

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

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

3

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23 **Running title: ISGylation regulates macrophage responses**

24

25

26 **Abstract**

27 Interferon stimulated gene 15 (ISG15) is a ubiquitin like modifier frequently induced
28 during virus infections and involved in versatile host defense mechanisms. Not
29 surprisingly, many viruses including SARS-CoV-2 have evolved de-ISGylating
30 activities to antagonize its effect. In this study we compared ISG15-driven macrophage
31 responses upon infection by influenza, Zika and SARS-CoV-2 viruses. ISG15 and its
32 modifying enzymes were upregulated in human macrophages after infection with all
33 three viruses. While influenza and Zika viruses induced cellular ISGylation, SARS-
34 CoV-2 triggered hydrolysis of ISG15 modifications instead, to generate free,
35 extracellular ISG15 from macrophages and dendritic cells, but not from bronchial
36 epithelial cells. Extracellular ISG15 was released independent of the conventional
37 secretory pathway or cell death, but instead, depended on a non-classical autophagy-
38 related secretory process. Increase of extracellular ISG15 was also reflected in serum
39 samples from COVID-19 patients. The high ratio of free versus conjugated ISG15 in
40 SARS-CoV-2 infected cells triggered macrophage polarization towards a M1
41 phenotype, increased secretion of pro-inflammatory cytokines, e.g. MCP-1 (CCL2), IL-
42 1β , $TNF\alpha$ and IL-6, and attenuated antigen presentation. Depleting ISG15 conjugating
43 enzymes Ube1L and HERC5 further increased free ISG15 and exacerbated this effect.
44 We could recapitulate this phenomenon by expressing the wild-type but not the
45 catalytically inactive PLpro de-ISGylating enzyme of SARS-CoV-2. Proteomic
46 analyses of the secretome from SARS-CoV-2 infected macrophages revealed that
47 besides ISG15, it displayed significant enrichment in non-classical secretory proteins
48 and inflammatory responses, which was further amplified by free ISG15. Collectively,
49 our results indicate that increased proportions of free ISG15 dramatically alter
50 macrophage responses and is likely a key feature of cytokine storms triggered by
51 highly pathogenic respiratory viruses such as influenza and SARS-CoV-2.

52

53 Introduction

54 Viruses that cause acute infections of the human respiratory tract such as influenza
55 and coronaviruses are responsible for high rates of morbidity and mortality. The
56 seasonal flu is estimated to affect 3-5 million cases worldwide. The ongoing pandemic
57 of SARS-CoV-2 has already affected >20 million people. Interferons (IFN) are the first
58 line of defense against several pathogens ¹. They are produced and secreted by the
59 host to boost innate immune responses. Many viruses activate type-I IFN (IFN α/β)
60 signaling, which results in upregulation of a large group of interferon-stimulated genes
61 (ISGs) that exert broad antiviral activities and contribute to lung inflammation ^{2,3}.
62 Although hosts deficient in type-I IFN are more susceptible to virus infections ⁴, an
63 excess of type-I IFN or aberrant cytokine response may lead to extensive lung damage
64 and host death, as is commonly observed in highly pathogenic cases of influenza ⁵ and
65 coronaviruses ^{6,7}. Mice lacking IFN α receptors (IFNAR) have a higher survival rate to
66 influenza infection than wild-type animals ⁸, again pointing to a dysregulation of IFN
67 signaling in the pathogenesis of severe cases.

68 Macrophages are cells of the innate immune system that play a critical role in
69 modulating disease severity during virus infections. They can be infected by influenza
70 and coronaviruses, and are the major producers of pro-inflammatory cytokines, such
71 as TNF α , IFN β , IP-10 (CXCL10), MCP-1 (CCL2), which have an impact on the
72 pathogenesis and clinical outcomes in the host ⁹⁻¹². Regulation of cytokines in
73 macrophages is essential. It has been proposed that overproduction of cytokines,
74 commonly defined as “cytokine storms”, aggravates lung damage with uncontrolled
75 extravasation of immune cells, including macrophages or monocytes into infection
76 sites ^{6,13}, although the exact sequence of events are not yet completely understood.

77 Interferon stimulated gene 15 (ISG15) is a ubiquitin-like modifier that is part of the
78 first line of defense against pathogens, with broad-spectrum antiviral activity. Post
79 translational modifications by ubiquitin and ISG15 are frequently targeted by viruses to
80 perturb host immune responses ¹⁴. ISG15 and its modifying enzymes are highly up-
81 regulated by type-I IFN ¹⁵. ISG15 can be conjugated (ISGylated) to other proteins in a
82 process termed ISGylation or be secreted in its free form. Among the hundreds of
83 modifiable substrates, many have immune-related functions ¹⁶⁻¹⁸, and ISG15 (free or
84 conjugated) has been shown to protect the host against infections ¹⁹.

85 The function of ISG15 in virus infection has so far been investigated in influenza
86 virus infected epithelial cells. ISG15 modified influenza NS1 protein inhibited virus

87 replication whereas modification of Tsg101, a member of the ESCRT complex inhibited
88 transport of viral proteins^{18,20-22}. Its impact in cells of the innate immune system such
89 as macrophages is not known, with only one report suggesting that ISG15 in mice
90 peritoneal macrophages is essential for influenza virus-triggered phagocytosis²³. The
91 fate of ISGylation in Zika or coronavirus-infected macrophages has not been reported
92 so far.

93 In this study, we show that ISG15 and its modifying enzymes are upregulated in
94 influenza, Zika and SARS-CoV-2 infected human macrophages. While influenza and
95 Zika viruses triggered both cellular ISGylation and release of modest levels of free
96 ISG15, infection by SARS-CoV-2 was able to reduce intracellular ISG15 conjugates
97 while dramatically increasing secretion of free ISG15, a phenomenon reflected in
98 serum samples from patients. The increased ratio of free to conjugated ISG15 in
99 SARS-CoV-2 infected cells was accompanied by aberrant macrophage immune
100 responses, which included polarization towards the M1 phenotype, secretion of pro-
101 inflammatory cytokines, and downregulation of IFN-production and antigen
102 presentation. Depletion of Ube1L or HERC5 that specifically prevented ISGylation and
103 increased free ISG15, exacerbated the hyper-induction of pro-inflammatory cytokines
104 such as MCP-1 (CCL2), IL-6, and IL-1 β , without affecting virus replication. This effect
105 was recapitulated by expressing the wild-type but not the catalytically inactive SARS-
106 CoV-2 PLpro de-ISGylating enzyme alone, indicating that it is necessary and sufficient
107 to dysregulate macrophage responses. We verified this phenomenon in a quantitative
108 analysis of the secretome which revealed enrichment of non-classical secretory
109 components and cytokine responses in SARS-CoV-2 infected macrophages.
110 Collectively our data underscore the critical impact of altered free versus conjugated
111 ISG15 on macrophage function, underpinning the onset of lymphopenia and cytokine
112 storms during infections by highly pathogenic respiratory viruses.

113 **Results**

114

115 **Virus infection induces bulk ISGylation in human macrophages**

116 ISG15 is produced during virus infections downstream of Type-I IFNs^{24,25}.
117 Upregulation of ISG15 and modifying enzymes, including the E1 activating enzyme
118 Ube1L, E2 conjugation enzyme UbCh8, E3 ligase HERC5, and de-ISGylase USP18,
119 have been reported, albeit only in virus infected epithelial cells^{20,26}. How these genes

120 are regulated in virus-infected human macrophages, is not well-understood. To
121 determine whether ISG15 and its modifying enzymes are expressed and upregulated
122 in virus infected macrophages, we performed RT-qPCR to quantify the mRNA levels
123 of ISG15, Ube1L (E1), UbcH8 (E2), HERC5 (E3), and USP18. We treated
124 macrophages with IFN β as positive control, where ISG15 and the conjugating
125 enzymes were all induced as anticipated (**Fig 1a-i**). In those infected by human
126 influenza A (H1N1) or avian influenza A (H9N2) virus, expression of all mRNAs, with
127 the exception of Ube1L, was upregulated (**Fig 1a-e**). In those infected by either Zika
128 or SARS-CoV-2, both (+)RNA viruses, the fold-induction was comparable to that in
129 influenza infected cells (**Fig 1f-i**). Intracellular ISG15 protein, measured by ELISA, was
130 equivalently upregulated after infection by influenza and Zika viruses, and more
131 significantly with SARS-CoV-2 (**Fig 1j, k**). These results indicate that ISG15 and
132 modifying enzymes are expressed in macrophages and markedly induced following
133 virus infection.

134 ISG15 is a ubiquitin-like (Ubl) modifier that is known to exert its antiviral function
135 via post-translational modification of substrates¹⁸. In influenza-infected macrophages,
136 bulk ISGylation in cell lysates was detected for both avian (H9N2/Y280), mammalian-
137 adapted (H9N2/Y280-PB2-627K) or pandemic (H1N1/CA04) influenza strains (**Fig 1l**).
138 Similarly, cellular ISGylation was significantly induced in Zika-infected macrophages
139 (**Fig 1l**). Interestingly, although the conjugating enzymes were induced upon SARS-
140 CoV-2 infection, cellular ISGylation remained significantly low (**Fig 1l**), suggesting that
141 SARS-CoV-2 is able to trigger de-ISGylation of cellular substrates.

142 Although type-I IFN signaling is a well-known pathway that induces ISG15 and
143 ISGylating enzymes¹⁸, type-I IFN independent mechanisms have also been proposed
144^{27,28}. For example, DNA damage by UV or genotoxic drugs could induce p53-mediated
145 expression of ISG15 and its modifying enzymes²⁸. To investigate whether virus
146 infection-induced ISG15 expression was dependent on type-I IFN signaling, we
147 infected macrophages in the presence of neutralizing antibodies directed against type-
148 I IFN receptor (anti-IFNAR2) or isotypic control. ISG15 expression was determined by
149 RT-qPCR (**Fig S1a**) and Western blotting (**Fig S1b**). In the presence of anti-IFNAR,
150 ISG15 mRNA expression as well as free and conjugated ISG15 was diminished
151 compared to isotypic control, implying that this was primarily dependent on type-I IFN
152 signaling in macrophages.

153 **Free ISG15 is secreted from virus-infected macrophages via unconventional**
154 **mechanisms**

155 Apart from its function as a Ubl protein modifier, ISG15 is also known to function as a
156 free, non-conjugated protein ²⁹, which can be secreted into the extracellular space ³⁰.
157 Mice harboring a deletion of Ube1L (E1) (which abolishes ISGylation) survived better
158 than ISG15^{-/-} (which abolishes both free and conjugated ISG15) animals, suggesting
159 that both free and conjugated forms of ISG15 play protective roles in virus infections
160 ¹⁹. We were able to detect extracellular ISG15 after infection by each of the viruses,
161 but not from cells stimulated by type-I IFN (**Fig 2a**). Highest amounts of secreted ISG15
162 was detected from SARS-CoV-2 infected cells (**Fig 2b**). In contrast, infection with UV-
163 inactivated Zika or SARS-CoV-2 did not trigger secretion of ISG15 at all, indicating that
164 live virus is necessary for this process (**Fig 2c**).

165 The process of ISG15 secretion is not well characterized and has been proposed
166 to occur from granulocytes via unconventional mechanisms ^{31,32}. To assess its mode
167 of secretion from virus-infected macrophages, we used Brefeldin A treatment to block
168 the conventional secretory pathway (**Fig 2d, e**). Brefeldin A treatment did not affect
169 secretion (**Fig 2d**), nor did it appreciably alter intracellular levels of ISG15 (**Fig 2e**),
170 indicating that it is likely secreted via unconventional mechanisms. We also treated
171 cells with caspase inhibitors to block cell death (**Fig 2g**). Cell viability of mock and
172 influenza virus-infected macrophages was virtually identical (**Fig 2f**), and inhibition of
173 caspases with Z-YVAD-FMK and Z-DEVD-FMK did not have any significant effect on
174 ISG15 secretion (**Fig 2g**), indicating that it is not dependent on the activation of
175 apoptotic or necrotic pathways. Instead, depleting components of secretory
176 autophagosomes/lysosomes ³³ abolished ISG15 secretion (**Fig 2h, i, S2a-b**).
177 Collectively, these data indicate that virus-infections can specifically trigger secretion
178 of free ISG15 via autophagy-dependent unconventional mechanisms, with the highest
179 amounts detected from SARS-CoV-2 infected macrophages.

180 **Free ISG15 is specifically secreted by monocytic cells during virus infections**

181 Epithelial cells, macrophages and dendritic cells (DCs) in the respiratory tract are all
182 essential infection targets of influenza and coronaviruses ^{34,35}. On the other hand, Zika
183 displays broad tissue tropism including monocytes, monocyte-derived macrophages
184 and dendritic cells as well as neural cells and hepatocytes ³⁶. The tissue tropism of

185 SARS-CoV-2 is less well understood; however, both lung epithelial cells and
186 macrophages get infected, although replication in macrophages appears to be limited
187 ³⁷. We detected a comparable extent of influenza virus replication and induction of
188 ISG15 mRNA expression in Normal Human Bronchial Epithelial (NHBE), DCs, and
189 macrophages (**Fig 3a, b**). Secretion of ISG15 was however detectable only from DCs
190 and macrophages, with the highest amounts from the latter (**Fig 3c**). These
191 observations were not limited to the avian influenza virus strain used. Human influenza
192 virus (H1N1/WSN), could also replicate in all three cell types and enhance ISG15 gene
193 expression, but triggered ISG15 secretion only from macrophages and dendritic cells
194 (**Fig 3a-c**). This phenomenon was also recapitulated in Zika and SARS-CoV-2 infected
195 iPSC-derived macrophages and DCs. Despite comparable replication among the
196 different cell types, secretion of ISG15 occurred from macrophages and DCs infected
197 with either SARS-CoV-2 or with Zika virus (**Fig 3d, e**), with the highest amounts
198 released from SARS-CoV-2 infected macrophages (**Fig 3f**). These results indicate that
199 respiratory viruses such as influenza and coronaviruses both of which have the
200 potential to cause “cytokine storms” trigger secretion of ISG15 from immune cells to a
201 significantly greater extent compared to Zika virus, which is well-adapted to replicate
202 in macrophages and therefore likely able to circumvent macrophage-mediated immune
203 responses.

204 **SARS-CoV-2 infection triggers aberrant immune responses in macrophages**

205 The dramatically reduced ISGylation (**Fig 1**) and increase in secreted ISG15 (**Fig 2**)
206 from SARS-CoV-2 infected macrophages prompted us to investigate the general
207 macrophage immune responses in SARS-CoV-2 infection. Clinical samples from
208 Covid-19 patients have already indicated aberrant early immune responses in SARS-
209 CoV-2 infection, often accompanied by lymphopenia and secretion of pro-inflammatory
210 cytokines ^{38,7}. We infected iPSC-derived macrophages with SARS-CoV-2 at MOI 2. At
211 24h post infection, surface presentation of both MHC-I and MHC-II was significantly
212 downregulated specifically in the virus-infected population, but not with UV-inactivated
213 virus control or with dsRNA transfection, a phenomenon that was also evident in Zika-
214 infected cells (**Fig 4a, 4b, S3a, S3b**). To assess other immune responses, we
215 measured macrophage polarization, cytokine secretion profiles and phagocytic activity
216 (**Fig 4c-e**). Polarization was measured in iPSC-derived macrophages infected with
217 SARS-CoV-2 and markers compared with those that were either differentiated into an

218 M1 state using M-CSF, LPS and IFN- γ or an M2-state using M-CSF and IL-4 (**Fig 4c**).
219 SARS-CoV-2 infected macrophages displayed a strong M1-like pro-inflammatory
220 phenotype (**Fig 4c**). We also measured induction of a selected set of cytokines which
221 have been reported to be altered in SARS-CoV-2 infection. Secretion of pro-
222 inflammatory cytokines such as IL-1 β , MCP-1 and IL-6 was significantly upregulated
223 in SARS-CoV-2 infection. On the other hand, that of IFN-I and IFN-II was significantly
224 downregulated, recapitulating the clinical pathology in COVID-19 patients (**Fig 4d**).
225 Similarly, phagocytic activity of SARS-CoV-2 infected macrophages resembled
226 reduced activity observed in M1- but not M2-macrophages (**Fig 4e**). Collectively, these
227 data indicate that infection by SARS-CoV-2 results in aberrant macrophage responses,
228 downregulating antigen presentation and triggering secretion of inflammatory
229 cytokines, which might underpin the consistent symptoms of lymphopenia and cytokine
230 storm observed in Covid-19 patients.

231 **Increasing ratio of free versus conjugated ISG15 drives pro-inflammatory** 232 **cytokine responses from virus infected macrophages**

233 To decouple the role of ISGylation from free ISG15 in virus-infected macrophages, we
234 systematically knocked-down ISG15, Ube1L, HERC5 and USP18 by transfecting
235 macrophages with DsiRNAs 72 hours prior to infecting with either H9N2/Y280, Zika or
236 SARS-CoV-2 virus as specified. Knockdown efficiency of ISG15 and its modifying
237 enzymes was verified in IFN-I treated cells by immunoblotting. The results confirmed
238 that all DsiRNA targets were significantly depleted in comparison to control cells (**Fig**
239 **5a, S4a-c**). In ISG15 knockdown cells, as predicted, both free and ISGylated forms
240 were downregulated, whereas in Ube1L and HERC5 knockdown cells, only the
241 conjugated forms were downregulated (**Fig 5b**). USP18 is the cellular de-ISGylating
242 enzyme and a negative regulator of type-I IFN response; as anticipated, we observed
243 an upregulation of ISGylated material in USP18-depleted macrophages (**Fig 5b**).
244 Depletion of either ISG15, ISGylating enzymes, or USP18 did not have any significant
245 effect on replication of influenza, Zika or SARS-CoV-2 in macrophages (**Fig 5c**). In
246 contrast, depletion of ISGylation alone, but not ISG15, stimulated secretion of pro-
247 inflammatory cytokines, particularly MCP-1, IL-6 and IL-1 β from virus-infected cells in
248 general (**Fig 5d-f**), but most significantly from SARS-CoV-2 infected cells (**Fig 5f**).
249 Interestingly, production of IFN-I and II from Zika and SARS-CoV-2 infected
250 macrophages displayed the reverse effect. Cells depleted in ISG15 conjugating

251 enzymes did not have any significant effect on IFN-I or II production, whereas USP18-
252 depleted cells displayed a modest increase (**Fig 5f**). Depletion of ISG15 and
253 Ube1L/HERC5 but not USP18 also resulted in reduced phagocytic activity in virus-
254 infected macrophages, indicating that ISGylation is important for this effector function
255 of macrophages as reported previously (**Fig S5a, b**). Collectively, these data indicate
256 that skewing the ratio towards a higher proportion of free ISG15 to its conjugated form
257 drives hyperproduction of pro-inflammatory cytokines often detected in severe
258 respiratory infections.

259 **SARS-CoV-2 PLpro de-ISGylase can recapitulate aberrant macrophage** 260 **phenotype of SARS-CoV-2 infection**

261 A number of viruses including coronaviruses encode deubiquitylases and de-
262 ISGylases in their genome. To evaluate whether expressing the viral de-ISGylase itself
263 was sufficient to induce aberrant macrophage responses, we expressed the wild-type
264 and catalytically inactive SARS-CoV-2 PLpro in iPSC-derived macrophages (**Fig 6a,**
265 **b**). We measured cellular ISGylation upon IFN-I treatment in cells expressing either
266 the empty control vector or those expressing either the wild-type or the mutant variants
267 of PLpro. Dose-dependent expression of the wild-type, but not the mutant PLpro
268 resulted in hydrolysis of bulk cellular ISGylation and a concomitant increase in free
269 ISG15 in IFN-I treated cells, indicating that it is indeed an active de-ISGylase (**Fig 6c**).
270 To investigate whether the PLpro enzyme was sufficient to alter macrophage
271 responses, we measured surface expression of MHC-I and secretion of the panel of
272 cytokines described in Fig 5 in cells transfected with dsRNA. Expression of the wild-
273 type but not the mutant PLpro was able to recapitulate downregulation of MHC-I
274 presentation (**Fig 6d, e**). Expression of the wild type and catalytically mutant variants
275 of USP18 - the cellular de-ISGylase – confirmed the ISGylation-dependent
276 downregulation of MHC-I (**Fig 6f, g**). PLpro-expressing cells displayed increased
277 secretion of pro-inflammatory cytokines such as MCP-1, IL-6, TNF α and IL-1 β along
278 with free ISG15, and attenuated secretion of IFN-I and II (**Fig 6h, i**). To validate our
279 findings in clinical settings we collected serum samples from patients, which also
280 displayed increased amounts of free ISG15 at their first week of disease onset (**Fig**
281 **6j**). Collectively, the results suggest that the de-ISGylating activity encoded by SARS-
282 CoV-2 can disrupt early immune responses in macrophages which likely underpin the
283 severe lymphopenia and cytokine storm that often accompanies severe COVID-19.

284 **Quantitative analyses of ISG15-dependent responses in SARS-CoV-2 infected**
285 **macrophages**

286 Aberrant cytokine responses from PLpro expressing cells prompted us to
287 systematically analyse the ISG15-dependent secretome from SARS-CoV-2 infected
288 macrophages and compare that with IFN γ treated macrophages as shown in
289 schematic (**Fig 7a**). We performed a quantitative mass spectrometry based proteomic
290 analysis of the extracellular protein profile (secretome) of SARS-CoV-2 infected and
291 IFN γ treated macrophages using established strategies of label free quantitation. We
292 selected 24 h post SARS-CoV-2 infection or IFN γ treatment as the timepoint for
293 analyses in macrophages expressing non-targeting (NT) DsiRNA or those targeting
294 ISG15 or Ube1L. We defined the secretome as proteins released via all mechanisms,
295 including classical, non-classical and exosomal pathways. Using LC-MS/MS mass
296 spectrometry and MaxQuant proteomics software package for computational analyses
297 we detected relative protein abundances in the conditioned media of control, SARS-
298 CoV-2 infected or IFN γ treated macrophages. For increased confidence in the protein
299 identification numbers we required that a protein be identified on the basis of at least
300 two unique peptides and quantified in a minimum of two replicates.

301 We identified 489, 428 and 502 protein in IFN γ treated NT, ISG15-deficient and
302 Ube1L-deficient macrophages, whereas 508, 485 and 544 proteins in SARS-CoV-2
303 infected macrophages (**Fig 7b**). Principal component analysis of the secretome
304 response in the NT, ISG15-deficient and Ube1L-deficient cells showed a clear
305 separation of the ISG15-deficient cells from NT and Ube1L-deficient cells, which
306 clustered together (**Fig 7c**). For the secretome data sets, the first three principal
307 components captured 89% (PC1: 51%, PC2: 25%, PC3: 13%) variability in the data.
308 Pairwise comparison showed the highest overlap between NT and Ube1L-deficient
309 SARS-CoV-2 infected macrophages. We evaluated the responses of the common
310 proteins identified in all the conditions. Hierarchical clustering analyses of these
311 common proteins revealed that the secretome of NT and Ube1L-deficient clustered
312 together while that of ISG15-deficient cells was significantly different from the others
313 (**Fig 7d**). Functional enrichment analyses revealed that the most prominent enrichment
314 in the secretome of SARS-CoV-2 infected cells were of the inflammatory responses,
315 cytokine secretion, non-classical secretory processes and exosomes, which strongly
316 correlated specifically with Ube1L-deficient cells that inhibited the conjugated but not
317 the extracellular free form of ISG15 (**Fig 7e**). These data therefore provide a systematic

318 overview of the core macrophage processes regulated by cellular ISG15 in response
319 to SARS-CoV-2 infection.

320 **Discussion**

321 In-vitro and in-vivo studies have established that macrophages are one of the major
322 determinants of pathogenesis during respiratory virus infections ³⁹⁻⁴², driven by the
323 production of interferons and interferon stimulated genes. However, the role of ISG15
324 in its free or conjugated form, in macrophage-mediated immune responses is not well
325 studied. Here, we compared the ISG15-dependent responses of human macrophages
326 to influenza, Zika and SARS-CoV-2 virus infections. All these viruses transcriptionally
327 upregulated ISG15 and ISGylating enzymes, which was accompanied by increased
328 bulk protein ISGylation in influenza and Zika, but not in SARS-CoV-2 infected cells.
329 This is particularly intriguing since SARS-CoV-2 encodes for a papain-like protease
330 (PLpro), which is a putative de-ISGylase. SARS-CoV-2 infected cells displayed a
331 skewed ratio of free versus conjugated ISG15, accompanied by heightened secretion
332 of pro-inflammatory cytokines despite reduced IFN production and antigen
333 presentation. This phenomenon was exacerbated by depleting ISG15 conjugating
334 enzymes Ube1L or HERC5, which prevented substrate ISGylation but not free ISG15.
335 These data suggest that ISG15 regulates macrophage inflammatory responses either
336 via protein ISGylation to inhibit secretory processes or via free ISG15 signaling that
337 may induce cytokine production, or a combination of the two.

338 Secretion of ISG15 was specific to virus-infected antigen presenting cells, but not
339 epithelial cells, consistent with its role in immune modulation. Extracellular free ISG15
340 can act as an adjuvant for CD8⁺ cytotoxic T-cells ⁴³ and can also influence infiltration
341 or activation of immune cells such as neutrophils ⁴⁴⁻⁴⁶. Early recruitment of NK and
342 cytotoxic CD8⁺ T-cells is vital to the host to control virus infection as well as lung
343 inflammation ⁴⁷⁻⁴⁹. Other reported immunomodulatory activities of extracellular ISG15
344 include anti-tumor activities of dendritic cells (DCs) ⁵⁰, and triggering Type-II IFN
345 response in NK and T-cells essential for immunity against mycobacteria ⁵¹.

346 Our results demonstrate that the combined effect of substrate-modified and free
347 form of ISG15 critically affect the global immune response of macrophages during virus
348 infections, unlike the respiratory tract epithelial cells where ISG15 primarily functions
349 as an antiviral factor to limit viral replication ²⁰⁻²². Depletion of Ube1L or HERC5 that
350 specifically prevented the conjugated, but not free ISG15, stimulated secretion of pro-

351 inflammatory cytokines from virus infected macrophages, such as MCP-1, TNF α , IL-6,
352 all of which have been implicated in the cytokine storm caused by highly pathogenic
353 influenza and severe Covid-19. MCP-1 (CCL2) and IL-6 have been consistently found
354 to be a predictor of severe pathogenesis in respiratory virus infections. Uncontrolled
355 MCP-1 secretion has also been implicated in increasing the severity of inflammatory
356 disorders of the lung ⁵² and can regulate infiltration of immune cells, including
357 monocytes, T-cells and NK cells ⁵³. Mice treated with pioglitazone, an agonist of the
358 peroxisome proliferator-activated receptor- γ (PPAR- γ), was shown to reduce morbidity
359 and mortality associated with influenza virus infection, correlated with reduced MCP-1
360 secretion ⁵⁴. Blocking MCP-1 can also reduce immune cell infiltration and ameliorate
361 influenza pathogenesis in a viral replication independent manner ⁵⁵. We previously
362 found that, in an in vivo mouse model, more pathogenic strains of influenza virus tend
363 to induce a higher amount of MCP-1 ⁵⁶. More recently anti-IL-6 therapy has proved to
364 be beneficial for treating severe COVID-19 cases ⁵⁷.

365 Knockdown experiments also demonstrated that ISGylation is required to trigger
366 macrophage effector functions in virus-infected human macrophages. We found that
367 knockdown of ISG15 and Ube1L resulted in downregulation of phagocytic activities in
368 human macrophages, in line with previous studies in mouse peritoneal macrophages
369 ²³.

370 Several viruses encode deubiquitylating and de-ISGylating activities in their
371 genome that can counter host antiviral immunity. SARS-CoV-2 itself encodes a papain
372 like protease which is a putative de-ISGylase ⁵⁸. Our data indicate that infection by
373 SARS-CoV-2 removes ISG15 modifications from cellular substrates, confirming its
374 intrinsic de-ISGylating ability. Reduced substrate ISGylation and increased free ISG15
375 was accompanied by hyperactivation of pro-inflammatory cytokines and reduced
376 antigen presentation, both of which are key features of severe COVID-19. Expression
377 of the wild-type but not catalytically inactive SARS-CoV-2 PLpro alone was able to
378 trigger dramatic cellular de-ISGylation, recapitulating these results. Collectively, these
379 data indicate that SARS-CoV-2 PLpro is able to perturb immune responses in
380 macrophages, which may underpin the loss of CD8+ T-lymphocytes and increased
381 inflammation often seen in severe COVID-19 ⁷.

382 Here we have characterized the role of ISG15 in virus-infected macrophages,
383 which underscore the importance that the conjugated and free form plays in driving
384 immune responses. Further characterization of the separable biochemical functions in
385 animal models are called for to assess the specific core functions of ISG15 in response

386 to infections. Delineating the roles of free and conjugated forms of ISG15 should
387 provide a better understanding of the pro- and antiviral impact of ISG15 in virus
388 pathogenesis and ascertain whether aberrant ISG15-dependent macrophage effector
389 responses underpin the cytokine storm and lymphopenia in COVID-19.

390 **Materials and Methods**

391 **Plasmid Construction**

392 The papain-like protease domain sequence is obtained from the SARS-CoV-2
393 complete genome (NCBI genome databank; NC_045512.2). Protein sequence for
394 PLpro domain (amino acids, 746-1059) of pDONR207 SARS-CoV-2 Nsp3 (Addgene;
395 #141257) was cloned into pCAGGs vector with C-terminal Flag-tag. Catalytic mutant
396 (C117A) was generated by site-mutation PCR and verified with sequencing.

397 **Virus cultures**

398 Influenza virus gene segments were amplified by PCR using Pfu Turbo DNA
399 polymerase (Stratagene Cat#600250) and cloned into pHW2000 vector (a gift from
400 Robert G. Webster, St. Jude Children's Research Hospital). Individual plasmids
401 containing the eight viral genome segments were co-transfected using TransIT-LT1
402 (MIR2300, Mirus Bio) into 293T (ATCC Cat#CRL-3216, RRID:CVCL_0063)/MDCK
403 (ATCC Cat#CCL-34, RRID:CVCL_0422) co-cultures. Recombinant viruses generated
404 from the transfection system were propagated in embryonated eggs and quantified by
405 plaque assay. Zika virus (strain MR766) and SARS-CoV-2 stocks were prepared by
406 determining tissue culture infective dose 50% (TCID₅₀/ml) in Vero E6 cells challenged
407 with 10-fold serial dilutions of infectious supernatants for 90 min at 37°C. Cells were
408 subsequently incubated in DMEM with 2.5% FCS.

409 **Primary cell culture and preparation for infection**

410 Buffy coat packs from healthy donors were kindly provided by the Hong Kong Red
411 Cross Blood Transfusion Service and autologous plasma was collected following
412 centrifugation from the top layer. The study received ethical approval from the
413 Institutional Review Board of the LKS Faculty of Medicine of the University of Hong
414 Kong (Ref no: UW 17-050). The remaining portion was mixed with RPMI1640 medium
415 (GIBCO Cat#23400021), overlaid onto Ficoll-Paque Plus density medium (GE

416 Healthcare Life Sciences Cat#17144003) and centrifuged at 1,000 x g for 20 minutes
417 without braking. Peripheral blood mononuclear cells (PBMCs) were collected from the
418 media-Ficoll interface. Plastic-adherent monocytes were cultured in 5% heat-
419 inactivated autologous plasma in RPMI1640 medium and allowed to differentiate for
420 14 days into macrophages. For dendritic cells differentiation, 50ng/mL GM-CSF
421 (Peprotech Cat#300-03), 10ng/mL IL-4 (Peprotech Cat#200-04), 5% heat-inactivated
422 autologous plasma in RPMI1640 medium were used. Normal human bronchial
423 epithelial cells (NHBE) were cultured in BEGM BulletKit Growth Media (Lonza Cat#CC-
424 3170). The day before influenza virus infection, cells were harvested in trypsin/EDTA
425 and 0.1×10^6 cells were seeded in 24-well cell culture plates (TPP).

426

427 **iPSC-derived macrophages**

428 iPSC-derived CD14⁺ monocytes generated from skin fibroblasts (from ATCC) were
429 resuspended in macrophage differentiation base medium (RPMI 1640; 10% heat
430 inactivated fetal calf serum, 2 mM L-glutamine; 100U/ml penicillin/streptomycin)
431 supplemented with 100 ng/ml M-CSF. Cells were counted and seeded at a density of
432 150,000 precursor cells/well of a 6-well plate. Cells were cultured at 37°C for 6 days to
433 differentiate into mature macrophages. At day 7, cell density was verified to be 2-3
434 times that of initial number of precursors. Mature differentiated macrophages were
435 infected with either Zika or SARS-CoV-2 for downstream functional assays.

436

437 **Collection of plasma samples**

438 Patients with RT-PCR confirmed COVID-19 disease at the Infectious Disease Centre
439 of the Princess Margaret Hospital, Hong Kong, were invited to participate in the study
440 after providing informed consent. The study was approved by the institutional review
441 board of the Hong Kong West Cluster of the Hospital Authority of Hong Kong (approval
442 number: UW20-169). Day 1 of clinical onset was defined as the first day of the
443 appearance of clinical symptoms. Specimens of heparinized blood were collected from
444 the patients, and the plasma were separated by centrifugation. The plasma was then
445 heat inactivated at 56°C for 30 minutes and stored at -80°C until use.

446

447 **Virus infection**

448 Cells were infected with the indicated strains of viruses at 37°C in the corresponding
449 culture medium under serum-free condition for 1 hour. The virus inoculum was then
450 removed, cells were washed with warm PBS, and replenished with medium

451 supplemented with 100U/ml penicillin, 100µg/ml streptomycin (GIBCO
452 Cat#15140122), and 1 µg/L TPCK-treated trypsin (Sigma Cat#T1426) in case of
453 influenza.

454 **Gene silencing by RNA interference**

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

460 **Western blotting**

461 Cells in 24-well plates were lysed on ice for 15 minutes in 100µl PBS pH 7.4, containing
462 1% IGEPAL and complete protease inhibitor cocktail (Roche). Following brief
463 centrifugation (10 minutes at 13,000 x g), cleared lysates were separated by SDS-
464 PAGE (12%) and proteins were transferred to PVDF membranes for Western blotting
465 with anti-ISG15 (Boston Biochem Cat#A-830) and anti-GAPDH (Abcam Cat#ab8245,
466 RRID:AB_2107448) antibodies, followed by horseradish peroxidase (HRP)-
467 conjugated secondary antibody (Jackson ImmunoResearch Cat#115-035-003,
468 RRID:AB_10015289). Protein bands were visualized by ECL™ Prime Western
469 Blotting System (GE Healthcare Life Sciences Cat#RPN2232) using the Image Quant
470 LAS 4000 mini machine (GE Healthcare Life Sciences).

471 **ELISA**

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

481 **Cytokine quantification**

482 Proinflammatory cytokines and chemokines concentrations were measured by a
483 cytometric beads assay kit (Biolegend Cat#740003), as advised by the manufacturer.
484 Briefly, cytokine/chemokine standards or cleared culture supernatants were mixed with
485 capture beads together with biotin-conjugated antibody cocktail for 2 hours.
486 Phycoerythrin (PE)-conjugated streptavidin was then added to the mixture and
487 incubated for 30 minutes. Beads were pelleted by centrifugation at 3000 x g for 5
488 minutes, washed, fixed in 4% formaldehyde for 15 minutes, centrifuged at 3,000 x g
489 for 5 minutes, and resuspended in 250µL 1X wash buffer for flow cytometry (BD
490 LSRFortessa) acquisition. Results were analyzed with FCAP array version 3.0 (BD).
491 Specific capture beads groups were first separated by forward scatter (FSC) and side
492 scatter (SSC) dot-plot graphs and further gated by allophycocyanin (APC) fluorescent
493 channel. PE fluorescence read outs were then curve-fitted to a standard curve to
494 estimate the concentration of analytes.

495 **Phagocytosis assay**

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

506 **Drug treatment**

507 Macrophages (2×10^5) were seeded in 24-well plates in 500µL RPMI1640-
508 supplemented with 5% autologous plasma, 100U/ml penicillin, and 100µg/ml
509 streptomycin (GIBCO Cat#15140122). Twenty-four hours later, media were discarded
510 and replaced with fresh serum-free medium (GIBCO Cat#12065074), which was

511 changed daily for 2 days. On the day of experiment, macrophages were pre-treated or
512 post-treated with the indicated drug concentrations as detailed.

513 **LDH assay**

514 LDH-Cytotoxicity Colorimetric Assay Kit II (BioVision Cat#K313) was used to estimate
515 cell death based on the amount of lactate dehydrogenase (LDH) leakage into the cell
516 culture media. Macrophages were cultured as described above; supernatants were
517 collected, cleared by centrifugation at 1,000 x g for 5 minutes, added to 100 μ L LDH
518 reaction mix in 96-well plates and incubated for 30 minutes at room temperature,
519 followed by absorbance measurement at 450nm. A standard curve was generated
520 from mixing cleared media from detergent treated cells (100% lysis) and untreated
521 cells (0% lysis) at different ratios (8.1, 2.7, 0.9, 0.3, 0.1, 0% lysis) and the obtained
522 results were expressed as percentage of lysis. All measurements were done in
523 duplicates and the average absorbance values from test samples (following
524 subtraction of background signal) were used for calculations.

525

526 **Protein identification and quantification by Tandem Mass spectrometry**

527

528 All LC-MS analyses were performed by a ThermoFisher Orbitrap Lumos instrument
529 that was operated in a data dependent acquisition mode to switch between Orbitrap
530 full scan MS and LTQ MS/MS. Mass spectra were analysed by MaxQuant version
531 1.4.1.2 and the Andromeda search engine. The maximum mass deviation allowed for
532 the monoisotopic precursor ions was 4.5 ppm for monoisotopic precursors and 0.5Da
533 for fragment ions. Trypsin was set as the digestion enzyme with a maximum of two
534 allowed missed cleavages. Cysteine carbamidomethylation was set as a fixed
535 modification, and N-terminal acetylation and methionine oxidation were allowed as
536 variable modifications. The spectra were searched using the Andromeda search
537 engine against the mouse Uniprot sequence database. Protein identification required
538 at least two unique peptides per protein group. The data were filtered for a 1% FDR at
539 the peptide and protein level. We used the Database for Annotation, Visualization
540 and Integrated Discovery to assign Gene Ontology (GO) annotations for cellular
541 component, molecular function and biological process. Principal component analysis
542 (PCA) was performed using the Perseus software version 1.5.0.9 on the proteins
543 common between the cells expressing non-targeting DsiRNA or those targeting ISG15

544 or Ube1L. Hierarchical clustering analyses were performed using Genesis. Protein
545 expression fold change values were log transformed (base 2). Log transformed fold
546 change data showed bell shaped distributions and were symmetric around 0.

547 **Statistical analysis**

548 Results are shown as mean \pm SEM or mean \pm sd as indicated, of experiments
549 performed in at least 3 independent biological replicates. Statistical differences
550 between groups were determined by the Mann-Whitney *U* test, with a confidence limit
551 for significance set at 0.05 or less.

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563 **Author Contributions Statement**

564 HHYL, CKPM, and SS designed and conducted the study. HHYL, QT, DM, JH, ES,
565 CKPM, LYLS, WWSN performed and analyzed experiments. HP, BK, AD, AP-F
566 generated critical reagents for the study. JSMP managed the clinical experiments. SS,
567 HHYL and CKPM wrote the manuscript.

568 **Conflict of Interest Statement**

569 The authors declare that the research was conducted in the absence of any
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571 interest.

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579

580 **Figure legends**

581 **Fig 1. ISG15 and modifying enzymes are induced during virus infections**

582 **a-e.** Macrophages were infected with the indicated influenza virus strains at a MOI 2.
583 Changes in mRNA expression levels of ISG15 (a) and ISG15 modifying enzymes
584 Ube1L UbcH8, HERC5, USP18 (b-e) against mock infection were quantified by qPCR
585 **f-i.** Macrophages were infected with Zika or SARS-CoV-2 at MOI 2. At indicated time
586 intervals changes in mRNA expression levels of ISG15 (f) and ISG15 modifying
587 enzymes Ube1L UbcH8 and HERC5 (g-i) against mock infection were quantified by
588 qPCR **j, k.** Intracellular ISG15 protein levels in influenza infected cells (j) or Zika or
589 SARS-CoV-2 infected macrophages (k) were quantified by ELISA. Data are displayed
590 as means \pm SEM of at least three independent donors. * $p < 0.05$ by the Mann-Whitney
591 U Test vs. mock-infected cells. **l.** ISGylation in virus-infected macrophages was
592 measured for influenza, Zika and SARS-CoV-2. Macrophages were infected with the
593 indicated wild-type and mutated strains of influenza virus (*left panel*), Zika (*middle*
594 *panel*) and SARS-CoV-2 (*right panel*) at a MOI 2. Lysates were collected in 1%
595 IGEPAL PBS pH 7.4, separated by SDS-PAGE and visualized by Western blotting
596 using an anti-human ISG15 antibody. Gapdh levels were measured as loading control.
597 The blot is representative of results obtained from three independent donors.

598

599 **Fig 2. ISG15 is secreted from virus-infected macrophages via non-conventional**
600 **secretory autophagosomal pathway.**

601 **a.** Macrophages were infected with the indicated influenza viral strains at a MOI 2. At
602 indicated time intervals changes in extracellular levels of ISG15 were quantified by
603 ELISA. **b, c.** iPSC-derived macrophages were infected with either Zika or SARS-CoV-
604 2 (b) at a MOI 2 or UV-inactivated Zika or SARS-CoV-2 (c). Changes in extracellular
605 levels of ISG15 were quantified by ELISA. **d, e.** Brefeldin (5 μ M), which inhibits
606 conventional secretion pathway, was added 1 h post-infection and had no effect on
607 ISG15 secretion (d) and protein expression (e) 24 h post infection. **f.** LDH assay was
608 performed using LDH-Cytotoxicity Colorimetric Assay Kit II (BioVision) to estimate cell
609 death based on the amount of LDH leakage into the cell culture media, 24 h post
610 infection following the manufacturer's protocol. **g.** Caspase inhibitors Z-YVAD-FMK
611 and Z-DEVD-FMK were added to virus-infected macrophages (1h post infection), and
612 ISG15 secretion measured 24h post infection. **h, i.** Depletion of proteins implicated in
613 secretory autophagosomes was performed by DsiRNA in iPSC-derived macrophages

614 and verified by immunoblotting (**Fig S2a, b**). Non-targeting and depleted cells were
615 infected with SARS-CoV-2 (MOI 2; 24h) and secretion of ISG15 measured by ELISA.
616 All data are displayed as means \pm SEM of at least three independent donors. * $p < 0.05$
617 by the Mann-Whitney U Test vs mock-infected cells.

618

619 **Fig 3. ISG15 is specifically secreted by monocytic cells during virus infections.**

620 **a.** Normal human bronchial epithelial cells (NHBE), macrophages (Mac) and dendritic
621 cells (DCs) were infected with the indicated wild-type and mutated influenza virus
622 strains at a MOI 2. Total RNA was collected at 24 hours post-infection and reverse
623 transcribed into cDNA using oligo-d(T)23VN primer. Relative changes of expression
624 levels against mock infection were quantified by qPCR using gene specific primers.

625 **b.** Quantification of ISG15 copy number was done by RT-qPCR using ISG15 specific
626 primers. **c.** Cell culture media were collected at 24 hours post-infection. ISG15 protein
627 was quantified by ISG15 sandwich ELISA. All data are displayed as mean \pm SEM of at

628 least three independent donors. * $p < 0.05$ by the Mann-Whitney U Test vs. mock-

629 treated cells **d.** NHBE, iPSC-derived macrophages (Mac) and dendritic cells (DCs)

630 were infected with either Zika or SARS-CoV-2 at a MOI 1. Total RNA was collected at
631 24- and 48-hours post-infection; relative changes of expression levels against mock

632 infection were quantified by qPCR using gene specific primers **e.** Quantification of

633 ISG15 copy number was done by RT-qPCR using ISG15 specific primers **f.**

634 Supernatants from infected cells were collected at 24 and 48 hours post-infection.

635 ISG15 protein was quantified by ISG15 sandwich ELISA. All data are displayed as

636 mean \pm sd of at least three independent experiments. * $p < 0.05$ by the Mann-Whitney

637 U Test vs. mock-treated cells.

638

639 **Fig 4. Immune dysfunction in SARS-CoV-2-infected macrophages**

640 **a.** Surface staining of MHC-I in iPSC-derived macrophages infected with SARS-CoV-

641 2 (MOI 2, 24 h). Controls included were antibody isotype, UV-inactivated virus and

642 dsRNA. Cells were gated on viral N+ (in red; 75% of population) and surface MHC-I.

643 Bystander cells are depicted in black. **b.** Surface staining of MHC-II in iPSC-derived

644 macrophages infected with SARS-CoV-2 (MOI 2, 24 h). Cells were gated on viral N+

645 (in red; 73% of population) and surface MHC-II+ cells. Bystander population is depicted

646 in black. Controls included were antibody isotype, UV-inactivated virus and dsRNA

647 treated cells **c.** iPSC-derived macrophages were stimulated to M1 or M2 by

648 differentiating for 48 hours in the presence of M-CSF+LPS+IFN- γ and M-CSF+IL-4
649 respectively, or infected with SARS-CoV-2 (MOI 2, 48h). Expression of key markers of
650 polarization was measured by RT qPCR. **d.** Secretion of indicated cytokines was
651 measured using cytometric bead arrays following the manufacturer's guidelines and
652 flow cytometry. **e.** Quantification of phagocytosis of M1- or M2-stimulated phagocytes
653 was compared with SARS-CoV-2 infected macrophages (MOI 2, 48 h). Error bars
654 represent mean \pm s.d; [$*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$; Two-way
655 ANOVA with Tukey's multiple comparison test].

656

657 **Fig 5. Role of free versus conjugated ISG15 on viral replication and cytokine**
658 **secretion.**

659 **a.** Macrophages were transfected with ISG15, Ube1L, HERC5 or USP18 DsiRNA for
660 72 hours; depletion was verified in IFN-I treated cells by immunoblotting. **b.** Cellular
661 ISGylation was measured in influenza, Zika and SARS-CoV-2 infected cells (MOI 2),
662 24 hours post infection **c.** Total RNA was collected at indicated time intervals from
663 influenza, Zika and SARS-CoV-2 infected cells, and reverse transcribed into cDNA
664 using uni-12 primer. Quantifications of absolute copy number were done by RT-qPCR
665 using universal vRNA specific primers. For infectivity assay, culture medium was
666 removed at 6 and 24 hours post-infection from influenza-infected cells and TCID₅₀
667 infectivity assays were done in MDCK cells as described in the Materials and Methods
668 section. TCID₅₀/mL value was calculated by the Spearman Kärber method. Data are
669 displayed as means \pm SEM of at least three independent donors. There were no
670 statistical differences by the Mann-Whitney U Test vs. control cells treated with non-
671 targeting (NT) DsiRNA **d.** Macrophages were transfected with either non-targeting
672 (NT), ISG15, Ube1L, or USP18 DsiRNA for 72 hours prior to H9N2/Y280 influenza
673 virus infection at MOI 2. Indicated cytokines were quantified by cytometric beads
674 assay. Data are displayed as means \pm SEM of at least three independent donors. $*p$
675 < 0.05 by the Mann-Whitney U Test vs. control **e-f.** iPSC-derived macrophages
676 transfected with either non-targeting (NT), ISG15, Ube1L, HERC5 or USP18 DsiRNA
677 for 72 hours were infected with either Zika (e) or SARS-CoV-2 (f) at MOI 2 for 24 hours.
678 Indicated cytokines were quantified by cytometric beads assay. Data are displayed as
679 means \pm s.d of at least three independent experiments. $*p < 0.05$ by the Mann-Whitney
680 U Test vs. control (NT cells).

681

682 **Fig 6. Dysregulation of antigen presentation and interferon response in**
683 **macrophages expressing SARS-CoV-2 PLpro**

684 **a, b.** Schematic of SARS-CoV-2 PLpro (wild-type and mutant) and their expression in
685 macrophages verified by immunoblotting **c.** Bulk ISGylation in IFN-I treated
686 macrophages expressing either the empty vector, wild-type PLPro, or mutant PLpro in
687 a dose-dependent manner **d, e.** Surface staining of MHC-I in iPSC-derived
688 macrophages expressing either wt HA-PLpro (d) or the catalytic mutant HA-PLpro
689 (C117A) (e) of SARS-CoV-2. Cells were then treated with dsRNA to induce surface
690 expression of MHC-I. For both samples (wt, mutant) >90% of cells stained positive for
691 HA-(PLpro) and dsRNA **f, g.** Same as (d, e) in cells expressing USP18 (wild-type or
692 C64R/C65R mutant). **h.** Secretion of indicated cytokines was measured using
693 cytometric beads assay following the manufacturer's guidelines and flow cytometry.
694 Error bars represent mean \pm sd from three independent experiments. Data are
695 displayed as means \pm s.d. *p < 0.05 by the Mann-Whitney U Test vs. control (empty
696 vector matched cells). **i.** Supernatants from cells described in (h) were collected and
697 ISG15 was quantified by ISG15 sandwich ELISA. All data are displayed as mean \pm sd
698 of at least three independent experiments. *p < 0.05 by the Mann-Whitney U Test vs.
699 control cells. **j.** Elevation of ISG15 was found in the plasma samples collected from the
700 COVID-19 patients at their first week of disease onset. *p < 0.05 by the Mann-Whitney
701 U Test vs. healthy donors.
702

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

705 **a.** Schematic of the label-free strategy of LC-MS/MS used to study the ISG15-
706 dependent secretome of SARS-CoV-2 infected cells. iPSC-derived macrophages were
707 either transfected with non-targeting DsiRNA or those targeting ISG15 or Ube1L.
708 Conditioned media was collected from control, SARS-CoV-2 infected or IFN γ treated
709 cells. Proteins were extracted from each of the samples, separated by SDS-PAGE and
710 digested with trypsin for LC-MS/MS as described in the Materials and methods **b.** Total
711 numbers of proteins quantified in atleast two biological replicates **c.** Principal
712 component analysis was performed using the Perseus software. Filled squares
713 represent control cells (NT DsiRNA), empty squares ISG15-depleted, filled circles
714 Ube1L-depleted. The uninfected cells are shown in gray and infected cells are shown

715 in red **d**. The heatmap represents the hierarchical clustering of the common proteins
716 in the secretome for IFN γ treated or SARS-CoV-2 infected cells. The color key
717 represents changes (log₂ scale) from dark blue indicating the largest decreases to red
718 indicating the largest increases **e**. Functional annotation of the common proteins
719 identified in all samples was performed by the DAVID software.

720

721 **References**

- 722 1. McNab, F., Mayer-Barber, K., Sher, A., Wack, A. & O'Garra, A. Type I interferons
723 in infectious disease. *Nat. Rev. Immunol.* **15**, 87–103 (2015).
- 724 2. Schneider, W. M., Chevillotte, M. D. & Rice, C. M. Interferon-stimulated genes: a
725 complex web of host defenses. *Annu. Rev. Immunol.* **32**, 513–545 (2014).
- 726 3. Makris, S., Paulsen, M. & Johansson, C. Type I Interferons as Regulators of
727 Lung Inflammation. *Front Immunol* **8**, 259 (2017).
- 728 4. García-Sastre, A. Induction and evasion of type I interferon responses by
729 influenza viruses. *Virus Res.* **162**, 12–18 (2011).
- 730 5. Taubenberger, J. K. & Morens, D. M. The pathology of influenza virus infections.
731 *Annu Rev Pathol* **3**, 499–522 (2008).
- 732 6. Channappanavar, R. & Perlman, S. Pathogenic human coronavirus infections:
733 causes and consequences of cytokine storm and immunopathology. *Semin*
734 *Immunopathol* **39**, 529–539 (2017).
- 735 7. Tay, M. Z., Poh, C. M., Rénia, L., MacAry, P. A. & Ng, L. F. P. The trinity of
736 COVID-19: immunity, inflammation and intervention. *Nature Reviews*
737 *Immunology* **20**, 363–374 (2020).
- 738 8. Davidson, S., Crotta, S., McCabe, T. M. & Wack, A. Pathogenic potential of
739 interferon $\alpha\beta$ in acute influenza infection. *Nat Commun* **5**, 3864 (2014).
- 740 9. Cheung, C. Y. *et al.* Induction of proinflammatory cytokines in human
741 macrophages by influenza A (H5N1) viruses: a mechanism for the unusual
742 severity of human disease? *Lancet* **360**, 1831–1837 (2002).

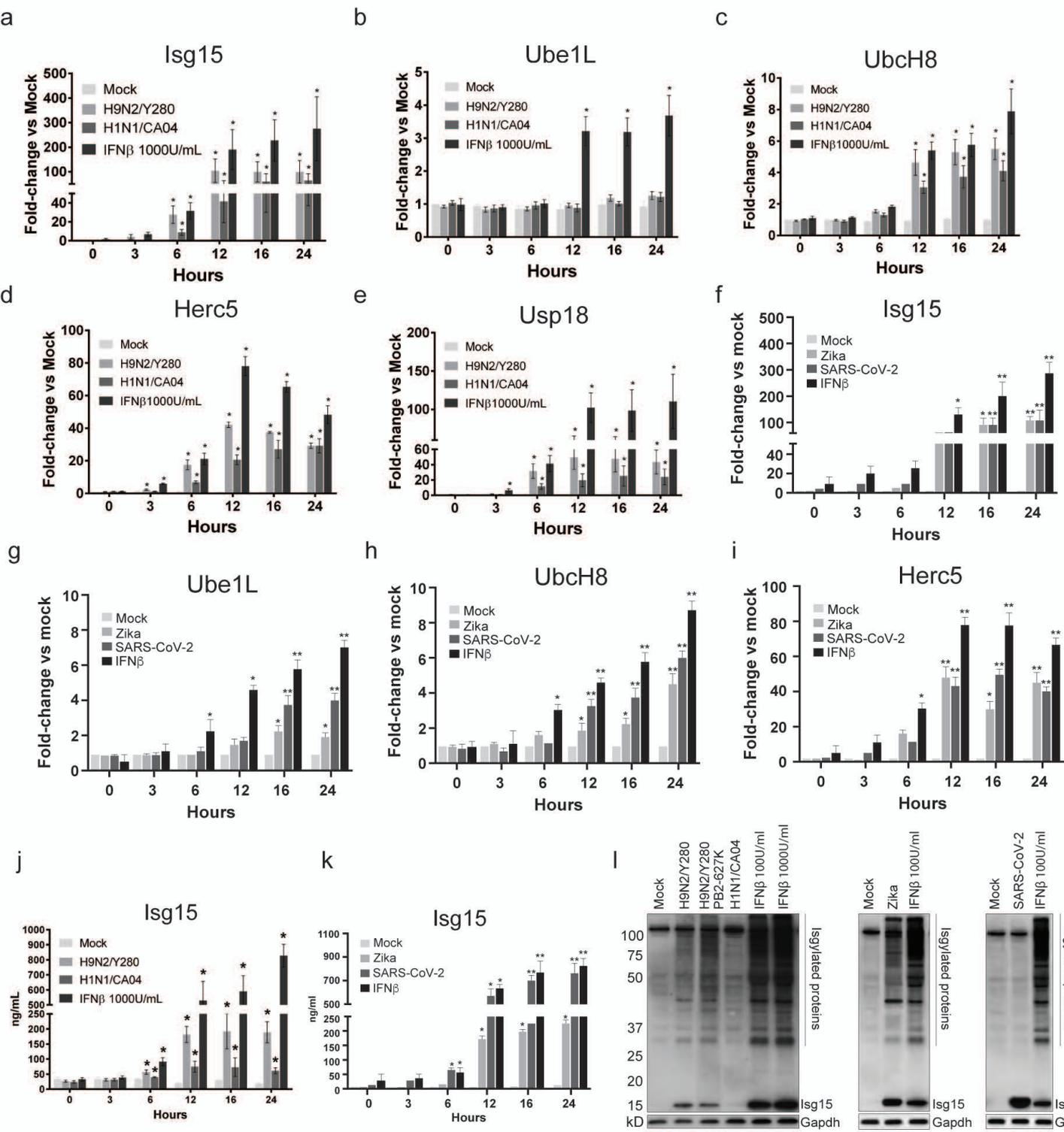
- 743 10. Zhou, J. *et al.* Differential expression of chemokines and their receptors in adult
744 and neonatal macrophages infected with human or avian influenza viruses. *J.*
745 *Infect. Dis.* **194**, 61–70 (2006).
- 746 11. Oslund, K. L. & Baumgarth, N. Influenza-induced innate immunity: regulators of
747 viral replication, respiratory tract pathology & adaptive immunity. *Future Virol* **6**,
748 951–962 (2011).
- 749 12. McGonagle, D., Sharif, K., O'Regan, A. & Bridgewood, C. The Role of Cytokines
750 including Interleukin-6 in COVID-19 induced Pneumonia and Macrophage
751 Activation Syndrome-Like Disease. *Autoimmun Rev* **19**, 102537 (2020).
- 752 13. Short, K. R., Kroeze, E. J. B. V., Fouchier, R. A. M. & Kuiken, T. Pathogenesis of
753 influenza-induced acute respiratory distress syndrome. *Lancet Infect Dis* **14**, 57–
754 69 (2014).
- 755 14. Jahan, A. S. *et al.* OTUB1 Is a Key Regulator of RIG-I-Dependent Immune
756 Signaling and Is Targeted for Proteasomal Degradation by Influenza A NS1. *Cell*
757 *Reports* **30**, 1570-1584.e6 (2020).
- 758 15. Zhang, D. & Zhang, D.-E. Interferon-stimulated gene 15 and the protein
759 ISGylation system. *J. Interferon Cytokine Res.* **31**, 119–130 (2011).
- 760 16. Giannakopoulos, N. V. *et al.* Proteomic identification of proteins conjugated to
761 ISG15 in mouse and human cells. *Biochem. Biophys. Res. Commun.* **336**, 496–
762 506 (2005).
- 763 17. Wong, J. J. Y., Pung, Y. F., Sze, N. S.-K. & Chin, K.-C. HERC5 is an IFN-induced
764 HECT-type E3 protein ligase that mediates type I IFN-induced ISGylation of
765 protein targets. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 10735–10740 (2006).
- 766 18. Sanyal, S. *et al.* Type I interferon imposes a TSG101/ISG15 checkpoint at the
767 Golgi for glycoprotein trafficking during influenza virus infection. *Cell Host*
768 *Microbe* **14**, 510–521 (2013).
- 769 19. Morales, D. J. *et al.* Novel mode of ISG15-mediated protection against influenza
770 A virus and Sendai virus in mice. *J. Virol.* **89**, 337–349 (2015).
- 771 20. Hsiang, T.-Y., Zhao, C. & Krug, R. M. Interferon-induced ISG15 conjugation
772 inhibits influenza A virus gene expression and replication in human cells. *J. Virol.*
773 **83**, 5971–5977 (2009).

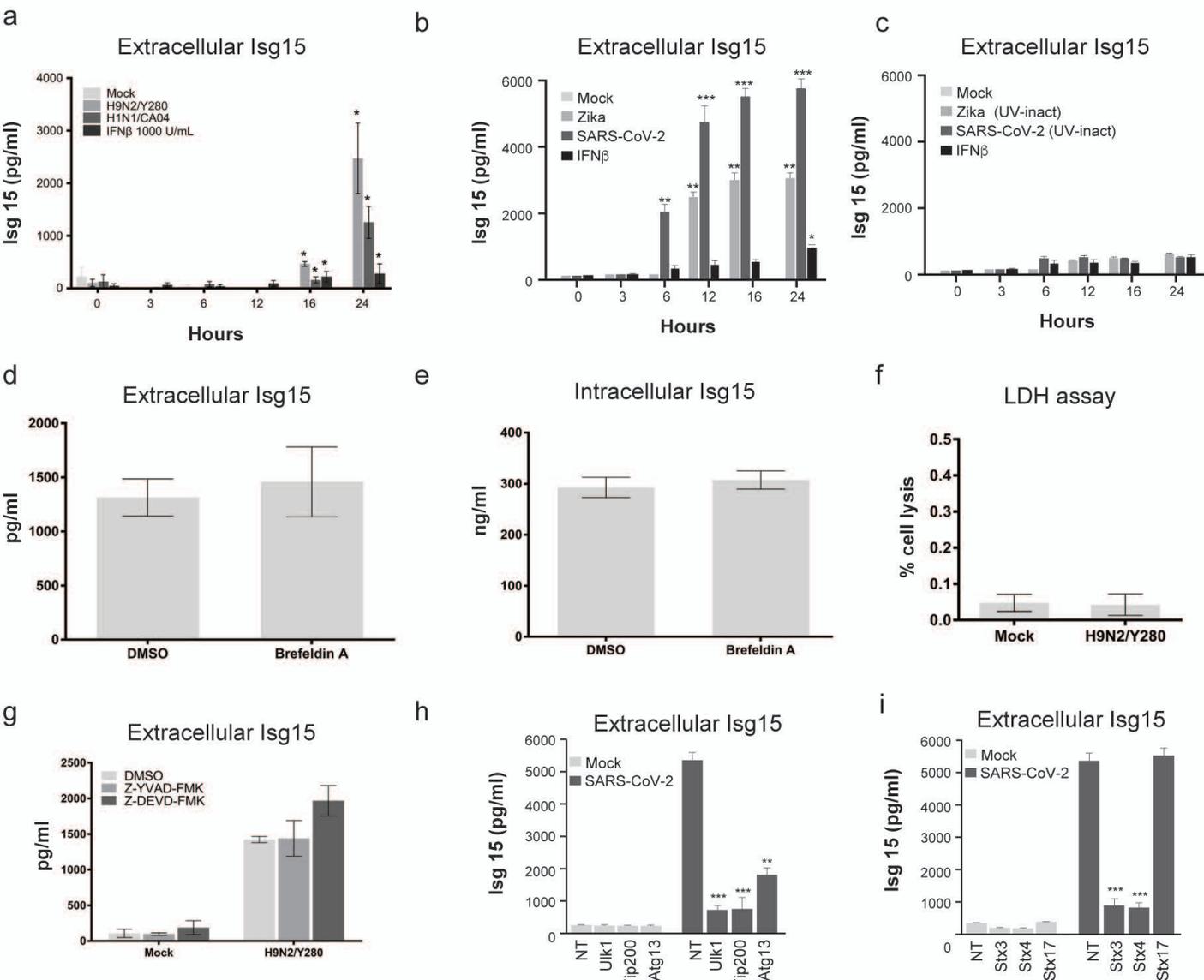
- 774 21. Tang, Y. *et al.* HERC5 attenuates influenza A virus by catalyzing ISGylation of
775 viral NS1 protein. *J. Immunol.* **184**, 5777–5790 (2010).
- 776 22. Zhao, C., Hsiang, T.-Y., Kuo, R.-L. & Krug, R. M. ISG15 conjugation system
777 targets the viral NS1 protein in influenza A virus-infected cells. *Proc. Natl. Acad.*
778 *Sci. U.S.A.* **107**, 2253–2258 (2010).
- 779 23. Yángüez, E. *et al.* ISG15 regulates peritoneal macrophages functionality against
780 viral infection. *PLoS Pathog.* **9**, e1003632 (2013).
- 781 24. Farrell, P. J., Broeze, R. J. & Lengyel, P. Accumulation of an mRNA and protein
782 in interferon-treated Ehrlich ascites tumour cells. *Nature* **279**, 523–525 (1979).
- 783 25. Haas, A. L., Ahrens, P., Bright, P. M. & Ankel, H. Interferon induces a 15-
784 kilodalton protein exhibiting marked homology to ubiquitin. *J. Biol. Chem.* **262**,
785 11315–11323 (1987).
- 786 26. Kroeker, A. L., Ezzati, P., Halayko, A. J. & Coombs, K. M. Response of primary
787 human airway epithelial cells to influenza infection: a quantitative proteomic
788 study. *J. Proteome Res.* **11**, 4132–4146 (2012).
- 789 27. Radoshevich, L. *et al.* ISG15 counteracts *Listeria monocytogenes* infection. *Elife*
790 **4**, (2015).
- 791 28. Park, J. H. *et al.* Positive feedback regulation of p53 transactivity by DNA
792 damage-induced ISG15 modification. *Nat Commun* **7**, 12513 (2016).
- 793 29. Dos Santos, P. F. & Mansur, D. S. Beyond ISGylation: Functions of Free
794 Intracellular and Extracellular ISG15. *J. Interferon Cytokine Res.* **37**, 246–253
795 (2017).
- 796 30. Hermann, M. & Bogunovic, D. ISG15: In Sickness and in Health. *Trends*
797 *Immunol.* **38**, 79–93 (2017).
- 798 31. Swaim, C. D., Scott, A. F., Canadeo, L. A. & Huibregtse, J. M. Extracellular
799 ISG15 Signals Cytokine Secretion through the LFA-1 Integrin Receptor. *Mol. Cell*
800 **68**, 581-590.e5 (2017).
- 801 32. Perng, Y.-C. & Lenschow, D. J. ISG15 in antiviral immunity and beyond. *Nat.*
802 *Rev. Microbiol.* **16**, 423–439 (2018).
- 803 33. Ponpuak, M. *et al.* Secretory autophagy. *Curr. Opin. Cell Biol.* **35**, 106–116
804 (2015).

- 805 34. Matrosovich, M. N., Matrosovich, T. Y., Gray, T., Roberts, N. A. & Klenk, H.-D.
806 Human and avian influenza viruses target different cell types in cultures of
807 human airway epithelium. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 4620–4624 (2004).
- 808 35. Manicassamy, B. *et al.* Analysis of in vivo dynamics of influenza virus infection in
809 mice using a GFP reporter virus. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 11531–11536
810 (2010).
- 811 36. El Costa, H. *et al.* ZIKA virus reveals broad tissue and cell tropism during the first
812 trimester of pregnancy. *Sci Rep* **6**, 35296 (2016).
- 813 37. Hui, K. P. Y. *et al.* Tropism, replication competence, and innate immune
814 responses of the coronavirus SARS-CoV-2 in human respiratory tract and
815 conjunctiva: an analysis in ex-vivo and in-vitro cultures. *Lancet Respir Med*
816 (2020) doi:10.1016/S2213-2600(20)30193-4.
- 817 38. Chen, G. *et al.* Clinical and immunological features of severe and moderate
818 coronavirus disease 2019. *J. Clin. Invest.* **130**, 2620–2629 (2020).
- 819 39. Channappanavar, R. *et al.* Dysregulated Type I Interferon and Inflammatory
820 Monocyte-Macrophage Responses Cause Lethal Pneumonia in SARS-CoV-
821 Infected Mice. *Cell Host & Microbe* **19**, 181–193 (2016).
- 822 40. Tate, M. D., Pickett, D. L., van Rooijen, N., Brooks, A. G. & Reading, P. C.
823 Critical role of airway macrophages in modulating disease severity during
824 influenza virus infection of mice. *J. Virol.* **84**, 7569–7580 (2010).
- 825 41. Yu, W. C. L. *et al.* Viral replication and innate host responses in primary human
826 alveolar epithelial cells and alveolar macrophages infected with influenza H5N1
827 and H1N1 viruses. *J. Virol.* **85**, 6844–6855 (2011).
- 828 42. Wang, J. *et al.* Innate immune response of human alveolar macrophages during
829 influenza A infection. *PLoS ONE* **7**, e29879 (2012).
- 830 43. Villarreal, D. O. *et al.* Ubiquitin-like Molecule ISG15 Acts as an Immune Adjuvant
831 to Enhance Antigen-specific CD8 T-cell Tumor Immunity. *Mol. Ther.* **23**, 1653–
832 1662 (2015).
- 833 44. Fujisawa, H. Inhibitory role of neutrophils on influenza virus multiplication in the
834 lungs of mice. *Microbiol. Immunol.* **45**, 679–688 (2001).

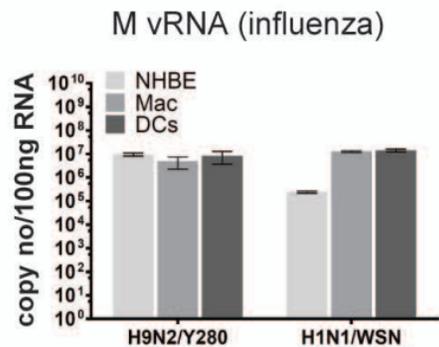
- 835 45. Tate, M. D. *et al.* The role of neutrophils during mild and severe influenza virus
836 infections of mice. *PLoS ONE* **6**, e17618 (2011).
- 837 46. Camp, J. V. & Jonsson, C. B. A Role for Neutrophils in Viral Respiratory Disease.
838 *Front Immunol* **8**, 550 (2017).
- 839 47. Wen, W. *et al.* Immune cell profiling of COVID-19 patients in the recovery stage
840 by single-cell sequencing. *Cell Discov* **6**, 31 (2020).
- 841 48. Liu, B. *et al.* Interleukin-18 improves the early defence system against influenza
842 virus infection by augmenting natural killer cell-mediated cytotoxicity. *J. Gen.*
843 *Virol.* **85**, 423–428 (2004).
- 844 49. Wang, Z. *et al.* Recovery from severe H7N9 disease is associated with diverse
845 response mechanisms dominated by CD8⁺ T cells. *Nat Commun* **6**, 6833 (2015).
- 846 50. Padovan, E. *et al.* Interferon stimulated gene 15 constitutively produced by
847 melanoma cells induces e-cadherin expression on human dendritic cells. *Cancer*
848 *Res.* **62**, 3453–3458 (2002).
- 849 51. Bogunovic, D. *et al.* Mycobacterial disease and impaired IFN- γ immunity in
850 humans with inherited ISG15 deficiency. *Science* **337**, 1684–1688 (2012).
- 851 52. Rose, C. E., Sung, S.-S. J. & Fu, S. M. Significant involvement of CCL2 (MCP-1)
852 in inflammatory disorders of the lung. *Microcirculation* **10**, 273–288 (2003).
- 853 53. Deshmane, S. L., Kremlev, S., Amini, S. & Sawaya, B. E. Monocyte
854 chemoattractant protein-1 (MCP-1): an overview. *J. Interferon Cytokine Res.* **29**,
855 313–326 (2009).
- 856 54. Aldridge, J. R. *et al.* TNF/*i*NOS-producing dendritic cells are the necessary evil of
857 lethal influenza virus infection. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 5306–5311
858 (2009).
- 859 55. Narasaraju, T., Ng, H. H., Phoon, M. C. & Chow, V. T. K. MCP-1 antibody
860 treatment enhances damage and impedes repair of the alveolar epithelium in
861 influenza pneumonitis. *Am. J. Respir. Cell Mol. Biol.* **42**, 732–743 (2010).
- 862 56. Li, W. *et al.* The PB2 mutation with lysine at 627 enhances the pathogenicity of
863 avian influenza (H7N9) virus which belongs to a non-zoonotic lineage. *Sci Rep* **7**,
864 2352 (2017).

- 865 57. Mazzone, A. *et al.* Impaired immune cell cytotoxicity in severe COVID-19 is IL-6
866 dependent. *J. Clin. Invest.* (2020) doi:10.1172/JCI138554.
- 867 58. Swaim, C. D. *et al.* Modulation of Extracellular ISG15 Signaling by Pathogens
868 and Viral Effector Proteins. *Cell Rep* **31**, 107772 (2020).
- 869

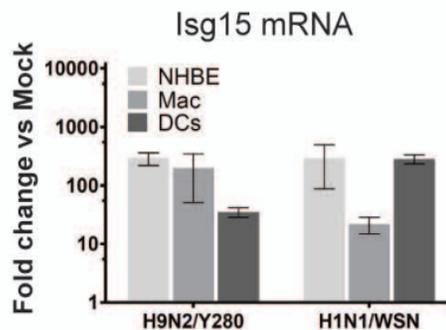




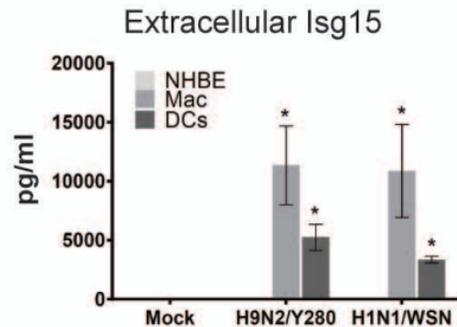
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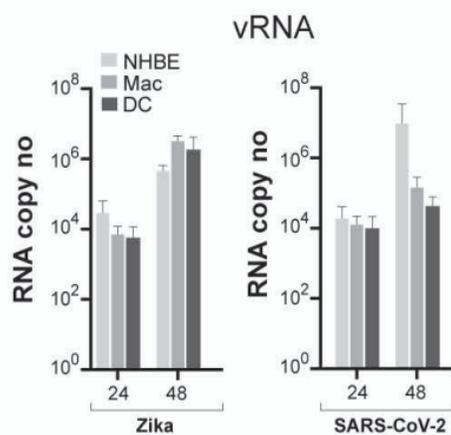
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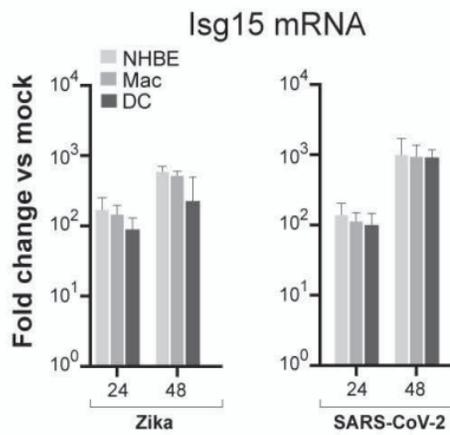
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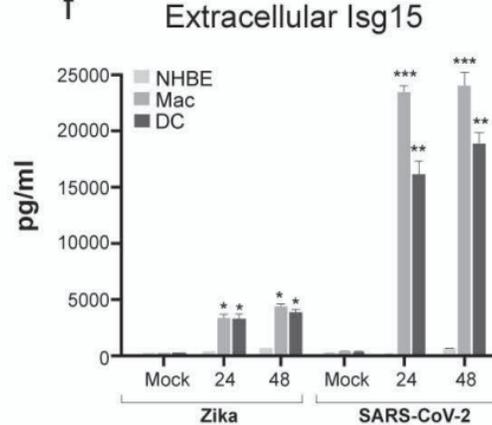
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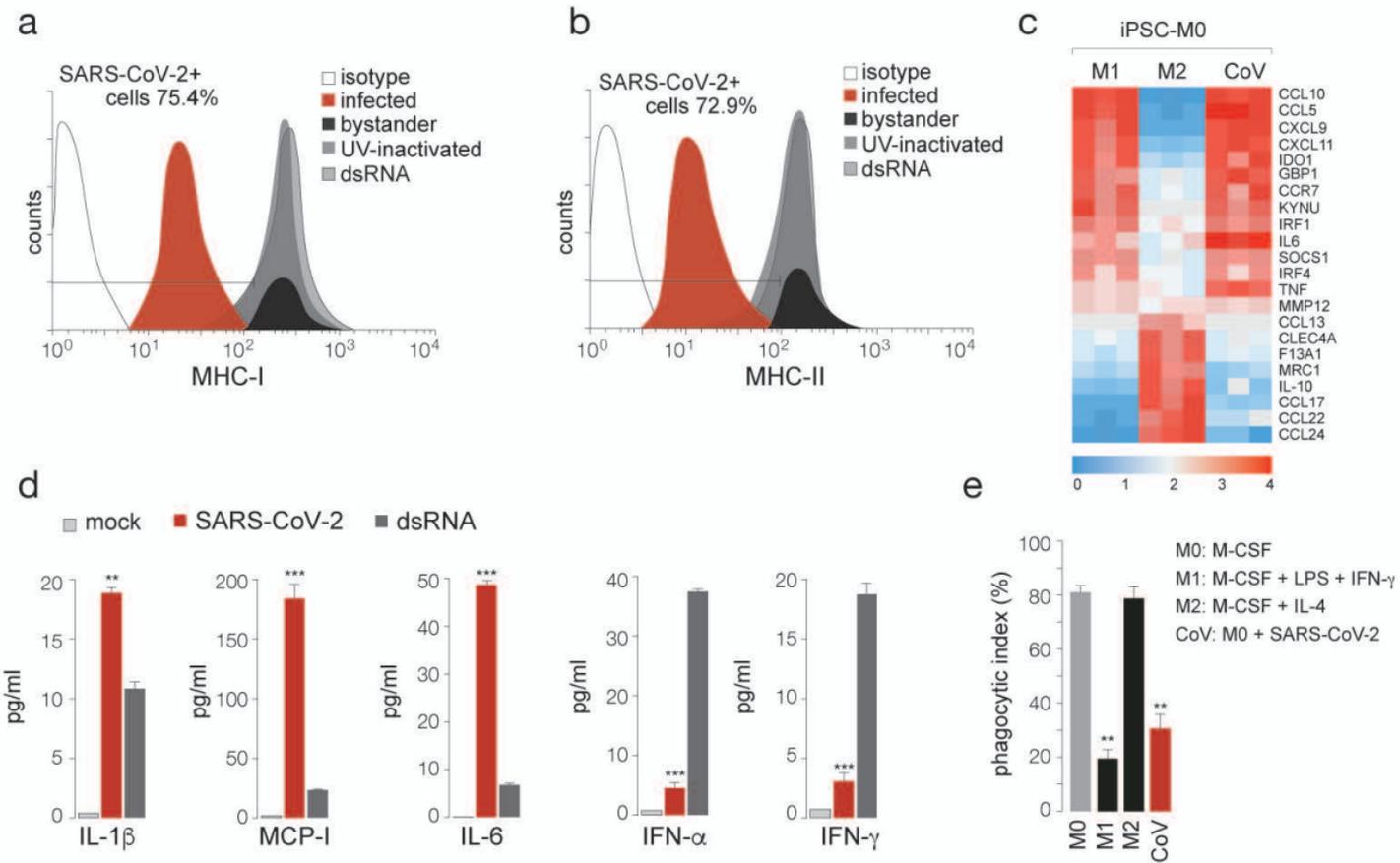


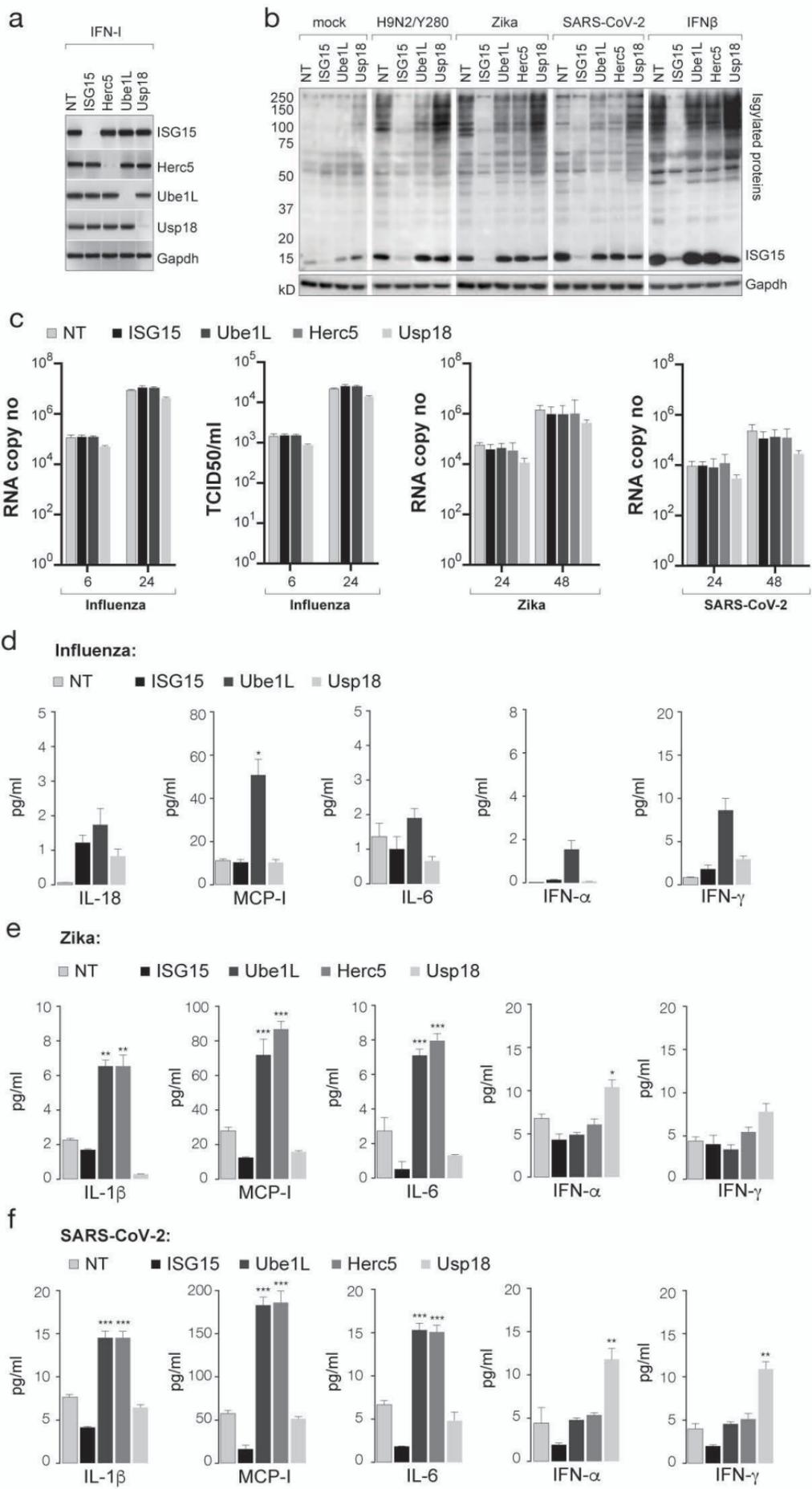
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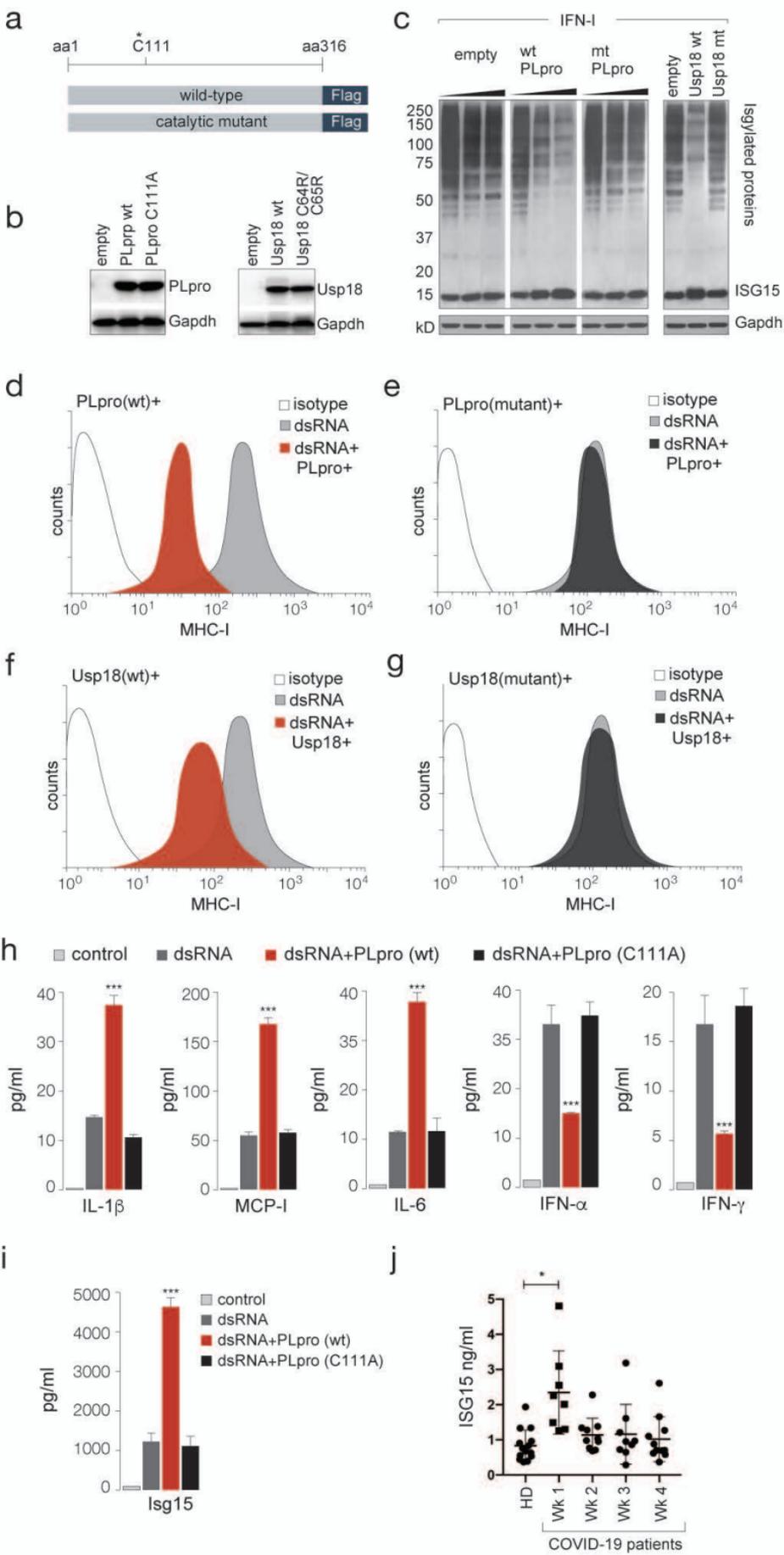


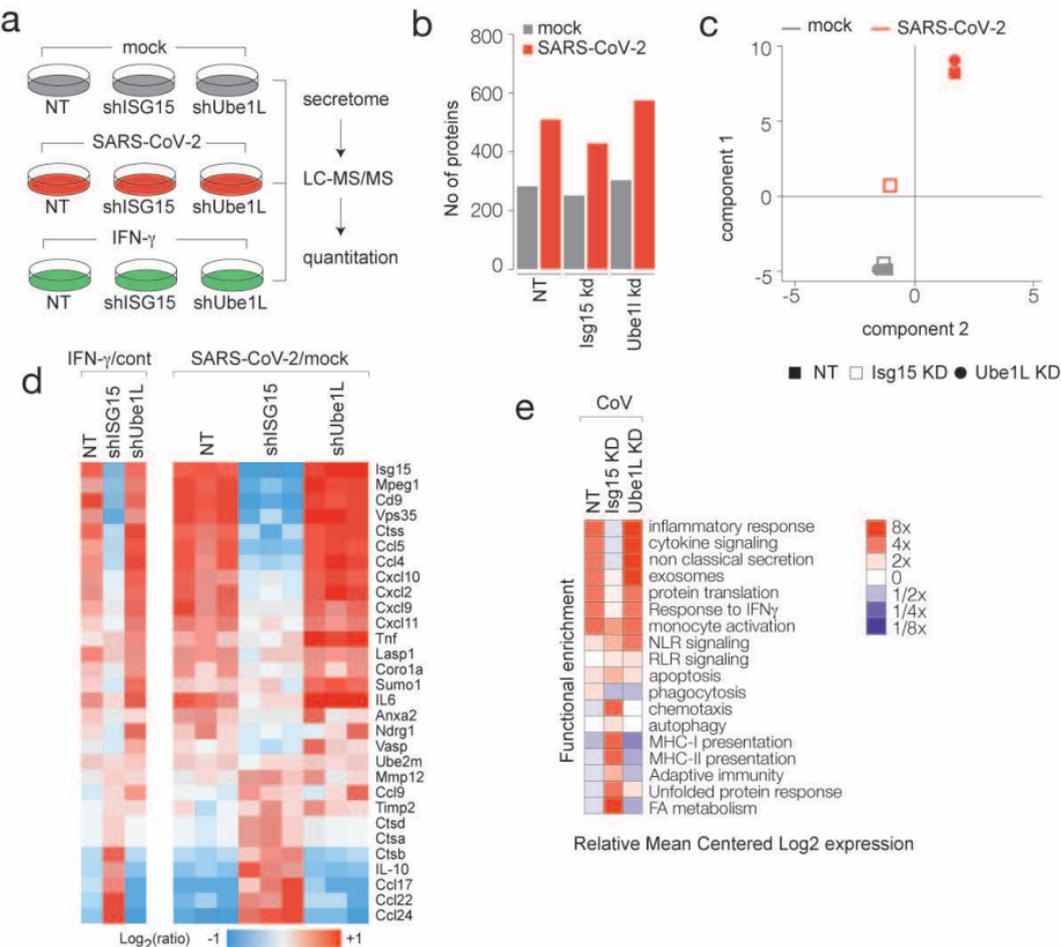
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Figures

Figure 1

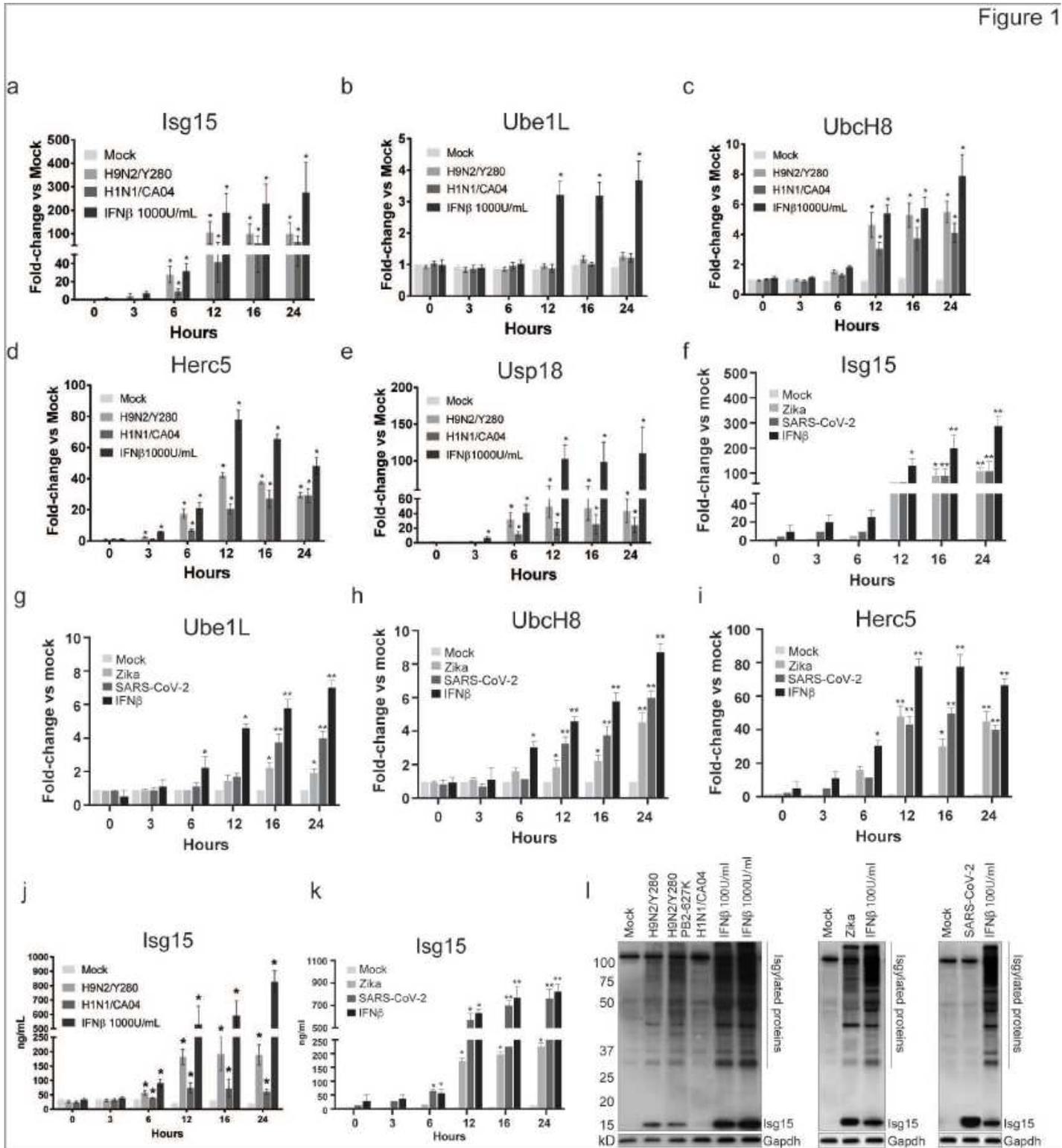


Figure 1

ISG15 and modifying enzymes are induced during virus infections a-e. Macrophages were infected with the indicated influenza virus strains at a MOI 2. Changes in mRNA expression levels of ISG15 (a) and ISG15 modifying enzymes Ube1L Ubch8, HERC5, USP18 (b-e) against mock infection were quantified by

qPCR f-i. Macrophages were infected with Zika or SARS-CoV-2 at MOI 2. At indicated time intervals changes in mRNA expression levels of ISG15 (f) and ISG15 modifying enzymes Ube1L UbcH8 and HERC5 (g-i) against mock infection were quantified by qPCR j, k. Intracellular ISG15 protein levels in influenza infected cells (j) or Zika or SARS-CoV-2 infected macrophages (k) were quantified by ELISA. Data are displayed as means \pm SEM of at least three independent donors. * $p < 0.05$ by the Mann-Whitney U Test vs. mock-infected cells. l. ISGylation in virus-infected macrophages was measured for influenza, Zika and SARS-CoV-2. Macrophages were infected with the indicated wild-type and mutated strains of influenza virus (left panel), Zika (middle panel) and SARS-CoV-2 (right panel) at a MOI 2. Lysates were collected in 1% IGEPAL PBS 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 donors.

Figure 2

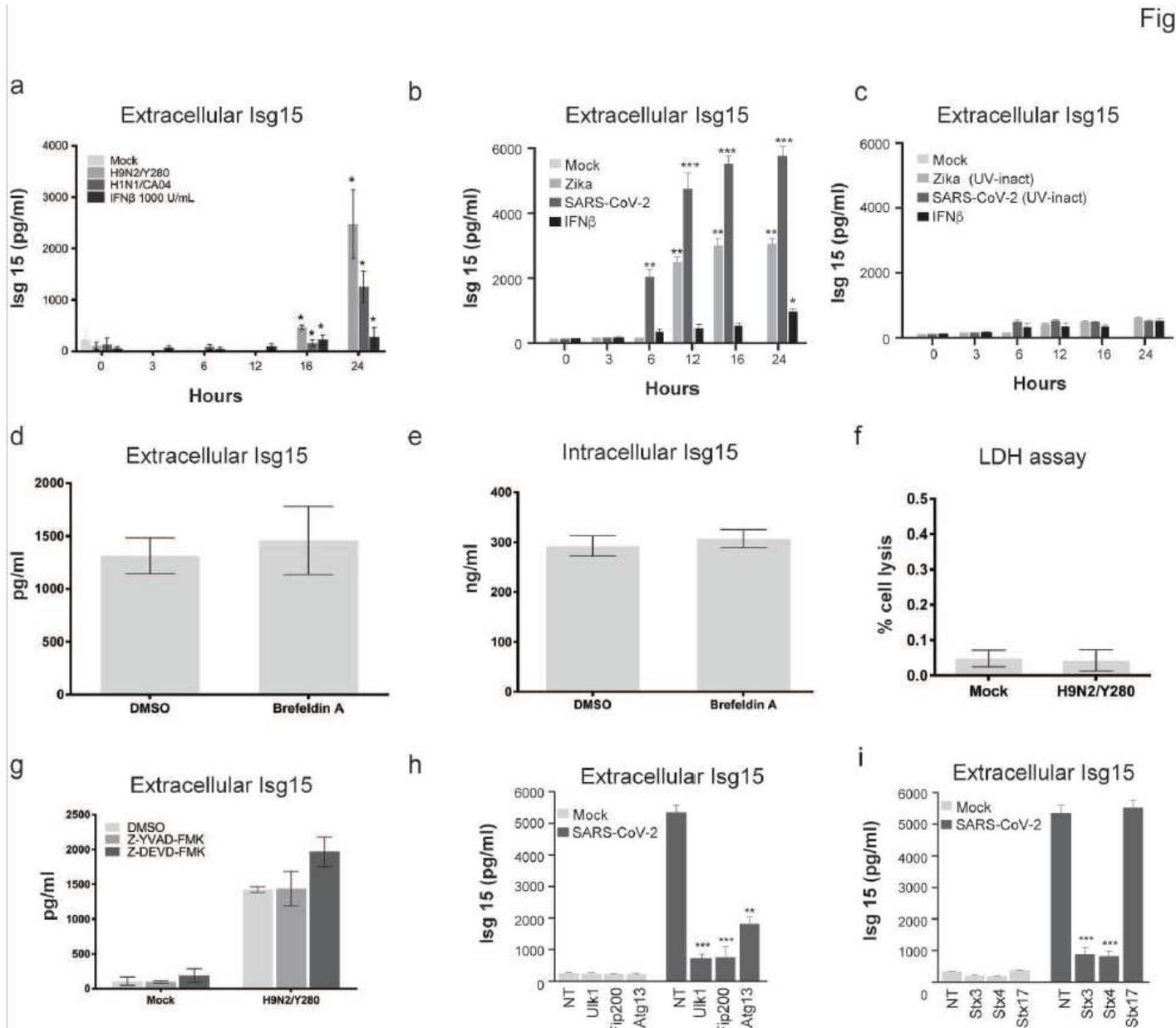


Figure 2

ISG15 is secreted from virus-infected macrophages via non-conventional secretory autophagosomal pathway. a. Macrophages were infected with the indicated influenza viral strains at a MOI 2. At indicated time intervals changes in extracellular levels of ISG15 were quantified by ELISA. b, c. iPSC-derived macrophages were infected with either Zika or SARS-CoV-2 (b) at a MOI 2 or UV-inactivated Zika or SARS-CoV-2 (c). Changes in extracellular levels of ISG15 were quantified by ELISA. d, e. Brefeldin (5 μ M), which inhibits conventional secretion pathway, was added 1 h post-infection and had no effect on ISG15 secretion (d) and protein expression (e) 24 h post infection. f. 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, 24 h post infection following the manufacturer's protocol. g. 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. h, i. Depletion of proteins implicated in secretory autophagosomes was performed by DsiRNA in iPSC-derived macrophages and verified by immunoblotting (Fig S2a, b). Non-targeting and depleted cells were infected with SARS-CoV-2 (MOI 2; 24h) and secretion of ISG15 measured by ELISA. All data are displayed as means \pm SEM of at least three independent donors. *p < 0.05 by the Mann-Whitney U Test vs mock-infected cells.

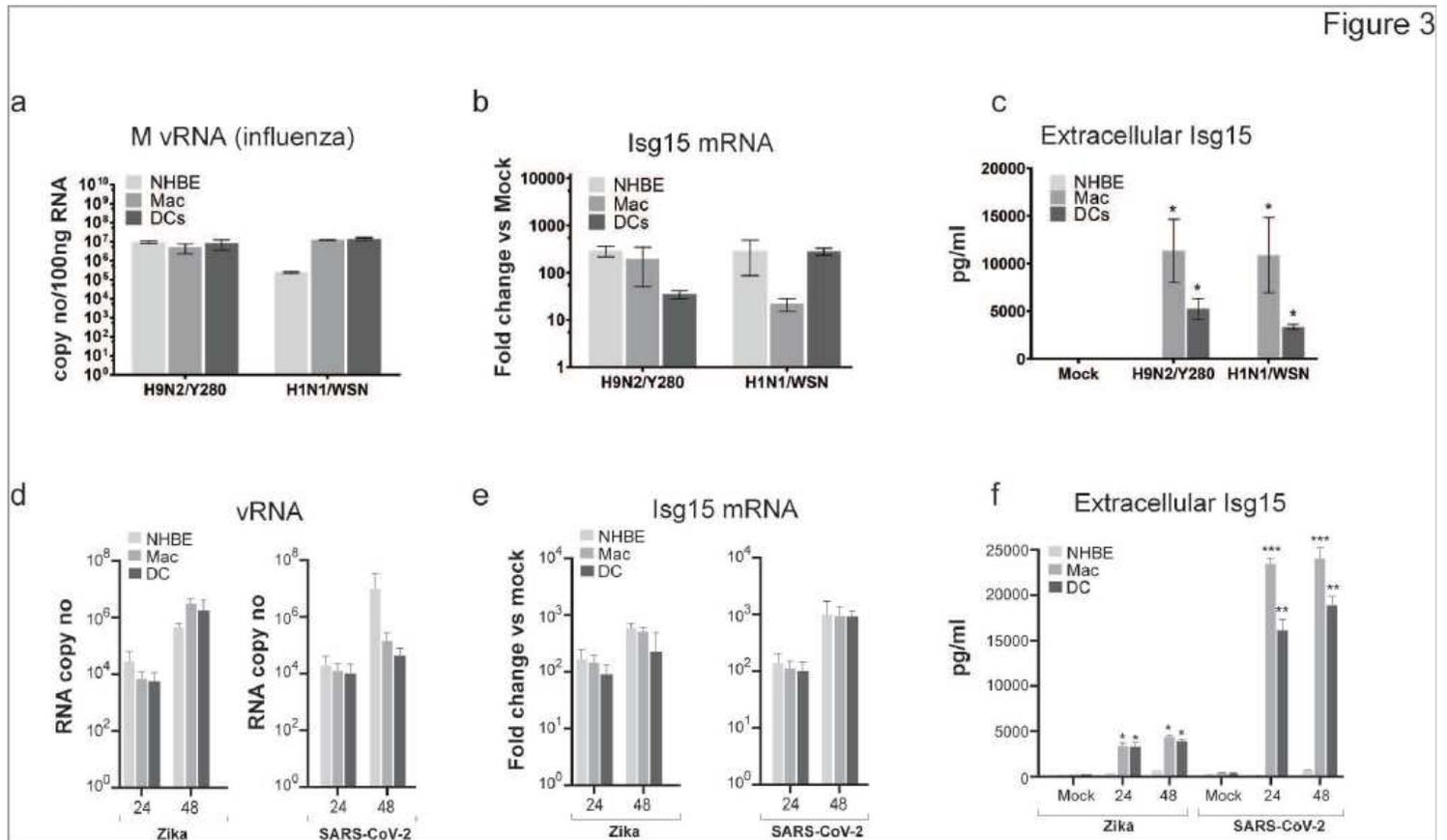


Figure 3

ISG15 is specifically secreted by monocyctic cells during virus infections. a. Normal human bronchial epithelial cells (NHBE), macrophages (Mac) and dendritic cells (DCs) were infected with the indicated wild-type and mutated influenza virus strains at a MOI 2. Total RNA was collected at 24 hours post-infection and reverse transcribed into cDNA using oligo-d(T)23VN primer. Relative changes of expression

levels against mock infection were quantified by qPCR using gene specific primers. b. Quantification of ISG15 copy number was done by RT-qPCR using ISG15 specific primers. c. Cell culture media were collected at 24 hours post-infection. ISG15 protein was quantified by ISG15 sandwich ELISA. All data are displayed as mean \pm SEM of at least three independent donors. * $p < 0.05$ by the Mann-Whitney U Test vs. mock treated cells d. NHBE, iPSC-derived macrophages (Mac) and dendritic cells (DCs) were infected with either Zika or SARS-CoV-2 at a MOI 1. Total RNA was collected at 24- and 48-hours post-infection; relative changes of expression levels against mock infection were quantified by qPCR using gene specific primers e. Quantification of ISG15 copy number was done by RT-qPCR using ISG15 specific primers f. Supernatants from infected cells were collected at 24 and 48 hours post-infection. ISG15 protein was quantified by ISG15 sandwich ELISA. All data are displayed as mean \pm sd of at least three independent experiments. * $p < 0.05$ by the Mann-Whitney U Test vs. mock-treated cells.

Figure 4

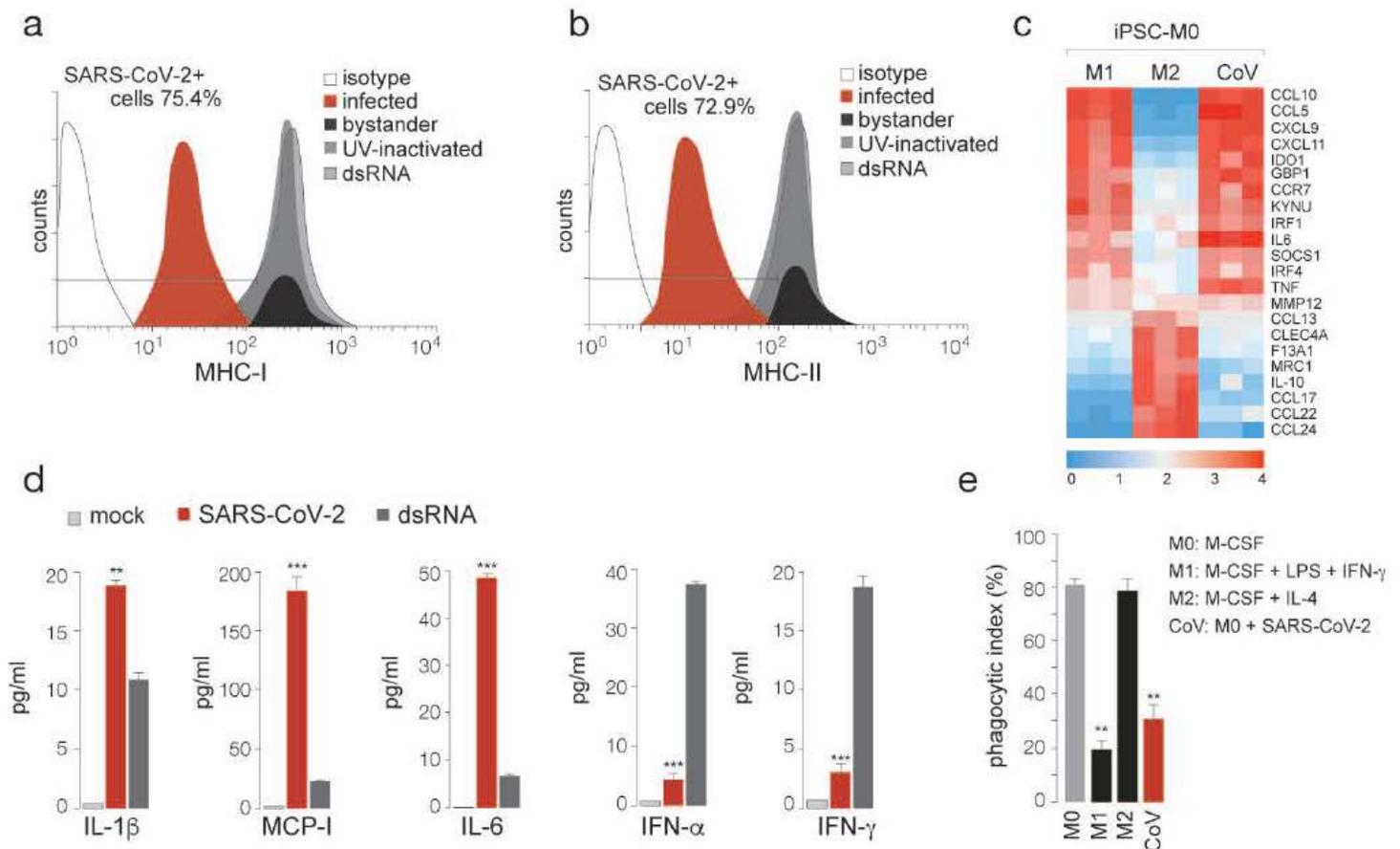


Figure 4

Immune dysfunction in SARS-CoV-2-infected macrophages a. Surface staining of MHC-I in iPSC-derived 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. Bystander cells are depicted in black. b. Surface staining of MHC-II in iPSC-derived 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. Bystander population is depicted in black. Controls included were antibody isotype, UV-inactivated virus and dsRNA treated cells. c. iPSC-derived 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 arrays following the manufacturer's guidelines and flow cytometry. 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 \pm s.d; [*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; Two-way ANOVA with Tukey's multiple comparison test].

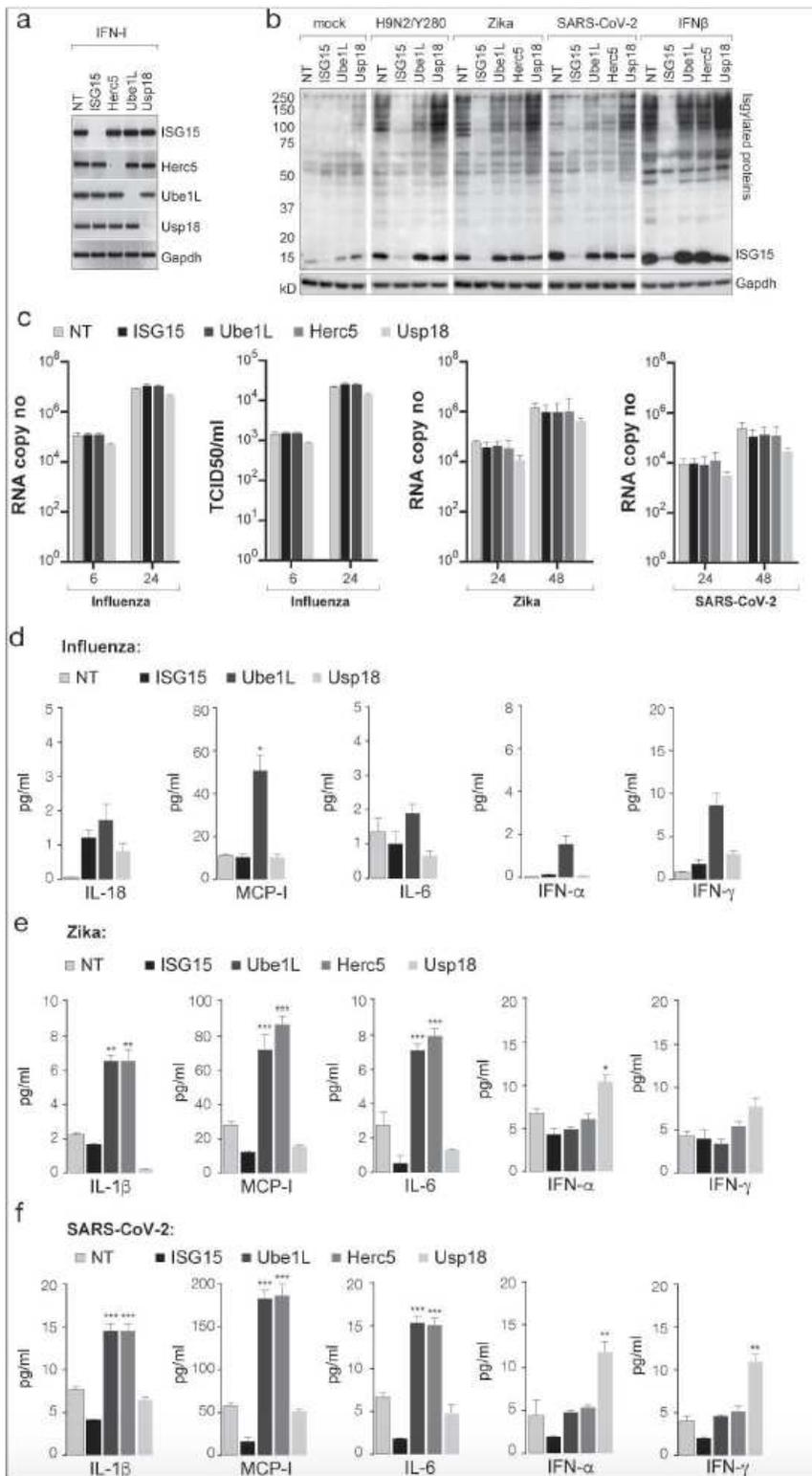


Figure 5

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 influenza, Zika and SARS-CoV-2 infected cells (MOI 2), 24 hours post infection c. Total RNA was collected at indicated time intervals from influenza, Zika and SARS-CoV-2 infected cells, and reverse transcribed into cDNA using uni-12 primer.

Quantifications of absolute copy number were done by RT-qPCR using universal vRNA specific primers. For infectivity assay, culture medium was removed at 6 and 24 hours post-infection from influenza-infected cells and TCID50 infectivity assays were done in MDCK cells as described in the Materials and Methods section. TCID50/mL value was calculated by the Spearman Kärber method. Data are displayed as means \pm SEM of at least three independent donors. There were no statistical differences by the Mann-Whitney U Test vs. control cells treated with non targeting (NT) DsiRNA d. Macrophages were transfected with either non-targeting (NT), ISG15, Ube1L, or USP18 DsiRNA for 72 hours prior to H9N2/Y280 influenza virus infection at MOI 2. Indicated cytokines were quantified by cytometric beads assay. Data are displayed as means \pm SEM of at least three independent donors. * $p < 0.05$ by the Mann-Whitney U Test vs. control e-f. iPSC-derived macrophages transfected with either non-targeting (NT), ISG15, Ube1L, HERC5 or USP18 DsiRNA for 72 hours were infected with either Zika (e) or SARS-CoV-2 (f) at MOI 2 for 24 hours. Indicated cytokines were quantified by cytometric beads assay. Data are displayed as means \pm s.d of at least three independent experiments. * $p < 0.05$ by the Mann-Whitney U Test vs. control (NT cells).

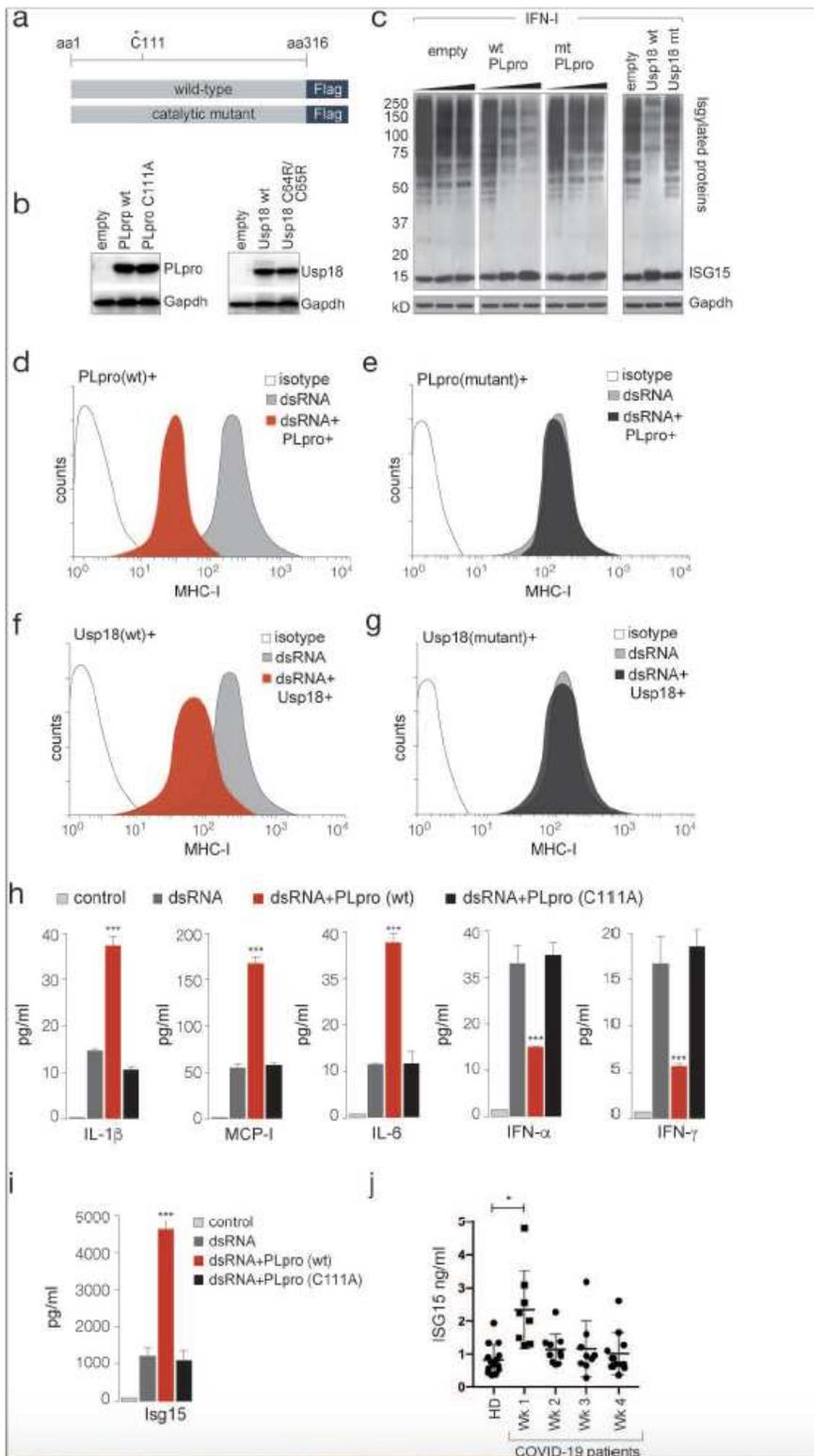


Figure 6

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 d, e. Surface staining of MHC-I in iPSC-derived macrophages expressing either wt HA-PLpro (d) or the catalytic mutant HA-PLpro (C117A)

(e) of SARS-CoV-2. Cells were then treated with dsRNA to induce surface expression of MHC-I. For both samples (wt, mutant) >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 \pm sd from three independent experiments. Data are displayed as means \pm s.d. * p < 0.05 by the 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 \pm sd of at least three independent experiments. * p < 0.05 by the Mann-Whitney U Test vs. control cells. j. Elevation of ISG15 was found in the plasma samples collected from the COVID-19 patients at their first week of disease onset. * p < 0.05 by the Mann-Whitney U Test vs. healthy donors.

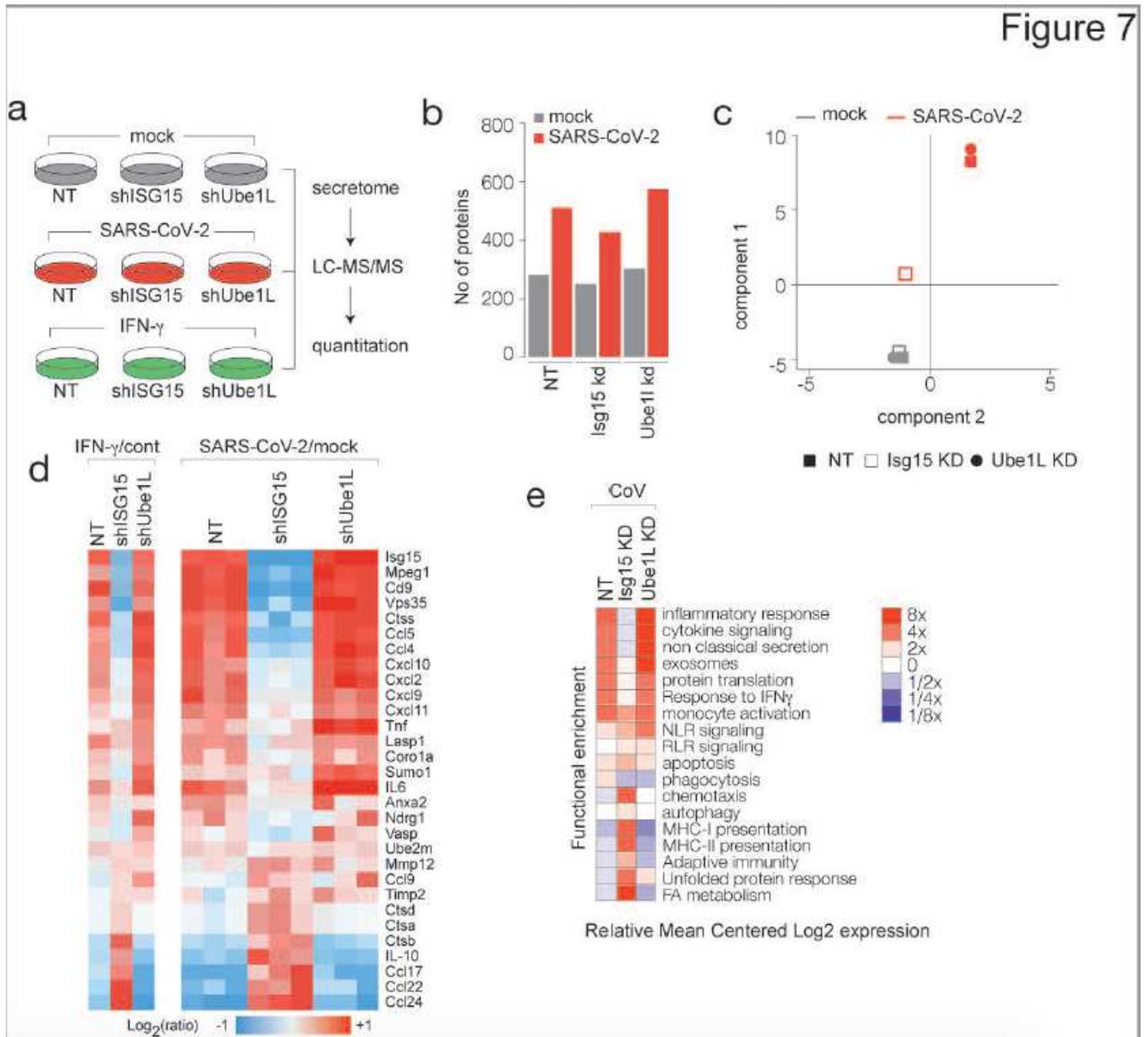


Figure 7

Quantitative analyses of ISG15-dependent responses in SARS-CoV-2 infected macrophages a. Schematic of the label-free strategy of LC-MS/MS used to study the ISG15- dependent secretome of SARS-CoV-2 infected cells. 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 IFN γ 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 b. Total numbers of proteins quantified in at least two biological replicates c. 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 d. The heatmap represents the hierarchical clustering of the common proteins in the secretome for IFN γ 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 e. Functional annotation of the common proteins identified in all samples was performed by the DAVID software.

Supplementary Files

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