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44 **ABSTRACT**

45

46 Influenza causes considerable morbidity and mortality, but current therapies have limited efficacy. We  
47 hypothesized that investigating the metabolic signaling during influenza may help to design innovative  
48 antiviral approaches. Using bronchoalveolar lavages of infected mice, we demonstrated that influenza  
49 virus induces a major reprogramming of lung metabolism. We focused on mitochondria-derived  
50 succinate that we found to accumulate both in the respiratory fluids of virus-challenged mice and of  
51 patients with influenza pneumonia. We found that succinate displays a potent antiviral activity *in vitro*  
52 as it inhibits the multiplication of influenza A/H1N1 and A/H3N2 strains and strongly decreases virus-  
53 triggered metabolic perturbations and inflammatory responses. Moreover, mice receiving succinate  
54 intranasally showed reduced viral loads in lungs and had increased survival compared to control animals.  
55 The antiviral mechanism involves a succinate-dependent post-translational modification, *i.e.*  
56 succinylation, of the viral nucleoprotein (NP) at the highly conserved K87 residue. Succinylation of NP  
57 could alter its electrostatic interactions with viral RNA and could further impair the formation and  
58 trafficking of viral ribonucleoprotein complexes. Hence, succinate efficiently disrupts the influenza  
59 replication cycle; this opens up new avenues for improved treatment of influenza pneumonia.

60

61

62 **Keywords:** antiviral / influenza / metabokine / signaling / virus

63

## 64 INTRODUCTION

65

66 In 1918, more than 50 million people died from severe influenza viral pneumonia. One century later,  
67 and despite considerable progress in medicine, annual epidemics are still estimated to result in 3 to 5  
68 million cases of severe illness worldwide and up to 650,000 deaths (Taubenberger *et al*, 2019,  
69 Lampejo, 2020). Influenza viruses are the etiological agents and are classified into four types (A, B, C  
70 and D) of which influenza A virus (IAV) is clinically the most significant.

71 Vaccination against influenza constitutes the most effective preventive strategy. However, the short  
72 duration of vaccine-induced immunity coupled with the intrinsic antigenic drift of IAV negatively affect  
73 vaccine efficiency (Jang and Seong, 2019). Besides, drugs targeting IAV neuraminidase (NA) are  
74 currently recommended by the WHO but their efficacy is largely disputed (Jefferson *et al*, 2014 ; Duwe,  
75 2017). Hence, the development of innovative antiviral drugs is required to better treat IAV pneumonia  
76 (Pizzorno *et al*, 2019). To this end, we need a better understanding of the mechanisms of IAV-host cell  
77 interactions that may lead to IAV-triggered lung hyper-inflammation. Indeed, several studies suggest  
78 that such an excessive and deleterious inflammatory response is responsible for the life-threatening “flu”  
79 syndrome (Le Goffic *et al*, 2006 ; Taubenberger *et al*, 2019).

80 Interestingly, accumulating data support the view that metabolic pathways may promote or inhibit  
81 immune and inflammatory responses (Infantino *et al*, 2019; Rambold & Pearce, 2018; Williams &  
82 O’Neill, 2018), in addition to their role in energy production and macromolecular biosynthesis.  
83 Succinate is of special interest in this context. It is an intermediate of the tricarboxylic acid (TCA) cycle  
84 in mitochondria. Succinate is mainly produced from succinyl coenzyme A by succinyl coenzyme A  
85 synthetase (Grimolizzi & Arranz, 2018). It can also connect intracellular metabolic status and  
86 intercellular communication, as it can be released into the extracellular space through plasma membrane  
87 transporters of the SLC13 family (Grimolizzi & Arranz, 2018; Murphy & O’Neill, 2018). As a result,  
88 succinate can accumulate extracellularly in certain pathophysiological situations such as chronic  
89 inflammatory diseases (Ryan *et al*, 2019). Thus, the relationship between succinate and inflammatory  
90 signaling in leukocytes is increasingly studied (Tannahill *et al*, 2013; Mills *et al*, 2016; Littlewood-

91 Evans *et al*, 2016; Zaslona & O'Neill, 2020; Rubic *et al*, 2008; Jha *et al*, 2015). In contrast, such effects  
92 of succinate on non-immune cells such as the epithelial cells that line the airway mucosa have not been  
93 explored to a similar extent. This is important since airway epithelial cells are the main site of infection  
94 for respiratory pathogens such as IAV, and their role in controlling immune and inflammatory responses  
95 is pivotal. Furthermore, we and others have previously demonstrated that these cells are the source of  
96 major inflammatory mediators contributing to IAV-related lung injury (Gregory & Kobzik, 2015; Si-  
97 Tahar *et al*, 2009; Simmons & Farrar, 2008; Denney & Ho, 2018).

98 In view of the pro-inflammatory properties of succinate (Tannahill *et al*, 2013; Mills *et al*, 2016;  
99 Littlewood-Evans *et al*, 2016; Zaslona & O'Neill, 2020; Rubic *et al*, 2008; Jha *et al*, 2015), we initially  
100 speculated that succinate could contribute to the deleterious immunopathological response associated  
101 with severe IAV pneumonia. Strikingly, the opposite situation was observed. Using *in vitro* and *in vivo*  
102 approaches, we reveal that succinate restricts IAV replication and downstream inflammatory signaling.  
103 The underlying mechanism involves a specific post-translational modification and nuclear retention of  
104 the IAV nucleoprotein (NP). Hence, we demonstrate that succinate is a key player in the antiviral defense  
105 of the lung mucosa.

## 106 **RESULTS**

107

### 108 **Influenza infection increases succinate levels in airways**

109 Despite the growing interest in immunometabolism (Pearce & Pearce, 2018), little is known about  
110 metabolic reprogramming upon IAV infection (Wendt *et al*, 2021; Keshavarz *et al*, 2020; Smallwood  
111 *et al*, 2017; Bahadoran *et al*, 2020; Tian *et al*, 2019). Here, we conducted an unbiased quantification  
112 of the lung metabolome of mice four days after the infection by a sub-lethal dose of IAV  
113 (A/Scotland/20/74 (H3N2)). From the nuclear magnetic resonance (NMR) spectra of the  
114 bronchoalveolar lavage (BAL) fluids, we focused on succinate for two main reasons: (i) it was among  
115 the top ten most altered metabolites in the BAL fluids of IAV-infected mice, compared to control  
116 animals (fold change = 2.2, FDR-adjusted p-value =  $8 \cdot 10^{-7}$ , Figure 1a) and (ii) succinate is  
117 increasingly recognized as a potent immunoregulatory mediator (Tannahill *et al*, 2013; Mills *et al*,  
118 2016; Littlewood-Evans *et al*, 2016; Zaslona & O'Neill, 2020; Rubic *et al*, 2008; Jha *et al*, 2015).

119 In agreement with the data from the mouse experiments (Figure 1a), succinate level in respiratory  
120 fluids (*i.e.* tracheal aspirates) was significantly higher in critically ill patients with influenza infection  
121 than in patients not infected by IAV but with similar severity and lung inflammation (Figure 1b).  
122 This second group of patients included critically ill individuals ventilated for coma (cardiac arrest,  
123 stroke or drug overdose) with similar comorbidities and severity score, as assessed by IL-6 and IL-  
124 8/CXCL8 measurements in the respiratory fluids (Figure 1c), the leucocyte count in respiratory fluids  
125 and the regular presence of positive bacterial culture (Figure 1d). The heterogeneity of succinate  
126 levels in the fluids of IAV-infected patients can partly be ascribed to the variability at the time of  
127 hospital admission and the subsequent symptoms-to-sampling time (median [IQR] = 9 [7; 18] days).  
128 Overall, we demonstrated that IAV infection was associated with high levels of succinate in lungs  
129 of mice and humans.

130

### 131 **Succinate limits the secretion of inflammatory mediators and restores metabolic dysregulation in** 132 **IAV-infected lung epithelial cells**

133 To decipher the potential role of succinate in IAV-infected lungs, we first compared at 20 h post-  
134 infection (pi) the gene expression profiles of IAV-infected human bronchial epithelial cells, exposed  
135 or not to succinate. The volcano plot in Figure 2a (*left panel*) shows that IAV infection increased the  
136 expression of multiple gene pathways, including canonical immune and inflammatory pathways. The  
137 volcano plot in the *right panel* indicates that succinate treatment of IAV-infected cells results in a  
138 drastic downregulation of those pathways. As illustrated in Figure 2a and in [Appendix](#) figure S1,  
139 those altered pathways included, but were not limited, to “inflammasome signaling”, “TREM1  
140 signaling”, “acute phase response signaling”, “role of PRR in recognition of bacteria and viruses”,  
141 “iNOS signaling”,...). Of note is that succinate does not induce any cell cytotoxicity at the doses we  
142 used ([Appendix](#) Figure S2a-b).

143 We considered that the decrease of inflammatory pathways mediated by succinate in the context of  
144 IAV infection was an important observation for two reasons: first, because inflammation is a key  
145 promoter of IAV pneumonia and lung function impairment (Gregory & Kobzik, 2015; Si-Tahar *et al*,  
146 2009; Simmons & Farrar, 2008; Denney & Ho, 2018); second, because so far succinate was mostly  
147 considered to be pro-inflammatory (Tannahill *et al*, 2013; Mills *et al*, 2016; Littlewood-Evans *et al*,  
148 2016; Zaslona & O’Neill, 2020; Rubic *et al*, 2008; Jha *et al*, 2015). Using a semi-quantitative approach  
149 (*i.e.* protein-array blots), we confirmed that succinate limits the secretion of various inflammatory  
150 mediators (Figure 2b). Then, we quantified the diminution of critical inflammatory cytokines by  
151 succinate and found that this metabolite decreases down to 75% IAV-mediated secretion of IL-6, IL-  
152 8, IP-10/CXCL10 and RANTES/CCL5 (Figure 2c). Consistently, malonate (*i.e.* a selective inhibitor  
153 of succinate dehydrogenase (SDH) which favors endogenous succinate accumulation (Mills *et al*,  
154 2016; Potter & Dubois, 1943)) also inhibited IAV-induced inflammatory response (as assessed by IL-  
155 6 quantification (not illustrated)).

156 Because viruses are known to take control of the host metabolism to replicate more efficiently  
157 (Keshavarz *et al*, 2020), we also wanted to examine whether succinate could prevent IAV-induced  
158 metabolic reprogramming. We first measured the IAV-induced changes in glycolysis and  
159 mitochondrial oxidative phosphorylation using a Seahorse™ analyser. We observed that IAV  
160 significantly impaired the oxygen consumption rate (OCR) (*i.e.* a key metric of mitochondrial

161 oxidative phosphorylation) and the extracellular acidification rate (ECAR) (*i.e.* an index of the  
162 glycolytic activity) (Figure 2d-e, pink dots). After succinate treatment, IAV-induced metabolic  
163 dysregulation tended to be corrected, with a trend towards the normalization of both glycolysis and  
164 mitochondrial oxidative phosphorylation (Figure 2d-e, green dots).  
165 Together, these results showed that succinate inhibited both the inflammatory and metabolic  
166 perturbations secondary to IAV infection.

167

### 168 **Succinate blocks the multiplication of IAV in lung epithelial cells**

169 Next, we wondered whether the effects of succinate in IAV-infected cells were due to its  
170 immunomodulatory rather than its anti-infective properties. To answer this question, we challenged  
171 lung epithelial cells with synthetic double-stranded RNA poly(I:C) (PIC), which is a potent agonist  
172 of TLR3 signaling that mimics IAV-triggered immune responses (Guillot *et al*, 2005). Poly(I:C)  
173 induced IL-6 and IL-8 secretion at a similar or even higher level than IAV does. However, succinate  
174 did not inhibit poly(I:C)-induced secretion of these cytokines and chemokines (Figure 3a). We further  
175 compared the production of viral particles in the supernatants of IAV-infected epithelial cells  
176 exposed or not to succinate, in terms of viral neuraminidase (NA) enzymatic activity or viral titers.  
177 We observed a marked reduction of IAV load upon succinate treatment **in distinct human airway**  
178 **epithelial cells (such as BEAS-2B (Figure 3b-c), 16HBE14o- and A549 cells (Appendix S4)).** These  
179 results were further confirmed by a decrease of neo-virion budding and release, as assessed by  
180 electronic microscopy (Figure 3d **and Appendix Figure S3**). Interestingly, the treatment of IAV-  
181 infected lung epithelial cells with succinate or malonate resulted in a similar reduction of NA activity  
182 (Figure 3e). **We confirmed and extended these findings by showing that succinate impairs IAV**  
183 **multiplication even more strongly in multicycle replication condition (using a MOI=10<sup>-3</sup>; ~5-fold**  
184 **decrease) than in single cycle replication condition (using a MOI=1; ~2-fold decrease; Figure EV1a-**  
185 **b).** Overall, we demonstrated that succinate inhibits IAV multiplication.

186

### 187 **Succinate protects mice from IAV pneumonia**

188 We further explored whether we could confirm *in vivo* the anti-influenza effect of succinate that we  
189 revealed *in vitro*. Mice were infected intranasally with a LD50 of A/Scotland/20/74 (H3N2) IAV and  
190 received or not succinate (4 mg/animal). Figure 4a-c shows that viral load and viral protein expression  
191 in lung tissues were lower in succinate-treated mice at 4 days pi. To assess concomitantly the possible  
192 modulation of the immune/inflammatory response by succinate, we first evaluated the relative  
193 expression of 111 mediators in the BAL compartment (Figure 4d and Appendix Figure S5). Eighty-four  
194 of these mediators were increased upon IAV infection, and almost all, including chemokines, cytokines  
195 and metalloproteases, were markedly reduced in succinate-treated animals (Figure 4d). A more accurate  
196 quantification of IL-6 and KC by ELISA confirmed the semi-quantitative protein-array findings (Figure  
197 4e). Consistently, the amount of inflammatory cells such as neutrophils and myeloperoxidase (a protein  
198 primarily released by neutrophils) were significantly reduced in succinate-treated animals (Figure 4f-g;  
199 see also Appendix Figure S6 for the leukocyte gating strategy)).

200 IAV-mediated damage of the lung mucosa results from a combination of intrinsic viral pathogenicity  
201 and an inappropriate regulation of host immune mediators (Le Goffic *et al*, 2006; Simmons & Farrar,  
202 2008; Blanc *et al*, 2016). Therefore, by histopathological scoring, we explored the effect of succinate  
203 on lung tissue aspect. We observed a significant decrease of IAV-induced alveolar wall thickening, and  
204 hyaline membrane and epithelial necrosis in mice that received succinate compared to control, infected  
205 animals (Figure 4h). Finally, mice infected by a LD50 of IAV started to lose weight at 5 days pi (Figure  
206 4i) and ultimately 40 % succumbed (Figure 4j). Mice that received succinate did not lose weight and all  
207 survived.

208

### 209 **The anti-IAV effect of succinate involves a retention of the NP protein in the nuclear compartment**

210 To better understand how succinate inhibits the production of viral particles, we studied its impact  
211 on the different stages of the replication cycle of IAV by first using experimental conditions yielding  
212 a single round of IAV infection (MOI=1). After entry, the virus cycle starts by the transcription of  
213 viral mRNAs (Figure 5a). These viral mRNA serve as a template for the translation of viral proteins.  
214 We observed a comparable amount of viral M1 mRNAs in non-treated and succinate-treated cells,  
215 as assessed by RT-qPCR (Figure 5b). By contrast, when lung epithelial cells were infected under

216 experimental conditions that support multicycle IAV replication ( $\text{MOI}=10^{-3}$ ), succinate induces less  
217 viral M1 transcripts due to a lower number of re-infecting virus particles (Figure EV1, panel c).  
218 The effect of succinate on the subsequent stages of the viral cycle was examined using single  
219 replication conditions. A similar viral protein expression pattern was found in non-treated and  
220 succinate-treated cells, as assessed by western-blot analysis of the viral NP, NS1, PB2, PA, M1 and  
221 M2 proteins; (Figure 5c). The lack of a succinate effect on IAV mRNA and protein expression led  
222 us to analyze the impact of such metabolite on viral proteins trafficking. We especially examined the  
223 effect of succinate on (i) the nuclear import (or nucleo-cytoplasmic shuttling) of newly synthesized  
224 viral proteins and (ii) the nuclear export of newly assembled viral ribonucleoproteins (vRNPs)  
225 complexes (Figure 5a). We performed real-time imaging using an NS1-GFP-tagged recombinant  
226 IAV (A/WSN/33 (H1N1)), which shows similar replication and virulence properties to its wild-type  
227 counterpart (not illustrated). In the absence of succinate treatment, we typically observed a  
228 fluorescent signal in the cytosol of IAV-infected lung epithelial cells at ~6 h pi. This was followed  
229 by progressive nuclear accumulation of the signal (at ~10 h pi). Ultimately, we observed a diffuse  
230 fluorescence signal, as well as vacuolization accompanying cell death at >22 h pi (Figure 5d-e; see  
231 also [Movie EV1](#) for visualization of the complete process). In contrast, in succinate-treated cells, the  
232 fluorescent signal seemed to be more restricted to the nuclear compartment and cytoplasmic  
233 vacuolization accompanying cell death was reduced (Figure 5d-e and [Movie EV2](#)). Thorough  
234 quantification carried out on 60 cells confirmed that the nuclear/cytoplasmic fluorescence ratio was  
235 significantly higher in succinate-treated cells than in control cells (Figure 5f).

236 We next studied whether the nuclear retention of NS1 by succinate could be extended to other viral  
237 proteins. Using confocal microscopy, we monitored at 20h pi the intracellular localization of NS1 as  
238 well as of PA, PB2, M2, M1 and NP proteins in lung epithelial cells infected by IAV  
239 (A/Scotland/20/74 (H3N2)) and exposed or not to succinate. We first confirmed that NS1 shows a  
240 selective nuclear accumulation in succinate-treated cells. Remarkably, we extended this observation  
241 to NP by confocal microscopy (Figure 6a) [as well as by flow cytometry \(Figure EV2\)](#). Those findings  
242 were further supported by the accurate quantification of NS1 as well as of NP relative nuclear

243 intensities (Figure 6b). In contrast, PB2, PA, M2, M1 proteins were essentially localized in the  
244 cytosolic compartment in both control- and succinate-treated lung epithelial cells (Figure 6c).  
245 Interestingly, when lung epithelial cells were infected by a distinct IAV strain, *i.e.* A/PR/8/34 (H1N1),  
246 succinate caused nuclear retention only of NP and not of NS1 (Figure 7a) while it did inhibit viral  
247 multiplication, as assessed by the measurement of NA activity ( $p < 0.05$ ; Figure 7b). Succinate also  
248 caused nuclear retention of NP in cells infected with an NS1-deficient PR8 strain of virus (*i.e.* “PR8  
249  $\Delta$ NS1”) (Figure 7c). Taken together, these data suggested that NP retention, and not NS1, is key in the  
250 anti IAV-effect of succinate.

251

### 252 **Succinate reduces the assembly of vRNPs but does not alter the CRM1-dependent transport** 253 **pathway**

254 Since NP has been shown to interact with the CRM1-mediated nuclear export pathway (Elton *et al.*,  
255 2001), we examined whether succinate could affect CRM1 protein expression or function. As shown  
256 in **Figure EV3a**, CRM1 levels in IAV-infected cells remained the same, whether these cells were  
257 exposed or not to succinate. Next, we compared the effect of succinate and leptomycin B (LMB), a  
258 specific CRM1 inhibitor, on the trafficking of the viral NEP/NS2 protein. This last protein is known to  
259 be translocated into the cytosol in a CRM1-dependent manner (Kudo *et al.*, 1999, 1998). As expected,  
260 nuclear export of NEP/NS2 was inhibited by LMB. In contrast, succinate did not alter NEP/NS2  
261 localization, indicating that succinate does not mimic the CRM1-dependent effects of LMB (**Figure**  
262 **EV3b**). Hence, these data suggest that succinate causes nuclear retention of NP, in a CRM1-independent  
263 way.

264 NP is a major structural component of vRNPs. Viral (v)RNAs are encapsidated by NP with  
265 approximately 12 nucleotides *per* NP molecule (Dou *et al.*, 2018). NP oligomerization is likely to be  
266 involved in the higher order structure of vRNPs (Elton *et al.*, 1999). We used a non-denaturing western-  
267 blotting approach to test whether succinate could alter NP oligomerization. **Figure EV3c** clearly shows  
268 that this is not the case.

269

270 **Succinate induces succinylation of NP at K87, a highly conserved amino acid involved in vRNA**  
271 **binding**

272 Next, we evaluated the impact of succinate on the interaction of NP with the vRNA. To this end, we  
273 used confocal microscopy and a monoclonal antibody that specifically recognizes the “NP-vRNA”  
274 complexes, but not unbound NP (Eisfeld *et al*, 2011), as illustrated in Figure 8a (*upper right panel*).  
275 Remarkably, succinate clearly reduced the fluorescence signal that marks NP-vRNA complexes but not  
276 the fluorescence signal associated to the total form of NP (Figures 8a (*lower panels*) and 8b). These  
277 results suggested that succinate reduces the assembly of vRNPs. We further questioned what could be  
278 the underlying molecular mechanism.

279 As a precursor of succinyl-coenzyme A, succinate can promote protein post-translational modification  
280 (PTM) *via* succinylation on lysine residues (Park *et al*, 2013; Xie *et al*, 2012; Yang & Gibson, 2019).  
281 Lysine succinylation can cause a mass change (~100 Da) and converts a positively charged side chain  
282 into a negatively charged one, potentially causing changes in protein structure and function. Therefore,  
283 we used liquid chromatography coupled to untargeted tandem mass spectrometry (LC-MS/MS) to  
284 examine whether succinate induced the succinylation of IAV proteins, especially NP. Notably, of all  
285 IAV proteins, only NP showed lysine succinylation in cells treated with succinate. Moreover, NP  
286 succinylation occurred at a unique residue, *i.e.* K87 (Figure 8c and Appendix Figure S7).

287 In regard to these latter data as well as those highlighting the impact of succinate on vRNPs assembly  
288 (Figure 8a), it is of interest to point out that K87 residue is located in the RNA-binding groove of NP  
289 (Ye *et al*, 2006). Hence, two types of experiments were further performed to assess the functional  
290 impact of succinate-induced NP succinylation on (i) the formation of NP-vRNA complexes and (ii)  
291 the intracellular trafficking of NP.

292 In line with lysine succinylation of K87, fluorescence anisotropy analysis showed that a recombinant  
293 K87E mutant NP (which bears a negative charge similar to a succinyl residue) interacts less with 6-  
294 mer and 12-mer polyUC RNA strands than wild-type NP does (Figure 8d). Moreover, reverse genetic  
295 experiments showed that an IAV mutant strain bearing a K87E NP is not replicative (not illustrated),  
296 suggesting that the positively charged K87 is critical in IAV life cycle.

297 Conversely, we used reverse genetics and confocal microscopy to monitor the localization of NP  
298 protein in lung epithelial cells infected either by a wild-type IAV strain (coined “NP K87”) or an IAV  
299 mutant (coined “NP K87R”; *i.e.* a virus which can normally replicate as assessed by PFU assay and  
300 qPCR (Appendix Figure S8) but carries an NP that cannot be succinylated at the aminoacid position  
301 87, due to the lysine-to-arginine substitution (Park *et al*, 2013; Xie *et al*, 2012; Yang & Gibson, 2019)).  
302 Cells were exposed or not to succinate. We first confirmed that succinate induces a significant  
303 nuclear retention of NP in cells infected with the “NP K87” IAV strain (Figure 8e, *upper panels*).  
304 By contrast, succinate could not impair the intracellular trafficking of NP in cells infected by the  
305 mutant “NP K87R” IAV strain (Figure 8e, *lower panels*). Those findings were supported by an  
306 accurate quantification of NP relative nuclear intensities (Figure 8f). Besides, viral titer  
307 measurements in epithelial cells infected by “NP K87R” strain indicated that this mutant virus is  
308 significantly more resistant to succinate treatment than wild-type K87 IAV ( $p < 0.01$ ; Figure 8g).  
309 Altogether, our results suggest that succinate-induced alterations of IAV life cycle involves a  
310 succinylation of NP at the lysine residue 87.

311

## 312 **DISCUSSION**

313

314 In the present study, we highlight the interplay between immunometabolism and antiviral immunity.  
315 Using a combination of *in vitro* and *in vivo* approaches, (i) we demonstrated that succinate accumulates  
316 in the airways of IAV-infected mice as well as in patients with influenza illness; (ii) we found that this  
317 metabolite displays a potent and specific antiviral effect; (iii) we revealed that succinate induces a unique  
318 succinylation of the viral NP residue K87, which probably impairs vRNPs assembly and accounts for  
319 the observed retention of NP in the cell nucleus and disruption of the viral replication cycle; (iv) we  
320 showed that instillation of succinate in the airways protects hosts from IAV-triggered acute pneumonia.  
321 Hence, we have confirmed and extended previous studies that revealed profound metabolic changes in  
322 lung fluids of patients (Cui *et al*, 2016b) as well as in mice (Cui *et al*, 2016a; Smallwood *et al*, 2017;  
323 Chandler *et al*, 2016) infected by IAV. Our findings are consistent with studies suggesting that viruses  
324 can reprogram host cell metabolic pathways (Eisenreich *et al*, 2019). Remarkably, our PCA and  
325 multivariate statistics showed that succinate is one of the predominant metabolites that accumulates in  
326 the lungs of IAV-infected hosts. Also, we established that succinate accumulation was restricted to the  
327 lung airspaces as succinate levels were not increased in the lung parenchyma compartment (not  
328 illustrated). This latter finding suggests that succinate modulates influenza pathogenesis by acting on  
329 cells of the top layer of the lung mucosa. However, further studies are needed to clarify which cell(s)  
330 is/are the major source of succinate accumulation in the airways and whether this is a repercussion of a  
331 dysregulated TCA catabolism and/or a higher extracellular transport of succinate.

332 Of note is that extracellular succinate increases in several pathological contexts with local tissue levels  
333 reaching up to 20 mM (Littlewood-Evans *et al*, 2016; Graham *et al*, 2013). This concentration is  
334 consistent with the non-cytotoxic and active amount of succinate that demonstrates an antiviral effect in  
335 our study.

336 TCA cycle-derived metabolites are not mere intermediates for ATP synthesis but have recently been  
337 demonstrated to play a critical immunomodulatory role (Zasłona & O'Neill, 2020). In particular,  
338 succinate has been identified as a crucial pro-inflammatory signal that regulates the transcription factor  
339 HIF-1 $\alpha$  in several leukocyte subtypes (Tannahill *et al*, 2013; Mills *et al*, 2016; Littlewood-Evans *et al*,

2016; Zaslona & O'Neill, 2020; Rubic *et al*, 2008; Jha *et al*, 2015). Regarding non-leukocyte compartments, succinate is considered an ulcerative, pro-inflammatory molecule in intestinal mucosa (Connors *et al*, 2018). In contrast, extracellular succinate might also mediate anti-inflammatory responses in neural stem cells as well as in adipose tissue resident macrophages (Keiran *et al*, 2019). Now, we extend these findings by revealing a downregulatory effect of succinate in IAV-triggered inflammation in lung epithelium.

IAV is known to replicate within the nucleus of lung epithelial cells (Dou *et al*, 2018). To this end, the viral proteins need to fold correctly, oligomerize and/or associate with each other. The neo-synthesized vRNPs have to be properly transported across the nuclear membrane and through the cytoplasm and be packaged into new virions (Dou *et al*, 2018). Here, we have clearly shown that succinate induces a nuclear retention of NP, a major component of vRNPs.

NP is a highly basic protein whose surface displays many positively charged amino acids such as lysines, which bind the negatively charged vRNAs. Therefore, NP is the main structural component of the vRNPs. X-ray crystallographic analysis of NP structure has previously characterized an “intrinsically disordered region (IDR)-2” (72-DERRNKYLEEHPSAGKDPKKT-92), located in an RNA-binding groove that exhibits the highest conservation ratio among human, swine, and avian IAVs (Kakisaka *et al*, 2016). In that regard, K87 is extremely conserved (>99.9%) among 2400 (H5N1), 23400 (H3N2) and 18800 (H1N1) IAV strains (source: Influenza research database; <https://www.fludb.org>; selection criteria: (i) complete coding region sequences; (ii) no geographical or temporal restrictions; (iii) no host discrimination (iv) no elimination of duplicates; not illustrated).

IAV is known to interact with the host cellular machinery, including the post-translational modifications (PTMs) components. The most common PTMs include phosphorylation, ubiquitination, SUMOylation, acetylation, methylation, NEDDylation, and glycosylation. Many PTMs foster influenza virus infection, whereas others can contribute to antiviral defense (Hu *et al*. 2020).

Remarkably, succinate triggers a unique succinylation modification of K87 in NP, whereas none of the other ten major IAV proteins were found to be succinylated. Our findings further support the hypothesis that K87 is a critical amino acid in the “IDR-2” of NP, in agreement with previous data showing that a K87A change in NP results in a decreased binding affinity for RNA (Kakisaka *et al*, 2016; Tang *et al*,

368 2021). Moreover, we found that (i) a NP with a K87E mutation (which bears a negative charge,  
369 mimicking a succinyl residue) interacts less efficiently than the wild-type NP with a synthetic RNA and  
370 (ii) succinate treatment results in a lower RNA-NP assembly. All these data are schematized in **Appendix**  
371 **Figure S9**. This is in line with the fact that IAV life cycle is not impaired by succinate-induced lysine  
372 succinylation in epithelial cells infected by a virus strain bearing an NP K87R substitution. Taken  
373 together, these data strongly point to the fact that succinylation of the NP-K87 could alter the  
374 electrostatic environment of the vRNA interaction site and could further impact the formation of vRNPs  
375 particles.

376 Concomitantly, we found that succinate treatment does not modify the accumulation of viral mRNAs or  
377 viral proteins, indicating that transcription and replication of the viral genome by the viral polymerase  
378 in the nucleus of infected cells is essentially unaffected, despite the altered NP-RNA interaction. In the  
379 presence of succinate, the CRM1-dependent nuclear export machinery is not inhibited either. However,  
380 NP appears to be retained in the nucleus, the levels of vRNP-associated NP in cytoplasm are decreased,  
381 and budding of viral particles and the production of infectious virions are strongly impaired. In view of  
382 these data, we propose a hypothetical model (Figure 9) in which succinylation of NP at the highly  
383 conserved K87 residue promotes a charge shift in the RNA-binding groove. This alters the  
384 encapsidation of vRNAs with NP in a way that preserves transcription/replication of the viral  
385 genome and the nuclear export of a vRNPs moiety (as suggested by the cytoplasmic staining of  
386 NEP/NS2 and the polymerase subunits at late time-points of the infection). **It remains to be**  
387 **determined whether NP succinylation affects the packaging of the viral genome into viral**  
388 **particles. However,** this proposal is consistent with the evidence that packaging of the  
389 segmented viral genome is governed not only by a complex network of RNA-RNA interactions  
390 but also by specific RNA-NP interactions (Bolte *et al*, 2019; Moreira *et al*, 2016).

391 **Regarding a limitation of our study, we have to mention a similar survival of mice infected by wild-type**  
392 **IAV or by the mutant K87R IAV. This suggests that *in vivo*, the antiviral effect of succinate is likely the**  
393 **result of not only the impairment of IAV trafficking in lung epithelial cells but also the induction of**  
394 **additional protective signals in lung cell subsets, such as resident and/or infiltrated leukocytes.**

395 Determination of the underlying signaling mechanisms and the cells involved in the global antiviral  
396 effect of succinate will therefore require further exploration.

397 In conclusion, our study demonstrates that IAV infection increases succinate levels in the airways and  
398 that this metabolite reduces IAV production through mechanisms that involves a specific post-  
399 translational modification. This further contributes to an inhibition of viral multiplication as well as  
400 viral-induced inflammatory response. More importantly, IAV-infected mice treated intranasally with  
401 succinate show increased resistance to the development of acute pneumonia. Hence, our study paves the  
402 way for the development of safe and promising succinate-based treatment of IAV infection.

403 **METHODS**

404

405 **Viruses.** Mouse adapted-influenza A/Scotland/20/74 (H3N2) was generously given by Pr. Sylvie van  
406 der Werf's team (Pasteur Institute, Paris, France). The influenza A/PR/8/34 (H1N1) wild-type and  $\Delta$ NS1  
407 strains were kindly provided by Dr. Georg Kochs (Freiburg University, Germany). Influenza A/WSN/33  
408 (H1N1) wild-type and NS1 flag-tagged viruses were produced in Dr. Bernard Delmas' team (INRAe,  
409 Jouy-en-Josas, France).

410 **Animal infection and fluid collection.** C57Bl/6 female mice were purchased from the Centre d'Elevage  
411 R. Janvier (Le Genest Saint-Isle, France) and were used at about 8 weeks of age. Mice were treated in  
412 accordance with the European animal welfare regulation. Mice were bred in an animal facility in  
413 pathogen-free conditions. Mice were fed normal mouse chow and water *ad libitum* and were reared and  
414 housed under standard conditions with air filtration. When needed, C57Bl/6 mice were challenged  
415 intranasally with 150 pfu of A/Scotland/20/74 (H3N2) IAV. At 4 days post-infection, airways were  
416 washed four times with 0.5 ml of PBS for BAL collection. After centrifugation, BAL fluids were stored  
417 at -80°C for subsequent measurement of inflammatory mediators and pellets were recovered in PBS 2%  
418 FBS. Erythrocytes were discarded using a red blood cell lysis buffer and leukocytes were counted and  
419 analyzed by flow cytometry. Lungs were crushed with 2 mL of PBS using Gentle MACS™ M tube and  
420 the Gentle MACS™ Octo Dissociator (Miltenyi Biotech). Lungs extracts were centrifugated 5 min at  
421 500 g to collect supernatants which were further stored at -80°C for subsequent Western-blotting.

422 **Metabolomic analysis of respiratory fluids using <sup>1</sup>H-nuclear magnetic resonance.** Freeze-dried BAL  
423 samples were mixed with 210 µl of 0.2 M potassium phosphate buffer in deuterium oxide (D<sub>2</sub>O) 99%,  
424 pH=7.4. Samples were spiked with 8 µL of 3-trimethylsilylpropionic acid (0.05 % wt in D<sub>2</sub>O) as an  
425 internal reference, and then samples were transferred to conventional 3 mm NMR tubes. <sup>1</sup>H-NMR  
426 spectra were obtained with an AVANCE III HD 600 Bruker spectrometer equipped with a TCI  
427 cryoprobe (Bruker). Standard water suppressed <sup>1</sup>H-NMR spectra were acquired at 298K using a  
428 “noesypr1d” pulse sequence with a relaxation delay of 20 s and 256 scans. Spectra were processed using  
429 Topspin software (Bruker). <sup>1</sup>H-NMR spectra were automatically reduced to ASCII files using the AMIX

430 software package (Analysis of Mixture, version 3.8, Bruker). Spectral intensities were scaled to the total  
431 spectral intensity, and the resulting data were analyzed by multivariate and univariate statistics. The  
432 NMR assignments were done using spectra online databases such as HMDB (<http://www.hmdb.ca>) and  
433 ChenomX NMR Suite (version 8.1 evaluation, ChenomX). The identification of succinate was carried  
434 out by supplementing a sample with exogenous succinate. Spectra analysis carried out with and without  
435 supplementation, allowed us to ensure signal specificity.

436 Non-supervised multivariate principal component analyses (PCA) were performed with data generated  
437 by <sup>1</sup>H-NMR (BAL) using SIMCA v14 software (Umetrics). The scores were plotted in 2D vs the two  
438 first principal components to visualize possible clusters. Succinate quantification in BAL and tracheal  
439 aspirates was performed using Metaboanalyst software ([www.metaboanalyst.ca](http://www.metaboanalyst.ca)).

440 ***Tracheal Aspirate collection.*** Aspirates were collected during routine tracheal suctioning of  
441 mechanically ventilated patients. Tracheal aspirate samples were collected from patients hospitalized at  
442 the University Hospital of Tours; 9 with influenza pneumonia and 7 not infected with influenza virus.  
443 Samples were dissociated in 3 volumes of PBS *per* gram, and 1 mM dithiothreitol, with stirring for 30  
444 min. After centrifugation at 500 *g* for 10 min, the supernatants were stored at -80°C until use.

445 ***Cytokine array and ELISA.*** Cytokine array and DuoSet ELISA (Human IL-6, IL-8/CXCL8,  
446 IP10/CXCL10, RANTES/CCL5 and mouse IL-6, KC, MPO) were performed according to the  
447 manufacturer's (R&D Systems) instructions.

448

449 ***Cell culture.*** Experiments were performed using human bronchial epithelial BEAS-2B cells, except for  
450 live imaging which used human alveolar epithelial A549 cells, plaque assays which used Madin-Darby  
451 Canine Kidney (MDCK) cells and reverse genetics which used co-culture of MDCK and HEK-293T  
452 cells. All cells were mycoplasma-free, cultured in either F-12K Medium (BEAS-2B), **DMEM**  
453 **(16HBE14o-)** or MEM (A549, HEK-293T and MDCK) supplemented with 10% FBS and 100 U/mL  
454 penicillin, 100 µg/mL streptomycin. BEAS-2B cells were infected in medium without FBS for 4 hours  
455 with A/Scotland/20/74 (H3N2) IAV at MOI=1 (except for SEM analysis, for which an MOI=5 was  
456 applied, and in experiments using A/PR/8/34 (H1N1) wild-type and deltaNS1 viruses which used an

457 MOI=4). Cells were also stimulated in medium with FBS with 2 µg/mL of Poly (I:C). Four hours after  
458 the challenge, cells were washed with PBS and incubated for 4 h or 20 h with different concentrations  
459 of succinate or malonate, or with 10 nM of Leptomycin B. **For multicycle replication analysis, BEAS-**  
460 **2B cells were infected with IAV at low MOI (*i.e* MOI=10<sup>-3</sup>) for 4h and treated or not with succinate for**  
461 **20h. 2 µg/mL of TPCK-treated Trypsin were added simultaneously to succinate.** Supernatants were  
462 centrifuged 5 min at 500 g and stored at -80°C for further analysis.

463

464 ***Cell proliferation and cytotoxicity assays.*** Cells in 96-well plates were washed twice with PBS and  
465 incubated for 1 h at 37°C with 100 µL of MTS reagent diluted 1/5 for the cell proliferation test.  
466 Optical density was measured at 490 nm. For the cytotoxicity assay, cells were stained for 15 min at  
467 4°C with Live/Dead or with anti-Annexin V-FITC (1/20) and Propidium Iodide (1/100) before flow  
468 cytometry analysis.

469 ***Microarrays.*** Profiling of human bronchial epithelial BEAS-2B cells was performed using Agilent's  
470 SurePrint G3 human gene expression microarray kit. A single-color design provided two types of  
471 comparison: (i) IAV-infected *versus* mock-infected cells, and (ii) succinate-treated infected cells  
472 *versus* mock-infected cells. Cy3-labeled cRNAs were prepared from 100 ng of total RNA using a  
473 one-color Low Input Quick Amp labeling kit. Specific activities and cRNA yields were determined  
474 using a NanoDrop ND-1000 instrument. For each sample, 600 ng of Cy3-labeled cRNA (specific  
475 activity of >9 pmol Cy3/µg cRNA) was fragmented at 60°C for 30 min. cRNA was hybridized to the  
476 microarrays for 17 h at 65°C in a rotating hybridization oven as described previously (Barthelemy *et*  
477 *al*, 2018). After hybridization, microarrays were washed and then dried immediately. After washing,  
478 the slides were scanned using a G2565CA scanner system (Agilent Technologies), with a resolution  
479 of 3 µm and a 20-bit dynamic range. The resulting TIFF images were analyzed with Feature  
480 Extraction Software, using a GE1\_107\_Sep09 protocol. Identification of differentially expressed  
481 genes and functional investigations were done using GeneSpring software. Differentially expressed  
482 genes were identified using a moderated t test, and a P value cutoff of 5% was applied. A fold change  
483 cutoff of >2 was added to select genes expressed differentially between control and treated/infected

484 conditions. For further analysis, data files were uploaded into the Ingenuity Pathways Analysis (IPA)  
485 software (Ingenuity Systems). The right-tailed Fisher's exact test was used to calculate a P value for  
486 the probability that each canonical pathway assigned to that data set is due to chance alone.

487 **SeaHorse.** Cellular oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) data  
488 were obtained using a Seahorse XF96 flux analyzer from Seahorse Bioscience (Agilent  
489 Technologies) as we described previously (Kouzi *et al*, 2020). Experiments were performed  
490 according to the manufacturer's instructions. Briefly, bronchial epithelial BEAS-2B cells were  
491 seeded in XF96 cell culture plates at 30,000 cells *per* well and cultured overnight, then cells were  
492 infected with IAV for 18 h in the presence or absence of succinate. On the day of analysis, culture  
493 media were replaced with XF Base medium supplemented with glutamine (2 mM) and lacking  
494 bicarbonate (pH 7.4). Cells were then incubated at 37°C in a non-CO<sub>2</sub> incubator for 1 h and  
495 measurements were performed as described in the figure legends. Sequential injection of glucose (10  
496 mM), oligomycin (1 μM), dinitrophenol (DNP) (100 μM) and rotenone/ antimycin A (0.5 μM) were  
497 added according to the suppliers' technical specifications and these permitted the determination of  
498 the main respiratory and glycolytic parameters. Data were acquired with the Seahorse Wave  
499 Controller and analyzed with Seahorse Wave Desktop Softwares.

500 **Neuraminidase (NA) assay.** The assay is based on the release of a 4-methylumbelliferone fluorescent  
501 product from the 2'-(4-Methylumbelliferyl)- $\alpha$ -D-N-acetylneuraminic acid sodium salt hydrate (MU-  
502 NANA) substrate as a measure of NA activity. 67 μL of cell supernatant was incubated with 33 μL  
503 of MU-NANA (50 μM) in black 96-well micro-plates. Fluorescence was immediately measured in a  
504 kinetic assay over 1 h at Ex = 355 nm and Em = 460 nm.

505 **IAV titration by plaque-forming units assay.** Titrations in cell supernatants, BALs and mouse lungs  
506 were performed as previously described (Blanc *et al*, 2016).

507 **Transmission and Scanning Electron Microscopy.** Cells were washed with PBS, detached using  
508 trypsin and centrifuged. Cells were fixed by incubation for 24 h in 4% paraformaldehyde, 1%

509 glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). Samples were further washed in PBS and post-  
510 fixed by incubation with 2% osmium tetroxide for 1 h. Next, samples were fully dehydrated in a  
511 graded series of ethanol solutions and propylene oxide. An impregnation step was performed with a  
512 mixture of (1:1) propylene oxide/Epon resin and then samples were left overnight in pure resin. They  
513 were further embedded in Epon resin for 48 h at 60°C. Ultra-thin sections (90 nm) of these blocks  
514 were obtained using a Leica EM UC7 ultramicrotome (Wetzlar, Germany). Sections were stained  
515 with 2% uranyl acetate and 5% lead citrate. Observations were made with a transmission electron  
516 microscope (JEOL 1011) and analyzed with a Digital Micrograph.

517 For scanning electron microscopy, samples were fully dehydrated in a graded series of ethanol  
518 solutions and dried in hexamethyldisilazane (HMDS, Sigma, St-Louis, MO). Dried samples were  
519 sprinkled onto carbon disks and coated with 40 Å platinum, with a GATAN PECS 682 apparatus  
520 (Pleasanton, CA), before observation under a Zeiss Ultra plus FEG-SEM scanning electron  
521 microscope (Oberkochen, Germany). IAV particles were colored in pink by digital computer  
522 processing.

523 **Western-blotting.** Cells in 6-well plates were lysed with 150 µL of RIPA buffer (150 mM sodium  
524 chloride, 50 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid, 1% Triton X100, 1% sodium  
525 deoxycholic acid, 0.1% sodium dodecyl sulphate) and a protease inhibitor cocktail (diluted 1/200).  
526 Samples were centrifuged for 15 min at 12,000 g to eliminate debris, then protein concentrations  
527 were measured using a Pierce™ Protein BCA Assay Kit. Ten µg of total proteins were diluted with  
528 reducing or non-reducing Laemmli buffer, heated at 100°C for 5 min, then loaded onto 4/12% SDS-  
529 PAGE. Proteins were subsequently transferred to nitrocellulose membranes, and probed with anti-  
530 NP (1/500), anti-NS1 (1/1000), anti-PA (1/1000), anti-PB2 (1/500), anti-M1 (1/1000), anti-M2  
531 (1/1000), anti-CRM1 (1/500) or anti-β-actin (1/5000). Bound antibodies were revealed with an anti-  
532 rabbit IgG for NS1 and PB2 and anti-mouse IgG for other proteins (HRP linked) and ECL detection  
533 reagents. An automated imaging system (MF ChemiBis 3.2, DNR BioImaging Systems) was used  
534 for detection, and the FUJI FILM MultiGauge software was subsequently used for analysis and  
535 quantification.

536

537 ***RNA isolation and RT-qPCR.*** Cells in 6-well plates were lysed with 350  $\mu$ L of “RA1” buffer  
538 (included in the Macherey-Nagel RNA extraction kit) and  $\beta$ -mercaptoethanol diluted 1/100. Total  
539 RNAs from cells or from purified A/Scotland/20/74 (H3N2) IAV were extracted using the  
540 NucleoSpin® RNA kit, including a step of genomic DNA digestion with DNase. Nucleic samples  
541 were quantified using a Nanodrop 2000 UV-visible spectrophotometer. Single-stranded cDNA was  
542 synthesized from 500 ng total RNA for each sample with the High Capacity cDNA reverse  
543 transcription kit (Applied Biosystems), using the specific sense IAV M1 primer (5'-TCT AAC CGA  
544 GGT CGA AAC GTA-3'). mRNA levels were determined using quantitative real-time PCR with a  
545 LightCycler 480 instrument (Roche Diagnostics). PCR was carried out using 10 ng of reverse-  
546 transcribed total RNA as the template, 10  $\mu$ M (each) forward and reverse primers (sense: 5'-AAG  
547 ACC AAT CCT GTC ACC TCT GA-3'; antisense: 5'-CAA AGC GTC TAC GCT GCA GTC C-3'),  
548 and 10  $\mu$ L SYBR® Premix Ex Taq in a final volume of 20  $\mu$ L. Each reaction was performed in  
549 duplicate in white 96-well plates. The thermal protocol consisted of an initial denaturation step at  
550 95°C for 30 s followed by 40 cycles of denaturation at 95°C for 5 s and primer annealing and  
551 extension at 60°C for 20 s (reading at 83°C). Melting curves were generated for each amplified  
552 cDNA to check the reaction specificity. For quantification analyses, serial dilutions of IAV cDNA  
553 (from 40 ng to 0.5 ng cDNA) were used to create a standard curve.

554

555 ***Flow cytometry analysis.*** BAL were dispensed into round bottomed 96-well plates and were  
556 centrifuged at 300 *g* at 4°C for 5 min. One well was seeded for each of the following controls:  
557 unstained cells, single-stained cells, isotype controls. Samples were further stained using specific  
558 antibodies and appropriate isotype controls as listed in the supplementary table.

559 **Adherent epithelial cells were removed from their substrate by treatment with Trypsin a few minutes at**  
560 **37°C. Trypsin was inhibited by adding 800  $\mu$ L of culture media and cells were centrifugated at 500 *g***  
561 **for 5 min. Cell pellet was re-suspended in staining buffer (50 mM EDTA, 5% FCS in PBS), dispensed**  
562 **into round bottomed 96-well plates (100 000 cells in 100  $\mu$ L), and were then centrifuged at 300 *g* at 4**  
563 **°C for 5 min. For nucleoprotein staining, cells were permeabilized with the Cytotfix/Cytoperm™**

564 **Solution Kit or True Nuclear™ Transcription Factor Buffer Set, following manufacturer's instructions.**  
565 **Samples were further incubated for 1h at 4 °C with anti-NP-FITC antibody diluted 1/300e or isotype**  
566 **control.** Flow cytometry data were acquired on a MACSQuant® Analyzer and analyses were  
567 performed using the VenturiOne software.

568 ***Histopathology.*** Lungs were collected immediately after euthanasia and airways were washed and  
569 placed in 4% paraformaldehyde in PBS. Lung sections of approximately 4 µm thickness were cut and  
570 stained with hematoxylin-eosin. A study pathologist examined the tissue sections using light microscopy  
571 on a Leica Diaplan microscope in a blinded experimental protocol. All histopathological findings were  
572 graded in a semi-quantitative fashion on a scale of 0 to 4 (0: absent, 1: mild, 2: moderate, 3: severe, 4:  
573 extremely severe). All lung preparations and analyses were performed at the LAPV (Amboise, France).

574 ***Generation of recombinant fluorescent IAV and time-lapse imaging.*** The 8 plasmid reverse  
575 genetics system (Hoffmann *et al*, 2002) was used to generate a recombinant virus expressing an eGFP  
576 reporter protein fused to the NS1 protein of the influenza A/WSN/33 (H1N1) virus. The NS1-eGFP-  
577 WSN virus was rescued using a previously described procedure (Vidy *et al*, 2016) and was propagated  
578 in MDCK cells. For long term time-lapse microscopy, A549 cells grown on cell culture in micro-  
579 wells were infected with NS1-eGFP-WSN at an MOI of 0.5 pfu/cell. Pictures were taken every  
580 10 min starting at 1 h post-infection and continuing until 24 h post-infection, on a Nikon BioStation  
581 microscope using BioStation IM software. The intensity of the GFP associated fluorescence *per*  
582 infected cell was quantified using Image J software.

583 ***Confocal fluorescence microscopy.*** Cells were grown in 12-well plates with a cover slide in the  
584 bottom of the well. After different treatments, cells were fixed using 4% formaldehyde for 30 min at  
585 room temperature and then permeabilized in PBS 0.1% Triton X-100 for 30 min at room temperature.  
586 Following a 1 h saturation step with PBS 1% bovine serum albumin, 0.1% Tween 20, cells were  
587 stained for 2 h at room temperature with the following antibodies: anti-NP-FITC (1/30), anti-NS1  
588 (1/200), anti-M2 (1/150), anti-M1 (1/150), anti-PA (1/50), anti-PB2 (1/200), anti-NEP/NS2 (1/100)  
589 or anti-NP mAb clone 3/1 (1/1000). An anti-rabbit-AF488 was used for 2 h at room temperature as

590 the secondary antibody for NS1, NEP/NS2 and PB2, and an anti-mouse-AF488 was used for other  
591 proteins. Then actin was stained with ActinRed 555 reagent for 30 min and nuclei were stained with  
592 the NucBlue reagent for 5 min. Samples were analyzed with a Leica SP8 confocal microscope and  
593 Leica LasX Life Sciences Software. Relative nuclear intensity was determined by using the Intensity  
594 Ratio Nuclei Cytoplasm Tool, RRID:SCR\_018573).

595 ***Succinylation analysis by LC-MS/MS.*** Human bronchial epithelial BEAS-2B cells were grown in 10  
596 culture dishes (150 mm) *per* condition. After different treatments, cells were washed with 5 mL of cold  
597 PBS, then lysed with 5 mL of urea buffer (8 M urea, 200 mM ammonium bicarbonate). The lysates were  
598 clarified by centrifugation at 12,000 g for 15 min at 4°C. Protein concentrations were measured with a  
599 Nano Drop spectrophotometer to take samples corresponding to 8 mg of proteins. Reduction and  
600 alkylation of proteins were performed by incubation for 1 h at 37°C with 10 mL of DTT 10 mM (5mM  
601 final) and then 30 min at room temperature with 4 mL of iodoacetamide 55 mM (10 mM final). After  
602 addition of 20 mL ammonium bicarbonate 200 mM, proteins were digested for 2 h at 37°C with  
603 Trypsin/LysC diluted 1/1000. 800 µL of TPCK-trypsin at 1 mg/mL were added and the samples were  
604 incubated overnight at 37°C. Five mL of 50% formic acid was added to allow purification of peptides  
605 over Sep-Pak® C<sub>18</sub> columns. Briefly, columns were equilibrated with 3x2 mL of 70% ACN + 0.1%  
606 formic acid and were washed with 3x2 mL of 0.1% formic acid. Samples were loaded, and columns  
607 washed as previously. Elution was done with 6 mL 40% ACN + 0.1% formic acid. Eluates were frozen  
608 (-80°C) overnight and lyophilized. Immunoaffinity purification of succinylated peptides was performed  
609 with a PTMScan® Succinyl-Lysine Motif kit according to the manufacturer's instructions. Immuno-  
610 isolate peptide samples were then loaded onto a homemade C18 StageTip for desalting. Peptides were  
611 eluted using 40/60 MeCN/H<sub>2</sub>O + 0.1% formic acid and vacuum concentrated to dryness before being  
612 reconstituted in injection buffer (2% MeCN/ 0.3% TFA) for nano-LC-MS/MS analysis. Liquid  
613 chromatography (LC) was performed with an RSLCnano system (Ultimate 3000, Thermo Scientific)  
614 coupled online to an Orbitrap Exploris™ 480 mass spectrometer (Thermo Scientific). Peptides were  
615 trapped on a C18 Nano Trap column with buffer A (2/ 98 MeCN/ H<sub>2</sub>O in 0.1% formic acid) at a flow  
616 rate of 3.0 µL/min over 4 min. Separation was performed on a C18 Acclaim PepMap™ RSLC column

617 regulated to a temperature of 50°C with a linear gradient of 2% to 25% buffer B (100% MeCN in 0.1%  
618 formic acid) at a flow rate of 300 nL/min over 91 min. MS full scans were performed in the ultrahigh-  
619 field Orbitrap mass analyzer in m/z ranges of 375–1500 with a resolution of 120,000 at m/z 200. The  
620 top 25 most intense ions were subjected to Orbitrap for further fragmentation via high energy collision  
621 dissociation (HCD) activation and a resolution of 15,000 with the AGC target set to 100%. We selected  
622 ions with charge state from 2<sup>+</sup> to 6<sup>+</sup> for screening. Normalized collision energy (NCE) was set at 30 and  
623 the dynamic exclusion was 45 s. Mass spectrometry proteomics raw data have been deposited with the  
624 ProteomeXchange Consortium via the PRIDE (Perez-Riverol *et al*, 2019) partner repository with the  
625 dataset identifier PXD018419 (username: reviewer53974@ebi.ac.uk, password: hTdy0ILL). For  
626 identification, the data were searched against the *Homo Sapiens* (UP000005640) and the *influenza A*  
627 *virus* (UP000162741) UniProt databases using Sequest-HT through proteome discoverer (version 2.2 or  
628 2.4). Enzyme specificity was set to trypsin and a maximum of three-missed cleavages sites were allowed.  
629 Oxidized methionine, N-terminal acetylation, Succinyl-Lysine and carbamidomethyl cysteine were set  
630 as variable modifications. The maximum mass deviation allowed was set to 10 ppm for monoisotopic  
631 precursor ions and 0.02 Da for MS/MS peaks. The resulting files were further processed using myProMS  
632 (Pouillet *et al*, 2007). FDR calculation employed Percolator and was set to 1% at the peptide level for  
633 the whole study. To quantify the succinylated peptide that contained lysine K87, we extracted from the  
634 MS survey of the nano-LC-MS/MS raw files the ion chromatogram (XIC) signal using the retention  
635 time and m/z values of the well-characterized tryptic peptide ions using the Xcalibur softwares  
636 (manually). XIC areas were integrated in Xcalibur under the QualBrowser interface using the ICIS  
637 algorithm. Boxplots were constructed from two sets of three independent experiments (IAV and IAV +  
638 Succinate). Thus, three data points were used for each box. The whiskers correspond to the minimum  
639 and maximum values, while the last point is the median. The first and third quartiles are merely the  
640 average values between the median and the extrema. It should be noted that boxplots do not indicate  
641 actual distributions (there are no values inside a box, except the median), but rather they are used to  
642 show different tendencies between two conditions.

643 **Fluorescence anisotropy measurements.** The full-length influenza virus NP (strain A/WSN/1933)  
644 gene, cloned in pET22b (Novagen), was used. The point mutation that gives the K87 > E change was  
645 introduced by PCR, by site directed mutagenesis and full-plasmid amplification, followed by DpnI  
646 restriction digestion. Sequencing was done by Eurofins Genomics. Production in *Escherichia coli* BL21  
647 RIL (DE3) cells and purification of wild-type NP and K87E were carried out as previously described  
648 (Chenavas *et al*, 2013; Labaronne *et al*, 2016). Fluorescence anisotropy measurements were done in a  
649 384-well microplate, and acquisitions were performed at room temperature after a 5 min incubation on  
650 a Clariostar plate reader (BMG Labtech) with polarization filters. NP was serially diluted in buffer (20  
651 mM Tris-HCl pH 7.5, 150 mM NaCl and 5 mM  $\beta$ -mercaptoethanol) and titrated into 5 nM polyUC RNA  
652 (Integrated DNA Technologies) 3'-labelled with 6-fluorescein (FAM). After blank subtraction of the  
653 RNA alone, a triplicate of each titration was fitted to the Prism (GraphPad) binding equation "Single  
654 binding site with Hill slope (h)".

655 **Reverse genetics.** 500 ng of each of the 8 bidirectional pRF483 plasmids encoding all the genomic  
656 segments of the A/Scotland/20/74 (H3N2) virus were transfected by LTX lipofectamine (Invitrogen) in  
657 a mix of HEK293T and MDCK cells. The reconstitution of viruses with an NP bearing either the wild-  
658 type K87, the K87R or the K87E mutations was performed in parallel. Negative controls of each virus  
659 were also prepared by transfection of cells with all genomic-encoding plasmids, with the exception of  
660 the PB1 expression plasmid. After 24 h, the cells were washed and incubated for 48 h in MEM medium  
661 supplemented with 1  $\mu$ g/mL of trypsin-TPCK. The viruses released were further amplified in MDCK  
662 cells in MEM medium supplemented with 1  $\mu$ g/mL of trypsin-TPCK and titrated after 72 h. NP sequence  
663 of rescued viruses (K87 and K87R) were verified by sequencing using the following primers (sense: 5'-  
664 ATG GCG TCC CAA GGC-3'; antisense: 5'-TTA ATT GTC GTA CTC CTC TGC-3'; Eurofins  
665 Genomics)).

666

667 **Statistical analysis.** Sample size estimate for animal studies were based on the experience of our group  
668 on IAV-infected mice model. There was no randomization or blinding procedure. Sample size for the  
669 human investigation could not be calculated because of the exploratory nature of this research but it was

670 estimated according to previous studies with a similar design (Guillon *et al*, 2019, 2015, 2016).  
671 Statistical analyses were performed using GraphPad Prism. Data are reported as mean  $\pm$  SEM. Statistical  
672 values, including the number of replicates (n) and the statistical test used, can be found in the figure  
673 legends. \*p < 0.05, \*\*p < 0.005, \*\*\*p < 0.0005, \*\*\*\*p < 0.0001. For *in vitro* experiments, n = the  
674 number of separate experiments. For *in vivo* work, n = the number of individual animals.

675

676 **Study approvals.** All animal experimentations were performed according to the ethical guidelines and  
677 were approved by our local and national ethics committee (CEEA.19, #201604071220401-4885). **Study**  
678 **of human samples was approved by the IRB of the Tours University Hospital/French national bioethics**  
679 **authority (CPP-37 2012-R21) (DC-2014-2285). Informed written consent was obtained from all**  
680 **subjects. The study was conformed to the principles set out in the VMA Declaration of Helsinki and the**  
681 **Department of Health and Human Service Belmont Report.**

682

683 **Data Availability.** MIAME (minimum information about microarray experiment) compliant data were  
684 deposited in Array-Express at EMBL (access number: E-MTAB-10031). Succinylation data provided  
685 by LC-MS/MS were deposited in PRIDE (Project Name: Influenza A virus-triggered succinylation in  
686 lung epithelial cells; Project accession: PXD018419 (username: reviewer53974@ebi.ac.uk, password:  
687 hTdy0ILL)).

688 **CONTRIBUTIONS**

689 **AG**, Conceptualization, Formal analysis, Supervision, Validation, Visualization, Writing - original  
690 draft; Writing - review & editing; **DD, AC**, Investigation, Methodology, Data curation, Formal  
691 analysis, Validation, Visualization; Writing - review & editing; **TB, JB, FP, VV, AW, LM, CC, LND,**  
692 **AA, FD, AT, ALG, FF, AD**, Investigation, Methodology, Validation ; review & editing; **JMC, TC,**  
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719

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912

913 **FIGURES LEGENDS**

914

915 **Figure 1. Influenza virus infection increases succinate levels in airways.**

916 (a) C57Bl/6 mice were infected (n=9) or not (n=8) intranasally with 150 pfu of influenza A virus (IAV;  
917 A/Scotland/20/74 (H3N2)). Bronchoalveolar lavage fluids (BALs) were collected 4 days post-infection.  
918 Samples were further processed for metabolomic analysis by NMR. *Left panel* shows a volcano-plot  
919 representing the comparison of “IAV-infected” *versus* “control, non-infected” animals. The graph plots  
920 the  $-\log(p\text{-value})$  against the fold change ( $\log_2(\text{ratio})$ ) for individual metabolites. Vertical dashed blue  
921 lines mark the two-fold change and horizontal dashed blue line marks the cut-off p-value of 0.0001.  
922 Metabolites in the upper right square in red are significantly more abundant in “IAV-infected” compared  
923 to “control, non-infected” samples. Succinate is one of these compounds with a FC=2.2 and a FDR-  
924 adjusted p-value=8.10<sup>-7</sup>. **The boxplots whiskers correspond to the minimum and maximum values,**  
925 **while the central band is the mean. The first and third quartiles (boxes) are merely the average values**  
926 **between the median and the extrema.**

927

928 (b) shows succinate quantification by NMR from tracheal aspirates collected in mechanically-ventilated  
929 patients with (pink bar, n=9) or without (orange bar, n=7) diagnostic of IAV pneumonia. \* $P < 0.05$ .

930 (c) IL-6 and IL-8 measurements by ELISA from these same tracheal aspirates.

931 (d) Main baseline characteristics of the patients included in the study.

932 Data information: Data are  $\text{mean} \pm \text{SEM}$  and statistical analysis was performed using the Mann-Whitney  
933 U-test.

934

935 **Figure 2. Succinate reverses the inflammatory response and the metabolic modifications induced**  
936 **by influenza virus.**

937 Bronchial epithelial (BEAS-2B) cells were infected or not with influenza A/Scotland/20/74 (H3N2)  
938 virus (IAV) at MOI=1 for 4 h and treated or not with succinate (Suc; 4 mg/mL / 24.7 mM) for 20 h.

939 (a) volcano-plot showing the most significantly regulated canonical pathways determined by microarray  
940 analysis compared with mock-treated cells. Each dot represents a specific canonical pathway as

941 determined by GSEA. Pathway representations result from the magnitude of regulation (z-score, x-axis)  
942 and significance ( $-\log_{10}$  adjusted p-value, y-axis). The dashed horizontal line indicates the statistical  
943 significance threshold ( $p \leq 0.01$  after adjustment with the Bonferroni correction). The two vertical  
944 dashed lines show the z-score threshold (-2: repressed; 2: induced). Colored spots characterize the most  
945 highly regulated canonical pathways endowed with statistical significance.

946 **(b)** Representative inflammatory protein-array blots obtained from the supernatants of control- or IAV-  
947 infected- or IAV-infected and succinate-treated cells. The table on the right side indicates the 8 most  
948 regulated mediators by succinate.

949 **(c)** Quantification by ELISA of IL-6, IL-8, IP-10 and RANTES in the supernatants of cells infected or  
950 not by IAV and treated or not with succinate.

951 **(d, e)** Panels show the basal replotted ECAR and OCR **(d)**, and glycolytic capacity (ECAR max) and  
952 maximal respiration (OCR max) **(e)** in cells infected with IAV and treated (green symbols) or not (pink  
953 symbols) with succinate. Data were normalized with respect to mock-infected, untreated cells (orange  
954 symbols), and were also normalized for DNA content.

955 Data information: Data are the mean  $\pm$  SEM of 6 (panel **c**) or 4 (panels **a, d, e**) independent experiments.  
956 Statistical analysis was performed using the Kruskal-Wallis test with Dunn's post-test **(c, d, e)** ( $*P <$   
957  $0.05$ ) and ANOVA with Holm-Sidak's post-test **(a)** ( $*P < 0.01$ ).

958

959 **Figure 3. Succinate does not inhibit the secretion of inflammatory mediators induced by Poly(I:C),**  
960 **but blocks the multiplication of influenza virus in lung epithelial cells.**

961 Bronchial epithelial (BEAS-2B) cells were challenged with PBS (as control) or with 2  $\mu\text{g/mL}$  Poly(I:C)  
962 (PIC) or with influenza A/Scotland/20/74 (H3N2) virus (IAV) at MOI=1 for 4 h and treated or not with  
963 succinate (Suc) for 20 h.

964 **(a)** Levels of IL-6 and IL-8/CXCL8, as measured by ELISA in the supernatants of cells stimulated with  
965 (PIC) and subsequently treated or not with succinate.

966 **(b-e)** A neuraminidase (NA) activity test **(b)**, a Plaque-Forming Units assay **(c)** and scanning electron  
967 microscopy **(d)** were used to assess the production of physical **(b, d)** and infectious **(c)** viral particles in

968 the supernatants of IAV-infected cells, treated or not with succinate. IAV particles budding **are colored**  
969 **in purple**. Scale bar: 1  $\mu\text{m}$ .

970 **(e)** A neuraminidase activity test was also applied in cells infected or not by IAV and treated or not with  
971 malonate (a succinate dehydrogenase inhibitor), in comparison with succinate.

972 Data information: Data are represented as the mean  $\pm$  SEM of 4 **(a)** or 6 **(b, c, e)** independent  
973 experiments. Statistical analysis was performed using the Kruskal-Wallis test with Dunn's post-test (**\*P**  
974 **< 0.05**).

975

#### 976 **Figure 4. Succinate protects mice from influenza infection**

977 8-week-old female C57Bl/6 mice were infected intranasally with 150 pfu of influenza A/Scotland/20/74  
978 (H3N2) virus (IAV) and treated or not simultaneously with 4 mg of succinate (Suc; by the intranasal  
979 route).

980 **(a-h)** Some mice were euthanized at 4 days post-infection and lungs as well as BAL fluids were collected  
981 to determine: **(a)** the viral load by a plaque forming unit assay; **(b) the viral protein expression by**  
982 **Western blotting followed by a relative quantification (c), (d)** the levels of 111 additional mediators  
983 using a specific protein-array; **(e, g)** the levels of KC, IL-6 and MPO by ELISA; **(f)** the number of  
984 immune and inflammatory cells by flow cytometry; **(h)** tissue lesions in lung sections stained with  
985 hematoxylin-eosin and further assessed by microscopy. Scale bar: x6/20  $\mu\text{m}$ . All data are represented  
986 as the mean or the mean  $\pm$  SEM and are cumulative of **2 independent experiments with 5 animals each**.  
987 **(i, j)** In separate experiments, body weight loss (n=8) **(i)** and animal survival (n=10) **(j)** were monitored  
988 daily. **Data are represented as the mean  $\pm$  SEM.**

989 Data information: Statistical analysis was performed using the Mann-Whitney test **(a, c, h, i)**, the log  
990 rank test **(j)** and the Kruskal-Wallis with Dunn's post-test **(e, f, g)**, (**\*P < 0.05**).

991

#### 992 **Figure 5. Succinate impairs the intracellular trafficking of influenza virus.**

993 **(a)** Schematic representation of the IAV replication cycle. After endocytosis, the viral ribonucleoprotein  
994 (vRNP) complexes are transported into the nucleus. Viral RNAs (vRNAs) serve as templates for the  
995 synthesis of messenger RNAs (mRNAs) and complementary RNAs (cRNAs) are used for the replication

996 of vRNAs. mRNAs are exported in the cytoplasm for translation. Some proteins are transported back to  
997 the nucleus to form new vRNPs with neosynthesized vRNAs. Newly synthesized vRNPs are exported  
998 in the cytoplasm *via* the CRM1 protein. HA, NA, M2 proteins and the vRNPs are transported to the  
999 plasma membrane for assembly and budding of the progeny virions.

1000 **(b-f)** Human bronchial epithelial BEAS-2B cells were infected with A/Scotland/20/74 (H3N2) virus  
1001 (IAV) at MOI=1. After 4 h, cells were treated or not with succinate (Suc; 4 mg/ml) up to 20 h. The effect  
1002 of succinate on IAV transcription **(b)** and protein expression **(c)** were assessed by RT-qPCR to quantify  
1003 the M1 viral mRNA and Western blotting to detect viral proteins ( $\beta$ -actin was used as a loading control),  
1004 respectively. **(d-f)** Human alveolar epithelial A549 cells were infected with the recombinant influenza  
1005 A/WSN/33 virus expressing a fusion NS1-eGFP protein at MOI= 0.5 for 4 h, then treated with 4 mg/mL  
1006 of succinate. A549 cells were monitored for 24 h using a BioStation IM-Q device. **(d)** Single-cell  
1007 dynamics of the nuclear/cytoplasmic fluorescence ratio. **(e)** Single-cell dynamics of cell death as  
1008 assessed by morphological analysis. **(f)** Quantification of the nuclear/cytoplasmic fluorescence ratio  
1009 measured at 13h post-succinate treatment. Data are represented as the mean  $\pm$  SEM of 3 biological  
1010 replicates **(b-c)** or 3 independent experiments **with 3 technical replicates each (d-f)**. Statistical analysis  
1011 was performed using the Mann-Whitney test **(b-e)** or t-test **(f)**, (\* $P < 0.05$ ).

1012

1013 **Figure 6. The anti-influenza virus effect of succinate involves the nuclear retention of NP and NS1**  
1014 **proteins.**

1015 **(a-c)** Human bronchial epithelial BEAS-2B cells were infected with influenza A/Scotland/20/74 (H3N2)  
1016 virus (IAV) at MOI=1 for 4 h, then washed and treated or not with succinate (Suc; 4 mg/mL) for 20 h.  
1017 **(a, c)** Expression of IAV proteins (*i.e.* PA, PB2, M2, M1, NP, NS1) was analyzed by confocal  
1018 microscopy using specific antibodies. Viral proteins are stained in green, DNA in blue and actin in red.  
1019 For a given antibody, pictures were taken on the exact same day with the same laser power settings,  
1020 Pictures are representative of five independent experiments. Scale bar 10  $\mu$ M. **(b)** Relative nuclear  
1021 intensity of NS1 or NP was determined by using the Intensity Ratio Nuclei Cytoplasm Tool. Seven  
1022 (NS1) or nine (NP) random images were collected *per* treatment with at least 5 cells *per* field. **Data are**

1023 represented as the mean  $\pm$  SEM. Statistical analysis was performed using the Mann-Whitney test, (\* $P <$   
1024 0.05).

1025

1026 **Figure 7. Nuclear retention of NP -and not NS1- is a key mechanism underlying the anti-IAV**  
1027 **effect of succinate.**

1028 (a-c) Human bronchial epithelial BEAS-2B cells were infected for 4 h with influenza A/PR/8/34 (H1N1)  
1029 virus, either wild-type (PR8 WT; at MOI=10 (a, b)) or carrying a deletion of the NS1 coding sequence  
1030 (PR8 $\Delta$ NS1; at MOI=4 (c)), and subsequently treated or not for 20 h with 4 mg/mL of succinate (Suc).  
1031 Expression of NP (a, c) and NS1 (a) proteins was analyzed by confocal immunofluorescence  
1032 microscopy. Viral proteins are stained in green, DNA in blue and actin in red. For a given antibody,  
1033 pictures were taken on the exact same day with the same laser power settings. They are representative  
1034 of three independent experiments; scale bar 10  $\mu$ M. (b) Neuraminidase (NA) activity in the supernatants  
1035 of epithelial cells infected by PR8 WT treated or not with succinate. Data are represented as the mean  $\pm$   
1036 SEM of 4 independent experiments. Statistical analysis was performed using the Student's t-test, (\* $P <$   
1037 0.05).

1038

1039 **Figure 8. Succinate reduces the assembly of influenza virus vRNPs by inducing succinylation of**  
1040 **NP at K87, a highly conserved amino acid probably involved in vRNA binding.**

1041 Human bronchial epithelial BEAS-2B cells were infected with the influenza A/Scotland/20/74 (H3N2)  
1042 virus (IAV) at MOI=1 for 4 h, and subsequently treated or not with 4 mg/mL of succinate (Suc) for 20  
1043 h.

1044 (a, b) Cells were stained using a monoclonal antibody that specifically recognizes "NP-vRNA"  
1045 complexes, but not RNA-free NP, and confocal microscopy (a) was performed (green staining). **The**  
1046 **value raw integrated density (RawIntDen, which is the sum of all pixel values in the ROI (region of**  
1047 **interest)) was further measured (b).**

1048 (c) Cells were lysed and proteins were digested to peptides with Lys-C and trypsin. Then, succinylated  
1049 peptides were isolated directly from protease-digested protein extracts by immunoaffinity purification  
1050 (IAP) using an antibody specific for the succinyl-Lysine motif. Next, the modified peptides were further

1051 analyzed by LC-MS/MS. Box plots show the distribution of intensities of the succinylated NP  
1052 “YLEEHPSAGK87succDPK” peptide, computed as the integrated mass spectrometry peak area of the  
1053 modified K87 succinyl peptide (n=3). The boxplots whiskers correspond to the minimum and maximum  
1054 values, while the central band is the median. The first and third quartiles (boxes) are merely the average  
1055 values between the median and the extrema.

1056 (d) Interaction of recombinant of wild-type NP (NP K87) or a K87E mutant protein (NP K87E) with the  
1057 indicated synthetic polyUC RNAs as measured by fluorescence anisotropy.

1058 (e) Human bronchial epithelial BEAS-2B cells were infected with a A/Scotland/20/74 (H3N2) virus  
1059 (IAV) carrying a wild-type NP (NP WT) or with the corresponding mutated virus bearing a NP with a  
1060 K87R substitution (NP K87R). Cells were infected by either virus at MOI=1 for 4 h, then washed and  
1061 treated or not with 4 mg/mL of succinate for 20 h. Localization of NP proteins was analyzed by confocal  
1062 immunofluorescence microscopy. Scale bar: 10  $\mu$ M. Pictures are representative of three independent  
1063 experiments.

1064 (f) Relative nuclear intensity of NP was determined by using the Intensity Ratio Nuclei Cytoplasm Tool.

1065 (g) Plaque-Forming Unit assays determined the production of infectious viral particles in cell  
1066 supernatants.

1067 Data information: Data are represented as the mean  $\pm$  SEM of 3 biological replicates with (g) or without  
1068 (a, c-e) 2 technical replicates each. 8 (b) or at least 10 (f) random images were analysed *per* treatment  
1069 with a minimum of 5 cells *per* field. Statistical analysis was performed using the Mann-Whitney test (b,  
1070 c, d, f) or paired t-test (g), (\* $P < 0.05$ ).

1071

## 1072 **Figure 9. Hypothetical model of the anti-influenza activity of succinate**

1073 Succinate triggers a unique succinylation modification of K87 in the NP of IAV which further alters the  
1074 electrostatic environment of the vRNA binding site. As a result, the formation of vRNPs particles is  
1075 impaired. This further contributes to an inhibition of viral multiplication as well as viral-induced  
1076 inflammatory response. *In vivo*, IAV-infected mice treated intranasally with succinate are more resistant  
1077 to the development of acute pneumonia.

1078

1079 **EXPANDED FIGURES LEGENDS**

1080

1081 **Figure EV1. Anti-influenza effect of succinate is more potent in multicycle replication condition**  
1082 **than in single cycle replication condition.**

1083 BEAS-2B were infected with influenza A/Scotland/20/74 (H3N2) virus (IAV) at MOI=1 (single cycle  
1084 replication) or at MOI=10<sup>-3</sup> (multicycle replication) for 4h and treated or not with succinate (Suc) for  
1085 20h. 2 µg/mL of TPCK treated Trypsin were added simultaneously to succinate to promote multicycle  
1086 replication.

1087 **(a, b)** Plaque-Forming Unit assays determined the production of infectious viral particles in cell  
1088 supernatants.

1089 **(c)** The effect of succinate on IAV transcription was assessed by RT-qPCR to quantify M1 viral mRNA.

1090 Data information: Data are represented as the mean ± SEM of 3 independent experiments. Statistical  
1091 analysis was performed using paired t-test **(a, b)** or one sample t-test **(c)**, (\**P* < 0.05).

1092

1093 **Figure EV2. Nucleoprotein localisation determined by flow cytometry.**

1094 BEAS-2B cells were infected with influenza A/Scotland/20/74 (H3N2) virus (IAV)) at MOI 1 for 4h  
1095 and treated or not with succinate (Suc) for 20h. Nucleoprotein (NP) expression was assessed by flow  
1096 cytometry using two different permeabilization buffers, one allowing whole cell fluorescent staining of  
1097 NP, the other only the NP-associated to the cytoplasmic compartment (by subtraction, nuclear-  
1098 associated NP signal was extrapolated). Data are represented as the mean ± SEM of 4 independent  
1099 experiments. Statistical analysis was performed using ANOVA with Sidak's post test, (\**P* < 0.05).

1100

1101 **Figure EV3. Succinate does not alter the CRM1-dependent transport pathway or the NP**  
1102 **oligomerization.**

1103 **(a, b)** Human bronchial epithelial BEAS-2B cells were infected or not with the A/Scotland/20/74  
1104 (H3N2) virus (IAV) at MOI=1 for 4 h, and subsequently treated or not with 4 mg/mL of succinate for  
1105 20 h **(a)** or with 10 mM of the CRM1 inhibitor leptomyacin B (LMB; *middle panels* in **b**). The expression  
1106 of CRM1 was analyzed by Western blotting **(a)**. Expression of the viral protein NEP/NS2 was analyzed

1107 by confocal fluorescence microscopy **(b)**. NEP/NS2 is stained in green, nuclear DNA in blue and actin  
1108 cytoskeleton in red. Scale bar: 10  $\mu$ M.

1109 **(c)** The formation of NP oligomers and monomers was assessed by Western blotting under non-reducing  
1110 and reducing conditions.

1111 Data information: Pictures are representative of three independent experiments.

1112

1113 **Expanded Movies EV1. Intracellular trafficking of influenza virus, in absence of succinate**

1114 **treatment.** A549 cells grown on cell culture in micro-wells were infected with NS1-eGFP-WSN at  
1115 an MOI of 0.5 pfu/cell. Pictures were taken every 10 min starting at 1 h post-infection and continuing  
1116 until 24 h post-infection.

1117

1118 **Expanded Movies EV2. Succinate impairs the intracellular trafficking of influenza virus.**

1119 A549 cells grown on cell culture in micro-wells were infected with NS1-eGFP-WSN at an MOI of  
1120 0.5 pfu/cell and treated with 4mg/ml of succinate. Pictures were taken every 10 min starting at 1 h  
1121 post-infection and continuing until 24 h post-infection.

1122

1123

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1125

1126 **APPENDIX FIGURES LEGENDS**

1127

1128 **Appendix Figure S1. Effect of succinate on gene expression in influenza virus-infected human**  
1129 **bronchial epithelial cells.**

1130 Human bronchial epithelial BEAS-2B cells (4 replicates *per* condition) were infected or not (“Ctrl”)  
1131 with the A/Scotland/20/74 (H3N2) virus (IAV) at MOI=1 and were treated or not with succinate  
1132 (4mg/mL). After 24 h of infection, cells were lysed and total RNA was purified and processed to  
1133 hybridize pangenomic microarrays. Differentially expressed genes (p-value  $\leq 2$ ) between two conditions  
1134 (“IAV-infected cells” *vs.* “control cells” and “IAV-infected cells treated with succinate” *vs.* “control  
1135 cells”) were then selected to perform a gene ontology analysis. Significantly enriched pathways were  
1136 identified using Ingenuity Pathway Analysis (IPA) and are represented as a bar plot representing the  
1137 right-tailed Fisher’s exact test that was used to calculate a p-value (probability that each canonical  
1138 pathway assigned to that data set is due to chance alone).

1139

1140 **Appendix Figure S2. Succinate is not cytotoxic.**

1141 Human bronchial epithelial BEAS-2B cells were treated or not with increasing doses (**a**) or 4 mg/mL  
1142 (**b**) of succinate for 24 h. Cell proliferation and cytotoxicity were further assessed by an MTS test (**a**)  
1143 and by flow cytometry using a Live/Dead staining/Annexin V/Propidium Iodide co-staining (**b**),  
1144 respectively. Data are represented as the mean  $\pm$ SEM. Statistical analysis was performed using the  
1145 Kruskal-Wallis test (**a**, n=3) and the multiple t-test (**b**, n=6).

1146

1147 **Appendix Figure S3. Scanning and transmission electron microscopy of IAV-infected lung**  
1148 **epithelial cells, treated or not by succinate.**

1149 Bronchial epithelial (BEAS-2B) cells were infected with influenza A/Scotland/20/74 (H3N2) virus  
1150 (IAV) at MOI=5 for 4 h and treated or not with succinate (Suc) for 20 h. Scanning (**a**) and transmission  
1151 (**b**) electron microscopy were used to assess the production of physical viral particles in the supernatants  
1152 of IAV-infected cells, treated or not with succinate. Scale bar: 1  $\mu$ M.

1153

1154 **Appendix Figure S4. Antiviral effect of succinate in distinct airway epithelial cell lines.**

1155 Human lung epithelial (a) BEAS-2B, (b) 16HBE14o- or (c) A549 cells were infected with influenza  
1156 A/Scotland/20/74 (H3N2) virus (IAV) at MOI=1 for 4h and treated or not with succinate (Suc) for 20h.  
1157 Plaque-Forming Units assay determined the production of infectious viral particles in cell supernatants.  
1158 Data are represented as the mean  $\pm$  SEM of 3 independent experiments. Statistical analysis was  
1159 performed using paired t-test.

1160

1161 **Appendix Figure S5. Datasheet of mouse XL cytokine array kit.**

1162 *Upper panels:* High resolution scans of original mouse XL cytokine arrays, *Middle panel:* overlay  
1163 template and coordinates; *Lower panels:* reading appendix.

1164

1165 **Appendix Figure S6. Flow cytometry gating strategy.**

1166 Surface gating used to define immune cell subsets in Figure 4.

1167

1168 **Appendix Figure S7. Mass spectrometry methodology and profiles.**

1169 (a) Human bronchial epithelial BEAS-2B cells were infected with A/Scotland/20/74 (H3N2) virus  
1170 (IAV) at MOI=1 for 4 h, and subsequently treated or not with 4 mg/mL of succinate for 20 h. Cells were  
1171 lysed and proteins were digested to peptides with Lys-C and trypsin. Succinylated peptides were isolated  
1172 directly from protease-digested cellular protein extracts by immunoaffinity purification (IAP) using an  
1173 antibody specific for the succinyl-Lysine motif, and the modified peptides were further analyzed by LC-  
1174 MS/MS. (b) Representative MS/MS spectra of succinylated and non-succinylated NP peptides.

1175

1176 **Appendix Figure S8. Similar growth of wild-type K87 and mutant K87R IAV strains.**

1177 Human bronchial epithelial BEAS-2B cells were infected with influenza A/Scotland/20/74 (H3N2)  
1178 (IAV) strains carrying a wild-type NP (“WT”) or a NP with a K87R substitution (“K87R”). Cells were  
1179 infected by either virus at MOI=1 for 4 h, then washed and left untreated for 20 h. Plaque-Forming Units  
1180 assay determined the production of infectious viral particles in cell supernatants. Data are represented

1181 as the mean  $\pm$  SEM of 4 independent experiments. Statistical analysis was performed using the Mann-  
1182 Whitney test.

1183

1184 **Appendix Figure S9. Impact of the NP structure on its interaction capacity with viral RNA.**

1185 *Panel 1:* In NP, the Lysine (K)87 is a positively charged amino acid which is key in the interaction with  
1186 negatively charged vRNA. *Panel 2:* Conversely, glutamic acid (E) is a negatively charged residue. As  
1187 a result, NP K87E mutant interacts less with vRNAs. *Panel 3:* In succinate-treated cells, the addition of  
1188 a succinyl group to the NP K87 residue neutralizes the positive charge of lysine and imparts a negative  
1189 charge, thus altering the NP-vRNA interaction as well.

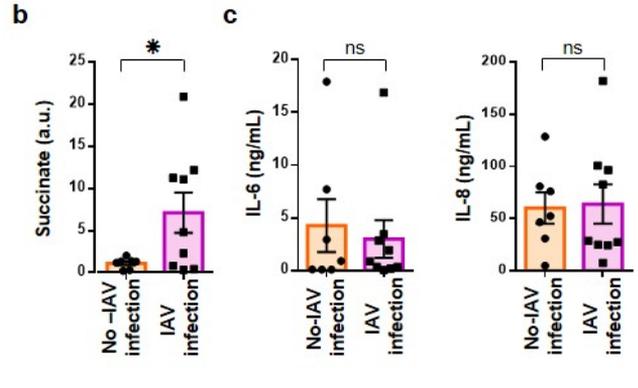
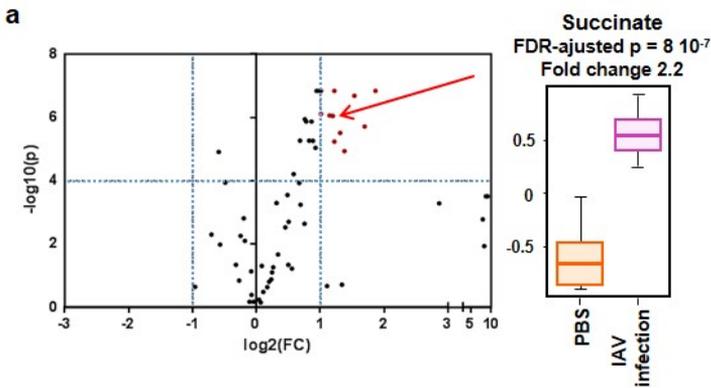
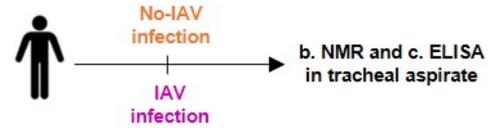
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1191 **Appendix Table S1. List of all reagents and resources used**

1192

1193

# Guillon et al. Figure 1

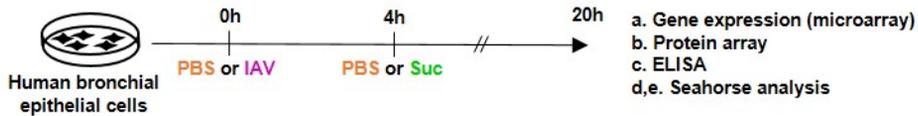


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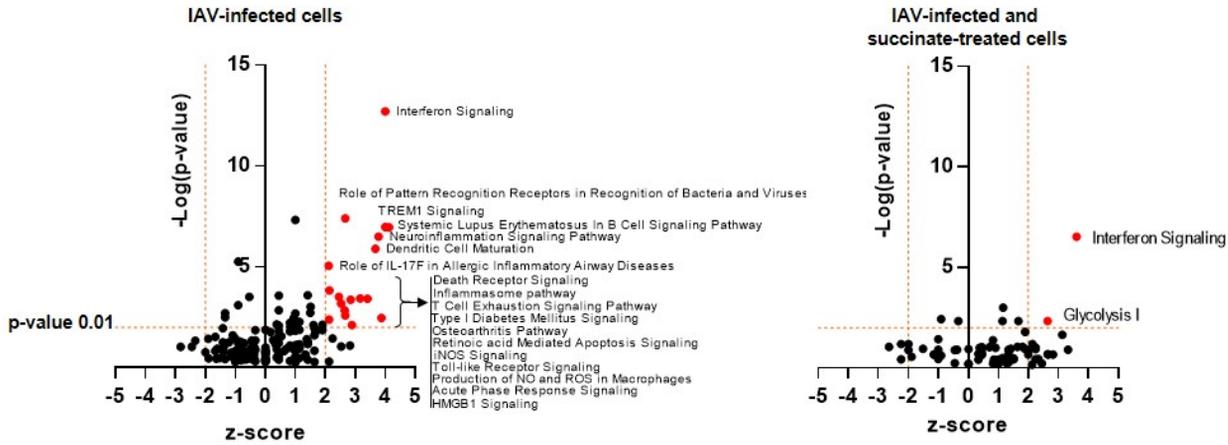
Patients' characteristics	No-IAV infection (n=7)	IAV infection (n=9)	<i>P</i> *
Indication for mechanical ventilation	Coma	Respiratory failure	
Age (y.o.), median [IQR]	57[36-65.5]	57 [44 – 62.5]	0.698
Sexe, men/women	4/3	7/2	0.60
SAPS II, median [IQR]	44.5 [36-62.8]	42[29-45]	0.323
Charlson Comorbidity Index, median [IQR]	1.5[0.75-3]	2[1-3]	0.68
<b>Chronic respiratory disease</b>			
COPD	0/7	0/9	na
Asthma	0/7	0/9	na
Current smoker	1/7	2/9	1.0
<b>Body Mass Index (kg/m<sup>2</sup>), median [IQR]</b>	32.5[24.5-36.4]	26.9[24.2-27.5]	0.25
<b>Time flu symptoms-to-sample, median [IQR]</b>	na	9[7-18]	na
<b>Time ventilator support-to-sample, median [IQR]</b>	4.0[1.5-7.0]	5.0[4.0-7.0]	0.99
<b>Leucocyte counts</b>			
<b>in blood (G/L), median [IQR]</b>	10.6[7.1-13]	15.4[6.9-17.5]	0.28
<b>in tracheal aspirate (n/FOV**)</b>	40[25-55]	30[20-150]	0.36
<b>Microbial examination of tracheal aspirate</b>			
Positive bacterial culture	5/7	2/9	0.13
Positive PCR for Influenza	na	9/9	na

\*Fisher's exact test for contingency or two-tailed Test for median comparison. \*\* Count by microscope field of view (mean of 5 counts)

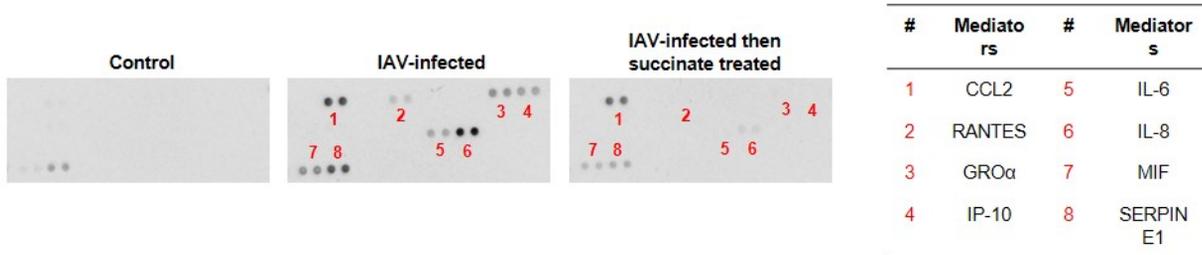
# Guillon *et al.* Figure 2



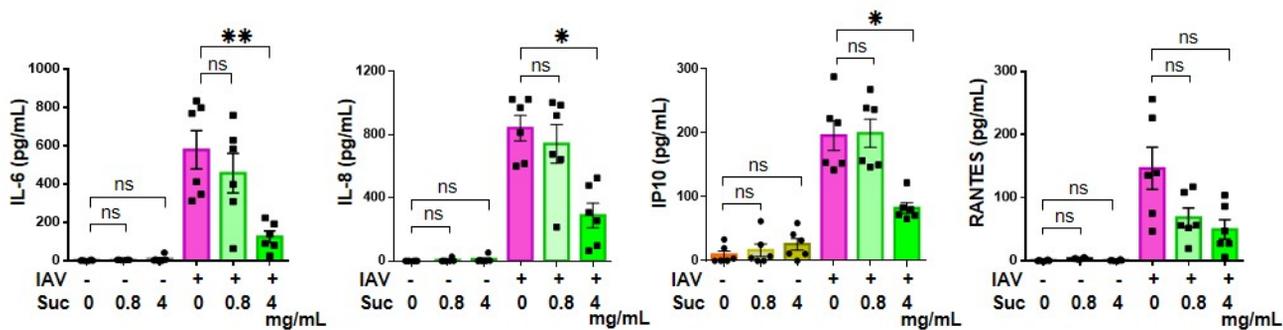
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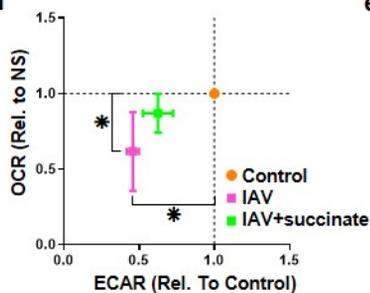
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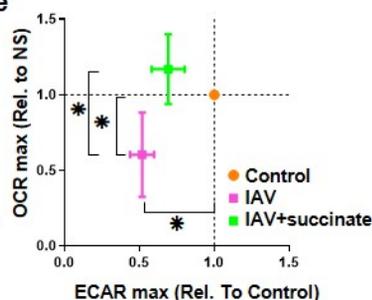
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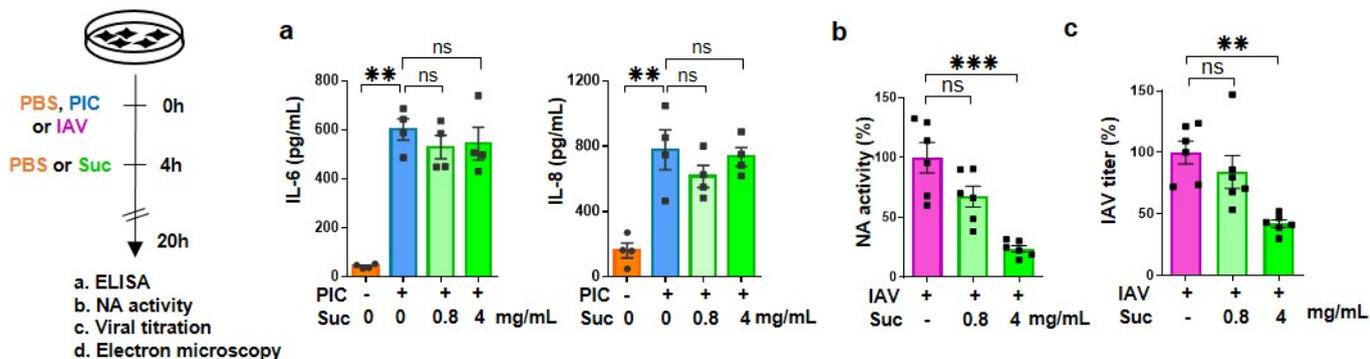
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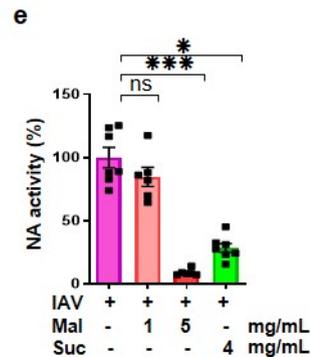
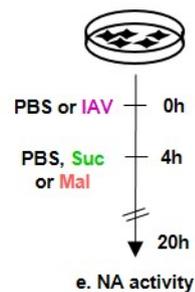
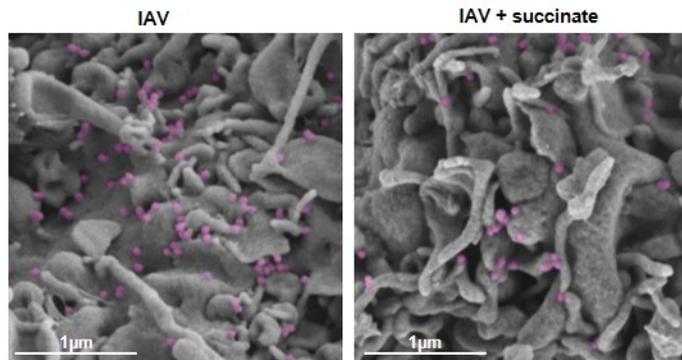
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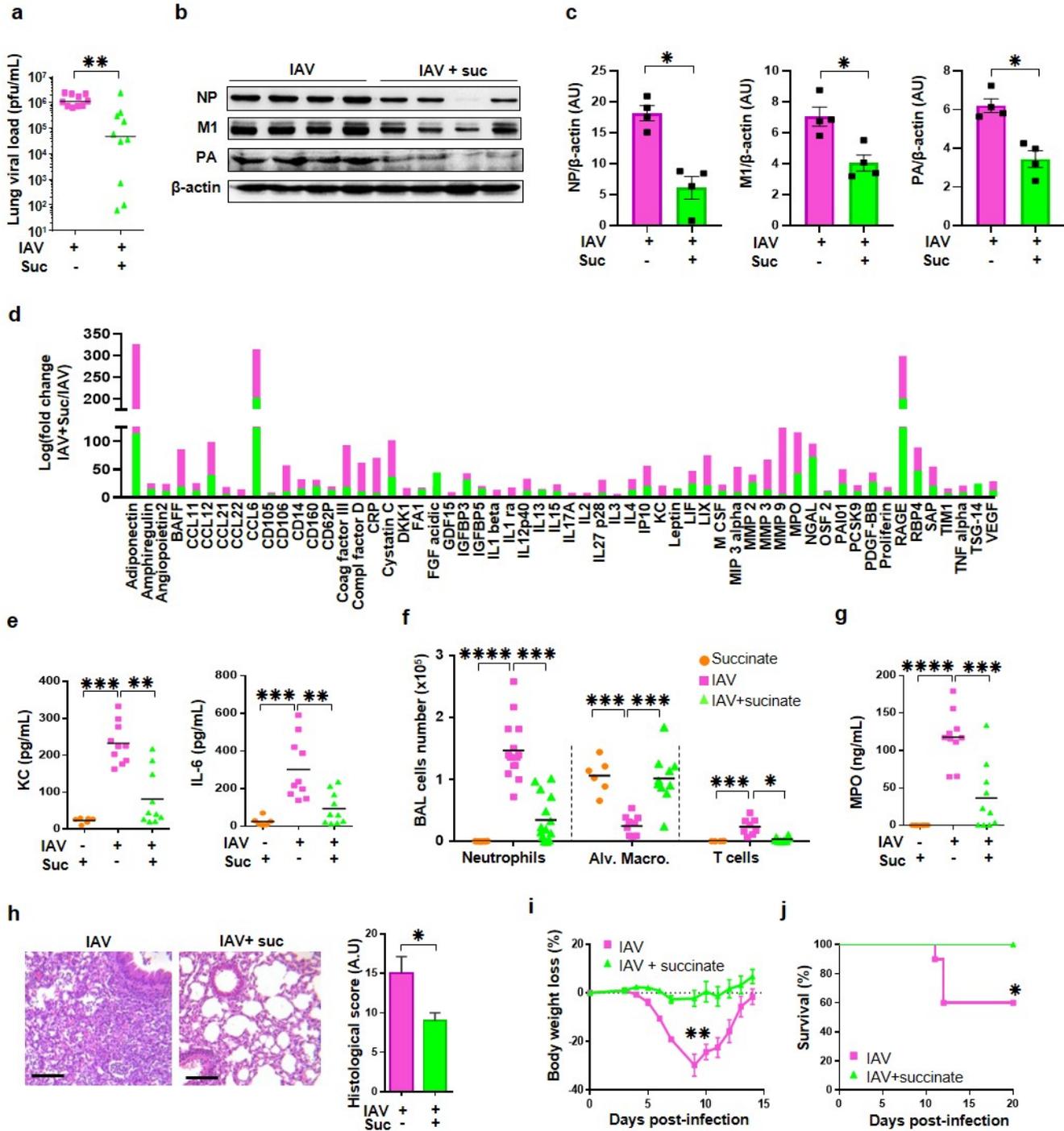
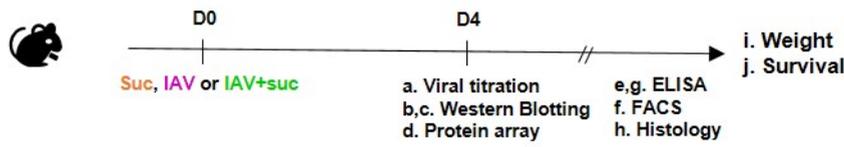
# Guillon *et al.* Figure 3



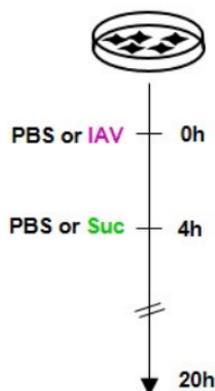
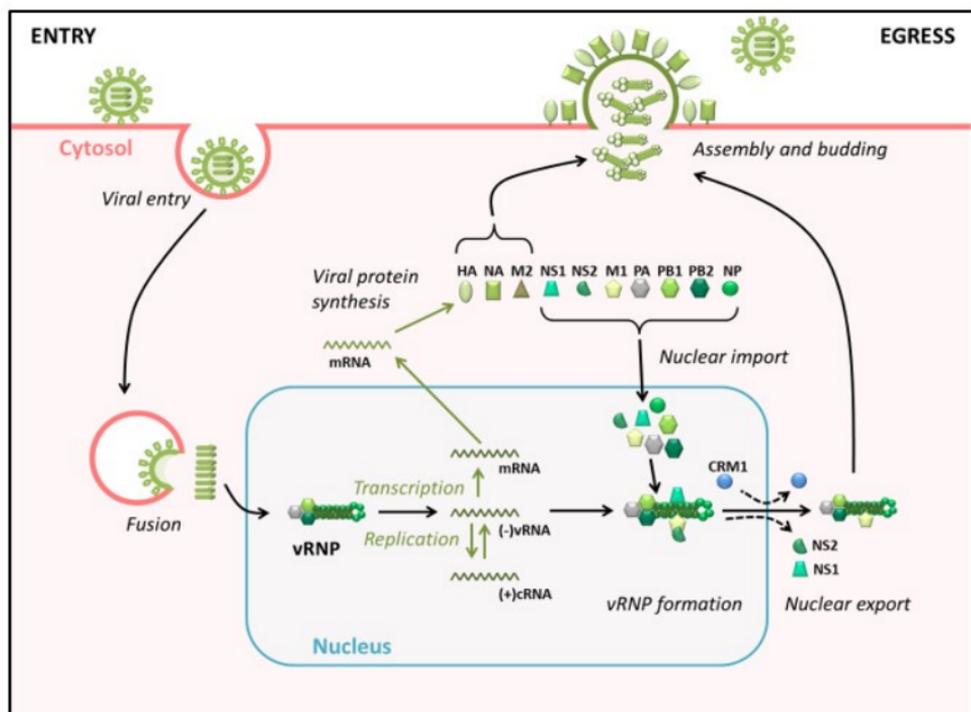
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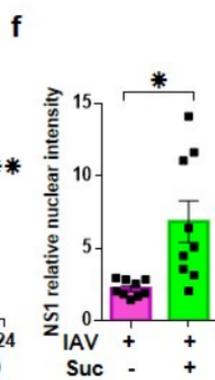
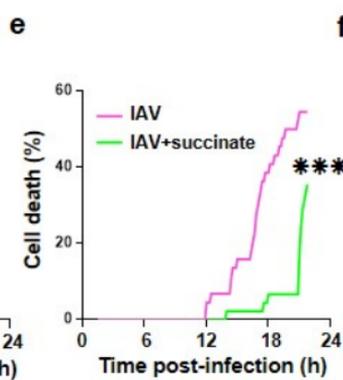
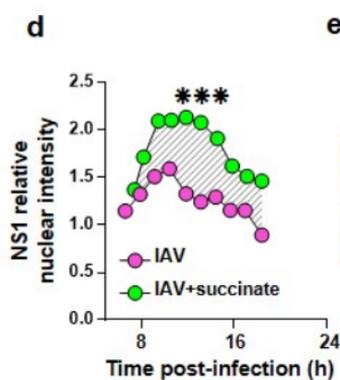
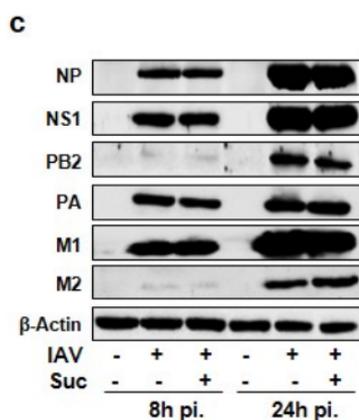
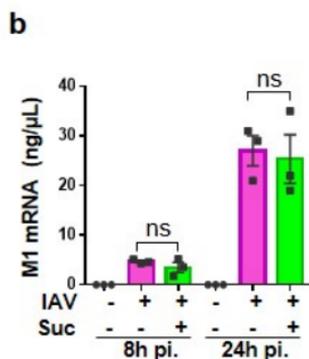
# Guillon *et al.* Figure 4



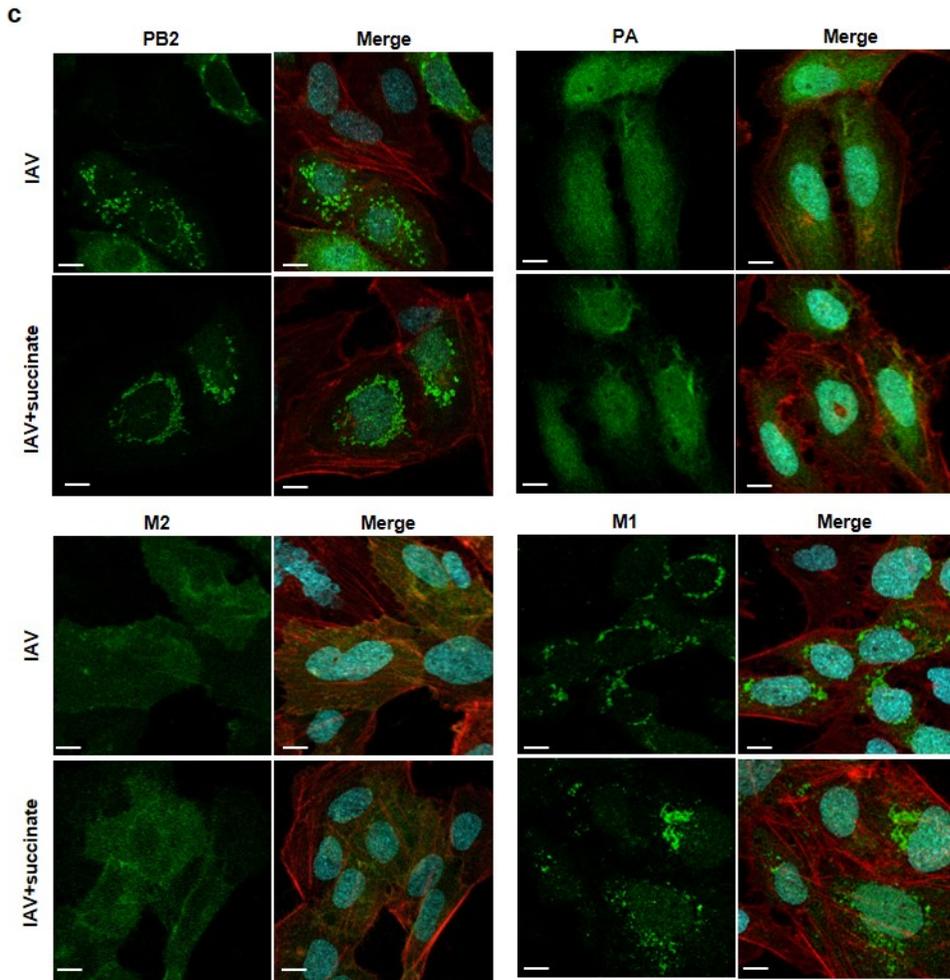
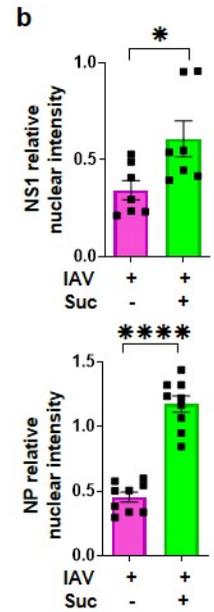
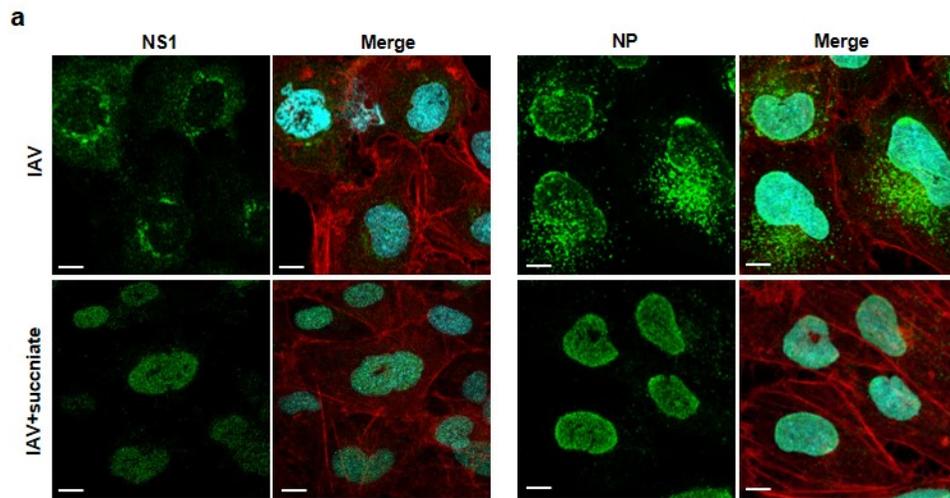
a

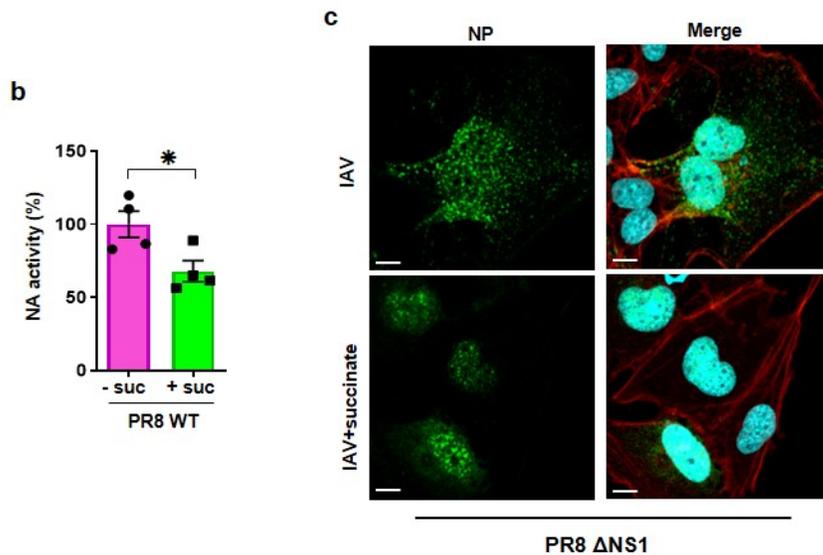
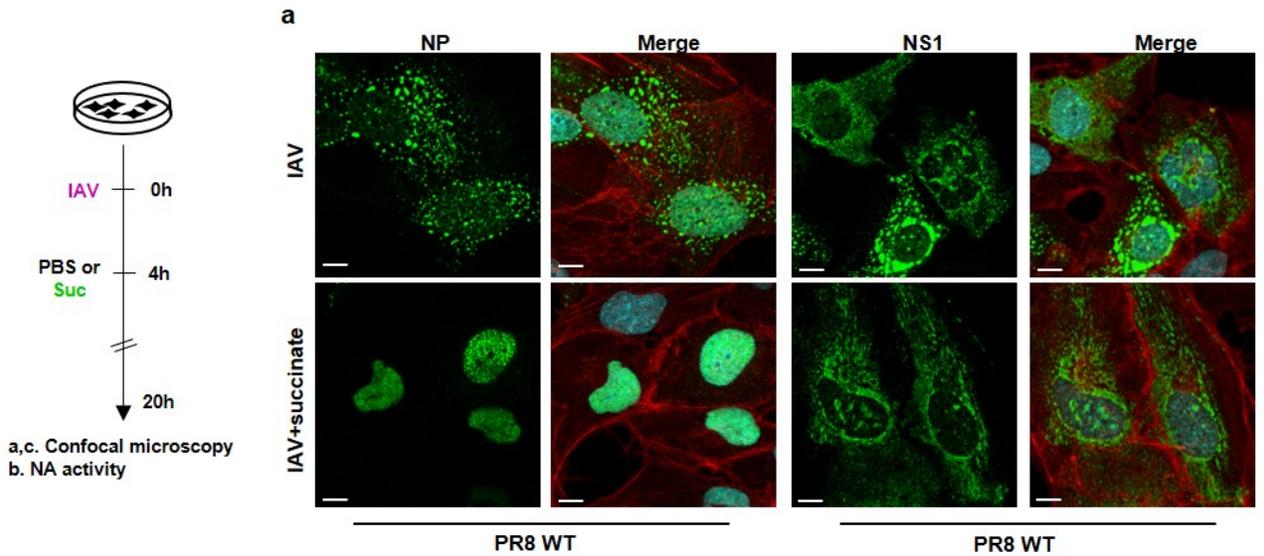


b. qRT-PCR  
c. Western Blotting  
d,e,f. Live imaging

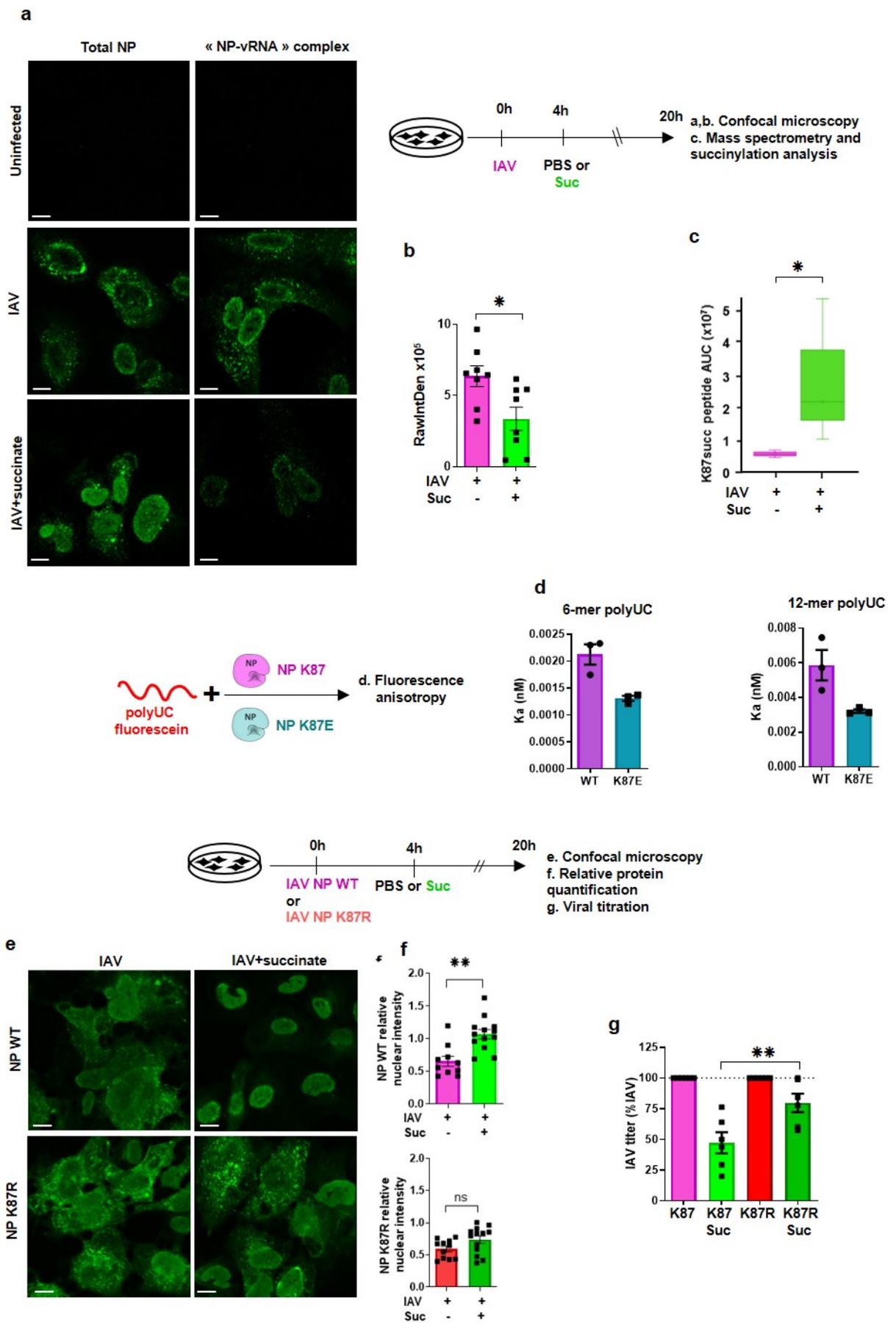


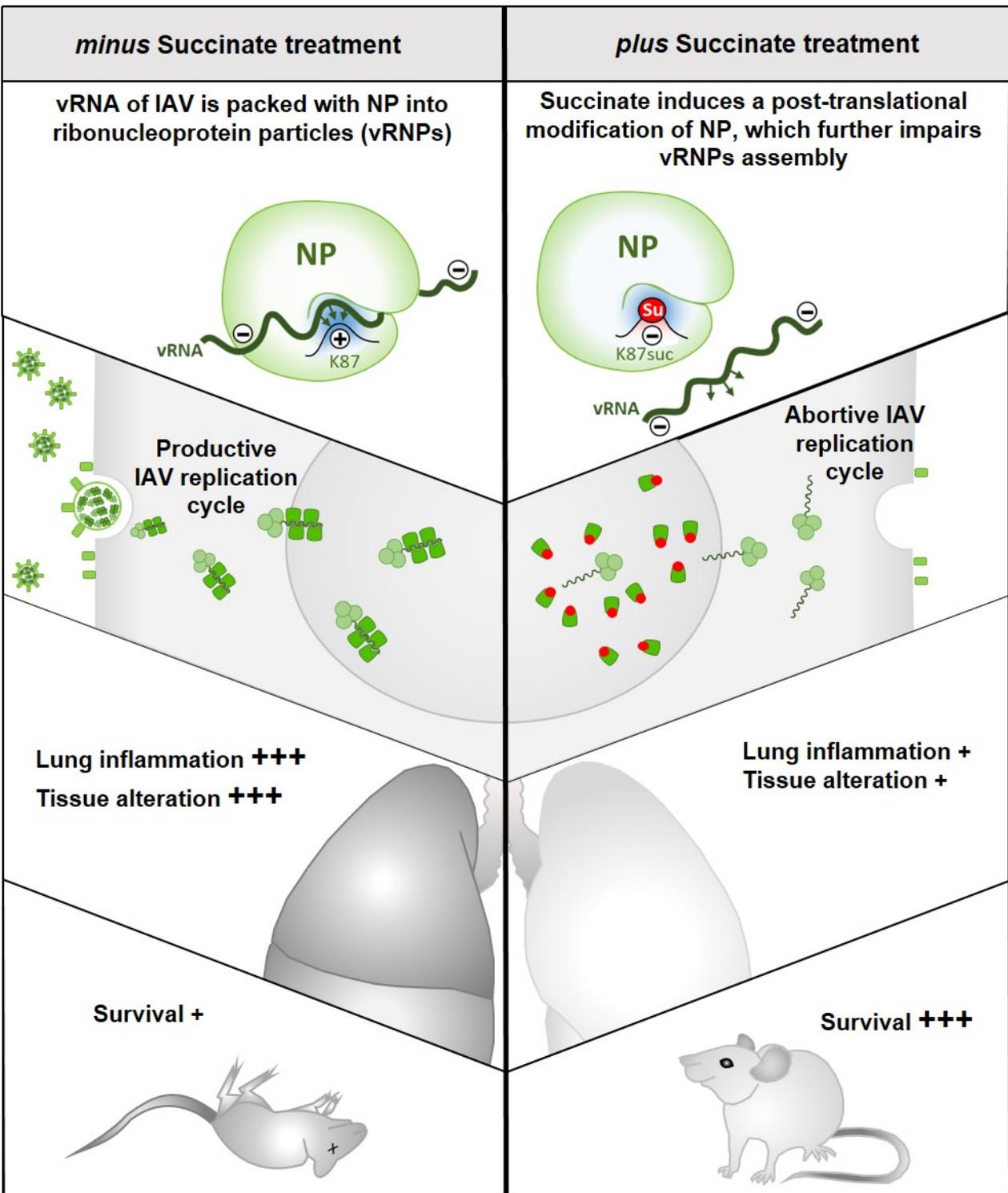
# Guillon *et al.* Figure 6





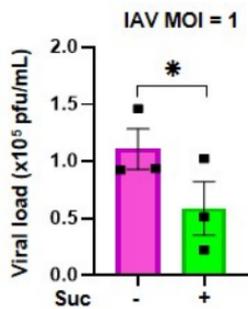
# Guillon et al. Figure 8



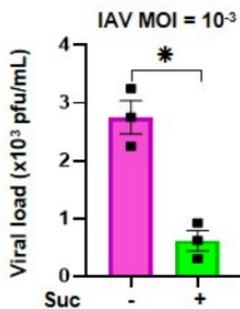


# Guillon *et al.* Figure EV 1

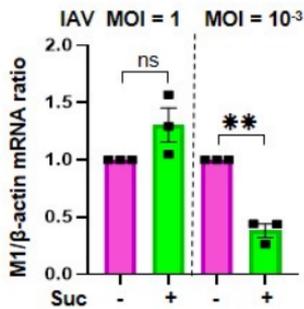
**a**



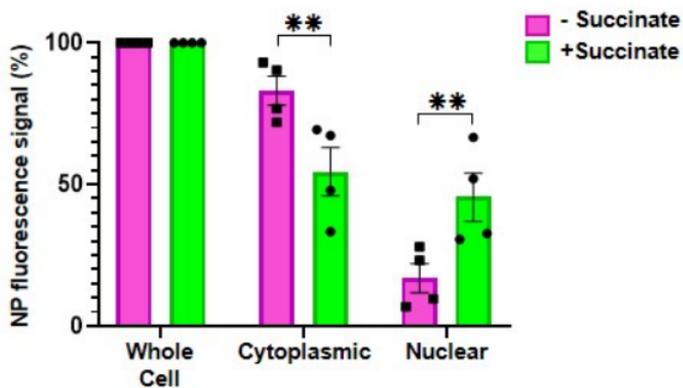
**b**



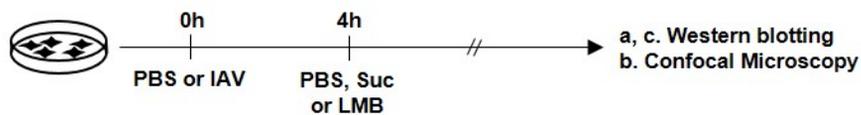
**c**



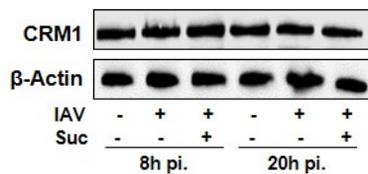
# Guillon *et al.* Figure EV2



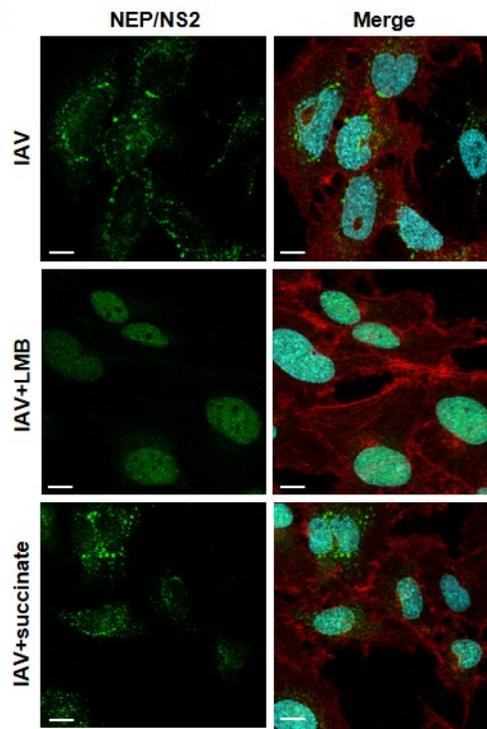
# Guillon *et al.* Figure EV3



**a**



**b**



**c**

