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ORF3 of porcine circovirus 2 enhances the in vitro and in vivo spread of the of the virus

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ABSTRACT

The ORF3 protein of the pathogenic porcine circovirus 2 (PCV2) causes apoptosis of the virus-infected cells. In PCV2-infected piglets, ORF3 induces B and CD4 T lymphocyte depletion and lymphoid organ destruction and the ORF3-deficient PCV2 is attenuated in its pathogenicity (*Virology*, 383 (2009), 338). In addition to its role in causing the apoptosis of the immune cells, characteristic of the PCV2 infection associated disease conditions, the ORF3 also plays a role in the systemic dissemination of the PCV2 infection. Our experiments here show that ORF3 expedites the spread of the virus by inducing the early release of the virus from the infected cells. Further, in PCV2-infected mice, the ORF3-induced apoptosis also aids in recruiting macrophages to phagocytize the infected apoptotic cells leading to the systemic dissemination of the infection. The apoptotic activity of the ORF3 of PCV2 hence lends advantage to the spread of the virus.

Introduction

Porcine circovirus 2 (PCV2) is the primary causative factor of post weaning multisystemic wasting syndrome (PMWS) in Swine (Allan et al., 1998; Ellis et al., 1998). Many other conditions like porcine dermatitis and nephropathy syndrome (PDNS), congenital tremors (CT) are also described to be associated with the PCV2 infection, which are collectively known as porcine circovirus associated diseases (PCVAD) (Gillespie et al., 2009). The PMWS primarily affects weanling piglets at 3 to 15 weeks age and has a high morbidity rate of up to 60% (Segales and Domingo, 2002). In the affected farms, the mortality ranges from 15% to 20% and occasionally reaches up to 80%. The PMWS is characterized by generalized lymphadenopathy, lymphocytic depletion and multinucleated giant cell formation in lymph nodes, and lymphoid organ destruction, with inflammatory lesions in multiple organs involving liver, kidney, lungs, which show histological lesions of histiocytic infiltration (Allan et al., 2004). The PCV2 infection leads to the decrease in the lymphocyte counts and development of a compromised immune status and is found as a co-infection with other pathogens (Nielsen et al., 2003; Allan et al., 2004; Darwich et al., 2002, 2004; Segales et al., 2004). The PCV2 genome encodes for three major open reading frames (ORF), which code for the replication associated proteins (ORF1), capsid protein (ORF2), and an apoptosis inducing protein (ORF3), which is not essential for the replication of the virus (Cheung, 2003; Liu et al., 2005). The study of the virus by molecular and epidemiological analysis, by various groups across the globe, points out two virus associated factors related to the replication cycle of the virus; polymorphisms in the capsid region of the virus and the ORF3 protein, which induces apoptosis in virus-infected cells (Cheung et al., 2007; Finsterbusch and Mankertz, 2009; Kiupel et al., 2005; Timmusk et al., 2008). The polymorphisms in the ORF2 protein could play a role in the cellular entry of the virus, which is thought to be mediated by the interaction of the capsid with cell surface Heparan Sulphate and Chondroitin Sulphate (Misinzo et al., 2006). Alternatively, the ORF2 polymorphisms could also affect the dynamics of virus assembly or the structural stability of the virus, which is known to have a high degree of thermal stability (O'Dea et al., 2008). Thus polymorphisms of ORF2 could lend a higher degree of fitness to viruses carrying certain "alleles" of ORF2. This is indeed reflected by the widespread temporal distribution of certain PCV2 genotypes, based on ORF2, observed in the epizootiological data (Cheung et al., 2007; Cortey et al., 2010; Gagnon et al., 2007; Wiederkehr et al., 2009). The second factor, the apoptosis induced by the ORF3, causes the loss of lymphocytes and lymphoid organ destruction in PCV2-infected piglets (Shibahara et al., 2000; Darwich et al., 2002; Nielsen et al., 2003; Liu et al., 2005, 2006; Timmusk et al., 2008; Karuppannan et al., 2009). The lymphocytes play a pivotal role in the development of the PCV2 infection associated diseases. The development of the PMWS is thought to be positively correlated with the proliferation of the lymphocytes induced by vaccination or co-infection with other

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infectious agents in the experimental disease models of PMWS (Krakowka et al., 2001, 2007; Ladekjaer-Mikkelsen et al., 2002). The replication of PCV2 in the lymphocytes eventually leads to their apoptosis. However, infection of mutant PCV2, deficient in ORF3, expression does not cause lymphocyte depletion or PCV2 associated pathogenesis in experimentally infected piglets, revealed by histological and systemic evidence (Karuppannan et al., 2009). The ORF3 of the non-pathogenic PCV1 and the ORF3 of the pathogenic PCV2 share less than 60% identity at the amino acid level. The ORF3 of PCV2 interacts with Pirh2, an E3 ligase involved in the ubiquitination of p53, resulting in the decreased levels of Pirh2 and an increase in levels of p53, and leading to apoptosis of the virus-infected cells (Liu et al., 2007; Karuppannan et al., 2010). Our previous studies have shown that SPF piglets inoculated with ORF3-deficient PCV2 show a delay in the onset of systemic viremia with a decreased level of peak viremia compared with the wild-type PCV2 inoculated specific pathogen free piglets (Karuppannan et al., 2010). We hypothesize that the apoptosis induced by the ORF3 of PCV2 could also have a role in the rapid spread of the virus, leading to high viral load, which is associated with the development of PCV2 associated diseases. Any factor that enhances the spread of the virus could increase its evolutionary fitness. Indeed, retrospective epidemiological analyses in Sweden have shown that specific ORF3 based phylogroups of PCV2 virus are associated with PCV2 induced disease (Timmusk et al., 2008). Here we examine the role of PCV2 ORF3 in the systemic spread of the virus using in vitro and in vivo experiments.

Results

Growth kinetics of wild-type PCV2 and ORF3-deficient PCV2

Our earlier experiments, in vitro in PK-15 cells and in vivo in mice and piglets, have shown that the growth kinetics of ORF3-deficient mutant PCV2 always lagged behind that of the wild-type PCV2 (Liu et al., 2005, 2006; Karuppannan et al., 2009). In order to examine the pattern of virus spread in the PK-15 cell culture, the kinetics of the replication of the wild-type PCV2 virus and the ORF3 mutant PCV2 virus in PK-15 cells were measured by quantifying the viral genome copy numbers using quantitative real-time PCR assay. Parallel cultures of PK15 cells in 35-mm cell culture dishes were infected with PCV2 or ORF3-deficient PCV2 virus, at a multiplicity of infection (MOI) of 0.1. The virus genome copy numbers in the infected cultures from the cell free supernatant, attached cells and the total virus copy numbers in the supernatant along with the cells were separately quantified, at 18, 24, 36, 48, 72, and 96 h after infection. The results showed that the cell free virus numbers in the wild-type PCV2-infected cells were higher than in the ORF3 mutant PCV2-infected cultures (Fig. 1). This difference was pronounced in the early time points after infection compared with the later time points after infection. However, the cell associated virus levels of the wild-type and ORF3 mutant PCV2 at early time points were similar. The difference in the cell associated virus levels between the two viruses increased after 36 h post infection until they reached almost similar levels at 72 h post infection. These results clearly show that the higher replication kinetics of wild-type PCV2 is associated with a higher cell free virus at the earlier time points after infection.

Role of ORF3-induced apoptosis in the spread of the virus

Many viruses are known to affect the apoptotic machinery of their host cells in the course of enhancing their evolutionary fitness (Devireddy and Jones, 1999; Dey et al., 2002; Dobner et al., 1996; Granja et al., 2004; Jeurissen et al., 1992; Noteborn et al., 1994; Roulston et al., 1999). The ORF3 of the PCV2 induces apoptosis in the infected cells (Liu et al., 2005). To examine the possible role of the apoptotic function of ORF3 in the spread of virus, the PCV2 replication kinetics were assayed in the presence of 50 μM of zVAD, a pan-caspase inhibitor, which is known to block the p53 mediated apoptosis. The apoptotic induction by ORF3, measured by quantifying the caspase 3 activity with a colorimetric assay, was inhibited by the zVAD and the caspase 3 activity was similar in the ORF3 mutant PCV2 and in the zVAD treated wild-type PCV2 infection (Fig. 2). The replication kinetics of the wild-type PCV2 and the ORF3-deficient PCV2 were quantified in the presence of the inhibitor (Fig. 3). The results showed that the cell free virus levels and total virus replication kinetics in the wild-type PCV2-infected cells were reduced in the presence of the inhibitor. However, the ORF3 mutant PCV2 did not show any change in its original replication kinetics in the presence of the inhibitor and especially, the cell free virus levels of the ORF3 mutant PCV2 were also not affected by the inhibitor. Further, in the presence of the inhibitor the replication kinetics of the wild-type PCV2 was similar to that of the ORF3 mutant PCV2 indicating that the apoptotic activity of the ORF3 contributes to the enhanced spread of the wild-type PCV2 to the supernatant.

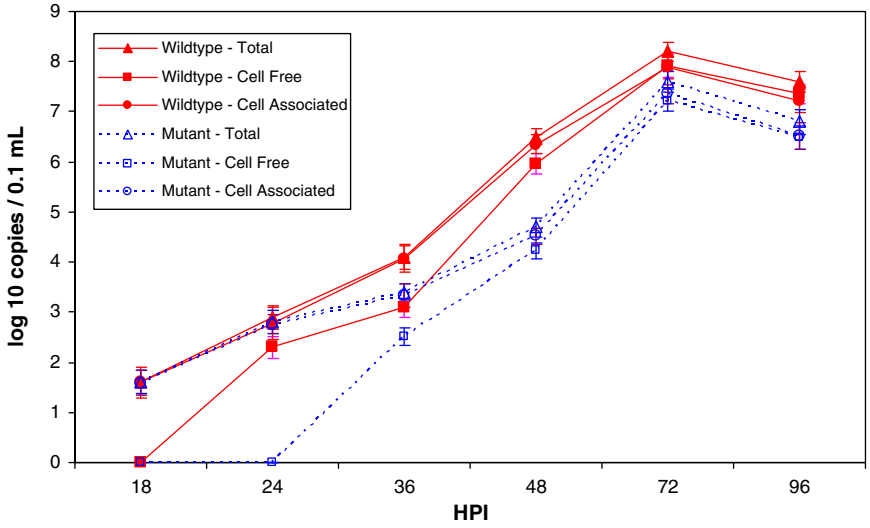

Fig. 1. The wild-type PCV2 is released into the cell culture supernatant earlier than the ORF3-deficient mutant PCV2.The copy number of the viral genome after infection with wild-type PCV2 or mutant PCV2 at 18, 24, 36, 48, 72, and 96 h after infection were quantitated by real-time PCR. The viral genome copy numbers in the total cell culture or in the cell associated fraction and the cell free fraction were quantified separately (HPI; hours post infection).

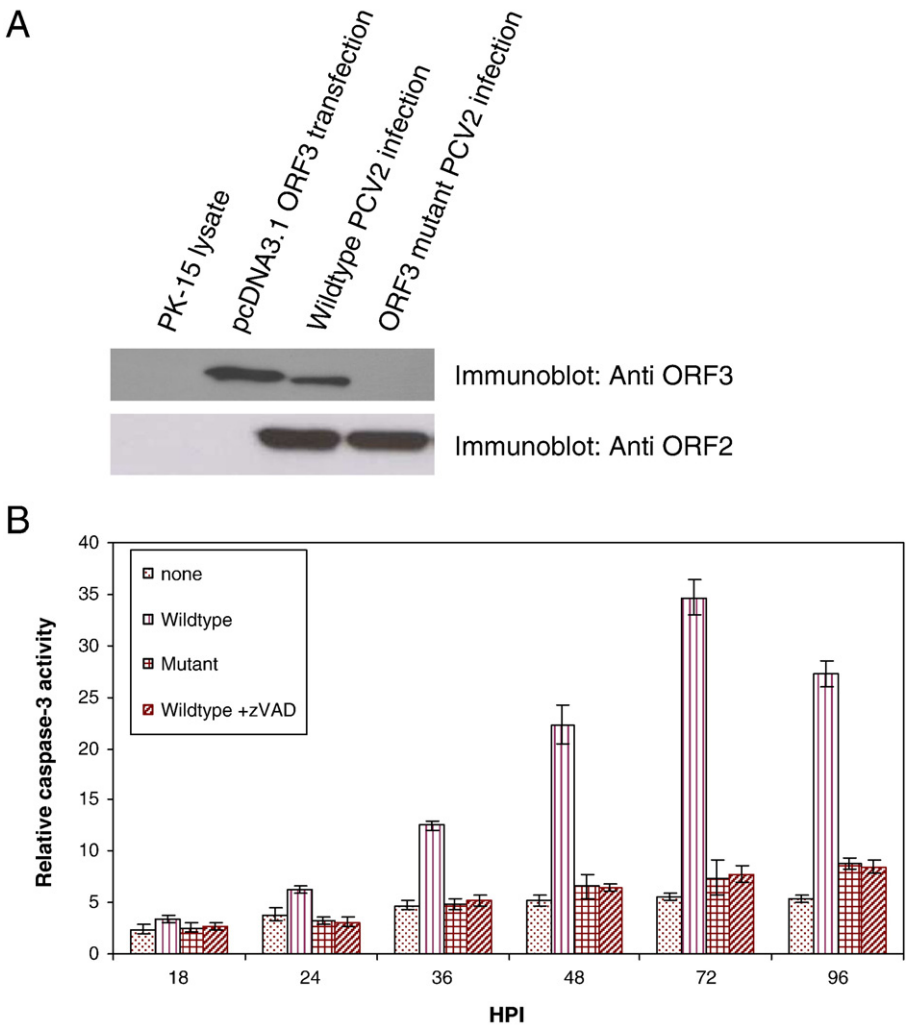

Fig. 2. A, Expression levels of ORF3 in wild-type PCV2 and ORF3 mutant PCV2 virus-infected PK-15 cells were assayed by Western blot. pcDNA3.1 ORF3 expression plasmid transfected PK-15 cells were used as a control. B, Caspase 3 activity induced by PCV2 is abolished by zVAD, a pan-caspase inhibitor. The ratio of caspase 3 activity in PK-15 cells at 18, 24, 36, 48, 72, and 96 h after infection with wild-type PCV2, mutant PCV2, wild-type PCV2 in the presence of 50 μM zVAD and uninfected control, was measured using a colorimetric assay (HPI; hours post infection).

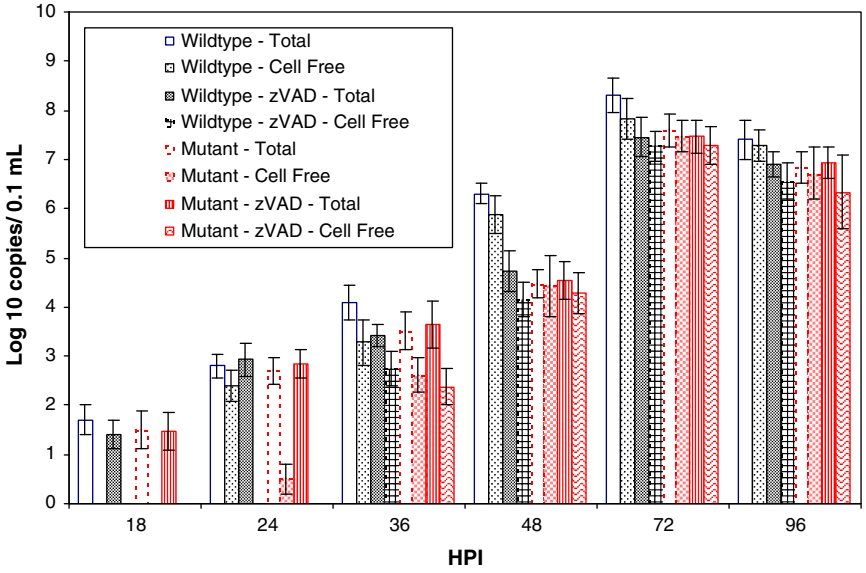

Fig. 3. The release of PCV2 virus from the infected cells to the cell culture supernatant is inhibited in the presence of caspase inhibitor zVAD. The copy number of the viral genome after infection with wild-type PCV2 or mutant PCV2 in the presence or absence of zVAD was quantitated by real-time PCR at 18, 24, 36, 48, 72, and 96 h after infection. The viral genome copy numbers in the total cell culture and the cell free fraction were quantified separately (HPI; hours post infection).

Mixed culture of ORF3-deficient PCV2 with a chimeric PCV1-2 virus

PCV1 is a non-pathogenic contaminant of the PK15 cell line and has been well documented to be a persistent non lytic infection even in other cell lines (Stevenson et al., 1999; Victoria et al., 2010). The ORF3 of the PCV1 significantly varies from the ORF3 of PCV2 and does not cause apoptosis when expressed in cells (our unpublished observations). A chimeric PCV1-2 viral genome with the ORF2, origin of replication of the PCV1 and the ORF1, ORF3 of PCV2 was constructed and the virus was regenerated. In the context of above findings, an assay was performed to examine if in mixed infection of the ORF3-deficient PCV2 and the PCV1-2 chimeric virus, the ORF3 of PCV2 expressed from the PCV1-2 chimeric virus can enable an early spread of the ORF3 mutant PCV2 virus to the cell free supernatant. The ORF3-deficient PCV2 was quantified by using primers specific for PCV2 ORF2 by a quantitative PCR assay. The replication kinetics of the ORF3 mutant PCV2 in mixed culture with the chimeric PCV1-2 expressing the PCV2 ORF3 was higher compared to the pure ORF3 mutant PCV2 infection (Fig. 4). The mixed culture with the PCV1-2 chimera displayed an early increase in the cell free virus levels of the ORF3-deficient PCV2 compared to the pure culture of ORF3-deficient PCV2, and this increase was reversed in the presence of 50 μM of zVAD in mixed virus infection cultures, showing that the apoptosis induced by the ORF3 is necessary for this spread of the virus into the cell free supernatant. This again directly shows that ORF3 plays a role in the exit of the virus from the infected cells.

Role of ORF3-induced apoptosis in the in vivo spread of the virus

We next wanted to examine if the ORF3-induced apoptosis plays a role in the spread of the virus in an in vivo infection. Many reports have shown that in vivo administration of zVAD in mice blocks p53 induced apoptosis (Emamaullee et al., 2007; Nussbaum and Whitton, 2004; Sarkar et al., 2006). To study the role of ORF3-induced apoptosis in PCV2 virus spread, four groups of BALB/c mice were inoculated with 10^5 $TCID_{50}$ of wild-type PCV2 or ORF3-deficient PCV2 virus in the presence or absence of zVAD. The zVAD regimen used here has been previously shown not to adversely affect the health of the mice or affect the immune response of mice to viral infection (Nussbaum and Whitton, 2004). The systemic spread of the wild-type PCV2 virus, assayed by serum viremia, was reduced in the presence of the zVAD (Fig. 5). On day 7 post infection the serum viremia could not be detected in the group infected with wild-type virus in the presence of zVAD and at all subsequent time points, the viremia in this group was less compared to the group infected with wild-type PCV2 in the absence of zVAD. However, the kinetics of systemic virus spread of the groups infected with ORF3-deficient PCV2 virus was not altered by the presence of the inhibitor zVAD. These observations show that the apoptosis induced by ORF3 plays a role in the systemic spread of the virus.

Role of macrophages in the spread of PCV2 viremia

Phagocytic cells play a central role in the clearance of apoptotic cells (Erwig and Henson, 2008). The apoptosis induced by PCV2 ORF3 in infected cells could facilitate the macrophage mediated systemic spread of the virus. Indeed, in the analysis of natural PCV2 infections in piglets, with severe disease manifestation, macrophages have been found to contain a high load of PCV2 virus and this has also been correlated with a high level of systemic PCV2 viremia (Darwich et al., 2004; Harding et al., 2008; Krakowka et al., 2002; Sanchez et al., 2004; Stevenson et al., 2001; Wallgren et al., 2009). This phenomenon could also be observed in mice models of PCV2 infection, where macrophages were found to contain PCV2 antigen (Kiupel et al., 2001). When we examined the virus levels in macrophages in the infected mice, the wild-type PCV2-infected mice displayed a higher level of virus in the macrophages compared to ORF3-deficient PCV2-infected mice (Fig. 6). However, in the presence of zVAD, the wild-type PCV2-infected mice showed lower virus levels in the macrophages, comparable to the levels in mice infected with ORF3-deficient PCV2 virus. The ORF3-deficient virus-infected mice on the other hand did not show any alteration in the virus levels in macrophages in the presence of zVAD. These results show that the apoptosis induced by ORF3 of PCV2 helps in enhancing the systemic spread of the virus by increasing the uptake of the virus by macrophages.

The ability of macrophages to support the replication of PCV2 is a debated issue (Yu et al., 2007; Perez-Martin et al., 2007; Sanchez et al., 2004; Darwich et al., 2004; Vincent et al., 2003). We next examined if the macrophages could support the replication of the virus or merely serves to disseminate the virus. RNA was isolated from purified CD11b-positive splenic cells from the different groups of mice and were examined for the levels of PCV2 replicase mRNA. The results showed that PCV2 replicase (ORF1) RNA expression could not be

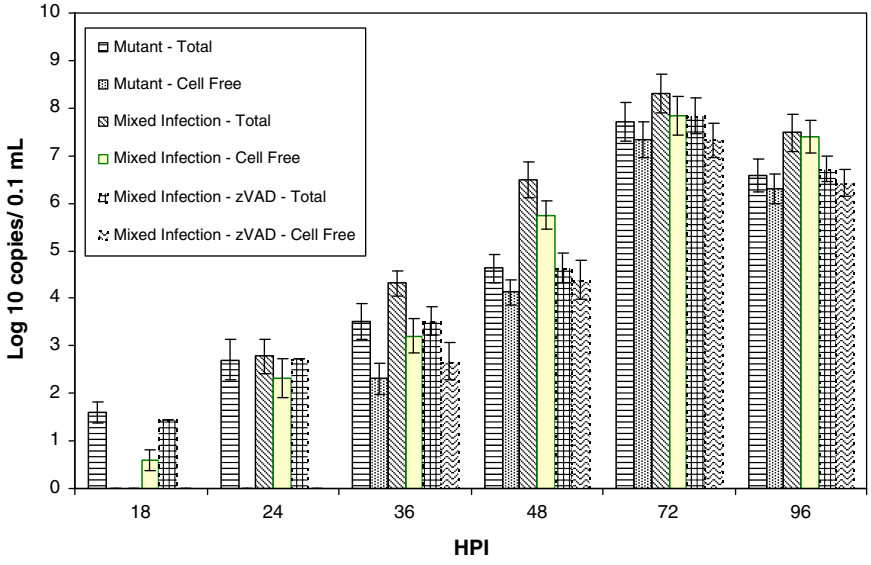

Fig. 4. The release of mutant PCV2 from the infected cells is enhanced by complementation of the ORF3 expression. The copy number of the viral genome after infection with mutant PCV2 or mutant PCV2 in the presence of chimeric PCV1 expressing the ORF3 of PCV2, were quantitated by real-time PCR at 18, 24, 36, 48, 72 and 96 h after infection. The viral genome copy numbers in the total cell culture and the cell free fraction were quantified separately (HPI; hours post infection).

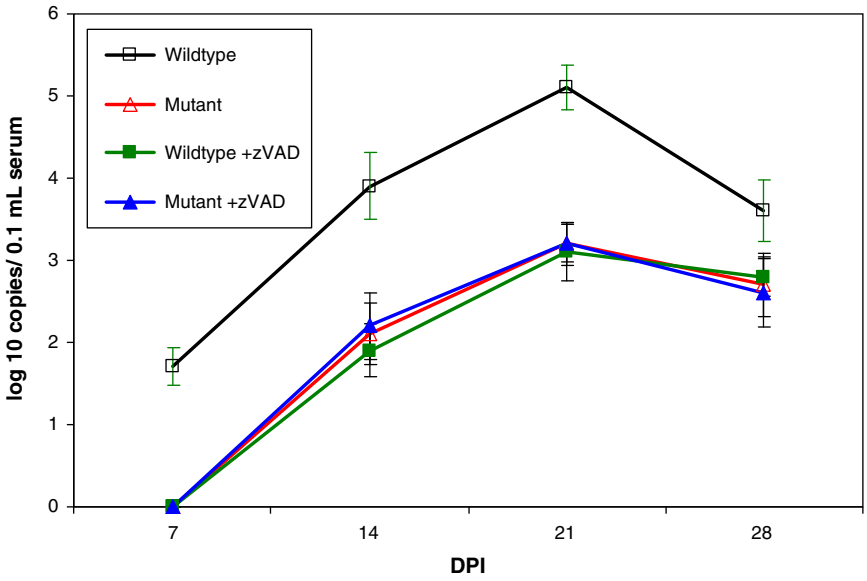

Fig. 5. The apoptosis induced by ORF3 of PCV2 enhances the spread of the virus in vivo. The serum viremia of mice infected with wild-type PCV2 or mutant PCV2 in the presence or absence of zVAD was quantified using real-time PCR assay at 7, 14, 21, and 28 days after infection (DPI; days post infection).

detected in the CD11b-positive splenic cells in mice from any of the groups (data not shown). This shows that PCV2 does not replicate in the macrophage cells, at least in the mice model of infection. Further, previous studies have shown that TNFα levels are elevated in the PMWS affected pigs and could contribute to the progression of the disease (Sipos et al., 2005; Kim et al., 2006; Shi et al., 2010). The in vitro infection of primary porcine alveolar macrophages with PCV2 also leads to an elevation of TNFα secretion (Chang et al., 2006). Supporting this observation, intramuscular injection of the ORF3 expression plasmid in mice has been shown to cause mortality and a significant elevation in the TNFα levels (An et al., 2008). We examined the levels of TNFα mRNA in the macrophages of mice in the different groups. The wild-type PCV2-infected mice showed an elevated TNFα mRNA level, relative to the mice infected with wild-type virus in the presence of zVAD (Fig. 7). Mice infected with the ORF3 mutant PCV2, in the presence or absence of zVAD, showed significantly lower levels of TNFα mRNA in the macrophages compared to the wild-type PCV2-infected mice. This indicates that ORF3-induced apoptosis and macrophage recruitment lead to elevated TNFα levels, which has been linked with PMWS progression in PCV2-infected piglets.

Discussion

Porcine circovirus 2 is the principal infectious agent implicated in the development of PMWS and other PCVAD (Allan et al., 1998; Ellis et al., 1998). The major virus associated factors that are implicated in the spread and pathogenesis of the virus are the polymorphisms in the capsid of the virus and the apoptosis induced by the ORF3 protein in the virus-infected cells (Cheung et al., 2007; Timmusk et al., 2008; Finsterbusch and Mankertz, 2009; Shibahara et al., 2000; Liu et al., 2005, 2006; Timmusk et al., 2008; Karuppannan et al., 2009). Viruses are genomic parasites that evolve to usurp cellular proteins and pathways for their benefit. Some viruses inhibit apoptosis in the host cells, while others induce apoptosis (Devireddy and Jones, 1999; Dey et al., 2002; Dobner et al., 1996; Granja et al., 2004; Jeurissen et al., 1992; Noteborn et al., 1994; Everett and McFadden, 1999). The PCV2 ORF3 protein induces apoptosis in the infected cells in a p53-dependent manner (Liu et al., 2005). The ORF3 protein interacts with Pirh2, an E3 ligase specific for p53. This interaction leads to the sequestration of Pirh2 in the cytoplasm and a decrease in its half-life, leading to the increased cellular levels of p53 and apoptosis (Liu et al., 2007; Karuppannan et al., 2010). Our

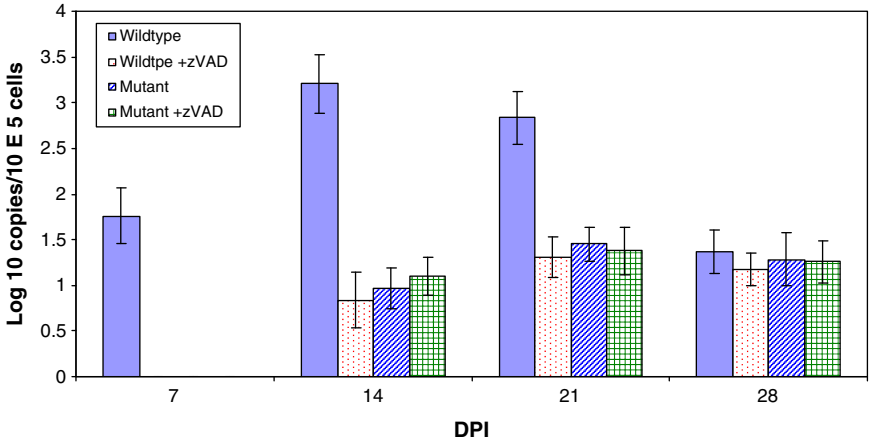

Fig. 6. The apoptosis induced by ORF3 of PCV2 enhances the viral load in macrophages in infected mice. The virus load in macrophages of mice infected with wild-type PCV2 or mutant PCV2 in the presence or absence of zVAD was quantified using real-time PCR assay at 7, 14, 21, and 28 days after infection (DPI; days post infection).

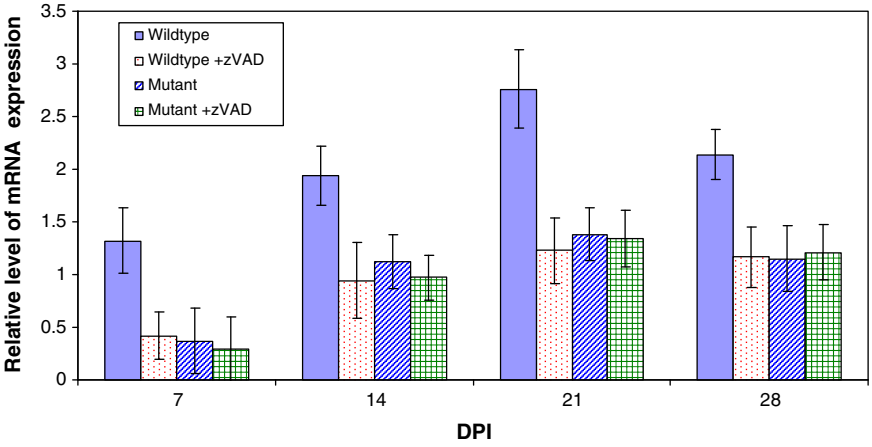

Fig. 7. ORF3 of PCV2 contributes to elevated TNFα expression in macrophages of infected mice. The relative expression level of TNFα mRNA in macrophages of mice infected with wild-type PCV2 or mutant PCV2 in the presence or absence of zVAD was quantified using real-time PCR assay at 7, 14, 21, and 28 days after infection (DPI; days post infection).

previous studies of ORF3-deficient PCV2 infection in mice and SPF piglets showed an absence of lymphoid organ depletion and reduction in lymphocyte counts in the course of the viral infection, usually found in infections with wild-type PCV2 (Karuppannan et al., 2009). Further, these studies showed delayed kinetics of systemic viremia in infections with the ORF3-deficient PCV2 virus along with the reduction in the level of peak systemic viremia compared to the wild-type PCV2 infection. However, the ORF3 of PCV2 is not essential for the replication of the virus both in vitro and in vivo (Liu et al., 2005, 2006). These observations indicate that the apoptosis induced by the ORF3 of PCV2 may play a role in enhancing the systemic spread of the virus without directly affecting the replication of the virus. Here, we have examined the role of ORF3-induced apoptosis in the spread of the virus using in vitro and in vivo experiments.

Our in vitro experiments reported here show that the apoptosis induced by the ORF3 of PCV2 enables the earlier release of the wild-type PCV2 virus to the cell free supernatant compared to the ORF3-deficient PCV2 virus (Figs. 1, 3). The released virus re-infects other cells, and this amplification cycle results in the observed enhancement in the replication kinetics. The pan-caspase inhibitor zVAD inhibits the apoptosis induced by the ORF3 protein and delays the release of the wild-type PCV2 virus into to the cell free supernatant and leads to a delayed virus replication kinetics (Figs. 2, 3). This is similar to the virus release pattern and replication kinetics of the mutant PCV2 deficient in ORF3 expression. On the same note, the PCV1, which does not induce apoptosis in the infected cells, is known as a persistent, un-evident virus infection in cell lines of porcine origin (Stevenson et al., 1999), and recently contamination of PCV1 has been observed even in cell lines used for production of live attenuated vaccines for human use (Victoria et al., 2010). Ultra-structural studies of PK-15 cells persistently infected with PCV1 shows that the assembled virus accumulates as dense para-crystalline arrays in the cytoplasm of the infected cells (Stevenson et al., 1999). Our experiments here show that in the absence of the ORF3, the mutant PCV2 also accumulates in the cytoplasm of the infected cells, possibly until the replication of the virus overwhelms the cellular homeostasis resulting in the release of the virus. In mixed infections with ORF3-deficient PCV2 and the PCV1-2 chimeric virus expressing the PCV2 ORF3, the replication kinetics of ORF3-deficient PCV2 is increased aided by the early release of cell free virus, and this increase is reversed by the presence of zVAD (Fig. 4). Thus the ORF3-induced apoptosis of PCV2-infected cells enhances the release of the virus and enables its spread. Similarly, mice infected with wild-type PCV2 reveals a faster in vivo spread compared to infection with ORF3-deficient PCV2, as measured by the serum viremia (Fig. 5). The higher rate of virus spread of the wild-type PCV2 is reduced by the co-administration of zVAD, a pan-caspase inhibitor that is known to block p53 induced apoptosis when administered in vivo. This shows that apoptosis induced by PCV2 ORF3 facilitates the in vivo spread of the virus. Supporting this view, infection studies in mice and SPF piglets display lower virus replication kinetics of ORF3-deficient PCV2 compared with the wild-type PCV2 (Liu et al., 2006; Karuppannan et al., 2009). Further in cases of natural infections of PCV2 in piglets with high levels of serum viremia that develop to PMWS, macrophages are found to contain a high viral antigen load (Darwich et al., 2004; Harding et al., 2008; Krakowka et al., 2002; Sanchez et al., 2004; Stevenson et al., 2001; Wallgren et al., 2009). This was also observed in mice experimentally infected with PCV2, which display a high viral load in macrophages (Kiupel et al., 2001). However, whether the PCV2 actively replicates in the macrophages is a debated issue, as some reports show the absence of the replicative form of the viral genome and absence of nuclear antigenic signal in these cells (Perez-Martin et al., 2007; Darwich et al., 2004; Vincent et al., 2003). Macrophages along with other phagocytic cells bring about the clearance of apoptotic cells (Erwig and Henson, 2008) and could inadvertently expedite the systemic dissemination of the PCV2 virus by engulfing virus-infected apoptotic bodies and transporting them to the regional lymph nodes, which are thought to be initial sites for PCV2 replication in natural infection (Yu et al., 2007; Vincent et al., 2003). In our observations, mice infected with wild-type PCV2 have a higher viral load in the macrophages compared to mice infected with the ORF3-deficient PCV2 or the wild-type PCV2-infected mice treated with zVAD (Fig. 6). This again shows a direct relationship between the PCV2 induced apoptosis and the spread of the virus through the macrophages. Further, the cytokine secretion by macrophages is known to be influenced by the presence of apoptotic cells (Lucas et al., 2003). Our observations here show that the macrophages in wild-type PCV2-infected mice have an elevated level of TNFα (Fig. 7), which is typically observed in PCV2-infected piglets with PMWS (Sipos et al., 2005; Kim et al., 2006, Shi et al., 2010). This elevation of TNFα is not observed in macrophages of mice infected with ORF3-deficient PCV2. The ORF3 mediated elevation of TNFα was also observed in other studies, when plasmid expression vector encoding ORF3 was injected into mice, leading to severe symptoms and death (An et al., 2008). The signaling mechanism involved in this cytokine induction needs to be further elucidated. In summary, the apoptosis induced by ORF3 of PCV2 hastens the exit of the virus from the infected cells, enables the macrophage mediated systemic spread of the virus, and also influences the cytokine secretion by the macrophages.

The role of virus induced apoptosis in the lymphocyte depletion and the progression of PCV2 associated diseases has been reported in many studies (Shibahara et al., 2000; Nielsen et al., 2003; Liu et al., 2005, 2006; Timmusk et al., 2008; Karuppannan et al., 2009). Our previous studies have shown that ORF3-induced apoptosis leads to

lymphocyte depletion in PCV2-infected mice and piglets (Liu et al., 2006; Karuppannan et al., 2009). However, the role of apoptosis in PCV2 pathogenesis has also been contested by other reports (Mandrioli et al., 2004; Resendes et al., 2004). On the same note, a recent report of ORF3-deficient PCV2 infection in conventional PCV2 free piglets claims that apoptosis induced by ORF3 does not play a significant role in the pathogenicity of the virus; however, on the contrary, their data shows reduced viral replication kinetics and lower peak viremia level in the ORF3-deficient PCV2-infected piglets compared to wild-type PCV2-infected piglets, supporting our findings (Juhan et al., 2010). Our observations here directly show that the ORF3 mediated apoptosis expedites the exit of the virus from the infected cells, which otherwise could be accumulated in the infected cells. The apoptosis mediated by ORF3 also leads to the rapid systemic dissemination of PCV2 infection by recruiting the macrophages, which could seed the draining lymph nodes with the virus, accelerating the progression of the viral spread. The ORF3, which is not directly involved in the replication of the virus, thus plays an important role in the systemic spread of the viral infection. The involvement of specific immune cells in the systemic pathogenesis of the PCV2 could be further explored in "knockout mice" lacking specific components of the immune system. Further studies to examine the role of ORF3 in the spread of PCV2 virus from one piglet to another are necessary, as the ORF3 may also enhance the shedding of the virus from piglets with high serum viremia.

Materials and methods

Cell culture and viruses

The PK-15 cells are of porcine origin and free of PCV1 contamination (Liu et al., 2005). The cells were maintained in minimal essential medium supplemented with 5% heat inactivated fetal bovine serum (FBS), 5% L-glutamine, 100U/ml of Penicillin G, and 100 µl/ml streptomycin at 37 °C in a humidified 5% CO_2 incubator.

The PCV2 BJW strain virus was used for the experiments (Liu et al., 2005). The chimeric PCV1-2 virus was constructed by inserting the ORF2 and origin of replication of the PCV1 (DQ472015) in place of its PCV2 (BJW strain) counterparts. The viral genome was synthesized from a commercial source (Genscript, USA) and the virus regenerated as described previously (Liu et al., 2005).

The virus infections were performed in parallel cultures of PK-15 cells in 35-mm dishes containing 1 ml of cell culture media. Briefly, the cells were seeded overnight and infected at a multiplicity of infection (MOI) of 0.1 with the indicated virus. At various time points after the infection, the virus genomes in the cell culture supernatant or in the attached cells or the total virus numbers were quantified as follows. The cell culture supernatants along with the floating cells were collected and the viral genome isolated with QIAamp DNA kit (Qiagen) according to the manufacturer's instructions. The attached cells were harvested separately by scrapping-off with a sterile cell-scarper and re-suspended in 1 ml PBS and the viral genome isolated as mentioned above. Alternatively the scraped cells were suspended in the cell culture supernatant and the total viral genome in both the cell free and cell associated compartments was isolated. The viral genome copy numbers were enumerated using real-time quantitative PCR as described below.

Quantitative real-time PCR

Briefly, viral genome was isolated using the QIAamp DNA (QIAGEN) and the copy number was estimated by using LightCycler ® real-time PCR machine (Roche Applied Sciences) and DyNAmo™ SYBR® Green qPCR Kit (Finnzymes). The primers used were specific for ORF2 in the PCV2 genome and amplified a 250-bp region. The primers and PCR parameters used were the same as described earlier (Liu et al., 2006).

Plasmids and transfection

The ORF3 of PCV2 BJW strain was cloned in pcDNA3.1+ expression vector at BamHI and XhoI restriction sites. The following primers were used for the amplification of the ORF3 gene. ORF3 forward 5′-CAGAGGATCCATGGTAACCATCCCACCACTTG-3′, ORF3 reverse 5′-CAGACTCGAGTTACTTATCGAGTGTGGAGCTC-3′. The PK-15 cells were transfected with the plasmid by using Transfectin reagent (Biorad).

Western blot analysis

The whole-cell lysates from virus-infected or untreated PK15 cells were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membranes (Biorad). The membranes were blocked for 1 h at room temperature in blocking buffer TBST (20 mM Tris–HCl [pH 7.4], 150 mM NaCl, 0.1% Tween 20) containing 5% skim milk powder to prevent nonspecific binding and then incubated with mouse polyclonal anti-ORF3 antibody (Liu et al., 2005) or anti-ORF2 specific mouse monoclonal antibody (8C2) at room temperature for 1 h. The membranes were then washed three times with TBST and incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-mouse secondary antibody (DAKO) diluted in blocking buffer (1:2000). After washing the membrane, the proteins were visualized using Super Signal West Femto Chemiluminescent substrate (Pierce) and Amersham Hyperfilm (GE Amersham).

Assay for caspase activity

For quantifying the apoptosis induction by ORF3 in PK-15 cells after viral infection, a colorimetric assay specific for active caspase 3 was performed (caspACE assay system, PROMEGA) by following the manufacturer's instructions. Briefly, the cells were harvested at different time points after virus infection using a sterile cell scraper, washed in PBS, and resuspended in the supplied cell lysis buffer. The lysate was used for the colorimetric assay, which measured the cleavage of Ac-DVED-pNA substrate by active caspase 3. The caspase 3 activity in virus-infected cells was normalized to that of uninfected PK-15 cells.

Mice infections studies

The experimental design consisted of four groups of 8-week-old BALB/c mice with sixteen mice per group. Each group of mice was housed in an individual room and handled separately. The mice were each inoculated, both intranasally and intraperitoneally, with 10^5 50% tissue culture infective doses of the PCV2. The zVADfmk (BD biosciences) was dissolved in DMSO vehicle to a final DMSO concentration of 20%. The zVAD was administered intra peritoneally at the rate of 1 mg/dose, once in 3 days, starting a day before the inoculation of the virus and was continued up to 25 days post inoculation. The regimen was adapted from a previous study on Lymphocytic chorio meningitis virus (LCMV) infection in BALB/c mice model, which showed that the zVAD does not affect the course of the immune response against viral infection (Nussbaum and Whitton, 2004). Four mice from each group were sacrificed at 1-week intervals and 0.5 ml of peripheral blood was collected from each mouse, for the estimation of viremia. The viral genomice DNA was isolated using QIAamp Blood DNA kit as mentioned above and quantified by real-time PCR. The spleen was dissected out for the isolation of the macrophage lineage cells. To describe the procedure briefly, the spleen cells were crushed out of the capsule and collected into heparinized PBS and the RBC were lysed using a commercial kit (UTI Lyse kit, Dako) and the macrophages were subsequently isolated with micro beads conjugated to mouse antiCD11b antibodies using magnetic cell sorting kit (MACS, Miltenyi biotec) by following the manufacturer's

instructions. Flow cytometric analysis showed that more than 95% of the purified cells were CD 11b-positive. The isolated cells were used to isolate the total RNA using Trizol Reagent (Invitrogen) following the manufacturer's instructions. The isolated RNA was treated with RNAse free DNAseI to remove any contaminated DNA. The levels of PCV2 Replicase RNA were estimated from the reverse transcribed RNA by real-time PCR using the following primers Rep forward 5′-GCGGCAGCACCTCG-3′ and Rep reverse GTC 5′-CTTCCTCATTACCCTCC-3′. The TNFα and GAPDH mRNA levels in the RNA fraction, after being reverse transcribed, were estimated by real-time PCR assay with the following primers; TNFα forward primer 5′-TGATCCGCGACGTGGAA-3′, TNFα reverse primer 5′-ACCGCCTGGAGTTCTGGAA-3′, GAPDH forward primer 5′-GCACAGT-CAAGGCCGAGAAT-3′, GAPDH reverse primer 5′-GCCTTCTCCATGGTGGT-GAA-3′.

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