Modulation of extracellular ISG15 signaling by pathogens and viral effector proteins

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Highlights
- ISG15 is released from multiple cell types to signal to LFA-1-expressing lymphocytes
- Mutational analysis separates ISG15 secretion from LFA-1 binding and ISGylation
- Intracellular conjugation of ISG15 negatively modulates its secretion
- Viral de-ISGylases, including SARS-CoV-2 PL<sup>pro</sup>, positively modulate ISG15 secretion

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In Brief
Swaim et al. characterize cell types and immune agonists that stimulate ISG15 secretion and signaling to lymphocytes. Intracellular conjugation of ISG15 is shown to inhibit secretion, whereas viral de-ISGylating enzymes, including SARS-CoV-2 PL<sup>pro</sup>, enhance ISG15 secretion, suggesting a potential role for ISG15 in pro-inflammatory responses associated with viral infections.
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INTRODUCTION

ISG15 is a ubiquitin-like modifier that also functions extracellularly, signaling through the LFA-1 integrin to promote interferon (IFN-γ) release from natural killer (NK) and T cells. The signals that lead to the production of extracellular ISG15 and the relationship between its two core functions remain unclear. We show that both epithelial cells and lymphocytes can secrete ISG15, which then signals in either an autocrine or paracrine manner to LFA-1-expressing cells. Microbial pathogens and Toll-like receptor (TLR) agonists result in both IFN-β-dependent and -independent secretion of ISG15, and residues required for ISG15 secretion are mapped. Intracellular ISGylation inhibits secretion, and viral effector proteins, influenza B NS1, and viral de-ISGylases, including SARS-CoV-2 PLpro, have opposing effects on secretion of ISG15. These results establish extracellular ISG15 as a cytokine-like protein that bridges early innate and IFN-γ-dependent immune responses, and indicate that pathogens have evolved to differentially inhibit the intracellular and extracellular functions of ISG15.

SUMMARY

ISG15 is a ubiquitin-like modifier that also functions extracellularly, signaling through the LFA-1 integrin to promote interferon (IFN-γ) release from natural killer (NK) and T cells. The signals that lead to the production of extracellular ISG15 and the relationship between its two core functions remain unclear. We show that both epithelial cells and lymphocytes can secrete ISG15, which then signals in either an autocrine or paracrine manner to LFA-1-expressing cells. Microbial pathogens and Toll-like receptor (TLR) agonists result in both IFN-β-dependent and -independent secretion of ISG15, and residues required for ISG15 secretion are mapped. Intracellular ISGylation inhibits secretion, and viral effector proteins, influenza B NS1, and viral de-ISGylases, including SARS-CoV-2 PLpro, have opposing effects on secretion of ISG15. These results establish extracellular ISG15 as a cytokine-like protein that bridges early innate and IFN-γ-dependent immune responses, and indicate that pathogens have evolved to differentially inhibit the intracellular and extracellular functions of ISG15.

INTRODUCTION

ISG15 is a ubiquitin-like protein (Ubl) consisting of two ubiquitin-like domains connected by a short linker (Narasimhan et al., 2005). Expression of ISG15 is induced by type I interferon (IFN-α/β) signaling (Farrell et al., 1979), although other innate immune activators can lead to expression of ISG15 (Ashley et al., 2019; Lertsooksawat et al., 2019; Radoshevich et al., 2015). ISG15 was the first Ubl modifier to be described (Haas et al., 1987; Loeb and Haas, 1992), and ISGylation of host and/or pathogen proteins has been best characterized as an antiviral defense mechanism. Viruses that are inhibited by ISGylation include influenza A, Ebola, hepatitis B and C, HIV, human papillomavirus (HPV), vaccinia, West Nile, and Zika (Morales and Lenschow, 2013). Consistent with its antiviral function, many viruses, including Nairoviruses, coronaviruses, and foot and mouth disease virus (FMDV), have devised mechanisms for inhibiting or reversing ISGylation, including by expression of ISG15 deconjugating enzymes (Frias-Staheli et al., 2007; Lindner et al., 2007; Mielech et al., 2014; Swatek et al., 2018; Dzimianski et al., 2019a), while the influenza B virus NS1 (NS1B) proteins bind non-covalently to ISG15 and sequester ISGylated proteins (Zhao et al., 2016).

Hundreds of cellular and viral proteins are ISGylated in type I IFN-stimulated cells (Zhao et al., 2005). The basis of the extreme range of ISGylation substrates is that Herc5, the major E3 enzyme for ISG15, is ribosome-associated and broadly ISGylates proteins co-translationally in a near-stochastic manner (Durfee et al., 2010). Like ISG15, the E1, E2, and E3 enzymes for ISG15, Ube1L/Ubα7 (Yuan and Krug, 2001), Ube2L6 (Kim et al., 2004; Zhao et al., 2004), and Herc5 (mouse Herc6) (Dastur et al., 2006; Oudshoorn et al., 2012; Ketscher et al., 2012) are all induced at the transcriptional level by type I IFN signaling, as is the major deconjugating enzyme for ISG15, Usp18 (mouse Ubp43) (Malakhov et al., 2002). The biochemical effects of ISGylation on both viral and cellular proteins and the basis of its antiviral effects remain important areas of investigation. We previously proposed that viral proteins, rather than cellular proteins, are likely to be the key biologically relevant targets of ISGylation, and that oligomeric viral structural proteins may be particularly sensitive to inactivation by ISGylation (Durfee et al., 2010). Evidence for this was recently provided for influenza A virus, where a relatively low level of ISGylation of the viral nucleocapsid protein (NP) had a dominant-negative effect on the ability of the complex to package viral RNAs (Zhao et al., 2016).

Early publications indicated that ISG15 had a non-canonical function as an extracellular signaling molecule, stimulating the release of IFN-γ from lymphocytes (O’Cunha et al., 1996; Recht et al., 1991). A confirmation of the relevance of this finding came with the discovery of ISG15-deficient patients who developed mycobacterial infections after receiving the live-attenuated Bacillus-Calmette-Guérin (BCG) tuberculosis vaccine (Bogunovic et al., 2012). All previously identified patients who were classified as “Mendelian susceptibility to mycobacterial disease” (MSMD) patients were deficient in either producing or responding to IFN-γ, and like the ISG15-deficient patients, most were identified...
RESULTS

A Cell-Based Reporter of ISG15 Secretion

ISG15 has been directly detected in the cell culture supernatants of various cell types (D’Cunha et al., 1996; Knight and Cordova, 1991), and we previously showed that purified ISG15 protein, when added directly to cell culture media, induced the secretion of IFN-γ from peripheral blood mononuclear cells (PBMCs) and T or NK-derived cell lines (Jurkat and NK-92 cells, respectively) (Swaim et al., 2017). We sought to bridge these lines of experimentation in a assay that would reflect both release of ISG15 from cells and a biologic readout based on IFN-γ secretion. Jurkat cells are defective for IFN-γ expression; however, it can be ectopically expressed by transfection of an IFN-γ expression plasmid; transfected cells will then secrete IFN-γ on addition of purified ISG15 to the culture media (Swaim et al., 2017). To determine whether Jurkat cells were also able to serve as a source of extracellular ISG15, we co-transfected cells with plasmids expressing IFN-γ and ISG15. If ectopically expressed ISG15 was secreted by the Jurkat cells, it would be predicted to result in autocrine-like signaling via LFA-1, which would in turn stimulate secretion of the ectopically expressed IFN-γ. As shown in Figure 1A, there was a low level of IFN-γ secretion when cells were transfected with only the IFN-γ-expressing plasmid; while co-transfection with the WT ISG15-expressing plasmid enhanced IFN-γ secretion approximately 2.5-fold. Co-expression of IFN-γ with a variant of ISG15 (Y96L/Q102D) that cannot engage the cell surface receptor for ISG15 (LFA-1) did not induce IFN-γ secretion above background. These results suggest that expression of ISG15 in Jurkat cells is sufficient for secretion of ISG15, which can then signal in an autocrine manner to induce IFN-γ secretion.

To determine whether expression of ISG15 in an epithelial cell type leads to extracellular signaling, ISG15 was expressed ectopically in HEK293T cells in a transwell culture system, with transfected HEK293T cells separated by a 0.4-μm membrane from NK-92 cells in suspension. As shown diagrammatically in Figure 1B, if diffusible extracellular ISG15 was produced by the HEK293T cells, it would be expected to cross the barrier and signal to the NK-92 cells. The use of the transwell system prevented the potential for direct cell–cell signaling events, as well as potential killing of the HEK293T cells by the NK-92 cells. As shown in Figure 1C, in the absence of IL-12 and without transfection of the HEK293T cells, there was almost no detectable IFN-γ produced by the NK-92 cells. In the presence of IL-12 alone, there was a modest level of IFN-γ secretion, and this was significantly enhanced when the HEK293T cells were transfected with the WT ISG15-expressing plasmid. There was no stimulation of IFN-γ secretion when the HEK293T cells were transfected with the plasmid expressing the Y96L/Q102D ISG15 variant. These results indicate that an epithelial cell is able to release ISG15 into the extracellular space, which can then signal to an LFA-1-expressing cell type (e.g., NK-92 cells).

Determinants of ISG15 Required for Release from Cells

We used the HEK293T/NK-92 transwell assay to map determinants of ISG15 required for secretion of ISG15. A set of surface residue variants of ISG15 was created previously and used to
map the residues necessary for interacting with LFA-1 (Swaim et al., 2017). A subset was expressed by transfection in HEK293T cells in order to identify variants specifically defective for secretion of ISG15. Variants of interest would be defective for signaling to NK-92 cells in the transwell system (Figure 2A) but active for signaling when added as purified proteins directly to IL-12-stimulated NK-92 cells (Figure 2B). Three variants met these criteria: L72A, S83A, and L85F (red bars in Figures 2A and 2B). Although these variants appeared to signal slightly less than WT ISG15 when added to NK-92 cells as purified proteins (Figure 2B), the differences relative to WT ISG15 were not statistically significant for the L72A or S83A variants and only weakly significant for the L85F variant. All three secretion-defective variants retained the ability to be conjugated intracellularly (Figure S1A). To directly compare their expression and secretion with wild-type (WT) ISG15, they were expressed by transfection in HEK293T cells and detected by immunoblotting of total cell extracts (to detect intracellular ISG15) and by immunoprecipitation and immunoblotting of the cell culture supernatants from transfected cells (to detect extracellular ISG15). As shown in Figure 2C, all of the proteins were expressed at similar levels intracellularly, but only the WT ISG15 was detected in the cell culture supernatant. Note that ISG15 proteins expressed intracellularly remained unconjugated, because the cells do not express the conjugating enzymes under these conditions (see Figure S1A). Together, these results indicate that L72A, S83A, and L85F variants are defective for secretion, yet they are competent for receptor binding and for intracellular ISGylation.

The L72, S83, and L85 residues lay on a surface that spans the linker between the N- and C-lobe Ubl domains of ISG15 (Figure 2D). As shown in Table S1 (top), L72 and L85 in human ISG15 are invariant across 22 other primate ISG15 sequences examined, whereas S83 was conserved in 18 of 22 sequences. Across 107 non-primate mammalian ISG15 sequences examined (Table S1, bottom), L85 was again invariant, whereas L72 and S83 were conserved in 103 and 82 of these sequences, respectively. L72, S83, and L85 are outside of the region critical for binding to the α2 domain of CD11a/αL, consistent with the fact that alteration of these residues did not impair IFN-γ secretion when the proteins were added directly to NK-92 cell culture media (Figure 2B). It should also be noted that the Y96L/Q102D variant, defective for LFA-1 binding (and used as a negative control in Figure 1A), was secreted similarly to WT ISG15 (Figure S1B). A feature of the linker region of ISG15 is a highly conserved cysteine residue; however, alteration of this residue (C78S) did not affect secretion or signaling in the transwell assay. We note it was reported that two cysteines of mouse ISG15 (the linker cysteine and C144) were required for an IFN-γ response to Toxoplasma gondii infection; however, that study reported only the effect of simultaneous alteration of both residues, and C144 is not conserved in

Figure 1. ISG15 Is Secreted from Jurkat and HEK293T Cells
(A) Jurkat cells were transfected with cDNAs encoding IFNG and/or ISG15 or ISG15 Y96L/Q102D. Cell culture supernatants were collected 48 h post-transfection, and IFN-γ was detected by ELISA.
(B) The transwell system for monitoring ISG15 secretion and signaling. ISG15 is expressed by transfection of HEK293T cells (lower chamber); secreted ISG15 will cross the 0.4-μm membrane and signal to NK-92 cells (upper chamber). Upper chamber culture supernatants are collected 48 h post-transfection and assayed for IFN-γ by ELISA.
(C) Lower chamber HEK293T cells were transfected as indicated with ISG15 or Y96L/Q102D-expressing plasmids, with NK-92 cells (± IL-12) in the upper chamber. Cell culture supernatants were collected 48 h post-transfection and assayed for IFN-γ. Here and in all subsequent experiments, bars represent the average of three experiments, and error bars represent standard error of the mean.

Ordinary one-way ANOVA was performed between each treatment condition and the IFNG alone (A) or IL-12 alone (C). *p = 0.01, **p = 0.001, ***p = 0.0001, ****p < 0.0001. ns, non-significant changes.
Figure 2. Determinants Required for ISG15 Secretion

(A) FLAG-tagged ISG15 variants were transfected into HEK293T cells in the lower level of a transwell plate, with NK-92 cells in the upper chamber (± IL-12, as indicated), and secreted IFN-γ was measured by ELISA. L72A, S83A, and L85F (red bars) were deficient in inducing IFN-γ secretion from the NK-92 cells. Ordinary one-way ANOVA was performed between each treatment condition and the IL-12 alone condition. *p = 0.01, **p = 0.001, ***p = 0.0001, ****p < 0.0001. ns, non-significant changes.

(B) Purified ISG15 protein and the indicated ISG15 variants were added directly to the cell culture media of NK-92 cells (± IL-12), and IFN-γ secretion was monitored by ELISA. ISG15 variants L72A, S83A, and L85F (red bars) and wild-type (WT) ISG15 stimulated IFN-γ secretion, whereas the Y96L/Q102D (LFA-1 binding mutant) did not.
human ISG15 (Napolitano et al., 2018). Together, the results presented here identify determinants of ISG15 required for secretion that are separable from those required for LFA-1 receptor interactions, and both of these sets of determinants are separable from those required for intracellular conjugation.

**Bacterial Pathogens and PAMPs (Pathogen-Associated Molecular Patterns) Stimulate the Production of Extracellular ISG15**

To identify biological factors that lead to the synthesis and secretion of extracellular ISG15, we treated human PBMCs with live Mycobacterium bovis BCG, heat-killed **Mycobacterium tuberculosis**, or heat-killed **Salmonella typhimurium**, in the absence or presence of purified IL-12. As shown in Figure 3A, none of these agents, by themselves, elicited significant IFN-γ production, but all were synergistic with IL-12 in stimulating production of IFN-γ. The magnitude of IFN-γ release and degree of synergism with IL-12 was similar to that seen when purified ISG15 was added directly to the culture medium (blue bars in Figure 3A). An anti-ISG15 antibody added to the culture medium greatly reduced IFN-γ production by the bacterial pathogens, indicating that IFN-γ production was dependent on production and secretion of ISG15 from one or more cell types within the PMBC population. Primary human NK and T cells responded similarly to IL-12 and heat-killed **M. tuberculosis** (Figure 3B), although the absolute amount was significantly greater with NK cells than T cells, consistent with previous results (Bogunovic et al., 2012). Addition of anti-ISG15 antibody to the culture medium greatly reduced IFN-γ production by the bacterial pathogens, indicating that IFN-γ production was dependent on production and secretion of ISG15 from one or more cell types.

**Mycobacterium bovis** BCG, heat-killed **Mycobacterium tuberculosis**, or heat-killed **Salmonella typhimurium**, in the absence or presence of purified IL-12. As shown in Figure 3A, none of these agents, by themselves, elicited significant IFN-γ production, but all were synergistic with IL-12 in stimulating production of IFN-γ. The magnitude of IFN-γ release and degree of synergism with IL-12 was similar to that seen when purified ISG15 was added directly to the culture medium (blue bars in Figure 3A). An anti-ISG15 antibody added to the culture medium greatly reduced IFN-γ production by the bacterial pathogens, indicating that IFN-γ production was dependent on production and secretion of ISG15 from one or more cell types.
isolated primary splenocytes from control C57B6 mice or ISG15-deficient (ISG15<sup>−/−</sup>) or LFA-1-deficient mice (CD11a<sup>−/−</sup>). As shown in Figure 3C, splenocytes from WT mice responded to heat-killed <i>M. tuberculosis</i> and <i>S. typhimurium</i> similarly to human PBMCs, producing IFN-γ in synergy with IL-12. Both the ISG15<sup>−/−</sup> and CD11a<sup>−/−</sup> splenocytes showed no production of IFN-γ above the level seen in either untreated splenocytes or splenocytes treated only with IL-12. It should be noted that ISG15 null mice have a normal distribution of immune cells, and that free ISG15 (Osiak et al., 2005), when added to ISG15 null mouse splenocytes with IL-12, elicited IFN-γ responses similar to that of WT mice (Figure S2B). These results confirm that both ISG15 and its cell-surface receptor, LFA-1, are essential for a robust IFN-γ response to heat-killed <i>M. tuberculosis</i> and <i>S. typhimurium</i>.

Poly(I:C), a TLR3 agonist, and Pam3CSK4, a bacterial lipoprotein mimetic and TLR1/TLR2 agonist, were both synergistic with IL-12 in inducing IFN-γ production from PBMCs (Figure 4A). Mouse splenocytes from control and ISG15<sup>−/−</sup> and LFA-1<sup>−/−</sup> mice were also assayed for IFN-γ production in response to these PAMPs (Figure 4B). Control splenocytes showed a robust response to both PAMPs in the presence of IL-12, whereas the response from the ISG15<sup>−/−</sup> and CD11a<sup>−/−</sup> splenocytes was significantly reduced relative to the WT splenocytes but slightly above the level seen with IL-12 alone. Directly treating primary NK cells or NK-92 cells with IFN-β also led to IFN-γ production, and this was blocked by the anti-ISG15 antibody (Figure 4C). This is consistent with IFN-β driving expression of both the IFNG and ISG15 genes in NK cells, as shown previously for the IFNG gene (Mack et al., 2011; Miyagi et al., 2007). Together, these results indicate that ISG15 acts as a secreted cytokine that bridges pathogen recognition to type II IFN responses.

**Multiple Routes to Induction of ISG15**

ISG15 is primarily induced by type I IFNs; however, there are reports of type I IFN-independent induction of ISG15,
including by *M. tuberculosis* (Kimmey et al., 2017; Manzanillo et al., 2012). Therefore, we examined mouse splenocytes from mice deficient for the type I interferon receptor (IFNAR1<sup>−/−</sup>) for IFN-γ production in response to poly(I:C), Pam3CSK4, and *heat-killed S. typhimurium* and *M. tuberculosis* (Figure 5A). Control splenocytes responded to all of these agonists to produce IFN-γ. The IFNAR-deficient mice did not respond to poly(I:C) or heat-killed *S. typhimurium*, but retained the response to Pam3CSK4 and *heat-killed M. tuberculosis*. These results indicate that ISG15-dependent signaling can be triggered by mechanisms independent of type I IFN signaling. This was further confirmed with NK-92 cells using the Vaccinia B18R protein, a type I IFN binding protein that blocks IFNAR signaling. As shown in Figure S3, B18R added to the culture media strongly inhibited poly(I:C) and *S. typhimurium*-induced IFN-γ production from NK-92 cells, but it had no effect on Pam3CSK4 or *M. tuberculosis*-induced IFN-γ production.

MyD88 is an adaptor protein required for signaling by all TLRs, with the exception of the viral TLR sensor, TLR3. To determine whether ISG15-dependent IFN-γ production in response to *M. tuberculosis* was TLR dependent, we tested a cell-permeable MyD88 inhibitor peptide for its ability to block *M. tuberculosis*-mediated IFN-γ secretion by NK-92 cells. As shown in Figure 5B, the MyD88 inhibitor blocked the IFN-γ response to both heat-killed *M. tuberculosis* and Pam3CSK4, but did not block the response to the TLR3 agonist poly(I:C). Together, these results indicate that the ISG15-dependent response to heat-killed *M. tuberculosis* in NK-92 cells is independent of type I IFN, yet dependent on one or more TLRs. Figure 5C confirms that poly(I:C), Pam3CSK4, and heat-killed *S. typhimurium* and *M. tuberculosis* all led to the production of extracellular ISG15 as determined by an ISG15 ELISA assay of cell culture supernatants from human PBMCs.

**Modulation of ISG15 Secretion by ISGylation, Influenza NS1B, and vDIGs**

To address whether ISGylated proteins produced intracellularly can reach the extracellular space, we used the HEK293T/NK-92 transwell assay, expressing ISG15 by transfection in HEK293T cells, with or without co-expression of the ISG15 E1 (Uba7), E2 (UbcH8), and E3 enzymes (WT Herc5 or the active-site C994A mutant), and assaying for IFN-γ release from NK-92 cells. As shown in Figure 6A, expression of ISG15 alone led to robust IFN-γ secretion by the NK-92 cells, whereas expression of ISG15 with the conjugation enzymes resulted in levels...
of IFN-γ secretion seen with IL-12 alone. When WT Herc5 was substituted with the catalytically inactive Herc5 C994 mutant, IFN-γ secretion was again similar to that seen when ISG15 was expressed alone. Figure 6B demonstrates that ISG15 conjugates were formed intracellularly only when ISG15 was expressed with the E1, E2, and WT Herc5 enzymes, and as expected, the level of intracellular free (unconjugated) ISG15 was decreased when conjugation occurred. An ISG15 immunoprecipitation and immunoblot of the cell culture supernatants from the same transfected cells showed that free ISG15 was not detected when it was expressed with the E1/E2/E3 (WT) enzymes, but was detected when expressed alone or with the E1/E2/E3 (C994A) enzymes. In addition, under conditions of conjugate formation, there was no detection of high-molecular-weight conjugates appearing in the cell culture supernatant. These results indicate that formation of intracellular ISG15 conjugates inhibits the secretion of ISG15, most likely by decreasing the pool of free ISG15 available for secretion, and that high-molecular-weight conjugates are not secreted to an appreciable degree.

NS1B binds non-covalently to both free and conjugated forms of ISG15 (Yuan and Krug, 2001; Zhao et al., 2016). One reported function of this interaction is to prevent ISGylated forms of the viral NP from being incorporated into oligomeric nucleocapsid complexes and impeding viral replication (Zhao et al., 2016). We hypothesized that an additional or alternative function of NS1B might be to sequester free ISG15 to prevent it from being secreted, thus inhibiting IFN-γ and/or other cytokine responses. To test this, we expressed ISG15 in the HEK293T/NK-92 transwell system with or without co-expression of NS1B or a variant that does not bind ISG15 (W36A/Q37A) (Guan et al., 2011). As shown in Figure 6C, co-transfection of HEK293T cells with ISG15 and either WT NS1B or the W36A/Q37A mutant (M) prevented ISG15-mediated IFN-γ release from the NK-92 cells, whereas the W36A/Q37A variant did not. Consistent with this, Figure 6D shows that free ISG15 was not detectable in the cell culture supernatant when co-expressed with NS1B, but was detectable when co-expressed with the W36A/Q37A variant. These results indicate, in addition to abrogating the effects of intracellular ISGylation, NS1B inhibits the secretion and extracellular signaling function of ISG15.

Several types of viruses encode proteases that deconjugate ISG15 and/or ubiquitin from target proteins. Considering that intracellular ISGylation inhibited ISG15 secretion, we hypothesized that vDIGs might result in increased ISG15 secretion and signaling to NK cells. The ERVE Nairovirus encodes an OTU domain protease (vOTU) that has strong de-ISGylase activity and low to no activity toward ubiquitin conjugates (Capodagli et al., 2013). The picornavirus FMDV Lbpro protease hydrolyzes ISG15 conjugates with high specificity relative to ubiquitin conjugates (Swatek et al., 2018). Lbpro is unique in that it does not hydrolyze the isopeptide bond formed at the ε-amino group of an ISGylated lysine residue, but rather cleaves the peptide bond proximal to the last two residues (Gly-Gly) of ISG15, liberating...
ISG15-ΔGG (an ISG15 variant that was shown to be active for secretion and signaling; Figure 2A). The severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) coronaviruses encode a papain-like protease (PLpro) that has a strong preference for ISG15 over ubiquitin (Baez-Santos et al., 2014; Daczkowski et al., 2017), and the recently discovered SARS-CoV-2 PLpro enzyme is 83% identical and 90% similar to SARS PLpro. The ability of the EREV vOTU, FMDV LBpro, and SARS-CoV-2 PLpro proteases to enhance ISG15 secretion when co-expressed with the ISGylating enzymes was tested in the HEK293T/NK-92 transwell system. As shown in Figure 7A, expression of each of the vDIGs restored ISG15-dependent IFN-γ secretion by NK-92 cells that was otherwise inhibited by expression of the ISGylating enzymes. All of these vDIGs are cysteine proteases, and the C-to-A active-site mutants of the enzymes (vOTU C43A, LBpro C51A, and PLpro C110A) were unable to restore extracellular ISG15 signaling. Figure 7B shows an immunoblot of cell lysates for intracellular ISG15 with and without co-expression of the vDIGs and vDIG C-A mutants, as well an immunoprecipitation/immunoblot for secreted ISG15. Consistent with the IFN-γ ELISA results, each of the vDIGs reversed intracellular conjugates and led to restoration of detection of extracellular ISG15, whereas the C-A mutants did not. Together, these results indicate that the vDIGs tested here counter the intracellular conjugation function but enhance, rather than inhibit, the extracellular signaling function of ISG15. The latter suggests that vDIG-expressing viruses are either relatively insensitive to the downstream effects of ISG15...
extracellular signaling, or that they have alternative mechanisms for limiting cytokine responses. In addition, it is possible that ISG15-induced pro-inflammatory cytokine production, although predicted to be anti-viral, may be partially responsible for cytokine release syndrome (“cytokine storms”), which mediates many of the harmful effects of viral infection.

**DISCUSSION**

Human and murine ISG15 share two separable biochemical functions as Ubl modifiers and as extracellular signaling proteins. Human ISG15 has an additional intracellular function unrelated to conjugation, in which it regulates the stability of Usp18, which in turn regulates signaling through the type I IFN receptor, independently of its catalytic activity (Bogunovic et al., 2012; Meuwissen et al., 2016; Speer et al., 2016). A challenge is to understand how these functions are temporally related and which function(s) are critical in response to specific infectious agents and immune stimulants. Here, we have focused on expression, secretion, and signaling of extracellular ISG15 in response to pathogen components. A simple but important advance in this study was that a cell-based biological readout of ISG15 secretion was utilized (e.g., LFA-1-dependent IFN-γ release from primary NK cells or the NK-92 cell line) rather than relying solely on detection of ISG15 in the extracellular space. The biological readout ensured that extracellular ISG15 was functional for initiating downstream cytokine (IFN-γ) secretion. Based on our findings, we propose that extracellular ISG15 signaling to lymphocytes (primarily NK cells) bridges early innate immune responses to type II IFN responses, and that viral effector proteins have differential effects on this function of ISG15.

Pattern recognition receptors detect intracellular pathogens, leading to the production and secretion of effector molecules, including type I IFNs and IL-12. These molecules activate the innate immune response by increasing pathogen killing and clearance, and initiate the process of activating IFN-γ-dependent humoral immune responses. The IL-12/ISG15 synergy is particularly important for mounting the immune response to MSMD pathogens, including mycobacterial and *Salmonella* infections (Bustamante et al., 2014). We demonstrated here that ISG15 expression can be induced by several immune stimulants, in both a type I IFN-dependent and -independent manner. Examples of IFN-β-dependent inducers of ISG15 expression included poly(I:C) and heat-killed *Salmonella*, whereas Pam3CSK4 and heat-killed *M. tuberculosis* induced ISG15 expression independently of type I IFN. Thus, although ISG15 expression is not strictly dependent on type I IFN, our results indicate that extracellular ISG15 is a critical mediator between initial pathogen recognition and the transition to type II IFN responses.

Intracellular ISGylation was shown to be inhibitory to extracellular signaling, providing insight into the relationship between these two functions. The simplest interpretation of this result is that ISGylation limits the pool of free ISG15 available for secretion. In a previous study, we showed that a purified ISG15 fusion protein (ISG15-BirA), which mimics an ISG15 conjugate, could signal through LFA-1 on NK-92 cells when added directly to the culture media (Swaim et al., 2017). Although this indicates that ISG15 conjugates may, in some cases, be able to productively engage LFA-1, results shown here indicate that it is unlikely that a significant amount of ISGylated proteins ever reaches the extracellular space. Interestingly, it was noted previously that IFN-β stimulation of A549 lung carcinoma cells resulted in rapid expression of free ISG15, with an approximate 12-h lag before the appearance of significant amounts of ISGylated proteins (Loeb and Haas, 1992; Yuan and Krug, 2001). This lag in conjugate accumulation correlates with the delay in expression of Uba7, UbcH8, and Herc5, relative to ISG15, in IFN-β-treated cells (Dastur et al., 2006). It is therefore conceivable that a delay in conjugation allows free unconjugated ISG15 to be secreted at early time points after IFN-β stimulation, essentially jump-starting secretion of IFN-γ before secretion is diminished by intracellular ISGylation. Further temporal regulation of the ISG15 system by expression of the type I IFN-induced cellular deconjugase (human Usp18/mouse Ubp43), as well the role of free intracellular ISG15 in downregulating signaling at the type I IFN receptor in human cells, remains to be explored.

We have shown that ISG15 is secreted from both lymphocytes and epithelial cells, suggesting that the ISG15 secretion mechanism is operational in a wide range of cell types. Several observations suggest that ISG15 secretion is non-conventional (i.e., not dependent on endoplasmic reticulum [ER] transit): it does not have a signal peptide sequence, and the fact that cytosolic viral de-ISGylating enzymes enhanced secretion indicates that the source of secreted ISG15 was not a pool of ISG15 that was translated into the ER. Importantly, it is unlikely that ISG15 is being released simply by a cell death or lysis, because ISG15 autocrine signaling was demonstrated in lymphocytes. In addition, extracellular ISG15 signaling was inhibited by intracellular ISGylation, despite the ability of ISGylated proteins to stimulate IFN-γ secretion from NK-92 cells when added directly to cell culture medium (Swaim et al., 2017); if ISG15 were being released by cell lysis, then one would expect conjugates to also appear in the extracellular space and signal. Specific amino acid alterations of ISG15 were identified that inhibited ISG15 secretion (L72A, S83A, and L85F), and these variants may prove useful in defining the mechanism of secretion.

The only cell types that are so far known to respond to extracellular ISG15 are cells that express LFA-1, which are limited to lymphocytes and other leukocytes. As shown previously, engagement of LFA-1 enhances cytokine secretion, including, but not limited to, IFN-γ, dependent on Src-family kinase activity (Swaim et al., 2017). Other cytokines that show enhanced secretion in response to ISG15 include other pro-inflammatory cytokines, such as CXCL1, CXCL5, IL-1, and IL-6 (Østvik et al., 2020), as well as IL-10, an anti-inflammatory cytokine (Dos Santos et al., 2018; Swaim et al., 2017). It will be important to determine both the full range of immune agonists that induce ISG15 expression and secretion, and the full spectrum of cytokines that are under the influence of extracellular ISG15.

The NS1B effector protein binds to the N-terminal Ubi domain of ISG15 and the linker region between the Ubi domains (Guan et al., 2020). This effect on cell growth correlated with activation of the ISGylation machinery, in which NS1B suppressed ISGylated proteins (Shulman et al., 2020). This model suggests that extracellular ISG15-induced signaling can be modulated by viral effector proteins, and that ISGylation can itself act as an epigenetic mechanism.
Interestingly, the binding of NS1B occludes L72 of ISG15, one of the residues that are critical for ISG15 secretion. Although it was first reported that NS1B inhibited ISGylation (Yuan and Krug, 2001), more recent work indicates that NS1B binds and sequesters ISGylated proteins, including the viral NP. Sequestration of ISGylated NP prevents this pool of NP protein from being incorporated into and disrupting formation of oligomeric NP-viral RNA complexes (Zhao et al., 2016), consistent with a model for the function of ISG15 proposed earlier (Durfee et al., 2010). The results presented suggest that influenza B is also sensitive to the extracellular functions of ISG15, and that the NS1B protein is capable of interfering with both of these core functions of ISG15.

Several types of animal viruses encode deubiquitinating enzymes (DUBs) that antagonize innate immune signaling by reversing ubiquitination of key signaling proteins, including RIG-I, MAVS, IKK, TBK1, and IκBα (Bailey-Elkin et al., 2017). In some cases, these enzymes also recognize ISG15 conjugates or have high specificity for ISG15 conjugates (Daczkowski et al., 2017; Dzimianski et al., 2019b). Here, we analyzed enzymes from three viruses (the ERVE vOTU domain protease, Lbpro from the FMDV, and PLpro from the recently described SARS-CoV-2 coronavirus) and determined that their de-ISGylation activity reversed the inhibitory effect of intracellular ISGylation on ISG15 secretion and extracellular signaling. Highlighting the importance of the de-ISGylating function of Lbpro, recent results indicate that defects in Lbpro de-ISGylase activity result in decreased FMDV replication in vitro and in vivo (Medina et al., 2020).

The SARS-CoV-2 PLpro enzyme is 83% identical to the SARS-CoV-1 PLpro enzyme, which has been shown to have robust de-ISGylase activity (Daczkowski et al., 2017; Lindner et al., 2007), and the results presented here demonstrate that SARS-CoV-2 PLpro expression also leads to deconjugation of ISG15 in cells. In addition to de-ISGylation activity, both the FMDV and coronavirus enzymes proteolytically process viral polyproteins, an essential function. Therefore, small-molecule inhibitors of PLpro might have a dual therapeutic effect of inhibiting cleavage of viral polyproteins and blocking intracellular de-ISGylation (Baéz-Santos et al., 2015). Further, the results presented here suggest inhibiting PLpro would also limit the secretion and extracellular signaling of ISG15. Limiting ISG15 secretion might be expected to be detrimental in combating the infection, because pro-inflammatory cytokines are important in recruiting and activating cells of the adaptive immune response. On the other hand, if some of the most severe and deadly consequences of infection are a result of cytokine release syndrome, then limiting ISG15 secretion and signaling by therapeutically inhibiting PLpro might be beneficial to coronavirus disease 2019 (COVID-19) patients.

ISG15 secretion and its relationship to IFN-γ and other pro- and anti-inflammatory cytokine responses has significant implications for functional links to adaptive immune responses and immune cell recruitment. Characterization of the separable biochemical determinants essential for the specific functions of ISG15, ISGylation, secretion, and receptor interaction, will allow for the creation of animal models in which the specific activities of ISG15 are individually assessed and pave the way for understanding the core functions of ISG15 in response to specific viral and microbial pathogens, including MSMD pathogens, influenza, and SARS-CoV-2.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2020.107772.

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AUTHOR CONTRIBUTIONS

C.D.S., K.J.M., and S.K. performed experiments and contributed to experimental approach and design. L.A.C., D.J.L., and J.M.H. contributed to experimental approach and design and writing of the paper. C.D.S. also contributed to writing.

DECLARATION OF INTERESTS

The authors declare no competing interests.
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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| M2 Flag             | Sigma  | CAT#F3165; RRID:AB_439685 |
| Anti-ISG15 monoclonal | Invitrogen | CAT#7H29L24; RRID:AB_2784562 |
| Anti-ISG15 monoclonal | Invitrogen | CAT#2H25L4; RRID:AB_2784595 |
| Anti-ISG15 polyclonal | Sigma  | CAT#SAB1408653; RRID:AB_10742647 |
| Rabbit IgG isotype control | Thermo Scientific | CAT#02-6102; RRID:AB_2532938 |
| **Bacterial and Virus Strains** |        |            |
| BL21 GST-3xFLAG ISG15 and variants | Swaim et al., 2017 | N/A |
| BCG Karlson and Lessel | ATCC  | CAT#35734 |
| **Biological Samples** |        |            |
| Human: LRS chambers with blood | We Are Blood | N/A |
| Human: PBMC | Immunospot | CAT# CTL-UP1 |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| Mouse IL-12 | R&D Systems | CAT#419-ML |
| Human IL-12 | BD PharMingen | CAT#554613 |
| IL-2 | Peprotech | CAT# 200-02 |
| IFN-β | Bayer | betaseron |
| PreScission Protease | GE Healthcare | CAT#27-0843-01 |
| Isopropyl-β-D-1-thiogalactopyranoside | Fisher Scientific | CAT#BP1755-10 |
| Pam3CSK4 | InvivoGen | CAT#tlrl-pm3s-1 |
| Heat-killed *M. tuberculosis* | InvivoGen | CAT#tlrl-hkmt-1 |
| Poly tC HMW | InvivoGen | CAT#tlrl-pic |
| Heat-killed *S. typhimurium* | InvivoGen | CAT#tlrl-hkst2 |
| Recombinant Viral B18R protein CF | R&D Systems | CAT#8185-BR-025 |
| MYD88 inhibitor peptide | Novus | CAT#NBP2-29328 |
| **Critical Commercial Assays** |        |            |
| Human IFN-γ ELISA | Thermo Scientific | CAT#EHIFNG |
| Human ISG15 ELISA | Aviva Biosciences | OKCA02472 |
| Mouse IFN-γ ELISA | Thermo Scientific | CAT#EM1001 |
| Lonza SE nucleofection | Lonza | CAT#V4XC-2024 |
| Pan T cell isolation kit | Miltenyi Biotec | CAT#130-096-535 |
| NK cell isolation Kit | Miltenyi Biotec | CAT#130-092-657 |
| **Deposited Data** |        |            |
| Mendeleys Data | this paper | https://doi.org/10.17632/4fvd98cr38.1 |

### Experimental Models: Cell Lines

| Human: NK-92 cells | ATCC | CAT#CRL-2407 |
| Human: Jurkat cells | ATCC | CAT#TIB-152 |
| Human: HEK293T cells | ATCC | CAT#CRL-3216 |

### Experimental Models: Organisms/Strains

| Mouse: C57B6 spleens | Jackson Laboratory | CAT#JR 644 |
| Mouse: C57B6 CD11a<sup>-/-</sup> spleens | Jackson Laboratory | CAT#JLR 5257 |
| Mouse: C57B6 ISG15<sup>-/-</sup> spleens | Osiak et al., 2005 | K.-P. Knobelach |
| Mouse: C57B6 IFNAR1<sup>-/-</sup> spleens | Jackson Laboratory | CAT#JR 644 |

(Continued on next page)
### RESOURCE AVAILABILITY

#### Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jon Huibregtse (huibregtse@austin.utexas.edu).

#### Software and Algorithms

| Resource | Source |
|----------|--------|
| Prism 8  | Graphpad |
Material Availability
All newly generated reagents for this study are available at the institution of the lead contact.

Data and Code Availability
Raw western blot data uploaded to Mendeley Data at https://doi.org/10.17632/4fvk98cr38.1

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell Culture
Human peripheral blood mononuclear cells (PBMCs) were obtained from Immunospot and donors were uncharacterized with respect to sex or age. Primary NK and T cells were isolated from leukoreduction system chambers (LRS chambers) obtained from We Are Blood (Austin, TX); T cells were isolated from three male donors (ages 50, 58, and 71 years) and NK cells were isolated from two 49 year old female donors and a 57 year old male donor. Primary NK cells, primary T cells, HEK293T, Jurkat, and NK-92 cells were maintained at 37°C, 5% v/v CO2 in a humidified incubator. HEK293T cells were maintained in DMEM (Corning) supplemented with 10% FBS (Sigma) and 1% Penicillin-Streptomycin (Corning). Jurkat cells were cultured in RPMI 1640 (Sigma), 10% FBS (Sigma), 1% Penicillin-Streptomycin. NK-92 cells were cultured in NK media; Alpha MEM without nucleosides (GIBCO) with 2 mM L-glutamine (Fisher), 1.5 g/l sodium bicarbonate (Sigma), 0.2 mM inositol (Sigma), 0.1 mM 2-mercaptoethanol, 0.02 mM folic acid (Fisher), 100U/ml recombinant IL-2 (Peprotech), 12.5% horse serum (GIBCO) and 12.5% FBS (Corning).

PBMC, T and NK cell isolation
PBMCs from healthy individuals were isolated from Leukoreduction System (LRS) Chambers obtained from We Are Blood (Austin, TX). The LRS chamber blood was diluted 1:1 with PBS+2% FBS and transferred to a SepMate tube (Stem Cell Technologies) containing an equal volume of Ficoll Plaque (GE Healthcare). The tube was centrifuged at 1,200 x g for 10 minutes at room temperature. The top layer containing the enriched mononuclear cells (MNCs) was poured off to a separate tube. The enriched MNCs were washed twice with PBS containing 2% FBS. To eliminate any red blood cells (RBCs) present in the MNC fraction, cells were lysed with 1X RBC lysis buffer (Biolegend). The MNCs were then counted and either frozen in RPMI 1640 containing 20% FBS and 5% DMSO or plated in RPMI 1640 at 1x10⁶ cells per mL. CD3+ T cells were isolated from PBMCs by magnetic cell separation using a Pan T cell isolation kit (Miltenyi Biotec) as per manufacturer’s protocol. CD56+ NK cells were isolated from PBMCs by magnetic cell separation using a NK cell isolation kit (Miltenyi Biotec) as per manufacturer’s protocol. Cells were sub-cultured in RPMI 1640 with 20% human serum (Omega Scientific) at 1x10⁶ cells per mL.

Mouse splenocyte isolation and treatment
Spleens from four WT C57B6 (JR00664), CD11a−/− (JR005357) (Ding et al., 1999), and IFNAR1−/− (JR028288) (Prigge et al., 2015) mice (two male and two female mice for each strain) were obtained from Jackson Laboratory. Each spleen was placed in a 60mM dish with 10 mL PBS. ISG15−/− mice were described previously (Knobeloch et al., 2005). Spleens were poked with a 26-gauge needle and crushed with the flat end of a 3 mL syringe. Cells and detritus were collected into a 15mL conical tube. Large debris settled to the bottom and cells were transferred to a second conical tube and spun at 500 x g for 5 minutes. Cell pellets were resuspended in RPMI 1640. Splenocytes were counted and plated in RPMI 1640 at 1x10⁶ cells per mL and incubated overnight at 37°C with 5% CO2.

BCG culture
Bacillus Calmette-Guérin (BCG) Karlson Lessel was obtained from ATCC and grown in Middlebrook 7H9 medium with ADC enrichment at 37°C with shaking. Cultures were monitored by A600 in a spectrophotometer and kept in log phase. BCG was added to PBMC and NK-92 cultures at a concentration of 100 cfu per sample.

METHOD DETAILS

Human PBMC, NK-92, and mouse splenocyte treatments and IFN-γ ELISAs
Human PBMC, NK-92 cells and mouse splenocytes were rested overnight in RPMI 1640 10%, FBS and NK media without IL2, or RPMI 1640 at a concentration of 1x10⁶ cells/mL. Rested cells were treated with 30ng/µL heat-killed M. tuberculosis, 10⁶ cells/mL heat-killed S. typhimurium, 10ng/mL Pam3CSK4, 1 mg/mL poly I:C, 18 ng/mL mL-12 or 18 ng/mL hIL-12 and 2400 ng/mL bacterially purified ISG15 (mouse or human; prepared as described in Swaim et al., 2017) for 48 hours. Supernatants were collected and monitored for IFN-γ production by ELISA (Thermo Fisher). B18R 10ng/mL (R&D Systems), MYD88 inhibitor peptide or control peptide 100µM (Novus), anti-ISG15 3µg/mL (Sigma) or Control IgG 3µg/mL (Thermo-Fisher) were used as indicated.

ISG15 ELISA assays
ISG15 ELISAs were performed on PBMCs cultured in RPMI 1640 without serum (serum proteins interfere with ELISA detection of ISG15; data not shown). 1x10⁶ PBMCs/mL were plated for 16 hours and then treated with heat-killed M. tuberculosis, heat-killed
S. typhimurium, Pam3CSK4 or poly I:C for 48 hours. Supernatants were collected and ISG15 concentrations were measured by the Aviva ISG15 Elisa Kit according to the recommended protocol (Aviva Biosciences).

Jurkat transfections

Jurkat cells were seeded at 5x10⁵ cells/mL two days before transfection. Cells were counted and resuspended at 1x10⁶ cells/100µL of Lonza SE. Nucleofection reagent with 2 µg/sample of the indicated DNA. Cells were transfected with a Lonza Nucleofector 4d using program cl-120. Freshly transfected cells were rested for 3 hours in RPMI 1640 with 10% serum, then plated at a density of 1x10⁶ cells/mL and cultured for 48 hours. Cell culture supernatants were analyzed for human IFN-γ by ELISA (Thermo Scientific).

293T NK-92 transwell assay

1x10⁵ HEK293T cells per well were seeded in the lower chamber of 24-well Corning transwell plates (0.4 µm membrane; Fisher Scientific). At the same time, NK-92 cells (1x10⁶ cells per ml) were transferred to media without IL-2 for 16 hours. HEK293T cells were transfected with 100ng of plasmid expressing FLAG-ISG15 or the indicated ISG15 variant, with or without plasmids expressing NS1B (synthesized by GenScript from Influenza B virus B/Yamanashi/166/1998 sequence), ISG15 conjugation components (Ube1L, UbcH8, Herc5), or the indicated vDIG (ERVEV vOTU, FMDV LbPro, SARS-CoV2 PLpro), using X-tremeGENE HP DNA transfection reagent (Roche). 0.3 mL of ISG15 antibodies (at 1x10⁶ per mL) were added to the upper chamber of the transwell chamber, with or without IL-12 as indicated, and cultured for 48 hours. Cell culture supernatants were collected from the upper transwell chamber and assayed for IFN-γ production by ELISA (Thermo Scientific).

ISG15 immunoprecipitations from cell culture supernatants

HEK293T cells were seeded in 10 cm plates at 1.3x10⁶ cells/mL in 10 mL DMEM with 10% FBS and 1% penicillin streptomycin the day before transfection. 3µg of indicated ISG15 or mutant were transfected, using X-tremeGENE HP DNA transfection reagent (Roche), into one 10 cm plate and grown for 48 hours. Cell culture supernatants were collected and cleared of any cell debris by centrifugation at 1000xg. These cell culture supernatants were further cleared of IgG from the serum by two one-hour bindings with Protein A Sepharose (Invitrogen) at room temperature. 20uL of Sera-Mag SpeedBead Protein A/G magnetic beads (GE Life Sciences), at 1000xg. These cell culture supernatants were further cleared of IgG from the serum by two one-hour bindings with Protein A Sepharose (Invitrogen) at room temperature. 20uL of Sera-Mag SpeedBead Protein A/G magnetic beads (GE Life Sciences), at 1000xg. These cell culture supernatants were further cleared of IgG from the serum by two one-hour bindings with Protein A Sepharose (Invitrogen) at room temperature. 20uL of Sera-Mag SpeedBead Protein A/G magnetic beads (GE Life Sciences), at 1000xg. These cell culture supernatants were further cleared of IgG from the serum by two one-hour bindings with Protein A Sepharose (Invitrogen) at room temperature. 20uL of Sera-Mag SpeedBead Protein A/G magnetic beads (GE Life Sciences), at 1000xg. These cell culture supernatants were further cleared of IgG from the serum by two one-hour bindings with Protein A Sepharose (Invitrogen) at room temperature. Beads were isolated with a neodymium magnet and washed 3X with 0.1% NP40 in PBS pH 7.0. ISG15 was eluted from the beads by boiling in 80 µL of 1X loading dye and 40 µL was run on a NuPAGE 4%-12% Bis-Tris gel and transferred to nitrocellulose for western blotting. ISG15 was detected with the M2 Flag antibody (Sigma).

ISG15 purification

ISG15 proteins were purified as GST fusion proteins in BL21 E. coli. Overnight starter cultures were grown at 37°C for 16 hours. Cultures were diluted 1:10 and cultured with shaking for 2 hours at 37°C. Expression of proteins was induced with 100 µM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 hours at 30°C. Cells were collected by centrifugation, resuspended in 10 mL PBS with 1% Triton X-100, and sonicated for 30 s. Lysates were spun at 15,000xg for 10 minutes, and supernatants were incubated with Glutathione Sepharose (GE Healthcare) for 2 hours with rotation at 4°C. Beads were collected and washed 3X with PBS plus 1% Triton X-100 and 3X with 50mM Tris pH 8.0, 150mM NaCl, 0.01% Triton X-100, and 2.5mM EDTA. GST fusion proteins (on beads) were subjected to site-specific cleavage with PreScission Protease (GE Healthcare) to remove the GST tag. Beads were removed and protein concentration in the supernatant was quantified by SDS-PAGE using a Li-Cor Odyssey Imager.

Cloning of de-ISGylating enzymes

The SARS-CoV-2 PLpro open reading frame (corresponding to nsp3 amino acids 774-1061) was synthesized by Genscript and was cloned into pcDNA-NTAP using BamHI and NotI restriction sites. C-A mutations for SARS-CoV-PLPro and NS1B (synthesized by GenScript from Influenza B virus B/Yamanashi/166/1998 sequence), ISG15 conjugation components (Ube1L, UbcH8, Herc5), or the indicated vDIG (ERVEV vOTU, FMDV LbPro, SARS-CoV2 PLpro), using X-tremeGENE HP DNA transfection reagent (Roche). 0.3 mL of ISG15 antibody (Invitrogen 7H29L24) and 20 mL of 0.1% NP40 in PBS pH 7.0 were added to the cell culture supernatants and rocked for 2 hours at room temperature. Beads were isolated with a neodymium magnet and washed 3X with 0.1% NP40 in PBS pH 7.0. ISG15 was eluted from the beads by boiling in 80 µL of 1X loading dye and 40 µL was run on a NuPAGE 4%-12% Bis-Tris gel and transferred to nitrocellulose for western blotting. ISG15 was detected with the M2 Flag antibody (Sigma).

Cytokines, inhibitors and small molecules

Cytokines used include mouse IL-12, (R&D Systems), human IL-12 (BD PharMingen), human IL-2 (Peprotech), IFN-β (Betaseron, Bayer), B18R (R&D systems), MYD88 inhibitor peptide or control peptide (Novus), PAM3CSK4, poly I:C, heat killed M. tuberculosis, heat killed S. typhimurium (InvivoGen).

Antibodies and bead reagents

Antibody reagents used here were anti-M2 FLAG antibody (Sigma), anti-ISG15 polyclonal (Sigma), anti-ISG15 monoclonal (Invitrogen 2H25L4, 7H29L24), Protein A Sepharose (Invitrogen), Sera-Mag SpeedBead Protein A/G (GE Life Sciences).
QUANTIFICATION AND STATISTICAL ANALYSIS

Graphpad Prism 8 software was used to plot all ELISA data. Bars represent three biological replicates of technical duplicates. Error bars are standard error of the mean (SEM). Ordinary one-way ANOVA was performed on each ELISA dataset. Asterisks indicate p values as follows: * = 0.01, ** = 0.001, *** = 0.0001, **** < 0.0001, and non significant changes are indicated by ns.
Supplemental Information

Modulation of Extracellular ISG15 Signaling
by Pathogens and Viral Effector Proteins

Caleb D. Swaim, Larissa A. Canadeo, Kristen J. Monte, Swati Khanna, Deborah J. Lenschow, and Jon M. Huibregtse
Supplementary Figure Legends

Supplementary Figure 1 related to Figure 2. Non-secreted ISG15 mutants are conjugated, and non-signaling mutants are secreted. **A.** HEK293T cells were transfected with FLAG-ISG15 or the indicated non-secreted FLAG-tagged ISG15 variants (L72A, S83A, L85F), in the absence or presence of the conjugation enzymes (Ube1L, Ube2L6, and Herc5). Total cell extracts were analyzed by immunoblotting with anti-FLAG antibody. Molecular weight markers are indicated (kDa) and the position of free FLAG-ISG15 is indicated. **B.** The Y96L/Q102D ISG15 variant, which is defective for binding to LFA-1, is secreted from HEK293T cells. HEK293T cells were transfected with FLAG-ISG15 WT or FLAG-ISG15 Y96L/Q102D. Cell culture supernatants were monitored by anti-ISG15 immunoprecipitation and immunoblotting for extracellular FLAG-ISG15. **C.** Uncropped western blots corresponding to main Figure 2C. HEK293T cells were transfected with FLAG-tagged WT ISG15 or the L72A, S83A or L85F variants. Cell lysates were monitored by anti-FLAG immunoblotting for FLAG tagged ISG15 variant (right). Cell culture supernatants were monitored by anti-ISG15 immunoprecipitation and immunoblotting with anti-FLAG antibody. Only WT FLAG-ISG15 was detected in the supernatant of transfected cells (Left).

Supplementary Figure 2 related to Figure 3. Microbial pathogens stimulate ISG15-dependent IFN-γ secretion from NK-92 cells. **A.** NK-92 cells were treated as indicated with IL-12, purified ISG15 protein, live BCG, heat killed *M. tuberculosis*, or heat killed *S. typhimurium* in the presence and absence of anti-ISG15 antibody (I) or control antibody (C). Cell culture supernatants were assayed for IFN-γ secretion by ELISA. Bars represent an average of three experiments and error bars are standard error of the mean. **B.** Splenocytes from WT and ISG15−/− mice were treated with bacterially purified ISG15 and IL-12 for 48 hours. Cell culture supernatants were monitored for IFN-γ by ELISA. Ordinary one-way ANOVA was performed between each treatment condition and the IL-12 alone condition. Asterisks indicate p-values as
Supplementary Figure 3 related to Figure 5. B18R inhibits IFN-γ production by PBMCs in response to poly I:C and *S. typhimurium*, but not Pam3SK4 or *M. tuberculosis*. PBMCs were treated as indicated with IL-12, Poly I:C, Pam3CSK4, heat killed *M. tuberculosis*, or heat killed *S. typhimurium*, or IFN-β and the type I interferon-binding protein B18R from vaccinia virus. Cell culture supernatants were assayed for IFN-γ secretion by ELISA. Bars represent an average of three experiments and error bars are standard error of the mean. Ordinary one-way ANOVA was performed between each treatment condition and the IL-12 alone condition. Asterisks indicate p-values as follows: *=0.01, **=0.001, ***=0.0001, ****<0.0001, and non-significant changes are indicated by ns.
Supplementary Figure 2 related to Figure 3

A.

NK-92 Cells

![Bar graph showing IFN-γ production in NK-92 cells with different treatments.]

B.

![Bar graph showing IFN-γ production in WT and ISG15 -/- mice.]

Legend:
- Mock
- ISG15
- BCG
- Heat Killed M. tuberculosis
- Heat Killed S. typhimurium

Antibody: - - I C - - I C - - I C - - I C - - I C

IFN-γ pg/ml

IL-12: - - - - + + + + - - - - + + + + - - - - + + + +
Supplementary Figure 3 related to Figure 5

PBMCs

- Mock
- IFN-β
- Poly IC
- Pam3CSK4
- Heat Killed S. typhimurium
- Heat Killed M. tuberculosis

IFN-γ pg/ml

IL-12: - - + + + + + + + + + +
B18R: - + - + - + - + - + - +

ns
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