

1 **Omicron-induced interferon signalling prevents influenza A virus**
2 **infection**

3 **Running title: Omicron inhibits influenza A virus infection**

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35 **Abstract**

36 Recent findings in permanent cell lines suggested that SARS-CoV-2 Omicron
37 BA.1 induces a stronger interferon response than Delta. Here, we show that BA.1 and
38 BA.5 but not Delta induce an antiviral state in air-liquid interface (ALI) cultures of
39 primary human bronchial epithelial (HBE) cells and primary human monocytes. Both
40 Omicron subvariants caused the production of biologically active type I (α/β) and III
41 (λ) interferons and protected cells from super-infection with influenza A viruses.
42 Notably, abortive Omicron infection of monocytes was sufficient to protect monocytes
43 from influenza A virus infection. Interestingly, while influenza-like illnesses surged
44 during the Delta wave in England, their spread rapidly declined upon the emergence
45 of Omicron. Mechanistically, Omicron-induced interferon signalling was mediated via
46 double-stranded RNA recognition by MDA5, as MDA5 knock-out prevented it. The
47 JAK/ STAT inhibitor baricitinib inhibited the Omicron-mediated antiviral response,
48 suggesting it is caused by MDA5-mediated interferon production, which activates
49 interferon receptors that then trigger JAK/ STAT signalling. In conclusion, our study 1)
50 demonstrates that only Omicron but not Delta induces a substantial interferon
51 response in physiologically relevant models, 2) shows that Omicron infection protects
52 cells from influenza A virus super-infection, and 3) indicates that BA.1 and BA.5 induce
53 comparable antiviral states.

54

55 **Keywords:** SARS-CoV-2; COVID-19; interferon; monocytes; influenza; super-
56 infection; antiviral state; BA.1; BA.5; Delta

57

58 **Introduction**

59 SARS-CoV-2, the coronavirus that causes COVID-19, has caused the worst
60 pandemic since the Spanish Flu in 1918-1920 [Bastard et al., 2022]. Virus-induced
61 interferon signalling has been shown to be critically involved in determining COVID-
62 19 severity [Bastard et al., 2022; Chiale et al., 2022]. Individuals with defects in their
63 interferon response are predisposed to life-threatening COVID-19 [Bastard et al.,
64 2022; Chiale et al., 2022], and a particularly pronounced interferon-related innate
65 immune response is anticipated to contribute to the lower COVID-19 severity observed
66 in children [Borrelli et al., 2021].

67 Recent findings suggested that SARS-CoV-2 Omicron BA.1 displays a lower
68 interferon antagonism than Delta [Bojkova et al., 2022; Bojkova et al., 2022a]. BA.1
69 and Delta viruses showed a similar replication pattern in interferon-deficient Vero cells,
70 but BA.1 replication was attenuated relative to Delta in interferon competent Calu-3,
71 Caco-2, and Caco-2-F03 (a highly SARS-CoV-2-susceptible Caco-2 subline [Bojkova
72 et al., 2022b]) cells [Bojkova et al., 2022; Hu et al., 2022; Shuai et al., 2022]. Moreover,
73 BA.1 induced a more pronounced interferon response than Delta [Bojkova et al., 2022;
74 Bojkova et al., 2022a].

75 Here, we investigated Delta, BA.1, and BA.5 replication in air-liquid interface
76 (ALI) cultures of primary human bronchial epithelial (HBE) cells and primary human
77 monocytes. To examine whether SARS-CoV-2-induced interferon induction results in
78 a biologically relevant antiviral state, we further determined the impact of Delta, BA.1,
79 and BA.5 infection on influenza A virus replication in ALI HBE cultures (H1N1) and
80 monocytes (H1N1, H5N1), as interferon signalling is considered to be a major
81 influenza A virus restriction factor [McKellar et al., 2021].

82 **Results**

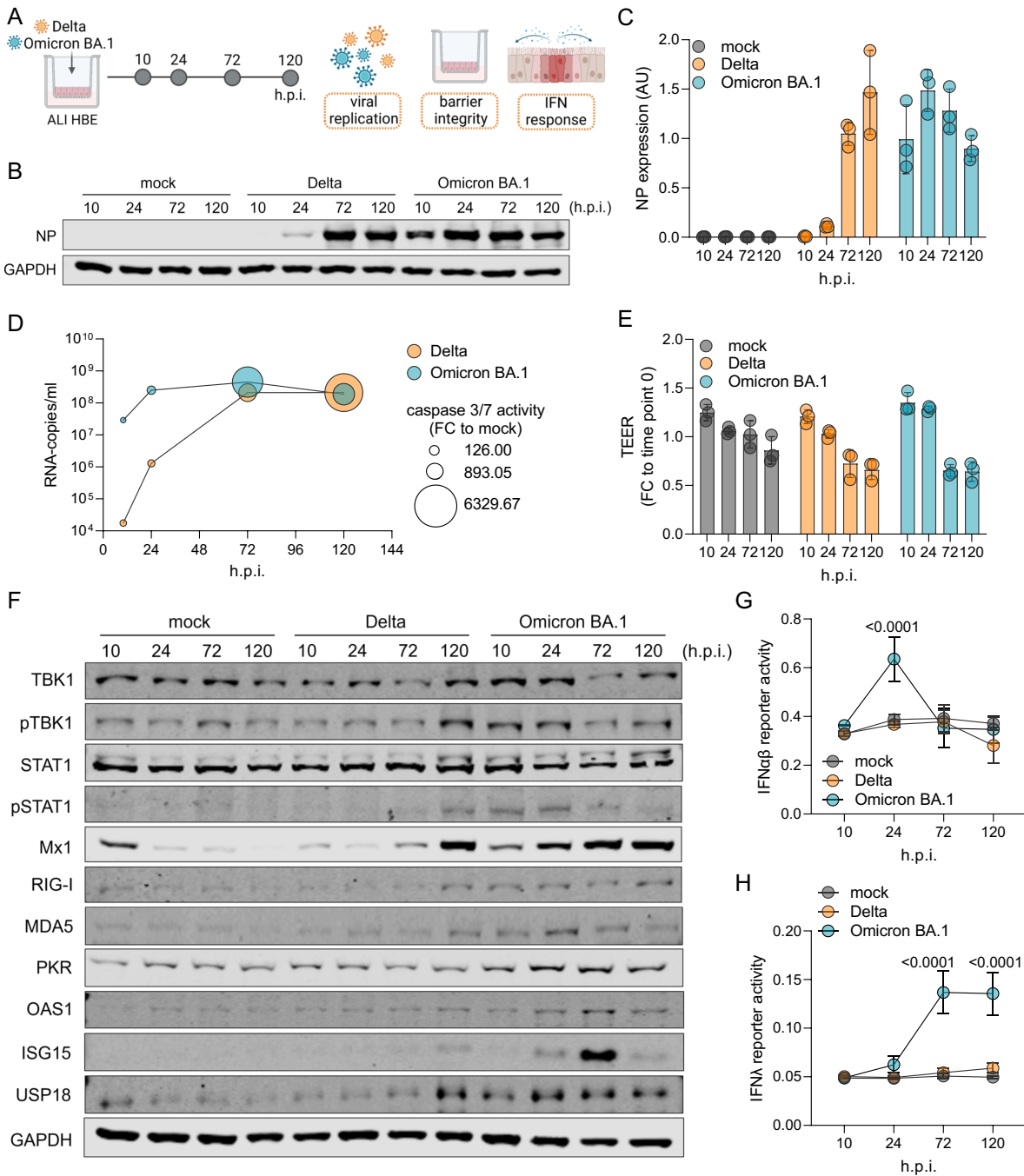
83 **Infection kinetics and interferon response induction by Omicron BA.1 and Delta** 84 **in air-liquid-interface (ALI) cultures of primary human bronchial epithelial (HBE)** 85 **cells**

86 BA.1 displayed faster replication kinetics than Delta in ALI HBE cultures (Figure
87 1), as indicated by high SARS-CoV-2 nucleoprotein (NP), genomic RNA levels, and
88 caspase 3/7 activity (which reflects SARS-CoV-2 replication independently of whether
89 the virus causes cytotoxicity resulting in a cytopathogenic effect in a cell culture model
90 [Bojkova et al., 2022b]) (Figure 1B-D). However, the replication of both SARS-CoV-2
91 variants resulted in comparable peak NP and genomic RNA levels (Figure 1B-D).
92 While the BA.1 levels declined after a peak (at 24h post infection for NP and 72h for
93 genomic RNA), Delta levels continued to increase until 120h post infection (Figure 1B-
94 D). These findings are in accordance with previous findings showing that BA.1
95 replicates faster than other variants in bronchial cells [Hui et al., 2022]. Independently
96 of the replication kinetics BA.1 and Delta caused similar reductions of the ALI HBE
97 barrier integrity (Figure 1E).

98 Also in agreement with previous findings [Alfi et al., 2022; Bojkova et al., 2022;
99 Bojkova et al., 2022a], BA.1 induced a stronger interferon response than Delta, as
100 indicated by the abundance and phosphorylation levels of a range of proteins involved
101 in interferon signalling (Figure 1F). Moreover, only BA.1 but not Delta induced the
102 secretion of biologically active interferon- α/β (Figure 1G) and - λ (Figure 1H) by ALI
103 HBE cultures, as demonstrated using HEK-reporter cell lines.

104

Figure 1



105

106 **Figure 1. Infection kinetics and interferon response induction by Omicron BA.1**

107 **and Delta in air-liquid-interface (ALI) cultures of primary human bronchial**

108 **epithelial (HBE) cells. (A) Schematic depiction of the experimental set-up. (B)**

109 **Immunoblot of cellular SARS-CoV-2 nucleoprotein (NP) levels in BA.1- and Delta (MOI**

110 **1)-infected ALI HBE cultures at different time points post infection. (C) Quantification**

111 **of NP levels (mean \pm SD) by ImageJ. (D) SARS-CoV-2 genomic RNA copy numbers**

112 (Y axis) and caspase 3/7 activity (bubble size) determined in the apical medium of
113 BA.1- and Delta (MOI 1)-infected at different time points post infection. (E) Evaluation
114 of barrier integrity by measurement of TEER in BA.1- and Delta (MOI 1)-infected ALI
115 HBE cultures at different time points post infection. Bars represents mean \pm SD of
116 three biological replicates. (F) Immunoblot indicating cellular levels of proteins
117 involved in interferon signalling in BA.1- and Delta (MOI 1)-infected ALI HBE cultures
118 at different time points post infection. (G,H) Interferon (IFN)- α/β (G) and IFN λ (H)
119 responses induced in HEK reporter cell lines by apical washes from BA.1- and Delta
120 (MOI 1)-infected ALI HBE cultures collected at different time points post infection. P
121 values were determined by one-way ANOVA with subsequent Tukey's test.

122

123 Interferon- α/β peaked at 24h post infection (Figure 1G), which was followed by
124 a return to basal levels, whereas interferon- λ remained elevated until 120h post
125 infection (Figure 1H). Short-term interferon type I (α/β) responses cause a protective
126 antiviral response, while long-term interferon activity is associated with potentially
127 deleterious inflammation [Forero et al., 2019; King & Sprent, 2021]. In contrast,
128 sustained interferon type III (λ) responses inhibit respiratory virus replication at
129 epithelial barriers in the respiratory tract and prevent excessive inflammation [Forero
130 et al., 2019; Prokunina-Olsson et al., 2020; King & Sprent, 2021]. Hence, the interferon
131 type I and III responses observed in BA.1-infected ALI HBE cultures add further
132 evidence explaining why Omicron is less pathogenic than other SARS-CoV-2 variants
133 like Delta [Wang et al., 2022].

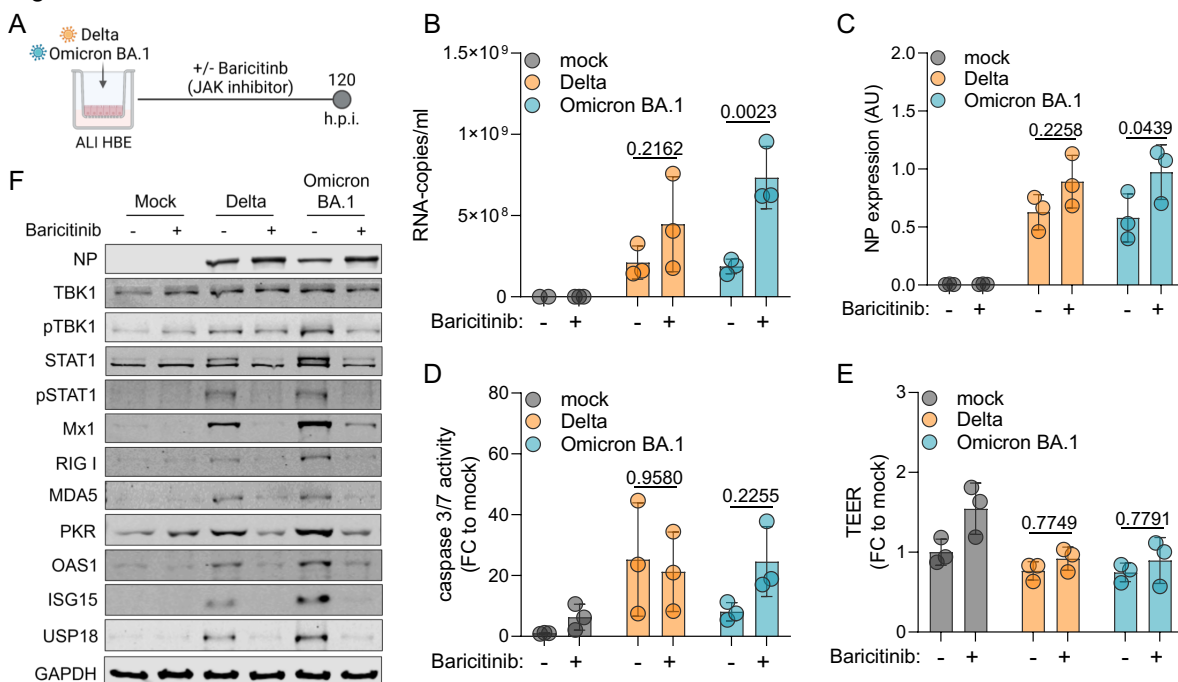
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135 **JAK/ STAT inhibition suppresses BA.1-induced interferon signalling and**
 136 **increases BA.1 replication in air-liquid-interface (ALI) human bronchial**
 137 **epithelial (HBE) cell cultures**

138 Interferon signalling can be induced in a STAT1-dependent and -independent
 139 manner [Rani et al., 2010; Bastard et al., 2022; Chiale et al., 2022]. In hibition of JAK/
 140 STAT signalling using the JAK inhibitor baricitinib significantly increased BA.1
 141 replication in ALI HBE cultures (Figure 2), as indicated by genomic RNA copy numbers
 142 (Figure 2B) and cellular NP levels (Figure 2C). In contrast, Delta only displayed a non-
 143 significant trend towards higher genomic RNA copy numbers (Figure 2B) and cellular
 144 NP levels (Figure 2C) in the presence of baricitinib. Baricitinib did not exert significant
 145 effects on BA.1- and Delta-mediated caspase 3/7 activation (Figure 2D) and the ALI
 146 HBE barrier function (Figure 2E), although there was a non-significant trend towards
 147 enhanced caspase 3/7 activity in BA.1-infected ALI HBE cultures (Figure 2D).

148

Figure 2



149

150 **Figure 2. Inhibition of JAK/STAT signalling promotes Omicron BA.1 replication.**

151 (A) Schematic depiction of the experimental set-up. (B) SARS-CoV-2 genomic RNA
152 copies in apical wash of air-liquid-interface (ALI) human bronchial epithelial (HBE)
153 cultures 120 h post SARS-CoV-2 (MOI 1) infection in the absence or presence of the
154 JAK inhibitor baricitinib (1 μ M). Bars represent mean \pm SD of three biological replicates.
155 (C) NP levels in SARS-CoV-2 (MOI 1)-infected ALI HBE cultures in the absence or
156 presence of baricitinib (1 μ M) as determined by immunoblotting. Bars represent mean
157 \pm SD of three biological replicates. (D) Caspase 3/7 activation in apical washes of
158 SARS-CoV-2 (MOI 1)-infected ALI HBE cultures in the absence or presence of
159 baricitinib (1 μ M) 120h post infection. Bars display mean \pm SD of three biological
160 replicates. (E) Barrier integrity in SARS-CoV-2 (MOI 1)-infected ALI HBE cultures in
161 the absence or presence of baricitinib (1 μ M) measured by TEER at 120h post
162 infection. Mean \pm SD of three biological replicates is presented. (F) Immunoblot
163 indicating cellular levels of proteins involved in interferon signalling in SARS-CoV-2
164 (MOI 1)-infected ALI HBE cultures at 120h post infection in the presence or absence
165 of baricitinib (1 μ M). All P values were calculated by Student's t-test.

166

167 Western blot analysis confirmed that baricitinib not only increased BA.1
168 replication but also suppressed BA.1-induced interferon signalling (Figure 2F). Taken
169 together, these findings indicate that the pronounced interferon response induced by
170 BA.1 is mediated via STAT1 and that it attenuates BA.1 replication.

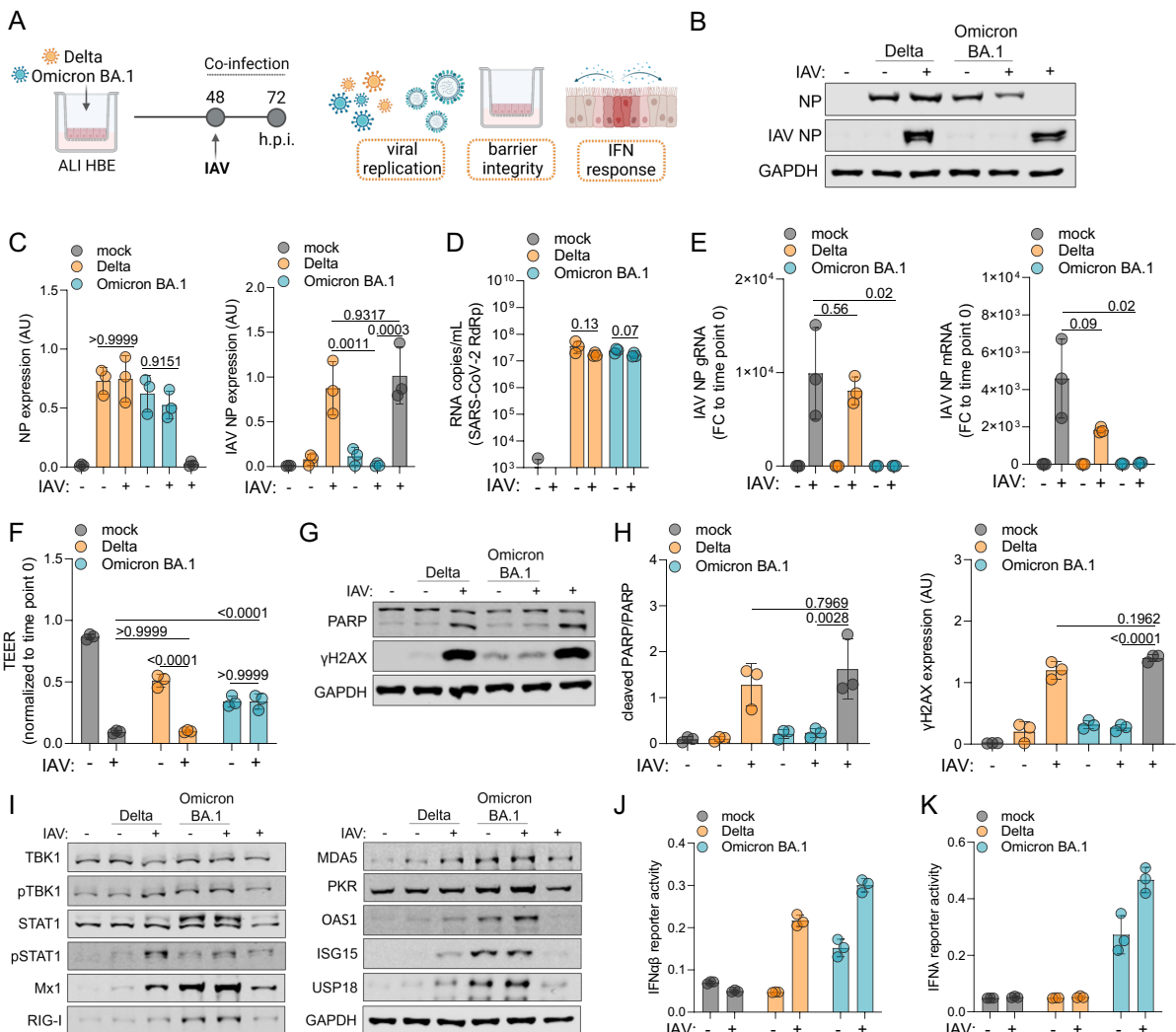
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172 **BA.1-induced interferon signalling protects air-liquid-interface (ALI) human**
 173 **bronchial epithelial (HBE) cell cultures from H1N1 influenza A virus super-**
 174 **infection**

175 Next, we here infected ALI HBE cultures with BA.1 or Delta (both MOI 1) for
 176 48h prior to infection with H1N1 influenza A virus (MOI 2) (Figure 3A) to examine
 177 whether the BA.1-induced interferon response may induce an antiviral state that
 178 interferes with H1N1 replication. Controls confirmed that both BA.1 and Delta
 179 replication as well as BA.1- and Delta-induced interferon induction were comparable
 180 to the data presented in Figure 1 (Suppl. Figure 1).

181

Figure 3



182

183 **Figure 3 Omicron BA.1 but not Delta infection prevents H1N1 influenza A virus**
184 **(IAV) replication.** (A) Schematic of the experimental set-up. (B) Immunoblot of the
185 SARS-CoV-2 nucleoprotein (NP) and H1N1 IAV NP levels 24 h post infection with
186 H1N1 strain A/NewCaledonia/20/99 (MOI 2). (C) Quantification of immunoblots from
187 (B). Bars represent mean \pm SD of three biological replicates. P values were calculated
188 by two-way ANOVA. (D) Genomic RNA copies of the SARS-CoV-2 RNA-dependent
189 RNA polymerase (RdRp) in the apical wash of ALI HBE cultures 24 hours post
190 infection with H1N1 (MOI 2). Values represent means \pm SD of three biological
191 replicates. P values were determined by Student's t-test. (E) IAV NP genomic RNA
192 (gRNA, left) or mRNA (right) levels 24h post infection with H1N1 (MOI 2). Bars display
193 means \pm SD of three biological replicates. P values were determined by Student's t-
194 test. (F) Barrier integrity measure by transepithelial electric resistance (TEER) in
195 single- or co-infected ALI cultures. Bars display means \pm SD of three biological
196 replicates. P values were calculated by two-way ANOVA. (G) Immunoblot of PARP
197 cleavage and γ H2AX after single- or co-infection. (H) Quantification of the
198 immunoblots from (G). Values represent the mean \pm SD of three biological replicates.
199 P values were calculated by two-way ANOVA. (I) Immunoblots displaying the levels of
200 proteins involved in interferon signalling in single- and co-infected ALI HBE cultures.
201 (J, K) Interferon (IFN) α/β (J) or IFN λ (K) signalling in HEK-reporter cell lines incubated
202 with apical washes of single- and co-infected ALI HBE cultures 24 hours post infection
203 with H1N1 (MOI 2).

204

205 Determination of H1N1 nucleoprotein (NP) levels indicated that only BA.1 but
206 not Delta suppressed H1N1 infection in ALI HBE cultures (Figure 3B, Figure 3C).

207 H1N1 super-infection did not significantly reduce SARS-CoV-2 levels in ALI HBE
208 cultures (Figure 3D).

209 Genomic H1N1 NP RNA and H1N1 NP mRNA levels confirmed that only BA.1
210 caused a significant reduction of H1N1 replication (Figure 3E). Moreover, only BA.1
211 infection prevented H1N1-induced cytotoxicity as indicated by transepithelial electric
212 resistance (TEER) measurement (Figure 3F). Influenza A virus replication is
213 associated with the induction of apoptosis and DNA damage induction in host cells [Li
214 et al., 2015; Ampomah & Lim, 2020], and PARP cleavage and γ H2AX levels also
215 confirmed that BA.1 infection suppressed H1N1-induced cytotoxicity (Figure 3G,
216 Figure 3H).

217 The analysis of proteins involved in interferon signalling showed that also in the
218 presence of H1N1 only BA.1 induced a pronounced interferon response (Figure 3I). In
219 agreement, only BA.1-infected (but not of Delta-infected) ALI HBE cultures produced
220 biologically active type I (α/β) and III (λ) interferons, as demonstrated using HEK-
221 reporter cell lines (Figure 3J, Figure 3K). Taken together, these findings indicate that
222 only BA.1 but not Delta induces an interferon-mediated antiviral state in ALI-HBE
223 cultures that protects them from H1N1 infection.

224

225 **Inhibition of JAK/ STAT signalling promotes H1N1 influenza A virus replication** 226 **in BA.1-infected cells**

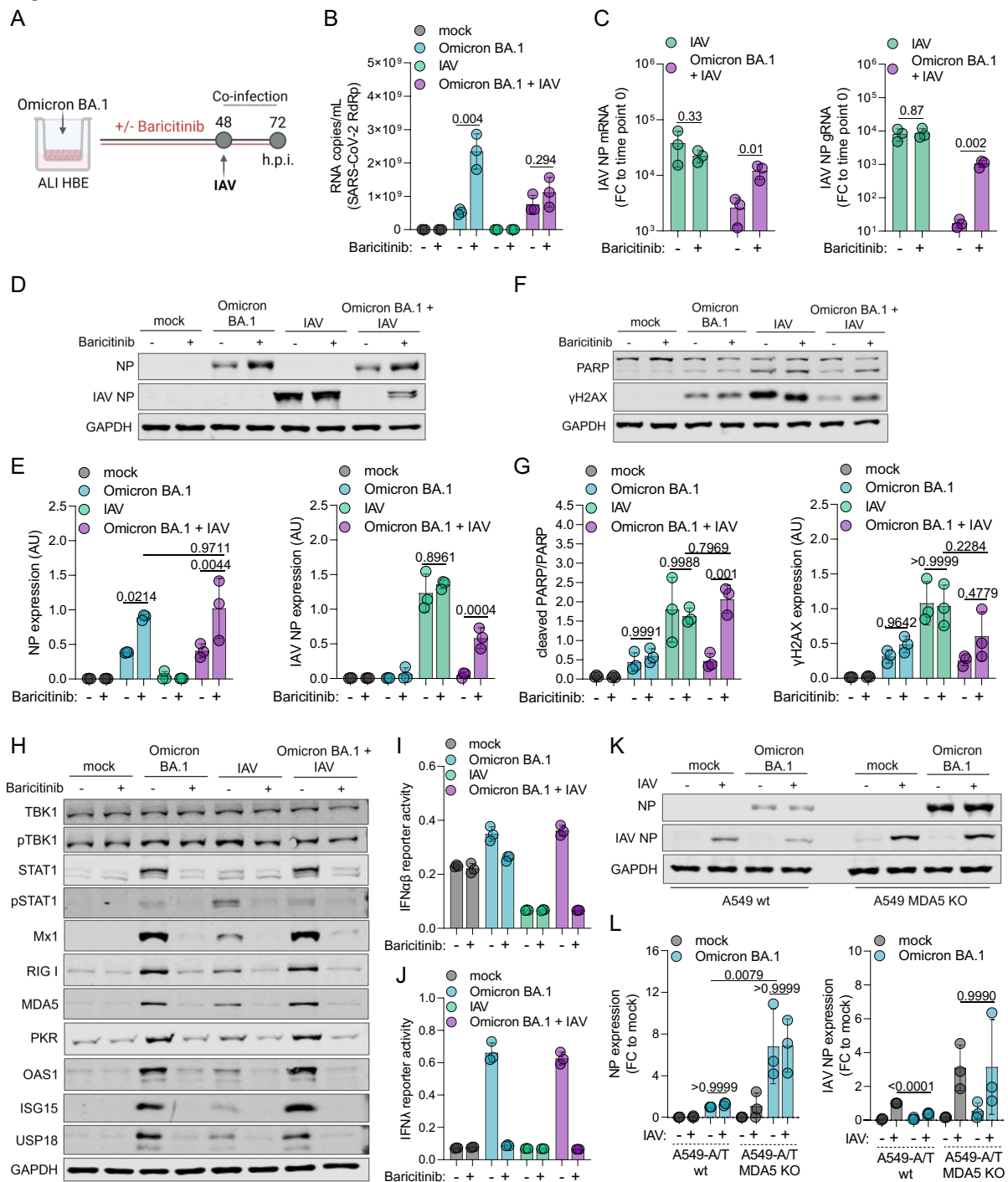
227 Next, we investigated the effect of the JAK inhibitor on the BA.1-mediated
228 suppression of H1N1 replication (Figure 4A). In agreement with the data presented in
229 Figure 2 and Figure 3, baricitinib increased BA.1 replication as indicated by genomic
230 RNA copies of the viral RNA-dependent RNA polymerase gene (Figure 4B). Moreover,
231 baricitinib prevented the BA.1-mediated inhibition of H1N1 replication as indicated by

232 H1N1 NP mRNA and genomic RNA levels (Figure 4C) and H1N1 NP protein levels
233 (Figure 4D, Figure 4E).

234 In line with our previous findings, baricitinib also antagonised BA.1-induced
235 suppression of H1N1-induced apoptosis as indicated by PARP cleavage and H1N1-
236 induced DNA damage as indicated by cellular γ H2AX levels (Figure 4F, Figure 4G).
237 Furthermore, baricitinib abrogated the BA.1-mediated protection of the ALI HBE
238 barrier integrity from H1N1-induced cytotoxicity (Suppl. Figure 2).

239

Figure 4



240

241 **Figure 4. Inhibition of JAK/ STAT signalling prevents Omicron BA.1-mediated**

242 **suppression of H1N1 influenza A virus (IAV) replication in air-liquid-interface**

243 **(ALI) human bronchial epithelial (HBE) cultures. (A) Experimental set-up. (B)**

244 **Genomic RNA copy numbers (RNA-dependent RNA polymerase gene = RdRp) in**

245 **apical washes of BA.1 (MOI 1)-infected ALI HBE cultures 72h post infection in the**

246 absence or presence of baricitinib 1 μ M. Values represent mean \pm SD of three
247 biological replicates. P values were determined by Student's t-test. (C) IAV NP mRNA
248 (left) or genomic RNA (right) levels 24h post H1N1 (MOI 2) infection in the absence or
249 presence of baricitinib 1 μ M. Bars represent mean \pm SD of three biological replicates.
250 P values were determined by Student's t-test. (D) Immunoblot indicating BA.1 NP and
251 IAV NP protein levels 72 h post BA.1 infection in the absence or presence of baricitinib
252 1 μ M. (E) Quantification of the immunoblot results from (D) by ImageJ. Values
253 represent mean \pm SD of three biological replicates. P values were calculated by two-
254 way ANOVA. (F) Immunoblot indicating PARP cleavage and γ H2AX protein levels 72
255 h post BA.1 infection in the absence or presence of baricitinib 1 μ M. (G) Quantification
256 of the immunoblot results from (F) by ImageJ. Bars represent the quantification of the
257 ratio between cleaved and total PARP (left) and cellular γ H2AX levels (right). Values
258 represent mean \pm SD of three biological replicates. P values were calculated by two-
259 way ANOVA. (H) Immunoblot displaying levels of proteins involved in interferon
260 signalling in single- and co-infected ALI HBE cultures in the absence or presence of
261 baricitinib 1 μ M. (I, J) Interferon (IFN) α/β (I) or $-\lambda$ (J) activity in HEK reporter cell lines
262 incubated with apical washes of ALI HBE cultures 72h post infection. (K) BA.1 NP and
263 IAV NP protein levels in ACE2/ TMPRSS2-transduced A549 (A549-A/T) cells (A549-
264 A/T wt) or A549-A/T MDA5 knock-out (KO) cells infected with BA.1 at MOI 0.01 for
265 24h and followed by influenza A virus (IAV) H1N1 (MOI 2) infection for an additional
266 24h. (L) Quantification of immunoblot results from (K) by ImageJ. Values represent
267 the mean \pm SD of three biological replicates. P values were calculated by two-way
268 ANOVA.
269

270 As indicated by the cellular levels of proteins involved in interferon signalling,
271 the BA.1-induced interferon was not affected by of H1N1 (Figure 4H). Moreover,
272 baricitinib inhibited interferon signalling in response to ALI HBE infection with either
273 single virus and after co-infection with both viruses (Figure 4H) and suppressed
274 interferon- α/β and - λ production (Figure 4I, Figure 4J).

275 The pattern recognition receptor MDA5 was previously shown to be critically
276 involved in the SARS-CoV-2-mediated, and in particular the BA.1-mediated, interferon
277 response [Yin et al., 2021; Bojkova et al., 2022; Bojkova et al., 2022a]. In agreement,
278 BA.1-mediated inhibition of H1N1 infection was abrogated in MDA5 knock-out cells
279 (Figure 4K, Figure 4L).

280 Taken together, our data show that BA.1-mediated suppression of H1N1
281 replication depends on the presence of MDA5 and is anatagonised by inhibition of
282 JAK/ STAT signalling by baricitinib.

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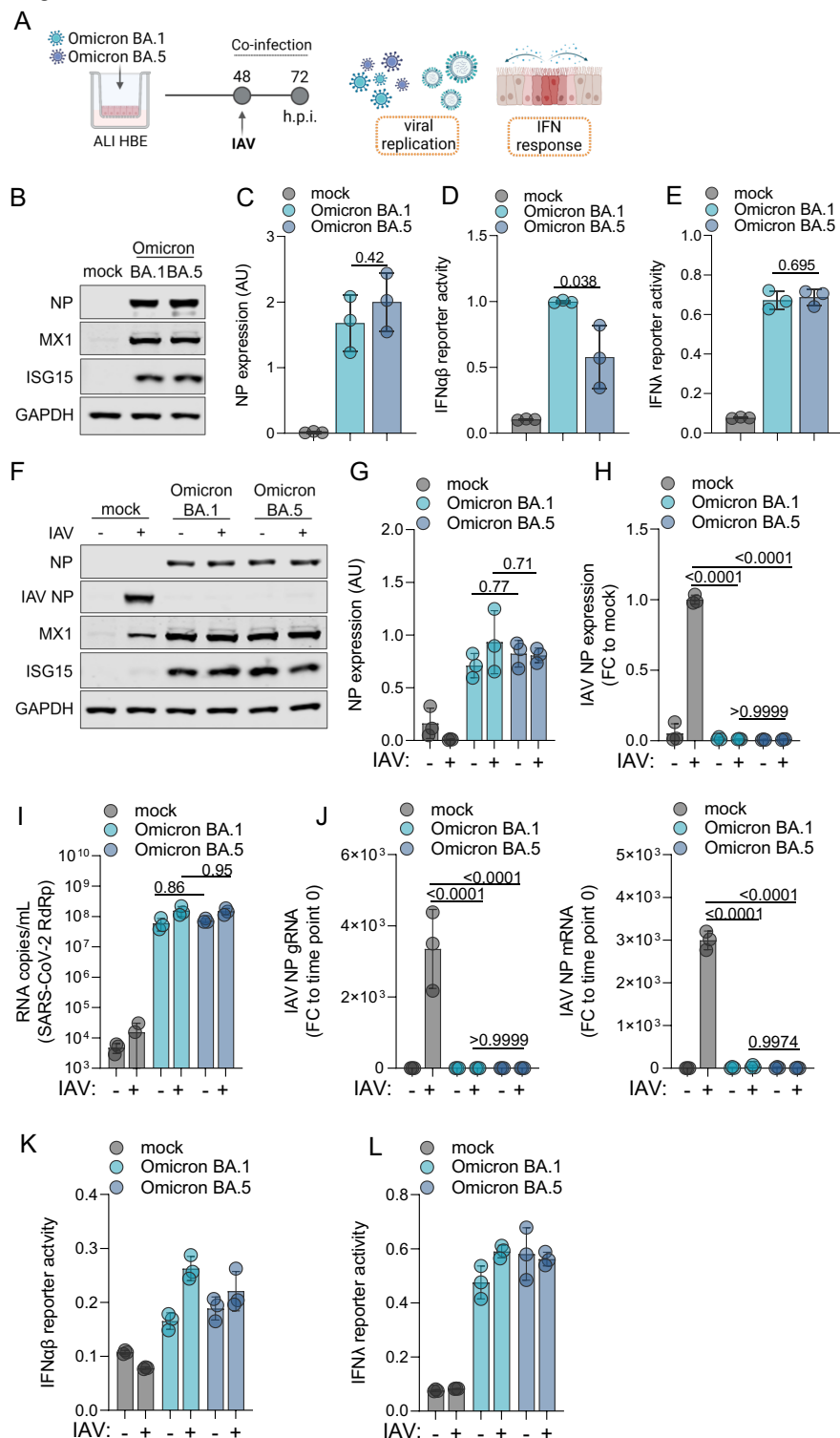
284 **Similar suppression of H1N1 influenza A virus replication by BA.1 and BA.5**

285 Next, we compared the effects of BA.1 on interferon signalling and H1N1
286 replication to those of the Omicron subvariant BA.5 (Figure 5A), which is currently
287 dominant in many parts of the world [Shrestha et al., 2022].

288 BA.1 and BA.5 infection of ALI HBE cultures resulted in similar SARS-CoV-2
289 NP protein levels (Figure 5B, Figure 5C) and induced similar interferon responses as
290 indicated by the cellular levels of the interferon-stimulated gene products MX1 and
291 ISG15 (Figure 5B) as well as interferon- α/β (Figure 5D) and - λ (Figure 5E) production
292 in BA.1- and BA.5-infected ALI HBE cultures.

293

Figure 5



294

295 **Figure 5. Effects of Omicron BA.1 and BA.5 on interferon signalling and H1N1**

296 **influenza A virus replication in air-liquid-interface (ALI) human bronchial**

297 **epithelial (HBE) cell cultures. (A) Experimental design. (B) Cellular levels of the**

298 SARS-CoV-2 nucleoprotein (NP) and proteins involved in interferon signalling (MX1,
299 ISG15) in BA.1- and BA.5 (MOI 1)-infected ALI HBE cultures 48h post infection. (C)
300 Quantification of the NP immunoblot results from (B). Values represent mean \pm SD
301 from three biological replicates. P values were determined by Student's t-test. (D,E)
302 Interferon- α/β (D) or - λ (E) promotor activity in HEK reporter cell lines incubated with
303 apical washes of BA.1- or BA.5-infected ALI HBE cultures 48h post infection. (F)
304 SARS-CoV-2 NP (NP), influenza A virus NP (IAV NP), MX1, and ISG15 protein levels
305 in BA.1 (MOI 1)-infected, BA.5 (MOI 1)-infected, IAV H1N1 (MOI 2)-infected, BA.1/
306 IAV co-infected, or BA.5/ IAV co-infected ALI HBE cultures. (G,H) Quantification of
307 SARS-CoV-2 NP (G) and IAV NP (H) levels from (F) by ImageJ. (I) Genomic SARS-
308 CoV-2 RNA (RNA-dependent RNA polymerase/ RdRp gene) levels in BA.1-infected,
309 BA.5-infected, BA.1/ IAV co-infected, and BA.5/ IAV co-infected cells 72h post
310 infection. Values represent mean \pm SD from three biological replicates. (J) Genomic
311 IAV NP (gRNA) copy numbers and IAV NP mRNA levels in BA.1-infected, BA.5-
312 infected, BA.1/ IAV co-infected, and BA.5/ IAV co-infected cells 72h post infection. (K,
313 L) Interferon- α/β (K) or - λ (L) promotor activity in HEK reporter cell lines incubated with
314 apical washes of BA.1-infected, BA.5-infected, BA.1/ IAV co-infected, and BA.5/ IAV
315 co-infected ALI HBE cultures 72h post infection.

316

317 H1N1 co-infection did not significantly affect cellular SARS-CoV-2 NP levels or
318 cellular MX1 and ISG15 levels (Figure 5F, Figure 5G). However, both Omicron
319 subvariants suppressed H1N1 replication as indicated by cellular NP levels (Figure
320 5F, Figure 5H). These findings (limited impact of influenza A virus infection on SARS-
321 CoV-2 replication, BA.1- and BA.5-mediated suppression of H1N1 replication) were
322 confirmed by the determination of genomic SARS-CoV-2 RNA copy numbers (Figure

323 5I), genomic influenza A virus RNA copy numbers (Figure 5J), and H1N1 NP mRNA
324 levels (Figure 5J). Both variants also induced similar interferon responses in the
325 presence or absence of H1N1 as indicated by interferon- α/β (Figure 5K) and - λ (Figure
326 5L) production.

327 Taken together, BA.1 and BA.5 induce comparable interferon-mediated
328 antiviral states in ALI HBE cultures that prevent H1N1 replication.

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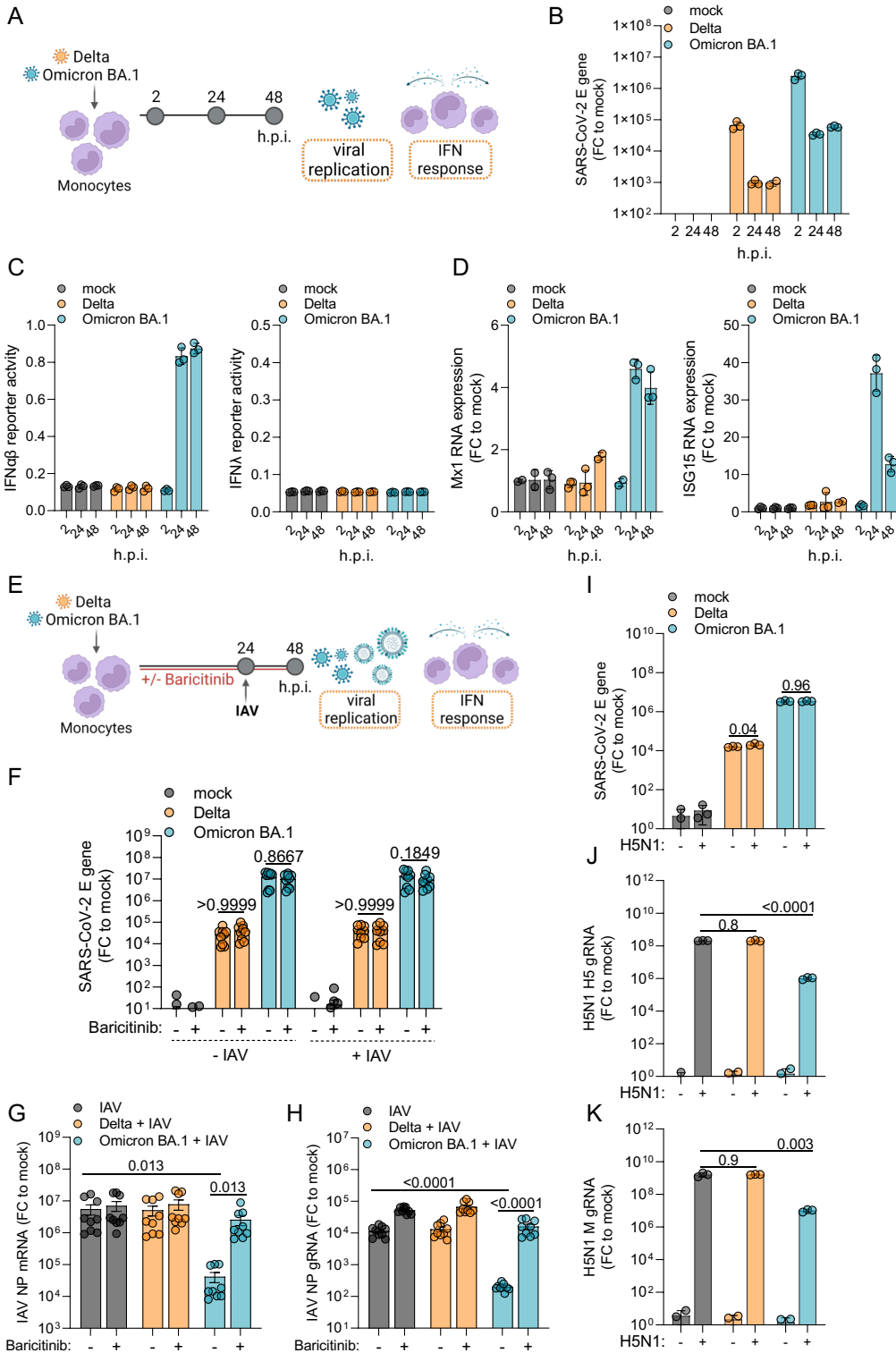
330 **BA.1-induced interferon signalling prevents H1N1 and H5N1 influenza A virus** 331 **replication in primary human monocytes**

332 Influenza A viruses can replicate in peripheral blood mononuclear cells,
333 including CD14⁺ monocytes [Lersritwimanmaen et al., 2015; Lee et al., 2017], and
334 interferon signalling in monocytes has been suggested to be a critical determinant of
335 influenza A virus pathogenesis [Hartshorn, 2020; Fourati et al., 2022]. Highly
336 pathogenic avian influenza A H5N1 virus has been described to replicate more readily
337 in monocytes and macrophages than H1N1 [Yu et al., 2011; Hoeve et al., 2012; Cline
338 et al., 2017; Lee et al., 2017; Lamichhane & Puthavathana, 2018; Lamichhane et al.,
339 2018; Westenius et al., 2018]. Hence, we finally investigated the impact of BA.1 and
340 Delta on H1N1 and H5N1 virus infection in primary human monocytes (Figure 6A).

341 The determination of SARS-CoV-2 E mRNA levels by quantitative PCR showed
342 that BA.1 and Delta did not replicate in primary human monocytes (Figure 6B), which
343 confirmed previous findings showing that SARS-CoV-2 causes abortive infections in
344 monocytes [McLaughlin et al., 2021; Junqueira et al., 2022]. Only BA.1 induced a
345 pronounced interferon response as indicated by interferon α/β signalling in a HEK
346 reporter cell line incubated with supernatants from infected monocytes (Figure 6C),
347 MX1 mRNA levels, and ISG15 mRNA levels (Figure 6D). In contrast to the findings in

348 ALI HBE cultures (Figure 1H, Figure 2K, Figure 4J, Figure 5L), supernatants from
 349 BA.1-infected monocytes did not induce interferon- λ activity in a HEK reporter cell line
 350 (Figure 6C).

Figure 6



351

352 **Figure 6. Impact of Omicron BA.1 and Delta on H1N1 and H5N1 influenza A virus**
353 **(IAV) infection of primary human monocytes.** (A) Experimental design underlying
354 (B-D). (B) SARS-CoV-2 E gene expression levels as determined by quantitative PCR.
355 Values represent mean \pm SD of three biological replicates. (C) interferon- α/β (IFN α/β)
356 (left) or IFN λ (right) activity induced by supernatants of Delta and BA.1 (MOI 1)-
357 infected primary human monocytes in HEK-reporter cell lines. (D) Mx1 (left) and ISG15
358 (right) mRNA levels in Delta and BA.1 (MOI 1)-infected primary human monocytes as
359 indicated by quantitative PCR. (E) Experimental design underlying (F-H). (F) SARS-
360 CoV-2 E mRNA levels in monocytes infected with Delta (MOI 1), BA.1 (MOI 1), Delta
361 plus H1N1 IAV (MOI 2), or BA.1 plus H1N1 IAV (MOI 2) in the absence or presence
362 of baricitinib 1 μ M. P values were determined by Student's t-test. (G, H) H1N1 IAV NP
363 mRNA (G) or genomic mRNA (H) levels in monocytes infected with Delta (MOI 1),
364 BA.1 (MOI 1), Delta plus H1N1 IAV (MOI 2), or BA.1 plus H1N1 IAV (MOI 2) in the
365 absence or presence of baricitinib 1 μ M 24h post H1N1 IAV infection. P values were
366 calculated by two-way ANOVA. (I-K) Impact of BA.1 and Delta infection on highly
367 pathogenic avian H5N1 IAV virus infection in monocytes. SARS-CoV-2 genomic E
368 RNA levels (I), genomic H5N1 H5 RNA levels (J), and genomic H5N1 IAV M levels (K)
369 in monocytes infected with Delta (MOI 1), BA.1 (MOI 1), Delta plus H5N1 strain
370 A/Vietnam/1203/04 (MOI 1), or BA.1 plus H5N1 (MOI 1). P values were calculated by
371 two-way ANOVA. All values represent mean \pm SD of monocyte preparations derived
372 from three different donors (A-H) or one donor (I-K), which were each tested in three
373 biological replicates.

374

375 Next, we tested the impact of BA.1 and Delta on H1N1 replication in primary
376 human monocytes in the absence or presence of the JAK inhibitor baricitinib (Figure

377 6E). Neither baricitinib nor H1N1 infection affect SARS-CoV-2 E mRNA levels (Figure
378 6F).

379 Similarly as in ALI HBE cultures, however, BA.1 (but not Delta) infection
380 reduced H1N1 infection as indicated by H1N1 NP mRNA (Figure 6G) and H1N1
381 genomic NP RNA levels (Figure 6H), which was prevented by baricitinib (Figure 6G,
382 Figure 6H).

383 Highly pathogenic H5N1 avian influenza A virus also did not affect SARS-CoV-
384 2 levels in monocytes as indicated by SARS-CoV-2 genomic E RNA levels (Figure 6I).
385 However, the determination of H5N1 virus genomic H5 (Figure 6J) and M (Figure 6K)
386 levels showed that abortive BA.1 but not Delta infection inhibited H5N1 replication in
387 primary human monocytes.

388 Taken together, abortive BA.1 but not Delta infection induced an interferon
389 response and reduced H1N1 and H5N1 replication in monocytes. The JAK/ STAT
390 inhibitor prevented BA.1-mediated H1N1 and H5N1 inhibition in monocytes, indicating
391 that BA.1-mediated influenza A virus inhibition is caused by BA.1-induced interferon
392 signalling.

393

394 **Comparison of the circulation of influenza-like illnesses during the Delta and** 395 **BA.1 infection waves in England**

396 An analysis comparing the spread of influenza-like illnesses (includes cases of
397 clinically diagnosed influenza with or without a virus test confirmation) and COVID-19
398 in England showed that both Delta and influenza-like illnesses surged after all
399 restrictions were removed on 19th July 2021. When BA.1 became the dominant variant,
400 however, the number of influenza-like illnesses strongly declined and has not surged
401 since (Suppl. Figure 3). These data fit with our findings showing that BA.1 but not Delta

402 induces an interferon response that prevents influenza A virus infection. Similarly, an
403 analysis of data from the John Hopkins Microbiology Laboratory found a notable
404 decrease in influenza A virus infections during the Omicron BA.1 wave between
405 November 2021 and February 2022 [Eldesouki et al., 2022]. However, future research
406 will have to analyse a possible causative relationship in more detail.

407

408

409

410 Discussion

411 Here, we investigated the impact of Delta and the Omicron subvariants BA.1
412 and BA.5 on interferon signalling in ALI HBE cultures and primary human monocytes.
413 Our results consistently showed that only Omicron causes pronounced production of
414 biologically active type I and type III interferons (Suppl. Figure 4). The kinetics of the
415 interferon type I (α/β) and type III (λ) responses provided additional mechanistic
416 evidence explaining the reduced pathogenicity of Omicron relative to Delta [Wang et
417 al., 2022]. BA.1 infection caused an early, transient interferon- α/β peak, which is
418 anticipated to mediate a protective antiviral response but also to avoid deleterious
419 inflammatory processes associated with prolonged interferon type I activation [Forero
420 et al., 2019; King & Sprent, 2021]. Moreover, BA.1 infection caused a sustained
421 interferon λ response known to inhibit virus replication and prevent excessive
422 inflammation in the respiratory tract [Forero et al., 2019; Prokunina-Olsson et al., 2020;
423 King & Sprent, 2021].

424 The Omicron-induced interferon response generated an antiviral state that
425 protected infected cells from super-infection with influenza A viruses, showing that the
426 Omicron-induced interferon response is of functional relevance. Our analysis of the
427 spread of influenza-like illnesses during the Delta and BA.1 infection waves (Suppl.
428 Figure 3) and data from the John Hopkins Microbiology Laboratory [Eldesouki et al.,
429 2022] found that the levels of influenza A virus transmission levels strongly declined
430 with the emergence of BA.1, but a causative relationship remains to be established.

431 Despite consistent BA.1-mediated interferon induction across the different cell
432 types, the relative replication kinetics of Omicron and Delta differed between the
433 models. BA.1 replicated less effectively than Delta in Caco-2F03 and Calu-3 [Bojkova
434 et al., 2022; Bojkova et al., 2022a], but (in agreement with other findings [Hui et al.,

435 2022]) faster than Delta in ALI HBE cultures. This probably reflects the contribution of
436 many factors to SARS-CoV-2 replication. The spike (S) proteins of the BA.1 and Delta
437 isolates that we used differ in 13 amino acid positions in the S receptor binding domain
438 (Suppl. Figure 5). For example, BA.1 S is known to interact differently with its cellular
439 receptor ACE2, to utilise ACE2 from a broader range of species as receptor, and to
440 mediate increased virus uptake [Cameroni et al., 2022; Hong et al., 2022; Li et al.,
441 2022; Meng et al., 2022; Rathnasinge et al., 2022; Willett et al., 2022; Zhang et al.,
442 2022].

443 Increased BA.1 uptake may also contribute to the enhanced interferon
444 response. Mechanistically, the BA.1-induced interferon response is, in agreement with
445 previous studies in other cell types [Yin et al., 2021; Bojkova et al., 2022; Bojkova et
446 al., 2022a], primarily mediated by the recognition of double-stranded RNA by the
447 pattern recognition receptor MDA5, as suggested by the lack of a BA.1-induced
448 interferon in MDA5 knock-out cells. In this context, virus uptake via the endosomal
449 pathway was described to result in greater activation of pattern recognition receptors
450 [Peacock et al., 2022].

451 BA.1 infection also induced an interferon-mediated antiviral state preventing
452 influenza A (H1N1, H5N1) infection in monocytes despite only establishing an abortive
453 infection, demonstrating that the antiviral state does not depend on a complete virus
454 replication cycle resulting in the production of infectious virus. In agreement, UV-
455 inactivated BA.1 had previously been shown to trigger a detectable interferon
456 response in lung organ cultures, although this response was weaker than that induced
457 by the replication-competent virus [Alfi et al., 2022].

458 The JAK/ STAT inhibitor baricitinib inhibited the Omicron-mediated antiviral
459 response. This suggests that the Omicron-induced antiviral state is caused by MDA5-

460 mediated production of interferon which activates interferon receptors that then trigger
461 JAK/ STAT signalling [Li et al., 2020].

462 In addition to these findings, we also showed that BA.5, which is currently the
463 dominant variant in many parts of the world [Shrestha et al., 2022], induces a
464 comparable interferon response to BA.1 and that the BA.1- and BA.5-induced
465 interferon responses protect infected cells from super-infection with influenza A
466 viruses. The latter finding demonstrates that the BA.1- and BA.5-induced interferon
467 signalling is of functional relevance. Baricitinib prevented the BA.1- and BA.5-induced
468 suppression of influenza A virus replication, providing evidence that the BA.1- and
469 BA.5-induced interferon response is responsible for the BA.1 and BA.5-induced
470 influenza A virus inhibition.

471 In summary, we show that BA.1 and BA.5 (but not Delta) induce a functionally
472 relevant pronounced interferon response that suppresses influenza A virus replication.
473 Further research will have to show the relevance of our findings in the context of
474 SARS-CoV-2/ influenza virus co-infection. Data on the severity of SARS-CoV-2/
475 influenza A virus co-infections are inconsistent in cell culture and animal models
476 [Andrés et al., 2022; Kim EH et al., 2022; Kim HK et al., 2022; Oishi et al., 2022] and
477 in humans [Cuadrado-Payán et al., 2020; Yue et al., 2020; Alosaimi et al., 2021; Stowe
478 et al., 2021; Xiang et al., 2021; Krumbein et al., 2022; Swets et al., 2022]. This may
479 not be a surprise, given the differences in interferon signalling between different
480 SARS-CoV-2 variants that we present here and that were described in previous
481 studies [Alfi et al., 2022; Bojkova et al., 2022; Bojkova et al., 2022a; Guo et al., 2022;
482 Thorne et al., 2022]. Future studies may have to include more different virus variants
483 and strains to establish a clearer picture.

484 In conclusion, our findings show that 1) BA.1 and BA.5 induce comparable
485 interferon responses in ALI HBE cultures, 2) the Omicron-induced interferon-response
486 is of functional relevance as it protects infected cells from influenza A virus replication,
487 and 3) abortive BA.1 infection of monocytes is sufficient to produce a protective
488 interferon response. Moreover, the kinetics of the Omicron-induced interferon
489 response (early and transient type I response, sustained type III response) provide
490 additional mechanistic evidence explaining why Omicron infections are usually
491 associated with less severe disease than Delta infections.
492

493 **Methods**

494 **Cell lines**

495 HEK293 (HEK-Blue™ reporter cells, InvivoGen) -IFN- α/β and -IFN- λ cells were
496 cultivated in DMEM (Gibco, ThermoFisher Scientific) with 10% heat-inactivated foetal
497 bovine serum (Sigma), Pen-Strep (100 U/ml-100 μ g/ml) (Sigma), 100 μ g/ml
498 Normocin™ (InvivoGen) and the required selective antibiotic for each cell line (IFN-
499 α/β : Blastocidin and Zeocin; IFN- λ , Blastocidin, Puromycin, and Zeocin) at 37°C and 5%
500 CO₂.

501 A549-ACE2/TMPRSS2 cells (Invivogen) were grown in DMEM supplemented with 2
502 mM L-glutamine, 4.5 g/l glucose, 10% (v/v) heat-inactivated fetal bovine serum (FBS;
503 30 min at 56 °C), PenStrep (100 U/ml-100 μ g/ml), 100 μ g/ml Normocin, 10 μ g/ml of
504 Blastocidin, 10 μ g/ml of Blastocidin, 100 μ g/ml of Hygromycin, 0.5 μ g/ml of Puromycin,
505 and 100 μ g/ml of Zeocin. A549-ACE2/TMPRSS2 MDA5 KO cells (Invivogen) were
506 grown in DMEM supplemented with 2 mM L-glutamine, 4.5 g/l glucose, 10% (v/v) heat-
507 inactivated fetal bovine serum (FBS; 30 min at 56 °C), PenStrep (100 U/ml-100 μ g/ml),
508 100 μ g/ml Normocin, 10 μ g/ml of Blastocidin, 100 μ g/ml of Hygromycin, 0.5 μ g/ml of
509 Puromycin, and 100 μ g/ml of Zeocin.

510 All cell lines were regularly tested for mycoplasma contamination.

511

512 **Air-liquid interface cultures**

513 Lung tissue for the isolation of primary epithelial cells was provided by the Hannover
514 Medical School, Institute of Pathology (Hannover, Germany). The use of tissue was
515 approved by the ethics committee of the Hannover Medical School (MHH, Hannover,
516 Germany, number 2701–2015) and was in compliance with The Code of Ethics of the
517 World Medical Association. Primary bronchial epithelial cells were isolated from the

518 lung explant tissue of a patient with lung emphysema as described previously [van
519 Wetering et al., 2000]. All patients or their next of kin gave written informed consent
520 for the use of their lung tissue for research.

521 Basal cells were expanded in Keratinocyte-SFM medium supplemented with bovine
522 pituitary extract (25 µg/mL), human recombinant epidermal growth factor (0.2 ng/mL,
523 all from Gibco, Schwerte, Germany), isoproterenol (1 nM, Sigma),
524 Antibiotic/Antimycotic Solution (Sigma-Aldrich), and MycoZap Plus PR (Lonza,
525 Cologne, Germany) and cryopreserved until further use.

526 For differentiation, the cells were thawed and passaged once in PneumaCult-
527 Ex Medium (StemCell Technologies, Cologne, Germany) and then seeded on
528 transwell inserts (12-well plate, Sarstedt, Nümbrecht, Germany) at 4×10^4 cells/insert.
529 Once the cell layers reached confluency, the medium on the apical side of the
530 transwell was removed, and medium in the basal chamber was replaced with
531 PneumaCult ALI Maintenance Medium (StemCell Technologies), including
532 Antibiotic/Antimycotic Solution (Sigma-Aldrich) and MycoZap Plus PR (Lonza). During
533 a period of four weeks, the medium was changed and the cell layers were washed
534 with PBS every other day. Criteria for successful differentiation were the development
535 of ciliated cells and ciliary movement, an increase in transepithelial electric resistance
536 indicative of the formation of tight junctions, and mucus production.

537

538 **SARS-CoV-2 variants preparation**

539 The SARS-CoV-2 isolates Omicron BA.1 (B.1.1.529: FFM-SIM0550/2021,
540 EPI_ISL_6959871, GenBank ID OL800702), Delta (B.1.167.2: FFM-IND8424/2021,
541 GenBank ID MZ315141), and Omicron BA.5 (GenBank ID OP062267) were isolated
542 in Caco-2-F03 cells as previously described [Cinatl et al., 2004; Bojkova et al., 2021]

543 and stored at -80°C . All variants underwent maximum two passages. Virus titres were
544 determined as TCID₅₀/mL.

545

546 **Influenza A virus strains H1N1 and H5N1**

547 The H1N1 influenza strain A/NewCaledonia/20/99 was received from the World
548 Health organisation (WHO) Influenza Centre (National Institute for Medical Research,
549 London, UK) and stocks were prepared by cultivation on MDCK cells (ATCC, CCL-34)
550 in medium containing 2µg/ml trypsin. Virus stocks were stored at -80°C . Virus titres
551 were determined as TCID₅₀/mL.

552 The H5N1 influenza strain A/Vietnam/1203/04 was received from the World
553 Health organisation (WHO) Influenza Centre (National Institute for Medical Research,
554 London, UK). Virus stocks were prepared by infecting Vero cells, and aliquots were
555 stored at -80°C . Virus titres were determined as TCID₅₀/mL.

556

557 **Barrier integrity measurement**

558 For trans-epithelial electrical resistance (TEER) measurement, medium was
559 added to the apical side 30min prior to measurement with a chopstick electrode
560 connected to a Volt-Ohm-meter (Millicell® ERS-2, Merck, Darmstadt, Germany)
561 according to the manufacturer's instructions. Blank inserts served as baseline. The
562 apical medium was removed after the measurement.

563

564 **Activation of caspase 3/7**

565 Caspase 3/7 activity was measured using the Caspase-Glo assay kit
566 (Promega, Madison, WI, USA), according to the manufacturer's instructions. Briefly,
567 100 µL of Caspase-Glo reagent were added to each well containing 100 µL of tested

568 sample, mixed, and incubated at room temperature for 30 min. Luminescence intensity
569 was measured using an Infinite M200 microplate reader (Tecan).

570

571 **Co-infection assay in ALI HBE**

572 ALI HBE cultures were infected with SARS-CoV-2 variant at MOI 1 from the
573 apical site. The inoculum was incubated for 2 h, then removed and cells were washed
574 three times with PBS. H1N1 A/NewCaledonia/20/99 at MOI 2 was added 48 h post
575 SARS-CoV-2 infection. The inoculum was incubated for 2 h, then removed and cells
576 were washed three times with PBS.

577

578 **Detection of extracellular and intracellular RNA**

579 SARS-CoV-2 RNA from the apical washes of the ALI HBE culture was isolated
580 using QIAamp Viral RNA Kit (Qiagen, Hilden, Germany) according to the
581 manufacturer's instructions. RNA was subjected to OneStep qRT-PCR analysis using
582 the Luna Universal One-Step RT-qPCR Kit (New England Biolabs, Frankfurt am Main,
583 Germany) and a CFX96 Real-Time System, C1000 Touch Thermal Cycler (Bio-Rad,
584 Feldkirchen, Germany). Primers were adapted from the WHO protocol²⁹ targeting the
585 open reading frame for RNA-dependent RNA polymerase (RdRp): RdRP_SARSr-F2
586 (GTG ARA TGG TCA TGT GTG GCG G) and RdRP_SARSr-R1 (CAR ATG TTA AAS
587 ACA CTA TTA GCA TA) using 0.4 µM per reaction. Standard curves were created
588 using plasmid DNA (pEX-A128-RdRP) as previously described [Bojkova et al., 2020].

589 Intracellular RNA isolation was carried out according to manufacturer's protocol
590 (RNeasy 96 QIAcube HT Kit, Qiagen, Hilden, Germany). Detection of selected targets
591 was performed with Luna® Universal One-Step RT-qPCR (New England BioLabs Inc.)
592 according to manufacturers protocol using specific primers: TBP (fw:5'-

593 ATCAGAACAACAGCCTGCC-3'; rev: 5'- GGTCAGTCCAGTGCCATAAG-3'); SARS-
594 CoV-2 E gene (fw:5'- ACAGGTACGTTAATAGTTAATAGCGT-3'; rev:5'-
595 ATATTGCAGCAGTACGCACACA-3'); ISG15 (fw:5'-GAGAGGCAGCGAACTCATCT-
596 3'; rev:5'-AGGGAC ACCTGGAATTCGTT-3'); MX1 (fw:5'-
597 TTTTCAAGAAGGAGGCCAGCAA-3'; rev:5'-TCAGGAACTTCCGCTTGTCG-3');
598 H5N1 H5 gene (fw:5'-GCCATTCCACAACATACACCC-3'; rev:5'-
599 CTCCCCTGCTCATTGCTATG-3'); H5N1 M gene (fw:5'-
600 TTCTAACCGAGGTCGAAACG-3'; rev:5'-ACAAAGCGTCTACGCTGCAG-3'); IAV
601 NP-mRNA (fw:5'- GACTCACATGATGATCTGGCA-3'; rev:5'-
602 CTTGTTCTCCGTCCATTCTCA-3'); IAV NP-gRNA (fw:5'-
603 AACGGCTGGTCTGACTCACATGAT-3'; rev:5'-
604 AGTGAGCACATCCTGGGATCCATT-3').

605

606 Immunoblot analysis

607 Whole-cell lysates were prepared using Triton-X sample buffer containing
608 protease inhibitor cocktail (Roche). The protein concentration was assessed by using
609 DC Protein assay reagent (Bio-Rad Laboratories). Equal protein loads were separated
610 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and proteins were
611 transferred to nitrocellulose membranes (Thermo Scientific). For protein detection the
612 following primary antibodies were used at the indicated dilutions: GAPDH (Cell
613 Signaling, #2118, 1:4000), γ H2AX (Cell Signaling, #9718, 1:1000), H1N1 (Influenza A
614 Virus) Nucleoprotein (Bioss, #bs-4976R, 1:4000), ISG15 (Santa Cruz Biotechnology,
615 #sc-166755, 1:200), MDA5 (Cell Signaling, #5321, 1:1000), Mx1 (Cell Signaling,
616 #37849, 1:1000), OAS1 (Cell Signaling, #14498, 1:1000), PARP (Cell Signaling,
617 #9542, 1:1000), PKR (Cell Signaling, #12297, 1:1000), SARS-CoV-2 Nucleocapsid

618 (Sino Biological, #40143-R019, 1:10000), STAT1 (Cell Signaling, #9172, 1:1000),
619 phospho-STAT1 Y701 (Cell Signaling, #9171, 1:1000), TBK1 (Cell Signaling, #3013,
620 1:1000), phospho-TBK1 S172 (Cell Signaling, #5483, 1:1000), USP18 (Cell Signaling,
621 #4813, 1:2000) and RIG1 (Cell Signaling, #3743, 1:1000). Protein bands were
622 visualised using IRDye-labeled secondary antibodies at dilution 1:40000 (LI-COR
623 Biotechnology, IRDye®800CW Goat anti-Rabbit, #926-32211 and IRDye®800CW
624 Goat anti-Mouse IgG, #926-32210) and Odyssey Infrared Imaging System (LI-COR
625 Biosciences).

626

627 **Detection of type I and type III IFNs production**

628 Detection of type I and type III IFNs in supernatants was carried out with HEK-
629 Blue™ IFN- α/β (type I) and HEK-Blue™ IFN- λ (type III) cells according to
630 manufacturer's protocol. HEK cells were washed twice with PBS and detached in
631 presence of PBS by tapping the flask. Cells were subsequently centrifuged at 200g for
632 5 min and resuspended in Test Medium (DMEM, 4.5 g/l glucose, 2 mM L-glutamine,
633 10% (v/v) heat-inactivated FBS, 50 U/ml penicillin, 50 μ g/ml streptomycin, 100 μ g/ml
634 Normocin™) at 280.000 cells/ml. 20 μ l supernatant were added in 96 well plates and
635 180 μ l cell suspension was added to the wells. Cells and supernatant were incubated
636 at 37°C and 5% CO₂ for 24h. After incubation, 20 μ l supernatant was removed and
637 incubated with 180 μ l QUANTI-Blue™ Solution for 1-3 h. SEAP (secreted embryonic
638 alkaline phosphatase) levels were determined using a spectrophotometer at 620nm.

639

640 **PBMCS isolation**

641 Human peripheral blood mononuclear cells were isolated from buffy coats of
642 healthy donors (RK-Blutspendedienst Baden-Württemberg-Hessen, Institut für

643 Transfusionsmedizin und Immunhämatologie Frankfurt am Main, Germany). After
644 centrifugation on a Ficoll (Pancoll, PAN-Biotech, Aidenbach, Germany) density
645 gradient, mononuclear cells were collected from the interface, washed with PBS, and
646 plated on cell culture dishes (Cell+, Saarstedt, Nümbrecht, Germany) in RPMI1640
647 (Gibco, ThermoFisher Scientific, Waltham, MA, USA) supplemented with 100 IU/mL
648 penicillin and 100 g/mL streptomycin. After incubation for 90 min(37°C, 5% CO₂), non-
649 adherent cells were removed, and the medium was changed to RPMI1640
650 supplemented with 100 IU/mL penicillin, 100 µg/mL of streptomycin, and 3% human
651 serum (RK-Blutspendedienst Baden-Württemberg-Hessen, Institut für
652 Transfusionsmedizin und Immunhämatologie Frankfurt am Main, Germany).

653

654 **Co-infection assay in human PBMCs**

655 Human PBMCs were infected with SARS-CoV-2 variants at MOI of 1 for 2 h in
656 infection medium (RPMI1640 supplemented with 100 IU/mL penicillin and 100 g/mL
657 streptomycin, 1% heat-inactivated fetal bovine serum) at 37°C at 5% CO₂. Afterwards
658 the cells were washed twice with PBS and incubated for 24 h in infection medium. For
659 co-infection, the cells were either infected with influenza A virus H1N1/New
660 Caledonia/20/99 (MOI 2), influenza A virus H5N1 A/Vietnam/1203/04 (MOI 1) or
661 treated with medium for 2 h, washed twice and incubated again for 24 h. After each
662 washing step and after 48 h supernatant samples were taken and cells were lysed for
663 RNA isolation.

664

665 **Statistics**

666 Results are expressed as the mean ± standard deviation (SD) of the number of
667 biological replicates indicated in figure legends. Statistical significance is depicted

668 directly in graphs and the statistical tests used for the calculation of p values are
669 indicated in the figure legends. GraphPad Prism 9 was used for visualisation of the
670 data and for calculation of statistical significance.
671

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678 **Competing interests**

679 The authors declare no competing interests.

680

681

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