Assessment of the Phenotype and Functionality of Porcine CD8 T Cell Responses following Vaccination with Live Attenuated Classical Swine Fever Virus (CSFV) and Virulent CSFV Challenge

Giulia Franzoni, Nitin V. Kurkure, Daniel S. Edgar, Helen E. Everett, Wilhelm Gerner, Kikki B. Bodman-Smith, Helen R. Crooke, Simon P. Graham

Virology Department, Animal Health and Veterinary Laboratories Agency, Addlestone, United Kingdom; Department of Microbial and Cellular Sciences, University of Surrey, Guildford, United Kingdom; Nagpur Veterinary College, Maharashatra Animal and Fishery Sciences University, Nagpur, India; Department of Pathobiology, Institute of Immunology, University of Veterinary Medicine Vienna, Vienna, Austria

Vaccination with live attenuated classical swine fever virus (CSFV) induces solid protection after only 5 days, which has been associated with virus-specific T cell gamma interferon (IFN-γ) responses. In this study, we employed flow cytometry to characterize T cell responses following vaccination and subsequent challenge infections with virulent CSFV. The CD3⁺ CD4⁻ CD8⁺ T cell population was the first and major source of CSFV-specific IFN-γ. A proportion of these cells showed evidence for cytotoxicity, as evidenced by CD107a mobilization, and coexpressed tumor necrosis factor alpha (TNF-α). To assess the durability and recall of these responses, a second experiment was conducted where vaccinated animals were challenged with virulent CSFV after 5 days and again after a further 28 days. While virus-specific CD4 T cell (CD3⁺ CD4⁺ CD8α⁻) responses were detected, the dominant response was again from the CD8⁺ T cell population, with the highest numbers of these cells being detected 14 and 7 days after the primary and secondary challenges, respectively. These CD8 T cells were further characterized as CD44hi CD62L⁻ and expressed variable levels of CD25 and CD27, indicative of a mixed effector and effector memory phenotype. The majority of virus-specific IFN-γ⁺ CD8 T cells isolated at the peaks of the response after each challenge displayed CD107a on their surface, and subpopulations that coexpressed TNF-α and interleukin 2 (IL-2) were identified. While it is hoped that these data will aid the rational design and/or evaluation of next-generation marker CSFV vaccines, the novel flow cytometric panels developed should also be of value in the study of porcine T cell responses to other pathogens/vaccines.

Classical swine fever (CSF) is one of the most important viral infectious diseases of domestic pigs and wild boars. CSF is caused by the classical swine fever virus (CSFV), a highly contagious, small, enveloped, single-stranded RNA virus belonging to the family Flaviviridae (1). Since 1990, outbreaks of CSF in the European Union have been controlled through a stamping-out slaughter policy, epidemiological and virological investigations, and movement restriction for pigs and pig products (1, 2). Owing to the economic losses caused by the stamping-out policy, there is pressure to develop alternative strategies to control CSF outbreaks and to minimize the need for mass culling (2).

Live attenuated CSF vaccines, such as C-strain viruses, provide a rapid onset of complete protection but pose problems in discriminating infected from vaccinated animals. Many studies have aimed to develop marker subunit vaccines, but they frequently fail to show an appropriate level of efficacy for use under emergency outbreak conditions (1, 3, 4). An understanding of the immunological basis of rapid protection afforded by live attenuated C-strain vaccine would aid the development of the next generation of marker CSFV vaccines, both through the identification of vaccine candidate antigens and through informing the selection of appropriate delivery systems/adjuvants to trigger protective responses. While the immunological effector mechanisms are not well defined, C-strain-induced protection may precede the appearance of neutralizing antibody but not gamma interferon (IFN-γ)-secreting cells in peripheral blood, suggesting that cellular immunity is responsible (5). Moreover, it has recently been shown that there is a close temporal correlation between the induction of CSFV-specific T cell IFN-γ responses and rapid protection induced by a C-strain vaccine (6). In addition to the secretion of IFN-γ, which has been shown to exert direct antiviral effects on CSFV in vitro (7), vaccine-induced CSFV-specific T cells have also been shown to have the capacity to lyse infected cells with specificities mapped to the structural protein E2 and the nonstructural protein NS3 (8–10). Flow cytometric studies have identified both CD4 and CD8 T cells as the cellular source of CSFV-specific IFN-γ, with the latter coexpressing the cytolytic molecule perforin (7, 11). Further evidence for a protective role for T cells stems from recent subunit vaccine studies which have shown that the protective capacity of a CSFV E2 DNA vaccine was associated with T cell IFN-γ rather than neutralizing antibody responses (12) and that inclusion of a defined NS3 T cell epitope improved the immunogenicity and the degree of protection afforded by a peptide-based CSF vaccine (13).

The goal of an efficient vaccine is to generate memory CD4 and/or CD8 T cells capable of recognizing and rapidly expanding to combat infection (14). While challenging, it has been proposed...
that the efficacy of T-cell-based vaccines could be improved by manipulating the generation and maintenance of distinct memory T cell subsets which in recent years have been identified in humans and mice through the application of multiparameter flow cytometry (15). The two main categories of memory T cells are classified as effector memory (TEM) and central memory (TCM) (16) and can be distinguished with the lymph node homing markers CD62L and CCR7, with CD62L⁻CCR7⁻ and CD62L⁻CCR7⁻ cells representing the TCM and TEM, respectively (17). The adhesion molecules CD44 and CD11a are further used to differentiate naive from effector/memory CD8 T cells (17). Moreover, the simultaneous detection of cytokines (e.g., IFN-γ, tumor necrosis factor alpha [TNF-α], interleukin 2 [IL-2]) and other effector molecules/markers (e.g., CD107a mobilization) has enabled assessment of T cell polyfunctionality and has been attributed to cells capable of exerting multiple functions with greater protective capacity (18). Recent studies using such approaches have suggested that effector memory and/or polyfunctional CD8 T cells play an important role in immunity to other viruses belonging to the family Flaviviridae, such as hepatitis C virus (HCV) (19–21) and yellow fever virus (YFV) (22). To provide an improved understanding of the role that T cells may play in immunity to CSFV, we deployed novel flow cytometric antibody panels to quantitatively and qualitatively assess the T cell response induced following CSFV-strain vaccination and subsequent CSFV challenge infection. We additionally assessed whether the T cell responses of vaccinated and challenged animals were sustained and/or readily recalled upon subsequent rechallenge infection and the impact that this rechallenge had on their phenotype/function.

MATERIALS AND METHODS

Ethics statement. All animal work was approved by the Animal Health and Veterinary Laboratories Agency Ethics Committee, and all procedures were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 under project license permit numbers PPL 70/6559 and 70/7403. To minimize animal suffering, careful preparation of clinical score sheets and regular observation were conducted which informed euthanasia decisions based on predefined humane endpoints. However, none of the animals experienced clinical signs necessitating euthanasia. All animals were euthanized on predetermined days by intramuscular (i.m.) administration of ketamine-xylazine (Rompun) sedative followed by intravenous administration of 20% sodium pentobarbital solution.

Viruses. A commercially available live attenuated C-strain CSFV (AC Riemser Schweinepestvakzine; Riemser Arzneimittel AG, Riems, Germany) was used for vaccinations. This CSFV C-strain vaccine was attenuated by multiple passages through rabbits followed by adaptation to culture in porcine kidney cells (23) and had previously been shown able to protect pigs against CSFV challenge after only 5 days (6). For challenge infections, the CSFV Brescia strain (kindly provided by Alexandra Meindl-Böhmer, University of Veterinary Medicine, Hannover, Germany) was selected as it had been shown in recent studies to be a highly virulent strain (24–26). The virulent CSFV AfIort 187 strain (AHLVCA Reference Laboratory Virus Archive, Addlestone, United Kingdom) was selected for use in in vitro restimulation assays, since this virus is of the same subgenotype (1.1) as C-strain and Brescia and, importantly, readily grows to high titers in PK15 cells, enabling its use in restimulation assays at a multiplicity of infection (MOI) of 1, which we had previously shown to induce a better recall of T cell responses than lower MOIs (7). All three viruses were propagated in vitro by inoculation of subconfluent PK15 cell monolayers. PK15 cells were maintained in Eagle’s minimum essential medium (E-MEM) (Invitrogen, Paisley, United Kingdom) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin (Invitrogen), and 10% fetal bovine serum (FBS) (Autogen Bioclear, Calne, United Kingdom). After 4 days, the supernatant was collected and pooled with a freeze-thawed cell lysate. The resultant pool was clarified by centrifugation at 524 × g for 10 min, aliquoted, and stored at −80°C. Virus titers were obtained by serial dilution of the virus suspension on PK15 cells followed by immunoperoxidase staining to identify infection rates as described previously (27). Mock virus supernatant was prepared in an identical manner from uninfected PK15 cells.

Vaccination and challenge of pigs with CSFV. In the first experiment (experiment 1), 42 Large White/Landrace crossbreed pigs of 8 to 10 weeks of age were randomly assigned to four groups. Numbers of animals per group, the treatments administered, and the days that animals were sampled and responses were analyzed are summarized in Table 1. Five days prior to challenge, animals in groups 1 (n = 6) and 2 (n = 12) were inoculated intranasally (i.n.) with 3 × 10⁵ 50% tissue culture infective doses (TCID₅₀) of C-strain CSFV (1 ml divided equally between each nostril) and administered using a mucosal atomization device (MAD-300; Wolfe Tory Medical, USA), those in group 3 (n = 12) were inoculated with 2 ml of reconstituted vaccine into the brachiocephalic muscle (as recommended by the manufacturer, Riemser Arzneimittel AG), and those in group 4 (n = 12) were intranasally inoculated with 1 ml of mock virus supernatant as described for groups 1 and 2. On day 0 postchallenge, groups 2 to 4 were inoculated intranasally with 10⁵ TCID₅₀ of CSFV Brescia strain as described above and group 1 received a similar inoculation of mock virus supernatant. On days −2 and 0 postchallenge, three animals were euthanized from groups 2 to 4, and on days 3 and 5 postchallenge, three animals were euthanized from all four groups and blood samples were collected for analysis of CSFV-specific T cell responses. A second experiment (experiment 2) utilized 11 Large White/Landrace pigs, 6 months of age. Eight animals were vaccinated at day −5 postchallenge by intramuscular inoculation of 10⁵ TCID₅₀ of C-strain CSFV and challenged at day 0 and day 28 by intranasal inoculation of 10⁵ and 10⁶ TCID₅₀ of CSFV Brescia, respec-

### Table 1 Experimental design of first vaccination/challenge experiment

| Expt group | Procedure(s) at day postchallenge: | PM/bleed (n = 3) | PM/bleed (n = 3) |
|------------|-------------------------------|-----------------|-----------------|
| 1, vaccine (i.n.) only (n = 6) | Vaccination with C-strain, 10⁵ TCID₅₀ i.n. | PM/bleed (n = 3) | PM/bleed (n = 3) |
| 2, vaccine (i.n.) plus challenge (n = 12) | Vaccination with C-strain, 10⁵ TCID₅₀ i.n. | PM/bleed (n = 3) | PM/bleed (n = 3) |
| 3, vaccine (i.m.) plus challenge (n = 12) | Vaccination with C-strain, 10⁵ TCID₅₀ i.m. | PM/bleed (n = 3) | PM/bleed (n = 3) |
| 4, challenge only (n = 12) | Mock vaccination i.n. | PM/bleed (n = 3) | PM/bleed (n = 3) |
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were inoculated with 1 ml (100 50% protective doses [PD50]) of the re-

Landrace cross male pigs, 8 to 10 weeks of age, were utilized. Four pigs

experiment (experiment 3) was performed, and the experimental design

inoculations of mock virus supernatant on each occasion. A third animal

tively, as described above. Three negative-control animals received similar

inoculations of mock virus supernatant into the brachiocephalic muscle (as recom-

mended by the manufacturer, Riemser Arzneimittel AG), and two uncon-

constituted C-strain vaccine into the brachiocephalic muscle (as recom-

mended by the manufacturer, Riemser Arzneimittel AG), and two unvac-

vaccinated pigs were used as negative controls and housed in a separate pen.

Peripheral blood leukocytes were enumerated every 3 or 7 days during

and continued until the termination of the experiment (day 5 postchal-

mometer readings and recorded once daily. For experiment 1, tempera-

attened and scored as 0 (normal), 1 (slightly altered), 2 (distinct clinical

spected twice daily (a.m. and p.m.), and 9 parameters relevant for indica-

uation kit, according to the manufacturer's protocols (Qiagen, Crawley,

United Kingdom). CSFV viral copy numbers were assessed by quantita-

tion of CSF (liveliness, body tension, body shape, breathing, walking, skin,

placed twice daily, and temperatures were monitored by rectal ther-

mometer readings and recorded once daily. For experiment 1, tempera-

ature (RT) in the dark (details of all the antibodies used in this work are

isothiocyanate (FITC) monoclonal antibody (MAb) (0.1 mg/ml, K252-

BioLegend MAb11 0.5 IgG1 Pacific Blue 1/10

TNF-α BioLegend CC302 1.0 IgG1 Unconjugated 1/10

IL-2 Invitrogen A150D 3F1 1.0 IgG2a Unconjugated 1/4

Mouse IgG1 BD Pharmingen A85-1 0.2 IgG2b eFluor450 1/16

IgG1 isotype control AbD Serotec NA 0.05 IgG1 APC 1/20

a MAb dilution following Zenon labeling.

b Isotype control used neat to control CD107a staining and 1/10 for IFN-γ staining.

c NA, not applicable.

**TABLE 2** Monoclonal antibodies for flow cytometric analysis of porcine T cell responses

| MAb          | Source       | Clone        | Conc (mg/ml) | Isotype | Labeling   | Working dilution |
|--------------|--------------|--------------|--------------|---------|------------|-----------------|
| CD45         | AbD Serotec  | K252-1E4     | 0.1          | IgG1    | FITC       | Neat            |
| CD107a       | AbD Serotec  | 4E9/11       | 0.05         | IgG1    | Alexa Fluor 647 | Neat            |
| CD2α         | BD Pharmingen| 76-2-11      | 0.2          | IgG2a   | PE         | 1/10            |
| CD4          | BD Pharmingen| 74-12-4      | 0.2          | IgG2b   | PerCP-Cy5.5 | 1/10            |
| CD8α         | Southern Biotech | BK23-6E6   | 0.5          | IgG1    | Unconjugated | 1/2             |
| γδ-TcR1-5N4  | VMRD         | PGBL22A      | 1.0          | IgG1    | Unconjugated | 1/2             |
| CD25         | AbD Serotec  | K231.3B2     | Not determined | IgG1    | Unconjugated | 1/5             |
| CD27         | In-house     | b30c7        | Not determined | IgG1    | Unconjugated | 1/4             |
| CD44         | eBioscience  | IM7          | 0.2          | IgG2b   | eFluor450   | 1/16            |
| CD62L        | AbD Serotec  | CC32         | 1.0          | IgG1    | Unconjugated | 1/100           |
| IFN-γ        | AbD Serotec  | CC302        | 0.05         | IgG1    | FITC or Alexa Fluor 647 | 1/10            |
| TNF-α        | BioLegend    | MAb11        | 0.5          | IgG1    | Pacific Blue | 1/10            |
| IL-2         | Invitrogen   | A150D 3F1    | 1.0          | IgG2a   | Unconjugated | 1/4             |
| Mouse IgG1   | BD Pharmingen| A85-1        | 0.2          | Rat IgG1 | APC        | 1/20            |
| IgG1 isotype control | AbD Serotec  | NA          | 0.05         | IgG1    | FITC or Alexa Fluor 647 | Neat, 1/10       |

Purification and cryopreservation of PBMC. Heparinized venous blood was collected on days −5, −2, 0, 2, and 5 postchallenge during experiment 1; on days −5 and 0 and every 7 days until day 56 postchall-

ence during experiment 2; and on days 0, 3, 6, 9, 12, 15, and 18 postvac-

ination during experiment 3. Peripheral blood mononuclear cells (PBMC) were prepared by diluting 20 ml of blood in 10 ml of phosphate-

buffered saline (PBS) (Invitrogen), layering it over 20 ml of Ficoll-

Paque Plus; GE Healthcare, Uppsala, Sweden), and centrifuging it at 1,455 × g for 30 min at room temperature (RT), without braking, in a

to a precooled (4°C) Cryo 1°C freezing container (Nalgene; Fisher

ue, United Kingdom). PBMC were aspirated from the plasma-Ficoll interface and washed three times in PBS by centrifugation at 930 × g for 5 min at 4°C. PBMC were resuspended in RPMI 1640 medium supplemented with 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin (cRPMI), and cell density was determined by analyzing 50 μl of cell suspension using a volumetric flow cytometer (Miltenyi Biotech) and gating on events with typical forward scatter (FSC) and side scatter (SSC) for PBMC. Cells were cryopreserved for later analyses. PBMC were adjusted to a density of 1 × 10^7 to 2 × 10^7 cells/ml, resuspended in cold (4°C) 10% dimethyl sulfoxide (DMSO) (Sigma, Poole, United Kingdom) in FBS, and transferred to precooled (4°C) labeled cryotubes. These tubes were immediately trans-

ferred to a precooled (4°C) Cryo 1°C freezing container (Nalgene; Fisher

Scientific, Loughborough, United Kingdom) containing 250 ml of 100% isopropyl alcohol (4°C), which was placed in a −80°C freezer for a mini-

mum of 4 h to a maximum of 24 h. Cryotubes were then transferred to a liquid nitrogen storage container.

Stimulation of peripheral blood mononuclear cells. To resuscitate cryopreserved cells, cryovials were rapidly thawed in a 37°C water bath and cells were transferred to tubes containing 10 ml of prewarmed (37°C) cRPMI. Cells were washed by centrifugation, 930 × g for 5 min at RT, and resuspended in fresh warm cRPMI. Cell densities were calculated using flow cytometry as described above and adjusted to 1 × 10^7 cells/ml, and 100 μl was transferred to wells of a 96-well round-bottom microtiter plate (Costar; Fisher Scientific). PBMC were stimulated with 100 μl of CSFV strain Alfort 187 (29).

**TABLE 2** Monoclonal antibodies for flow cytometric analysis of porcine T cell responses
Characterization of CSFV-Specific CD8 T Cell Responses

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virus/mock supernatants for 6 h before the addition of CD107a-Alexa Fluor 647 or IgG1 isotype control-Alexa Fluor 647 MAb (both from AbD Serotec; 10 μl/well) (Table 2), brefeldin A (as above), and monensin (Golgi Stop; BD Bioscience; 0.2 μl/well) and then cultured for a further 14 h.

Multiparameter cytometric analysis of peripheral blood mononuclear cells. Cells were harvested and washed in Dulbecco’s PBS without Mg2+ and Ca2+ (Invitrogen). To assess viability, cells were stained with Trypan Blue and stained with CD45-FITC or -Alexa Fluor 647 (Innogen) (76-2-11; BD Pharmingen), CD4-peridinin chlorophyll protein (PerCP)-Cy5.5 (74-12-4; BD Pharmingen), and CD3 (BB238E6; Southern Biotech, Cambridge Bioscience, Cambridge, United Kingdom). CD3 MAbs was labeled using the Zenon Alexa Fluor 647 IgG1 labeling kit, according to the manufacturer’s instructions (Invitrogen). To directly assess the contribution of γδ T cells to CSFV-specific IFN-γ responses, CSFV-mock-virus-stimulated PBMC from three representative C-strain-vaccinated/Brescia-challenged pigs from each of experiments 1 (day 5 postchallenge) and 2 (day 21 postchallenge) were stained with CD8α-phycocerythrin (PE) (76-2-11; BD Pharmingen), CD4-peridinin chlorophyll protein (PerCP)-Cy5.5 (74-12-4; BD Pharmingen), and CD3 (BB238E6; Southern Biotech, Cambridge Bioscience, Cambridge, United Kingdom). CD3 MAbs was labeled using the Zenon Alexa Fluor 647 IgG1 labeling kit.

Since results showed that CSFV-specific IFN-γ T cells were localized solely in CD3+ γδ-TcR1− CD4+ CD8αlow and CD3+ γδ-TcR1− CD4− CD8αhigh T cell populations (see Fig. 2 and also Fig. S1 in the supplemental material), subsequent stainings were conducted with the omission of CD3 MAbs and used only CD4 and CD8 MAbs to discriminate these two T cell populations. The following MAbs were used to assess surface expression of activation/memory markers: CD25 (K231.3B2; AbD Serotec), CD27 (b30c7 [31]), CD44-eFluor450 (IM7; eBioscience, Hatfield, United Kingdom), and CD62L (CC32; AbD Serotec). Since cryopreservation of human PBMC had been reported to induce a decrease in CD62L expression (32), an experiment was conducted to compare expression of CD62L, CD44, CD25, and CD27 on antigen-specific IFN-γ+ CD8 T cells stimulated from fresh versus cryopreserved/thawed PBMC. Two pigs previously infected with porcine reproductive and respiratory syndrome virus (PRRSV) were used, and PBMC were stimulated with a synthetic PRRSV peptide previously shown to be recognized by CD8 T cells from these animals (H. Mokhtar and S. P. Graham, unpublished data). Staining with CD25, CD27, and CD62L MAbs was visualized by incubation of cells with allophycocyanin (APC)-conjugated rat antimouse IgG1 (BD Biosciences) for 10 min, and then cells were washed twice with FACS buffer.

Surface-stained cells were fixed and permeabilized using CytoFix/CytoPerm solution (BD Bioscience) for 20 min at 4°C. After two washes in BD Perm/Wash buffer (BD Biosciences), PBMC were incubated with cytokine-specific MAbs at RT for 10 min in the dark. The MAbs used were IFN-γ–FITC or -Alexa Fluor 647 (CC302; AbD Serotec), TNF-α–Pacific Blue (Mab11; Biolegend, Cambridge Bioscience), and IL-2 (A105D 3F1; Invitrogen), labeled using the Zenon Alexa Fluor 647 mouse IgG2a labeling kit (Invitrogen). IgG1–FITC or -Alexa Fluor 647 isotype control MAbs were used to control staining with IFN-γ MAb. Unstained cells were used as controls for IL-2 and TNF-α. The cells were given two final washes in BD Perm/Wash buffer and resuspended in FACS buffer prior to flow cytometric analysis. Further details of all the antibodies used in this study are reported in Table 2. All the washes used centrifugation at 930 × g for 2 min, and cells were analyzed on a MACSQuant (Miltenyi Biotec) or a CyAn ADP (Beckman Coulter) flow cytometer. Cells were analyzed by exclusion of doublets followed by gating on viable cells (Live/Dead Fixable Dead Cell stain negative) in the lymphocyte population, and defined lymphocyte subpopulations were then gated upon and their expression of cytokines was assessed. Gates were set using the corresponding isotype/ unstained controls, and values were corrected by subtraction of the percent positive events in the biological negative control (mock virus supernatant stimulated). The number of singlet, live lymphocytes acquired for analysis ranged from 200,000 to 400,000, which translated to 20,000 to 80,000 CD8 T cells being analyzed. To determine the absolute numbers of IFN-γ-producing cells, the PBMC density in peripheral blood was assessed by CD45 staining as described above except with the exclusion of granulocytes, which were distinguished by their forward and side scatter characteristics. This value was then used to convert the percentage of IFN-γ-secreting cells within the viable PBMC gate to a cell density/μl of blood.

Data analysis and statistics. Graphical and statistical analysis was performed using GraphPad Prism 5.04 (GraphPad Software Inc., La Jolla, CA). Data were represented as means with standard errors of means (SEM) quoted to indicate the uncertainty around the estimate of the group mean. The differences between mean values were analyzed by a two-tailed paired t test or a one-way analysis of variance (ANOVA) followed by Bonferroni’s posttest or Dunnett’s test, and a P value of <0.05 was considered statistically significant.

RESULTS

CD8 T cells are the primary source of virus-specific IFN-γ after C-strain vaccination and challenge with virulent CSFV. The rapid onset of protection conferred by the C-strain vaccine has previously been correlated with the induction of a broadly cross-reactive T cell IFN-γ response (7). To characterize this early response, an in vivo experiment was conducted where pigs were vaccinated with the C-strain vaccine and challenged after 5 days with the virulent Brescia strain. Vaccinated pigs were solidly protected against CSF. In contrast, unvaccinated challenge control pigs developed clinical signs of the disease, viremia, and leukopenia (Fig. 1). PBMC from vaccinated and challenged control pigs were collected longitudinally during the experiment, at days −2, 0, 3, and 5 postchallenge, and cryopreserved. CSFV-specific IFN-γ release from different lymphocyte populations was assessed using flow cytometry in order to identify the cellular source (Fig. 2A). As shown in Fig. 2B, CD8 T cells, as defined by the CD3+ CD4− CD8αlow phenotype (33), were included as the initial source of CSFV-specific IFN-γ after vaccination with the C-strain virus. Neither CD4+ CD8α− nor CD4− CD8αhigh T cells, which in swine have been proposed to represent naïve and activated/memory populations, respectively (33), showed a significant increase in CSFV-specific IFN-γ+ cells over the time period analyzed. CD3− CD8α− NK cells and CD8αlow− T cells, which are mostly γδ T cells (33), did not show significant IFN-γ expression after virus stimulation at any of the time points examined. To exclude the possibility that CD8α− γδ T cells falling within the CD3− CD4− CD8αhigh gate were contributing to the IFN-γ+ cells detected, assays were performed with a pan-γδ T cell MAb and utilizing PBMC from 3 representative vaccinated and challenged pigs (see Fig. S1 in the supplemental material). Both challenged and unchallenged vaccinated animals, but not the challenge-only pigs, displayed significant virus-specific CD8 T cell IFN-γ responses from 8 days after vaccination (day 3 postchallenge) (P < 0.05). Interestingly, CD8 T cells from vaccinated but unchallenged animals showed a higher frequency of IFN-γ+ CD8 T cells than did vaccinated and challenged animals at 8 days postvaccination (P < 0.05; Fig. 2C). The frequency of virus-specific CD8 T cells increased most markedly in the vaccinated and challenged pigs on day 5 postchallenge (10 days after vaccination), with the greatest number of cells being observed in those vaccinated intranasally (P < 0.05).

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In order to further characterize this early T cell response, the ability of the IFN-γ-expressing CD8 T cells to degranulate and release other antiviral cytokines upon stimulation with CSFV was investigated. Translocation of lysosome-associated membrane protein 1 (LAMP1/CD107a) to the cell membrane has been confirmed as a marker of cytotoxic degranulation by CD8 T and NK cells (34). While CD107a mobilization has been reported as a marker for porcine NK cell degranulation (35), this is the first report of its application to assess porcine CD8 T cell degranulation. PBMC isolated 5 days postchallenge were stimulated with CSFV. A proportion of IFN-γ-expressing CD8 T cells from all vaccinated animals expressed CD107a on their surface, suggestive of cytotoxic activity against CSFV-infected antigen-presenting cells (Fig. 3A). Assessment of the coexpression of IFN-γ and TNF-α by CD8 T cells after virus stimulation showed individual variability between animals (data not shown), but on average, the majority of CSFV-specific CD8 T cells expressed IFN-γ and not TNF-α (Fig. 3B). Comparison of TNF-α and CD107a expression on IFN-γ+ CD8 T cells between experimental groups showed a significantly greater number of TNF-α+ IFN-γ+ cells in all vaccinated groups than in the challenge control group (P < 0.05), but only the intranasally vaccinated and challenged animals showed a greater number of TNF-α+ IFN-γ+ CD8 T cells (P < 0.05). This group was also the only one which expressed a greater number of both CD107a+ and CD107a−expressing IFN-γ+ CD8 T cells. There were no significant differences in the proportions of TNF-α+ IFN-γ− or CD107a+ IFN-γ− CD8 T cells between mock- and CSFV-stimulated conditions (data not shown).

CSFV C-strain-induced CD8 T cell responses are sustained and boosted upon later rechallenge infection. To further investigate the possible role for T cells in providing protection against CSFV, another experiment was conducted whereby C-strain-vaccinated animals were challenged with the Brescia strain after 5 days and then rechallenged with a 10-fold-higher dose after 28 days. All animals were protected against both challenge infections with no reduction in leukocyte counts or clinical signs of CSF being observed (data not shown). PBMC were collected longitudinally during the experiment, and the CSFV-specific IFN-γ response was again investigated ex vivo using flow cytometry (Fig. 4). Significant virus-specific CD8 (CD3+ CD4− CD8α(high)) (Fig. 4A) and “memory” CD4 (CD3+ CD4− CD8α(low)) (Fig. 4B) T cell responses were detected in vaccinated and challenged animals, but not control animals, from days 7 and 14 postchallenge, respectively. No virus-specific IFN-γ expression was detected in “naïve” CD4 T cell (CD3+ CD4− CD8α(high)), CD8α(low)− T cell, or NK cell populations (data not shown). As described above, the contribution of γδ T cells was directly assessed on a subset of pigs using a T-cell receptor (TcR) δ-chain MAb, and no CSFV-specific increase in IFN-γ+ γδ T cells was detected (see Fig. S1 in the supplemental material). The CD8 T cell response showed an initial peak on day 14 postchallenge, contracted to a degree, and then peaked again 7 days after the second challenge infection (day 35) (Fig. 4A). The percentage of cells remaining in the contracted CSFV-specific CD8 T cell population following this second peak was similar to that observed after the first, at ~0.4%. Individual differences between animals were observed in the kinetics of the response, with pigs which showed the greatest peak in response to the first challenge infection displaying less evidence of a recall in response to the second challenge (data not shown). The kinetics of the memory CD4 T cell IFN-γ response was largely similar to the CD8 response except that the frequency of cells was lower and was accompanied with an increase, albeit to lower levels, in the per-
CD8 T cells are the primary source of CSFV-specific IFN-γ following vaccination and challenge. At days −2, 0, 3, and 5 postchallenge, PBMC were stimulated in vitro with CSFV or mock virus supernatant and IFN-γ expression was assessed by flow cytometry. (A) Gating strategy used to interrogate responses in singlet, live lymphocytes and representative data from pig 51, vaccine (i.n.)/challenge group, day 5 postchallenge, are displayed. T cell populations were defined as CD3⁺CD8⁺⁰⁺CD4⁺, CD3⁺CD8⁺⁰⁺CD4⁺, CD3⁺CD8⁺⁰⁺CD4⁺, and CD3⁺CD8⁺⁰⁺CD4⁺, and NK cells were defined as CD3⁻CD8⁺⁰⁺CD4⁻. Finally, intracytoplasmic IFN-γ expression was assessed in each population and compared to staining with an isotype control MAb. (B) Representative data of the expression of IFN-γ versus CD8α on live, singlet CD3⁺ CD4⁺ T cells after CSFV stimulation (pig 51, vaccine [i.n.]/challenge group, day 5 postchallenge). (C) Mean numbers of IFN-γ-producing cells/μl blood in response to CSFV stimulation for each lymphocyte population following correction for the IFN-γ expression in response to mock virus supernatant. The mean data from three randomly selected pigs/group/time point are presented, and error bars represent SEM.
percentage of IFN-γ-expressing CD4 T cells in response to the mock virus supernatant, an effect not observed in the control animals despite these animals having been inoculated with this supernatant in place of the vaccine/challenge viruses. Subtraction of the response to mock virus supernatant from the CSFV-stimulated responses and conversion of this percentage to the number of IFN-γ-expressing cells per μl of blood clearly revealed that the CD8 T cell population was the major contributor to the CSFV-specific T cell response (Fig. 4C). The comparison of fluorescence intensity measurements revealed no differences between IFN-γ expression levels of CD8 (Fig. 4A) and memory CD4 T cells, and the levels did not differ significantly over time (data not shown).

Following challenge, CSFV C-strain-induced CD8 T cell responses are polyfunctional and show a putative mixed effector/effectormemory phenotype. Phenotypic and functional analysis was performed on PBMC previously cryopreserved at the peaks following primary and secondary challenge infections, days 14 and 35 post-primary challenge, respectively, and PBMC were compared to cells collected from pigs at the peak of response post-C-strain vaccination (day 9) (28). Due to the limited number of cryopreserved cells, for some parameters only 5 randomly chosen vaccinated and challenged pigs out of 8 were analyzed. On day 9 postvaccination, and days 14 and 35 postchallenge, the majority of IFN-γ-expressing CD8 T cells expressed high levels of the cell adhesion and indicative effector memory marker CD44 (Fig. 5A) and lacked expression of the peripheral lymph node homing receptor, L-selectin/CD62L (Fig. 5B). Evaluation of the expression of CD44 and CD62L at the other time points postchallenge revealed that CD44^{high}CD62L^{−} was the dominant phenotype throughout (data not shown). In contrast, CSFV-specific T cells from both groups and selected time points displayed variable levels of both CD25 and CD27 (Fig. 5C and D). There were no significant differences in expression of these markers by IFN-γ^{+}CD8 T cells between mock- or CSFV-stimulated conditions (data not shown). To assess the potential effect of previous cryopreservation on porcine CD8 T cell memory/activation marker expression, which might have influenced these data, a separate experiment was conducted to directly compare expression of CD62L, CD44, CD25, and CD27 by antigen-specific IFN-γ^{+} CD8 T cells stimulated from fresh versus cryopreserved/thawed PBMC (see Fig. S2 in the supplemental material). Following stimulation with a model antigenic peptide, the phenotype of specific IFN-γ^{+} CD8 T cells did not differ between fresh and previously cryopreserved PBMC, with a CD62L^{−}CD44^{high}CD25^{−}CD27^{+} phenotype being discerned in both cases. However, when considering the entire CD8 T cell population, there was a small but measurable decrease in the proportion of cells expressing CD62L, CD25, and CD27 following cryopreservation.

The polyfunctionality of CSFV-specific CD8 T cells at the peaks of response postvaccination and vaccination/challenge was...
again investigated by their ability to degranulate and coexpress cytokines upon virus stimulation. As had been observed in the earlier experiment, only a proportion (~50%) of IFN-γ+ CD8 T cells from the vaccinated-only group had mobilized CD107a, indicative of cytotoxic degranulation (Fig. 5E). In contrast, the majority of cells from vaccinated and challenged animals, on both day 14 and day 35, showed evidence of degranulation. Coexpression of TNF-α and IL-2 by CSFV-specific IFN-γ+ CD8 T cells was assessed using the gating strategy illustrated in Fig. 6A. Interestingly, significant variability was observed in the cytokine profile between individual animals, groups, and time points (Fig. 6B). Nine days postvaccination, the majority of CSFV-specific CD8 T cells secreted only IFN-γ, with a significant minority (19.4 to 42% of CSFV-specific IFN-γ+ CD8 T cells) coexpressing TNF-α. Only 2 of the 4 animals were found to present a small proportion of CD8 T cells expressing all three cytokines (2.9% and 5.1% of CSFV-specific IFN-γ+ CD8 T cells). Analysis of the vaccinated and challenged animals showed that on day 14 postchallenge, cells coexpressing IFN-γ and TNF-α were the largest population in 2/5 animals (43.6% and 49.9% of CSFV-specific IFN-γ+ CD8 T cells), with the single IFN-γ expressers being the major population in the other animals. In 4 of the 5 animals, there was a significant population of cells expressing all three cytokines, which ranged from 9.1% to 29.6% of CSFV-specific IFN-γ+ CD8 T cells. On day 35 postchallenge (7 days post-rechallenge), there was an increase in the relative proportion of single IFN-γ expressers in three animals, while in the other two the greatest increase was observed in the triple expressers (pig 8) and IFN-γ and TNF-α dual expressers (pig 10). There were no significant differences in the proportions of TNF-α+ IFN-γ−, IL-2+ IFN-γ−, or CD107α+ IFN-γ− CD8 T cells between mock- and CSFV-stimulated conditions (data not shown). Assessment of the relative IFN-γ expression levels, by
FIG 5 Phenotypic and functional characterization of CSFV-specific CD8 T cells. PBMC from pigs vaccinated with C-strain (animal experiment 3) or C-strain-vaccinated pigs challenged with CSFV Brescia (animal experiment 2) were stimulated with CSFV and mock virus supernatant 9 days after vaccination or 14 days after challenge (day 14) and 7 days after rechallenge (day 35), respectively. The expression of CD44 (A), CD62L (B), CD25 (C), CD27 (D), and CD107a (E) on IFN-γ+ singlet, live CD8+ high CD4+ T cells was assessed by flow cytometry. Data represent the mean mock-virus stimulation-corrected percent IFN-γ+ CD8 T cells for 8 or 16 replicate cultures (2 replicates for four vaccine-only pigs and eight vaccinated and challenged pigs, respectively), and error bars represent SEM. Values were compared using a two-tailed unpaired t test, and significance is indicated by *** (P < 0.001), ** (P < 0.01), and * (P < 0.05).
Characterization of polyfunctional cytokine expression by CSFV-specific CD8 T cells. PBMC from pigs vaccinated with C-strain CSFV (animal experiment 3; n = 4) or C-strain-vaccinated pigs challenged with CSFV Brescia (animal experiment 2; n = 5) were stimulated with CSFV and mock virus supernatant 9 days after vaccination or 14 days after challenge (day 14) and 7 days after rechallenge (day 35), respectively. The expression of TNF-α and IL-2 by CSFV-specific IFN-γ+ singlel, live CD8εhigh CD4− T cells was determined by flow cytometry. (A) Representative dot plots. (B) Mock-corrected mean proportions of IFN-γ-secreting CD8 T cells expressing either of the two other cytokines, for duplicate cultures/animal. (C) Mean fluorescence intensity (MFI) of IFN-γ staining of each population is presented. Data represent the mean mock-virus stimulation-corrected percent IFN-γ+ CD8 T cells for 8 or 10 replicate cultures (two replicates for four vaccinated pigs and eight vaccinated and challenged pigs, respectively), and error bars represent SEM. Statistical analyses were performed using a one-way ANOVA followed by Dunnett’s multiple comparison test against CD8 T cells secreting only IFN-γ. Significance is indicated by *** (P < 0.001), ** (P < 0.01), and * (P < 0.05).
comparing fluorescence intensity measurements, revealed that on
day 9 postvaccination and days 14 and 35 postchallenge, the triple-
cytokine-expressing CD8 T cells produced significantly larger
amounts of IFN-γ than did the single and dual expressers
(Fig. 6C).

DISCUSSION
For the first time, our results provide detailed phenotypic and
functional data on the T cell response induced by vaccination with
a live attenuated C-strain vaccine and boosted upon subsequent
challenge infections. We demonstrate that CD8 T cells (CD3+ CD8αhigh CD4−) are the initial source of CSFV-specific IFN-γ after vaccination with the C-strain vaccine and can be detected in
the blood as early as 8 days postvaccination. IFN-γ-producing
CD8 T cells were detected in all vaccinated groups, independently
of the route of vaccine administration (intranasal [i.n.] or intramuscular [i.m.]); in contrast, infection of unvaccinated animals
did not result in a detectable T cell IFN-γ response against the
virus and animals developed clinical signs of the disease, leukopenia, and viremia. Our results are in accordance with previous
publications, where it has been reported that a CSFV-specific T cell
IFN-γ response can be identified in the blood as early as 6 or 8 days
after C-strain vaccination (5–7), and confirm the correlation be-
tween the T cell IFN-γ response and protection against CSF. The
inability to detect a CSFV-specific IFN-γ response in unvacci-
nated challenge control pigs is in accordance with earlier studies
conducted by our group utilizing moderately virulent CSFV
strains (6, 7) but contrasts with a previous report where IFN-γ-
producing CD4 and CD8 T cells were detected in unvaccinated pigs
challenged with a virulent strain of CSFV (11). While CD8 T
cells were detected as the first source of IFN-γ produced in re-
sponse to CSFV, they were also shown to be the major producers,
with 400 to 1,200 CSFV-specific CD8 T cells/μl being detected in
blood. In comparison, virus-specific CD4 T cells were present at 2-
to 6-fold-lower numbers, which is in general accordance with our
earlier studies (7). Moreover, rechallenge of animals resulted in
a significant boosting of specific CD8 T cell numbers while CSFV-
responding CD4 T cells remained relatively constant in number.
Interestingly, vaccinated/challenged pigs showed an increase in
the percentage of IFN-γ-expressing memory CD4 T cells in re-
sponse to culture with the mock virus supernatant. We might
speculate that this is due to reactivity against an (as yet) unidenti-
fied antigen present in the PK15 lysate. The presence of CSFV may
have adjuvanted the response to this antigen so that it was ob-
served only following vaccination and challenge and not seen in
control animals inoculated with the mock virus supernatant.

We next addressed the memory/activation phenotype of the
CSFV-specific CD8 T cells, since it has become apparent in recent
years that the efficacy of T-cell-based vaccines is highly dependent
upon the quality of the memory population induced. CSFV-spe-
cific IFN-γ− CD8 T cells expressed high levels of the cell adhesion
glycoprotein CD44 and lacked expression of the lymph node
homing receptor CD62L. Based upon current models of CD8 T
cell differentiation, this would classify these cells as either effectors
(TEFF) or TEM cells, which traffic via lymphoid tissues, rather
than TCM cells which express CD62L (17). CD27, a costimulatory
receptor that provides a critical survival signal to activated T cells
(36), is required for CD8 memory T cell formation and responsi-
siveness. CD27 expression is lost on human CD8 T EFF cells, and
expression increases upon differentiation to memory cells with
low levels expressed on TEM cells and higher levels on TCM cells
(37, 38). A recent study of CD27 expression by porcine CD4 T cells
has shown that antigen-specific IFN-γ-expressing CD4+ CD8α+ T
cells may be either CD27− or CD27+ (39). Compared to CD27−
CD4+ CD8α+ T cells, CD27+ CD4+ CD8α+ T cells respond to
polyclonal activation with enhanced cytokine production and re-
duced proliferation. They also lack or show reduced expression of
lymph node homing receptors, and it has therefore been proposed
that CD27+ and CD27− porcine CD4 T cells may represent TCM
and TEM, respectively. While a comparable analysis of CD27 ex-
pression by porcine CD8 T cells is still to be conducted, a longitudi-
nal study of CD27 expression on porcine CD8 T cells from birth
to 6 months of age suggested that early effector cells expressed low
levels of CD27, which was absent on late effector or memory cells
(40). Thus, we may only speculate at this time that the variable
level of CD27 expression on CSFV-specific CD8 T cells reflects
a mixture of effector and memory phenotypes. Variable expression
of CD25, the alpha-chain of the IL-2 receptor that is expressed on
the surface of activated lymphocytes (11, 41, 42), was also seen.
Heterogeneity in expression of CD25 on CD8 T cells has been
described in an acute murine virus infection model, with low
CD25 expression associated with generation of long-lived mem-
ory and high expression associated with terminal effector differ-
entiation (43). This expression pattern may therefore also imply a
mixed CSFV-specific T EFF and TEM population and explain the
previously described lack of association between enhanced CSFV-
specific IFN-γ production and upregulation of CD25 on CD8 T
cells (11). Since this analysis was conducted following in vitro
restimulation of previously cryopreserved PBMC, it remains to be
determined whether these phenotypes accurately represent those
of CSFV-specific CD8 T cells in vivo. We have recently identified
a number of CD8 T cell epitopes on CSFV (G. Franzoni and S. P.
Graham, unpublished data), and together with the recent develop-
ment of porcine major histocompatibility complex (MHC) class I tetramers (44), tools could be developed to confirm the
phenotype of CSFV-specific CD8 T cells by direct ex vivo exami-
nation. We finally assessed the polyfunctionality of the CSFV-
specific CD8 T cell population since this may be central to their
protective capacity. Based on current models, CD8 TEM cells have
been shown to have high ex vivo cytotoxic potential and rapidly
produce cytokines upon reexposure to antigens, whereas CD8
TCM cells possess a greater proliferative capacity (15). Interest-
ingly, we found that early postchallenge or in the absence of chal-
lenge only a proportion of CSFV-specific IFN-γ CD8 T cells mo-
bilized CD107a, compared to later time points postchallenge,
where the majority expressed CD107a on their surface. CD107a is
expressed on the cell surface following activation-induced degran-
ulation, and it has been described as a marker for cytotoxic CD8 T
cell activity whose expression has been associated with loss of per-
forin following antigen stimulation (34). The data presented here
suggest that CSFV-specific IFN-γ-expressing cells may also have
cytotoxic capacity and consolidate our previous results where we
showed that IFN-γ expression was restricted to CD8 T cells ex-
pressing intracellular perforin (7) and other earlier studies that
demonstrated CSFV-specific cytotoxic activity by T cell cultures
isolated from vaccinated pigs (8–10). While IFN-γ-secreting TEM
cells have been implicated in protection against a variety of intra-
cellular pathogens, simultaneous production of IFN-γ, TNF-α,
and IL-2 detected at the single-cell level by multiparameter flow
cytometry has been correlated with enhanced vaccine-induced
protection (45–51). Moreover, data suggest that such polyfunctional CD8 T memory cells are critical to long-term protection against other viruses belonging to the family *Flaviviridae* (20, 22).

In the present study, we observed that the majority of CD8 T cells produced only IFN-γ or IFN-γ and TNF-α, with only a small percentage coexpressing IFN-γ, TNF-α, and IL-2. As seen with CD107a mobilization, there appeared to be a trend toward an increase in functionality following challenge, although interanimal variability was observed. Since all animals were solidly protected against the challenge infections, we were unable to correlate this with the cells coexpressing TNF-α, similar to that observed in other systems (48, 52, 53).

Overall, our data support the hypothesis that CD8 T\textsubscript{EFF}/T\textsubscript{EM} cells play a key role in the protection afforded by C-strain CSFV in the early period preceding the appearance of neutralizing antibodies and are also readily recalled by later challenge. This information and the novel flow cytometric panels developed and employed for the first time in the pig to characterize these responses will hopefully aid the development and evaluation of next-generation vaccines against CSFV and more broadly against other porcine pathogens.

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