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1 **Major Article**

2 **Formyl peptide receptor 2 plays a deleterious role**

3 **during influenza A virus infections**

4

5 Sergey Tcherniuk¹, Nicolas Cenac², Marjorie Comte³, Julie Frouard³, Elisabeth Errazuriz-
6 Cerda⁴, Angel Galabov⁵, Pierre-Emmanuel Morange¹, Nathalie Vergnolle², Mustapha Si-
7 Tahar⁶, Marie-Christine Alessi¹ and Béatrice Riteau¹

8

9 ¹Institut National de la Santé et de la Recherche Médicale (Inserm), UMR_S 1062, 13005
10 Marseille, France Inra, UMR_INRA 1260, 13005 Marseille, France Aix Marseille Université,
11 13005 Marseille, France. ²IRSD, Université de Toulouse, INSERM, INRA, INP-ENVT,
12 Université de Toulouse 3 Paul Sabatier, Toulouse, France. ³EA4610, Lyon, France; ⁴Centre
13 Commun d'Imagerie Quantitative Lyon Est (CIQLE), SFR Santé Lyon-Est, University of
14 Lyon, France. ⁵The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of
15 Sciences, Sofia, Bulgaria. ⁶INSERM U1100 Université François Rabelais, Tours, France.

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29 **Corresponding author:** Beatrice Riteau: beatrice.riteau@laposte.net

30

31

32 **ABSTRACT**

33 Background: Pathogenesis of influenza A virus (IAV) infections is a multifactorial process
34 including the replication capacity of the virus and a harmful inflammatory response to
35 infection. Formyl Peptide Receptor 2 (FPR2) emerges as a central receptor in inflammatory
36 processes controlling resolution of acute inflammation. Its role in virus pathogenesis has not
37 been investigated yet.

38 Methods: We used pharmacologic approaches to investigate the role of FPR2 during influenza
39 A virus infection *in vitro* and *in vivo*.

40 Results: *In vitro*, FPR2 expressed on A549 cells was activated by IAV which harbor its ligand
41 Annexin-A1 in their envelope. FPR2 activation by IAV promoted viral replication through an
42 extracellular-regulated kinase (ERK)-dependent pathway. *In vivo*, activating FPR2 by
43 administering the agonist WKYMVm-NH₂ decreased survival and increased viral replication
44 and inflammation after IAV infection. This effect was abolished by treating the mice with
45 U0126, a specific ERK pathway inhibitor, showing that the deleterious role of FPR2 also
46 occurs through an ERK-dependent pathway, *in vivo*. In contrast, administration of the FPR2
47 antagonist WRW4 protected mice from lethal IAV infections.

48 Conclusion: These data show that viral replication and IAV pathogenesis depend on FPR2
49 signaling and suggest that FPR2 may be a promising novel strategy to treat influenza.

50

51 **Key words: Influenza virus, Flu, Host-immune response, Formyl Peptide receptor 2**

52 INTRODUCTION

53

54 Influenza is an acute respiratory disease responsible for seasonal epidemics and sporadic
55 pandemic outbreaks, leading to significant mortalities in humans [1, 2]. The most severe
56 complication is acute pneumonia, which develops rapidly and may result in respiratory failure
57 and death. Influenza A virus (IAV) pathogenesis is a multifactorial process, involving
58 increased viral replication competence and a harmful inflammatory response. Lipid mediators
59 lipoxins, protectins and resolvins that play a key and active role in the resolution of acute
60 inflammation have received a particular interest in infectious disease lately [3, 4].
61 Surprisingly, the lipid mediator protectin D1 does not affect inflammatory processes during
62 influenza but inhibits IAV replication and protects mice from severe infection [5]. To date,
63 the contribution of inflammatory pro-resolving receptors that mediate the lipid signaling
64 cascade of lipoxins and protectins mediators to the pathogenesis of IAV infections remains to
65 be determined. Deciphering their role is of crucial importance as these receptors has multiple
66 ligands mediating different functions and these receptors can be used as a novel strategy to
67 fight against severe influenza.

68

69 One receptor of interest is the Formyl Peptide Receptor 2 (FPR2/FPRL1/AXL). FPR2 belongs
70 to the seven transmembrane domain of G protein-coupled receptors. FPR2 initiates an active
71 resolution of acute inflammation by binding anti-inflammatory lipid mediators or cellular
72 proteins such as the most prominent lipoxin A4 (LXA4) or the glucocorticoid-modulated
73 protein Annexin-A1 (ANXA1). Although the involvement of FPR2/LXA4 in the resolution of
74 inflammatory responses is now well-recognized both *in vitro* and *in vivo* [6-8], a distinct
75 function of FPR2 includes the detection of bacterial formyl peptides and induction of pro-
76 inflammatory responses [9-11]. Due to the nature of this ligand, FPR2 has also been attributed

77 the label of “pattern recognition receptor”. Thus, FPR2 is emerging as a central checkpoint
78 receptor in inflammatory processes, whose function will most likely depend on accessible
79 ligands and the time course of infection. Studies on the involvement of FPR2 in viral
80 pathogenesis have not been documented so far.

81

82 FPR2 could contribute to the pathogenesis of IAV infections by promoting virus replication
83 and /or a harmful inflammatory response. At present it is unknown whether one or both of
84 these two mechanisms of FPR2 contribute to the pathogenesis of IAV infections. Our findings
85 show that FPR2 plays an important role in modulating lung inflammation and IAV
86 replication, mainly through activation of the ERK pathway. In addition, these results suggest
87 that FPR2 may be explored as a novel target to treat IAV infections.

88

89

90 **METHODS**

91

92 **Ethics statement**

93 Experiments were performed according to recommendations of the “National Commission of
94 Animal Experiment (CNEA)” and the “National Committee on the Ethic Reflexion of Animal
95 Experiments (CNREEA)”. The protocol was approved by the committee of animal
96 experiments of the Faculty of Marseille la Timone (Permit number: G130555). All animal
97 experiments were also carried out under the authority of license issued by “la direction des
98 services Vétérinaires” (accreditation number 693881479).

99 **Viruses and reagents**

100 The following reagents were used: IAV A/PR/8/34 (H1N1), A/WSN/33 (H1N1) and
101 A/Udorn/72 (H3N2) IAV, Protease-Activated-receptor 4 agonist AYPGKF-NH₂ (Bachem,
102 Bubendorf Switzerland), antagonist of FPR2 WRW4 and agonist of FPR2 WKYVMm-NH₂
103 (R&D Systems, Lille, France), MEK inhibitor U0126 (Promega, Charbonnières, France),
104 siRNA targeting ANXA1, monoclonal anti-ANXA1, polyclonal anti-A5, monoclonal anti-M2
105 and monoclonal anti-HA (Santa Cruz Biotechnology, Heidelberg, Germany), cholera toxin B
106 subunit and monoclonal anti-tubulin (Sigma Aldrich, Lyon, France), polyclonal anti-ERK and
107 anti-p-ERK (Cell Signalling, Saint Quentin, France), ELISA kits for mouse IL-6 (R&D
108 Systems) and IFN- β (Invitrogen, Cery Pontoise, France).

109

110 **Cell culture**

111 The human alveolar A549 and the Madin-Darby canine kidney (MDCK) cell lines were
112 obtained from the American Type Culture Collection (ATCC). MDCK cells were maintained
113 in EMEM (Lonza, Levallois Perret, France) supplemented with 10% Fetal Bovine Serum

114 (Lonza, France), 2 mM L-glutamine, and penicillin-streptomycin (PS). A549 cells were
115 grown in DMEM (Lonza, France) supplemented with 10% FBS, 2 mM L-glutamine and PS.

116

117 **Virus production, titration, purification and immunogold analysis**

118 Viruses were produced and titrated as previously described [12]. Purified virus particles were
119 obtained from MDCK cells supernatants as done previously [13]. Immunogold labeling of
120 ANXA1 and HA was performed on gradient-purified virus particles as previously described
121 [12].

122

123 **Flow cytometry, ELISA and western blot analysis**

124 A549 or MDCK cells were infected or not with A/PR/8/34, A/Udorn/72 or A/WSN/33 (MOI
125 of 1) for 24 hours, and the expression of FPR2 was assessed using flow-cytometry analysis as
126 previously described [14, 15]. ELISA was performed according to the manufacturers'
127 instructions. For western blot analysis, purified virions or cells were lysed and proteins were
128 analyzed, as previously described [16].

129

130 **ERK activation experiments**

131 Before lysis for western blot analysis, A549 cells were stimulated with the indicated
132 concentration of FPR2 agonist WKYMVm-NH₂ for 5 minutes at 37°C. Regarding the
133 specificity of the WRW4 antagonist (10 μM), cells were first pretreated for 20 minutes at
134 37°C with FPR2 antagonist WRW4 and stimulated with either 1 μM FPR2 agonist
135 WKYMVm-NH₂ or 200 μM PAR4 agonist AYPGKF for 5 minutes at 37°C. For the kinetic
136 of virus-induced ERK phosphorylation, A549 cells were stimulated or not with purified
137 A/WSN/33 virus (MOI 10) for the indicated time point before cell lysis. The effect of FPR2
138 blockade was assessed as followed: A549 cells were pre-incubated with the indicated

139 concentration of FPR2 antagonist WRW4 for 20 minutes at 37°C. Cells were then stimulated
140 or not with purified A/WSN/33 or A/Udorn/72 viruses (MOI 10) for 5 minutes in the presence
141 of 60 µM U0126 or vehicle. Stimulation of A549 cells with WT or A1-KD viruses (MOI of 1)
142 was performed for 5 minutes at 37°C in the presence of 60 µM U0126 or vehicle.

143

144 **Viral replication experiments**

145 A549 cells were infected with IAV A/WSN/33 (MOI 1) and stimulated with the indicated
146 concentration of FPR2 agonist, WKYMVm. For FPR2 blockade experiments, A549 cells
147 were first pre-incubated for 20 minutes with 10 µM otherwise indicated of FPR2 antagonist
148 WRW4 for 20 minutes at 37°C before infection. In some experiments, assays were performed
149 in presence of 60 µM U0126 or vehicle. Regarding experiments with IAV harboring KD
150 ANXA1, cells were infected with WT or ANXA1 KO virus at a MOI of 1 in presence of
151 U0126 (60 µM) or vehicle. In all conditions, virus titers were evaluated by plaque assay in the
152 supernatant 16 hours post-inoculation, otherwise indicated. Cell viability in presence of
153 agonist or antagonist of FPR2 was assessed by trypan blue staining 24 hours post-treatment.

154

155 **siRNA experiments**

156 Specific siRNA targeting ANXA1 was used to knock-down protein expression, in A549 cells.
157 Non-targeted siRNA was used as a control, as previously described [12]. Western blot
158 analysis was performed to control the transfection efficiency, 48 hours post-transfection. At
159 this step, control siRNA or targeting ANXA1 siRNA transfected cells were infected with IAV
160 (A/WSN/33 or A/Udorn/72, MOI 1) and supernatants containing the virus particles were
161 harvested 24 hours post-infection and used in experiments. Reduced expression of packaged
162 ANXA1 in the virions released in the supernatant of ANXA1-specific siRNA-treated cells
163 (referred to as A1-KD virus) compared to control viruses (referred to as WT virus) was

164 confirmed by loading 20µl of the corresponding supernatants on a gel followed by western
165 blot analysis.

166

167 **Mouse infection and treatment**

168 C57BL/6 mice were anesthetized with Ketamine/Xylazine (43/5 mg/kg) and inoculated
169 intranasally with 20 µl of a solution containing A/PR/8/34 virus. Inoculation was made with
170 500 PFU and 5000 PFU of A/PR/8/34 virus regarding stimulation experiments with the
171 agonist and antagonist of FPR2, respectively. 8 mg/kg FPR2 agonists, FPR2 antagonist or
172 U0126 were administered intraperitoneally. Mice were treated either at days 0, 2 and 4 post-
173 infection or at days 2 and 4 post-infection. For assessing virus replication, broncho-alveolar
174 lavages (BAL) were harvested from sacrificed mice and infectious virus titers were
175 determined by plaque assay, as previously described [12].

176

177 **Statistical analysis**

178 The Mann–Whitney test was used for statistical analysis, regarding viral replication and
179 cytokine production. Kaplan-Meir method was used to calculate the survival fractions in *in*
180 *vivo* experiments. Two survival curves were compared by the log-rank test (Mantel-Cox test).
181 Results were considered statistically significant at $p < 0.05$ (*).

182

183 **RESULTS**

184

185 **Formyl Peptide Receptor 2 promotes IAV replication**

186 Flow cytometry analysis showed increased FPR2 cell surface expression after infection of the
187 cells with A/Udorn/72, A/PR/8/34, or A/WSN/33 viruses (Figure 1A, left panel). Viral protein
188 M2 was only detected after virus infection. Despite low expression of FPR2 at the surface of
189 uninfected A549 cells, addition of the FPR2 agonist peptide WKYVMm-NH₂ (FPR2-AP) to
190 A549 cells triggered ERK phosphorylation (Figure 1A, right panel). Maximal effect was
191 observed at about 1 μ M, while the percentage of cell viability was not affected (Figure 1B,
192 left panel). Because ERK phosphorylation is essential for IAV infectivity [17], A549 cells
193 were infected with IAV and stimulated or not with the FPR2-AP. When exposed to the FPR2-
194 AP, infectious virus titers were significantly increased in a dose-dependent manner in the
195 supernatant of these cells compared to vehicle-treated cells (Figure 1B, right panel). Thus,
196 FPR2 activation promotes viral replication during IAV infections, *in vitro*.

197

198 **FPR2 antagonist inhibits viral replication**

199 Treatment of IAV-infected A549 cells with FPR2 antagonist WRW4 reduced viral production
200 in a dose- and time course-dependent manner (Figure 1C). WRW4 inhibited the FPR2-AP-
201 induced ERK activation but not the one mediated by PAR4 agonist peptide (Figure 1D, left
202 panel), suggesting that WRW4 blocks FPR2 signalling specifically. Furthermore, WRW4 had
203 no effect on A549 cell viability (Figure 1D, right panel). Thus, FPR2-signaling inhibition
204 blocks viral production in A549-infected cells.

205

206 **FPR2 activation by IAV increases viral replication through the ERK pathway**

207 Binding of purified virions to A549 cells for 5 or 10 minutes induced ERK activation (Figure
208 2A) that was prevented in a dose-dependent manner when cells were pre-incubated with FPR2
209 antagonist WRW4 (Figure 2B, Vehicle). Similar results were observed using IAV
210 A/Udorn/72. Thus, IAV-FPR2 recognition activated ERK. Then, A/WSN/33 or A/Udorn/72
211 virus-infected cells were exposed to the FPR2 antagonist in the presence or absence of the
212 ERK signaling-pathway inhibitor U0126. Control experiments showed that U0126 efficiently
213 blocked ERK phosphorylation mediated by IAV recognition of FPR2 on A549 cells (Figure
214 2B, U0126). As expected, WRW4 treatment decreased virus production by IAV-infected cells
215 (Figures 2C). U0126 also showed antiviral activity in cell culture against IAV. In the presence
216 of U0126 and WRW4, no additional antiviral effect was observed showing that U0126
217 treatment abolished the difference in viral replication between untreated and WRW4-treated
218 cells. Thus, FPR2 promotes IAV replication through an ERK-dependent pathway.

219

220 **Annexin 1 is incorporated into IAV particles**

221 The ligand on IAV particles that mediated FPR2 activation upon binding to A549 cells was
222 investigated. Proteins from purified IAV A/PR/8/34, A/Udorn/72, and A/WSN/33 were
223 analyzed by Western blot. Results indicated that all purified virions contained the M2 viral
224 protein and ANXA1 (Figure 3A). ERK was only detectable in uninfected and virus infected
225 lysates. Microscopic immunogold analysis also showed a specific immunogold staining of
226 ANXA1 and viral HA on all IAV particles (Figure 3B). Thus, ANXA1 is incorporated into
227 IAV particles. In addition, lipid rafts act as platforms for IAV budding, leading us to
228 investigate whether ANXA1 was upregulated at the cell surface and concentrates in rafts.
229 Results showed that ANXA1 was increased at the surface of infected cells and was enriched
230 in lipid rafts, the site of virus budding (4A-B). Thus, altogether, these results showed that

231 upon IAV infections, ANXA1 is translocated to the cell membrane, recruited to lipid rafts
232 allowing its incorporation in IAV particles during the budding process.

233

234 **Annexin 1 incorporated into IAV promotes viral replication**

235 Wild-type (WT) virions or virions for which ANXA1 was knock-down by silencing gene
236 expression (A/WSN/33 and A/Udorn/72 strains) were generated. Western blot analysis
237 confirmed that A549 cells transfected with the siRNA targeting ANXA1, express less
238 ANXA1, but did not affect A5 protein expression, compared to A549 cells transfected with a
239 siRNA control (Figure 5A). Viruses released by these cells were knock-down for ANXA1
240 levels (A1-KD virus) compared to siRNA control viruses (WT virus) (Figure 5B). Binding of
241 WT virus to A549 cells induced ERK activation (Figure 5C, Vehicle). This activation was
242 strongly impaired after incubation with A1-KD virus. Also, WT virus replicated more
243 efficiently compared to A1-KD virus (Figure D, Vehicle). In the conditions where U0126
244 efficiently blocked virus-induced ERK activation (Figure 5D, U0126), this difference in virus
245 fitness was abolished. Thus, ANXA1 incorporated in IAV is responsible for ERK activation
246 upon IAV binding to the cells and increases virus replication.

247

248 **FPR2 contributes to the pathogenesis of IAV infection**

249 Mice were infected with IAV A/PR/8/34 and treated or not with FPR2-AP, WKYMVm-NH₂.
250 Infected mice treated with the FPR2-AP displayed significant increased mortality rates,
251 compared to control-treated mice (Figure 6A, left panel). FPR2-AP had no effect on
252 uninfected mice (Figure 6A, right panel). At days 3 or 6 after inoculation, lung virus titers and
253 cytokine production were significantly increased after FPR2-AP treatment (Figure 6B-C).
254 Thus, FPR2-signaling enhanced IAV pathogenesis which correlated with increase viral titers
255 and inflammation in the lungs.

256

257 **The effect of FPR2 activation occurs through an ERK dependent pathway**

258 Infected mice were treated or not with the ERK inhibitor U0126 in presence or absence of
259 FPR2-AP treatment. In contrast to untreated mice, treatment of mice with U0126 abrogated
260 the effect of FPR2-AP on mortality rates after IAV infection (Figure 6D). Thus, the role of
261 FPR2 in IAV pathogenesis occurs through an ERK signaling pathway *in vivo*.

262

263 **FPR2 antagonist protects against IAV infection**

264 Mice treated with FPR2 antagonist WRW4 were more resistant to A/PR/8/34 infection than
265 vehicle-treated mice (Figure 7A). No effect was observed on uninfected mice. Protection
266 mediated by WRW4 correlated with reduced lung virus titers and inhibition of cytokines IL6
267 and INF β production (Figure 7B and 7C). Interestingly, when WRW4 was administered from
268 day 2 post inoculation onward, mice were also significantly protected from A/PR/8/34 and
269 A/Hong-Kong/68 virus infections (Figure 7D). Thus, inhibition of FPR2-signaling protect
270 mice from IAV replication in the lungs, inflammation and severe disease development.

271

272

273 **DISCUSSION**

274

275 The present study showed that FPR2 plays an important role during IAV infections. *In vitro*,
276 stimulation of FPR2 using specific agonists increased viral replication while blocking FPR2
277 with a specific FPR2 inhibitor did the opposite, indicating that FPR2-signaling plays a pro-
278 viral effect during IAV infections. Consistent with our *in vitro* studies, FPR2-AP promoted
279 virus replication and exacerbated the effects of IAV infection in infected mice. Moreover,
280 FPR2-antagonist-treated mice were protected from IAV infection showing that FPR2
281 activation contributes to the pathogenesis of IAV infection.

282 To our knowledge, a role for FPR2 in the pathogenesis of virus infections using *in vivo*
283 models has not been previously described. FPR2 activation did not exacerbate the effects of
284 IAV infection in mice treated with an inhibitor of ERK activation. Thus, ERK is playing a
285 permissive role for the effect of FPR2 activation in IAV infection. Our observation that FPR2
286 promoted an ERK-dependent proviral effect in lung epithelial cultures and in the lungs of
287 infected mice demonstrate a link between FPR2 signaling, ERK activation and the ability of
288 IAV to replicate. Interestingly, our results showed that FPR2 inhibition induces the late
289 production of protectins in the lungs of infected mice (S2 Fig.), suggesting that FPR2
290 signaling blocks the production of protectin generation. This observation is of particular
291 interest since protectin attenuates IAV replication through inhibition of virus RNA export [5],
292 a step which requires signalling through the ERK cascade [17]. Thus, it is possible that FPR2
293 signaling inhibits protectin generation, leading to RNA export and virus replication.

294 The endogenous activators of FPR2 in the airways are not well characterized. FPR2 expressed
295 by respiratory epithelial cells as well as leucocytes is susceptible to be activated by various
296 ligands of diverse classes and from different sources. The observation that ANXA1 is
297 incorporated into IAV particles and that ANXA1-deficient virions have no effect on FPR2-

298 signaling suggests that the first ligand that maybe involved at an early stage during IAV
299 infection could be ANXA1 which was incorporated into IAV particles. Interestingly, FPR2-
300 binding to peptides derived from the envelope protein gp41 of human immunodeficiency
301 viruses (HIV) acts as an efficient coreceptor for virus entry [18, 19]. Thus, several strategies
302 seems to be developed by different viruses to activate FPR2 for efficient replication,
303 highlighting an emerging role for FPR2 during viral infections. Possibly, the extent of
304 ANXA1 incorporation into IAV particles in a strain-dependent manner may explain
305 differences in pathogenicity of IAV strains through activation FPR2.

306 Formylated peptides are the prototypical ligands for FPR2 and to our knowledge, bacterial
307 and mitochondrial proteins are the only source of N-formyl peptides [10, 20]. However, a
308 broad range of non-N formyl and protein ligands have also been identified, including lipid
309 metabolites such as LXA4, in addition to cellular ANXA1. In this context, activation of FPR2
310 rather elicits anti-inflammatory and pro-resolving reactions in several models of acute
311 inflammation [21]. The fact that ANXA1 was incorporated into IAV suggests that as soon as
312 IAV infects a cell, FPR2 is activated. Thus, it is most likely that IAV developed mechanisms
313 to escape immune surveillance by inhibiting the host immune response through
314 ANXA1/FPR2 before acute inflammation occurs. This dampened early immune response
315 together with an increase virus replication might be responsible for a subsequent harmful
316 inflammation of the lungs. Indeed, excessive inflammation is a well-known contributor of
317 lung damage during severe influenza, a process that limits respiratory capacity and may
318 account for IAV pathogenesis in humans [1, 22]. Consistently, along with increased viral
319 replication, FPR2 exacerbated lung inflammation during IAV infection, in mice. To our
320 knowledge, the role of FPR2 in the inflammatory process of virus infections has not been
321 previously described. Increase inflammation mediated by FPR2 might also be the
322 consequence of a direct activation of the ERK pathway, a known signaling mediator of

323 inflammation [23]. FPR2 controls platelet/neutrophil aggregates leading to the rapid
324 generation of circulating LXA4 that subsequently further activates FPR2 [24]. Thus, the
325 involvement of a dysregulated platelet activation, known to promote acute lung injury during
326 influenza cannot also be ruled out in the deleterious role of FPR2 [25, 26].

327 Altogether our results show that FPR2 is an important receptor involved in IAV pathogenesis,
328 acting both at the level of IAV replication and inflammation. Our results also suggest that
329 inhibitors of FPR2 should be explored as a novel strategy for the treatment of IAV infections.

330

331 **FIGURES LEGENDS**

332

333 **Figure 1: Cell surface expression and function of FPR2 during IAV infections.**

334 (A, left panel) A549 cells were infected with A/PR/8/34, A/Udorn/72 or A/WSN/33 viruses
335 (MOI of 1). Twenty four hours post-infection, cell surface expression of FPR2 was evaluated
336 by flow cytometry analysis using an anti-FPR2 antibody (open histograms) or an isotype
337 control (closed histograms). The viral protein M2 protein was used as a positive control for
338 viral infection. Results are representative of two independent experiments. (A, right panel)
339 A549 cells were treated with the indicated concentrations of FPR2-AP for 5 minutes at 37°C.
340 Cells were then lysed and ERK phosphorylation was analyzed by western blot using an anti-
341 phospho ERK antibody (p-ERK). Total ERK protein was used as a loading control. (B, left
342 panel) A549 cells were treated with the indicated concentrations of FPR2-AP for 24 hours.
343 Cell viability was then estimated by trypan blue staining. Results show the mean values ±
344 standard deviations from three independent experiments. (B, right panel) A549 cells were
345 infected or not with IAV A/WSN/33 and treated with the indicated concentration of FPR2-
346 AP. Sixteen hours post-infection, infectious virus titers were determined by plaque assay. (C,
347 left panel) A549 cells were pre-treated with the indicated concentrations of FPR2-antagonist,
348 and infected with IAV A/WSN/33 at a MOI of 1. Thirty-six hours post-infection, infectious
349 virus titers were determined by plaque assay. (C, right panel) A549 cells were pre-treated with
350 10 μM of FPR2-antagonist and infected with A/WSN/33 virus at an MOI of 1. After the
351 indicated time points post-infection, infectious virus titers were determined by plaque assay.
352 (D, left panel) A549 cells were pre-incubated with FPR2-antagonist and treated with 1 μM of
353 FPR2-AP or 200 μM PAR4-AP. ERK activation was then evaluated by western blot analysis.
354 (D, right panel) A549 cells treated with the indicated concentrations of WRW4 FPR2-

355 antagonist for 24 hours. Cell viability was estimated by trypan blue staining. Results show the
356 mean values \pm standard deviations from three independent experiments.

357

358 **Figure 2: Influenza virus activates FPR2-induced ERK activation and virus replication**

359 (A) A549 cells were incubated with purified A/WSN/33 particles (MOI 10). After cell lysis,
360 ERK phosphorylation was analysed by western blot at the indicated time points post-
361 infection. (B) A549 cells were pretreated with the indicated concentration of FPR2-antagonist
362 for 20 minutes and incubated 5 minutes with purified A/WSN/33 or A/Udorn/72 particles
363 (MOI 10) in presence of vehicle or U0126 (60 μ M). After cell lysis, ERK activation was
364 analysed by western blot. (C) A549 cells were pre-incubated with FPR2-antagonist (10 μ M,
365 20 minutes) and infected with purified A/WSN/33 or A/Udorn/72 particles (MOI 10) in
366 presence of vehicle or U0126 (60 μ M). Thirty-six hours post-infection, infectious virus titers
367 were determined by plaque assay.

368

369 **Figure 3: ANXA1 incorporation into IAV particles**

370 (A) Proteins from purified A/PR/8/34, A/Udorn/72 and A/WSN/33 viruses were analysed by
371 western blot using anti-ANXA1, anti-M2 and anti-ERK antibodies. Aliquots of total proteins
372 from uninfected or A/PR/8/34 virus infected MDCK cells were used as controls. Protein
373 molecular weight was presented in kDa. Results are representative of three independent
374 experiments. (B) IAV A/PR/8/34, A/Udorn/72 and A/WSN/33 viruses were produced in
375 MDCK cells and purified by sucrose gradient ultracentrifugation. Electron microscopic
376 immunogold labeling was performed on the purified virions using anti-ANXA1 and anti-HA
377 antibodies. Bar is 50 nm. Results are representative of two independent experiments.

378

379

380 .

381 **Figure 4: ANXA1 expression at the cell surface and in the lipid rafts after IAV infection**

382 (A) Detection of cell surface ANXA1 in A549 (upper panels) or MDCK cells (lower panels)
383 after infection with A/Udorn/72, A/PR/8/34 or A/WSN/33 virus (MOI 1, 24 hours) by flow
384 cytometry using an anti-ANXA1 antibody (open histograms) or an isotype control antibody
385 (closed histograms). The viral protein M2 was used as a positive marker for viral infection.
386 Results are representative of two independent experiments. (B) A549 cells were left
387 uninfected or were infected with A/WSN/33 virus for 16 hours at a MOI of 1.

388

389 After cell lysis, the rafts domains were isolated by sucrose gradient ultracentrifugation.
390 Fractions 1-10 were then collected from the top of the tube and proteins within each fractions
391 were characterized by western blot analysis. Blots were probed with cholera-toxin B subunit
392 (GM1) or anti-ERK (ERK), anti-HA (HA0-HA2), anti-M2 and anti-ANXA1 (ANXA1)
393 antibodies. Fractions 3-4 and 9-10 correspond to rafts and soluble fractions, respectively.

394

395 **Figure 5: Effect of packaged ANXA1 on virus replication and involvement of the ERK**
396 **pathway**

397 (A) A549 cells were transfected with siRNA targeting ANXA1 at the indicated concentration
398 (siRNA) or 80 nM of control siRNA (-). Forty eight hours post-transfection, cells were lysed
399 and proteins from the lysates were analysed by western blot using an anti-ANXA1 or anti-A5
400 antibodies. (B) A1-KD and WT viruses were harvested from the supernateants of infected
401 A549 cells transfected with siRNA targeting ANXA1 or control siRNA, respectively. After
402 virus lysis, proteins from were characterized by western blot, using anti-ANXA1, anti-M2 and
403 anti-ERK antibodies. Lysates from uninfected or A/PR/8/34 virus infected A549 cells were
404 used as controls. (C) A549 cells were incubated 5 minutes with A1-KD virus or WT virus
405 (MOI 1) in presence of vehicle or U0126 (60 μ M). After cell lysis, proteins were analysed by

406 western blot using the anti p-ERK or anti ERK antibodies. (D) A549 cells were infected with
407 WT virus or A1-KD virus (MOI 1, 16 hours) in presence of vehicle or U0126 (60 μ M) and
408 infectious virus titers were determined by plaque assay.

409

410 **Figure 6: Effect of FPR2 activation on IAV pathogenicity**

411 (A) Time course of IAV-induced pathogenesis and death in mice in response to FPR2
412 stimulation. Mice were inoculated intranasally with A/PR/8/34 virus (500 PFU, n = 13-
413 15/group) and treated with vehicle or FPR2-AP (8 mg/kg upper panel). Alternatively, mice
414 were left uninfected and treated or not with FPR2-AP (n = 8-12/group, down panel). Results
415 show the average percent survival from 2 experiments. (B) infectious virus titers in the BAL
416 of infected mice treated or not with 8 mg/kg of FPR2-AP. Data are average \pm SD from 6
417 animals per group. (C) Levels of IFN- β and IL-6 in the BAL of A/PR/8/34 virus infected
418 mice treated with FPR2-AP WKYMVm-NH₂ (8mg/kg) or vehicle (1% DMSO); n= 3-
419 6/group. (D) After treatment with U0126 (8mg/kg) or vehicle (Untreated), mice were
420 inoculated with A/PR/8/34 virus (250 PFU, n = 6/group) and stimulated or not with FPR2-AP
421 (8 mg/kg). Mice were then followed for survival.

422

423 **Figure 7: Antiviral effect of FPR2 antagonist**

424 (A) Survival of A/PR/8/34 virus infected mice (n=7/group, upper panel) or uninfected mice
425 (down panel, n=12/group) after treatment with FPR2 antagonist at days 0, 2 and 4 post-
426 infection (B) Infectious lung virus titers in vehicle or FPR2 antagonist-treated mice. Data
427 represent mean \pm s.e.m of 5-6 individual mice per group. (C) IFN- β and IL-6 analysis in the
428 BAL of virus-infected mice (n= 3-5/group) treated with FPR2 antagonist or vehicle. (D) Mice
429 were inoculated with IAV A/PR/8/34 (n=18/group) or A/HK/68 (n=6/group), as indicated.
430 FPR2 treatment was initiated two days post-inoculation.

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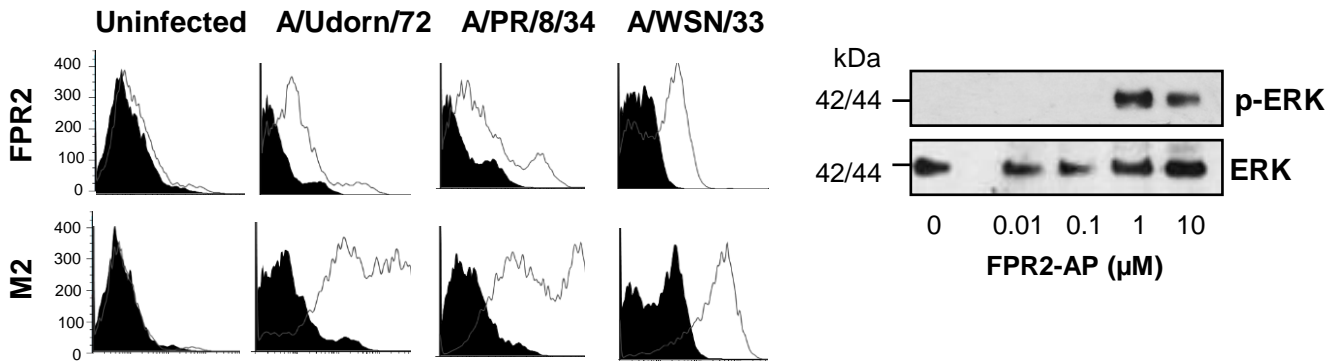
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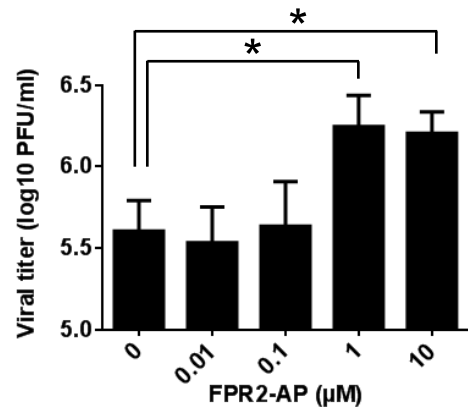
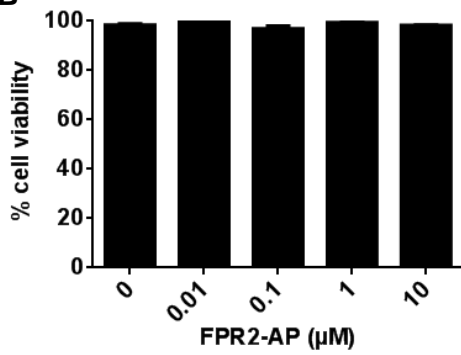
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Fig 1

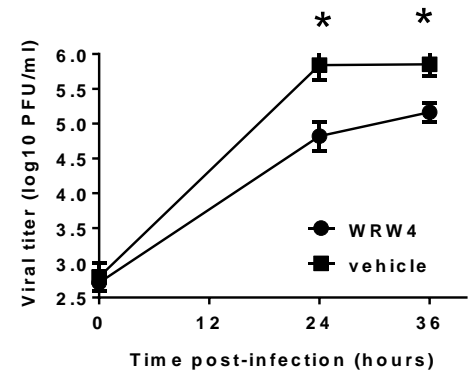
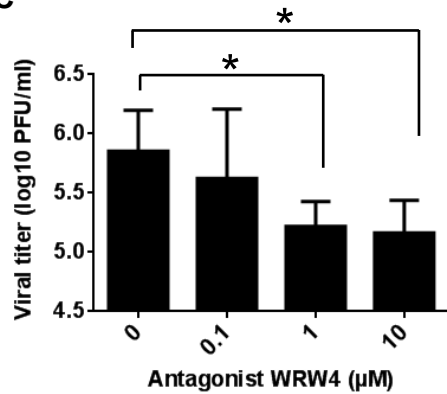
A



B



C



D

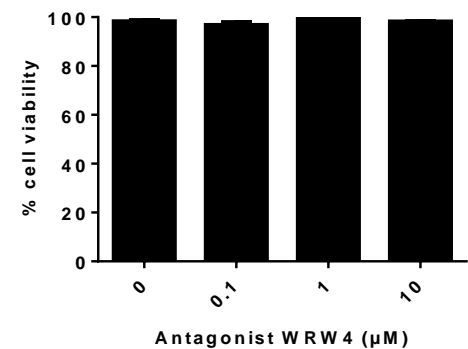
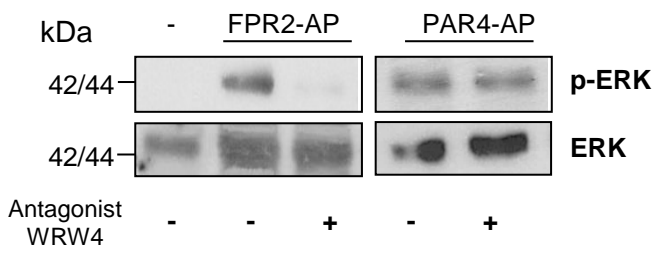
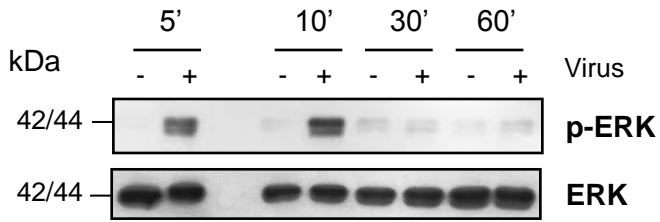


Fig 2

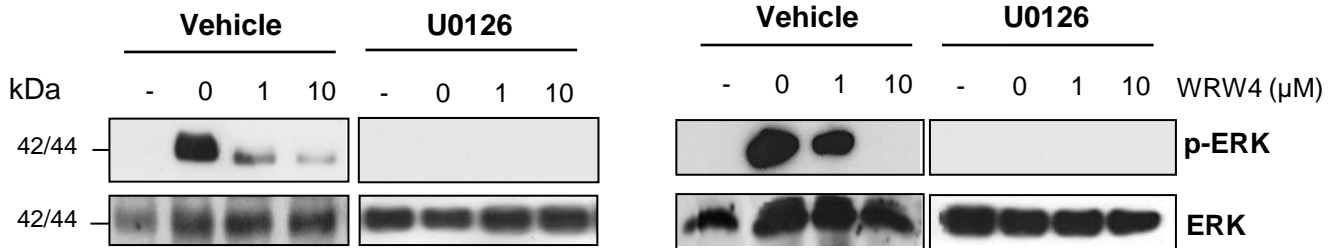
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B

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C

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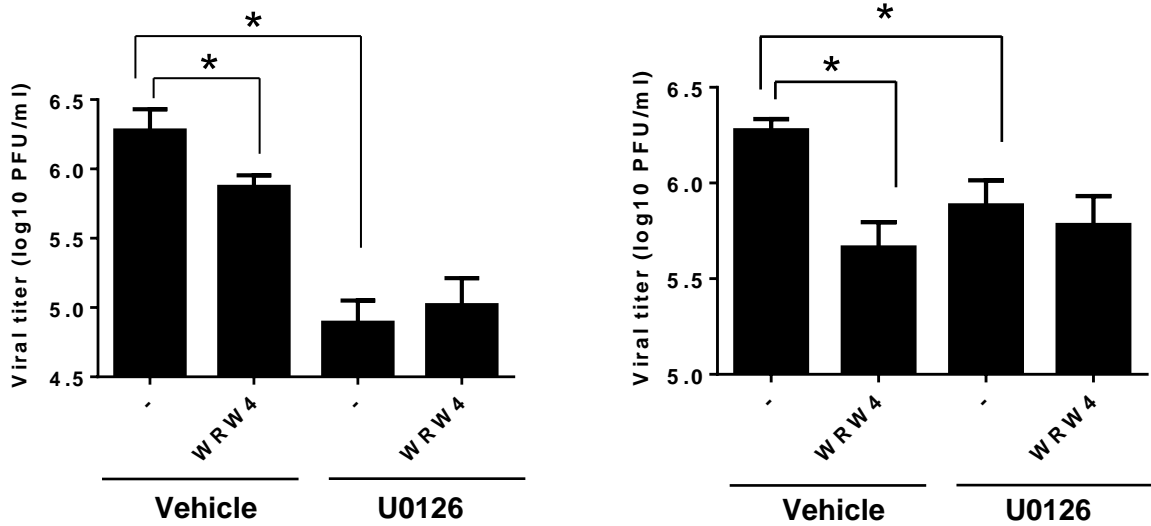


Fig 3

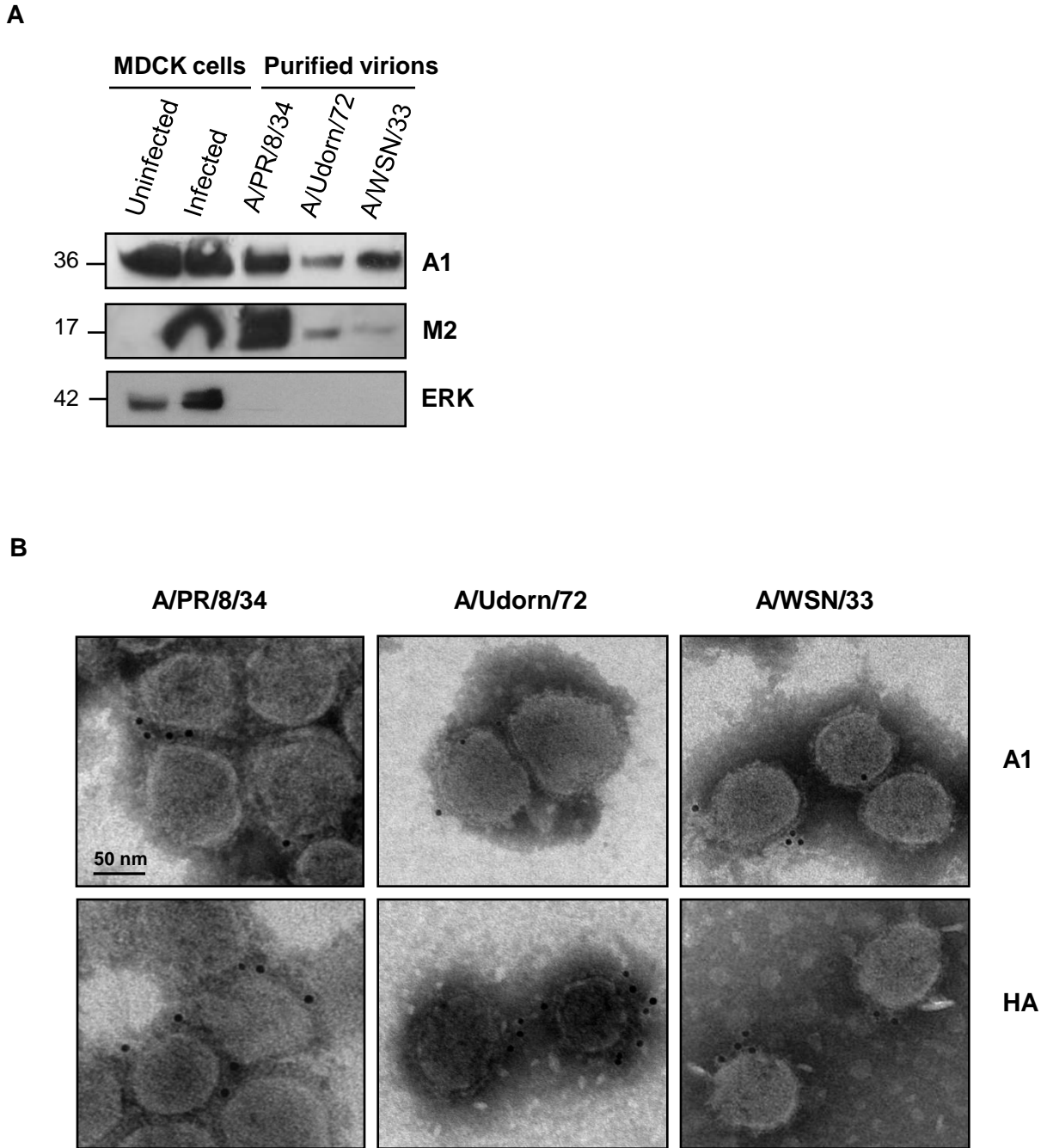
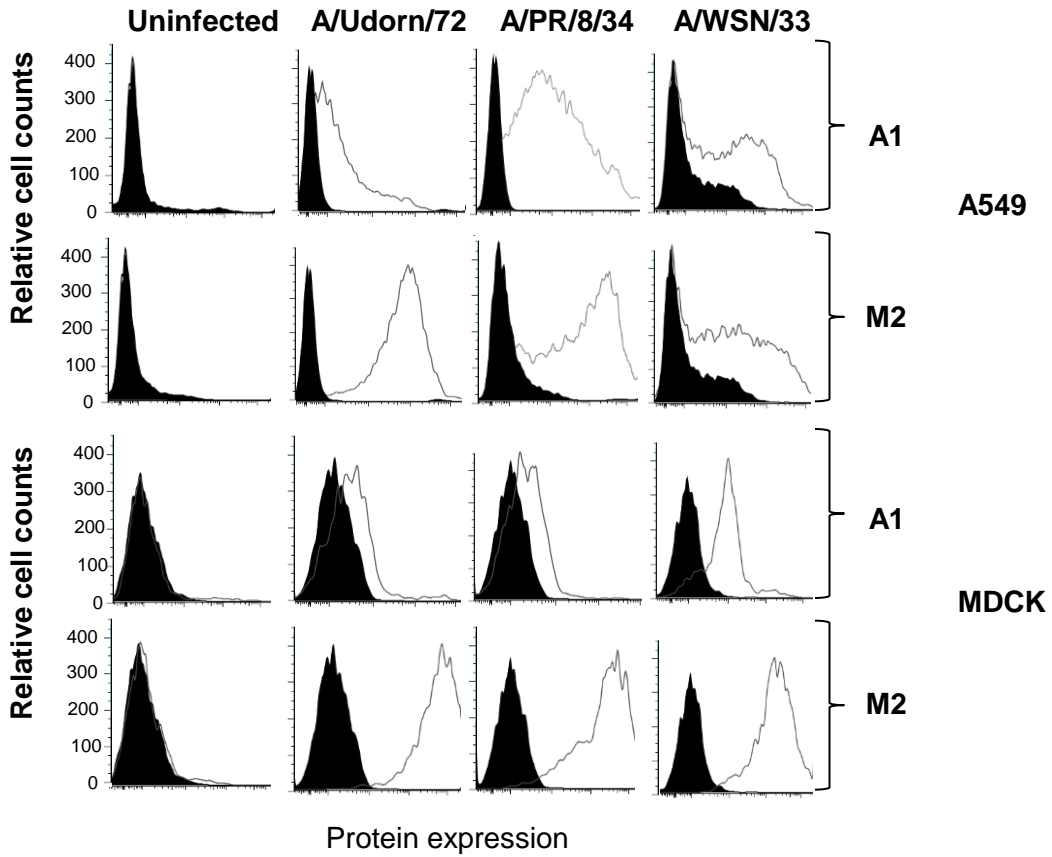


Fig 4

A



B

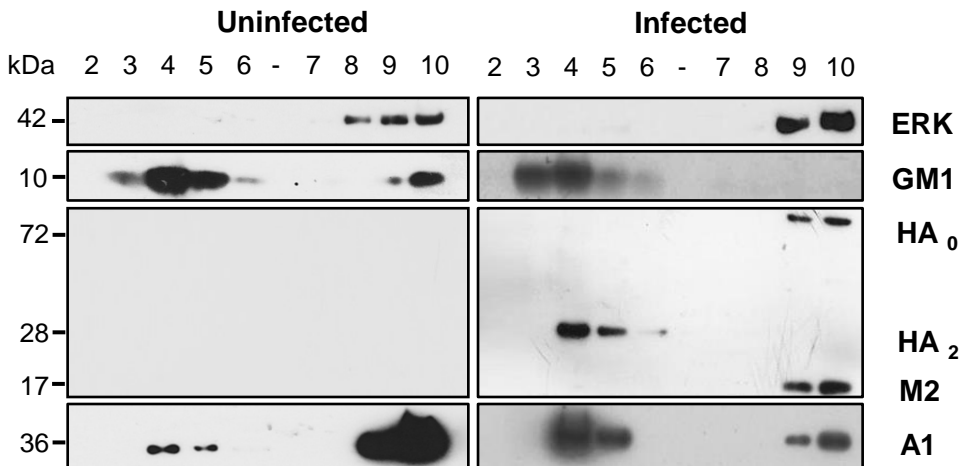


Fig 5

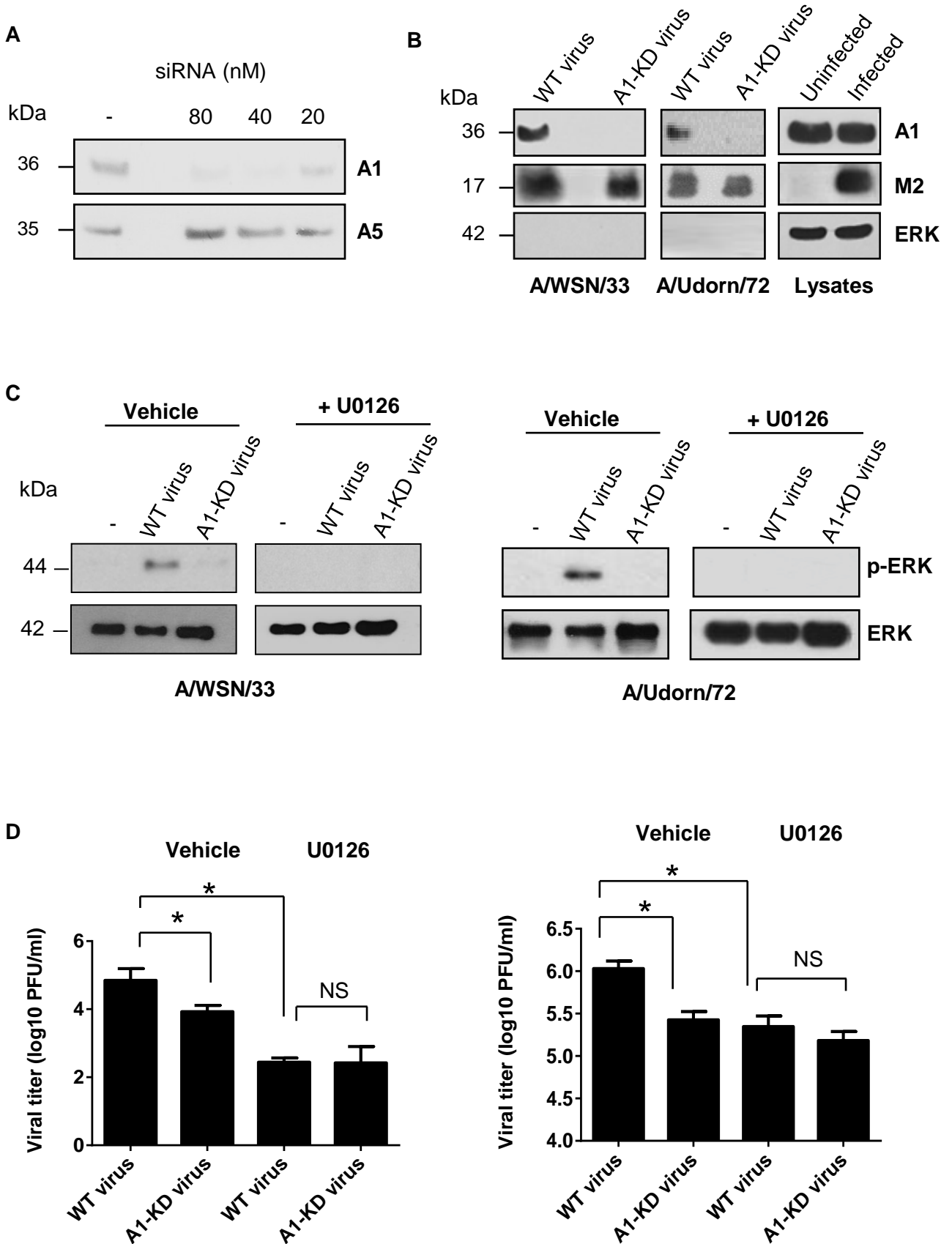


Fig 6