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Head Start Immunity: Characterising the early protection of C strain vaccine against subsequent classical swine fever virus infection

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In review

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Author contribution statement

HE, SG, and HC contributed to the performance of the animal experiments and generation of microarray data. RM performed bioinformatics analysis and prepared the manuscript. SG, FS and HC designed the experiments. All authors reviewed the manuscript.

Keywords

CSFV, C strain, ISG15, antiviral, Vaccination, innate immunity, Classical Swine Fever

Abstract

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Classical Swine Fever Virus (CSFV) is an ongoing threat to the pig industry due to its high transmission and mortality rates associated with infection. Live attenuated vaccines such as the CSFV C strain vaccine are capable of protecting against infection within 5 days of vaccination, but the molecular mechanisms through which this early protection is mediated have yet to be established. In this study, we compared the response of pigs vaccinated with the C strain to non-vaccinated pigs both challenged with a pathogenic strain of CSFV. Analysis of transcriptomic data from the tonsils of these animals during the early stages after vaccination and challenge reveals a set of regulated genes that appear throughout the analysis. Many of these are linked to the ISG15 antiviral pathway suggesting it plays a key role in the rapid and early protection conferred by C strain vaccination.

In review

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In review

1 # Head Start Immunity: Characterising the early protection of C strain
2 # vaccine against subsequent classical swine fever virus infection

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In review

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25 **Key Words:** swine, CSFV, innate, C Strain, ISG15, antiviral, vaccination

26 **Abstract**

27 Classical Swine Fever Virus (CSFV) is an ongoing threat to the pig industry due to its high
28 transmission and mortality rates associated with infection. Live attenuated vaccines such as the
29 CSFV C strain vaccine are capable of protecting against infection within 5 days of vaccination,
30 but the molecular mechanisms through which this early protection is mediated have yet to be
31 established. In this study, we compared the response of pigs vaccinated with the C strain to
32 non-vaccinated pigs both challenged with a pathogenic strain of CSFV. Analysis of
33 transcriptomic data from the tonsils of these animals during the early stages after vaccination
34 and challenge reveals a set of regulated genes that appear throughout the analysis. Many of
35 these are linked to the ISG15 antiviral pathway suggesting it plays a key role in the rapid and
36 early protection conferred by C strain vaccination.

In review

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50 **Introduction**

51 Classical Swine Fever (CSF) is a contagious, haemorrhagic and often fatal disease of suidae
52 such as pigs and wild boar, caused by the classical swine fever virus (CSFV). CSFV is an
53 enveloped, single stranded RNA virus that belongs to the pestivirus genus of the *Flaviviridae*
54 family (Moennig, 2000). The positive-sense RNA genome of approximately 12.3kb is
55 translated as a single polyprotein that is then cleaved by both host and native proteases to form
56 11 proteins, 4 of which are structural components of the virion (Blome et al., 2017). Of these
57 structural proteins 2 envelope glycoproteins, E1 and E2, are required for virus entry into the
58 cell through clathrin-dependent, receptor-mediated endocytosis (Shi *et al*., 2016). The primary
59 site of replication are the tonsils and oropharangyeal lymph nodes. From here, the virus is
60 transported through the lymphatic system to the primary lymph nodes, where further rounds of
61 replication occur until the virus eventually reaches all other organs in the body via the
62 circulatory system (Gavier-Widen *et al*., 2012). Interferon signalling is a key component of
63 how the innate immune system responds to challenge with CSFV. High levels of interferon-α
64 (IFN- α) are a characteristic feature of acute disease (Summerfield and Ruggli, 2015). The
65 levels of induction are associated with the virulence of the strain, with highly virulent strains
66 inducing the highest levels (Durand *et al*., 2009a; Durand *et al*., 2009b; Renson *et al*., 2010).
67 Despite the classical functional role of IFNs during viral infection, which is to induce the
68 expression of a cohort of antiviral proteins, these high levels of IFN-α are counterproductive,
69 do not limit virus replication, and lead to the development of disease-associated
70 immunopathology observed through severe lymphoid depletion, lymphocyte apoptosis and
71 thrombocytopenia. This immune dysfunction presents clinically as a viral haemorrhagic fever
72 (Summerfield and Ruggli, 2015).

In review

73 CSF is endemic to parts of South East Asia, Russia and South America. Within Europe,
74 stringent controls such as a stamping out policy, movement restrictions and epidemiological
75 surveillance measures have been in place since 1990 to prevent the spread of the disease,
76 however, sporadic outbreaks have occurred, for example in Lithuania and Latvia, and the recent
77 reoccurrence of CSF in Japan after 26 year absence highlight that CSFV remains an epizootic
78 threat (van Oirschot, 2003; Schulz *et al*., 2017). CSF is amenable to control by vaccination
79 with a number of different live attenuated vaccines available, the most widely used of which is
80 the C strain vaccine (van Oirschot, 2003). However, the inability to distinguish serologically
81 between animals that have been vaccinated or are infected with the virus (DIVA) means its use
82 as an outbreak control tool is limited in CSF-free countries (Blome *et al.,* 2017) . The C strain
83 vaccine was generated through serial passage in rabbits until it was no longer pathogenic. It
84 provides a rapid and complete protection of pigs against infection and also prevents viral
85 transmission within 5 days of vaccination (Leifer et al., 2009; Graham et al., 2012). The
86 immunological signalling cascades behind the early protection afforded by C Strain are poorly
87 understood, but precede the adaptive response, where $IFN\gamma^{+}$ $CD8^{+}$ cells precede the detection
88 of a humoral, virus neutralising response (Kaden and Lange, 2001; Dewulf *et al*., 2004;
89 Franzoni *et al*., 2013). As the C strain vaccine has been the most widely used vaccine for CSFV
90 to date, deciphering the precise innate immune signalling pathways underpinning its
91 effectiveness may help shape and optimise the current generation of marker and subunit
92 vaccines. To achieve a greater insight into the host response to vaccination with C strain,
93 porcine microarrays were utilised to analyse the differences in gene expression in tonsil tissue
94 between pigs that were vaccinated with C strain or given a mock inoculum. These pigs were
95 then subsequently challenged with a virulent strain of CSFV five days post immunisation, thus
96 before an effective adaptive response could be mounted. In this study we have examined

In review

97 transcriptional changes in tonsils at early time points to identify subsets of genes that may be
98 integral to this rapid protection and could support the induction of an early adaptive immune
99 response.

100 **Materials and Methods**

101 **Viruses.** C strain CSFV (AC Riemser Schweinepestvakzine, Riemser Arzneimittel AG, Riems,
102 Germany) and the virulent CSFV Brescia strain were propagated in PK15 cell monolayers.
103 Both mock virus and virus stocks were prepared, and titers were determined, as described
104 previously (Franzoni *et al.,* 2013).

105 **Ethics statement.** All animal work was approved by the Animal and Plant Health Agency
106 (APHA) Animal Welfare and Ethical Review Board, and all procedures were conducted in
107 accordance with the Animals (Scientific Procedures) Act 1986 (United Kingdom) under
108 project license permits PPL 70/6559. Each animal was euthanized on predetermined days by
109 stunning and exsanguination.

In review

110 **Animals.** Eighteen Large White/Landrace crossbreed pigs of 9 weeks of age were randomly
111 assigned to one of two groups. On day 0 the animals in group 1 (n = 9) were vaccinated with 2
112 ml of C strain vaccine into the brachiocephalous muscle (as recommended by the
113 manufacturer), and group 2 (n = 9) was intranasally inoculated with tissue culture supernatant
114 (mock). For intranasal inoculations 1ml per nostril was administered using a mucosal
115 atomization device (MAD300; Wolf Tory Medical, USA). On day 5 post vaccination (DPV),
116 both groups were inoculated intranasally with 10^5 $TCID_{50}$ of CSFV Brescia strain. EDTA anti-
117 coagulated blood samples were collected in Vacutainers (BD Biosciences) prior to and after
118 challenge from the external jugular vein. Three animals from each group were euthanized on
119 dpv 5 (prior to challenge), dpv 8 and dpv10 and the tonsils were collected.

120 **Clinical, hematological, and virological methods.** The animals were inspected by the APHA
121 Animal Sciences Unit staff twice daily (am and pm), and 10 parameters relevant to an
122 indication of CSF (temperature, liveliness, body shape and tension, breathing, walking, skin,
123 eye/conjunctiva, appetite, and defecation) were examined and scored as 0 (normal), to 3
124 (severely altered; known CSF sign) (Everett *et al.*, 2010) . A total clinical score for each animal
125 was assigned twice daily, and their temperatures were monitored by rectal thermometer
126 readings and recorded once daily. Peripheral blood leukocytes and CSFV RNA were monitored
127 in EDTA blood samples collected every 3 days using volumetric flow cytometry and real-time
128 reverse transcription-quantitative RT-PCR (RRT-qPCR), respectively (Everett *et al.*, 2010).

129 **Gene expression microarray analysis**

In review

130 At days 5, 8 and 10 post-vaccination animals were euthanized, the tonsils removed, chopped
131 into fine pieces and stored at -80ºC in RNAlater (Sigma-Aldrich). RNA was extracted using
132 MagMax 96 microarray total RNA isolation kit which includes a Turbo DNAse treatment to
133 remove contaminating genomic DNA. Elimination of genomic DNA was confirmed by q-PCR
134 detection of porcine *β-actin* gene with and without reverse transcription. The Ovation PicoSL
135 WTA System v2 kit (NuGEN, Leek, The Netherlands) was used to amplify cDNA from 50ng
136 total RNA. The MinElute Reaction Cleanup Kit (Qiagen) was used to purify cDNA, and 1 µg
137 was then labelled using a one-color DNA labelling kit (NimbleGen, Madison, USA). For each
138 sample, 4 µg labelled cDNA was hybridised to a custom NimbleGen 12 × 135 K porcine array
139 designed using the *Sus scrofa* 10.2 genome build and incorporating a total of 19,351 genes,
140 each represented on the array by a set of six different probes (116,106 probes in total) (Edwards
141 *et al.*, 2017) . The microarray also contained a large number (24,179) of random probes.
142 Hybridised arrays were scanned at 2 µm resolution on a microarray scanner (Agilent,
143 Wokingham, UK). Microarray images were processed using DEVA v1.2.1 software to obtain

a pair report containing the signal intensity values for each probe. To correct for differences in the overall intensity levels between slides robust multi-array (RMA) normalization was used. Data was then processed using GeneSpring GX using the manufacturer's guidelines. RMA normalized pair files were imported and empirical Bayesian unpaired comparison (moderated *t*-test, $P<0.05$) combined with a Westfall and Young Permutation to correct for multiple testing was carried out to generate a list of genes with significantly altered expression between C strain and mock inoculated pigs of greater than twofold. The raw microarray data (background-corrected signal) can be assessed at Gene Expression Omnibus (GEO accession GSE111486. Reviewer access code: mzurkuggzdylleh).

Gene Ontology and Pathway Analysis

In review

To aid in the analysis of the data, where possible human orthologue of porcine genes were used for further analysis. Gene Ontology analysis was performed using BiNGO within Cytoscape 3.2 (Shannon *et al.*, 2003; Maere *et al.*, 2005). BiNGO analysis was performed using a hypergeometric test with a Bejamini Hochberg False Discovery Rate correction and significance value of 0.05, the ontology file used was GO_Biological_Process. PANTHER Overrepresentation Analysis (release 20171205) was performed using the annotation Reactome version 58 (Release 20161207) using a Binomial test with a Bonferoni Correction for multiple comparison (Ashburner *et al.*, 2000; Gene Ontology, 2015). Network analysis was performed using NetworkAnalyzer tool in Cytoscape, nodes and label sizes are mapped based on betweenness centrality (Shannon *et al.*, 2003).

Results

166 **Vaccination, challenge and clinical observations**

167 Samples for transcriptomic analysis were generated from animals vaccinated, or mock
168 inoculated, 5 days prior to challenge with virulent CSFV (Franzoni *et al*., 2013). Tonsil samples
169 were collected prior to challenge at 5 dpv and also at day 8 and day 10 pv (3 and 5 days post-
170 challenge (dpc)) (Fig. 1A). C strain vaccinated animals were protected from the challenge with
171 no clinical signs or temperature increase detected. The mock inoculated animals had early
172 clinical signs of CSF from 4-5 dpc and elevated rectal temperatures (Fig. 1B and C). CSFV
173 RNA and leukopenia was detected in blood samples from 8 dpv in the mock inoculated animals
174 but not in vaccinated animals (Fig. 1D and E). This level of protection corresponds with
175 previous studies in that complete protection from challenge with CSFV was observed within 5
176 days of vaccination, thus before the onset of an adaptive immune response, which then rapidly
177 develops after challenge (Graham *et al.,* 2012).

In review

178 **Intramuscular vaccination produces a robust transcriptional response in tonsil cells of**
179 **naïve pigs**.

180 At day 5 post-vaccination (prior to challenge), when vaccinated pigs were compared to mock
181 inoculated pigs, 448 genes were differentially regulated; 255 genes were down-regulated and
182 193 genes upregulated (Table 1, Sup.Table 1). Gene Ontology analysis (Maere *et al*., 2005)
183 highlighted over representation of gene categories associated with response to virus among the
184 upregulated genes as expected since the C strain vaccine is a live, attenuated virus (Fig. 2A).
185 Among the downregulated genes a number of different metabolic processes were over-
186 represented (Fig. 2B).

187 At day 8 pv, i.e. 3 dpc and thus when viral RNA was detected in the unvaccinated animals (Fig.
188 1E) 138 genes were differentially regulated, with 118 genes significantly less expressed in

189 tonsils of C strain vaccinated pigs compared to mock inoculated pigs (Table 2, Sup. Table 2).
190 In terms of gene ontology over-representation, an inversion occurred whereby pathways
191 associated with response to virus were now overrepresented in those pigs that were not
192 vaccinated (Sup. Fig 1).

193 At 10 dpv, thus 5 dpc, 142 genes were differentially regulated, with 126 of these genes
194 expressed less in the vaccinated animals compared to the mock inoculated group (Table 3, Sup.
195 Table 3). Ontology analysis yielded similar observations as was seen at day 8 with an over-
196 representation of pathways associated with a response to a virus (Sup. Fig 2) in pigs that were
197 not vaccinated. Notably, among the few genes that were up regulated in the C strain vaccinated
198 pigs was *eomes*, a gene that encodes a transcriptional regulator known to play a role in $CD8^+$
199 T cell differentiation (Martinet *et al*., 2015). This corresponds with previously published data
200 where CSFV specific $CD8^+$ T cells were detected in the same animal cohort (Franzoni *et al*.,
201 2013).

In review

202 **Specific sub-sets of genes fluctuate in response to CSFV regardless of strain virulence.**

203 Analysis of all the significantly differentially expressed genes at day 5, 8 and 10 post-
204 vaccination revealed a cohort of genes that were differentially expressed at all of the time
205 points. This suggested that these genes were integral to the response to both the C strain vaccine
206 and the virulent CSFV strain Brescia. These genes were significantly upregulated in C strain
207 vaccinated pigs at 5 dpv (Fig. 3A). However, by day 8 and day 10 the expression of these genes
208 in vaccinated animals had alleviated suggesting they were no longer induced. Remarkably, this
209 same subset of genes was instead induced significantly in the mock inoculated animals at 8
210 days (Fig. 3B) and 10 days (Fig. 3C) post-vaccination (3 and 5 days post-challenge) (Fig3D).
211 The expression of these genes corresponds with exposure to either strain of the virus and may

212 play a key role in enabling vaccinated pigs to overcome challenge. Indeed, among this cohort
213 are a number of genes coding for antiviral effectors, such as IFIT1, IFIT2, IFIT3, IFIT5 which
214 encode proteins that directly interact with viral RNA preventing the initiation of translation
215 (Schmeisser *et al*., 2010; Ablasser and Hornung, 2011; Pichlmair *et al*., 2011; Cho *et al*., 2013;
216 Hsu *et al*., 2013; Wetzel *et al*., 2014)**,** as well as MX1 and MX2*,* proteins that can directly
217 prevent viral ribonucleoprotein complex formation (Jin *et al*., 1999; Salomon *et al*., 2007;
218 Verhelst *et al*., 2012; Cai *et al*., 2013; He *et al*., 2014; Zhang *et al*., 2015; Wang *et al*., 2016).
219 The increase in expression of the genes encoding these antiviral effectors, as well as other
220 proteins involved in the innate immune response, such as RSAD2 (Viperin), DDX60 and
221 DHX58, at the time of challenge may be integral to the early protection offered by C strain
222 vaccination.

223 **The ISG15 pathway is activated in response to C strain vaccination.**

224 The proteins encoded by the subset of genes differentially expressed across all three time points
225 were subjected to an interaction analysis using Cytoscape and pathways from the InnateDB
226 database. This network analysis revealed that many of the proteins within this cohort are
227 capable of directly interacting with at least one other protein in the cohort and also highlighted
228 ISG15 as the best connected node within the network (Fig. 4). This is likely to be expected
229 given the nature of ISG15, which functions in a pathway similar to the ubiquitination pathway,
230 in that ISG15 is conjugated to a range of host and non-host proteins modifying their function
231 in a process known as ISGylation (Zhao *et al.,* 2013). Indeed among our common cohort of
232 genes differently expressed at all time points were a number of known ISG15 conjugation
233 targets such as IFIT1-3, IFIT5, DHX58, MX1 (Zhao *et al*., 2005), as well as other components
234 of the ISG15 pathway including key enzymes HERC5 and USP18, which are directly involved

In review

235 in the ISGylation conjugation and deconjuguation process, respectively (Sadler and Williams,
236 2008).

237 Gene overrepresentation analysis using the Reactome Database identified the Interferon
238 signalling pathway and also identified the ISG15 pathway as being significantly
239 overrepresented across all time points ($p < 4.04E-09$, Sup. Table 4), albeit in different groups
240 at each time point. It was overrepresented in C strain vaccinated pigs at 5 dpv ($p < 5.99E-03$,
241 Sup. Table 5), but in mock inoculated pigs at day 8 ($p < 1.83E-07$, Sup. Table 6) and 10 dpv
242 ($p < 6.97E-06$, Sup. Table 7).

243 The early induction of the ISG15 pathway may play a key role in the early protection afforded
244 by the C strain vaccination as it ensures that an innate immune response that is producing
245 numerous antiviral effectors (IFIT1, IFIT2, IFIT3, IFIT5, MX1 and MX2) is elevated during
246 this early window.

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In review

255 **Discussion**

256 Since its introduction in the early 1960's the C strain vaccine, has proven remarkably effective
257 and is still the most used vaccine to control CSFV in endemic settings for example in SE Asia
258 (Brown and Bevins, 2018). It has been shown to stimulate an adaptive cell mediated immune
259 response within 8 - 10 dpv (Franzoni *et al*., 2013). However, vaccinated pigs are protected
260 already 5 dpv, with partial protection observed even earlier (Leifer *et al.,* 2009, Graham *et al.,*
261 2012). Understanding the molecular mechanisms underpinning this early immunity may aid in
262 the development of more effective, rapid vaccines and in the optimization of vaccines that are
263 currently available. In this study we used a transcriptomic approach to identify a subset of genes
264 that are regulated after both vaccination and challenge and that are linked to a distinct antiviral
265 pathway that is up-regulated during this early protective window.

266 Type I IFN is known to play a key role in generating a robust host immune response to viral
267 infection and the according expression of interferon stimulated genes (ISG) with vaccination
268 or challenge had been expected since it is known that CSFV is a strong inducer of type I IFNs
269 (Cao *et al*., 2015). The interaction of type I IFN with CSFV has been extensively studied, not
270 least as CSFV also exhibits ways to suppress type I IFN (Fiebach *et al*., 2011). However, the
271 interferon response comprises of over 300 ISGs (Der *et al*., 1998) and the precise mechanism
272 through which this signalling cascade mediates these numerous antiviral responses is yet to be
273 fully elucidated. We focussed here on a set of genes that was significantly regulated across
274 both studied conditions (vaccination and challenge) and was significantly regulated at all time
275 points studied in this early period post exposure to either virus.

276 Expression of the ISG15 gene has previously been shown to be induced in response to virulent
277 strains of CFSV *in vitro* (Cai *et al*., 2017; Li *et al*., 2018), however, this is the first study to

In review

278 demonstrate induction of the ISG15 pathway in response to vaccination with C strain *in vivo*
279 and specifically that this induction occurs in the tonsil, the primary site of CSFV replication.
280 Importantly, the ISG15 pathway was up-regulated in C strain vaccinated strains during the
281 window in which a protective immune response exists and the adaptive immunity develops.
282 Although the C strain vaccine was given intramuscularly it is well established that CSFV has
283 a tropism for tonsil tissue which is the primary site of replication of CSFV (Gavier-Widen *et*
284 *al*., 2012), the elevation of the ISG15 pathway in this specific tissue is ideally placed to prevent
285 challenge by the most likely natural route of infection.

286 ISG15 plays a central role in mediating IFN-induced host antiviral responses. ISG15 is a 15
287 kDa protein that is covalently attached to its target proteins via the action of a group of 3
288 enzymes (UBE1L, UBCH8 and HERC5), which are also induced in response to type I IFN.
289 This pathway is similar to that of ubiquitination, however unlike ubiquitination, conjugation of
290 ISG15 to host target proteins does not prime them for degradation but instead stabilises or
291 activates them. Over 150 host ISG15 conjugation targets have been identified thus far (Zhao *et*
292 *al*., 2005) Among this cohort of ISGylation targets are some anti-viral proteins whose mRNA
293 has been identified as differentially regulated through our analysis such as IFIT1, IFIT2, IFIT3,
294 IFIT5 MX1 and MX2. These proteins target a number of different aspects of the viral
295 replication cycle such as RNA translation and virion assembly (Jin *et al*., 1999; Salomon *et al*.,
296 2007; Ablasser and Hornung, 2011; Pichlmair *et al*., 2011; Cai *et al*., 2013; Cho *et al*., 2013).
297 Some of the proteins such as MX1, have direct antiviral activity against CSFV (He *et al*., 2014;
298 Zhang *et al*., 2015), other proteins are known to be active against other *Flaviviruses*, such as
299 IFIT2 which restricts growth of West Nile virus (Cho *et al*., 2013). Moreover, the free
300 unconjugated form of the ISG15 has antiviral activity and can protect mice against another
301 RNA (Toga-)virus, the Chikungunya virus infection (Werneke *et al*., 2011).

In review

302 Conjugation of ISG15 to viral proteins results in their loss of function and the evolutionary
303 importance of this pathway in controlling viral infection is demonstrated by the emerging
304 number of viral proteins that have evolved to disrupt this pathway. For example, the NS1
305 protein of influenza A and B viruses inhibits ISG15 conjugation (Yuan and Krug, 2001; Tang
306 *et al*., 2010; Zhao *et al*., 2010; Zhao *et al*., 2013) and NSP2 of porcine reproductive and
307 respiratory syndrome virus, another important pig pathogen, inactivates ISG15 (Sun *et al*.,
308 2012).

309 Further to those proteins directly linked with the ISG15 pathway, we also saw the upregulation
310 of a number of other ISGs. These included IFI44 which is known to have antiviral activity
311 although the precise mechanism of action remains to be characterised (Power *et al*., 2015) and
312 RSAD2 (Viperin) which inhibits many DNA and RNA viruses, including CSFV through
313 interaction with the E2 structural protein (Li *et al*., 2017). Importantly, RSAD2 has also been
314 implicated in DC maturation and CD4 cell activation (Sezin *et al*., 2017; Jang *et al*., 2018) and
315 may thus be one of the genes that links the innate and adaptive immune system. One porcine
316 gene LOC100157244 was differentially regulated that has not previously been characterised
317 but is predicted to be a ATP-dependent RNA helicase similar to DDX60. This protein may be
318 a novel component of the pig host's immune response to viral infection and future work needs
319 to focus on characterising this gene, as well as establishing if some of the other genes
320 upregulated that have not yet been directly related to the ISG15 pathway could represent as yet
321 uncharacterised ISG15 conjugation targets.

322 The role of IFN I in CSFV infection has been discussed (Summerfield and Ruggli, 2015) and
323 it is proposed that the type I IFNs contribute to the pathology of haemorrhagic fever. However,
324 it is well known that IFN I induce anti-viral effects in cells that have been treated before
325 infection, so that ISGs can be induced, and that a single dose IFN I does not induce a long

In review

326 lasting anti-CSFV effect (Fernandez-Sainz *et al*., 2015). In light of our analysis, we propose a
327 model whereby C strain vaccination is giving vaccinated pigs a head start during which a wide
328 range of innate antiviral effectors are produced, which serve to contain viral replication, should
329 exposure to a virulent strain of CSFV take place prior to the onset of adaptive responses. In
330 naïve hosts, a virulent strain of CSFV will replicate faster, as the innate response cannot
331 produce enough antiviral effectors in time to contain the infection (Fig. 5). While many of the
332 proteins described have been shown to have direct antiviral activity against CSFV, this
333 response is not necessarily specific to CSFV, but since C strain targets the tonsil, which is also
334 the primary site of CFSV replication, it is particularly effective at protecting against CSFV.
335 The up-regulation of the ISG15 pathway in unvaccinated pigs after CSFV Brescia challenge is
336 most likely associated with the failed attempt of the immune system to induce an antiviral
337 response after infection, contributing to clinical disease including leukopenia (Zhao *et al*.,
338 2013; Summerfield and Ruggli, 2015).

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In review

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In review

526 **Figure 1: C Strain Vaccination and Subsequent Challenge:** **(A)** Schematic outline of the
527 vaccine/challenge study highlighting key time points of vaccination and challenge. Three
528 animals per group were euthanized at day 5, 8 and 10 pv for sample acquisition **(B)** Mean
529 clinical score data from both C strain vaccinated and mock inoculated animals from before the
530 study commenced until completion. **(C)** Rectal temperatures of animals throughout the course
531 of the study. **(D)** Peripheral blood leukocyte counts in EDTA blood samples throughout the
532 study **(E)** CSFV RNA as detected in blood by reverse transcription-quantitative RT-PCR. Error
533 bars indicate SD.

534 **Figure 2: Overrepresented Gene Ontologies in tonsils 5 days after vaccination:** **(A)** Gene
535 ontologies overrepresented as determined by the BiNGO Cytoscape application in the subset
536 of genes upregulated in C-Strain vaccinated pigs at 5 days post vaccination. (**B**) Gene
537 ontologies overrepresented in the subset of genes downregulated in C- strain vaccinated pigs
538 at 5 days post vaccination. Hypergeometric Test used to determine significance ($p<0.05$). Level
539 of significance indicated by yellow to orange colouring.

540 **Figure 3: Differential expression of a cohort of genes at identified at each time point: (A)**
541 Expression of a cohort of 14 genes at day 5 post vaccination comparing the C Strain vaccinated
542 animals to those that received the mock inoculation. (**B)** Expression of a cohort of 14 genes at
543 day 8 post vaccination (3 dpc) comparing the C Strain vaccinated animals to those that received
544 the mock inoculation. **(C)** Expression of a cohort of 14 genes at day 10 post vaccination (5 dpc)
545 comparing the C Strain vaccinated animals to those that received the mock inoculation. **(D)**
546 Heat map showing the gene expression changes as they occurred over the course of the study.
547 Expression values are from 3 pigs per condition per time point. Significance was determined
548 using a moderated t-test $p< 0.05$ considered as significant.

In review

Figure 4: Network analysis of Co-expressed Genes: Network assembled from 14 gene cohort significantly differentially regulated at each time point. Network based on interactions defined in the InnateDB. Nodes and label sizes are mapped based on betweenness centrality. Network assembled using Cytoscape 3.2

Figure 5: Head Start Immunity Model: Upon vaccination with C strain, the induction of interferon results in induction of ISGs, including the ISG15 antiviral pathway activation resulting in the induction and activation via ISGylation of a wide variety of antiviral effectors. These antiviral effectors accumulate over the 5 days post vaccination, priming the host in an antiviral state and, for example via induction of RSAD2, instigating the adaptive immune response. If during this window a virulent strain of CSFV attempts to infect the host, the multitude of antiviral effectors are already present within the cell and can immediately prevent the replication of the virus and ultimately assist in preventing the establishment of infection. Without prior vaccination, replication of a virulent strain of CFSV is allowed as although the antiviral effectors of IFN and ISG15 pathways are induced by the virulent virus these cannot keep pace with the replication rate of virulent strains of CSFV and thus are not able to sufficiently control viral replication before adaptive responses can be activated, leading to the onset of clinical disease.

In review

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575 **Author Contributions**

576 HE, SG, and HC contributed to the performance of the animal experiments and generation of
577 microarray data. RM performed bioinformatics analysis and prepared the manuscript. SG, FS
578 and HC designed the experiments. All authors reviewed the manuscript.

579 **Conflict of Interest statement**

580 The authors declare that the research was conducted in the absence of any commercial or
581 financial relationships that could be construed as a potential conflict of interest

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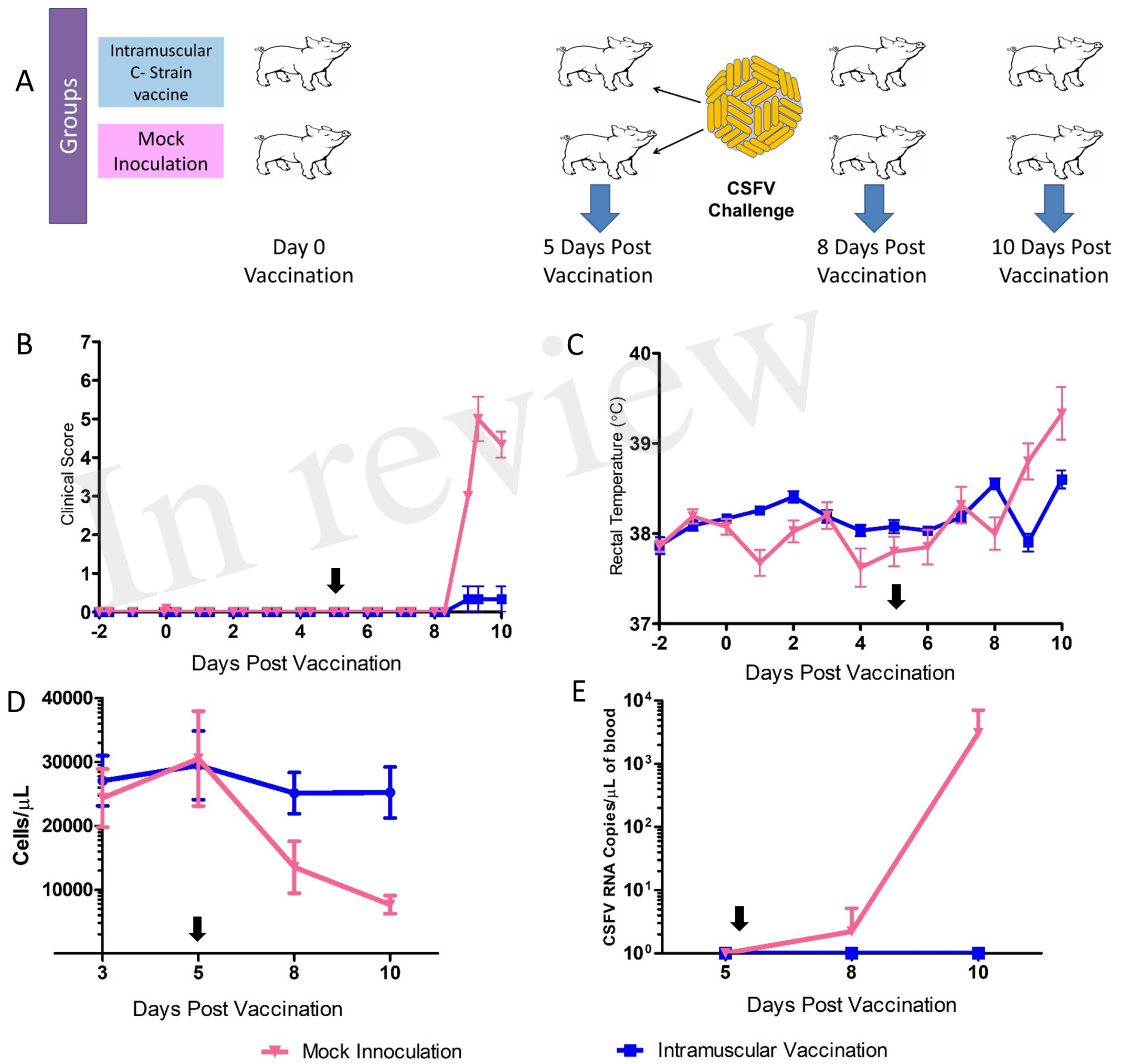
In review

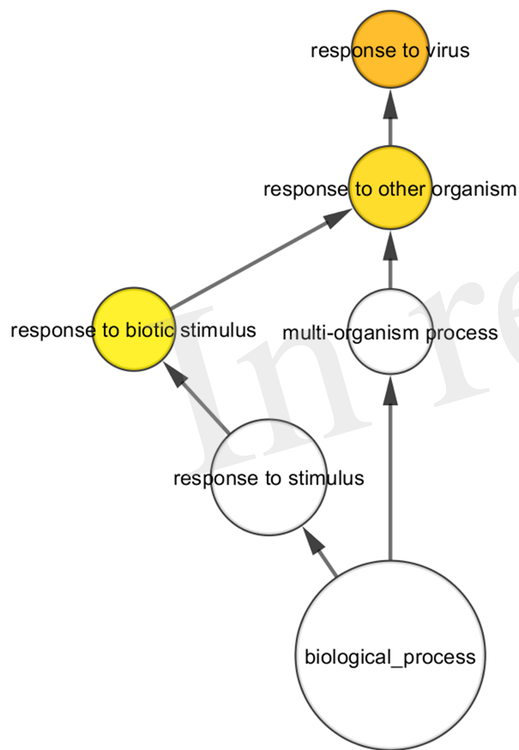


In review

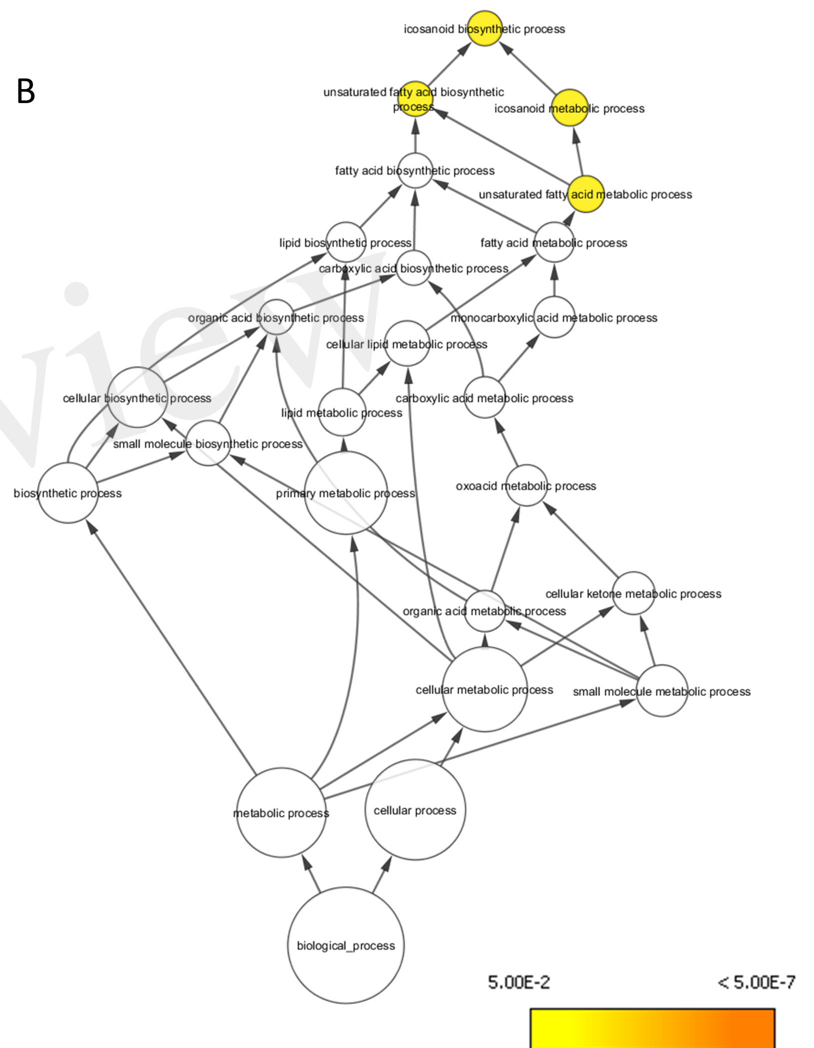
Figure 2.TIF

Figure 2

A



B



In review

Figure 3.TIF

Figure 3

A

Day 5
C-Strain Vaccine Vs Mock
LogFC
3
2
1
0
DDX60 DHX58 IFIT1 IFIT2 IFIT3 IFIT5 RSAD2 ISG15 LOC100157244 HERC5 IFI44 MX1 MX2 USP18

B

Day 8
C-Strain Vaccine Vs Mock
LogFC
0
-1
-2
-3
-4
DDX60 DHX58 IFIT1 IFIT2 IFIT3 IFIT5 RSAD2 ISG15 LOC100157244 HERC5 IFI44 MX1 MX2 USP18

C

Day 10
C-Strain Vaccine Vs Mock
LogFC
0
-2
-4
-6
DDX60 DHX58 IFIT1 IFIT2 IFIT3 IFIT5 RSAD2 ISG15 LOC100157244 HERC5 IFI44 MX1 MX2 USP18

D

Day 5 Mock
Day 5 Vaccine
Day 8 Mock
Day 8 Vaccine
Day 10 Mock
Day 10 Vaccine
DDX60 DHX58 IFIT1 IFIT2 IFIT3 IFIT5 RSAD2 ISG15 LOC100157244 HERC5 IFI44 MX1 MX2 USP18
LogFC
5
0
-5

In review

Figure 4.TIF

Figure 4

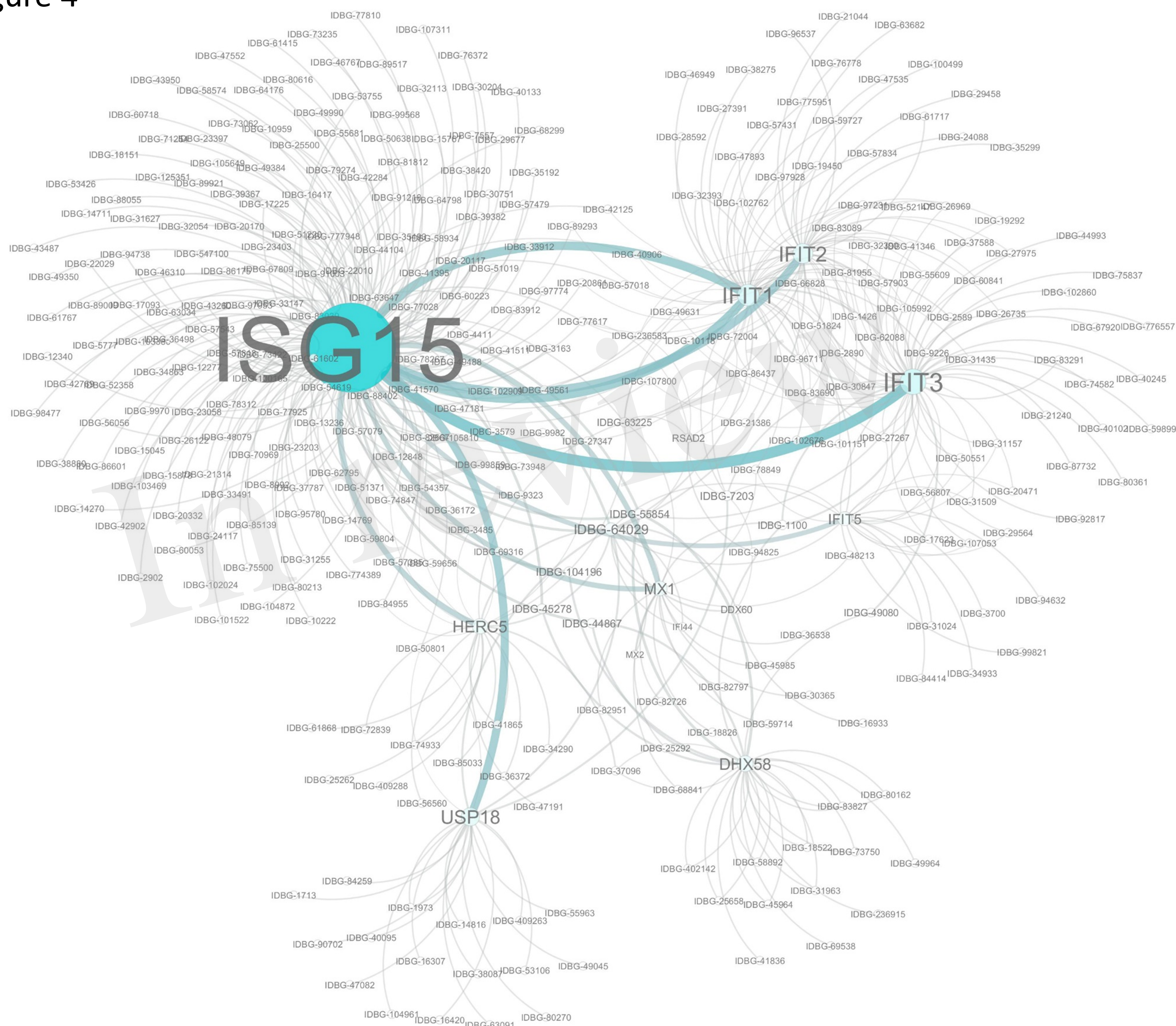
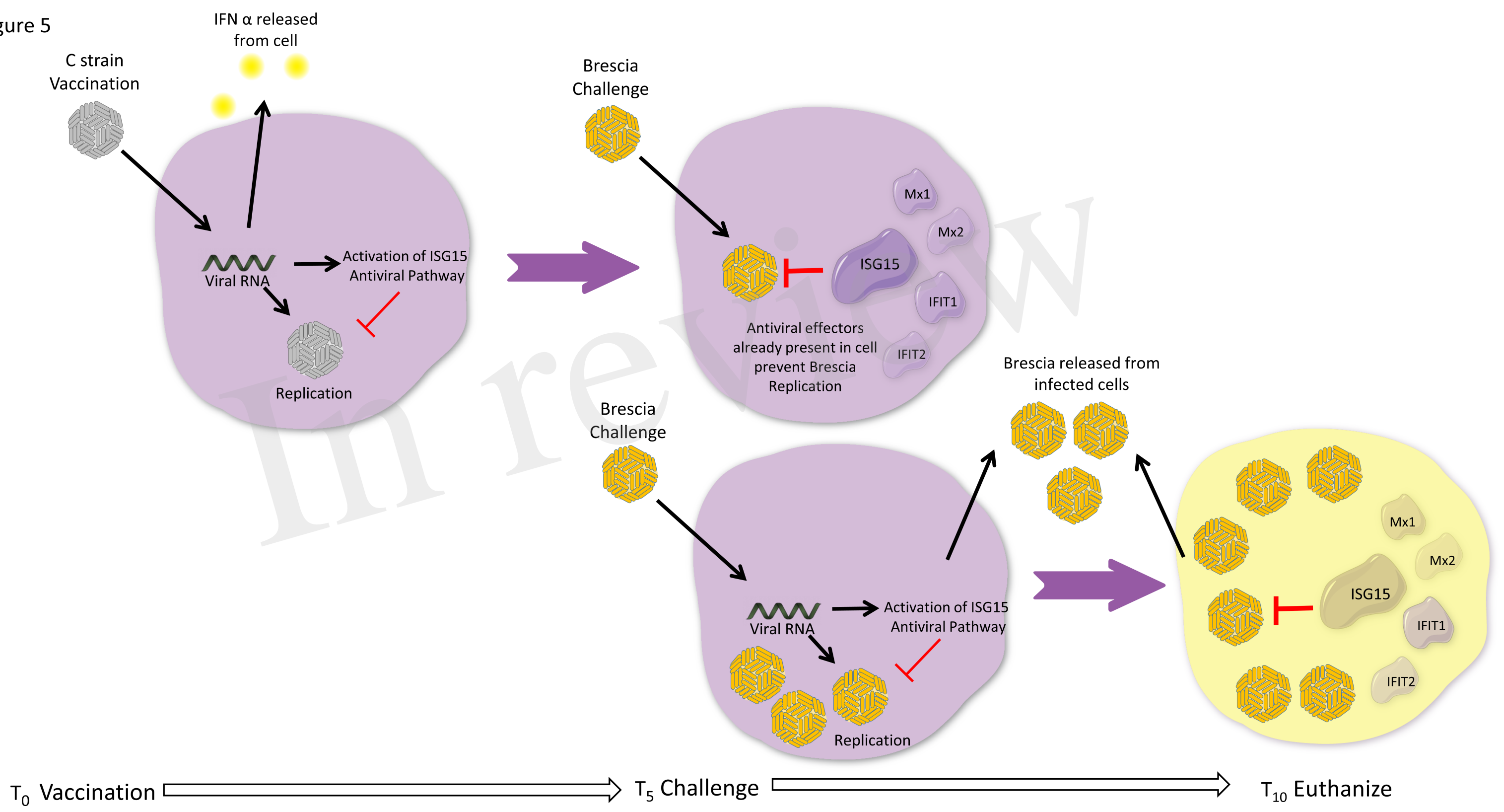


Figure 5.TIF



In review

Figure 6.TIF

Table 1: Genes differentially regulated in C strain vaccinated pigs compared to mock inoculated pigs at 5 days post vaccination. Top 20 up/down regulated genes shown

Genes Up	LogFC	Genes Down	LogFC
ifit2	2.553934	*pg-2*	-1.64178
pkia	2.373655	*c6h19orf33*	-1.64285
hgf	2.295012	*zfp36*	-1.66558
ano5	2.191831	*bcas4*	-1.67219
rsad2	2.176041	*ssc-mir-135-1*	-1.68395
adam7	2.075018	*tmem141*	-1.70127
rab27b	1.918744	*ddt*	-1.71247
kiaa1107	1.892113	*ndufa11*	-1.7917
loc100520366	1.842525	*loc100511639*	-1.80764
tmem178a	1.816612	*loc100626517*	-1.80975
ifit1	1.807552	*atox1*	-1.81446
epb41l4b	1.796457	*myadm*	-1.83981
c1h14orf37	1.785264	*ccl14*	-1.88256
tdrd1	1.777872	*loc100521485*	-1.89069
rpgrip1	1.77437	*ssc-mir-125b-2*	-1.92758
galntl5	1.714732	*dusp15*	-1.93288
ifi44	1.689456	*tmem160*	-1.94606
ttc39a	1.678465	*dpm3*	-1.95109
wdr35	1.673544	*scgb3a1*	-2.0114
pln	1.670908	*ndufb11*	-2.03292

In review

Figure 7.TIF

Table 2: Genes differentially regulated in C-Strain vaccinated pigs compared to mock vaccinated pigs, 8 days post vaccination (3 days post challenge) with CSFV. Top 20 Up/Down regulated genes shown.

Genes Up	LogFC	Genes Down	LogFC
npg1	2.47	*sprr1a*	-2.65
pgrmc2	1.97	*krt78*	-2.68
lyzl4	1.83	*lgals7*	-2.71
tex14	1.76	*oasl*	-2.73
pg-2	1.69	*krt78*	-2.74
lrg1	1.58	*gsta1*	-2.86
loc102158214	1.54	*cnfn*	-2.88
pcd1b	1.50	*sprp*	-2.89
loc102157463	1.45	*loc100516001*	-2.91
npg4	1.39	*cnfn*	-2.92
loc100739707	1.29	*ifit2*	-3.18
loc100522081	1.26	*csta*	-3.32
pcd1e	1.25	*olfm4*	-3.37
slc7a8	1.18	*sprr1a*	-3.43
loc100514211	1.17	*spink5*	-3.56
znf449	1.11	*tprg1*	-3.63
c1h9orf116	1.06	*csta*	-3.80
kcnip1	1.02	*krt23*	-3.86
pr39	1.01	*pheroc*	-4.05
tenm3	1.00	*cldn17*	-4.19

In review

Figure 8.TIF

Table 3: Genes differentially regulated in C-Strain vaccinated pigs compared to mock vaccinated pigs, 10 days post vaccination (5 Days post challenge with CSFV). Top 20 Up/Down regulated genes shown.

Genes Up	LogFC	Genes Down	LogFC
eomes	3.35	*loc100157995*	-2.98
dapl1	2.94	*ifi44*	-3.14
il21	2.83	*ifit5*	-3.19
gzmk	2.74	*cd101*	-3.20
rgs5	2.74	*loc100511472*	-3.24
loc100516016	2.65	*irg6*	-3.29
apitd1	2.57	*oas1*	-3.40
loc100512025	2.40	*loc100518694*	-3.46
pcdh15	2.34	*loc100525838*	-3.51
loc100153678	2.26	*dhx58*	-3.62
abca8	2.24	*irg1*	-3.77
loc100523628	2.12	*loc100511550*	-3.98
loc100521080	1.98	*ube2l6*	-4.15
cacnb4	1.90	*fcgr1a*	-4.15
loc100512149	1.81	*loc100512690*	-4.23
		usp18	-4.28
		cxcl11	-4.55
		oasl	-4.78
		slpi	-4.90
		ifit2	-5.72

In review