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Review

Identification and utility of innate immune system evasion mechanisms of ASFV

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ABSTRACT

The interferon (IFN) system is an early innate anti-virus host defense mechanism that takes place shortly after entry of the pathogen and long before the onset of adaptive immunity. Thus, African swine fever virus (ASFV), as an acute and persistent virus in pigs, is predicted to have evolved multiple genes for the manipulation and evasion of interferon. Although, ASFV is known to interfere with signaling pathways controlling the transcription of cytokines, surprisingly no individual virus gene manipulating the induction or impact of IFN has been described.

Since an initial bioinformatics search of the ASFV genome failed to identify potential antagonists of the IFN response, our strategy was to functionally screen early expressed, "unassigned" ASFV genes without existing homologies, particularly from MGFs 360 and 530, in luciferase reporter assays for their inhibition of the induction and impact of IFN. Specifically, we used reporter plasmids containing the luciferase gene under the control of: (1) the IFN-β promoter, to screen for inhibition of induction of type I IFN stimulated by the addition of Poly(I:C); (2) the ISRE DNA elements, to screen for the inhibition of the impact of type I IFN; and (3) the GAS DNA elements to screen for the inhibition of the impact of type II IFN.

Our initial experiments revealed six ASFV genes inhibiting one or more of the three luciferase assays. From these, we have selected a total of 3 genes for presentation. The ASFV A276R gene from MGF360 inhibited the induction of IFN-β via both the TLR3 and the cytosolic pathways, targeting IRF3, but not IRF7 or NF-κB. The ASFV A528R inhibited the induction of both NF-κB and IRF3 branches of the type I IFN induction signaling pathway and the impact of IFN response via both IFN type I and type II stimulation. The ASFV I329L gene is a functional viral TLR3 homologue inhibiting the induction of IFN at the level of TRIF. Thus, these genes reduce the IFN response by targeting different intracellular signaling intermediates. Their deletion from wild type virus may strengthen the host interferon response and so provide an attenuated form with more restricted virus spread after the initial infection, perhaps "buying" sufficient time to allow the development of a protective adaptive immune response. The demonstration of multiple ASFV genes for the evasion of IFN responses will demand technology to construct viruses with multiple gene deletions. An alternative would be a multigene DNA vaccine.

Finally, our work clearly demonstrates that unassigned viral genes may be viewed as a repository of host evasion strategies, only identifiable through functional assays. These may be considered to be "ready-made tools" for the experimental manipulation of cell biology and immune responses in health and disease and, as proof of concept, we have constructed a T-cell restricted transgenic mouse expressing the ASFV gene A238L, a dual inhibitor of NF-κB and NFAT activation. The resulting T cell restricted A238L transgenic mice developed a lymphoma with a phenotype reminiscent of some acute lymphoblastic lymphomas. In contrast, transgenic mice similarly expressing a mutant A238L solely inhibiting transcription mediated by NF-κB were indistinguishable from wild type mice, suggesting a transgene-NFAT-dependent transformation. Elucidation of the molecular events associated with the development of this virus host evasion molecule induced tumor may clarify some mechanisms of tumorigenesis in general, and in the development of T cell acute lymphoblastic leukemia in particular.

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Contents

1. Introduction

A major reason to define ASFV genes manipulating the host immunity is the hope of constructing an attenuated mutant virus vaccine with such genes deleted. In order to develop a rational virus vaccine strategy, however, the following four key points should be considered:

(1) The natural vertebrate host of ASFV is not the domestic pig where, typically, infection results in an acute infection and rapid death due to massive hemorrhage and lymphoid apoptosis. In the natural wild life vertebrate hosts, the warthog and bushpig, on the other hand, infection is unapparent, providing clear cut evidence that protection is possible.
(2) Protection in domestic pigs is isolate specific. Vaccination with a given virus isolate only protects against the same, or related, isolates. In the future, therefore, it may be necessary to tailor the vaccine to the target strain of virus, whatever it may be.
(3) Immunity to ASFV is complex, involving both serological (Gomez-Puertas and Escribano, 1997; Kollnberger et al., 2002) and cellular (Jenson et al., 2000; Leitao et al., 2001) responses and the recognition of multiple antigen determinants (Jenson et al., 2000; Kollnberger et al., 2002). Thus, construction of a vaccine comprising one or few viral proteins, in whatever delivery system, may not be a feasible strategy. Development of an attenuated virus or complex DNA vaccine, on the other hand, is a practical and potentially effective approach, offering the advantage of stimulating all arms of the immune response via multiple antigen determinants.
(4) The ASFV is adapted to infect both vertebrate and invertebrate (soft tick) hosts. From the point of view of possible mechanisms of protective immunity, only the innate response is common to these two hosts, and so it is reasonable to predict that the virus will have evolved genes for the manipulation of innate immunity. The characterization of viral genes evolved for the downregulation of innate immunity should therefore provide a rational basis for the construction of a live virus vaccine with one or more of these host evasion genes deleted. The predicted diminished acute phase and reduced mortality of the deletion mutant virus would "buy time" to allow the development of adaptive immunity and an associated memory response focused on potentially protective viral antigens still included in the deletion mutant virus.

In considering where to start the search for candidate genes for deletion, it is important to stress the acute nature of the disease. The innate immune response represents a rapid first line of defense, playing a very important role in keeping the virus load low, and thus controlling acute virus infections. In addition to its role in early protection against infection, components of the innate response shape the adaptive immune response and its subsequent effector phase. Interferon is an essential component of anti-virus innate immunity, inhibiting intracellular propagation and the intercellular transmission of the virus from early infected cells, thus inhibiting virus replication in both infected and nearby non-infected cells.

Given that the IFN system is a key player against viruses, in particular acute and persistent ones, ASFV must have evolved a number of counter strategies to antagonize this response (Randall and Goodbourn, 2008). The IFN mediated activation of the host cell of ASFV, the macrophage, would constitute a grave threat to ASFV, one that would be expected to exert an evolutionary pressure resulting in the evolution and selection of ASFV genes manipulating the IFN response. Surprisingly, however, no individual ASFV gene targeting IFN has been described. Therefore we have initiated a program to identify non-homologous ASFV genes downregulating IFN responses as possible candidates for the construction of a deletion mutant vaccine.

Thus, the principal focus of this review is the balance between innate immunity, IFN in particular, and reciprocal countermeasures by viruses in general, and ASFV in particular. We will then summarize our work on the detection and characterization of non-homologous ASFV genes manipulating the IFN system and finally, we will illustrate the potential of virus host evasion genes through the construction of transgenic mice expressing ASFV ORF A238L, a dual inhibitor of NF-κB and NFAT activation.

1.1. The interferon system

The interferons are absolutely crucial to the control of virus infections and constitute a principal focus of this review. Virus infection of a cell induces the development of an antiviral state within the infected cell and, due to the concomitant secretion of IFN, leads to the establishment of an anti-viral state in nearby cells. Thus there are two crucial activities of the IFN system: first its *induction*, and second, its *impact* upon binding to IFN receptors present on many cell types. In addition, and as mentioned above, both type I and type II IFN's are important in the coordination of the innate and adaptive immune responses to a viral infection. Whereas type I IFN is produced ubiquitously by virus infected cells, secretion of type II IFN is restricted to cells of the immune system.

The two principal classes of type I IFN, IFN-α and IFN-β, are induced directly in response to virus infection (Akira and Takeda,

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2004; Honda et al., 2006). In humans, 30 genes coding for type I IFN include 13 IFN-α genes, one IFN-β gene, one IFN-ω gene, one IFN-ε gene, one IFN-κ gene and 13 additional pseudogenes of the IFN-α and -ω families. The functional activities and cellular distribution of this complex gene family are yet to be completely defined. When type I IFN is induced and secreted by a virus infected cell, it signals through an ubiquitously expressed cell receptor composed of two chains: IFN-αR1 and IFN-αR2 (Chelbi-Alix and Wietzerbin, 2007; Hardy et al., 2004). The consequent potent antiviral impact is the transcription of over 300 antiviral ISG's that inhibit several steps of the viral life cycle. Furthermore, secreted type I IFN amplifies the original IFN signal, inducing an augmented antiviral state that results in secretion of high levels of cytokines and chemokines. As a result, cells of the innate immune system are recruited to virus-infected tissues, where they are activated and, in turn, facilitate the induction of the adaptive immune response (Le Bon and Tough, 2008)

'Immune interferon', otherwise known as type II IFN or IFN-γ, is secreted mostly by activated NK cells, Th1 lymphocytes, DC's and macrophages. It signals via a ubiquitously expressed cell receptor composed of the IFN-γR1 and IFN-γR2 subunits (Young and Bream, 2007), also inducing the transcription of ISG's. Although antiviral activity is not the primary biological function of IFN-γ, it stimulates cell-mediated immune responses that are critical for the development of immunity against pathogenic intracellular microorganisms, inducing the activation of macrophages for microbicidal activity and increasing the expression of major histocompatibility complex (MHC) for more effective antigen presentation. Type II IFN may also play a central role in the development of antitumor immune responses, and can amplify the induction of antiviral activity by IFN-α or -β.

Type I and type II IFNs often work together to activate a variety of innate and adaptive immune responses that result in the induction of effective antitumor immunity and the elimination of viral infections.

In 2003, a novel class of IFN's was identified and named type III IFN or IFN-λ. Type III IFN's have functional similarities with type I IFN's but, unlike type I IFN's, which exert antiviral activity on all cell types, type III IFN's primarily target epithelial cells, and consequently play an important role in innate antiviral defenses at the epithelial surfaces, which constitute a major portal of entry for many viral infections (Kotenko et al., 2003).

1.1.1. Induction of IFN

Type I IFN expression can be induced in essentially all cell types by several different mechanisms. However, the downstream kinases and transcription factors are common to all. Expression of type II IFN ("immune interferon") is restricted to cells of the lymphoid system, such as T lymphocytes, NK cells, DC's and macrophages.

The first step in the activation of the different mechanisms leading to type I IFN expression is the recognition of the viral infection by the host cell through conserved, germ-line encoded pathogen recognition receptors (PRR's). These receptors detect and distinguish invariant microbial molecular structures called Pathogen Associated Molecular Patterns (PAMP's) which are shared by all pathogens of a given class. The first potent PAMP inducing IFN to be identified was double-stranded RNA (dsRNA), a molecular pattern associated with viral infection, because it is produced by most viruses at some point in their replication.

The PAMP molecular signatures typically fulfill a conserved and essential functional role for the pathogen life cycle. Their selective recognition by host PRR's poses a special problem, as viruses usually replicate using host strategies and consequently generate molecular structures that resemble the molecular patterns found in the host (Diebold, 2010). The host's twofold solution to this is, first, the detection of minor structural differences and, second, to restrict the detection of the pathogen structures to subcellular compartments where the equivalent host cell structure is normally not found. For example, to circumvent the fact that viral nucleic acids are structurally similar to eukaryotic nucleic acids, their recognition occurs in intracellular compartments, where a particular form of host cell nucleic acids (i.e. uncapped RNA, dsDNA, dsRNA, etc.) is not normally present (Diebold, 2010).

PAMP's, according to their origin and nature, activate distinct classes of PRR's, which include Toll-like receptors (TLR's), retinoic acid inducible gene-I (RIG-I)-like receptors (RLR's), nucleotide binding and oligomerization domain-like receptors (NLR's), C-type lectin receptors (CLR's) and DNA receptors (cytosolic DNA sensors). This variety of PRR's, strategically distributed in different subcellular compartments, ensures the existence of a multiple sensor system that can detect and respond to almost any infection in almost any subcellular location of the host. During viral infections, nucleic acid- and glycoprotein viral-PAMP's interact with two major complementary PRR systems that detect most viral products. The TLR receptors TLR3, TLR4, TLR7/TLR8 and TLR9, have evolved to detect viral nucleic acids in the endosomes, while the ubiquitously expressed RLR's, and the more recently discovered DNA receptors, have evolved to detect nucleic acids in the cytosol (Stetson and Medzhitov, 2006). There are two principal RLR's, the retinoic acid inducible gene-I (RIG-I), and the melanoma differentiation-associated gene 5 (MDA5). Interplay and redundancy between TLR's and RLR's in different cell types during viral infection plays an important role in antiviral responses, as well in controlling adaptive immunity. While the cytoplasmic PRR's are responsible for limiting virus spread locally and for generating an inflammatory environment, the nucleic acid-sensing TLR's are crucial for orchestrating the adaptive anti-viral immune response that eventually leads to the elimination of the virus and virus-infected cells (Christensen and Thomsen, 2009; Diebold, 2010; Kawai and Akira, 2011).

Sensing the invading viral pathogen through the appropriate PRR(s) triggers multiple and distinct intracellular signaling pathways, activating the nuclear factor-kappa B (NF-κB), the interferon regulatory factor (IRF)-3 and IRF7 transcription factors. These play a major role in the induction of proinflammatory cytokines and chemokines that impact on both innate and adaptive immunity. The transcription factor IRF3 is particularly important in the induction of the antiviral response. Being ubiquitously expressed, IRF3 mediates the antiviral response and the induction of IFN-β in many varieties of infected cells. Signaling through IRF3 is required for type I IFN induction triggered by TLR3/TLR4 and the cytosolic RNA and DNA sensors. Constitutive expression of IRF7 is restricted to some lymphoid cells, particularly plasmacytoid DC's that express high amounts of IFN-α in response to activation via TLR7/8 and TLR9 (Paun and Pitha, 2007). Finally, IRF7 may be critical for the induction of IFN-α and IFN-β gene expression, functioning even in the absence of IRF3 (Honda et al., 2005).

The majority of type II IFN expression is not directly induced by invading pathogens but is instead a secondary consequence of the infection. Type II IFN is produced both in the early stages of infection by NK cells and macrophages (Darwich et al., 2009; Malmgaard, 2004) and at later stages by activated T lymphocytes (Boehm et al., 1997), by either receptor-mediated stimulation (through T cell receptors or NK cell receptors) or in response to early produced cytokines, such as IL-12, IL-18, and IFN-α/β (Malmgaard, 2004).

Type III IFN genes (IFN-λ) are expressed in response to many classes of viruses and to a variety of TLR agonists, in fact the same stimuli responsible for expression of type I IFN genes. Recently, types I and III IFN's were demonstrated to be induced by transcriptional mechanisms involving IRF's and NF-κB (Onoguchi et al., 2007; Osterlund et al., 2007). However, while IFN-β induction requires the coordinated action of a multifactor enhanceosome, and

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IFN-α expression is activated by multiple IRF-binding cis-promoter elements, the type III IFN's are induced through independent actions of IRF's and NF-κB (Thomson et al., 2009). Hence, it was proposed that type III IFN expression is more flexible than type I IFN expression, which could allow expression of type III IFN's in response to a wider range of stimuli than those inducing type I IFN's (Levy et al., 2011).

1.1.2. Response to IFN

The type I IFN's are critical mediators in the innate host response to viral infection and induce the expression of hundreds of ISG's that may have direct antiviral activity. In addition, they modulate innate and adaptive immunity by activating immature DC's, enhancing NK-cell function and promoting survival and effector functions of T and B lymphocytes (Christensen and Thomsen, 2009).

Although all type I, type II and type III IFN's bind to distinct receptors, they all activate a common intracellular signaling pathway, regulating many of the same biological activities, including a range of antiviral immune responses (Donnelly and Kotenko, 2010). The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) (JAK-STAT) pathway was the first signaling pathway shown to be activated by IFN's. Secreted IFN type I is recognized by cell surface specific receptors leading to the phosphorylation of both STAT1 and STAT2, which forms heterodimers that associate with IRF9 and translocate into the nucleus to bind ISRE elements present in the promoter region of ISG's. Type II IFN, on the other hand, also bind to specific cell surface receptors, but in this case leads to the phosphorylation of STAT1, which forms homodimers that translocate into the nucleus and bind to GAS elements of ISG's. More details on this signaling pathway are in Section 3.2, which describes the impact of ASFV gene A528R on the IFN response.

The induction of an anti-viral state by IFN is achieved by the rapid and efficient activation of the JAK-STAT pathways, leading to the expression of multiple proteins encoded by ISG's, which will limit virus replication and its subsequent spread to neighboring cells. The best characterized IFN inducible components are the enzymes dsRNA-dependent protein kinase (PKR), 2′,5′-oligoadenylate synthetase (2′5′OAS), and Mx proteins (Haller et al., 2009). In order to avoid damage to the host, the response to IFN must be tightly regulated and terminated once the viral threat is over. Toward this end, several negative regulators of the JAK-STAT signaling have been already described (Brierley and Fish, 2005; Droescher et al., 2011; Guo et al., 2010; Kisseleva et al., 2002; Levy and Darnell, 2002; Murray, 2007; Schmidt and Muller, 2003; Zimnik et al., 2009).

The JAK-STAT pathway only accounts for some of the biological functions exerted by IFN's in the organism. The PI3K pathway was already mentioned as necessary for the full activation of STAT1. In addition, this pathway can also induce downstream survival versus death pathways, in response to IFN. Other pathways, such as Crk-like protein (CRKL), and p38 kinase pathways, are also involved in IFN-mediated signaling (Platanias, 2005; van Boxel-Dezaire et al., 2006). Interferons also induce the activation of downstream signaling pathways that direct the expression of genes involved in the establishment of a pro-apoptotic state or cell-cycle arrest in target cells (Versteeg and García-Sastre, 2010).

1.1.3. Immunomodulation by IFN

The most studied property of the IFN system is its ability to establish an antiviral state. In addition, and with the ultimate goal of eliminating virus infected cells, this system also plays an important role in the control of several effector responses of both the innate and the adaptive immune system (Hervas-Stubbs et al., 2011; Stetson and Medzhitov, 2006).

2. Viral evasion of IFN

The continuous interaction between viruses and their respective hosts during the course of evolution, has shaped and determined the survival strategies evolved by viruses and their hosts. As intracellular pathogens, viruses must first enter the cell and then take control of its machinery in order to replicate, and prior to further transmission to new hosts. In turn, the vertebrate host has evolved an elaborate system of innate and adaptive antiviral immune mechanisms in order to recognize and destroy virus-infected cells. This vicious cycle of selective pressure on viruses has led to the evolution of multiple mechanisms of virus host evasion. There are two principal strategies employed by viruses to evade the host defense mechanisms: (1) to manipulate the various components of the early, innate immune response (IFN, apoptosis, cytokines, chemokines) and (2) to avoid recognition by the adaptive immune response, through continuous antigenic variation, and/or the absence or failure to stimulate a cytotoxic cellular or a neutralizing antibody response. There have been several excellent reviews on immune evasion by viruses (Alzhanova and Fruh, 2010; Bahar et al., 2011; Di Lorenzo et al., 2011; Engel and Angulo, 2012; Fischl and Bartenschlager, 2011; Jackson et al., 2011; Unterholzner and Bowie, 2008). Here we will focus on inhibition of the IFN response, also recently reviewed (Goodbourn and Randall, 2009; Randall and Goodbourn, 2008).

2.1. Inhibition of the induction of IFN

In order to replicate and spread in a host population, a virus depends on highly specific interactions of viral host evasion proteins with infected cells. These result in the subversion of multiple cellular signal transduction pathways controlling a wide variety of host cell functions. Several viral strategies for interfering with the synthesis and impact of cytokines and chemokines, particularly the inhibition of IFN, are already known. Downregulation of the IFN system, a powerful and first line of defense against virus infections is, unsurprisingly, a priority for most viruses. The viral strategies are numerous and include the inhibition of IFN production, the inhibition of IFN-mediated signaling pathways, and the blocking of the action of IFN-induced enzymes with antiviral activity.

Given that the induction of IFN proceeds in a cascade-like manner, viruses have evolved a wide variety of molecular mechanisms that act in concert at different steps in the relevant signal transduction pathways in order to subvert the IFN response. Many viral antagonists are multifunctional proteins that interact with multiple host components, thereby increasing the range and efficiency of their host evasion mechanisms. The exact host proteins manipulated by a given virus will reflect the biology of the infection and will be a major factor that will influence the pathogenesis of that virus infection.

Regarding the inhibition of IFN production, a number of viral strategies have been identified and characterized (see Table 1). Significantly, almost 50% of the viruses which have been studied interfere with multiple steps in the IFN response. This clearly illustrates the necessity for viruses to successfully circumvent the IFN response (Versteeg and García-Sastre, 2010).

Induction of type I IFN is initiated by the interaction of a virus component, the PAMP, with the various specialized and reciprocally interacting host cell PRR's that have evolved as an essential part of the innate immune response. In order to avoid recognition by PRR's, viruses usually minimize the production of PAMP's, thus reducing IFN production in response to the viral infection. This can be achieved by minimizing the production of dsRNA, the potent virus PAMP inducing IFN, through the regulation of virus transcription and replication or, in the case of paramyxoviruses

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Table 1
Inhibition of IFN production.

Virus (protein)	Mechanism
Encephalomyocarditis virus (leader protein)	Prevents IRF3 dimerization
Bunya virus (NSs)	Inhibit RNA polymerase II
Reovirus (σ3/A)	Sequesters dsRNA
Hepatitis C virus	Inhibits TLR signaling and MAVS
Influenza A virus (NS1)	Inhibits MDA5 and RIG-I
Human papilomavirus 16 (E6); Herpes simplex virus (ICP0); Bovine respiratory syncitial virus	Inhibit or degrade IRF3
Human papilomavirus (E7); Adenovirus (E3)	Interfere with IKK complex
Kaposis associated herpesvirus (IRF orthologues)	Interferes with IFN-β promoter activation
African swine fever virus (A238L)	Competitive non-functional IKB homologue.

Adapted from Versteeg and García-Sastre (2010).

Table 2
Inhibition of IFN signaling.

Virus (protein)	Mechanism
Human cytomegalovirus	Targets Jak1 for proteasomal degradation
Sendai virus; Bovine respiratory syncitial virus	Induces degradation of STATs
Hepatitis C virus; Sendai virus; Human cytomegalovirus	Sequesters STATs or alters their phosphorylation
Hepatitis C virus (Core); Herpes simplex virus (UL13, UL41)	Induces SOCS3

Adapted from Versteeg and García-Sastre (2010).

and picornaviruses, by capping viral RNA, rendering it indistinguishable from cellular mRNA (Randall and Goodbourn, 2008).

The majority of viral IFN antagonists exert their action by one of five different strategies: (1) a general inhibition of cellular gene expression, (2) the sequestration of components of the IFN signal transduction pathways, (3) the specific and limited proteolytic cleavage of components of the IFN signal transduction pathways, (4) the targeting of these components for degradation to peptides by the proteasome, or (5) the manipulation of reversible epigenic mechanisms controlling activation of components of the IFN signal transduction pathways, such as reversible phosphorylation or ubiquitination (Versteeg and García-Sastre, 2010).

By inhibiting the host cell gene expression and/or protein synthesis, viruses interfere with several cellular functions, including the IFN response. For example, the matrix (M) protein of vesicular stomatitis virus inhibits basal transcription, nuclear-cytoplasmic transport of RNAs and proteins, and inactivates translation factors. Another example, the NS1 protein of influenza A virus, inhibits processing and export of cellular mRNAs (Weber and Haller, 2007).

The sequestration of the ligands that bind to host cell PRR's is another efficient viral mechanism to inhibit the induction of IFN expression. Very well known examples are the NS1 protein of influenza A virus, and the VP35 protein of Ebola virus. These bind dsRNA, thereby inhibiting not only the induction of IFN but also dsRNA inducible proteins such as the anti-viral enzymes PKR and 5′OAS (Versteeg and García-Sastre, 2010).

Some viral antagonists directly inhibit components of the TLR and RLR signaling pathways, blocking IFN production and suppressing host antiviral signal propagation. The 3Cpro cysteine protease of coxsackievirus B3 cleaves two key adaptor molecules of the innate immunity: the mitochondrial antiviral-signaling protein (MAVS) and the Toll-interleukin-1 receptor (TIR)-domain-containing adaptor-inducing interferon-β (TRIF), thereby blocking TLR3, TLR4 and RLR signaling (Mukherjee et al., 2011). The hepatitis A virus uses a 3Cpro homologue to achieve the same result (Qu et al., 2011). The Vaccinia virus protein A46R not only inhibits TLR3 signaling through TRIF-mediated IRF3 activation, but is also capable of binding to other TIR adaptors such as myeloid differentiation primary response gene 88 (MyD88) and TIR domain containing adaptor protein (TIRAP), consequently interfering with the activation of the NF-κB and MAP kinases (Stack et al., 2005).

By inhibiting the post-translational attachment of ubiquitin or ubiquitin-like modifiers to host cell proteins, viruses are able to deregulate many cellular processes, including the generation of innate and adaptive immune responses to pathogens (Viswanathan et al., 2010). For example, the Npro protein of both bovine viral diarrhea virus and classical swine fever virus induces proteasome-dependent degradation of IRF3 (Hilton et al., 2006; La Rocca et al., 2005). The human immunodeficiency virus-1 proteins Vpr and Vif also mediate IRF3 ubiquitination, leading to proteasomal degradation (Okumura et al., 2008). Influenza A virus NS1 binds and inhibits TRIM25, an E3 ligase required for ubiquitin-dependent interaction between RIG-I and its adaptor MAVS. Thus, NS1 prevents activation of IRF3-dependent IFN secretion (Gack et al., 2009).

2.2. Inhibition of the impact of IFN

Interferon-mediated signaling not only induces the expression of host anti-viral proteins, but also stimulates antigen presentation through increased MHC expression. Given these crucial roles in anti-viral immunity, it is not surprising that viruses have evolved strategies to inhibit the signal transduction pathways triggered upon binding of IFN to its specific receptor.

Type I and type II IFN signal through distinct receptors, activating downstream components that can be either unique or common to both signaling pathways. Thus, viruses can block the impact of IFN at several levels, inhibiting only one or both of these two pathways (see Table 2). Poxviruses encode soluble versions of cellular cytokine and cytokine receptors which, through competition with the natural ligand, interfere with the normal function of the host cytokines or receptors. For example, the B8R protein of Vaccinia virus binds to soluble IFN-γ and prevents its binding to the cellular receptor. In this way, this virus simultaneously inhibits the antiviral effects due to signaling through the type II IFN receptor, and also the immunoregulatory functions of IFN-γ (Alcami and Smith, 1995).

Modulation of STAT activity is a very common viral strategy. For example, the Dengue virus NS5 protein mediates ubiquitination and proteasome-dependent degradation of STAT2 (Ashour et al., 2009). Members of paramyxoviruses encode two different but genetically related proteins, C and V, which interfere with STAT function. According to the strain of the virus, these IFN antagonists act by binding to STAT proteins inducing their degradation, or by inhibiting the JAK kinases (Weber and Haller, 2007).

Inhibition of signaling through the STAT proteins can also be indirect. The VP24 protein of Ebola virus interacts with the NPI-1 subfamily of karyopherin-α proteins (responsible for transporting dimerised phospho-STAT1 to the nucleus), thereby inhibiting nuclear accumulation of STAT1 (Reid et al., 2006). An indirect mechanism to block IFN signaling includes the rapid induction of the expression of suppressor of cytokine signaling 3 (SOCS3), a cellular inhibitor of the JAK-STAT pathway. This is a strategy explored by many viruses, such as the influenza A virus, respiratory syncitial virus, hepatitis C virus, and herpes simplex virus (Versteeg and García-Sastre, 2010).

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2.3. Inhibition of IFN-induced effector proteins

Viruses employ different strategies to either inhibit or prevent the activation of IFN-inducible antiviral effector proteins. The protein kinase RNA-activated (PKR), one of the major host responses to viral infection, is a priority virus target. Active PKR dimers phosphorylate eIF-2α, preventing the formation of the ternary translational complex and, as a result, repressing translation of RNAs. Since viruses require the machinery of the host cell for the translation of their own proteins, inhibition of PKR is mandatory (Roberts et al., 2009). Some viruses express RNA-binding proteins that sequester viral dsRNA, thus preventing the activation of PKR. The hepatitis C virus protein NS5a is able to directly interact with and inhibit PKR (Gale et al., 1997). Similarly, the cellular PKR inhibitor p58 (IPK) is activated during infection with influenza A, Tobacco mosaic, and Tobacco etch viruses and contributes to negative regulation of PKR by direct protein–protein interaction (Bilgin et al., 2003; Goodman et al., 2007). As an alternative strategy, the hepatitis C virus E2 glycoprotein competes with eIF-2α for binding to PKR, thus preventing the inactivation of translation by PKR (Taylor et al., 1999). Yet another virus approach is to encode small RNAs which compete with dsRNA for binding to PKR, hence inhibiting its activation (Elia et al., 1996; Gunnery et al., 1990; Mathews and Shenk, 1991; Vyas et al., 2003). Direct binding and inhibition of PKR have been employed by the vIRF2 protein of Kaposi's sarcoma-associated herpes virus, preventing PKR activation by inhibiting its autophosphorylation (Burýšek and Pitha, 2001).

The ISG15, an ubiquitin-like protein, has also been shown to be targeted by viruses. The N-terminal domain of the L protein of Crimean Congo hemorrhagic virus has de-ISGylating and de-ubiquitinating activity (Garcia-Sastre, 2007), while influenza B virus NS1 protein inhibits ISG15 by direct binding (Yuan and Krug, 2001). Finally, adenovirus, herpes simplex virus-1, Epstein–Barr virus, and human cytomegalovirus, are able to disrupt promyelocytic leukemia protein (PML) nuclear bodies by proteasome-dependent degradation, although the relevance of this is debated (Moller and Schmitz, 2003).

3. Modulation of the IFN response by ASFV

The importance of the continuously evolving dialog between the host IFN system and virus countermeasures is stressed by the fact that it is not the initial infection with ASFV that kills pigs, but rather the accumulated impact of subsequent virus spread and its associated pathogenicity. This virus spread is inhibited by the impact of IFN secreted from the early infected cells, inducing the development of an "anti-viral state" in both the infected and nearby non-infected cells. Thus ASFV, as an acute and persistent virus in pigs, is predicted to have evolved multiple genes for the manipulation and evasion of IFN. Although, ASFV is known to interfere with signaling pathways controlling the transcription of cytokines (Powell et al., 1996; Whittall and Parkhouse, 1997; Zhang et al., 2006), surprisingly no individual virus gene manipulating the induction or impact of IFN has been described. There is, however, strong evidence to suggest that such genes are indeed encoded in the ASFV genome. For example, modulation of the IFN response by ASFV has been inferred by comparing of transcriptional profiles of macrophages infected with wild type virus or a deletion mutant virus lacking six genes from multigene family 360 (MGF360) and two genes from MGF530. The results revealed an up-regulation of several mRNAs corresponding to ISG's when the cells were infected with the mutant virus, but not when infected with the wild type virus, suggesting that MGF360 and/or MGF530 include a gene, or genes, responsible for the observed inhibition of the IFN response. Indeed, and in contrast with the wild type virus infection, supernatants from mutant virus infected culture supernatants contained significantly increased amounts of IFN-α (Afonso et al., 2004). Consistent with this, the IFN-α induced MHC class I expression was down-regulated in porcine aortic endothelial cells infected with ASFV (Vallee et al., 2001). Also, using microarray analysis, changes in macrophage gene transcription after infection with a highly virulent ASFV isolate, has revealed increased expression of IFN-β and other proinflammatory cytokines and chemokines at 4 h post infection, followed by a significant decrease in the levels of their expression at 16 h post infection. These results were attributed to the impact of ASFV encoded proteins that efficiently downregulate the early innate immune response mounted by the host cell against the virus (Zhang et al., 2006). Although it has been recently reported that, in domestic pigs infected with a virulent strain of ASFV, serum levels of IFN-β increased continuously from 2 to 7 days p.i. (Karalyan et al., 2012), one must take into account that this particular cytokine is broadly expressed by several cell types upon viral infection, most of which are not susceptible to ASFV. As such, this study cannot exclude the downregulation of the IFN-β expression by ASFV, and the deletion of host evasion genes involved in this modulation remains an interesting hypothesis for extending the acute phase of infection and thus develop an attenuated vaccine.

Given the justified suspicion for the existence of ASFV genes evolved for the downregulation of IFN, and the feasibility of constructing an attenuated ASFV mutant with "anti-IFN" genes deleted, our first approach was a bioinformatic enquiry. Since this analysis failed to identify potential antagonists of the IFN response, our strategy was to functionally screen early expressed, "unassigned" ASFV genes without existing homologies, particularly from MGF360 and 530, in luciferase reporter assays for their inhibition of the induction and impact of IFN. Specifically, we used luciferase reporter plasmids (Boehm et al., 1997) containing the luciferase gene under the control of: (1) the human IFN-β promoter, to screen for inhibition of induction of type I IFN stimulated by the addition of Poly(I:C); (2) the ISRE DNA elements, to screen for the inhibition of the impact of type I IFN; and (3) the GAS DNA elements to screen for the inhibition of the impact of type II IFN (King and Goodbourn, 1994). The Vero cell line was simultaneously transfected with the cloned ASFV gene and one of the three luciferase reporters (IFN-β, ISRE or GAS), then treated with the appropriate stimulus (Poly(I:C) for IFN-β and type I and II IFN for ISRE and GAS assays, respectively) and, finally, the luciferase activity of control plasmid and experimental plasmid transfected cells was compared. The Vero cell line has the advantage of lacking the type I IFN locus (Mosca and Pitha, 1986), which facilitates the interpretation of the results, since the IFN amplification loop is absent in these cells and so control background levels are low.

Our initial experiments revealed six ASFV genes inhibiting one or more of the three luciferase assays. From these, we have selected a total of 3 genes for presentation below; two non-homologous ASFV genes, one from MGF360 (A276R) and the other from the MGF530 (A528R), and one ASFV gene (I329L) with borderline homology to cellular TLR3. The two genes selected from multigene families 360 and 530 (A276R and A528R, respectively) have not been deleted in the recombinant knockout Pr4 Delta 35 that has been shown to induce type I IFN in swine macrophages (Afonso et al., 2004). However, it cannot be excluded that more genes from these multigene families are also involved in the modulation of the interferon response.

Although we have not looked for ASFV genes evolved for the manipulation of cytosolic DNA receptors (Kerur et al., 2011; Wilkins and Gale, 2010), nor for any impact of the virus on the induction and impact of type III IFN, there is no doubt that this would be worthwhile in the future.

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3.1. The ASFV A276R gene from MGF360 inhibits the induction of IFN-β

As a member of MGF360, the ASFV A276R gene is a possible candidate for modulation of the IFN response (Afonso et al., 2004). In order to test this hypothesis, luciferase reporter assays using the IFN-β, the ISRE or the GAS luciferase reporters, were performed in cells expressing A276R. The results indicated that this viral gene was able to modulate the pathway(s) leading to the induction of IFN by Poly(I:C), but had no effect on the JAK-STAT pathway in response to either type I or type II IFN (data not shown).

Type I IFN expression can be induced by several different mechanisms. However, the downstream kinases and transcription factors are common to all. Upon Poly(I:C) recognition, by TLR3 or RLR's, the common downstream kinases IKKε and TBK-1 are activated and phosphorylate IRF3 and/or IRF7, leading to their dimerization, nuclear translocation and binding to the promoter of type I IFN. The NF-κB transcription factor is also activated by a TRAF6 dependent pathway through the IKKα/β/γ complex of kinases which, in turn, also translocates to the nucleus and binds to the type I IFN promoter. The assembled complex of the transcription factors IRF3 and/or IRF7, NF-κB, and AP-1, as well as associated structural elements and basal transcriptional machinery, is called the enhanceosome, a large, multi-subunit complex that binds the four positive regulatory regions (PRDI-IV) of the IFN-β promoter region.

Thus, in order to identify the intracellular target of A276R, luciferase assays using the IFN-β reporter were performed in Vero cells activated by ectopic expression of the potentially targeted signaling intermediates. As can be seen (Fig. 1), the induction of IFN-β by ectopic expression of RIG-I was found to be inhibited by cotransfection of A276R, demonstrating that A276R is able to modulate the cytosolic IFN induction pathway, in addition to the previously observed Poly(I:C)-TLR3 mediated stimulation.

Detection of dsRNA by TLR3 and RLR's (RIG-I and MDA5), initiate separate intracellular signaling pathways that terminate in the phosphorylation of the NF-κB and IRF3/IRF7 transcription factors, leading to the induction of IFN-β. To determine if A276R could be modulating induction of IFN-β at the level of either IRF3 or IRF7, or both IRF3 and IRF7, luciferase assays were performed in cells ectopically activated by overexpression of IRF3 or IRF7. In the presence of the control plasmid, activation of the IFN-β promoter was, as expected, induced. In contrast, the expression of A276R inhibited transcription of IFN-β stimulated by ectopic expression of IRF3, but not IRF7 (data not shown). Thus, the ASFV A276R inhibits the induction of IFN-β by both the TLR and cytosolic pathways, the latter through an impact at the level of IRF3, but not IRF7.

As the recognition of dsRNA by both the TLR3 and the cytosolic receptors may activate both the NF-κB and IRF routes of the IFN-induction signaling cascade, A276R could inhibit the activation of NF-κB in addition to the activation of IRF3. To explore this possibility, two further luciferase reporters were employed: (1) PRDII-luc, containing the NF-κB binding site of the IFN-β promoter, the positive regulatory domain PRDII (Visvanathan and Goodbourn, 1989) and (2) ISG15-luc, containing a multimer of the IRF-3-responsive ISG15 interferon-stimulated response element (Hilton et al., 2006). Again, the cells were transfected with either the empty vector or the A276R expressing vector, and there was inhibition of activation of the IRF3 responsive luciferase reporter (data not shown). The absence of any impact of A276R on the activation of NF-κB, demonstrates that the A276R-mediated inhibition of IFN-β induction is NF-κB independent, and is restricted to the IRF3 activation pathway.

In order to confirm that the A276R-mediated inhibition of IFN-β induction observed in the luciferase assays was also correlated with an inhibition of secretion of IFN-β, we determined the amount of IFN-β in the supernatants of cells transfected with control and A276R recombinant plasmids, with and without the addition of Poly(I:C). The IPAM porcine tissue culture adapted macrophage cell line was used for this experiment, not only because it is an appropriate cell, but also because Vero cells do not produce IFN. When compared to control cells, the expression of A276R in cells stimulated with Poly(I:C) clearly reduced the level of secreted IFN-β (Fig. 1B), confirming that A276R is impacting at both the transcriptional and/or translational level of IFN-β gene expression and, most importantly, reduces the effectiveness of the host cell type I IFN antiviral response.

In conclusion, the ASFV A276R protein impairs the induction of IFN-β through targeting IRF3, but not IRF7, in an NF-κB independent manner. However, the precise mechanism of this inhibition remains to be elucidated.

3.2. The ASFV A528R inhibits both the induction and impact of the IFN response

The ASFV A528R gene is a member of MGF530, and therefore also a potential candidate for an IFN evasion protein (Afonso et al., 2004). This viral gene was cloned and tested in luciferase assays and was found to inhibit induction of IFN-β (Fig. 2). In contrast to the ASFV A276R gene, the activation of the NF-κB transcription factor was also inhibited by expression of A528R (Fig. 2), suggesting that the signaling intermediate being targeted by this viral gene is common to both branches of the IFN induction signaling pathway.

Although the inhibition of cellular responses to type I or type II IFN is a very frequent viral strategy of immune evasion until now, surprisingly, no ASFV gene has been described that inhibits the JAK-STAT signaling cascade, the central transduction pathway mediating IFN anti-viral effects.

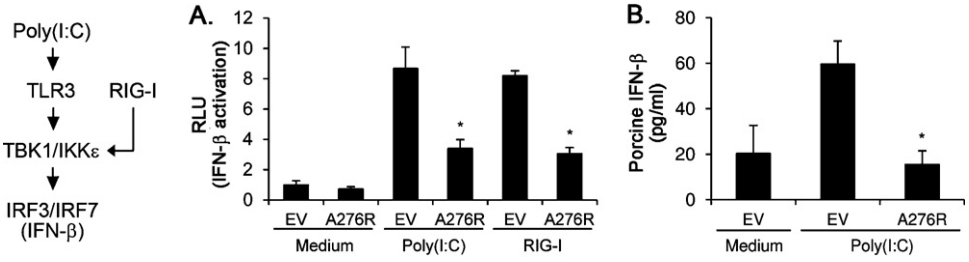

Fig. 1. The ASFV ORF A276R inhibits induction and expression of IFN-β. (A) Vero cells were co-transfected with the empty vector pcDNA3HA (EV) or pcDNA3HA-A276R plasmid, the β-galactosidase plasmid and the IFN-β promoter (IFN-β) luciferase reporter plasmid. Cells were either induced with 35 μg/ml Poly(I:C) for 5 h, or by co-transfection of RIG-I expression plasmid, or left untreated (Medium). Luciferase activity was normalized to β-galactosidase activity as a control for transfection efficiency. Data are expressed as means of Relative Luciferase Units (RLU) ± SD of triplicate well from one of three similar experiments. (B) IPAM cells were co-transfected with the empty vector pcDNA3HA (EV) or pcDNA3HA-A276R plasmid. Cells were induced with 100 μg/ml Poly(I:C) for 5 h or left untreated (medium). Supernatants were collected and IFN-β concentration (pg/ml) was measured by ELISA. Data are expressed as means ± SD of triplicate well from one of two similar experiments. Statistically significant difference was assessed by Student's *t*-test and is represented as $p \leq 0.05$ (*) or $p \leq 0.01$ (**).

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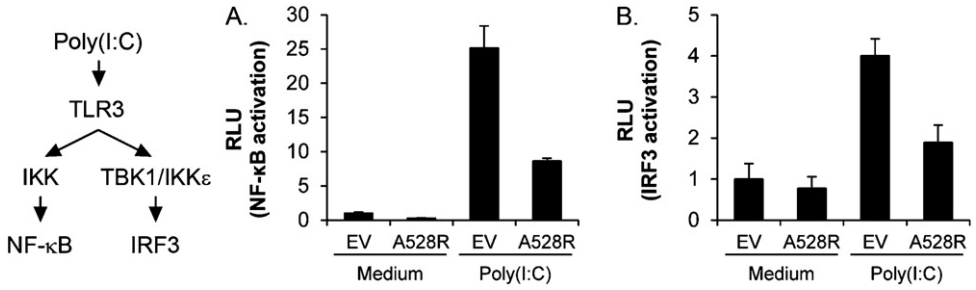

Fig. 2. The ASFV ORF A528R inhibits Poly(I:C)-mediated activation of IRF3 and NF-κB. Vero cells were co-transfected with the empty vector pcDNA3HA (EV) or pcDNA3HA-A528R plasmid, the β-galactosidase plasmid, and the (A) NF-κB binding sequence luciferase reporter plasmid or the (B) IRF3 binding sequence luciferase reporter plasmid. Cells were induced with 35 μg/ml Poly(I:C) for 5 h or left untreated (medium). Luciferase activity was calculated and expressed as in the legend to Fig. 1.

Type I IFN's are secreted factors that are recognized by a cell surface transmembrane receptor – the type I IFN receptor. This protein is a heterodimer composed of two subunits, IFN-α receptor 1 (IFN-αR1) and IFN-αR2, which cytoplasmic domains are associated with the inactive Janus tyrosine kinases, TYK2 and JAK1, respectively. Upon IFN binding to the receptor, TYK2 and JAK1 are activated and phosphorylate STAT2 at Tyr690 and STAT1 on Tyr701, respectively. The activated STAT's dissociate from the receptor forming a stable heterodimer and associate with IRF9, forming the interferon stimulated gene factor 3 (ISGF3) tertiary complex that translocates into the nucleus and binds to IFN-stimulated response elements (ISRE) present in the promoter region of IFN-stimulated genes (ISG's), inducing their transcription.

The type II IFN receptor is also a heterodimer composed of two subunits, the IFN-γ receptor 1 (IFN-γR1), which associates with JAK1, and the IFN-γR2, which constitutively associates with JAK2. Dimerization of the receptor, upon binding of IFN-γ, leads to association of JAK1 and JAK2 and subsequent activation, which in turn phosphorylates STAT1 at Tyr701. The phosphorylated STAT1 homodimer dissociates from the receptor and translocates into the nucleus, where it binds to unique elements of IFN-γ stimulated genes, the gamma-activation sequence (GAS), and induces transcription of ISG's.

As a potential IFN modulator, the ASFV A528R gene was also tested for its impact on the JAK-STAT signaling pathway. Using reporter plasmids containing the luciferase gene under the control of ISRE or GAS promoter elements and stimulating the transfected cells with IFN-β and IFN-γ, respectively, the presence of A528R protein clearly inhibited induction of both reporters, indicating that A528R is able to inhibit both type I (Fig. 3A) and type II (Fig. 3B) IFN signaling pathways.

Thus the ASFV gene A528R has evolved not only to inhibit the induction of IFN, but also to reduce the impact of both type I and type II IFN's. This viral gene is also a good example of a multifunctional host evasion protein that impacts on at least three signaling pathways. Identification of the intracellular targets and the mechanisms of inhibition are currently being pursued.

3.3. The ASFV I329L gene is a viral TLR3 homologue inhibiting the induction of IFN

The search for a TLR agonist in the ASFV genome was stimulated by the fact that the virus is adapted to survive in both vertebrate and invertebrate hosts. Only innate immunity, in particular TLR responses, is common to both hosts and, in addition, ASFV specifically infects macrophages in its porcine host.

Many viral genes evolved to mimic or block normal cellular functions have been acquired from the host. Although the homology search we performed at the level of entire genes to identify a possible ASFV TLR homologue was disappointingly negative, the ASFV I329L ORF was interestingly predicted to be a type I transmembrane protein containing four leucine-rich repeats (LRR's) in its extracellular domain. Since the presence of multiple LRR's is a structural feature of the extracellular domains of TLR family members, this clue prompted a more focused bioinformatics analysis of the viral protein. Another characteristic of the signaling domain of TLR family proteins is their intracellular Toll-interleukin-1 receptor (TIR) domain, which mediates the interaction between the TLR's and their adaptor proteins as an initial step in the intracellular signaling pathway. The TIR domain contains three conserved sequences (Box1, 2 and 3). A detectable, but very low, homology with Box1 and Box2 of the TLR3 intracellular TIR domain was observed (Fig. 4), raising the possibility that I329L might inhibit activation of IFN-β through an inhibitory interaction with the TIR motif of the TLR's and the corresponding downstream signaling adaptor proteins (de Oliveira et al., 2011).

This structural homology correlated with luciferase reporter assays which clearly demonstrated that I329L inhibits TLR3-mediated induction of IFN-β and activation of NF-κB (de Oliveira et al., 2011). The precise mechanism for the inhibition of TLR3

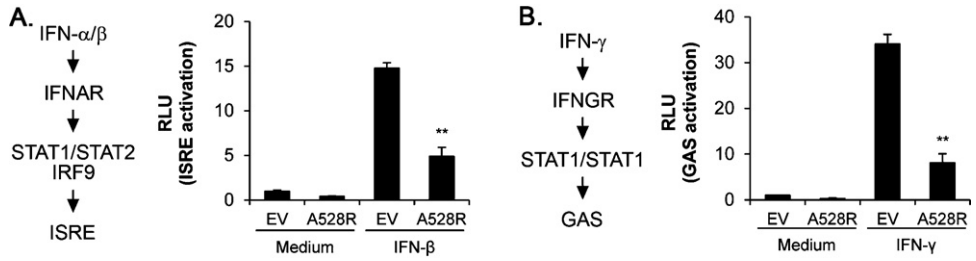

Fig. 3. The ASFV A528R inhibits signaling through type I and type II IFN receptors. Vero cells were co-transfected with the empty vector pcDNA3HA (EV) or pcDNA3HA-A528R expressing plasmid, the β-galactosidase plasmid and the (A) ISRE promoter or the (B) GAS promoter luciferase reporter plasmids. Seventy-two hours post-transfection, the cells were induced with (A) 1 U/μl human IFN-β or (B) 1 U/μl human IFN-γ for 5 h, or left untreated (medium) (A and B). Luciferase activity was calculated and expressed as in the legend to Fig. 1.

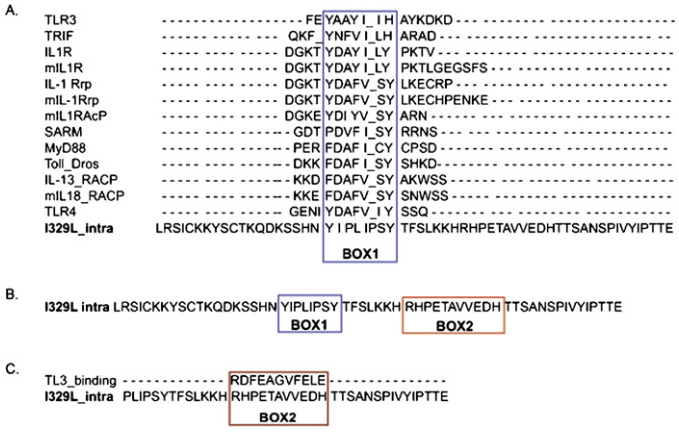

Fig. 4. The intracellular domain of ASFV protein I329L has a putative viral TIR domain. Analysis of peptide sequences was performed with ClustalW2. (A) Multiple alignments of several TIR motif-containing proteins and I329L intracellular domain indicating the conserved aminoacid sequence of the characteristic Box1. (B) Alignment between the Box2 of TLR3-TIR domain and I329L intracellular domain. (C) Sequence of the intracellular domain of I329L (amino acid residues 260–329) indicating the predicted Box1 and Box2 regions.
Adapted from de Oliveira et al. (2011).

signaling was not elucidated but the adaptor TRIF was tentatively proposed as a potential target for I329L. In fact, I329L-mediated inhibition could be observed by stimulating the pathway with either double stranded RNA (the synthetic TLR3 ligand, Poly(I:C)), or by ectopic expression of the intracellular signaling intermediate TRIF (Fig. 5A).

By interfering with the TLR3 signaling at the level of TRIF, I329L is predicted to inhibit activation of the transcription factors, required for IFN-β transcription (IRF3, IRF7 and NF-κB). Therefore, the amount of IFN-β secreted into the supernatants of cells expressing I329L and activated by ectopic expression of TRIF, was determined by ELISA. The very evident inhibition of IFN production resulting from the expression of I329L (Fig. 5B) supported the conclusions derived from the luciferase assays. Thus I329L is a viral TLR3 antagonist, reducing the effectiveness of the host type I IFN antivirus response.

Although the observed inhibition of NF-κB activation by I329L was consistent with an impact of I329L at the level of TRIF, an effect of I329L directly on NF-κB or other pathways leading to NF-κB activation was not ruled out. Therefore, additional luciferase assays were performed using a luciferase reporter driven by the specific binding sequence of NF-κB on the IFN-β promoter, and providing TNF-α as a TLR pathway independent stimulus for the activation of NF-κB. The results obtained demonstrated that I329L has no impact on the TNF-α receptor pathway, nor on the downstream activation of NF-κB, confirming the initial assumption that inhibition of this transcription factor by I329L occurs at the level of TRIF, upstream in the TLR3 pathway, and before the bifurcation into TRAF3/TRAF6 pathways (data not shown).

Upon binding its ligand, lipopolysaccharide (LPS), TLR4 initiates signaling transduction pathways through both MyD88 and TRIF adaptors. Signaling through MyD88 requires TIRAP (Kagan and Medzhitov, 2006) and culminates in the activation of NF-κB and the induction of an early pro-inflammatory response. Recently, TLR4 was demonstrated to be able to recognize viral proteins and, as a consequence, it is internalized into the endosomes (Husebye et al., 2006), where it interacts with TRIF via the TRIF-related adaptor molecule (TRAM) (Kagan et al., 2008). Through this pathway, TLR4 induces both an NF-κB dependent late pro-inflammatory response and, as well, type I IFN expression. Considering TRIF as the proposed target for the I329L-mediated inhibition of the induction of IFN-β, I329L would be predicted to inhibit NF-κB activation at the level of the TLR4 endosomal pathway. To test for this possibility, luciferase reporter assays were performed to assess the impact of I329L protein expression on the LPS-mediated activation of NF-κB transcription factor.

The results demonstrated that I329L is indeed able to inhibit NF-κB activation in LPS activated cells, specifically through a TRIF-dependent pathway (Fig. 6). Thus, by inhibiting the crucial adaptor protein TRIF, I329L is able to block stimulation of the host cell response by both viral nucleic acids and proteins (Husebye et al., 2006), thereby inhibiting secretion of IFN-β and the initiation of a pro-inflammatory response.

A modeling exercise on the I329L viral protein supported the idea that I329L might function as a TLR3 decoy, perhaps through the formation of TLR3-I329L heterodimers and, in doing so, inhibit the downstream signaling pathway (Henriques et al., 2011). In a recent report, Qi et al. demonstrated that TLR3 is proteolytically processed by cathepsins within the region of Loop1 (amino acids 336–343). This processing may not be essential for TLR3 signaling function, but it stabilizes the protein, increasing its half-life. Additionally, uncleaved and cleaved TLR3 locate to different endosomal compartments and respond distinctly to different ligands (Qi et al., 2012). Although the putative I329L extracellular domain (ECD) is considerably shorter than the uncleaved TLR3 counterpart, it aligns with the last ~340 out of the 680 residues of TLR3-ECD, precisely the ectodomain region of the proteolytically processed TLR3. This is the region that is brought into contact with the ligand, dsRNA, an event that is then followed by the association of the transmembrane helices and the functionally essential dimerization of the cytoplasmic TIR domains.

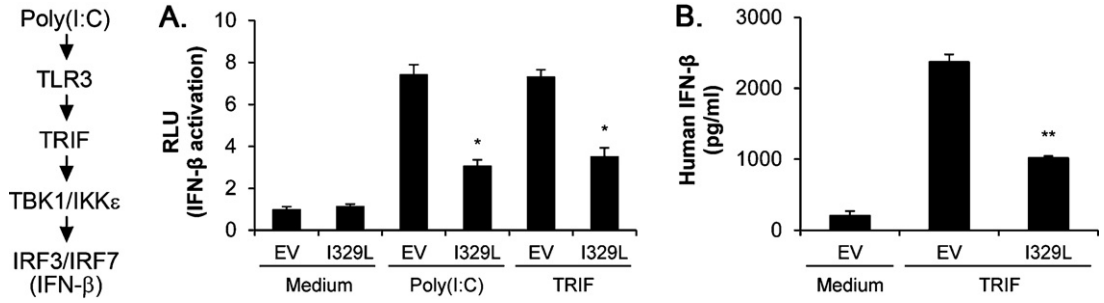

Fig. 5. The ASFV ORF I329L inhibits induction and expression of IFN-β. (A) HEK-293T cells stably expressing TLR3 protein, were co-transfected with the empty vector pcDNA3HA (EV) or pcDNA3-I329L-HA plasmid, the β-galactosidase plasmid and the IFN-β promoter (IFN-β) luciferase reporter. Cells were either induced with 35 μg/ml Poly(I:C) for 5 h, or by co-transfection of TRIF expression plasmid, or left untreated (medium). Luciferase activity was calculated and expressed as in the legend to Fig. 1. (B) HEK-293T cells were co-transfected with the empty vector pcDNA3HA (EV) or pcDNA3-I329L-HA plasmid. Cells were induced by co-transfection of TRIF expression plasmid. Supernatants were collected and IFN-β concentration (pg/ml) was measured by ELISA. Data are expressed as means ± SD of triplicate well from one of two similar experiments. Statistically significant difference was assessed by Student's *t*-test and is represented as $p \leq 0.05$ (*) or ≤0.01 (**).

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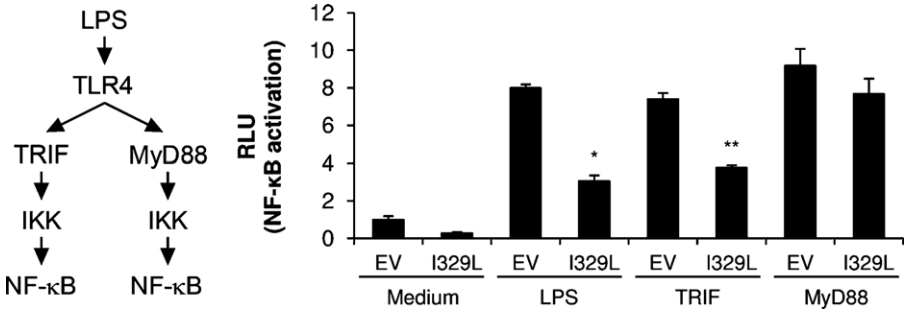

Fig. 6. The ASFV ORF I329L inhibits LPS-mediated activation of NF-κB through TRIF, and not MyD88, dependent pathway. Vero cells were co-transfected with the empty vector pcDNA3HA (EV) or pcDNA3-I329L-HA plasmid, the β-galactosidase plasmid and the NF-κB binding sequence (NF-κB) luciferase reporter. Cells were either induced with 100 ng/ml LPS for 5 h, or by co-transfection of TRIF or MyD88 expression plasmids, or left untreated (medium). Luciferase activity was calculated and expressed as in the legend to Fig. 1.

The modeling analysis of the dimerization of TLR3 intracellular domain suggested that I329L might also substitute for one of the TLR3 monomers, preventing the correct formation of the region of twofold symmetry where the adaptor TRIF is expected to bind (Fig. 7), with consequent disruption of the signaling function. It should be pointed out that this particular sequence contains the binding-adaptor loop (BB loop) which, on dimerization, shapes a twofold symmetrical exposed patch where the appropriate TLR adaptor is suggested to dock (Nyman et al., 2008). More precisely, experimental evidence has demonstrated the importance of the alanine in the BB loop for the binding of TLR3 directly to the adaptor TRIF. The other TLR's do not have this structure and require adaptors, such as TIRAP (MyD88 adaptor) or TRAM (TRIF adaptor) (Vercammen et al., 2008). Recent studies performed using the Vaccinia virus A46 protein, identified a small motif (VIPER) that targets the conserved BB loop proline of these TIR adaptors, disrupting TLR4:Mal and TLR4:TRAM interactions, thereby preventing TLR4 signaling (Stack and Bowie, 2012). This is in agreement with a previous prediction by Toshchakov et al. that cell-penetrating BB loop peptides, once inside the cell, could occupy the docking site of the cognate adaptor for its target and prevent binding of the native adaptor. This, in turn, would disrupt the formation of a functional signaling platform, inhibiting TLR signaling (Toshchakov et al., 2007). Clearly, TIR motif BB loops have been evolved by the virus as a strategy for preventing the formation of TLR signaling complexes.

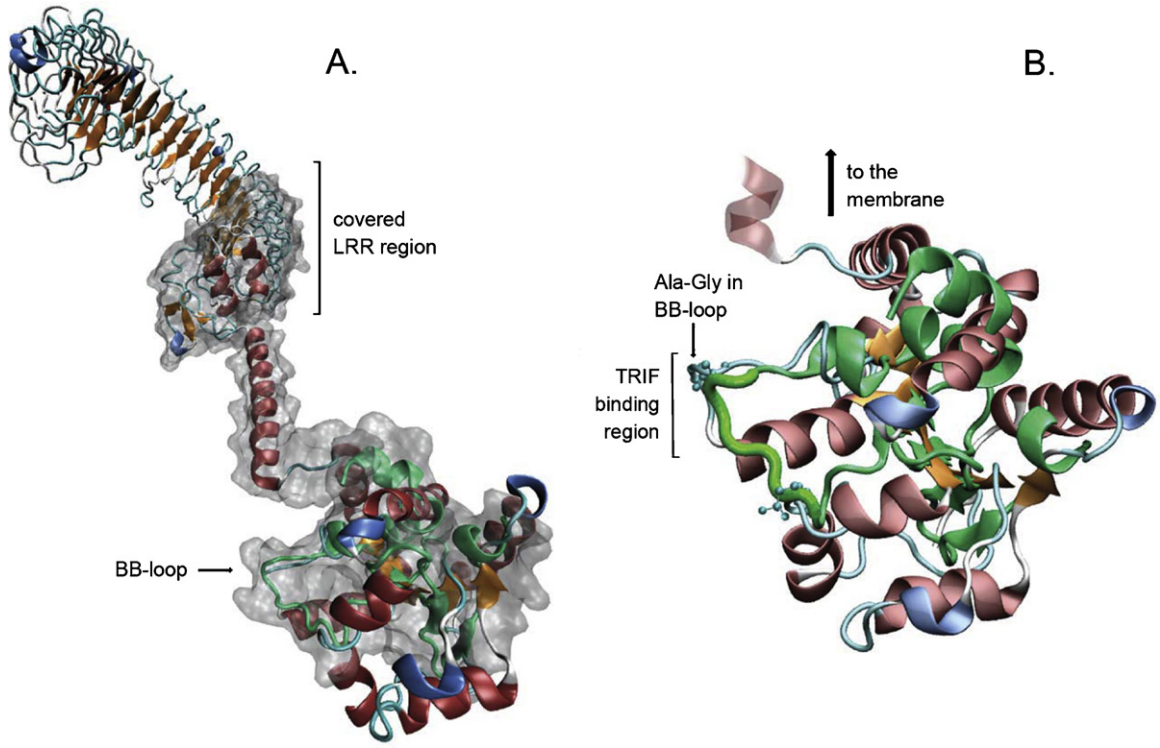

Fig. 7. The intracellular domain of I329L superposes to the TLR3-TIR domain. Models of the pI329L intracellular domain (green cartoon representation) superposed on the TLR3-TIR domain (cartoon representation is colored by secondary structure: pink rod, helix; yellow arrow, strand; and blue line, coil). The gray transparent surface delimits the regions of the receptor's domains for which the entire pI329L could have mimetic action; while the close-up of the intracellular region shows in more detail how one of the pI329L loops (tube representation in bright green) overlaps some key residues that are engaged in TLR3-TIR dimerization.
Adapted from Henriques et al. (2011).

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In conclusion, our modeling study provides a molecular basis for subversion of TLR3 signaling by I329L, with both intracellular and extracellular domains potentially capable of contributing.

4. Virus host evasion genes as 'ready-made tools' for genetic manipulation and therapy

Viruses have evolved multiple strategies to manipulate and evade host cell biology and immune responses. Paradoxically, however, virus host evasion genes potentially provide 'ready-made tools' to explore and manipulate the regulation of these basic cellular processes. We have therefore advanced this concept through construction of a mouse with transgene expression of A238L, an ASFV inhibitor of the transcription factors NF-κB and NFAT (Almeida et al., 2012). Although a number of mice lacking expression of NF-κB or NFAT have been constructed in order to define the roles of these transcription factors, their manipulation through transgenic expression of the viral host modification A238L gene, provides an alternative strategy.

One domain of A238L, with homology to IκBα, interacts with p65 of the NF-κB family of transcription factors, thereby inhibiting its activation (Powell et al., 1996; Revilla et al., 1998). Another domain interacts with calcineurin phosphatase (CanPase) (now known as protein phosphatase 3C, or PP3C), thus inhibiting activation of NFAT transcription factors (Granja et al., 2004; Miskin et al., 2000, 1998). A mutant A238L (mutA238L) no longer capable of interaction with CanPase, but still inhibiting the activation of p65, has been characterized (Miskin et al., 2000). A third function of A238L was more recently described and results in inhibition of p65/RelA acetylation and inhibition of protein kinase C-h-dependent transcriptional activation of p300 (Granja et al., 2006a,b, 2008, 2009). The p300 and CREB-binding protein (CBP) proteins play a key role in transcriptional regulation of a myriad of genes which do not bind directly to the promoters of such genes, but are recruited through interaction with several transcription factors, for example, NF-κB and NFAT (Garcia-Rodriguez and Rao, 1998; Gerritsen et al., 1997; Perkins et al., 1997).

Taking into account the possibility of pleiotropic effects of the A238L expression in many cell types, the virus transgene was restricted to T lymphocytes, as the development and function of these lymphocytes is very well understood, and provides an excellent system to explore the impact of the transgene. The resulting T lymphocyte restricted A238L transgenic mice developed transplantable, angiogenic thymic tumors, whose T lymphocytes were CD4+CD8+CD69− (mono-)oligoclonal lymphoblasts, with uncontrolled growth in the thymus and metastasis to both the secondary lymphoid organs (spleen and lymph nodes) and the non-lymphoid tissues, such as kidney, lung and liver. The absence of CD69 from the tumor cells suggests that they were derived from T lymphocytes at a stage prior to positive selection. Significantly, expression of Rag1, Rag2, TCRβ-V8.2, CD25, FoxP3, Bcl3, Bcl2, l14, Myc, IL-2, NFAT1 and Itk by purified CD4+CD8+CD69− thymocytes from A238L transgenic mice was consistent with the phenotype (Fig. 8).

In contrast, transgenic mice similarly expressing the mutant A238L, solely inhibiting transcription mediated by NF-κB, were indistinguishable from wild type mice. Similarly, expression profiles of CD4+CD8+CD69− thymocytes from the mutant A238L transgenic mice were comparable to those of wild type mice (Fig. 8).

These features, together with the demonstration of (mono-) oligoclonality of the tumor cells, suggest a transgene-NFAT-dependent transformation yielding a lymphoma with a phenotype reminiscent of some acute lymphoblastic lymphomas. Elucidation of the molecular events associated with the development of this virus host evasion molecule induced metastasizing, angiogenic tumor may clarify some mechanisms of tumorigenesis in general, and in the development of T lymphocyte acute lymphoblastic

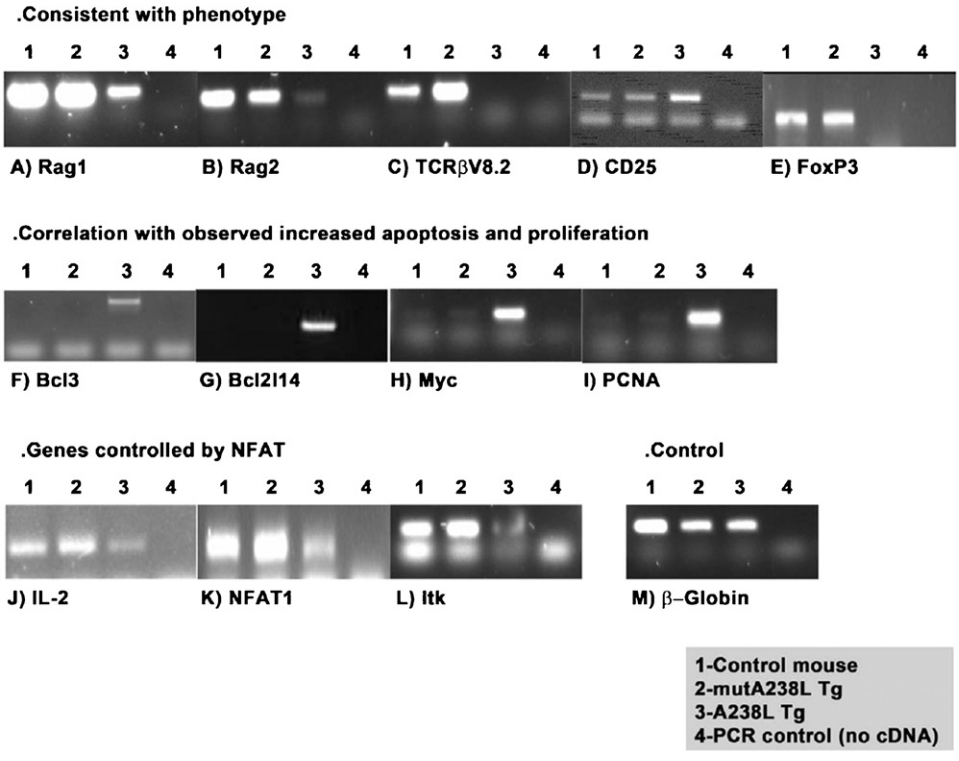

Fig. 8. Expression analysis of CD4+CD8+CD69− thymocytes demonstrates selective effect of the expression of the A238L transgene in the A238L transgenic mice. Semi-quantitative PCR analysis of the cDNA prepared from the mRNA of the FACS purified CD4+CD8+CD69− thymic cells of A238L and mutA238L transgenic and control mice shows that the A238L transgenic mice display a distinctive pattern of expression for a group of selective genes which correlates to the phenotype of the mice. β-Globin was used as the housekeeping gene. The results presented correspond to a pool of 5 mice per genotype.

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leukemia in particular. Indeed, the phenotype of the T lymphocyte restricted A238L transgenic mouse would have been predicted from the recent suggestion for targeting calcineurin activation as a therapeutic intervention in acute T cell lymphoblastic leukemia (Medyouf et al., 2007).

Finally, the work demonstrates that virus host evasion genes can be used as probes to manipulate the genetic program of mammalian cells in vivo. Virus strategies for manipulating transcription may complement the analysis of knock-out mice and also provide tools for understanding and manipulating mechanisms of transcription in health and disease.

5. Conclusions

ASFV is an important and devastating hemorrhagic veterinary pathogen, originally confined to Africa, but now out of control in Russia and thus threatening Europe and the rest of the world. There is no vaccine and, as immunity is complex, involving multiple immunological mechanisms and immune-determinants, a feasible route would be the construction of an attenuated host evasion gene(s) deletion mutant virus.

The fact that ASFV is not only an acute virus, but can also be persistent in both pigs and warthogs, strongly suggests that the virus must have evolved strategies to evade IFN responses. If so, then deletion of virus genes inhibiting the IFN response would be a rational approach for construction of a vaccine. An alternative would be a multigene DNA vaccine.

Surprisingly, however, until now no individual ASFV gene targeting IFN had been described, perhaps because bioinformatics' approaches have failed to identify any obvious potential inhibitory viral homologues. Our strategy, therefore, was to screen early expressed, non-homologous ASFV genes for inhibition of the induction and impact of IFN in functional assays.

Three of the six genes that we so identified are described above. They reduce the IFN response by targeting different intracellular signaling intermediates. Thus their deletion from wild type virus may strengthen the host IFN response and so provide an attenuated live virus vaccine with more restricted virus spread after the initial infection, perhaps "buying" sufficient time to allow the development of a protective adaptive immune response. The demonstration of multiple ASFV genes for the evasion of IFN responses, however, demands technology to construct viruses with multiple gene deletions, and, fortunately, this has recently been developed (Abrams and Dixon, 2012).

Finally, our work clearly demonstrates that unassigned viral genes may be viewed as a repository of host evasion strategies, only identifiable through functional assays. These may be considered to be "ready-made tools" for the experimental manipulation of cell biology and immune responses in health and disease, and, as proof of concept, we have constructed a T lymphocyte restricted transgenic mouse expressing the ASFV encoded dual inhibitor of NF-κB and NFAT activation, A238L. The resulting T lymphocyte restricted A238L transgenic mice developed a lymphoma with a phenotype reminiscent of some acute lymphoblastic lymphomas. Elucidation of the molecular events associated with the development of this tumor may clarify some mechanisms of tumorigenesis in general, and in the development of T lymphocyte acute lymphoblastic leukemia in particular.

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