# Altered ISGylation drives aberrant macrophage-dependent immune responses during SARS-CoV-2 infection

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**Abstract**

Interferon stimulated gene 15 (ISG15) is a ubiquitin-like modifier induced during infections and involved in host defense mechanisms. Not surprisingly, many viruses encode de-ISGylating activities to antagonize its effect. Here we show that infection by Zika, SARS-CoV-2, and influenza viruses induce ISG15-modifiying enzymes. While influenza and Zika viruses induce ISGylation, SARS-CoV-2 triggers de-ISGylation instead, to generate free ISG15. The ratio of free versus conjugated ISG15 driven by the PLpro enzyme of SARS-CoV-2, correlate with macrophage polarization towards a pro-inflammatory phenotype and attenuated antigen presentation. In vitro characterization of purified wild-type and mutant PLpro revealed its strong de-ISGylating over de-ubiquitylating activity. Quantitative proteomic analyses of PLpro substrates and secretome from SARS-CoV-2 infected macrophages revealed several glycolytic enzymes previously implicated in the expression of inflammatory genes and proinflammatory cytokines respectively. Collectively, our results indicate that altered free versus conjugated ISG15 dysregulate macrophage responses and likely contributes to cytokine storms triggered by SARS-CoV-2.

## Introduction

Interferons (IFN) are the first line of defense against virus infections[1], and are critical drivers of innate immune responses. Although hosts deficient in type-I IFN are more susceptible to virus infections[2], an excess of type-I IFN or aberrant cytokine response may lead to extensive tissue damage, as is commonly observed in highly pathogenic cases of influenza [3] and coronaviruses [4]. Mice lacking IFNα receptors (IFNAR) have a higher survival rate to influenza and coronavirus infections than wild-type animals[5,6], again pointing to a dysregulation of IFN signaling underpinning the immunopathology of severe cases.

Macrophages are cells of the innate immune system that play key roles in modulating disease severity during virus infections. They can be infected by a range of viruses, and are the major producers of pro-inflammatory cytokines, such as TNFα, IFNβ, IP-10, MCP-1, which have an impact on the pathogenesis and clinical outcomes in the host[7–10]. Regulation of cytokines in macrophages is essential - overproduction of cytokines, commonly referred to as “cytokine storms”, aggravates lung damage with uncontrolled extravasation of immune cells into infection sites [4,11], although the exact sequence of events are not yet completely understood.

Interferon stimulated gene 15 (ISG15) is a ubiquitin-like modifier that is part of the first line of defense against pathogens, with broad-spectrum antiviral activity. Post translational modifications by ubiquitin and ISG15 are frequently targeted by viruses to perturb host immune responses[12]. ISG15 can be conjugated to proteins in a process termed ISGylation or be secreted in its free form. Among the hundreds of modifiable substrates, many have immune-related functions[13–15], and ISG15 (free or conjugated) has been shown to protect the host against infections[16].

The fate of ISGylation in virus-infected macrophages has not been reported so far. The impact of ISG15 has only been investigated in influenza virus infected epithelial cells, where ISG15-modified influenza NS1 inhibited virus replication. In addition, ISGylated Tsg101, a member of the ESCRT complex inhibited transport of influenza virus proteins [15,17]. However, much of the immune response at the site of infection emanates from monocyte-derived and tissue-resident macrophages. Here we investigated how viruses interact with the immune activation pathways in infected macrophages using Zika and SARS-CoV2 as two (+)RNA viruses from distinct families. Zika is a member of the *Flaviviridae* family while SARS-CoV-2 is a coronavirus. Both Zika and SARS-CoV-2 are single-stranded, positive-sense RNA

viruses that replicate in the cytoplasm within membrane de-lineated replication organelles. Macrophages are permissive to infection by both viruses; however, they are the primary target cells of Zika virus, and support replication to high titres. We also compared these responses to those infected with influenza virus, which is an orthomyxovirus with a vastly different genome organization (negative-sense segmented RNA) and unlike the former, replicates in the nucleus.

Influenza and Zika viruses promoted cellular ISGylation while SARS-CoV-2 triggered secretion of ISG15. Expression of the wild-type but not the catalytically inactive SARS-CoV-2 papain like protease (PLpro) alone was sufficient to drive de-ISGylation and aberrant macrophage responses. Proteomic analyses revealed that glycolytic enzymes that regulate inflammatory responses are the primary substrates of PLpro de-ISGylase activity. The secretome from SARS-CoV-2 infected macrophages also revealed enrichment of non-classical secretory components and pro-inflammatory cytokines. Collectively our data underscore the critical impact of altered free versus conjugated ISG15 on macrophage function, potentially underpinning the onset of lymphopenia and cytokine storms during infections by SARS-CoV-2.

## Results

### Zika and SARS-CoV2 induces expression of ISGylation enzymes

ISG15 is produced during virus infections downstream of Type-I IFNs [18,19]. Upregulation of ISG15 and modifying enzymes, including the E1 activating enzyme Ube1L, E2 conjugation enzyme UbcH8, E3 ligase HERC5, and de-ISGylase USP18, have been reported, albeit only in virus infected epithelial cells [20,21]. To determine the magnitude of expression of ISG15 and its modifying enzymes in virus infected macrophages, we performed RT-qPCR to quantify the mRNA levels of ISG15, Ube1L (E1), UbcH8 (E2), HERC5 (E3), and USP18 upon Zika and SARS-CoV-2 infection. We treated macrophages with IFNβ as positive control, where ISG15 and its conjugating enzymes were all induced as anticipated (**Fig 1a-d**). In those infected by either Zika or SARS-CoV-2, all enzymes of the ISGylation pathway were significantly induced (**Fig 1a-e**). We also measured their expression in influenza A infected cells. Similar to the +RNA viruses, in cells infected by human influenza A (H1N1) or avian influenza A (H9N2) virus, expression of all mRNAs, with the exception of Ube1L, was induced to similar levels (**supplementary Fig S1a-e**). Intracellular ISG15 protein, measured by

ELISA, was equivalently upregulated after infection by influenza (**supplementary Fig S1f**), Zika virus and most significantly with SARS-CoV-2 (**Fig 1f**). These results indicate that ISG15 and modifying enzymes are expressed in macrophages and markedly induced following virus infection.

Cellular ISGylation was significantly induced in Zika-infected macrophages (**Fig 1g**). Interestingly, although the conjugating enzymes were induced upon SARS-CoV-2 infection, total protein ISGylation remained significantly low (**Fig 1g**), which suggests that SARS-CoV-2 is likely able to trigger de-ISGylation of cellular substrates. In influenza-infected macrophages too, bulk ISGylation in cell lysates was detectable for both avian (H9N2/Y280), mammalian-adapted (H9N2/Y280-PB2-627K) or pandemic (H1N1/CA04) influenza strains (**supplementary Fig S1g**).

Although type-I IFN signaling is a well-known pathway that induces ISG15 and ISGylating enzymes[15], type-I IFN independent mechanisms have also been proposed [22,23]. To investigate whether virus infection-induced ISG15 expression was dependent on type-I IFN signaling, we infected macrophages in the presence of neutralizing antibodies directed against type-I IFN receptor (anti-IFNAR2) or isotypic control. ISG15 expression was determined by RT-qPCR (**supplementary Fig S1h, i**) and Western blotting (**supplementary Fig S1j**). In the presence of anti-IFNAR, ISG15 mRNA expression as well as free and conjugated ISG15 was diminished compared to isotypic control. Collectively, these data indicate that in virus-infected macrophages, IFN-dependent signaling trigger expression and conjugation of ISG15, which in turn are hydrolyzed effectively by SARS-CoV-2.

**Free ISG15 is secreted via unconventional mechanisms**

Apart from its function as a Ubl protein modifier, ISG15 is also known to function as a free, non-conjugated protein[24], which can be secreted into the extracellular space[25]. Mice harboring a deletion of Ube1L (E1) (which abolishes ISGylation) has been reported to survive better than *Isg15*$^{-/-}$ (which abolishes both free and conjugated ISG15) animals, suggesting that both free and conjugated forms of ISG15 may play protective roles in virus infections[16]. We were able to detect extracellular ISG15 after infection by Zika, SARS-CoV-2 and influenza viruses, but not from cells stimulated by type-I IFN alone or by infection with UV-inactivated viruses (**Fig 2a, b**). Highest amounts of secreted ISG15 were detected from SARS-CoV-2 infected cells (**Fig 2a**). In contrast, infection with UV-inactivated Zika or SARS-CoV-2 did not trigger secretion

of ISG15 at all, indicating that live virus is necessary for this process (**Fig 2b**). Although a comparable extent of ISG15 mRNA induction was detected in Normal Human Bronchial Epithelial (NHBE), DCs, and macrophages (**supplementary Fig S3a, b**), secretion of ISG15 was detectable only from DCs and macrophages, with the highest amounts from the latter, in particular from those infected with SARS-CoV-2 (**supplementary Fig S3c-e**). These results indicate that respiratory viruses such as influenza and coronaviruses both of which have the potential to cause "cytokine storms" trigger secretion of ISG15 from immune cells to a significantly greater extent compared to Zika virus, which is well-adapted to replicate in macrophages and therefore likely able to circumvent macrophage-mediated immune responses.

The process of ISG15 secretion is not well-characterized and has been proposed to occur from granulocytes via unconventional mechanisms[26,27]. To exclude the possibility that the extracellular pool of ISG15 was due to cell lysis, apoptosis or via the conventional secretory pathway (**supplementary Fig S2a**), we measured LDH released from infected cells (**Fig 2c, supplementary Fig S2e**). We also measured extracellular ISG15 secretion upon treatment with either caspase inhibitors (**Fig 2d, supplementary Fig S2f**) or Brefeldin A to block the conventional secretory pathway (**Fig 2e, supplementary Fig S2c**). Neither of these treatments inhibited ISG15 secretion. Brefeldin A treatment did not affect ISG15 secretion from cells infected with either Zika, SARS-CoV-2 or influenza (**Fig 2e, supplementary Fig S2c**), nor did it appreciably alter intracellular levels of ISG15 (**supplementary Fig S2d**) in infected cells, indicating that it is likely secreted via unconventional mechanisms.

Different mechanisms of unconventional secretory pathways have been previously reported. Amongst these are a recently identified TMED10-dependent secretory pathway[28], LC3-dependent extracellular loading and secretion pathway (LDELS)[29], secretory autophagosomes[30] and lysosomes[31] (**Fig 2f**). Depletion of TMED10 did not appreciably alter ISG15 secretion from SARS-CoV-2 infected macrophages (**Fig 2g**). The key components of the LDELS pathway are LC3 and nSMase2[29]. Depletion of LC3 abolished secreted ISG15; however, loss of nSMase2 did not, suggesting that LC3 might regulate ISG15 secretion via a different process (**Fig 2h**). Secretory lysosomes have been proposed to be critical for egress of coronaviruses. However, inhibiting key components of the secretory lysosomal pathway also did not affect ISG15 secretion (**Fig 2i**). Instead, loss of secretory autophagosomal[30] factors abolished ISG15 secretion (**Fig 2j, k**). To characterize this pathway in further detail, we systematically depleted known components of the

secretory autophagy pathway[32]. Depletion of the ULK1 complex of the early autophagy pathway, Lyn kinase, that we recently showed to function in this pathway[32], Stx3/Stx4, the SNARE complex of secretory autophagy, but not Stx17 that functions in degradative autophagy blocked ISG15 secretion. Collectively, these data indicate that CoV-infection can specifically trigger secretion of free ISG15 via autophagy-dependent unconventional mechanisms, with the highest amounts detected from SARS-CoV-2 infected macrophages.

**SARS-CoV-2 infection triggers aberrant immune responses**

The dramatically reduced ISGylation (**Fig 1**) and increase in secreted ISG15 (**Fig 2**) from SARS-CoV-2 infected macrophages prompted us to investigate the general macrophage immune responses in SARS-CoV-2 infection. Clinical samples from Covid-19 patients have already indicated aberrant early immune responses in SARS-CoV-2 infection, often accompanied by lymphopenia and secretion of pro-inflammatory cytokines [33,34]. To measure macrophage effector functions we infected cells with SARS-CoV-2 at MOI 2. At 24h post infection, surface presentation of both MHC-I and MHC-II were significantly downregulated specifically in the virus-infected population, but not with UV-inactivated virus control or with dsRNA transfection, a phenomenon that was also evident in Zika-infected cells (**Fig 3a, 3b, supplementary Fig S4a, b**). The effect on MHC in influenza-infected cells was far more modest and in accordance to previous reports that show a more significant downregulation of MHC-I in Influenza B infected cells compared to influenza A[35] (**supplementary Fig S3c**). To assess other immune responses, we measured macrophage polarization, cytokine secretion profiles and phagocytic activity (**Fig 3c-e**). Polarization was measured in M0 macrophages infected with SARS-CoV-2 and markers compared with those that were either differentiated into a pro-inflammatory M1 state using M-CSF, LPS and IFN-$\gamma$ or a wound-healing M2-state using M-CSF and IL-4 (**Fig 3c**). SARS-CoV-2 infected macrophages displayed a strong M1-like pro-inflammatory phenotype (**Fig 3c**). We also measured induction of a selected set of cytokines which have been reported to be altered in SARS-CoV-2 infection. Secretion of pro-inflammatory cytokines such as IL-1$\beta$, MCP-1 and IL-6 was significantly upregulated in SARS-CoV-2 infection. On the other hand, that of IFN-I and IFN-II was significantly downregulated, recapitulating the early events in the immunopathology of COVID-19 patients (**Fig 3d**). Similarly, phagocytic activity of SARS-CoV-2 infected macrophages resembled reduced activity

observed in M1- but not M2-macrophages (**Fig 3e**). To determine whether these cytokines followed the same secretory mechanism as ISG15, we depleted secretory autophagosomal components (LC3, Ulk1 and Stx4) by DsiRNA treatments. Depletion of Stx17 was performed to inhibit degradative autophagy. Control and depleted cells were challenged with SARS-CoV-2 and released cytokines measured as described above. Although all cytokines displayed significant decrease in released amounts, none of them were completely abolished, suggesting that they are likely secreted via multiple pathways (**Fig 3f**). Interestingly, secretion of TNF$\alpha$ was not affected in the autophagy depleted cells, suggesting that all cytokines are not secreted via the same route. Collectively, these data indicate that infection by SARS-CoV-2 results in aberrant macrophage responses, downregulating antigen presentation and triggering secretion of inflammatory cytokines, which might underpin the consistent symptoms of lymphopenia and cytokine storm observed in COVID-19 patients.

**Substrate de-ISGylation drives pro-inflammatory cytokine responses**

To decouple the role of ISGylation from free ISG15 in virus-infected macrophages, we systematically knocked-down ISG15, Ube1L, HERC5 and USP18 by transfecting macrophages with DsiRNAs 72 hours prior to infecting with either Zika or SARS-CoV-2 virus as specified. Knockdown efficiency of ISG15 and its modifying enzymes was verified in IFN-I treated cells by immunoblotting. The results confirmed that all DsiRNA targets were significantly depleted in comparison to control cells (**Fig 4a, supplementary Fig S5a-c**). In ISG15 depleted cells, as predicted, both free and ISGylated forms were downregulated, whereas in Ube1L and HERC5 knockdown cells, only the conjugated forms were downregulated (**Fig 4b, supplementary Fig S5d**). USP18 is the cellular de-ISGylating enzyme and a negative regulator of type-I IFN response; as anticipated, we observed an upregulation of ISGylated material in USP18-depleted macrophages (**Fig 4b**). Interestingly, depleting Herc5 or Ube1L did not result in a significant increase in mono ISG15 in the lysates of infected cells, as compared to IFN-treated cells, which is likely due to increased secretion of ISG15. Depletion of either ISG15, ISGylating enzymes, or USP18 did not have any significant effect on replication of Zika (**Fig 4c**), SARS-CoV-2 (**Fig 4e**) or influenza (**supplementary Fig S5e**) in macrophages. On the other hand, depletion of ISGylation alone, but not ISG15, stimulated secretion of pro-inflammatory cytokines, particularly MCP-1, IL-6 and IL-1$\beta$, as well as free ISG15 from virus-infected cells in general (**Fig**

**4d, 4f, supplementary Fig S5f**), but most significantly from SARS-CoV-2 infected cells (**Fig 4f**). Interestingly, production of IFN-I and II from Zika and SARS-CoV-2 infected macrophages displayed the reverse effect. Cells depleted in ISG15 conjugating enzymes did not have any significant effect on IFN-I or II production, whereas USP18-depleted cells displayed a modest increase (**Fig 4f**). To further decouple the effect of conjugated versus free ISG15, we infected ISG15-depleted cells with SARS-CoV-2 and treated with purified exogenous ISG15 to measure its effect on cytokine secretion (**Fig 4g**). While ISG15-depleted cells displayed reduced secretion of cytokines, treatment with exogenous ISG15 triggered increased production of IL1β and IL-6; however, secretion of TNFα remained unaffected, suggesting that different cytokines might be secreted via distinct routes and free ISG15 possibly triggers a subset of them. Interestingly, secretion of IFNγ was also induced upon exogenous ISG15 treatment, contrary to the wild-type cells. The phenomenon of increased cytokine secretion by exogenous ISG15 treatment was partially inhibited by pre-treating cells with anti-LFA-1, which was previously reported as the receptor for secreted ISG15[26], supporting the role of free ISG15 in triggering inflammatory responses in a paracrine manner (**Fig 4g**). Depletion of ISG15 and Ube1L/HERC5 but not USP18 also resulted in reduced phagocytic activity in influenza-infected macrophages, indicating that ISGylation is important for this effector function of macrophages as reported previously (**supplementary Fig S6a, b**). Collectively, these data indicate that skewing the ratio towards a higher proportion of free ISG15 to its conjugated form drives hyperproduction of atleast a subset of pro-inflammatory cytokines often detected in severe respiratory infections.

## SARS-CoV-2 PLpro recapitulates aberrant macrophage phenotypes

A number of viruses including coronaviruses encode deubiquitylases and de-ISGylases in their genome. To evaluate whether expressing the viral de-ISGylase itself was sufficient to induce aberrant macrophage responses, we expressed the wild-type and catalytically inactive SARS-CoV-2 PLpro in macrophages (**Fig 5a, b**). Wild-type and mutant Usp18 was expressed in parallel as controls. We measured cellular ISGylation upon IFN-I treatment in cells expressing either the empty control vector or those expressing either the wild-type or the mutant variants of PLpro. Dose-dependent expression of the wild-type, but not the mutant PLpro resulted in hydrolysis of bulk cellular ISGylation and a concomitant increase in free ISG15 in IFN-I treated cells,

indicating that it is indeed an active de-ISGylase (**Fig 5c**). Expression of PLpro alone did not induce expression of cellular Usp18, suggesting that cellular de-ISGylation, atleast in PLpro expressing cells, was specifically due to PLpro activity (**Fig 5b**). However, since the mRNA levels of Usp18 increase in infected cells, it is likely that partial de-ISGylation occurs via Usp18 function upon infection. A recent study reported that Usp18 decreases antigen presentation in cancer cells[36]; a similar phenomenon may very well contribute to the immune dysregulation in virus-infected cells.

To investigate whether the PLpro enzyme was sufficient to alter macrophage responses, we measured surface expression of MHC-I and secretion of the panel of cytokines described in Fig 4 in cells transfected with dsRNA. Expression of the wild-type but not the mutant PLpro was able to recapitulate downregulation of MHC-I presentation (**Fig 5d, e**). Expression of the wild type and catalytically mutant variants of USP18 - the cellular de-ISGylase – also confirmed the ISGylation-dependent downregulation of MHC-I (**Fig 5f, g**). PLpro-expressing cells displayed increased secretion of pro-inflammatory cytokines such as MCP-1, IL-6, TNF$\alpha$ and IL-1$\beta$ along with free ISG15, and attenuated secretion of IFN-I and II (**Fig 5h, i**). To test these findings in clinical settings we collected serum samples from patients, which also displayed increased amounts of free ISG15 at their first week of disease onset (**Fig 5j**). Collectively, the results suggest that the de-ISGylating activity encoded by SARS-CoV-2 can disrupt early immune responses in macrophages which likely contribute to the lymphopenia and cytokine storm that often accompanies severe COVID-19.

**SARS-CoV-2 PLpro de-ISGylates glycolytic enzymes**

To further characterize the function of CoV2 PLpro, we bacterially expressed and purified both the wild type and C111A mutant variants of PLpro (**Fig 6a**). The purified enzymes were tested on lysates from macrophages and HeLa cells treated with IFN-I. In both cases, the wild-type enzyme was able to substantially hydrolyse ISGylated proteins (**Fig 6b**). We determined their substrate preference using propargylamide activity-based probes for both ubiquitin and ISG15. As previously reported PLpro displayed a significantly higher activity towards the ISG15 probe compared to ubiquitin (**Fig 6c**).

To delineate the effects of free and conjugated ISG15, we performed a proteome-wide analysis of host ISG15 sites targeted by SARS-CoV-2 PLpro. Our search strategy was based on the original ISG15-GlyGly peptidomics tool[37]. We used trypsin digestion

to reveal diglycines on the modified lysine residues of ISGylated proteins, to enrich modified peptides and locate ISG15 modification sites on target proteins by LC-MS/MS. However, because ubiquitin and NEDD8 leave the same diglycine adduct after trypsin digestion, we used *ISG15*$^{-/-}$ cells as a control to distinguish *bona fide* ISGylation sites.

We applied the ISG15-GlyGly peptidomics tool on HeLa cells producing high levels of ISGylated proteins upon stimulation with IFN-I. Lysates from wild-type and *ISG15*$^{-/-}$ cells were incubated with recombinant wild-type or mutant PLpro. Treatment with wild-type PLpro markedly reduced the levels of ISG15-conjugates compared to untreated or mutant PLpro-treated samples (**Fig 6d**). In addition, we observed a minor decrease in ubiquitination levels after wild-type PLpro treatment, in line with our activity-based assay (**Fig 6c**) and with previous reports showing weak activity of PLpro towards ubiquitin [38]. PLpro-treated samples were trypsin digested to generate diglycine-modified peptides that were enriched by immunoprecipitation and quantified by LC-MS/MS. Following statistical analysis and unsupervised hierarchical clustering, significantly regulated sites were grouped into three major clusters (**Fig 6e;** Supplementary Table 1). Replicate cell cultures clustered together by genotype and treatment, indicating the high reproducibility of our approach. In total, we uncovered 276 ISGylation sites on 181 human proteins that were induced upon stimulation with IFN-I (**Fig 6e**, cluster 1-2). These sites were mostly absent in *ISG15*$^{-/-}$ control cells, thereby marking them as *bona fide* ISGylation sites (**Fig 6e** – indicated in grey). Interestingly, more than half of the identified ISGylation sites were targeted by PLpro as indicated by their absence in the wild-type PLpro treated samples (**Fig 6e**, cluster 1a). In addition, a small subset of ISG15 sites was only partially removed by the viral protease which might be the result of low enzyme:substrate ratios used during PLpro treatment (**Fig 6e**, cluster 1b). Of note, baseline presence of some of these sites in the *ISG15*$^{-/-}$ cells might also result from co-regulation by ubiquitin. Finally, we found 20 ubiquitination sites on 16 proteins that were uniquely upregulated in *ISG15*$^{-/-}$ cells upon IFN-I treatment, with none of these sites targeted by PLpro (**Fig 6e**, cluster 3). To verify that proteome alterations in the *ISG15*$^{-/-}$ cells did not lead to false positive identifications of ISG15 sites (e.g. by downregulation of ubiquitinated proteins in these cells), we checked shotgun data of input to highlight differentially regulated proteins between wild-type and *ISG15*$^{-/-}$ cells upon IFN-I treatment (**Supplementary Figure S7**; Supplementary table 2). Comparison of protein intensities between wild-type and *ISG15*$^{-/-}$ samples revealed 282 significantly regulated proteins, irrespective of the

treatment with PLpro (**Supplementary Figure S7**, supplementary Table 3). Many of these proteins are upregulated in *ISG15*$^{-/-}$ cells, in line with previous reports showing an amplified IFN-I response in cells from *ISG15*$^{-/-}$ patients[39]. Interestingly, many of the upregulated ubiquitination sites in *ISG15*$^{-/-}$ cells (cluster 3), were also found on these upregulated ISGs (**Supplementary Figure S7**). In contrast, only 17 of the identified ISG15 sites (listed in Supplementary Table 1) were present on significantly regulated proteins which precludes any major effect of genotype on ISG15 site identification (**Supplementary Figure S7**). Collectively, our approach led to the high-confidence identification of 118 ISGylation sites on 95 proteins as targets of the SARS-CoV-2 protease PLpro. To validate some of the top scoring candidates, we radiolabelled macrophages expressing either the wild-type or mutant variants of PLpro with [$^{35}$S]cysteine/methionine. Endogenous proteins were immunoprecipitated using specific antibodies, resolved by gel electrophoresis and their ISGylated forms detected by autoradiography (**Fig 6f**). In line with the mass spectrometry data, we confirmed the ISGylation and PLpro-mediated de-ISGylation of glycolytic enzymes and the TAP transporter. Interestingly, only a very minor fraction of IRF3, which was previously reported to be the primary target of PLpro was found to be ISGylated.

Modification of glycolytic enzymes by ISG15 suppresses proinflammatory gene expression in adipocytes[40]. This phenomenon is linked to production of macrophage derived cytokines such as TNF, IL6, and pattern recognition receptors. ISGylation of glycolytic enzymes suppresses the glycolytic flux, resulting in attenuated expression of inflammatory genes. Expression of the cellular de-ISGylase Usp18 is able to rescue these defects. Our ISGylome data indicate that this is also the case with SARS-CoV-2 PLpro. Many of the highest scoring candidates for de-ISGylation were enzymes of the glycolytic pathway, indicating the PLpro very likely reverses the ISG15-dependent suppression of inflammation in infected cells and drives the M1 pro-inflammatory phenotype during SARS-CoV-2 infection.

**SARS-CoV-2 infected macrophages secrete pro-inflammatory cytokines**

Aberrant cytokine responses from PLpro expressing cells prompted us to systematically analyse the ISG15-dependent secretome from SARS-CoV-2 infected macrophages and compare that with IFNγ treated macrophages as shown in schematic. We performed a quantitative mass spectrometry based proteomic analysis of the extracellular protein profile (secretome) of SARS-CoV-2 infected and IFNγ

386 treated macrophages using established strategies of label free quantitation. We
387 selected 24 h post SARS-CoV-2 infection or IFNγ treatment as the timepoint for
388 analyses in macrophages expressing non-targeting (NT) DsiRNA or those targeting
389 ISG15 or Ube1L. We defined the secretome as proteins released via all mechanisms,
390 including classical, non-classical and exosomal pathways. Using LC-MS/MS mass
391 spectrometry and MaxQuant proteomics software package for computational analyses
392 we detected relative protein abundances in the conditioned media of control, SARS-
393 CoV-2 infected or IFNγ treated macrophages. For increased confidence in the protein
394 identification numbers we required that a protein be identified on the basis of at least
395 two unique peptides and quantified in a minimum of two replicates.
396 We identified 489, 428 and 502 protein in IFNγ treated NT, ISG15-deficient and
397 Ube1L-deficient macrophages, whereas 508, 485 and 544 proteins in SARS-CoV-2
398 infected macrophages (**Fig 7a**). Principal component analysis of the secretome
399 response in the NT, ISG15-deficient and Ube1L-deficient cells showed a clear
400 separation of the ISG15-deficient cells from NT and Ube1L-deficient cells, which
401 clustered together (**Fig 7b**). For the secretome data sets, the first three principal
402 components captured 89% (PC1: 51%, PC2: 25%, PC3: 13%) variability in the data.
403 Pairwise comparison showed the highest overlap between NT and Ube1L-deficient
404 SARS-CoV-2 infected macrophages. We evaluated the responses of the common
405 proteins identified in all the conditions. Hierarchical clustering analyses of these
406 common proteins revealed that the secretome of NT and Ube1L-deficient clustered
407 together while that of ISG15-deficient cells was significantly different from the others
408 (**Fig 7c**). Functional enrichment analyses revealed that the most prominent enrichment
409 in the secretome of SARS-CoV-2 infected cells were of the inflammatory responses,
410 cytokine secretion, non-classical secretory processes and exosomes, which strongly
411 correlated specifically with Ube1L-deficient cells that inhibited the conjugated but not
412 the extracellular free form of ISG15 (**Fig 7d**). Collectively, our data indicate that altered
413 free versus conjugated ISG15 results from the PLpro enzymatic function of SARS-
414 CoV-2, which also triggers increased expression of pro-inflammatory genes and
415 cytokines, skewing macrophages to the M1-state via perturbation of glycolytic flux. This
416 phenomenon can be exacerbated by depleting the cellular ISGylation enzymes without
417 affecting mono ISG15. Free ISG15 released from infected macrophages can
418 subsequently amplify cytokine secretion in a paracrine fashion via the LFA-1 receptor
419 and Src-family kinase activity as has been shown previously. These data therefore

provide a systematic overview of the core macrophage processes regulated by cellular ISG15 in response to SARS-CoV-2 infection.

## Discussion

In-vitro and in-vivo studies have established that macrophages are one of the major determinants of pathogenesis during virus infections [6,41–43], driven by the production of interferons and interferon stimulated genes. However, the role of ISG15 in its free or conjugated form, in macrophage-mediated immune responses is not well studied. Here, we compared the ISG15-dependent responses of human macrophages to influenza, Zika and SARS-CoV-2 virus infections. All these viruses transcriptionally upregulated ISG15 and ISGylating enzymes, which was accompanied by increased bulk protein ISGylation in influenza and Zika, but not in SARS-CoV-2 infected cells. This is particularly intriguing since SARS-CoV-2 encodes for a papain-like protease (PLpro), which is a putative de-ISGylase. SARS-CoV-2 infected cells displayed a skewed ratio of free versus conjugated ISG15, accompanied by heightened secretion of pro-inflammatory cytokines despite reduced IFN production and antigen presentation. Free ISG15 was secreted specifically from macrophages, but not epithelial cells, consistent with its role in immune modulation. Secretion was ISG15 and pro-inflammatory cytokines was exacerbated by depleting ISG15 conjugating enzymes Ube1L or HERC5, which prevented substrate ISGylation but not free ISG15. These data suggest that ISG15 regulates macrophage inflammatory responses either via protein ISGylation to inhibit secretory processes or via free ISG15 signaling that may induce cytokine production, or a combination of the two.

Our results demonstrate that the combined effect of de-ISGylation of metabolic enzymes and free form of ISG15 critically affect the global immune response of macrophages during virus infections, unlike the respiratory tract epithelial cells where ISG15 primarily functions as an antiviral factor to limit viral replication [17]. Depletion of Ube1L or HERC5 that specifically prevented the conjugated, but not free ISG15, stimulated secretion of pro-inflammatory cytokines from virus infected macrophages, such as MCP-1, TNF, IL-6, all of which have been implicated in the cytokine storm caused by highly pathogenic influenza and severe Covid-19. MCP-1 (CCL2) and IL-6 have been consistently found to be a predictor of severe pathogenesis in respiratory virus infections. Uncontrolled MCP-1 secretion has also been implicated in increasing the severity of inflammatory disorders of the lung and can regulate infiltration of

immune cells, including monocytes, T cells and NK cells. Depletion of ISG15 followed by treatment with exogenous free ISG15 in CoV2 infected cells recapitulated this phenotype for atleast a subset of cytokines.

Several viruses encode deubiquitylating and de-ISGylating activities in their genome that can counter host antiviral immunity. SARS-CoV-2 itself encodes a papain like protease which is a putative de-ISGylase [44]. Our data indicate that infection by SARS-CoV-2 removes ISG15 modifications from cellular substrates, confirming its intrinsic de-ISGylating ability. Substrate de-ISGylation combined with increased free ISG15 was accompanied by hyperactivation of pro-inflammatory cytokines and reduced antigen presentation, both of which are key features of severe COVID-19. Expression of the wild-type but not catalytically inactive SARS-CoV-2 PLpro alone was able to trigger dramatic cellular de-ISGylation, recapitulating these results. In vitro characterization of PLpro substrates further revealed that primarily metabolic enzymes of the glycolytic pathway are ISGylated during infection and targeted by CoV2 PLpro. ISGylation of glycolytic enzymes has been reported to suppress expression of thermogenic genes in obesity and polarization of macrophages to the M1 state. This feature can be reversed by loss of ISG15 or expression of cellular de-ISGylase Usp18. Besides, activation of the glycolytic pathway has been independently shown as critical for NK cell cytotoxicity and inflammatory response to cytomegalovirus infections[45]. The widespread removal of ISG15 modifications from glycolytic enzymes therefore indicates that the increased activation of this pathway drives the M1 proinflammatory phenotype in macrophages. Collectively, these data indicate that SARS-CoV-2 PLpro is able to perturb immune responses in macrophages, which may underpin the loss of CD8+ T-lymphocytes and increased inflammation often seen in severe COVID-19 [34].

The role of ISG15 in virus-infected macrophages underscore the importance that the conjugated and free form plays in driving immune responses. Extracellular free ISG15 can act as an adjuvant for $CD8^+$ cytotoxic T cells and can also influence infiltration or activation of immune cells such as neutrophils. Early recruitment of NK and cytotoxic $CD8^+$ T-cells is vital to the host to control virus infection as well as lung inflammation [46,47]. Other reported immunomodulatory activities of extracellular ISG15 include anti-tumor activities of dendritic cells (DCs) [48], and triggering Type-II IFN response in NK and T-cells essential for immunity against mycobacteria [49]. Further characterization of the separable biochemical functions in animal models are called for to assess the specific core functions of ISG15 in response to infections. Delineating the roles of free and conjugated forms of ISG15 should provide a better understanding

of the pro- and antiviral impact of ISG15 in virus pathogenesis and ascertain whether aberrant ISG15-dependent macrophage effector responses are universal features that underpin hyperinflammatory responses during infections by highly pathogenic viruses.

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**Author Contributions Statement**

FI, CKPM, and SS designed and conducted the study. DM, QT, DE, HHYL, FT, SWvL, JH, CKPM, LYLS, WWSN performed and analyzed experiments. HP, BK, AD, AP-F generated critical reagents for the study. SS and CKPM wrote the manuscript.

## Competing Interests Statement

The authors declare no conflict of interest.

## Figure legends

**Fig 1. The ISGylation machinery is induced in Zika and SARS-CoV-2 virus infected macrophages**

**a-e.** Macrophages were infected with Zika or SARS-CoV-2 at MOI 2. At indicated time intervals changes in mRNA expression levels of ISG15 modifying enzymes Ube1L UbcH8 and HERC5 and Usp18 (a-d), and ISG15 (e) against mock infection were quantified by qPCR **f.** Intracellular ISG15 protein levels in Zika or SARS-CoV-2 infected macrophages were quantified by ELISA. Data (a-f) are displayed as means ± SEM of three biologically independent experiments. [$*p < 0.05$, $**p < 0.01$, $***p < 0.001$ by two-tailed Mann-Whitney U Test vs. mock-infected cells; n=3]. **g.** ISGylation in virus-infected macrophages was measured for Zika and SARS-CoV-2. Macrophages were infected with Zika *(left panel)* and SARS-CoV-2 *(right panel)* at a MOI 2. Lysates were collected in 50 mM Hepes supplemented with 0.5% IGEPAL pH 7.4, separated by SDS-PAGE and visualized by Western blotting using an anti-human ISG15 antibody. Gapdh levels were measured as loading control. The blot is representative of results obtained from three independent experiments.

**Fig 2. ISG15 is secreted from virus-infected macrophages via unconventional LC3-derived secretory pathway.**

**a, b.** Macrophages were infected with either Zika or SARS-CoV-2 at MOI 2 (a) or with an equivalent dose of UV-inactivated Zika or SARS-CoV-2 (b). Changes in extracellular levels of ISG15 were quantified by ELISA. **c.** LDH assay was performed using LDH-Cytotoxicity Colorimetric Assay Kit II (BioVision) to estimate cell death based on the amount of LDH leakage into the cell culture media, at indicated timepoints post infection following the manufacturer's protocol **d.** Caspase inhibitors Z-YVAD-FMK and Z-DEVD-FMK were added to virus-infected macrophages (1h post infection), and ISG15 secretion measured 24h post infection. **e.** Brefeldin (5µM) or DMSO alone was added 1 h post-infection and ISG15 secretion measured 24 h post infection. **f.** Potential pathways of unconventional protein secretion. **g-k.** Depletion of TMED10 (h), LDELS components (LC3; nSMase2) (i), and secretory lysosomal proteins (Lamp1;

Arl8b) (j), early autophagy components (Ulk1 complex; Lyn kinase) (k), SNARE proteins of the secretory autophagy pathway (l) were performed by DsiRNA treatments and verified by immunoblotting. Non-targeting and depleted cells were infected with CoV2 (MOI 2; 24h) and secretion of ISG15 measured by ELISA. Error bars (a-k) represent means ± sd of 3 biologically independent experiments. [*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by the two-sided Mann-Whitney U Test vs mock-infected cells].

**Fig 3. Immune dysfunction in SARS-CoV-2-infected macrophages**

**a.** Surface staining of MHC-I in macrophages infected with SARS-CoV-2 (MOI 2, 24 h). Controls included were antibody isotype, UV-inactivated virus and dsRNA. Cells were gated on viral N+ (in red; 75% of population) and surface MHC-I. Uninfected cells (bystander; viral N-) are depicted in black. **b.** Surface staining of MHC-II in macrophages infected with SARS-CoV-2 (MOI 2, 24 h). Cells were gated on viral N+ (in red; 73% of population) and surface MHC-II+ cells. Uninfected population (bystander; viral N-) is depicted in black. Controls included were antibody isotype, UV-inactivated virus and dsRNA treated cells **c.** M0 macrophages were stimulated to M1 or M2 by differentiating for 48 hours in the presence of M-CSF+LPS+IFN-γ and M-CSF+IL-4 respectively, or infected with SARS-CoV-2 (MOI 2, 48h). Expression of key markers of polarization was measured by RT qPCR. **d.** Secretion of indicated cytokines was measured using cytometric bead array following the manufacturer's guidelines and flow cytometry. Error bars represent mean±s.d; **$p < 0.01$, ***$p < 0.001$; n=4 biologically independent experiments]. **e.** Quantification of phagocytosis of M1- or M2-stimulated phagocytes was compared with SARS-CoV-2 infected macrophages (MOI 2, 48 h). Error bars represent mean±s.d; [*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; Two-way ANOVA with Tukey's multiple comparison test, n=4 biologically independent experiments]. **f.** Cells depleted of secretory autophagosomal components (LC3, Ulk1, Stx4) were challenged with SARS-CoV-2 and indicated cytokines measured by cytometric bead array; Error bars represent mean±s.d; [*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; n=4 biologically independent experiments by two-tailed Mann Whitney U Test compared with control cells].

**Fig 4. Role of free versus conjugated ISG15 on viral replication and cytokine secretion.**

**a.** Macrophages were transfected with ISG15, Ube1L, HERC5 or USP18 DsiRNA for 72 hours; depletion was verified in IFN-I treated cells by immunoblotting. **b.** Cellular

ISGylation was measured in Zika and SARS-CoV-2 infected cells (MOI 2), 24 hours post infection. Immunoblots (a, b) are representative of 3 biologically independent experiments **c.** Total RNA was collected at indicated time intervals from Zika infected cells; quantifications of absolute copy number were done by RT-qPCR using universal vRNA specific primers. Data are displayed as mean ± SEM of 3 biologically independent experiments. *p < 0.05 by the Mann-Whitney U Test vs. control. **d.** Indicated cytokines were quantified by cytometric beads assay from macrophages transfected with either non-targeting (NT), ISG15, Ube1L, Herc5 or USP18 DsiRNA for 72 hours prior to infection. Free ISG15 was determined by ELISA. Data are displayed as means ± SEM of 3 biologically independent experiments. [*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by the two-tailed Mann-Whitney U Test vs. control] **e, f.** Same as c, d in macrophages infected with SARS-CoV-2. Data are displayed as mean ± s.d of 3 biologically independent experiments. *p < 0.05 by the two-tailed Mann-Whitney U Test vs. control (NT cells) **g** Macrophages were treated with ISG15 DsiRNA and infected with SARS-CoV-2 (MOI 2) in media supplemented with exogenous purified ISG15 (1µg/ml); cells were either untreated or pre-treated with anti-LFA-1 inhibitory antibody (500ng) for 1 hour prior to infection. Indicated cytokines were quantified by cytometric beads assay. Error bars represent mean±s.d; [*$p < 0.05$, **$p < 0.01$; n=3 biologically independent experiments], by the two-tailed Mann-Whitney U Test vs. control cells.

**Fig 5. Dysregulation of antigen presentation and interferon response in macrophages expressing SARS-CoV-2 PLpro**

**a, b.** Schematic of SARS-CoV-2 PLpro (wild-type and mutant) and their expression in macrophages verified by immunoblotting **c.** Bulk ISGylation in IFN-I treated macrophages expressing either the empty vector, wild-type PLpro, or mutant PLpro in a dose-dependent manner. Immunoblots (b, c) are representative of 3 biologically independent experiments. **d, e.** Surface staining of MHC-I in macrophages expressing either wt HA-PLpro (d) or the catalytic mutant HA-PLpro (C111A) (e) of SARS-CoV-2. Cells were then treated with dsRNA to induce surface expression of MHC-I. For both samples (wild-type, mutant C111A) >90% of cells stained positive for HA-(PLpro) and dsRNA **f, g.** Same as (d, e) in cells expressing USP18 (wild-type or C64R/C65R mutant). **h.** Secretion of indicated cytokines was measured using cytometric beads assay following the manufacturer's guidelines and flow cytometry. Error bars represent mean±SD from four independent experiments. Data are displayed as means ± s.d. *p < 0.05 by the two-tailed Mann-Whitney U Test vs. control (empty vector matched cells).

**i.** Supernatants from cells described in (h) were collected and ISG15 was quantified by ISG15 sandwich ELISA. All data are displayed as mean ± sd of at four independent experiments. *p < 0.05 by the two-tailed Mann-Whitney U Test vs. control cells. **j.** ISG15 levels in the plasma samples collected from the COVID-19 patients at their first week of disease onset. Error bars represent mean±SD *p < 0.05 by the two-tailed Mann-Whitney U Test vs. healthy donors (n=38 for patients and n=14 for healthy donors).

**Fig 6. Identification of substrates de-ISGylated by SARS-CoV-2 PLpro**

**a.** Coomassie stained gel of fractions collected from size exclusion chromatography for bacterially expressed and purified wild-type and mt (C111A) mutant SARS-CoV2 His-tagged PLpro **b**. Cell lysates from IFN-I treated macrophages or HeLa cells were treated with buffer alone (control lanes), purified WT or Mt (C111A) PLpro. Lysates were resolved by gel electrophoresis and visualized by western blotting **c**. Activity-based assay to determine de-ubiquitylating versus de-ISGylating activities of WT and C111A PLpro. 10μM of either HA-Ub-PA or ISG15-PA were treated with indicated concentrations of WT and mt (C111A) PLpro for 30 mins at RT. Usp18 was used as a positive control for de-ISGylase activity. Reaction products were resolved by gel electrophoresis and visualized by Coomassie staining **d**. Sample preparation for mass spectrometry to identify PLpro substrates. Western blot analysis of cellular lysates obtained from HeLa wild-type (WT) or *ISG15*$^{-/-}$ cells following 72h of IFN-I stimulation and treatment with recombinant wild-type (WT) or mutant (Mut) PLpro for 30 min. The same lysates were used for the actual ISG15-GlyGly peptidomics experiment. ISG15- and ubiquitin-conjugates were visualized by immunoblotting with anti-ISG15 (IB:ISG15) and anti-ubiquitin (IB:UBQ) antibody, respectively. Tubulin-α served as a loading control and was detected with anti-tubulin-α antibody (IB:Tubα). Images (a-d) are representative of 3 biologically independent experiments. **e**. Heatmap showing significantly regulated GlyGly(K) sites after unsupervised hierarchical clustering. Different genotypes (wild-type or ISG15 knockout (KO)) and protease treatments (wild-type (WT) or mutant (Mut) PLpro) are indicated. Colors indicate up- (red) or downregulated (blue) sites. On the right, the same heatmap is shown with originally missing values depicted in grey. Three major clusters can be observed which contain ISG15 sites targeted (cluster 1a-1b) or untargeted (cluster 2) by PLpro, or ubiquitin sites unaffected by PLpro treatment (cluster 3). **f** Validation of identified hits in macrophages expressing either WT or C111A mutant variants of PLpro, radiolabelled

with [$^{35}$S]cys/met and endogenous proteins immunoprecipitated with antibodies against indicated proteins. Autoradiograms are representative of 3 biologically independent experiments.

**Fig 7. Quantitative analyses of ISG15-dependent responses in SARS-CoV-2 infected macrophages**

**a.** iPSC-derived macrophages were either transfected with non-targeting DsiRNA or those targeting ISG15 or Ube1L. Conditioned media was collected from control, SARS-CoV-2 infected or IFNγ treated cells. Proteins were extracted from each of the samples, separated by SDS-PAGE and digested with trypsin for LC-MS/MS as described in the Materials and methods**.** Total numbers of proteins quantified in atleast two biological replicates **b.** Principal component analysis was performed using the Perseus software. Filled squares represent control cells (NT DsiRNA), empty squares ISG15-depleted, filled circles Ube1L-depleted. The uninfected cells are shown in gray and infected cells are shown in red **c.** The heatmap represents the hierarchical clustering of the common proteins in the secretome for IFNγ treated or SARS-CoV-2 infected cells. The color key represents changes (log2 scale) from dark blue indicating the largest decreases to red indicating the largest increases **d.** Functional annotation of the common proteins identified in all samples was performed by the DAVID software.

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## Methods

### Plasmid Construction

The papain-like protease domain sequence is obtained from the SARS-CoV-2 complete genome (NCBI genome databank; NC_045512.2). Protein sequence for PLpro domain (amino acids, 746-1059) of pDONR207 SARS-CoV-2 Nsp3 (Addgene; #141257) was cloned into pCAGGs vector with C-terminal Flag-tag. Catalytic mutant (C111A) was generated by site-mutation PCR and verified with sequencing. Plasmid expressing PLpro was cloned in pET28 expression vector using NcoI and XhoI restriction enzymes (kindly provided by Ivan Ahel) for bacterial expression. Catalytic dead mutant of PLpro C111A was made using Q5 site directed mutagenesis kit.

### Virus cultures

Influenza virus gene segments were amplified by PCR using Pfu Turbo DNA polymerase (Stratagene Cat#600250) and cloned into pHW2000 vector (a gift from Robert G. Webster, St. Jude Children's Research Hospital). Individual plasmids containing the eight viral genome segments were co-transfected using TransIT-LT1 (MIR2300, Mirus Bio) into 293T (ATCC Cat#CRL-3216, RRID:CVCL_0063)/MDCK (ATCC Cat#CCL-34, RRID:CVCL_0422) co-cultures. Recombinant viruses generated from the transfection system were propagated in embryonated eggs and quantified by plaque assay. Zika virus (strain MR766) and SARS-CoV-2 (Wu/01) stocks were prepared by determining tissue culture infective dose 50% ($TCID_{50}$/ml) in Vero E6 cells challenged with 10-fold serial dilutions of infectious supernatants for 90 min at 37ºC. Cells were subsequently incubated in DMEM with 2.5% FCS.

### Cells and Cell Lines:

HeLa *ISG15*$^{-/-}$ cells have been previously described[1].

### Primary cell culture and preparation for infection

For influenza infections, buffy coat packs from healthy donors were kindly provided by the Hong Kong Red Cross Blood Transfusion Service and autologous plasma was collected following centrifugation from the top layer. The study received ethical approval from the Institutional Review Board of the LKS Faculty of Medicine of the

University of Hong Kong (Ref no: UW 17-050). The remaining portion was mixed with RPMI1640 medium (GIBCO Cat#23400021), overlaid onto Ficoll-Paque Plus density medium (GE Healthcare Life Sciences Cat#17144003) and centrifuged at 1,000 x g for 20 minutes without braking. Peripheral blood mononuclear cells (PBMCs) were collected from the media-Ficoll interface. Plastic-adherent monocytes were cultured in 5% heat-inactivated autologous plasma in RPMI1640 medium and allowed to differentiate for 14 days into macrophages. For dendritic cells differentiation, 50ng/mL GM-CSF (Peprotech Cat#300-03), 10ng/mL IL-4 (Peprotech Cat#200-04), 5% heat-inactivated autologous plasma in RPMI1640 medium were used. Normal human bronchial epithelial cells (NHBE) were cultured in BEGM BulletKit Growth Media (Lonza Cat#CC-3170). The day before influenza virus infection, cells were harvested in trypsin/EDTA and 0.1 x $10^6$ cells were seeded in 24-well cell culture plates (TPP).

**iPSC-derived macrophages**

For Zika and CoV infections iPSC-derived CD14+ monocytes (from ATCC) were resuspended in macrophage differentiation base medium (RPMI 1640; 10% heat inactivated fetal calf serum, 2 mM L-glutamine; 100U/ml penicillin/streptomycin) supplemented with 100 ng/ml M-CSF. Cells were counted and seeded at a density of 150,000 precursor cells/well of a 6-well plate. Cells were cultured at 37ºC for 6 days to differentiate into mature macrophages. At day 7, cell density was verified to be 2-3 times that of initial number of precursors. Mature differentiated macrophages were infected with either Zika or SARS-CoV-2 for downstream functional assays.

**Collection of plasma samples**

Patients with RT-PCR confirmed COVID-19 disease at the Infectious Disease Centre of the Princess Margaret Hospital, Hong Kong, were invited to participate in the study after providing informed consent. The study was approved by the institutional review board of the Hong Kong West Cluster of the Hospital Authority of Hong Kong (approval number: UW20-169). Day 1 of clinical onset was defined as the first day of the appearance of clinical symptoms. Specimens of heparinized blood were collected from the patients, and the plasma were separated by centrifugation. The plasma was stored at -80°C until use.

**Virus infection**

Cells were infected with the indicated strains of viruses at 37°C in the corresponding culture medium under serum-free condition for 1 hour. The virus inoculum was then removed, cells were washed with warm PBS, and replenished with medium supplemented with 100U/ml penicillin, 100µg/ml streptomycin (GIBCO Cat#15140122), and 1 µg/L TPCK-treated trypsin (Sigma Cat#T1426) in case of influenza.

**Gene silencing by RNA interference**

All gene-specific Dicer-substrate small interfering RNA (DsiRNA) oligos were purchased from IDT (Supplementary Table 4). DsiRNA was transfected with Viromer BLUE transfection reagent (Lipocalyx Cat#VB-01LB-01) at a final concentration of 25nM. Forty-eight hours post-transfection, cells were either harvested for analysis or subjected to additional experimental procedures as described.

**ELISA**

Cellular and secreted ISG15 were quantified with human ISG15 ELISA kit (MBL Cat#CY-8085) according to the manufacturer's instructions. Briefly, 100µL of cleared cell lysate or culture medium were captured on ELISA assay plates coated with anti-ISG15 antibody. After extensive washing in buffer containing 0.2% Tween, 100µL HRP-anti-ISG15 antibody was added for ISG15 protein detection. Binding was visualized by adding 100µL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate and absorbance was measured at 450nm. For background correction, absorbance values at 550nm were also measured and plotted against ISG15 protein standards. ISG15 protein concentration was then calculated using a linear regression method.

**Cytokine quantification**

Proinflammatory cytokines and chemokines concentrations were measured by a cytometric beads assay kit (Biolegend Cat#740003), as advised by the manufacturer. Briefly, cytokine/chemokine standards or cleared culture supernatants were mixed with capture beads together with biotin-conjugated antibody cocktail for 2 hours. Phycoerythrin (PE)-conjugated streptavidin was then added to the mixture and incubated for 30 minutes. Beads were pelleted by centrifugation at 3000 x g for 5 minutes, washed, fixed in 4% formaldehyde for 15 minutes, centrifuged at 3,000 x g

for 5 minutes, and resuspended in 250µL 1X wash buffer for flow cytometry (BD LSRFortessa) acquisition. Results were analyzed with FCAP array version 3.0 (BD). Specific capture beads groups were first separated by forward scatter (FSC) and side scatter (SSC) dot-plot graphs and further gated by allophycocyanin (APC) fluorescent channel. PE fluorescence read outs were then curve-fitted to a standard curve to estimate the concentration of analytes.

**Phagocytosis assay**

Macrophages ($1x10^6$) were seeded in 35mm non-cell culture treated polystyrene dish in 500µL RPMI1640 supplemented with 5% autologous plasma, 100U/ml penicillin, and 100µg/ml streptomycin (GIBCO Cat#15140122). Macrophages were infected with the specified strains of influenza virus (MOI = 2) in fresh serum-free medium (GIBCO Cat#12065074). At 24 hours post-infection, $1x10^7$ blue, fluorescent latex beads (SpheroTech; 1µm, 10 beads/cells) were added and incubation continued for 1 hour at 37°C. Cells were then detached in 10mM EDTA at 4°C for 20 minutes, centrifuged at 250 x g for 5 minutes at 4°C and eventually fixed in 4% formaldehyde at room temperature for 15 minutes. Macrophages were pelleted at 500 x g for 5 minutes and resuspended in 250µL PBS, pH 7.4, for flow cytometry acquisition as described above.

**Drug treatment**

Macrophages ($2x10^5$) were seeded in 24-well plates in 500µL RPMI1640-supplemented with 5% autologous plasma, 100U/ml penicillin, and 100µg/ml streptomycin (GIBCO Cat#15140122). Twenty-four hours later, media were discarded and replaced with fresh serum-free medium (GIBCO Cat#12065074), which was changed daily for 2 days. On the day of experiment, macrophages were pre-treated or post-treated with the indicated drug concentrations as detailed.

**Protein expression and purification**

pET28 WT and mutant PLpro plasmids were transformed into Rosetta DE3 expression cells and grown in LB media supplemented with Kanamycin and Chloramphenicol. Culture was induced with 0.5 mM IPTG and 1 mM zinc chloride at 0.5-0.6 $OD_{600}$ and grown overnight at 16 °C. Protein purification was performed as described elsewherer[38]. Bacterial pellet was suspended in lysis buffer (50 mM Tris HCL pH 8, 150 mM NaCl, 10 mM imidazole and 2 mM DTT) and lysed using homogeniser.

Cleared lysate was incubated with pre-washed Ni-NTA agarose resin (50% slurry, Qiagen) at 4 °C for 1 h. Beads were further washed with increasing imidazole concentration (50 mM Tris HCL pH 8, 150 mM NaCl, 10-40 mM imidazole and 2 mM DTT). His-tagged PLpro protein was eluted in elution buffer (50 mM Tris HCL pH 8, 150 mM NaCl, 250 mM imidazole and 2 mM DTT). Eluted protein was dialysed overnight in dialysis buffer (20 mM Tris pH 8, 100 mM NaCl and 2 mM DTT) and further purified by size exclusion chromatography using Superdex75 column equilibrated with 20 mM Tris pH 8, 100 mM NaCl and 1 mM DTT.

**Activity based assay for PLpro**

HA tagged-Ub-PA and ISG15-PA were used to perform activity-based probe assay with PLpro proteins. Briefly, activity probes (Ub-PA and ISG15-PA) were diluted in probe buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl) and used at 10 µM final concentration in each reaction. WT and mutant PLpro protein were used at different concentration as indicated in figure and prepared in activation buffer (25 mM Tris HCl pH 7.5, 150 mM NaCl, 10 mM DTT). Reaction was initiated by mixing equal volumes of activated protein and activity probe and incubating the reaction at room temperature for 30 mins and stopped by addition of SDS loading dye. Samples were run on 4-12 % bis-tris protein gel (Invitrogen) and visualised by Coomassie staining.

**Secretome identification**

LC-MS analyses for secretome identification were performed by a ThermoFisher Orbitrap Lumos instrument that was operated in a data dependent acquisition mode to switch between Orbitrap full scan MS and LTQ MS/MS. Mass spectra were analysed by MaxQuant version 1.4.1.2 and the Andromeda search engine. The maximum mass deviation allowed for the monoisotopic precursor ions was 4.5 ppm for monoisotopic precursors and 0.5Da for fragment ions. Trypsin was set as the digestion enzyme with a maximum of two allowed missed cleavages. Cysteine carbamidomethylation was set as a fixed modification, and N-terminal acetylation and methionine oxidation were allowed as variable modifications. The spectra were searched using the Andromeda search engine against the human Uniprot sequence database. Protein identification required at least two unique peptides per protein group. The data were filtered for a 1% FDR at the peptide and protein level. Principal component analysis (PCA) was performed using the Perseus software version version 1.6.2.1 on the proteins common between the cells expressing non-targeting DsiRNA or those targeting ISG15 or Ube1L. Protein expression fold change values were log transformed (base 2). Log transformed fold change data showed bell shaped distributions and were symmetric around 0.

## Sample preparation for PLpro ISGylome

HeLa WT and *ISG15*$^{-/-}$ cells were cultured in Dulbecco's modified Eagle's medium (DMEM, 31966047, ThermoFisher Scientific) supplemented with 10% fetal bovine serum (FBS, 10270106, ThermoFisher Scientific) and maintained in a humidified incubator at 37°C and 5% $CO_2$. 5 million cells of each genotype were seeded in triplicate for each condition (WT or mutant PLpro) in 12x150 $mm^2$ culture dishes (Greiner Bio-One). Cells were stimulated for 72h with 500 U/mL IFN-α (11343504, ImmunoTools), washed in PBS and lysed in 50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% Triton-X-100, 1 mM PMSF (Sigma-Aldrich) and 1x Protease Inhibitor Cocktail (Roche). Crude lysates were sonicated by 3 bursts of 10 sec at 5W output followed by centrifugation at 16,000g for 15 min at 4°C. The protein content of the cleared lysates was measured by Bradford and 7.2 mg total protein of each replicate was treated with WT or mutant PLpro at a 1:50 (w/w) ratio for 30 min at 37°C. To quench the activity of PLpro, 5% SDS was added to each replicate followed by reduction with DTT (4.5 mM) for 30 min at 55°C and alkylation with chloroacetamide (10 mM) for 15 min at RT in the dark. Samples were subsequently prepared for MS analysis using the PTMScan® HS Ubiquitin/SUMO Remnant Motif (K-ε-GG) Kit (59322, Cell Signaling Technology). Briefly, all proteins were immobilized on S-trap midi columns (C02-mini-40, Protifi) and digested on-column with 1:100 (w/w) trypsin (V5111, Promega) at 37°C overnight. The resulting peptides were eluted from the column, dried in a vacuum concentrator and re-dissolved in 1x immunoprecipitation buffer prior to immunocapture of GG-modified peptides. At this point, an aliquot of 30 µg total peptide was taken for shotgun proteomics analysis. The remaining peptide solution was incubated with antibody-bead slurry for 2h at 4°C. Beads were washed and GG-modified peptides were eluted by adding 0.15% TFA. Captured peptides were desalted on reversed phase C18 OMIX tips (Agilent), dried under vacuum in HPLC inserts and stored at -20°C until further use.

## LC-MS/MS and data analysis for ISGylome determination

Dried GG-modified peptides were re-dissolved in 33 µL loading solvent A (0.1% TFA in water/ACN (98:2, v/v)) and 15 µL was injected for LC-MS/MS analysis on an Ultimate 3000 RSLCnano system in-line connected to a Q Exactive HF mass spectrometer (Thermo Scientific). Trapping was performed at 10 µL/min for 4 min in loading solvent A on a 20 mm trapping column (made in-house, 100-µm internal diameter (I.D.), 5-µm

beads, C18 Reprosil-HD, Dr. Maisch, Germany) and the sample was loaded on a 250 mm nanoEase™ analytical column (Waters, 75-µm internal diameter (I.D), 1.8-µm beads, C18 HSS T3). Peptides were eluted by a nonlinear increase from 1 to 55% MS solvent B (0.1% FA in water/acetonitrile (2:8, v/v)) over 135 min at a constant flow rate of 300 nL/min, followed by a 15 min wash reaching 97% MS solvent B and re-equilibration with MS solvent A (0.1% FA in water/acetonitrile (98:2, v/v)). The column temperature was kept constant at 40 °C in a column oven (CoControl 3.3.05, Sonation). The mass spectrometer was operated in data-dependent mode, automatically switching between MS and MS/MS acquisition for the 8 most abundant ion peaks per MS spectrum. Full-scan MS spectra (375–1500 m/z) were acquired at a resolution of 60,000 in the orbitrap analyzer after accumulation to a target value of 3,000,000. The 8 most intense ions above a threshold value of 8,300 were isolated for fragmentation at a normalized collision energy of 28% after filling the trap at a target value of 100,000 for maximum 120 ms. MS/MS spectra (200–2000 m/z) were acquired at a resolution of 15,000 in the orbitrap analyzer. From the aliquots for shotgun proteomics analysis, ~2 µg of peptides were injected on the same LC-MS/MS system, using similar settings as described above. Here, the 16 most intense ions above a threshold value of 13,000 were isolated for fragmentation after filling the trap at a target value of 100,000 for maximum 80 ms.

All data were analyzed in MaxQuant (version 1.6.17.0) using the Andromeda search engine with default search settings, including a false discovery rate set at 1% on the peptide and protein level. Separate searches were performed for the GG-peptidomics and shotgun samples. In both cases, spectra were searched against the human protein sequences in the Swiss-Prot database (database release version of January 2021), containing 20,621 sequences (www.uniprot.org). Mass tolerance was set at 4.5 ppm for precursor ions and 20 ppm or 0.5 Da for fragments ions of the GG-peptidomics and shotgun samples, respectively. Digestion mode was set to “trypsin allow P” enzyme specificity with up to three missed cleavages. Both searches included carbamidomethylation of cysteine residues as a fixed modification and oxidation of methionine and acetylation of protein N-termini as variable modifications. In addition, GG modification of lysine residues was added as a variable modification for the GG-peptidomics samples. The minimum score for identification of modified peptides was set to 30 which led to the discovery of 2143 GG-modification sites (listed in the GlyGly(K) site table). For the shotgun samples, only proteins with at least one unique

or razor peptide were retained resulting in 3130 identified human proteins (listed in the proteinGroups table).

GG-peptidomics data analysis was continued in Perseus (version 1.6.2.1) after uploading the GlyGly(K) site table from MaxQuant. The data was processed by removing reverse sequences, potential contaminants and sites with a localization probability less than 0.75. Afterwards, the site table was expanded and the intensities log2(x) transformed. The data was normalized through subtraction of the median intensity in each sample. Next, replicates were grouped and sites with less than three valid values in at least one replicate group were discarded. To allow statistical testing, missing data values were imputed from a normal distribution around the detection limit. The experimental design was uploaded into the site table by defining groups based on treatment (WT or mutant PLpro) and genotype (HeLa WT or *ISG15*$^{-/-}$). A two-way ANOVA analysis was done to compare the site intensities between treatment and genotype. Three p-values were calculated for each site, including a p-value for the effect of treatment and genotype and an interaction p-value. Sites with a p-value less than 0,01 for at least one of the three parameters were retained and used for unsupervised hierarchical clustering after Z-score normalization. Significantly regulated sites were grouped into clusters and visualized in a heatmap in Figure 6 with their intensity per replicate across the different experimental groups. The significantly regulated modification sites are listed in Supplementary Table 1.

In similar fashion, the shotgun proteomics data was further analyzed in Perseus after uploading the proteinGroups table from MaxQuant. Reverse sequences, potential contaminants and proteins only identified by site were removed. LFQ intensities were log2(x) transformed and the data was normalized by subtraction of the median intensity in each sample. Replicates were grouped and proteins with less than three valid values in at least one replicate group were discarded. Missing data values were imputed from a normal distribution around the detection limit. To reveal proteins that were significantly regulated, samples were grouped in the same way as described above, and a two-way ANOVA was performed to compare the LFQ intensities of the proteins in the treatment group with the genotype group. Proteins with a p-value less than 0,01 for at least one of the three parameters were considered to be significantly regulated. The LFQ intensities of these proteins are further shown in a heatmap in Supplementary Figure S7 after non-supervised hierarchical clustering. The significantly regulated proteins are reported in Supplementary Table 2. Since only two major clusters were observed in the heatmap, a t-test was performed (FDR = 0.05 and S0 = 1) to compare

protein intensities between all wild-type and *ISG15*$^{-/-}$ samples. Quantified proteins (n = 2,438) and the results of the t-test are listed in Supplementary Table 3 and shown in the volcano plot in Supplementary Figure S7.

## Statistical analysis

Results are shown as mean ± SEM or mean ± SD as indicated, of experiments performed in 3 or 4 independent biological replicates. Statistical differences between groups were determined by the two-tailed Mann-Whitney *U* test, with a confidence limit for significance set at 0.05 or less.

## Software and Code

FACS data was acquired on Attune NxT Flow Cytometer and BD LSRFortessa, LC-MS/MS data was acquired on an Ultimate 3000 RSLCnano system in-line connected to a Q Exactive HF mass spectrometer (Thermo Scientific). All FACS data were analysed using FlowJo 10. MS data were analyzed in MaxQuant (version 1.6.17.0) using the Andromeda search engine. GG-peptidomics data analysis was continued in Perseus (version 1.6.2.1). Ingenuity Pathway Analyses (version March 2020), Panther (version 16.0) and DAVID (version 6.8) were used for performing gene ontology analyses and GraphPad Prism (version 8) for statistical analyses.

## Data availability statement

Protein sequences used in this study were extracted from spectra were searched against the human protein sequences in the Swiss-Prot database (database release version of January 2021), containing 20,621 sequences (www.uniprot.org) (Human; including isoforms and unreviewed sequences). Mass spectrometry data has been deposited to the ProteomeXchange Consortium through the PRIDE repository under the identifier PXD026748. All accession codes of RNAi experiments have been provided in the supplementary information. Source files for immunoblots presented in the manuscript have been provided. Data generated or analysed during the current study are available through Figshare (https://figshare.com/s/e20b8cca1e02219f16b9).

## Reference

1. Kespohl, M. *et al.* Protein modification with ISG15 blocks coxsackievirus pathology by antiviral and metabolic reprogramming. *Science advances* **6**, eaay1109–eaay1109 (2020).