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Coronavirus virulence genes with main focus on SARS-CoV envelope gene

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ABSTRACT

Coronavirus (CoV) infection is usually detected by cellular sensors, which trigger the activation of the innate immune system. Nevertheless, CoVs have evolved viral proteins that target different signaling pathways to counteract innate immune responses. Some CoV proteins act as antagonists of interferon (IFN) by inhibiting IFN production or signaling, aspects that are briefly addressed in this review. After CoV infection, potent cytokines relevant in controlling virus infections and priming adaptive immune responses are also generated. However, an uncontrolled induction of these proinflammatory cytokines can lead to pathogenesis and disease severity as described for SARS-CoV and MERS-CoV. The cellular pathways mediated by interferon regulatory factor (IRF)-3 and -7, activating transcription factor (ATF)-2/jun, activator protein (AP)-1, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and nuclear factor of activated T cells (NF-AT), are the main drivers of the inflammatory response triggered after viral infections, with NF-κB pathway the most frequently activated. Key CoV proteins involved in the regulation of these pathways and the proinflammatory immune response are revisited in this manuscript.

It has been shown that the envelope (E) protein plays a variable role in CoV morphogenesis, depending on the CoV genus, being absolutely essential in some cases (genus α CoVs such as TGEV, and genus β CoVs such as MERS-CoV), but not in others (genus β CoVs such as MHV or SARS-CoV). A comprehensive accumulation of data has shown that the relatively small E protein elicits a strong influence on the interaction of SARS-CoV with the host. In fact, after infection with viruses in which this protein has been deleted, increased cellular stress and unfolded protein responses, apoptosis, and augmented host immune responses were observed. In contrast, the presence of E protein activated a pathogenic inflammatory response that may cause death in animal models and in humans.

The modification or deletion of different motifs within E protein, including the transmembrane domain that harbors an ion channel activity, small sequences within the middle region of the carboxy-terminus of E protein, and its most carboxy-terminal end, which contains a PDZ domain-binding motif (PBM), is sufficient to attenuate the virus. Interestingly, a comprehensive collection of SARS-CoVs in which these motifs have been modified elicited full and long-term protection even in old mice, making those deletion mutants promising vaccine candidates. These data indicate that despite its small size, E protein drastically influences the replication of CoVs and their pathogenicity. Although E protein is not essential for CoV genome replication or subgenomic mRNA synthesis, it affects virus morphogenesis, budding, assembly, intracellular trafficking, and virulence. In fact, E protein is responsible in a significant proportion of the inflammasome activation and the associated inflammation elicited by SARS-CoV in the lung parenchyma. This exacerbated inflammation causes edema accumulation leading to acute respiratory distress syndrome (ARDS) and, frequently, to the death of infected animal models or human patients.

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1. Introduction

An overview of the sensors detecting virus infection is presented first, followed by a description of the mechanisms elicited by CoV proteins to counteract innate immune responses. Some CoV proteins act as antagonists of interferon (IFN) production, whereas others inhibit IFN signaling. As a consequence, a collection of potent cytokines relevant in controlling virus infections and priming adaptive immune responses are generated (Le Bon and Tough, 2002).

Virus pathogenesis is frequently associated with an exacerbated induction of proinflammatory cytokines that is mainly driven by the activation of at least one of the following five pathways: IRF-3 and -7, ATF-2/jun, jun/fos (AP-1), NF-κB and NF-AT. Among them, the NF-κB pathway is the most frequently activated (Hatada et al., 2000; Mogensen and Paludan, 2001). NF-κB is a heterogeneous collection of dimers, composed of various combinations of members of the Rel family, which in eukaryotes include p50 (NF-κB1), p52 (NF-κB2), Rel (c-Rel), p65 (RelA) and RelB. An exacerbated immune response and a weak IFN response have been associated with virulent CoVs such as SARS-CoV and MERS-CoV (Baas et al., 2008; Lau et al., 2013; Smits et al., 2010).

The main focus of this review is the analysis of the role of the CoV envelope (E) protein in virus pathogenesis. E protein contains several active motifs despite its small size, between 76 and 109 amino acids depending on the CoV. The modification or deletion of E protein in different CoVs has led to viruses with different phenotypes and unique alteration of virus–host interactions, such as the induction of stress and unfolded protein responses, or changes in cellular ion concentrations due to the ion channel activity of E protein. All these activities have high impact on CoV pathogenesis (DeDiego et al., 2011; Nieto-Torres et al., 2014).

E protein PDZ-binding motif (PBM), which during SARS-CoV infection could potentially target more than 400 cellular PDZ motifs present within cellular proteins, confers to E protein virus pathogenicity modulating properties. Interestingly, deletion or modification of E protein PBM and internal regions within the carboxy-terminus of E protein most frequently results in attenuated CoVs that are good vaccine candidates (Jimenez-Guardeño et al., 2014; Regla-Nava et al., 2014). In addition, the identification of signaling pathways, such as NF-κB-mediated signaling, responsible for CoV pathogenicity has led to the selection of antivirals that considerably increase the survival of infected animal models (DeDiego et al., 2014).

1.1. Coronavirus proteins inhibiting type I interferon production

IFNs are potent cytokines relevant in the control of virus infections and in the priming of adaptive immune responses (Le Bon and Tough, 2002). Treatment with type I IFN inhibits CoV growth in tissue culture and in animal models such as cynomolgus macaques and mice (Barnard et al., 2006; Dahl et al., 2004; Fuchizaki et al., 2003; Haagmans et al., 2004; Kumaki et al., 2011; Mahlakoiv et al., 2012; Sainz et al., 2004; Stroher et al., 2004; Zheng et al., 2004). To circumvent the inhibition of virus replication, many viruses, including CoVs, encode viral proteins inhibiting IFN production or signaling (Table 1). However, most of the studies describing the IFN antagonist activity of coronavirus-encoded proteins have been conducted in cells transiently expressing the viral proteins. Therefore, additional analyses in the context of the virus infection are required.

Type I IFN production is controlled by two major pathways dependent on RNA helicases or toll-like receptors (TLRs) (Arpaia and Barton, 2011; Rathinam and Fitzgerald, 2011; Sen, 2001) (Fig. 1). RNA helicases containing the cytoplasmic CARD domain, retinoic acid-inducible gene 1 (RIG-I) and melanoma differentiation-associated protein 5 (MDA5), sense pathogen-associated molecular patterns (PAMPs) in the cell cytoplasm. On the other hand, toll like receptors detect PAMPs in the cell surface and in endosomal compartments.

The RNA helicases-dependent cytoplasmic IFN induction pathways use the adaptor molecule mitochondrial antiviral signaling protein (MAVS) (Fig. 1). MAVS promotes the activation of a complex comprising the proteins TNF receptor-associated factor 3 (TRAF-3), TRAF family member-associated NF-κB activator (TANK), TANK-binding kinase 1 (TBK-1) and IkappaB kinase ε (IKKε). Active TBK1 and IKKε directly phosphorylate the transcription factors IRF-3 and IRF-7, promoting homodimerization (Sharma et al., 2003). Then, the IRF-3 and IRF-7 dimers are imported into the nucleus, leading to IRF-3 and IRF-7-dependent transcription. In addition, MAVS triggers the NF-κB pathway through IKKα and IKKβ activation (Kawai and Akira, 2007).

The TLRs-dependent IFN induction pathways use the adaptor molecules TIR-domain-containing adapter-inducing IFN-β (TRIF) and myeloid-differentiation primary response 88 (MyD88) (Fig. 1) (Kawai and Akira, 2007). TRIF-dependent pathway leads to the activation of IRF-3 and -7, and NF-κB. The activation of IRF-3 and IRF-7 is mediated by the phosphorylation of these factors by TBK-1 and IKKε, which promote their activation, as described above. TRIF also mediates NF-κB activation through the activation of IKKα and IKKβ. MyD88-mediated pathway activates the transcription factors NF-κB, AP-1 and ATF-2/jun, through the activation of mitogen-activated protein kinases (MAPKs) (Herlaar and Brown, 1999; Whitmarsh and Davis, 1996). NF-κB is also activated in this pathway through IKKs (Kawai and Akira, 2007).

IRF-3 and IRF-7, with the help of other transcription factors like NF-κB, and AP-1, initiate the transcription of IFN-β and selected IFN-α genes. IFN-α and IFN-β proteins are then secreted from the cell and can act in either an autocrine or a paracrine fashion to amplify the IFN response (Fig. 1).

CoVs have devised a number of cell type-specific strategies to inhibit type I IFN production (Table 1; Fig. 1). These viruses encode a 2′-*O*-methylase (non-structural protein nsp16) that creates a 5′-cap structure analogous to the cellular mRNAs on the viral mRNAs, thereby escaping detection by MDA5 (Zust et al., 2011). MERS-CoV accessory protein 4a is a dsRNA binding protein that blocks IFN induction by suppressing PACT-induced activation of RIG-I and MDA5 (Niemeyer et al., 2013; Siu et al., 2014). The ORF4b encoded accessory proteins of MERS-CoV and two related bat CoVs localize to the cell nucleus and inhibit type I IFN production and NF-κB signaling pathway (Matthews et al., 2014). Interestingly, a MERS-CoV lacking 4a and 4b proteins grew about 10-fold lower than the parental virus in IFN competent infected-cells (Almazan et al., 2013). However, the specific effect of 4a and 4b proteins IFN antagonistic activity in virus growth and virulence still needs to be determined. SARS-CoV membrane (M) protein impairs the formation of TRAF3/TANK/TBK1/IKKε complex, inhibiting IFN-β production (Siu et al., 2009). SARS-CoV structural nucleocapsid (N) protein blocks IFN-β production after induction with Sendai virus and polyI:C, but not upstream of components such as RIG-I, MDA5, MAVS, IKKε, TBK1 or TRIF, indicating that N protein acts after these signaling mediators (Kopecky-Bromberg et al., 2007; Lu et al., 2011). SARS-CoV papain-like protease (PLP) domain of nsp3 inhibits RIG-I and TLR3-dependent IFN-β production, being this activity independent of the deubiquitinating and protease activities (Clementz et al., 2010), and most probably mediated by the interaction of PLP domain with the protein stimulator of IFN genes (STING), which is a protein that stimulates phosphorylation of IRF3 by the kinase TBK1 (Sun et al., 2012). The inhibition of IFN production has also been described for nsp3 PLP2 of HCoV-NL63 (Clementz et al., 2010; Sun et al., 2012), MHV (Wang et al., 2011; Zheng et al., 2008), and for the PLP domain of MERS-CoV, which blocks IFN production by inhibiting IRF3 phosphorylation and translocation into

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Table 1
Coronavirus proteins affecting innate immune responses.

Protein	CoV	Immune function	References
Nsp1	SARS-CoV	Antagonizes type I IFN production and signaling by inducing host mRNAs shut off, promoting the degradation of host mRNAs and preventing phosphorylation of STAT1	Wathelet et al. (2007), Kamitani et al. (2009), Huang et al. (2011), Tanaka et al. (2012)
		Upregulates CCL5, CXCL10, and CCL3 in human lung epithelial cells via the activation of NF-κB	Law et al. (2007)
Nsp3	SARS-CoV	Prevents IFN production by blocking IRF3 phosphorylation, most probably by interacting with STING	Devaraj et al. (2007), Frieman et al. (2009), Sun et al. (2012), Clementz et al. (2010)
	MHV	Antagonizes type I IFN	Zheng et al. (2008), Wang et al. (2011)
	MERS-CoV	Antagonizes type I IFN	Yang et al. (2014)
Nsp7	SARS-CoV	Antagonizes type I IFN	Frieman et al. (2009)
Nsp15	SARS-CoV	Antagonizes type I IFN	Frieman et al. (2009)
S	SARS-CoV	Induces the expression of IL6, IL8, CXCL10 and TNF through NF-κB activation in macrophages	Wang et al. (2007), Dosch et al. (2009)
M	SARS-CoV	Blocks IFN-β production by impairing the formation of TRAF3–TANK–TBK1/IKKε complex	Siu et al. (2009)
N	SARS-CoV	Antagonizes type I IFN production by blocking IRF-3 phosphorylation	Lu et al. (2011), Kopecky-Bromberg et al. (2007)
		Activates NF-κB and upregulates the expression of IL-6	Liao et al. (2005), Zhang et al. (2007)
		Activates AP-1	He et al. (2003)
		Induces the expression of IL8 via AP-1 activation	Chang et al. (2004)
3a	SARS-CoV	Downregulates the expression of the type I IFN receptor (IFNAR), leading to a blockade on type I IFN signaling	Minakshi et al. (2009)
		Increases NF-κB and JNK activity and upregulates TNF, IL8 and CCL5 production	Obitsu et al. (2009), Kanzawa et al. (2006)
3b	SARS-CoV	Antagonizes type I IFN production by blocking IRF-3 phosphorylation. Inhibits IFN signaling	Kopecky-Bromberg et al. (2007), Freundt et al. (2009)
		Induces transcriptional activity of AP-1, through activation of JNK and ERK pathways, leading to CCL2 upregulation	Varshney and Lal (2011), Varshney et al. (2012)
6	SARS-CoV	Antagonizes type I IFN production by blocking IRF-3 phosphorylation	Kopecky-Bromberg et al. (2007), Frieman et al. (2009)
		Inhibits IFN signaling by blocking the nuclear translocation of the transcription factor STAT1	Frieman et al. (2007)
7a	SARS-CoV	Activates NF-κB and upregulates the expression of the proinflammatory mediators IL8 and CCL5	Kanzawa et al. (2006)
Nsp3	NL63	Antagonizes type I IFN	Clementz et al. (2010)
Nsp1	MHV	Antagonizes type I IFN	Zust et al. (2007)
N	MHV	Acts as an interferon antagonist and prevents RNA degradation by inhibiting RNaseL activity	Ye et al. (2007)
2	MHV	Antagonizes type I IFN signaling and prevents activation of the cellular endoribonuclease RNase L	Zhao et al. (2011, 2012)
5a	MHV	Antagonizes type I IFN	Koetzner et al. (2010)
4a	MERS-CoV	Block interferon induction at the level of MDA5 activation presumably by direct interaction with double-stranded RNA	Niemeyer et al. (2013)
4b	MERS-CoV	Antagonizes type I IFN	Matthews et al. (2014)
7	TGEV	Reduces the expression of genes involved in the immune response, the interferon response, and inflammation	Cruz et al. (2013)
7a	FIPV	Antagonizes type I IFN	Dedeurwaerder et al. (2013)

the nucleus (Yang et al., 2014). SARS-CoV nsp7 and nsp15 block IFN-β production through an unidentified mechanism (Frieman et al., 2009). SARS-CoV proteins N, 3b and 6 prevent IFN production by blocking IRF-3 phosphorylation (Devaraj et al., 2007; Freundt et al., 2009; Frieman et al., 2009; Kopecky-Bromberg et al., 2007).

TGEV protein 7 inhibits IFN production as it has been shown that a TGEV lacking protein 7 grew with similar titers than the wt virus, but induced expression of genes involved in the immune response and interferon response to a higher extent than the wt virus (Cruz et al., 2013). In addition, protein 7 prevents host translational shut off and RNA degradation through the interaction with protein phosphatase 1 (PP1) (Fig. 1) (Cruz et al., 2011).

1.2. CoV proteins inhibiting type I IFN signaling

Type I IFN signaling starts with its binding to IFNAR receptors at the cell surface, which leads to the activation of the JAK–STAT pathway (Samuel, 2001) (Fig. 2). The members of the Janus Kinase (JAK) family JAK-1 and protein tyrosine kinase 2 (TYK-2) phosphorylate the signal transducer and activators of transcription (STATs) which become activated. Phosphorylated STAT1 and STAT2 recruit IRF-9, to form the IFN stimulated gene factor 3 (ISGF3) complex. The ISGF3 heterotrimer translocates to the nucleus and triggers the transcription of IFN-stimulated genes (ISGs) that will drive the antiviral response.

Coronaviruses have developed strategies to interfere with IFN signaling at different levels. SARS-CoV affects the initial stages of the cascade by down regulating the expression of IFNAR, and inhibiting the translocation of STAT1 to the nucleus, through proteins 3a and 6, respectively (Frieman et al., 2007; Kopecky-Bromberg et al., 2007; Minakshi et al., 2009). In addition, SARS-CoV nsp1 affects STAT1 phosphorylation and induces a host translational shut off promoting the degradation of cellular mRNAs, further affecting IFN antiviral signaling (Huang et al., 2011; Jauregui et al., 2013; Kamitani et al., 2009; Tanaka et al., 2012; Wathelet et al., 2007; Zust et al., 2007). SARS-CoV protein 3b inhibits IFN signaling without inhibiting STAT1 phosphorylation (Kopecky-Bromberg et al., 2007). SARS-CoV nsp1 antagonizes type I IFN production and signaling by inducing host mRNAs shut off, promoting the degradation of cellular mRNAs and preventing phosphorylation of STAT1 (Huang et al., 2011; Kamitani et al., 2009; Tanaka et al., 2012; Wathelet et al., 2007; Zust et al., 2007). Inhibition of downstream

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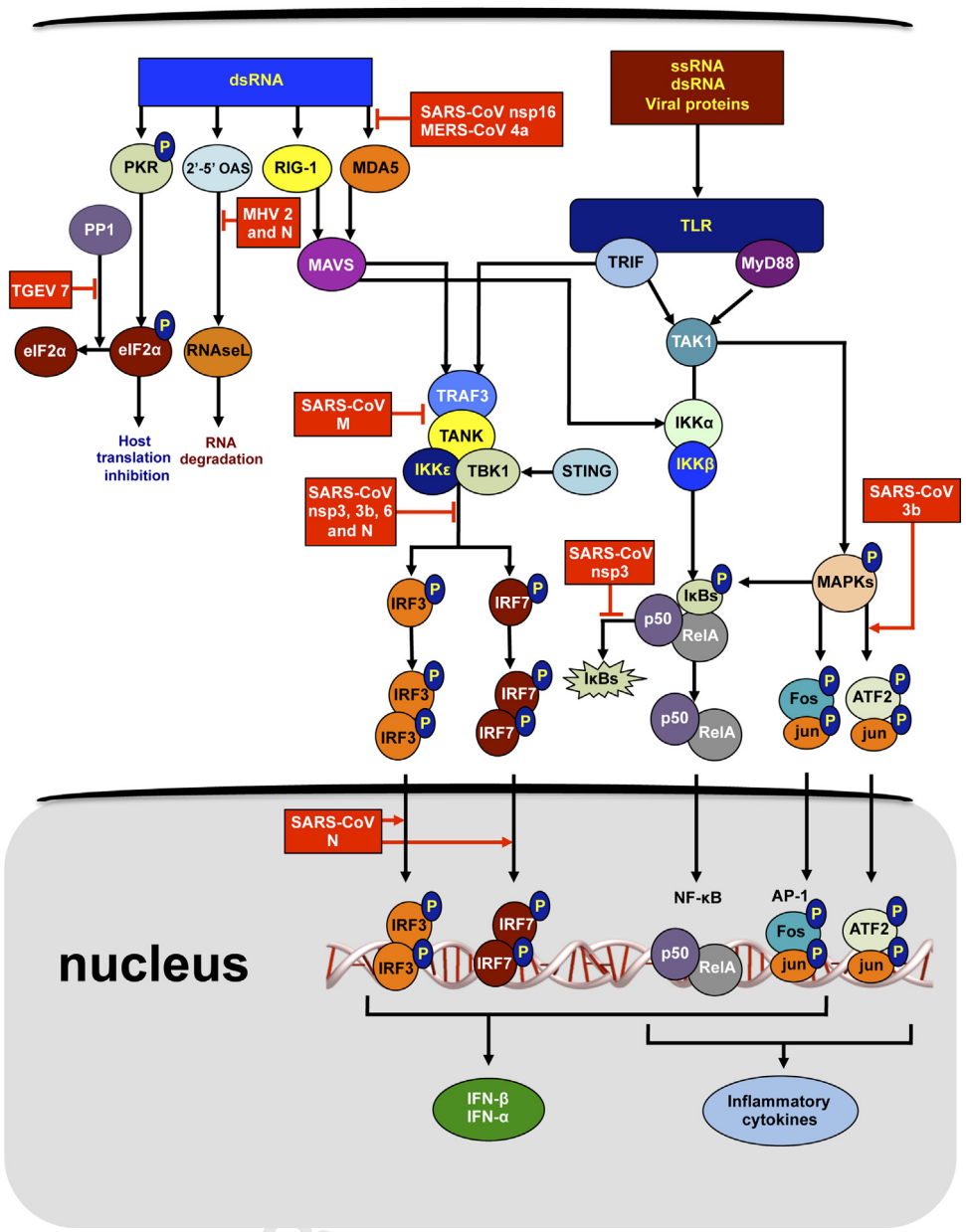

Fig. 1. Effect of coronavirus proteins on cellular signaling pathways associated with the innate immune response. PAMPs such as ssRNA, dsRNA, or viral proteins, trigger the activation of transcription factors leading to proinflammatory cytokine and type I IFN induction. PAMPs activate the PKR, leading to eIF2α phosphorylation and host translation inhibition, and 2′–5′ OAS, leading to RNase L triggering and RNA degradation. The activation of RIG-I and MDA-5 triggers the activation of IRF-3, IRF-7 and NF-κB through MAVS. In addition, TLRs activate the MyD88 and TRIF-dependent pathways, activating the transcription factors IRF-3, IRF-7, NF-κB, and AP-1. The steps inhibited or promoted by CoV proteins are indicated in red boxes. Beside these proteins, other proteins that inhibit or promote the IFN signaling and production and inflammatory cytokine expression, through an identified mechanism, are indicated in Table 1.

effectors of IFN signaling pathway (ISGs) has also been described during coronavirus infection. MHV N and ns2 as well as SARS-CoV N proteins prevent the activation of RNase L, blocking viral RNA degradation (Fig. 1) (Ye et al., 2007; Zhao et al., 2012).

Other CoV proteins confer IFN-resistance, however, whether they inhibit IFN production or signaling is unknown. MHV nsp1 is an efficient interferon antagonist in mice, as replication and spread of an nsp1 mutant virus were restored almost to wild-type levels in type I IFN receptor-deficient animals (Zust et al., 2007). MHV protein 5a or its homologues from related genus β coronaviruses, confer IFN-resistance to the virus (Koetzner et al., 2010). FIPV 7a protein protects the virus from the antiviral state induced by IFN, but it needs the presence of ORF3 encoded proteins to exert its antagonistic function (Dedeurwaerder et al., 2013).

1.3. Coronavirus proteins affecting the induction of proinflammatory signals

Proinflammatory cytokines and chemokines are a part of the necessary initial immune response to pathogens. However, an exacerbated immune response has been associated with the high virulence of SARS-CoV (Baas et al., 2008; Smits et al., 2010). Expression levels of proinflammatory cytokines, such as IL-1, IL-2, IL-6, and IL-8, and chemokines such as CXCL10 and CCL2 are elevated in peripheral blood and lungs of SARS patients, and associated with disease severity (Cameron et al., 2007; Chien et al., 2006; Jiang et al., 2005; Tang et al., 2005; Wong et al., 2004).

The most important signal transduction pathways activated by viruses leading to the expression of proinflammatory cytokines are

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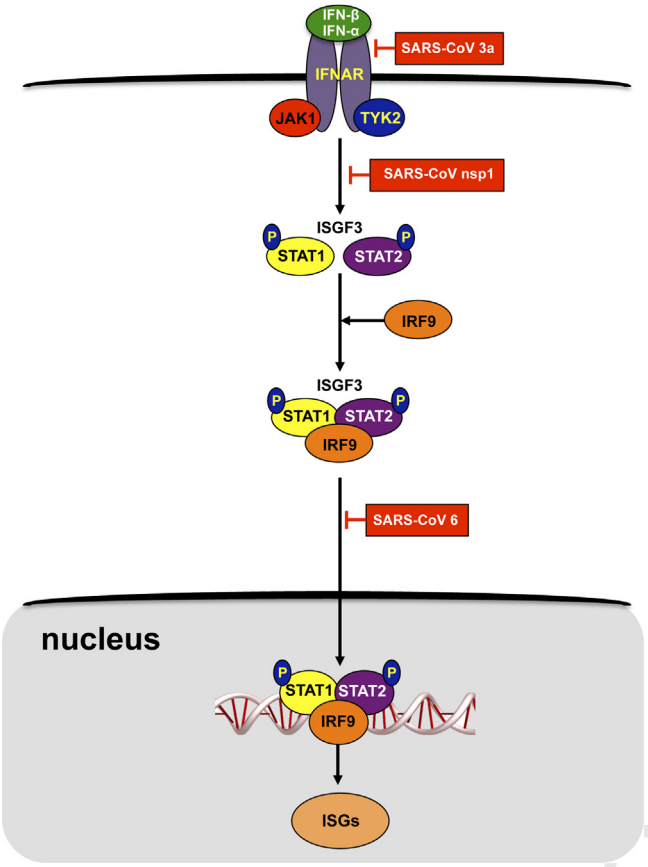

Fig. 2. Effect of coronavirus proteins on type I IFN signaling. The IFN-α and IFN-β proteins are secreted from the cell and amplify the IFN response activating the ISGF3 complex formed by STAT1, STAT2 and IRF-9, leading to the expression of the interferon-stimulated genes (ISG). The steps inhibited or promoted by CoV proteins are indicated in red boxes.

mediated by factors IRF-3 and -7, ATF-2/jun, AP-1, NF-κB and NF-AT (Mogensen and Paludan, 2001). The activation of these factors has been briefly described above. NF-AT is constitutively present in the cytoplasm in a latent phosphorylated form. Increasing levels of cytoplasmic calcium activate the calmodulin-dependent phosphatase calcineurin that activates NF-AT by dephosphorylation (Crabtree, 1999).

Activation of NF-κB is a hallmark of most infections including viral infections, leading to pathological outcomes. In fact, SARS-CoV-infected-aged macaques develop a more severe pathology, with an increase in differential expression of genes associated with inflammation, with NF-κB as a central player, and a reduction in the expression of type I IFN-β (Smits et al., 2010).

Several CoV-encoded proteins interfere with the production of inflammatory mediators. SARS-CoV nsp1 plays an important role in CCL5, CXCL10, and CCL3 upregulation in human lung epithelial cells via the activation of NF-κB (Law et al., 2007). The nsp3 PLP domain disrupts NF-κB signaling, most probably by inhibiting the degradation of phosphorylated IκB-α, which diminishes the induction of proinflammatory cytokines, leading to virus attenuation (Frieman et al., 2009). The structural SARS-CoV N protein activates NF-κB-driven transcription and upregulates the expression of IL-6 by facilitating the translocation of NF-κB from cytosol to nucleus (Liao et al., 2005; Zhang et al., 2007). In addition, the expression of N protein, but not the M protein, activates the AP-1 pathway (He et al., 2003). Similarly, SARS-CoV S protein induces the expression of TNF, IL6, IL8, and CXCL10 through NF-κB activation in macrophages (Dosch et al., 2009; Wang et al., 2007), and the expression of IL-8 via AP-1 in lung epithelial cells (Chang et al., 2004). The accessory protein 3a upregulates mRNA and fibrinogen levels in lung epithelial cells (Tan et al., 2005). In addition, 3a protein increases NF-κB and JNK activities and upregulates the TNF, IL8 and CCL5 production in murine macrophages and lung cell lines (Kanzawa et al., 2006; Obitsu et al., 2009). Similarly, SARS-CoV 7a protein also activates NF-κB and upregulates the expression of the proinflammatory mediators IL8 and CCL5 in a lung cell line (Kanzawa et al., 2006). The accessory protein 3b induces transcriptional activity of AP-1, through activation of JNK and ERK pathways, leading to CCL2 upregulation in a human hepatoma cell line (Varshney et al., 2012; Varshney and Lal, 2011). The presence of protein 7 in TGEV reduced the expression of proinflammatory genes, compared to a virus lacking this protein, indicating that TGEV protein 7 inhibits proinflammatory cytokine expression (Cruz et al., 2013).

In summary, several structural and non-structural SARS-CoV proteins affect the expression of proinflammatory signals, most frequently by modulating the NF-κB pathway and, to a lower extent, by affecting AP-1 signaling.

1.4. Virulence of recombinant coronaviruses lacking specific viral proteins

The generation of viral mutants lacking specific proteins or domains, or containing point mutations is an invaluable tool to study the contribution of a particular protein to the virulence of the virus. These systems offer advantages in comparison to over expression systems because in this case the studies are performed in the context of infection, in the presence of the other viral proteins, in a scenario in which the only difference is the presence of a single mutated or deleted viral protein. In contrast, the over-expression of specific proteins frequently yields overwhelming amounts of protein that may result toxic to the virus–host cell interaction required for a balanced virus replication.

To study the role of SARS-CoV group specific protein 6 during viral infection two approaches have been used. In one of them, SARS-CoV protein 6 has been expressed in the context of an attenuated mouse hepatitis virus (MHV). This recombinant virus grew more rapidly and to higher titers in cell culture and in the murine central nervous system than the control virus, leading to increased mortality in mice (Hussain et al., 2008; Netland et al., 2008; Pewe et al., 2005). In the other approach, a SARS-CoV lacking protein 6 was engineered. The SARS-CoV deletion mutant grew with lower titers but essentially maintained its virulence in transgenic mice expressing the human receptor for SARS-CoV hACE-2, as it killed 100% of the mice with a delay of 1 day (Zhao et al., 2009). These data indicated that SARS-CoV protein 6, in the context of the infection by the virus of which it is a structural component (Huang et al., 2007), does not seem to have a high influence on SARS-CoV virulence.

Infection of immune suppressed hamsters with recombinant SARS-CoV viruses bearing disruptions in the gene 7 coding region showed no significant changes in replication, tissue tropism, morbidity, or mortality suggesting that the 7a and 7b proteins are not essential for virus pathogenesis (Schaecher et al., 2008). Deletion of each of genes 3a, 6, 7a, and 7b from SARS-CoV did not affect virus growth in mice to a high extent (Yount et al., 2005). A SARS-CoV lacking the group specific genes 6, 7a, 7b, 8a, 8b, and 9b grew similarly to the parental virus and induced a slightly diminished weight loss and a delay in the time of death in transgenic mice expressing hACE-2, which are highly susceptible to the disease (DeDiego et al., 2008). Although further analysis using other animal models should be performed, these data suggested that the contribution of proteins 6, 7a, 7b, 8a, 8b and 9b to the virulence of SARS-CoV is limited.

A recombinant MHV with a deletion in nsp1 (a homolog of SARS-CoV nsp1) grew normally in tissue culture, but was severely

attenuated in vivo. Interestingly, replication and spread of the nsp1 deletion mutant virus was restored almost to wild-type levels in type I IFN receptor-deficient mice, indicating that nsp1 interferes efficiently with the type I IFN system in vivo (Zust et al., 2007). Similarly, a mutant virus lacking a conserved domain of MHV nsp1, showed no growth defects in cell culture, but was highly attenuated in vivo (Lei et al., 2013).

Deletion of group specific proteins ns2, HE, 4ab, and 5a from MHV led to attenuated viruses in the natural host, the mice (de Haan et al., 2002). A MHV mutant missing protein ns2 was unable to replicate in the liver or to induce hepatitis in wild-type mice, but was highly pathogenic in RNase L deficient mice, indicating that protein ns2 increases the pathogenicity of the virus by an RNase L dependent mechanism (Zhao et al., 2011, 2012).

Genus α CoVs such as TGEV missing gene 7, or FIPV lacking at the same time genes 3abc and 7ab showed modification of the inflammatory response and virulence. TGEV 7 protein deletion mutant increased proinflammatory responses and acute tissue damage after infection, leading to a more pathogenic virus (Cruz et al., 2011, 2013). In contrast, FIPV deletion mutant was attenuated in cats and induced protection against feline infectious peritonitis (Haijema et al., 2004). In this case the effect of FIPV proteins 3abc or 7b deletion on its virulence prevailed over the deletion of FIPV protein 7a, which is the protein equivalent to TGEV protein 7.

2. Requirement of coronavirus E protein in coronavirus replication and morphogenesis

CoV E protein is multifunctional, affecting several steps of the viral cycle. SARS-CoV can infect mouse brain, whereas in the absence of E protein this tissue tropism has not been observed (DeDiego et al., 2008). However, in this case, the involvement of E protein in entry is not necessarily required to explain the observed difference, as the restriction could operate at a later step. E protein expression is not involved in CoV genome replication, as both SARS-CoV with and without E protein synthesize the same amounts of genomic and subgenomic mRNAs (DeDiego et al., 2011).

The requirement of E protein in CoV morphogenesis has been under debate. In fact, E protein seems necessary for virus like particle formation using some experimental systems (Ho et al., 2004; Mortola and Roy, 2004) but not others (Huang et al., 2004). Different CoVs have shown variable requirements for E protein during morphogenesis, resulting in three different phenotypes. One of them is shown by genus α coronaviruses, like TGEV, and also by genus β MERS-CoV, which in the absence of E protein are replication-competent propagation-defective viruses (Almazan et al., 2013; Curtis et al., 2002; Ortego et al., 2002, 2007). Both TGEV and MERS-CoV missing E protein were propagated in packaging cells by providing E protein in trans, leading to high virus titers. In this case, the level of recovered viruses was proportional to the amount of E protein provided by the packaging cell line (Ortego et al., 2002). A second phenotype of CoVs missing E protein, is represented by genus β MHV, with a reduction of virus titers higher than 1000-fold (Kuo and Masters, 2003). The third phenotype was observed for genus β SARS-CoV, in which deletion mutants missing E protein only reduced their replication between 20 and 200-fold, leading to viruses that replicate both in cell culture and in vivo, and display an attenuated phenotype (DeDiego et al., 2007, 2008, 2014; Enjuanes et al., 2008). The assembled viral particles could be the base for safe vaccine candidates, once additional safety guards have been incorporated at a distal position in the CoV genome.

Whereas the deletion of E protein in different CoVs may affect virus production to different extents, it is clear that for CoVs such as SARS-CoV, E protein is not essential, since SARS-CoV missing E protein can produce virus titers close to 1×10^6 pfu per ml or per gram of tissue, in Vero E6 cells or in lungs of infected BALB/c mice, respectively, in a reproducible fashion (DeDiego et al., 2007, 2008; Fett et al., 2013). Nevertheless, the presence of E protein optimizes SARS-CoV yields. The contribution of E protein to CoV morphogenesis could be mediated through its interaction with other virus structural proteins within the virus envelope (M, 3a, 3b, 6, 7b, and 9b) (Arndt et al., 2010; Boscarino et al., 2008; Chen et al., 2009; Neuman et al., 2008; Pan et al., 2008; von Brunn et al., 2007). E protein has three potential palmitoylation residues in its carboxy-terminus. The palmitoylation of these sites is essential for the formation of vesicles including E protein that contribute to CoV morphogenesis (Boscarino et al., 2008; Lopez et al., 2008). Also, the extent of E protein palmitoylation affects its interaction with M protein (Boscarino et al., 2008).

The presence of E protein in CoVs particles is very low in general (around 20 molecules per virion) (Godet et al., 1992), although this could vary depending on the species (Liu and Inglis, 1991). Interestingly, E protein is highly abundant in the cytoplasm of infected cells, what may be due to its role in virus transport and morphogenesis (Ortego et al., 2007). A role in intracellular trafficking has been described for CoV E protein. The hydrophobic domain of IBV E protein seems important for the forward trafficking of cargo to the plasma membrane. In fact, E protein alters the host secretory pathway to the apparent advantage of the virus, increasing the efficacy of infectious virus release (Ruch and Machamer, 2011, 2012). Therefore E protein seems to play a role in virus egress.

E protein oligomerizes and forms ion channels that influence the electrochemical balance in some subcellular compartments of host cells, as described below.

3. Effect of SARS-CoV E gene deletion on viral pathogenesis

To study the effect of SARS-CoV E protein on viral pathogenesis, a SARS-CoV lacking the full-length E gene (rSARS-CoV-ΔE) was engineered. The deleted virus was attenuated in golden Syrian hamsters, and in transgenic mice expressing the SARS-CoV receptor hACE-2 (DeDiego et al., 2007, 2008). In addition, a mouse adapted SARS-CoV lacking E gene (rSARS-CoV-MA15-ΔE) was attenuated in conventional young and aged BALB/c mice (DeDiego et al., 2014; Fett et al., 2013), indicating that the expression of E gene increases virus pathogenicity. rSARS-CoV-ΔE titers decreased in *vivo*, in comparison to parental virus titers. However, intrinsic properties of E protein, and not just a decrease in virus titers, may increase the viral pathogenesis. In fact, viral mutants lacking E protein ion channel activity and PBM, grow similarly to the wt virus, and nevertheless are attenuated (see Sections 5 and 6) (Jimenez-Guardeño et al., 2014; Nieto-Torres et al., 2014).

To identify mechanisms leading to rSARS-CoV-ΔE attenuation, gene expression was compared in cells infected with the attenuated ΔE virus and in wt virus-infected cells. Stress response genes were preferentially upregulated during infection in the absence of E gene. Interestingly, expression of E protein in trans reduced the stress response in cells infected with rSARS-CoV-ΔE or with respiratory syncytial virus, or in cells treated with drugs, such as tunicamycin and thapsigargin, that elicit cell stress by different mechanisms (DeDiego et al., 2011). In addition, SARS-CoV E protein down-regulated the signaling pathway inositol-requiring enzyme 1 (IRE-1) of the unfolded protein response, and limited cell apoptosis. The expression of proinflammatory cytokines was lower in rSARS-CoV-ΔE-infected cells compared to rSARS-CoV-infected ones, suggesting that the increase in stress responses and the reduction of inflammation in the absence of the E gene contributed to the attenuation of rSARS-CoV-ΔE (DeDiego et al., 2011). These results were confirmed in mice. A reduced expression of proinflammatory cytokines, decreased number of neutrophils in lung

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infiltrates, and diminished lung pathology were observed in SARS-CoV-MA15-ΔE-infected mice, compared to the wt virus-infected ones (DeDiego et al., 2014), indicating that lung inflammation contributes to SARS-CoV virulence. Furthermore, infection with rSARS-CoV-ΔE resulted in a decreased activation of the transcription factor NF-κB. Importantly, treatment with NF-κB inhibitors, led to a reduction in inflammation in both SARS-CoV-infected cultured cells and mice, and significantly diminished lung pathology. These changes increased mice survival after SARS-CoV infection (DeDiego et al., 2014). These data indicated that NF-κB activation is a major contributor to the inflammation induced after SARS-CoV infection, and that drugs inhibiting NF-κB activation are promising antivirals to treat SARS-CoV induced disease, and most probably the inflammation caused by other pathogenic coronaviruses, such as MERS-CoV.

Interestingly, hamsters immunized with the attenuated rSARS-CoV-ΔE developed high serum-neutralizing antibody titers, and were protected after the challenge with homologous (Urbani) and heterologous (GD03) SARS-CoV strains (Lamirande et al., 2008). In addition, SARS-CoV missing E protein partially protected transgenic mice against challenge with virulent SARS-CoVs (Netland et al., 2010). Moreover, rSARS-CoV-MA15-ΔE totally protected young and old (up to 2 years) BALB/c mice against the virulent mouse adapted virus (Fett et al., 2013), by inducing high humoral and cellular immune responses. These data indicated that the viruses lacking E gene are promising live attenuated vaccine candidates.

4. SARS-CoV E protein amino and carboxy-terminus modification and virus attenuation

To identify SARS-CoV E protein domains and host responses that contribute to rSARS-CoV-MA15 virulence, several mutant viruses (rSARS-CoV-MA15-E*) containing amino acid substitutions in the amino-terminal domain, or small deletions covering the carboxy-terminus region of E protein, were constructed using a mouse adapted virus (Fig. 3) (Regla-Nava et al., 2014). Interestingly, amino acid substitutions in the amino-terminus, or deletion of central domains within the carboxy-terminal region of E protein led to viruses attenuated in mice, indicating that these domains are essential for SARS-CoV pathogenesis (Regla-Nava et al., 2014). Intranasal infection of mice with these attenuated mutants resulted in minimal lung damage and cellular infiltration compared to mock-infected mice, similar to what happened with rSARS-CoV-MA15-ΔE. The lower pathology induced by the attenuated SARS-CoV-MA15 without E protein, or by deletion of mutants of this protein, including small deletions in the carboxy-terminus, was associated with a significant reduction in the expression of proinflammatory cytokines in the lungs (Regla-Nava et al., 2014). Interestingly, a reduction in the number of neutrophils, which contribute to severe inflammation, and an increase in the number of T cells, which contribute to virus clearance (Zhao and Perlman, 2010), were found in the lungs of mice infected with the attenuated mutants compared to those infected with the virulent ones (Regla-Nava et al., 2014). These results indicate that increased levels of lung inflammation, exacerbated inflammatory cytokine expression, high levels of neutrophils, and decreased levels of T cells in the lungs, contributed to SARS-CoV virulence. Interestingly, the attenuated viruses missing E protein domains, completely protected mice against challenge with lethal virus, as happened with full-length E protein deleted virus, indicating that the viruses with small deletions in the carboxy terminus may also be the basis for promising vaccines.

5. SARS-CoV E protein PDZ binding domain and SARS-CoV virulence

A functional PDZ-binding motif (PBM) has been identified at the carboxy-terminus end of E protein using in vitro and in vivo approaches (Fig. 4) (Jimenez-Guardeño et al., 2014; Teoh et al., 2010). PDZ motifs are abundant modules involved in protein–protein interaction, which consist of 80–90 amino acids that recognize a specific peptide sequence (PBM) found in the extreme C-termini of target proteins (Hung and Sheng, 2002). In the human genome, more than 900 PDZ domains are found in over 400 proteins (Spaller, 2006). It has been described that proteins containing PDZ domains can be involved in cellular processes of relevance for viruses, such as cell–cell junctions, cellular polarity and signal transduction pathways (Javier and Rice, 2011). According to this data, several viruses, such as influenza A virus (Jackson et al., 2008), tick-borne encephalitis virus (TBEV) (Melik et al., 2012), and human papillomavirus (HPV) (Kiyono et al., 1997) encode proteins with PBMs that target cellular PDZ motifs carrying proteins during infection. Through these interactions, cellular pathways influence viral replication, dissemination in the host, and pathogenesis (Javier and Rice, 2011).

To identify SARS-CoV E protein cellular targets containing PDZ domains, yeast two-hybrid based studies were undertaken. The protein associated with Lin Seven 1 (PALS1), a tight junction-associated protein, was the first PDZ protein identified as a target of E protein PBM, and this interaction was confirmed using co-immunoprecipitation studies in mammalian cells (Teoh et al., 2010). PALS1 is a key component of the complex that controls polarity establishment and tight junction formation in epithelia. Studies using Vero E6 cells infected with SARS-CoV showed that E protein relocalized PALS1 to the ERGIC and Golgi region. In addition, the ectopic expression of E protein in MDCK epithelial cells led to delayed tight junction and polarity establishment. The results suggested that hijacking of PALS1 by E protein could play an important role in the pathology observed in SARS-CoV patients by altering lung epithelia integrity (Teoh et al., 2010).

We have recently shown that SARS-CoV E protein PBM is a molecular determinant of virulence (Jimenez-Guardeño et al., 2014). In this study, recombinant viruses missing E protein PBM were generated, leading to a fully attenuated phenotype in mice. Infection of mice with the recombinant viruses lacking the E protein PBM led to a decrease in the deleterious, exacerbated immune response triggered during SARS-CoV infection and a lower expression of inflammatory cytokines, without significantly affecting virus titers in mice lungs. To understand the molecular basis of this attenuation, host factors interacting with E protein PBM were identified using proteomic studies. A specific interaction of this motif with the cellular protein syntenin, a relevant scaffolding protein that participates in the activation of p38 mitogen-activated protein kinase (MAPK), was found (Jimenez-Guardeño et al., 2014). Interestingly, activated p38 MAPK, which mediates the expression of proinflammatory cytokines (Kumar et al., 2003; Underwood et al., 2000), was specifically reduced in mice infected with viruses missing E protein PBM, as compared with viruses containing this motif. These results highlight a novel mechanism of modulation of SARS-CoV pathogenesis by E protein. The interference with this signaling pathway will allow the development of therapies to reduce the exacerbated immune response triggered during SARS-CoV infection. Interestingly, bioinformatics analysis showed that other human CoV E proteins, such as that from MERS-CoV, HCoV-229E, HCoV-NL63, HCoV-OC43 and HCoV-HKU1 also encode a PBM in its carboxy-terminus. Therefore, the antiviral strategies described above to prevent SARS-CoV, most probably also apply to the reduction of the pathogenesis induced by other human CoVs. Furthermore, the generation of human attenuated coronaviruses

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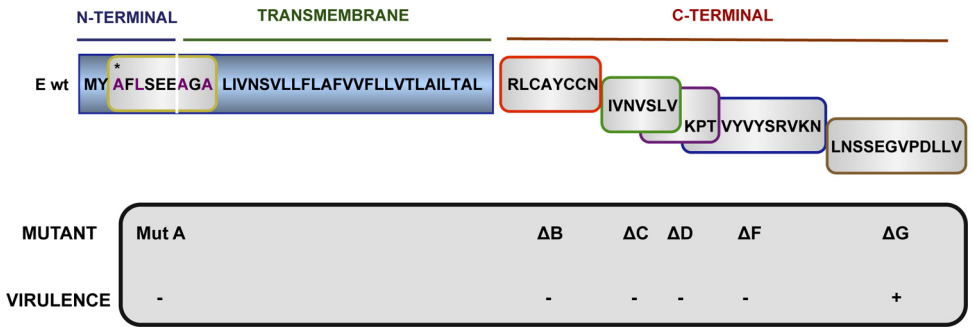

Fig. 3. Engineered rSARS-CoVs-MA15 with point mutations and deletions in E gene. The organization of E protein is shown. E protein sequence is divided into three domains: the amino terminal (N-terminal), the transmembrane and the carboxy-terminal (C-terminal). The figure illustrates the deletions and point mutations engineered within E protein. The asterisk (*) indicates mutations in the residues S3A, V5L, T9A, T11A. Gray box at the bottom indicates mutant virulence: (+) indicates a virulent phenotype and (−) indicates an attenuated phenotype.

by deleting E protein PBM could be the basis for the development of recombinant vaccines, as those described by deleting the whole SARS-CoV E protein or internal domains of this protein (Fett et al., 2013; Lamirande et al., 2008; Netland et al., 2010; Regla-Nava et al., 2014).

6. Ion channel activity of SARS-CoV E, 3a and 8a proteins and virulence

A wide range of animal viruses encode hydrophobic proteins that oligomerize in host cell membranes leading to structures with ion channel (IC) activity. These proteins, named viroporins, may influence viral replication and assembly, as well as virus particle entry and release from infected cells. Viroporins have a high impact on relevant host cell physiological processes (Nieva et al., 2012). Therefore, these proteins are useful targets to counteract viral infections.

Most of the RNA viruses encoding these proteins only have one viroporin in their genome (Castaño-Rodriguez et al., 2014). However, SARS-CoV encodes three proteins with IC activity: E, 3a and 8a, which indicates that SARS-CoV is the RNA virus expressing the highest number of viroporins known up to date.

The IC activity of E protein is the most extensively characterized among the three SARS-CoV viroporins using structural, functional and physiological assays. E protein has a single transmembrane domain topology and its monomers oligomerize in a pentameric ion conductive pore, as determined by linear dichroism and NMR studies (Parthasarathy et al., 2008; Pervushin et al., 2009; Torres et al., 2006).

The first functional evidence of SARS-CoV E protein acting as a viroporin was provided after its expression in bacteria, where E protein oligomerized and modified membrane permeability (Liao et al., 2004, 2006). Direct measurement of E protein IC activity was first reported using synthetic peptides representing full-length SARS-CoV E protein or its N-terminal 40 amino acids, including the transmembrane domain, in artificial lipid membranes (Wilson et al., 2004). This IC activity was confirmed, and mutations that suppressed this function were identified (Torres et al., 2007; Verdia-Baguena et al., 2012). In addition, compounds that inhibit the SARS-CoV E protein ion conductivity were described, although their efficacy in the context of a viral infection was not reported (Pervushin et al., 2009). Initially, it was considered that SARS-CoV E protein formed an IC with an enhanced selectivity for monovalent cations over monovalent anions, and for Na^+ over K^+ ions (Wilson et al., 2004). However, recent studies showed that the selectivity of SARS-CoV E protein IC was dependent on the charge of the lipid membranes in which the pore was reconstituted, which strongly suggested that the lipid head-groups are an integral component of the channel pore (Fig. 5) (Verdia-Baguena et al., 2012, 2013). This novel finding highlights the relevance of the lipid membrane composition in the SARS-CoV ion channel structure and activity.

The influence of SARS-CoV E protein IC activity in cell ion homeostasis is highly dependent on its subcellular localization. After SARS-CoV infection, E protein mainly accumulates in the

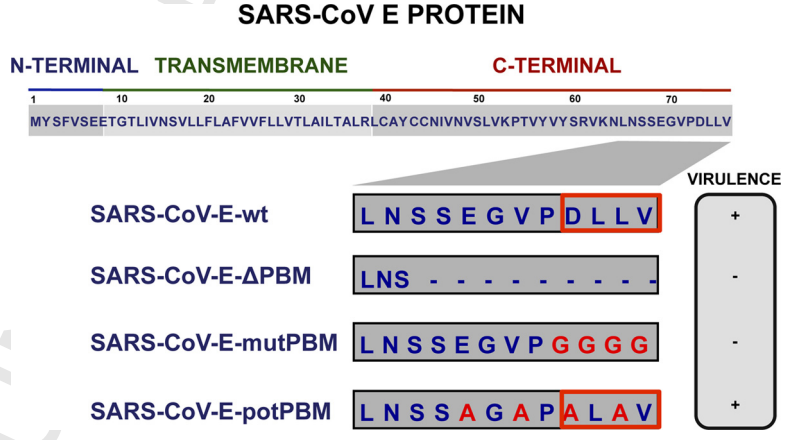

Fig. 4. Recombinant SARS-CoVs with E protein PBM truncated or mutated by reverse genetics. SARS-CoV E protein sequence and its corresponding domains are shown at the top. Below, sequences corresponding to the end of E protein are shown in boxes for the different virus mutants. SARS-CoV-E-wt, wild type sequence. In SARS-CoV-E-ΔPBM and SARS-CoV-E-mutPBM virus mutants, E protein PBM was eliminated by the introduction of deletions or point mutations, reducing or keeping the full protein length, respectively. In SARS-CoV-E-potPBM, four amino acids of E protein were replaced by alanine, to generate a new potential PBM. Red boxes highlight PBMs within E protein. Gray box on the right indicates the virulence of the mutants: (+) indicates a virulent phenotype and (−) indicates an attenuated phenotype.

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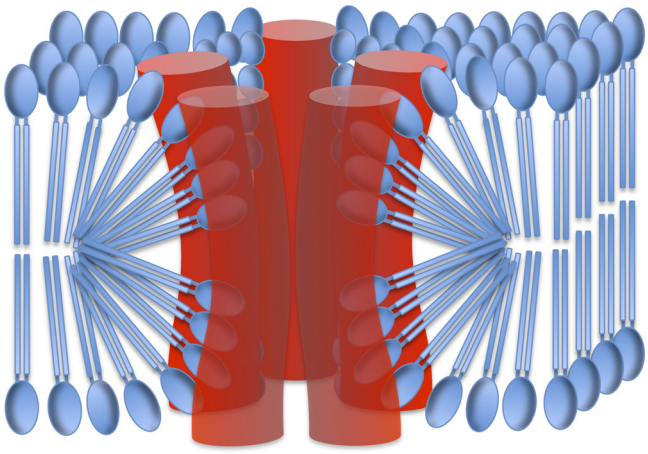

Fig. 5. Structure of SARS-CoV E protein proteolipidic ion channel. Phospholipids are represented in blue, and E protein monomers are shown as red cylinders. Note that lipid head groups (blue ellipses) also face the ion channel lumen.

endoplasmic reticulum–Golgi intermediate compartment (ERGIC) region of the infected cells, where virus morphogenesis and budding take place (Nieto-Torres et al., 2011). In artificial membranes, mimicking the ERGIC membrane composition, where E protein is mainly inserted, E protein showed a slight selectivity for cations over anions, with no preference for a specific cation (Verdia-Baguena et al., 2012). It has been suggested that E protein could also be located at the cell plasma membrane, which could influence cell depolarization (Liao et al., 2006; Pervushin et al., 2009). Efforts done by our group to identify the presence of E protein in the cell surface, or to detect IC activity in the cell surface by using patch-clamp technology showed the absence of this activity in the plasma membrane (Nieto-Torres et al., 2011). Accordingly, an additional study indicated that E protein does not form ion channels at the cell surface (Ji et al., 2009). Therefore, we have concluded that E protein ion channel activity is only shown in the intracellular structures, where E protein has been located (Nieto-Torres et al., 2011; Ruch and Machamer, 2012).

Ionic imbalances within cells can interfere with innate immunity and affect virus pathogenesis. Interestingly, disruption of ion gradients within the endoplasmic reticulum and Golgi apparatus by viral proteins with IC activity delayed protein transport preventing MHC molecules from reaching the plasma membrane (Cornell et al., 2007; de Jong et al., 2006). Recently, it has been described that ionic imbalances controlled by viroporins are sensed by the inflammasome, which triggers the activation of key pro-inflammatory cytokines such as IL-1β, a major determinant of disease progression (Ichinohe et al., 2010; Ito et al., 2012; McAuley et al., 2013; Triantafilou et al., 2013).

The introduction of point mutations that inhibited SARS-CoV E protein IC activity led to attenuated viruses, without significantly affecting virus production (Nieto-Torres et al., 2014). Furthermore, viruses in which E protein IC activity was suppressed quickly evolved by incorporating mutations that restored ion conductivity and a virulent phenotype (Nieto-Torres et al., 2014). After infection with viruses displaying E protein IC, increased damage within pulmonary epithelia and edema accumulation within lung airways (Fig. 6), the ultimate determinant of ARDS, was observed, compared to mice infected with viruses lacking E protein IC (Nieto-Torres et al., 2014). Enhanced liquid levels within lung airways avoid proper oxygen exchange leading to severe hypoxemia and eventually to death (Matthay and Zemans, 2011). Ionic balances play a central role in controlling liquid amounts present within air spaces. Lung epithelia create an osmotic gradient between the interior of the airways and the interstitial spaces. To resolve edema, a vectorial transport of Na^+ ions driven by epithelial sodium channels (ENaC) and Na^+/K^+ ATPase is established (Hollenhorst et al., 2011). Viruses displaying E protein ion channel activity caused an increased damage within pulmonary epithelia, which correlated with edema accumulation (Fig. 6) (Nieto-Torres et al., 2014). In addition, SARS-CoV E protein decreased the levels and activity of ENaC in lung epithelial cells, via the activation of distinct PKC isoforms, decreasing both ENaC exocytosis and endocytosis rates (Ji et al., 2009). These data indicated that the activation of PKC by SARS-CoV E protein, which may lead to decreased levels and activity of ENaC at the apical surface of lung epithelial cells, and the IC activity of E protein contribute to the lung edema observed after SARS-CoV infection.

Pulmonary epithelial damage is associated with a deleterious exacerbated inflammatory response triggered in the lungs after SARS-CoV infection. Evaluation of key inflammatory cytokines involved in epithelial damage and edema accumulation revealed that IL-1β, TNF and IL-6 amounts were increased in the lung airways of the mice infected with the viruses displaying E protein ion conductivity compared to the infection with the mutants lacking IC activity (Nieto-Torres et al., 2014). IL-1β is one of the most important proinflammatory cytokines involved in ARDS disease (Meduri et al., 1995; Pugin et al., 1996). IL-1β activation occurs when the inflammasome complex is stimulated by viral proteins with ion channel activity (Ichinohe et al., 2010; Ito et al., 2012; McAuley et al., 2013; Triantafilou et al., 2013). The inflammatory response elicited by IL-1β is accompanied by an increase in TNF, and both signals are amplified by the accumulation of IL-6, which are key events during ARDS progression after SARS-CoV infection (Tisoncik et al., 2012; Wang et al., 2005). We believe that this exacerbated deleterious response is a causal agent of the observed damage in the lung parenchyma of animals infected with the viruses displaying ion channel activity.

In summary, inhibition of SARS-CoV E protein IC activity, without significantly affecting virus growth, led to a virus inducing an attenuated pathogenesis. Attenuation correlated with a moderate inflammatory response leading to less epithelial damage and edema accumulation. These findings may have implications for the other viroporins encoded by SARS-CoV and, most importantly, for the identification of therapies to protect against highly pathogenic CoVs such as SARS-CoV and MERS-CoV, or other viruses encoding proteins with IC activity. For example, hexamethylene amiloride (HMA), an inhibitor of the HIV-1 Vpu protein ion channel activity, also inhibited SARS-CoV, HCoV-229E and MHV E protein ion channel conductance (Pervushin et al., 2009; Wilson et al., 2006) and, as a consequence, suppressed the replication of the wt HCoV-229E and MHV (Wilson et al., 2006). Therefore, this ion channel inhibitor may be an efficient antiviral compound to control the replication several members of the *Coronaviridae* family.

8a and 3a proteins are SARS-CoV viroporins as well, but their ion channel activities are much less studied than that of E protein. A 29 nt deletion occurred in ORF8 when the virus first infected human beings, splitting ORF8 into ORF8a and ORF8b. ORF8a encodes a 39-amino-acid-long polypeptide whose first 35 residues are identical to the N-terminal part of the ORF8 primary product (Oostra et al., 2007). ORF8a shows IC activity when reconstituted into artificial lipid bilayers (Chen et al., 2011), but this activity has not been identified in cells. A role for 8a protein in virus replication and in vitro apoptosis through a mitochondrial-dependent pathway has been suggested (Chen et al., 2007) but the experiments were performed with a HA tagged variant of 8a protein and some of the results are at variance with those previously reported (Oostra et al., 2007). A variant of SARS-CoV with a deletion of 415 nt resulting in the loss of ORF8, was isolated toward the end of the SARS epidemic and, in spite of this deletion, some of the infected patients died, suggesting that ORF8 is not essential for virus pathogenicity (Chiu et al., 2005).

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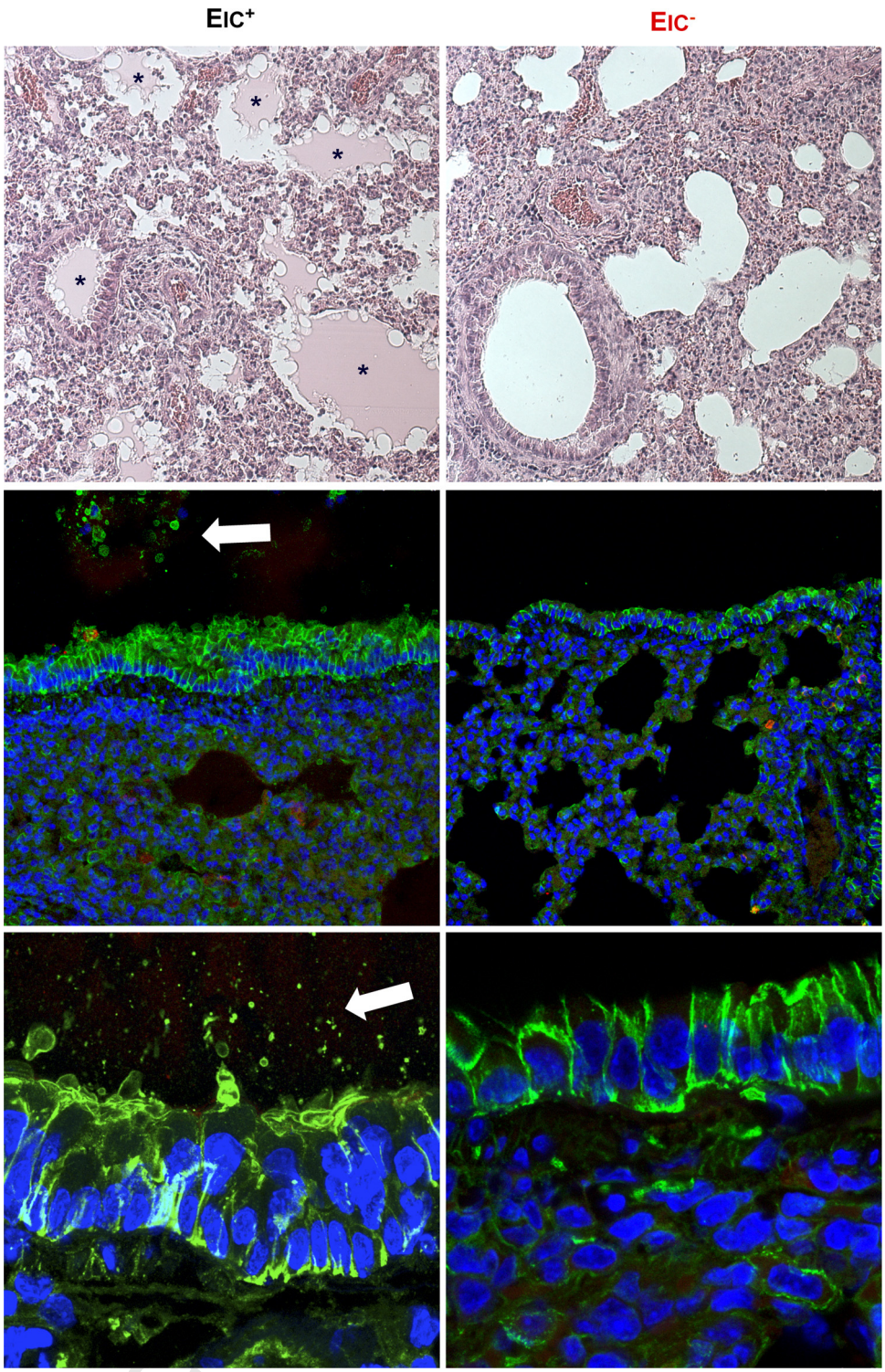

Fig. 6. Effect of SARS-CoV E protein ion channel activity in lung pathology. The lung histopathology in mice infected with a virus displaying (EIC$^+$) or lacking (EIC$^-$) E protein ion channel activity at 4 days post infection (dpi) is shown at the top. Lung sections were analyzed by hematoxylin and eosin staining at an original magnification of 20×. Airspaces where edema was accumulated are indicated with asterisks. Immunofluorescence staining of lung sections, and detail of bronchiolar epithelia at 4 dpi, at a magnification of 40× and 190×, respectively is shown at the bottom. Lung epithelia was labeled using an anti Na^+/K^+ ATPase antibody (green) and SARS-CoV infection was tracked with an anti-N protein antibody (red). Nuclei are shown in blue. Desquamated epithelial cells and cell debris are observed in lung airways after EIC$^+$ virus infection (white arrows).

3a protein is a 274 aa SARS-specific structural component of the virus with three transmembrane domains (TMDs) in its N-terminus. 3a protein forms a potassium ion channel after tetramerization via inter-monomer disulfide bridges (Cys133) (Lu et al., 2006). The IC activity of 3a protein has been characterized by the self-oligomerization of synthetic peptides corresponding to each of the three TMDs into artificial lipid bilayers. Only TMD2 and TMD3 peptides restored IC activity (Chien et al., 2013). However, additional studies are required to characterize the IC selectivity of the reconstituted viroporin. SARS-CoV 3a protein influences virus

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pathogenicity (McBride and Fielding, 2012). The implication of 3a protein in viral replication seems limited as deletion mutants missing this protein only show a modest reduction in virus replication (Castaño-Rodriguez et al., 2014; Yount et al., 2005). Nevertheless, the role of 3a protein in virus budding and release still warrants further investigation due to conflicting data (Akerstrom et al., 2007; Lu et al., 2006). Also, the influence of 3a protein in virus virulence requires additional studies. SARS-CoV infection induces an uncontrolled proinflammatory response leading to ARDS and respiratory failure (Smits et al., 2010, 2011). One of the most relevant functions of 3a protein in SARS-CoV virulence is the induction of a pro-inflammatory response (Kanzawa et al., 2006; Obitsu et al., 2009), similarly to what has been shown for SARS-CoV E protein (Nieto-Torres et al., 2014), and for other viroporins (Ichinohe et al., 2010; Ito et al., 2012; McAuley et al., 2013; Triantafilou et al., 2013). These data suggest that 3a protein and its IC activity could also be responsible for the enhancement of a proinflammatory response after SARS-CoV infection. Protein 3a also induces apoptosis (Lu et al., 2006). However, this activity was elicited by a 3a protein mutant deficient in oligomerization, therefore the IC activity of 3a protein does not seem responsible for the induction of apoptosis.

7. Conclusions

The effect of CoVs proteins on cellular signaling pathways, in particular those affected by E protein, has been revised. It has been shown that deletion of full-length E protein or modification of active motifs present in this protein have been essential to achieve two aims, the engineering of vaccine candidates that provide full-protection against homologous and heterologous CoVs, and the identification of drugs that interfere with exacerbated pathways responsible for disease severity. These drugs increase experimental animals survival and, therefore, are good candidates as antivirals in human health.

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