (CD14, $P = 0.030$; MD2, $P = 0.015$; Fig. 5B,D). There was no significant difference of surface TLR4 expression between cells from A549 spheroids and monolayers (data not shown). In HepG2 cell cultures, we found no difference in cell surface CD14 or TLR4 between the spheroids and monolayers. However, a significant elevation of surface MD2 protein expression in HepG2 spheroid cells was detected compared with monolayer cells ($P = 0.038$; Fig. 5E,F).

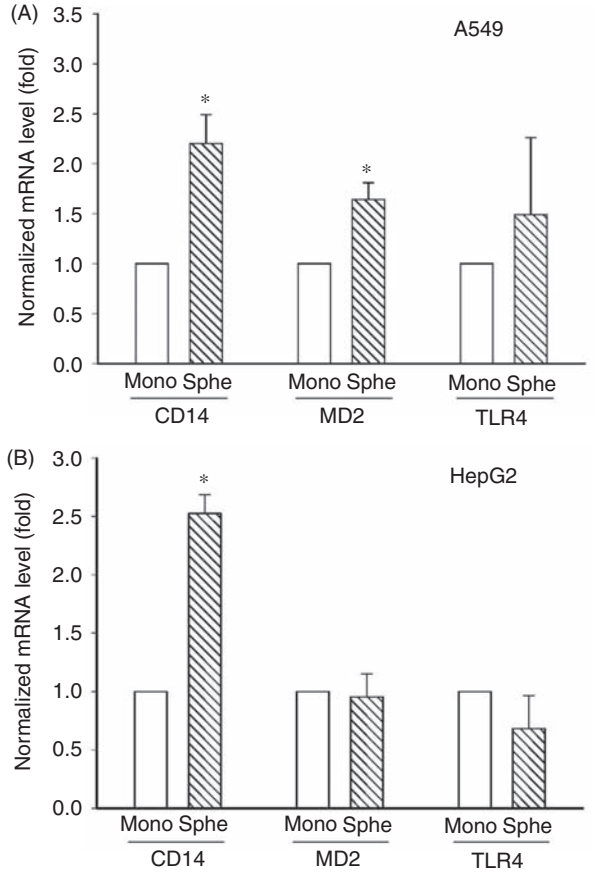

Fig. 4. Quantitative RT-PCR analysis of LPS receptor mRNA expression in monolayers and spheroids from A549 (A) and HepG2 (B) cells. GAPDH mRNA level was used as an internal control. Results are obtained from three independent experiments. $*P < 0.05$ versus monolayer control.

Discussion

Current *in vitro* studies of microbial pathogenesis in non-leukocyte cells are predominantly based on monolayer culture models. However, several lines of evidence have documented significant functional differences between monolayer and 3-D cultures during the development of inflammation.[20,32,33] This study has shown that 3-D spheroid cultures may be a more *in vivo*-like model to study inflammatory responses.

Dynamic remodeling of actin filaments is critical in cell maturity, tissue structuring and functionality.[34] The formation of cortical actin bands and junctional F-actin at the points of cell–cell contact has been associated with intercellular signaling communication and functional performance in different cells.[29,35] Additionally, epithelial cells and hepatocytes cultured in monolayers become flattened and display reductions in cell–cell contact and subsequent tissue-specific functions, which are different from their *in vivo* characteristics.[19,20,23,29] In the present study, significant cortical F-actin and junctional F-actin were observed in the cells of 3-D A549 and HepG2 cultures. However, their monolayer counterparts show stress fibers of actin cytoskeleton in the cytoplasm. Notably, there is marked F-actin at the cell base area but not at the cell–cell contact points. The decreased junctional F-actin in monolayer cultures may explain the diminished cell–cell adhesion and communication.[29,35] E-Cadherin is one of the most well-studied adherin junction proteins that is important in the maintenance of cell adhesion, tissue integrity and functionality in epithelium, as well as in hepatocytes.[20,36] Similar to F-actin, E-cadherin levels were significantly enhanced at the cell–cell contact points in A549 and HepG2 spheroids compared with their monolayer controls. We also found that the dissociation of spheroids by trypsin/EDTA or Accutase is much more difficult and takes longer than for monolayers, implying that the contact between cells in 3-D cultures is more stringent than that in 2-D cultures. Our findings suggest that the 3-D culture model is more close to primary tissue, since dispersal of the solid tissue into a single-cell suspension is demanding due to the tight connection between cells within their native 3-D tissue structure.[37]

One of the means by which the lung epithelial cells and hepatocytes respond to a pathogen is to release a number of inflammatory mediators, including pro-inflammatory cytokines.[14,16] Interleukin-6 and IL-8 are two important cytokines involved in the regulating network of host immune systems. IL-6 is a pro-inflammatory cytokine important in fever and the acute phase reaction, while IL-8 acts as a chemical signal attracting neutrophil migration to the site of inflammation. Here, we used these two cytokines as markers to investigate the inflammatory response to LPS in A549 and HepG2 cell cultures. We found significantly up-regulated cytokine secretion induced by 24–72 h of LPS stimulation in A549 spheroids compared with monolayers. The HepG2 monolayer cells show no response to LPS, but 3-D cultures recover the hepatocyte ability to respond with IL-8 release after 3–6 h of treatment with the stimulus. A previous report also showed that LPS can stimulate the release of adrenocorticotropic hormone from mouse pituitary cell spheroids but not monolayers.[33] Interestingly, the disappearance of IL-8 induction in HepG2 spheroids after 24 h of LPS stimulation is possibly due to the LPS uptake/detoxification by hepatocytes.[38] Furthermore, our recent experiments observed that peptidoglycan from *Staphylococcus aureus* can cause higher levels of cytokine secretion from spheroids of A549 and pharyngeal epithelial cells than monolayer cultures (Liu *et al*., unpublished data), suggesting that the differences in cytokine responses between 3-D and 2-D cultures are not specific to LPS.

Our data are in general agreement with a recent study documenting that the secretion of several inflammatory

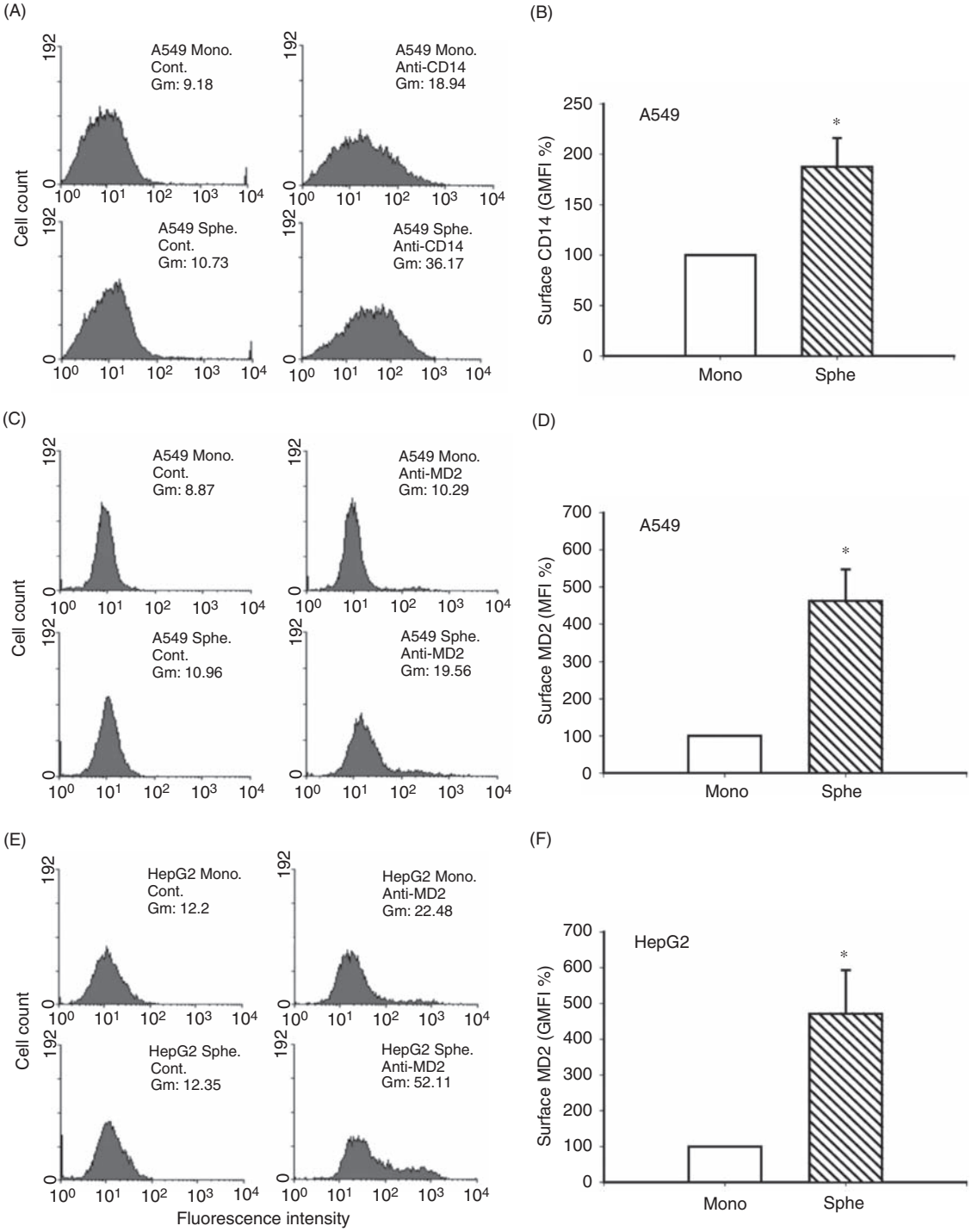

Fig. 5. Flow cytometric analysis of cell-surface LPS receptor expression of cells from monolayers and spheroids. Histograms of CD14 and MD2 expression in A549 cell cultures were shown in (A) and (C), respectively (Gm, geometric mean). (E) shows the histograms of MD2 expression in HepG2 cell cultures. (A, C and E) are representatives from three independent experiments. Statistical comparison of LPS receptor levels between monolayer and spheroid cells presented in geometric mean fluorescence intensity (GMFI) ratio was displayed in (B) CD14 for A549, (D) MD2 for A549 and (F) MD2 for HepG2, respectively. $*P < 0.05$ versus monolayer control.

mediators, including tumor necrosis factor (TNF)-α, IL-6, IL-12 and IL-10, are significantly higher in 3-D A549 aggregates than monolayers after 6 h of infection with *Pseudomonas aeruginosa*.[20] In order to show the kinetics of pro-inflammatory response to LPS, the time course (up to 72 h) of cytokine release from 2-D and 3-D cultures was examined in our experiments. The results demonstrate that A549 spheroids have enhanced and

sustained cytokine response to LPS compared with monolayers. Production of TNF-α was also tested, but no significant LPS-induced TNF-α release was found in any of the cultures used. Possibly, the TNF-α secretion in 3-D A549 aggregates stimulated by *P. aeruginosa*[20] is due to other bacterial component(s), rather than LPS. Taken together, the results from this study and another[20] indicate that the overall level of inflammatory cytokine response to bacterial components in 3-D cultures is higher than monolayer cultures.

It has been shown that primary tissue cultures derived from human bronchial and conjunctival epithelium have greater up-regulation in the expression of inflammation-related molecules than corresponding transformed cell lines grown in monolayer cultures when treated with the pro-inflammatory stimulus TNF-α, IL-1β or interferon-γ.[32,39] Importantly, persistently high levels of pro-inflammatory cytokines in the circulation have been repeatedly reported in animal models and patients with severe inflammatory conditions.[40,41] Therefore, we suggest that the 3-D cultures with up-regulated and sustained inflammatory response to stimuli are more like the primary tissue and represent more *in vivo*-like cell characteristics during the development of inflammation compared with 2-D cultures.

Lipopolysaccharide-induced inflammatory responses are mediated via ligand binding to a receptor complex and initiation of inflammatory signaling pathways. It is known that lung epithelium and hepatocytes express LPS receptors.[14,15] The regulation of LPS receptors during inflammation has been studied and both up- and down-regulation of LPS receptor expression in cells from different origins have been reported.[18,42–44] In addition, it is clearly established that the expression levels of CD14, TLR4 or TLR4–MD2 complex correlate with the systemic immune responsiveness. For instance, CD14 and TLR4–MD2 knockout mice have reduced LPS susceptibility for cytokine release, immune cell activation and lethal toxicity.[5,6] *In vitro* studies also revealed that the levels of cell-surface CD14 and TLR4 are in line with the inducibility of LPS in pro-inflammatory cytokine production in murine peritoneal macrophages and human bladder epithelial cells.[7,10]

These findings led us to speculate that the enhanced cytokine response to LPS in 3-D cultures may be due to the regulation of LPS receptor(s). Indeed, A549 and HepG2 cells grown as spheroids show variably increased LPS receptor expression compared with monolayer cells. The regulation of membrane CD14 and MD2 in A549 cells from spheroids is totally consistent with the mRNA expression profile. The HepG2 spheroids present an increase in surface-bound MD2 with an unchanged MD2 transcript level compared with HepG2 monolayers, suggesting a possible post-transcriptional gene regulation.[44] Frleta and co-workers[44] also found a CD40-mediated up-regulation of TLR4–MD2 complex at the cell surface without the mRNA change in murine dendritic cells. On the other hand, although the molecular mechanism involved in the difference of LPS receptor expression between 3-D and 2-D cultures need to be further investigated, it has been shown that the expression of membrane-bound CD14 in human gingival fibroblasts is decreased after subculturing and recovered to the highest level at cell confluent stage as monolayers, indicating that the rate of cell–cell contact may affect CD14 expression.[45] The present study showing that 3-D cultures with improved cell–cell contact enhance the expression of CD14 and MD2 molecules further support the concept.[45]

Conclusions

Our results demonstrate that A549 and HepG2 cells grown as spheroids exhibit enhanced cell–cell contact, LPS receptor expression and pro-inflammatory cytokine responses to LPS compared with their monolayer cultures. We suggest that the 3-D cultures more closely resemble *in vivo* and primary tissue inflammation responses. Further studies are warranted to delineate the potential difference in the intracellular signaling mediated by LPS between spheroids and monolayers. Although the 3-D model cannot totally mimic the complexity of the *in vivo* tissue environment, it could be used as an alternative to study specific aspects related to infection pathogenesis in lung epithelial cells and hepatocytes. It will be also interesting to use a recently developed ultrasound trap-based technique to form epithelial aggregates in a unilayer or thin disc-like format in suspension away from the influence of solid substrata[27,46] and investigate the biological phenomenon of such an epithelial culture. Better understanding of the biological basis of the novel culture model will be valuable for its potential application in studies of molecular mechanisms of inflammatory disease.

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