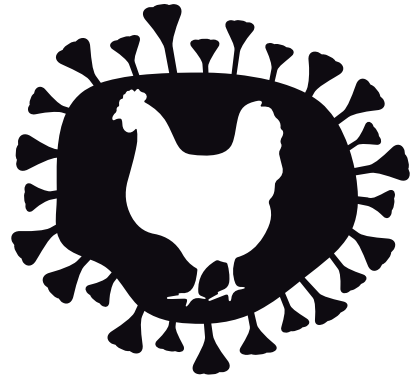
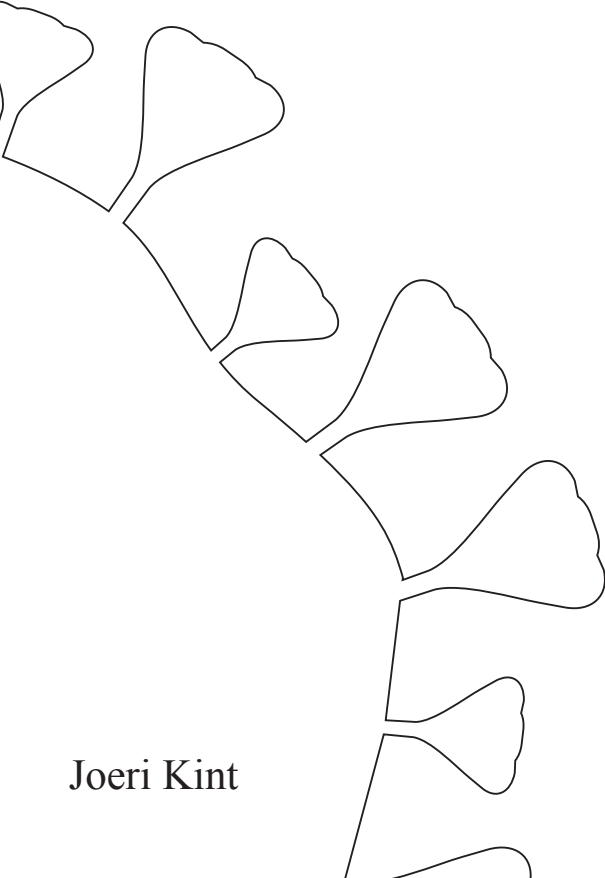


Activation
and Evasion
of the
Type I Interferon Response by
Infectious Bronchitis Virus

Roles of the accessory proteins



Joeri Kint



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Voor pa en ma

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Chapter

1

General Introduction

1.1 VIRUS

Coronaviruses

Coronaviruses infect many animal species and are associated with a range of respiratory, enteric, hepatic, and neurological diseases of varying severity. Coronaviruses belong to the family of *Coronaviridae*, which falls under the order of Nidovirales. This order of viruses is characterised by a similar genome organisation and by the production of subgenomic or nested (Latin: *nidus*) mRNAs. The *Coronaviridae* possess the largest known RNA genomes of all viruses, which consist of a monopartite, 25 to 32 kb, positive sense, single stranded RNA molecule, which is capped and polyadenylated. The *Coronaviridae* are further divided in two subfamilies; the *Torovirinae* and the *Coronavirinae*. The *Coronavirinae* in turn, are divided into the genera *Alpha-*, *Beta-*, *Gamma-* and *Deltacoronaviruses* on the basis of pairwise comparisons of conserved domains in the genome (1-3). Sequence-based modelling of coronavirus evolution, indicates that the last common coronavirus ancestor diverged several million years ago (4) into two major lineages; the bat-borne ancestor of the *Alpha-* and *Betacoronaviruses* and the avian-borne ancestor of the *Gamma-* and *Deltacoronaviruses* (5) (Fig. 1).

Coronavirus virion, genome organisation and replication.

Coronaviruses are enveloped viruses and their name is derived from the club-shaped trimeric spike proteins (S) that protrude from the envelope and make the virion look like a crown (Latin, *corona*). The spike protein is the main antigenic determinant of the virus and it mediates both binding to and entry into the host cell. The viral envelope also contains the membrane (M) and the envelope (E) proteins. Inside the envelope resides the ssRNA genome, which is tightly associated with the nucleocapsid (N) proteins (6) (Fig. 2A). More than two-thirds of the genome of coronaviruses consists of two large replicase genes, ORF1a and ORF1b. When the genomic RNA is released into the host cell, ORF1a and ORF1b are translated directly from the genomic RNA via a ribosomal frameshift mechanism (Fig. 2B). Depending on the genus, these polyproteins autoproteolytically cleave themselves into 15 or 16 non-structural proteins (nsps), (7) (Fig 2B, *Gammacoronavirus genome*). The variation in number of nsps is explained by the fact that the genome of both *Gamma-* and *Deltacoronaviruses* do not contain nsp1. One of the first steps during coronavirus infection is the assembly of membrane-bound replication transcription complexes (RTC) that transcribe the (+) strand RNA genome into full length (-) stranded copies of the genome, which in turn, serve as template for production of (+) strand RNA genomes. Additionally, the RTCs transcribe subgenomic (-) strand RNAs from the genomic (+) RNA, through a process of discontinuous transcription involving the transcription regulation sites (TRS) and the 5' leader sequence (Reviewed in (8)). From these subgenomic (-) RNAs, many (+) stranded RNAs are produced that serve as mRNAs for translation of the spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins (Fig. 2B). In addition to these structural proteins, coronaviruses encode between one and eight accessory proteins, a number which varies between genera and even between individual strains within a genus (9). These accessory proteins have generally been found dispensable for viral replication *in vitro* (10-15), but some of them have been shown important for coronavirus infection *in vivo* (10, 16). Functional studies have revealed that coronavirus accessory proteins modulate various cellular processes, such as apoptosis, cell proliferation, and DNA synthesis. However, most accessory proteins

have been found to modulate the interferon response, which is an important host-defence mechanism against viruses (summarised in Table 1).

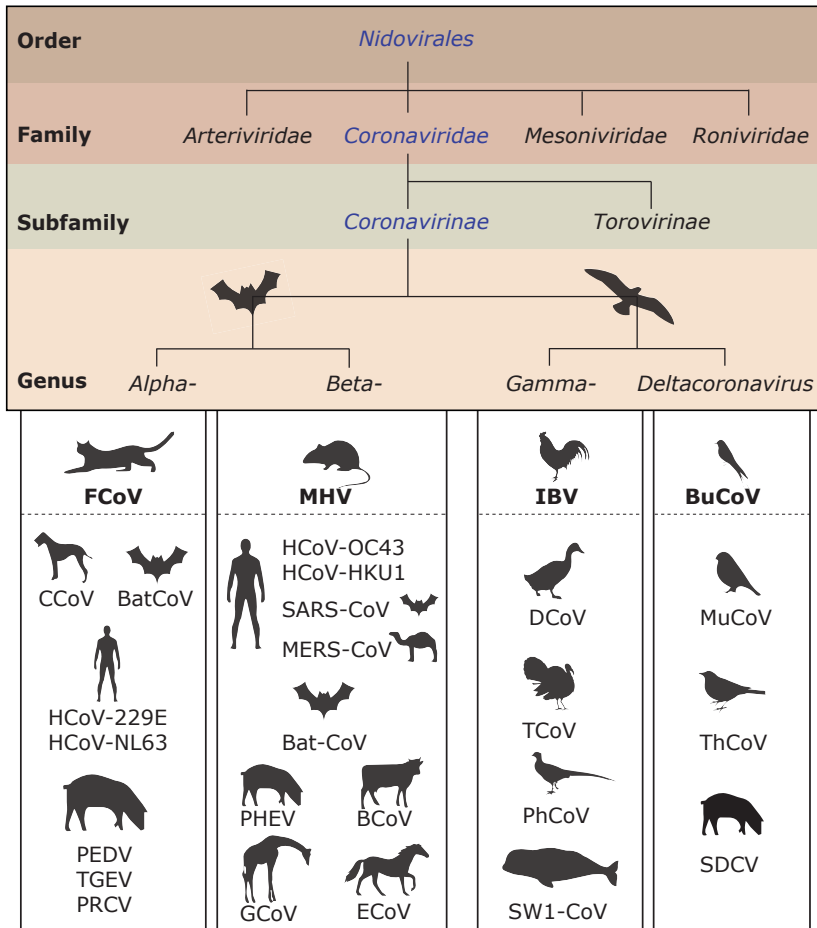


Fig. 1 Taxonomy and host species of coronaviruses

The top panel displays the taxonomical organisation of coronaviruses. The probable host species of the common ancestors of both *Alpha-* and *Beta-* and *Gamma-* and *Deltacoronaviruses* is also indicated. Bottom panels show prominent members of each genus and their host species. Type species are displayed at the top in a bold typeset. FCoV, Feline coronavirus; CCoV, Canine coronavirus; HCoV, human coronavirus; PEDV, porcine epidemic diarrhea virus; TGEV, transmissible gastroenteritis; PRCV, porcine respiratory coronavirus; MHV, mouse hepatitis virus; SARS-CoV severe acute respiratory syndrome coronavirus; MERS-CoV, Middle East respiratory syndrome coronavirus; PHEV, porcine hemagglutinating encephalomyelitis virus; BCoV, bovine coronavirus; GCoV, Giraffe Coronavirus; IBV, infectious bronchitis virus; DCoV, duck coronavirus; TCoV, turkey coronavirus; PhCoV, pheasant coronavirus; SW1-CoV, Belugawhale coronavirus SW1; BuCoV, Bulbul coronavirus; MuCoV, Munia coronavirus; ThCoV, Thrush coronavirus; SDCV, swine *Deltacoronavirus*.

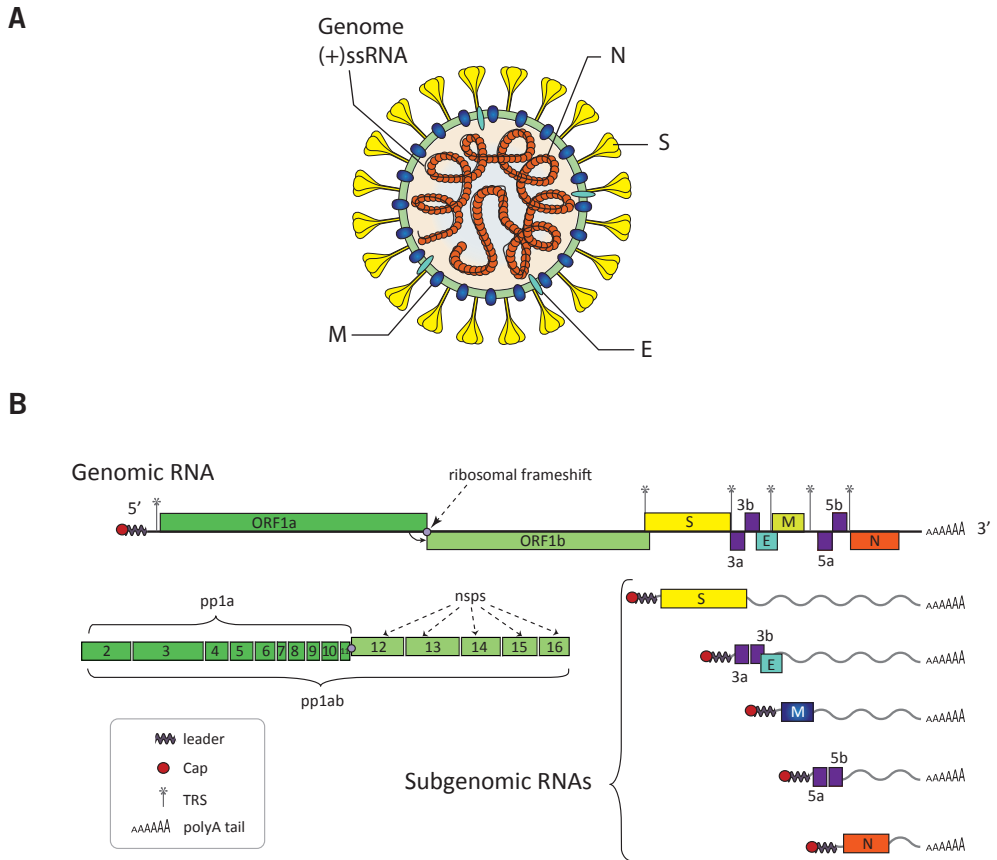


Fig. 2 Virion structure and genome organisation of infectious bronchitis virus

(A) Schematic drawing of the IBV virion, displaying the location of the structural proteins within the virion. The (+) ssRNA genome is fully covered by nucleocapsid proteins. (B) All coronaviruses share a similar genome organisation; this is a graphical representation of the genomic RNA and the subgenomic RNAs of *Gammaparvovirus* IBV, strain Beaudette. The components of the virion are not drawn to scale. Two open reading frames (ORF1a and ORF1b) are translated directly from the genome to yield two large polyproteins, pp1a and pp1ab, the latter via a ribosomal frameshift mechanism. These polyproteins cleave autoproteolytically into 15 non-structural proteins (nsps). Subgenomic (-) strand RNAs are transcribed from the genomic (+) RNA, through a process of discontinuous transcription involving the transcription regulation sites (TRS) and the 5' leader sequence. From these subgenomic (-) RNAs, many (+) stranded RNAs are produced from which the four structural proteins, spike (S), membrane (M), envelope (E) and nucleocapsid (N) as well as (at least) four accessory proteins (purple), are translated. Both the genomic and the subgenomic RNAs are capped and polyadenylated.

Table 1. Current knowledge on how coronavirus accessory proteins influence the host

Coronavirus	Accessory protein	Influence on the host
SARS-CoV	3a	Activates NF- κ B and Il-8 reporters (17), induces apoptosis in Vero cells (18)
	3b	Inhibits activation of Ifn β and NF- κ B reporters and inhibits translocation and phosphorylation of IRF3 (19, 20)
	6	Inhibits activation of Ifn β and NF- κ B reporters and inhibits translocation and phosphorylation of IRF3(19). Inhibits nuclear translocation but not phosphorylation of STAT1 (21)
	7a	Activates NF- κ B and Il-8 reporters (17), induces apoptosis in Vero cells (18)
	7b	?
	8a	Induces apoptosis and promotes viral replication (22)
	8b	Stimulates DNA synthesis (23)
	9b	Induces apoptosis (24)
MHV	NS2	Antagonises the antiviral OAS-RnaseL pathway (25)
	HE	Virulence factor <i>in vivo</i> , not present in all strains (26)
	4	?
	5a	Increases resistance to IFN-treatment <i>in vitro</i> , mechanism unknown (27)
TGEV	3a	?
	3b	?
	7	Inhibits phosphorylation of eIF2 α (28) Inhibits transcription and translation of antiviral genes, including Ifn β (16)
FIPV	3	? not necessary for replication <i>in vitro</i> and <i>in vivo</i> (29)
	7a	Confers resistance to IFN-treatment via unknown mechanism (30)
	7b	?
MERS-CoV	3	-
	4a	Inhibits activation of Ifn β reporter and translocation of IRF3 (31). Binds dsRNA (31). Inhibits activation of Ifn β and ISRE reporters (32)
	4b	Decreases activation of Rig-I/IRF3 and NF- κ B reporters (33)
	5	Reduces activation of Ifn β reporter (32)
IBV	3a	Not necessary for replication <i>in vivo</i> (34) and <i>in vitro</i> (13, 14, 35, 36)
	3b	
	5a	
	5b	

'?' indicates that no function has been elucidated thus far.

Infectious Bronchitis Virus

The *Gammacoronavirus* infectious bronchitis virus (IBV) is the causative agent of a highly contagious respiratory disease (37). IBV was first described in the USA in the 1930's, and has since then spread to almost every country with a poultry industry. In fact, infectious bronchitis has become one of the most important diseases in the poultry industry, and is responsible for large economic losses worldwide (38). IBV enters the avian host through the respiratory tract where it leads to nasal discharge, sneezing, and rales. Depending on the strain, IBV can spread to other epithelial surfaces such as the gastrointestinal tract, the kidneys and the oviduct, causing reduced growth of chickens and a decrease in egg production and quality (38-43). IBV-associated mortality primarily occurs when animals get simultaneously infected with another pathogenic virus, bacterium or mycoplasma. Such co-infections occur frequently, since IBV facilitates entry of other pathogens by compromising epithelial barriers during infection.

Serotypes

Control of IBV is hindered by the continuous occurrence of new serotypes of the virus (44), (Fig. 3). Mutation of the virus can be attributed to the error rate of the Coronavirus RNA-dependent RNA-polymerase (45) and by the high frequency of recombination between different strains of IBV (46-49). These effects are aggravated by the large population sizes of its hosts in the poultry industry and the possibility that IBV can be carried over great distances by migratory birds (47, 50).

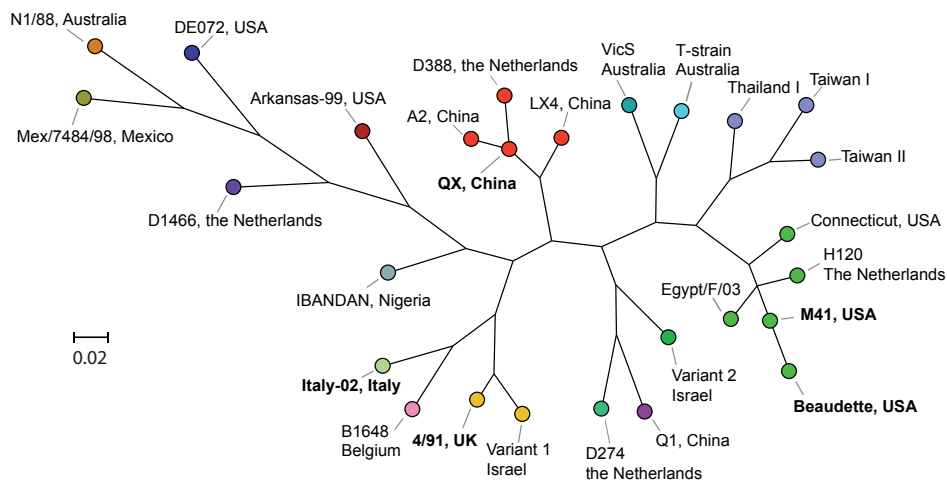


Fig. 3 Phylogenetic tree of various IBV isolates, including the location where they were isolated

The phylogenetic tree was constructed by neighbour joining analysis using the Bionumerics software package, using 500bp sections of the S gene (bp 20,447-20,924 as based on the genome sequence of Ark DPI (48)). IBV isolates used in the current study are indicated in bold. This figure was kindly supplied by J.J. de Wit and R. Dijkman, Gezondheidsdienst voor dieren, Deventer, The Netherlands. Branch lengths represent nucleotide substitutions per position. Colours were chosen arbitrarily.

Vaccines and challenges

Because of the economic impact of infectious bronchitis on the poultry industry, much effort has gone into the development of vaccines against IBV. The first vaccines, developed in the 1950's (41), consisted of live viruses that were attenuated by multiple passages in embryonated chicken eggs. Depending on the field pressure and the life expectancy of the chicken involved (broiler, breeder or layer), different vaccination schemes may be applied. Commonly, animals are primed by spraying them with a live-attenuated virus. This treatment can be repeated after a every few weeks to further boost the immune response. To establish lasting immunity during the laying period, all layers receive an intramuscular injection with an inactivated IBV vaccine before the onset of lay. It is common practise to combine multiple strains of IBV in one vaccine, since cross-protection between strains is limited (38, 42, 51, 52).

All commercially-available IBV vaccines contain either inactivated or attenuated viruses, which are produced in embryonated chicken eggs. Although this method of production has proven very successful, the use of embryonated eggs is associated with various safety and supply issues.

The eggs used for manufacturing of IBV vaccines frequently contain viral or bacterial pathogens, which can subsequently get introduced into the vaccines made using these eggs. To prevent such pathogens from ending up in the final product, a range of costly quality control tests need to be performed on a regular basis. Another safety concern about existing live IBV vaccines is their potential to revert to virulence. Current vaccine strains have been attenuated through serial passaging in embryonated eggs. Attenuation of a virus through this method relies on introduction of random mutations to the genome, making it unclear which mutation(s) is/are responsible for the attenuation, let alone why the virus is attenuated. Several studies have shown that these IBV vaccine strains can revert to virulence in the field through mutation and recombination with field strains (47, 53-55). In addition to these disadvantages, the supply of eggs can be halted by issues with bacterial or viral infections at the egg-supplier or by a ban on egg-transport, following an outbreak of avian influenza.

A considerable improvement to the safety and supply issues of current IBV-vaccines would be to abandon the use of embryonated eggs, for example by shifting to the use of subunit vaccines. Several of such subunit vaccines have been tested, but all of them provided inadequate protection against challenge with virulent strains of IBV (56-59). Another strategy that has proven successful for production of viral vaccines is based on the use of cell lines for virus production. Production of IBV using cell lines was achieved by adaption of the avirulent IBV Beaudette strain to propagation in Vero and HeLa cell lines. Unfortunately, vaccination using Beaudette does not provide protection against virulent strains of IBV (60, 61). A third method, which could increase the safety of current IBV vaccines while maintaining production in embryonated eggs, would be to minimise the chance of reversion of vaccine strains to a virulent form. This could be achieved by using IBV strains that carry deletions in multiple virulence determining regions within the genome. These rationally-attenuated vaccines have been developed for several viruses (reviewed in (62)) and indeed, attempts to create rationally-attenuated, replication-competent coronaviruses have yielded promising results (63, 64). However, the development of such a vaccine for IBV requires extensive knowledge about the interaction between virus proteins and host cells. Currently, knowledge about which parts of the genome could be specifically targeted to attenuate IBV is too restricted to develop a rationally-attenuated vaccine against IBV.

1.2 HOST

The type I interferon response protects against RNA viruses.

The innate immune response is an evolutionarily conserved part of the immune system providing immediate defence against invading pathogens. The innate immune response consists of a network of antimicrobial mechanisms, of which the type I interferon (IFN) response is an essential defence mechanism against viruses. The IFN response to RNA viruses is triggered by detection of aberrant RNA molecules, generated by the replicating virus. Detection of these molecules drives production of antiviral proteins and secretion of type I interferon cytokines, which act as a danger-signal to neighbouring cells. Binding of IFN by a neighbouring cell induces the production of antiviral proteins, thereby establishing an antiviral state.

The type I interferon response in detail

Two families of pattern recognition receptors (PRRs) have been shown to be involved in the recognition of RNA viruses, namely the membrane-bound Toll-like receptors (TLRs) and the cytosolic RIG-I-like receptors (RLRs) (65). The primary ligands for the activation of these PRRs are double-stranded RNA (dsRNA) and 5'-triphosphorylated RNA, normally absent from uninfected host cells. Activation of TLRs or RLRs by these molecules activates a signaling cascade that, via the transcription factors (Interferon Regulatory Factor) IRF3 and IRF7, activates transcription of type I interferons (IFN α and IFN β) and a set of antiviral genes (Fig. 4). Interferons are readily secreted by the infected cell, and act as warning signal by binding to the ubiquitously expressed IFN receptor (IFNAR) on neighbouring cells. Binding of IFN induces phosphorylation of Janus kinases (JAK and TYK), which in turn, phosphorylate STAT1 and STAT2 (Signal Transducers and Activators of Transcription), causing them to dimerize and associate with IRF9 to form the interferon-stimulated gene factor 3 (ISGF3) complex. Subsequent nuclear translocation and binding of ISGF3 to interferon stimulated response promoter elements (ISRE) activates transcription of antiviral genes known as interferon-stimulated genes (ISGs) (66, 67). Hundreds of ISGs have been described (68) and those that have been characterised in more detail, such as Mx, MDA5, TLR3, PKR, OAS, ADAR and IRF1 are involved in detection and signaling of viral infection and inhibition of viral translation, replication, entry, and budding (69). To provide a rapid response against viral infection, transcription of a subset of interferon-stimulated genes (ISGs) is also activated upon stimulation of pattern recognition receptors, independently of IFN (70).

The type I interferon response to RNA viruses in chicken

The interferon response is an evolutionarily conserved defence mechanism against invading pathogens. In many respects the chicken interferon system, although not studied in much detail, resembles that of humans. Similar to the human genome, the chicken genome encodes for one copy of *Ifn β* and multiple paralogs of *Ifn α* . However, differences between the chicken and mammalian interferon system also exist (Reviewed in (71)). For example, chickens lack the ssRNA sensor TLR8 and stimulation of chicken TLR7 with ssRNA results in expression of *Il1 β* and *Il8*, but not *Ifn α* or *Ifn β* (72).

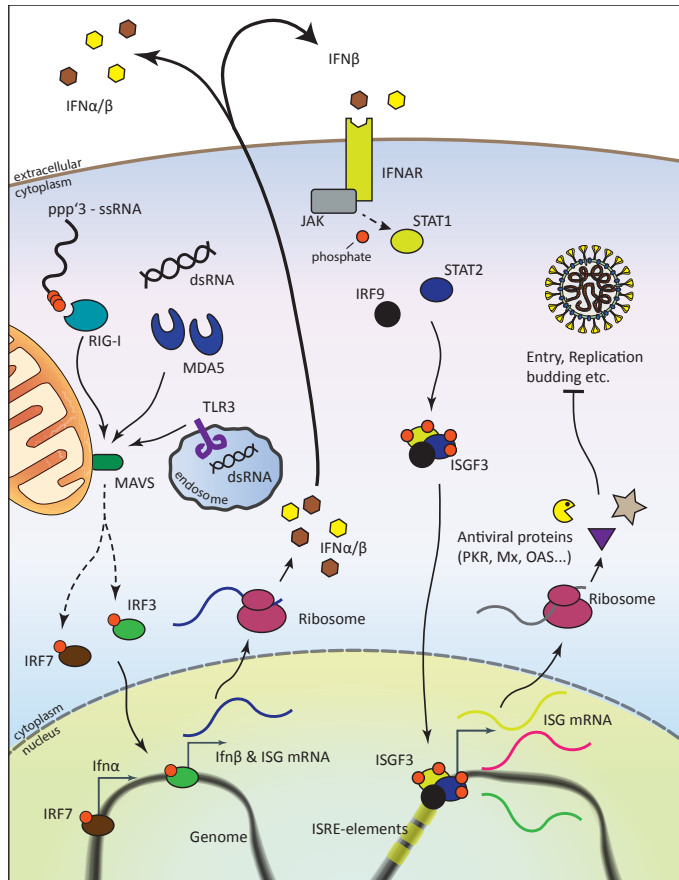


Fig. 4 Schematic overview of the type I interferon response

Overview explained in paragraph 1.2: The type I interferon response in detail. Dotted lines indicate pathways that have been highly simplified to improve clarity

Contrary to chicken TLR7, stimulation of human TLR7 does induce expression of *Ifnα/β* via the transcription factor IRF7. This difference between chicken and mammalian TLR7 may be related to the fact that chickens encode a transcription factor that resembles both mammalian IRF3 and IRF7 (named IRF3/7) (73). The dsRNA-sensor TLR3 is also expressed by chicken cells (74) and like its mammalian counterpart, recognizes dsRNA (75), and activates transcription of *Ifnβ* probably via IRF3/7 (76). Rather unexpectedly, the cytoplasmic RNA sensor RIG-I, which is present in ducks, seems to be absent from the chicken genome (77). However, the cytoplasmic dsRNA pattern recognition receptor MDA5 is present in chickens and responds to dsRNA (78). In line with this, the adaptor protein for both RIG-I and MDA5, termed mitochondrial antiviral-signaling protein (MAVS), has been identified in chicken cells (79). In chicken cells, detection of influenza virus was shown to depend on recognition by MDA5, whereas in mammalian cells detection of influenza is mediated by RIG-I (79-81). These observations indicate that chicken MDA5 may have a broader sensitivity than its mammalian counterpart (78, 82).

1.3 INTERACTION

Coronaviruses and the type I IFN response

The IFN response is a powerful mechanism in combating virus infections, including coronaviruses. However, our understanding of the power and importance of the IFN response in combating coronaviruses *in vivo* comes primarily from studies on mouse hepatitis virus (MHV) infection in mice. IFN receptor-deficient mice (IFNAR^{-/-}) quickly succumb to a normally sub-lethal MHV infection, indicating that without an active IFN response mice cannot sufficiently combat the virus and recover from infection (64, 83, 84). In general, to avoid clearance, coronaviruses have evolved various strategies to interfere with the IFN response.

Coronaviruses prevent activation of the IFN response

Although large amounts of dsRNA accumulate in coronavirus-infected cells (85-87), *Alpha-* and *Betacoronaviruses*, including human coronavirus 229E (HCoV-229E), severe acute respiratory syndrome coronavirus (SARS-CoV) and mouse hepatitis virus (MHV), only mildly activate the type I interferon response (88-94). In both fibroblasts and hepatocytes, MHV and SARS-CoV do not induce expression of type I interferons. Stimulation of infected cells with dsRNA (poly I:C) however, does induce translocation of IRF3 and expression of interferon, indicating that recognition of dsRNA is not blocked in coronavirus-infected cells. The fact that coronaviruses induces considerable accumulation of dsRNA without triggering the IFN response, suggests that dsRNA is somehow hidden from pattern recognition receptors. In fact, almost all (+) stranded RNA viruses induce so-called membrane-bound replicative organelles, in which viral RNA synthesis is thought to take place (95). Coronaviruses are no exception, and the membrane structures that they induce are associated with the viral replication-transcription complexes, indicating that these membrane structures indeed form the site of viral RNA synthesis (96). As a matter of fact, coronavirus-induced double-membrane vesicles (DMV's) have been shown to contain dsRNA (85) suggesting that DMV's shield dsRNA from recognition by PRRs (85, 97). In addition to hiding dsRNA from PRRs, several coronavirus proteins have been shown to 'actively' interfere with activation of IFN. In overexpression studies, activation of *Ifn*-reporters was prevented by both *nsp1* (64, 98, 99) and *nsp3* (19, 94) of MHV and SARS-CoV. Using similar assays it was shown that the SARS-CoV nucleocapsid as well as accessory proteins ORF3b and ORF6 of SARS-CoV (19) and 4a and 4b of Middle East respiratory syndrome coronavirus (MERS-CoV) (100, 101) inhibited activation the IFN response. Taken together, coronaviruses seem to use a combination of hiding and active countermeasures to prevent activation of the IFN response.

Coronaviruses resist the activated IFN response

Most coronaviruses are relatively resistant to treatment with IFN, compared to other viruses such as Sendai virus (family Paramyxoviridae) or vesicular stomatitis virus (family Rhabdoviridae). Insensitivity towards IFN could be another mechanism used by coronaviruses to evade the host immune response. MHV and feline coronavirus (FCoV) show the highest resistance to IFN (93, 102), followed by SARS-CoV (103-105). MERS-CoV on the other hand is 50 to 100 times more sensitive to IFN treatment than SARS-CoV (106, 107), a difference that has been ascribed to the ability of SARS-CoV,

but not MERS-CoV, to inhibit nuclear translocation of STAT1 (106). Phosphorylation and subsequent translocation of STAT1 is an essential step in the activation of ISGs. Accessory protein ORF6 of SARS-CoV has been shown to inhibit translocation of STAT1 by tethering nuclear import factors at the ER/Golgi membrane, thus preventing expression of IFN-activated genes (19, 98, 108). Besides blocking IFN signaling, coronaviruses also antagonise IFN-activated antiviral proteins. For example, accessory protein ns2 of MHV inhibits the IFN-activated OAS-RNase L antiviral pathway (109), the nucleocapsid of MHV and the nsp2 protein of IBV seem to antagonise PKR (110, 111), while accessory proteins 5a of MHV and 3 and 7a of FCoV confer resistance to IFN treatment via unknown mechanisms (110, 112, 113).

Coronaviruses inhibit host-translation

Like most viruses, coronaviruses are dependent on host-cell machinery for translation of their proteins. To maximise production of viral proteins and limit production of host cytokines and other antiviral proteins, some viruses have evolved strategies to favour the production of viral proteins over the production of host proteins (114). *Betacoronaviruses*, such as MHV, SARS-CoV and several Bat coronaviruses limit host-translation using their nsp1 protein (115-117), which induces degradation of host, but not viral mRNA (116, 118, 119). Nsp1 of the *Alphacoronavirus* TGEV inhibits host-translation through an unknown mechanism that does not seem to involve degradation of host mRNA (117, 120). Strikingly, MHV-nsp1-mutant viruses are still pathogenic to type I IFN receptor-deficient (IFNAR^{-/-}) mice but are severely attenuated in IFN competent individuals. This indicates that inhibition of host-translation by nsp1 is crucial for coronaviruses such as MHV to interfere with the innate immune response. Contrary to *Alpha-* and *Betacoronaviruses*, the genomes of *Gamma-* and *Deltacoronaviruses* lack an nsp1 homologue (3, 121-123), raising the question whether also these viruses counteract host-translation. One study on IBV *Gammacoronavirus* showed that this virus does not reduce host-translation (111). The lack of nsp1 coupled to the lack of translational inhibition by *Gammacoronaviruses*, could indicate that *Gammacoronaviruses* have evolved alternative ways, compared to *Alpha-* and *Betacoronaviruses*, to counteract the innate immune response of their host.

1.4 OUTLINE OF THE THESIS

Infectious bronchitis virus (IBV) is an economically-important pathogen of poultry, but little is known about how it interacts with the avian innate immune response, neither at the level of the animal, nor at the level of individual cells. This lack of knowledge has hindered the understanding of IBV pathology and the development of rationally-attenuated vaccines against this disease. The overall aim of this thesis is to understand how IBV avoids clearance by the innate immune system of the cell and, more specifically, to investigate the potential role of the accessory proteins herein. In **chapter 1** we provide a framework for this thesis by describing background information on coronaviruses, the type I IFN response and our current knowledge on the antagonism of the IFN response by coronaviruses. In **chapter 2** we study if and to what extent IBV activates the type I IFN response in chicken cells. In addition to this we test whether the accessory proteins of IBV influence the kinetics of activation of the type I IFN response. We also investigate which pattern recognition receptor of the chicken is involved in the detection of IBV. In **chapter 3** we quantify the sensitivity of IBV to IFN treatment, and we investigate whether IBV can inhibit signaling of IFN, as previously reported for some *Betacoronaviruses*. In addition, we assess the potential influence of accessory proteins on both IFN sensitivity and signaling of IFN. In **chapter 4** we investigate whether IBV, similar to *Alpha-* and *Betacoronaviruses* is able to inhibit translation of host-cell proteins, and evaluate the potential role of accessory proteins in this process. In **chapters 5 and 6** we describe the specific methods used in this thesis for quantification of IBV, expression of ISGs and production of interferon by chicken cells. The results described in these chapters answer several questions about the activation and evasion of the type I interferon response by IBV. In **chapter 7** I place the findings of this thesis within the framework of known work on innate immune evasion by coronaviruses. I also discuss the potential implications of my findings for the understanding of IBV pathology and the development of live rationally-attenuated vaccines against this disease. In conclusion, this thesis provides further insight into how IBV avoids and antagonises the innate immune system, and is the first study that attributes a function to the accessory proteins of IBV.

Chapter

2

Activation of the Chicken Type I Interferon Response by Infectious Bronchitis Coronavirus

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ABSTRACT

Coronaviruses from both the *Alphacoronavirus* and *Betacoronavirus* genera interfere with the type I interferon (IFN) response in various ways, ensuring the limited activation of the IFN response in most cell types. Of the gammacoronaviruses that mainly infect birds, little is known about the activation of the host immune response. We show that the prototypical *Gammacoronavirus*, infectious bronchitis virus (IBV), induces a delayed activation of the IFN response in primary renal cells, tracheal epithelial cells, and a chicken cell line. In fact, *Ifn β* expression is delayed with respect to the peak of viral replication and the accompanying accumulation of double-stranded RNA (dsRNA). In addition, we demonstrate that MDA5 is the primary sensor for *Gammacoronavirus* infections in chicken cells. Furthermore, we provide evidence that accessory proteins 3a and 3b of IBV modulate the response at the transcriptional and translational levels. Finally, we show that, despite the lack of activation of the IFN response during the early phase of IBV infection, the signaling of nonself dsRNA through both MDA5 and TLR3 remains intact in IBV-infected cells. Taken together, this study provides the first comprehensive analysis of host-virus interactions of a *Gammacoronavirus* with avian innate immune responses.

INTRODUCTION

Coronaviruses constitute a large family of positive-stranded RNA viruses and cause a range of human and veterinary diseases. Infectious bronchitis virus (IBV) is the prototype avian coronavirus from the *Gammacoronavirus* genus and the causative agent of a highly contagious respiratory disease of major economic importance to the poultry industry (38). IBV enters the avian host through the respiratory tract, where it causes destruction of the epithelium leading to respiratory distress and initiation of secondary bacterial infections. Depending on the strain, IBV can also spread to other epithelial surfaces such as the gastrointestinal tract, the kidneys and the oviduct, the latter causing problems in egg production and quality (38-43). Contrary to coronaviruses from the *Alphacoronavirus* and *Betacoronavirus* genera, including human coronavirus HCoV-229E, severe acute respiratory syndrome (SARS-CoV), Middle East respiratory syndrome (MERS-CoV) and mouse hepatitis virus (MHV), very little is known about how *Gammacoronaviruses* including IBV evade or interfere with innate immune responses of their host.

Innate immune responses consist of a network of antimicrobial mechanisms, of which the type I interferon (IFN) response is an essential defence mechanism against viruses. Typically, the type I IFN response, from hereafter referred to as IFN response, is initiated upon activation of host pattern recognition receptors (PRRs), present in all animal cells. Two families of PRRs have been shown to be involved in the recognition of RNA viruses namely the membrane-bound Toll-like receptors (TLRs) and the cytosolic RIG-I-like receptors (RLRs) (65). The primary ligands for activation of these PRRs are double-stranded RNA (dsRNA) and 5' triphosphate-containing RNA, normally absent from uninfected host-cells. Activation of RLRs leads to the transcription of genes encoding type I interferons (IFN α and IFN β). These interferons are secreted from the infected cell providing a signal for the infected as well as the neighbouring cells that induce the transcription of anti-viral effector genes collectively called interferon stimulated genes (ISGs).

The ability of a virus to replicate and produce infectious progeny depends for a large part on its ability to avoid induction or counteract the IFN response of its host. Indeed, a common feature of alpha- and betacoronaviruses, including HCoV-229E, SARS-CoV, and MHV, is their limited activation of the IFN response (88-93). This limited activation can be partially explained by intracellular membrane rearrangements that might shield dsRNA and other viral components from recognition by host PRRs (85, 97). In addition, coronavirus nsp16 displays 2'-O-methylase activity, which results in 2'-O-methylation of a ribose moiety on the 5' cap of coronavirus mRNAs, making them indistinguishable from host mRNAs (124). Furthermore, many other coronavirus proteins, such as nsp1, nsp3, the nucleocapsid and many of the accessory proteins have been shown to interfere with the IFN response in various ways (reviewed in (9, 125)).

Interaction between gammacoronaviruses and innate immune responses of their avian hosts is poorly understood. Early studies on gammacoronaviruses in chicken suggest that IBV-induced IFN production is variable and dependent on both virus strain and cell type. (126-129). Further, two transcriptional studies on tissues collected after *in vivo* and *in ovo* IBV infections, found only limited upregulation of ISGs at 1 - 3 days post-infection (130-132). Functional studies using IBV Beaudette showed that it induced cell-cycle arrest and apoptosis (133, 134), that IBV interacts with eIF3f (135) and that IBV inhibits protein kinase R activation, thereby maintaining protein synthesis (111). Although these studies did provide a

number of details on the interactions between IBV and the host cell, most experiments were carried out in Vero cells. This non-avian cell line is one of the very few cell lines in which the IBV-Beaudette strain has been adapted to grow, facilitating *in vitro* experiments. Vero cells, however, lack the *Ifn β* gene, preventing them from mounting a type I IFN response (136, 137), reducing the value of Vero cells for research on innate immune responses to IBV. In addition, the Beaudette strain is non-pathogenic *in vivo* with limited replication in host tissues (61), reducing the value of these *in vitro* studies for translation to *in vivo* situations. For these reasons, we used pathogenic isolates of IBV to infect primary chicken cells, and a chicken cell line, as these isolates are known to infect, spread and cause clinical disease *in vivo*.

In the current study, we show that IBV infection leads to a significant induction of *Ifn β* transcription through an MDA5-dependent activation of the IFN response, albeit delayed with respect to both virus replication and accumulation of dsRNA. This delayed induction of *Ifn β* was further confirmed through RNA FISH analysis showing that accumulation of *Ifn β* mRNA is restricted to IBV-infected and not neighbouring uninfected cells. Although the time lag between accumulation of dsRNA and induction of *Ifn β* transcription might suggest that IBV interferes with recognition of dsRNA, we observed that sensing of exogenous (non-self) dsRNA remained functional in IBV-infected cells. Using mutant IBV viruses we demonstrate that both accessory proteins 3a and 3b are involved in limiting *Ifn β* expression, as both 3a and 3b null viruses induced increased *Ifn β* expression. Nevertheless, 3a and 3b seem to have a differential effect on IFN protein production, infection with 3a null virus induced lower IFN levels whereas a 3b null virus increased IFN production compared to the parental virus. Altogether, our data suggest that IBV delays but does not prevent detection by MDA5, and that accessory proteins 3a and 3b modulate the IFN response in avian cells. This is the first study addressing immune evasion and interference strategies of IBV in chicken and not in mammalian cells, providing information essential to further understanding of the pathogenesis of *Gammacoronaviruses*.

MATERIALS AND METHODS

Cells

Chicken embryonic kidneys were aseptically removed from 17- to 19-day-old chicken embryo's (Charles River Laboratories Inc.). A cell suspension was obtained by trypsinisation for 30 min at 37°C and filtered through a 100 µm mesh. The resulting chicken embryo kidney (CEK) cells were seeded at 4×10^5 cells/cm² in 199 medium (Invitrogen) supplemented with 0.5% fetal bovine serum (FBS) and 1% PenStrep (Gibco, Invitrogen). Chicken tracheal cells were isolated from 8- to 10-week-old chickens (white leghorn). Tracheas were collected in ice-cold PBS, washed and stripped from adipose tissue. Trachea were filled with a solution of 3.5 U/ml protease type XIV (Sigma), 4 U/ml DNase I (Qiagen) and 1% PenStrep in Eagle's minimum essential medium (EMEM), sealed with clamps, and incubated overnight at 4°C. The next day, cells lining the luminal side of the trachea were flushed out with cold EMEM, filtered through a cell strainer and seeded at 4×10^5 cells/cm² in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS and 1% PenStrep. The RIG-I^{wt}, RIG-I^{KO}, MDA5^{wt} and MDA5^{KO} murine embryonic fibroblasts (MEFs) were provided by Prof. S. Akira (138). The MAVS^{wt} and MAVS^{KO} MEFs were provided by Z.J. Chen (139). DF-1, CEC-32 and MEF cells were cultured in DMEM (Gibco, Invitrogen) supplemented with 10% FBS and 1% PenStrep. All cells were incubated in a humidified incubator at 37°C and 5% CO₂.

DF-1 *I*fnβ-luc reporter cell line

DF-1 cells were transfected using Fugene (Promega) according to the manufacturer's instructions with a construct expressing firefly luciferase under the control of the -110bp proximal region of the human *IFNβ* promoter (140). Stably expressing cells were selected over a period of 3 weeks using geneticin (500 µg/ml). DF-1 *I*fnβ-luc stable cells were cultured in DMEM supplemented with 10% FBS and 1% PenStrep and were not further subcloned.

Viruses

IBV-M41, IBV-QX and IBV-Italy-O2, Rift Valley Fever Virus clone 13 (RVFV C113) and Infectious Pancreatic Necrosis Virus (IPNV) were obtained from Merck Animal Health, Boxmeer, The Netherlands. Sindbis-green fluorescent protein (GFP) was a kind gift from J. Fros, (Laboratory of Virology, Wageningen University). IBV Beaudette, strain Beau-R, as well as the generation of the ScaUG3a, ScaUG3b, ScaUG3ab and ScaUG5ab Beau-R null viruses has been published previously (13, 14, 141). In these mutant IBV viruses, the start codons of the indicated accessory genes were mutated to stop codons. All IBV strains were amplified and titrated on CEK cells. Sindbis-GFP was amplified on baby hamster kidney (BHK) cells and titrated on CEK cells. RVFV C113 was amplified and titrated on Vero cells, an African green monkey cell line. IPNV was amplified and titrated on the CHSE-214, Chinook-salmon cell line. IPNV was inactivated by 20 min UV exposure on a 48W BXT-26-M instrument (Uvitec).

Poly I:C stimulation and RNase treatment

Polyinosinic-poly(C) [p(I:C)] sodium was purchased from Sigma, dissolved in nuclease-free water and

stored at -80°C . p(I:C) was either directly added to the medium or transfected using Lipofectamine 2000 (Invitrogen) as per manufacturer's instructions. DF-1 cells (3×10^5 /well) were cultured in 24 well plates and transfected with 500 ng p(I:C). RNase treatment of CEK cell culture supernatant was performed by addition of 10 $\mu\text{g/ml}$ RNase A (Invitrogen) before IBV infection or before stimulation with 2 $\mu\text{g/ml}$ p(I:C).

RNA isolation and cDNA synthesis

Approximately 8×10^5 CEK cells or 3×10^5 DF-1 cells were lysed in RLT buffer (Qiagen) at various time points after treatment or infection. RLT cell lysis buffer was spiked with 1 ng/sample of luciferase mRNA (Promega) immediately prior to RNA isolation. Luciferase expression will later be used as external reference gene for normalization during the gene expression analysis. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions, including an on-column DNase treatment with RNase-free DNase (Qiagen). Before cDNA synthesis of 0.5–1 μg total RNA, a second DNase treatment was performed using DNase I, amplification grade (Invitrogen). Synthesis of cDNA was performed using SuperScript III (Invitrogen) using random primers. cDNA samples were further diluted 1:50 in nuclease-free water before real-time quantitative PCR analysis.

Gene expression analysis

Real-time quantitative PCR was performed on a Rotor-Gene 6000 (Corbett Research), using Brilliant SYBR Green quantitative PCR (Stratagene) and primers (79, 142-144) as listed in Table 1. Cycle thresholds and amplification efficiencies were calculated by the Rotor-Gene software (version 1.7). The relative expression ratio of the target gene was calculated using the average reaction efficiency for each primer set and the cycle threshold (C_t) deviation of sample vs. control at time point 0h, as described in (145). For calculation of the fold change of IBV total RNA, C_t deviation was calculated versus C_t 30, as no IBV was present in the non-infected cells that were used as control in all the experiments. Because expression of various housekeeping genes was unstable during virus infections at time points later than 24 hours (data not shown), gene-expression ratios were normalised using an external reference gene (luciferase).

Immunohistochemistry

CEK cells were seeded on fibronectin-coated glass Biocoat coverslips (BD Biosciences) at a density of 1×10^5 cells/cm². After incubation at 37°C for 48 hours, cells were infected with IBV strain M41 at an MOI of 1, and fixed at different time points with 3.7% paraformaldehyde and permeabilized using 0.1% Triton X-100. Infected cells were probed with anti-dsRNA antibody (English & Scientific Consulting) and polyclonal chicken serum raised against IBV M41 was obtained from Merck AH. Detection was done performed using Alexa 488 goat anti-mouse antibody (Invitrogen) and FITC labelled goat anti-chicken antibody (Kirkegaard and Perry laboratories). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Cells were imaged using a Zeiss Primo Vert microscope and Axiovision software. Image overlays were made in ImageJ.

Table 1 siRNAs and primers used in this study

Gene product	Category	Orientations ^a	Sequence (5'-3')	Accession no.	Reference
<i>Tlr3</i>	siRNA	S	UCGAAUACUUGGCUUUAAA	NM_001011691	
		AS	UUUAAAGCCAAGUAUUCGA		
<i>ctrl</i>	siRNA	S	AGGUAGUGUAAUCGCCUUG	--	
		AS	CAAGGCGAUUACACUACCU		
<i>Mda5</i>	siRNA	S	ACACUGGUAUCAAGUUAUU	GU570144	
		AS	AAUAAACUUGAUACCAGUGU		
<i>Ifnβ</i>	RQ primer	FW	GCTCTCACCACCACCTTCTC	NM_001024836	
		RV	GCTTGCTTCTTGTCCTTGCT		
<i>Ifna</i>	RQ primer	FW	ATCTGTGCTCACGCTCTTCT	XM_004937096	142
		RV	GGTGTGCTGGTGTCCAGGATG		
<i>Irf3</i>	RQ primer	FW	CAGTGCTTCTCCAGCACAAA	NM_205372	
		RV	TGCATGTGGTATTGCTCGAT		
<i>Irf1</i>	RQ primer	FW	CAGGAAGTGGAGGTGGAGAA	NM_205415	
		RV	TGGTAGATGTCGTTGGTGCT		
<i>Tlr7</i>	RQ primer	FW	TTCTGGCCACAGATGTGACC	NM_001011688	142
		RV	CCTTCAACTTGGCAGTGCAG		
<i>Tlr3</i>	RQ primer	FW	TCAGTACATTTGTAACACCCCGCC	NM_001011691	142
		RV	GGCGTCATAATCAAACACTCC		
<i>Mda5</i>	RQ primer	FW	TGGAGCTGGGCATCTTTCAG	GU570144	
		RV	GTTCCCACGACTCTCAATAACAGT		
<i>Mx</i>	RQ primer	FW	TTGTCTGGTGTGCTCTTCCT	GQ390353	
		RV	GCTGTATTTCTGTGTGCGGTA		
<i>Oas</i>	RQ primer	FW	CACGGCCTCTTCTACGACA	NM_205041	143
		RV	TGGCCATACGGTGTAGACT		
<i>Il8</i>	RQ primer	FW	TTGGAAGCCACTTCAGTCAGAC	NM_205498	143
		RV	GGAGCAGGAGGAATTACCAGTT		
<i>Pkr</i>	RQ primer	FW	CCTCTGTGGCCTTACTGTCA	NM_204487	144
		RV	AAGAGAGGCAGAAGGAATAATTGCC		
<i>Adar</i>	RQ primer	FW	TGTTTGATGCTGTGAG	AF403114	
		RV	AGATGTGAAGCCGTGTTG		
<i>Isg12</i>	RQ primer	FW	TAAGGGATGGATGGCGAAG	NM_001002856	
		RV	GCAGTATCTTTATTGTTCTCAC		
<i>Mhc-I</i>	RQ primer	FW	CTTCATTGCCTTCGACAAAG	NM_001031338	143
		RV	GCCACTCCACGCAGGT		
<i>Ifnar2</i>	RQ primer	FW	GCTTGITTCGTCAGCATT	AF082665	143
		RV	TTCGCAATCTCCAGTTGT		
IBV-N	RQ primer	FW	GAAGAAAACCAGTCCAGA	AY851295	
		RV	TTACCAGCAACCCACAC		
Luciferase	RQ primer	FW	TGTTGGGCGGTTATTTATC	X65316	
		RV	AGGCTGCGAAATGTTCACT		

^a S, sense; AS, antisense; FW, forward; RV, reverse; RQ primer, primer used in real-time quantitative PCR

RNA fluorescence *in situ* hybridization

RNA fluorescence *in situ* hybridization (FISH) was performed according to previously described protocols (146-148). A set of forty RNA FISH probes (20 bp), each labelled with one CAL Fluor Red 610 fluorophore and targeting chicken *Ifnβ* (NM_001024836), was designed using the Stellaris probe designer (Biosearch Technologies). The coding sequence of chicken *Ifnβ* is 601 bp, therefore to accommodate the optimum number of fluorescent probes (48; explained in reference (146)), the 3'UTR was included in the probe design tool. CEK cells were grown on fibronectin-coated coverslips (BD Biosciences) at a density of 2×10^5 cells/cm². After incubation at 37°C for 48 hours, cells were infected with IBV M41, and at the indicated time points fixed in 70% ethanol at 4 °C. Hybridisation of the probes was performed using the manufacturer's protocol for adherent cells. Imaging was performed using a Roper (Evry, France) Spinning Disc Confocal System on a Nikon Eclipse Ti microscope using a 100 × Plan Apo oil immersion objective (numeric aperture, 1.4) and a 491 nm laser line. Z-stacks were collected with 0.25 μm Z-intervals. For each channel, maximum Z-stack projections were made and processed with ImageJ.

chIFN bioassay.

Bioactive chicken type I interferon (chIFN) was measured using a bioassay based on the CEC-32 quail reporter cell line expressing luciferase under the control of the chicken *Mx* promoter (149) (kindly provided by Prof. Peter Staeheli). Briefly, CEC-32 were incubated with serial dilutions of chIFN-containing samples for 6 hours, after which luciferase activity was quantified and IFN concentrations calculated using a chIFN standard. To avoid influence of IBV on the assay, samples were heat inactivated at 56°C for 30 min, which did not influence type I chIFN bioactivity.

Gene silencing

siRNAs targeting chicken *Tlr3* and *Mda5* were designed by and purchased from Microsynth, Switzerland (sequences are in Table 1). Transfections were performed using siLentFect (Biorad) at a final siRNA concentration of 20 nM. For one well, 160 ng siRNA was combined with 1 μL siLentFect in 100 μL OptiMEM (Gibco) and incubated for 20 min. The siRNA complexes were added to 2×10^5 DF-1 cells grown in 500 μL medium per well in a 24 well plate. siRNA complexes were left on the cells for 48 hours before further experiments were performed.

Statistics

All statistical analyses were performed in GraphPad Prism 5.0. RT-qPCR fold changes first were log transformed and then used for statistical analysis. For all tests, equality of variance was assessed using Bartlett's test. Significant differences ($P < 0.01$) were determined by a one way or two-way ANOVA (indicated in the figure legend) followed by a Bonferroni *post hoc* test.

RESULTS

IBV delays the onset of an IFN response during infection of primary chicken cells

To investigate the kinetics of viral replication and IFN induction upon infection with the avian *Gammacoronavirus* IBV, we infected primary CEK cells (131) with the IBV M41 strain. To monitor the kinetics of the IFN response in relation to IBV replication, we quantified transcription of *Ifnβ* and a set of genes involved in innate immunity, extracellular IFN protein production, virus titres and IBV RNA in M41-infected CEK cells. In line with previous observations (87), progeny virus was produced after 6 hpi and virus titres reached a maximum around 24 hpi (Fig. 1A). Total intracellular IBV RNA levels reflected the kinetics of infectious IBV virus in the supernatant (Fig. 1A), reaching maximum levels around 24 hpi. *Ifnβ* expression was delayed with respect to the peak of viral replication and remained low until 18 hpi, after which it was strongly upregulated, peaking around 36 hpi (Fig. 1B). IFN protein activity levels were quantified using a chicken IFN-specific *Mx*-luc cell-based bioassay showing accumulation of IFN from 36 hpi onwards (Fig. 1B). Concomitant with *Ifnβ*, a subset of genes involved in innate immunity, including *Mx*, *Oas* and *Il8*, were upregulated whereas others, such as *Tlr7*, *Adar*, *Isg12*, *MHC-I* and *Ifnar2* appeared not, or only marginally affected by IBV infection (Fig. 1C). Pattern recognition receptors *Mda5* and *Tlr3* and the transcription factor *Irf3* were also upregulated (Fig. 1C), which is of interest given the role of these PRRs in virus recognition.

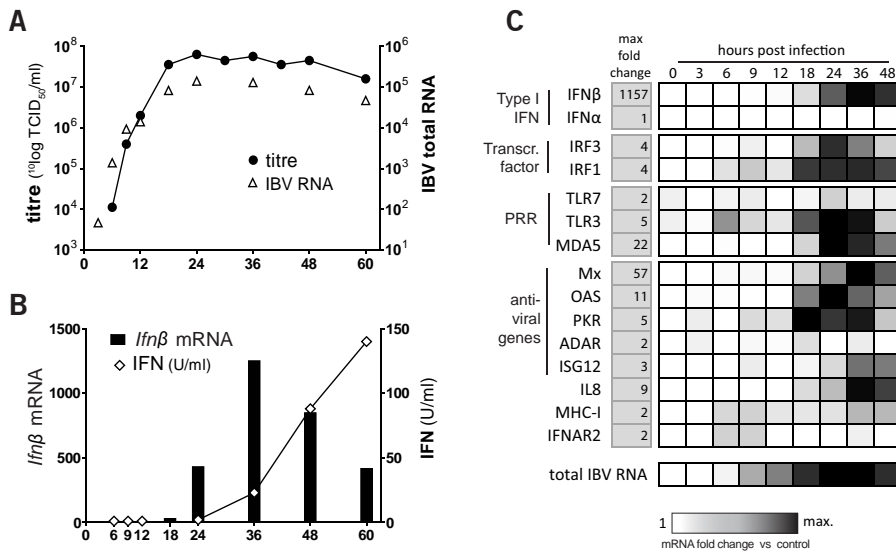


Fig. 1 IBV infection delays *Ifnβ* upregulation

Chicken embryo kidney (CEK) cells were infected with IBV M41 at an MOI of 0.1. (A) Replication of IBV was quantified by titration in cell culture supernatants of infected cells; in a parallel experiment, intracellular IBV RNA was quantified using RT-qPCR. (B) *Ifnβ* mRNA levels were determined using RT-qPCR and IFN protein levels using a chicken IFN-specific *mx*-luc cell-based bioassay, respectively. (C) Expression of genes involved in the antiviral response. All gene expressions were calculated as fold changes relative to uninfected control cells and normalised against an external reference gene (luciferase). For IBV total RNA, fold changes were calculated relative to C₁ 30. Depicted are the results of a representative experiment out of three independent experiments.

The delayed IFN response is independent from the cell type or virus strain

Ifn β transcription during infection with coronaviruses such as MHV and SARS-CoV is generally low (89, 90, 92, 93, 105), and was shown to be dependent on cell type and virus strain (83). The delayed induction of *Ifn β* transcription observed in IBV M41-infected CEK cells prompted us to investigate whether induction of *Ifn β* would be dependent on the cell type or IBV strain. Epithelial cells isolated from trachea of 10-week-old SPF chickens and DF-1 chicken fibroblast cells were infected with IBV M41 or IBV Beaudette (Beau-R, (141)). At several time points after infection, *Ifn β* levels were monitored by RT-qPCR (Fig. 2A and 2B). In both cell types *Ifn β* transcription followed the same kinetics observed in CEK cells (Fig. 1B), indicating that induction of *Ifn β* by IBV is independent of cell type. To study whether induction of *Ifn β* transcription differs between different strains of IBV, we also infected CEK cells with the QX and ItO2 strains of IBV (Fig. 2C). Although we observed some differences in absolute levels of *Ifn β* upregulation induced by QX, ItO2 and M41, kinetics of *Ifn β* transcription were similar, suggesting that delayed induction of *Ifn β* transcription could be considered a general feature of IBV infection in chicken cells.

To assess whether CEK and DF-1 cells do have the intrinsic ability to express *Ifn β* earlier than 18h, we stimulated these cells with extracellular polyI:C (pI:C), transfected pI:C (t[pI:C]) or with the dsRNA virus Infectious Pancreatic Necrosis Virus (IPNV). We found that stimulation of CEK cells with pI:C could induce *Ifn β* transcription as early as one hour after stimulation (Fig. 3A). In DF-1 cells, stimulation with IPNV, and t[pI:C], but not pI:C, induced *Ifn β* already at 4h (Fig. 3B). The observation that DF-1 cells do not respond to stimulation with extracellular dsRNA, is in accordance with previous findings and is most likely due to the lack of surface expression of TLR3 (150). In addition, a 12h infection of CEK cells with Sindbis, IPNV or Rift Valley Fever Virus clone 13 (RVFV C113) induced a clear transcription of *Ifn β* (Fig. 3C). These results suggest that delayed expression of *Ifn β* is a specific feature of IBV infection and not an intrinsic characteristic of chicken cells.

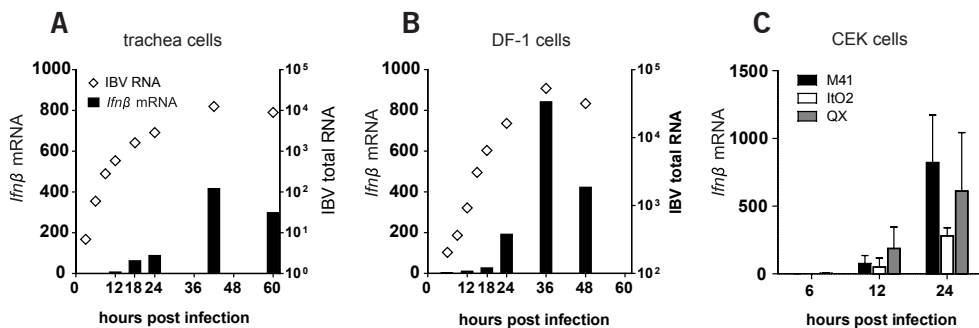


Fig. 2 Delayed induction of *Ifn β* transcription is independent of cell type or IBV strain

(A) Epithelial cells from adult chicken trachea were infected with IBV M41 at MOI 0.1. (B) Fibroblast DF-1 cells were infected with IBV Beau-R at MOI 0.1. (C) CEK cells were infected with IBV strains M41, QX and ItO2, at an MOI 0.1. Intracellular IBV total RNA (open diamonds) and *Ifn β* mRNA (bars) are depicted as fold changes as assessed by RT-qPCR. Gene expression of *Ifn β* was calculated as fold changes relative to uninfected control cells and normalised against an external reference gene (luciferase). For IBV total RNA, fold changes were calculated relative to C_1 30. Error bars indicate standard deviation.

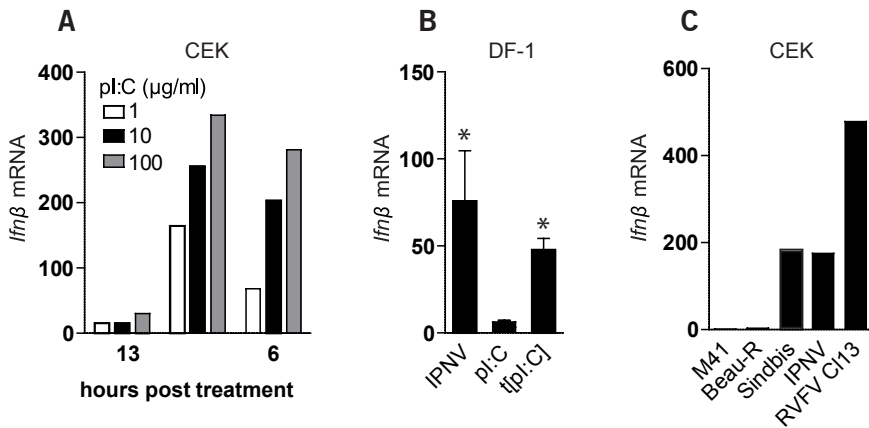


Fig. 3 Chicken cells have the intrinsic ability to respond rapidly to dsRNA

(A) CEK cells were seeded in 24 well plates and 48 hours later stimulated with extracellular poly I:C for the indicated times. (B) DF-1 cells were infected with IPNV, a non-replicating dsRNA virus, or stimulated with extracellular pI:C or transfected pI:C (t(pI:C)). Four hours later, *Ifnβ* fold changes were determined by RT-qPCR. Bars represent the mean (plus standard deviation) of triplicate wells from a representative experiment. Asterisks indicate significant differences ($P < 0.01$) with respect to the non-stimulated control as assessed by one-way ANOVA followed by a Bonferroni post-hoc test. (C) CEK cells were infected with IBV M41 (MOI 1), IBV Beau-R, (MOI 1), Sindbis-GFP (MOI 1), IPNV (MOI 50) and RVFV C113 (MOI 5). Depicted are *Ifnβ* fold changes at 12 hpi relative to uninfected control cells as assessed by RT-qPCR.

The intracellular pattern recognition receptor MDA5 is the primary sensor of IBV

In general, dsRNA has been shown to be the canonical inducer of *Ifnβ* during infection with *Alpha*- and *Betacoronaviruses* (83, 124). To determine which pattern recognition receptor (PRR) would be involved in sensing (ds)RNA of the *Gammacoronavirus* IBV, leading to subsequent *Ifnβ* transcription, we first examined the possibility that IBV-(ds)RNA could be sensed extracellularly by, for example, cell-surface receptors. To investigate this, CEK cells were infected with IBV M41 in the presence of RNase A and *Ifnβ* expression was analysed. As a positive control, CEK cells were stimulated with pI:C in the presence or absence of RNase A. The IFN response to pI:C was greatly inhibited by addition of RNase A, which had no effect on *Ifnβ* levels induced by infection with IBV M41 (Fig. 4A). These data suggest that *Ifnβ* upregulation during the late stage (>18 hpi) of IBV infection could be the result of sensing of IBV-(ds)RNA by an intracellular rather than an extracellular pattern recognition receptor. This is consistent with our observation that IBV infection can be detected by DF-1 cells, which show only a marginal upregulation of *Ifnβ* transcription in response to extracellular dsRNA (see Fig. 3B). In general, dsRNA can be recognised by membrane-bound TLR3 and cytosolic RLRs such as MDA5 and RIG-I. Genome mining strongly indicates that chickens do not express a *RIG-I* homologue (77), leaving TLR3 and MDA5 as the two PRRs potentially involved in dsRNA sensing. Silencing of *Mda5*, but not *Tlr3*, in DF-1 cells resulted in a 70% decrease in *Ifnβ* transcription (Fig. 4B). Similar results were obtained with an *Ifnβ*-luc DF-1 reporter cell line in which silencing of *Mda5*, but not *Tlr3*, resulted in a 70% decrease in luciferase activity by the reporter cells (Fig. 4C). Because no antibody against chicken MDA5 is currently available for protein detection, successful knockdown was evaluated using

RT-qPCR demonstrating a silencing efficiency for both *Tlr3* and *Mda5* of approximately 60% (data not shown). Replication of IBV at the investigated time point was not affected by knockdown of neither *Tlr3* nor *Mda5*, as measured by both virus titre and intracellular IBV total RNA (Fig. 4D). These results indicate that MDA5 is the primary PRR responsible for sensing *Gammacoronavirus* IBV-(ds)RNA in chicken cells.

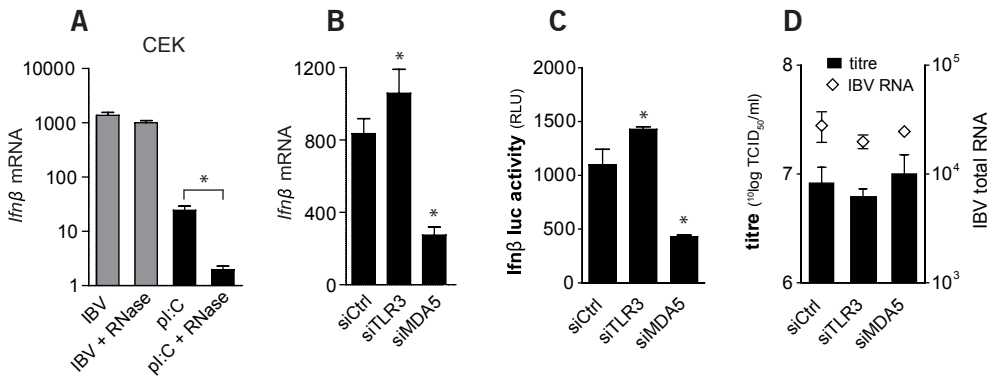


Fig. 4 MDA5, and not TLR3, is the prime sensor of IBV

(A) CEK cells were infected with IBV M41 for 24 hours, in the presence or absence of RNase A. *Ifnβ* expression was analysed by RT-qPCR. Stimulation with pl:C in the presence or absence of RNase A was included as a positive control. (B,D) DF-1 cells and (C) DF-1 *Ifnβ*-luc reporter cells were transfected with siRNAs against *Tlr3*, *Mda5* or a control siRNA and 48 hours later infected with IBV M41 (MOI 0.1). (B) *Ifnβ* mRNA, (C) *Ifnβ*-luciferase activity, and (D) IBV titres and intracellular RNA were analysed 18 hpi. Bars represent the mean (plus standard deviation) of triplicate wells from a representative experiment. Asterisks indicate significant differences ($P < 0.01$) with respect to the non-RNaseA-treated control (A) or to the siRNA control (B-C), as assessed by one-way ANOVA followed by a Bonferroni post-hoc test.

Early accumulation of dsRNA in IBV-infected cells does not result in early induction of *Ifnβ*

Having assessed that chicken cells can indeed promptly respond to stimulation with dsRNA (Fig. 3) and having identified MDA5 as the primary sensor involved in the detection of IBV (Fig. 4), we investigated whether there would be a temporal difference between IBV-induced accumulation of dsRNA and the upregulation of *Ifnβ* transcription in CEK cells. Indeed, dsRNA could clearly be detected, even at low MOI of 0.01, by 12 hpi (Fig. 5A). In contrast, *Ifnβ* levels at this time point remained low (Fig. 5B) even in cell cultures infected at higher MOIs of 1 or 10 and despite the increased abundance of dsRNA. To further investigate the time lag between early accumulation of dsRNA and late *Ifnβ* expression, we performed a time course analysis. Foci of dsRNA could be detected as early as 3 hours post-infection only in IBV-infected cells (Fig. 5C, inset 3hpi) indicating that dsRNA starts accumulating very early in IBV-infected cells but apparently only leads to late (>18 hpi) *Ifnβ* transcription.

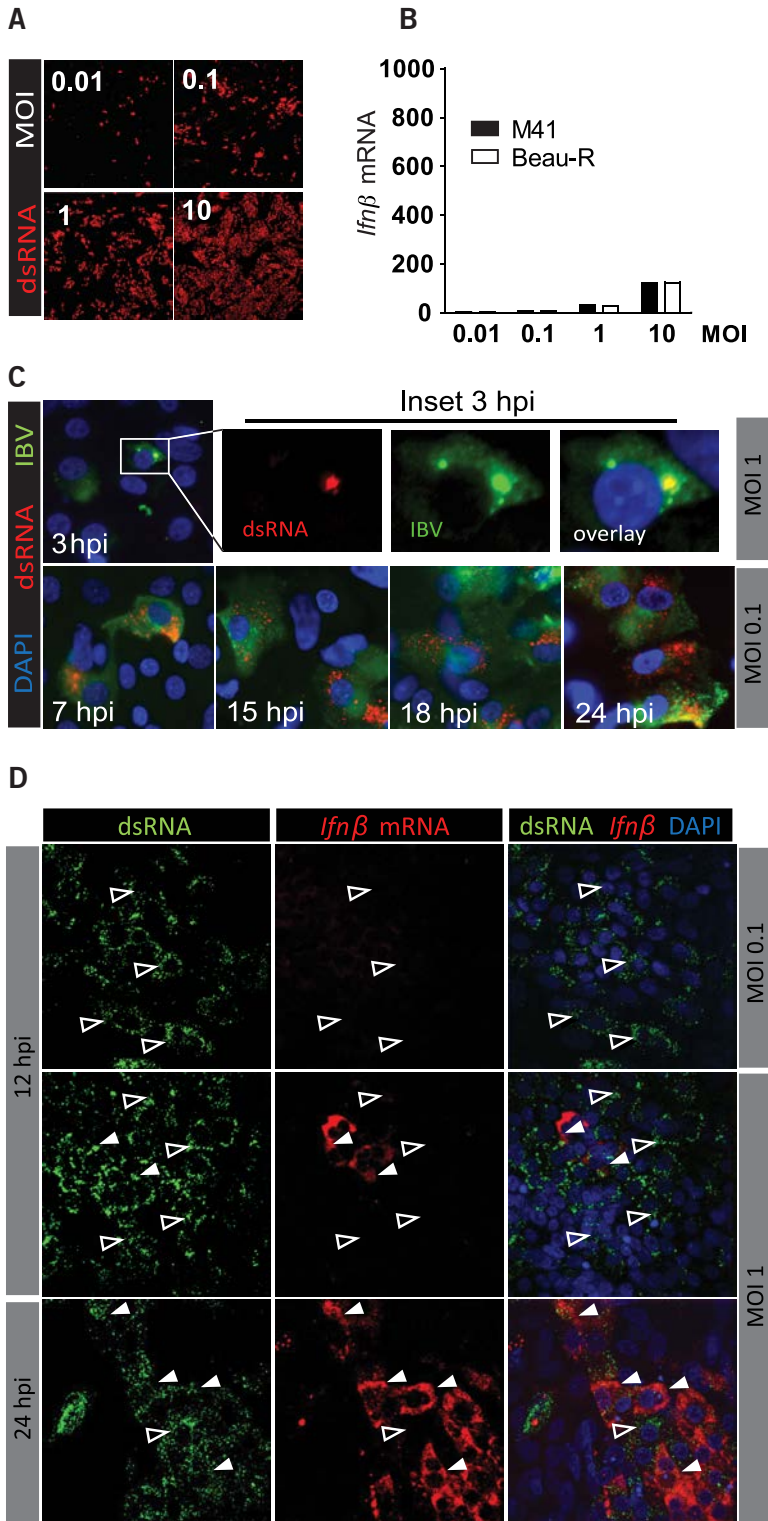
Primary CEK cells consist of a heterogeneous mix of cell types. Even at high MOI, IBV M41 infects only ~70% of the cells, indicating that not all cells are permissive to IBV M41 infection. In order to assess whether the time lag between accumulation of dsRNA and *Ifnβ* expression could be due to the induction of *Ifnβ* in bystander rather than IBV-infected cells, we used RNA fluorescent *in*

situ hybridisation to visualise *Ifnβ* mRNA in IBV-infected CEK cell cultures (Fig. 5D). At 12 hpi and low MOI (0.1), with most cells showing clear foci of dsRNA, none of the IBV-infected cells displayed an accumulation of *Ifnβ* mRNA. At 12 hpi and a higher MOI, a few cells stained positive for *Ifnβ* mRNA and only later, at 24 hpi, did most IBV-infected cells also stain positive for *Ifnβ* mRNA, the kinetics of which closely following that observed in Fig. 5A. In all cases, detection of *Ifnβ* mRNA was restricted to cells that contained dsRNA. Altogether our data shows that IBV-infected, but not adjacent uninfected cells, upregulate *Ifnβ* transcription in response to IBV infection. The significant time lag between accumulation of dsRNA and *Ifnβ* transcription further suggests the presence of a mechanism adopted by IBV to circumvent the onset of an IFN response.

Accessory proteins 3a and 3b regulate IFN transcription and protein production

To investigate whether the accessory proteins of IBV might play a role in the observed delay in *Ifnβ* transcription, we infected CEK cells with IBV scAUG3ab and scAUG5ab null viruses and the parental Beau-R virus (scAUG viruses possess a scrambled AUG start codon resulting in transcription but not translation of either ORFs 3a and 3b or 5a and 5b (13, 14)). Infection with the scAUG3ab, but not a scAUG5ab null virus resulted by 24 hpi in increased upregulation of *Ifnβ* expression (Fig. 6A). Indicating that either one, or a combination of, accessory proteins 3a and 3b play a role in down regulating *Ifnβ* transcription. The difference in *Ifnβ* transcription between the scAUG3ab and the parental (Beau-R) virus could not be ascribed to differences in kinetics of virus replication, as all viruses displayed similar growth kinetics until 24 hpi (Fig. 6B). To determine whether 3a, 3b or both accessory proteins are involved in the observed down regulation of the IFN response, we quantified *Ifnβ* transcription and IFN protein production in CEK cells infected with scAUG3a, scAUG3b and scAUG3ab mutant viruses, and compared the values observed in cells infected with Beau-R (Fig. 6C and 6D). Infection with all mutant viruses led to an increased transcription of *Ifnβ* when compared to the Beau-R (Fig. 6C), indicating that the presence of either one of the two accessory proteins is sufficient to limit *Ifnβ* transcription. The kinetics of *Ifnβ* transcription in response to AUG3a/b differs between Fig. 6A and Fig. 6C. In Fig 6C there is a significant difference in *Ifnβ* transcription between AUG3a/b and Beau-R, which is absent in Fig 6A. This difference can probably be attributed to variation in the kinetics of *Ifnβ* transcription between primary CEK cells isolated from embryos originating from different flocks. Nonetheless, this difference does not affect the conclusion that knockout of 3a and 3b leads to an increase in transcription of *Ifnβ*.

No significant differences in IFN protein production were observed between cells infected with the Beau-R and the scAUG3ab double null virus, except at 36 hpi. However, infection with scAUG3b virus led to an increase in IFN protein levels, whereas infection with the scAUG3a virus led to a decrease in IFN when compared to both Beau-R and scAUG3ab double null virus (Fig. 6D). Taken together, these results indicate that accessory proteins 3a and 3b both play a role in the inhibition of *Ifnβ* transcription but have distinct and opposing effects on protein production. Accessory protein 3b seems to be involved in limiting IFN protein activity whereas 3a is involved in promoting it.



◀ Fig. 5 Early accumulation of dsRNA in IBV-infected cells does not result in early induction of *Ifnβ*

CEK cells were infected with IBV M41 or IBV Beau-R at the indicated MOIs. At time point 12 hpi (A) dsRNA was visualised in M41-infected cells using an antibody against dsRNA. (B) Expression of *Ifnβ* mRNA was analysed by RT-qPCR. (C) CEK cells were infected with IBV M41 and accumulation of dsRNA was visualised at the indicated time post infection. (D) RNA fluorescent *in situ* hybridisation of *Ifnβ* mRNA in IBV M41-infected CEK cells. Open arrowheads indicate cells that contain dsRNA and no *Ifnβ* mRNA. Solid white arrowheads indicate cells that contain both dsRNA and *Ifnβ* mRNA.

Signaling of non-self dsRNA remains intact in IBV-infected cells

Since IBV showed the intrinsic ability to delay *Ifnβ* transcription in several cell types (Fig. 1 and Fig. 2) even in the presence of high levels of intracellular dsRNA (Fig. 5), we investigated the ability of IBV to interfere with sensing of non-self dsRNA by TLR3 or MDA5. We infected CEK cells with IBV M41 and subsequently used extracellular poly I:C to trigger TLR3 signaling (Fig. 7A). Stimulation with pI:C alone led to a significant increase in *Ifnβ* transcription whereas stimulation with pI:C following an infection with IBV led to an enhanced increase in *Ifnβ* transcription in an MOI-dependent manner. These results indicated that IBV infection does not interfere with TLR3-mediated *Ifnβ* transcription, on the contrary IBV infection appears to result in a synergistic activation of the TLR3 pathway triggered by pI:C. Next, we investigated whether IBV infection could interfere with MDA5-mediated transcription of *Ifnβ*. Although transfection of pI:C into the intracellular compartment is a commonly used ligand of MDA5, this method induced very little transcription of *Ifnβ* in primary CEK cells, because of low transfection efficiency (data not shown). As an alternative route to stimulate MDA5 in primary chicken cells, we investigated the use of either RVFV C113 or IPNV, that induce *Ifnβ* transcription in CEK cells (Fig 3C). RVFV C113 is a (-) ssRNA virus with a truncated IFN antagonist (151), for which RIG-I, but not by MDA5 or TLR3, was previously shown to be the most likely PRR in mammalian cells (152, 153). Since chickens, as opposed to most mammals, do not have a *RIG-I* homologue, the most likely PRR for RVFV in CEK cells would be MDA5. IPNV is a birnavirus with a dsRNA genome that naturally infects salmonids but has been shown to enter but not replicate in cells of warm-blooded animals (154). To date, the PRR responsible for sensing IPNV dsRNA has not been described. Knockdown experiments in DF-1 *Ifnβ*-luc reporter cells, using siRNAs against chicken MDA5 or TLR3, revealed that MDA5, but not TLR3, is the prime PRR for IPNV (Fig. 7B). These findings were confirmed using MEFs (mouse embryo fibroblasts) from knockout mice deficient in expression of either MDA5, RIG-I or the downstream adaptor protein MAVS. Here, knockout of either MDA5 or MAVS abrogated sensing of IPNV as shown by a strong reduction of *Ifnβ* transcription, whereas knockout of RIG-I did not (Fig. 7C). Both IPNV and RVFV C113 were subsequently used to investigate whether IBV infection could interfere with MDA5-mediated transcription of *Ifnβ* in CEK cells.

Using quantification of *Ifnβ* transcription by RT-qPCR as read out, we could show that IBV infection does not interfere with MDA5-mediated signaling of IPNV (Fig. 7D) or RVFV C113 (Fig. 7E), in fact it had a synergistic effect on *Ifnβ* transcription as previously observed for TLR3-mediated signaling (Fig. 7A). Similar results were obtained when stimulating IBV-infected DF-1 cells with IPNV or t[pI:C] (Fig. 7F), indicating that the observed synergistic effect is not specific to CEK cells. Taken together, IBV infection very efficiently prevents sensing of IBV (ds)RNA, but our results indicate that it does not interfere with sensing and downstream signaling of other non-self (ds)RNA ligands.

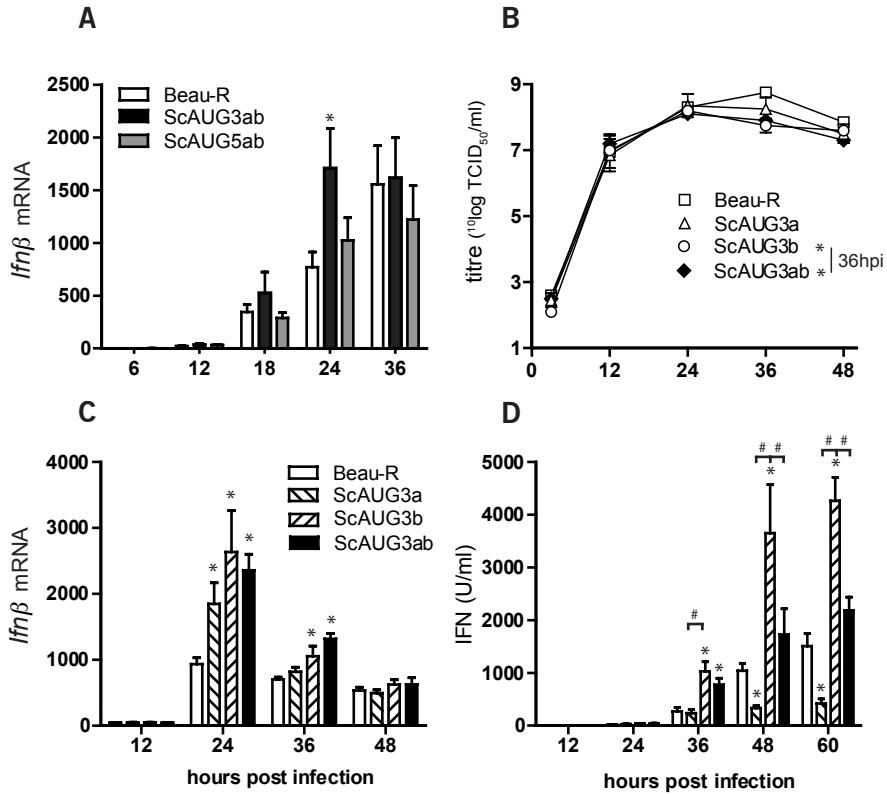


Fig. 6 Accessory proteins 3a and 3b are involved in regulation of IFN transcription and protein production (A) CEK cells were infected with IBV Beau-R 3a/3b (scAUG3ab) or 5a/5b (scAUG5ab) null viruses (MOI 0.1). *Ifnβ* levels were determined using RT-qPCR. (B-D) CEK cells were infected with scAUG3a, scAUG3b or scAUG3ab null IBV viruses (MOI 0.1). In the same cultures (B) *Ifnβ* mRNA, (C) virus titres and (D) type I IFN protein were quantified. Bars represent the mean (plus standard deviation) of triplicate wells from a representative experiment. Significant differences ($P < 0.01$) relative to the Beau-R virus at the same timepoint (*) or between the indicated bars (#) as assessed by a two-way ANOVA followed by a Bonferroni post-hoc test.

DISCUSSION

In this study we performed a comprehensive analysis of the kinetics of IBV infection in avian cells and studied the mechanisms by which IBV interferes with the onset of the type I IFN response. We show that infection with the *Gammacoronavirus* IBV leads to a considerable activation of the type I IFN response, albeit delayed with respect to the peak of viral replication and accumulation of viral dsRNA. Using an siRNA knockdown approach we show that MDA5 is the main receptor involved in the induction of *Ifn β* expression during IBV infection. We present evidence that IBV accessory proteins 3a and 3b play a role in the modulation of the delayed IFN response, by regulating interferon production both at the transcriptional as well as translational level. In addition, we show that although IBV alone effectively prevents *Ifn β* induction in IBV-infected cells, it does not block *Ifn β* induction upon stimulation of IBV-infected cells with other RIG-I, MDA5 or TLR3 ligands. To our knowledge, this study provides the most comprehensive analysis of the interplay between a *Gammacoronavirus* and the avian type I IFN response.

Much of our current knowledge about the interaction of coronaviruses with the innate immune response (reviewed in (155)) comes from studies in mice and mouse cells using mouse hepatitis virus (MHV). MHV activated IFN production only in specific cell types and an efficient IFN response was only mounted by plasmacytoid dendritic cells (156), bone marrow derived macrophages (90, 157) and oligodendrocytes (90). In a recent study on SARS-CoV and MERS-CoV in an epithelial lung cell line, ISGs started to be upregulated at 12 hpi (158), when virus titres were already reaching their maximum. The kinetics of IFN response observed in our study are in line with aforementioned studies, however it must be noted that in most cell types, infection with *Alpha* or *Betacoronaviruses* induced very little, if any, *Ifn β* transcription (88-93, 105). This suggests that all coronaviruses are able to modulate the activation of the type I IFN response.

We found that IBV infection is detected by various chicken cell types, but until now it was unknown which PRR was involved. MHV has been shown to be detected by MDA5 and not RIG-I or TLR3 in brain macrophages (83), by both MDA5 and RIG-I in an oligodendrocyte derived cell line (90) and by TLR7 in plasmacytoid dendritic cells (156). Analysis of the chicken genome suggests that chicken lack a *RIG-I* homologue (77), and basal expression of *Tlr7* was found to be very low in CEK cells (data not shown). We therefore silenced the remaining candidate RNA sensors MDA5 and TLR3, and were able to show that MDA5, but not TLR3, is involved in the sensing of IBV. Silencing of *Mda5* did not lead to an increase in replication of IBV, suggesting that IBV might have developed strategies to counteract the activated IFN response.

We recently reported membrane rearrangements in chicken cells infected with IBV (87), similar to those found in cells infected with *Betacoronaviruses*. In theory, the formation of intracellular membrane rearrangements might partly explain the discrepancy observed in the kinetics of dsRNA accumulation and *Ifn β* upregulation. Indeed, for SARS-CoV it has been shown that virus-induced double membrane vesicles (DMVs) contain dsRNA (85), suggesting that coronaviruses might exploit membrane structures to shield dsRNA from recognition by host PRRs (86). However, the kinetics of *Ifn β* transcription were not investigated in these studies. The presence of coronavirus-induced DMVs has been demonstrated as early as 2 hpi in SARS-CoV-infected cells (85). Although we did not demonstrate the presence of DMVs in IBV-infected chicken cells at time points earlier than

7 hpi (87), it is likely that DMVs could also be present at earlier time points. As such, the timing of DMV formation in coronavirus-infected cells could suggest that membrane rearrangements play a role in the delayed activation of the IFN response by shielding dsRNA from cellular PRRs.

In addition to membrane rearrangements, coronavirus-encoded proteins, including numerous accessory genes, have been shown to interfere with the type I IFN response pathway (reviewed in (9, 125)). To investigate the possible role of IBV accessory proteins in the regulation of the IFN response we made use of our previously constructed mutant IBV Beau-R viruses that do not express either one or more of the four accessory proteins 3a, 3b, 5a and 5b. Previously we have demonstrated the accessory genes of IBV are not essential for replication (13, 14).

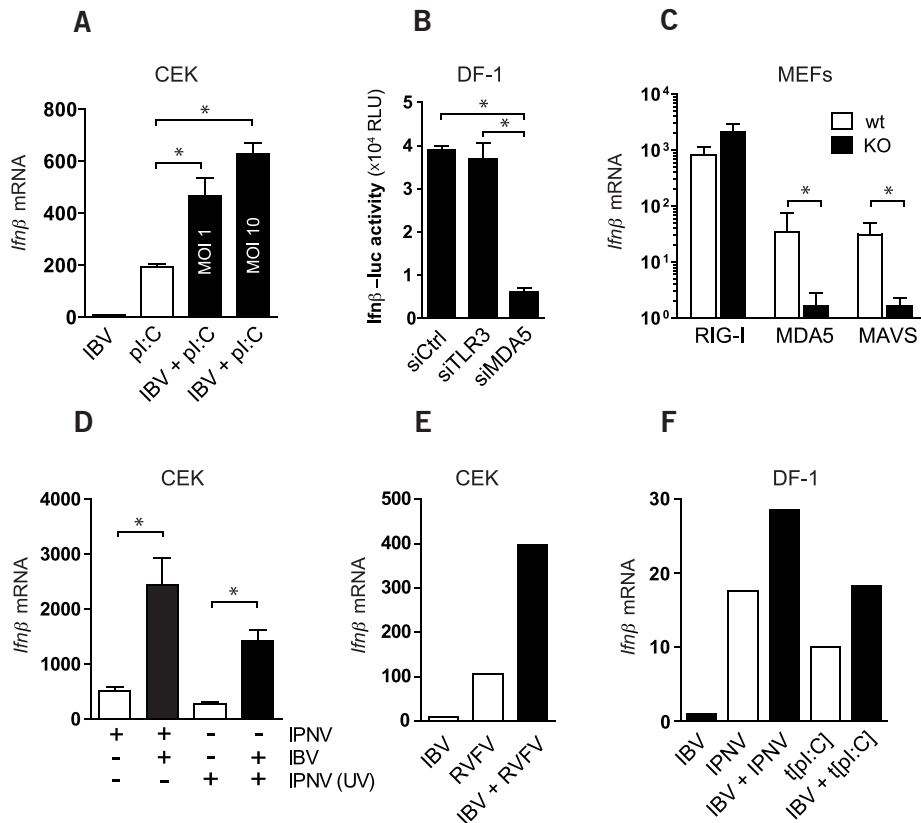


Fig. 7 Signaling of non-self RNA remains intact in IBV-infected cells

(A) CEK cells were infected with IBV M41 for 3 hours and stimulated with extracellular poly I:C (50 μ g/ml) for an additional 3 hours after which *Ifnβ* transcription was analysed by RT-qPCR. (B) DF-1 *Ifnβ*-luc reporter cells were transfected with siRNAs against *Tlr3*, *Mda5* or a control siRNA and 48 hours later infected with IPNV (MOI 50); at 6 hpi luciferase activity was quantified. (C) Knockout (KO) and wild-type (wt) MEFs were infected with IPNV (MOI 50) for 8 hours. (D) CEK cells were infected with IBV M41 (MOI 10) for 6h and super-infected with IPNV or UV-inactivated IPNV (MOI 50) for an additional 6 h. (E) CEK cells were co-infected with IBV M41 (MOI 5) and RVFV clone 13 (MOI 5) and sampled at 6 hpi. (F) DF-1 cells were infected with IBV Beau-R (MOI 1) for 3 h and super-infected with IPNV (MOI 50) or transfected with pI:C (tipl:C), 500 ng/well for an additional 4 h. (C-F) *Ifnβ* levels were quantified by RT-qPCR. Bars represent the mean (plus standard deviation) of triplicate wells. Significant differences ($P < 0.01$) are indicated by (*) as assessed by one-way ANOVA followed by a Bonferroni post-hoc test

In addition to membrane rearrangements, coronavirus-encoded proteins, including numerous accessory genes, have been shown to interfere with the type I IFN response pathway (reviewed in (9, 125). To investigate the possible role of IBV accessory proteins in the regulation of the IFN response we made use of our previously constructed mutant IBV Beau-R viruses that do not express either one or more of the four accessory proteins 3a, 3b, 5a and 5b. Previously we have demonstrated the accessory genes of IBV are not essential for replication (13, 14). In the present study we show that infection of CEK cells with 3a or 3b null viruses as well as a 3a/3b double null virus led to increased *Ifn β* transcription compared to Beau-R. Because the kinetics of *Ifn β* transcription of 3a, 3b and 3a/3b null viruses are comparable to the parental virus, we conclude that, 3a and 3b are probably not responsible for the delay in *Ifn β* transcription, suggesting that IBV utilises additional strategies to delay transcription of *Ifn β* . Apart from their effect on *Ifn β* transcription, 3a and 3b seem to have opposing effects on IFN protein production by IBV infected cells. Infection with the 3b null virus resulted in increased IFN production whereas infection with the 3a null virus resulted in reduced IFN levels compared to the Beau-R virus. Together with the observation that IFN production induced by the 3a/3b double null virus is comparable to that induced by Beau-R virus, our data suggests that accessory proteins 3a and 3b antagonise each other to tightly regulate IFN production (Fig. 6B).

Using the eukaryotic linear motif server (159), we identified a Protein phosphatase 1 (PP1)-binding ¹⁷KISF²⁰ domain in the IBV 3b protein sequence. The canonical PP1-binding motif is [R/K][V/I/L]X[F/W], in which x can be any amino acid except proline (160). Interestingly, *Alphacoronavirus* TGEV accessory protein 7 (TGEV-7) has been shown to bind PP1 via a binding motif similar to that found in IBV 3b (15). Similar to IBV scAUG3b, infection with TGEV- Δ 7 led to increased mRNA and protein levels of IFN β (16). The fact that both TGEV-7 and IBV 3b contain a PP1 binding domain indicates that interaction with PP1 could be a common strategy of coronaviruses to inhibit the host innate immune response. The mechanism by which interaction of coronavirus accessory proteins with PP1 counteracts the innate immune response still needs to be determined. One clue might come from the PP1-binding domain of Measles virus V, which was recently shown to be essential for inhibition of MDA5 signaling (161, 162). Measles V protein binds PP1 and inhibits dephosphorylation of MDA5, which is required for activation and subsequent signaling by MDA5. Motif analysis for IBV 3a protein did not reveal the presence of relevant motives that might explain the observed activity of 3a on IFN regulation. We conclude that both accessory proteins 3a and 3b limit *Ifn β* transcription but have distinct and opposing effects on protein production. Whereas 3a seems to promote IFN production, 3b seems to be involved in limiting IFN protein production, possibly through a similar mechanism as described for protein 7 of TGEV. The fact that IBV 3a and 3b have opposing roles in regulating IFN production, indicates that CoV's tightly regulate IFN production to balance their own survival with that of the host. This hypothesis is supported by the observation that field isolates lacking 3a and 3b display reduced virulence *in vitro* as well as *in vivo* (34). Elucidation of the exact mechanisms of action of 3a and 3b will be the subject of further investigation.

To investigate whether IBV interferes with a general sensing of (ds)RNA ligands or downstream signaling that leads to *Ifn β* transcription, we stimulated IBV-infected cells with TLR3, RIG-I, and MDA5 ligands. Surprisingly, we found that infection with IBV did not reduce *Ifn β* transcription but rather increased *Ifn β* levels upon stimulation with these PRR ligands. Similar to IBV, MHV has been shown unable to inhibit expression of *Ifn β* induced by either t[pi:C] or Sendai virus (163,

164), but in these studies no synergistic effect was observed. Currently, we can only speculate about the cause of this synergistic effect. It appears that IBV infection ‘arms’ the *Ifn β* induction pathway, without actually triggering it, possibly by enhancing the activity of one or more components of the pathway leading to *Ifn β* upregulation. One possibility is that IBV-proteins interact with host-proteins that regulate this pathway through ubiquitination and phosphorylation (reviewed in (165)). The fact that stimulation with either TLR3 or MDA5 ligands resulted in exacerbated transcription of *Ifn β* indicates that IBV influences a component which is downstream of both MDA5 and TLR3.

Taken together, our study provides the first comprehensive analysis of host-virus interactions of a *Gammacoronavirus* with the avian innate immune response. We show that the *Gammacoronavirus* IBV, induces activation of the type I IFN response in primary chicken renal cells, tracheal epithelial cells and in a chicken cell line. We show that activation of the IFN response is dependent on MDA5 but is delayed with respect to the peak of virus replication. We demonstrate that *Ifn β* transcription is restricted to IBV-infected, dsRNA-containing cells and provide evidence that accessory proteins 3a and 3b of IBV are involved in regulating transcription as well as protein production of type I IFN.

Acknowledgements

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Chapter

3

Infectious bronchitis virus inhibits STAT1 signaling and requires accessory proteins for resistance to type I IFN activity in chicken cells

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ABSTRACT

The innate immune response is the first line of defence against viruses and the type I interferon (IFN) response is a critical component of this response. Similar to other viruses, the *Gammacoronavirus* infectious bronchitis virus (IBV) has evolved under evolutionary pressure to evade and counteract the IFN response to enable its survival. Previously, we reported that IBV induces a delayed activation of the IFN response and that accessory proteins 3a and 3b modulate the IFN response. In the present work, we describe the resistance of IBV to IFN and the potential role of accessory proteins herein. We identify that accessory proteins 3a and 3b are involved in resistance to IFN, as their absence renders IBV less resistant to IFN treatment. In addition to this, we find that independent of the presence of accessory proteins, IBV inhibits IFN-mediated phosphorylation and translocation of STAT1. In summary, we show that IBV uses multiple strategies to counteract the IFN response.

INTRODUCTION

Infectious bronchitis virus (IBV) is a member of the genus *Gammacoronavirus*, a group of viruses from the order of *Nidovirales* characterised by a large positive-stranded RNA genome (1). IBV is the causative agent of infectious bronchitis, which is one of the most important viral diseases in chickens, causing a highly contagious respiratory disease that can spread to the gastrointestinal or the urogenital tract (38, 166). Despite widespread application of inactivated and live-attenuated vaccines, IBV remains one of the most reported diseases in poultry farms worldwide. Notwithstanding the widespread nature and economic importance of this virus, interactions between IBV and the host immune response remain poorly understood.

During the immune response to viruses, the type I interferon response plays a pivotal role. Recently, we have shown that IBV induces delayed activation of the interferon response (167) in a manner similar to several members of the genus *Betacoronavirus*, including mouse hepatitis virus (MHV), severe acute respiratory syndrome-associated coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) (88, 89, 157, 158). The observation that coronaviruses delay activation of the IFN response and limit production of IFN, suggests that IFN has the ability to hinder their propagation. In apparent contrast, most coronaviruses are relatively resistant to treatment with IFN *in vitro* (93, 113), one exception being MERS-CoV, which was shown to be highly sensitive to IFN β *in vitro* (101, 107). Although previous studies suggest that treatment with IFN could hinder propagation of IBV, based on reduced plaque formation (168) and reduced syncytia formation (169), quantitative data on the resistance of IBV to IFN is lacking.

It is unknown which of the IBV proteins confer resistance to IFN, if any. Various studies have demonstrated that accessory proteins of coronaviruses play an important role in the resistance to the IFN-induced antiviral response (15, 19, 32, 100, 101, 109, 112, 113). Accessory proteins are a group of small (50–300 aa) proteins that are not essential for virus replication *in vitro*. The number of accessory proteins varies between coronaviruses, and amino acid sequences of accessory proteins from different genera show very limited similarity, suggesting that their function is virus- or host specific. IBV has been shown to express at least four accessory proteins, 3a, 3b, 5a and 5b, which are translated from two polycistronic mRNAs. Recently, we showed that both 3a and 3b limit transcription of *Ifn β* and that 3b limits production of IFN protein *in vitro* (167). Additional roles of IBV accessory proteins have remained elusive.

In the present study we show that IBV, is relatively resistant to treatment with either IFN α or IFN β , but that simultaneous knockout of 3a and 3b makes IBV less resistant to treatment with type I IFN. In addition, we show that IBV inhibits phosphorylation and translocation of the IFN-activated transcription factor STAT1 and inhibits subsequent IFN-mediated activation of an ISG promoter, at least during late stages of the infection. However, using mutant viruses we demonstrate that the presence of accessory proteins 3a, 3b, 5a and 5b is not required for either inhibition of STAT1 translocation or activation of an ISG promoter. We discuss two strategies by which IBV counteracts the type I IFN response: one based on counteracting the IFN-mediated antiviral response using accessory proteins 3a and 3b and another based on blocking of IFN-mediated activation of antiviral genes through inhibition of STAT1 translocation. This study demonstrates that the *Gammacoronavirus* IBV has evolved multiple strategies to counteract activation of, and clearance by the type I IFN response.

MATERIALS AND METHODS

Cells

Chicken embryonic kidneys (CEK) were aseptically removed from 17- to 19-day-old chicken embryos (Charles River, SPAFAS). A cell suspension was obtained by trypsinisation for 30 min at 37 °C and filtered through a 100 µm mesh. The resulting CEK cells were seeded at 4×10^5 cells/cm² in a 1:1 mix of 199 and F10 medium (Invitrogen) supplemented with 0.5% foetal bovine serum (FBS, SAFC) and 1% penicillin-streptomycin (PenStrep; Gibco, Invitrogen). DF-1 chicken fibroblast cells, the African green monkey Vero cells and baby hamster kidney (BHK) cells were cultured in DMEM (Gibco, Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% PenStrep. All cells were incubated in a humidified incubator at 37 °C and 5% CO₂.

Viruses

Sindbis virus (SinV) was a kind gift from Dr. G. Pijlman, (Laboratory of Virology, Wageningen University). IBV Beaudette, strain Beau-R, as well as the generation of the ScAUG3a, ScAUG3b, ScAUG3ab and ScAUG5ab Beau-R null viruses were described previously (13, 14, 141). In the null IBV viruses, the start codons of the indicated accessory genes were mutated to stop codons. All IBV strains were amplified and titrated on CEK cells. SinV was amplified on BHK cells and titrated on CEK cells.

Immunohistochemistry

Vero cells were cultured on 8 well Lab-Tek #1.0 borosilicate coverglasses (Sigma-Aldrich) whereas CEK cells were cultured in 24-well culture plates. Briefly, cells were fixed with 3.7% paraformaldehyde and permeabilized using 0.1% Triton X-100 in phosphate-buffered saline (PBS). SinV infection was detected using a mouse monoclonal antibody against dsRNA (English & Scientific Consulting) and IBV infection using antibodies against the IBV-nucleocapsid (N) protein (Prionics). Tyr701-phosphorylated STAT1 (pSTAT1) was detected using the rabbit monoclonal MA5-15071 (Thermo Scientific) and total STAT1 was detected using the rabbit polyclonal sc-346 (Santa Cruz Biotechnology). Visualization was performed using Alexa-488 or -568 labelled goat-anti-mouse or goat-anti-rabbit antibodies (Invitrogen). Antibodies were diluted 1:1000 in PBS supplemented with 5% FBS, except the anti-pSTAT1 which was diluted 1:500. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, 0.5 µg/ml; Sigma). Cells were imaged using a Zeiss Primo Vert microscope and Axiovision software. Image overlays and cross-sections were made in ImageJ.

Interferon sensitivity assay

CEK, DF-1, or Vero cells at 100% confluency were pre-treated for 6 hours with different concentrations of recombinant chicken IFN α or IFN β produced in HEK293 cells (170), or recombinant human IFN β (CalBioChem). Infection was carried out using different viruses at MOI 0.01 for two hours, after which cells were washed and new medium containing the same concentration of interferon was added. Supernatants were collected for titration at 18 hours post infection (hpi) (CEK) or 24 hpi (DF-1).

ISG54-luciferase reporter assays

Vero or DF-1 cells were seeded at 80-90% confluence in 96 well plates and transfected using FuGENE HD (Promega) at a 1:3.5 ratio of DNA:FuGENE HD according to manufacturers' specifications. Per well, 100 ng of ISG54-luciferase reporter plasmid (kind gift from David E. Levy (171)) was transfected, together with 2 ng pRL-SV40 Renilla plasmid (Promega) to correct for differences in transfection efficiency and transcription. At least 24 hours later, cells were infected and 18 hpi, stimulated with 1000 U/ml IFN for an additional 6 hours. Firefly and Renilla luciferase activities were quantified using the Dual-Glo Luciferase Assay (Promega) and a Filtermax F5 luminometer (Molecular Devices).

Western Blot

Vero cells in 24 well plates at 90% confluency were infected with IBV Beau-R at MOI 1. At 18 hpi, cells were stimulated with human IFN β (10,000 U/ml) for 30 min and subsequently lysed in lysis buffer (20 mM Tris, 100 mM NaCl, 1mM EDTA, 0.5% Triton X-100 and 1 mM PMSF, pH 8.0). Samples were boiled for 10 minutes in Laemmli loading buffer, clarified by centrifugation at 5000 x g for 5 min and separated on a 10% SDS-PAGE gel. Proteins were transferred onto a Whatman Protran nitrocellulose membrane (GE Healthcare) by semi-dry blotting (Trans-Blot SD Semi-Dry Transfer Cell, Bio-Rad). Blotted membranes were blocked overnight in 5% non-fat dry milk (w/v) in TBS/Tween (20 mM Tris, 500 mM NaCl, 0.05% Tween-20 (v/v), pH 8.0) at 4 °C. The blotted membranes were incubated with primary antibodies (rabbit anti-STAT1 sc-346, Santa Cruz Biotechnology 1:1000; rabbit anti-pSTAT1 MA5-15071, Thermo-Scientific 1:500; rabbit anti- β -tubulin, Abcam, Ab6046 1:2000) in 5% non-fat dry milk in TBS/Tween for 1 h at 37 °C followed by incubation with a goat-anti-rabbit-HRP antibody (Bio-Rad) at a 1:1000 dilution in the same buffer for 1 h at 37 °C. Chemiluminescence of bound anti-rabbit-HRP antibody was detected with WesternBright ECL (Advansta) and visualized using Lumni-film (Roche).

Statistics

Statistical analyses were performed in GraphPad Prism 6.0 or IBM SPSS 19. Equality of variance was assessed using Bartlett's test. Significant differences were determined by a one-way ANOVA followed by a Bonferroni post-hoc test or by a two-way ANOVA when indicated.

RESULTS

IBV is relatively resistant to treatment with type I IFN

To test resistance of IBV to type I IFN, we treated primary chicken embryo kidney cells with recombinant chicken IFN and subsequently infected them with IBV Beau-R, or with the IFN-sensitive Sindbis virus as control. Immunofluorescence staining indicated that propagation of IBV was less affected by treatment with IFN α and IFN β than propagation of the IFN-sensitive Sindbis virus (Fig. 1A). To investigate the kinetics of IBV resistance to IFN, we treated CEK cells with increasing concentrations of IFN α and IFN β , and determined the effect on propagation by titration of Beau-R (Fig. 1B). The titre of Beau-R decreased in a dose-dependent manner and the effect of IFN β on the titre of Beau-R was more pronounced than that of IFN α . Similar to other coronaviruses, relatively high concentrations of IFN (>1000 U/ml) were required to hinder propagation of IBV Beau-R which suggested that IBV, like other coronaviruses, is relatively resistant to IFN and raised the possibility that IBV actively counteracts the type I IFN response.

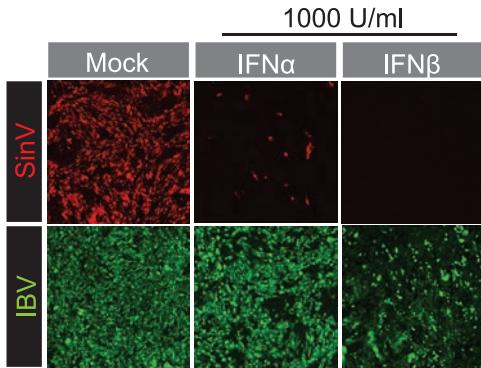
Accessory proteins 3a and 3b contribute to IFN resistance

For coronaviruses other than IBV, the accessory proteins have been implicated in counteracting the type I IFN response. To investigate whether the accessory proteins of IBV contribute to resistance to IFN, we stimulated CEK cells with a high concentration of IFN, and infected them with 3a/3b and 5a/5b null viruses (scAUG3a/3b and scAUG5a/5b). These viruses do not express the indicated accessory proteins owing to a mutation in the AUG start codon. IFN treatment reduced titres of scAUG3a/3b more than either scAUG5a/5b or the parental Beau-R virus (Fig. 1C), suggesting that 3a/3b null virus is more sensitive to treatment with IFN. To further investigate IFN-sensitivity of 3a/3b null virus, we stimulated DF-1 cells with increasing concentrations of IFN α or IFN β (Fig. 1D and E). Again, scAUG3a/3b was more sensitive to treatment with either IFN α or IFN β than scAUG5a/5b or the parental Beau-R, indicating that accessory proteins 3a and 3b could play an important role in conferring resistance of IBV to treatment with type I IFN.

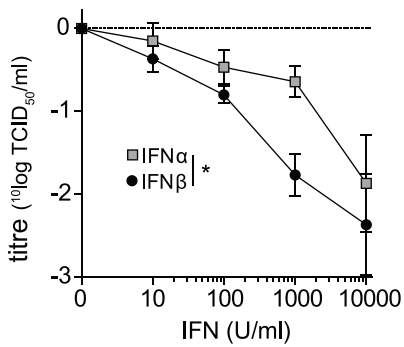
Fig. 1 Accessory proteins 3a/3b confer resistance to treatment of IBV with type I IFN ▶

Cells were pre-stimulated with the indicated concentration of IFN α or IFN β for 6 h, and subsequently infected with the indicated viruses at MOI 0.01. At 2 hpi cells were washed and IFN-containing medium was added. (A) CEK cells were infected with Sindbis virus (SinV) or IBV (Beau-R). At 24 hpi, cells were fixed and stained for dsRNA (red) or IBV-N (green). (B) CEK cells were infected with Beau-R in the presence of the indicated concentrations of IFN. Virus titers at 18 hpi are expressed relative to the titres measured in mock-treated, IBV-infected cells. Symbols indicate the means (\pm SEM) of triplicate measurements from two independent experiments and error bars indicate standard error of the mean (SEM). Asterisk (*) indicates significant differences ($P < 0.05$) between IFN α and IFN β treatment as assessed by a two-way ANOVA. (C) CEK cells were IFN-treated, virus-infected and sampled as described in (B), using Beau-R and accessory protein-null viruses in the presence of 10,000 U/ml of IFN. Symbols indicate the means (\pm SEM) of triplicate measurements from two independent experiments. Asterisk (*) indicates significant difference ($P < 0.001$) compared to the parental virus as assessed by one-way ANOVA followed by a Bonferroni post-hoc test. (D and E) DF-1 cells were IFN-treated and virus-infected as described in (B). Virus titers at 24 hpi are expressed relative to titres measured in mock-treated, IBV-infected cells. Symbols indicate the mean (\pm SEM) of triplicate wells from a representative experiment of two biological replicates. Asterisk (*) indicates significant difference ($P < 0.01$) between the 3a/b null virus and the other viruses as assessed by two-way ANOVA.

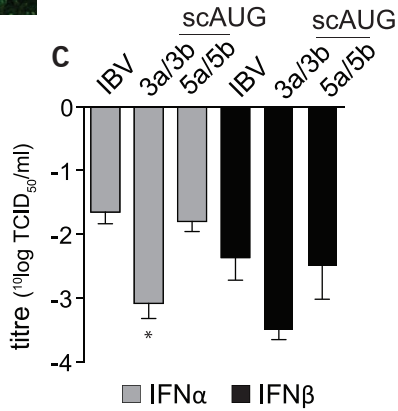
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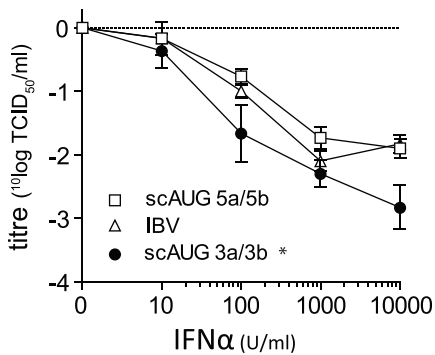
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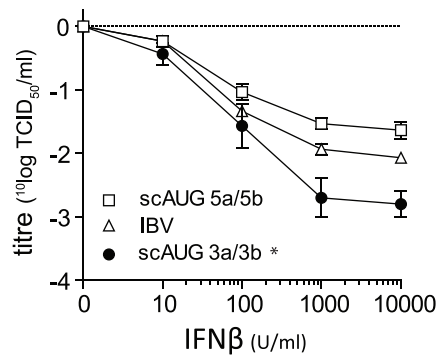
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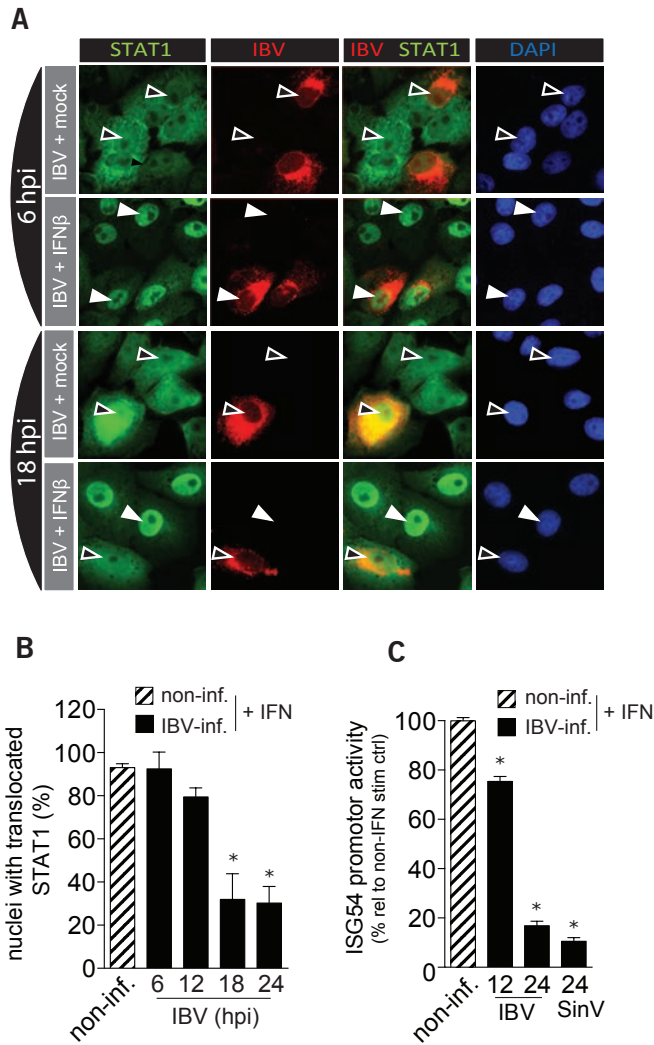


Fig. 2 IBV prevents translocation of STAT1 and IFN signaling at late stages of infection

(A) Vero cells were infected with IBV-Beau-R (MOI 1 for 6 h and MOI 0.1 for 18h) and at 6 or 18 hpi cells were stimulated with 1000 U/ml IFN β for 30 min before fixation and staining for IBV-N and STAT1. White arrowheads indicate nuclear accumulation of STAT1, black arrowheads indicate absence of STAT1 accumulation in the nucleus. (B) In IFN β -treated wells, the percentage of nuclei showing translocation of STAT1 was determined in non-infected (non-inf.) cells and in IBV-infected cells at the indicated time-points. Each bar indicates the mean percentage of nuclei showing translocation of STAT1 as determined in 50 - 400 cells from multiple images of a representative experiment of two biological replicates. Error bars indicate standard deviation (SD). (C) DF-1 cells were transfected with an ISG54-Firefly luciferase construct, and 24 hours later infected with Beau-R or SinV (MOI 5 and 0.5, respectively); at 6 or 18 hpi, cells were stimulated with 1000 U/ml IFN β for an additional 6 h. Firefly and Renilla luciferase activity was quantified and ISG54 promoter activity at 12 and 24 hpi was calculated as percentage relative to duplicate wells not treated with IFN β . Firefly luciferase values were normalised to SV40-Renilla luciferase to correct for differences in transfection efficiency and protein translation. Bars indicate the mean (+ SD) of triplicate wells from a representative example of three biological replicates. Asterisks (*) indicate significant differences ($P < 0.001$) with respect to non-infected cells, as assessed by one-way ANOVA followed by a Bonferroni post-hoc test.

IBV prevents IFN signaling late during infection

Next, we wanted to investigate how accessory proteins 3a and 3b contribute to IFN resistance. One possibility is that IBV 3a/3b interfere with signaling of IFN, in a similar way to accessory protein ORF6 of SARS-CoV which was shown to block IFN signaling through inhibition of nuclear translocation of STAT1 (108). To investigate whether also IBV is able to inhibit nuclear translocation of STAT1, we used Vero cells, as commercially available STAT1 antibodies did not detect chicken STAT1. Vero cells were infected with IBV and translocation of STAT1 was induced at 6 and 18 hpi by stimulation with IFN β . Localisation of STAT1 in the nucleus of IBV-infected cells was visualised by immunostaining against IBV-N (red) and STAT1 (green) (Fig. 2A). In mock-treated cells (no stimulation with IFN β), nuclear translocation of STAT1 was not visible, neither in infected nor in non-infected cells (black arrowheads), indicating that IBV infection alone does not induce translocation of STAT1. At 6 hpi IBV did not prevent IFN β -induced translocation of STAT1 (white arrowheads). At 18 hpi however, IFN β -induced translocation of STAT1 was strongly reduced in IBV-infected cells (Fig 2A, bottom row of images). This indicated that IBV-mediated inhibition of STAT1 translocation is a time-dependent event.

To substantiate the observed time-dependency of IBV-mediated inhibition of STAT1 translocation, we quantified translocation of STAT1 in both infected and non-infected IFN β -treated cells. In non-infected cells, treatment with IFN β led to translocation of STAT1 in more than 90% of the cells (Fig. 2B, striped bar), regardless of time point (6-24 hpi) or presence of neighbouring cells infected with IBV (not shown). Translocation of STAT1 in mock-treated cells was comparable between IBV-infected and non-infected cells (<5%, data not shown), indicating that IBV alone did not induce translocation of STAT1. In contrast, in IBV-infected cells, treatment with IFN β did not always lead to translocation of STAT1. The inhibition seen in IBV-infected cells was time-dependent: at time points between 6 and 12 hpi translocation of STAT1 was not different from non-infected cells, whereas at later time points, between 12-18 hpi onwards, STAT1 translocation was strongly inhibited (Fig. 2B, black bars).

To verify whether the observed time-dependency of IBV-mediated inhibition of STAT1 translocation would correlate with an inhibition of transcription of ISGs, we used an IFN reporter assay based on the human *ISG54* promoter, which contains multiple copies of the STAT1-binding interferon-regulated regulatory element (ISRE) (171). Indeed, at early time points (12 hpi) we observed only a marginal inhibition of luciferase production, whereas at later time points (24 hpi) IBV strongly inhibited the IFN-mediated production of luciferase to the same extent as Sindbis virus, a well-known inhibitor of STAT signaling (Fig. 2C). We interpret inhibition of luciferase activity as the result of a reduction in IFN-mediated *ISG54* promoter activity and thus conclude that IBV inhibited the transcription of ISGs by inhibiting translocation of STAT1, but only during later stages of infection.

IBV inhibits phosphorylation of STAT1

A crucial step in IFN-induced translocation of STAT1 is its phosphorylation. Only phosphorylated STAT1 (pSTAT1) can associate with STAT2 and IRF9 to form the transcription factor ISGF3, which binds to ISRE promoter elements. To investigate whether IBV is able to block phosphorylation of STAT1, we visualised IFN β -induced phosphorylation of STAT1 in IBV-infected cells (18 hpi), using a pSTAT1-specific antibody. pSTAT1 could not be detected in mock-treated cells, even when infected with IBV (Fig. 3A, upper panel; left). Cells treated with IFN β however (Fig. 3A, lower panel), showed

nuclear translocation of pSTAT1, but only in cells not infected with IBV. In IBV-infected cells, in contrast, translocation of pSTAT1 was severely reduced. In addition to reduced levels of nuclear pSTAT1 (*i.e.* reduced translocation), we also observed reduced levels of cytoplasmic pSTAT1 in IFN β -stimulated cells, infected with IBV (Fig. 3B, delineated area). A cross-section of IBV-infected areas versus non-infected areas confirmed the general lack of pSTAT1 signal in IBV-infected cells (Fig 3B). To determine whether IBV selectively inhibits phosphorylation of STAT1 rather than affecting total STAT1 protein levels, we performed a western blot analysis (Fig. 3C). Levels of total STAT1 were comparable between IBV-infected and non-infected monolayers, whereas IFN-mediated phosphorylation of STAT1 was reduced in infected compared to non-infected monolayers, confirming that IBV prevents phosphorylation of STAT1. In the western blot, we observed a residual signal for pSTAT1 in IFN β -stimulated, IBV-infected monolayers, most likely due to the presence of non-infected cells. Taken together, our data suggest that IBV prevents IFN-induced phosphorylation of STAT1.

IBV accessory proteins are not responsible for inhibition of phosphorylation and translocation of STAT1.

The *Betacoronavirus* SARS-CoV mediates inhibition of STAT1 translocation by its accessory protein ORF6 (106, 108). To test whether the IBV accessory proteins are also involved in inhibition of phosphorylation and translocation of STAT1, we used 3a/3b and 5a/5b null viruses (scAUG3a/3b and scAUG5a/5b) that do not express the indicated accessory proteins owing to a mutation in the AUG start codon. First, we investigated nuclear translocation of pSTAT1 in cells infected with 3a/3b and 5a/5b null virus under circumstances similar to those shown before (see Fig. 3) for the parental Beau-R virus. We found that translocation of pSTAT1 was inhibited by both 3a/3b and 5a/5b null viruses in a manner similar to that observed for the parental virus (Fig. 4A). Quantification of the percentage of cells showing pSTAT1 in the nucleus substantiated that translocation of pSTAT1 to the nucleus was inhibited to the same extent by 3a/3b null, 5a/5b null and the parental IBV Beau-R virus (Fig. 4B).

Second, we investigated whether the accessory proteins of IBV are involved in inhibition of STAT1 phosphorylation. Western blot analysis indicated that wild-type Beau-R had a more pronounced inhibitory effect on STAT1 phosphorylation than the 5a/5b null virus, whereas the inhibitory effect on pSTAT1 of the 3a/3b null virus was intermediate (Fig. 4C). To verify the results of the western blot analysis, we performed immunostaining which indicated that the parental Beau-R virus and the 3a/3b and 5a/5b null viruses, all reduced STAT1 phosphorylation to the same extent (Fig. 4D). To examine the apparent discrepancy between the western blot analysis and the immunostaining, we investigated the efficiency of replication of Beau-R versus 3a/3b and 5a/5b null viruses in Vero cells. To do so, we quantified the percentage of infected cells in microscopic images (Fig 4E) in parallel to quantification of virus titre in supernatants of infected cells (Fig 4F). These experiments indicated that replication of the 5a/5b null virus was lower than that of either Beau-R or 3a/3b null, which is in agreement with a previous report showing that replication of 5a/5b null is reduced in Vero, but not in CEK cells (172). Reduced replication of 5a/5b null virus in Vero cells provides an explanation for its reduced inhibitory effect on IFN-mediated phosphorylation of STAT1 in the western blot analysis. In short, we conclude that it is likely that phosphorylation of pSTAT1 is inhibited to the same extent by 3a/3b null, 5a/5b null and the parental Beau-R virus.

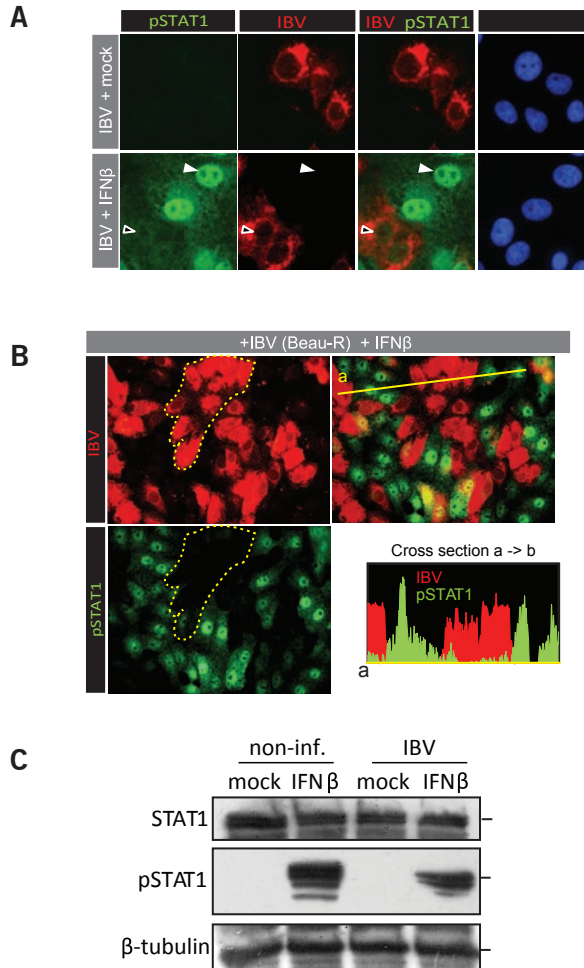


Fig. 3 IBV prevents translocation and phosphorylation of STAT1

Vero cells were infected for 18 h with IBV Beau-R (MOI 0.1) and subsequently stimulated with 1000 U/ml IFNβ for 30 min before fixation and staining for IBV-N and pSTAT1. White arrowheads indicate translocation of pSTAT1, black arrowheads indicate absence of pSTAT1 from the nucleus. (B) to verify the overall decrease of pSTAT1, an area containing IBV-infected cells within an IFNβ-stimulated monolayer is delineated by a dotted line in the top left panel and overlaid on the bottom left panel. Cross section: fluorescence intensity plot of pSTAT1 and IBV-N along the yellow line indicated in the top right panel of (B). (C) western blot analysis of non-infected (non-inf.) and IBV-infected monolayers that were either mock- or IFNβ-treated as described in (A). Staining was performed using antibodies against STAT1 and Tyr701-phosphorylated STAT1. Staining against β-Tubulin was included as a loading control.

Third, we investigated to which extent the null viruses would inhibit IFN-mediated activation of the ISG54 promoter and found no differences between the null viruses and wild-type Beau-R, in both Vero and DF-1 cells (Fig. 4G). Taken together, our data indicate that the inhibition of phosphorylation and translocation of STAT1 and activation of ISG54 promoter, observed after infection with IBV, is independent of the accessory proteins 3a, 3b, 5a and 5b.

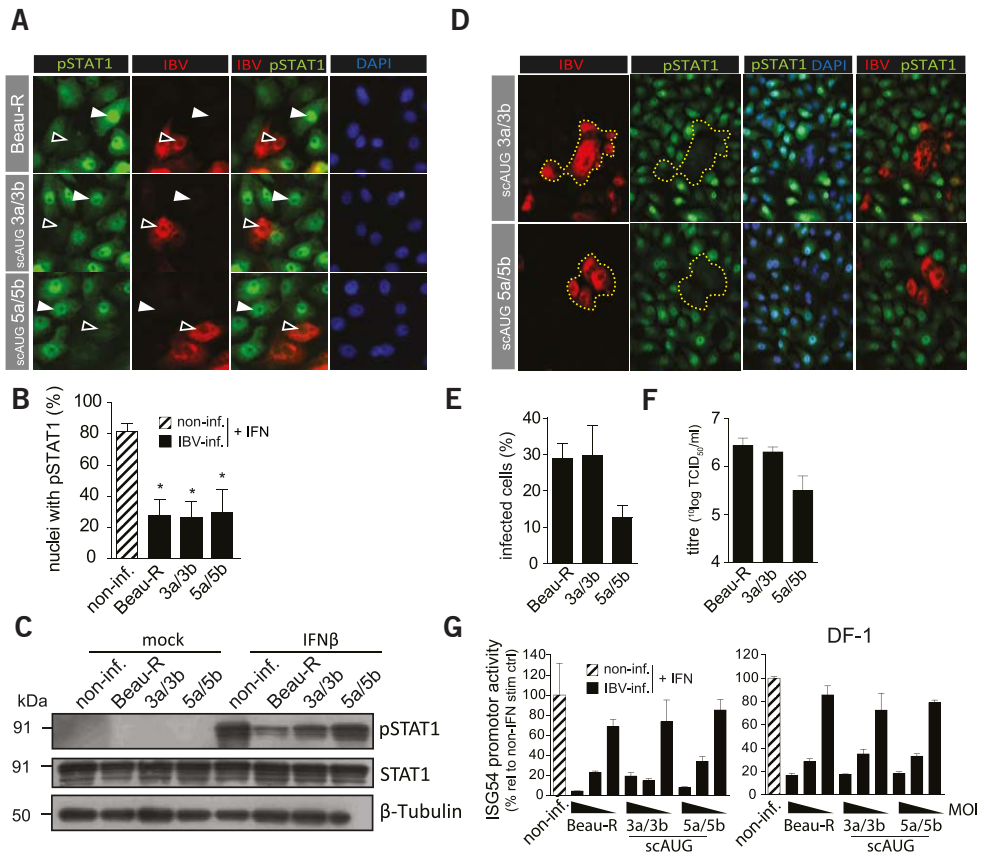


Fig. 4 IBV accessory proteins are not required for inhibition of translocation of pSTAT1 and ISG promoter activation

(A) Vero cells were infected with Beau-R, 3a/3b or 5a/5b null viruses (MOI of 0.1) and at 18 hpi, stimulated with 1000 U/ml IFN β for 30 min, and stained for IBV-N and pSTAT1. White arrowheads indicate translocation of pSTAT1, black arrowheads indicate absence of accumulation of pSTAT1 in the nucleus. (B) In parallel, we determined the percentage of nuclei showing translocation of pSTAT1 in the wells treated as described in (A). Each bar indicates the mean (+ SD) percentage of nuclei showing translocation based on 100 - 300 cells from multiple images of a representative experiment of two biological replicates. Asterisks (*) indicate significant differences ($P < 0.01$) with respect to non-infected cells, as assessed by one-way ANOVA followed by a Bonferroni post-hoc test. (C) Western blot analysis of IBV-infected and non-infected Vero cells that were either mock- or IFN β -treated. Staining was performed using an antibody against Tyr701-phosphorylated STAT1, and an antibody against β -tubulin was used as loading control. (D) Vero cells were infected with the indicated viruses (MOI 0.1) and at 18 hpi, stimulated with 1000 U/ml IFN β for 30 min, and stained for IBV-N and pSTAT1. The area delineated by the yellow dotted line indicates the overall decrease in pSTAT1 staining in IBV-infected cells. (E) Quantification of the percentage of IBV-infected cells in microscopic images of cells infected with the indicated viruses at MOI 0.1 and stained using IBV-N-specific antibody at 18 hpi. For each virus, at least 500 cells divided over 10 microscopic fields were analysed. (F) Virus titres in supernatants from Vero cells infected for 18 h with the indicated viruses at MOI 0.01. (G) Vero and DF-1 cells were transfected with an ISG54-Firefly luciferase construct, and 24 h later infected with Beau-R, 3a/3b or 5a/5b null viruses at various MOI: 5, 0.5, 0.05. At 18 hpi, cells were stimulated with 1000 U/ml IFN β for an additional 6 h. After a total of 24 h, Firefly and Renilla luciferase activity was quantified and ISG54 promoter activity calculated as percentage relative to duplicate wells not treated with IFN β . Firefly luciferase values were normalised to SV40-Renilla luciferase to correct for differences in transfection efficiency and protein translation. Bars indicate the mean (+ SD) of triplicate wells of a representative example of $n=3$ biological replicates.

DISCUSSION

In this study we investigated the *in vitro* sensitivity of the *Gammacoronavirus* IBV to treatment with IFN, and the potential role of IBV accessory proteins in conferring resistance to the host's type I IFN response. We found IBV to be relatively resistant to treatment with IFN and showed that simultaneous knockout of the accessory proteins 3a and 3b decreased resistance of IBV to treatment with IFN. Independent of the counteracting activity of 3a and 3b on the IFN response we found that IBV interferes with IFN signaling by inhibition of phosphorylation and nuclear translocation of STAT1. In summary, this study demonstrates that the *Gammacoronavirus* IBV has evolved multiple strategies to antagonise the innate immune response.

The coronaviruses MHV, SARS-CoV, MERS-CoV and IBV have all been shown to induce modest and delayed transcription of *Ifn β* (91, 158). *Alpha-* and *Betacoronaviruses* (not *Gamma-* and *Deltacoronaviruses*) encode the nsp1 protein that decreases transcription of *Ifn β* and inhibits synthesis of host proteins thereby further reducing production of IFN (64, 119, 120, 167, 173). The observation that coronaviruses employ multiple strategies to limit production of IFN seems to suggest that IFN could be detrimental to the propagation of coronaviruses. However, treatment of both MHV and Feline coronavirus (FCoV) with IFN (1000 U) reduces their propagation by approximately 1 log only, indicating that these viruses are relatively resistant to IFN (93, 102). In comparison, SARS-CoV is at least 10 times more sensitive (103-105), and MERS-CoV even 1000 times more sensitive to IFN treatment than MHV (106, 107). We found that propagation of IBV was reduced by 0.5-2.5 log upon treatment with IFN (1000 U) suggesting that IBV is relatively resistant to IFN.

Compared to MHV and FCoV, SARS-CoV is relatively sensitive to IFN treatment. However, MERS-CoV is 50 to 100 times more sensitive than SARS-CoV (106, 107). The difference in sensitivity between the latter two viruses has been ascribed to the ability of SARS-CoV to inhibit nuclear translocation of pSTAT1 (106). Considering the relative resistance of IBV to treatment with IFN we investigated whether IBV, similar to SARS-CoV, would inhibit nuclear translocation of pSTAT1. We observed that at time points earlier than 18 hpi, IBV did not inhibit nuclear translocation of pSTAT1 or activation of a STAT1-responsive promoter (ISG54). In contrast, from 18 hpi onwards, IBV inhibited both IFN-mediated pSTAT1 translocation and activation of the ISG54-promoter. Of interest, SARS-CoV has been shown to inhibit STAT1 translocation as early as 8 hpi, whereas MERS-CoV did not inhibit STAT1 translocation (106). In another study, MHV did not inhibit IFN-mediated translocation of STAT1-GFP at 9 hpi, but inhibited IFN-mediated ISG expression at 11 hpi and rescued Sendai virus (SeV) from the antiviral effects of IFN β when MHV was present prior to SeV infection and for a total period of 16 h (88). Our data indicate a time-dependent inhibition of IFN signaling by IBV, a phenomenon that has not been reported for other coronaviruses, although it cannot be excluded that for the *Betacoronaviruses* MHV and possibly MERS-CoV, inhibition of pSTAT1 translocation could be a relatively late event similar to what we observed for the *Gammacoronavirus* IBV.

For SARS-CoV, it has been shown that accessory protein ORF6 is responsible for blocking nuclear translocation of STAT1 by tethering nuclear import factors at the ER/Golgi membrane, inhibiting expression of STAT1-activated genes (19, 98, 108). In the present study we showed that IBV inhibits phosphorylation of STAT1 and that, in contrast to SARS-CoV, the presence of accessory proteins of IBV was not required for inhibition of STAT1-mediated signaling. Our data

suggest that IBV and SARS-CoV may exploit different strategies to inhibit translocation of STAT1.

Taking together the ability of IBV to significantly delay transcription of *Ifn β* up until 12-18 hpi and delay subsequent translation of IFN until 36 hpi (167) and the inhibition of pSTAT1 translocation at times points >18 hpi, we suggest there could be a correlation between the timing of *Ifn β* transcription by the host cell and inhibition of IFN signaling induced by IBV. Although there is no proof of causality, we hypothesize that changes in the host cell trigger the relocation of, or conformational changes in IBV proteins, which in turn activate their anti-IFN activity. Further research is needed to verify this hypothesis.

In general, coronavirus accessory proteins can antagonise the IFN response at various steps. For example, proteins 4a and 4b of MERS and 3b of SARS inhibit activation of *Ifn β* (19, 100, 101), whereas protein 7 of TGEV and 3b of IBV inhibit transcription and translation of *Ifn β* (15, 16, 167). Notwithstanding these and other steps to counteract and/or avoid activation of the IFN response (reviewed in (155)). Accessory proteins not only inhibit activation of the IFN response, but they also antagonise the antiviral effect of IFN. ORF6 of SARS-CoV inhibits IFN-signaling by blocking translocation of STAT1 (108), ns2 of MHV inhibits the IFN-activated OAS-RNase L antiviral pathway (109) and 5a of MHV and 7a of FCoV also confer resistance to IFN treatment but via presently unknown mechanisms (112, 113). Using IBV accessory protein null viruses, here we showed that simultaneous knockout of protein 3a and 3b rendered IBV more sensitive to IFN treatment. In a previous study we found that 3a and 3b decrease transcription of *Ifn β* and modulate production of IFN protein (167). The mechanism by which accessory proteins 3a and 3b confer resistance to IFN treatment remains unclear although, in the present study, we could show that 3a and 3b do not interfere with STAT1-mediated signaling.

To explain the role of 3a and 3b in counteracting the type I IFN response, we hypothesise that these two accessory proteins interact with host-proteins involved in both the induction of *Ifn β* and the IFN-induced antiviral response. Host proteins that meet these criteria are, for example, the dsRNA-activated antiviral proteins PKR and OAS, the latter of which was also shown to be involved in the antiviral response against MHV

Chapter

4

Accessory protein 5b of infectious bronchitis virus inhibits production of host protein, including IFN

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Manuscript in preparation

ABSTRACT

We previously showed that infectious bronchitis virus (IBV, genus *Gammacoronavirus*) induces both *Ifn β* transcription and IFN protein production in chicken cells albeit substantially delayed with respect to the peak of viral replication. Other studies have proven the intrinsic ability of chicken cells to respond with production of high levels of IFN protein to dsRNA, or influenza virus infection, but we consistently observed low-to-negligible amounts of IFN upon IBV infection. We also previously showed that very high doses of IFN are required to effectively hinder IBV replication. All together, the late kinetics of IFN production, the low amount of IFN produced in response to IBV and the high doses of IFN required to hinder viral replication, suggest that IBV has devised mechanisms to inhibit IFN production by chicken cells. In the present study we investigate whether the ability of IBV to delay transcription and limit protein production is restricted to type I IFN only, or whether IBV, similarly to *Alpha-* and *Betacoronaviruses*, adopted a strategy based on a more general inhibition of translation of host-cell proteins. Many viruses in fact, inhibit translation of host-cell proteins, a process commonly referred to as “host shutoff”. In *Alpha-* and *Betacoronaviruses*, non-structural protein 1 (nsp1) is responsible for virus-induced host shutoff. *Gamma-* and *Deltacoronaviruses*, however, lack nsp1 and it has remained unclear whether and how these viruses inhibit translation of host-cell proteins. Here, we confirm that also IBV induces host shutoff and, using viruses mutant for the accessory proteins of IBV, we show that accessory protein 5b contributes to this process by inhibiting translation of host proteins, including translation of type I IFN. Taken together, our data identify the accessory protein 5b of IBV *Gammacoronavirus* as the potential functional equivalent of nsp1 found in *Alpha-* and *Betacoronaviruses*. Our findings suggest that inhibition of translation of host-cell proteins is an evolutionarily conserved defense mechanism found in most if not all coronaviruses. This study increases our understanding of innate immune evasion by IBV and provides a novel target for the development of improved vaccines against this important pathogen.

INTRODUCTION

The type I IFN (IFN) response is essential for clearance of coronavirus infections *in vivo* (64, 83, 84), whereas coronaviruses have evolved multiple mechanisms to delay and antagonise the antiviral response (114). Previously (Chapter 2, this thesis), we demonstrated that in chicken cells infected with the *Gammacoronavirus* IBV, transcription of *Ifn β* mRNA and translation of IFN is substantially delayed with respect to the peak of viral replication (176). In the same study we reported that the accessory proteins 3a and 3b both play a role in the inhibition of *Ifn β* transcription while having opposing effect on *Ifn β* translation; infection with a 3b null virus resulted in increased IFN production whereas infection with a 3a null virus resulted in reduced IFN levels compared to the wild type virus, suggesting that accessory proteins 3a and 3b antagonise each other's effects to tightly regulate IFN production. In other studies where researchers used the same reporter assay as used by us in our studies to quantify IFN protein production, production of high levels (up to 10.000 units/ml) of IFN were reported for chicken cells when stimulated *in vitro* with dsRNA analogues (79) or *in vivo* when infected with highly pathogenic avian influenza viruses (177). The observation that IBV delays activation of the IFN response and limits production of IFN, prompted us to investigate to what extent IBV is affected by treatment with IFN. We showed that IBV, compared to the IFN-sensitive Sindbis virus, is relatively resistant to treatment with either IFN α or IFN β (chapter 3, this thesis). In fact, high doses of IFN protein (1000 to 10.000 units/ml) were required to reduce replication of IBV by 1-2 logs. The discrepancy between the amount of IFN protein produced during IBV infection (150-1000 units/ml, (176)) and the amount of IFN required to inhibit viral replication, combined with the delayed kinetics of IFN production, suggests that IBV must have developed mechanisms to limit IFN protein production.

Whether the effects observed on gene transcription and translation during IBV infection are restricted to the regulation of type I IFN only, or whether IBV has adopted a broader strategy based on general inhibition of host-protein translation is currently unknown. For example, *Alpha-* and *Betacoronaviruses* antagonise the overall antiviral response of their host cells through inhibition of host-protein translation, including that of type I IFN, a mechanism mediated by their nsp1 protein and best known as "host shutoff". For example, *Betacoronaviruses* such as mouse hepatitis virus (MHV), severe acute respiratory syndrome coronavirus (SARS-CoV) and several bat coronaviruses all inhibit translation of host-cell proteins via a mechanism mediated by nsp1 (115-117); inducing degradation of host, but not viral mRNA (116, 118, 119). In contrast, transmissible gastroenteritis coronavirus (TGEV, genus *Alphacoronavirus*) and several bat *Betacoronaviruses* inhibit translation of host-cell proteins via a mechanism mediated by nsp1, that does not seem to involve degradation of host mRNA (117, 120). In contrast to *Alpha-* and *Betacoronaviruses*, the genome of *Gamma-* and *Deltacoronaviruses* is devoid of a nsp1 homologue (3, 121-123), raising the question whether these viruses have evolved alternative strategies to inhibit translation of host mRNA, including inhibition of type I IFN. The few studies performed on IBV have remained inconclusive; Wang *et al.* suggested that IBV does not significantly inhibit translation of host proteins as assessed by metabolic labelling (111), but also reported that the spike protein of IBV decreases host-translation through interaction with eIF3F (135). These conflicting data, combined with our previous work on the kinetics of IBV infection and type I IFN response, left unanswered the question whether or not IBV inhibits translation of host-cell proteins in general or of

IFN in particular. In the present study, we show that IBV does indeed inhibit translation of host-cell proteins in general, including type I interferon, and we present evidence that accessory protein 5b is, at least partly, responsible for this process of IBV-induced host shutoff. Taken together, our results suggest that Gammacoronavirus accessory protein 5b may be functionally equivalent to Alpha- and Betacoronaviruses nsp1. As such, this study closes a gap in the understanding of Gammacoronaviruses and shows that evolutionarily-distant coronaviruses use similar strategies to ensure their survival.

MATERIALS AND METHODS

Cells

Chicken embryonic kidneys (CEK) were aseptically removed from 17- to 19-day-old chicken embryos (Charles River, SPAFAS). A cell suspension was obtained by trypsinisation for 30 min at 37 °C and filtered through a 100 µm mesh. The resulting CEK cells were seeded at 4×10^5 cells/cm² in a 1:1 mix of 199 and F10 medium (Invitrogen) supplemented with 0.5% fetal bovine serum (FBS, SAFC) and 1% penicillin-streptomycin (PenStrep; Gibco, Invitrogen). DF-1 chicken fibroblast cells, the African green monkey Vero cells, baby hamster kidney (BHK) cells and the chIFN-reporter CEC-32 cells were cultured in DMEM (Gibco, Invitrogen) supplemented with 10% FBS and 1% PenStrep. All cells were incubated in a humidified incubator at 37 °C and 5% CO₂.

Viruses

IBV-M41, IBV-QX and IBV-Italy-O2, Rift Valley Fever Virus clone 13 (RVFV C113) were obtained from Merck Animal Health, Boxmeer, The Netherlands. IBV-M41, IBV-QX and IBV-Italy-O2 were adapted to propagation in CEK-cells by serial passaging. Sindbis virus (SinV) was a kind gift from dr G. Pijlman, (Laboratory of Virology, Wageningen University). IBV Beaudette, strain Beau-R, as well as the generation of the ScAUG3a, ScAUG3b, ScAUG3ab and ScAUG5ab Beau-R null viruses has been described previously (21-23). In these mutant IBV viruses, the start codon of the indicated accessory genes was mutated to a stop codon. All IBV strains were amplified and titrated on CEK cells. SinV was amplified on BHK cells and titrated on CEK cells. RVFV C113 was amplified and titrated on Vero cells.

cDNA synthesis, RNA isolation and gene expression analysis

Approximately 8×10^5 CEK cells were lysed in RLT buffer (Qiagen) at various time points after infection. RLT cell lysis buffer was spiked with 1 ng/sample of luciferase mRNA (Promega) immediately prior to RNA isolation as external reference gene for normalization during the gene expression analysis. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions, including an on-column DNase treatment (Qiagen). Prior to cDNA synthesis, a second DNase treatment was performed using amplification grade DNase I (Invitrogen), and subsequently 0.5 - 1.0 µg RNA was used for cDNA synthesis using SuperScript III (Invitrogen) and random hexamer primers. cDNA samples were diluted 1:50 in nuclease-free water before real-time quantitative PCR analysis on a Rotor-Gene 6000 (Corbett Research), using Brilliant SYBR Green quantitative PCR (Stratagene) and primers (142-144, 178, 179) as listed in Table 1. Cycle thresholds and amplification efficiencies were calculated using the Rotor-Gene software (version 1.7). The relative expression ratio of the target gene was calculated using the average reaction efficiency for each primer set and the cycle threshold (C_t) deviation of sample vs. control at time point 0h, as previously described (145). Because expression of various housekeeping genes was unstable during virus infections at time points later than 24 hours (data not shown), gene-expression ratios were normalised using an external reference gene (luciferase).

Table 1 Primers used in this study

Gene product	Orientation ^a	Sequence (5'-3')	Accession no.	Reference
<i>Ifnβ</i>	FW	GCTCTCACCACCACCTTCTC	NM_001024836	
	RV	GCTTGCTTCTTGTCCTTGCT		
<i>Ifnα</i>	FW	ATCTGCTGCTCACGCTCCTTCT	XM_004937096	142
	RV	GGTGTGCTGGGTGCCAGGATG		
<i>Irf3</i>	FW	CAGTGCTTCTCCAGCACAAA	NM_205372	
	RV	TGCATGTGGTATTGCTCGAT		
<i>Tlr3</i>	FW	TCAGTACATTTGTAACACCCCGCC	NM_001011691	142
	RV	GGCGTCATAATCAAACACTCC		
<i>Mda5</i>	FW	TGGAGCTGGGCATCTTTCAG	GU570144	
	RV	GTTCCACGACTCTCAATAACAGT		
<i>Mx</i>	FW	TTGTCTGGTGTGCTCTTCCT	GQ390353	
	RV	GCTGTATTCTGTGTTGCGGTA		
<i>Oas</i>	FW	CACGGCCTCTTCTACGACA	NM_205041	143
	RV	TGGGCCATACGGGTAGACT		
<i>I18</i>	FW	TTGGAAGCCACTTCAGTCAGAC	NM_205498	143
	RV	GGAGCAGGAGGAATTACCAGTT		
<i>Pkr</i>	FW	CCTCTGCTGGCCTTACTGTCA	NM_204487	144
	RV	AAGAGAGGCAGAAGGAATAATTGCC		
<i>Adar</i>	FW	TGTTTGATGGCTGTTGAG	AF403114	
	RV	AGATGTGAAGTCCGTGTTG		
<i>Mhc-1</i>	FW	CTTCATTGCCTTCGACAAAG	NM_001031338	143
	RV	GCCACTCCACGCAGGT		
<i>Isg20</i>	FW	TCTGGAAAGGTGGTGGTT	EU602349	
	RV	AAGGGGATTTGGATGTGT		
<i>Caspase 3</i>	FW	GTTAGAAACGCAAACCTGA	NM_204725	
	RV	TGAAGATACGAAACCAAACCA		
<i>Rpl17</i>	FW	TGGATTCTCTGGTGATTGAG	XM_004949013	
	RV	CTTCTTCTCTGGCTTGG		
<i>Gapdh</i>	FW	CATCACAGCCACACAGAAG	NM_204305	
	RV	GGTCAGGTCAACAACAGAGA		
<i>Eef1α1</i>	FW	CTGATTGTGCTGCCTGATT	NM_204157	
	RV	TTCGTATCTCTTCTGGCTGT		
ASFV	FW	AAAGGAACAATGGACTCTGGTCA	AF134508	178
	RV	CACTTCTTACTACCATGTCCCTCCAAT		
SinV	FW	CCCAGGAACCCGCAAGTATG	GM893992	179
	RV	CGTGAGGAAGATTGCGGTTT		
IBV-N	FW	GAAGAAAACCAGTCCCAGA	AY851295	
	RV	TTACCAGCAACCCACAC		
Luciferase	S	TGTTGGGCGGTTATTTATC	X65316	
	AS	AGGCTGCGAAATGTTCACTACT		

^a FW, forward; RV, reverse

Chicken type I IFN bioassay.

Bioactive chicken type I interferon (chIFN) was measured as described previously (180) using a bioassay based on the CEC-32 quail reporter cell line expressing luciferase under the control of the chicken *mx* promoter (149) (kindly provided by Prof. Peter Staeheli). Briefly, CEC-32 cells were incubated with serial dilutions of chIFN-containing samples for 6 hours, after which luciferase activity was quantified and IFN concentrations calculated using a chIFN standard. To avoid influence of virus on the assay, samples were heat-inactivated at 56 °C for 30 min, which did not influence bioactivity of chIFN.

Luciferase activity

CEK cells (4×10^6) were electroporated using the Amaxa nucleofector II (solution V, program W001), applying 2 µg pGL3-Firefly luciferase reporter plasmid (pGL3-FFluc, Promega), and subsequently seeded at 100.000 cells/well in 96-well plates. Vero and DF-1 cells at 80 - 90% confluence in 96-well plates were transfected with 100 ng pGL3-FFluc per well using FuGENE HD (Promega) at a 1:3.5 ratio of DNA:FuGENE according to manufacturers' specifications. At 24 hours post-transfection, cells were infected at an MOI 10 with IBV-M41 (CEK) or Beau-R (DF-1 and Vero), and 22 hours later luciferase activity was quantified using the Bright-Glo Luciferase Assay (Promega) and a Filtermax F5 luminometer (Molecular Devices).

Transfection of accessory proteins and cytotoxicity assay

The constructs used for the overexpression of accessory proteins of IBV were generated as described previously (181). Briefly, the open reading frame (ORF) of the Beau-R 3a, 3b, 5a and 5b proteins were cloned in the EcoRI site of the pFLAG-CMV-2 vector (Sigma-Aldrich), in frame with the N-terminal FLAG tag sequence encoded by the vector.

Vero and DF-1 cells at 80 - 90% confluence were transfected in 96-well plates as described above, using 10 ng pRL-SV40 Renilla luciferase plasmid (Promega) and 90 ng pFLAG-Beau-R 3a/3b/5a/5b or pEGFP-MHV-nsp1 per well. At 18 hours post transfection, luciferase activity was quantified using the Renilla Luciferase Assay (Promega) and a Filtermax F5 luminometer (Molecular Devices). In parallel wells, cytotoxicity of accessory proteins was quantified using the CellTiter 96 cell proliferation assay (Promega). Briefly, at 18 hours post transfection, 20 µl Aqueous one solution was added to each well and incubated at 37 °C for 4 hours. Absorbance at 485 nm was measured using a FilterMax F5 luminometer. The absorbance value for 0% cell viability was established by incubating non-transfected cells for 15 minutes in 2% Triton X-100 (BioRad) in medium, prior to addition of the Aqueous one solution. To visualise expression of Beau-R accessory proteins, the above mentioned Fugene transfection method was used to transfect Vero cells, cultured on 8-well Lab-Tek #1.0 borosilicate coverglasses (Sigma-Aldrich) at 60% confluence. At 18 hours post-transfection, cells were fixed with 3.7% paraformaldehyde and permeabilized using 0.1% Triton X-100 in PBS. FLAG-tagged accessory proteins were detected using anti-FLAG M2 antibody (Sigma-Aldrich) and visualization was performed using Alexa-488 labelled goat-anti mouse antibody (Invitrogen). Antibodies were diluted 1:1000 in phosphate-buffered saline (PBS) supplemented with 5% FBS. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, 0.5 µg/ml, Sigma). Cells were imaged using a Zeiss Primo Vert microscope and Axiovision software. Image overlays were obtained in ImageJ.

RNA stability assay

Stability of host mRNAs was quantified by comparing the fold change in gene expression between infected and non-infected cells after treatment with 10 µg/ml Actinomycin D (ActD). To this extent, CEK cells (8×10^5) were seeded in 24-well plates and infected with Beau-R at MOI 10 or mock treated; 5 hours later ActD was added to all cells and incubation was continued for an additional 6 hours. Before (IBV-5h; ActD-0h) and after ActD treatment (IBV-11h; ActD-6h), samples were collected for RNA isolation, cDNA synthesis and RT-qPCR. Fold changes were calculated relative to the samples taken prior to ActD addition (ActD-0h) and normalised to an external reference gene (luciferase, Promega) which was added as mRNA to the RLT cell-lysis buffer.

Radioactive labelling of de-novo translated proteins

Approximately 2×10^5 Vero cells were seeded in 6-well plates and 24 hours later infected with either Beau-R or scAUG-3ab, scAUG-5ab, scAUG-5a, scAUG-5b Beau-R null viruses at a MOI of 20. Cell lysates were collected at 6, 12, and 24 hours post infection. Forty-five minutes prior to the indicated time points, cells were starved in Met/Cys deficient medium for 30 minutes and incubated with ^{35}S -trans-label (Amersham) for 15 minutes. Cells were washed three times in phosphate-buffered saline (PBS) to wash away free label. Cells were released using trypsin, suspended in complete medium, spun down 5 minutes at $300 \times g$, washed once with PBS and lysed in TEN-L buffer (40 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM EDTA, 1% NP40 and protease inhibitor cocktail [Roche]). Lysates were cleared for 15 minutes at $20,000 \times g$ and incorporation of label was quantified using the scintillation counter (Beckman). Equal amount of labelled proteins (based on scintillation counts) were separated on a sodium dodecylsulphate- polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently used for fluorography as previously described (115). For quantification of protein translation levels, the gel was imaged using a Storm 860 PhosphorImager (Molecular Dynamics) and quantification was performed using ImageJ software.

Statistics

Statistical analyses were performed in GraphPad Prism 6.0. Significant differences were determined using an unpaired t test or a one-way ANOVA followed by a Bonferroni post-hoc test when indicated.

RESULTS

IBV inhibits production of type I IFN

Although chicken cells are able to produce high levels of IFN protein both *in vitro* (79) and *in vivo* (177), we showed that infection with IBV, despite the transcription of a considerable amount of *Ifnβ* mRNA, leads to the production of only low concentrations of IFN (176). Furthermore, we showed (chapter 3, this thesis) that high concentrations of IFN are needed to significantly inhibit IBV replication in chicken cells. To address the question as to whether IBV infection indeed limits production of IFN protein, we infected chicken cells with IBV and compared *Ifnβ* transcription and IFN protein production with that of different RNA viruses known to either trigger IFN protein production or actively inhibit host-protein translation: as a positive control for IFN production, we used rift valley fever virus clone 13 (RVFV C113), which has a truncated IFN antagonist (the NSs protein) (182), and was previously shown to induce transcription of *Ifnβ* in chicken embryo kidney (CEK) cells (176). As a positive control for inhibition of host translation, we used Sindbis virus (SinV) (183) that was also previously shown to propagate in chicken cells and induce transcription of *Ifnβ* (176). To examine the effect of IBV on *Ifnβ* transcription and IFN protein production, we used a pathogenic IBV-M41 strain adapted to CEK cells.

Virus replication in CEK cells was examined by RT-qPCR using virus-specific primers, detecting large amounts of total viral RNA in both SinV- and IBV-infected cells, but much less in RVFV C113-infected cells (Fig. 1A). These data confirmed previous observations that SinV and IBV propagate well in chicken cells (184, 185) whereas the RVFV C113 mutant strain does not. Despite the quantitative differences in virus replication, quantification of *Ifnβ* mRNA in the same samples showed that all three viruses triggered considerable transcription of *Ifnβ* (Fig. 1B). In contrast to *Ifnβ*, transcription of *Ifnα* was not upregulated in chicken cells by IBV (176), RVFV C113 or Sindbis at any of the time points studied (data not shown). In parallel to the quantification of *Ifnβ* mRNA, we quantified IFN protein and found that in both SinV- and RVFV C113-infected cells, accumulation of IFN in the supernatant correlated to transcription of *Ifnβ*. In contrast, in the supernatant of IBV-infected cells, only negligible amounts of IFN could be detected (Fig. 1C). As predicted based on the observation that SinV strongly inhibits translation of host-cell proteins (183), production of IFN by SinV-infected cells was markedly lower than IFN production by RVFV-infected cells. The observation that IBV-infected cells produce significant amounts of *Ifnβ* mRNA, but only negligible amounts of IFN protein, indicated that IBV limits IFN production by inhibiting translation of *Ifnβ* mRNA.

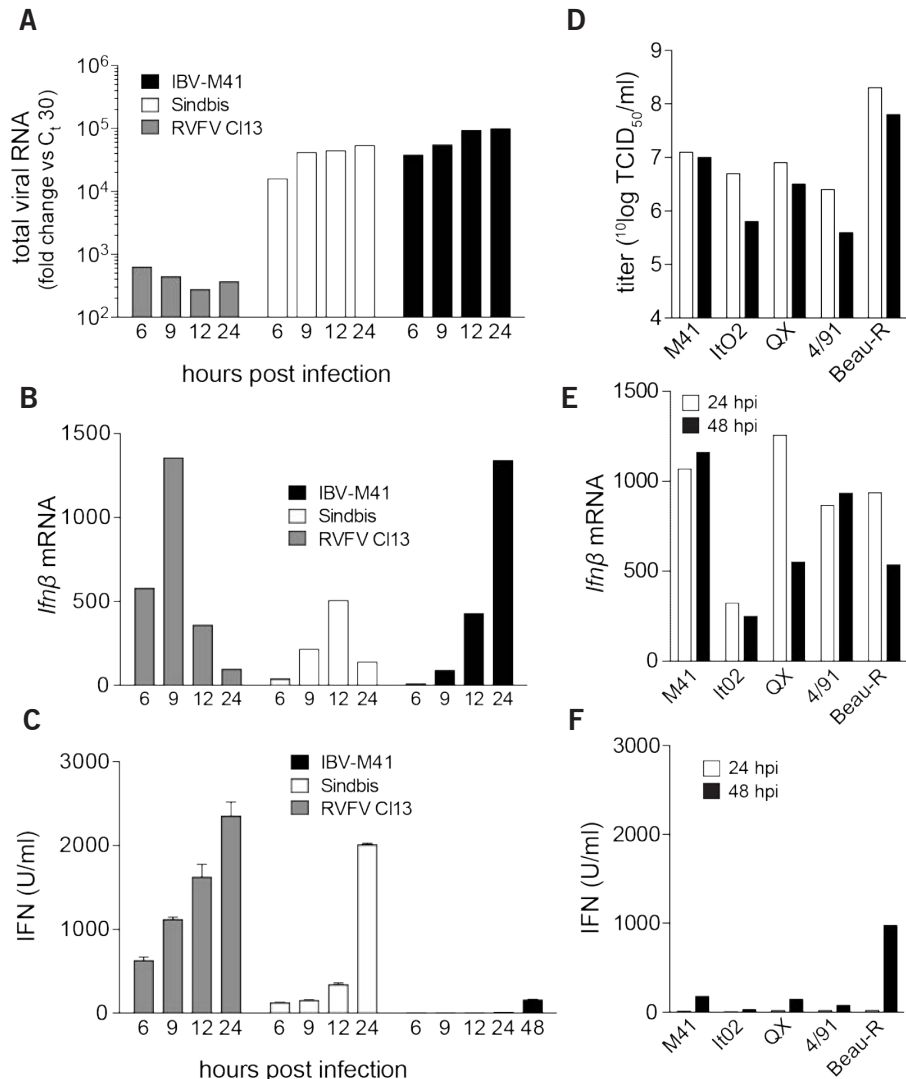


Fig. 1 IBV induces transcription, but limits production of type I IFN

Chicken embryo kidney (CEK) cells were infected with IBV-M41, RVFV CI13 (MOI 5) or Sindbis (MOI 1). At the indicated time points (A) total viral RNA, (B) *Ifnβ* mRNA were determined and (C) IFN protein in the cell culture supernatant was quantified. Values represent results of one representative experiment, which was performed twice with comparable results. Error bars indicate standard deviation of triplicate wells. (D - F) CEK cells were infected with the indicated strains of IBV (MOI 1). (D) Virus titres, (E) *Ifnβ* mRNA and (F) IFN protein were determined at 24 and 48 hpi.

To assess whether the observed inhibition of IFN production could be a specific trait of the IBV-M41 strain or a general feature of IBV, we quantified both *Ifnβ* mRNA and IFN protein concentrations in supernatants of CEK cells infected with five different strains of IBV. All strains had previously been adapted to grow in CEK cells, as also confirmed by titration (Fig. 1D). Subsequent RT-qPCR analysis confirmed that all five strains of IBV induced transcription of *Ifnβ* mRNA, although with some

differences between strains (Fig. 1E). Again, only very low concentrations (<25 U/ml) of IFN protein were detected in culture supernatants, with the exception of supernatants from Beau-R-infected CEK cells collected at 48 hpi (Fig. 1F). The higher concentration of IFN protein in Beau-R supernatants could be attributed to the ability of this particular strain to infect almost 100% of the cells as it is very well-adapted to growth in CEK cells. Despite the difference between strains, the results clearly indicate that inhibition of translation of *Ifn β* mRNA is a general feature of IBV viruses.

IBV inhibits translation but not transcription of host mRNA

Many viruses, including *Alpha*- and *Betacoronaviruses* inhibit translation of host-mRNA (114). The observation that IBV inhibited translation of *Ifn β* mRNA could be the result of a general inhibition of translation by IBV, leading to a general inhibition of protein expression. To study whether besides IFN, IBV infection would also inhibit expression of other proteins we investigated the effect of IBV on expression of Firefly luciferase in three different cell types; to this end CEK, DF-1 and Vero cells were transfected with a luciferase plasmid. The results showed that infection with IBV reduces luciferase expression by 30 – 50% in all three cell types (Fig. 2A). Although the reduction in luciferase activity was not complete, this clearly indicated that besides IFN, IBV also inhibits the expression of other proteins. Considering that transfected cells are relatively difficult to infect with IBV and that IBV-M41 only infects a subset of CEK cells which typically consist of a mixed cell population, to further investigate whether IBV inhibits general host-cell translation, we visualised *de novo* protein synthesis in IBV-infected cells using ³⁵S metabolic labelling (Fig. 2B). Results indicated that at 6 hpi, both host and viral proteins were translated, whereas at 12 hpi translation of host proteins was severely reduced compared to mock-infected cells, while translation of viral proteins was unaffected. At 24 hpi, however, both translation of host and viral proteins was markedly reduced compared to mock-infected cells, which probably can be attributed to the extensive cytopathic effect (CPE) observed at this time point. Taken together, these data indicate that the inhibitory effect of IBV is not limited to synthesis of IFN only, but extends to a more general host translational shut off.

After having established that IBV inhibits general synthesis of host proteins, we investigated the underlying mechanisms. For the *Betacoronaviruses* SARS-CoV and MHV it was shown that inhibition of protein synthesis is accompanied by degradation of host mRNA (116, 118, 119). To investigate whether also IBV would negatively affect stability of host mRNA we restricted *de novo* transcription in IBV-infected cells and compared mRNA levels of a number of genes before and after treatment with actinomycin D (ActD) (Fig. 2C). Indeed, inhibition of *de novo* transcription by ActD induced a decrease in mRNA levels of all genes studied. Although statistical analysis indicated no significant ($P=0.12$) difference between the change in mRNA levels in infected (black bars) versus mock-treated cells (white bars), the rate of degradation of most mRNAs was lower in IBV-infected cells than in mock-treated cells, clearly suggesting that IBV does not increase host mRNA degradation. Interestingly, of all genes studied, *Ifn β* mRNA was least degraded in IBV-infected cells indicating a stabilising effect of IBV on *Ifn β* mRNA. Taken together, our results indicate that the mechanism by which IBV decreases host translation is not based on degradation of host mRNA and it therefore appears different from the mechanisms adopted by some *Betacoronaviruses*.

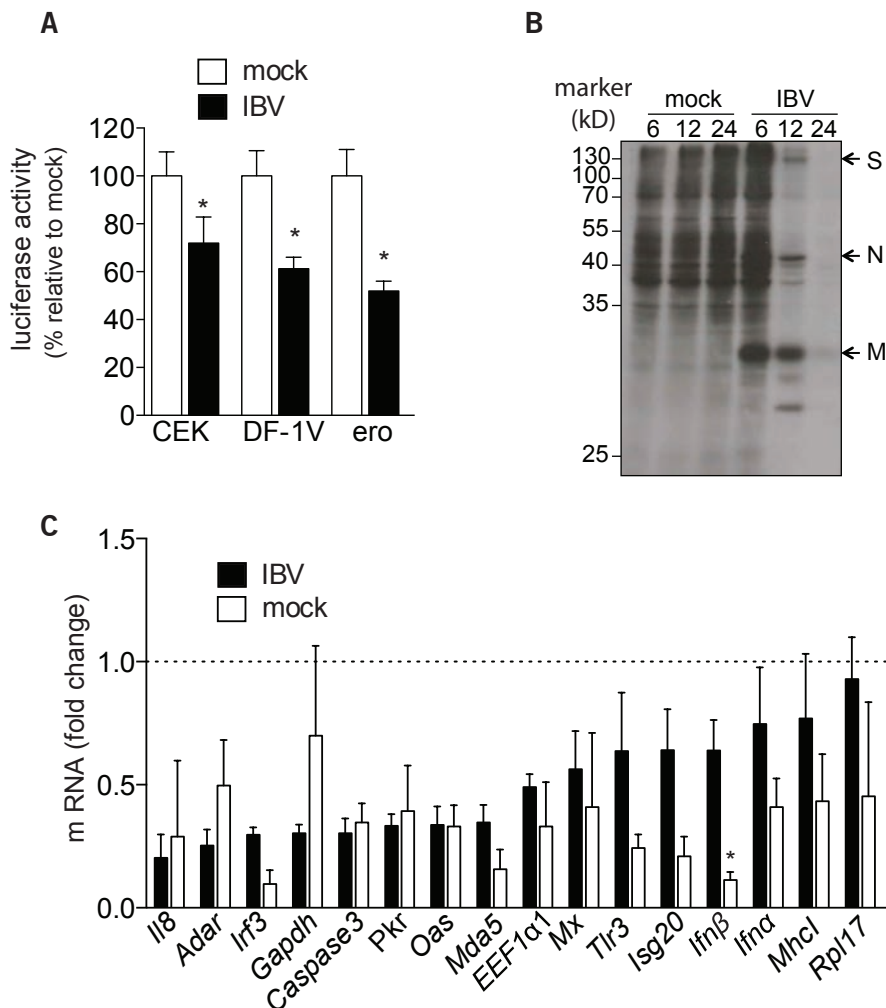


Fig. 2 IBV-induced host-protein shutoff does not require degradation of host mRNA

(A) CEK cells were transfected with pGL3 SV40-Firefly luciferase plasmid and 24 hrs later cells were infected with IBV-M41 (MOI 10). DF-1 and Vero cells were transfected with the same plasmid and 24 hrs later infected with Beau-R (MOI 10). At 22 hpi, luciferase activity was quantified. Bars represent mean luciferase activity of triplicate measurements from two independent experiments. Error bars indicate standard deviation and asterisks (*) indicate statistically significant differences ($P < 0.0001$) compared to the respective mock-infected cells, as determined using an unpaired Student's t-test. (B) Vero cells were infected with Beau-R (MOI 20), and at the indicated time after infection, newly synthesised proteins were radioactively labelled using ^{35}S methionine. Cells were lysed, total protein content quantified based on scintillation counts and separated using SDS-PAGE after which ^{35}S was visualised using a phosphoimager. (C) CEK cells were infected with Beau-R (MOI 10) and five hours later *de novo* transcription was inhibited using Actinomycin D (ActD) for an additional six hours, after which mRNA levels were quantified by RT-qPCR. The dotted line indicates the relative gene level before ActD treatment. Bars indicate the fold change of the gene induced by actinomycin D treatment, compared to before treatment. Bars indicate the mean of triplicate wells from a representative experiment out of two biological replicates. Error bars indicate standard deviation. Asterisk (*) indicates significant difference ($P < 0.05$) between IBV-infected and mock-treated cells as assessed by one-way ANOVA followed by a Bonferroni post-hoc test.

Accessory protein 5b inhibits translation of host-cell proteins

Alpha- and *Betacoronaviruses* mediate host shutoff via their nsp1, but IBV, like other *Gamma-* and *Deltacoronaviruses*, does not encode an nsp1 homologue, leaving unresolved the question of which IBV protein could be responsible for the observed inhibition of translation of host-cell proteins. Besides genes that encode for structural and non-structural proteins, all coronaviruses have genes that encode a variable number of accessory proteins that are dispensable for virus replication *in vitro*, but may play a role in virulence *in vivo* (10, 11, 186). *Gammacoronaviruses* possess at least four accessory proteins, namely 3a, 3b, 5a, and 5b. To examine whether one or more of the IBV accessory proteins could be involved in inhibition of translation of host-cell proteins, we investigated whether individual IBV accessory proteins could inhibit expression of luciferase protein in cell culture. To this end, we overexpressed all four IBV accessory proteins as Flag-tagged constructs (Fig. 3A) together with a construct expressing Renilla luciferase, in both DF-1 and Vero cells. As a positive control for inhibition of translation we included a MHV nsp1 construct. Our results show that accessory protein 5b in particular, substantially inhibited luciferase activity in both cell types (Fig. 3B). Inhibition of luciferase activity could not be ascribed to direct cytotoxicity since no difference in cell viability could be observed upon transfection of any of the constructs (Fig. 3B). In contrast to the clear effect of 5b, expression of accessory protein 3a only moderately inhibited luciferase activity and only in DF-1 cells, whereas accessory protein 3b induced a slight increase in luciferase activity in both cell types. In conclusion, these results suggest that accessory protein 5b of IBV, similar to nsp1 of MHV, might be involved in inhibition of translation of host-cell proteins.

To verify whether accessory protein 5b indeed would be the best candidate to investigate the ability of IBV to inhibit general translation of host-cell proteins, we first compared the levels of IFN protein produced by chicken cells infected with 3a, 3b, 3a/3b, 5a, 5b, and 5a/5b null viruses. These mutantviruses do not express the indicated accessory proteins owing to a mutation in the AUG start codon of the respective ORFs (13, 14). CEK cells were infected with Beau-R wild type virus or mutant viruses and at various time points after infection, IFN protein in the cell culture supernatants was quantified (Fig. 3C). Interestingly, at 24 and 36 hpi, only cells infected with the 5b null virus produced elevated levels of IFN protein whereas only at 36 hpi, cells infected with the 3b null virus also began to express significantly more IFN protein. Altogether these results confirm the importance of accessory protein 5b in mediating inhibition of translation of host-cell proteins.

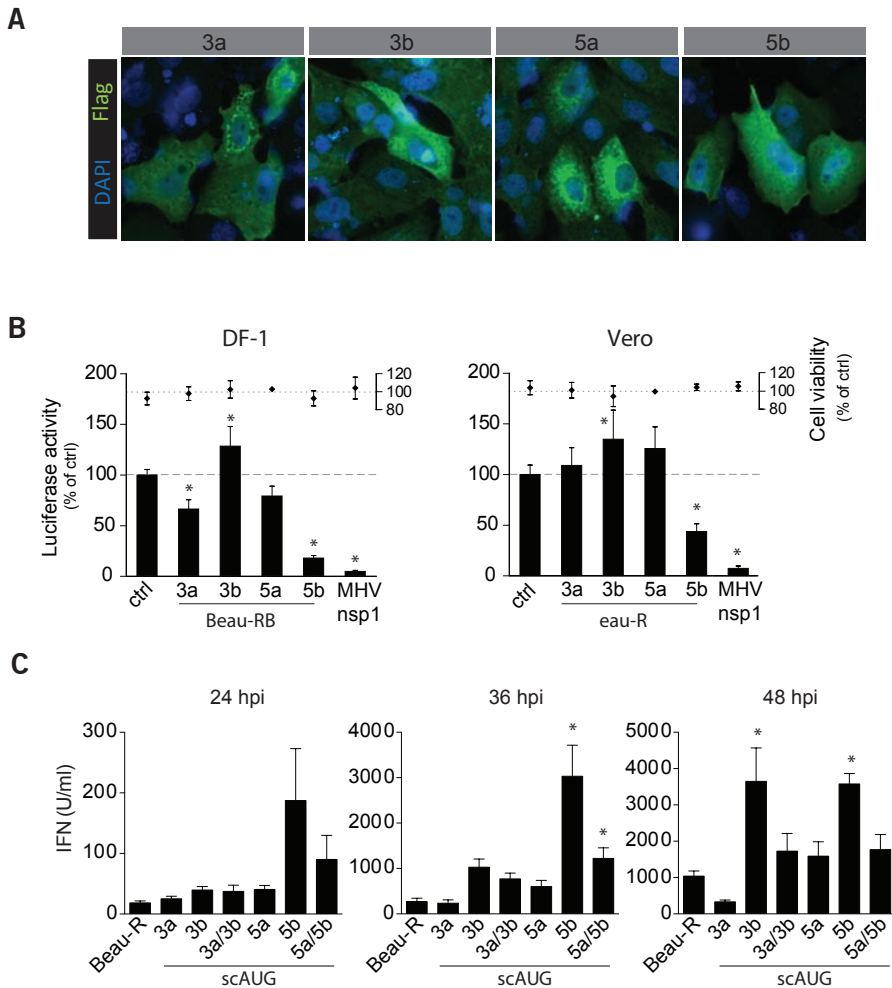


Fig. 3 Accessory protein 5b plays a role in limiting IFN production

(A) Detection of IBV accessory proteins. Vero cells were transfected with plasmids expressing Flag-tagged accessory proteins and 22 hrs later proteins were detected using a Flag-specific antibody. (B) DF-1 and Vero cells were seeded in 96-well plates and transfected with 10 ng Renilla luciferase expressing plasmid plus 90 ng of plasmid expressing the indicated accessory protein of Beau-R, MHV nsp1 or empty plasmid (ctrl). At 18 hours post transfection, luciferase activity was quantified and plotted on the left Y-axis. In parallel wells, cytotoxicity of each construct was investigated using the cell titer 96 cytotoxicity assay. Results are plotted on the right Y-axis. For both luciferase and cytotoxicity, values represent the mean of quadruplicate measurements from two independent experiments. Error bars indicate standard deviation and asterisks indicate significant differences ($P < 0.001$) compared to the control, as assessed by a one-way ANOVA followed by a Bonferroni post-hoc test. (C) CEK cells were infected with Beau-R or Beau-R-null viruses not expressing the indicated accessory proteins (MOI 0.1). The concentration of type I IFN protein was determined in the supernatant at the indicated time points. Values represent the mean of one experiment performed in triplicate and error bars indicate standard deviation. Asterisks indicate significant differences ($P < 0.01$) compared to the parental Beau-R virus at the same time point, as assessed by a one-way ANOVA followed by a Bonferroni post-hoc test.

Considering the kinetics of IFN protein production observed in cells infected with the 5b null virus (Fig. 3B) and the kinetics of host-cell protein shutoff (Fig. 2B), we used mutant viruses to verify whether, besides inhibition of IFN protein, accessory protein 5b would also inhibit general translation of host-cell proteins. We again visualised *de novo* protein synthesis by repeating the ³⁵S metabolic labelling experiment using 5a, 5b, 5a/5b and 3a/3b null viruses (Fig. 4A). Protein synthesis was significantly inhibited at 12 hpi in cells infected with wild-type Beau-R, and in cells infected with 3a/3b and 5a null viruses but not in cells infected with 5a/5b- and 5b-null viruses (Fig. 4B). At 24 hpi, also the 5a/5b null virus significantly inhibited protein synthesis, but not to the same extent as the other viruses whereas the 5b null virus remained unable to inhibit *de novo* protein synthesis (Fig. 4C). Differently from the effect on translation of host proteins, at 12 hpi the translation of viral proteins remained unaffected (Fig. 4D). Nevertheless, under the conditions studied, infection with 5a/5b and 5b null viruses did not strongly inhibit *de novo* protein synthesis, supporting the observation that 5b inhibits general translation of host-cell proteins, including that of type I IFN. Taken together, our results indicate that accessory protein 5b of IBV is at least partly responsible for the IBV-induced host shut off.

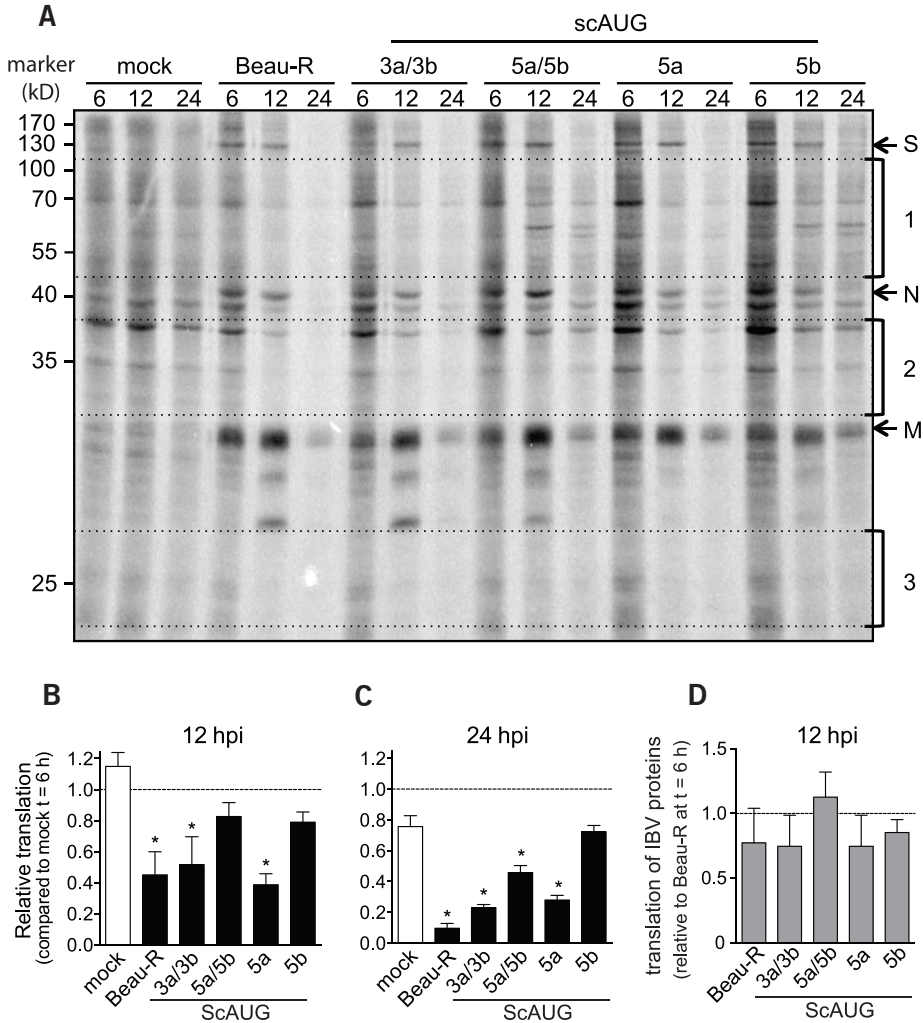


Fig. 4 Accessory protein 5b inhibits host-protein translation

(A) Vero cells were mock treated or infected with the indicated mutant viruses not expressing one or two accessory proteins (MOI 20). At the indicated time points after infection, *de novo* synthesized proteins were labelled with ^{35}S methionine for one hour. Subsequently, cells were lysed and proteins were separated using SDS-PAGE and ^{35}S -labelled proteins were visualized using a phosphorimager. Areas containing host proteins are indicated with 1, 2 and 3. Virus proteins are indicated with S, N and M. (B) Synthesis of host-proteins at 12 hpi and (C) at 24 hpi in mock- and virus-infected cells expressed as ratio relative to mock-infected cells at 6 hpi (dotted line). Host translation was approximated by quantification of ^{35}S signal intensity in the areas that contain mostly host-proteins, indicated with 1, 2 and 3 on the right side of Fig. 4A. (D) Synthesis of IBV-proteins at 12 hpi relative to Beau-R infected cells at 6 hpi. IBV-translation was approximated by quantification of ^{35}S signal intensity of IBV-S, -N and -M proteins, as indicated on the right side of Fig. 4A. Bars indicate the mean of the three values determined for either virus or host proteins at the indicated time point and error bars indicate standard deviation. Asterisks (*) indicate significant differences ($P < 0.001$) compared to mock, as assessed by one-way ANOVA followed by a Bonferroni post-hoc test.

DISCUSSION

In this study we investigated the ability of the *Gammacoronavirus* IBV to interfere with the host antiviral response through inhibition of translation of host-cell proteins and studied the role of accessory proteins in this process. Previously, we demonstrated that IBV is relatively resistant to treatment with IFN and that very high doses (1000 to 10.000 U/ml) of IFN are necessary to significantly inhibit viral growth by 1-2 logs (chapter 3, this thesis). In another study, we investigated the kinetics of *Ifn β* transcription and showed that IBV infection elicits delayed, but substantial transcription of *Ifn β* mRNA in avian cells (176). Despite the substantial *Ifn β* transcription, only low levels (100 - 150 U/ml) of IFN protein could be detected in supernatants from IBV-infected chicken cells. In the current study, using as positive controls RVFV clone 13, a virus with a defective IFN antagonist, and Sindbis, a virus known to cause host shutoff, we were able to confirm that IBV effectively limits production of IFN protein in chicken cells, independently of the IBV strain used.

The ability of a virus to delay and antagonise the antiviral response determines its ability to avoid clearance by the host and coronaviruses have evolved multiple mechanisms to this purpose. One mechanism used by *Alpha-* and *Betacoronaviruses* is inhibition of host-protein translation, including that of type I IFN, a mechanism which is mediated by viral nsp1 protein. *Gamma-* and *Deltacoronaviruses* lack nsp1 and it has remained unclear whether and how these viruses inhibit host-protein translation. Only few studies have been performed on IBV by Wang *et al.* but produced apparently inconsistent data; it appeared that IBV does not significantly inhibit translation of host proteins as assessed by metabolic labelling (111), but also that the spike protein of IBV decreases host-translation through interaction with eIF3F (135). Considering the lack of IFN protein production during IBV infection observed in our study we investigated whether IBV specifically limited production of IFN only or whether IBV induced a more general inhibitory mechanism based on host-cell protein shutoff. Using the same cell line and the same IBV strain used by Wang *et al.* (18), we could show that IBV induces an efficient host shutoff. The apparent discrepancy between our and the previous study by Wang *et al.* is probably due to the difference in MOI used. To achieve host shutoff measured by ³⁵S metabolic labelling it is crucial to infect all cells simultaneously, for which reason we have used an MOI of 20, which is 10-fold higher than the MOI used by Wang *et al.* Based on our results, we conclude that IBV does indeed induce a general host shutoff, indicating that such a defense mechanism is evolutionary conserved between *Gammacoronaviruses* and *Alpha-* or *Betacoronaviruses*.

During infection with *Beta-*, but not *Alphacoronaviruses*, host shutoff is accompanied by degradation of host mRNAs (118, 119). To investigate whether IBV infection induces degradation of mRNA, we inhibited *de novo* transcription and quantified the decrease in host-mRNA levels in IBV-infected and non-infected cells. In contrast to what is known for MHV and SARS-CoV (118, 119), we did not observe a degradation of host mRNAs. The observation that IBV does not degrade host mRNAs may suggest that IBV induces host shutoff via a mechanism similar to *Alphacoronaviruses*, which also do not induce degradation of host mRNA (120). Interestingly, some mRNAs, such as *Ifn β* , *Isg20* and *Tlr3* showed increased rather than decreased stability in IBV-infected cells. The increased mRNA stability of these particular mRNAs could be the result of stress granule (SG) formation as it has been observed for MHV (115). Although formation of SGs

has been associated with increased mRNA stability, activation of this stress pathway has thus far not been studied for IBV and awaits further investigation before a clear conclusion can be drawn.

Because the genome of *Gamma*- and *Deltacoronaviruses* does not encode for an nsp1 homologue, it has remained unclear which IBV protein could play a role in the above-described IBV-induced host protein shutoff. Based on the hypothesis that one of the four canonical accessory proteins might be involved in this process, we overexpressed the IBV-accessory proteins and found that only 5b consistently inhibited the activity of a constitutively expressed host protein. Although maybe difficult to compare in quantitative terms, inhibition by IBV 5b seemed less pronounced than inhibition by MHV-nsp1. Also, similar differences in inhibition efficiency have been reported for nsp1 proteins from various *Betacoronaviruses* (117). Next, we investigated the role of IBV accessory proteins on IFN protein production using mutant viruses that do not express one or more of the accessory proteins. We found that both 5a/5b- and 5b-null viruses induced up to 90 times more type I IFN in chicken cells than the parental Beau-R virus. The difference in induction of type I IFN between the IBV 5b-null virus and Beau-R-infected cells is comparable to the difference observed between a SARS-CoV-nsp1 mutant virus and the parental virus (187). We previously showed that infection with accessory protein 3b null viruses leads to increased IFN production but only late (>36hpi) after IBV infection (176). Here, metabolic labelling analysis indicated that inhibition of translation of host-proteins by IBV occurs early, already at 12 hpi. The observation that infection with null virus for accessory protein 5b, but not 3b, led to increased IFN protein production already at 24 hpi, together with the observation that 5b overexpression efficiently inhibited luciferase activity, suggests that accessory protein 5b in particular, might play a role in IBV-induced host shutoff.

Using radioactive labelling of *de novo* protein synthesis, we found that both 5a/5b- and 5b-null viruses were less efficient at inhibiting host translation than the parental virus (Fig. 3). The difference in inhibition efficiency between the 5b-null virus and the parental virus, resembles the difference in IFN production between an nsp1-mutant and the parental SARS-CoV (187). Taken together, these observations suggest that IBV 5b may be the functional equivalent of *Alpha*- and *Betacoronavirus* nsp1.

The conclusion that IBV 5b and accessory protein nsp1 of *Alpha*- and *Betacoronavirus* share a functional resemblance would answer a long-standing question on the absence of nsp1 in *Gammacoronaviruses* and whether this absence is compensated for by another viral protein. As such, this study marks a significant step forward in the understanding of coronavirus biology and demonstrates that mechanisms aimed at altering host antiviral responses may have been functionally conserved during evolution. Although the exact mechanism by which 5b inhibits host-translation remains to be elucidated, our *in vitro* results indicate that accessory protein 5b may be an important virulence factor of *Gammacoronaviruses* and a potential target for the design of rationally-attenuated vaccines against IBV.

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Chapter

5

Quantification of infectious bronchitis coronaviruses by titration in vitro and in ovo

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ABSTRACT

Quantification of the number of infectious viruses in a sample, is a basic virological technique. In this chapter we provide a detailed description of three techniques to estimate the number of viable infectious avian coronaviruses in a sample. All three techniques are serial dilution assays, better known as titrations.

1. INTRODUCTION

Technological advances in particle analysis have made it possible to quantify the number of virus particles in a sample with increasing accuracy. Techniques such as specialized flow cytometry (188), dynamic light scattering (189), quantitative capillary electrophoresis (190) and fluorescence correlation spectroscopy (191) can determine the number of particles in a sample within hours. The choice of technique depends on the sort of virus and the matrix in which it is suspended. All aforementioned techniques differentiate particles on the basis of physical properties such as size or antibody affinity. As a consequence particle analysis cannot differentiate infectious from non-infectious virus particles.

There is only one technique available that can reliably quantify the number of infectious particles in a sample. This technique, developed many decades ago (192), exploits the fact that virus can propagate in biological systems such as embryonated eggs or cell cultures. Propagation of a virus is generally accompanied by changes in cell morphology (referred to as cytopathic effect or CPE), which can be visualised using a microscope, or even by eye. Some viruses do not induce CPE, in which case an antibody based assay (immunofluorescence or ELISA) is needed to determine presence or absence of virus. During a titration assay, tissue cultures or embryonated eggs are incubated with tenfold serial dilutions of a virus containing sample and several days later the cytopathic effect is scored. From these scores, the virus titre is calculated using the methods described by Spearman and Kaerber (193, 194) or Reed and Muench (195). The virus titre is defined as the reciprocal of the dilution at which 50% of the inoculated embryos or tissue cultures show CPE. In this chapter we use the method of Spearman and Kaerber to calculate the titre, as this calculation can cope with unequal group sizes. Unequal group sizes frequently arise when eggs are lost to a-specific death of the embryo or bacterial infection.

Coronaviruses in general have a narrow host range and many clinical isolates only replicate in primary cells. Replication of most field isolates of infectious bronchitis coronavirus is restricted to embryonated eggs or tracheal organ culture. Most isolates however, can be adapted to propagation in primary chicken kidney (CK) cells. Adaptation typically requires several passages and selects for viral subpopulations and can induce mutations (185). Passaging of IBV in either embryonated eggs or primary cell cultures leads to attenuation of the virus *in vivo* (196-198). The most striking example is the IBV Beaudette strain, which has been passaged hundreds of times in eggs and primary chicken kidney cells (199, 200). Although IBV Beaudette propagates very well on eggs, CK cells and even in Vero cells, the virus is highly attenuated *in vivo* and vaccination using Beaudette provides little protection against infection with pathogenic strains of IBV (61).

For quantification of IBV field isolates, embryonated chicken eggs are the most suitable substrate. A protocol on titration of IBV on embryonated chicken eggs is provided in the first part of this protocol. IBV strains which have been adapted to grow in cultures of primary chicken cells can be titrated on these cells using either the TCID₅₀ method or plaque titration. Protocols for both methods are provided in this chapter. Plaque forming unit (PFU) titration yields more accurate and reproducible results than the TCID₅₀ method, it is however more labour intensive. Both methods are presented in this chapter.

2. MATERIALS

2.1 Titration of Avian Infectious Bronchitis virus in fertilized eggs by EID₅₀.

1. Fertilized specific pathogen free (SPF) eggs, 9 to 11-day-old (*see Note 1*)
2. Diluent: Tryptose 2.5% w/v supplemented with 1000 IU/ml of Penicillin and 1000µg/ml of Streptomycin
3. Disinfectant: 70% alcohol in water
4. Egg shell drill or punch
5. Sterile 1 ml syringes
6. Needles, preferably 25 G; 16 mm
7. Hobby glue or melted wax to seal the inoculation site
8. Egg candling light
9. Egg incubator with rocking
10. Positive control sample with known titre

2.2 Tissue culture infective dose (TCID₅₀) titration.

1. 96-well plates containing 80 - 100% confluent CK cells
2. Titration medium: 1:1 mix of medium M199 and Ham's F-10 nutrient mixture supplemented with 0.5% fetal calf serum (FCS), 0.1% w/v tryptose phosphate broth, 0.1% w/v sodium bicarbonate, 0.1% w/v HEPES, 100 U/mL penicillin, 100 µg/mL streptomycin.
3. Multistepper pipette (Socorex or equivalent)
4. Inverted microscope.
5. Positive control sample with known titre.

2.3 Plaque forming unit titration.

1. Six well plates containing 60 - 70% confluent CK cells
2. 1x BES cell culture medium: EMEM, 10% w/v tryptose phosphate broth, 0.2% w/v BSA, 20 mM N,N bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 0.4% w/v sodium bicarbonate, 2 mM L-glutamine, 250 U/mL nystatin, 100 U/mL penicillin, 100 µg/mL streptomycin.
3. 2x BES cell culture medium: 2x EMEM, 20% w/v tryptose phosphate broth, 0.4% w/v BSA, 40 mM N,N bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 0.8% w/v sodium bicarbonate, 4 mM L-glutamine, 500 U/mL nystatin, 100 U/mL penicillin and 100 µg/mL of streptomycin.
4. Sterile phosphate buffered saline (PBS).
5. 2% w/v agarose in water (autoclaved)
6. 10% w/v formaldehyde in PBS
7. 0.1% w/v crystal violet in water
8. Microwave
9. Water bath
10. Small spatula
11. Positive control sample with known titre.

3. METHODS

3.1 Titration of Avian Infectious Bronchitis virus in fertilized eggs.

1. Candle the eggs using the candling light and draw a line on the shell marking the edge of the air sac. Draw an X approximately 5mm above this line, which marks the inoculation site.
2. Assign 10 eggs per dilution and select those dilutions (at least three) that include the 50% end point of the sample.
3. Prepare tenfold serial dilutions in diluent.
4. Disinfect the eggs by spraying them with disinfectant
5. After the eggs have dried drill or pierce a hole in the egg shell at the marked inoculation site.
6. Inoculate ten eggs per dilution each with a 0.2 ml volume via the allantoic cavity by holding the syringe and needle vertically and by inserting the needle approximately 16 mm into the egg (see Fig. 1A, B).

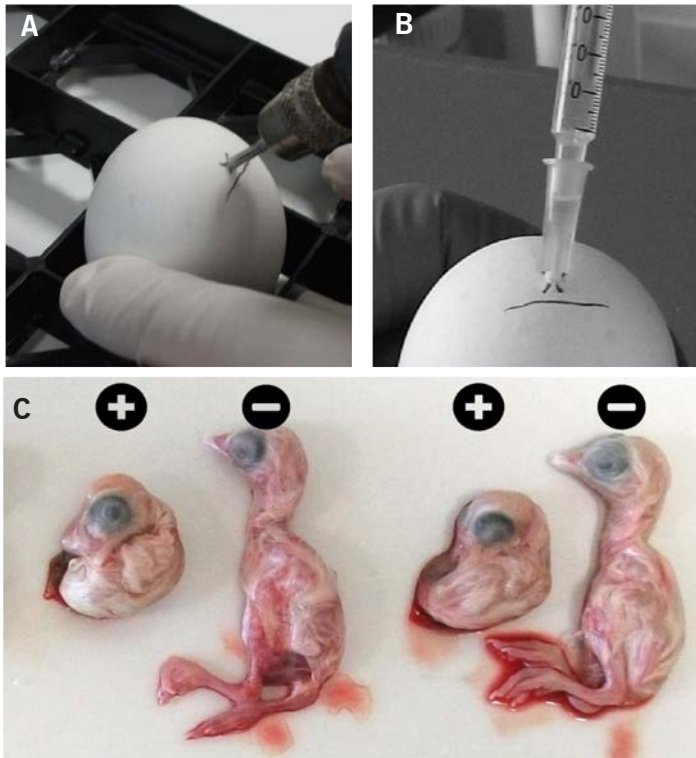


Fig. 1 Egg inoculation into the allantoic cavity.

(A) Drill or pierce a hole in the egg 5 mm above the edge of the air sac. (B) Inoculate each egg with a 0.2 ml volume by inserting the needle approximately 16 mm into the egg. (C) Typical IBV induced malformations 2 days post infection with strain 4/91. *Plus signs* indicate infected embryos with IBV, *minus* indicates non-infected embryos

7. After inoculation, the hole in each egg is sealed with hobby glue or melted wax.
8. Incubate the eggs in an egg incubator with rocking at 37.8°C and 60-65% humidity.
9. Candle the eggs after 24 h of incubation. Embryo mortality occurring up till 24 h post inoculation is considered non-specific and therefore these eggs are discarded.
10. Incubate the eggs for 6 more days in an egg incubator with rocking at 37.8 °C and 60-65% humidity. Candle the eggs at the end of the incubation period to identify embryos that have died. Subsequently, macroscopically evaluate all surviving embryos for the presence of lesions characteristic for IBV infection (stunting and curling; Fig. 1C). Embryos that died and embryos that exhibit lesions characteristic for IBV infection are considered positive. (*see Note 2*)
11. Virus titers in the original sample, expressed as $^{10}\log EID_{50}/ml$ are calculated using the method described by Spearman and Kaerber (193, 194), using the following formula:

$$Titre = \left(x_0 - \frac{d}{2} + d \sum \frac{r}{n} \right)$$

In which:

- x_0 : logarithm of the inverse value of the lowest dilution at which all embryos are positive.
- d : logarithm of the dilution factor ($d = 1$ when using tenfold serial dilutions).
- n : number of eggs used per dilution.
- r : number of positive eggs at that dilution.
12. Example of calculation of virus titre

Using the result of the titration depicted in Fig. 2 , the virus titre is calculated as follows:

$$Titre (^{10}\log EID_{50} / 0.2ml) = \left(4 - \frac{1}{2} + 1 \cdot \left(\frac{5}{10} + \frac{1}{9} \right) \right) =$$

$$5.1 \ ^{10}\log EID_{50} / 0.2ml = 10^{5.1} / 0.2 = 5.8 \ ^{10}\log EID_{50} / ml$$

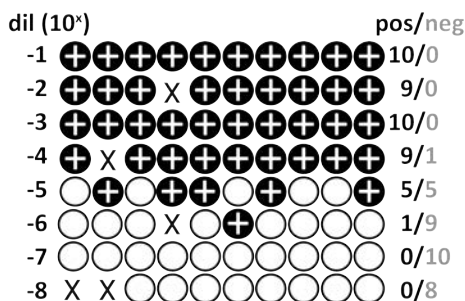


Fig. 2 Schematic result of an egg titration

Each circle represents one egg and crosses indicate aspecific death. Plus signs indicate embryos with IBV specific malformations

3.2 Tissue culture infective dose (TCID₅₀) titration.

1. CK cells are seeded in 96-well plates at 7.5×10^4 cells/well, one or two days before the titration. One plate is needed per sample. At the time of titration, the monolayer should be nearly confluent.
2. Prepare tenfold serial dilutions of the samples in titration medium. (*see Note 3*).
3. Empty the medium from the 96-well plate containing CK cells in a waste container, and gently tap the plate dry on a stack of tissues.
4. Fill the wells of column 1 and 12 of the 96w-plate with 100 μ l/well titration medium. These are the negative control wells (Fig. 3A).
5. Starting with the highest dilution, dispense 100 μ l/well in row H using the multi stepper pipette. Proceed with filling the ascending dilutions in rows G till A.
6. Incubate the infected CK cell monolayers for 3-4 days at 37 °C and 5% CO₂.
7. After 3-4 days incubation, score the CK cell monolayer in all wells for IBV specific CPE using a microscope. Although the CPE may vary per IBV strain, it is generally characterized by clusters of rounded cells on top of the monolayer. At low dilutions the monolayer may be partly destroyed, exemplified by the IBV Beaudette strain (Fig. 3B).
8. Titers are calculated using the method described by Spearman and Kaerber (subheading 3.1, step 11) and are expressed in $^{10}\log$ (TCID₅₀)/ml.

3.3 Plaque forming unit titration.

Infection of cells

1. CK cells are seeded into 6-well plates one or two days before the titration. When performing the titration, the monolayer should 60-70% confluent.
2. Prepare tenfold serial dilutions of virus in 1x BES.
3. Remove media from cells and wash once with sterile PBS.
4. Remove PBS from the cells and add 500 μ l of diluted virus to each well. Duplicate wells should be inoculated for each dilution.
5. Incubate cells at 37 °C for 1 hour to allow virus attachment.
6. Melt 2% agar in a microwave and then transfer to a 42 °C water bath. Allow the agar to equilibrate in temperature.
7. Mix the partially cooled agar with 2x BES pre-warmed to 37 °C to generate 1x BES + 1% agar. Keep at 42 °C until needed to prevent premature setting (*see Note 4*).
8. Remove virus inoculum and overlay cells with 2.5 ml of the 1x BES/agar mix.
9. Leave cells at room temperature for approximately 5 minutes until agar has solidified.
10. Incubate at 37 °C and 5% CO₂ for 3 days for plaques to develop.

Staining cells and determining titre

1. Overlay agar with 1 ml per well 10% formaldehyde in PBS.
2. Incubate at room temperature for 1 hour.
3. Remove formaldehyde and ensure disposal according to local regulations.
4. Using a small spatula, flick off the agar from the cells (see Note 5).
5. Wash cells by shaking the plate upside down in a sink containing water.
6. Add 0.5 ml 0.1% crystal violet to each well.
7. Incubate at room temperature for 10 minutes.
8. Remove crystal violet and dispose of according to local regulations.
9. Wash plate by shaking upside down in a sink of water.
10. Pat plate dry and leave upside down at room temperature to fully dry.
11. Plaques should be clearly visible as holes in the monolayer (Fig. 4). Count the number of plaques per well at the dilution with clearly defined individual (not overlapping) plaques (typically 10-50 plaques/well). Ensure duplicate wells are counted and an average taken.
12. Determine titre using the following equation:

$$\text{Titre (PFU/ml)} = \frac{\text{average number of plaques}}{\text{dilution factor} \times \text{inoculum volume (0.5ml)}}$$

13. For most accurate results, the plaque assay should be repeated three times and the average titre determined.

4. Notes

1. SPF eggs should be used for titration of IBV, as non-SPF eggs may contain IBV specific antibodies that can interfere with the replication of IBV.
2. Non-egg adapted IBV isolates may induce very little IBV specific aberrations of the embryos. When titrating such viruses the eggs are incubated for an additional 2-3 days after inoculation. Candle the eggs at the end of the incubation period to identify embryos that have died. Subsequently, collect allantoic fluid of each of the surviving embryos and test them in a monoclonal based antigen capture ELISA as described in (201, 202). Dead embryos and those of which the allantoic fluid contains IBV as established by ELISA are considered positive.
3. IBV titres usually do not exceed $10^{8.8}$ TCID₅₀/ml, therefore 10^8 should be adequate as highest dilution. When the virus titre of the sample is known, select a number of tenfold dilutions (at least 3) that include the 50% end-point dilution. If the titre is unknown select a broader range of tenfold dilutions that most likely include the 50% end-point dilution. For a TCID₅₀ titration typically all the wells of the 96-well plate are used.
4. Alternative methods also exist for mixing media and agar. If there is concern regarding the overlay setting too quickly or risk of contamination from the water bath, hot agar can be mixed directly with cold media (4 °C). Once the mixture feels warm to the touch, rather than hot, it can be added to the cells.

5. The simplest method for removing agar from the cells is to hold the plate upside down with the lid removed. The small spatula is inserted between the agar and the wall of the well. Once the base of the well is reached, a small amount of pressure is applied to remove the agar being careful not to scrape off the cells. The whole agar plug should then fall out easily.

Chapter

6

Quantification of interferon signaling in avian cells

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ABSTRACT

Activation of the type I interferon (IFN) response is an essential defense mechanism against invading pathogens such as viruses. This chapter describes two protocols to quantify activation of the chicken IFN response through analysis of gene expression by real-time quantitative PCR and by quantification of bioactive IFN protein using a bioassay.

INTRODUCTION

The type I interferon response (IFN response) is an important part of the immune reaction against viruses. Interferon alpha and beta (IFN α and IFN β) are the prototypical type I interferons and can be produced by most animal cells. Production of IFN α/β is triggered upon stimulation of pattern recognition receptors, such as Toll like receptors (TLRs) or RIG-I like receptors (RLRs). Upon production, IFN α and IFN β are rapidly secreted to the extracellular compartment, where they can bind to the ubiquitously expressed IFN receptor. Binding of IFN to the receptor activates the JAK/STAT signaling pathway, leading to the formation of the ISGF3 transcription complex consisting of a STAT1, STAT2 and IRF9. In the nucleus, the ISGF3 complex induces transcription of hundreds of IFN-stimulated genes (ISGs)(203). Many of these genes encode proteins that interfere with the replicative cycle of viruses at various stages (reviewed in ref. (204)). The IFN response is a potent antiviral mechanism, therefore most viruses have been evolutionarily selected to counteract it and coronaviruses are no exception (reviewed by Zhong et al. (125)).

In this chapter we describe two protocols to quantify activation of the IFN response. We have found these protocols useful to study if and how viruses counteract the IFN response in chicken cells. The first protocol describes how to quantify activation of the IFN response at the transcriptional level using real-time quantitative PCR (RT-qPCR) on *Ifn* and IFN-stimulated genes. The second protocol describes quantification of bioactive type-I IFN protein (both IFN α and IFN β) by the use of a reporter cell line. This bioassay can be used to quantify IFN secreted in response to virus infection and, when combined with transcription analysis of *Ifn α* and *Ifn β* these assays can provide an integral picture of activation of the chicken IFN response.

1.1 Quantitation of transcription of chicken *Ifn*-related genes.

Similar to most mammalian cell lines, activation of the interferon response in most chicken cells is characterised by upregulation of *Ifn β* . Like the human genome, the chicken genome encodes only one copy of the *Ifn β* gene, whereas at least ten isoforms of *Ifn α* are present (205, 206). Similar to mammals, production of chicken IFN α is mainly mediated by monocytes, other cells mainly produce IFN β in response to viral infection (207). Because avian coronaviruses replicate mainly in epithelial cells, we monitor activation of the type I interferon response by quantification of *Ifn β* . Similar to mammalian cells, *Ifn β* is upregulated upon activation of either TLR or RIG-I like receptors (RLRs), but not in response to stimulation with IFN. Concomitant with *Ifn β* , many ISGs are also upregulated, indicating that the term interferon stimulated genes is somewhat misleading (208).

Studying the expression of ISGs can be useful, therefore we have provided a list of avian-specific primers for use in real-time quantitative PCR (RT-qPCR; Table 1) (142-144). Protocols for RNA isolation, cDNA synthesis and real-time PCR are plenty and every lab has its own protocols. In this chapter we describe briefly the methods used in our lab. For a detailed overview of RT-qPCR techniques and theoretical background, please refer to Forlenza *et al.* (145).

1.2 Quantitation of chicken type I IFN protein using a bioassay.

The chicken interferon bioassay was developed in the laboratory of Prof. P. Staeheli (149). It is based on a quail cell line (CEC-32) that contains the luciferase gene downstream of a part of the inducible

chicken *mx* promoter. Stimulation of these cells with type I interferon readily induces activation of the *mx* promoter and subsequent production of the firefly luciferase enzyme. Firefly luciferase can be easily quantified using commercially available luciferase assay kits. Here we provide a step-by-step protocol for measuring IFN concentrations using this bioassay.

Table 1 Chicken-specific real-time qPCR primers, including accession numbers of the sequences used to design the primers

Gene product	Orientation ^a	Sequence (5'-3')	Accession no.	Reference
<i>Ifnβ</i>	FW	GCTCTCACCACCACCTTCTC	NM_001024836	
	RV	GCTTGCTTCTTGCTCTTGCT		
<i>Ifna</i>	FW	ATCCTGCTGCTCACGCTCCTTCT	XM_004937096	142
	RV	GGTGTGCTGGGTGCCAGGATG		
<i>Irf3</i>	FW	CAGTGCTTCTCCAGCACAAA	NM_205372	
	RV	TGCATGTGGTATTGCTCGAT		
<i>Irf1</i>	FW	CAGGAAGTGGAGGTGGAGAA	NM_205415	
	RV	TGGTAGATGTCGTTGGTGCT		
<i>Tlr7</i>	FW	TTCTGCCACAGATGTGACC	NM_001011688	142
	RV	CCTCAACTTGGCAGTGCAG		
<i>Tlr3</i>	FW	TCAGTACATTTGTAACACCCCGCC	NM_001011691	142
	RV	GGCGTCATAATCAAACACTCC		
<i>Mda5</i>	FW	TGGAGCTGGGCATCTTTCAG	GU570144	
	RV	GTTCCACGACTCTCAATAACAGT		
<i>Mx</i>	FW	TGTCTGGTGTGCTCTTCCT	GQ390353	
	RV	GCTGTATTTCTGTGTTGCGGTA		
<i>Oas</i>	FW	CACGGCTCTTCTACGACA	NM_205041	143
	RV	TGGCCATACGGGTAGACT		
<i>Il8</i>	FW	TTGGAAGCCACTTCAGTCAGAC	NM_205498	143
	RV	GGAGCAGGAGGAATTACCAGTT		
<i>Pkr</i>	FW	CCTCTGCTGGCCTTACTGTCA	NM_204487	144
	RV	AAGAGAGGCAGAAGGAATAATTGCC		
<i>Adar</i>	FW	TGTTGTGATGGCTGTTGAG	AF403114	
	RV	AGATGTGAAGTCCGTGTTG		
<i>Isg12</i>	FW	TAAGGGATGGATGGCGAAG	NM_001002856	
	RV	GCAGTATCTTTATTGTTCTCAC		
<i>Mhc-I</i>	FW	CTTCATTGCCTTCGACAAAG	NM_001031338	143
	RV	GCCACTCCACGCAGGT		
<i>Ifnar2</i>	FW	GCTTGTGTTTCGTCAGCATT	NP_990189	143
	RV	TTCGCAATCTCCAGTGT		
Luciferase	FW	TGTTGGGCGGTTATTATC	X65316	
	RV	AGGCTGCGAAATGTTCTACT		

^a FW, forward; RV, reverse

MATERIALS

2.1 RNA isolation, cDNA synthesis and RT-qPCR on avian cells.

1. Cells and virus, as per experiment
2. RNeasy Mini Kit (QIAGEN)
3. RNase-free DNase set (QIAGEN)
4. Bioanalyser (Agilent Technologies) or agarose gel electrophoresis equipment.
5. Spectrophotometer (NanoDrop or equivalent)
6. DNase I, Amplification Grade
7. Reverse Transcriptase (Invitrogen SuperScript® III or equivalent)
8. PCR machine (for cDNA synthesis)
9. Nuclease-free water
10. Luciferase mRNA
11. Random hexamers
12. 2X SYBR® Green I mix
13. Quantitative-PCR machine (Qiagen Rotor-Gene Q or equivalent)
14. Primers (Table 1)

2.2 Quantitation of chicken type I IFN protein using a bioassay.

1. Culture medium: DMEM, 10% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin
2. Stimulation medium: DMEM, 1% FCS, 100 IU/ml penicillin 100 µg/ml streptomycin
3. CEC-32 chicken IFN reporter cells in 96-well plates at 70 - 90% confluency (provided by P. Staeheli, *see Note 1*) (149).
4. Recombinant chicken interferon alpha (chIFN α ; Labome)
5. Multichannel pipet (8 x 200 µL)
6. Firefly luciferase assay buffer (Promega Bright-Glo™ or equivalent)
7. Luminometer

METHODS

3.1 RNA isolation, cDNA synthesis and RT-qPCR on avian cells.

1. Perform the experiment in 24-well plates (*see Note 2*). Infect or treat cells as desired.
2. When appropriate, cells are lysed by adding 350 µl RLT buffer spiked with 1 ng/sample of luciferase mRNA prior to RNA isolation. (*see Note 3*)
3. Total RNA is isolated using the RNeasy Mini Kit according to the manufacturer's instructions, including an on-column DNase treatment with RNase-free DNase.
4. Verify RNA integrity on a 1% agarose gel or using a Bioanalyser.
5. Determine RNA concentration using a spectrophotometer (NanoDrop or equivalent).
6. Prior to cDNA synthesis, perform a second DNase digestion step using DNase I.

7. Synthesis of cDNA is performed on 0.5 – 1 µg total RNA using Reverse transcriptase and random hexamers according to the manufacturer's instructions. Incubation steps are performed in a regular PCR machine or, alternatively in a waterbath.
8. After cDNA synthesis, samples are further diluted 1:50 in nuclease-free water before qPCR analysis.
9. Per sample, prepare a master mix containing 7 µl 2x Sybr-Green I Mix and 2µl primer mix (2.1 µM forward and reverse primer).
10. Combine 9µl master mix and 5 µl diluted cDNA per PCR tube.
11. Real-time quantitative PCR is performed on a qPCR machine, such as Rotor-Gene Q, 35-40 cycles, 60 °C annealing temperature, 20 s extension time.
12. Cycle thresholds and amplification efficiencies are calculated using the software pertaining to the qPCR machine, such as Rotor-Gene 6000.
13. Using equation 1 (Eq. 1), the relative expression ratio of the target gene is calculated using the average reaction efficiency for each primer set and the cycle threshold (C_t) deviation of sample vs control at time point 0h (*see Note 4*).

$$R_{\text{Ifn}\beta} = \frac{E_{\text{Ifn}\beta}^{(C_t \text{ Ifn}\beta(\text{calibrator}) - C_t \text{ Ifn}\beta(\text{sample}))}}{E_{\text{GAPDH}}^{(C_t \text{ GAPDH}(\text{calibrator}) - C_t \text{ GAPDH}(\text{sample}))}}$$

With:

- | | |
|------------|--|
| R | = fold change of the target gene relative to the control |
| Calibrator | = control cells at time point 0 (zero) |
| E | = average amplification efficiency for that set of primers |
| C_t | = cycle threshold |

3.2 Quantification of chicken type I IFN protein using a bioassay.

1. If the samples contain virus, heat inactivate at 56 °C for 30 min prior to performing the assay. This treatment inactivates coronaviruses but retains bioactivity of type I IFN (*see Note 5*).
2. Fill a sterile 96-well plate with 50 µl stimulation medium/well.
3. Add 50 µl chIFN α standard (50 U/ml) or test sample to the first row (vortex before adding).
4. Make serial twofold dilutions in the plate using a multichannel pipet (Fig. 1, *see Note 6*).
5. Remove the medium from the CEC-32 cells which have been cultured in the 96-well plate (*see Note 7*).
6. Transfer the content of the plate containing the diluted samples and standard to the CEC-32 cells (*see Note 8*).
7. Incubate plates at 37 °C and 5% CO₂ for 6 h.
8. Use a Firefly luciferase assay kit to detect luciferase activity, according to manufacturer's instructions.

dil. factor	sample #										IFN standard	
	1	2	3	4	5	6	7	8	9	10	IFN α (U/ml)	
2											25	25
4											12.5	12.5
8											6.3	6.3
16											3.1	3.1
32											1.6	1.6
64											0.8	0.8
128											culture medium	
256												

Fig. 1 Layout of a 96-well plate to accommodate ten samples and an interferon standard

3.3 Calculation of IFN concentration from luminescence data

1. To calculate the units of interferon in the original sample, a workflow is provided in Fig. 2.
2. Transfer the measurements from the luminometer to a spreadsheet program (Microsoft Excel or equivalent).
3. Calculate the average value of the background luminescence and subtract this value from all wells (Fig. 2, point A).
4. Calculate the average of the wells incubated with the diluted interferon standard and plot them in a scatter plot. This graph is the standard curve (B).
5. Make a new graph using only the data points that fall within the linear range of the standard curve, usually 1 - 12.5 or 1 - 6 U/ml.
6. Plot a linear trend line through these data points and display the equation on the chart (C).
7. Next, all luminescence values that fall within the linear range of the standard curve are selected (here 2 - 12 U/ml).
8. Calculate the IFN concentration in each well using the equation from the standard curve (D).
9. Multiply by the dilution factor to obtain the concentration of IFN in the undiluted samples (E).
10. Finally, calculate the average IFN concentration of the wells that fall within the linear range (usually two or three wells per sample). This value corresponds to the final concentration of type I interferon in the original sample (F).

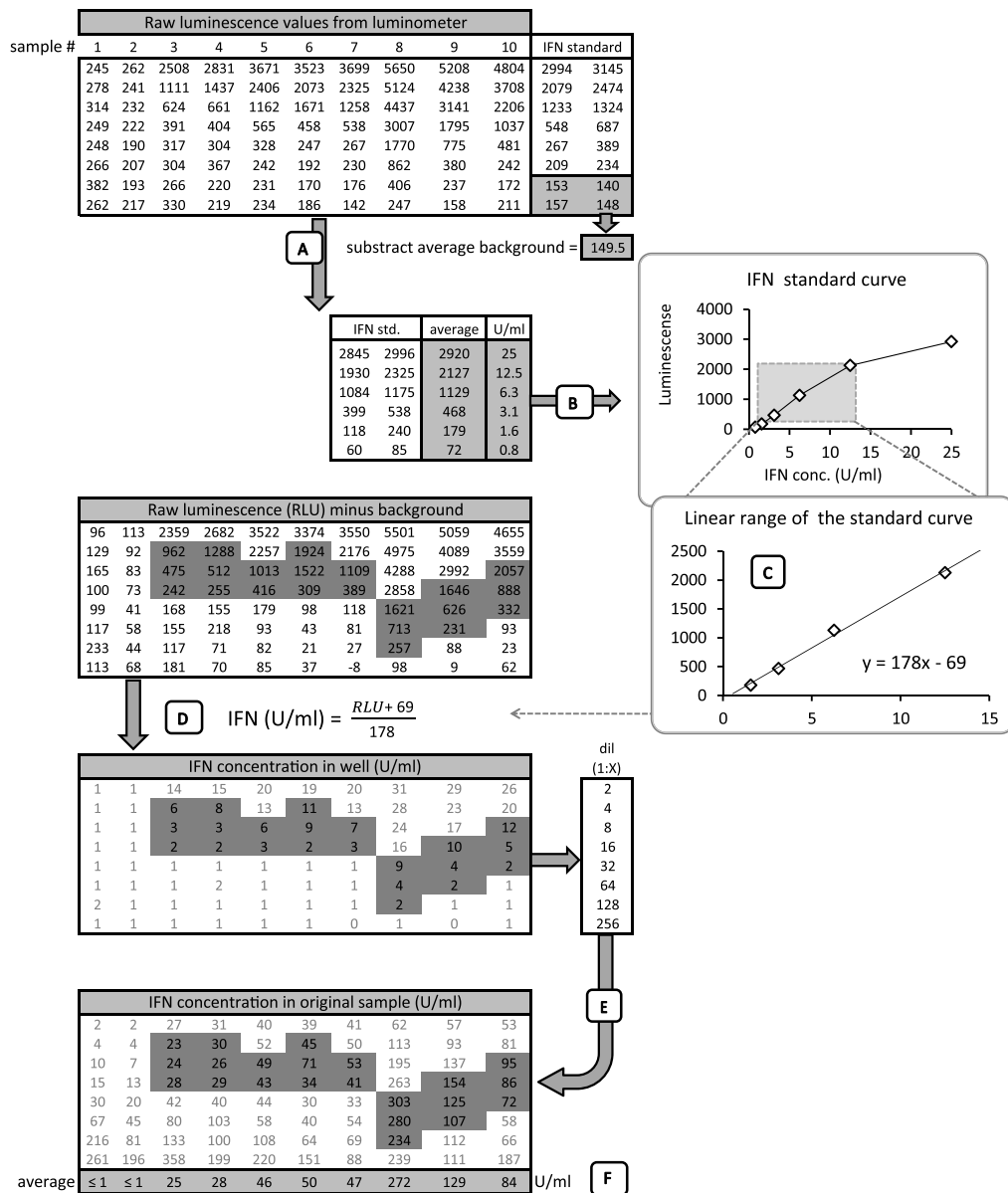


Fig. 2 Workflow on how to calculate the concentration of IFN in the original sample from readout of the luminometer.

Notes

1. For more detail on the construction of CEC-32 chicken-IFN reporter cells, *see ref (149)*.
2. To obtain enough RNA, each well of a 24-well plate should contain around 3×10^5 avian cells.
3. For normalisation, a housekeeping gene such as GAPDH is generally used. It is advised to ensure that the reference gene selected is stable under the conditions of each experiment by performing stability analysis. When the mRNA level of the reference gene is not stable during the experimental procedure, such as during prolonged infection with a virus, we use an external reference gene for normalisation. The external reference gene we use is luciferase, which is added as mRNA (commercially available) to the RLT lysis buffer (1 ng/sample) prior to RNA isolation and cell lysis. This guarantees that the external reference RNA and the host RNA are subject to the same treatment prior to cDNA synthesis
4. To calculate the fold change of IBV total RNA, C_t deviation is calculated versus a fixed C_t value (e.g., $C_t = 30$), because no IBV is present in the non-infected cells that are used as control in all the experiments.
5. Interferon containing samples can be stored at 4 °C overnight. Storage at -20 °C ensures long time (> months) stability. One freeze-thaw cycle can reduce the IFN-activity of a sample by 40%. To avoid repeated freeze-thawing of samples, avoid measuring the same sample twice. To achieve this, ensure that at least one of the dilutions of the samples falls within the linear range of the standard curve.
6. To select the appropriate dilutions it is advisable to perform a pilot experiment. One can either make an extensive twofold serial dilution series (for example 2 - 1024 times dilution), or use tenfold pre-dilutions. In our hands IFN production by avian cells rarely exceeds 5000 U/ml, therefore a maximum of 1000 times pre-dilution should suffice.
7. Medium is removed simply by emptying the 96-well plate in the waste and gently tapping it dry on a stack of tissues. Sterility is not an issue, the cells will only be incubated for another 6 h.
8. CEC-32 cells should *not be allowed to dry out!* Make sure you transfer the samples to the cells within minutes. Drying of the monolayer will decrease the luminescence and thereby negatively influence the assay. When transferring the diluted samples to 96-well plate with CEC-32 cells, start with the lowest concentration and work your way up the dilutions. In this way the same tips can be used for multiple dilutions. Do not forget to add medium to the negative controls.

Acknowledgements

We would like to thank Peter Staeheli for supplying the CEC-32 chicken type I interferon reporter cell line.

Chapter

7

General Discussion

INTRODUCTION

The antiviral type I IFN response (IFN response) has been shown to be crucial for the control of coronavirus infection (65, 84, 85). To secure their persistence in the host, coronaviruses have been evolutionarily selected to evade and antagonise the IFN response. In this chapter, I will discuss our findings on how the infectious bronchitis virus (IBV) counteracts the IFN response within the current landscape of knowledge. To provide a framework for this discussion, the IFN response is divided in four stages, at which we found that IBV antagonises or evades the IFN response (Fig. 1). Firstly, we describe that IBV delays detection by the host (Chapter 2). Secondly, we found that IBV decreases translation of host-proteins including that of IFN (Chapter 4). Thirdly, we observed that IBV inhibits signaling by IFN, and fourthly we conclude that IBV resists the IFN-induced antiviral state (Chapter 3).

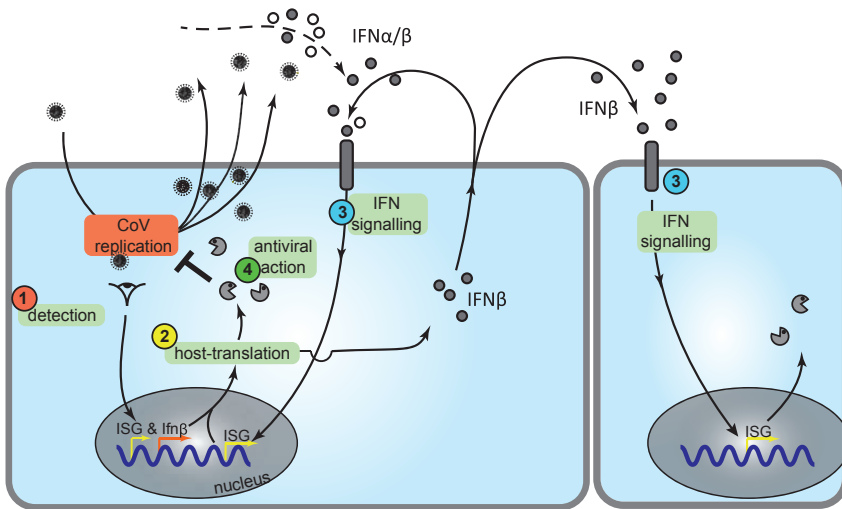
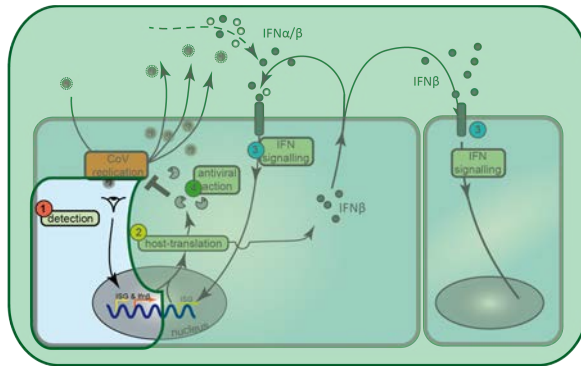


Fig. 1 Antagonism of the type I IFN-response by IBV.

This figure provides a framework for the discussion on the four points at which IBV antagonises the IFN-response as identified in this thesis. 1) IBV delays detection by the host. 2) IBV decreases translation of host-proteins including that of IFN. 3) IBV inhibits signaling by IFN. 4) IBV resists the IFN-induced antiviral state. This figure is included at the start of every paragraph to help guide the reader through the discussion.

In the following paragraphs I will discuss the findings of this thesis for each of the four points at which IBV antagonises the IFN response. In addition, I will discuss some of the remaining questions on how IBV antagonises the IFN response, and I will put forward suggestions on how to answer these questions. Fig. 1 is included at the start of every paragraph to guide the reader through the discussion.



1. IBV DELAYS DETECTION BY THE HOST

A common feature of *Alpha*- and *Betacoronaviruses*, including human coronavirus 229E (HCoV-229E), severe acute respiratory syndrome coronavirus (SARS-CoV), and mouse hepatitis virus (MHV), is their limited activation of the IFN response (88, 90-93). In fact, these coronaviruses have been shown to evade detection by the host-cell in various ways. First of all, coronavirus RNAs are modified by two virus encoded methyltransferases, providing a cap structure that makes them undistinguishable from host mRNAs (reviewed in ref. (155)). In addition to this, coronavirus-infection induces extensive rearrangements of intracellular membranes (reviewed in ref. (95)). These membrane structures have been described for many coronaviruses, including IBV, and they have been suggested to shield virus induced double-stranded RNA (dsRNA) from detection by pattern-recognition receptors (PRRs) (86, 87, 163). Shielding of dsRNA from PRRs is essential for coronaviruses, because dsRNA is produced in large quantities during infection (86, 176, 209). In addition to these hiding strategies, coronaviruses have been shown to block the signaling pathways downstream of PRRs. For example, nsp 3 of both MHV and SARS-CoV inhibits signaling by PRRs through deubiquitination of signaling molecules that are downstream of these PRRs (210, 211). Additionally, the nucleocapsid, ORF3b and ORF6 proteins of SARS-CoV and MHV have all been shown to interfere with activation of the IFN response via unknown mechanisms (reviewed in ref. (125)).

At the start of this thesis, knowledge about activation of the IFN response by IBV was limited. Two transcriptional studies found that infection with IBV induced upregulation of various immune-related genes, but not of *Ifna* or *Ifnb* (type I IFN) in the lungs and trachea of chickens (130, 131). Another study demonstrated upregulation and protein production of *Ifnγ* (type II IFN) but not *Ifna* or *Ifnb* in the lungs of IBV-infected chickens (212). Contrary to *Ifna* and *Ifnb*, production of *Ifnγ* does not depend on activation of intracellular PRRs, as it is mainly produced by NK cells and activated T cells. Taken together, these studies provided no clear evidence for activation of the type I IFN response upon IBV-infection.

In chapter 2 we quantified activation of the type I IFN response *in vitro* and found that IBV-infection induces transcription of *Ifnb* (type I IFN) in chicken epithelial cells, but only after the peak of viral replication. We also show that transcription of *Ifnb* is dependent on the pattern recognition receptor MDA5, showing that MDA5 is the primary PRR involved in detection of IBV. Although virus titres reached a maximum between 18 – 24 hours post infection (hpi), we did not observe transcription of *Ifnb*,

during the first 12 - 18 hours of the infection. Because *Ifnβ* transcription seemed to be delayed in respect to the peak of viral replication, we investigated whether IBV blocks *Ifnβ* transcription during the first 12 hours of infection. Stimulation of IBV-infected cells with dsRNA (poly I:C) or superinfection with other RNA viruses indicated that IBV does not interfere with recognition, signaling, or transcription of *Ifnβ* during the first 12 hours of infection (Fig. 2).

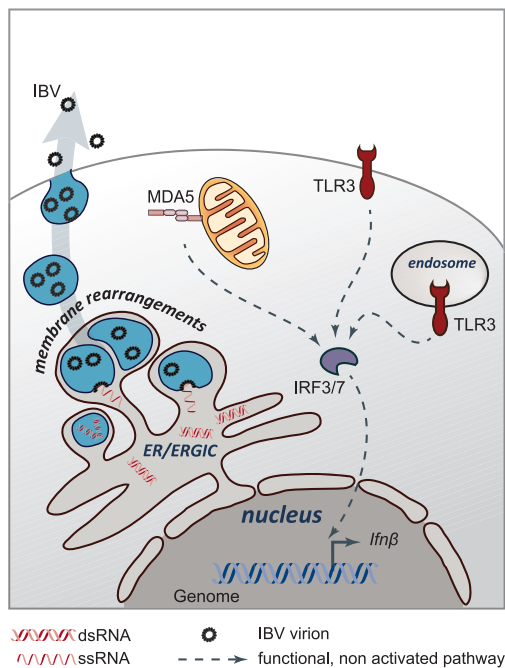
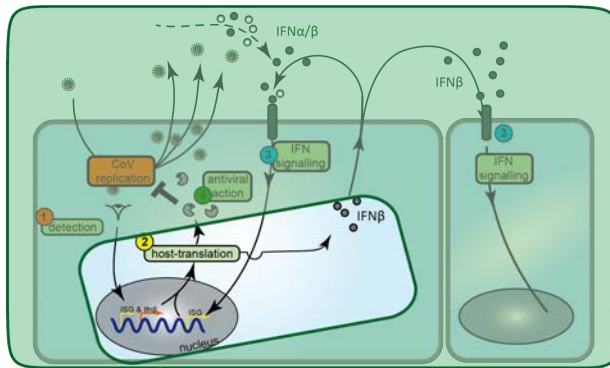


Fig. 2 PRR signaling during IBV infection.

During the first 12 hours of the infection, IBV replicates and new progeny virus is released from the cell. During this period, virus-induced dsRNA accumulates in the cell, but does not lead to activation of the IFN response. This suggests that IBV either avoids or prevents detection of dsRNA by the host cell. Our experiments (chapter 2) suggest that during the first 12 hours signaling of non-self RNA (dashed lines) via RNA pattern recognition receptors (e.g. MDA5 and TLR3) remains fully functional and can be activated by stimulation with dsRNA or heterologous RNA viruses. We suggest that during this period, IBV induced dsRNA is not recognised by PRRs, because it is protected by virus-induced membrane rearrangements.

Our observation that IBV infection does not reduce *Ifnβ* transcription in response to dsRNA or RNA viruses probably does not exclude the possibility that IBV could influence PRR signaling before 12 hpi. The nsp3 protein of MHV for example, has been shown to interfere with activation of the IFN response (213) however, signaling of poly I:C is not inhibited in MHV-infected cells (163). In fact, we investigated whether nsp3 of IBV can interfere with activation of the IFN response. We found that overexpression of IBV-nsp3-GFP did not inhibit activation of an *Ifnβ* reporter (data not shown). This result was unexpected, as inhibition of *Ifnβ*-signaling by nsp3 is mediated via deubiquitination of host proteins, and nsp3 of IBV has recently been shown to have deubiquitinating activity (214). Further research will be needed to definitively establish whether nsp3 of IBV antagonises activation of the type I IFN response.



2. IBV DECREASES PRODUCTION OF ANTIVIRAL PROTEINS INCLUDING IFN.

Many viruses inhibit translation of host proteins (host-translation), a process referred to as host shutoff (114). Host shutoff is beneficial for the virus as it increases the capacity of the host-cell to produce viral proteins. Simultaneously host shutoff hinders the innate immune response by preventing production of antiviral host proteins, including cytokines such as IFN α and IFN β (Fig. 1). Coronaviruses from the *Alpha-* and *Betacoronavirus* genus have also been shown to inhibit translation of host proteins.

In chapter 2 we found that accessory protein 3b of IBV decreases the production of IFN protein late during the infection of chicken cells (36 – 60 hpi). In this chapter we also found that 3a-null virus induces less IFN than the parental virus and much less IFN production than 3b-null virus. We speculate that decreased IFN production by 3a-null virus is the result of increased translation of 3b. Both protein 3a and 3b are translated from the same subgenomic mRNA 3 of IBV and translation of 3b is dependent on ‘leaky scanning’, in which the ribosome skips the first start-codon (IBV-3a) to start translation at the second start codon (IBV-3b) (215). The scrambled start-codon of the 3a null-virus could thus increase translation of 3b, which in turn further inhibits translation of IFN. The expression of the third (E) protein encoded by this tricistronic RNA is probably not influenced by scrambling of the upstream start codon, because translation of this protein is initiated via an internal ribosome entry site (IRES) (216).

In the same experiment we found that infection with 3b-null virus induced high levels of *Ifn β* mRNA at 24 hpi, indicating that IBV imposes an additional block on the translation of *Ifn β* mRNA. Indeed, in chapter 4 we show that IBV-infection induces a general decrease in translation of host-proteins as early as 12 hpi. Using accessory gene null-mutants we further show that accessory protein 5b of IBV is essential for establishment of this host shutoff, although the mechanism by which 5b decreases host translation remains to be investigated. Interestingly, removal of 5b does not affect replication of IBV *in vitro*, indicating that host shutoff is not essential for viral replication.

Like accessory proteins 3b and 5b of IBV, accessory protein 7 of TGEV (TGEV-7) has been shown to decrease transcription and translation of IFN (17). In chapter 2 we report that both TGEV-7 and IBV-3b contain a PP1 binding domain, a domain that was shown to be important for the function of TGEV-7. In apparent contrast to its inhibitory effect on production of IFN, TGEV-7 has been shown

to increase total host-translation, decrease phosphorylation of eIF2 α , and stabilise host-mRNA (16). Thus far, our observations on IBV3b-null virus are in line with the observations on TGEV-7-null virus as both induce increased translation and transcription of IFN and both contain a PP1 binding domain.

Taken together, we conclude that IBV inhibits translation of host proteins, including that of type I IFN. We demonstrate that accessory protein 5b plays an essential role in the overall inhibition of host-translation and we show that accessory protein 3b inhibits production of IFN protein. The mechanism(s) by which IBV-3b and 5b decrease translation of host-proteins merits further investigation. Additionally, it remains to be investigated whether IBV-3b only inhibits production of IFN, or also of other host-proteins. Because of the similarities between our observations on IBV-3b null and previous observations on TGEV-7-null virus, it would be interesting to compare the IBV-3b and TGEV-7 accessory proteins. For example, by investigating whether 3b, like TGEV-7 also stabilises host mRNA, increases general host translation and inhibits phosphorylation of eIF2 α .

Potential role of stress-granules and processing bodies in IBV-induced host shutoff.

In chapter 4 we demonstrated that IBV inhibits protein production, without inducing degradation of host mRNA. Interestingly, some host mRNAs, such as that of *Irf3* showed increased stability in IBV-infected cells. The reason for this increased stability is unclear, but one possible explanation could be that host mRNAs are recruited to stress granules (SG) in IBV infected cells. SGs are temporary repositories of mRNAs and they are formed in response to stress-induced translational arrest (217, 218). Stress granules have been shown to prevent degradation of mRNAs by cellular ribonucleases. Increased phosphorylation of eIF2 α , which frequently accompanies virus-induced host shutoff, is one of the triggers for SG formation (219). Many viruses have been shown to modulate formation of SG, but in most cases it is unclear whether SG formation is beneficial to the host or to the virus (reviewed in (220)). Stress-granules have been observed in TGEV and MHV infected cells (115, 221) and in the case of TGEV, formation of SG coincided with decreased viral RNA synthesis, suggesting that SGs are detrimental to the virus. To evaluate the possible role of SG during IBV infection, we studied SG formation in IBV infected cells with an antibody specific for the stress granule-marker Ras GTPase-activating protein-binding protein 1 (G3BP) (Fig. 3).

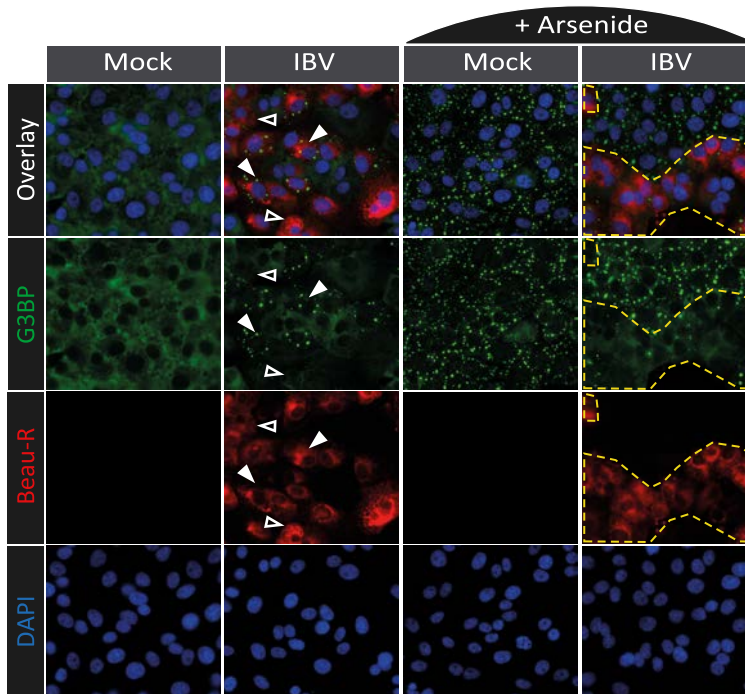


Fig. 3 IBV modulates formation of stress granules.

Vero cells were infected with IBV at MOI 0.1 or left untreated (mock). At 18 hpi cells were either left untreated (mock) or stimulated with arsenide for 30 minutes to induce formation of stress granules. Subsequently, cells were stained with antibodies against the nucleocapsid of IBV and against G3BP, a marker for stress granules. White arrowheads indicate IBV-infected cells that do not contain stress granules, and open arrowheads indicate IBV-infected cells that contain stress granules. The dotted line in the right column of images delimits an area of IBV-infected cells to show that IBV inhibits formation of canonical (arsenide induced) stress granules.

Our data showed that at aggregates of G3BP could be detected in a subset (~10 - 25%) of IBV-infected Vero cells. Next, we induced formation of canonical SGs in IBV infected cells and found that formation of canonical stress granules was strongly reduced in IBV-infected cells.

These results indicate that IBV modulates the localisation of G3BP and inhibits the formation of canonical SGs. Preliminary experiments indicate that IBV-induced SG-like aggregates of G3BP are in fact different from canonical SGs. Unlike canonical, arsenide induced stress granules, IBV-induced SG-like aggregates did not dissolve upon treatment with cycloheximide (data not shown). Our observations on SG formation in IBV-infected cells are highly reminiscent of observations made on vaccinia virus lacking the E3L double-stranded RNA (dsRNA)-binding protein (vaccinia Δ E3L), which also induced non-canonical SGs (222). In this study SG formation was associated with increased phosphorylation of eIF2 α , decreased host-translation and decreased virus replication (222).

To evaluate if modulation of stress-granules is essential for inhibition of host-translation by IBV, we investigated SG-modulation by IBV-5a/5b null-virus, because this virus allows more translation than the parental virus (chapter 4). We found no differences in aggregation of G3BP- or inhibition of arsenide-induced SG formation between cells infected with 5a/5b null-virus or the parental virus (data not shown).

In short, IBV modulates SG formation, but accessory protein 5b, which inhibits host-translation does not seem to be involved in modulation of SG formation. These results indicate that inhibition of translational by IBV does not depend on activation or inhibition of stress granule-formation.

MHV infection induces a general decay of host-mRNAs, which has been associated with the formation of processing bodies (PB) (115). processing bodies are cytoplasmic granules present in all eukaryotic cells and are linked to mRNA silencing and degradation (218, 223). IBV infection does not induce general degradation of host-mRNA, which might suggest that, contrary to MHV, IBV does not induce formation of processing bodies. Using immunofluorescent microscopy, we investigated the localisation of 5'-3' exoribonuclease 1 (XRN1), which is a marker for processing bodies (Fig. 4).

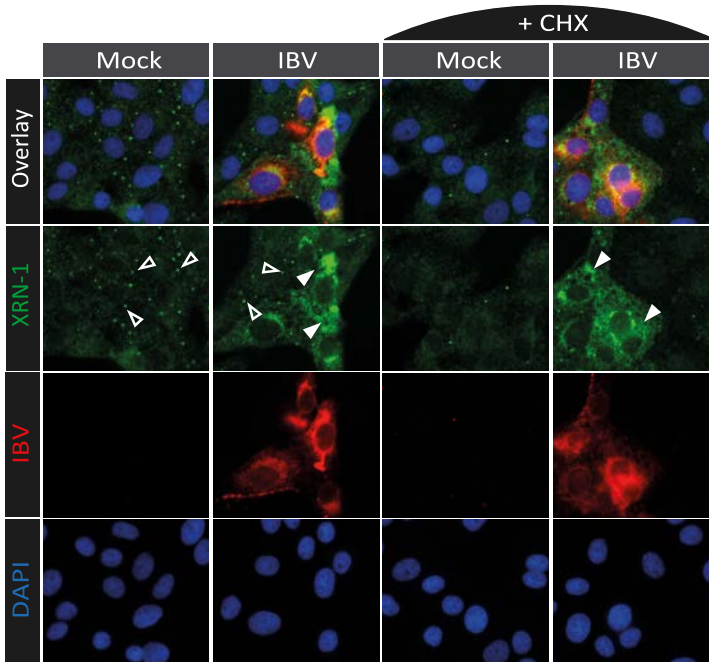
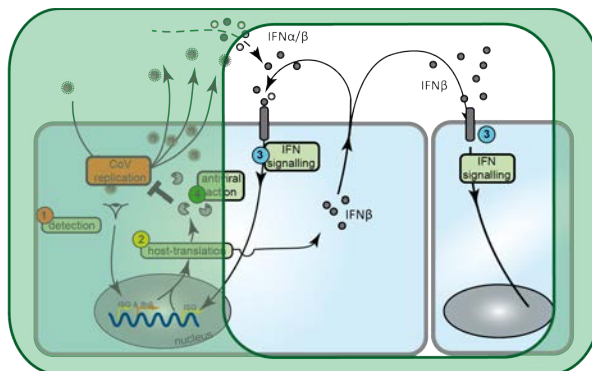


Fig. 4 IBV modulates distribution of processing bodies

Vero cells were infected with IBV at MOI 0.1 or left untreated (mock) at 18 hpi cells were either mock treated or stimulated with cycloheximide (CHX) for 30 minutes to dissolve processing bodies. Subsequently, cells were stained with antibodies against the nucleocapsid of IBV and against the exonuclease XRN1, a marker for P-bodies. Open arrowheads indicate non-infected cells showing canonical localisation of XRN1 in processing bodies. White arrowheads indicate IBV-infected cells that show non-canonical localisation of XRN1.

In mock treated cells, XRN1 is localised in a limited number of discrete cytoplasmic foci, whereas in IBV-infected cells XRN1 was more abundant and differently localised compared to mock treated cells. Treatment of cells with cycloheximide (inhibitor of translation) dissolves canonical processing bodies, leading to a diffuse localisation of XRN1 (223). In IBV-infected cells cycloheximide did not induce significant alteration in the localisation of XRN1, suggesting that except stress granules, IBV also modulates formation of processing bodies. Processing bodies are sites of mRNA degradation, and although speculative, modulation of PB formation by IBV could reduce degradation of host mRNAs. This would offer an explanation for increased stability of host mRNA in IBV-infected cells observed in chapter 4.

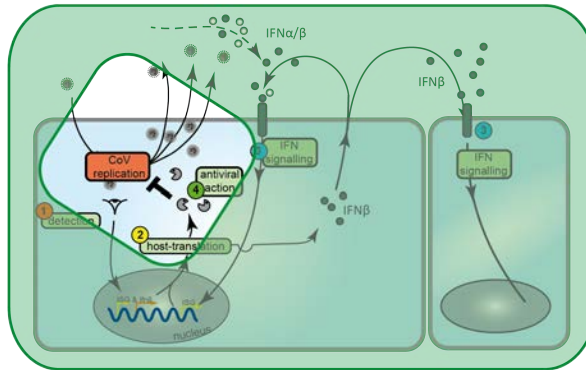


3. IBV INHIBITS IFN-SIGNALING.

In chapter 3 we show that IBV blocks IFN-induced signaling by inhibiting phosphorylation and subsequent nuclear translocation of STAT1. Phosphorylation of STAT1 is the first step in activation of its function as transcription factor and is mediated by two Janus kinases, JAK1 and TYK2. The mechanism by which IBV inhibits STAT1-phosphorylation is unclear; however, as phosphorylation of STAT1 is dependent on JAK1 and TYK2 it is reasonable to assume that IBV somehow interferes with the function of these kinases. We did not observe STAT1-degradation products in Western blot, therefore we can exclude that reduced phosphorylation of STAT1 is caused by degradation of the protein. To elucidate how IBV inhibits phosphorylation of STAT1 will require the identification of the viral protein(s) that mediate this inhibition. In chapter 3 we exclude the involvement of the accessory proteins of IBV by showing that IBV-3a/3b and IBV-5a/5b null viruses also inhibit phosphorylation of STAT1. The next step would be to test whether the individual structural and non-structural proteins of IBV inhibit phosphorylation and translocation of STAT1 when over-expressed in Vero cells. The IBV protein responsible for inhibition of STAT1 phosphorylation could then be used to identify interacting host proteins, which could provide clues on the potential mechanism of inhibition.

In chapter 3 we also describe that STAT1-signaling is not inhibited during the first 12 hours of IBV infection in Vero cells. The first progeny virus is released approximately 10 hours after IBV-infection of Vero cells, which is about four hours later than in chicken cells (4, 172). The replication cycle of IBV in Vero cells is thus completed within 10 hours, and except for the accessory proteins, all IBV-proteins are required for generation of progeny virus (15, 224). As a consequence, all IBV proteins are present in Vero cells before 10 hpi, indicating that the time-dependency of STAT1-inhibition (not before 12 hpi) must be the result of a post-translational event. One possibility is that the localization of viral protein(s) that influence STAT1-phosphorylation changes over time, possibly by disintegration of virus-induced membrane structures.

In support of this hypothesis, we observed that activation of *Ifnβ* transcription by double stranded RNA or heterologous RNA viruses is not inhibited during the first 12 hours of IBV infection (chapter 2, ref. (176)). Combined, these observations suggest that during the first 12 hours of infection, IBV effectively 'hides' from the host-cell and does not inhibit signaling from the interferon receptor or PRRs. Later during the infection, virus induced membrane structures may disintegrate, thereby allowing detection of IBV, and re-localisation of host or viral proteins to inhibit STAT1 signaling.



4. IBV ANTAGONISES THE IFN-INDUCED ANTIVIRAL STATE.

In this thesis, we find that IBV efficiently inhibits the production of IFN *in vitro*, suggesting that IBV does not encounter high concentrations of IFN during infection. If this were the case, IBV does not need to be resistant to the IFN induced antiviral state. However, during *in vivo* infection this situation might be different, as birds are often simultaneously infected with various pathogens. In fact, IBV-associated mortality is almost exclusively observed in birds infected with another virus, bacterium or mycoplasma. Such co-infections occur frequently, as IBV-infection compromises epithelial barriers and thereby facilitates infection by other pathogens. Within this context it is likely that IBV might frequently encounter high concentrations of IFN and thus needs to be resistant to the IFN-induced antiviral state. In line with this view, coronaviruses such as MCoV and feline coronavirus (FeCoV) have been shown to be relatively resistant to IFN treatment *in vitro* (93, 113). Although the mechanisms by which these viruses resist IFN treatment is poorly understood, it has been shown that several accessory proteins of coronaviruses are important for resistance to IFN treatment. Previous studies on IBV have demonstrated that plaque formation (168) and syncytia formation (169) is reduced by IFN treatment, but quantitative data on IFN sensitivity of IBV is lacking.

In chapter 3 we describe that IBV is relatively resistant to IFN treatment. In addition, we show that IBV-3a/3b null virus is less resistant to IFN treatment. The observation that IBV-3a/3b null virus is more sensitive to IFN, raises the question as to what extent individual 3a and 3b proteins contributed to the IFN resistance of IBV-3a/3b null virus. To address this question we investigated the resistance of IBV-3a/3b, IBV-3a and IBV-3b null viruses to IFN treatment in DF-1 cells by quantifying viral genomes and infectious particles in the supernatant 24 hours post infection. Our preliminary results (Fig. 5), indicate that both the 3a- and 3a/3b null viruses were less resistant to IFN treatment than the parental virus, whereas IFN-resistance of the 3b-null virus was comparable to the parental virus. These results indicate that accessory protein 3a of IBV is primarily responsible for resistance to IFN. Interestingly, during infection of primary trachea organ culture (TOC), the titre of both IBV-3a null and IBV-3a/3b null viruses declined more rapidly than the titre of the parental virus or IBV-3b null virus (15). In view of our findings, the decrease in titre of IBV-3a null and IBV-3a/3b null viruses in TOC could be the result of increased sensitivity of both viruses to IFN produced by cells of the TOC.

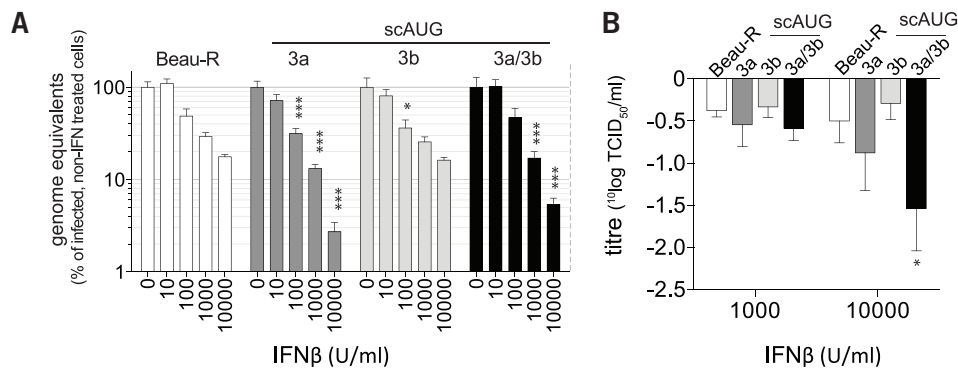


Fig. 5 Accessory protein 3a contributes to IFN resistance in chicken cells.

DF-1 cells were stimulated with chicken IFN α or IFN β for 6 hours and subsequently infected with Beau-R or accessory protein null viruses (scAUG) at MOI 0.01 in the presence of IFN. (A) At 24 hpi, virus was quantified by RT-qPCR using primers against IBV-N on RNA isolated from cell supernatant. Bars represent the percentage of genome equivalents relative to non-IFN treated cells infected with the same virus. Error bars indicate standard deviation and asterisks indicate significant differences (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) compared to the parental Beau-R virus as determined using a two-way ANOVA followed by a Holm-Sidak post-hoc test. (B) In a duplicate experiment, virus in the supernatant was quantified by titration and the titre relative to non-IFN treated cells was calculated. Values represent the mean of triplicate wells from a representative experiment, error bars indicate standard deviation and asterisks indicate significant differences ($P < 0.01$) compared to the parental Beau-R virus as determined using a two-way ANOVA followed by a Holm-Sidak test for multiple-comparisons.

Currently, we can only speculate about the mechanism by which IBV-3a antagonises the effect of the IFN-induced antiviral state. For MHV it was demonstrated that its accessory protein ns2, antagonises the 2'-5'-oligoadenylate synthetase (OAS) ribonuclease (RNase) L pathway (109). This pathway is a potent antiviral response that is activated by double-stranded RNA. Interestingly, accessory protein 3a has previously been shown to partially co-localise with dsRNA in IBV-infected chicken cells, which could indicate that 3a, just as MHV ns2 antagonises the OAS-RNase L antiviral pathway (181).

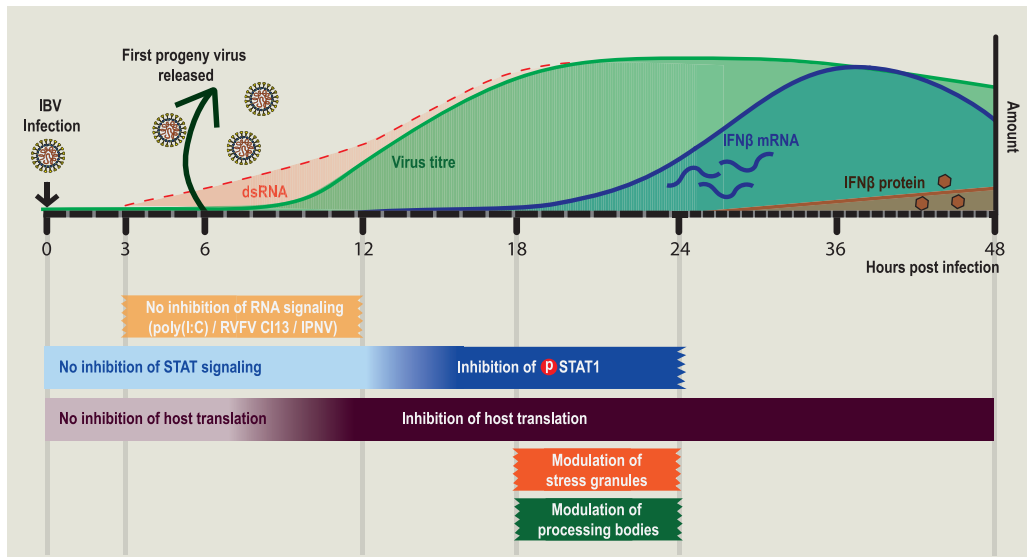


Fig. 6 Timing of events during IBV-infection in vitro.

To visualise the temporal relationship between the findings described in this thesis, we provide a schematic overview of the timing of events that (may) influence the IFN response during IBV-infection in vitro. We indicate in which cell type the finding was made, because kinetics of infection are different between chicken and Vero cells. At timepoint 0, cells are infected by IBV. At three hours post infection (hpi), dsRNA can be detected in chicken cells (Chapter 2). The first progeny virus starts to be released from infected chicken cells around 6 hpi – 10 hpi in Vero cells- (4). Between 6 and 12 hpi, inhibition of host-translation by IBV is detected in Vero cells (Chapter 4). From 6 hpi until 18 hpi the number of infectious viruses in the supernatant of chicken cells increases exponentially (Chapter 2). Transcription of *Ifnβ* in chicken cells starts around 18 hpi (Chapter 2) During the first 12 hours of the infection, signaling of dsRNA, heterologous RNA viruses (chicken cells), and type I IFN (STAT signaling –Vero cells) is not inhibited. At 18-24 hpi IBV modulates the formation of stress granules and processing bodies in Vero cells (data about modulation of these structures before and after this period is lacking). Starting at 36 hpi, limited amounts of IFN protein are detected in the supernatant of chicken cells.

6. DESIGN OF RATIONALLY ATTENUATED VACCINES FOR IBV: IMPLICATIONS OF OUR FINDINGS.

In this thesis we show the involvement of three of the four accessory proteins of IBV in antagonism of the type I interferon response. Proteins 3b and 5b inhibit production of IFN protein and 3a is most probably responsible for increased resistance to IFN. The interferon antagonistic properties of accessory proteins 3a, 3b and 5b could make them interesting targets for rational attenuation of IBV. However, the work described in this thesis was carried out *in vitro*, using cell lines and primary cells isolated from chickens and chicken embryos. In addition to this, all conclusions regarding the functions of the accessory proteins of IBV in this thesis are based on experiments using the IBV Beaudette strain, which is attenuated and non-pathogenic *in vivo*. To investigate the role of the accessory proteins on the pathogenicity of IBV *in vivo*, will require the generation of pathogenic strains of IBV such as M41 or QX, that do not express one or more accessory proteins.

It will be very interesting to see what influence deletion of the accessory proteins of IBV strains such as M41 or QX will have on their pathogenicity in chickens. In general, deletion of coronavirus accessory proteins leads to attenuation of the virus in the natural host (11, 12, 186). This has been confirmed for the accessory proteins of MHV, FeCoV and TGEV. Field isolates of IBV that lack three or four of the canonical accessory proteins have been identified, and these viruses were attenuated *in vivo* (35). This observation suggests that deletion of one or more accessory proteins of pathogenic IBV viruses will indeed lead to their attenuation *in vivo*.

It remains to be investigated whether the functions we describe in this thesis for the accessory proteins of IBV Beaudette, translate to pathogenic strains of IBV, such as M41 or QX. To visualise the differences between the accessory proteins of different *Gammacoronaviruses*, we aligned the protein sequences of the accessory proteins of turkey coronavirus (Turkey-CoV), duck coronavirus (Duck CoV) and four IBV strains, including M41 and QX. The result of these alignments is summarised in similarity and identity matrices (Fig. 7).

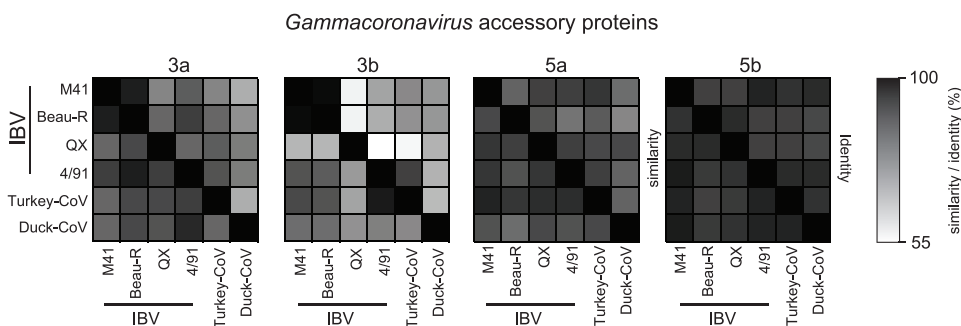


Fig. 7 Identity and similarity matrices of *Gammacoronavirus* accessory proteins.

The protein sequences of six *Gammacoronaviruses* were aligned using ClustalW. The shade of grey indicates the percentage of similarities and identity between the protein sequences of the indicated viruses. Genbank numbers of the sequences used in the alignment: M41, AY851295; Beau-R, NC_001451; QX, JQ088078; 4/91, KF377577; Turkey CoV, EU022526; Duck CoV, JF705860.

When comparing the amino-acid identities of the accessory proteins of different *Gammacoronaviruses*, it becomes apparent that 5b is the most conserved of the four accessory proteins (minimum of 88% identity). Accessory protein 3b on the other hand shows the least degree of similarity between the different viruses (minimum of 56% identity). The identity matrices also show a high degree of amino acid identity between the accessory proteins of the apathogenic Beaudette (Beau-R) and the pathogenic M41 strain. This may indicate that our findings on the accessory proteins of Beau-R could translate to the accessory proteins of pathogenic IBV strains such as M41.

In view of the results presented in this thesis, preventing expression of one or more accessory proteins of pathogenic IBV viruses may possibly attenuate these viruses to such an extent that they can be used as a live vaccine strain. However, the fact that all accessory proteins are located in the same region of the viral genome increases the risk of 'reversion to virulence'. In theory, an accessory protein-null virus could recombine with another IB virus in the field and regain its accessory proteins and thus, its pathogenicity. To minimise this risk, additional attenuating mutations could be introduced in distant parts of the genome such as the replicase gene. IBV viruses lacking accessory proteins could possibly even prove safe enough for vaccination *in ovo*. During *in ovo* vaccination the rationally attenuated live IB virus would be injected into the egg a few days before hatching. Because vertical transmission of IBV (from chicken to egg) has never been reported, the chances that a live attenuated vaccine would recombine with field strains during *in ovo* vaccination are very small.

In conclusion, the work described in this thesis demonstrates for the first time how IBV evades, activates, and antagonises the IFN response. It is the first study that describes a function for the IBV accessory proteins and shows that they play a role in antagonism of the type I IFN response.

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Summaries and acknowledgements

Summary

Samenvatting

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SUMMARY

Viruses are intracellular parasites that exploit the machinery of the host cell to replicate. To defend themselves against invading viruses, animal cells have evolved an anti-viral mechanism, known as the type I interferon response. Through natural selection viruses have in turn evolved mechanisms to counteract or evade the type I IFN response. Coronaviruses are a large group of positive-stranded RNA viruses that cause a range of human and veterinary diseases. Infectious bronchitis virus (IBV) is a member of the genus *Gammacoronavirus* and it is the causative agent of a highly contagious respiratory disease of poultry. To date, only few studies have investigated the interaction between IBV and the type I IFN response.

In this thesis, we describe for the first time the activation of the type I interferon response (IFN response) by the *Gammacoronavirus* IBV, and the repressive role of accessory proteins therein. In **Chapter 1** I provide a general introduction into coronaviruses in general and the *Gammacoronavirus* IBV in particular. I also introduce the IFN response, and highlight differences between the mammalian and chicken IFN response. Finally, I review current knowledge on the roles of coronavirus accessory proteins in counteraction of the IFN response. In **Chapter 2** we describe our studies which demonstrated that activation of the IFN response by IBV is dependent on the intracellular double-stranded RNA sensor MDA5. We show that detection of IBV-infection by MDA5 is delayed with respect to the peak of viral replication, and demonstrate that this delay is not due to inhibition of dsRNA detection by IBV. Using mutant viruses that cannot express accessory proteins (null viruses), we found that accessory proteins 3a and 3b of IBV mediate transcription and translation of *Ifn β* mRNA.

The observation that IBV delays the activation of the IFN response, prompted us to investigate the sensitivity of IBV to IFN treatment in **Chapter 3**. Here we show that IBV is relatively resistant to treatment with type I IFN, as relatively high doses of type I IFN are required to decrease propagation of the virus. Next, we studied which viral protein(s) contribute to resistance of IBV to type I IFN and found that absence of accessory proteins 3a and 3b increased sensitivity of IBV to type I IFN, via a presently unknown mechanism. In addition, we observed that independent of accessory proteins 3a and 3b, IBV blocks signaling of IFN by inhibiting phosphorylation and translocation of the transcription factor STAT1. To explain the delayed kinetics of IFN production observed in Chapter 2, we investigated whether delayed protein production was restricted to IFN, or whether IBV, like *Alpha-* and *Betacoronaviruses*, inhibits general translation of host proteins (i.e. induces host shutoff). In **Chapter 4** we demonstrate that IBV-induced transcription of *Ifn β* mRNA leads to the production of relatively little IFN protein. We discovered that limited production of IFN protein by IBV-infected cells is the result of general inhibition of host translation, confirming that IBV induces a shutoff of host-protein production. This finding indicates that evasion of the innate immune system by *Gammacoronaviruses* may be more similar to that of *Alpha-* and *Betacoronaviruses* than previously thought. Using accessory protein null viruses we discovered that accessory protein 5b of IBV is essential for the inhibition of host-protein synthesis by IBV. In **Chapter 5** and **Chapter 6** we describe the methods used in this thesis to quantify the number of infectious virus particles of IBV as well as methods used to quantify the activation of the type I IFN response in chicken cells. Although the studies described in this thesis have answered several questions about the interaction of IBV with the type I IFN response of its host, they have also raised new questions to be addressed in future research. In the final

Chapter of this thesis (**Chapter 7**), I discuss a number of remaining questions and future perspectives regarding evasion of the IFN response by IBV. Finally, I explore the possible implications of our findings on the *in vivo* pathogenicity of IBV and on the rational design of attenuated IBV vaccines.

In conclusion, the work described in this thesis demonstrates for the first time how IBV evades, activates, and antagonises the IFN response. Also, this thesis comprises the first study that describes a function for the accessory proteins of IBV and shows that these poorly understood proteins play an important role in antagonism of the type I IFN response.

SAMENVATTING

Virussen zijn intracellulaire micro-organismen die de machinerie van hun gastheer cel nodig hebben om zichzelf te repliceren. Om zich te verdedigen tegen binnendringende virussen hebben dierlijke cellen verscheidene anti-virale verdedigingsmechanismen ontwikkeld, waarvan de ‘type I interferon respons’ één van de belangrijkste is. Door natuurlijke selectie hebben virussen op hun beurt manieren gevonden om de type I interferon respons tegen te gaan of te ontwijken. Coronavirussen zijn de veroorzakers van verschillende ernstige menselijke- en veterinaire ziekten. Ze worden gekenmerkt door hun erfelijk materiaal wat bestaat uit een enkel, zeer groot, enkelstrengs positief-RNA molecuul. Het infectieuze bronchitis virus (IBV) is een coronavirus van het genus *Gammacoronavirus*, en veroorzaakt één van de meest besmettelijke respiratoire pluimvee ziektes.

Tot op heden zijn slechts weinig studies uitgevoerd naar de interactie tussen IBV en de type I interferon respons (IFN respons). In dit proefschrift beschrijven we voor de eerste keer de activatie van de IFN respons door IBV, en de repressieve rol die de accessoire eiwitten van IBV hierin spelen. In **Hoofdstuk 1** introduceer ik: coronavirussen, de werking van de IFN respons en de verschillen tussen de IFN response bij zoogdieren en kippen. Daarnaast geef ik in een overzicht aan wat er bekend is over de rol van de accessoire eiwitten van *Alpha-* en *Betacoronavirussen* in het tegengaan van de IFN respons.

In **Hoofdstuk 2** tonen we aan dat activering van de IFN-respons in kippencellen tijdens een IBV-infectie afhankelijk is van de intracellulaire sensor MDA5 welke aanwezig is in de gastheer cel. We laten ook zien dat detectie van IBV-infectie door MDA5 vertraagd is ten opzichte van de piek van virale replicatie. We tonen daarnaast aan dat de vertraging van de herkenning van IBV niet te wijten lijkt te zijn aan inhibitie van MDA5. Met behulp van mutante virussen die geen accessoire eiwitten kunnen maken, laten we zien dat accessoire eiwitten 3a en 3b van IBV betrokken zijn bij het reguleren van transcriptie en translatie van type I IFN.

De waarneming dat IBV de activatie van de IFN-respons vertraagt, bracht ons ertoe om in **Hoofdstuk 3** de gevoeligheid van IBV voor de aanwezigheid van IFN te onderzoeken. Het bleek dat IBV relatief ongevoelig is voor behandeling van gastheer cellen met type I IFN, aangezien zeer hoge doses IFN nodig zijn om de groei van IBV te reduceren. Vervolgens hebben we onderzocht welke virale eiwitten bijdragen aan de weerstand van IBV tegen IFN. We vonden dat accessoire eiwitten 3a en 3b van IBV ook belangrijk zijn voor de ongevoeligheid voor IFN, aangezien het ontbreken van 3a en 3b zorgt voor verhoogde gevoeligheid van IBV voor IFN. Het mechanisme waarmee 3a en 3b de IFN response tegengaan is vooralsnog onbekend. Daarnaast vonden we dat, zelfs zonder 3a en 3b, IBV de signalering van IFN tegen gaat door het remmen van fosforylatie en translocatie van de IFN-geactiveerde transcriptiefactor STAT1.

In **Hoofdstuk 2** vonden we dat IFN productie tijdens IBV infectie vertraagd wordt. Om een verklaring te vinden voor deze vertraging hebben we onderzocht of IBV de productie van alleen IFN uitstelt of dat het virus de algemene productie van eiwitten door de gastheer cel uitschakelt, net als *Alpha-* en *Betacoronavirussen* dat doen. In **Hoofdstuk 4** vonden we dat late activatie van $I\text{f}\beta$ transcriptie na IBV-infectie vrijwel niet leidt tot productie van IFN eiwit. We ontdekten dat de beperkte productie van IFN eiwit door IBV-geïnfecteerde cellen een gevolg is van de algemene inhibitie van translatie van mRNA naar eiwit door de gastheer cel. Deze bevinding laat voor de eerste keer zien dat *Gammacoronavirussen*, net als *Alpha-* en *Betacoronaviruses* eiwitsynthese in de

gastheer cel onderdrukken. Met behulp van de eerdergenoemde mutante virussen ontdekten we dat het accessoire eiwit 5b van IBV essentieel is voor het remmen van de eiwitsynthese in de gastheer cel.

In **Hoofdstuk 5** en **Hoofdstuk 6** beschrijven we in detail de procedures waarmee we infectieuze virusdeeltjes van IBV hebben gekwantificeerd en beschrijven we de methoden om de activering van de type I IFN-respons in kippencellen te kwantificeren.

Hoewel dit proefschrift meerdere vragen beantwoordt over de interactie van IBV met de type I IFN respons van zijn gastheer cel, roept het ook nieuwe vragen op. In het laatste hoofdstuk van dit proefschrift (**Hoofdstuk 7**) bespreek ik een aantal van deze vragen, evenals voorlopige resultaten van experimenten om ze te beantwoorden. Tenslotte belicht ik de mogelijke implicaties van de bevindingen van onze *in vitro* experimenten voor de pathogeniciteit van IBV *in vivo* en de daaruit voortvloeiende ontwikkeling van nieuwe-, rationeel verzwakte IBV vaccins.

In dit proefschrift tonen we voor het eerst aan hoe IBV de type I IFN respons ontwijkt, activeert en tegenwerkt. Het is bovendien de eerste studie welke een functie beschrijft van de accessoire eiwitten van IBV en daarmee laat zien dat deze verder slecht gekarakteriseerde eiwitten een belangrijke rol spelen bij het tegengaan van de door IBV geïnduceerde IFN-respons.

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Was getekend,

Joeri Kint

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About the author

List of publications

Training activities

Curriculum Vitae

LIST OF PUBLICATIONS

Accessory protein 5b of infectious bronchitis virus inhibits production of host protein, including IFN

Joeri Kint, Martijn A. Langereis, Helena J. Maier, Paul Britton, Frank van Kuppeveld, Joseph Koumans, Geert F. Wiegertjes, Maria Forlenza
Manuscript in preparation

Infectious bronchitis virus inhibits STAT1 signaling and requires accessory proteins for resistance to type I IFN activity in chicken cells

Joeri Kint, Annemiek Dickhout, Jasmin Kutter, Helena J. Maier, Paul Britton, Joseph Koumans, Geert F. Wiegertjes, Maria Forlenza
Submitted for publication

Quantification of infectious bronchitis coronaviruses by titration in vitro and in ovo

Joeri Kint, Helena J. Maier, Erik Jagt
Methods in Molecular Biology, 2015 Volume 1282 pages 89 - 98

Quantification of interferon signaling in avian cells

Joeri Kint, Helena J. Maier, Maria Forlenza
Methods in Molecular Biology, 2015 Volume 1282 pages 251 – 259

Activation of the chicken type I interferon response by infectious bronchitis coronavirus

Joeri Kint, Marcela Fernandez-Gutierrez, Helena J. Maier, Paul Britton, Martijn A. Langereis, Joseph Koumans, Geert F. Wiegertjes, Maria Forlenza
Journal of Virology, 2015 Volume 89 no. 2 pages 1156 - 1167

Targeting Membrane-Bound Viral RNA Synthesis Reveals Potent Inhibition of Diverse Coronaviruses Including the Middle East Respiratory Syndrome Virus

Lundin A, Dijkman R, Bergström T, Kann N, Adamiak B, Hannoun C, Kindler E, Jónsdóttir H R, Muth D, **Kint J**, Forlenza M, Müller M A, Drosten C, Thiel V, Trybala E
PLoS Pathogens, 2014, Volume 10, Issue 5, page e1004166

TRAINING ACTIVITIES

Conferences and meetings

- Vaccine Technology II, Albufeira, Portugal, 2008
- NCMLS New Frontiers in Pattern Recognition, Nijmegen, 2009
- 3rd Dutch Stem Cell Meeting, Utrecht, 2010
- XIIth International Nidovirus Symposium, Acme, Michigan, USA, 2011
- Dutch Society for immunology (NVVI) conference, Noordwijkerhout, 2010
- 12th Congress of the International Society of Developmental and Comparative Immunology, Fukuoka, Japan, 2012
- XIIIth International Nidovirus Symposium, Salamanca, Spain, 2014
- Annual WIAS science day, Wageningen 2011 - 2014
- WIAS seminar: It makes sense to know your enemy, 2010
- WIAS seminar: the embryonic life of chickens: factors that influence development, 2010
- WIAS seminar: Allergenicity in food allergy, 2011
- WIAS seminar: Mucosal factors regulating allergy, 2013
- Dutch Annual virology symposium, Amsterdam, 2010 - 2014

Presentations

- WIAS Science Day, awarded the annual WIAS presentation prize, 2013
- 12th Congress of the International Society of Developmental and Comparative Immunology, Fukuoka, Japan, 2012
- Invited lecture at Laboratory of Virology, Wageningen University, 2012
- Invited lecture at Molecular Virology Laboratory, Leiden University Medical Center, 2011

Courses, workshops and teaching

- WIAS Introduction Course, Wageningen University, 2010
- Philosophy of science and ethics course, Dieren, 2010
- New cells for new Vaccines IV, Wilmington, USA, 2009
- Fish Immunology Workshop, Wageningen, 2010
- Research and technology in bacterial vaccine development, MSD AH, Boxmeer, 2010
- Advanced Immunology course, Utrecht University, 2011
- Dutch Society for Immunology (NVVI) Course, Lunteren, 2013
- Flow Cytometry, BD Biosciences, Wageningen University, 2012
- Design of Experiments course, Boxmeer, 2007
- High-Impact Writing in Science, Wageningen University, 2013
- Intellectual property and business development - MSD Animal health, Boxmeer, 2012
- Writing PhD research proposal, 2010
- Supervision of three Bachelor and two Master thesis students, 2010 - 2014
- Supervision of internship Master student, 2014

Total study load: 44.5 ECTS (approximately 1250 hours)

CURRICULUM VITAE

Wat weinig mensen weten is dat, naast de wereldberoemde acteur en zanger Nicholas Tse uit Hong Kong, op 29 Augustus 1980 nóg een grootheid geboren werd: de auteur van dit boek.

Joeri Kint zag het licht te Hulst in Zeeuws-Vlaanderen als zoon van secretaresse Trees en elektricien Jo. Joeri ging naar de katholieke lagere school St. Willibrordus, was zelfs misdienaar (waar zijn die foto's?) en versleet zijn dagen met lego-, judo- en modelvliegtuigjes bouwen.

Het Reynaertcollege was ten tijde van de eerste twee Paarse kabinetten het leerhuis voor Joeri. Hij volgde het Atheneum van 1992 tot 1998. Over zijn uitstekende schoolprestaties aldaar hoeven niet uit te wijden, het is namelijk veel leuker om zijn extra-curriculaire activiteiten tijdens zijn middelbare schooltijd te bespreken. Die modelvliegtuigjes werden vervangen door heuse delta vliegtuigen. Joeri haalde zelfs zijn eerste brevet. Verder was hij op het strand aan het vliegeren. Niet mijn keuze, maar goed, je woont in Zeeland. Wat wel mijn goedkeuring kan wegdragen is zijn toenmalige liefde voor Rave & (Happy) hardcore muziek die beluisterd werd op veel te grote, zelf in elkaar gesoldeerde boxen. Zoon van een elektricien! In de vijfde klas ging Joeri twee maanden (!) naar Argentinië voor een jeugduitwisseling. Tot slot ging hij, naast zijn werk in een Grieks restaurant, geregeld tot veel te laat uit in shabby discotheken in België.

Voordat Joeri ging studeren in Wageningen heeft hij een jaar lang in Maringa in Brazilië gewoond (via de Youth exchange van de Rotary). Heel Brazilië gezien, veel caipirinhas gedronken, portugees leren spreken (Saude!) en de avond voor zijn vertrek verkozen tot Exchange Student of the Year (whatever that may be).

In 1999 start hij met Bioprocestechnologie aan de Wageningse universiteit. Tijdens zijn studie volgde hij een stage bij Virologie in Wageningen op het onderwerp *Identification of viral suppressors of RNAi in mammalian viruses*. Daarna ging hij terug naar Brazilië om aan Instituto Oswaldo Cruz (FioCruz) te werken aan het project *Characterisation of neutralising antibodies against Hepatitis B*. Na naar Utrecht te zijn verhuisd heeft hij nog eens 6 maanden stage gelopen bij het Nederlands Vaccin Instituut te Bilthoven. Hij heeft daar zijn tanden gezet in de *Determination of critical process parameters for the cultivation of Bordetella Pertussis*. Voor al zijn stages haalde hij een 8 of hoger, wat natuurlijk voortreffelijk is.

Naast zijn studie en stages had de promovendus ook nog tijd voor een zwik andere activiteiten. Ongelofelijk maar waar: Joeri werkte bij Morres Meubel als verkoper (zou U een bank bij hem aanschaffen?). Daarnaast gaf hij zogenaamde mobiele practica op middelbare en lagere scholen en organiseerde hij de bijscholingsdagen voor de technisch onderwijs assistenten (TOAs). Hij was voorzitter van de studievereniging van de Biotechnologen (Codon) en organisator van buitenlandse studiereizen naar Madrid & Duitsland. Ook was hij organisator van de Java comedy night in het Scheldetheater in Terneuzen. *Wat?!* Terwijl een gemiddelde persoon al uitgeteld op de net bij Joeri aangeschafte bank zou liggen, had Joeri ook nog energie voor hobby's zoals hardlopen, skiën, reizen en in de kroeg hangen.

In 2006 reist Joeri naar de oksel van de Peel, alwaar hij bij Intervet te Boxmeer wordt aangenomen door René Aerts in het Trainee programma. In datzelfde jaar verhuist hij naar Nijmegen en begint hij op de BAPPP-afdeling. Hij mag een jaar later naar Salamanca waar een grote productie faciliteit van Intervet staat. Hij heeft zich daar onsterfelijk gemaakt met een gigantische typefout in een presentatie. (Vraag dit maar aan Joeri zelf, want dat kan ik hier niet openbaren.) Ondanks deze blunder mag hij daarna op zijn visite kaartje schrijven: Assistant Project Leader development of viral vaccine production processes.

Vier jaar na Wageningen te hebben verlaten keert Joeri weer terug bij zijn Alma Mater en start zijn promotie onderzoek. Helemaal hippe publieke-private samenwerking met de niet hippe titel: Activation and Evasion of the Type I Interferon Response by Infectious Bronchitis Virus. Bij Kippen. Enfin. Hier ligt het prachtige resultaat.

Sinds november 2014 reist hij weer dagelijkse naar Boxmeer als Project Leader Process development & support of production processes for viral poultry vaccines, verslijt hij kilometers op de duitse Autobahn om bij zijn geliefde in Dresden te zijn en geeft hij nog één keer per jaar les op zijn oude middelbare school in Hulst. Good on ya' mate!

Mark Geels

CURRICULUM VITAE

What only very few people know is that besides the world-famous actor and singer from Hong Kong Nicholas Tse, another famous person was born on August 29th 1980: the author of this book. Joeri Kint burst into this world in Hulst in the Zeeland province as the son of secretary Trees and electrician Jo. Joeri spent his primary education on the devout catholic St. Willibrordus School where he even was an altar boy (where are those pictures?) while passing away the rest of his days playing with Lego and model planes and being a judoka on the tatami.

During the nineties, while the liberals and socialists reigned over the Netherlands, Joeri went to the Reynaertcollege high school in Hulst. Although we could talk about his excellent results, it is simply much more fun to discuss his extracurricular activities. He dropped the small-plane thing and switched to serious hanggliding. Somebody even gave him a license. He also liked to fly the kite on the beach. Not my cup of tea, but hey, what do you do when you live in Zeeland (= Sea-land...)? What I certainly can appreciate is the love for rave and (happy) hardcore music that blossomed during this period and was listened to on DIY loudspeakers personally soldered together. Son of an electrician. He spent two whole months (!) in Argentina during high school on an exchange visit. The rest of his time he worked at a Greek restaurant and he was spotted on many occasions in shabby Belgian discotheques just across the border.

Prior to going to college, Joeri lived a year in Maringa in Brazil through a youth exchange of the Rotary. He saw every corner of the country, drank some caipirinhas, learned Portuguese (saude!) and got elected as Exchange Student of the Year (*whatever that may be*).

In 1999 he started as a freshman in college majoring in Bioprosesstechnology. He performed several internships. His first internship was at the virology department on a project called *Identification of viral suppressors of RNAi in mammalian viruses*. Then the prospect of returning to samba soccer on the beach in Rio led him back to Brazil to do an internship at Instituto Oswaldo Cruz (FioCruz) called *Characterisation of neutralising antibodies against Hepatitis B*. Back in the Netherlands he moved to Utrecht and interned at the Dutch Vaccine Institute. He worked on the *Determination of critical process parameters for cultivation of Bordetella Pertussis*. He scored an 8 or higher for all his internships which truly is exceptional.

His extra-curricular activities were equally impressive (or shocking). Unbelievable but true, Joeri worked as a salesman in a furniture shop. *Would you buy a couch from Joeri?* Besides this, he taught so-called mobile practical lab courses on elementary and high schools and he organised refresher courses for technical support staff on high schools. He managed to become chair of the Student Faculty organisation (Codon) and organised exchange trips for students to Madrid and Germany. He also was one of the organisers of the Java comedy night in the Scheldetheater in Terneuzen. *What?! Where?!* And while a normal person would need a breather on the couch (freshly bought from Joeri), Joeri found enough time and energy for hobbies such as running, skiing, travelling and drinking beer in the pub.

In 2006, Joeri was hired as a trainee at Intervet in Boxmeer. Boxmeer, for those who do not know, is at the end of the world. That year he moved to Nijmegen to cut the commute short and starts working at the Biological All Purpose Pilot Plant or BAPPP. A year later he works for a month in the Intervet facilities in Salamanca, Spain. It is during that visit that he makes an epic typo during a presentation that I cannot repeat because there are small children in the room here but you should ask him about it. Despite this epic fail he successfully finishes the work and becomes an *Assistant Project Leader development of viral vaccine production processes* at Intervet.

Four years after having left Wageningen, Joeri returns to his Alma Mater and starts his PhD research project. State-of-the-art public private collaboration with the not-so-exciting title: Activation and Evasion of the Type I Interferon Response by Infectious Bronchitis Virus. In chickens. Enfin. It is the beautiful result that counts and lies before you.

In the fall of 2014, he restarts his daily commute to Boxmeer but this time as a *Project Leader Process development & support of production processes for viral poultry vaccines*. He also burns many miles on German Autobahns to be with his loved one in Dresden and he still teaches once a year at his old high school in Hulst. Good on ya' mate!

Mark Geels

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