Legionella pneumophila inhibits type I interferon signaling to avoid cell-intrinsic host cell defense.

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Abstract:

The host type I interferon (IFN) response protects against a *Legionella pneumophilia* infection. Other pathogens inhibit type I IFN-mediated cell signaling; however, the interaction between this signaling pathway and *L. pneumophila* has not been described. This study demonstrates that *L. pneumophila* inhibits the IFN-β signaling pathway in an IFN-β dose-dependent manner. However, *L. pneumophila* does not inhibit IFN-γ-mediated cell signaling. The addition of IFN-β to *L. pneumophila*-infected macrophages limited bacterial growth in a nitric oxide (NO)-dependent pathway since inhibiting NO production via a chemical inhibitor increased bacterial growth. Finally, this study demonstrates that the type IV secretion system of *L. pneumophila* is required to inhibit IFN-β-mediated cell signaling. In conclusion, this study describes a novel host cell signaling pathway inhibited by *L. pneumophila,* which improves the survival of bacteria in infected macrophages.

30 **Introduction:**

31 *Legionella* species are ubiquitous in the environment and have adapted to survive within
32 freshwater amoebas (Rowbotham, 1980; Boamah et al., 2017). The bacteria rely on their type 4
33 secretion system (T4SS), named the defective in organelle trafficking and intracellular
34 multiplication (Dot/Icm), to inject proteins into the host cell to manipulate host response
35 pathways and increase their chances of survival (Berger and Isberg, 1993; Brand et al., 1994;
36 Segal and Shuman, 1998; Vogel et al., 1998). *Legionella* can infect a wide variety of amoeba
37 species and, thus, through evolution, carry multiple versions of bacterial effectors to
38 successfully modify the response of various hosts (Park et al., 2020). Humans are accidental
39 hosts that can be exposed to *Legionella* via the aerosolization of contaminated water supplies,
40 which often results in respiratory infections (McDade et al., 1977).

41 Type I interferons (IFNs), such as IFN-β, bind to the heterodimeric interferon-α/β
42 receptor (IFNAR) and induce cell signaling, leading to the upregulation of hundreds of IFN-
43 stimulated genes (ISGs) (Stetson and Medzhitov, 2006; Lazear et al., 2019). Type I IFNs are
44 critical for the host defense against viral pathogens (Stetson and Medzhitov, 2006; Lazear et al.,
45 2019). However, during bacterial infections, the activity of type I IFNs is more complex, where in
46 some cases, they mediate host resistance, and in other cases, they increase the susceptibility
47 (Boxx and Cheng, 2016; Peignier and Parker, 2021). *L. pneumophila* has been shown to induce
48 IFN-β production by host cells via various cytosolic surveillance pathways, including the nucleic
49 acid surveillance pathway (Liu and Shin, 2019). Type I IFNs then signal through IFNARs
50 expressed on the cell surface of infected and bystander cells, contributing to *L. pneumophila*
51 resistance observed in mice (Lippmann et al., 2011). Multiple studies have demonstrated that
52 cell-intrinsic host defense mechanisms, including the production of nitric oxide (NO) (Plumlee et
53 al., 2009) and itaconic acid (IA) (Naujoks et al., 2016), are triggered by exogenous IFN-β and
54 subsequent IFNAR signaling during *ex vivo L. pneumophila* infections (Schiavoni et al., 2004;

55 Opitz et al., 2006; Plumlee et al., 2009; Majoros et al., 2016). Thus, the IFN-β response plays a
56 role in determining the outcome of *L. pneumophila*.
57 Here, we demonstrate that *L. pneumophila* has a capacity to inhibit IFN-β-mediated cell
58 signaling. Additionally, we show that the ability to reduce IFN-β signaling depends on the T4SS
59 of *L. pneumophila* and thus is likely to be mediated by secreted effectors. The inhibition of the
60 IFN-β response pathway improves the intracellular survival of *L. pneumophila* by limiting the
61 cellular production of NO.
62

63 **Materials and Methods:**

64 **Cell culture and mice**

65 *Ifnb*$^{-/-}$ mice were originally obtained from Dr. E. Fish (University of Toronto) and have
66 been previously described (Deonarain et al., 2000). Bone marrow-derived macrophages
67 (BMDMs) were prepared from bone marrow cells flushed from the tibias and femurs of these
68 mice. The BMDMs were cultured in Gibco Dulbecco's Modified Eagle Medium (DMEM)
69 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin,
70 and 20% L929 conditioned medium for six days prior to infection. L929 conditioned medium was
71 used for all the BMDM infection experiments. RAW 264.7 and RAW-Lucia ISG-KO-IRF3 cells
72 (InvivoGen, rawl-koirf3) were grown in DMEM supplemented with 10% heat-inactivated FBS.
73 RAW-Lucia ISG-KO-IRF3 cells were plated in FBS-free DMEM 16 hours before the infection
74 since the absence of FBS reduces the background activation of the integrated ISRE-Luciferase
75 gene cassette.

76 **Ethics statement**

77 All animals were handled in accordance with the NIH guidelines for the housing and care
78 of laboratory animals. Studies were approved by the Institutional Animal Care and Use
79 Committee at the University of Maryland.

80 **Bacteria**

81 *L. pneumophila* Philadelphia-1 strain Lp02 were cultured at 37°C in liquid N-(2-
82 87Acetamido)-2-aminoethanesulfonic acid (ACES)-buffered yeast extract (AYE) medium or on
83 solid charcoal ACES-buffered yeast extract (CYET) containing 0.4 mg/ml L-cysteine (Sigma),
84 0.135 mg/ml ferric nitrate (Sigma), and 0.1 mg/ml thymidine (Sigma). The *L. pneumophila* Lp02
85 *dotA- deficient* mutant was previously described (Berger and Isberg, 1993).

86 **Cellular infections**

87 *L. pneumophila* were grown in AYE medium to the post-exponential phase (A600 = 3.7–
88 4.1). Raw 264.7 cells, RAW-Lucia ISG-KO-IRF3 cells, or BMDMs were infected with *L.*

89 *pneumophila* for two hours at the indicated multiplicity of infections (MOI). The cells were
90 washed three times with phosphate-buffered saline (PBS) and incubated in DMEM media
91 supplemented with or without IFN for the given time.
92
93 **IFN-β enzyme-linked immunosorbent assay (ELISA)**
94 The levels of secreted IFN-β were measured by ELISA (R&D Systems). RAW 264.7
95 cells were infected with *L. pneumophila* at an MOI of 0.05 for four hours. The supernatant of *L.*
96 *pneumophila* infected and uninfected cells were collected 24-, 48-, 72-, and 96-hours post-
97 infection (h.p.i). The ELISA was performed according to the manufacturer's instructions, and the
98 IFN-β signal was measured by a plate reader (BioTek ELx800).
99
100 **Luciferase Reporter Assay**
101 RAW-Lucia ISG-KO-IRF3 cells were infected with *L. pneumophila* at an MOI of 10 for 20
102 hours. IFN-β (R&D Systems) or IFN-γ (S&D Systems), at the indicated concentrations, was
103 added to the post-infection media at 0 h.p.i. The supernatant from the infected and uninfected
104 cells was collected, and the amount of type I IFN signaling was indirectly measured using
105 QUANTI-Luc Gold, following the manufacturer's protocol.
106
107 **Bacterial Survival**
108 Macrophages were infected with *L. pneumophila* at the indicated MOI. Cells were
109 collected at the provided time points and lysed in sterile water. The bacteria were enumerated
110 based on the colony forming units (CFUs) from plating cellular lysates on the CYE medium agar
111 plates.
112
113 **Antibody neutralization**

114 Antibody neutralization studies were performed using *InVivo*Plus anti-mouse IFNAR1
115 and *InVivo*MAB mouse IgG1 isotype control antibodies (Bio X Cell). Antibodies were added to
116 the infected cells at 0 h.p.i. at a final concentration of 20 μg/ml.
117
118 **NO assay**
119 The production of NO in response to IFN-β was quantified using a Griess assay kit
120 (Promega) following the manufacturer's protocol. Briefly, the supernatants from the infected
121 cells with or without IFN-β were collected at the indicated time points. The production of nitrite, a
122 derivative of NO, was measured at 548 nm using a microplate reader (BioTek). The nitrite
123 concentration was determined using a sodium nitrite standard curve.
124
125 **Statistical Analysis**
126 All statistical analyses were performed using GraphPad 9.0. For all the experiments, the
127 data are shown as the mean ± standard deviation (*SD*) of three biological replicates unless
128 otherwise noted. Significance is indicated by a *p*-value less than or equal to 0.05.

129 **Results:**

130 To test if *L. pneumophila* infections activated IFN-β production, RAW 264.7 cells were
131 infected with *L. pneumophila* at an MOI of 0.05 for four hours, rinsed, and the amount of
132 secreted IFN-β was measured at 24-hour intervals via an ELISA, where each data point is
133 equivalent to a single round of infection. After 24 hours, 163.5 ± 55.2 pg/ml of IFN-β was
134 detected and stayed constant through 48 h.p.i,, which was a ten-fold increase compared with
135 the uninfected cells. At 72 h.p.i., the level of IFN-β increased to 342.4 ± 103.9 pg/ml and
136 continued to rise to 460.9 ± 109.5 pg/ml by 96 h.p.i. (Figure 1A). These data indicate that *L.*
137 *pneumophila* induces IFN-β production in RAW 264.7 macrophages and that IFN-β
138 accumulates over time after the infection.

139 Since it has been previously reported that *Mycobacterium tuberculosis* and *Shigella*
140 *sonnei* inhibits IFNAR signaling in infected cells (Banks et al., 2019; Alphonse et al., 2022) we
141 aimed to determine if *L. pneumophila* inhibited type I interferon signaling. RAW-Lucia ISG-KO-
142 IRF3 cells were used for these studies because they are deficient in *irf3*$^{-/-}$ which means that
143 infection with *L. pneumophilia* will not induce production of endogenous IFN-β. Consequently,
144 the only IFN-β detected by the IFN-β-inducible reporter gene is the IFN-β which we are adding
145 exogenously to the cells. Cells were infected with *L. pneumophila* in the presence of increasing
146 amounts of IFN-β (200–2000 pg/ml) for 20 hours to measure the effect *L. pneumophila* has on
147 IFN-β-mediated cell signaling. The secretion of luciferase into the supernatant is representative
148 of the activity of the IFN-β responsive promotor. The secreted luciferase was measured via a
149 chemiluminescence assay. Comparing the response of infected cells to the uninfected cells,
150 cells challenged with *L. pneumophila* showed lower levels of IFN-β-associated gene expression
151 at all the doses of IFN-β tested (Figure 1B). The reduction in the transcriptional response was
152 most significant at the lower doses of IFN-β, with a 75% decrease relative to the uninfected
153 cells. At the highest IFN-β dose (2000 pg/ml), the transcriptional response between the infected
154 and uninfected cells was reduced by 20% (Figure 1C). However, the IFN-β signaling pathway

155 reduction was reduced in a dose-dependent manner, suggesting that as the IFN-β concentration
156 increases, *L. pneumophila*'s ability to impede the signaling pathway is overwhelmed.
157 The IFN-β and IFN-γ signal transduction pathways share some of their intracellular
158 signaling components and thus we asked the question if *L. pneumophila* can also inhibit IFN-γ
159 signaling. The RAW-Lucia ISG-KO-IRF3 reporter construct is also induced by IFN-γ signaling
160 and could thus serve as a model to study the potential of *L. pneumophila* to inhibit IFN-γ
161 signaling. An increasing amount of IFN-γ (200–2000 pg/ml) was added to *L. pneumophila*-
162 infected or uninfected cells, and the luciferase production was analyzed (Figures 1C and D).
163 Overall, there was no reduction in the IFN-γ response in the infected cells; however, at IFN-γ
164 doses of 1000 and 2000 pg/ml, there was a significant increase in transcription response in the
165 *L. pneumophila*-infected cells compared with the uninfected cells (Figures 1D and E). These
166 results indicate that *L. pneumophila* partially inhibits IFN-β signaling while not affecting IFN-γ
167 signaling.
168 Since IFN-β has been shown to contribute to the clearance of *L. pneumophila* in mice
169 (Lippmann et al., 2011), *ifnb*$^{-/-}$-derived BMDMs were infected with *L. pneumophila* and treated
170 with IFN-β to determine the effects of IFN-β on bacterial survival within macrophages. The CFUs
171 were determined from *L. pneumophila*-infected *ifnb*$^{-/-}$-derived BMDMs that were untreated or
172 treated with 1000 pg/ml IFN-β. The untreated *ifnb*$^{-/-}$-derived BMDMs cells showed a delayed
173 reduction compared with the IFN-β-treated cells in the percentage of CFUs starting at 48 h.p.i.
174 and continued until 96 h.p.i. (Figure 2A). The reduction in the percentage of CFUs was
175 significantly different between the IFN-β treated and untreated groups, indicating that IFN-β
176 signaling decreases *L. pneumophila* survival and enhances *L. pneumophila* clearance in *ifnb*$^{-/-}$-
177 derived BMDMs. The decrease in CFUs in the untreated *ifnb*$^{-/-}$-derived BMDMs could indicate
178 that another innate cellular immune response independent of IFN-β, such as TNFα (Liu et al.,
179 2008), contributes to the clearance of *L. pneumophila* in these cells.

180 To determine if the protective role of IFN-β is due to IFN-receptor signaling, *L.*
181 *pneumophila*-infected RAW-Lucia ISG-KO-IRF3 cells were treated with IFNAR1-neutralizing
182 antibodies with or without IFN-β. The RAW-Lucia ISG-KO-IRF3 cells were infected with *L.*
183 *pneumophila* at an MOI of 1, and then 20 µg/ml of IFNAR1-neutralizing or isotype control
184 antibodies were added. The IFN-β treated groups were then treated with 1000 pg/ml IFN-β. In
185 contrast to the BMDMs, RAW-Lucia ISG-KO-IRF3 cells have been shown to support the
186 intracellular growth of *L. pneumophila* (Gilchrist et al., 2020). The addition of IFN-β to the RAW-
187 Lucia ISG-KO-IRF3 cells did not affect the growth for the first two time points (24 and 48 h.p.i.),
188 but at 72 and 96 h.p.i. there was a significant reduction in the CFUs in the isotype IFN-β-treated
189 cells compared with the untreated isotype cells (Figure 2B). However, blocking the IFNAR
190 receptor by the IFNAR1-neutralizing antibodies inhibited the IFN-β-mediated reduction in the
191 CFUs (Figure 2B). These results indicate that IFN-β plays a role in the clarence of *L.*
192 *pneumophila* in macrophages and that this clarence results from signaling through the IFNAR1
193 receptor.
194 Nitric oxide (NO) has been shown to have cell-intrinsic anti-bacterial activity and limits
195 the growth of *L. pneumophila* (Plumlee et al., 2009). NO production has been shown to be
196 induced via the IFN-β signaling pathway in RAW 264.7 cells in response to lipopolysaccharide
197 through the induction of inducible nitric-oxide synthase (iNOS) (Jacobs and Ignarro, 2001).
198 Therefore, the NO production was analyzed using a Griess assay, which detects nitrite as a
199 proxy for the induced production of NO. *L. pneumophila*-infected RAW-Lucia ISG-KO-IRF3 cells
200 showed a slight increase in nitrite levels in the IFN-β-treated cells (1000 pg/ml) compared with
201 untreated cells at 24 h.p.i. (Figure 3A). After 48 hours, the IFN-β treatment increased the nitrite
202 level to about 18 μM; however, the nitrite level in the untreated cells was about 5 μM. The IFN-β
203 treatment led to nitrite production of about 25 μM at 96 hours, whereas the level in the untreated
204 cells remained low and was around 8 μM (Figure 3A). These results indicate that IFN-β

205 signaling induced NO production in the RAW-Lucia ISG-KO-IRF3 cells and that this production
206 is reduced in cells infected with *L. pneumophila* through the *L. pneumophila*-associated
207 inhibition of the IFNAR signaling pathway.

208 To determine if the reduced NO production influenced the clearance of *L. pneumophila,*
209 Raw-Lucia ISG-KO-IRF3 cells were infected with *L. pneumophila* and then were treated with or
210 without the iNOS inhibitor L-NMMA and with or without IFN-β. First, it was determined that L-
211 NMMA had a dose-dependent effect on the nitrite production of RAW-Lucia ISG-KO-IRF3 cells.
212 At the highest dose of L-NMMA, the nitrite production was approximately 12 μM, about 15% of
213 the nitrite produced by the untreated cells (Figure 3B). Next, RAW-Lucia ISG-KO-IRF3 cells
214 were infected with *L. pneumophila* and treated with or without IFN-β and with the solvent control
215 (DMSO) or 12 μM of L-NMMA to observe the effect of NO production on the survival of *L.*
216 *pneumophila* (Figure 3C). For all the experimental groups, a similar bacteria growth was
217 observed up to 48 h.p.i. At 72 h.p.i., there was a ten-fold reduction in the CFUs observed in the
218 DMSO and IFN-β-treated cells compared with the DMSO-treated cells. In contrast, the L-
219 NMMA-treated cells had a similar growth regardless of whether IFN-β was added (Figure 3C).
220 At the final measured timepoint of 96 h.p.i., the CFU difference between the DMSO and IFN-β-
221 treated cells was reduced about 100-fold compared with the DMSO-treated cells. However,
222 there was no significant fold reduction in the CFUs between the L-NMMA DMSO and L-NMMA
223 IFN-β-treated cells (Figure 3C). These results suggest that IFNAR signaling stimulates cellular
224 NO production, which leads to the clearance of *L. pneumophila*, and through the inhibition of the
225 IFN-β signaling pathway, *L. pneumophila* gains some resistance to the cellular anti-bacterial
226 response.

227 *L. pneumophila* T4SS Dot/Icm is a critical virulence factor that deploys effector proteins
228 to the host cell to manipulate host cellular processes (Berger and Isberg, 1993; Brand et al.,
229 1994; Segal and Shuman, 1998; Vogel et al., 1998). One mechanism by which *L. pneumophila*

230 might inhibit IFN-β signaling is through the activity of a secreted effector. To test this hypothesis,
231 an *L. pneumophila dotA*- deficient mutant was used to explore the importance of the Dot/Icm
232 system and, thus, the effectors in inhibiting the IFN-β signaling pathway. RAW-Lucia ISG-KO-
233 IRF3 cells were infected with wild-type *L. pneumophila* or a *dotA*-deficient mutant at an MOI of
234 10 and were treated with 600, 1000, or 2000 pg/ml of IFN-β for 20 hours (Figure 4A). The IFN-
235 β-mediated signaling was reduced in the cells infected with the wild-type bacteria by
236 approximately 60% for all the tested IFN-β concentrations (Figure 4A). In contrast, the *dotA*-
237 deficient mutant did not reduce the IFN-β-mediated signaling as much as the wild-type *L.*
238 *pneumophila* (Figure 4A). In addition to the wild-type and *dotA*- deficient mutant, RAW-Lucia
239 ISG-KO-IRF3 cells were infected with heat-inactivated wild-type and *dotA*- deficient mutant
240 bacteria to explore if the IFN-β inhibition requires live bacteria or if the inhibition is a result of
241 pre-existing/heat resistant bacterial lipids. The heat inactivation was achieved by incubating the
242 bacteria at 70°C for 30 minutes. The two heat-inactivated bacterial strains did not inhibit the IFN-
243 β signaling compared with the uninfected Raw-Lucia ISG-KO-IRF3 cells (Figure 4A). Overall,
244 these results suggest that the inhibition of the IFN-β pathway results from an effector secreted
245 through the Dot/Icm T4SS.
246 Next, the effect of impairing the T4SS on the capacity of infected cells to produce NO
247 upon IFN-β stimulation was analyzed. Uninfected RAW-Lucia ISG-KO-IRF3 cells treated with
248 100 ng/ml LPS and 1000 pg/ml IFN-β were used as a positive control. The wild-type *L.*
249 *pneumophila*-infected cells had significantly less NO production than the dot*A*- deficient mutant
250 and the positive control upon treatment with 1000 pg/ml IFN-β (Figure 4B). The level of NO
251 produced was not significantly different between the *dotA- deficient* mutant *L. pneumophila*-
252 infected cells and the positive control. Overall, these results demonstrate that the Dot/Icm T4SS
253 is necessary for the bacteria to inhibit IFN-β-mediated cell signaling and suggest that one or

254 more *L. pneumophila* effectors secreted into the host cell are responsible for inhibiting the
255 IFNAR signaling pathway.
256
257 **Discussion**
258 Type I IFNs have mainly been shown to play a protective role during viral infections
259 (Lazear et al., 2019); however, the role of type I IFNs is more complex in bacterial infections
260 since IFN-β has been shown to produce protective and detrimental effects depending on the
261 bacterial genus (Boxx and Cheng, 2016; Peignier and Parker, 2021). Previous studies have
262 identified other bacteria that inhibit IFN-β signaling through various means. For instance, *M.*
263 *tuberculosis* has been shown to inhibit IFN-β signaling by limiting the activation of IFNAR-
264 associated tyrosine kinase (Banks et al., 2019). Conversely, *Shigella* has been shown to inhibit
265 IFN signaling through the effector OspC, which binds to calmodulin, inhibiting calmodulin kinase
266 II and Stat1 phosphorylation (Alphonse et al., 2022). While previous studies have focused on
267 IFN-β and other bacteria, our work is the first to demonstrate that IFN-β plays a protective role
268 against an *L. pneumophila* infection and contributes to the clarence of *L. pneumophila.*
269 However, our results also demonstrate that *L. pneumophila* employs mechanisms to inhibit IFN-
270 β signaling to enhance their survival.
271 Our study suggests that *L. pneumophila* employs an effector(s) to inhibit the IFN-β
272 response since a T4SS-deficient mutant fails to inhibit IFNAR signaling. Future studies should
273 focus on defining the effector(s) and the mechanisms by which *L. pneumophila* disrupts the IFN-
274 β signaling pathway. *L. pneumophila* has adapted to survive in amoebas not human
275 macrophages. Additionally, amoebas do not have an equivalent receptor to IFNAR, or a class of
276 receptors associated with tyrosine kinases (Goldberg et al., 2006). The evolutionary advantage
277 for *L. pneumophila* to inhibit IFNAR-signaling when amoebas lack equivalent receptors is
278 currently unknown. However, amoebas, such as *Dictyostelium sp.* and *Acanthamoeba sp.,*
279 express a homolog to the mammalian STAT proteins (Kawata et al., 1997; Kicinska et al.,

280 2014), and STAT1 and STAT2 are secondary signaling components activated via
281 phosphorylation by receptor-associated kinases during IFNAR signaling (Lazear et al., 2019). In
282 addition, the amoeba STAT1 is closely related to the human and mouse STAT2 (Kicinska et al.,
283 2014), which is the STAT isoform involved in type I, but not type II, IFN signaling (Villarino et al.,
284 2020). Consequently, *L. pneumophila* could have evolved mechanisms to suppress STAT1-
285 mediated transcriptional regulation which allows the growth of *L. pneumophila* in amoebas, and
286 these mechanisms might have a similar effect on mammalian STAT2. Due to the homology of
287 mammalian STAT2 with the amoeba STAT1 (Kicinska et al., 2014), we hypothesize that IFNAR
288 signaling could be inhibited at the STAT2 level; however, future studies are needed to elucidate
289 the role of STAT2 in *L. pneumophila*-mediated inhibition of IFN-β signaling.
290

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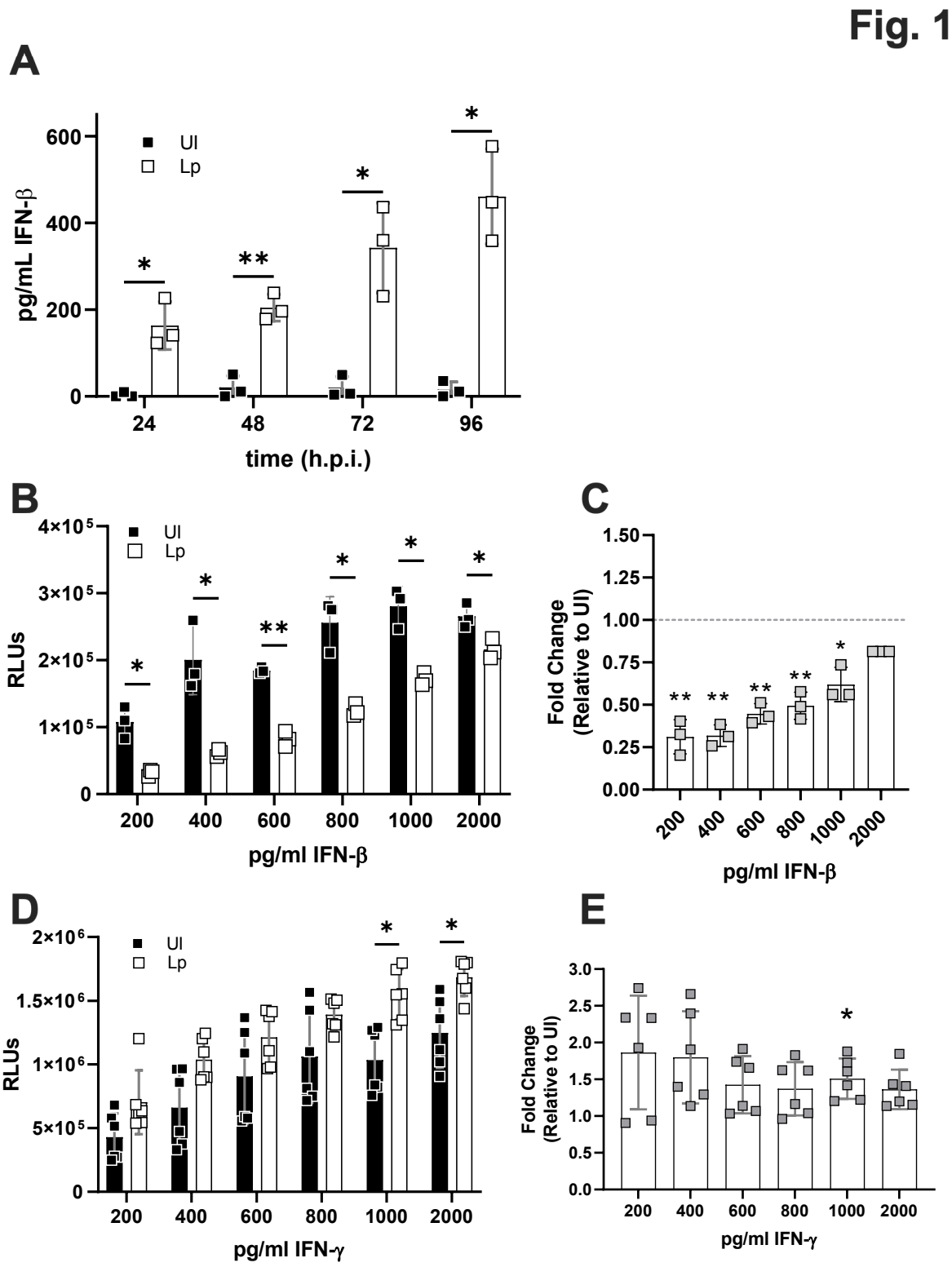
295



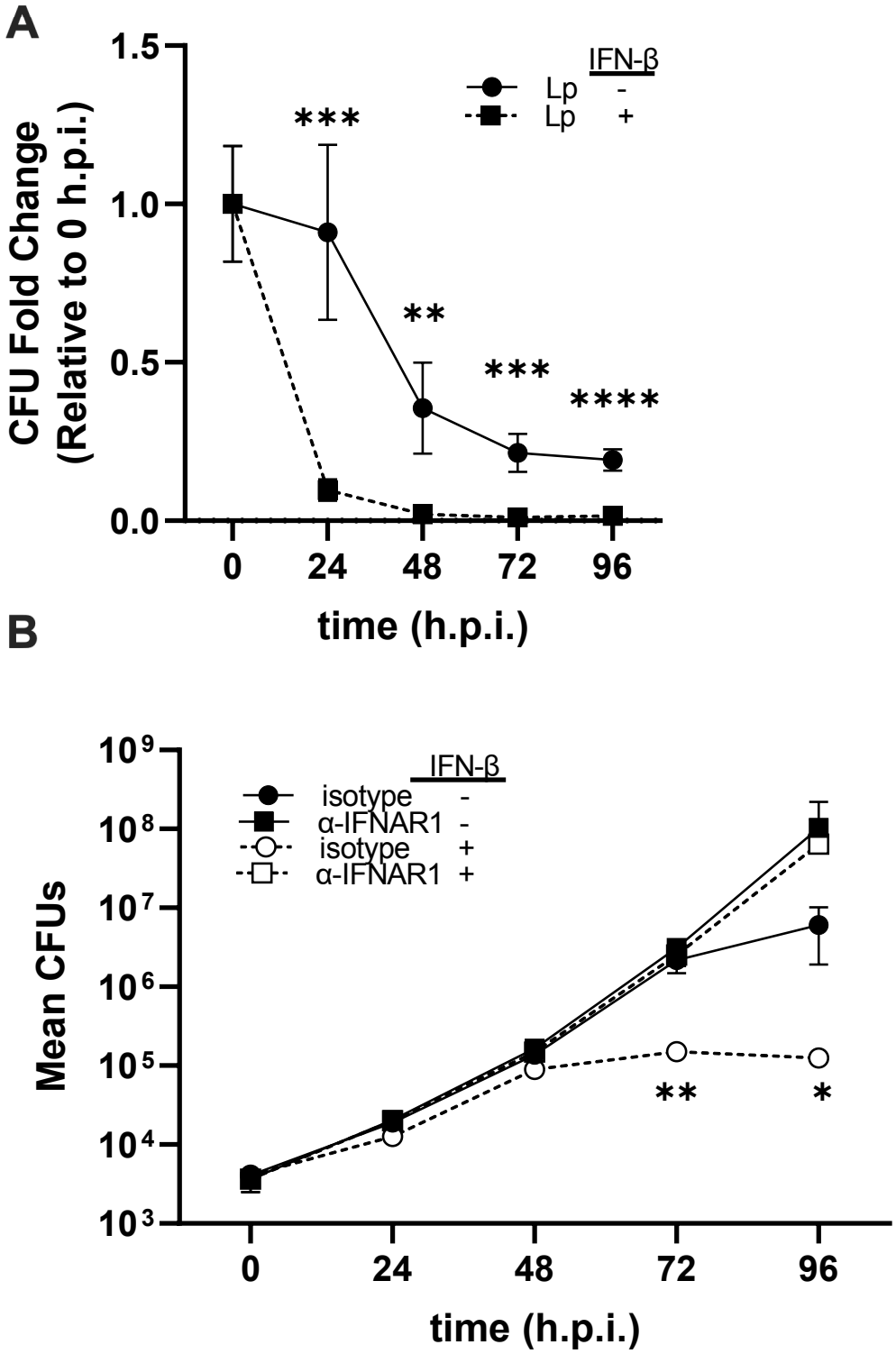
296

297 **Figure 1: *Legionella pneumophila* inhibits type I but not type II IFN signaling.** (A) RAW

298 264.7 cells were infected with *L. pneumophila* (Lp), and the amount of secreted IFN-β was

299 detected by an ELISA. (B) Infected or uninfected cells were challenged with increasing
300 quantities of IFN-β, and the reporter ISG-response was measured after 20 hours post-infection
301 (h.p.i.). (C) The fold change of the raw data in B compared with the uninfected cells. (D) Infected
302 or uninfected cells were challenged with increasing quantities of IFN-γ, and the reporter ISG-
303 response was measured after 20 hours post-infection (h.p.i.). (E) The fold change of the raw
304 data in D compared with the uninfected cells. Individual points indicate the averages of
305 independent experiments. Statistical analyses were done using multiple unpaired *t*-tests with
306 Welch correction for each time point (A) or each dose concentration of IFN-β or IFN-γ (B, D).
307 The fold change is relative to the uninfected cells for the same treatment dose of IFN-β or IFN-
308 γ. Statistical analyses were done using multiple unpaired *t*-tests with Welch correction, and each
309 fold change was compared to the fold change of the uninfected cells for each treatment dose of
310 IFN-β or IFN-γ (C, E). Significance is indicated as follows: $^{*}p \leq 0.05$, and $^{**}p \leq 0.01$.
311

Fig. 2


A
CFU Fold Change
(Relative to 0 h.p.i.)
1.5
1.0
0.5
0.0
IFN-β
Lp -
Lp +
***
**
***
****
0
24
48
72
96
time (h.p.i.)
B
Mean CFUs
10^9
10^8
10^7
10^6
10^5
10^4
10^3
IFN-β
isotype -
α-IFNAR1 -
isotype +
α-IFNAR1 +
**
*
0
24
48
72
96
time (h.p.i.)

312

313 **Figure 2: IFN-β-treatment increases cell-intrinsic resistance to *L. pneumophila*.** (A) Bone
314 marrow-derived macrophages (BMDMs) from ifnb$^{-/-}$ mice were infected with *L. pneumophila* (Lp)
315 and treated with 1000 ng/ml IFN-β throughout the infection. The number of colony-forming units
316 (CFUs) was counted at the listed time points and normalized to the CFUs of the untreated
317 control cells at 0 hours post-infection (h.p.i.). (B) RAW-ISG-KO-IRF3 cells were infected with *L.*
318 *pneumophila* (Lp) and treated with 1000 ng/ml IFN-β throughout the infection. Isotype control
319 antibodies or IFNAR1-blocking antibodies and IFN-β were added to cells simultaneously. The
320 points are the mean ± standard deviation (*SD*) of two biological replicates. (A) An asterisk
321 indicates multiple unpaired *t*-tests with Welch correction relative to the untreated cells and (B)
322 comparing isotype ± IFN-β to the untreated isotype cells or α-IFNAR1 ± IFN-β to the untreated
323 α-IFNAR1 at each time point, where *$p \leq 0.05$ and **$p \leq 0.01$.
324

Fig. 3

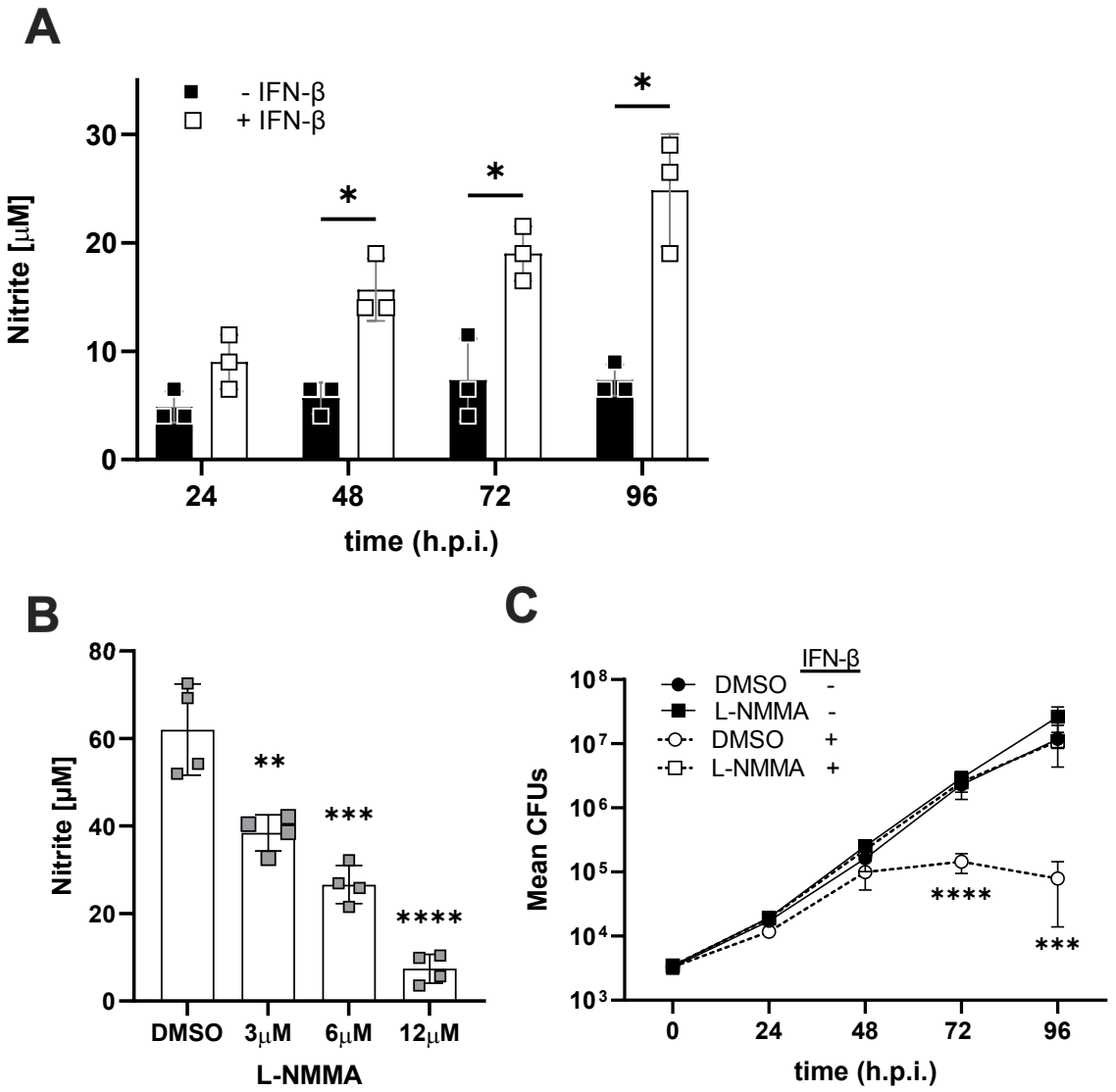
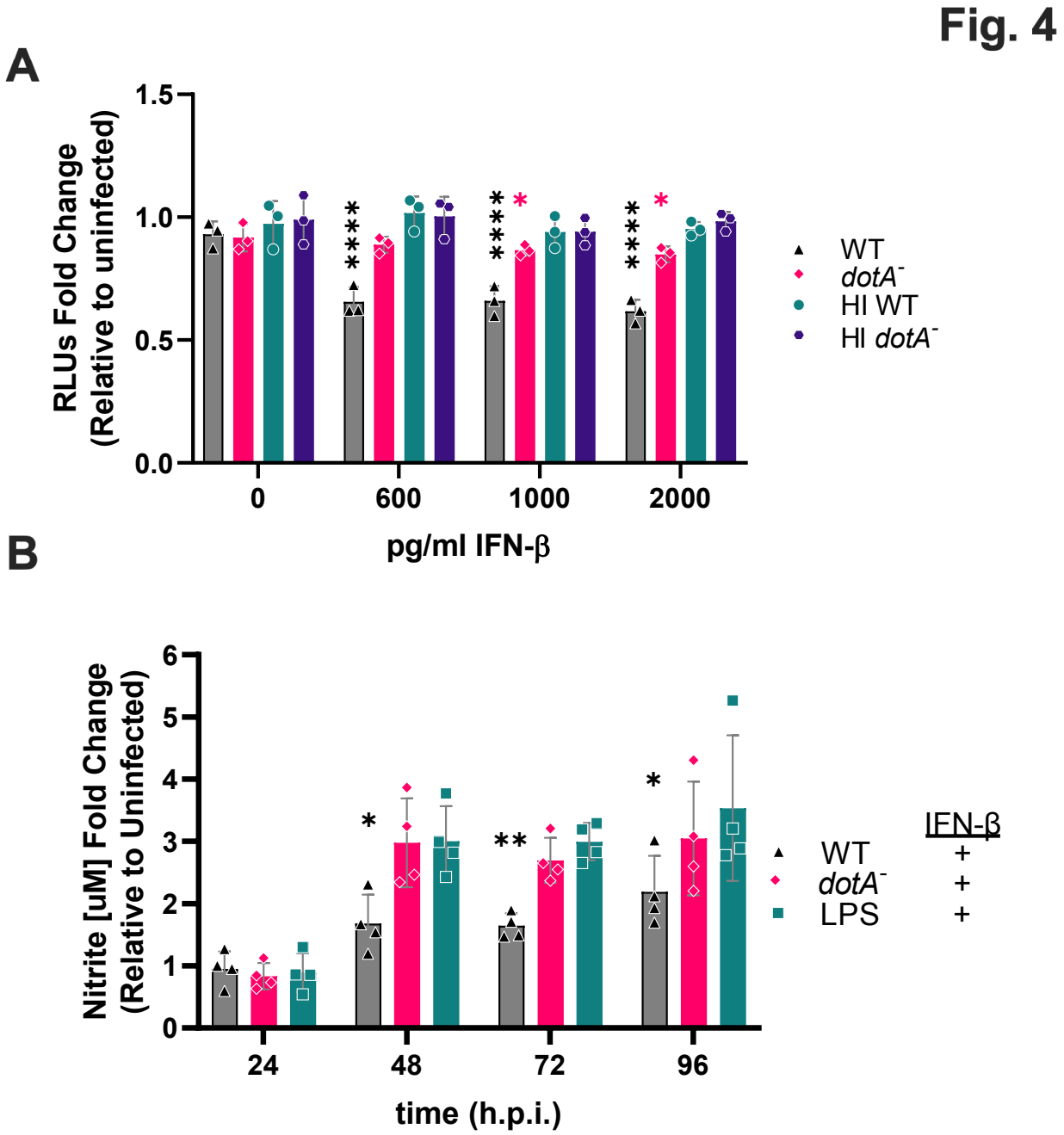


Figure 3: IFN-β-treatment increases cell-intrinsic resistance to *L. pneumophila* via increased NO production. (A) RAW-ISG-KO-IRF3 cells were infected with *L. pneumophila* (Lp) at an MOI of 10. The amount of produced nitrite was measured using a Griess assay and shown as the averages of three individual experiments. (B) The dose-response analysis of the iNOS2 inhibitor L-NMMA on RAW-ISG-KO-IRF3 cells is the averages of four independent experiments. (C) RAW-ISG-KO-IRF3 cells were infected with *L. pneumophila* (Lp) at MOI of 1 and treated with 1000 ng/ml IFN-β and solvent control (DMSO) or L-NMMA. The averages of three

333 independent experiments are shown. Statistical analyses were done using an unpaired *t*-test
334 with Welch correction per row with individual variances (A), a repeat-measure (RM) one-way
335 ANOVA (B), or an unpaired *t*-test with Welch correction comparing DMSO ± IFN-β or L-NMMA ±
336 IFN-β (C). Significance is indicated as follows: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq$
337 0.0001.
338



339

340 **Figure 4: The inhibition of IFN-β signaling by *L. pneumophila* is dependent on the type IV**

341 **secretion system.** (A) RAW-ISG-KO-IRF3 cells were infected with wild-type *L. pneumophila*

342 (WT), the *L. pneumophila dotA*- deficient mutant (*dotA*$^-$), the heat-inactivated wild-type *L.*

343 *pneumophila* (HI WT), or the heat-inactivated *dotA*- deficient mutant (HI *dotA*$^-$) at an MOI of 10.

344 All experimental groups were treated with 1000 ng/ml IFN-β. The fold differences between the

345 wild-type, *dotA*- deficient mutant, heat-inactivated wild-type *L. pneumophila,* and heat-
346 inactivated *dotA*- deficient mutant were determined. Data points are shown as the averages of
347 three independent experiments. (B) RAW-ISG-KO-IRF3 cells were infected with the wild-type *L.*
348 *pneumophila* (WT) or the *L. pneumophila dotA*- deficient mutant (*dotA*$^{-}$) at an MOI of 10 and
349 treated with or without 1000 ng/ml IFN-β. The production of NO was measured via a Griess
350 assay, and the data points are shown as the averages of three independent experiments. A two-
351 way ANOVA with Geisser-Greenhouse correction and Tukey's multiple comparisons test was
352 performed on all data. Significance is indicated as follows: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$,
353 and ****$p \leq 0.0001$.
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