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ISG15: Its Roles in SARS-CoV-2 and Other Viral Infections

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Abstract

Interferon (IFN)-stimulated gene 15 (ISG15), a ubiquitin-like pleiotropic protein and one of the most abundant ISGs, has been studied extensively; however, its roles in SARS-CoV-2 and other viral infections have just begun to be elucidated. Emerging evidence suggests that ISG15 –either in its conjugated or unconjugated 'free' form– acts both intracellularly and extracellularly, and exerts anti- or pro-viral effects. To counteract ISG15's antiviral roles, viruses have evolved sophisticated tactics. Here, we discuss recent advances in ISG15's physiological functions as a post-translational modifier or 'cytokine-like' molecule during SARS-CoV-2 and other viral infections. Furthermore, we highlight the detailed mechanisms viruses use to block ISG15-dependent antiviral defenses. A comprehensive understanding of ISG15 biology in the context of virus infection may spur new therapeutic approaches for a range of viral infectious diseases.

Keywords

ISG15; ISGylation; innate immunity; SARS-CoV-2; hyperinflammation; viral evasion

ISG15's multifaceted roles in virus infection and immunity

The broad array of clinical manifestations and variable disease progression associated with coronavirus disease 2019 (**COVID-19**; see Glossary) still represent a public health concern, even more than three years after the emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1–3]. SARS-CoV-2 and its **variants of concern (VOCs)** induce a plethora of disease symptoms in humans, varying from asymptomatic or mild infections to severe illness including lung damage and multi-organ failure as well as long-term sequelae [4, 5]. Recent research has revealed that aberrant cytokine responses characterized by impaired early type I interferon (*e.g.*, IFN-α/β) responses and prolonged secretion of proinflammatory cytokines and chemokines (such as TNF, IL-1β, IL-6, IL-8, and CCL2) are major determinants of COVID-19 severity and the associated mortality [6–10].

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IFN-mediated host responses lead to rapid immune defense against invading viral pathogens. Upon detection of viral RNA or DNA (major pathogen-associated molecular patterns (PAMPs)), innate sensor proteins or **pattern-recognition receptors (PRRs)**, including RIG-I-like receptors and cyclic GMP–AMP synthase (cGAS), initiate downstream antiviral signaling pathways that lead to type I and type III IFN gene expression [11, 12]. The binding of type I and type III IFNs, respectively, to the IFN-α/β receptor (IFNAR) and IL-10R2/IFN-λR1 receptor complex on the surface of the infected cell and also neighboring cells stimulates **autocrine and paracrine signaling**, which prompts the induction of many **IFN-stimulated genes (ISGs)** [13–16]. Among these ISGs, ISG15 is a key orchestrator of host immune defense programs during viral infection [17, 18]. The ISG15 protein, which has only been identified in higher vertebrates [19–21], shares conformational and functional homology (overall sequence similarity 59.3%) with ubiquitin (Ub), leading to its designation as an Ub-like (**UBL**) protein [22]. It contains two nearly identical ubiquitin-like β-grasp domains interconnected through a short linker – a central coil hinge. Each domain comprises four β-sheets and one α-helix, and the carboxyl-terminal sequence of the ISG15 precursor protein is cleaved off during maturation to expose the UBL diglycine motif (LRGG) involved in covalent conjugation to target proteins [23–26]. Intracellular ISG15 protein accumulates after induction of IFN signaling in response to the detection of a range of viruses including respiratory viruses (*e.g.*, SARS-CoV-2 and influenza viruses) [27, 28] and mosquito-transmitted viruses (*e.g.*, Zika and West Nile viruses) [29, 30]. Inside cells, ISG15 can exert antiviral effects by functioning as a post-translational modifier of host and viral proteins. It is conjugated to target proteins via a three-step biochemical cascade termed **'ISGylation'** that involves a concerted action of 1) an E1 enzyme (Ube1L/UBA7) that activates ISG15; 2) an E2 conjugating enzyme (Ube2L6/UbcH8); and 3) an E3 ligase (*e.g.*, HERC5, EFP/TRIM25, ARIH1) which catalyzes the final step of ISG15 conjugation to the substrate protein [23, 31–34]. Covalent conjugation of ISG15 to lysine residues in target host proteins regulates protein translation, stability or trafficking, cytokine production and immune modulation, cytoskeleton dynamics, and DNA damage responses (among many other processes) [35]. Ubiquitin-specific peptidase 18 (USP18/UBP43), a host enzyme bearing isopeptidase activity, catalyzes the removal of conjugated ISG15 from ISGylated proteins [36, 37]. Besides its role in host or viral protein ISGylation, intracellular ISG15 – in its unconjugated form – regulates IFNAR signaling and demonstrates an unexpected proviral effect as evidenced in certain human patients with in-born ISG15 deficiency [38]. Moreover, unconjugated ISG15 can be secreted from mammalian cells into the extracellular milieu [39]. Recent studies illuminated that extracellular ISG15 can induce IFN-γ secretion, which points towards an immunomodulatory, 'cytokine-like' function of ISG15 [40]. Lack of free ISG15 impairs IFN-γ immunity and increases mycobacterial disease susceptibility [41, 42]. More recently, it has been reported that SARS-CoV-2 infection of induced pluripotent stem cell (iPSC)-derived human macrophages bolstered ISG15 secretion via the deISGylating activity of the viral papain-like protease (PLpro), which in turn, contributed to aberrant macrophage activation and excessive production of proinflammatory cytokines. This activity of the free, extracellular ISG15 has been proposed to have implications in the hyperinflammation seen in severe COVID-19 (Box 1) [9, 43].

While recent studies underscore that ISG15 has both antiviral and proviral roles, detailed mechanistic insights into its biological functions, including its role as an extracellularly secreted molecule, have just begun to be elucidated [40, 44–46]. In particular, a detailed characterization of ISG15's target proteins and substrates is warranted. Along these lines, certain functions of ISG15 are species-, cell type- and/or virus-specific; therefore, elucidating the underlying mechanisms for these differential effects is an exciting avenue for future studies, and may provide important insights for designing novel antiviral or immunomodulatory therapeutics.

In this review, we summarize the roles of ISG15 in RNA virus or DNA virus infection and antiviral innate immunity, and draw parallels to SARS-CoV-2 infection and COVID-19 immunopathology. We discuss the emerging antiviral effector functions of ISG15 and also highlight its unexpected proviral effect illuminated by studies on inborn ISG15 deficiency in humans. We further discuss the recent advances in the proposed 'cytokine-like' function of free ISG15, and its relevance for hyperinflammation, a critical clinical manifestation of severe COVID-19. Finally, we summarize how viruses including SARS-CoV-2 perturb the ISGylation-deISGylation circuit to reprogram host innate immunity. A comprehensive understanding of ISG15's immunomodulatory roles, and viral dysregulation thereof, may promote the development of next-generation antiviral therapeutics.

The antiviral vs. proviral roles of ISG15

The antiviral functions of ISG15 in mice

The *in vivo* role of ISG15 has been examined both in murine models (deficient in ISG15 or specific components of the ISGylation machinery) and in human patients with inborn ISG15 deficiency. Intriguingly, these studies revealed primarily antiviral activities for ISG15 in mice, while the studies in *ISG15*-deficient humans showed a dominant proviral function (as described in detail in the later section).

Studies using *Isg15* and/or *Ube1l* knockout (KO) murine models, as well as cells derived from these mice, indicated that ISG15 has antiviral activity against a range of viruses [44]. For example, $Isg15^{-/-}$ and $Ube1l^{-/-}$ mice showed increased virus susceptibility and replication of Sindbis virus, influenza A virus (IAV), influenza B virus (IBV), and certain herpesviruses (herpes simplex virus type 1 and murine gammaherpesvirus 68) compared to wild-type mice [47–49]. Similar observations of ISG15's antiviral activity via conjugation were made for $Isg15^{-/-}$ and $Ube1l^{-/-}$ mice presenting with coxsackievirus B3 (CVB3)-induced lethality due to exacerbated **myocarditis** and heart failure. Complementation of $Isg15^{-/-}$ cardiomyocytes with ISG15 expression substantially reduced viral titers, and infection of $Usp18^{C61A/C61A}$ knock-in mice, in which the ISG15-specific isopeptidase activity is ablated, led to enhanced ISG15 conjugation in cardiac homogenates coinciding with ameliorated CVB3 myocarditis [50]. $Isg15^{-/-}$ and $Ube1l^{-/-}$ mice infected with murine norovirus (MNV-1, an enteric pathogen) exhibited enhanced viral burden in various organs despite no significant difference in weight loss or clinical signs. Bone marrow-derived dendritic cells and macrophages isolated from these mice, upon type I IFN priming, supported greater viral replication than the treated WT cells, indicating ISG15 conjugation-mediated virus restriction [51]. While these studies are instrumental in unveiling ISG15's

broad antiviral role via conjugation, the ISGylation targets and mechanisms underlying the antiviral phenotypes during infection with a specific virus remain less well understood.

Direct conjugation of viral proteins by ISG15

One of the chief antiviral functions of ISG15, in its conjugated form, has been attributed to the modification of viral proteins involved in various steps of the viral replication cycle including: *1)* viral entry/fusion; *2)* uncoating and RNA release; *3)* viral replication complex formation, transcription, and translation; *4)* post-translational modification and processing of newly synthesized viral proteins; *5)* viral egress and trafficking; *6)* viral assembly and budding; and *7)* viral release and maturation (Figure 1). Studies on HERC5, which is a major E3 ligase for ISG15 conjugation, revealed co-translational ISGylation of newly synthesized proteins which included also viral proteins [52]. Mechanistically, ISGylation can dominant-negatively interfere with viral protein stability, multimerization or interactions, thereby inhibiting specific steps of viral lifecycles. For example, IAV NS1, IBV NP, human papillomavirus virus (HPV) capsid protein L1, human cytomegalovirus (HCMV) pUL26, and CVB3 2A protease (2Apro) undergo ISGylation, which blocks their functions (Figure 1) [27, 50, 52–54]. IAV NS1 is a major virulence factor important for type I IFN antagonism and thereby effective IAV replication [55]. Two studies revealed that IAV NS1 is ISGylated upon ectopic expression of the ISGylation machinery components in human embryonic kidney (HEK293T) cells. ISG15 conjugation at lysine-41 (K41) of NS1 inhibits its nuclear translocation by importin-α, thereby blocking NS1 functions. In the second study, ISGylation of NS1 (at multiple lysines across the protein) disrupted NS1 dimerization and its ability to bind RNA (*e.g.*, double-stranded RNA and U6 small nuclear RNA) [27, 56]. Similarly, infection of human adenocarcinoma alveolar basal epithelial (A549) cells expressing either WT or mutant ISG15 (harboring carboxy-terminal Ala-Ala instead of the Gly-Gly motif required for ISG15 conjugation) revealed that ISG15 conjugation of IBV NP protein blocks its **oligomerization** and the formation of viral ribonucleoprotein (vRNP). Mechanistically, ISGylated IBV NP functions as a dominant-negative inhibitor of the oligomerization of unmodified NP, which ultimately impedes effective viral RNA synthesis and replication [53]. ISGylation of CVB3 2Apro was shown to inhibit the 2Apro-mediated cleavage of mammalian eukaryotic translation initiation factor 4γ1 (eIF4G1) in mouse cardiomyocytes, thereby blocking virus-induced host translational shut-off to restrict CVB3 replication [50]. Together, these findings show that viral protein ISGylation can serve to directly block crucial steps in the viral lifecycle or reinstate host antiviral responses, though the precise mechanisms can be host-species and cell-type dependent. It is also noted that whereas ectopic expression of the ISGylation machinery can lead to ISGylation of many proteins, only ~5% of newly translated proteins are ISGylated by the endogenous ISGylation machinery upon IFN stimulation [52]. Therefore, in-depth studies under physiological settings are warranted to carefully validate the ISG15 substrates identified in overexpression systems.

Modulation of host PRR activity by ISGylation

Conjugated ISG15 can also restrict virus infection by positively regulating host PRR-mediated IFN induction (Figure 2). ISGylation at residues K23 and K43 within the caspase activation and recruitment domains (CARDs) of the cytosolic RNA receptor MDA5

promotes its multimerization and activation; this ultimately drives innate immune responses against several RNA viruses including coronaviruses, picornaviruses, and flaviviruses [28]. Ariadne RBR E3 ubiquitin protein ligase 1 (ARIH1) catalyzes mono-ISG15 modification at the K187 site of the DNA sensor cGAS, promoting its oligomerization and ability to elicit antiviral and proinflammatory cytokine production (Figure 2) [57]. Understanding in molecular detail the roles of host protein ISGylation is important because it may unveil novel regulatory mechanisms of immunity (and potentially other cellular pathways) involved in the defense against a range of viral pathogens.

USP18 negatively regulates IFNAR signaling independently of deISGylation

Several *in vivo* studies demonstrated that USP18, apart from its 'classical' isopeptidase activity, plays an important role as a negative-feedback regulator of IFNAR signaling independently of its protease activity. Both *Usp18*$^{-/-}$ and *Usp18*$^{-/-}$/*Isg15*$^{-/-}$ mice (bred on a mixed genetic background (129, C57BL/6, Swiss Webster)) displayed reduced survivability due to hypersensitive responses to proinflammatory stimuli such as LPS and poly(I:C) [58]. In contrast, *Usp18*$^{-/-}$ mice on only C57BL/6 background developed hydrocephalus (inflammation of the white matter region in the brain) and succumbed 2–19 weeks after birth, suggesting that USP18 deficiency can lead to hyperinflammation independent of heightened ISGylation, at least in certain contexts (*i.e.* mouse background). In accord, *USP18*-deficient mice developed cell-autonomous activation of white matter microglia from postnatal day 10 onwards, and the brain homogenates showed robust induction of myelo-attracting and myelo-activating chemokines [59]. Notably, *Usp18*$^{C61A/C61A}$ knock-in mice devoid of only USP18's isopeptidase activity did not develop brain abnormalities or reduced survivability, indicating that USP18 serves other functions in regulating IFN and inflammatory cytokine responses [60]. Similarly, while USP18-deficient mice exhibited reduced virus replication following lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus (VSV), Sindbis virus, and human immunodeficiency virus (HIV) infections [44, 60–63], these effects were also observed upon combinatorial ISG15 deficiency, signifying a deISGylation-independent role of USP18. Subsequent mechanistic studies showed that USP18 regulates the sensitivity of IFNAR signaling by competing with JAK1 for IFNAR2 binding [64, 65]. As such, USP18 deficiency sensitizes IFNAR signaling in multiple murine cell types such as microglial cells and macrophages, leading to increased and prolonged phosphorylation and activation of STAT1 and STAT2 and thereby elevated expression of ISGs restricting the virus (Figure 2) [59, 66].

USP18 stabilization by ISG15 underlies the proviral effect of ISG15 in humans

Clinical studies have supported the apparently unexpected proviral role of ISG15 during pathogenic infections. Human patients with inherited ISG15 deficiency develop **type I interferonopathy** with unique clinical manifestations such as intracranial calcifications [38] or dermatological lesions [67]. Serological tests however from these patients infected with both DNA viruses and RNA viruses did not manifest increased susceptibility to infection, virus replication, or viral disease severity [38]. Further investigation of type I IFN-primed patient fibroblasts revealed enhanced and prolonged resistance to a panel of DNA viruses and RNA viruses including HSV-1, IAV, HCMV, Sendai virus (SeV, a murine paramyxovirus), and even the highly pathogenic Rift Valley fever virus (RVFV)

and Nipah virus (NiV). Notably, the resistance of these patients' cells to virus infection coincided with an elevated expression of ISGs, a phenomenon reminiscent of that observed in USP18-deficient murine cells. Altogether, these results suggested that ISG15-deficient individuals may show a comparatively mild disease course in response to viral infections, supporting a proviral effect of ISG15 [38]. Extending the mechanistic role of USP18 in the negative regulation of IFNAR signaling, it was found that USP18 underwent accelerated proteasomal degradation (mediated by the E3 ligase S-phase kinase-associated protein 2 (SKP2) and likely others) in immortalized human fibroblasts as well as fibrosarcoma and epithelial cell lines lacking ISG15. Transduction of ISG15-deficient patient fibroblasts with lentiviral constructs expressing either WT ISG15 or its unconjugatable mutant (diglycine motif deletion; ΔGG) restored USP18 expression and attenuated the aberrant upregulation of ISGs, indicating the stabilization of USP18 by unconjugated intracellular ISG15 [38] (Figure 2). Therefore, both USP18 and ISG15 participate in a negative-feedback loop to restrain IFNAR signaling, and ISG15 deficiency in human patients phenocopies the prolonged type I IFN signaling and enhanced ISG levels seen in the context of murine USP18 deficiency [38, 67, 68] (Figure 2). Interestingly, the antiviral effect of ISG15 in mice, opposed to its dominant proviral function in humans, is presumably due to the biochemical and functional differences of mouse vs. human ISG15. Recent findings suggested that mouse ISG15 is unable to stabilize USP18, despite that the interaction between ISG15 and USP18 is conserved among several different species [36, 68]. Further investigation is crucial to determine the differential regulation of IFN signaling by human and mouse ISG15. Additional research will also be essential to elucidate the host species- and cell-type-specific effects of ISG15–both antiviral and proviral ones–, and to understand how these different functions of ISG15 contribute to virus restriction and immune homeostasis.

The intracellular vs. extracellular roles of ISG15

Regulation of antiviral responses by intracellular ISG15

Antiviral innate immune signaling relies on a coordinated series of protein posttranslational modifications to fine-tune the magnitude of cytokine responses and to maintain immune homeostasis [12]. Intracellularly, conjugated and free ISG15 have been shown to positively and negatively regulate innate immune signaling pathways, respectively. For example, in addition to the innate immune sensors MDA5 and cGAS, transcription factors such as IFN-regulatory factor 3 (IRF3) and STAT1 also undergo ISG15 conjugation/ISGylation and play significant roles in promoting the innate immune response [69–71]. ISGylation of IRF3 by HERC5 at K190, K360, and K366 enhances antiviral immune responses by constraining IRF3 degradation. Reconstitution of *IRF3*$^{-/-}$ murine primary embryonic fibroblasts (MEFs) with an ISGylation-deficient IRF3 mutant showed a stronger interaction with the peptidyl-prolyl isomerase Pin1 and elevated levels of polyubiquitination followed by degradation during SeV infection [70]. STAT1 ISGylation was shown to sustain STAT1's phosphorylation state and activation (Figure 3). In a model of alcohol-induced liver injury associated with hepatitis C virus (HCV) infection, exposure of HCV-infected hepatocellular carcinoma Huh7.5 cells to acetaldehyde dampened ISGylation of STAT1, leading to its K48-linked polyubiquitination and proteasomal degradation [69]. Interestingly, free ISG15 can reportedly also positively regulate IFNAR signaling by facilitating the activation and nuclear

translocation of STAT1 and STAT2 during pseudorabies virus (PRV) infection of porcine kidney epithelial cells (PK15), hinting at host-species-specific regulatory mechanisms of IFNAR signaling by ISG15 [72].

Both ISG15 and the host protein kinase R (PKR) are induced by IFNs, linking the ISGylation machinery and the translational regulation pathway. PKR, in general, inhibits cellular mRNA translation by phosphorylating eukaryotic translation initiation factor 2A (eIF2α) to execute its antiviral effect against virus infection. Proteomic analysis identified PKR as a major target for ISGylation [35, 71], and mutational analysis revealed K69 and K159 as ISGylation sites [73] (Figure 3). ISGylation of PKR in response to exogenous IFN or LPS stimulation was sufficient to drive PKR constitutive activation, suggesting a potentially unique regulatory circuit of PKR activity [73]. It however remains to be fully determined whether PKR ISGylation has any implication in direct restriction of virus replication. On the other hand, the binding of free ISG15 to the sensor RIG-I reportedly facilitates the LRRC25-mediated autophagic degradation of RIG-I and thereby downregulates innate signaling (Figure 3) [74, 75].

Intracellular free ISG15 can also mediate virus restriction by modulating viral protein function. For example, Ebola virus matrix protein VP40 is regulated by ISG15 to restrict the budding and release of Ebola virus-like particles (VLPs). Ectopic expression of ISG15 in HEK293T cells counteracted VP40 ubiquitination catalyzed by the host E3 ligase NEDD4, which has been shown to be a crucial step in promoting virion release [76, 77]. In the context of HIV-1 infection, ISG15 appears to play both antiviral and proviral roles affecting multiple steps in the viral lifecycle [62, 78, 79]. Ectopically expressed ISG15 impaired HIV-1 budding and release by suppressing ubiquitination of Gag and its association with the cellular protein tumor susceptibility gene 101 (TSG101) that is key to initiating viral assembly/budding via the cellular endosomal sorting complexes required for transport (ESCRT) pathway [62]. Inversely, type I IFN-primed ISG15-deficient human fibroblasts and CRISPR-edited ISG15 KO primary $CD4^+$ T cells were shown to be more resistant to single-cycle HIV-1 infection compared to IFN-primed control cells due to enhanced ISG expression, echoing ISG15's proviral effect that acts through USP18 stabilization [78]. A recent study highlighted a novel antiviral mechanism mediated by free ISG15 during Peste des petits ruminants virus (PPRV) infection. ISG15 interacts with both PPRV nucleoprotein (N) and phosphoprotein (P), impeding the formation of a functionally-active N-P complex, which ultimately suppresses virus replication [80]. Defining the mechanistic roles and contributions of free ISG15 to virus restriction and antiviral immunity is an important avenue of future research because it may offer new ways to target viral pathogens therapeutically.

Regulation of other host defense pathways by ISGylation

Recent global ISGylome screenings have expanded the catalog of known ISG15-target proteins in human cells and mice [71, 81]. However, the vast majority of these potentially new ISGylation target proteins have not yet been validated. Interestingly, ISG15 conjugation often targets critical sites for protein-protein interactions [81], but the functional consequences require further investigation. In the context of autophagy, which has many

fundamental functions but also exerts host defenses against certain viruses (and other pathogens), *in vitro* and *in vivo* studies unveiled a link between protein ISGylation and increased basal or infection-induced autophagy. Identified ISGylated proteins with key functions in autophagy include the mammalian target of rapamycin (mTOR), WIPI2, a phosphatidylinositol-3-phosphate binding protein required for starvation-induced autophagy, activating molecule in BECN1-regulated autophagy protein 1 (AMBRA1), and Ras-related protein Rab-7a (RAB7) [44, 81, 82]; however, the mechanisms by which ISGylation regulates autophagy during specific virus infections are largely unknown. Together, these findings highlight a crucial role for protein ISGylation in fine-tuning autophagy responses during pathogenic assault, and it is tempting to speculate that other important cell-intrinsic host defense pathways are also regulated by ISG15 conjugation.

Cytokine-like functions of extracellular ISG15 in IFN-γ immunity

Despite its structural and functional similarities with Ub (which functions only intracellularly), ISG15 possesses an intriguing feature: a non-canonical function as an extracellular signaling protein. The balance between unconjugated and conjugated intracellular ISG15 (and their corresponding functions) is controlled by the ISGylation/deISGylation machinery of the cell as well as by viral interference. Disruption of the ISGylation/deISGylation circuit can lead to the secretion of free ISG15, which has immunomodulatory functions. However, the mechanisms of how extracellular ISG15 exerts its immunomodulatory effects, and the relevant secretory pathway(s) for ISG15 secretion, have not yet been fully elucidated. Targeting the specific secretory pathway(s) to limit the amount of freely-circulating ISG15 may lead to the development of novel COVID-19 therapeutics (Box 1) [45].

ISG15 can be secreted by human primary monocytes, fibroblasts, neutrophils, and plasmablasts in a manner that is type I IFN-dependent or - independent. Once secreted, ISG15 binds to the cell surface receptor lymphocyte function-associated antigen-1 (LFA-1) [45, 83], facilitating activation of Src family kinases which then stimulate IFN-γ and IL-10 secretion by natural killer (NK) cells and T lymphocytes via autocrine or paracrine signaling pathways (Figure 3). Apart from viral infections, murine infection with *Toxoplasma gondii* triggered the extracellular release of ISG15 (in a dimeric or multimeric form), which stimulated IL-1β production by $CD8\alpha^+$ dendritic cells at the site of infection [84]. Recently, specific residues in ISG15 that are likely involved in mediating its secretion have been mapped, and specific ISG15 mutants that retained protein conjugation and LFA-1 binding abilities but lacked secretory function have been defined [45].

The critical role of extracellular ISG15 is probably best evidenced by a small cohort of human patients presenting Mendelian susceptibility to mycobacterial diseases. These patients contain homozygous genetic lesions that ablate ISG15 expression and developed higher susceptibility to *Salmonella* and *Mycobacterium* infections due to IFN-γ deficiency [42]. Mechanistically, the absence of extracellular ISG15 in the blood impairs IFN-γ production by NK cells and T cells. Investigating the relevance of extracellular ISG15 in other disease contexts may unveil novel insights into ISG15 biology and may lead to new therapeutic intervention approaches.

Regulation of inflammatory responses by unconjugated ISG15

Several studies have highlighted a role for unconjugated ISG15 in dampening proinflammatory cytokine and chemokine expression during virus infection. For example, *Isg15*$^{-/-}$ neonatal mice challenged with Chikungunya virus (CHIKV) exhibited increased pathogenesis compared to WT mice, which was however not due to higher viral replication but caused by a cytokine storm-like phenomenon. Further, antiviral protection in this context was not through Ube1L-catalyzed ISGylation (of note, *Ube1l*$^{-/-}$ mice showed no increased lethality upon CHIKV infection), suggesting an atypical role for unconjugated ISG15 [85]. Similarly, infection of *Isg15*$^{-/-}$ mice with a mutant vaccinia virus (VVΔE3L) lacking the E3 protein (which binds ISG15 and subverts restriction by ISG15) led to significant mortality accompanied by heightened inflammatory responses in the lungs despite limited viral replication [86]. This effect was further attributed to free ISG15 which is proposed to facilitate suppression of lung inflammation upon VVΔE3L infection [87]. It remains to be elucidated whether in these contexts, unconjugated ISG15 functions intracellularly or extracellularly, and what cell types play a pertinent role in producing excessive proinflammatory cytokines. Along these lines, the precise mechanism(s) by which ISG15 restricts viral infection independent of Ube1L-mediated conjugation needs further investigation.

Viral tactics antagonizing ISG15 or the ISGylation machinery

Extensive studies have shown that viruses have evolved immune evasion strategies that antagonize the ISG15 conjugation of viral proteins or block other ISG15-mediated antiviral effects. These strategies include: *1)* transcriptional induction of cellular ISG15-deconjugating enzymes, *2)* sequestration of ISGylated proteins, *3)* suppressing ISG15 transcription and/or ISG15 conjugation, and *4)* utilizing viral enzymes that have direct de-ISGylating activities (Figure 3).

Many viruses employ mechanisms to block or reverse ISGylation by upregulating the gene expression of cellular ISG15-deconjugating enzymes [46]. The IBV NS1 sequesters ISGylated NP proteins to prevent their incorporation into vRNP, which would otherwise impede vRNP assembly [53, 88, 89]. HCV and HCMV, among many other viruses, suppress ISG15 transcription [44]. Vaccinia virus E3L [86, 87] and HCMV pUL26 bind to ISG15 to thwart ISG15 conjugation, although the detailed mechanisms are currently unknown [54]. HCMV pUL26 [54] and Kaposi's sarcoma-associated herpesvirus (KSHV) vIRF1 [90] bind to HERC5 (a major E3 ISG15-protein ligase) to prevent ISG15 conjugation (Figure 3). Viruses have also evolved tactics to remove ISGylation from host or viral proteins by employing viral de-ISGylating enzymes. Prominent examples are coronaviruses encoding papain-like proteases (PLpro) [28, 91], as well as nairoviruses [92] and arteriviruses [93] which utilize ovarian tumor domain (OTU) proteases [94–96]. Interestingly, whereas SARS-CoV-2 PLpro preferentially targets and cleaves ISG15 from substrates, which results in the de-ISGylation and inactivation of MDA5 and other host proteins [28, 97], PLpro from SARS-CoV predominantly targets oligo- or poly-Ub chains [97, 98]. These virus-adapted immune-escape strategies illuminate the concurrent evolution of viral proteins antagonizing the ISGylation machinery. How viruses target and manipulate specifically conjugated ISG15

warrants future investigation. It would be also a new arena to explore a potential role for viral effector proteins in targeting free ISG15 secretion and the secretory signaling pathways to disturb immune surveillance. Moreover, it will be important to define the relative contribution of viral antagonism of ISG15's antiviral roles to pathogenesis.

Concluding Remarks

Free unconjugated ISG15 and ISGylation have been explored in several pathogenic infections, and their roles have recently been investigated in SARS-CoV-2 infection and disease progression. New therapeutics discovered via target-based approaches aimed at targeting SARS-CoV-2 PLpro's de-ISGylating activity, may diminish COVID-19 immunopathology by boosting antiviral IFN and ISG responses via MDA5 and IRF3. Whether SARS-CoV-2 PLpro affects the ISG15 conjugation of other proteins in the infected host cell, and whether de-ISGylation of these substrates contributes to COVID-19 disease progression and severity should be explored in future studies. Whether the potential adjuvant effect of free ISG15 has therapeutic potential also warrants further investigation. Important questions remain about the ideal model systems for studying the functions of free vs. conjugated ISG15, how ISG15 is secreted, and which roles it plays *in vivo* after its secretion. A recombinant mutant SARS-CoV-2 encoding de-ISGylation-impaired PLpro activity might help illuminate these questions. Mapping the ISGylome in SARS-CoV-2 and other viral infections will be useful for identifying novel ISGylated host (immune) proteins and also potential targets of viral antagonism (see Outstanding Questions). Defining the interplay between ISGylation and other innate immune signaling pathways (*e.g.*, Toll-like receptor or NOD-like receptor signaling) is another area of exciting new research. These future studies are expected to provide key information on the multifaceted cellular functions of ISG15 and may offer new insights into approaches to circumventing uncontrolled inflammatory responses and into pan-antiviral strategies for controlling current and future global pandemics.

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Glossary

Autocrine and paracrine signaling

Autocrine signaling is when a cell releases a hormone/chemical substance that acts on the same cell through receptor binding, whereas paracrine signaling refers to cell-to-cell signaling between either neighboring cells or target cells in close vicinity.

COVID-19

Coronavirus disease 2019, a respiratory disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged in December 2019. COVID-19 can cause mild fever, cold, cough, severe respiratory illness, and pneumonia including mortality.

De-ISGylating enzyme

The ISGylation process can be reversed or attenuated by host or viral enzymes called de-ISGylating enzymes, which break the covalent conjugation of ISG15 with target proteins. In the case of viruses, these enzymes often help escape the immune surveillance machinery, or can dysregulate proinflammatory cytokine responses.

ISG
An arsenal of proteins that are induced by type I, II, and III IFNs and have primarily antiviral effector activities. Some ISG products amplify innate signaling, perpetuating feed-forward regulatory loops, or they serve as innate immune receptors.

ISGylation
ISGylation refers to the covalent conjugation of the ubiquitin-like molecule interferon-stimulated gene (ISG15) to lysine residues on substrates through a biochemical enzymatic cascade; this posttranslational modification increasingly occurs during pathogen infection or in response to proinflammatory stimuli.

Cytokine storm
Also known as cytokine release syndrome that is characterized by a dysregulated or hyper-activated innate immune response. In severe COVID-19, excessive secretion of proinflammatory cytokines and chemokines causes local or systemic inflammation and multiorgan failure, leading to death.

Myocarditis
Inflammation of the heart muscle, caused by the uncontrolled body's immune system in response to an infection or some other stimuli.

Oligomerization
An arrangement of several units of the same polypeptide chain (called 'homo-oligomerization'), or of two or more distinct proteins (termed 'hetero-oligomerization'). Oligomerization often increases the stability of the protein(s), or it is necessary to initiate signal transduction or to recruit additional interaction partners.

Pattern-recognition receptor (PRR)
A class of receptors (e.g., cGAS, MDA5 and RIG-I) that act in first-line defense by recognizing pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). The activation of PRRs is necessary to initiate innate immunity and ultimately induces adaptive immune signaling.

Type I interferonopathy
A disorder characterized by dysregulation of type I IFN responses, which is often caused by mutations in innate immune sensors or signaling proteins (such as MDA5 and RIG-I) or by deficiency of ISG15 stimulating aberrant upregulation of IFNs, cytokines, and ISGs.

Variants of concern (VOCs)
More recently emerged SARS-CoV-2 variants that have higher transmissibility or infectivity compared to the original SARS-CoV-2 strain (Wuhan). Some VOCs also have acquired the

ability to evade COVID-19 treatments, diagnostics, or previous immunity from vaccination or natural infection.

UBL

Ubiquitin-like protein. A group of small regulatory proteins (such as ISG15 and SUMO) that, similar to ubiquitin, function in post-translational modifications of proteins. Typically, UBLs are enzymatically conjugated to substrate proteins, regulating their enzymatic activities, interactomes, stabilities or cellular localizations. Some UBLs can also bind to proteins noncovalently, regulating their functions.

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Text Box 1.

Imbalance of free vs. conjugated ISG15 in COVID-19 hyperinflammation

SARS-CoV-2 papain-like protease (PLpro), which is part of the multifunctional non-structural protein 3 (Nsp3), acts as a de-ISGylating enzyme and actively removes conjugated ISG15 from host proteins (*e.g.*, MDA5 and IRF3) to antagonize antiviral IFN responses [28, 97]. Interestingly, recent studies unveiled that the PLpro activity can perturb the intracellular balance between the conjugated and free ISG15, which, in turn, dysregulates human macrophage activation and cytokine responses. Not only did this imbalance induce a shift in macrophage polarization to the inflammatory M1 phenotype, but it also prompted the secretion of deconjugated ISG15 via autophagy-dependent unconventional secretory pathways, which was not observed during infections with ZIKV or IAV [43]. Both events led to the aggravated production of proinflammatory cytokines such as IL-1β and IL-6, which likely contributes to SARS-CoV-2-associated hyperinflammation and determines COVID-19 disease severity [43]. Nevertheless, whether there is any direct interconnection between extracellular ISG15 and systemic inflammation in COVID-19 patients is yet to be identified. Global secretome analysis, and the use of recombinant ISG15 variants devoid of secretion activity, would help define the role of secreted ISG15 in inflammatory responses and perhaps other cellular processes. It is also important to study the role of free vs conjugated ISG15 in immunomodulation of different cell types. Modulating the conjugated vs free ISG15 circuit, or targeting the secretory pathway of free circulating ISG15, may lead to the design of novel immunomodulatory drugs to reduce inflammatory responses in COVID-19 and perhaps other viral diseases.

In regards to antiviral therapeutics, targeting the PLpro activity is particularly attractive because of a three-pronged effect: direct inhibition of viral polyprotein cleavage; reversion of innate-signal antagonism by PLpro; and alleviation of hyperinflammation induced by ISG15 dysregulation. High-throughput screening of clinically approved drugs and pharmacologically-active compounds reported that YM155, Tanshinone I, Cryptotanshinone, and GRL0617 can inhibit SARS-CoV-2 PLpro [99]. GRL0617 selectively inhibits the activity of PLpro by noncovalently binding to the ubiquitin-specific protease (USP) domain of PLpro. In addition, GRL0617 can also block the binding of ISG15's C-terminus to PLpro and inhibits PLpro-mediated de-ISGylation activities [100]. Studies to evaluate the inhibitory efficacies of these compounds in suitable preclinical models will be needed to develop new therapeutic strategies for bench-to-bedside translational research. Such strategies are expected to ameliorate SARS-CoV-2-induced pathology and restore IFN- and ISG15-dependent antiviral pathways.

Outstanding Questions

- How does ISG15 conjugation target specific viral and host proteins?
- What are the ideal model systems to explore the functions of free vs. conjugated ISG15?
- How is ISG15 secreted, and what roles does it play *in vivo* after secretion? What are the major secretory pathways involved in ISG15 secretion and what triggers them to secrete ISG15?
- How does coronavirus PLpro modulate ISG15 secretion *in vivo*?
- How does intracellular unconjugated ISG15 modulate cellular pathways to limit pathogen burden or induce damage during infection?
- Is there any role of unbound ISG15 in systemic inflammation in COVID-19, such as in the brain/lung/liver/spleen/gastrointestinal tract?
- Does extracellular ISG15 induce hyperinflammation in chronic viral infections and in pathology other than COVID-19?
- What are the molecular mechanisms behind other ISG15 targets involved in COVID-19 immunopathology?
- What are the determinant factors for ISG15's species-specific effect on the IFNAR-USP18 axis?

Highlights

- The ubiquitin-like protein, interferon-stimulated gene 15 (ISG15) functions as an intracellular post-translational modifier whose conjugation to viral or host proteins regulates their functionalities.
- ISG15 in its unconjugated (free) form fine-tunes intracellular signaling cascades and, upon extracellular secretion, functions ‘cytokine-like’ contributing to immunomodulation.
- Viruses from diverse families have evolved tactical strategies to manipulate ISG15-mediated antiviral responses.
- Perturbation of both intracellular and extracellular ISG15 functions by SARS-CoV-2 is implicated in COVID-19 hyperinflammation.

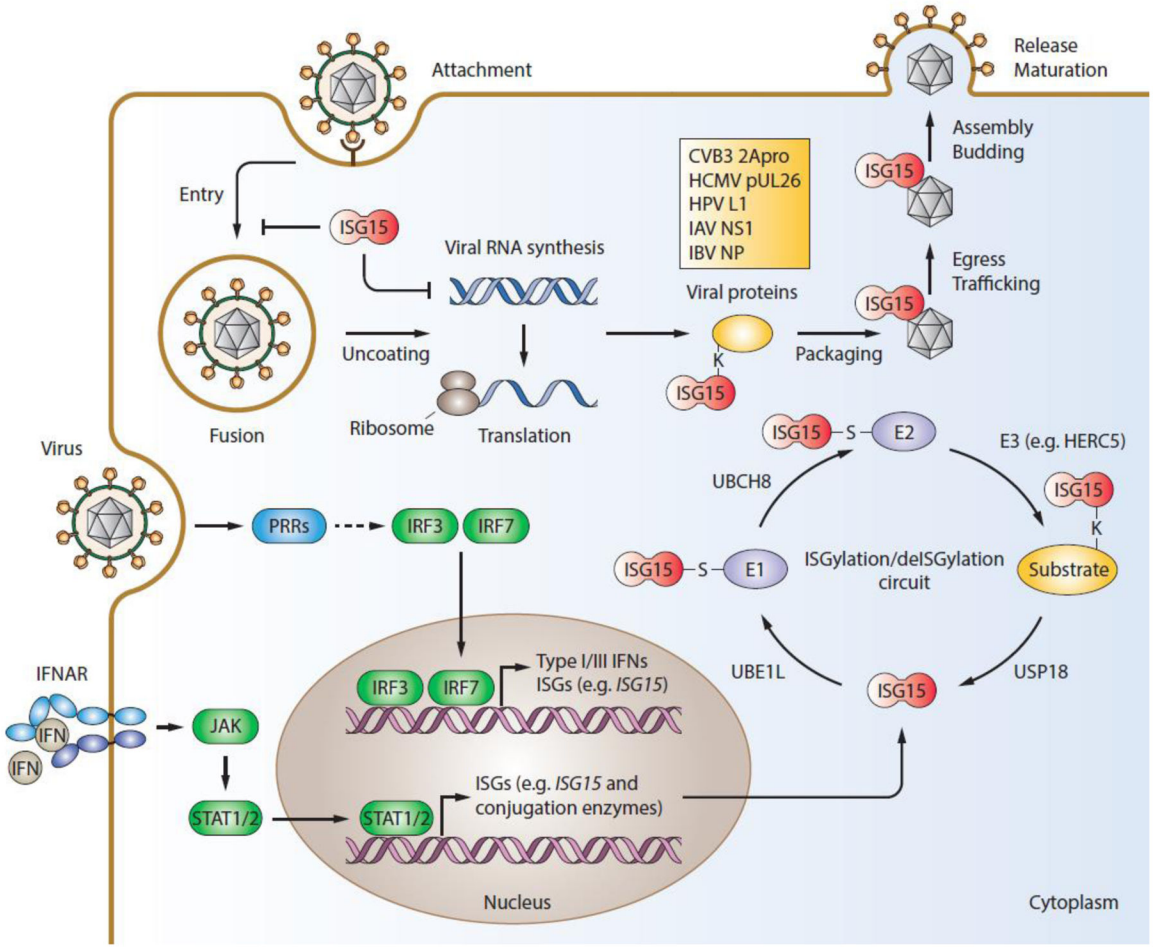

Figure 1. ISG15's role in direct virus restriction.

ISG15, among many other ISGs, is upregulated after virus infection as part of innate signaling elicited by PRRs (such as RIG-I, MDA5 or cGAS) and downstream type I and type III IFN signal transduction (via the JAK-STAT1/2 axis). It is conjugated to substrate proteins via a cascade of enzymatic activities termed 'ISGylation' involving 1) an E1 activating enzyme (Ube1L/UBA7); 2) an E2 conjugating enzyme (Ube2L6/UbcH8); and 3) an E3 ligase (*e.g.*, HERC5). The host isopeptidase USP18/UBP43 catalyzes the removal of conjugated ISG15 from ISGylated proteins. In addition to regulating host proteins, the antiviral activity of ISG15 has been attributed to the inhibition of distinct steps of the viral lifecycle such as viral attachment, entry and uncoating, viral RNA synthesis and protein translation, packaging, egress and trafficking, virion assembly and budding, as well as virion release and maturation. Although the precise mechanisms of how ISG15 blocks these key steps of virus replication are largely undetermined, ISG15 conjugation of specific viral proteins has emerged as a major mode of ISG15's antiviral action. Here, newly synthesized viral proteins are often targeted for ISGylation. Known ISG15-target proteins of viruses include viral capsid proteins such as HPV L1, essential effectors of viral replication complexes and viral IFN antagonists (*e.g.*, IBV NP and IAV NS1), and viral proteins involved in host shut-off such as Coxsackievirus B3 protease 2A (CVB3 2Apro).

ISG15 conjugation of some of these viral proteins impedes their interactions with host-cell proteins or certain RNA species.

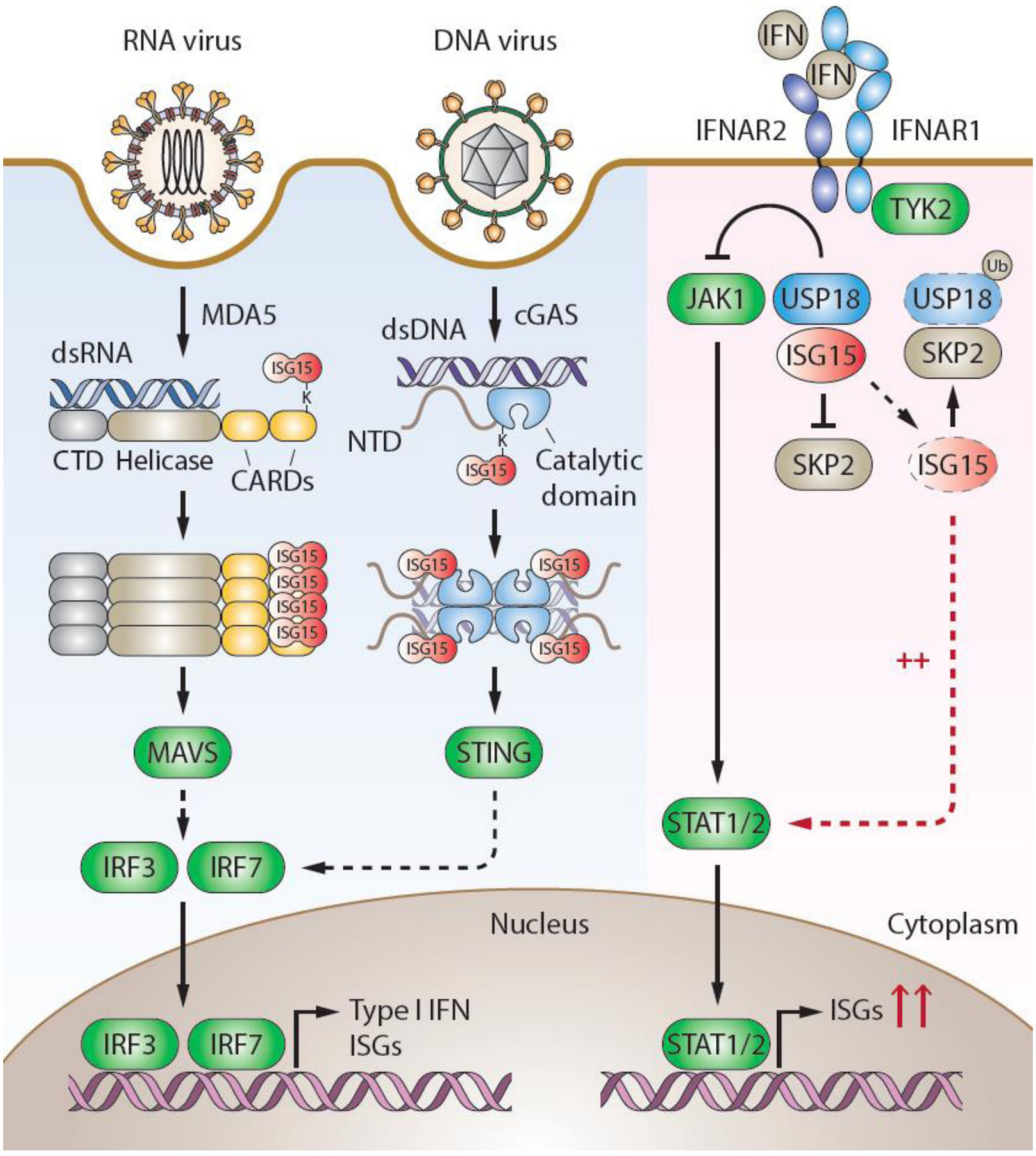

Figure 2. Dual roles of ISG15 in the type I interferon response

ISG15 positively and negatively regulates type I IFN-mediated innate immune responses. ISGylation promotes PRR-mediated signaling in response to RNA virus (*e.g.*, sensed by MDA5) or DNA virus (detected by cGAS) infection. When activated by RNA-PAMPs, MDA5 undergoes ISGylation in its first CARD (at K23 and K43), facilitating MDA5 oligomerization and inducing downstream signaling via MAVS, which localizes to mitochondrial outer membranes (not depicted). Upon binding of immunostimulatory DNA, cGAS oligomerization and activation are facilitated by mono-ISGylation at K187, catalyzed by the E3 ligase ARIH1 (not depicted); this then potentiates IFN gene expression by activating STING at the endoplasmic reticulum (not depicted). Downstream of MAVS and STING, kinases (TBK1/IKKε) activate IRF transcription factors which, once localized to the nucleus, drive the expression of IFNs, proinflammatory cytokines, and a subset of ISGs. Contrasting its role in facilitating PRR activation, ISG15 negatively regulates IFNAR

signaling. Secreted IFNs bind to the IFNAR1/2 complex on the host-cell surface and initiate signaling via the JAK-STAT axis that activates TYK2 and JAK1. These kinases recruit and phosphorylate STAT1 and STAT2, which ultimately drive the expression of ISGs (including ISG15, USP18, and E1, E2, and E3 enzymes). USP18 acts as a negative regulator of IFNAR signaling by competing with JAK1 for IFNAR2 binding. In human cells, free ISG15 binding to USP18 decelerates its proteasomal degradation mediated by the E3 ligase SKP2, reinforcing the inhibition of IFNAR signaling. ISG15 deficiency destabilizes USP18 and thus sensitizes IFNAR signaling, leading to enhanced and prolonged STAT activation and ISG expression.

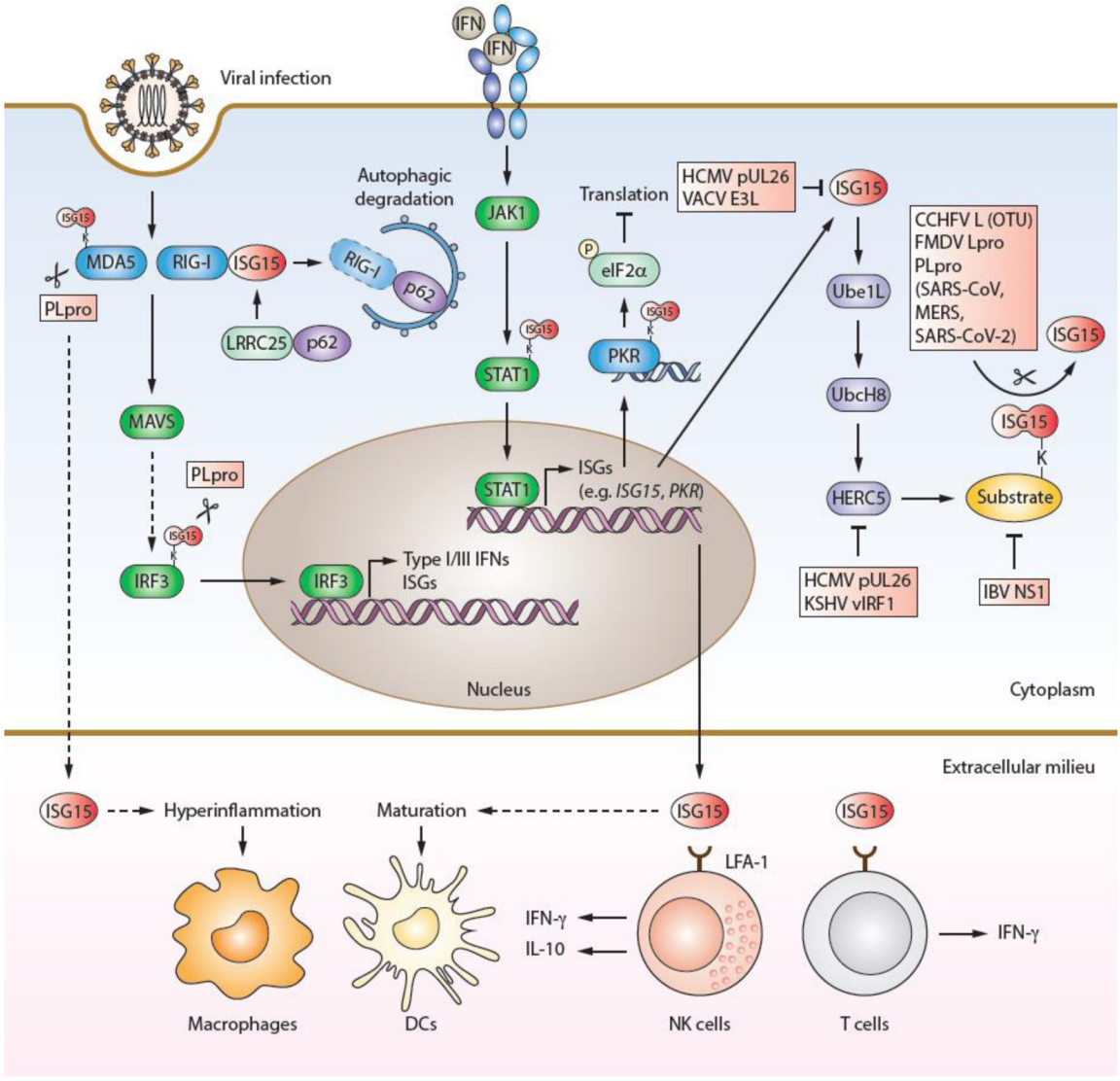

Figure 3. Regulation of antiviral immunity by intracellular and extracellular ISG15 and its perturbation by viral antagonism

Intracellular ISG15 –either in its conjugated or unconjugated free form– regulates antiviral innate signaling by targeting key sensors or transcription factors. In addition to MDA5, PKR, IRF3 and STAT1 are positively regulated by ISGylation. In contrast, RIG-I is negatively regulated by free ISG15, which recruits LRRC25 and leads to RIG-I degradation via autophagy. On the other hand, viruses have evolved divergent tactics to dysregulate ISG15-mediated antiviral immunity. HCMV and HCV (among many other viruses) attenuate ISG15 transcriptional upregulation. HCMV pUL26 and VACV E3L inhibit ISG15 conjugation by binding to ISG15 via unknown mechanisms. Interactions of HCMV pUL26 and KSHV vIRF1 with HERC5 impede its E3 ligase activity, blunting ISGylation reactions. IBV NS1, through sequestering ISGylated NP, counteracts the ISG15-mediated inhibition of viral ribonucleoprotein complex assembly. OTU proteases of nairoviruses and arteriviruses and PLpro enzymes of coronaviruses have direct deISGylating activity by targeting the diglycine conjugation motif of ISG15. Specifically, the PLpro of SARS-CoV-2 removes

conjugated ISG15 from MDA5 and IRF3, thereby inhibiting innate immune signaling. Certain cell types secrete free ISG15 after viral infection, and the extracellular ISG15 can stimulate IFN-γ production via engagement with the LFA-1 receptor on NK and T cells or can promote dendritic cell maturation. SARS-CoV-2 PLpro has been shown to perturb the ISGylation-deISGylation circuit in human macrophages, which aggravates ISG15 secretion contributing to macrophage M1 polarization and exacerbated production of proinflammatory cytokines and chemokines.