Modulation of lipopolysaccharide receptor expression by lactate dehydrogenase-elevating virus

Dan Su, Thao Le-Thi-Phuong and Jean-Paul Coutelier

The Unit of Experimental Medicine, de Duve Institute, Université Catholique de Louvain, 1200 Bruxelles, Belgium

Lactate dehydrogenase-elevating virus (LDV) exacerbates mouse susceptibility to endotoxin shock through enhanced tumour necrosis factor (TNF) production by macrophages exposed to lipopolysaccharide (LPS). However, the in vivo enhancement of TNF production in response to LPS induced by the virus largely exceeds that found in vitro with cells derived from infected animals. Infection was followed by a moderate increase of Toll-like receptor (TLR)-4/MD2, but not of membrane CD14 expression on peritoneal macrophages. Peritoneal macrophages from LDV-infected mice unresponsive to type I interferons (IFNs) did not show enhanced expression of TLR-4/MD2 nor of CD14, and did not produce more TNF in response to LPS than cells from infected normal counterparts, although the in vivo response of these animals to LPS was strongly enhanced. In contrast, the virus triggered a sharp increase of soluble CD14 and of LPS-binding protein serum levels in normal mice. However, production of these LPS soluble receptors was similar in LDV-infected type I IFN-receptor deficient mice and in their normal counterparts. Moreover, serum of LDV-infected mice that contained these soluble receptors had little effect if any on cell response to LPS. These results suggest that enhanced response of LDV-infected mice to LPS results mostly from mechanisms independent of LPS receptor expression.

INTRODUCTION

Some viral infections strongly exacerbate mouse sensitivity to endotoxin and the severity of septic shock (Nansen et al., 1997; Doughty et al., 2001). This is the case for lactate dehydrogenase-elevating virus (LDV), a common mouse nidovirus that triggers a strong, but transient activation of innate immune cells (Le-Thi-Phuong et al., 2007a). Concomitant exposure to LDV and lipopolysaccharide (LPS) leads to a synergistic secretion of pro-inflammatory cytokines such as tumour necrosis factor (TNF) and gamma interferon (IFN-γ), which are strongly involved in the pathogenesis of endotoxin shock.

At the cellular level, upon exposure to LPS, mouse macrophages secrete large amounts of TNF. The complex formed by LPS and the soluble LPS-binding protein (LBP) is firstly recognized by membrane CD14 (Miyake, 2007). It is then loaded to the Toll-like receptor (TLR)-4/MD2 receptor complex. TLR-4 dimerization is followed by signal transduction that involves MyD88 and the NF-κB pathway, leading to the secretion of pro-inflammatory molecules, including TNF. In addition, TLR-4 internalization in the endosome leads to the activation of the interferon regulatory factor 3 pathway (reviewed by McGettrick & O’Neill, 2010). Soluble LPS receptors such as sCD14 can either increase or decrease cellular responses to LPS, depending on their concentration and on the cell type involved (Tapping & Tobias, 2000).

Due to its function in loading LPS onto TLR-4/MD2, it is not surprising that modulations of CD14 expression may affect the cellular response to the endotoxin. Such a modulation can be regulated by cytokines. Th2 anti-inflammatory cytokines like interleukin (IL)-4 and IL-13 decrease CD14 expression on human monocytic cells and fibroblasts, leading to an increased response of these cells to LPS (Tamai et al., 2002, 2003).

Macrophages derived from mice acutely infected with LDV display a stronger ability to secrete TNF when exposed to LPS than cells obtained from uninfected animals (Le-Thi-Phuong et al., 2007a). The mechanisms responsible for this synergy between the viral infection

†These authors contributed equally to this work.

‡Present address: 46 Golf Links Road, Glenroy, 3046 VIC, Australia.
and LPS have not been determined. Here, we tested the hypothesis that LDV infection results in a modulation of CD14 and/or TLR-4/MD2 expression, as well as of LBP, leading to an increased capacity of these cells to respond to LPS. Instead, our data suggest that enhanced TNF production in response to LPS observed in LDV-infected mice does not result from an increase in LPS receptor expression.

RESULTS

Effect of LDV infection on LPS receptor expression

Since LPS triggers an enhanced TNF response in peritoneal cells from LDV-infected mice (Le-Thi-Phuong et al., 2007a), CD14 expression was first measured in these cells. As shown in Fig. 1(a), CD14 gene expression in adherent peritoneal cells surprisingly decreased significantly at 1 day after LDV infection when compared with cells from control animals (P<0.0092). At 4 days after infection, it returned to control levels.

The effect of LDV infection on CD14 expression was also measured in spleen cells. In contrast to what was observed with adherent peritoneal cells, the infection was followed by a transient increase in CD14 gene expression in splenocytes (Fig. 1b). This enhanced expression was observed at 1 day after LDV infection (significant difference, P=0.0286 by Mann–Whitney test) and returned below basal level 4 days after virus inoculation (P=0.0286).

To confirm that LDV infection induces a decrease in CD14 expression on peritoneal cells, flow cytometry analysis was performed. As shown in Fig. 2(a) for pooled peritoneal cells from 129/Sv mice, membrane expression of CD14 decreased as soon as 15 h after infection and remained low for at least 2 days. A similar downregulation of CD14 expression on peritoneal cells was observed in BALB/c mice 1 day after infection with LDV (Fig. 2b, P=0.0286). To determine whether it was related to a decrease in the number of macrophages, peritoneal cells obtained from these controls or LDV-infected BALB/c animals were labelled with anti-F4/80 antibody. The proportion of F4/80-positive cells in peritoneal cells was slightly lower in infected mice when compared with controls (6.0±2.4 and 11.1±3.9 %, respectively, no significant difference with P=0.0697). Moreover, the proportion of these F4/80-positive peritoneal cells that expressed CD14 was similar in control and infected mice (76.2±5.2 and 70.6±3.5 %, P=0.1233). In contrast, the level of CD14 expression on F4/80-positive cells, measured after double-labelling and indicated by the mean fluorescence intensity corresponding to the binding of anti-CD14 antibody, was significantly decreased after infection (Fig. 2c, P=0.0393). Therefore, although LDV triggers at best a modest drop in peritoneal macrophages, the remaining macrophages still express CD14, but at a reduced level.

In addition to CD14, TLR-4/MD2 expression was also measured by flow cytometry analysis on peritoneal cells obtained from control and LDV-infected 129/Sv mice. As shown in Fig. 2(d), LDV infection resulted in a moderate increase rather than in a down-modulation of TLR-4/MD2 on peritoneal cells (P=0.0286). Therefore, the enhancing effect of LDV infection on peritoneal macrophage sensitivity to LPS might be related to an effect of the virus on TLR-4/MD2, but not on CD14 membrane expression on these cells.

The effect of LDV infection on CD14 expression was also measured in spleen cells from 129/Sv mice. In contrast to gene expression, membrane CD14 expression was significantly decreased in these cells at 1 day after infection (Fig. 3a, P=0.0022), to an extent similar to the drop in expression observed on peritoneal cells. However, TLR-4/MD2 expression was not significantly modified by the infection (Fig. 3a, P=0.0649). This confirmed that LDV infection has divergent effects on CD14 and TLR-4/MD2 membrane expression.

Similarly, a moderate decrease of CD14 expression was observed on BALB/c pooled F4/80-positive spleen and liver cells at 1 day after LDV infection (Fig. 3b).
Effect of LDV infection on TNF production and LPS receptor expression by cells from mice deficient for type I IFN receptor

Because mice deficient for type I IFN receptor (called here IFNAR KO mice) display, after LDV infection, a strong enhancement of susceptibility to endotoxin shock, when compared with normal counterparts (Le-Thi-Phuong et al., 2007a), we measured also the effect of the virus on macrophages in these animals.

As reported before, TNF production by peritoneal cells from infected normal 129/Sv mice was increased when compared with cells from uninfected animals (Fig. 4a). A similar increase of TNF production induced by LDV was observed with cells from IFNAR KO mice. However, surprisingly, peritoneal cells from both control and infected IFNAR KO animals did not produce more TNF than cells from 129/Sv counterparts (Fig. 4a, \( P < 0.2222 \) and \( 0.9048 \), respectively), although in vivo TNF production triggered by LPS administration has been shown to be dramatically enhanced in mice deficient for type I IFN receptor (Le-Thi-Phuong et al., 2007a).

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Soluble LPS ligands after LDV infection

A discrepancy between CD14 gene and membrane protein expression such as that observed in spleen cells of normal...
mice infected with LDV might be due to increased shedding and release of soluble CD14 (sCD14). Therefore, sCD14 was measured in sera of control and infected 129/Sv mice. As shown on Fig. 5(a) with sera obtained 1 day after virus inoculation, LDV infection resulted in an early and very strong increase of sCD14 levels (significant difference between control and LDV-infected mice, \( P < 0.0022 \)).

Since LBP can also modulate LPS binding to cell membrane, it was measured in the serum of mice infected with LDV. Similarly to sCD14, LBP levels were significantly (\( P = 0.0286 \)) enhanced 1 day after LDV infection (Fig. 5b). Interestingly, LDV-induced LBP production was similar in 129/Sv and IFNAR KO animals (Fig. 5c, \( P = 0.8021 \)). A slight decrease of sCD14 was observed in LDV-infected IFNAR KO animals when compared with 129/Sv mice, which was not significant in this experiment (\( P = 0.3206 \)), although it was in another.

To determine whether these soluble LPS ligands contained in the serum of LDV-infected mice may modulate TNF production by macrophages exposed to LPS, serum from control or infected mice was added to the in vitro assay of TNF production. As shown in Fig. 6(a), a strong enhancement of TNF production was again observed when macrophages from LDV-infected mice were used, when compared with macrophages from control animals (\( P = 0.0279 \)). However, addition of serum did not enhance this response to LPS. Although not significant, the only minor difference that was observed was a slight decrease of TNF production when macrophages from LDV-infected mice were exposed to LPS in the presence of serum from LDV-infected animals.

Soluble LPS receptors might also modulate the response to LPS of other cells than macrophages (Ulevitch & Tobias, 1999; Tapping & Tobias, 2000). We therefore analysed the response of liver endothelial cells freshly isolated from control and acutely infected mice (Fig. 6b). Although TNF production by these cells was low, a significant enhancement was observed in the presence of LPS (\( P = 0.0286 \)). It was not possible to formally exclude that this low TNF production originated from contaminant macrophages. However, no significant difference was observed between cells from control and infected animals in contrast to what had been observed with purified macrophages (Fig. 4a). Moreover, addition of serum, from both control and infected mice, did not modify the endothelial cell response.

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**Fig. 4.** Effect of LDV infection on cells from IFNAR KO mice. (a) Peritoneal cells freshly obtained from either uninfected or LDV-infected 129/Sv (open columns) and IFNAR KO (closed columns) mice were incubated for 4 h with LPS (10 ng ml\(^{-1}\)). TNF-\( \alpha \) was assayed in individual supernatants (results shown as mean ± SEM for groups of four to five animals). (b) CD14 expression was measured by flow cytometry on total peritoneal cells obtained from groups of four control IFNAR KO mice or of animals 1 day after LDV infection. Results are shown as means of geometric mean fluorescence intensity (log scale) ± SEM. (c) CD14 expression was measured by flow cytometry on spleen cells obtained from the same groups of four control or LDV-infected IFNAR KO mice. Results are shown as means of geometric mean fluorescence intensity (log scale) ± SEM. (d) TLR-4/MD2 expression was measured by flow cytometry on peritoneal and spleen cells of the same groups of four control and LDV-infected IFNAR KO mice. Results are expressed as geometric means of fluorescence intensity (means ± SEM).
DISCUSSION

Cell surface expression of LPS receptor has been analysed after infection with different viruses. CD14 expression is enhanced after pig infection with porcine reproductive and respiratory syndrome virus (Van Gucht et al., 2005), leading to exacerbation of LPS-induced respiratory disease (Van Gucht et al., 2003, 2004), and after cell infection with respiratory syncytial virus (Raza et al., 1999), which may result in increased susceptibility to bacterial infection. However, no such enhancement of surface CD14 expression on cells showing increased response to LPS was observed here after LDV infection. In contrast, like some other viruses, such as respiratory syncytial virus and dengue virus that may enhance TLR-4 expression, and therefore sensitize cells to LPS (Monick et al., 2003; Azeredo et al., 2010), LDV infection triggered also such an effect, although moderate. This could be mediated by the high production of pro-inflammatory cytokines, including IFN-γ that follows LDV infection (Markine-Goriaynoff et al., 2002; Le-Thi-Phuong et al., 2007a). Therefore, the increased TNF response of macrophages from LDV-infected animals might at best correspond to a modulation of TLR-4/MD2, but not of membrane CD14 expression.

Interestingly, LDV-infected mice deficient for type I IFN receptor displayed only a moderate increase of CD14 expression on spleen cells, but not on peritoneal macrophages when compared with immunocompetent counterparts. Moreover, the in vitro TNF production by macrophages in response to LPS was similar in cells derived from infected IFNAR KO and 129/Sv animals, although the in vivo TNF response was largely increased in mice unable to respond to type I interferons (Le-Thi-Phuong et al., 2007a). These observations indicate a lack of correlation between macrophage expression of LPS receptor, intensity of the in vitro TNF production in response to LPS challenge of these
cells and in vivo TNF production after administration of the same endotoxin. This may therefore suggest that the major effect of LDV on macrophage response to endotoxin is mediated by factors that are present in vivo, but not in vitro, a hypothesis that fits well with the observation that the two to fourfold increase in in vitro TNF production by macrophages from LDV-infected mice is largely inferior to more than 100-fold enhancement of TNF levels measured in vivo (Le-Thi-Phuong et al., 2007a).

Although membrane CD14 expression on spleen cells is decreased after LDV infection, CD14 gene expression in the same cells is enhanced and correlates with a surge in the serum of infected mice of sCD14. Moreover, LDV infection strongly increases serum levels of LBP. Interestingly, enhanced levels of sCD14 have been found in patients after viral infection (Lien et al., 1998). These molecules can induce response to LPS in cells that do not express surface CD14, such as endothelial cells (Ulevitch & Tobias, 1999; Tapping & Tobias, 2000) and their role might therefore be suspected in the enhanced TNF production elicited by LPS administration to LDV-infected mice. However, two observations argued against a major role of these soluble receptors in the effect of LDV: their similar levels in LDV-infected IFNAR KO and 129/Sv animals despite a wide range of differences in susceptibility to LPS (Le-Thi-Phuong et al., 2007a); and the lack of in vitro enhancement of cell response to LPS after addition of serum from LDV-infected mice containing high levels of sCD14 and LBP. It must moreover be stressed that high concentrations of these soluble receptors have also been reported to decrease cellular responses to LPS (Tapping & Tobias, 2000; Gutsmann et al., 2001; Chaby, 2004).

It therefore remains to understand the mechanisms that explain the very strong enhancement of in vivo TNF production in LDV-infected mice challenged with LPS. These mechanisms might involve the response of still unidentified cells or soluble mediators, local conditions that would result in optimal concentrations of soluble LPS receptors in the microenvironment of responsive cells and/or the involvement of other molecules expressed by macrophages after LDV infection.

**METHODS**

**Animals.** Specific-pathogen-free 129/Sv and BALB/c mice were bred at the Ludwig Institute for Cancer Research (Brussels) by G. Warnier and used when 8–12-weeks-old. IFN-κRKO mice, on the 129/Sv genetic background (Müller et al., 1994), were received from M. Aguet (Zurich, Switzerland) and bred similarly.

**Virus.** Mice were infected by intraperitoneal injection of approximately 2 x 10⁷ 50 % infectious doses (ID₅₀) of LDV (Riley strain; ATCC) in 500 μl saline.

**LPS.** LPS was from *Escherichia coli* (0111:B4; Sigma).

**Liver endothelial cell purification.** Liver endothelial cells were purified after perfusion of liver with perfusion buffer containing HEPES (10 mmol l⁻¹), KCl (3 mmol l⁻¹), NaCl (130 mmol l⁻¹), NaH₂PO₄ (1 mmol l⁻¹) and d-glucose (10 mmol l⁻¹) (pH 7.4), with EGTA (1.9 mg ml⁻¹), followed by perfusion buffer with CaCl₂ (5 mmol l⁻¹) and collagenase IV (0.03 % w/v) as described by Benten et al. (2005). Endothelial cells were isolated on discontinuous 70–30 % Percoll gradient.

**RT-PCR.** mRNA was prepared from lysed adherent cells as reported previously (El Azami El Idrissi et al., 1998). CD14 gene expression was measured by RT-PCR with primers described by Liu et al. (2006). As an internal control, housekeeping hypoxanthine guanine phosphoribosyltransferase (HPRT) gene expression was measured using primers described by Peterson et al. (1994). The ratios between CD14 and HPRT expression were calculated and expressed as arbitrary units (El Azami El Idrissi et al., 1998).

**Flow cytometry.** Flow cytometry analysis of CD14 expression was performed using fluorescein-conjugated- or phycoerythrin-conjugated anti-CD14 antibody (1 μg for 10⁶ cells; Sa2-8 antibody from ImmunoSource). For TLR-4/MD2 expression analysis, cells were labelled with phycoerythrin-conjugated anti-TLR-4/MD2 antibody (0.5 μg for 10⁶ cells; Sa2-8 antibody from ImmunoSource). Macrophage determination was performed by labelling with fluoresceinated anti-mouse F4/80 antibody (ImmunoSource). After fixation in 0.62 % paraformaldehyde, fluorescence was analysed with a FACScan flow cytometer (Becton Dickinson).

**TNF assay.** TNF was measured by bioassay, using WEHI-164 clone 13 cells, as described previously (Grohmann et al., 2000; Le-Thi-Phuong et al., 2007a).

**Soluble LPS ligand assays.** sCD14 was measured by Biometec with an enzyme immunoassay kit. LBP was quantified with an ELISA test kit from Cell Sciences.

**ACKNOWLEDGEMENTS**

The authors are indebted to J. Van Snick for helpful discussions and critical reading of this manuscript, and to N. Ouled Haddou and A. Tonon for expert technical assistance. This work was supported by the Fonds National de la Recherche Scientifique (FNRS), Fonds de la Recherche Scientifique Médicale (FRSM), Loterie Nationale, Fonds Spéciaux de Recherche (UCL), the State-Prime Minister’s Office - S.S.T.C. (interuniversity attraction poles) and the ‘Actions de recherche concertées’ from the Communauté française de Belgique – Direction de la Recherche scientifique (concerted actions), Belgium. J.-P. C. is a research director with the FNRS.

**REFERENCES**


de Waal Malefyt, R., Figdor, C. G., Huijbens, R., Mohan-Peterson, S., Bennett, B., Culpepper, J., Dang, W., Zurawski, G. & de Vries, J. E. (1993). Effects of IL-13 on phenotype, cytokine production, and