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Short communication

Lipopolysaccharide-induced impairment of classical swine fever virus infection in monocytic cells is sensitive to 2-aminopurine

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Abstract

Lipopolysaccharide (LPS) impairs classical swine fever virus (CSFV) replication in monocytic cells, which are primary targets for CSFV and mediators of virus-induced immunomodulation. Although soluble antiviral factors including interferons (IFN) were not detected, IFN-α and IFN-β mRNA were induced. The serine threonine protein kinase inhibitor 2-aminopurine, impeded this antiviral activity. These results indicate that the LPS-induced antiviral state employs signaling pathways, in which the double-stranded RNA-dependent protein kinase (PKR) is actively involved. © 2002 Published by Elsevier Science B.V.

Keywords: Classical swine fever virus; LPS; Monocytes/macrophages; PKR (double-stranded RNA-dependent protein kinase); 2-aminopurine; IFN; Poly(I)·poly(C); Antiviral effect

1. Introduction

Classical swine fever virus (CSFV), a member of the family *Flaviviridae*, genus *Pestivirus*, is the causative agent of an important and often fatal disease of pigs. Monocytes and macrophages (Mø) play an important role in the pathogenesis of classical swine fever (CSF). They are among the main host cells for CSFV infection (Cheville and Mengeling, 1969; Ressang, 1973; Knoetig et al., 1999), are potential vectors in the spread of CSFV to different tissues, and are major reservoirs for the virus. Furthermore, the pro-inflammatory factors prostaglandin E2 and interleukin-1, which have been identified as potential pathogenic factors, are secreted after CSFV infection of monocytic cells (Knoetig et al., 1999). Yet, monocytic cells are also important regulators and mediators of the early innate immune defenses.

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CSFV can induce acute, subacute or chronic diseases. Both viral and host factors are thought to be important in determining the outcome of classical swine fever (Trautwein, 1988). However, the host factors involved are still unclear, as indeed are the viral properties conferring virulence. Age, immunocompetence and the nutritional condition can influence the severity of the disease (van Oirschot, 1988). In light of the monocytic target cell playing a key role in the pathogenesis, their activation-dependent intrinsic antiviral activity was of interest. LPS was chosen as a model for monocytic cell activation through pattern recognition receptors, since such pathways are likely to influence monocytic cell activity in field situations.

1.1. Lipopolysaccharide (LPS) activation of monocytic cells impairs CSFV replication

Monocytes were obtained by 2 h plastic adherence of peripheral blood mononuclear cells (PBMC) cultured at 4×10^6/ml (McCullough et al., 1993), and Mø by culture of these monocytes for 3–4 days in DMEM, 30% (v/v) porcine plasma, 2 mM L-glutamine and 25 mM HEPES (McCullough et al., 1999). The virulent CSFV strain Brescia, was used for the infections (Summerfield et al., 1998). Treatment of both monocytes and Mø with 10 μg/ml LPS (*Escherichia Coli*-derived; Sigma Chemical Co., Buchs, Switzerland) for 2–24 h before infection impaired CSFV replication. This resulted in a 1–3 log reduction in virus yield (Fig. 1A, which shows the results with Mø; monocytes gave similar results). There was a variation between monocytes and Mø in their response to the LPS in terms of an induced antiviral effect. In general, monocytes tended to display a stronger antiviral effect, but this also varied between different preparations of cells. Nevertheless, a two-sample Student's *t*-test

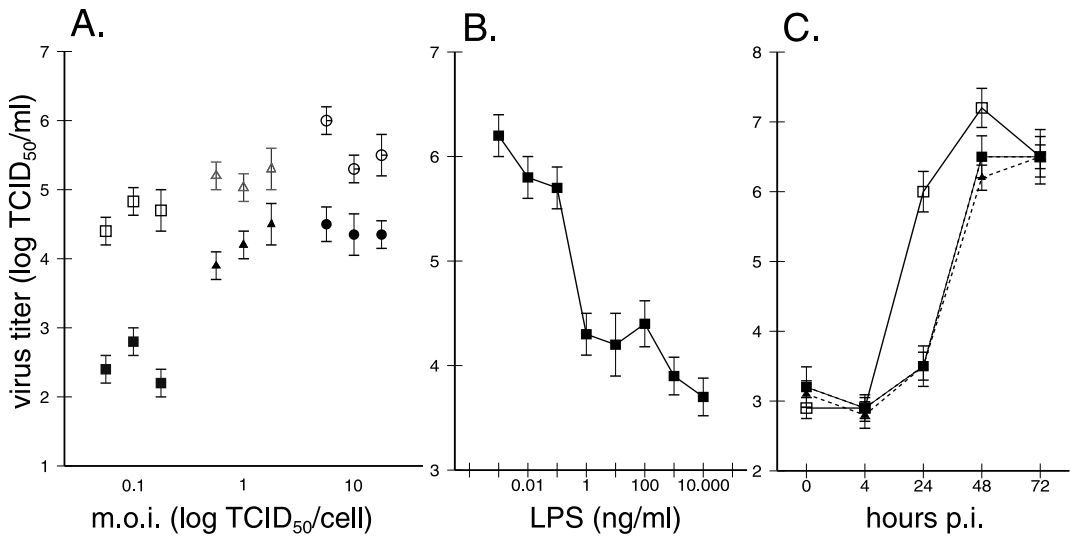

Fig. 1. Effect of LPS treatment on the production of infectious CSFV by Mø. (A) CSFV replication, following infection with an m.o.i. of 0.1 (squares), 1 (triangles) or 10 (circles) $TCID_{50}$/cell, in untreated (open symbols) and LPS-treated (10 μg/ml, 2 h before infection; filled symbols) Mø (0.5×10^6/ml) from different pigs. Titres of supernatant samples collected 24 h p.i. are shown as mean ± the standard error (SE) from triplicate wells. Three experiments (shown as the three points) were performed for each m.o.i. Similar results were obtained with monocytes. (B) LPS dose-dependent impairment of CSFV infection in Mø. Three-day-old Mø cultures were treated with LPS (0.01 ng to 10 μg/ml) for 2 h before infection with a m.o.i. of 1 $TCID_{50}$/cell. Data are from one representative experiment out of three, and the mean ± SE from triplicate supernatant samples collected 24 h p.i. are shown. (C) Kinetics of CSFV replication in porcine monocytes: CSFV replication in untreated cells (open squares), cells treated with LPS (10 μg/ml) 2 h prior to infection (solid squares), and cells treated with LPS 2 h prior to infection with replenishment of LPS every 24 h (triangles). All cultures were infected with CSFV at an m.o.i. of 0.1 $TCID_{50}$/cell. Data are from one representative experiment out of four, and the mean ± SE from triplicate samples are shown.

Table 1
Effect of antiviral cytokines and polyIC on CSFV replication

	Mø treatment[a]	% NS3 positive cells[b]	Virus yield (log 10 $TCID_{50}$/ml)[b]
Experiment 1	Medium	70 ± 3	5.8 ± 0.3
	LPS 10 μg/ml	28 ± 2	4.3 ± 0.2
	TNF-α 0.2 μg/ml	72 ± 4	5.6 ± 0.2
	LPS 10 μg/ml + αTNF-α (3 μg/ml)	29 ± 2	4.8 ± 0.1
	IL-6 50 U/ml	77 ± 4	5.5 ± 0.3
Experiment 2	Medium	72 ± 2	6.1 ± 0.3
	LPS 10 μg/ml	7 ± 1	4.1 ± 0.1
	IFN-α 100 U/ml	6 ± 1	4.3 ± 0.1
Experiment 3	Medium	73 ± 2	5.9 ± 0.2
	LPS 10 μg/ml	6 ± 1	3.7 ± 0.1
	LPS 10 μg/ml + αIFN-α (1 μg/ml)	9 ± 1	3.9 ± 0.3
	IFNα 100 U/ml	8 ± 2	4.4 ± 0.1
	pIC 20 μg/ml	6 ± 1	4.0 ± 0.2
Experiment 4	Medium	80 ± 2	6.3 ± 0.1
	LPS 10 μg/ml	13 ± 2	3.8 ± 0.1
	LPS 10 μg/ml + αIL6 (5 μg/ml)	76 ± 2	6.1 ± 0.2

[a] Treatment with the indicated stimuli was for 2 h before infection. After virus adsorption, non-adsorbed virus was removed and fresh medium supplemented with the respective cytokines or antibodies was added. All experiments were repeated at least three times and representative data are shown.

[b] Infection was at a m.o.i. of 1 $TCID_{50}$/cell. Analyses were performed 24 h p.i. Data are from triplicate samples ± SEM.

was applied to a total of 21 experiments in which comparison was made of CSFV replication in LPS-treated and untreated cells. From this, the LPS-induced antiviral state was clearly statistically significant ($P < 0.01$). The generation of both cell free virus (Fig. 1A) and cell-associated virus (data not shown) was impaired. The effect of LPS on progeny CSFV production was less pronounced at the higher m.o.i. (Fig. 1A), but the reduction was still at least 90%. The antiviral state was also dose-dependent, being effected by as little as 10 ng/ml LPS (Fig. 1B).

Blocking CD14—a major LPS binding receptor—with the *anti*-CD14 mAb MY4 (Coulter clone, 10 μg/ml) reversed the 65–85% of the LPS-induced impairment of CSFV replication (data not shown). This demonstrated that the LPS-mediated effect was CD14-dependent.

An optimal antiviral state was achieved by pretreatment of the cells with LPS for 2–18 h before infection. When LPS was added for less than 2 h before infection or only after infection, the impairment of CSFV infection/replication was less evident or undetectable (data not shown). If the virus infection was permitted to continue beyond 24 h p.i., it was noted that the levels of progeny virus eventually reached those obtained in untreated cultures (Fig. 1C). This was noted even when LPS was refreshed every 24 h, demonstrating that LPS did not abrogate CSFV replication, but only delayed the onset of the virus infection process.

1.2. Role of antiviral cytokines in inhibition of CSFV replication

LPS is known to induce the secretion of several cytokines with antiviral properties, such as TNF-α, IL-6 and IFNs (Vilcek and Sen, 1996). Supernatants from LPS-stimulated monocytic cell cultures contained higher amounts of TNF-α (tested by ELISA; Endogen, Woburn, MA) and IL-6 (assessed using a bioassay, Aarden et al., 1987) than samples from untreated cells. Nevertheless, supplementing non-stimulated monocytic cell cultures with recombinant porcine TNF-α and IL-6 (both Endogen) had no effect on CSFV replication or de novo synthesis of viral NS-3

(Table 1). The latter was detected by flow cytometry using mAb C16 (kindly provided by Dr I. Greiser-Wilke, Hannover Veterinary School, Germany; Greiser-Wilke et al., 1992) as previously described (Knoetig et al., 1999). Similarly, addition of neutralizing *anti*-TNF-α mAbs (MP-390-392; Endogen) or polyclonal *anti*-IL6 antibodies (Endogen) to LPS-treated monocytic cell cultures infected with CSFV did not alter the effect of LPS on virus replication (Table 1). Therefore, it was concluded that neither TNF-α nor IL-6 were mediating the LPS-induced *anti*-CSFV state in monocytic cells.

No or very low (<1 unit) IFN activity was detectable in the supernatants from mock or CSFV infected cells, in the presence or absence of LPS; IFN activity was identified using a bioassay based on the antiviral effect against vesicular stomatitis virus in Madin-Darby bovine kidney (MDBK) or porcine kidney cells (PK-15) (data not shown). Furthermore, CSFV-infected monocytic cells were as susceptible to vesicular stomatitis virus infection as uninfected cells (data not shown). Addition of a pool of *anti*-IFN-α mAbs (K9 and F17, each 1 μg/ml; Diaz de Arce et al., 1992) did not strongly influence the LPS-induced antiviral state (Table 1), again indicating no direct major role for LPS-induced IFN. However, when recombinant porcine IFN-α (kindly provided by C. La Bonnardiere, INRA, Jouy-en-Josas, France; Lefevre et al., 1990) was added to monocytic cells before infection with CSFV, both the percentage of infected cells and virus titers were clearly reduced (Table 1). In fact, similar results were obtained using LPS, IFN-α and the synthetic double stranded RNA polyinosinic–polycytidylic acid (pIC; Sigma; Table 1). Such results were demonstrating that the LPS-induced antiviral state had a cell-associated nature, confirmed by the observation that it could not be transferred. Addition of supernatants from LPS-treated cells, wherein the LPS had been removed 24 h before harvest, to fresh Mø had no effect on CSFV infection/replication (data not shown).

1.3. IFN induction in monocytic cells

Although IFNs could not be detected in the supernatants, IFN levels in culture supernatants may have been below the limits of detection, but still capable of conferring antiviral activity on these cells. LPS-treated cells did express detectable IFN-α and IFN-β mRNA as determined by RT-PCR, similar to that induced by 20 μg/ml pIC (Fig. 2). The RT-PCR was performed as previously described using published IFN-α, IFN-β primers (Dozois et al., 1997), and β-actin primers (Basta et al., 1999) as internal controls. After reverse transcription (50 °C, 30 min) and 94 °C for 90 s, PCR was performed using 30 cycles of amplification (30 s, 94 °C, 30 s, 55 °C, 1 min, 68 °C, and a final incubation for 5 min at 68 °C). The induction of IFN type I mRNA transcription without detectable IFN bioactivity could result from an LPS-induced desensitization of the PBMC (Ziegler-Heitbrock, 1995).

1.4. Inhibition of the LPS-induced antiviral state by 2-aminopurine

Due to the reported capacity of LPS to activate PKR (Gusella et al., 1995), which is part of the intracellular pathways associated with antiviral defense (Clemens and Elia, 1997), it was of interest to determine what role PKR might play in the LPS-mediated *anti*-CSFV activity. Consequently, 2-aminopurine (2-AP, Sigma), a serine threonine protein kinase inhibitor and established inhibitor of PKR (Gusella et al., 1995; Hu and Conway, 1993; Thomis and Samuel, 1993), was employed. This drug probably acts as a competitive inhibitor of ATP during autophosphorylation following

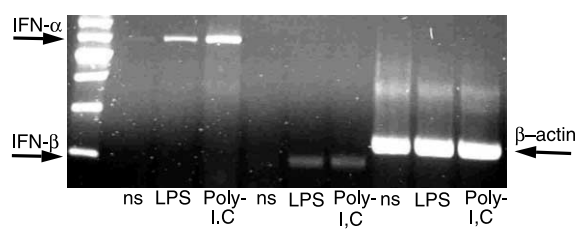

Fig. 2. Induction of IFN mRNA by LPS and pIC in Mø. RT-PCR amplification products of IFN-α mRNA (left), IFN-β mRNA (middle) and β-actin mRNA (right) isolated from 5-day-old Mø, which were untreated (ns), LPS-stimulated (10 μg/ml) or pIC-treated (20 μg/ml) for 16 h. Data are from one representative experiment out of three.

PKR activation (Hu and Conway, 1993). 2-AP was added to monocytes for 1 h at 37 °C before LPS treatment for another 2 h. The antiviral state induced by LPS was prevented in a 2-AP dose-dependent manner (Fig. 3A). Further addition of 2-AP during the 24 h culture period after virus infection increased this effect (Fig. 3A). In addition, the strong LPS-mediated inhibition of viral NS3 protein expression was also partially reversed (Fig. 3B). The effect of 2-AP was reproducibly seen in a total of four experiments and was statistically significant following analysis by the Student's t-test ($P < 0.01$). Interestingly, 2-AP also partially reversed the antiviral activity induced by pIC, but no effect was found against the antiviral effect of IFN-α (data not shown).

LPS stimulation of Mø has been reported to induce an early autophosphorylation of PKR, starting 1 h post stimulation, demonstrating that the effect of LPS on PKR activation is likely to be direct (Gusella et al., 1995). With the CSFV infection, it was noted that 2-AP treatment of the cells for only 2 h before infection was sufficient to reverse the LPS-mediated antiviral activity, again indicating a direct link between PKR induction and LPS activation. Downstream of PKR activation, a number of antiviral pathways can be induced. Particularly important is the phosphorylation of the alpha subunit of the protein synthesis initiation factor eIF2. This results in the shutdown of (viral) protein synthesis, and can certainly be related to the observed reduction in CSFV protein levels. In addition to this effect, a secondary role for IFN, which is induced following PKR activation, can be assumed (Der and Lau, 1995; Zinn et al., 1988). Taken together, the present results show for the first time that the

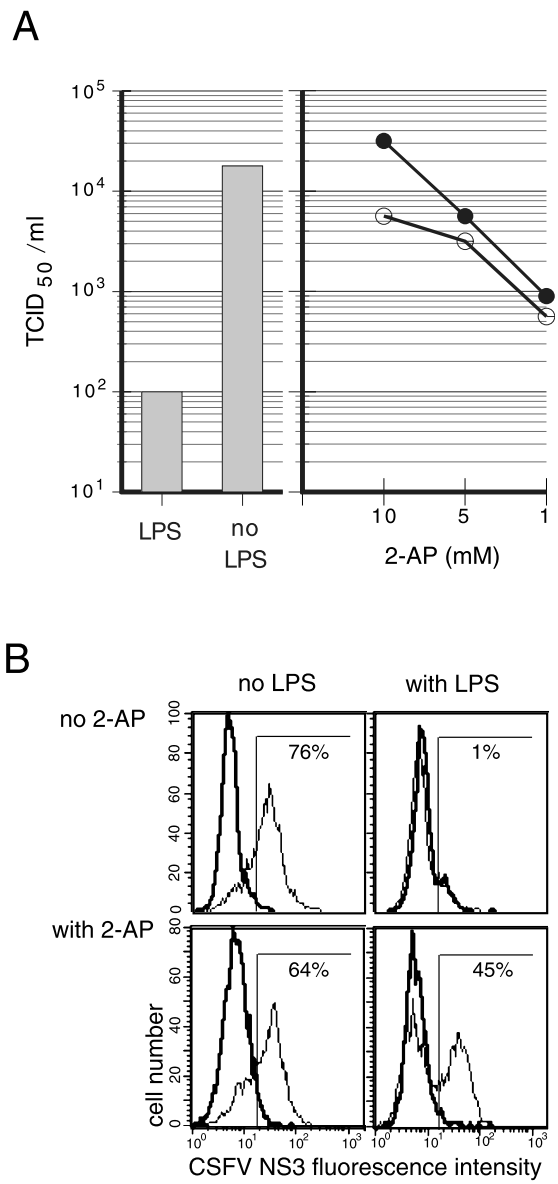

Fig. 3.

Fig. 3. (A) 2-AP reversal of LPS-mediated inhibition of CSFV replication in monocytes. Monocytes were treated with LPS (1 μg/ml) 2 h prior to infection with CSFV (1 $TCID_{50}$/cell), and cultured for a further 24 h. Supernatants were titrated for the presence of infectious CSFV (left graph). In certain of the LPS-treated cultures, different concentrations of 2-AP were added 1 h before LPS stimulation (right graph, open dots), or 1 h before LPS treatment plus during the subsequent LPS stimulation (2 h) and the virus infection/replication period (24 h) (filled dots). Again, supernatants were tested for virus titers. (B) CSFV NS3 protein expression in monocytic cells treated with 2-AP (10 mM, lower plots) and LPS (1 μg/ml; right-hand plots) as described above. Overlay histograms of mock-treated cells (bold line) over infected cells (light line) are shown. The experiment was repeated four times and similar results obtained.

LPS-mediated antiviral effect against CSFV is dependent on serine threonine protein kinase activation, likely PKR activation. It is also possible that this is a major mechanism involved in LPS activation-dependent inhibition of many other viruses.

With respect to CSF pathogenesis, it can be concluded that CSFV does not induce an antiviral response in monocytic cells. Furthermore, the susceptibility of monocytic cells to CSFV infection would be dependent on their activation status. PKR induction and/or autophosphorylation would be critical in determining the permissiveness of the cells to virus infection, and thus the clinical outcome of infection.

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