1 **Comparative immunopathogenesis and biology of recently discovered porcine circoviruses**

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Abstract:

Porcine circoviruses are important pathogens of production swine. Porcine circovirus type 1 (PCV1) is non-pathogenic, and discovered as a contaminant of a porcine kidney cell line, PK-15. The discovery of pathogenic variant, PCV2, occurred in the late 90's in association with post-weaning multi-systemic wasting disease syndrome (PMWS), which is characterized by wasting, respiratory signs and lymphadenopathy in weanling pigs. A new PCV type, designated as PCV3, was discovered in 2016, in pigs manifesting porcine dermatitis and nephropathy syndrome (PDNS), respiratory distress and reproductive failure. Pathological manifestations of PCV3 Infections include systemic inflammation, vasculitis and myocarditis. A 4th PCV type, PCV4, was identified in 2020 in pigs with PDNS, respiratory and enteric signs. All the pathogenic PCV types are detected in both healthy and morbid pigs. They cause chronic, systemic infections with various clinical manifestations. Dysregulation of the immune system homeostasis is a pivotal trigger for pathogenesis in porcine circoviral infections. While the study of PCV3 immunobiology is still in its infancy lessons learned from PCV2 and other circular replication-associated protein (Rep)-encoding single stranded(ss) (CRESS) DNA viruses can inform the field of exploration for PCV3. Viral interactions with the innate immune system, interference with dendritic cell function coupled with the direct loss of lymphocytes compromises both innate and adaptive immunity in PCV2 infections. Dysregulated immune responses leading to the establishment of a pro-inflammatory state, immune complex associated hypersensitivity, and the necrosis of lymphocytes and immune cells are key features of PCV3 immunopathogenesis. A critical overview of the comparative immunopathology of PCV2 and PCV3/4, and directions for future research in the field are presented in this review.

Key words: porcine, circoviruses, PCV2, PCV3, PCV4 immunity, cell mediated immunity, innate immunity, antibody, immunopathogenesis

Introduction:

Porcine circovirus (PCVs) type 1 was initially discovered as a non-pathogenic contaminant of a porcine kidney (PK-15) cell line (Tischer, Gelderblom, Vettermann, & Koch, 1982). The association of a pathogenic variant, PCV2, with a post-weaning multi-systemic wasting disease (PMWS) of piglets was established after its isolation from pigs with clinical signs of wasting, respiratory disease and lympadenopathy (Ellis et al., 1998). Thereafter, two other PCV types, designated as PCV3 and PCV4 were identified in 2015 (Palinski et al., 2017; Phan et al., 2016) and 2019 respectively (H. H. Zhang et al., 2020). While the prevalence of PCV3 is reported in several parts of the world, PCV4 has been detected in China, Korea and Malaysia so far (Opriessnig, Karuppannan, Castro, & Xiao, 2020), but was absent in swine samples from Spain and Italy (Franzo et al., 2020).

As both pigs with subclinical and overt clinical signs harbored PCV2, the initial scientific debate was focused on whether PCV2 was a primary or opportunistic pathogen. However, based on both observational and experimental science, it is now established that PCV2 is the primary cause of a number of disease manifestations such as wasting, lymphadenopathy, respiratory distress, reproductive failure, jaundice and diarrhea, collectively known as porcine circovirus associated diseases (PCVAD). Typical lesions in PCV2 infected pigs involve the lymphoid system. Enlarged lymph nodes, lymphoid depletion with loss of T and B cells both in the lymphoid organs and in the circulation, followed by histiocytic replacement and an increase in the number of monocytes and macrophages are common findings on necropsy (Ellis et al., 1998; Ramamoorthy & Meng, 2009). Therefore, regardless of the clinical manifestation, the development of immunopathology is fundamental to inducing clinical disease in other organ systems in both single PCV2 infections and coinfections with other agents.

Porcine dermatitis and nephropathy syndrome (PDNS) is an immune complex mediated disease of pigs, which is characterized by purple patchy lesions distributed over the abdomen, hind quarters, and

66   ears, coupled with wasting and loss of condition. Typical lesions on necropsy include systemic necrotizing

67   vasculitis, especially of the skin and kidneys with petechial hemorrhages, glomerulonephritis, edema, and

68   fluid accumulation in the body cavities. Based on epidemiological associations, the etiology of PDNS was

69   previously attributed to PCV2 (Langohr et al., 2010) or porcine reproductive and respiratory disease

70   syndrome virus (PRRSV), and PCV2 co-infections (Choi & Chae, 2001). However, typical signs of PDNS

71   were not reproducible in experimental models. In 2015, two independent studies based on viral

72   metagenomic technology confirmed the presence of a novel porcine circovirus, now designated at PCV3, in

73   the tissues of pigs manifesting PDNS and reproductive failure (Palinski et al., 2017) or disseminated multi-

74   organ inflammation, myocarditis and respiratory signs (Phan et al., 2016). Previously suspect etiological

75   agents such as PCV2, PRRSV, and other major swine pathogens were not detected in the tissues of the

76   pigs investigated, indicated that PCV3 is likely to be the primary causative agent of the disease

77   manifestations. Very little is currently known about the pathogenesis of PCV4 currently except that it is

78   epidemiologically associated with respiratory, enteric and PDNS signs (H. H. Zhang et al., 2020). In the

79   case of PCV3, dysregulation of the inflammatory response and immune homeostasis leading to immune-

80   complex mediated hypersensitivity appears to be central to pathogenesis (Jiang et al., 2019; Palinski et al.,

81   2017). Thus, immune dysregulation is focal to the pathogenesis of both PCV2 and 3, with subsequent

82   clinical manifestations in other organ systems following the disruption of immune homeostasis.

83   The availability of metagenomic sequencing technology has led to an explosion in the discovery of

84   circular replication-associated protein (Rep)-encoding single stranded(ss) (CRESS) DNA viruses.

85   Circoviruses are a part of these small, genetically diverse, ubiquitous viruses, which fall under class II of the

86   Baltimore classification system. They are distributed across 10 families, and infect a very wide host range

87   including prokaryotes, plants and eukaryotes. The members of the CRESS viruses are unified by the

88   presence of universally conserved replicase proteins with motifs for rolling circle amplification and

89   endonuclease/ helicase functions. The capsid proteins of CRESS viruses, however, are not very diverse

90   (Krupovic et al., 2020). Additionally, members of the CRESS family of viruses share characteristics such as

91   being difficult to culture in the laboratory and straddling the fence in their identity as primary or opportunistic

92   pathogens (Shulman & Davidson, 2017). Among the CRESS viruses, porcine circoviruses and chicken

93   anemia virus (CAV) are the only members with established pathogenicity, while Anelloviruses, including

94   swine torque teno viruses (TTSuVs) are suspected to be opportunistic pathogens that play a role in

95   enhancing co-morbidities (Webb, Rakibuzzaman, & Ramamoorthy, 2020). Therefore, it is very likely that

96   circoviruses share some aspects of their immune biology with other CRESS viruses, to support a

97   ubiquitous life style and confer the ability to selectively trigger disease under appropriate conditions.

98   While the current case definitions of PCV2 and PCV3 are based upon the organ system affected,

99   given that both PCV2 and 3 can infect immune cells, and result in inflammation, immune-complex formation

100   and immune dysregulation, the immune system can be considered the primary but underestimated target of

101   pathogenic porcine circoviruses. Both PCV2 and 3 are widely prevalent in swine populations in pork-

102   producing countries globally, indicating they are highly successful in transmission and colonization of their

103   hosts, and avoiding host immune responses to successfully establish as a state of co-existence or

104   selectively induce disease in the host. The major replicase and capsid proteins of the newly discovered

105   PCV3 are less than 50% similar at the amino acid level to PCV2 proteins. Similar to PCV3, mink and bat

106   circoviruses cluster more closely with PCV4 than PCV2, with PCV4 proteins also having less than 50%

107   sequence similarity to the PCV2 Rep and Cap proteins (Palinski et al., 2017). Therefore, the newly

108   discovered porcine circoviruses are likely to be distinct from PCV2 in their biology and mechanisms of

109   pathogenesis. Besides its role as a primary pathogen, PCV2 plays an important role in exacerbating

110   coinfections. Similarly, there are several reports of the co-detection of PCV3 with PCV2, PCV4 (Sun et al.,

111   2021), pseudorabies virus (Tian et al., 2020), classical swine fever (Zheng et al., 2020), PEDV (H. Y. Han

et al., 2019) and PRRSV (Chen et al., 2019) among other agents. While not fully understood, the incidence of co-infections with PCV2 and the possible immune mechanisms involved are reviewed in detail elsewhere (Saade et al., 2020). There is currently little information regarding the molecular immunology of PCV3 and 4 coinfections. Hence, this topic is not reviewed herein. Although information on host immune responses to PCV3 and 4, or prevention by vaccination, is limited, prior information findings on PCV2 will likely inform the directions for future research the recently discovered porcine circoviruses. Therefore, this review provides a condensed, comparative analysis of the current status of knowledge on viral immunity and immuno-subversion mechanisms for PCV2 and 3, while drawing parallels from findings for other CRESS viruses, and identifying gaps in knowledge. A limitation of the review is that it is not an exhaustive summary of all published literature on the topic.

Viral components and immunopathogenesis: Given the extremely small size of CRESS viruses and their limited coding capacity, the identification of non-essential viral proteins which function exclusively as virulence factors is difficult. Practically all viral components, such as viral DNA, mRNA, cDNA and proteins interact with and regulate the host immune system. Although the members of CRESS viruses are genetically diverse, their viral components, share common patterns of genome and protein structure and function, regardless of whether the virus has known pathogenic potential or not. The genome of PCV2, 3 and 4 are approximately 1769bp, 2000bp and 1770bp respectively in size with 2 intergenic regions, one of which contains the origin of replication which is characterized by a conserved nonanucleotide motif (Ramamoorthy & Meng, 2009; Ssemadaali, Ilha, & Ramamoorthy, 2015). Viral DNA constitutes an important pathogen associated molecular pattern (PAMP) whose interaction with pathogen recognition receptors (PRRs) such as for Toll Like Receptor (TLR) 9 or 7 influence early viral immunity, and consequently, the downstream adaptive immune responses (Rocchi et al., 2009; Wikstrom et al., 2007). Not only the composition of viral DNA but also the various structural conformations can influence outcomes.

135 Many structural forms such as single stranded and double stranded replicative intermediates detected

136 during active replication in cells infected with circoviruses and gemini viruses (Faurez, Dory, Grasland, &

137 Jestin, 2009) and the replication competent sub-genomic molecules produced during active replication of

138 torque teno viruses and Gemini viruses (de Villiers, Borkosky, Kimmel, Gunst, & Fei, 2011) exist and can

139 differentially regulate host immunity. While equivalent information is not yet available for PCV3, it can be

140 expected that the single and double stranded DNA produced during PCV3/4 replication in host cells with

141 likely interact with hos PRRs in a similar manner, although the low level of genetic relatedness between

142 PCV2 and PCV3 also suggests that PCV3 DNA may have its own unique mechanisms of host immune

143 modulation which needs to be explored further.

144 While mRNA is generally identified as self by the innate immune system, it has been shown that

145 mRNA can act as an endogenous ligand for TLR3 (Kariko, Ni, Capodici, Lamphier, & Weissman, 2004),

146 and that non-self-single stranded RNA can be recognized by TLR 7, 8 and other endosomal sensors

147 (Linares-Fernandez, Lacroix, Exposito, & Verrier, 2020). Although PCV2 is predicted to contain 11 open

148 reading frames, only 5 viral proteins (Rep, Rep", ORF1, ORF2, ORF3 and ORF4) have been

149 experimentally characterized thus far (Hamel, Lin, & Nayar, 1998). However, with the availability of long-

150 read sequencing, the transcriptome of PCV1 was found to generate nine previously undetected RNA

151 molecules and was considerably more complex than previously thought. (Moldovan et al., 2017). However,

152 it is not clear if the newly discovered transcripts are translated into proteins. While the transcriptome of

153 PCV3 and 4 are as yet uncharacterized, the availability of NGS technology can help to rapidly identify and

154 assign function to viral mRNA, and study their contribution to molecular pathogenesis. Among the

155 mammalian CRESS viruses, a human TTV strain encoded a micro-RNA (miRNA) which inhibits interferon

156 signaling (Kincaid, Burke, Cox, de Villiers, & Sullivan, 2013). However, PCV2 encoded miRNAs were not

157   detected in PCV2 infected pigs (Nunez-Hernandez, 2015 #19); and information for PCV3 and 4 is as yet

158   unavailable.

159   Viral proteins play a dual role in acting as virulence factors which can subvert host immunity and

160   mediate pathogenesis, while also serving as protective antigens in stimulating effective vaccine or infection

161   derived immunity. The porcine circovirus ORF1 encodes the replicase protein, which is largely conserved in

162   circoviruses. However, the transfer of the PCV2 ORF2 encoded capsid protein into the backbone of the

163   non-pathogenic PCV1 resulted in attenuation of the chimeric virus, indicating that the PCV2 replicase or

164   non-coding genomic DNA can influence viral pathogenicity (Fenaux, Opriessnig, Halbur, Elvinger, & Meng,

165   2004). Indeed, the efficacy of a pseudorabies vaccine delivered in conjunction with the PCV2 replicase and

166   origin of replication was lower than a vaccine encoding the pseudorabies protective antigen alone (Faurez

167   et al., 2012), indicating that the circoviral replicase proteins can dampen immune responses. Although

168   structural similarities exist between the PCV2 and 3 replicase proteins, the PCV3 replicase is more closely

169   related to the bat circoviruses at the genetic level (55%) than to the porcine circoviruses, necessitating a

170   more thorough exploration of the structure and function of the PCV3 capsid protein and its role in the

171   adaptation of PCV3 to pigs. Moreover, unlike the previously known porcine circoviruses, PCV3 is also

172   found in several non-porcine species like dogs, cattle, ticks, and mice (Franzo et al., 2019). If more solid

173   evidence for productive infection in these species is established, understanding the molecular basis for the

174   promiscuity of PCV3 in adapting to a broad host range would be critical, given the demonstrated

175   pathogenic potential of PCV3 in pigs.

176   The PCV2 capsid protein, encoded by ORF2, on the other hand, is both necessary and sufficient

177   for protective immunity against clinical PCVAD in vaccinated pigs. It is also most commonly used as a

178   diagnostic antigen for serological studies on PCV2. Therefore, antibody responses to the PCV2 capsid

179   protein have been studied extensively (Afghah, Webb, Meng, & Ramamoorthy, 2017). While the role of the

180   PCV3 capsid protein in mediating protective immunity is as yet uncharacterized, it is implicated in

181   downregulating host IL-12 responses (Du et al., 2018), disrupting cell cycle regulation (T. Wang et al.,

182   2019) and autophagy (C. Han et al., 2020; Klaumann et al., 2018). The proteins encoded by the PCV2

183   ORF3, 4 and 5 can induce or prevent apoptosis depending on the stage of the viral life cycle (Pan, 2018

184   #16). Analogous proteins in PCV3 are as yet undiscovered, but the PCV3 ORF3 encodes a protein with

185   231 amino acids. Further details on how porcine circoviruses interact with the host immune system is

186   presented below, incorporating the latest and available information for PCV3 and PCV4.

187   **Innate immune responses:** In PCV2 infected pigs, PCV2 capsid antigen is commonly detected within

188   innate immune cells such as macrophages, dendritic cells and follicular dendritic cells (Krakowka et al.,

189   2002). Indeed, the interaction of PCV2 with the innate immune system is likely the first and most crucial

190   trigger of immunopathogenesis. Early *in vitro* studies on the effects of PCV2 on dendritic cells (DCs)

191   indicated that internalization or infection of conventional DC's, which are involved in antigen presentation,

192   neither affects their viability or function. On the other hand, type I interferon secretion in plasmacytoid DCs

193   (pDCs) was significantly compromised. Plasmacytoid DCs are a subset of DCs which respond to pathogen

194   associated molecular patterns via the production of type I interferons (Vincent et al., 2005). Similarly,

195   antigen presentation and maturation capabilities were reduced in PCV2 infected monocyte derived

196   macrophages (Yang et al., 2018). Differential gene expression analysis of pigs with either clinical PMWS or

197   subclinical PCV2 infection revealed activated granulocytes and monocytes, dysregulated pro-inflammatory

198   responses, and downregulated cell-cycle check point genes in pigs with PMWS but not in subclinically

199   infected pigs. The trigger for the transcriptional dysregulation in pigs manifesting PCVAD is as yet

200   unidentified but suspected to originate from other coinfecting agents (Van Renne, Wei, Pochet, &

201   Nauwynck, 2018). Both PCV2 and TTV viral DNA contain oligodeoxynucleotides (ODNs) containing CpG

202   motifs which are both inhibitory and stimulatory in nature (Wikstrom et al., 2007). In cells infected with

203 PCV2 and TTVs, viral DNA occurs in both single stranded and double stranded intermediate

204 conformations. Viral DNA interacts with TLR9, TLR 7, and possibly other sensors of single (Vijay &

205 Chande, 2018) and double stranded DNA to induce or suppress type I interferon stimulation in pDCs

206 (Faurez et al., 2009; Rocchi et al., 2009; Wikstrom et al., 2007). Single stranded PCV2 DNA was

207 demonstrated to have a stimulatory effect on type I interferon production, while dsDNA had an inhibitory

208 effect (Wikstrom, 2007 #22). Secretion of IFN-γ, presumably from NK cells in early infection, leads to

209 activation of pDC's and IFN-α production in a feedback loop (Baumann, McCullough, & Summerfield,

210 2013). The upregulation of IL-10 is a common feature in many chronic viral infections, and is also observed

211 in PCV2 infections, but not in infections with the non-pathogenic PCV1. In vitro studies have shown that

212 innate immune cells are an important source of IL-10 (Wu et al., 2019), and upregulation of IL-10 led to

213 downregulated Th1 responses, IL-12 and IFN- γ in response to a secondary infection. Interestingly, the

214 secretion of IL-10 was not triggered by viral proteins in an inactivated vaccine preparation, indicating that

215 the interaction of viral DNA and PRRSs is responsible for the innate regulation of IL-10 (Kekarainen,

216 Montoya, Dominguez, Mateu, & Segales, 2008; Wu et al., 2019). From the viral perspective, PCV2 viral

217 replication is promoted by host interferons due to the presence of an interferon stimulated response

218 element in the viral genome (B. Huang, Zhang, Lu, Li, & Lv, 2018; Ramamoorthy, Opriessnig, Pal, Huang,

219 & Meng, 2011). Taken together, these findings indicate that the interaction of viral nucleic acids with TLR9,

220 TLR7 or other sensors of single and double stranded DNA is central to the interaction of PCV2 with the

221 innate immune system. Findings for human TTVs are similar to that of PCV2. Multiple forms of TTV DNA

222 including ssDNA, ds DNA intermediates, defective replicative genomes and the presence of CpG motifs in

223 viral DNA are reported. Activation of TLR-9, and a direct downregulation of NFkB signaling by the ORF2

224 protein are suspected to play a role viral immunopathogenesis (Rocchi et al., 2009). In addition, virally

225 encoded micro RNAs (miRNA) are reported to downregulate type I interferon responses (Kincaid, 2013

226 #26) (Table 1) (Fig1).

227   While studies regarding the innate immune responses to PCV3 and 4 are in their infancy, the

228   similarity in the mechanisms of circoviral replication supports the premise that the production of ssDNA and

229   dsDNA intermediates during viral replication and interaction with host PRRs will be similar to PCV2.

230   However, PCV3 is genetically more closely related to bat circoviruses for the rep gene and avian

231   circoviruses for the cap gene (Palinski et al., 2017). Avian and mammalian circoviruses show differing

232   patterns of genome composition, dinucleotide frequency and codon bias, with avian circoviruses in general

233   having a higher CpG content. However, in birds, TLR 9 is absent, and TLR 21 carries out the function of

234   TLR9 (Franzo, Segales, Tucciarone, Cecchinato, & Drigo, 2018). Therefore, although PCV3 has evolved to

235   adapt to a mammalian host, the interaction of PCV3 with the mammalian innate immune system can be

236   considerably different from that of PCV2. A comparison of CpG content among circoviral genomes showed

237   that the overall CpG content was low in PCV3 (Li, Wang et al. 2018) and CpG dineuclotides were under-

238   represented in the PCV3 cap gene but not the rep gene ((Franzo et al., 2018; Greenbaum, Levine, Bhanot,

239   & Rabadan, 2008; G. Li et al., 2018). In a parallel scenario, the adaptation of avian influenza viruses to

240   humans resulted in an overall reduction of CpG content, and a more efficient evasion of innate immune

241   sensing of viral nucleic acids and thus, a dampening the innate immune response towards the influenza

242   viral strain (Greenbaum, Levine et al. 2008). The PCV3 capsid protein is reported to inhibit type I interferon

243   production by stearic inference with the STAT2, the host ISRE and IRF9-S2C complex (Shen, 2020 #35;

244   Zhang, 2020 #57). The PCV3 capsid protein induces autophagy in PK-15 cells (S. C. Geng, Li, & Fang,

245   2020), similar to PCV2, which exploits autophagy pathways to promote replication in PK-15 cells (Lv et al.,

246   2020). Since the capsid protein is the most important protective antigen for PCV2, the PCV3 capsid protein

247   is also likely to be targeted as the primary protective antigen for PCV3, the implications of the above

248   findings for the design of PCV3 vaccines remains to be explored (Table 1) (Fig1).

249   In contrast, based on the proteomic analysis of the lung tissue of 4-week-old SPF piglets infected

250   with PCV3 derived from an infectious clone (Jiang et al., 2019), PCV3 infection upregulated interferon

251   stimulated genes (ISG) such as OAS1, Mx1, Mx2, IFIT3, and ISG15, immunoglobulins, complement, pro-

252   inflammatory and acute phase proteins, and proteins involved in the phagosome pathway (H. Jiang et al.,

253   2020). While the contribution of these proteins to PCV3 pathogenesis remains to be explored, these

254   findings are similar to *in vitro* studies on PCV2, where infection of PK-15 cells results in RIG-1 and cGAS

255   signaling, interferon β production, and downstream ISG expression (Dvorak, Puvanendiran, & Murtaugh,

256   2018; B. Huang et al., 2018). While in vivo studies on PCV3 infection are limited, when tissue

257   homogenates from infected pigs were used as the inoculum, there were no significant differences in the

258   systemic levels of IFN-α, IFN-γ, or TNF-α between infected pigs and uninfected controls. In this study,

259   PCV3 infected pigs did not develop overt clinical signs but had microscopic lesions consistent with PCV3

260   infection (Temeeyasen et al., 2021). In a second study where the PCV3 viral inoculum was derived from an

261   infectious clone, infected piglets showed signs of pneumonia and PDNS. The proliferative ability of PBMCs

262   in response to stimulation with mitogens was significantly diminished in infected pigs by day 7 post infection

263   and persisted until day 28 post infection, for the duration of the study. Additionally, abundant eosinophilia,

264   coupled with increased levels of proinflammatory chemokines and cytokines TNF-α, IL-1β, IFN-γ, IL-6, and

265   CCL5 in the serum, and generalized inflammation of tissues was detected in infected pigs. Similar to PCV2

266   and other chronic viral infections IL-23α and IL-10 continued to increase for the duration of the study.

267   Innate anti-viral cytokines such as IFN- α and IFN- β were not measured in this study (Jiang et al., 2019).

268   Therefore, for PCV3, the induction of a chronic proinflammatory state, leading to dysregulation of cytokine

269   homeostasis and innate immunity are the primary triggers for the consequent clinical manifestations of

270   disease (Table 1) (Fig1).

271

272 **Cell-mediated immune responses:** Lymphoid depletion associated with a decline in the numbers of

273 lymphocytes in the circulation and lymphoid organs coupled with apoptosis is highly characteristic of PCV2

274 infections. All T cell subsets, especially memory T cells, B cells, and NK cells, are reported to be affected

275 (Nielsen et al., 2003). Similar to TTVs and chicken anemia virus (de Smit & Noteborn, 2009), a delicately

276 interwoven regulation of pro and anti-apoptotic pathways are observed during various stages of the viral

277 infection cycle. Apoptosis is mediated by the viral proteins encoded by ORF2, 3 and 4 and involves both

278 the intrinsic and extrinsic pathways (Pan et al., 2018). Studies aimed at better understanding the

279 mechanisms by which lymphopenia occurs in PCVAD have explored the role of apoptosis in PCV2

280 pathogenesis and are reviewed in detail elsewhere (Pan et al., 2018).Immuno-subversion of the innate

281 immune system, can indirectly lead to compromised antigen presentation, reduced T cell stimulation and

282 secretion of immunosuppressive cytokines, to negatively impact adaptive immunity against PCV2 (Yang et

283 al., 2018). However, direct effects of PCV2 infection such as dysregulation of positive and negative

284 selection in thymocytes leading to hypo-responsive helper T cells are also reported (Klausmann et al.,

285 2015). PCV2 can infect and replicate in T lymphoblasts, with some strains replicating better in

286 lymphoblasts than others (Wei, Van Renne, & Nauwynck, 2019). Induction of regulatory T cells consequent

287 to the activation of the PD1-PD-L1 axis compounds immuno-suppression, and leads to lymphocyte anergy

288 in PCV2 infected pigs (Richmond et al., 2015). Levels of IL-1, IL-12p40, IL-4, and IFN-γ are consistently

289 reported to be downregulated in pigs with PCVAD, corresponding with the upregulation of IL-10 (Darwich,

290 2003 #44). The PCV2 capsid protein is implicated in downregulating IL-12p40 by inhibition of NF-KB and

291 selected host miRNAs, which then translates to diminished protection against coinfecting agents like

292 PRRSV (Du, 2018 #45). As piglets with low levels of antibodies against PCV2 are still protected against

293 clinical signs, cell-mediated immune responses, especially Th1 mediated responses involving CD4^{+}T cells,

294 CD8^{+}T cells, IL-12, IFN-γ, TNF-α and are critical for protection. Both the capsid and replicase proteins

295   stimulate antigen specific IFN-γ secreting cells (Fort et al., 2010; Jung, Kim, Lee, Jang, & Chang, 2019;

296   Koinig et al., 2015) (Table 2) (Fig1).

297   While information on cell mediated immune responses to PCV3 and 4 is very limited, pathological

298   lesions in PCV3 infected pigs involve generalized and systemic inflammatory changes, especially of the

299   cardiovascular, respiratory, and urinary systems, in conjunction with lymphoid dysplasia, necrosis, and

300   infiltration of tissues with phagocytic cells as a sequela of inflammation. The patterns of lesions observed in

301   field cases (Palinski et al., 2017; Phan et al., 2016) are also reproduced in experimental infections of pigs

302   with PCV3 (Jiang et al., 2019; Mora-Diaz et al., 2020). Therefore, unlike PCV2 infections, dysregulation of

303   inflammatory responses, and not virally mediated primary lymphopenia, may be central to PCV3 immuno-

304   pathogenesis. In one study where infective material from pigs with clinical signs was used to infect

305   caesarian-derived, colostrum-deprived (CD/CD) pigs, the lymphocyte counts between infected and

306   uninfected pigs was not significantly different, as measured by flow cytometry of PBMCs. Nor were

307   cytokines like IL-4, IL-10, and IFN-γ detected in the serum of both groups (Temeeyasen et al., 2021).

308   However, in a second study where recombinant PCV3 was used to infect conventional weanling piglets, an

309   inability of PBMCs to respond to mitogen stimulation was evident by day 7 post-infection, and persisted for

310   the 28-day duration of the study. However, recall responses to PCV3 antigens were not assessed in this

311   study. Hence, it is not clear if virus-specific cell mediated immunity is diminished or if the lymphocyte

312   anergy is reversible following the establishment of chronic infection (Jiang et al., 2019). In this study, IL-12

313   levels peaked at day 7 and then declined to insignificant levels by day 21 in PCV3 infected pigs,

314   corresponding to an increase in IL-10 and IL-23, which could suppress Th1 responses. However, IFN-γ

315   levels in the serum of infected pigs increased until day 21. Proteomic analysis of lung tissue from PCV3

316   infected pigs showed that SLA-DRB1, an MHC-II protein, was the most highly upregulated protein in PCV3

317   infected pigs when compared to uninfected pigs at a ratio of 9.35. Other proteins from the SLA-I and II loci

318   were also significantly upregulated. Proteins from SLA-III locus which are associated with immune

319   functions, such as proteins related to heat shock, inflammation and the complement cascade were also

320   upregulated. As the samples were examined at 28 days post infection, it appears that PCV3 infection does

321   not downregulate $CD8^+$T cell and $CD4^+$T cell markers (H. Jiang et al., 2020). However, a more detailed

322   characterization of cell mediated immunity against PCV3 is required to more fully understand viral

323   pathogenesis (Table 2) (Fig1).

324   **Antibody based immune evasion, pathology, and protection:**

325   The pathognomonic lesions of PDNS, which are characterized by vasculitis and glomerulonephritis

326   and thrombotic hemorrhages, are typical hypersensitivity reactions mediated by antigen-antibody immune

327   complexes. The formation of immune complexes is associated with both PCV3 and PCV2 infections (Jiang

328   et al., 2019; Langohr et al., 2010). Immune complex formation is a normal physiological process for the

329   clearance viral antigens. Immunopathology due to immune complex formation has been attributed to the

330   production of excess antigen or antibody, and impaired Fcγ receptor mediated functions such as antibody-

331   dependent cytotoxicity (ADCC), B cell selection, antigen presentation, or phagocytosis. Prolonged immune-

332   complex Fcγ receptor signaling can lead to the dysregulation of other T and B cell functions, causing

333   hyperreactivity, production of autoantibodies, T cell exhaustion, delayed class switching, and diminished

334   IgA or IgG production leading to impaired virus neutralization (T. T. Wang & Ravetch, 2015). Proteomic

335   analysis of lung tissue of PCV3 infected pigs showed that an SLA-II related protein was the most highly

336   upregulated protein detected in the analysis (H. Jiang et al., 2020), which could have both positive and

337   negative implications for immuno-pathology and protection. Immune complex accumulation can result in

338   vascular inflammation, alterations in coagulation pathways, complement and phagocytic activation leading

339   to tissue damage, and fibrotic thrombosis in the microvasculature. Thus, the consequences of antibody

340   mediated immuno-pathology in chronic viral infections, such as PCV2 and PCV3/4, can be multi-

341 dimensional and appear share a commonality in CRESS viruses. The accumulation of immune complexes

342 is also observed in individuals who are persistently infected with torque teno viruses, leading to speculation

343 that TTVs can promote autoimmune disease, especially under immune compromised conditions (Maggi &

344 Bendinelli, 2009) (Table 3) (Fig1).

345   Although the role of antibodies can be perceived as a double-edged sword in porcine circoviral

346 infections, there is universal scientific consensus that antibody responses against PCV2 are critical for

347 protection against PCVAD. The role of neutralizing antibodies in mediating protection is supported by the

348 observation that piglets become susceptible to PCV2 just as maternal immunity wanes at weaning

349 (Hedegaard & Heegaard, 2016). Both the major viral proteins, the replicase and capsid proteins, are

350 immunogenic. However, the capsid protein alone is considered necessary and sufficient for protection

351 against PCV2. Antibodies against the PCV2 capsid protein can be detected as early as 7 days post-

352 infection. However, significant virus neutralizing antibody responses are not detected until after 2 weeks

353 post infection (Ramamoorthy & Meng, 2009), likely as a consequence of impaired innate immune

354 responses as described above. Further, antibody-based immunity to PCV2 is complicated by the

355 phenomenon of immunodominance, wherein selected antigenic epitopes within the PCV2 capsid dominate

356 the early antibody response. Early antibodies that map to these dominant epitopes are largely non-

357 protective. Immunodominance of the non-protective epitopes persist into the chronic stages of infection

358 (Ilha, Nara, & Ramamoorthy, 2020; Rakibuzzaman et al., 2020), contributing to viral immune evasion and

359 establishment of infection. It is suggested that minor variations in PCV2 amino acid sequences can

360 contribute to partial immune escape, and that the diversity of viral quasi species is significantly greater in

361 pigs showing clinical signs of PCVAD when compared to sub-clinically infected pigs, presumably as the

362 immune system is not compromised in asymptomatic pigs (Correa-Fiz et al., 2020). Indeed, previous

363 findings show that single amino acid changes can influence the strain and subtype specific antibody

364 responses for PCV2 (Constans, Ssemadaali, Kolyvushko, & Ramamoorthy, 2015), while conserved

365 sequences contribute to the cross-neutralizing effects of antibodies (L. Huang et al., 2020). Despite the

366 increasing diversity of PCV2 subtypes, evidence for serological and cell-mediated cross-reactivity has

367 remained consistent and likely accounts for the broad vaccine mediated protection observed in the field

368 (Table 3) (Fig1). However, subtle differences in antigenicity at the epitope level but may influence

369 protection and viral evolution in the field, not just for PCV2 but also for TTVs. Superinfection with new TTV

370 strains is not prevented despite the presence of strong antibody responses, indicating a deficiency in the

371 production of functional, virus neutralizing antibodies. Thus, an individual can be simultaneously infected

372 with multiple TTV strains, which can potentially exacerbate disease conditions under immune-compromised

373 conditions (Maggi & Bendinelli, 2009).

374   The high rates of nucleic acid-based detection of PCV3 in swine herds across the world are

375 mirrored by high sero-prevalence rates which range from about 50% (Deng et al., 2018; S. Geng et al.,

376 2019; Palinski et al., 2017; Y. Wang et al., 2020) to 20-80% (Zhang et al., 2019), with no significant

377 differences observed between healthy and sick animals in a majority of studies. While PCV3 DNA is

378 detected in several non-porcine species such as cattle, dogs and rodent's and insects, it is not clear if

379 seroconversion occurs in these species (Zhai et al., 2019). Consistent with the above-described effects of

380 impaired Fcγ receptor mediated functions on antibody responses, CD/CD pigs which were experimentally

381 infected with natural isolates of PCV3 in combination with keyhole limpet hemocyanin (KLH), mounted

382 strong IgM responses by day 7 post-infection. The IgM responses persisted for the duration of the study for

383 28 days. However, significant PCV3-specific IgG responses were not detected for the duration of the study.

384 Antibody responses were measured with a bacterially expressed PCV3 capsid protein based indirect ELISA

385 in this study (Mora-Diaz et al., 2020). The lack of strong IgG responses was also recorded in a second

386 study where CD/CD pigs were infected with tissue lysates from PCV3 PCR positive pigs. However, in this

387   study, anti-PCV3 IgG antibodies were detectable by 14 days post infection when pigs were administered

388   PCV3 in combination with KLH but not in pigs infected with PCV3 alone. The IgG response persisted for

389   the duration of the study (Temeeyasen et al., 2021). In contrast, when 4 and 8-week-old conventional pigs

390   were infected with recombinant PCV3 derived from an infectious clone in combination with or without KLH,

391   anti-PCV3 IgG responses were not detected until day 14. However, the anti-PCV3 IgG responses

392   continued to increase for the duration of the study in both groups. Therefore, PCV3 likely interferes with or

393   delays antibody type switching, and the effect is unmitigated by immunostimulants. In this study, the IgG

394   levels correlated inversely with serum viral loads indicating that antibodies play a role in PCV3 clearance

395   (Jiang et al., 2019). However, virus neutralization responses were not evaluated in any of these studies on

396   PCV3 experimental infection. Taken together, these findings suggest that evasion of innate immunity could

397   lead to delayed virus neutralizing IgG responses and compromised isotype switching. However, additional

398   studies are required to characterize the antibody response to PCV3 in detail, and to compare the results

399   from experimental studies with natural field infections, where coinfection with more than one pathogen is

400   very likely.

401   Recombinant viral antigens for serological assay development, and PCV3 capsid protein specific

402   monoclonal antibodies for the detection of the viral antigen in tissues and biological samples are now

403   available (X. Li et al., 2018; Palinski et al., 2017). However, the functionality of the monoclonal antibodies in

404   neutralizing PCV3 is as yet unknown. The recently published cryo-EM structure of the PCV3 capsid protein

405   demonstrated conservation in symmetry between PCV2 and PCV3. The major differences between the two

406   PCV2 types were located in the CD loop, exterior to the viral surface. The PCV3 CD loop was shorter, less

407   conserved, and more flexible than the PCV2 CD loop. The N terminal arginine rich domain was also more

408   flexible for PCV3. The PCV3-specific monoclonal antibody generated by the same research group mapped

409   to residues 128-143 (Bi et al., 2020). In a second study where linear B cell epitopes of the PCV3 capsid

410 protein were mapped using monoclonal antibodies and overlapping peptides, three immunodominant

411 regions were identified in the PCV3 capsid protein. Three conserved linear B cell epitopes

412 [57]NKPWH[61], [140]KHSRYFT[146], and [161]QSLFFF[166.] were identified. In addition, epitope [140]KHSRYFT[146] was

413 conserved between the different PCV types, and corresponded to a previously identified immunodominant,

414 non-neutralizing epitope for PCV2. Only the first amino acid varied from the previously identified PCV2

415 epitope (Ilha et al., 2020; Trible, Ramirez, et al., 2012; Trible, Suddith, et al., 2012). Serological assays for

416 PCV4 are as yet unavailable. Given the sequence divergence between the PCV types, it is not surprising

417 that serological cross- reactivity has not been reported, despite the presence of the conserved

418 immunodominant B cell epitope (M. Jiang et al., 2020). Characterization of B and T cell epitopes for PCV3

419 and PCV4 is critical to advancing the current understanding antibody responses and to inform rational

420 vaccine design in the future (Table 3) (Fig1).

421 **Prevention by vaccination:** Vaccination of production swine against PCV2 is a well-established practice.

422 Several commercial vaccines including subunit, inactivated and chimeric PCV1-2 vaccines against PCV2

423 are available; and are very successful in preventing clinical PCVAD. Consistent with serological cross-

424 reactivity between subtypes, the PCV2a capsid antigen is included in a majority of commercial vaccines.

425 Vaccination elicits cross protection clinical PCVAD regardless of the subtype in circulation in the herd.

426 Strong virus neutralizing antibody responses against the PCV2 capsid protein are positively correlated with

427 protection (Kolyvushko, Rakibuzzaman, Pillatzki, Webb, & Ramamoorthy, 2019). However, cell mediated

428 associated with IFN-γ secreting cells is also important for preventing PCVAD (Zanotti, Martinelli, Lelli, &

429 Amadori, 2015). Therefore, effective vaccine mediated immunity requires the stimulation of both humoral

430 and cell mediated immune responses. While PCV2 vaccines are very useful in preventing economic losses

431 by conferring protection against clinical disease, they do not induce sterilizing immunity and may actually

432 influence viral evolution in the field. The current status of PCV2 vaccines and vaccine mediated immunity is

433 reviewed extensively elsewhere (Afghah et al., 2017; Franzo & Segales, 2020).

434 Currently, there is little published information regarding the prevention of PCV3 and 4, but are likely

435 to be the focus of future research as a clearer picture of case definitions, economic impact, and contribution

436 of PCV3 and 4 to disease emerges (Hess, 2019). Given the low levels of genetic and antigenic similarity

437 between PCV2 and PCV3/4, significant cross-protection between the two PCV types is unlikely. A recent

438 estimation of the influence of PCV3 on PCV2 viremia in PCV2 vaccinated herds showed that there were no

439 significant differences in PCV2 viral loads between vaccinated/ PCV3 positive pigs and vaccinated/ PCV3

440 negative pigs. Neither were PCV3 viral loads higher in PCV2 unvaccinated pigs with high levels of PCV2

441 circulation (Wozniak, Milek, Baska, & Stadejek, 2019). However, further studies are needed to determine

442 the effects of coinfection and vaccination in the field. While patents for a standard subunit (Hause, 2018)

443 and an inactivated vaccine (Kegong, Xiangdong, Yan, Jinzhong, & Xuke, 2020) are issued, commercial

444 vaccines against PCV3 are not available in the U.S market as yet. A custom RNA vaccine technology, the

445 Merck Sequivity platform, has been applied in individual farms with PCV3 associated reproductive failure.

446 Viral sequences obtained from samples collected from individual farms are used to synthesize the custom

447 mRNA particle vaccines (Hess, 2019). The efficacy of the Sequivity PCV3 mRNA vaccines in preventing

448 PCV3 infections or reducing the impact of other co-infections is as yet unknown. The demonstration of

449 Koch's postulates for PCV3, the development of *in vitro* culture methods and animal models are critical

450 milestones in PCV3 research. They lay the ground work for future studies on the evaluation of disease

451 burden and impacts in the field, gaining an understanding of the correlates of protection, and development

452 of effective vaccines to reduce possible economic impacts of PCV3/4 to the industry.

453 **Conclusions:** With the continued expansion of the family of porcine circoviruses the etiology of clinical

454 manifestations of PCAVD, such as PDNS and systemic inflammation, are now clearer. The recently

455   discovered PCV3 is no exception to the pattern of widespread distribution in production swine previously

456   associated with PCV2. Despite the variety of clinical manifestations, modulation of host immune responses

457   and disruption of immune homeostasis are pivotal triggers for the subsequent clinical and pathological

458   manifestations of PCV2 and PCV3. Thus, targeting interventions towards immune events which occur in

459   early infection may be critical for preventing the downstream sequalae. In the case of PCV2, infection of

460   antigen presenting cells leading to downregulation of the early antiviral response likely takes an enormous

461   toll on ability of the host to mount a robust adaptive immune response, which is indispensable for viral

462   clearance and resolution of infection. Further lymphopenia and apoptotic events in the immune cell

463   population can directly help establish chronic infections or induce clinical disease for PCV2 and indirectly

464   influence the ability of PCV2 infected pigs to respond to coinfections.

465   While the study of immune responses against PCV3 is in its infancy, and no information is

466   available for PCV4, based on the commonly reported clinical signs of generalized inflammation,

467   reproductive failure, cardio vasculitis, and necrosis of immune cells in tissues, the key mechanisms by

468   which PCV3 engages the host immune system may differ from PCV2. The ability of PCV3 to induce

469   uncontrolled and systemic inflammation is a significant difference from PCV2 and the likely cause of

470   reproductive failure, as maintaining immune homeostasis is critical for the successful maintenance of

471   pregnancy. Although upregulation of IL-10 and IL-23, likely resulting in a downregulation of the Th1

472   response, is a shared immune signature between PCV2, PCV3 and other chronic viral infections,

473   deciphering the mechanisms by which PCV3 achieves a pro-inflammatory state needs further investigation

474   to enable the development of effective preventive measures. Further, the avian and bat origins of PCV3

475   and its subsequent adaptation to the mammalian system, coupled with its detection in several non-porcine

476   species indicate a level of adaptability which could pose a concern if PCV3 is determined to be a significant

477   pathogen of pigs or cause disease in other species. Therefore, investment in further studies to fully

478   understand the biology of PCVs are critical to prevent economic losses and possible viral evolution which

479   could result in adaptation to other hosts or increases in pathogenicity.

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487 **Conflict of Interest:** The authors declare that they have no conflict of interest to disclose

488 **Author's contributions:** The authors have mutually agreed to the sequence of authorship, listed in the

489 order of contribution.

490 **Ethics statement**: As this is a review article and not an original research article, approvals for ethical

491 research conduct are not applicable. However, all the journal requirements for ethical publication have

492 been adhered to.

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499 **Table 1: Immuno-subversion and-pathology of the innate immune system**

Virus	Mechanism	Reference
PCV2	Reduced type I interferon production, antigen presentation, and maturation in dendritic cell subsets	(Vincent et al., 2005; Yang et al., 2018)
	Inhibition of type I interferons by inhibitory CpG motifs and double stranded intermediates of viral DNA	(Wikstrom et al., 2007)
	Upregulated IL-10 responses, dampening IL-12, and IFN- γ responses to secondary infections	(Kekarainen et al., 2008; Wu et al., 2019)
	Binding of host interferons to an interferon stimulated response element in the viral genome to promote viral replication	(B. Huang et al., 2018; Kekarainen et al., 2008; Ramamoorthy et al., 2011; Wu et al., 2019)
PCV3	Reduced CpG content in viral DNA leading to diminished innate sensing of viral nucleic acids	(Franzo et al., 2018; Greenbaum et al., 2008)
	Inhibition of type I interferon signaling by the PCV3 capsid protein	(Shen et al., 2020; P. Zhang et al., 2020)
	Induction of autophagy by the capsid protein to promote viral replication	(S. C. Geng et al., 2020)
	Eosinophilia, cytokine storm with proinflammatory chemokines and cytokines, TNF-α, IL-1β, IFN-γ, IL-6, and CCL5	(Jiang et al., 2019)
	Upregulated IL-23α and IL-10 responses, possibly downregulating Th1 responses	(Jiang et al., 2019)
PCV4	Unknown	

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502 **Table 2: Cell mediated immuno-subversion and-pathology**

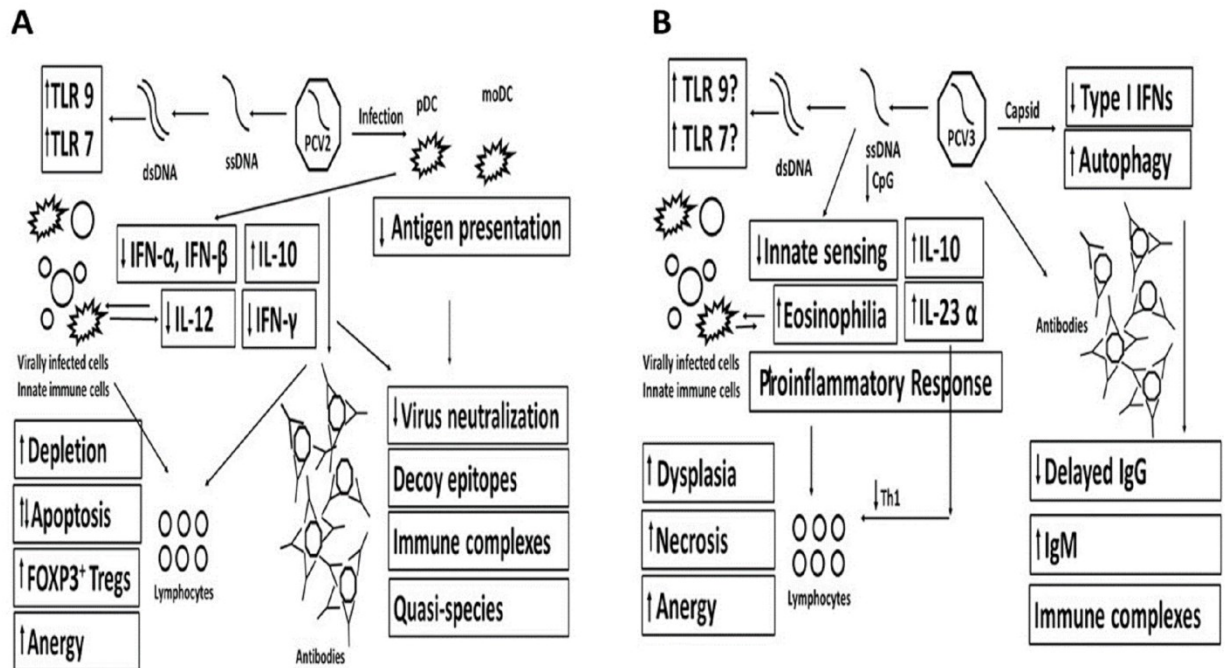| Virus	Mechanism	Reference
PCV2	Depletion of T cell, memory T cells, B cells and NK cells involving apoptotic pathways	(Nielsen et al., 2003; Pan et al., 2018)
	Dysregulated selection and T cell hypo-responsiveness	(Klausmann et al., 2015)
	Activation of the PD1-PDL1 axis, Induction of regulatory T cells, and T cell anergy	(Richmond et al., 2015)
	Downregulation of IL-1, IL-12p40, IL-4 and IFN-γ. Upregulation of IL-10 in clinically infected pigs	(Darwich et al., 2003)
PCV3	Dysregulated inflammation leading to lymphoid dysplasia, necrosis of lymphocytes	(Jiang et al., 2019; Mora-Diaz et al., 2020; Palinski et al., 2017; Phan et al., 2016)
	Diminished lymphocyte proliferation responses to mitogens	(Jiang et al., 2019)
	Upregulation of IL-10 and IL-23	(Jiang et al., 2019)
PCV4	Unknown	

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505 **Table 3: Antibody based immuno-subversion and-pathology**

Virus	Mechanism	Reference
PCV2	Delayed virus neutralization responses	(Ramamoorthy & Meng, 2009)
	Decoy epitopes, immunodominance	(Ilha et al., 2020; Rakibuzzaman et al., 2020)
	Immune complex formation	(Langohr et al., 2010)
	Viral quasi species and antigenic diversity	(Franzo & Segales, 2020; Ssemadaali et al., 2015)
PCV3	Strong and persistent IgM responses	(Mora-Diaz et al., 2020; Temeeyasen et al., 2021)
	Delayed IgG responses and possibly type switching	(Jiang et al., 2019; Mora-Diaz et al., 2020)
	Immune complex formation, hypersensitivity reactions leading to clotting disorders, fibrotic thrombosis and necrosis of tissue and lymphocytes	(Jiang et al., 2019; T. T. Wang & Ravetch, 2015)
PCV4	Unknown	

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Fig 1: Mechanisms of immunopathogenesis of PCV2 and PCV3. Details are as provided in the text.

Briefly **A. PCV2 immunopathogenesis:** Infection of antigen presenting cells such as DC's downregulates

type I interferon production and interferes with antigen presentation. Viral DNA with immunostimulatory and

suppressive properties engages host PRRs such as TLR 9. Increase in levels of IL-10 and dampening of

the Th1 responses leads to chronic infection. Delay in virus neutralizing antibody responses and directing

the antibody response to non-protective immunodominant epitopes of the capsid protein help to evade

antibody mediated immune responses. **B. PCV3 immunopathogenesis**: The PCV3 capsid protein

downregulates type I interferon responses and triggers pro-apoptotic pathways. Infection results in a highly

proinflammatory state that leads to tissue lesions and necrosis of immune cells. Upregulation of IL-10 and

IL-23 helps to establish chronic infections, possibly by downregulating Th1 responses. The accumulation of

antigen-antibody complexes leads to impaired Fcγ receptor mediated functions, delayed IgG production

and type switching and evasion of antibody mediated immunity.

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