Review

Laboratory animal models to study foot-and-mouth disease: a review with emphasis on natural and vaccine-induced immunity

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Laboratory animal models have provided valuable insight into foot-and-mouth disease virus (FMDV) pathogenesis in epidemiologically important target species. While not perfect, these models have delivered an accelerated time frame to characterize the immune responses in natural hosts and a platform to evaluate therapeutics and vaccine candidates at a reduced cost. Further expansion of these models in mice has allowed access to genetic mutations not available for target species, providing a powerful and versatile experimental system to interrogate the immune response to FMDV and to target more expensive studies in natural hosts. The purpose of this review is to describe commonly used FMDV infection models in laboratory animals and to cite examples of when these models have failed or successfully provided insight relevant for target species, with an emphasis on natural and vaccine-induced immunity.

Introduction

Foot-and-mouth disease virus (FMDV: family *Picornaviridae*; genus *Aphthovirus*) is known to naturally infect a wide variety of cloven-hoofed domesticated and wild animal species, causing an acute disease characterized by vesicular lesions of the tongue, snout, buccal cavity, feet and teats (Grubman & Baxt, 2004). Despite causing extensive lesions, the cycle of infection in the individual animal is short, and foot-and-mouth disease (FMD) usually resolves without the need for treatment and is seldom lethal in adults (Arzt *et al.*, 2011b). However, the highly contagious nature, wide dissemination and significant economic impact of FMD have made it one of the most feared livestock diseases and a major research focus for more than a century. Progress towards the development of effective tools for FMD control has been hampered by several factors including the cost and logistics of large-animal experimentation in specialized high-containment facilities, incomplete knowledge of the host's immune systems and lack of immunological reagents compared to biomedical rodent species and humans. These factors delayed the production of vaccines on an industrial scale and this major research goal was subsequently only achieved in the 1950s (Lombard *et al.*, 2007). In a review, Brown (2003) highlighted that this milestone could not have been achieved without certain significant advances in our knowledge of FMD. The first significant advance was the demonstration by Loeffler & Frosch (1897) that the disease was caused by a virus and the second was the establishment of FMD laboratory animal models, including the guinea-pig model (Waldman & Pape, 1920) followed by the suckling mouse model (Skinner, 1951). Although not without their flaws, these FMD laboratory animal models have helped elucidate several mechanisms of FMD pathogenesis, which would have been difficult to achieve directly in target species. These models have provided an accelerated time frame at significantly reduced costs to develop and test vaccine candidates and continue to be a useful tool for interrogating FMDV immune responses. However, we now know that porcine and ruminant immune systems and responses to pathogens are significantly different compared with laboratory animals and there are occasions when prophylactic strategies proven effective in FMD laboratory animal models have completely failed in natural hosts. Although one could argue these failures demonstrate the models are of limited value and FMDV data generated in laboratory animals are controversial, these scenarios have highlighted the gaps in our understanding and may identify responses to FMDV and immune mechanisms that are particular to natural hosts. There are clear examples of data obtained from FMD laboratory

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animal models that have been extrapolated and applied to target species. The goal of this review is to highlight the strengths and limitations of FMD laboratory animal models, focusing on natural and vaccine-induced immunity.

Historical overview of FMDV pathogenesis in laboratory animals

As early as 1890, there were reports of FMDV-infected animals that were not members of the order Artiodactyla (as reviewed by Arkwright & Burbury, 1925). Rabbits in stalls with FMDV-infected cattle were found to have oral vesicles. The report by Waldman & Pape (1920) followed, demonstrating that guinea pigs could be inoculated by scarification on the planter surface of the metatarsus with vesicular fluid from infected cattle. Challenged animals developed generalized disease, including salivation, weight loss and secondary vesicles on the fore-feet, tongue and oral cavity. The disease was passaged successfully by intracutaneous inoculation through 19 guinea pigs without loss of virulence. Animals recovered from infection after 7 days and were immune from rechallenge with the same strain (Arkwright & Burbury, 1925). These investigators also reported that disease transmission from inoculated to healthy guinea pigs did not occur, even when infected and naive animals were placed in the same cage. Following the demonstration of susceptibility in non-ungulate species, a number of studies were performed to determine the potential role of rats, mice, rabbits and birds in FMD epidemiology. These animals can be experimentally infected following parenteral challenge, with secondary mouth or foot lesions reported in rats, rabbits and chickens (Arkwright & Burbury, 1925; Beattie *et al.*, 1928; Bedson *et al.*, 1927; Skinner, 1954). Contact infection was only demonstrated in rabbits, and it is probable infection occurred through existing skin abrasions (Beattie *et al.*, 1928). Therefore, depending on their susceptibility to infection, animals can be divided into three categories: (i) animals susceptible to FMDV infection which play a role in the natural epidemiology of the disease, like cattle, sheep, goats, pigs and African buffalo, (ii) animals susceptible to FMDV infection that can play a role in the epidemiology but only under some circumstances (for example capybaras, deer, camels and a number of other animal species in the order Artiodactyla) or (iii) animals susceptible to infection only under experimental conditions that do not play a role in the epidemiology of the disease; mice, guinea pigs and rabbits belong to the last category (Alexandersen & Mowat, 2005; Gomes & Rosenberg, 1984).

It is clear that guinea pigs are the best laboratory animal to model the pathogenesis of FMDV epithelial vesiculation (di Girolamo *et al.*, 1985). Similar to natural hosts, extensive vesicles develop at the inoculation site within 24 h, the vesicles rapidly rupture and the epithelium is desquamated. Secondary vesicles develop on the tongue or mouth, leading to salivation, food refusal and weight loss. Within 4 to 5 days, these vesicles begin to heal and desquamation is completed in about 3 weeks (Knudsen *et al.*, 1979). Similar to the natural host, animals are pyrexic for a short period and viraemia is cleared rapidly, coinciding with a rapid antibody response, with serum neutralizing antibody (SNA) titres detectable from 3 days post-infection (p.i.). Mortality rates in guinea pigs are low, reported to be of the order of 5 % (Arkwright & Burbury, 1925; Knudsen *et al.*, 1979). Due to the reproducibility of the FMDV response, guinea pigs have been used extensively to produce antiserum, which has been used to develop sensitive diagnostic and serotyping assays (Ferris, 1988). Guinea pigs have also been used extensively for FMDV vaccine efficacy trials (Cartwright *et al.*, 1982; Guo *et al.*, 2005; Yao *et al.*, 2008). It is noteworthy that natural FMDV isolates need to be adapted to the guinea pig by serial injection in the footpad (Aramburu, 1949; Knudsen *et al.*, 1979). Although adaptation has been shown to alter viral antigenicity and receptor recognition, guinea-pig-adapted virus can productively infect natural hosts and kill suckling mice (Núñez *et al.*, 2007). Similar to natural hosts, the guinea-pig model is also limited by the lack of immune reagents, genetic engineering and knockout technology.

Following the failure of earlier attempts to produce clinical disease, the mouse as a model for FMDV was discounted until the 1950s. During a series of experiments investigating FMDV susceptibility of the cotton rat, Skinner (1951) inoculated 3-week-old mice intracerebrally due to the limited available stock of cotton rats. A large number of mice surprisingly died, and Skinner went on to demonstrate that unweaned mice 1 to 2 weeks old could be infected intraperitoneally leading to a fatal infection characterized by muscular paralysis and degenerative changes in the myocardium and skeletal muscles (Platt, 1956; Skinner, 1951; Subak-Sharpe *et al.*, 1963). Clinical signs included paralysis of the hindquarters, respiratory distress within 24 to 48 h p.i. and death shortly thereafter. Susceptibility rapidly wanes with increasing age, and infection in mice older than 3 weeks is typically subclinical (Campbell, 1970; Fernández *et al.*, 1986; Skinner, 1951). Skinner's report is considered a major milestone in FMDV research as he established a critical research tool for FMDV isolation and titration, and for serum neutralization tests; the suckling mouse model was eventually superseded by *in vitro* cell culture systems (Skinner *et al.*, 1952). It is now clear that adult mice are also susceptible to experimental FMDV infection. Following intraperitoneal (IP) challenge, virus replicates primarily in the pancreas and, similar to target species, the viraemic period is short, lasting between 48 and 72 h p.i. with production of SNA coinciding with viral clearance (Borca *et al.*, 1986; Charleston *et al.*, 2011; Fernández *et al.*, 1986; Lefebvre *et al.*, 2010). The exception to this short period of virus replication is the 'carrier state', which is considered unique to ruminants and is defined as the period after 28 days p.i. in which infectious FMDV may be detected in oesophageal–pharyngeal fluid (OPF) (OIE, 2012). FMD pathogenesis in adult mice is dependent on the mouse strain, FMDV strain and, similar to the natural host

(Arzt *et al.*, 2014), the route of challenge (Table 1). C57BL/6 mice are the most susceptible common laboratory strain for FMDV infection. These data are in agreement with our unpublished results comparing the susceptibility of C57BL/6 and BALB/c mice to FMDV O UKG 34/2001 IP challenge. C57BL/6 mice challenged with 10^3 $TCID_{50}$ developed clear signs of disease, including respiratory distress, neurological signs and wasting; by comparison, no clear signs were detected in BALB/c mice challenged with a higher dose of 10^6 $TCID_{50}$ (unpublished data). The underlying reasons for differences in susceptibility are not clear, but may be useful to help determine the underlying genetic susceptibility or resistance to FMDV in large animals. Infection of susceptible mouse strains can lead to a lethal systemic infection in adults, with virus replicating in all major organs, including the heart, lung, brain, kidney, liver, spleen and thymus (Salguero *et al.*, 2005; Sanz-Ramos *et al.*, 2008). FMDV has also been shown to induce the formation of vesicles following subcutaneous inoculation into the footpad of susceptible mouse strains, with similar histological features to those described in natural hosts (Salguero *et al.*, 2005). Similar to natural hosts, it is difficult to make a clear judgement on the virulence of different FMDV serotypes in mice due to the myriad FMDV strains. It is clear from the literature that virulence is strain dependent. For example, García-Núñez *et al.* (2010) demonstrated that FMDV A/Arg/00 does not cause death in adult C57BL/6 mice even at 10^7 p.f.u.; by contrast, FMDV A/Arg/01 was lethal at doses as low as 10^2 p.f.u. In cattle, FMDV A/Arg/00 showed only low virulence; by contrast, FMDV A/Arg/01 caused severe lesions and calf deaths. Therefore, field observations of differences in virulence in target species were reproducible in the adult mouse model (García-Núñez *et al.*, 2010). Consequently, the mouse model can provide data on strain virulence to guide further experiments in cattle and may be a useful tool to characterize new emerging FMDV strains in a cost-effective manner.

The two common features of the FMDV mouse model that warrant further review are viral replication in the myocardium and pancreas, and their associated pathologies (Fig. 1). Death in young livestock, documented in calves, piglets and lambs, is a fairly common feature of FMD epizootics (Alexandersen & Mowat, 2005). Generally, the only gross pathological changes seen in these young animals are in the myocardium and death is often attributed to myocarditis (Donaldson *et al.*, 1984; Gulbahar *et al.*, 2007). In addition, the rare manifestation of FMDV-associated death in adults, known as 'malignant FMD', is characterized by lesions and degeneration of the myocardium (Arzt *et al.*, 2011a; Shimshony *et al.*, 1986). Both viral myotropism, leading to direct cell injury, and the immune response of the host are likely to play a role in the pathogenesis of this syndrome. However, as reviewed by Arzt *et al.* (2011a), there has been little specific investigation into this syndrome and the pathogenic mechanisms remain unknown. There are clear age-related host factors playing a role in FMD pathogenesis in the mouse as susceptibility, characterized by muscular paralysis and degenerative changes in the myocardium and skeletal muscles, rapidly wanes with increasing age. The marked myopathic affinity that FMDV has in young mice warrants further investigation, as it may prove a useful model to investigate age-related susceptibility and myotropism in target species. In addition, myocarditis is a common feature of FMDV infection in susceptible adult mice (BALB/c mice; Fig. 1a) and dilated cardiomyopathy has been reported as a common sequela in highly susceptible C57BL/6 strains (Salguero *et al.*, 2005). Age-related susceptibility and myotropism have been described for other viral infections in mice, the most studied being the coxsackieviruses (CAVs). Like FMDV, CAV infection can result in a marked myositis, with older mice seemingly more resistant (Mclaren & Sanders, 1959). It has been shown that certain CAVs utilize $\alpha v\beta 3$ integrin as a receptor, and that age-restricted expression of $\alpha v\beta 3$ integrin on skeletal muscle cells is likely responsible for murine CAV age-related myotropism (Goldberg & Crowell, 1971; Roivainen *et al.*, 1994). Age-dependent receptor expression by striated muscle cells may play a role in age-related susceptibility of mice to FMDV, and indeed in other animals. FMD experiments in adult mice may also provide insight into the viral factors, host immune factors and genetic susceptibility to malignant FMD in target species. Similar to FMD pathogenesis in the mouse model, myocarditis is reported to occur more frequently in target species than indicated by case fatality alone, and may be a common feature of FMDV infection (Korn & Potel, 1954).

The pancreas is considered the preferred site for FMDV replication in adult mice; at 24 h p.i. the highest viral load is in the pancreas (Bachrach, 1968; Fernández *et al.*, 1986; Sanz-Ramos *et al.*, 2008). FMDV causes acute pancreatitis in adult mice, affecting more severely the acinar tissue of the exocrine pancreas (Sanz-Ramos *et al.*, 2008). Severe pancreatic injury is still clearly visible following clearance of virus from the tissue at 21 days p.i., with histological changes suggestive of progression to chronic pancreatitis (Fig. 1). These changes include ablation of acinar cells, vacuolization of the exocrine pancreas, cellular infiltration, atrophy of the endocrine pancreas and fibrosis. Although not demonstrated, these changes are likely to be associated with loss of pancreatic function. Comparable pancreatic pathology has been described during a lethal outbreak of malignant FMD in gazelle (Berkowitz *et al.*, 2010). It has also been suggested that disruption of the pancreas accounts for the biochemical changes reported for cattle with heat-intolerance syndrome, a frequently reported sequel to FMD in endemic settings (Arzt *et al.*, 2011a; Barasa *et al.*, 2008; Catley *et al.*, 2004; Ghanem & Abdel-Hamid, 2010). Hyperglycaemia and hypoinsulinaemia have been reported during the acute stages of FMD in cattle and there are reports in cattle of FMDV causing pancreatic necrosis (Manocchio, 1974; Nai, 1940; Yeotikar *et al.*, 2003). The unambiguous pancreatic tropism of FMDV in the mouse model, combined with the available evidence for

Table 1. Susceptibility of common laboratory mouse strains to FMDV infection

Mouse strain	Age (days)	Virus strain	Virus propagation	Range of challenge virus titre	Volume inoculated (μl)	Route of infection	Minimum lethal dose (MLD)	Proportion of deaths with MLD (%)	Time of first death (days p.i.)	References
BALB/c	56–70	C1 C-S8c1	BHK-21	$10–10^5$ p.f.u.	100	Foot pad	10^3 p.f.u.	100	4	Salguero *et al.* (2005)
	49–63	C1 C-S8c1	BHK-21	10^3 $TCID_{50}$	100	IP	10^3 $TCID_{50}$	90	–	Kamstrup *et al.* (2006)
	49–63	C1 Noville	–	$10–10^5$ $TCID_{50}$	100	IP	None	None	None	Lefebvre *et al.* (2010)
	49–63	O1 Manisa 8/69	Calf kidney	$10–10^5$ $TCID_{50}$	100	IP	None	None	None	Kamstrup *et al.* (2006); Lefebvre *et al.* (2010)
	49–63	Asia1 Shamir	Calf kidney	$10–10^5$ $TCID_{50}$	–	IP	None	–	4	Lefebvre *et al.* (2010)
	49–63	Asia1 Shamir 3/89	Calf kidney	10^3 $TCID_{50}$	100	IP	10^3 $TCID_{50}$	89	–	Kamstrup *et al.* (2006)
	49–63	A-22 Iraq 24/64	Calf kidney	10^3 $TCID_{50}$	100	IP	None	None	None	Kamstrup *et al.* (2006)
	49–63	SAT1 Bot 1/68	BHK-21	10^3 $TCID_{50}$	100	IP	10^3 $TCID_{50}$	50	1	Kamstrup *et al.* (2006)
	49–63	SAT2 Zim 5/81	BHK-21	10^3 $TCID_{50}$	100	IP	10^3 $TCID_{50}$	60	–	Kamstrup *et al.* (2006)
	49–63	SAT3 Zim 4/81	BHK-21	10^3 $TCID_{50}$	100	IP	10^3 $TCID_{50}$	100	1	Kamstrup *et al.* (2006)
	3–4	O OM III	BALB/c suckling mice	20–100 $SMLD_{50}$	100	SC	20 $SMLD_{50}$	100 (6/6)	2	Yang *et al.* (2008)
	56–70	O1 Campos	BHK-21	$10^{7.8}$ $SMLD_{50}$	500	IP	None	None	None	Fernández *et al.* (1986)
C57BL/6	56–70	C1 C-S8c1	BHK-21	$10–10^5$ p.f.u.	50	Foot pad	10^5 p.f.u.	100	3	Salguero *et al.* (2005)
	56–70	C1 C-S8c1	BHK-21	$10–10^5$ p.f.u.	100	IP	10 p.f.u.	100	2	Salguero *et al.* (2005)
	56–70	C1 C-S8c1 MARLS	BHK-21	$10–10^5$ p.f.u.	50	Foot pad	None	100	None	Salguero *et al.* (2005)
	56–70	SAT1	BHK-21	$10–10^5$ p.f.u.	50	Foot pad	10 p.f.u.	100	2	Salguero *et al.* (2005)
	56–70	A22	BHK-21	$10–10^5$ p.f.u.	50	Foot pad	10^3 p.f.u.	33	4	Salguero *et al.* (2005)
	63–70	A/Arg/00	BHK-21	$10^3–10^7$ p.f.u.	100	IP	None	None	None	García-Núñez *et al.* (2010); Molinari *et al.* (2010)
	63–70	A/Arg/01	BHK-21	$10^2–10^6$ p.f.u.	100	IP	10^2 p.f.u.	100	2	García-Núñez *et al.* (2010); Molinari *et al.* (2010)
CF-1	7	A/Arg/00	BHK-21	0.06–585 p.f.u.	50	IM	6 p.f.u.	10	6	García-Núñez *et al.* (2010)
	7	A/Arg/01	BHK-21	0.03–333 p.f.u.	50	IM	3 p.f.u.	10	4	García-Núñez *et al.* (2010)
	56–70	O1 Campos	BHK-21	$10^{7.8}$ $SMLD_{50}$	500	IP	None	None	None	Fernández *et al.* (1986)
SCID	21–28	C1 Noville	–	$10–10^5$ $TCID_{50}$	–	IP	10 $TCID_{50}$	100 (3/3)	4	Lefebvre *et al.* (2010)
	21–28	O1 Manisa	–	$10–10^5$ $TCID_{50}$	100	IP	None	None	None	Lefebvre *et al.* (2010)
	21–28	A22	–	$10–10^5$ $TCID_{50}$	100	IP	10^2 $TCID_{50}$	67 (2/3)	6	Lefebvre *et al.* (2010)
	21–28	Asia1 Shamir	–	$10–10^5$ $TCID_{50}$	100	IP	10 $TCID_{50}$	100 (3/3)	3	Lefebvre *et al.* (2010)
Swiss	56	C1 C-S8c1	BHK-21	10^5 p.f.u.	100	Foot pad	10^4 p.f.u.	30	4	Salguero *et al.* (2005)
	3–7	O1 K	BHK-21	$700–7 \times 10^4$ p.f.u.	100	IP	10^2 p.f.u.	72	2	(Rodríguez-Pulido *et al.* (2011a)
	56–70	O1 Campos	BHK-21	$10^{7.8}$ $SMLD_{50}$	500	IP	None	None	None	Fernández *et al.* (1986)
SJL/J	56–70	C1 C-S8c1	BHK-21	10^6 p.f.u.	100	Foot pad	None	None	None	Salguero *et al.* (2005)

BHK, Baby hamster kidney cells; IM, intramuscular; SC, subcutaneous; SMLD, suckling mouse lethal dose; –, no data.

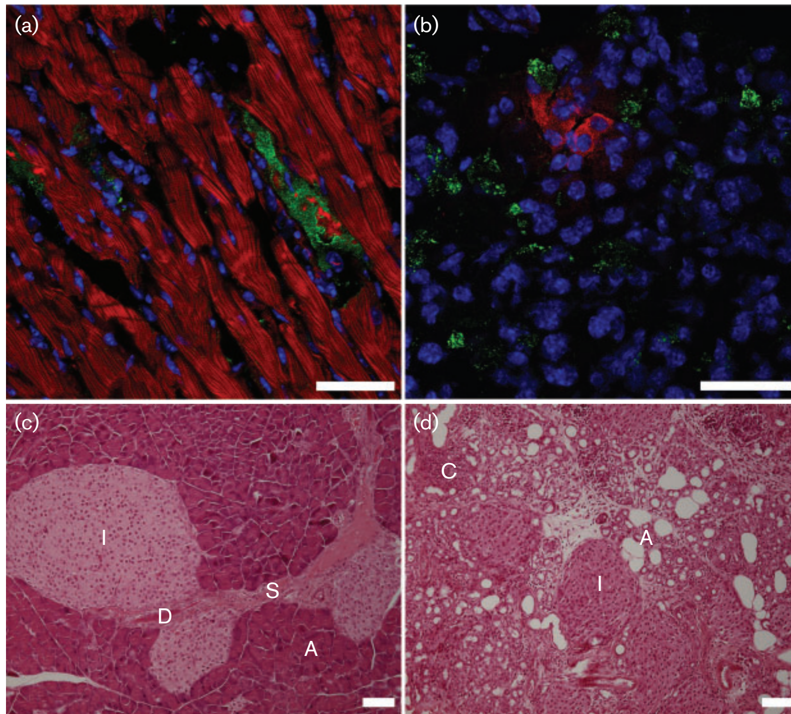

Fig. 1. (a) Striated ventricle muscle fibres of a BALB/c mouse 1 day after IP challenge with FMDV O UKG 34/2001. FMDV capsid (green) is localized to cardiomyocytes [red, phalloidin; blue, 4′,6-diamidino-2-phenylindole (DAPI)]. (b) Pancreas of a C57BL/6 mouse 1 day after IP challenge with FMDV O UKG 34/2001. FMDV capsid (green) is detectable in the pancreas [red, insulin (islets of Langerhans); blue, DAPI]. No FMDV capsid was detected in pancreas samples at 28 or 46 days post IP challenge (data not shown). (c) Pancreas of a C57BL/6 mouse 21 days post ovalbumin IP inoculation. Routine haematoxylin and eosin (H & E) stain demonstrates the normal morphology of the pancreas: A, glandular acinar cells of the exocrine pancreas; D, interlobular duct; I, islets of Langerhans of the exocrine pancreas; S, septa of the collagenous capsule. (d) Pancreas of a C57BL/6 mouse 21 days after IP challenge with FMDV O UKG 34/2001. Routine H & E stain demonstrates the chronic pathology following FMDV infection: A, acinar cells; C, cellular infiltration; I, islets of Langerhans. Bars: (a, b) 40 μm, (c, d) 100 μm.

pancreatic pathology in target species, justifies additional investigation of FMDV-induced pancreatitis. Exploring viral and host mechanisms for FMDV-induced pancreatitis is supported further by the potential contribution of this pathology to chronic long-term metabolic sequelae of FMD, which are major contributors to the impacts of FMD upon livestock productivity (Barasa *et al.*, 2008). There are several aspects of FMDV pathogenesis in the mouse model which are similar to those described in natural hosts. The similarities described herein provide support for the mouse as a model to investigate the role of host genetic factors and viral factors involved in FMD pathogenesis. However, the major contribution of the FMD mouse model has been an improved understanding of the immune response.

Humoral immunity to FMDV infection and vaccination

The interaction of FMDV with the immune system of target species remains incompletely understood, partly due to the cost and logistics of large-animal experimentation but mainly due to the paucity of immune reagents and incomplete knowledge of their immune systems. Consequently, laboratory animal models are an essential tool for investigating viral and host factors that contribute to FMD pathogenesis. In selecting a laboratory animal to model FMDV immunity, a number of factors must first be considered: animals must be susceptible to infection, support viral replication and the immune response must play an active role in controlling infection. Mice are the most widely used laboratory animal to model FMDV immune responses; the reasons for this are largely practical in terms of cost, coupled with the availability of immune reagents and our ability to manipulate mice genetically. Although adult mice are not susceptible to natural infection and do not develop discernible FMD lesions, following IP inoculation FMDV replication leads to viraemia and elevated SNA titres (Borca *et al.*, 1984; Fernández *et al.*, 1986). In addition, Borca *et al.* (1984) demonstrated that immunity can be transferred by immune cells to immunosuppressed mice, and viral clearance coincided with the onset of SNA titres. These data confirm an active role of the immune response and highlight the importance of humoral immunity in the FMD murine model.

The significance of humoral immunity in controlling FMDV infection is well documented and antibodies form the major mechanism of protection (Loeffler & Frosch, 1897). It is also accepted that SNA titres determined by using *in vitro* virus neutralization test (VNT) assays correlate with protection in vaccinated livestock, although exceptions do occur when protection predicted by VNT is not observed, and vice versa (Doel, 1996). Natural infection induces a rapid and long-lived immunity in cattle that is characterized by the maintenance of high titres of SNA, for example up to 4.5 years (Cunliffe, 1964), and protection from challenge has been demonstrated up to 5.5 years after initial infection (Garland, 1974). By contrast, current inactivated vaccines induce a comparatively short duration of immunity, with revaccination recommended at least every 6 months (Doel, 1996). The precise reasons for this discrepancy are unknown and understanding infection-induced immunity in order to enhance vaccine-induced immunity has been a major research target. The primary

response to infection in cattle is characterized by serum IgM detectable between 3 and 7 days post intradermolingual challenge, reaching a peak between 5 and 14 days p.i., then slowly declining to an undetectable level by 56 days p.i. Recently, Pega *et al.* (2013) demonstrated that the early IgM response forms the major component of the *in vitro* virus-neutralizing activity in cattle serum during the first 6 days p.i. However, isotype switching occurs rapidly with specific IgG1 and IgG2 detected from 4 days p.i. and reaching maximal levels from 14 days p.i. (Collen, 1994; Doel, 2005; Juleff *et al.*, 2009; Pega *et al.*, 2013; Salt *et al.*, 1996). IgA is initially detected in serum and OPF from 7 days p.i., reaching a peak serum titre between 7 and 14 days p.i. (Collen, 1994; Doel, 2005; Salt *et al.*, 1996). The IgA titre in serum slowly declines from 14 days p.i. except in 'carriers', where a significant second late response is detected around 28 days p.i. In contrast to serum titres, a second late IgA response is detected from 28 days p.i. in OPF of all infected cattle independent of their 'carrier state'. Thereafter, the OPF IgA titre either declines to undetectable levels or persists in animals classified as 'carriers' (Parida *et al.*, 2006; Salt *et al.*, 1996). Virus-neutralizing activities of both serum and OPF are higher in carrier than non-carrier animals, consistent with continued immune stimulation (McVicar & Sutmoller, 1974). Although similar early B-cell responses have been reported in both contact- and needle-challenged swine (Eblé *et al.*, 2007; Pacheco *et al.*, 2010b), the duration of immunity has been shown in some cases to be short lived, with convalescent animals succumbing to rechallenge 3 to 6 months after first exposure (Gomes, 1977; McKercher & Giordano, 1967) and it is generally accepted that the duration of immunity in convalescent pigs is significantly shorter than in cattle (Doel, 1996).

Vaccination protects cattle and pigs from the development of clinical disease but not typically from subclinical infection. Vaccination of cattle with FMDV antigen using either oil or aluminium hydroxide/saponin formulations is also characterized by a rapid antibody response, with FMDV-specific IgM detected from 3 to 4 days post vaccination (p.v.), IgG1 and IgG2 from 4 to 6 days p.v. and SNA titres detected as early as 3 to 4 days p.v. (Abu Elzein & Crowther, 1981; Carr *et al.*, 2013). These data are consistent with studies demonstrating protection from challenge from 4 days post high-potency vaccination (Barnett & Carabin, 2002). Similar to cattle, the onset of immunity in pigs following high-potency oil-adjuvanted emergency vaccination is surprisingly rapid and seems to correlate with a rapid B-cell response (Eblé *et al.*, 2007; Pacheco *et al.*, 2010b) with protection from challenge as early as 3 to 5 days p.v. (Barnard *et al.*, 2005). Although the antibody response to vaccination varies depending on the antigen dose, quality and type of adjuvant used, there appear to be consistent differences from the infection response. Compared with the short-lived IgM responses p.i., higher and longer-lasting serum IgM titres have been reported for both cattle (80 days p.v.) and pigs (84 days p.v.) (Abu Elzein & Crowther, 1981; Cox *et al.*, 2003). While infected cattle develop a rapid IgG and IgA response in OPF, vaccinated cattle only develop an IgG response; IgA is not detected after vaccination or even at subsequent revaccination and low titres have only been detected following multiple administrations (Francis *et al.*, 1983; Garland, 1974). In contrast to cattle, both serum and salivary IgA can be detected in pigs from 7 days p.v., and a correlation has been described between mucosal IgA titres and protection against contact exposure in pigs (Eblé *et al.*, 2007).

Similar to cattle and pigs, a rapid SNA response is elicited by FMDV infection in mice. However, the response is more comparable to cattle as high titres are maintained for prolonged periods. López *et al.* (1990) demonstrated high titres maintained to 500 days post IP infection; the response was protective as mice were resistant to rechallenge with homologous virus. The antibody response to FMDV infection was first characterized in detail in mice, before reagents were available for target species. There is still a lack of reagents for a number of antibody isotypes in target species and immunoglobulin genes are still being characterized in livestock, especially for pigs, where the specificity of available reagents is a major concern (Pacheco *et al.*, 2010b). Following IP challenge of mice, FMDV-specific serum IgM titres can be detected from 3 days p.i., IgG1 and IgG3 titres from 7 days p.i. and IgG2a and IgG2b from 14 days p.i. (Collen *et al.*, 1989; Pérez Filgueira *et al.*, 1995). IgG2b has been shown to be the dominant IgG subclass in response to IP challenge, followed by IgG1, IgG2a and IgG3, respectively (Pérez Filgueira *et al.*, 1995). Low serum IgA titres have been reported; however, the mucosal FMDV immune response in mice has not been described despite this region being the most common site for primary virus replication in target species (Pacheco *et al.*, 2010a). As in natural hosts, FMD vaccines prepared with inactivated virus and adjuvants induce lower antibody titres which persist for less time than those induced by live virus. Despite the short duration of immunity, these vaccines are effective at protecting mice against challenge with lethal doses of FMDV (Salguero *et al.*, 2005). The antibody isotype profile of mice in response to vaccination is different from that in response to infection; the response can also be altered by the vaccine formulation or by addition of immune modulators to more closely resemble the infection responses. Antibody responses of mice immunized with conventional oil or aluminium hydroxide formulated FMD vaccines are dominated by either IgG1 or IgG2a, respectively, and these were the first isotypes to be elicited in each case (Pérez Filgueira *et al.*, 1995). For both formulations, low titres of IgG2b were transiently detected at 60 days p.v. Incorporating immune modulators, for example lipopolysaccharides, enhanced the antibody response, especially the IgG2b response, and augmented resistance to viral challenge at 210 days p.v. (Berinstein *et al.*, 1991, 1993). FMDV-specific IgA has been detected in saliva of subcutaneously vaccinated mice, a response which can be enhanced by incorporating immune modulators and which may merit further investigation due

to the correlation of vaccine-induced mucosal IgA titres with protection from challenge in pigs (Batista *et al.*, 2010; Eblé *et al.*, 2007). Incorporating immune modulators significantly elevated titres of the complement-fixing IgG2a and 2b subclasses and increased protection against challenge. Of note, IgG1 titres were not significantly affected by incorporating immune modulators (Batista *et al.*, 2010).

In contrast to farm animals, there is extensive knowledge on the regulation of antibody isotype switching by helper T-cells in the murine immune response, the role of cytokines in directing B-cell responses and the interactions of antibodies with Fc receptors on different cell types (Mosmann & Coffman, 1989). Antibody isotype profiles are restricted by the nature of the antigen and by the form in which the antigen is processed and presented to the immune system; an understanding of this process is important when considering the mechanism of immune protection. Protective humoral immunity to pathogens is contributed by distinct B-cell subsets with unique activation requirements and response signals. In the mouse, IL-4 preferentially induces class switching to IgG1 and transforming growth factor β induces switching to IgG2b, the predominant isotype generated in FMDV-infected mice. T-helper 2 cells produce both of these cytokines and may play a role in driving a T-dependent B-cell response dominated by IgG2b and IgG1 (Hoyler *et al.*, 2013). Rapid synthesis of the complement-fixing IgG subclasses 2a, 2b and 3 would agree with McCullough *et al.* (1992), who proposed that effective protection is achieved through antibody-enhanced phagocytosis of FMDV by cells of the reticuloendothelial system. In addition, the early induction of isotype class switching leading to FMDV-specific serum IgG1 and IgG3 by 7 days p.i. will drive the interaction of FMDV immune complexes with the high affinity receptor FcγRI expressed on monocytes, macrophages and dendritic cells, modulating the adaptive immune response (van der Poel *et al.*, 2011). Vaccination studies in mice demonstrated that immune modulators could enhance complement-fixing IgG subclasses and augment resistance to virus challenge (Batista *et al.*, 2010; Pérez Filgueira *et al.*, 1995). These data provide support for complement-mediated phagocytosis playing a significant role in viral clearance; however, no direct correlation has been made between the different antibody isotypes elicited and efficacy of protection in FMD laboratory animal models or target species. It is possible that immune mechanisms in the mouse model leading to long-lasting humoral immunity are similar to those in target species. A major drawback that must be considered is the established IP route of challenge in the mouse model. It is difficult to relate protection afforded by vaccination in mice as challenge virus will interact with different cell populations in the peritoneal cavity, which may not reflect the natural challenge routes in target species. In relation to antibodies and comparing cross-species interactions with FMDV, it is now clear that bovine antibodies have a number of unusual characteristics compared with other vertebrates (Wang *et al.*, 2013). The unusual structure of the exceptionally long heavy-chain complementary determining region 3 may allow bovine antibodies to bind antigenic targets that are difficult for mouse antibodies to access, such as channels and pores (Wang *et al.*, 2013). It is noteworthy that mouse monoclonal antibodies have been used to identify antigenic sites on the FMDV capsid and these sites are located on structural protrusions on the virus surface, formed by loops connecting β-barrel structures of the three outer capsid proteins (Baxt *et al.*, 1989; Kitson *et al.*, 1990; Mateu *et al.*, 1990; Sanyal *et al.*, 1997). Five neutralizing antigenic sites on the capsid of serotype O FMDV have been mapped using mouse monoclonal antibodies and the G-H loop of VP1 was identified as immunodominant, and as a consequence the G-H loop region has been a major target for synthetic peptide vaccine studies (Crowther *et al.*, 1993). A significant component of the research on these experimental vaccines was performed in mice and guinea pigs; the peptide vaccines induced high titres of SNA and protection from severe challenge infection in the FMDV small laboratory animal models (as reviewed by Brown, 1992). However, the antibody response in cattle and pigs was poor; the non-responsiveness was studied in inbred mice and was overcome by incorporating T-helper cell epitopes (Francis *et al.*, 1987). These constructs performed well in mice, guinea pigs and pigs, providing protection from infectious challenge and high titres of SNA (Wang *et al.*, 2002). The difference between the response in these species and the response reported in cattle is dramatic. Vaccinated cattle developed antibodies to the peptide, as determined by ELISA; however, the majority of animals did not develop SNA titres as determined by VNT and all animals developed clinical FMD upon challenge at 21 days p.v. (Rodriguez *et al.*, 2003). It is clear from large-scale FMDV peptide vaccine studies in cattle that efficacy is difficult to achieve (Taboga *et al.*, 1997). One could speculate that differences in antibody responses in cattle compared with other species are due to the structure of cattle antibodies and how they interact with FMDV. Further work is justified to explain these incongruous antibody responses and to investigate antigenic sites on the FMDV capsid which are recognized by antibodies from target species.

Importance of cell-mediated immunity in response to FMDV infection

A number of research groups have attempted to ascertain the role of T-cells during FMDV infection and the majority of these studies have been in mice. Borca *et al.* (1986) were the first to describe a protective immune response in mice that was independent of T-cells. Athymic nude mice, which cannot generate mature T-cells, were challenged intraperitoneally and presented near-identical curves of viraemia, SNA responses and tissue viral clearance compared with those of their heterozygous littermates. These investigators also demonstrated that adoptive transfer of enriched splenic B-cells from previously challenged mice, harvested

at 8 days p.i., aborted viraemia in irradiated recipients. By contrast, adoptive transfer of enriched splenic T-cells from immune donors was totally ineffective in protecting against FMDV. The same laboratory demonstrated that the prolonged immune memory following FMDV infection in mice was not dependent on T-cells (López *et al.*, 1990). Athymic mice and their euthymic littermates were FMDV infected intraperitoneally; both groups showed a prolonged SNA response up to 240 days p.i. and remained protected against rechallenge. However, the kinetics of the SNA response differed markedly between euthymic and athymic mice. Both groups presented similar titres 8 days p.i.; however, from 14 days p.i. the titres in athymic mice were significantly lower and continued to decrease to 240 days p.i. By contrast, the titres in euthymic mice continued to increase from 14 to 240 days p.i. Athymic mice may therefore have succumbed to higher titre challenge based on the association between SNA titres and protection. These data support a functional role for T-cells in maintaining high titres of SNA in mice post FMDV infection, yet T-cells were not essential for maintaining protective immunity in this challenge model. Further support for T-cells in the anti-FMDV antibody response is provided by Collen *et al.* (1989), who demonstrated a significantly lower frequency of FMDV-specific IgG antibody secreting cells in the spleen of athymic mice compared with euthymic mice during the first 12 days after intravenous FMDV challenge. Interestingly, sera from both groups contained similar FMDV-specific IgM, IgG2a, IgG2b, IgG3 and IgA titres at 7 and 10 days p.i.; however, IgG1 titres were significantly lower at both time points in athymic mice. These data suggest that isotype class switching in response to FMDV infection can occur in the absence of T-cells in mice. However, it must be recognized that low numbers of functional T-cells have been demonstrated in athymic nude mice and Collen *et al.* (1989) detected low numbers of splenic T-cells in their athymic nude mice (Ikehara *et al.*, 1984).

Although the relevance of immune mechanisms in mice which lead to rapid and protective FMDV antibody responses to the situation in target species is unclear, they have served to focus research efforts. Borca's data demonstrating that FMDV is a T-independent antigen in mice, combined with a number of reports of no or very low *in vitro* proliferation of peripheral blood T-cells despite the development of high SNA titres in FMDV-challenged cattle, led researches to question the role that T-cells play (Doel, 1996). This role has been investigated recently in cattle using subset-specific antibody depletion (Juleff *et al.*, 2009). Partial CD8$^+$ T-cell depletion and complete WC1$^+$ $\gamma\delta$ T-cell depletion had no discernible effect on the kinetics of infection, clinical signs and immune response to FMDV. The failure to achieve complete CD8$^+$ depletion was not unexpected as mAb-mediated depletion of these cells is notoriously difficult; consequently, their role cannot be described in target species using currently available reagents (Naessens *et al.*, 1998). Although FMDV-specific MHC class I-restricted CD8$^+$ T-cell responses have been reported in infected or vaccinated cattle (Guzman *et al.*, 2008), data from the mouse model suggest these cells do not play a major role, a conclusion supported by the partial CD8$^+$ depletion studies reported by Juleff *et al.* (2009), and evidence of a role for cytolytic T-cells in the immune response to FMDV is still lacking. In contrast to mice, $\gamma\delta$ T-cells are considered a major T-lymphocyte population in ruminants. It is noteworthy that WC$^-$ cells, which represent approximately 30 % of the mononuclear cell population in bovine splenic red pulp, would not have been affected by the WC1$^+$ depletion protocol (Machugh *et al.*, 1997). Complete CD4$^+$ T-cell depletion inhibited antibody responses to a G-H loop peptide and non-structural polyprotein 3ABC, but did not affect the rapid isotype-switched SNA response, clinical response or virus clearance (Juleff *et al.*, 2009). Therefore, CD4$^+$ T-cells do not play a major role in the resolution of acute FMD in cattle; however, other T-cell subsets may have contributed to the response, including isotype class switching, and the outcome might have been different if multiple T-cell subsets were depleted simultaneously. In addition, depletion was only temporary; therefore, the contribution of T-cell-mediated responses to the maintenance of long-lived serological memory, typically described in FMDV-infected cattle, remains unclear.

The immune mechanisms leading to the rapid and protective T-independent antibody response have been investigated by Ostrowski *et al.* (2007) in mice. Both virus localization and FMDV-mediated modulation of dendritic cell (DC) functionality are reported to play a major role. These investigators demonstrated *in vitro* that FMDV-infected bone marrow-derived DCs (BMDCs) can directly stimulate splenic marginal zone B-cells (CD9$^+$ 'innate B-lymphocytes') to secrete anti-FMDV IgM in a process dependent on DC-derived IL-6 and B-cell-derived IL-10, but independent of T-cells. However, T-cell help was required to induce class switching to different IgG isotypes in their *in vitro* model (Ostrowski *et al.*, 2007). It is noteworthy that both IL-10 and IL-6 have been shown to promote innate-like B-lymphocyte proliferation and terminal differentiation during the development of an immune response against other pathogens (Montes *et al.*, 2006). IL-10 can also play an immunosuppressive role by suppressing antigen-presenting cell (APC) and T-cell function by inhibiting chemokine secretion and MHC class II expression (Pestka *et al.*, 2004). Although Collen *et al.* (1989) demonstrated isotype class switching in athymic mice in response to FMDV infection, it is still not clear if infection induces isotype class switching *in vivo* in the complete absence of T-cells. Ostrowski *et al.* (2007) only detected IgM isotype up to 6 days p.i. in athymic mice; by contrast, IgG1 and IgG3 were detectable in euthymic mice although titres were still low at this early time point and no data were provided for later time points. These reports provide further support for T-cell functions to achieve high SNA titres and for long-lived IgG responses p.i. Similar

T-cell dependency has been reported for other acute cytopathic viral infections in mice, for example vesicular stomatitis virus, where the production of neutralizing IgG antibody is dependent on T-cells, while early infection is characterized by a rapid T-independent neutralizing IgM response (Ostrowski *et al.*, 2007).

Interestingly, although FMDV infection of mouse BMDCs is abortive, infected cells lose their ability to stimulate T-cells and differentiate towards a macrophage-like phenotype (Ostrowski *et al.*, 2005). In fact, a generalized suppression of T-dependent responses has been observed *in vivo* in mice between 3 and 5 days p.i., thought to be mediated in part by MHC class II and CD40 downregulation on DCs and by IL-10 (Ostrowski *et al.*, 2005). These results are supported by Langellotti *et al.* (2012), who recently demonstrated in mice that FMDV infection induces a reduction in splenic plasmacytoid dendritic cells (pDCs) and conventional DCs ($CD11c^{+}/CD8\alpha^{+/-}$) and lymphocyte proliferation is inhibited during early infection, with inhibition thought to be associated with IFN-α induction. Significantly increased levels of IFN-α protein were detected by ELISA in plasma of FMDV-infected mice at 1 day p.i., with levels returning to background by 3 days p.i. (Langellotti *et al.*, 2012). Similar to the mouse, FMDV infection of porcine pDCs, monocyte-derived DCs (MODCs) and BMDCs is abortive (Guzylack-Piriou *et al.*, 2006; Harwood *et al.*, 2008; Rigden *et al.*, 2002). Although porcine MODCs have been reported to respond *in vitro* by increasing expression of MHC class II and CD86, consistent with phenotype maturation, data generated *in vitro* from cells derived from infected pigs are more consistent with the suppressive responses described *in vitro* and *in vivo* in mice (Summerfield *et al.*, 2009). FMDV infection impaired MODC function; infected cells produced no IFN-γ, less IFN-α and substantial amounts of IL-10, and these investigators demonstrated that IL-10 was responsible for *in vitro* T-cell inhibition (Diaz-San Segundo *et al.*, 2009; Nfon *et al.*, 2008). Diaz-San Segundo *et al.* (2009) also demonstrated significant amounts of IL-10 in serum of FMDV-infected swine and proposed that a reduction of T-cell activity by IL-10 may actually result in a more potent induction of SNA and support T-independent antibody responses. This hypothesis is consistent with the dependency of the FMDV-innate B-cell response in mice on IL-10 (Ostrowski *et al.*, 2005). The impairment of porcine MODC function during FMDV infection *in vitro* is consistent with reports in mice. In addition, the generalized suppression of T-dependent responses in mice 3 to 5 days p.i. is consistent with reports in swine that T-cell function is affected during acute FMDV infection, characterized by T-cell unresponsiveness and lymphopenia (Bautista *et al.*, 2003; Diaz-San Segundo *et al.*, 2006, 2009). Comparable to the mouse, serum IFN-α protein is also detectable in pigs from 2 to 3 days p.i. and lymphopenia is reported to coincide with the serum IFN-α response and peak viraemia (Nfon *et al.*, 2010). Similar to splenic pDC and conventional DC numbers in infected mice, circulating pDC numbers in pigs transiently decline during FMDV infection (Nfon *et al.*, 2010). Porcine pDCs are susceptible to FMDV infection, but only in the presence of antibody and their response is characterized by secretion of high levels of IFN-α (Guzylack-Piriou *et al.*, 2006). There is a report of FMDV, type C serotype, productively infecting T- and B-cells resulting in lymphopenia (Diaz-San Segundo *et al.*, 2006); however, the rapid recovery from lymphopenia in mice and swine is more consistent with altered cell migration than cell loss and subsequent repopulation (Golde *et al.*, 2011). IFN-α could play a role in the observed lymphopenia as type-I IFN has been shown in mice to directly regulate lymphocyte recirculation, leading to a transient blood lymphopenia (Kamphuis *et al.*, 2006). As described for mice, type-I IFN may also promote B-cell responses and downregulate T-cell responses. Nfon *et al.* (2010) also proposed that the short-lived IFN-α response may contribute to the resolution of FMDV viraemia prior to induction of specific immunity; this hypothesis is supported by data on prophylactic administration of IFN by adenovirus vectors, which rapidly induces a FMDV-protective state in swine (Dias *et al.*, 2011). FMDV is highly sensitive to the effects induced by type-I IFNs *in vivo* and *in vitro* (reviewed by Summerfield *et al.*, 2009). In addition to endosomal sensors of RNA in cells of the immune system, for example DCs and toll-like receptors (TLR) 3, 7 and 8, which are likely to play an important role, it has been shown that IP inoculation of RNA transcripts corresponding to FMDV S, IRES and 3′-non-coding regions can trigger type-I IFN in suckling mice and reduce their susceptibility to subsequent infection (Rodríguez-Pulido *et al.*, 2011a, b). These results suggest the presence of pathogen-associated molecular patterns in the FMDV genome that are able to induce innate immunity in mice leading to rapid antiviral responses involving type-I IFNs. Of particular interest, it has been demonstrated recently in mice that type-I IFN contributes to T-cell-independent antibody responses to pathogens by promoting participation of follicular B-cells and, therefore, enhancing the overall magnitude of the antibody response to one that is class-switched and dominated by IgG isotypes (Swanson *et al.*, 2010). Clearly, innate immunity can drive the humoral immune response to pathogens, and the innate immune response to FMDV remains a major knowledge gap (Summerfield *et al.*, 2009).

In contrast to data derived from mice and pigs, FMDV infection of bovine MODCs is productive and infected cells die, losing their ability to stimulate T-cell proliferation *in vitro* (Robinson *et al.*, 2011). One would expect these interactions to lead to generalized suppression of T-dependent responses and lymphopenia during acute infection *in vivo*, as reported for mice and pigs. Yet this does not seem to be the case as there are no reports of generalized immunosuppression during the acute phase of FMDV infection in cattle (Windsor *et al.*, 2011). Compared with the significant levels of the inhibitory cytokine IL-10 and serum IFN-α protein levels in pigs and mice, only transient and low titres of biologically active type-I IFN and IL-10 have been reported during acute infection in cattle (Reid *et al.*, 2011; Windsor *et al.*, 2011). In

addition, cattle did not develop leukopenia and proliferative responses of peripheral blood mononuclear cells (PBMCs) to either mitogen or third party antigen were not suppressed (Windsor *et al.*, 2011). However, as reported previously, animals do not develop significant FMDV-specific T-cell responses during the resolution of acute infection and up to 19 days p.i. (Garcia-Valcarcel *et al.*, 1996; Windsor *et al.*, 2011). Robinson *et al.* (2011) proposed that the poor FMDV-specific T-cell response during acute infection was the direct result of FMDV immune-complex-mediated depletion of APCs at sites of infection, leaving the animal able to respond normally to third party antigens, consistent with no generalized immunosuppression (Windsor *et al.*, 2011). The absence of leukopenia and generalized immunosuppression may also be associated with the comparatively low levels of type-I IFN and IL-10 during acute infection in cattle. High levels of these cytokines during acute infection could also explain the more severe clinical signs generally described following FMDV infection in pigs (Alexandersen *et al.*, 2003). Interestingly, the FMDV-specific T-cell proliferative response has been reported to gradually increase from 28 days p.i. in cattle, a response attributed to the carrier state and the presence of persisting virus in ruminants (Collen, 1991). As proposed for the mouse model, the T-independent immune response leading to resolution of acute FMD may, therefore, be followed by a T-dependent phase required for maintenance of serological memory.

Importance of cell-mediated immunity in response to FMDV vaccination

Compared to the immune response elicited by live virus, the complexity of the response elicited by inactivated vaccine virus preparations is far lower. Live FMDV induces potent and long-lived systemic and mucosal antibody responses due to its ability to replicate, deliver RNA to endosomal compartments and initiate innate immune responses (Zabel *et al.*, 2013). Engineering vaccine formulations to mimic natural infection could provide more robust and long-lasting immunity, especially at mucosal surfaces. However, present knowledge of immune responses in target species offers little insight into the importance of different T-cell subsets in the antiviral responses. In contrast to infection, FMDV vaccination induces rapid T-cell responses, and FMDV-specific $CD4^+$ T-cell proliferation has been detected in cattle as early as 7 days p.v. (Carr *et al.*, 2013; Doel, 1996). Similar results have been reported in mice (Ostrowski *et al.*, 2005) and inactivated FMDV has been shown to increase $CD8^+$ and regulatory T-cell ($CD4^+CD25^+Foxp3^+$) numbers in the spleen (Langellotti *et al.*, 2012). In addition, porcine $\gamma\delta$ T-cells have been shown to proliferate and express cytokine and chemokine mRNA in response to FMDV antigen *in vitro*, and similar proliferative responses have been reported for bovine $CD8^+$ and $WC1^+$ $\gamma\delta$ T-cells, although $CD4^+$ T-cells are the predominant PBMC type that respond specifically to FMDV antigen *in vitro* (Carr *et al.*, 2013; Takamatsu *et al.*, 2006). The importance of T-cells in the FMD vaccine response was first demonstrated in the mouse model. Piatti *et al.* (1991) demonstrated by adoptive transfer of cells from FMDV-antigen-immunized mice, that doses of B-cells 20 times lower than those shown to be sufficient to abort viraemia alone are effective when FMDV-primed T-cells are present. Therefore, FMDV-specific T-cells can enhance anti-FMDV B-cell responses when lower doses of antigen are administered. Interestingly, T-cells sensitized with an unrelated T-cell-dependent antigen, keyhole limpet haemocyanin, did not enhance the response, suggesting the dependence is antigen-specific (Piatti *et al.*, 1991). The importance of stimulating $CD4^+$ T-cell responses in order to achieve optimal antibody responses to vaccination has recently been demonstrated in cattle (Carr *et al.*, 2013). Depleting $CD4^+$ T-cells significantly reduced SNA titres and delayed isotype class switching to FMD-killed vaccines; therefore, in contrast to the response to infection, $CD4^+$ T-cells clearly fulfil an important facilitator role. As reviewed recently by Golde *et al.* (2011), a detailed knowledge of the antigenic regions recognized not only by B-cells, but also by T-cells of target species, is crucial to design novel vaccines to support serological memory.

Similar to infection, FMDV-antigen localization and interactions with DCs are likely to play a major role in the protective immune responses induced by vaccination. As demonstrated in mice, cattle and pigs, FMDV-infected DC populations do not stimulate FMDV-specific T-cell proliferation; by contrast, DCs loaded with UV-inactivated FMDV (UV-FMDV) stimulate a significant proliferative response *in vitro* and can significantly boost antibody responses *in vivo* when adoptively transferred to FMDV-primed mice (Ostrowski *et al.*, 2005; Robinson *et al.*, 2011; Summerfield *et al.*, 2009). Similar to the results for FMDV-infected DCs, Ostrowski *et al.* (2007) demonstrated *in vitro* that UV-FMDV-loaded BMDCs could directly stimulate splenic $CD9^+$ B-cells. However, IgM was detected later at 7 days p.v. compared with 3 days p.i., and at significantly lower titres. FMDV-infected BMDCs also stimulated IgG2a, IgG2b and IgG3; by comparison, the only class-switched isotype elicited by UV-FMDV was IgG2a (Ostrowski *et al.*, 2007). UV-FMDV also elicited IgM responses in splenocyte cultures derived from athymic mice, but similar to the response to infectious virus, no IgG subclasses were detected. Therefore, in comparison to the response induced by FMDV-infected BMDCs, DCs loaded with UV-FMDV are significantly less efficient in directly stimulating innate $CD9^+$ B-cells to secrete T-independent antibodies and the delayed response is typical of a T-dependent immune response. The cytokine profiles were also distinct; UV-FMDV-loaded mouse BMDCs did not induce IL-10 secretion upon co-culture with splenocytes and secretion of IL-6 was significantly lower than that by FMDV-infected BMDCs (Ostrowski *et al.*, 2005). Both of these cytokines were shown to be essential *in vitro* for robust anti-FMDV antibody responses and could in part explain the differences in kinetics, magnitude and isotype profile of the antibody responses (Ostrowski *et al.*, 2007). By contrast, UV-FMDV-loaded

BMDCs induced IL-2 secretion *in vitro* and IFN-γ secretion both *in vivo* and *in vitro*. Compared with IL-6 and IL-10, neutralizing IFN-γ in culture did not impair the secretion of anti-FMDV antibodies (Ostrowski *et al.*, 2005, 2007). Contrary results have been reported for splenic CD11c^{+} cells derived from mice 3 days after IP immunization with binary ethyleneimine inactivated FMDV (BEI-FMDV) as used in most current inactivated vaccines (Langellotti *et al.*, 2012). In contrast to reports by Ostrowski *et al.* (2005, 2007), BEI-FMDV increased the production of the pro-inflammatory cytokines IL-6, IL-10 and TNF-α while infection only induced poor levels of IL-6 and IL-10 but significantly more IFN-α. In fact, Langellotti *et al.* (2012) reported that BEI-FMDV failed to stimulate T-cell proliferation and concluded that BEI-FMDV induces a regulatory state that inhibits effector mechanisms. This is in contrast to reports by Ostrowski *et al.* (2005) that UV-FMDV improved the functionality of BMDCs, favouring the development of typical T-dependent responses. The reasons for these discrepancies are not clear. It is noteworthy that FMDV O1 Campos was used in both systems; however, different methods were used for FMDV inactivation and the investigators also isolated different DC populations. The report by Ostrowski *et al.* (2005) is consistent with bovine and porcine data that UV-FMDV-loaded DCs are highly efficient APCs and that DC targeting could improve both T- and B-cell responses to FMDV antigen (Robinson *et al.*, 2011; Summerfield *et al.*, 2009). One could speculate the results would have been different if BEI-FMDV was used as opposed to UV-FMDV, as viral RNA remains mostly intact following BEI-treatment (Brown, 2001) and the results in target species would be more aligned to the regulatory state reported by Langellotti *et al.* (2012). The conflicting data generated in mice using UV-FMDV and BEI-FMDV warrant further investigation.

As described previously in this review, immune modulators can be incorporated into vaccine preparations to enhance mucosal and circulating antibody responses in mice and augmented resistance to FMDV challenge for long periods (Berinstein *et al.*, 1991, 1993). Targeting cells of the innate immune system in order to induce rapid and long-lasting protective immunity remains an active area of research. Targeting innate immunity in combination with conventional vaccination offers a means to achieve early cross-serotype protection before onset of vaccine-induced adaptive immunity. Based on *in vitro* and *in vivo* observations that IFN is effective against FMDV, IFN-inducers were initially tested in mice for their ability to induce innate protection against FMDV. A single IP administration of poly I : C was shown to protect suckling mice from lethal FMDV challenge; protection was effective for 48 h after administration and survival correlated with serum IFN titres (Richmond & Hamilton, 1969). These experiments were extended to target species to test whether protection against FMDV challenge could be similarly induced. Unexpectedly, administering poly I : C intravenously to both cattle and goats failed to offer any degree of protection against FMDV, and similar results were reported following IP administration of poly I : C to pigs (Cunliffe *et al.*, 1977; McVicar *et al.*, 1973). Therefore, data obtained from the mouse model were considered of limited value and irrelevant for target species. Despite this discouraging experience, the mouse model demonstrated proof-of-principle that protection against FMDV challenge could be achieved *in vivo* by stimulating innate immune responses. Researchers continued to target innate immune responses to induce rapid protection, and success has been demonstrated recently following administration of adenovirus (Ad5) vectors expressing type-I or type-III IFN to pigs (Dias *et al.*, 2011; Perez-Martin *et al.*, 2014) and type-III IFN, but not type-I IFN, to cattle (Perez-Martin *et al.*, 2012). It is unclear why type-I IFN protected pigs but did not protect cattle as pre-treatment of bovine cell cultures with porcine or bovine IFN-α or -β inhibits FMDV replication (Chinsangaram *et al.*, 2001). Similar to many other viruses, FMDV has developed mechanisms to antagonize the IFN response, for example the viral proteases L^{pro} and $3C^{pro}$ inhibit IFN production; however, type-I IFN is readily detected in serum after FMDV infection in cattle, pigs and mice (de los Santos *et al.*, 2007; Wang *et al.*, 2010, 2012). Recently $3C^{pro}$ has been shown to inhibit the IFN signalling pathway by blocking STAT1/STAT2 nuclear translocation and knockout mice, for example different STAT deficient strains, may be valuable for identifying innate signalling pathways relevant for FMDV pathogenesis (Akira, 1999; Du *et al.*, 2014). Recently, structural domains predicted to enclose stable double-stranded RNA in the 5′- and 3′-non-coding regions of the FMDV genome have been shown to trigger type-I IFN in suckling mice (Rodríguez-Pulido *et al.*, 2011a). These RNAs were also able to induce an antiviral state in porcine cells and reduce susceptibility to challenge when administered intraperitoneally to suckling mice. Recently, Venezuelan equine encephalitis virus empty replicon particles (VRPs) have been shown to induce an innate immune response that can protect C57BL/6 mice from lethal FMDV challenge, a response dependent on a functional type-I IFN system and IFN-γ-inducible protein 10 (Diaz-San Segundo *et al.*, 2013). Interestingly, Diaz-San Segundo *et al.* (2013) demonstrated that VRPs induce a more potent protective innate response *in vitro* than the Ad5 vector, which has been used as a vector for FMD vaccines with variable results (Moraes *et al.*, 2002). It will be interesting to follow how these studies of the innate response against FMDV translate to the target species, and if they offer further support for the mouse model.

Duration of protective immunity

In contrast to infection, current inactivated FMD vaccines formulated with adjuvant elicit short-lived protection in target species and in laboratory animal models. Although there are occasional exceptions, SNA titres correlate with vaccine-induced protection in cattle, pigs and mice. The mechanism for maintaining long-lived protective serological immunity post viral infections remains a major knowledge gap. As serum antibodies have a short half-life,

reported to be less than 3 weeks in adult mice (Talbot & Buchmeier, 1987; Vieira & Rajewsky, 1988), continual replenishment is required either by long-lived plasma cells, activation of memory B-cells to differentiate into plasma cells or on-going recruitment and differentiation of naive B-cells into antibody secreting plasma-blasts and plasma cells to maintain protective humoral immunity (Wrammert & Ahmed, 2008). Various mechanisms have been proposed to explain the maintenance of serological immunity after FMDV infection. These hypotheses include constant antigenic boost due to virus persistence in carrier animals, induction of more efficient immune mechanisms during infection compared with vaccination and quantitative differences due to greater antigen mass after infection compared with vaccination (Gebauer *et al.*, 1988; López *et al.*, 1990; Piatti *et al.*, 1991). Although a laboratory animal model for FMDV persistence has not been described, there are data generated in the mouse that support the importance of persisting virus or antigen to maintain serological memory. Splenocytes from donor mice infected 135 days previously, which were irradiated before cell transfer, were shown to induce a strong anamnestic immune response in FMDV pre-immunized recipient mice (Wigdorovitz *et al.*, 1997). Irradiation suppressed the transferred splenocytes so any new anti-FMDV antibody detected in the new host must have been produced by its own immune system. These authors concluded that FMDV antigen present in the irradiated cell population induced the anamnestic immune response in the pre-sensitized recipients. No live virus could be isolated from the transferred spleen cells and no viral RNA was detected by reverse transcription PCR. The response was dependent on donor cells presenting FMDV epitopes and was MHC class II restricted and dependent on recipient T-cell function. Similar anamnestic responses were induced when splenocytes from 0.5 μg BEI-FMDV-immunized donors were transferred 15 days p.v., but not at 30 days p.v. By comparison, splenocytes from donors immunized with 30 μg BEI-FMDV did induce responses at 30 days p.v., consistent with delayed antigen clearance at higher antigen doses (Wigdorovitz *et al.*, 1997). López *et al.* (1990) also reported that repeated transfer of splenocytes from infected animals was able to induce antibody responses against FMDV in normal recipients and protect against challenge. These authors suggested that FMDV or antigen may persist throughout life after infection in mice. These data from the mouse model could be explained by the observations in ruminants that virus particles are trapped by follicular dendritic cells within the germinal centres (GCs) of lymphoid tissue for long periods of time, potentially stimulating the long-lasting immune responses characteristic for FMDV infection (Juleff *et al.*, 2008, 2012). One could speculate that persisting virus or antigen, or the establishment of the ‘carrier state’ could explain the distinctive second late IgA responses and late T-cell responses after 28 days p.i. in ruminants (Doel, 1996) and a degree of antigen retention is crucial for serological memory. FMDV retention in GCs has been reported in both carrier and non-carrier ruminants and this condition may be a common sequel to infection (Juleff *et al.*, 2012). It is unclear if these virus depots contribute to viral repopulation and replication in other cells in the oropharynx, contributing to the ‘carrier state’. Further studies in natural hosts and appropriate mouse models may answer these questions. FMDV retention in GCs could explain the IgA response detected from 28 days p.i. in OPF of all infected cattle independent of their ‘carrier state’ and intermittent virus replication may be required for the second late serum IgA response described in carrier cattle (Parida *et al.*, 2006; Salt *et al.*, 1996).

Piatti *et al.* (1991) demonstrated that the duration and magnitude of the immune response in mice immunized intraperitoneally with inactivated virus in PBS correlated directly with the mass of antigen used, and at high antigen doses there was no difference in the immune response elicited or maintenance of SNA titres over 200 days compared with experimental infection. Similar results were reported by López *et al.* (1990) and Wigdorovitz *et al.* (1997). These data provide support for the hypothesis that the amount of antigen in contact with the immune system is responsible for the differences observed between vaccination and infection. As yet, very little is known about the FMDV plasma and memory B-cell responses in laboratory animals or target species and the generation and maintenance of serological memory remains a major knowledge gap. Similar to natural hosts, experimental FMDV infection in mice is characterized by a short viraemic period, rapid clearance of infectious virus and life-long serological memory. Therefore, the mouse may be a suitable model to identify mechanisms responsible for persistent antibody responses to FMDV.

Conclusion

Several laboratory animal species have been used to model FMD, each with their particular advantages and disadvantages. Although clinical disease is less overt in mice compared with other laboratory animals and data generated in the mouse are controversial and even contradictory at times, these models have provided robust data to extend our understanding of FMD in natural hosts. Arguably, the major disadvantages of the FMD mouse model are the unnatural routes of experimental infection or vaccination and the uncertainty of the relevance for target species. There is no doubt that data generated in laboratory animals need to be assessed in the context of the target species. However, interpreting data generated in the target species is also complicated by the various different routes and methods of experimental challenge. Therefore, data from one natural host species are not always applicable to other hosts. It is clear from this review that data generated in the mouse can often be reconciled with available data from target species and the models have successfully predicted immune responses to FMDV in cattle and pigs. Significant knowledge gaps remain in our understanding of FMD pathogenesis, and even basic knowledge of the development

of anti-FMDV antibody responses contains substantial gaps (Arzt *et al.*, 2011a, b). The following FMD knowledge gaps will benefit from research in small laboratory animals:

1. What are the mechanisms for maintaining serological memory to FMDV?

2. What are the mechanisms of virus neutralization *in vivo*, what is the role of different antibody isotypes and what role do subneutralizing or non-neutralizing antibodies play?

3. Besides DCs and B-cells, what is the role of other cell types at various stages of infection?

4. What other factors besides virus binding and entry are responsible for cellular susceptibility?

5. What are the determinants of tissue tropism beyond integrins, and what innate and adaptive factors contribute to tropism?

6. What factors are responsible for genetic resistance to FMDV infection?

7. What factors are responsible for age-dependent susceptibility to FMDV infection?

8. Which other innate immune factors are essential for a protective response to FMDV infection?

9. What are the virus and host factors responsible for malignant FMD and viral myotropism?

10. What processes are responsible for long-term metabolic disturbances associated with FMDV infection, for example heat-intolerance syndrome?

Although data from these studies are unlikely to be conclusive, they will undoubtedly provide preliminary data to direct studies in target species and will add substantial basic science value by improving understanding of viral infections.

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