Rhinovirus exposure impairs immune responses to bacterial products in human alveolar macrophages.

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This article has an online data supplement.
Abbreviation:
LPS lipopolysaccharide
LTA lipoteichoic acid
RV rhinovirus
Abstract

Rhinovirus infection is responsible for considerable morbidity and mortality as the major cause of exacerbations of asthma, and is also known to induce exacerbations of cystic fibrosis and chronic obstructive pulmonary disease. Exacerbations of these diseases are also frequently associated with bacterial and atypical bacterial infection. Alveolar macrophages are the major immune cells in the airways and are important in defence against bacterial infections. Therefore we investigated whether rhinovirus modifies cytokine release, the pattern recognition receptor (PRR) expression and phagocytosis by human alveolar macrophages in response to bacterial products. Viable rhinovirus was detected in macrophages up to 3 days after exposure and viral RNA expression persisted to 10 days. Infectious but not UV-inactivated rhinovirus increased TNF-\(\alpha\) and IL-8 release by macrophages. In contrast, infectious rhinovirus impaired lipopolysaccharide and lipoteichoic acid induced TNF-\(\alpha\) and IL-8 secretion by macrophages. Rhinovirus-induced impairment of macrophage antibacterial immune responses did not involve IL-10, PGE\(_2\), or down-regulation of TLR2. Furthermore the macrophage phagocytic response to labelled bacterial particles, but not to latex beads, was impaired. In conclusion, we have identified impairment of cytokine responses to bacterial lipopolysaccharide and lipoteichoic acid by alveolar macrophages in response to infectious rhinovirus. Virus induced impairment of antibacterial host defence has important implications in the pathogenesis of exacerbations of respiratory diseases.
Introduction

Acute exacerbations of the chronic respiratory disorders asthma, cystic fibrosis and chronic obstructive pulmonary disease (COPD) are the major cause of morbidity, mortality and health care costs related to these diseases. The pathogenesis of acute exacerbations is poorly understood and therefore a better assessment of the underlying mechanisms will help to develop new therapeutic strategies.

Viral respiratory tract infections are the major precipitants of asthma exacerbations in both children[1-4] and adults [5-10]. There is increasing evidence that exacerbations of chronic obstructive pulmonary disease (COPD)[11;12] and cystic fibrosis [13] are also induced by viral infections. Of the different virus types associated with exacerbations of each of these diseases, rhinoviruses (RV) account for approximately two thirds of viruses identified [4-7;10;14].

Bacterial infection is also associated with the majority of exacerbations of COPD [15] and cystic fibrosis [16]. A recent study has identified viral and bacterial co-infection in one quarter of COPD exacerbations, and reported that exacerbations with co-infection were of increased severity [17]. Atypical bacteria have also been shown to be associated with exacerbations of COPD [18-20]. The role of bacterial infections in asthma exacerbations is more controversial, but asthmatics have recently been shown to have increased susceptibility to invasive bacterial infection [21], and importantly atypical bacterial infection was reactivated in virus induced asthma exacerbations[22] and related to exacerbation frequency [23]. Finally we have recently shown that an antibiotic therapy active against atypical bacteria is effective in the treatment of asthma exacerbations [24].

The possibility arises that virus-induced exacerbations may be further worsened by the occurrence of a concomitant bacterial superinfection [25]. The occurrence of bacterial superinfection as a consequence of influenza viral infection is well documented. Combined viral and bacterial infection is likely to be due in part to increased bacterial adherence to infected epithelial cells as demonstrated in-vitro for influenza virus [26], respiratory syncytial virus [27] and RV [28]. However, little else is known about the possibility of RV infection increasing risk of bacterial infection.
RV infects the lower respiratory tract of infants [29] and adults [30], with the major site of infection occurring in bronchial epithelial cells [30]. The importance of epithelial cells in anti-viral immunity has recently been shown in that epithelial cells from asthmatic subjects have reduced RV-induced production of interferon β, with consequent increased RV replication [31]. However, leukocytes present in the airway are also important in host defence against infections. The predominant leukocyte found at this location is the alveolar macrophage and these have also been shown to be deficient in interferon production in asthma [32]. In support of their anti-microbial role, macrophages produce inflammatory cytokines to recruit cells of the adaptive immune system, express a number of innate pattern recognition receptors (PRRs) capable of detecting bacterial products, and phagocytose bacterial organisms. In addition, we have recently shown that the production of TNF-α by macrophages in response to RV requires viral replication [33].

RV has been shown to bind, enter and activate alveolar macrophages, though productive replication was not demonstrated [34]. In this study we investigate the hypothesis that RV infection of alveolar macrophages down-regulates their anti-bacterial responses, thereby increasing the host’s susceptibility to bacterial infections.

To test this hypothesis, in the absence of the existence of a small animal model of RV infection, isolated human alveolar macrophages exposed to either infectious or UV-inactivated RV were stimulated with the gram negative and gram positive bacterial products lipopolysaccharide (LPS) or lipoteichoic acid (LTA) and the production of pro-inflammatory cytokines TNF-α and IL-8, PRR expression and phagocytic ability was assessed.
Materials and Methods

For full details see online supplement at http://www.thoraxjnl.com-supplemental

Isolation and in vitro culture of alveolar macrophages

Alveolar macrophages were isolated from resected lung tissue by parenchymal lavage, and plated in 10% FCS in RPMI medium (5 x 10⁵/ml). Following washing adherent cells (i.e. alveolar macrophages) from each subject were cultured in the presence of medium alone, or infected / stimulated with RV, bacterial lipopolysaccharide (LPS) and lipoteichoic acid (LTA) both from Sigma (Australia).

RV propagation and titration

Stocks of human RV-16 and human RV-2 were amplified by growth in Ohio HeLa cells and UV inactivated (UVi) as previously described [35;36]. Following exposure to RV, virion production was assessed by titration assay [35] and RT-PCR [37].

RV exposure and toxin stimulation of alveolar macrophages.

Alveolar macrophages were exposed to RV at a multiplicity of infection (MOI) of 0.1 or 1.at 37°C for one hour. To determine bacterial toxin responsiveness to LPS, infected cells were stimulated for 24 hours with LPS derived from Escherichia coli (10ng/ml) at either 1 or 4 days post infection or LTA derived from Staphylococcus aureus (10 ng/ml) at 1 day post infection.

ELISA

ELISA kits for eotaxin, TNF-α, IL-8, IL-10, and INF-γ were purchased from R&D Systems Europe, Abingdon, UK. ELISAs were carried out according to the manufacturer’s instructions. The detection limits of these assays are: 15.6 pg/ml for all except IL-10 (33pg/ml), and eotaxin (25pg/ml).

Cell labelling for flow cytometry
Antibody binding and subsequent flow cytometric analysis was performed to assess the cell surface expression of TLR2 (Santa Cruz, USA), TLR4 (Santa Cruz, USA), and CD14 (BD, North Ryde, Australia). Appropriate Isotype controls were purchased from BD (North Ryde, Australia). Briefly, alveolar macrophages were detached by scraping, resuspended in calcium- and magnesium-free PBS/FBS and incubated with the antibodies for one hour at 4°C. The cells were washed twice, and resuspended in PBS/FBS prior to analysis.

**Phagocytosis assay**

Phagocytosis of labelled bacterial particles was assessed using the Vibrant Phagocytosis Assay Kit (Invitrogen, Australia) according to manufacturers’ instructions. Phagocytosis of latex beads (Polysciences 0.5 µM Fluoresbrite Microparticles, Polysciences, Inc., Warrington, USA), was determined using flow cytometry.

**Flow cytometry**

Fluorescence was analysed by FACScan flow cytometry (Becton Dickinson, San Jose, CA). Macrophages (10,000 events) were acquired by gating on forward (FSC) and side angle scatter (SSC) properties.

**Statistical methods and analysis of results.**

For experiments in which measurements were compared between the constitutive and experimental response observed in cells from the same donor, repeated measures ANOVA with Dunnett’s post test or paired Students t-test were used. Post hoc tests were carried out only on data tables that were shown to be significantly different by ANOVA. Data were analysed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, California, USA). A probability level of 95% (p≤0.05) was considered as the threshold for statistical significance.
Results

Patient demographics
Alveolar macrophages were isolated from 46 subjects: 31 male (mean [SD] age 63 [11.6] years) and 15 female (59.9 [14.4]). The clinical diagnoses were lung cancer in 32 (69.6%), other thoracic malignancies in 9 (19.6%), interstitial lung disease in 1 (2%), emphysema in 1 (2%) and unknown in 3 (6.5%).

Replication of RV in human alveolar macrophages

RV PCR and titration assays were used to examine if RV was capable of replicating within alveolar macrophages. In alveolar macrophages infected with an initial minimal virus load of 0.1 or 1 (data not shown) MOI RV, RV RNA was detected up to 10 days post infection (figure 1a), and was significantly reduced at 2 days post infection, (figure 1b), with further reductions at either 5 or 10 days. To examine if the persistence of infectious RV also occurred, we assessed the levels of infectious RV from lysed alveolar macrophages by RV titration assay. A significant reduction of the presence of live infectious RV was observed at all time points in comparison to the amount of RV present following the 1 hour infection period. Two days post infection, the level of live RV was almost zero (figure 1c).

Only infectious RV induces TNF-α and IL-8 release by human alveolar macrophages.

RV-16 induced the release of TNF-α from alveolar macrophages over 2,5 and 10 days post infection. Exposure of alveolar macrophages to RV at an MOI of 1 significantly induced the release of TNF-α at all three time points in comparison to non-infected control cells (figure 1d). When cells were infected with RV at an MOI of 0.1 the release of TNF-α was significantly increased only at day 2 (figure 1d).

In our experiments UVi-RV failed to induce TNF-α and IL-8 release from alveolar macrophages (figure 2a and 2b). Neither interferon gamma nor eotaxin were present
in the culture medium (data not shown). These data suggest that RV induction of TNF-α and IL-8 by alveolar macrophages is replication dependent.

**RV down regulates alveolar macrophage immune responses to bacterial products**

We then examined alveolar macrophage responses to gram negative and gram positive bacterial products in the presence and absence of prior RV infection. We examined TNF-α and IL-8 secretion (measured within the same tissue culture medium) from alveolar macrophages isolated from 16 subjects, exposed to RV-16 for 1 day and then further stimulated with LPS for 1 day. As depicted in figure 3a, LPS induced TNF-α release. This LPS-induced secretion was significantly inhibited when the cells had been pre-treated with live infectious RV for 24 hours (p<0.001). A similar inhibitory, and even more significant effect of RV infection on LPS-induced IL-8 release by alveolar macrophages was observed for IL-8, as shown in figure 3b (p<0.0001).

The experiments were repeated in alveolar macrophages which had been exposed to RV for 4 days, followed by LPS stimulation for 1 day. We observed that under this condition the LPS stimulated secretion of TNF-α and IL-8 by alveolar macrophages infected with RV was again significantly reduced in comparison to LPS alone (figure 3c and d) demonstrating that impairment of LPS responses persisted beyond the ability to detect live virus within macrophages. Interestingly, the LPS-stimulated cytokine release decreased with increased time in culture. We speculate that this is related to the *in-vitro* age of the cells.

**Effect of exposed UVi RV-16 on LPS-induced cytokine release by alveolar macrophages.**

In contrast to infection with RV, pre-treatment with UVi RV-16 (24 hours) did not reduce LPS-induced TNF-α secretion (p>0.05, n=8) or IL-8 secretion (p>0.05, n=8 figure 4b), indicating that the impairment of LPS induced cytokine release by rhinovirus was likely replication dependent.
Minor Group RV also down regulate LPS-induced cytokine release by alveolar macrophages.

A minor group RV, RV-2, was used (see online supplement) in a set of similar experiments. Pre-treatment of alveolar macrophages with RV-2 for 24 hours significantly inhibited the LPS-induced secretion of TNF-α and IL-8, in comparison to LPS stimulation alone, further supporting the observation that rhinovirus mediated inhibition of cytokine release was replication dependent.

RV inhibits lipoteichoic acid induced cytokine release by human alveolar macrophages.

LTA stimulation (1ng/ml) increased TNF-α release (11.3(10.6) ng/ml) by macrophages compared to control cells (0.02(0.002) ng/ml), however this was not statistically significant (p=0.3297, n=7). However, LTA significantly induced IL-8 release (76.2(19.2) ng/ml) by macrophages compared to controls (4.6(0.96) ng/ml p<0.05 n=7) (see online supplement Figure E1a).

Whilst not statistically significant, the LTA-induced TNF-α secretion by alveolar macrophages exposed to infectious RV-16 was reduced (from 11.3(10.6) to 3.1(2.5) ng/ml). In contrast, UVi RV-16 exposed alveolar macrophage (initial MOI of 1) caused a small but significant reduction in the secretion of IL-8 in response to LTA, when compared to LTA alone (p<0.05, n=7). However exposure to infectious RV-16 demonstrated an even greater inhibition in LTA induced IL-8 when compared to UVi RV-16 exposed cells (p<0.01, n=7) (see online supplement Figure E1b).

Modulation of alveolar macrophage receptors for bacterial products by RV.

To investigate the possibility that exposure to RV-16 downregulated the expression of cellular receptors for bacterial products (PRRs) on macrophages was assessed using flow cytometry. In untreated cells the expression of TLR2 (LTA receptor) but not CD14 or TLR4 (LPS receptors) was detectable by flow cytometry. Exposure to RV
for one day did not downregulate cell surface TLR2 expression in comparison to non-infected cells (p>0.05, n=5) (see online supplement Figure E2.)

**RV dependent secretion of anti-inflammatory mediators by alveolar macrophages.**

As cell surface TLR2 expression was not altered by RV infection, we next investigated whether RV impairment of anti-bacterial responses was mediated by RV induction of the anti-inflammatory mediators IL-10 and PGE₂. IL-10 was not induced by RV exposure of alveolar macrophages (constitutive production 29.7(5.1) pg/ml versus RV-16 MOI 1 26.4(3.4) pg/ml, p=0.18, n=7). Based on preliminary data we assessed the anti-inflammatory role of PGE₂ [38] on RV-impaired cytokine secretion in macrophages, and the production of PGE₂ was inhibited by the cyclooxygenase-2 (COX-2) inhibitors indomethacin (2.5 μM) and nimesulide (1.5 μM). As shown in the online supplement figure E3 the COX-2 inhibitors did not reduce the RV-dependant reduction in LPS-induced cytokine release.

**RV inhibits phagocytosis of *E. coli* bioparticles but not latex beads in alveolar macrophages.**

An important innate immune response to bacteria by alveolar macrophages is phagocytosis. Since RV exposure impaired the macrophages’ ability to secrete TNF-α and IL-8 in response to bacterial products, we examined if RV exposure also down-regulated phagocytosis by alveolar macrophages. As shown in the online supplement figure E4 RV reduced the phagocytosis of *E. coli* bioparticles (P=0.027, n=3) but not latex beads (p>0.05, n=5).
Discussion

Infectious rhinovirus (RV) and RV RNA were detected in alveolar macrophages up to 3 and 10 days post exposure. We have shown that infectious RV but not UVi RV increased TNF-α and IL-8 release by alveolar macrophages and furthermore infectious RV reduced their ability to respond to LPS or LTA. This RV-dependent impairment of the macrophage immune response was not mediated by autocrine production of the anti-inflammatory cytokines IL-10 and PGE₂, or by down regulation of the cell surface receptor for LTA. It is unlikely that RV infection induced a non-specific cellular downregulation since the phagocytic ability of the infected alveolar macrophages to ingest latex beads was not altered. However since phagocytosis of *E. coli* bioparticles was reduced this further supports the notion that bacterial innate immune responses are reduced in alveolar macrophages infected with rhinovirus.

Since infectious RV virions could be isolated from infected macrophages up to 3 days after RV exposure our data suggest that RV either actively infects human alveolar macrophages or is taken up via phagocytosis and is not immediately eliminated. Furthermore, RV RNA persisted in alveolar macrophages up to 10 days post RV exposure, indicating that RV survives and may even replicate at a low level for a limited time. These observations are in accordance with Gern et al (1996), who reported that there was no increase in RV virions within 24 hours in alveolar macrophages [43]. Thus, it seems that the macrophage is able to allow but limit the replication of RV and perhaps act as a viral sink. If we assume that no RV replication occurs in the alveolar macrophage, and the observed decrease of infectious virions represents the natural RV decay, the detection of RV RNA should mirror these events. Our findings suggest that RV is replicating in alveolar macrophages at a lower rate than that at which the virus is eliminated from the macrophage over this time period.

UVi-RV is incapable of replicating, however it may bind and activate the host receptor in susceptible cells. Here we have shown that exposure of alveolar macrophages to UVi-RV failed to induce the production of TNF-α and IL-8, and therefore it can be assumed that their production was linked to ongoing RV replication. Others have shown replication independent responses in a variety of cells [39] [40] [41] [42]. A cell type specific response to RV infection at the MOI used is
further supported by our observation that, using the same UVi-RV stocks and infection procedure, UVi-RV induced similar cytokine secretion to infectious RV in human airway smooth muscle cells [36]. However it is also possible that interaction between RV-16 and ICAM-1 in alveolar macrophages may be altered following UV irradiation. Previous reports which have examined cytokine release from alveolar macrophages in response to exposure to UVi-RV have shown that both TNF-α [34] and MCP-1 (via P38 MAPK) [43] were induced, perhaps suggesting that UV-inactivation does not alter RV-ICAM interactions. In monocytes, IP-10 (via the JAK/STAT pathway) production has also been demonstrated following exposure to replication defective rhinovirus [44]. The differences between these reports and our results could be accounted for by the 10 fold greater MOI of rhinovirus used in their studies[34] [43]. Further study will clearly be needed to clarify the importance of rhinovirus replication in alveolar macrophage function.

Cell death was not induced following viral exposure of alveolar macrophages, in agreement with other studies [34]. In addition the anti-inflammatory cytokine IL-10 was not up regulated by RV infection of alveolar macrophages, in contrast to RV-14 exposure of blood monocytes in which significant up-regulation of IL-10 occurred [45].

As a in-vitro model of bacterial infection we used LPS and LTA, components of the bacterial cell wall from gram negative and gram positive bacteria respectively, to stimulate RV exposed and non-exposed alveolar macrophages. RV exposure resulted in a reduced ability to mount an immune response against bacterial products. This is a novel finding which provides a potential mechanism by which RV infection may foster bacterial superinfection. Even 4 days post RV exposure the bacterial toxin induction of TNF-α and IL-8 was impaired. This indicates a long lasting effect of RV infection and might be related to the sustained presence of RV RNA in the host cell. Using UVi-RV, only LTA-induced IL-8, but not TNF-α, secretion was inhibited compared to toxin stimulation alone. However considerably greater impairment of LTA-dependent IL-8 release was observed when cells were exposed to infectious RV. This suggests that LTA-induced IL-8 production is partly ICAM-1 dependent, but largely replication dependent.
We were unable to detect cell surface expression of either CD14 or TRL4. This is most likely the result of the staining technique we used, as CD14 expression upon alveolar macrophages is undetectable unless autofluorescence is blocked [46]. We chose not to block autofluorescence, since this procedure would have permeabilized the cell membrane thereby introducing the possibility that intracellular and extracellular CD14 would be detected. Other studies indicate that CD14 cell surface expression on alveolar macrophages (5-50%) is considerably lower than that found upon blood monocytes [47;48]. However we were able to detect the cell surface expression of TLR 2, the cellular receptor for LTA [49;50], and found no downregulation following RV exposure. We concluded that bacterial toxin receptor down-regulation by RV is unlikely to be the cause of the observed impairment of cytokine release.

We further assessed if the effect of RV infection on alveolar macrophages reflected a general down regulation of the host cells’ function using phagocytosis as an indicator. RV exposure did not modulate the phagocytic ability of alveolar macrophages to ingest latex beads when compared to non-RV exposed macrophages, however phagocytosis of bacterial particles was significantly impaired. This supports our hypothesis that RV infection of alveolar macrophages would promote the occurrence of a bacterial superinfection.

Since exposure to UVi RV did not alter the LPS induced secretion of TNF-α and IL-8 by alveolar macrophages we can assume that the RV dependent TNF-α and IL-8 down-regulation observed was not mediated through ICAM-1. To confirm this we exposed alveolar macrophages to a minor group RV (which does not infect cells via ICAM1), RV-2, and found that LPS-induced TNF-α and IL-8 secretion was reduced as was found with RV-16.

In summary this study provides evidence for a novel unidentified mechanism by which RV can impair the innate immune response in alveolar macrophages and may thereby provide an environment that facilitates additional bacterial infection.
Acknowledgments

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Disclosure

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Figure 1. Rhinovirus RNA survives in alveolar macrophages and induces cytokine release. a) agarose gel electrophoresis showing RV RNA and the GAPDH (control) from human alveolar macrophages (0.5 x 10^6) that were exposed to infectious rhinovirus virions (MOI 0.1 and 1) at day zero for 1 hours and then at day 2, 5, and 10. The image is representative of four independent experiments carried out in alveolar macrophages derived from four different volunteers. A more detailed densitometric analysis of the ratio of RV and GAPDH RNA levels is depicted in panel b) Each symbol represents the data point of a single experiment, and the resulting mean value is indicated by a line for each time point. The ratio of rhinovirus / GAPDH PCR product was normalised in each data set, defining the level of RV mRNA (initial MOI 0.1) detected immediately post the one hour infection period as “100” (day 0). RV PCR product was only detected in 3 of the 4 samples tested at day 10. c) The presence of infectious RV-16 virion from alveolar macrophage lysates was detected by virus titration assay over a time period of 72 hours. Each bar represents the mean (S.E.) of data derived from alveolar macrophages of three volunteers. Estimations at each time point was carried out in triplicate for each subject and the virus load of each experiment was assayed in duplicate and compared to the load of infectious rhinovirus (initial MOI 1) directly after exposure of alveolar macrophages at day zero. d) The effect of rhinovirus-16 (RV-16) exposure (MOI 1 and 0.1) of alveolar macrophages on TNF-α release into tissue culture medium was determined by ELISA (n=4).

Figure 2. The release of TNF-α and IL-8 into the cell free supernatant following exposure to UVi RV-16 or RV-16 (MOI 1) measured 48 hours post infection. a) TNF-α release from alveolar macrophages was induced by infectious RV only, in comparison to constitutive production (n=9). b) IL-8 release into the culture medium was induced following exposure to both UVi RV-16 or infectious RV-16 (MOI 1), in comparison to constitutive release (n=9).
Figure 3. LPS induced TNF-α and IL-8 release was impaired in rhinovirus-16 (RV) exposed alveolar macrophages. Macrophages were either mock infected or exposed to RV-16 (MOI 1) for one hour followed by washing and replacement of culture media and then cultured for a further one day (a and b) or four days (c and d) at which point they were stimulated with LPS (10 ng/ml) for a further one day. LPS-induced a) TNF-α release (n=16) and b) IL-8 release (n=16) from alveolar macrophages was reduced in cells which were exposed to RV-16 when compared to nonexposed control macrophages. Similarly, LPS induced c) TNF-α release (n=8) and d) IL-8 (n=8) release from alveolar macrophages was reduced in cells which had RV-16 exposure when compared to non exposed control macrophages.

Figure 4. The effect of prior exposure to UVi RV-16 upon LPS-induced TNF-α and IL-8 release. Macrophages were either mock infected or exposed to UVi RV-16 (initial MOI 1) and then cultured for a further one day at which point they were stimulated with LPS (10 ng/ml) for a further one day. For comparative purposes macrophages exposed to UVi RV-16 alone and infectious RV-16 (± LPS stimulation), from cells derived from the same subjects are included. UVi rhinovirus exposure did not reduce LPS stimulated a) TNF-α release and b) IL-8 release, in comparison to LPS stimulation alone. n=8 for all.
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Figure 1

(a) Gel electrophoresis showing GAPDH and RV bands for different time points and conditions. Control, RV MOI 0.1, RV MOI 1, PCR -ve.

(b) Graph showing ratio of RV/GAPDH and PCR product over days post RV exposure. Statistically significant at p<0.05.

(c) Bar chart showing AM lysate RV TCID50 at different time points (0 hours, 4 hours, 8 hours, 24 hours, 48 hours, 72 hours). Statistically significant at p<0.001.

(d) Graph showing TNF-α levels from day 0 to day 10. Statistically significant at p<0.01, p<0.05, p<0.01, p<0.01.
Figure 2

(a) TNFα (pg/mL)

(b) IL-8 (ng/mL)
Figure 4

(a) TNF-α (ng/ml) levels in different groups: Control, LPS, RV-16, RV-16 + LPS, UVi RV-16, UVi RV-16 + LPS.

(b) IL-8 (ng/ml) levels in different groups: Control, LPS, RV-16, RV-16 + LPS, UVi RV-16, UVi RV-16 + LPS.

Significance levels: p<0.05 and p<0.01.
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