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1 Characterisation of virus-specific peripheral blood cell cytokine responses following
2 vaccination or infection with classical swine fever viruses

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12

13 **Abstract**

14 Existing live attenuated classical swine fever virus (CSFV) vaccines provide a rapid
15 onset of complete protection but pose problems in discriminating infected amongst
16 vaccinated animals. With a view to providing additional information on the cellular
17 mechanisms that may contribute to protection, which in turn may aid the development
18 of the next generation of CSFV vaccines, we explored the kinetics of the cytokine
19 responses from peripheral blood cells of pigs vaccinated with an attenuated C-strain
20 vaccine strain and/or infected with a recent CSFV isolate. Peripheral blood cells were
21 isolated over the course of vaccination/infection and stimulated *in vitro* with C-strain
22 or UK2000/7.1 viruses. Virus-specific responses of peripheral blood cells isolated
23 from C-strain vaccinated pigs were dominated by the production of IFN- γ . IFN- γ
24 production in response to the C-strain virus was first detected in vaccinates 9 days
25 post-vaccination and was sustained over the period of observation. In contrast, cells

1 from challenge control animals did not secrete IFN- γ in response to stimulation with
2 C-strain or UK2000/7.1 viruses. Supernatants from UK2000/7.1 infected animals
3 contained significant levels of pro-inflammatory cytokines from day 8 post-infection
4 and these cytokines were present in both virus and mock stimulated cultures. The
5 results suggest that the C-strain virus is a potent inducer of a type-1 T cell response,
6 which may play a role in the protection afforded by such vaccines, whereas the pro-
7 inflammatory cytokine responses observed in cultures from infected pigs may reflect a
8 pathological pro-inflammatory cascade initiated *in vivo* following the replication and
9 spread of CSFV.

10

11 **Keywords:** Classical swine fever virus; Swine; Vaccination and infection; Interferon-
12 γ ; Cytokines.

13

14

1 **1. Introduction**

2 Classical swine fever (CSF) is a devastating disease that poses one of the
3 greatest risks to the swine industry worldwide. CSF is caused by the classical swine
4 fever virus (CSFV), a highly contagious, small enveloped, single-stranded RNA virus
5 that belongs to the family *Flaviviridae*. CSF has, since 1990, been controlled in the
6 EU through a 'stamping-out' slaughter policy but the presence of a CSFV reservoir in
7 European wild-boar populations, together with increasing public opposition against
8 stamping-out policies, has now led to an increased likelihood that vaccination may be
9 deployed as a last resort component of a control policy (Vandeputte and Chappuis,
10 1999; van Oirschot, 2003). Existing live attenuated CSFV vaccines, such as those
11 based on lapinised or culture attenuated C-strain viruses, provide a rapid onset of
12 complete protection but pose problems in discriminating infected amongst vaccinated
13 animals whereas questions remain about the efficacy of available marker sub-unit
14 vaccines for use under emergency outbreak conditions (van Oirschot, 2003).

15 The immunological mechanisms that underlie the rapid protection afforded by
16 live attenuated C-strain vaccines are not well defined, however protection may
17 precede the appearance of neutralising antibody but not IFN- γ secreting cells in
18 peripheral blood (Suradhat et al., 2001; Suradhat and Damrongwatanapokin, 2003),
19 suggesting that cellular immunity is responsible. A number of investigations have
20 attempted to characterise these cellular mechanisms; both virus-specific CD4⁺ and
21 CD8⁺ T cell IFN- γ responses occur following vaccination, with different studies
22 suggesting prominent roles for either population (Suradhat et al., 2005). Following C-
23 strain vaccination and challenge, CD6⁺CD8⁺ MHC class I restricted cytotoxic T
24 lymphocyte responses, directed against the non-structural viral protein NS3, were
25 evoked that could lyse virus-infected cells (Pauly et al., 1995). It was later

1 demonstrated that the NS3 specific cytotoxic T lymphocytes also secrete IFN- γ (Rau
2 et al., 2006). While IFN- γ appears to serve as a good marker for anti-CSFV cell-
3 mediated responses, its role in the primary response to virulent CSFV is less well
4 studied. Piriou et al. (2003) infected pigs with a sub-acute dose of CSFV that resulted
5 in the induction of a strong IFN- γ response. Suradhat et al. (2001) reported a virus
6 specific IFN- γ response in pigs 8 days after a virulent CSFV infection and yet all
7 animals died within 14 days.

8 Other cytokine responses induced by CSFV have been studied *in vitro* and *in*
9 *vivo* but the mechanisms underlying these responses and their contribution to
10 immunity/disease remain to be defined. *In vitro* infection of primary endothelial cells
11 with virulent CSFV induces an up-regulation in the transcription of pro-inflammatory
12 cytokines (Bensaude et al., 2004). Analyses of early cytokine responses following
13 CSFV infection have shown that different dendritic cell types express different
14 cytokine profiles. The dominant effect appeared to be highly elevated secretion of
15 TNF- α and IFN- α , which could also be detected in sera, and may play a role in the
16 disruption of immune responses (Jamin et al., 2008). In addition
17 immunohistochemical studies have demonstrated macrophages expressing
18 proinflammatory cytokines in both the spleen and liver of CSFV infected pigs
19 (Sanchez-Cordon et al., 2005, Nunez et al., 2005).

20 With a view to providing additional information concerning the cytokine
21 mediated mechanisms that may contribute to protection or pathology, and therefore
22 aid the development of the next generation of CSFV vaccines, we explored the
23 kinetics of the cytokine response from peripheral blood cells of pigs vaccinated with a
24 highly efficacious attenuated C-strain vaccine and/or infected with a moderately

1 virulent isolate from the UK CSF outbreak in 2000 (Sandvik et al., 2000; Everett et
2 al., this issue).

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1 2. Materials and methods

2

3 2.1 *Animals and viruses*

4 'High-health' status Large White/Landrace cross male pigs, 8-10 weeks of age
5 were purchased from a local commercial source. Lyophilized live attenuated Riemser
6 C-strain CSFV (AC Reimser Schweinepestvakzine, Reimser Arzneimittel AG,
7 Germany) was provided by the European Commission Vaccine Bank. For inoculation
8 of pigs, the virus was reconstituted in vaccine diluent as described by the
9 manufacturer. A CSFV isolate from the disease outbreak in 2000 (UK2000/7.1
10 Sandvik et al., 2000) was obtained from the Mammalian Virus Investigation Unit
11 (VLA-Weybridge, UK) (Everett et al., this issue). Additional virus stocks of both
12 UK2000/7.1 and C-strain were prepared for *in vitro* stimulation following inoculation
13 of sub-confluent PK-15 cell monolayers and virus titres determined as previously
14 described (Drew, 2008).

15

16 2.2 *Vaccination and infection with CSFV*

17 In two independent experiments, pigs were inoculated intra-nasally with $10^{5.3}$
18 TCID₅₀ (2ml divided equally between each nostril) of the UK2000/7.1 CSFV isolate
19 (n=6) (Expt. 1) or intramuscularly with 100PD₅₀ (1ml; <100 TCID₅₀) of the attenuated
20 C-strain of CSFV (n=4) (Expt. 2). For each experiment, two uninfected control
21 animals were housed in a separate pen from the vaccinated/infected animals. In a third
22 experiment, pigs were vaccinated with the C-strain virus as described above (n=6) and
23 then challenged with the UK2000/7.1 CSFV isolate after 5 days, a group of
24 unvaccinated pigs (n=4) were infected in parallel (Expt. 3). All work was carried out

1 in accordance with UK legislation pertaining to care and use of animals under
2 experimentation.

3

4 *2.3 Clinical, haematological and virological monitoring*

5 All animals were monitored daily from pre-vaccination/infection through to
6 day 15-21 post-vaccination/infection. Animals were euthanized by lethal injection of
7 anaesthetic when clinical scores reached or approached 15. Clinical scores were
8 recorded twice daily by animal technicians and a veterinarian with 10 clinical
9 parameters scored on a scale of 0-3 (Everett et al., this issue). Leukopenia was
10 monitored every 2-3 days by flow cytometry staining. EDTA blood (50µl) was stained
11 with 10µl of FITC conjugated anti-porcine CD45 (AbD Serotec, Kidlington, UK) for
12 15 min at room temperature. Erythrocytes were lysed and leukocytes fixed by addition
13 of 940µl of FACS Lysing Solution (BD Biosciences, Oxford, UK) and incubation for
14 10 min at room temperature. An aliquot of cell suspension was analyzed on a
15 MACSQuant flow cytometer (Miltenyi Biotec, Gergisch Gladbach, Germany) and
16 leukocyte counts obtained by gating on FITC positive events. Viral RNA was detected
17 in EDTA blood samples collected every 2-3 days; total RNA was extracted from
18 140µl of blood using QIAmp Virus RNA Mini Extraction kits as described by the
19 manufacturer (Qiagen, Crawley, UK) and CSFV viral copy numbers assessed by
20 quantitative RT-PCR (Hoffman et al., 2005. Everett *et al.*, this issue).

21

22 *2.4 In vitro stimulation of peripheral blood and lymphoid cells*

23 Heparinised blood was collected from pigs every 2-3 days post
24 vaccination/infection. For Experiment 1, peripheral blood mononuclear cells (PBMC)
25 were prepared by layering heparinised blood diluted 1:1 in HBSS over an equal

1 volume of Biocoll Separating Solution (Autogen Bioclear, Caine, UK) and
2 centrifuged at 2500rpm for 30 min at RT. PBMC were aspirated from the plasma-
3 Biocoll interface and washed three times in HBSS (Invitrogen, Paisley, UK) by
4 centrifugation at 1500 rpm for 5 min at RT. For Experiments 1 and 3, buffy coat cells
5 were prepared using a standard protocol. In brief, blood was centrifuged at 2500rpm
6 for 10 min and visible buffy coat material aspirated. Contaminating erythrocytes were
7 lysed by addition of 10ml of Pharmlyse Buffer (BD Biosciences) and incubation for
8 10 min at RT before being washed three times in HBSS.

9 At the termination of the Expt. 2, necropsies were carried out on the
10 vaccinated pigs and samples of the following lymphoid associated tissues were
11 obtained: bone marrow, spleen, tonsil, mandibular lymph node, inguinal lymph node,
12 mesenteric lymph node and thymus. The tissues were chopped and teased in PBS in a
13 petri-dish to liberate cells and a single cell suspension was obtained by passing the
14 suspension through a 70um cell sieve (BD Biosciences). Contaminating erythrocytes
15 were lysed and cells washed as described for the buffy coat cells.

16 Isolated buffy coat, PBMC and lymphoid tissue cells were finally resuspended
17 in RPMI-1640 medium supplemented with 10% FCS and antibiotics (Invitrogen).
18 Cell densities were determined by analysing 50µl of cell suspension using a
19 MACSQuant flow cytometer and gating on events with typical SSC and FSC
20 properties for buffy coat and PBMC. Cell densities were adjusted to 5×10^6 cells/ml
21 and 100µl or 1ml transferred to wells of a 96 well round-bottom or 24 well plate,
22 respectively. Cells were stimulated by the addition of an equal volume of medium
23 containing C-strain or UK2000/7.1 CSFV at a multiplicity of infection (MOI) of 1 or
24 0.1. To serve as a negative control, a mock inoculum prepared from uninfected PK-15

1 cells was added in an equivalent volume to the viruses. Plates were then incubated for
2 24-72 hours at 37°C in a humidified 5% CO₂ atmosphere.

3

4 *2.5 Measurement of cytokines in culture supernatants*

5 After incubation, the contents of wells were mixed by repeated pipetting and
6 then centrifuged at 1500rpm for 5min and cell free supernatants removed and
7 immediately stored at -80°C until analysed. IFN- γ was measured in the culture
8 supernatants diluted 1:2 in Standard Diluent Buffer using a swine IFN- γ ELISA
9 according to the manufacturers instructions (Biosource, Invitrogen) and absorbance at
10 440nm read using a FLUOstar OPTIMA microplate reader (BMG Labtech,
11 Aylesbury, UK). The presence of other cytokines in culture supernatants was analysed
12 using the Pierce Searchlight Porcine Cytokine Array (Perbio Science UK Ltd.
13 Cramlington, UK), which simultaneously measures porcine IL-1 β , IL-2, IL-4, IL-6,
14 IL-8, IL-10, IL-12p70, IFN- γ and TNF- α . Equal volumes of culture supernatants from
15 each group of vaccinated or infected pigs were pooled and diluted 1:2 in sample
16 diluent before addition to the plates. Plates were incubated and developed as described
17 by the manufacturer and analysed using the SearchLight CCD Imaging and Analysis
18 System (Perbio Science).

19

20 *2.6 Statistical analysis*

21 ANOVA was used for the analysis of fixed effects on different traits using
22 GraphPad Prism 5 (Prism 5 for Windows, Version 5.01, GraphPad Software, Inc. La
23 Jolla, USA).

24

1 3. Results

2 3.1 Outcome of vaccination and infection with CSFV viruses

3 Following infection with the CSFV isolate from a UK outbreak in 2000
4 (UK2000/7.1) (Expt. 1) or vaccination of pigs with the C-strain virus (Expt. 2),
5 clinical signs were measured using a clinical score system (Fig. 1A). On infection
6 with UK2000/7.1, pigs displayed signs of CSF from 7-14 days post-infection and
7 progressed to reach clinical scores between 10 and 15 by 13-19 days post-infection. In
8 contrast, no clinical signs were observed in any of the pigs vaccinated with the C-
9 strain virus. The development of leukopenia was monitored by quantitative flow
10 cytometry with mean leukocyte counts shown in Fig. 1B. Infection with the
11 UK2000/7.1 virus produced a leukopenia; leukocyte counts dropped below pre-
12 infection levels after 4-8 days post-infection reaching a relatively steady state of 5-
13 10,000 leukocyte/ μ l for the remaining period of observation. Leukocyte counts were
14 unaffected by immunisation with the C-strain virus. Viral RNA was first detected in
15 the blood of UK2000/7.1 infected pigs at 2-4 days post-infection and increased
16 exponentially, plateauing around day 11 post- infection with viral loads of above 10^6
17 virus copies/ μ l blood (Fig. 1C). No viral RNA could be detected in the blood of C-
18 strain vaccinated animals, but was detected in some tonsil and retropharyngeal lymph
19 node biopsies collected post-mortem (days 23-24 post-vaccination) (data not shown).
20 Challenge of C-strain vaccinated pigs 5 days post-vaccination (Expt. 3) did not result
21 in the development of clinical signs and no virus could be detected in peripheral
22 blood. In contrast, all challenge control animals developed severe clinical CSF
23 requiring euthanasia and had high levels of viraemia (data not shown)

24

25

1 3.2 Characterisation of virus specific IFN- γ responses by *in vitro* stimulation of PBL
2 from pigs vaccinated and challenged with CSFV viruses

3 Following vaccination of pigs with the C-strain virus (Expt. 2), virus specific
4 IFN- γ responses of peripheral blood leukocyte (PBL) were measured by ELISA after
5 *in vitro* stimulation and compared to responses from unvaccinated pigs (Fig. 2A).
6 IFN- γ production in response to stimulation with C-strain virus was first detected in
7 vaccinates 9 days post-vaccination ($p < 0.001$). This response declined but appeared
8 sustained over the period of observation (18 days-post vaccination). No significant
9 virus-specific IFN- γ responses were detected in non-vaccinated animals (Fig 2A).
10 PBL IFN- γ responses of pigs vaccinated 5 days previously were similarly determined
11 after challenge with the UK2000/7.1 strain and compared to responses from
12 unvaccinated pigs (Fig 2B) (Expt. 3). IFN- γ responses following *in vitro* stimulation
13 with C-strain virus peaked in vaccinated pigs 6 days post-challenge (11 days post-
14 vaccination) and then showed similar kinetics to that observed in the previous
15 vaccination experiment (Fig. 2B) ($p < 0.001$). In contrast, unvaccinated pigs failed to
16 display virus-specific IFN- γ responses following UK2000/7.1 infection. (Fig. 2B).
17 PBL's isolated 14 days post-challenge were stimulated with either C-strain or
18 UK2000/7.1 viruses. Unvaccinated animals failed to mount an IFN- γ response to
19 either virus while the vaccinated animals showed a weak response to UK2000/7.1 at
20 an MOI=0.1 ($p < 0.1$), which was similar to the response to the equivalent dose of C-
21 strain and less than the response to the C-strain virus at an MOI of 1 ($p < 0.01$) (Fig.
22 2C). The presence of C-strain viral RNA in the lymphoid tissues of the
23 nasopharyngeal region and absence in blood observed in Expt. 2 led us to examine the
24 distribution of CSFV-specific IFN- γ secreting cells in lymphoid organs well described
25 as key sites for viral expansion and/or persistence. From day 23-25 post-vaccination,

1 lymphoid cells from bone marrow, spleen, tonsil, blood, thymus and mandibular,
2 inguinal and mesenteric lymph nodes were stimulated with C-strain CSFV and IFN- γ
3 responses measured (Fig 2D). The most significant responses were obtained from
4 blood ($p < 0.05$) and spleen cell cultures ($p < 0.001$). The lack of response by cells from
5 the tonsil and lymph nodes draining the oronasal mucosae, which are likely to be the
6 first sites of replication for an incoming challenge virus, was surprising and may be
7 because the vaccine was administered by intramuscular inoculation rather than by the
8 intranasal route.

9
10

11 *3.3 Assessment of additional cytokine responses by in vitro stimulation of PBL from* 12 *pigs vaccinated or challenged with CSFV viruses*

13 Further analyses of other cytokine responses revealed highly divergent
14 responses between vaccinated and infected animals (Fig. 3). Stimulation of PBL from
15 vaccinates with the C-strain virus (Expt. 2) induced a cytokine response that was
16 dominated by IFN- γ (Fig. 3A & B). IL-8 production was also detected, however this
17 response appeared non-specific as it was also observed in all cultures analysed (Fig.
18 3B-D). Of the other cytokines measured, there was a small increase in the secretion of
19 IL-10 and TNF- α from day 12 post-vaccination, which appeared to be virus specific.
20 Analysis of PBMC culture supernatants from UK2000/7.1 infected pigs (Expt. 1)
21 showed that UK2000/7.1 virus stimulation resulted in TNF- α , IL-6 and IL-1 β being
22 detected from day 8 post-infection (Fig. 3C). However, these cytokine responses
23 were also observed, albeit at a slightly lower magnitude, in supernatants from mock
24 infected cultures (Fig. 3D). These responses followed the onset of viraemia as

1 detected by real-time qRT-PCR but were coincident with the detection of CSFV
2 infected cells in peripheral blood as assessed by flow cytometry (data not shown).

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1 4. Discussion

2 Experimental infection of pigs with UK2000/7.1 demonstrated this isolate to
3 be of moderate virulence, causing high viraemia and clinical symptoms, and appears
4 to be representative of CSFV strains causing recent outbreaks (Floegel-Niesmann et
5 al., 2003, Everett et al., this issue). Vaccination of pigs with the C-strain virus induced
6 no clinical signs and provided solid protection against the UK2000/7.1 virus after only
7 5 days. Thus, these viruses offered excellent model systems with which to analyse
8 cytokine responses with a view to attributing them to protection or pathology. As had
9 been previously described (Suradhat et al., 2001; Suradhat et al., 2007), C-strain
10 vaccination induced significant IFN- γ responses from peripheral blood leukocytes
11 following *in vitro* stimulation with CSFV, which peaked around day 9 and remained
12 elevated until the termination of the experiment on day 21. Challenge of vaccinated
13 animals induced a strong IFN- γ response and these animals completely controlled the
14 challenge infection. In contrast, cells from unvaccinated animals infected with the
15 UK2000/7.1 virus failed to mount a detectable IFN- γ response. It appears unlikely
16 that this was strain related since no response could be detected against either the
17 vaccine or challenge virus while cells from vaccinates responded to both. It therefore
18 also appears that there are significant cross-reactive T cell epitopes between the 2
19 viruses of different genotypes.

20 The absence of an IFN- γ response following infection conflicts with the data
21 of Suradhat et al. (2001) that showed an IFN- γ response from animals infected with a
22 virulent isolate. However, the present data supports the hypothesis that virus specific
23 IFN- γ responses provide a marker of T cell-mediated mechanisms that control CSFV.
24 Understanding the mechanisms by which virulent CSFV prevents the induction of this
25 response may be of value in the efforts to develop new vaccines that aim to trigger

1 protective cellular mechanisms. Both earlier studies and the present study have shown
2 that CSFV infection results in the rapid onset of leucopenia and it has been shown
3 that this depletion affects T lymphocytes (Graham, S.P., unpublished data, Pauly et
4 al., 1998, Summerfield et al., 2001). The inability to prime IFN- γ secreting virus-
5 specific T cells may be due to the depletion of cells prior to their activation or the
6 recently described phenomenon of apoptotic cell-induced dendritic cell mediated
7 suppression (Williams et al., 2008). Alternatively, the failure to prime these responses
8 may stem from the dysregulation of cytokine responses from infected dendritic cell
9 populations. It has been shown that plasmacytoid dendritic cells over-express
10 cytokines such as TNF- α and IFN- α in response to CSFV infection (Jamin et al.,
11 2008) whereas the Type I IFN response from myeloid derived cells is impaired
12 (Carrasco et al., 2004).

13 The present study showed evidence of dysregulated cytokine responses in
14 infected animals, with secretion of pro-inflammatory cytokines TNF- α , IL-1 β and IL-
15 6 from stimulated and unstimulated cultures following the onset of viraemia and
16 coincident with the appearance of clinical signs. It is unclear whether this pro-
17 inflammatory response is largely T cell independent and simply reflects a pro-
18 inflammatory cascade initiated *in vivo* following the dissemination of replicating
19 virus, as has been suggested from investigations during the early stages of CSFV
20 infection *in vivo* (Jamin et al., 2008). However, the pathology associated with acute
21 CSFV infection that is attributed to a pro-inflammatory cytokine storm is often absent
22 from animals born persistently infected due to *in utero* infection prior to the onset of
23 immunocompetence (Van Oirschot, 1979). It has previously been shown *in vitro* that
24 CSFV infection of myeloid dendritic cells does not affect their T cell stimulatory
25 capacity (Carrasco et al., 2004). It is therefore tempting to speculate that this

1 proinflammatory response has an adaptive component and that the delayed kinetics of
2 the response reflects the time required for induction of an adaptive response. IL-17-
3 producing CD4⁺ T cells (Th-17 cells) have recently been identified as a unique subset
4 of Th cells that develop along a pathway that is distinct from the Th1- and Th2-cells,
5 with both IFN- γ and IL-4 negatively regulating the generation of these Th-17 cells
6 (Bettelli et al., 2008). Th-17 cells promote inflammatory responses and are implicated
7 in a variety of pathological conditions. It could be speculated that the early cytokine
8 responses induced following CFSV infection favours priming of a Th17 response
9 leading to activation of granulocytes and macrophages and the resultant tissue
10 inflammation observed in clinical CSF.

11 Utilising a commercially available live attenuated CSFV vaccine and a
12 moderately virulent CSFV isolate, the present study established robust models to
13 study vaccine mediated protection and uncontrolled viral infection leading to clinical
14 disease. These models have been exploited to demonstrate that attenuated and virulent
15 viruses induce dramatically different cytokine responses which likely play important
16 roles in dictating whether protective immunity or pathology develops. With a view to
17 enhancing our understanding of the contribution of these responses, current studies
18 are focussing on the identification of the cells secreting these cytokines and extending
19 the panel of cytokines analysed to include cytokines such as IFN- α , TGF- β , IL-17 and
20 IL-22, which may play an important role in the induction of the responses measured in
21 the present study.

22

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1

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12

13 Conflict of interest statement

14 The authors declare no conflict of interests.

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1 **Figure Legends**

2

3 Fig. 1. Outcome of vaccination with a live attenuated C-strain CSFV or infection with
4 a recent CSFV isolate. Following infection with a CSFV isolate from a UK outbreak
5 in 2000 (UK2000/7.1) (n=6) (Expt.1) or vaccination of pigs with a C-strain virus
6 (n=4) (Expt.2), **(A)** clinical signs were measured using a clinical scoring system, **(B)**
7 leukocyte numbers were measured by quantitative flow cytometry and **(C)** viral loads
8 in peripheral blood determined by real-time qRT-PCR. Results are expressed as the
9 mean data for each group of pigs and error bars represent SEM.

10

11 Fig. 2. Characterisation of virus specific IFN- γ responses *by in vitro stimulation of*
12 *PBL from pigs* following vaccination with a live attenuated C-strain CSFV and
13 challenge with a recent CSFV isolate. **(A)** Following vaccination of pigs with a C-
14 strain CSFV (n=4) (Expt. 2), peripheral blood leukocyte (PBL) IFN- γ responses were
15 measured by ELISA following *in vitro* stimulation with C-strain virus (CSFV) or
16 mock inocula (Mock) and compared to responses from unvaccinated pigs. **(B)** PBL
17 IFN- γ responses of pigs, vaccinated 5 days previously (n=6), were similarly measured
18 after challenge with the UK2000/7.1 CSFV isolate and compared to responses from
19 unvaccinated pigs (n=4) (Expt. 3). **(C)** Fourteen days after UK2000/7.1 challenge,
20 PBL IFN- γ responses of vaccinated and unvaccinated pigs were measured against
21 mock, C-strain and UK2000/7.1 viruses (Expt. 3). **(D)** At the termination of Expt. 2,
22 lymphoid cells were isolated from: bone marrow, spleen, tonsil, blood, mandibular
23 (Mand LN), inguinal (Ing LN), and mesenteric lymph nodes (MLN) and thymus and
24 IFN- γ responses were measured following *in vitro* stimulation with mock or C-strain

1 CSFV. All results are expressed as the mean data for each group of pigs and error bars
2 represent SEM.

3

4 Fig. 3. Analyses of peripheral blood cell cytokine responses *by in vitro stimulation of*
5 *PBL from pigs* following vaccination with a live attenuated C-strain CSFV or
6 infection with a recent CSFV isolate. PBL and PBMC were isolated following
7 vaccination of pigs with the C-strain virus (n=4) (Expt. 2) or infection with the
8 UK2000/7.1 CSFV isolate (n=6) (Expt. 1), respectively. Cells from vaccinated pigs
9 were stimulated with (A) the C-strain virus or (B) mock stimulated, whereas cells
10 from infected pigs were stimulated with (C) the UK2000/7.1 virus or (D) mock
11 stimulated. Culture supernatants for each group were pooled on each occasion and
12 cytokine responses analysed using a chemiluminescent cytokine array.

13

14

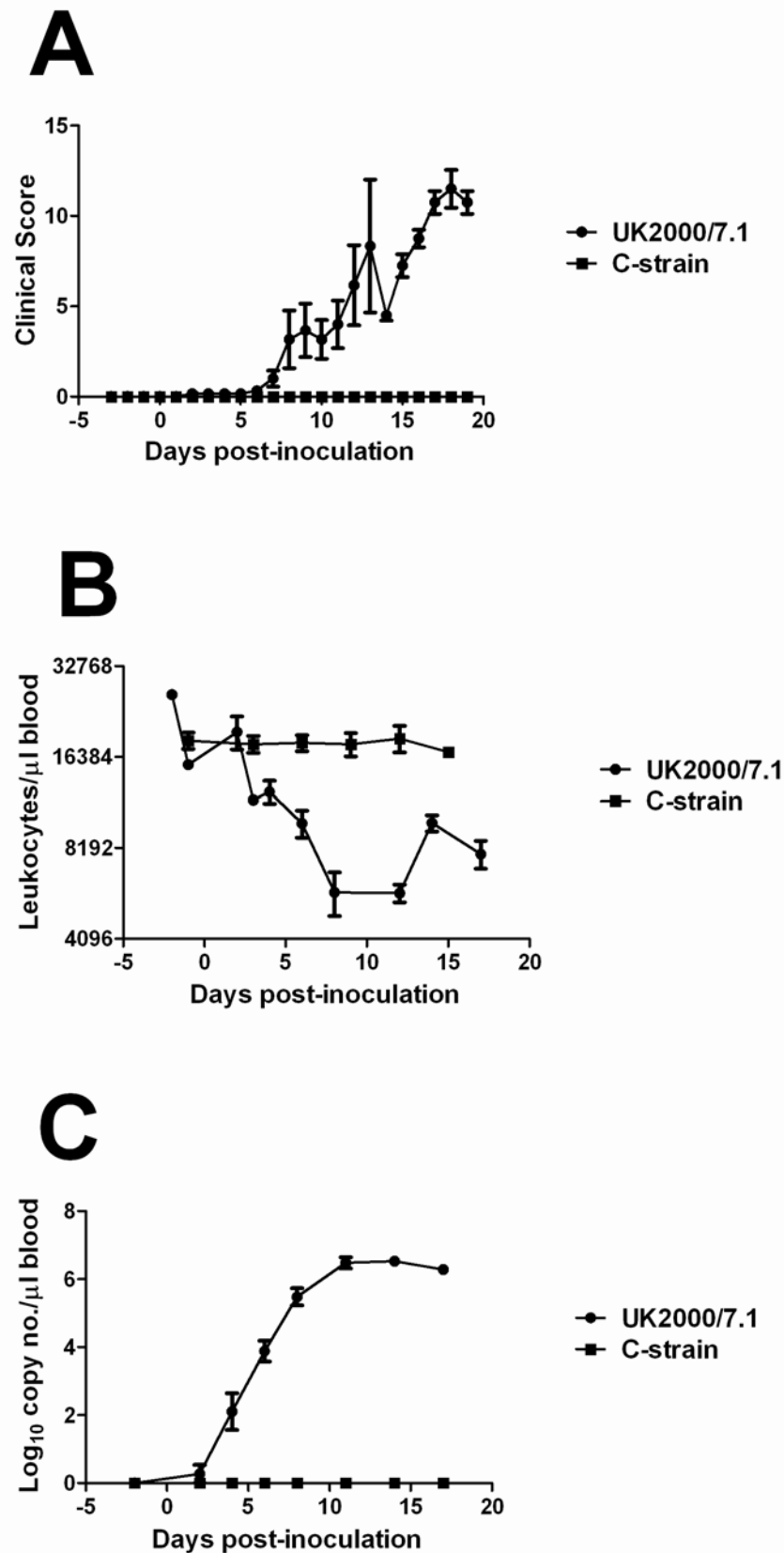
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1 FIG. 1

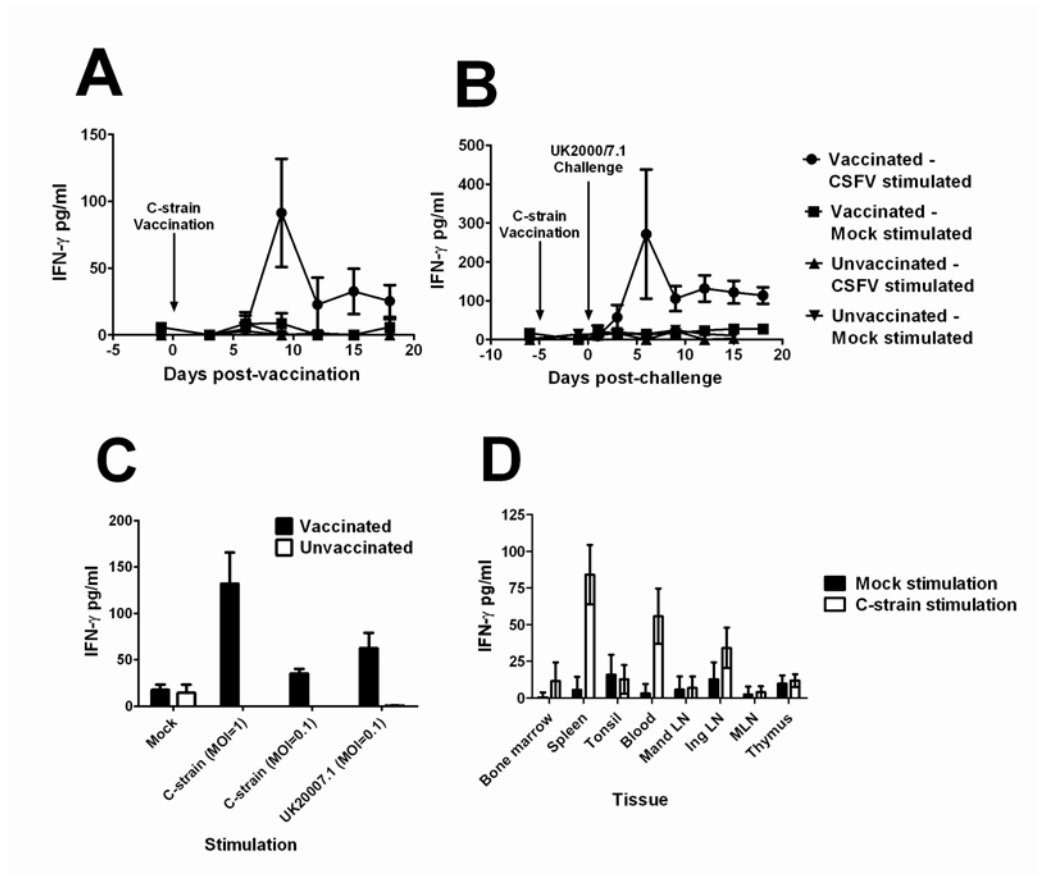
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1 FIG. 2

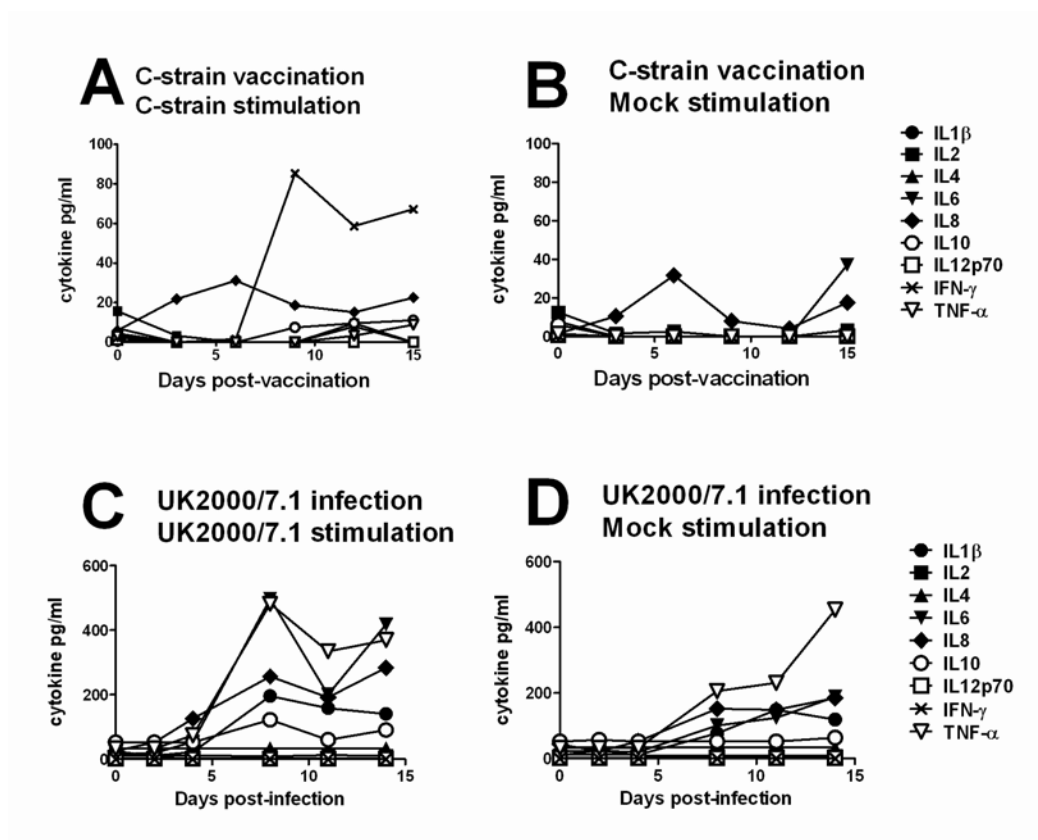


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1 FIG. 3



2

ACCEPTED MANUSCRIPT