Transboundary and Emerging Diseases

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REVIEW

Evidence of Activation and Suppression during the Early Immune Response to Foot-and-Mouth Disease Virus

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Summary

Foot-and-mouth disease virus causes a serious disease of livestock species, threatening free global trade and food security. The disease spreads rapidly between animals, and to ensure a window of opportunity for such spread, the virus has evolved multiple mechanisms to subvert the early immune response. The cycle of infection in the individual animal is very short, infection is initiated, disseminated throughout the body and infectious virus produced in <7 days. Foot-and-mouth disease virus has been shown to disrupt the innate response *in vitro* and also interacts directly with antigen-presenting cells and their precursors. This interaction results in suboptimal immune function, favouring viral replication and the delayed onset of specific adaptive T-cell responses. Detailed understanding of this cycle is crucial to effectively control disease in livestock populations. Knowledge-based vaccine design would specifically target and induce the immunological mechanisms of early protection and of robust memory induction. Specifically, information on the contribution of cytokines and interferon, innate immune cells as well as humoral and cellular immunity can be employed to design vaccines promoting such responses. Furthermore, understanding of viral escape mechanisms of immunity can be used to create attenuated viruses that could be used to develop novel vaccines and to study viral pathogenesis.

Introduction

Infection of susceptible livestock species with foot-and-mouth disease virus (FMDV) is characterized by the rapid onset of clinical signs within 2–5 days of exposure. Clinical signs of disease include fever, malaise and the development of vesicles on the coronary bands of the feet, in the mouth and on the tongue and teats. Viraemia is detectable in the same time frame and resolves very quickly, as do clinical signs, by which time an infected individual can have secreted enormous amounts of virus into the environment and infected countless other animals. To achieve such a rapid takeover of the host, FMDV must manipulate the early immune response to ensure a window of opportunity in which to replicate and spread, before the onset of effective adaptive immunity.

Understanding the host/pathogen interaction and the contributions of innate versus adaptive immune responses has become a central topic in FMDV research. An improved knowledge of the role of the immune response in disease progression and pathology is key to both understanding transmission between animals and the rational design of intervention strategies.

In this review, we will summarize current knowledge of the interplay between FMDV and the host immune system early in infection, i.e. within 48 h of onset of viraemia. We will aim to identify the key viral and cellular components of this interaction, and areas for further

research and of potential interest for vaccine development.

FMDV Manipulates Target Cells to Subvert Effective Immunity

The interaction of FMDV with the host begins via its infection of epithelial cells. Following binding to its cellular receptor, FMDV is endocytosed, and its genetic material enters the cell cytoplasm. The single strand of viral RNA genome encodes the capsid proteins and a number of non-structural (NS) proteins. The NS proteins are required not only for translation and replication of the genome but also for hijacking the cellular machinery of the host cell to synthesize progeny virus and subvert the immune response.

A number of FMDV proteins have specific functions that include inhibition of the innate response of infected cells. The leader protein (L^{pro}) is a papain-like proteinase, which initially functions to cleave itself from the viral polyprotein (Strebel and Beck, 1986; Kleina and Grubman, 1992). It then cleaves the cellular translation initiation factor eIF4G, causing shut-off of host cap-dependent mRNA translation while leaving intact the IRES-dependent translation mechanisms used by the virus (Devaney et al., 1988; Kirchweger et al., 1994). Further studies revealed that L^{pro} regulates type I interferon (IFN) production, not only at the level of translation (Chinsangaram et al., 1999) but also by limiting transcription of IFN-β (de Los Santos et al., 2006). *In vitro* studies revealed that FMDV infection results in L^{pro}-dependent degradation of nuclear factor κ-B (NF-κB), which affects transcription of IFN and many other factors involved in the innate immune response (de Los Santos et al., 2007; Zhu et al., 2010). While the precise mechanism of L^{pro}'s effects on NF-κB is undefined, nuclear localization and retention of L^{pro} is required; mutation of a conserved motif affecting L^{pro} nuclear retention rendered an FMDV strain attenuated both *in vitro* and *in vivo* (de los Santos et al., 2009). L^{pro} has also been shown to degrade IFN-regulatory factor 3/7 *in vitro* (Wang et al., 2010), which could potentially synergize with its effects on NF-κB in terms of immune disruption, although whether this mechanism operates during FMDV infection is unknown.

Cytotoxic T lymphocytes (CTL) are able to kill virus-infected cells following recognition of viral peptides bound to major histocompatibility complex (MHC) class I molecules on the infected cell surface. The existence of a CTL response to FMDV has only recently been uncovered (Guzman et al., 2008), most likely because FMDV devotes considerable resources to avoid stimulating just such a response. *In vitro* studies show that FMDV NS proteins 2B and 2C acting together, or their precursor 2BC, inhibit the secretory pathway (Moffat et al., 2005), paralleling the function of other picornavirus NS proteins, such as coxsackievirus and rhinovirus 2Bs (de Jong et al., 2008) and poliovirus 2B and 3A (Deitz et al., 2000). During poliovirus infection, these effects substantially reduce MHC class I expression at the cell surface to a level that protects cells from CTL-mediated lysis *in vitro* (Deitz et al., 2000). This intracellular alteration in protein trafficking also results in limiting the secretion of proinflammatory cytokines, for example IL-6, IL-8 and IFN-β (Dodd et al., 2001). A similar down-regulation of MHC class I at the cell surface has also been observed in FMDV-infected cell lines (Sanz-Parra et al., 1998). Down-regulation of MHC class I is a common strategy in viral infection and cancer, used to avoid CTL killing. The immune system, however, has evolved a coping strategy, using natural killer (NK) cells to destroy low MHC class I-expressing cells. Interestingly, during FMDV infection, a reduced capacity of NK cells to lyse target cells and secrete IFN-γ has been demonstrated (Toka et al., 2009).

Other NS proteins contribute to FMDV virulence, although their mechanisms of action remain unclear. For example, $3C^{pro}$-dependent histone H3 cleavage occurs in infected cells (Grigera and Tisminetzky, 1984) and is followed by a decrease in host RNA synthesis (Falk et al., 1990). Additionally, $3C^{pro}$ has been implicated in cleaving the translation factors eIF4A and eIF4G, thus exacerbating the shut-off of host cell translation (Belsham et al., 2000). A global effect on host cell transcription and translation should contribute to modulate the host response to viral infection.

FMDV Interacts with Professional Antigen-Presenting Cells

Antigen-presenting cells (APC) are the master regulators of immune responses, so perhaps it is no surprise that FMDV has evolved to prevent them functioning optimally. The virus is likely to first come into contact with APC as a result of its lytic infection of epithelial cells. This results in local tissue damage, causing inflammation and the release of so-called 'danger signals' (Gallucci and Matzinger, 2001) to alert the immune system to the attack. Inflammatory mediators cause local vasodilation and increased vascular permeability, enabling the recruitment of mononuclear cells to infection sites (Goldsby et al., 2000). These mononuclear cells then respond to the inflammatory microenvironment, differentiating towards macrophage or dendritic cell (DC)-like phenotypes.

Phagocytosis of FMDV by porcine macrophages has been demonstrated *in vitro* (Rigden et al., 2002), although following uptake the cells do not become productively infected (Baxt and Mason, 1995). These observations

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support the idea that porcine macrophages play a key role in resolution of disease via phagocytosis and destruction of antibody-opsonized FMDV (McCullough et al., 1992).

While the majority of studies on APC interactions with FMDV have been carried out in pigs, mice can be experimentally infected and have yielded potentially interesting data. Mouse bone marrow-derived DCs were infected by FMDV *in vitro* without detectable cytopathic effect or live virus production. FMDV caused down-regulation of MHC class II and CD40 in infected DC cultures, resulting in a decreased ability of the cells to stimulate T-cell proliferation to either allogeneic or FMDV antigens (Ostrowski et al., 2005). DC exposed to live, compared to inactivated FMDV, induced the potentially immunosuppressive cytokine, IL-10 in T cells incubated with infected DCs (Ostrowski et al., 2005). Moreover, repeating the experiment with DC–splenocyte co-cultures, the authors detected a threefold higher titre of T-independent neutralizing IgM when DCs were exposed to live FMDV compared to inactivated virus. They proposed that this was attributed to the induction of IL-6 in DC, which subsequently induced IL-10 secretion, but this time from B lymphocytes in the co-culture (Ostrowski et al., 2007).

The interpretation of these experiments deserves caution because of the species used; however, the work has recently been given significant support from a study in pigs (Diaz-San Segundo et al., 2009). Porcine monocytes infected *in vitro* were differentiated into DCs, but these infected cells produced APCs that secreted high amounts of IL-10 (as opposed to IFN-γ) in allostimulatory T-cell co-cultures. This may indeed be physiologically relevant as the amount of IL-10 in serum from FMDV-infected swine was higher than in naïve animals. Together, these studies propose a scenario whereby FMDV induces high levels of IL-10 production, either directly or indirectly through DCs. While this cytokine supports T-cell-independent antibody responses, it may suppress T-cell activation during acute infection.

The interaction of fully differentiated porcine DCs and FMDV is less clear. Early studies reported that 10% of skin DCs were infected *in vitro* by a wild-type FMDV isolate and were dying as a result (Gregg et al., 1995). Although the experimental conditions used were not explicitly defined, it is plausible that direct loss of a proportion of the APC population during infection could adversely affect the immune response to FMDV. In contrast, Rigden et al. (2002) reported, as unpublished data, similar findings in DCs as in pulmonary macrophages in which almost all cells exposed to wild-type FMDV subsequently expressed NS proteins. In contrast to the study by Gregg et al., Rigden et al. (2002) detected no evidence of cell death caused by the virus.

A later re-examination of the effects of FMDV exposure on porcine skin DCs [identified as Langerhans cells (Nfon et al., 2008)] indicated a complete lack of viral replication (Bautista et al., 2005). However, exposure of DCs to live, but not UV-inactivated FMDV, did induce the production of substantial quantities of type I IFNs. Despite being stimulated to produce IFNs, FMDV-exposed DCs surprisingly did not modulate their antigen uptake or surface molecule expression (Bautista et al., 2005), indicating that APC function remains intact following exposure to the virus. The situation appears to be slightly more complicated when considering skin DCs isolated during infection, as these cells exhibit defective IFN responses up until 42–50 days post-infection, despite not being infected themselves (Nfon et al., 2008). These Langerhans cells show stable to slightly increased expression of APC-related proteins, including MHC class II and CD80/CD86 and antigen-processing function, implying FMDV mediates maturation of these cells.

Monocyte-derived DCs (MoDC) are an *in vitro* model for the DC, which differentiate *in vivo* at sites of inflammation, such as FMDV lesions, and are generating increasing interest in the field. As in porcine skin DCs, MoDC also exhibit an IFN-α response to a range of FMDV strains *in vitro* (Nfon et al., 2008). The cells appear not to be infected by the virus either *in vitro* or *in vivo*, but as noted above, the virus is able to infect their CD172a^{+} progenitors and interfere with DC development (Diaz-San Segundo et al., 2009). Monocytes taken from pigs during the acute stages of FMDV infection also fail to differentiate into DCs capable of responding to IFN-inducing toll-like receptor (TLR) ligands *in vitro* (Nfon et al., 2008). However, like the Langerhans cells, monocytes from FMDV-infected pigs differentiate into DCs with stable expression of MHC class II and CD80/CD86 and process antigen, indicating that differentiation into APCs occurs. In another study, MoDCs from infected pigs did not stimulate a mixed lymphocyte reaction (MLR), indicating a compromise of DC functions (unpublished). One of the mechanisms by which FMDV directly disrupts IFN responses might be by selectively inhibiting the transcription of specific genes involved in this function (Rodríguez-Calvo et al., unpublished observation), although other possibilities also warrant investigation.

Plasmacytoid DCs (pDC) are distinct in lineage and function from the DC populations mentioned above as their main role is in the production of large quantities of IFNs in response to viral infection. Porcine pDCs are infected by FMDV–antibody complexes and express NS proteins and secrete IFN-α. In contrast, they do not become infected or produce IFN-α in response to virus alone (Guzylack-Piriou et al., 2006). In acutely infected swine, the blood pDC population is depleted, and the

remaining pDC are less able to produce IFN-α in response to TLR ligands or FMDV, although they recover this ability by day 7–9 post-infection (Nfon et al., 2010). Further studies are required to determine how pDC secrete IFN in response to FMDV immune complexes despite detectable production of viral proteins.

Stimulation of the Early Antibody Response by FMDV

The early B-cell response to FMDV infection is typified by a strong neutralizing antibody response, with serum IgM detectable as soon as 3–4 days post-infection in cattle, followed by IgA and then IgG peaking 1–2 weeks later (Collen et al., 1989; Salt et al., 1996; Juleff et al., 2009). In infected swine, a similar response is detected (Pacheco et al., 2010).

Early work in nude mice demonstrated that anti-FMDV IgM production occurred in the absence of T-cell help (Borca et al., 1986; Collen et al., 1989). This result has recently been confirmed in cattle by Juleff and colleagues, who went on to show that during infection, antibody isotype switching to high-affinity IgG can also occur in the absence of detectable T-cell help (Juleff et al., 2009). While some T-independent antibody responses may be initiated without DC help (Pape et al., 2007; Scandella et al., 2007), DC-derived factors such as B-cell-activating factor of the TNF family (BAFF) are likely to be required to induce class switching in naïve B cells (Litinskiy et al., 2002). While this has yet to be studied *in vivo* for FMDV, in a porcine *in vitro* model, both BAFF and IL-2 were essential for anti-FMDV recall IgG responses (Bergamin et al., 2007b). In this model, BAFF was DC derived, while IL-2 was added exogenously to represent the contribution of T-cell help. However, in some situations, DC may also secrete IL-2 (Granucci et al., 2001), which could potentially maintain the T independence of the antibody response. It should be noted, however, that expression of BAFF in the context of DNA vaccination of pigs did not improve antibody responses (Bergamin et al., 2007a). Although a role for DC has yet to be explicitly proven in the early B-cell response to FMDV, it is likely that stimulation of T-cell responses by DCs will be required for protection following vaccination to achieve induction of memory B cells.

FMDV Stimulation and Manipulation of the T-cell Response

In swine, there is some evidence that T-cell function may be broadly affected during acute FMDV infection. Peaking at day 2 post-infection, a transient lymphopenia is observed in infected animals, mainly affecting $CD8^+$ T cells, but beginning to resolve from day 4. However, even after resolution of the lymphopenia, T cells responded poorly to the mitogen concanavalin A, demonstrating an ongoing impairment in function (Bautista et al., 2003). This work was partly supported by a study of Diaz-San Segundo et al. (2006) who showed that C serotype FMDV could productively infect both T and B lymphocytes to a high level (30% and 60%, respectively), resulting in lymphopenia, again most profoundly affecting $CD8^+$ T cells and inducing mitogen unresponsiveness (Diaz-San Segundo et al., 2006). However, neither study addressed the mechanism of lymphopenia, for example Diaz-San Segundo et al. (2006) documented productive infection of lymphocytes without cell death, raising the possibility that the lymphopenia was not related to virus-mediated killing. In fact, it now seems that the observed lymphopenia is likely to represent IFN-induced lymphocyte egress from the blood into the affected sites or lymphoid tissues. Support for this hypothesis comes from murine studies in which administration of IFN-α *in vivo* results in a broad lymphopenia, which is not observed in IFNα/β receptor knockout mice (Kamphuis et al., 2006), as well as the detection of high levels of type I IFNs in the blood of infected swine (Nfon et al., 2010). It is important to note that a transient virus-induced IFN-mediated lymphopenia in the peripheral blood may not be a sign of immunosuppression but rather can be physiological and potentially beneficial to the host (Schattner et al., 1983; Kamphuis et al., 2006). In fact, the rapid recovery from lymphopenia detected in FMDV-infected swine is consistent with altered migration patterns in response to cytokine signals rather than loss and subsequent repopulation of lymphocytes.

Joshi and colleagues also detected infection of bovine lymphocytes exposed to FMDV *in vitro* and found an inhibited response to mitogen activation (Joshi et al., 2009). However, the situation is less clear-cut, as contact-challenged cattle exhibit normal lymphocyte numbers and subpopulations (Windsor et al., 2008). Moreover even during acute infection, T cells from infected cattle proliferate well to stimulation with either mitogens or non-FMDV recall antigens (Windsor et al., 2008). Interestingly, although proliferation assays were carried out up to day 19 post-infection, only a modest specific T-cell response to FMDV antigen was ever observed, although serum IL-10 levels remained low.

Overall, it is currently not possible to make clear statements about the nature and consequences of the interactions between FMDV and T cells. As detailed earlier, there are clear differences between results obtained *in vitro* and *in vivo* in cattle and between studies in pigs when different isolates are used. Clearly, quite different results could be obtained if the challenge viruses were

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harvested directly from animals or had been adapted to grow in cells which did not express the integrin molecules that act as viral receptors. There may be additional uncharacterized mutations that confer altered tropism *in vitro* and *in vivo*, which could account for the different experimental observations. This puzzle will not be resolved easily, and systematic studies to correlate virus genotype and phenotype will have to be performed *in vitro* and *in vivo*.

These studies serve to highlight the gap in our understanding of the importance and precise function of the T-cell response during FMDV infection. For example, while immunity to FMDV during infection is initially dominated by T-independent antibody production, in vaccinated cattle, antibody titres are not always predictive of protection, and in carrier animals, live virus persists in the face of high titres of neutralizing antibody (DiMarchi et al., 1986; McCullough et al., 1992; Juleff et al., 2008). In fact, T-cell responses to vaccination in cattle in combination with antibody responses may better predict protection from challenge (Glass and Millar, 1994; Hohlich et al., 2003), for example detection of established T-cell responses may predict rapid development of a protective memory immune response after rechallenge.

T-cell responses to FMDV further have the advantage of being serotype cross-reactive, making them attractive targets for vaccination strategies (Collen et al., 1998b). However, the success of subunit vaccines incorporating T-cell epitopes has been limited by a lack of fundamental understanding of T-cell responses to FMDV infection. T-cell epitopes have been identified within both structural and NS proteins during infection and vaccination (van Lierop et al., 1992; Collen et al., 1998b), but the use of such epitopes in vaccine strategies has met with, at best, limited success. The T-cell response in mice following DNA vaccination with epitopes from FMDV structural and NS proteins did not induce specific antibodies and yet was sufficient for protection from disease (Borrego et al., 2006). However, only partial protection of swine could be achieved by vaccination with recombinant vaccinia virus expressing the FMDV 3D protein (Garcia-Briones et al., 2004). A similar phenomenon was observed in cattle, where a range of peptide vaccines were tested but none induced protection in more than 40% of challenged animals (Taboga et al., 1997; Rodriguez et al., 2003). T cells are likely to be key to the development of long-term protective immune responses to FMDV by supporting and maintaining T-cell-dependent antibody responses. The studies described above suggest these T-cell responses are serotype cross-reactive, which may provide opportunities to develop vaccines to the different serotypes using promiscuous T-cell epitopes combined with specific B-cell epitopes.

Conclusion

In summary, in the early stages of infection, FMDV initially interacts with components of the innate immune system. These components are likely to include host mononuclear phagocytes, macrophages and multiple DC subsets. The initial response of these cells is production of type I IFNs, although the virus possesses several mechanisms to slow or inhibit this process. The innate interaction progresses to initiate the adaptive immune response, most likely beginning with the induction of T-cell-independent B-cell responses. The classical helper T-cell response is stimulated later in infection, and the subsequent induction of $CD8^+$ lymphocytes is still poorly understood (Collen et al., 1998a; Childerstone et al., 1999; Guzman et al., 2008).

Many details of FMDV's interaction with the early immune response remain unknown. There is accumulating evidence for a complex interaction of the virus with host APCs. This is especially important to understand because the ability of the virus to hijack DC functions early in infection represents the equivalent of storming the control room of the immune system. For example, it seems that DC-derived factors may be required for the induction of the T-independent antibody response (Bergamin et al., 2007b), but may also be suppressing T-lymphocyte activation (Ostrowski et al., 2005; Diaz-San Segundo et al., 2009). Moreover, it appears that the virus has evolved ways of subverting the innate, type I IFN response (Nfon et al., 2008) or differentiation of inflammatory APCs from their precursors (Diaz-San Segundo et al., 2009) or both. Given the highly acute and contagious nature of this infection, the evolution of this virus to disrupt APC functions may provide just the temporary escape that it needs to spread to the next host. Dissection of the FMDV–APC relationship will likely be critical to understanding immunity to FMDV and also to rational vaccine design for the future.

Outbreaks of FMDV in naïve populations of livestock resemble the spread of a wildfire in dry woodlands driven by hot winds. Immune evasion needs only be short lived, but must be immediate to allow FMDV to be a successful pathogen. Therefore, a unique characteristic of new vaccines being developed for outbreak responses should address the need to induce a rapid, innate response as well as the classical T-cell-dependent, adaptive B-cell response. Such vaccine formulations are not simple to design, as innate immune responses in mammals have evolved to be down-regulated by numerous mechanisms to avoid unnecessary inflammatory damage to the host. How to activate these responses well enough to protect against infection of livestock with FMDV is the subject of intense research at this time.

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