Understanding the Mechanisms of Interferon-Induced Protection against Foot-and-Mouth Disease

Fayna Diaz-San Segundo
Plum Island Animal Disease Center, North Atlantic Area, Agricultural Research Service, U.S. Department of Agriculture, Greenport, New York 11944 USA

Nestor Montiel
Plum Island Animal Disease Center, North Atlantic Area, Agricultural Research Service, U.S. Department of Agriculture, Greenport, New York 11944 USA.ORISE- PIADC Research Participation Program, Oak Ridge, TN 37831 USA

Teresa de los Santos
Plum Island Animal Disease Center, North Atlantic Area, Agricultural Research Service, U.S. Department of Agriculture, Greenport, New York 11944 USA

Marvin J. Grubman*
Plum Island Animal Disease Center, North Atlantic Area, Agricultural Research Service, U.S. Department of Agriculture, Greenport, New York 11944 USA

*Corresponding author. Mailing address: Plum Island Animal Disease Center, USDA, ARS, NAA, P.O. Box 848, Greenport, New York 11944. Phone: (631) 323-3329. Fax: (631) 323-3006. E-mail: marvin.grubman@gmail.com.

Keywords: foot-and-mouth disease, interferons, adenovirus vector, dendritic cells, natural killer cells

Running title: Understanding the mechanisms of IFN-induced protection against FMD.

1 Introduction

At the end of the nineteenth century, foot-and-mouth disease (FMD) became the first animal disease shown to be caused by a virus, earning a place in history. Although the earliest recognition of the clinical entity of FMD is generally credited to Fracastorius's observations in the 16th century (Fracastorius, 1546), it was not until 1897 when Loeffler and Frosch demonstrated that this disease was caused by a filterable agent (Loeffler & Frosch, 1897). In its acute form, FMD affects livestock and wild cloven-hoofed animals and is characterized by fever, lameness, and vesicular lesions of the feet, tongue, snout and teats. With high morbidity, low mortality rates, and its notorious debilitating effects, FMD is responsible for severe losses in livestock productivity, including weight loss, decreased milk production, and loss of draught power. The highly contagious nature of this disease and the severity of the associated economic impact caused by an outbreak have led to the recognition of FMD as the most important disease in the livestock industry, limiting trade of animals and animal products throughout the world (Leforban, 1999). In fact, countries that are FMD-free prohibit imports of livestock or animal products from countries where the disease is enzootic. The economic impact of an FMD outbreak can be significant, as observed during recent outbreaks in Europe, Japan, Taiwan, South Korea, and other countries, that resulted in losses of billions of dollars (Grubman & Baxt, 2004). Furthermore, worldwide concerns following the 2001 terrorist attacks in the United States has raised awareness about the possibility of terrorist groups or rogue states attempting to use FMD virus (FMDV) as a bioweapon to target the $100-billion/year U.S. livestock industry.

The virus spreads from animal to animal in a number of ways including direct contact between an infected animal and non-infected animals and indirect spread when an animal eats food that has been contaminated by the virus (from saliva, for example). The virus can also become airborne, particularly when it has been exhaled, thus an infection in one herd can quickly become widespread over the countryside (Alexandersen et al., 2003). However, experimental studies use direct inoculation including subcutaneous, intradermal, intramuscular and intravenous inoculation, intranasal instillation, and exposure to artificially created aerosols.

Current methods of disease control include limiting movement of susceptible animals and animal products, slaughtering of infected and susceptible in-contact animals, except in countries in which the disease is enzootic, disinfection of affected areas, and vaccination with a chemically inactivated whole virus antigen vaccine (Grubman & Baxt, 2004). However, in case of an outbreak occurring in previously FMD-free countries, the use of vaccination is problematic since The World Organization for Animal Health (OIE) favors slaughtering or vaccination followed by slaughtering, rather than vaccination alone in order to rapidly regain FMD free status. Countries that slaughter all infected and susceptible in-contact animals can regain FMD-free status, and thus re-engage in trade, by documenting and demonstrating absence of disease for the next three consecutive months after the last case. On the other hand, countries that do not slaughter vaccinated animals must wait six months to regain FMD-free status. Although the conventional inactivated vaccine and a newly developed recombinant replication-defective human adenovirus 5 (Ad5) FMDV subunit vaccine are effective in controlling disease, they require approximately 7 days to induce full protection in swine and cattle (Golde et al., 2005; Moraes et al., 2002; Pacheco et al., 2005; Grubman et al., 2010). Therefore, in the event of an outbreak, induction of rapid protection, prior to the development of vaccine-induced adaptive immunity, is critical and necessary to limit disease dissemination and potentially reduce the number of animals that need to be eliminated.

A number of studies have demonstrated that FMDV initially infects epithelial cells of the nasopharynx attaching to these cells via integrin receptors and then the virus spreads to epithelial cells in the lungs (Alexandersen et al., 2003; Arzt et al., 2011; Monaghan et al., 2005; O'Donnell et al., 2008). The viremic phase of FMDV infection generally occurs and peaks during the first 24 to 48 hours, and coincides with appearance of clinical signs. It is during this period that an infected individual can secrete copious amounts of virus into the

environment transmitting the disease to susceptible animals. To achieve such a rapid takeover of the host, FMDV manipulates the early immune response ensuring a window of opportunity to replicate and spread before the onset of effective adaptive immunity. Thus, understanding early host-pathogen interactions and the contributions of innate versus adaptive immune responses has become a central topic in FMDV research. The role of the immune response in disease progression and pathology is fundamental to an effective and rational design of early intervention strategies. This review will present a brief description of FMDV and the different functions of various viral proteins, with the main focus on our current knowledge of the strategies that FMDV utilizes to circumvent the host protective response and the results of stimulating host innate immunity to control disease.

2 The Agent

Foot-and-mouth disease virus is the prototype member of the *Aphthovirus* genus of the *Picornaviridae* family and contains a single-stranded, positive-sense RNA genome of approximately 8,500 nucleotides surrounded by an icosahedral capsid composed of 60 copies each of four structural proteins (VP1 [1D], VP2 [1B], VP3 [1C], and VP4 [1A]) (Fig. 1A) (Grubman and Baxt, 2004; Rueckert, 1996; Rueckert and Wimmer, 1984). The viral genome consists of a long open reading frame (ORF) flanked by 5' and 3' highly structured untranslated regions (5' and 3'UTRs) (Fig. 1A). Upon infection, the viral ORF is translated as a single polyprotein that is cotranslationally processed by three virus-encoded proteinases, leader (L^{pro}), 2A, and $3C^{pro}$, into the four structural proteins and a number of nonstructural (NS) proteins (Mason et al., 2003; Rueckert, 1996). L^{pro} is a papain-like proteinase that cleaves itself from the nascent polypeptide chain (Strebel & Beck, 1986) and as later explained in this chapter, interacts with the host, playing a critical role in virulence. 2A is a peptide of ~~19~~ 18 amino acids that, using a unique cleavage mechanism, is responsible for processing P1-2A from the remainder of the polyprotein (Donnelly et al., 2001a and 2001b). Most of the viral polyprotein is enzymatically processed by the cysteine-proteinase $3C^{pro}$ (Klump et al., 1984; Vakharia et al., 1987) which also plays an important role in virulence. $3C^{pro}$ is involved in the cleavage of various host proteins including histone H3, Sam68, NEMO, cytoskeleton proteins, and translation initiation factors eIF-4A and eIF-4G (Armer et al., 2008; Belsham et al., 2000; Falk et al., 1990; Lawrence et al., 2012; Wang et al., 2012). Other NS viral proteins are involved in various aspects of the viral replication cycle: 3D is the viral RNA-dependent RNA polymerase; 3B (also termed VPg) is a protein covalently linked to the 5' end of virion RNA that is involved in the initiation of RNA synthesis; 2C binds single-stranded RNA nonspecifically, has ATPase activity and its interaction with cellular protein Beclin1 modulates autophagy pathways allowing for virus survival (Gladue et al., 2012); 2B, 2C, and 3A are also involved in membrane rearrangements required not only for viral RNA replication, but for capsid assembly (Grubman & Baxt, 2004). Furthermore, recent evidence has demonstrated that some of the NS proteins or their precursors also have additional roles in controlling the host response including inhibition of protein trafficking by 2B and 2C or 2BC (Moffat et al., 2005, 2007). This latter function may be responsible for the decrease in surface expression of major histocompatibility class I molecules upon FMDV infection resulting in a delay in the host adaptive immune response (Sanz-Parra et al., 1998).

As mentioned above the leader proteinase is the first viral protein translated upon infection. L^{pro} cleaves itself from the polyprotein precursor and early in infection cleaves host translation initiation factor eIF-4G, resulting in the shut off of host cap-dependent mRNA translation (Fig. 1B; Devaney et al., 1988; Medina et al., 1993, Strebel & Beck, 1986). Translation of FMDV mRNA remains unaffected since it proceeds by a cap-independent mechanism, via an internal ribosome entry site (IRES) present within the 5' UTR of the viral genome that does not require intact eIF-4G (Belsham & Brangwyn, 1990; Kuhn et al., 1990). Thus, as a result of FMDV infection, host cell protein synthesis is rapidly suppressed without affecting translation of viral

mRNA, thereby diverting the cell protein synthesis machinery towards the production of large amounts of new virus.

L^{pro} is a virulence factor and viruses can be attenuated *in vitro* and *in vivo* by removing the leader coding region (leaderless virus) or by inserting mutations in some domains within the L^{pro} coding sequence (Chinsangaram et al., 1998; de los Santos et al., 2009; Diaz-San Segundo et al., 2012, Mason et al., 1997; Piccone et al., 1995, 2010). It has been shown that the attenuated phenotype of the leaderless virus results from its inability to block the host innate immune response, in particular the production of type I interferon (IFN-α/β) (Chinsangaram et al., 1999). FMDV L^{pro} also blocks the induction of IFN-β transcription (de los Santos et al, 2006), which requires the translocation of biologically active L^{pro} to the nucleus of infected cells and subsequent degradation of p65/RelA, a subunit of the transcription factor nuclear factor kappa B (NF-κB) (Fig. 1B; de los Santos et al, 2007). Structure-function analysis of L^{pro} has demonstrated that, in addition to the proteinase activity, an intact protein motif, SAP (SAF-A/B, acinus, and PIAS domain), is required for FMDV virulence (de los Santos et al., 2009). Others have demonstrated that L^{pro} is involved in the degradation of IFN regulatory factors (IRF)-3 and -7, affecting specific gene transcription (Fig. 1B; Wang et al., 2010) and causes de-ubiquitination of RIG-I, TBK-1, TRAF-1 and TRAF-6, affecting IFN and IFN stimulated gene (ISG) transcription (Fig. 1B; Wang et al., 2011). Thus, FMDV L^{pro} helps subvert the host innate response by inhibiting the induction of antiviral molecules at both transcriptional and translational levels thereby blocking the action of IFNs and other cytokines that would naturally prevent virus replication and dissemination.

3 Evading the Host Immune Response

As a successful pathogen, FMDV has developed several mechanisms to counteract or evade the host innate and adaptive immune response (Grubman et al., 2008; Golde et al., 2008).

Dendritic cells (DCs), a heterogeneous group of potent antigen-presenting cells (APCs), are the master regulators of the immune response and are functionally situated at the interphase between the innate and the adaptive immune system (Banchereau & Steinman, 1998). In order to fulfill their role as sentinels of the immune system, DCs express several families of specialized pattern recognition receptors (PRRs) which recognize virus specific products termed pathogen-associated molecular patterns (PAMPs). PRRs include Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and C-type lectin receptors (CLRs) all reacting directly with pathogen components (Kawai & Akira, 2011; Lee & Kim, 2007; Robinson et al., 2006; Trinchieri & Sher, 2007). The most common division of DCs is into two major groups, conventional DCs (cDCs) and plasmacytoid DCs (pDCs). While cDCs have been described to have a unique capacity for naive T cell priming (Banchereau & Steinman, 1998), pDCs have the unique property of secreting IFN-α/β in response to viruses and/or virus-derived nucleic acids (Reizis et al., 2011). The cross-talk between DCs and natural killer (NK) cells leads to the activation of NK cells to modulate the initial barriers of the cellular host defense against pathogens (Vivier et al., 2011). In addition, DCs interactions with T and B lymphocytes modulate the cellular and humoral adaptive immune response (Iwasaki & Kelsal, 2000; Wykes et al., 1998). Thus, it is not surprising that FMDV has evolved mechanisms to prevent these cell populations from functioning optimally throughout the course of infection. During the course of FMD, the virus interacts with different DCs populations, either by direct infection or as a consequence of DCs phagocytosing the virus in peripheral tissues for antigen presentation (Merad & Manz, 2009).

Several studies have documented the interaction between FMDV and different DC populations (Bautista et al., 2005; Diaz-San Segundo et al., 2009; Nfon et al., 2008; Ostrowski et al., 2005; Robinson et al., 2011). FMDV can infect cDCs *ex vivo* leading to the synthesis of viral NS proteins. Although in most cases this

interaction is abortive and no virions are produced in infected cDCs (Diaz-San Segundo et al., 2009; Ostrowski et al., 2005), it has been demonstrated that the presence of specific anti-FMDV antibodies enhances infection through the interaction of virus-antibody complexes with FcγR receptors located on the surface of DCs. In cattle, uptake of these complexes by DCs results in productive infection and cell death (Robinson et al., 2011). In addition, FMDV can modulate adaptive immune responses by means of its interaction with DCs. During acute infection the virus stimulates DCs to produce interleukin (IL)-10, thus directing adaptive immunity towards the development of a stronger humoral rather than a T-cell mediated response (Diaz-San Segundo et al., 2009; Robinson et al., 2011). It has been shown that interaction of FMDV with bovine cDCs, *in vitro*, results in productive infection; however attempts to recover virus from DCs derived from FMDV infected swine have been unsuccessful. Nevertheless, FMDV affects monocytes extracted from pigs during acute stages of FMDV blocking their ability to differentiate into mature cDCs (Diaz-San Segundo et al., 2009) and to respond to stimulation with TLR ligands (Nfon et al., 2008).

Langerhan cells (LCs), which are a DC subset found in all layers of the epidermis and express langerin (Valladeau et al., 2000), are also affected by FMDV (Bautista et al., 2005; Nfon et al., 2008). Although these cells constitutively express type I IFN, *in vitro* studies demonstrated that FMDV is able to attach and become internalized by these cells; however, there is no evidence of viral RNA replication or production of viral proteins (Bautista et al., 2005). Furthermore, LCs from FMDV infected pigs show a reduction in IFN-α production after *ex vivo* stimulation, although their ability to present antigen remains normal (Nfon et al., 2008). These findings suggest that whereas FMDV enhances pathogenesis in infected animals during the acute phase of infection, DCs antigen presentation is preserved as it is critical to the host for the relatively rapid induction of a strong adaptive immune response and recovery.

pDCs are not susceptible to FMDV *in vitro*, unless the virus is internalized as part of immune complexes bound to FcγRII surface receptors, and uptake of these complexes results in abortive virus replication (Guzylack-Piriou et al., 2006). However, during acute infection of swine, the pDC population is depleted in blood, and the remaining pDCs are less able to produce IFN-α in response to TLR ligands or FMDV (Nfon et al., 2010). Similarly, FMDV can be internalized by bovine pDCs as immune complexes recognized by FcγRII receptors, but the ability of these cells to produce large amounts of type I IFN is not affected (Reid et al., 2011).

Macrophages are part of the innate responses to virus infections and are fundamental for rapid "clean up" of viral pathogens at the sites of infection. Similar to pDCs, FMDV also utilizes an antibody-dependent internalization process via the FcγRII receptor to enter macrophages (Baxt & Mason, 1995; McCullough et al., 1988). Interestingly, it has been reported that, in the absence of productive infection of porcine macrophages, FMDV remains infectious for 10 to 24 hours after internalization by macrophages (Ridgen et al., 2002). Implications of these observations favor the role of macrophages as transporters and disseminators of viable virions to distant sites of the body where the virus can infect and replicate in other cells.

As mentioned before, NK cells occupy a critical position during the initial host responses against pathogens, particularly during virus infections (Vivier et al., 2011). *In vitro*, stimulation of NK cells with proinflammatory cytokines induced lysis of FMDV-infected cells and expression of IFN-γ (Toka, et al., 2009). In addition, Toka et al., (2009) also demonstrated that direct activation of cytokine-secreting accessory cells by TLR7 and TLR8 agonists and at least partial activation of NK cells by these compounds, caused cytotoxitciy against FMDV-infected cells through enhanced secretion of IFN-γ and storage of perforin granules (Toka, et al., 2009). Interestingly, the same group later demonstrated that during FMDV infection of swine there is a reduced capacity of NK cells to lyse target cells and secrete IFN-γ (Toka et al., 2009). The later *in vivo* results contrast with the former (*in vitro*) results, which is not entirely unexpected given the complexity of host-virus interactions and immune responses during FMDV infection. Natural killer cell dysfunction during the viremic phase of acute infection with FMDV suggest that the virus can effectively block NK function and evade the host's immune system allowing the virus to replicate and disseminate within the host. Several mechanisms attempt to explain, synergistically, the inhibition or lack of activation of NK cells during FMDV infection. Upregulation of TLR3 and more transiently of SOCS3 mRNA can lead to a block in IFN-α gene expression,

which is necessary for NK activation. Additionally, inhibitory effects on protein synthesis, specifically cytokines of importance in NK activation such as IL-12, IL-15, and IL-18, can render NK cells hyporeactive to FMDV. Although it is know that a number of surface receptors with inhibitory and activating functions modulate responses of NK cells to infected cells, measurement of mRNA expression for some of these molecules such as NKG2D, NKp80, and granzyme B was minimally altered in infected animals. Nevertheless, these cells were dysfunctional. Although productive infection of NK cells could not be demonstrated the authors did not rule out the possibility of low levels of viral replication. While specific mechanisms explaining NK cell dysfunction remain unresolved, it is evident that FMDV infection induces negative effects in NK cell function, including reduced cytotoxicity, impaired expression of NK cell receptors, and reduced capacity to secrete cytokines, ultimately allowing the virus to effectively evade the host antiviral responses to FMDV infection.

FMDV also has an effect on other lymphocytic populations. It has been demonstrated that there is a transient lymphopenia during the peak of viremia in infected swine and cattle (Bautista et al., 2003; Diaz-San Segundo et al., 2006; Perez-Martin et al., 2012). T and B cell subsets are affected and severe lymphoid depletion in lymphoid organs has been reported (Diaz-San Segundo et al., 2006). In addition, the T cell function during early stages of infection is significantly impaired (Bautista et al., 2003; Diaz-San Segundo et al., 2006; Ostrowski et al., 2005). One possible mechanism that can explain such diminished T cell responses could be related to the elevated amounts of IL-10 produced by the cDCs during infection (Diaz-San Segundo et al., 2009; Ostrowski et al., 2005), as IL-10 has been reported to have a role in inducing immunosuppression *in vivo* (Brooks et al., 2006). However, the exact mechanisms involved in FMDV-induced lymphopenia remain poorly understood. While infection of lymphocytes with FMDV has been described in swine and cattle (Diaz-San Segundo et al., 2006; Joshi et al., 2009), infection could not be associated with cell death, suggesting that lymphopenia during infection might not be related to virus-mediated killing.

Effects on the early host innate and subsequent adaptive immune responses, such as modulation of DC and NK cells and transient lymphopenia, may provide favorable conditions for the virus to both spread systemically within the animal and shed infectious particles into the environment. Although there is a rapid B cell depletion after FMDV infection, the host is still able to mount a very fast antibody response that clears virus within 4-5 days postinfection. Ultimately, the infected host will induce production of serum IgM detectable as early as 3-4 days post-infection followed by maximum IgA and then IgG titers 1 to 2 weeks later (Collen et al., 1989; Juleff et al., 2009; Pacheco et al., 2010; Salt et al., 1996). Using a murine experimental model, it was demonstrated that an effective neutralizing antibody response seems to occur despite absence of T-cell help. Spleen cells of mice were adoptively transferred into irradiated recipients and only B cells were able to control viremia in FMDV-infected animals (Borca et al., 1986). Similarly, it was shown that FMDV-infected DCs can stimulate $CD9^+$ B-cells producing neutralizing anti-FMDV immunoglobulin M antibodies without T-lymphocyte collaboration. Therefore, it is suggested that early protective thymus-independent antibody responses to FMDV is mediated, in mice, by splenic B lymphocytes (Otrowski et al., 2007). Similar results have been reported in cattle (Juleff et al., 2009). In this regard, the specific mechanism of B-cell activation in the absence of T-cell activation in swine and cattle requires further investigation.

4 FMDV is Susceptible to IFN

IFNs are the first line of the host innate immune defense against viral infection in mammals (Ank et al., 2006; Basler, and Garcia-Sastre, 2002; Frese et al., 2002). Three families of IFNs have been described, type I, II and III, based on their receptor specificity (Fensterl & Sen, 2009). Type I IFNs (IFN-α and IFN-β) signal through a heterodimeric receptor complex formed by IFNAR1/IFNAR2, type II IFN (IFN-γ) signals through the complex IFN-γR1/IFN-γR2, and type III IFNs bind the receptor complex IL-28Rα/IL-10Rβ. IFNs have some

overlapping biologic activities but unique functional roles in the innate and adaptive immune response. Type I IFNs, are primarily responsible for inducing direct antiviral responses in virus infected cells and do so with more potency than IFN-γ (Tan et al., 2005), but type I IFNs can also stimulate DC maturation and NK cell activation (Tilg, 1997). IFN-γ is mainly produced by activated T lymphocytes and NK cells (Schoenborn & Wilson, 2007). In addition to having antiviral activity, IFN-γ also activates components of the cell-mediated immune system such as cytotoxic T lymphocytes, macrophages, and NK cells, favoring Th1 responses. Type III IFNs are also induced in response to recognition of PAMPs and activation of transcription factors, such as NF-κB and IRF-3 and -7, and are mainly produced by DCs (Iversen et al., 2010). However, expression of the type III IFN receptor in a tissue-specific manner, particularly in epithelia, has been proposed as one of the mechanisms that some organisms utilize to prevent and protect themselves from viral invasion through the skin and mucosal surfaces (Sommereyns et al., 2008). Despite the receptor differences, the three families of IFN transduce signals through the JAK-STAT pathways and type I and type III IFNs induce redundant responses. After the IFN pathway is stimulated transcriptional up-regulation of hundreds of effector genes occurs to block viral infection and spread (Der at al., 1998; Takaoka & Yanai, 2006).

As explained above and similar to many other viruses, FMDV counteracts the innate immune response at least in part by blocking the expression of IFN (Chinsangaram et al., 1999; de los Santos et al. 2006). In fact, L^{pro} is the major FMDV protein responsible for this blocking effect. This was initially observed in supernatants from leaderless virus-infected primary cells, which contain higher levels of IFN-α/β antiviral activity than supernatants from wild-type (WT) virus-infected cells (Chinsangaram et al., 1999). Furthermore, it has been demonstrated that pretreatment of cell cultures with type I, type II, and type III IFNs can dramatically inhibit replication of all seven FMDV serotypes (Chinsangaram et al., 1999, 2001; Diaz-San Segundo et al., 2011; Moraes et al., 2007). To determine whether IFN could block FMDV replication *in vivo* in swine and/or cattle, replication defective human Ad5 vectors that express type I, II and III IFNs were constructed (Grubman et al., 2012). These vectors have the advantage of allowing for a sustained production of IFN in treated animals reducing the need for multiple inoculations of relatively high doses of IFN protein that could lead to undesirable adverse effects (Lukaszewski and Brooks, 2000; Qin et al., 1998; Santodonato et al., 2001). In addition, unlike humans, livestock do not have pre-existing antibodies to human Ad5 vectors and thus administration of this vector containing various foreign transgenes can efficiently induce an immune response to the transgenes in swine and cattle (Graham & Previc, 1992; Mayr et al., 1999). Swine pretreated with Ad5 vectors expressing either porcine IFN-α (Ad5-poIFN-α), porcine IFN-γ (Ad5-poIFN-γ) or porcine IFN-λ (Ad5-poIFN-λ) are completely protected when challenged with different FMDV serotypes one day post inoculation (Chinsangaram et al., 2003; Dias et al., 2011; Moraes et al., 2003, 2007; Perez-Martin et al., manuscript in preparation) and protection lasts 3-5 days (Moraes et al., 2003). Interestingly, the synergistic actions of type I in combination with type II IFN can block virus replication *in vivo*; swine inoculated with a combination of Ad5-poIFN-α and Ad5-poIFN-γ, at doses that alone do not protect against FMDV, are completely protected against clinical disease and do not develop viremia (Moraes et al., 2007). In cattle, the use of type I or type II IFNs has had only limited success (Wu et al., 2003). However, treatment of cattle with an Ad5 vector expressing type III IFN (Ad5-boIFN-λ3) followed by exposure to aerosolized FMDV, resulted in protection for at least 9 days post challenge (Perez-Martin et al., 2012).

5 IFNs Induce Tissue-Specific Innate Immune Cell Infiltration and Stimulate ISGs Expression against FMDV

Although the critical importance of the IFN system in regulating FMDV pathogenesis is now well established, it is still unclear how IFN inhibits FMDV replication and dissemination. The action of IFNs can involve upregulation of hundreds of IFN-stimulated genes (ISGs) and activation of different aspects of the immune

system. It has been recently demonstrated that the actions of IFN can not only be virus-specific, but also ISGs- and tissue specific (Fensterl et al., 2012; Schoggins & Rice, 2011).

In vitro studies demonstrated that at least two ISGs, double-stranded-RNA-dependent protein kinase (PKR) and 2', 5' oligoadenylate synthetase (OAS)/RNase L, are involved in inhibition of FMDV replication (Chinsangaram et al., 2001; de los Santos et al., 2006). Subsequently in Ad5-IFN-treated animals, we showed that numerous ISGs were induced and that protection against FMDV correlated with an increase of the 10-kDa IFN-γ-inducible protein (IP-10) mRNA expression in peripheral blood mononuclear cells (PBMCs) as well as in all analyzed FMDV infected tissues (Fig. 2; Diaz-San Segundo et al., 2010; Moraes et al., 2007;), including isolated skin DCs and NK cells (Fig. 2; Diaz-San Segundo et al., 2010,2011). This chemokine is known to be involved in the recruitment, proliferation, and activation of NK cells (Taub et al., 1995) and has been demonstrated to have a protective effect in mice infected with a number of viruses including mouse hepatitis virus (Trifilo et al., 2004), coxsackievirus B3 (Yuan et al., 2009), dengue virus (Chen et al., 2006), and respiratory syncytial virus (Lindell et al., 2008), but not in Theiler's murine encephalomyelitis virus (TMEV) infected mice (Tsunoda et al., 2004). To determine whether IP-10 has a role in the IFN-induced protection against FMDV, we used a mouse model for FMDV developed by Salguero et al. (2005). IP-10 knockout (ko) or WT C57Bl/6 mice were treated with murine IFN-α (muIFN-α) or PBS 4 h prior to challenge. Although IFN treatment protected 100% of WT C57Bl/6 mice, protection was significantly reduced to only 30% survival when the IP-10 gene was absent, (Diaz-San Segundo et al., submitted). These results indicated that IP-10 is directly involved in protection induced by IFN against FMDV.

The major replication organ for FMDV and the main site of macroscopic lesions in infected animals is the skin (Alexandersen et al., 2003). We have observed that the totality of animals inoculated with an Ad5 vector expressing IFN not only show statistically significant higher numbers of epidermal DCs, but also these cells appear larger and displayed more dendrites 24 h after IFN treatment (Fig. 3; Diaz-San Segundo et al., 2010). LCs migrate selectively to areas with higher concentrations of MIP-3α (via CCR6), a chemokine that is secreted by keratinocytes (Charbonnier et al., 1999). In studies in swine inoculated with Ad5-poIFN-α, we found systemic antiviral activity in plasma and high levels of poIFN-α in serum (Fig. 4), and there are increased mRNA levels of MIP-3α in keratinocytes and skin DCs 14 h after inoculation (Fig. 2). Other IFN-regulated chemokines including monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1α, and IP-10 are not only involved in migration, but also in epidermal DC maturation (Fujita et al., 2005). We showed that upregulation of both, MCP-1 and IP-10, occurs in keratinocytes and LCs of Ad5-poIFN-α treated animals (Fig. 2). Furthermore, the IP-10 receptor, chemokine (C-X-C motif) receptor (CXCR) 3, is also upregulated in skin DCs (Fig. 2). These results indicate that the migratory capacity of skin DCs is enhanced following IFN treatment. Most importantly, this enhancement correlates with protection against FMDV.

In addition, several studies have shown that IFN-α can induce rapid differentiation of monocytes into activated DCs (Longhi et al., 2009). In order to verify the correlation between these upregulated chemokines and swine skin DCs maturation, we analyzed skin DC status in pigs treated with type I IFN. DC maturation is a multi-step process characterized by phenotypic changes including: redistribution of major histocompatibility complex (MHC) molecules from intracellular endocytic compartments to the cell membrane surface, down-regulation of antigen internalization, increase in antigen processing, increase in the surface expression of co-stimulatory molecules (CD80/86), and morphological changes (*e.g.* formation of dendrites). As previously described, there is an increase in the number of LCs and a significant increase in size and number of dendrites in the skin of Ad5-IFN-α-treated swine. Furthermore, we found that skin DCs extracted from pigs treated with Ad5-poIFN-α showed increased antigen processing capacity (Fig. 5a), increased expression of CD80/86 (Fig. 5b), and decreased phagocytic activity (Fig. 5c). However, no changes were observed in micropinocytosis activity or in the expression of MHC II molecules (Fig. 5d). These data indicate that IFN-α induces partial maturation of skin DCs. Moreover, upregulation of mRNA levels of IL-18 and IL-15 at 24 and 48 h respectively after treatment with IFN-α has been observed in the skin (Diaz-San Segundo et al., 2010). These two cytokines are produced by activated DCs (Andoniou et al., 2005, Lucas et al., 2007) and are functionally

involved in porcine NK cell proliferation and activation (Toka et al., 2009). In recent *ex vivo* experiments in which skin DCs from pigs were treated with type I IFN, we also found upregulation of IL-18 and IL-18R mRNA expression was also noticed (Fig. 2). Furthermore, supernatants obtained from the *ex vivo* assay used to purify skin DCs, showed increased levels of IL-18 with significant amounts by 24 h after treatment (Fig. 4B). IL-18 was also detected in the serum of IFN-treated animals at 24 h post-treatment (Fig 4A), indicating that IL-18 secretion by DCs was systemic. Increased expression of IL-18R was also observed in enriched populations of NK cells obtained from PBMCs extracted from IFN-α-treated swine (Fig. 2). These results suggest that IFN-α dependent expression of IL-18 and IL-15 by DCs might be responsible for enhanced NK activity. Infiltration of NK cells in the skin of IFN treated swine was not detected by immunophenotype analysis ($CD2^+/CD8^+/CD3^-$) using an immunofluorescence technique; however an increase in the number of these cells in draining lymph nodes was noticeable (Diaz-San Segundo et al., 2010). We also detected upregulation of perforin and granzyme A in draining lymph nodes, as well as genes associated with NK cell regulation (NKp80, NKG2A and NKG2D) (Fig. 2). However, whether NK cells in the draining lymph node display an increased killing activity after IFN treatment requires further research. It has been previously described that NK cells are rapidly recruited to lymph nodes upon DC activation (Longhi et al., 2009). It is possible that IFN-induced DCs maturation is involved in NK cells recruitment to the lymph nodes via IL-18 and IL-15 stimuli. In fact, we also observed upregulation of IL-15 and IL-18 mRNA levels in draining lymph nodes of IFN treated animals (Fig. 2 and Diaz-San Segundo et al., 2010).

Cytotoxicity of NK cells can be enhanced by many activating cytokines. Type I IFN is one of the most potent activators of NK cells in both humans and in mice (Biron et al., 1999); however, as mentioned earlier, the underlying mechanism of enhanced NK cytotoxicity remains incompletely understood. It has been proposed that IL-15 produced by accessory DCs in response to type I IFN can activate NK cells (Lucas et al., 2007). Furthermore, type I IFN-induced granzyme B and perforin were found to be required for NK cell activation in response to vaccinia virus infection in mice (Martinez et al., 2008). These results suggest that type I IFN can activate NK cells by direct and indirect mechanisms. To this end, it is evident that intricate pathways involving NK cell cytokine responses that depend on cross-talk with activated/mature DCs exists, and systemic antiviral effects induced by FMDV infection add complexity to the current understanding of virus-host interactions. Further studies are required to understand the role of NK cells in protection against FMDV infection.

6 Concluding Remarks

Similar to most viruses, FMDV is highly sensitive to the action of IFNs. Acting in a concerted manner hundreds of IFN stimulated genes control viral infection through the induction of direct antiviral activity and the modulation of multiple cellular responses. Concurrently, FMDV has evolved numerous strategies to effectively counteract the effects of IFN, thus establishing infection. The studies described herein will contribute to a better understanding of the intricate relationships between FMDV and the host immune response and will hopefully lead to the development of new and improved disease control strategies.

Acknowledgements

This research was supported in part by the Plum Island Animal Disease Research Participation Program administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and the U.S. Department of Agriculture (appointment of Nestor Montiel), by CRIS project number 1940-32000-057-00D, ARS, USDA (M. J. Grubman and T. de los Santos) and by grants through an interagency agreement with the Science and Technology Directorate of the U.S. Department of Homeland Security under the Award Numbers HSHQDC-09-X00373 and HSHQDC-11-X-00189 (M. J. Grubman and T. de los Santos). We thank the animal care staff at PIADC for their professional support and assistance.

References

Alexandersen, S., Zhang, Z., Donaldson, A. I. & Garland, A. J (2003). The pathogenesis and diagnosis of foot-and-mouth disease. *Journal of Comparative Pathology*, 129, 1-36.

Andoniou, C. E., van Dommelen, S. L. H., Voigt, V., Andrews, D. M., Brizard, G., Asselin-Paturel, C., Delale, T., Stacey, K. J., Trinchieri, G., & Degli-Esposti., M. A. (2005). Interaction between conventional dendritic cells and natural killer cells is integral to the activation of effective antiviral immunity. *Nature Immunology*, 214, 331-341.

Ank N., West, H., & Paludan, S. R. (2006). IFN-lambda: novel antiviral cytokines. *Journal of Interferon Cytokine Research*, 26, 373-379.

Armer, H., Moffat, K., Wileman, T., Belsham, G. J., Jackson, T., Duprex, W. P., Ryan, M., & Monaghan, P. (2008). Foot-and-mouth disease virus, but not bovine enterovirus, targets the host cell cytoskeleton via the nonstructural protein 3Cpro. *Journal of Virology*, 82, 10556-10566.

Arzt, J., Juleff, N., Zhang, Z, & Rodriguez, L. L. (2011). The pathogenesis of foot-and-mouth disease I: viral pathways in cattle. *Transboundary and Emerging Diseases*, 58, 291-304.

Banchereau, J., & Steinman, R. M. (1998). Dendritic cells and the control of immunity. *Nature.* 392, 245-252.

Basler, C. F., & Garcia-Sastre, A. (2002). Viruses and the type I interferon antiviral system: induction and evasion. *Internal Reviews of Immunology*, 21, 305-337.

Bautista, E. M., Gregg, D., & Golde, W. T. (2002). Characterization and functional analysis of skin-derived dendritic cells from swine without a requirement for in vitro propagation. *Veterinary Immunology and Immunopathology*, 88, 131-148.

Bautista, E. M., Ferman, G. S., & Golde, W. T. (2003). Induction of lymphopenia and inhibition of T cell function during acute infection of swine with foot and mouth disease virus (FMDV). *Veterinary Immunology and Immunopathology* 92, 61-73.

Bautista, E. M., Ferman, G. S., Gregg, D., Brum, M. C., Grubman, M. J., & Golde, W. T. (2005). Constitutive expression of alpha interferon by skin dendritic cells confers resistance to infection by foot-and-mouth disease virus. *Journal of Virology*, 79, 4838-4847.

Baxt, B., & Mason. P. W. (1995). Foot-and-mouth disease virus undergoes restricted replication in macrophage cell cultures following Fc receptor-mediated adsorption. *Virology* 207, 503-509.

Belsham, G. J., & Brangwyn, J. K. (1990). A region of the 5' noncoding region of foot-and-mouth disease virus RNA directs efficient internal initiation of protein synthesis within cells: involvement with the role of L protease in translational control. *Journal of Virology*, 64, 5389-5395.

Belsham, G. J., McInerney, G. M., & Ross-Smith, N. (2000). Foot-and-mouth disease virus 3C protease induces cleavage of translation initiation factors eIF4A and eIF4G within infected cells. *Journal of Virology*, 74, 272-280.

Biron, C. A., Nguyen, K. B., Pien, G. C., Cousens, L. P., & Salazar-Mather, T. P. (1999). Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annual Reviews of Immunology*, 17, 189-220.

Borca, M.V., Fernandez, F.M., Sadir, A.M., Braun, M., & Schudel, A. A. (1986). Immune response to foot-and-mouth disease virus in a murine experimental model: effective thymus-independent primary and secondary reaction. *Immunology,* 59, 261–267.

Brooks D. G., Trifilo, M. J., Edelmann, K. H., Teyton, L., McGavern, D. B., & Oldstone. M. B. A. (2006). Interleukin-10 determines viral clearance or persistence in vivo. *Nature Medicine,* 12, 1301-1309.

Charbonnier, A. S., Kohrgruber, N., Kriehuber, E., Stingl, G., Rot, A., & Maurer, D. (1999). Macrophage inflammatory protein 3alpha is involved in the constitutive trafficking of epidermal langerhans cells. *Journal of Experimental Medicine,* 190, 1755-1768.

Chen, J. P., Lu, H. L., Lai, S. L., Campanella, G. S., Sung, J. M., Lu, M. Y., Wu-Hsieh, B. A., Lin, Y. L., Lane, T. E., Luster, A. D., & Liao, F. (2006). Dengue virus induces expression of CXC chemokine ligand 10/IFN-γ-inducible protein 10, which competitively inhibits viral binding to cell surface heparin sulfate. *Journal of Immunology,* 177, 3185-3192.

Chinsangaram, J., Beard, C., Mason, P. W., Zellner, M. K., Ward, G., & Grubman, M. J. (1998). Antibody response in mice inoculated with DNA expressing foot-and-mouth disease virus capsid proteins. *Journal of Virology*, 72, 4454-4457.

Chinsangaram, J., Piccone, M. E. & Grubman, M. J. (1999). Ability of foot-and-mouth disease virus to form plaques in cell culture is associated with suppression of alpha/beta interferon. *Journal of Virology,* 73, 9891-9898.

Chinsangaram, J., Koster, M., & Grubman, M. J. (2001). Inhibition of L-deleted foot-and-mouth disease virus replication by alpha/beta interferon involves double-stranded RNA-dependent protein kinase. *Journal of Virology,* 75, 5498-5503.

Chinsangaram, J., Moraes, M. P., Koster, M., & Grubman, M. J. (2003). Novel viral disease control strategy: adenovirus expressing alpha interferon rapidly protects swine from foot-and-mouth disease. *Journal of Virology,* 77, 1621-1625.

Collen, T., Pullen, L. & Doel, T. R. (1989). T-cell-dependent induction of antibody against foot-and-mouth- disease virus in a mouse model. *Journal of General Virology*, 70, 395–403.

de los Santos, T., de Avila Botton, S., Weiblen, R. & Grubman, M. J. (2006). The leader proteinase of foot-and-mouth disease virus inhibits the induction of beta interferon mRNA and blocks the host innate immune response. *Journal of Virology,* 80, 1906-1914.

de los Santos T., Diaz-San Segundo, F., & Grubman, M. J. (2007). Degradation of nuclear factor kappa B during foot-and-mouth disease virus infection. *Journal of Virology*, 81, 12803-12815.

de los Santos T., Diaz-San Segundo, F., Zhu, J., Koster, M., Dias, C. C., & Grubman, M. J. (2009). A conserved domain in the leader proteinase of foot-and-mouth disease virus is required for proper subcellular localization and function. *Journal of Virology,* 83, 1800-1810.

Der, S. D., Zhou, A., Williams, B. R. G., & Silverman, R. H. (1998). Identification of genes differentially regulated by interferon α, β, or γ using oligonucleotide arrays. *Proceedings of the Natural Academy of Science*, 95, 15623-15628.

Devaney, M. A., Vakharia, V. N., Lloyd, R. E., Ehrenfeld, E., & Grubman, M. J. (1988). Leader protein of foot-and-mouth disease virus is required for cleavage of the p220 component of the cap-binding protein complex. *Journal of Virology,* 62, 4407-4409.

Dias, C. C., Moraes, M. P., Diaz-San Segundo, F., de los Santos, T., & Grubman, M. J. (2011). Porcine type I interferon rapidly protects swine against challenge with multiple serotypes of foot-and-mouth disease virus. *Journal Interferon Cytokine Research*, 31, 227-236.

Diaz-San Segundo, F., Dias, C. C., Moraes, M. P., Weiss, M., Pérez-Martín, E., Owens, G, Custer, M., Kamrud, K., de los Santos, T., & Grubman, M. J. (2012). Venezuelan equine encephalitis replicon particles can induce rapid protection against foot-and-mouth disease virus. *Submitted.*

Diaz-San Segundo, F., Weiss, M., Pérez-Martín, E., Dias, C. C., Grubman, M. J., & de los Santos, T. (2012). Inoculation of swine with foot-and-mouth disease SAP-mutant virus induces early protection against disease. *Journal of Virology,* 86, 1316-27.

Diaz-San Segundo, F., Weiss, M., Pérez-Martín, E., Koster, M. J., Zhu, J., Grubman, M. J., & de los Santos, T. (2011). Antiviral activity of bovine type III interferon against foot-and-mouth disease virus. *Virology.* 413, 283-292.

Diaz-San Segundo, F., Moraes, M. P., de Los Santos, T., Dias, C. C., & Grubman, M. J. (2010). Interferon-induced protection against foot-and-mouth disease virus infection correlates with enhanced tissue-specific innate immune cell infiltration and interferon-stimulated gene expression. *Journal of Virology*, 84, 2063-2077.

Diaz-San Segundo, F., Rodriguez-Calvo, T., de Avila, A., & Sevilla, N. (2009). Immunosprression during acute infection with foot-and-mouth disease virus in swine is mediated by IL-10. *PLoS One* 4, 1-11.

Díaz-San Segundo, F., Salguero, F. J., de Avila, A., de Marco, M. M., Sanchez-Martín, M. A., & Sevilla, N. (2006). Selective lymphocyte depletion during the early stage of the immune response to foot-and-mouth disease virus infection in swine. *Journal of Virology*, 80, 2369-2379.

Donnelly, M. L., Hughes, L. E., Luke, G., Mendoza, H., ten Dam, E., Gani, D., & Ryan, M. D. (2001a). The 'cleavage' activities of foot-and-mouth disease virus 2A site- directed mutants and naturally occurring '2A-like' sequences. *Journal of General Virology*, 82, 1027-1041.

Donnelly, M. L., Hughes, L. E., Luke, G., Mendoza, H., ten Dam, E., Gani, D., & Ryan, M. D. (2001b). Analysis of the aphthovirus 2A/2B polyprotein 'cleavage' mechanism indicates not a proteolytic reaction, but a novel translational effect: a putative ribosomal'skip'. *Journal of General Virology*, 82, 1013-1025.

Falk, M. M., Grigera, P. R., Bergmann, I. E., Zibert, A., Multhaup, G., & Beck, E. (1990). Foot-and-mouth disease virus protease 3C induces specific proteolytic cleavage of host cell histone H3. *Journal of Virology*, 64, 748-56.

Fracastorius, H. (1546). De alijs differentijs contagionis. *De sympathia et antipathia rerum Liber unus. De Contagione et Contagiosis Morbis et Curatione (libri iii)*, pp. 36–38. Heirs of L.A. Junta Book 1.

Fenster,l V., & Sen, G. C. (2009). Interferons and viral infections. *Biofactors,* 35, 14–20.

Fensterl, V., Wetzel, J. L., Ramachandran, S., Ogino, T., Stohlman, S. A., Bergmann, C. C., Diamond, M. S., Virgin, H. V. & Sen, G. C. (2012). Interferon-induced Ifit2/ISG54 protects mce from lethal VSV neuropathogenesis. *PLoS Pathogens,* 8, e1002712.

Frese, M., Schwärzle, V., Barth, K., Krieger, N., Lohmann, V., Mihm, S., Haller, O., & Bartenschlager, R. (2002). Interferon-gamma inhibits replication of subgenomic and genomic hepatitis C virus RNAs. *Hepatology,* 35, 694-703.

Gladue D. P., O'Donnell, V., Baker-Branstetter, R., Holinka, L. G., Pacheco, J. M., Fernandez Sainz, I., Lu, Z., Brocchi, E., Baxt, B., Piccone, M. E., Rodriguez, L., & Borca, M. V. (2012). Foot and mouth disease virus non structural protein 2C interacts with Beclin1 modulating virus replication. *Journal of Virology,* 86, 12080-12090.

Golde, W. T., Pacheco, J. M., Duque, H., Doel, T., Penfold, B., Ferman, G. S., Gregg, D. R. & Rodriguez, L. L. (2005). Vaccination against foot-and-mouth disease virus confers complete clinical protection in 7 days and partial protection in 4 days: Use in emergency outbreak response. *Vaccine,* 23, 5775-5782.

Golde, W. T., Nfon, C. K. & Toka, F. N. (2008). Immune evasion during foot-and-mouth disease virus infection of swine. *Immunological Reviews,* 225, 85-95.

Graham, F. L. & Prevec, L. (1992). Adenovirus-based expression vectors and recombinant vaccines. *Biotechnology,* 20, 363-390.

Grubman, M. J., & Baxt, B. (2004). Foot-and-mouth disease. *Clinical Microbiology Reviews,* 17, 465-493.

Grubman, M. J., Moraes, M. P., Díaz-San Segundo, F., Pena, L., & de los Santos, T. (2008). Evading the host immune response: how foot-and- mouth disease virus has become an effective pathogen. *FEMS Immunology and Medical Microbiology,* 53, 8-17.

Grubman, M. J., Moraes, M. P., Shutta, C., Barrera, J., Neilan, J., Ettyreddy, D., Butman, B. T., Brough, D. E., & Brake, D. A. (2010). Adenovirus serotype 5-vectored foot-and-mouth disease subunit vaccines: the first decade. *Future Virology,* 5, 51-64.

Grubman, M. J., Diaz-San Segundo, F., Dias, C. C., Moraes, M. P., Perez-Martin, E. & de los Santos, T. (2012). Use of replication-defective adenoviruses to develop vaccines and biotherapeutics against foot-and-mouth disease. *Future Virology,* 7, 767-778.

Guzylack-Piriou L., Bergamin, F., Gerber, M., McCullough, K. C., & Summerfield, A. (2006). Plasmacytoid dendritic cell activation by foot-and-mouth disease virus requires immune complexes *European Journal of Immunology,* 36, 1674-1683.

Haller, O., Staeheli, P., & Kochs, G. (2007). Interferon-induced Mx proteins in antiviral host defense. *Biochimie.* 89, 812-818.

Iversen, M. B., Ank, N., Melchjorsen, J. & Paludan, S. R. (2010). Expression of type III interferon (IFN) in the vaginal mucosa is mediated primarily by dendritic cells and displays stronger dependence on NF-kappaB than type I IFNs. *Journal of Virology,* 84, 4579-4586.

Iwasaki, A., & Kelsall, B. L. (2000). Localization of distinct Peyer's patch dendritic cell subsets and their recruitment by chemokines macrophage inflammatory protein (MIP)-3alpha, MIP-3beta, and secondary lymphoid organ chemokine. *Journal of Experimental Medicine,* 191, 1381–1394.

Joshi, G., Sharma, R. & Kakker, N. K. (2009). Phenotypic and functional characterization of T-cells and in vitro replication of FMDV serotypes in bovine lymphocytes. *Vaccine,* 27, 6656-6661.

Juleff, N., Windsor, M., Lefevre, E. A., Gubbins, S., Hamblin, P., Reid, E., McLaughlin, K., Beverley, P. C., Morrison, I. W. & Charleston, B. (2009). Foot-and-mouth disease virus can induce a specific and rapid CD4+ T-cell-independent neutralizing and isotype class-switched antibody response in naïve cattle. *Journal of Virology,* 83, 3626-3636.

Kawai, T., & Akira, S. (2011). Regulation of innate immune signalling pathways by the tripartite motif (TRIM) family proteins. *EMBO Molecular Medicine,* 3, 513-327.

Kleina, L. G., & Grubman, M. J. (1992). Antiviral effects of a thiol protease inhibitor on foot-and-mouth disease virus. *Journal of Virology,* 66, 7168-7175.

Kochs, G., & Haller, O. (1999). Interferon-induced human MxA GTPase blocks nuclear import of Thogoto virus nucleocapsids. *Proceedings of the Natural Academy of Science*, 96, 2082-2086.

Kuhn, R., Luz, N. & Beck, E. (1990). Functional analysis of the internal translation initiation site of foot- and-mouth disease virus. *Journal of Virology*, 64, 4625-4631.

Klump, W., Marquardt, O. & Hofschneider, P. H. (1984). Biologically active protease of foot-and-mouth disease virus is expressed from cloned viral cDNA in Escherichia coli. *Proceedings of the Natural Academy of Science*, 81, 3351-3355.

Lawrence P., Schafer, E. A., & Rieder, E. (2012). The nuclear protein Sam68 is cleaved by the FMDV 3C protease redistributing Sam68 to the cytoplasm during FMDV infection of host cells.*Virology*. 425, 40-52.

Lee M. S., & Kim, Y. J. (2007). Signaling pathways downstream of pattern-recognition receptors and their cross talk. *Annual Reviews of Biochemistry*, 76, 447–480.

Leforban, Y. (1999). Prevention measures against foot-and-mouth disease in Europe in recent years. *Vaccine*, 17, 1755-1759.

Lindell, D. M., Lane, T. E., & Lukacs, N. W. (2008). CXCL10/CXCR3-mediated responses promote immunity to respiratory syncytial virus infection by augmenting dendritic cell and CD8+ T cell efficiency. *European Journal of Immunology*, 38:2168-2179.

Loeffler, F., & Frosch, P. (1897). Summarischer Bericht ueber der Ergebnisse der Untersuchungen zur Erforschung der Maul- und Klauenseuche. ZentBl. Bakt. *Parasitkde* 22, 257–259.

Longhi, M. P., Trumpfheller, C., Idoyaga, J., Caskey, M., Matos, I., Kluger, C., Salazar, A. M., Colonna, M., & Steinman, R. M. (2009). Dendritic cells require a systemic type I interferon response to mature and induce CD4+ Th1 immunity with poly IC as adjuvant. *Journal of Experimental Medicine*, 206, 1589-1602.

Lucas, M., Schachterle, W., Oberle, K., Aichele, P., & Diefenbach, A. (2007). Natural killer cell-mediated control of infections requires production of interleukin 15 by type I IFN-triggered dendritic cells. *Immunity*, 26, 503–517.

Lukaszewski, R. A., & Brooks, T. J. (2000). Pegylated alpha interferon is an effective treatment for virulent Venezuelan equine encephalitis virus and has profound effects on the host immune response to infection. *Journal of Virology*, 74, 5006–5015.

Martinez, J., Huang, X. & Yang, Y. (2008). Direct action of type I IFN on NK cells is required for their activation in response to vaccinia viral infection in vivo. *Journal of Immunology*, 180, 1592–1597.

Mason, P. W., Piccone, M. E., McKenna, T. S., Chinsangaram, J., & Grubman, M. J. (1997). Evaluation of a live-attenuated foot-and-mouth disease virus as a vaccine candidate. *Virology*, 227, 96-102.

Mason, P. W., Chinsangaram, J., Moraes, M.P., Mayr, G.A., & Grubman, M. J. (2003). Engineering better vaccines for foot-and-mouth disease. *Developments in Biologicals (Basel)*, 114, 79-88.

Mayr, G. A., Chinsangaram, J., & Grubman, M. J. (1999). Development of replication-defective adenovirus serotype 5 containing the capsid and 3C protease coding regions of foot-and-mouth disease virus as a vaccine candidate. *Virology*, 263, 496-506.

McCullough, K.C., Parkinson, D. & Crowther, J. R. (1988). Opsonization-enhanced phagocytosis of foot-and-mouth disease virus. *Immunology*, 65, 187-191.

Medina, M., Domingo, E., Brangwyn, J. K. & Belsham, G. J. (1993). The two species of the foot-and-mouth disease virus leader protein, expressed individually, exhibit the same activities. *Virology*, 194, 55-359.

Merad, M., & Manz, M. G. (2009). Dendritic dells homeostasis. *Blood*, 113, 3418-3427.

Moffat, K., Howell, G,. Knox, C,. Belsham, G. J., Monaghan, P., Ryan, M. D. & Wileman, T. (2005). Effects of foot-and-mouth disease virus nonstructural proteins on the structure and function of the early secretory pathway: 2BC but not 3A blocks endoplasmic reticulum-to-Golgi transport. *Journal of Virology*, 79, 4382-4395.

Moffat, K., Knox, C., Howell, G., Clark, S. J., Yang, H., Belsham, G. J., Ryan, M., & Wileman, T. (2007). Inhibition of the secretory pathway by foot-and-mouth disease virus 2BC protein is reproduced by coexpression of 2B with 2C, and the site of inhibition is determined by the subcellular location of 2C. *Journal of Virology*, 81, 1129-1139.

Monaghan, P, Gold, S, Simpson, J, Zhang, Z, Weinreb, P. H., Violette, S. M., Alexandersen, S., & Jackson, T. (2005). The avb6 integrin receptor for foot-and-mouth disease virus is expressed constitutively on the epithelial cells targeted in cattle. *Journal of General Virology*, 86, 2769-2780.

Moraes, M. P., Chinsangaram, J., Brum, M. C. S., & Grubman, M. J. (2003). Immediate protection of swine from foot-and-mouth disease: a combination of adenoviruses expressing interferon alpha and a foot-and-mouth disease virus subunit vaccine. *Vaccine*, 22, 268-279.

Moraes, M. P., de los Santos, T., Koster, M., Turecek, T., Wang, H., Andreyev, V. G., & Grubman, M. J. (2007). Enhanced antiviral activity against foot-and-mouth disease virus by a combination of type I and II porcine interferons. *Journal of Virology*, 81, 7124-7135.

Moraes, M. P., Mayr, G. A., Mason, P. W. & Grubman, M. J. (2002). Early protection against homologous challenge after a single dose of replication-defective human adenovirus type 5 expressing capsid proteins of foot-and-mouth disease virus (FMDV) strain A24. *Vaccine*, 20, 1631-1639.

Nfon, C. K., Dawson, H., Toka, F. N. & Golde, W. T. (2008). Langerhans cells in porcine skin. *Veterinary Immunology and Immunopathology*, 126, 236-247.

Nfon, C. K., Ferman, G. S., Toka, F. N., Gregg, D. A., & Golde, W. T. (2008). Interferon-alpha production by swine dendritic cells is inhibited during acute infection with foot-and-mouth disease virus. *Viral Immunology*, 21, 68-77.

Nfon, C. K., Toka, F. N., Kenney, M., Pacheco, J. M., & Golde, W. T. (2010). Loss of plasmacytoid dendritic cell function coincides with lymphopenia and viremia during foot-and-mouth disease virus infection. *Viral Immunology*, 23, 29-41. Erratum in: *Viral Immunology*, 23, 339.

O'Donnell, V., Pacheco, J. M., Gregg, D., & Baxt, B. (2008). Analysis of foot-and-mouth disease virus integrin receptor expression in tissues from naive and infected cattle. *Journal of Comparative Pathology*, 141, 98-112.

Okuse, C., Rinaudo, K J., Farrar, A., Wells, F., & Korba, B. E. (2005). Enhancement of antiviral activity against hepatitis C virus in vitro by interferon combination therapy. *Antiviral Research*, 65, 23-34.

Ostrowski, M., Vermeulen, M., Zabal, O., Geffner, J. R., Sadir, A. M. & Lopez, O. J. (2005). Impairment of thymus-dependent responses by murine dendritic cells infected with foot-and-mouth disease virus. *Journal of Immunology*, 175, 3971-3979.

Ostrowski, M., Vermeulen, M., Zabal, O., Zamorano, P. I., Sadir, A. M., Geffner, J. R. & Lopez. O. J. (2007). The early protective thymus-independent antibody response to foot-and-mouth disease virus is mediated by splenic $CD9^+$ B lymphocytes. *Journal of Virology*, 81, 9357-9367.

Pacheco, J. M., Brum, M. C. S., Moraes, M. P., Golde, W. T., & Grubman, M. J. (2005). Rapid protection of cattle from direct challenge with foot-and-mouth disease virus (FMDV) by a single inoculation with an adenovirus vectored FMDV subunit vaccine. *Virology*, 337, 205-209.

Pacheco, J.M., Butler, J.E., Jew, J., Ferman, G.S., Zhu, J., & Golde, W.T. (2010). IgA antibody response of swine to foot-and-mouth disease virus infection and vaccination. *Clinical and Vaccine Immunology*, 17, 550–558.

Perez-Martin E., Weiss, M., Diaz-San Segundo, F., Pacheco, J. M., Arzt, J., Grubman, M. J., & de los Santos, T. (2012). Bovine type III interferon significantly delays and reduces the severity of foot-and-mouth disease in cattle. *Journal of Virology*, 86, 4477-4487.

Piccone, M. E., Rieder, E., Mason, P. W., & Grubman, M. J. (1995). The foot and- mouth disease virus leader proteinase gene is not required for viral replication. *Journal of Virology*, 69, 5376-5382.

Piccone, M. E., Pacheco, J. M., Pauszek, S. J., Kramer, E., Rieder, E., Borca, M. V., & Rodriguez, L. L. (2010). The region between the two polyprotein initiation codons of foot-and-mouth disease virus is critical for virulence in cattle. *Virology*, 396, 152-159.

Qin, X.Q., Tao, N., Dergay, A., Moy, P., Fawell, S., Davis, A., Wilson, J.M., & Barsoum, J. (1998). Interferon-beta gene therapy inhibits tumor formation and causes regression of established tumors in immune-deficient mice. *Proceedings of the Natural Academy of Science*, 95, 14411–14416.

Reid, E., Juleff, N., Gubbins, .S, Prentice, H., Seago, J., & Charleston B. (2011). Bovine plasmacytoid dendritic cells are the major source of type I interferon in response to foot-and-mouth disease virus in vitro and in vivo. *Journal of Virology*, 85, 4297-4308.

Reizis, B., Bunin, A., Ghosh, H, S., Lewis, K. L., & Sisirak V. (2011). Plasmacytoid dendritic cells: recent progress and open questions. *Annual Review in Immunology*, 29, 163-183.

Rigden, R. C., Carrasco, C. P., Summerfield, A., & McCullough, K. C. (2002). Macrophage phagocytosis of foot-and-mouth disease virus may create infectious carriers. *Immunology*, 106, 537-548.

Roberts, P. J., & Belsham, G. J. (1995). Identification of critical amino acids within the foot-and-mouth disease virus leader protein, a cysteine protease. *Virology*, 213, 140-146.

Robinson, M. J., Sancho, D., Slack, E. C., LeibundGut-Landmann, S., & Reis e Sousa. C. (2006). Myeloid C-type lectins in innate immunity. *Nature Immunology*, 7, 1258-1265.

Robinson, L., Windsor, M., McLaughlin, K., Hope, J., Jackson, T., & Charleston, B. (2011) Foot-and-mouth disease virus exhibits an altered tropism in the presence of specific immunoglobulins, enabling productive infection and killing of dendritic cells. *Journal of Virology*, 85, 2212-2223.

Rueckert, R. R. (1996). Picornaviridae: the viruses and their replication. *In "Field's Virology" (B. N. Fields, D.M. Knipe, and P.H. Howley, Ed.)*, pp. 609-654. Lippincott-Raven, Philadelphia and New York.

Rueckert R. R., & Wimmer, E. (1984). Systematic nomenclature of picornavirus proteins. *Journal of Virology*, 50, 957-959.

Salguero, F. J., Sánchez-Martín, M. A., Díaz-San Segundo, F., de Avila, A., & Sevilla, N.. (2005). Foot-and-mouth disease virus (FMDV) causes an acute disease that can be lethal for adult laboratory mice. *Virology*. 332:384-396.

Salt, J. S., Mulcahy, G., & Kitching, R. P. (1996). Isotype-specific antibody responses to foot-and-mouth disease virus in sera and secretion of 'carrier' and 'non-carrier' cattle. *Epidemiology and Infection*, 117, 349-360.

Santodonato, L., Ferrantini, M. Palombo, F., Aurisicchio, L., Delmastro, P., La Monica, N., Di Marco, S., Ciliberto, G., Du, M. X., Taylor, M. W., & Belardelli, F. (2001). Antitumor activity of recombinant adenoviral vectors expressing murine IFN-alpha in mice injected with metastatic IFN-resistant tumor cells. *Cancer Gene Therapy*, 8, 63–72.

Sanz-Parra, A., Sobrino, F., & Ley, V. (1998). Infection with foot-and-mouth disease virus results in a rapid reduction of MHC class I surface expression. *Journal of General Virology*, 79, 433-436.

Schoenborn, J. R., & Wilson,, C. B. (2007). Regulation of interferon-gamma during innate and adaptive immune responses. *Advances in Immunology*, 96, 41–101.

Schoggins, J. W., & Rice, C. M. (2011). Interferon stimulated genes and their antiviral effector functions. *Current Opinion on Virology*, 6, 519-525.

Skern, T., Fita, I. & Guarne, A. (1998). A structural model of picornavirus leader proteinases based on papain and bleomycin hydrolase. *Journal of General Virology*, 79, 301-307.

Sommereyns, C., Paul, S., Staeheli, P., & Michiels, T. (2008). IFN-lambda (IFN-lambda) is expressed in a tissue-dependent fashion and primarily acts on epithelial cells in vivo. *PLoS Pathogens*, 4, e1000017.

Strebel, K., & Beck, E. (1986). A second protease of foot-and-mouth disease virus. *Journal of Virology*, 58, 893-899.

Summerfield, A., & McCullough, K. C. (2009). The porcine dendritic cell family. *Developmental and Comparative Immunology*, 33, 299-309.

Takaoka, A., & Yanai, H. (2006). Interferon signalling network in innate defence. *Cell Microbiology*, 8, 907-922.

Tan, H., Derrick, J., Hong, J., Sanda, C., Grosse, W., M., Edenberg, H. J., Taylor, M., Seiwert, S., & Blatt, L. M. (2005). Global transcriptional profiling J. demonstrates the combination of type I and type II interferon enhances antiviral and immune responses at clinically relevant doses. *Journal of Interferon Cytokine Research*, 25, 632–649.

Taub, D. D., Sayers, T. J., Carter, C. R. D., & Ortaldo, J. R. (1995). α and β chemokines induce NK cell migration and enhance NK-mediated cytolysis. *Journal of Immunology*, 155, 3877-3888.

Tilg, H. (1997). New insights into the mechanisms of interferon alpha: an immunoregulatory and anti-inflammatory cytokine. *Gastroenterology*, 112, 1017-1021.

Toka, F. N., Nfon, C. K. Dawson, H., Estes, D., & Golde, W. T. (2009). Activation of porcine natural killer cells and lysis of foot-and-mouth disease virus infected cells. *Journal of Interferon Cytokine Research*, 29, 179-192.

Toka, F. N., Nfon, C. K. Dawson, H. & Golde, W. T. (2009). Accessory-cell-mediated activation of porcine NK cells by toll-like receptor 7 (TLR7) and TLR8 agonists. *Clinical Vaccine Immunology*, 16, 866-878.

Toka, F. N., Nfon, C. K. Dawson, H., & Golde, W. T. (2009). Natural killer cell dysfunction during acute infection with foot-and-mouth disease virus. *Clinical Vaccine Immunology*, 16, 1738-1749.

Trifilo, M. J., Montalto-Morrison, C., Stiles, L. N., Hurst, K. R., Hardison, J. L., Manning, J. E., Masters, P. S., & Lane, T. E. (2004). CXC chemokine ligand 10 controls viral infection in the central nervous system: evidence for a role in innate immune response through recruitment and activation of natural killer cells. *Journal of Virology*, 78, 585-594.

Trinchieri, G,, & Sher, A. (2007). Cooperation of Toll-like receptor signals in innate immune defense. *Nature Reviews on Immunology*, 7, 179-190.

Tsunoda, I., Lane, T. E., Blackett, J., & Fujinami, R. S. (2004). Distinct roles for IP-10/CXCL10 in three animal models, Theiler's virus infection, EAE, and MHV infection, for multiple sclerosis: implication of differing roles for IP-10. *Multiple Sclerosis*, 10, 26-34.

Valladeau, J., Ravel, O., Dezutter-Dambuyant, C., Moore, K., Kleijmeer, M., Liu, Y., Duvert-Frances, V., Vincent, C., Schmitt, D., Davoust, J., Caux, C., Lebecque, S., & Saeland, S. (2000). Langerin, a novel C-type lectin specific to Langerhans cells, is an endocytic receptor that induces the formation of Birbeck granules. *Immunity*, 12, 71-81.

Vakharia, V. N., Devaney, M. A., Moore, D. M., Dunn, J. J., & Grubman, M. J. (1987). Proteolytic processing of foot and mouth disease virus polyproteins expressed in a cell free system from clone derived transcripts. *Journal of Virology*, 61, 3199-31207.

Vivier, E., Raulet, D. H., Moretta, A., Caligiuri, M. A., Zitvogel, L., Lanier, L. L., Yokoyamal, W. M. & Ugolini, S. (2011). Innate or adaptive immunity? The example of natural killer cells. *Science*, 331, 44-49.

Wang, D., Fang, L. Luo, R., Ye, R., Fang, Y., Xie, L., Chen, H., & Xiao, S. (2010). Foot-and-mouth disease virus leader proteinase inhibits dsRNA-induced type I interferon transcription by decreasing interferon regulatory factor 3/7 in protein levels. *Biochemical and Biophysical Research Communication*, 399, 72-78.

Wang, D., Fang, L., Li, K., Zhong, H., Fan, J., Ouyang, C., Zhang, H., Duan, E., Luo, R., Zhang, Z., Liu, X., Chen, H., & Xiao, S. (2012). Foot-and-mouth disease virus 3C protease cleaves NEMO to impair innate immune signalling. *Journal of Virology*, 86, 9311-9322.

Wang, D., Fang, L., Liu, L., Zhong, H., Chen, Q., Luo, R., Liu, X., Zhang, Z., Chen, H., & Xiao, S. (2011). Foot-and-mouth disease virus (FMDV) leader proteinase negatively regulates the porcine interferon-λ1 pathway. *Molecular Immunology*, 49, 407-412.

Schoenborn, J. R., & Wilson, C. B. (2007). Regulation of interferon-gamma during innate and adaptive immune responses. *Advances Immunology*, 96, 41-101.

Wu, Q., Brum, M. C. S., Caron, L., Koster, M., & Grubman, M. J. (2003). Adenovirus-mediated type I interferon expression partially protects cattle from foot-and-mouth disease. *Journal of Interferon Cytokine Research*, 23, 371-380.

Wykes, M., Pombo, A., Jenkins, C. & MacPherson, G. G. (1998). Dendritic cells interact directly with Naive B lymphocytes to transfer antigen and initiate class switching in a primary T-dependent response. *Journal of Immunology*, 161, 1313–1319.

Yuan, J., Liu, Z., Lim, T., Zhang, H., He, J., Walker, E., Shier, C., Wang, Y., Su, Y., Sall, A., McManus, B., & Yang, D. (2009). CXCL10 inhibits viral replication through recruitment of natural killer cells in coxsackievirus B3-induced myocarditis. *Circulation Research*, 104, 628-638.

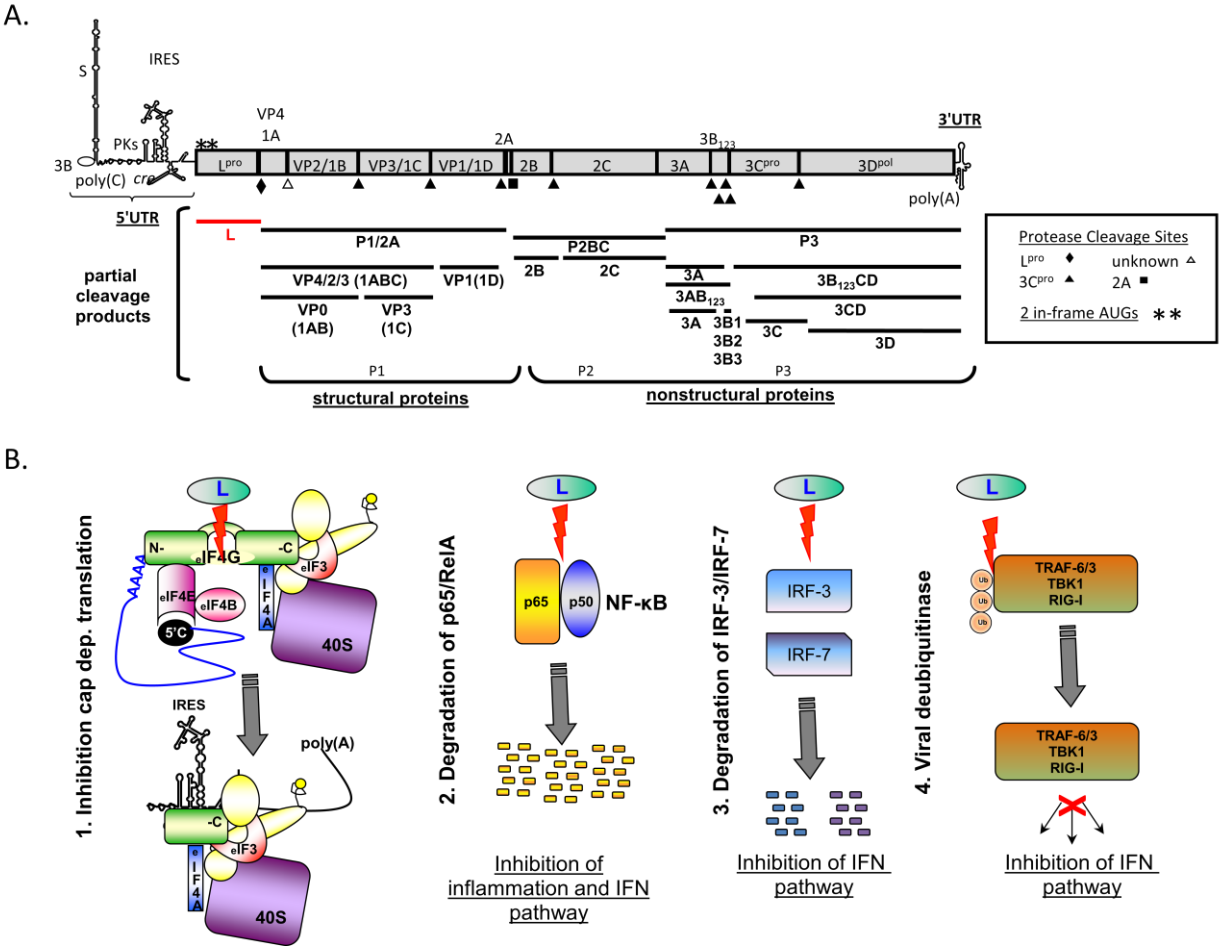

Figure 1: A. Schematic map of the FMDV genome. The ORF is shown in the boxed area, with the viral proteins named according to the nomenclature of Rueckert and Wimmer (1984), lines indicate RNA structures. Thick lines below the genome diagram indicate prominent partial cleavage products. S fragment, short fragment of the genome, poly(C), poly cytosine tract, PK, pseudoknot, *cre*, *cis*-acting replicative element, IRES, internal ribosome entry site. The sites of the primary cleavages and the proteases responsible are indicated (adapted from Mason et al., 2003). **B**. Schematic diagram illustrating the known biological activities of FMDV L^{pro} which affect the host response. 1. L^{pro} cleaves the translation initiation factor eIF4G resulting in the shutoff of host cap dependent protein synthesis. eIF4A, eIF4B, eIF4G, eIF4E and eIF3 are translation initiation factors. 40S and 60S are ribosomal subunits. 2. L^{pro} is directly or indirectly involved in the cleavage of the transcription factor NF-kB. p65/p50 represents the NF-kB heterodimer. 3. L^{pro} is involved in the degradation of IFN regulatory factor (IRF)-3 and IRF-7, resulting in the inhibition of the IFN pathway. 4. L^{pro} acts as a viral deubiquitinase removing ubiquitin (Ub) moieties from TNF receptor-associated factor 6 (TRAF-6), and TRAF-3, retinoic acid-inducible gene I (RIG-I) and TANK-binding kinase 1 (TBK1), key signaling molecules in activation of type I IFN response. (adapted from Grubman et al., 2008).

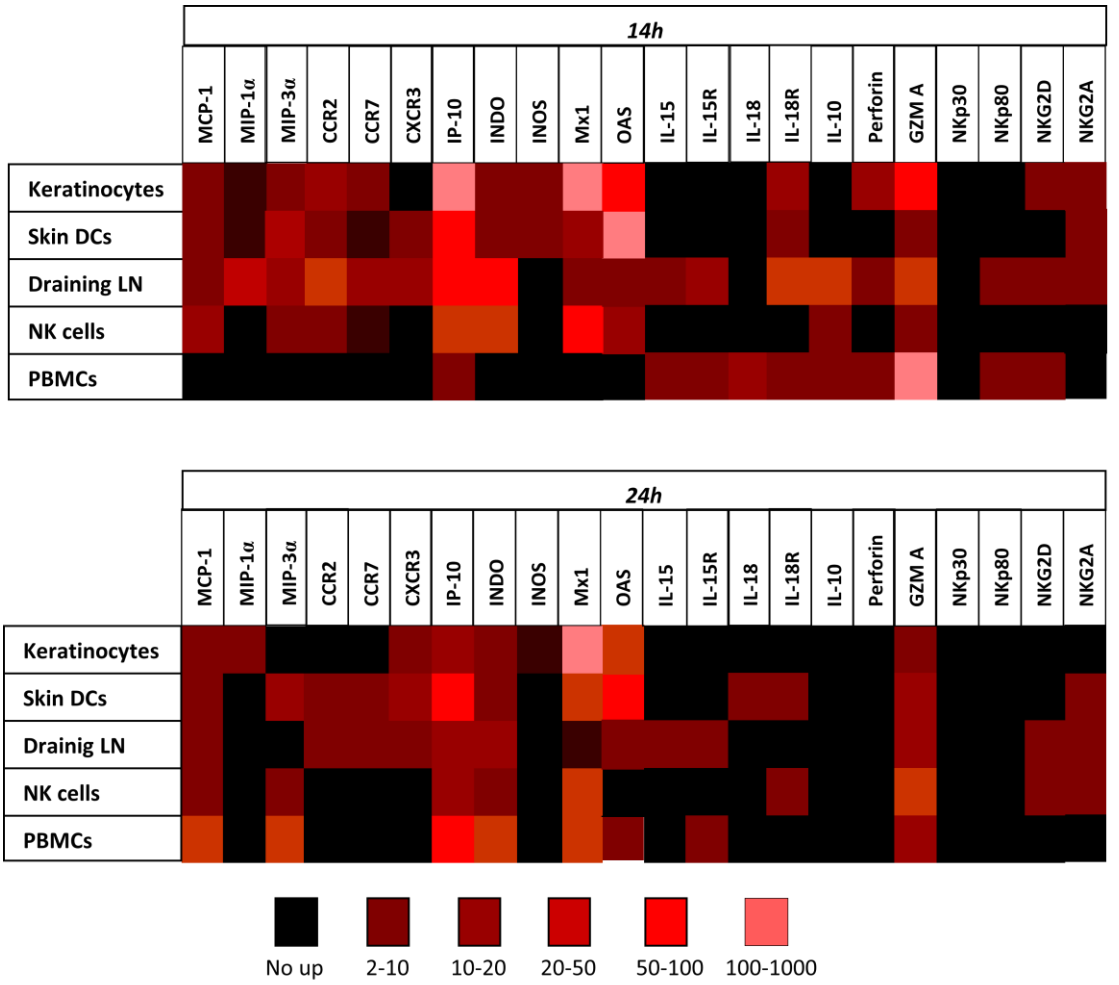

Figure 2: Induction of interferon stimulated genes (ISGs) in PBMCs, keratinocytes, skin DCs, NK cells, and the draining lymph node (LN) of swine. Real time RT-PCR was used to analyze upregulation of ISGs in different organs. Relative mRNA levels were determined by comparative cycle threshold analysis utilizing as a reference the samples at 0 dpi from the control group and upregulation of genes at different time-points are represented in different shades of red as indicated in the figure.

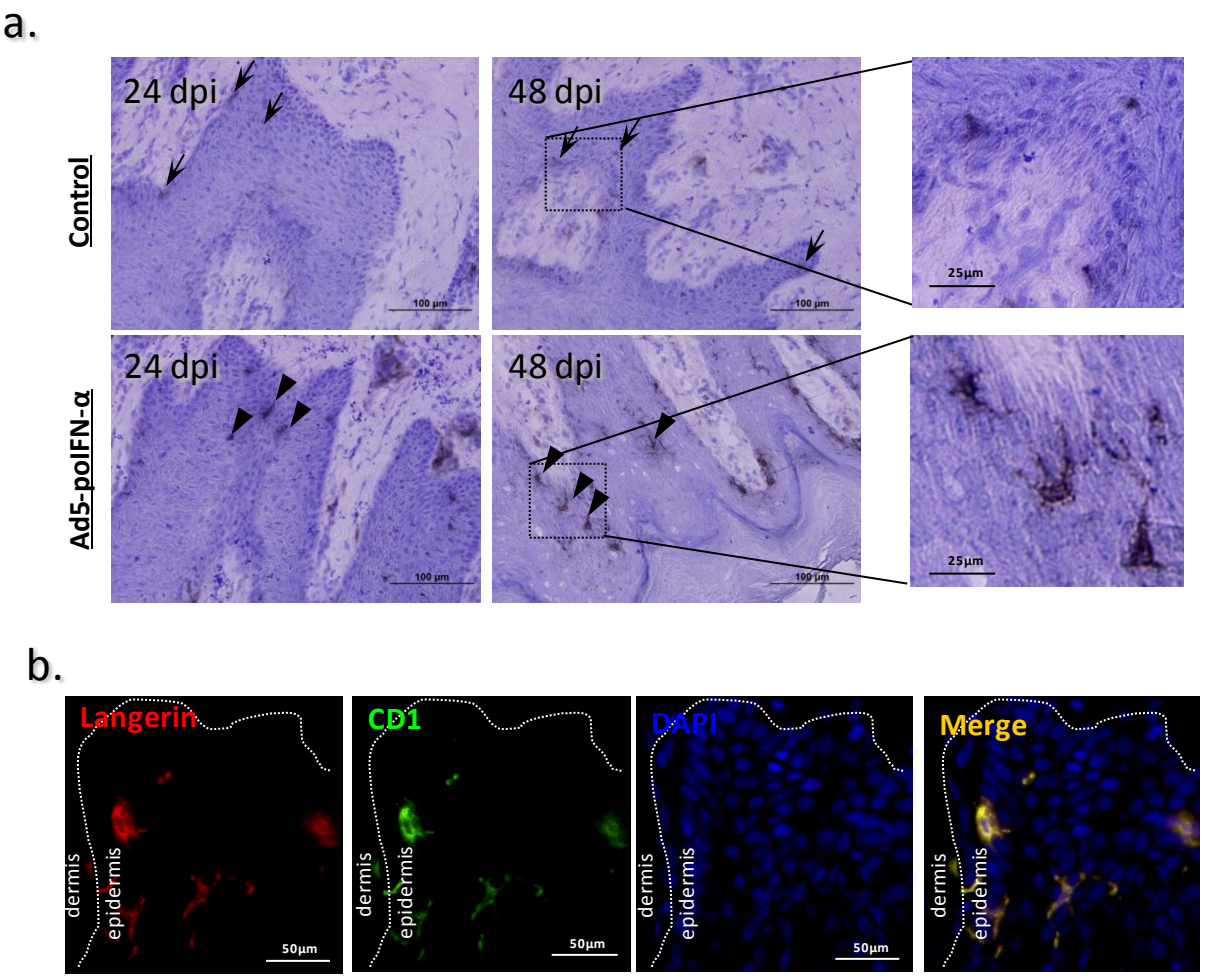

Figure 3: Immunohistochemical detection of DCs in skin from the heel-bulb of swine. a. Skin from control non-treated animals or Ad5-poIFN-α-inoculated swine was harvested 24 and 48 h after Ad5 treatment. **a.** $CD1^+$ cells were detected by immunohistochemistry (IHC) using the avidin-biotin-peroxidase complex technique and developed with 3,3´-diaminobenzidine which gives a brown product where primary antibody binds (dark brown); sections were counterstained with Harry's hematoxylin. Arrows indicate positive staining for DCs in control animals; these cells were small and appeared localized in the stratum basale of the epidermis. Arrowheads indicate the positive staining of DCs in treated animals; these cells appeared larger than positive cells in control animals, showed dendrites, and were localized along the stratum spinosum. **b.** Double-Immunofluoresence (IFA) detection of CD207/Langerin (red) and CD1 (green) in the epidermis of the heel-bulb. Nuclei were stained with DAPI (blue) (adapted from Diaz-San Segundo et al., 2010).

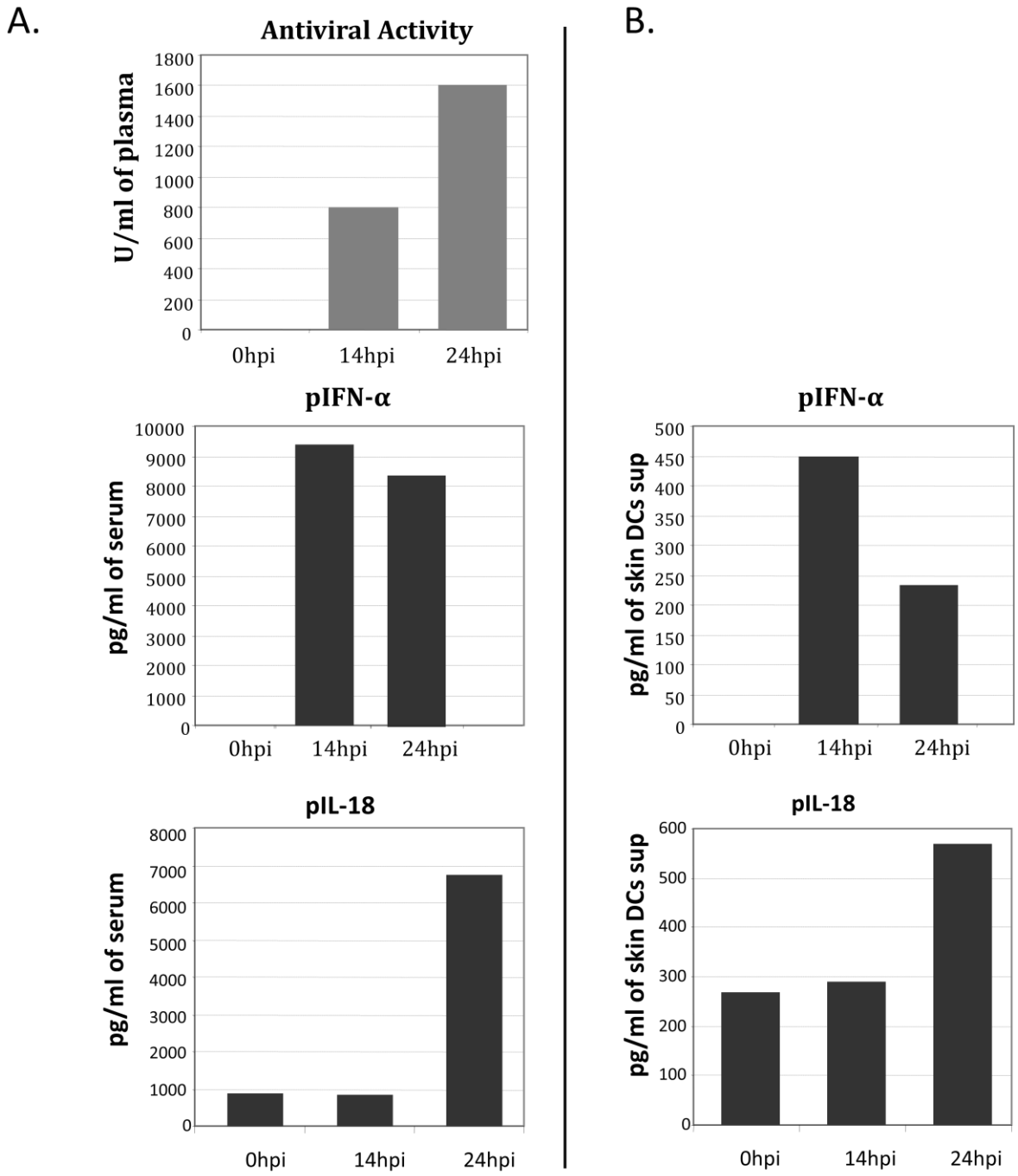

Figure 4: Effect of IFN treatment in swine. Systemic effect (serum and plasma) (A.) or effects in skin DCs (B.) were evaluated by measuring antiviral activity or levels of poIFN-α and IL-18 in plasma, serum or supernatant of skin DCs, 14 and 24 h after IFN treatment. Antiviral activity was detected in a porcine cell line and is expressed as Units (U) per milliliter of plasma. Amounts of IFN-α and IL-18 were detected by ELISA and are expressed in picograms per milliliter (pg/ml).

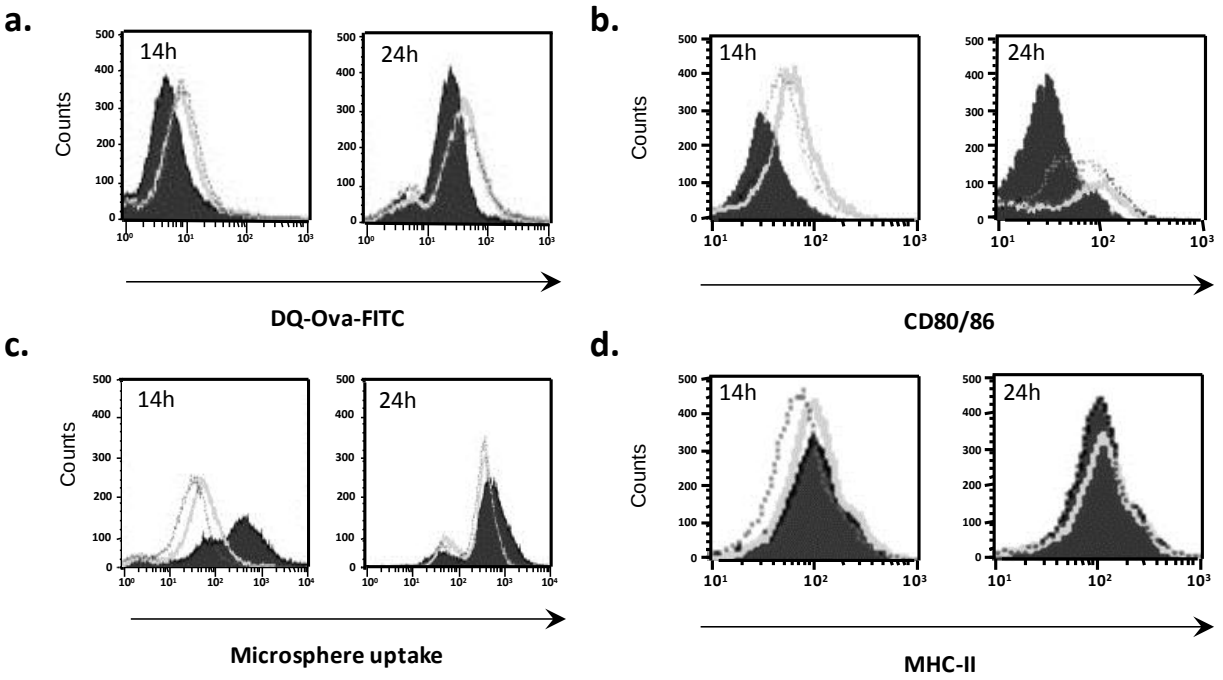

Figure 5: Maturation of skin DCs induced by IFN treatment. Antigen processing (a.), co-stimulatory molecules expression (CD80/86) (b.), phagocytosis (c.), and expression of MHC-II (d.) were evaluated on swine skin DCs extracted at 14 or 24 h after type I IFN treatment. **a.** Isolated skin DCs were incubated with 2 µg/ml DQ-OVA-FITC at 37 ℃ for 2 h. DQ-OVA processing in DCs was assessed by comparing the intensity of FITC signal of cells from treated animals (open histograms) with that obtained from a control untreated pig (filled histogram). **b.** CD80/86 surface staining was analyzed by flow cytometry from freshly isolated skin DCs from treated and control animals at 14 and 24 h after treatment. **c.** Isolated skin DCs were incubated with fluorescent labeled polystyrene microspheres of 2 µm diameter at 37 ℃ for 2 h. Phagocytosis was assessed by comparing the intensity of microspheres signal of cells from treated animals (open histograms) with that obtained from a control untreated pig (filled histogram). **d.** MHC-II surface staining was analyzed by flow cytometry from freshly isolated skin DCs from treated and control animals at 14 and 24 h after treatment. Analysis was performed on a FACSCalibur® flow cytometer using the Cell Quest® software (Becton Dickinson, San Jose, CA).