1 The pneumococcal two-component system VisRH is linked to enhanced
2 intracellular survival of *Streptococcus pneumoniae* in influenza-infected
3 pneumocytes.

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15

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26

27 **Abstract**

28 The virus-bacterial synergism implicated in secondary bacterial infections caused by
 29 *Streptococcus pneumoniae* following infection with epidemic or pandemic influenza A virus (IAV)
 30 is well documented. However, the molecular mechanisms behind such synergism remain largely
 31 ill-defined. In pneumocytes infected with influenza A virus, subsequent infection with *S.*
 32 *pneumoniae* leads to enhanced pneumococcal intracellular survival. The pneumococcal two-
 33 component system VisRH appears essential for such enhanced survival. Through comparative
 34 transcriptomic analysis between the $\Delta visR$ and *wt* strains, a list of 179 differentially expressed
 35 genes was defined. Among those, the *clpL* protein chaperone gene and the *psaB* Mn^{+2}
 36 transporter gene, which are involved in the stress response, are important in enhancing *S.*
 37 *pneumoniae* survival in influenza-infected cells. The $\Delta visR$, $\Delta clpL$ and $\Delta psaB$ deletion mutants
 38 display increased susceptibility to acidic and oxidative stress and no enhancement of intracellular
 39 survival in IAV-infected pneumocyte cells. These results suggest that the VisRH two-component
 40 system senses IAV-induced stress conditions and controls adaptive responses that allow survival
 41 of *S. pneumoniae* in IAV-infected pneumocytes.

42 **Author summary**

43 *S. pneumoniae* is an inhabitant of the human nasopharynx that is capable of causing a variety of
 44 infections contributing to an estimated 1.6 million deaths each year. Many of these deaths occur
 45 as result of secondary *S. pneumoniae* infections following seasonal or pandemic influenza.
 46 Although *S. pneumoniae* is considered a typical extracellular pathogen, an intracellular survival
 47 mechanism has been more recently recognized as significant in bacterial pathogenesis. The
 48 synergistic effects between influenza A and *S. pneumoniae* in secondary bacterial infection are
 49 well documented; however, the effects of influenza infections on intracellular survival of *S.*
 50 *pneumoniae* are ill-defined. Here, we provide evidence that influenza infection increases *S.*
 51 *pneumoniae* intracellular survival in pneumocytes. We demonstrate that the poorly understood
 52 VisRH signal transduction system in pneumococcus controls the expression of genes involved in
 53 the stress response that *S. pneumoniae* needs to increase intracellular survival in influenza A-

54 infected pneumocytes. These findings have important implications for understanding secondary
55 bacterial pathogenesis following influenza and for the treatment of such infections in influenza-
56 stricken patients.

57

58 **Introduction**

59 The World Health Organization (WHO) estimates that seasonal influenza virus infections result in
60 about 1 billion infections, 3 to 5 million cases of severe disease, and between 300,000 and
61 500,000 deaths around the world every year. Oftentimes, influenza infections are complicated by
62 secondary bacterial infections, particularly caused by *S. pneumoniae*. About 11-35% of
63 laboratory-confirmed cases of influenza infection are associated with secondary *S. pneumoniae*
64 infections [1]. Such secondary infections ultimately exacerbate the severity of respiratory
65 symptoms resulting in excess morbidity and mortality [2,3]. Highlighting the importance of *S.*
66 *pneumoniae*, it has been proposed that the majority of 40-50 million deaths during the 1918
67 Spanish influenza pandemic were associated to *S. pneumoniae* secondary bacterial infections
68 [4,5]. The *S. pneumoniae* is a Gram-positive bacterium of great significance on human health,
69 being the causal agent of otitis, sinusitis, as well as severe diseases such as community-acquired
70 pneumonia, sepsis, and meningitis [6]. More recently, about 34% of the deaths associated with
71 the 2009 pandemic influenza were also linked to secondary bacterial infections, with *S.*
72 *pneumoniae* as the most commonly associated bacterial pathogen (in addition to *Staphylococcus*
73 *aureus* and *Streptococcus pyogenes*) [7,8].

74 A myriad of concomitant events and factors are thought to be associated with the promotion of
75 secondary bacterial infections following infection with influenza virus: 1) influenza infections
76 produce damage of pulmonary epithelial cells, decreasing the mucociliary clearance and favoring
77 bacterial adherence and infection [9]; 2) the virus' neuraminidase results in the desialylation of
78 mucins, which increases pneumococcal adherence [10]; and 3) macrophages and neutrophils
79 infected with influenza virus show impaired phagocytosis of pneumococci [11]. Although these
80 and perhaps other virus-induced modifications on different host cells and tissues [1-3,8,12] can

81 contribute to secondary *S. pneumoniae* infections, the precise molecular mechanisms of
82 synergism between influenza viruses and *S. pneumoniae* remain poorly understood.

83 *S. pneumoniae* is considered a typical extracellular pathogen. However, mounting evidence
84 suggests a significant role of the replication and survival *S. pneumoniae* inside host cells for
85 disease progression and pathogenesis. In this regard, Ercoli *et al* [13] described that intracellular
86 replication of *S. pneumoniae* in splenic macrophages acts as a bacterial reservoir for septicemia.
87 Ogawa *et al* [14] characterized autophagic vesicles that contain pneumococci during the first
88 hours of bacterial infection of human nasopharyngeal epithelial cells and mouse embryonic
89 fibroblasts. The same work also showed that the bacterial protein Ply, a cholesterol-binding,
90 thiol-activated cytolysin, provides advantages for the bacteria to escape from endosomal
91 elimination at early stages of infection. We previously reported that the two-component systems
92 (TCSs) ComDE and CiaRH are involved in the pneumococcal stress response to acidic
93 conditions and in the intracellular survival of *S. pneumoniae* in pneumocytes [15]. In addition, we
94 recently reported that the crosstalk signaling between the serine/threonine kinase StkP and
95 ComE controls H₂O₂ production in *S. pneumoniae* modulating its intracellular survival in
96 pneumocytes [16].

97 In this report, we studied how IAV infection affects the intracellular survival of *S. pneumoniae* in
98 an in vitro pneumocyte IAV-*S. pneumoniae* superinfection model. We observed that *S.*
99 *pneumoniae* exhibits increased intracellular survival in IAV-infected cells. In *S. pneumoniae*, we
100 identified the two-component system VisRH as a mediator of such increased survival. We found
101 that VisRH controls the expression of 179 pneumococcal genes, such as *clpL* and *psaB*, which
102 encode a molecular chaperone and a Mn⁺² transporter, respectively. We show that *clpL* and *psaB*
103 expression is required in response to acidic and oxidative stress and for bacterial survival in IAV-
104 infected pneumocytes.

105

106 **Results**

107 **Enhanced intracellular survival of *S. pneumoniae* in influenza virus-infected pneumocytes**

108 We previously demonstrated that the *S. pneumoniae* R801 strain can survive inside pneumocytes
 109 for several hours [16]. To further define whether a concomitant influenza virus infection would
 110 affect *S. pneumoniae* intracellular survival, we established an in vitro IAV-*S. pneumoniae*
 111 superinfection model in human-derived A549 pneumocyte cells. As a model virus, we utilized the
 112 laboratory-adapted influenza A/Puerto Rico/8/1934 (H1N1) virus (IAV), which has been
 113 extensively shown to infect A549 cells [17]. A549 cells were inoculated with a multiplicity of
 114 infection (MOI) of either 1, 5, or 10 of the IAV strain. Virus replication was allowed to progress for
 115 24 h before infection with the *S. pneumoniae* R801 strain at a MOI of 30. Flow cytometry using
 116 Annexin-V-ACP/PI labeling to test necrosis/apoptosis levels revealed that a MOI of 10 of IAV led
 117 to ~5% increase in the number of necrotic/apoptotic cells compared to non-infected cells (Fig
 118 S1A) and ~15% after bacterial superinfection using a bacterial MOI of 30 (Fig S1B), as described
 119 [16]. In further studies, we used IAV at a MOI of 10 and *S. pneumoniae* at a MOI of 30 in the
 120 superinfection model. Gentamicin was used to eliminate extracellular bacteria before evaluation
 121 of intracellular *S. pneumoniae* following the classical protection assay [16]. Prior IAV inoculation
 122 consistently increased bacterial survival by ~2 fold in A549 pneumocytes (Fig 1A). The synergism
 123 between these two pathogens, as defined in this case as enhanced *S. pneumoniae* survival in
 124 IAV-infected cells, was also observed in mouse embryonic fibroblasts (MEF) and in cervical
 125 cancer cells (HeLa) (Fig 1A), suggesting that this phenomenon is cell-line independent.

126 **The VisRH two-component system mediates enhanced pneumococcal survival in**
 127 **influenza-infected cells.** *S. pneumoniae* requires ComE and CiaR response regulators to
 128 control the acid stress response and intracellular survival in non-IAV infected A549 pneumocytes
 129 [15,16]. We hypothesized that pneumococcal two-component systems (TCSs) sense
 130 physiological changes induced by IAV-infection of pneumocytes and mediate adaptive
 131 responses that lead to increased intracellular bacterial survival. Next, we considered that the
 132 intracellular changes induced by IAV-infection generate stress conditions sensed by *S.*
 133 *pneumoniae* via TCSs other than ComE and CiaR [15,16]. From a previous systematic screening
 134 of insertion-duplication histidine kinase (*hk*) mutants of *S. pneumoniae* [15] (Table S1), we
 135 focused the search on *hk* mutations that were null for pneumococcal intracellular survival in the

136 absence of IAV infection. When non-IAV infected A549 pneumocytes were inoculated with the *S.*
 137 *pneumoniae* *hk* mutants, most of them showed no changes in intracellular survival compared to
 138 the *wt* strain, including the *hk01::ery* mutant (Fig S2). However, in the context of IAV infection, the
 139 *hk01::ery* mutant showed impaired pneumococcal intracellular survival compared to the *wt* strain
 140 (Fig 1B), indicating that their components participate in sensing the IAV-infected environment.
 141 The *hk01::ery* mutant corresponds to TCS01, one of the least studied TCSs but previously
 142 identified as a virulent marker in *S. pneumoniae* [18-20]. TCS01, hereafter renamed VisRH (for
 143 virus-induced stress) contains the VisH histidine kinase and the VisR response regulator.
 144 Deletion mutants for the *visR* ($\Delta visR$) and *visH* ($\Delta visH$) genes obtained using the Janus cassette
 145 [21] (Table S1), showed similar impairment in intracellular survival as the *hk01::ery* mutant
 146 compared to the *wt S. pneumoniae* strain in IAV-infected A549 pneumocytes (Fig 1B). In
 147 contrast, the reconstructed revertant of the $\Delta visR$ mutant (*wr visR*⁺) recovered the *wt* phenotype
 148 (Fig 1B). These results confirmed that *S. pneumoniae* needs VisRH for increased intracellular
 149 survival in IAV-infected A549 pneumocytes.

150 **VisRH controls the acidic stress response of *S. pneumoniae***

151 *S. pneumoniae* needs an acidic stress response for intracellular survival in pneumocytes [15,16]
 152 and survives in acidic autophagic vesicles of Detroit 562 human nasopharyngeal epithelial cells
 153 and in mouse embryonic fibroblasts (MEFs) [14]. In IAV-infected cells, *S. pneumoniae* is likely to
 154 survive in acidic autophagic vesicles, which implies exposure to the acidic environment and
 155 increased ROS production induced by IAV [14]. Since bacterial TCSs typically respond to
 156 changes in environmental conditions, we hypothesized that VisRH senses IAV-induced
 157 physiological changes at the intracellular level, resulting in an adaptive stress response that
 158 improves *S. pneumoniae* survival in autophagic vesicles in IAV-infected pneumocytes [22]. The
 159 $\Delta visR$ mutant was incubated in culture media at pH 4.8 for 1 h showing a 10³-fold decrease in
 160 bacterial cell viability compared to the *wt*. In contrast, the *wr visR*⁺ revertant recovered the acidic
 161 tolerance (Fig 2A). The $\Delta visR$ mutant behaved similarly as the control *atpC*^{A49T} mutant, which
 162 contains a point mutation at position 49 of the subunit ϵ of the F₀F₁-ATPase (a proton pump that
 163 controls intracellular pH) and is unable to respond to acidic stress in acidified media [15,23].

164 These results suggest that VisRH is required for the acidic stress response of *S. pneumoniae*.
 165 To further define the role of vesicle acidification in *S. pneumoniae* survival, A549 cells were
 166 treated with Bafilomycin A1 (100 nM, 3h), a known v-ATPase inhibitor that halts lysosomal
 167 acidification [24] and prevents the fusion between endosome/autophagosome and lysosome, and
 168 simultaneously inoculated with *S. pneumoniae*. Intracellular survival of the pneumococcal *wt*
 169 strain showed a significant increase when A549 cells were exposed to Bafilomycin A1, as
 170 described [25]. In contrast, when Bafilomycin A1-treated or non-treated A549 pneumocytes were
 171 infected with either the control *atpC*^{A49T} mutant or the $\Delta visR$ mutant cells (Fig 2B), *S. pneumoniae*
 172 showed no increased survival suggesting that the $\Delta visR$ mutant is unable to respond to the pH
 173 variation in vesicles. Since IAV infection also leads to inhibition of the autophagosome/lysosome
 174 fusion step [26], VisRH is likely involved in the regulation of stress genes required for
 175 pneumococcal adaptation to IAV-induced acidic stress conditions.

176 **VisRH is involved in the oxidative stress response of *S. pneumoniae***

177 In a separate study, we previously reported that the StkP/ComE pathway is involved in the
 178 regulation of the oxidative stress response that affects the intracellular survival of *S. pneumoniae*
 179 in pneumocytes [16]. Additionally, previous reports had indicated that the oxidative stress
 180 response is controlled by TCS04 [27], suggesting a complex regulatory system that likely involves
 181 the participation of other signal transduction systems. To test the putative role of VisRH in the
 182 oxidative stress response of *S. pneumoniae*, we examined the hydrogen peroxide resistance of
 183 the $\Delta visR$ mutant (20 mM H₂O₂ in BHI media for 1 h), which was reduced by approximately 30
 184 times while the *wr visR*⁺ (revertant) displayed a hydrogen peroxide resistance similar to *wt* (Fig
 185 2C). As a control, we tested the $\Delta sodA$ mutant (Table S1), a strain deficient in the oxidative stress
 186 response that displayed a 10-fold decrease in H₂O₂ resistance compared to the *wt* (Fig 2C)
 187 [28,29]. These observations suggest a role of VisRH in the oxidative stress response. IAV-
 188 infection of A549 cells leads to enhanced reactive oxygen species (ROS) production and
 189 alteration of the antioxidant defense [30,31]. By measuring the intracellular ROS levels using
 190 H₂DCF-DA, we reproduced this phenotype in our model. We found that ROS production
 191 increased by 33% in IAV-infected cells compared to mock-infected cells (Fig S3). In this context,
 192 the intracellular survival of the $\Delta visR$, $\Delta sodA$ and *wt* strains was determined in IAV-infected A549

cells. Both the $\Delta visR$ and $\Delta sodA$ mutants showed reduced survival rates compared to the wt in IAV-infected cells (Fig 2D), suggesting that VisRH oxidative stress response is relevant for the viral-bacterial synergism. To further explore the effects of ROS production on the intracellular survival mechanism of *S. pneumoniae*, A549 cells were treated with 5 mM N-acetyl-L-cysteine (NAC, 1h prior to *S. pneumoniae* inoculation), a potent ROS inhibitor [32]. In the absence of IAV infection, the NAC-treated A549 cells lead to increased survival of the *S. pneumoniae* wt strain (~2-fold) compared to non-NAC-treated A549 cells. In contrast, the $\Delta visR$ and $\Delta sodA$ mutants were less sensitive to the effects of low ROS biosynthesis (inhibited by NAC). Overall, VisRH likely senses ROS production to activate an oxidative stress response that allows *S. pneumoniae* to survive into autophagosomes.

VisR regulates expression of pneumococcal stress genes

Bacterial response regulators control gene expression to develop an adaptive response to stress conditions [22]. In order to identify the VisR-regulated genes, we compared the transcriptomes of the $\Delta visR$ and wt strains by RNAseq analysis. These strains were grown in acidified media at the exponential growth phase, and total RNA was purified and analyzed as described [16]. The transcriptomic analysis revealed the differential expression of 179 genes, 65 were down-regulated and 114 were up-regulated (Fig S4; Table S2; Fig 3A). We identified that VisR controls, directly or indirectly, the expression of stress genes such as those coding for molecular chaperones, redox homeostasis, as well as genes involved in cation and metabolite transport, cell wall biosynthesis, amino acid biosynthesis, purine/pyrimidine, central metabolism, ribosomal and translation structures, among others (Fig. 3B and Table S2). The expression of *visH* showed a 3-fold decrease in the $\Delta visR$ mutant, suggesting that the VisRH TCS is auto-regulating its own genes (Fig 3C).

We focused on stress genes and confirmed by RT-qPCR that in the $\Delta visR$ mutant there was decreased expression of *visH* (17.9 times), *clpL* (3.9 times) and *psaB* (2.4 times), and increased transcription of *murN* (2.3 times), *glyA* (3.2 times), and *aroC* (3 times) compared to the wt (Fig 3C). The *clpL* gene encodes for a molecular chaperone (heat shock protein) involved in stress response [33,34], *murN* encodes for an enzyme of cell-wall biosynthesis [35], *glyA* encodes for a glycine hydroxymethyltransferase [36], *psaB* encodes for a subunit of a manganese ABC

222 transporter related to oxidative resistance [27,29], and *aroC* encodes for chorismate synthase
223 involved in aromatic amino acid biosynthesis in bacteria [37].

224 To determine a putative correlation between the transcript and the protein levels, we compared
225 the proteomes of the $\Delta visR$ mutant and *wt*. We used protein extracts obtained from bacterial cells
226 grown in the same conditions described for RNAseq assays. By LC-MS/MS, we detected 925
227 proteins in total, we found differential expression of 33 down-regulated and 33 up-regulated
228 proteins, and we confirmed the absence of VisR in the $\Delta visR$ mutant (Fig 4A). The full list of
229 differentially expressed proteins is available (Table S3), and a volcano plot showed proteins
230 differentially expressed with a fold change greater than 2 (Fig S5). When these data were
231 compared with those obtained by RNAseq analysis, we obtained a correlation between the
232 expression of the *clpL*, *psaB*, *dpr* (codes an iron-containing ferritin) [38], *trxA* (encodes for a
233 thioredoxin) [39], *groES* (codes for molecular chaperones) [40], and *nrdD* (codes for a
234 ribonucleotide reductase) [41] genes with the expression of their corresponding encoded proteins
235 (Fig 4B). We found that expression of ClpL and PsaB are repressed in the $\Delta visR$ mutant, and
236 these proteins have been previously involved in stress response in *S. pneumoniae* [29,34].

237

238 **ClpL and PsaB are involved in the pneumococcal stress response and in the synergistic** 239 **mechanism between influenza A and *S. pneumoniae***

240 Since the RNAseq and proteomic data pointed to many stress-related genes under control of
241 VisRH, we focused our study in two particular stress genes, *clpL* and *psaB*. The *clpL* gene codes
242 for a chaperone that is known to be induced by heat shock [33,34]. However, experimental
243 evidence showed ClpL is mainly induced under acidic stress (Fig S6). SDS-PAGE comparison of
244 protein extracts from cells grown in ABM (pH 7.8) or incubated in ABM (pH 5.9) showed
245 increased expression of a 78-kDa band under acidic conditions (Fig S6A). Protein sequencing of
246 this band revealed two peptides of 11 and 14 amino acids with 100% homology with the amino
247 acid sequence of ClpL (Fig S6B). ClpL is predicted to have 701 amino acids and a theoretical
248 molecular weight of 77.6 kDa, in line with our observations in SDS-PAGE (78-kDa). To analyze
249 the *clpL* transcript levels under acidic conditions, the *wt* cells were exposed at either pH 5.9 or pH

250 7.8, and total RNA was purified and treated as described [16]. We detected an increase of 70
251 times in the *clpL* transcript when cells were exposed to pH 5.9 (Fig 5A) indicating that the rise in
252 ClpL expression is linked to adaptive changes at transcriptional levels that are triggered under
253 acidic conditions.

254 To define the role of ClpL in the pneumococcal stress response, we constructed a $\Delta clpL$ mutant
255 (Table S1), which displayed a decrease of 10^4 times in its tolerance to acidified media (exposure
256 to THYE pH 4.8 for 1 h) compared with the *wt* (Fig 5B). This mutant had the same acid sensitivity
257 as the $\Delta visR$ mutant, as showed before. With the purpose to determine the effect of oxidative
258 stress, the $\Delta clpL$ cells were also exposed to H_2O_2 , which displayed a reduction in H_2O_2
259 susceptibility of 200 times compared with *wt*, indicating that this chaperone is not only a heat
260 shock protein [33] but is also involved both in acidic and oxidative stress responses (Fig 5C).

261 To determine the contribution of ClpL in our cellular infection model, A549 cells were infected with
262 the $\Delta clpL$ mutant, and it displayed that its intracellular survival capacity was similar to *wt*.
263 However, when A549 cells were treated with 100 nM Bafilomycin A1 or were previously infected
264 with IAV, conditions that expose pneumococci to the acidic environment of autophagosomes, the
265 $\Delta clpL$ mutant did not show an increased survival as *wt* did (Fig 5D). Altogether, these findings
266 indicate that ClpL is involved in the acidic stress response, which is in turn required for increased
267 intracellular survival of *S. pneumoniae* in IAV-infected pneumocytes.

268 Based in the RNAseq assays, we were also interested in the *psaB* gene that encodes for a Mn^{+2}
269 transporter in *S. pneumoniae*. It was reported that the $\Delta psaB$ mutant displays susceptibility to
270 oxidative stress [29]. We hypothesized that lack of *psaB* could influence the intracellular survival
271 of *S. pneumoniae* in IAV-infected cells due to the virus' ROS production. The $\Delta psaB$ mutant strain
272 was 400-fold more sensitive to acidic stress (Fig 5C) and showed 10^4 times more susceptibility to
273 20 mM H_2O_2 , in line with previous studies [28,29]. In contrast to the $\Delta clpL$ mutant, $\Delta psaB$
274 displayed an impaired intracellular survival in non-IAV infected cells (Fig 5E). However, both
275 mutants failed to exhibit increased intracellular survival in IAV-infected or NAC-treated A549 cells,
276 resembling the phenotype observed for the $\Delta visR$ mutant (Fig 5E). These observations confirm
277 that ClpL and PsaB are necessary for the IAV-*S. pneumoniae* synergistic mechanism.

278 **Influenza A-S. pneumoniae synergism occurs only in autophagy-proficient cells**

279 Previous reports showed that IAV induces autophagy but blocks the last step of the autophagic
 280 process [26] [42]. *S. pneumoniae* [14,43] also induces autophagy but survives in autophagic
 281 vesicles [14]. In order to determine whether autophagy is affected in our IAV-*S. pneumoniae*
 282 superinfection model, the potential increased accumulation of LC3-II due to autophagy inhibition
 283 was evaluated. As controls of autophagy assays, A549 cells treated with either Bafilomycin A1, a
 284 well-known inhibitor of the late phase of autophagy as well [44], or rapamycin, a well-known
 285 autophagy inducer [45] showed increased LC3-II levels by Western blot analysis (Fig S7A-B).
 286 When A549 cells were infected with either the IAV, the pneumococcal *wt* strain, or superinfected,
 287 increased LC3-II levels were observed (Fig S7A-B), consistent with previous results [46]. In a
 288 separate study, mKate2-hLC3 vectors [47] were transfected into A549 cells and subsequently
 289 infected by either IAV, *S. pneumoniae* or superinfected. Confocal microscopy results indicated
 290 that any of these treatments induced remarkably high mKate2-hLC3 punctation in A549 cells (Fig
 291 S7C), indicating autophagy induction.

292 To confirm the functional role of autophagy in this viral-bacterial synergism, IAV-infected mouse
 293 embryonic fibroblasts (MEF *atg5-KO*), which are deficient in autophagy [48], were superinfected
 294 with the pneumococcal *wt* strain. As control, similar treatments were performed in the parental
 295 cell line (MEF *wt*), which are autophagy-proficient cells. A significant increase in the intracellular
 296 survival of *S. pneumoniae* in IAV-infected MEF cells was observed similar to that observed in
 297 A549 cells (Fig 6A). In contrast, *S. pneumoniae* superinfection of IAV-infected MEF *atg5-KO* cells
 298 showed a significant decrease in bacterial intracellular survival compared to the bacterial infection
 299 only (Fig 6B). Similarly, the $\Delta visR$ mutant showed lower intracellular survival in non-IAV infected
 300 MEFs *atg5-KO* relative to non-IAV infected MEFs *wt*, although it was equally deficient in both
 301 MEFs *wt* and MEFs *atg5-KO* previously infected with IAV (Fig 6A-B), as observed in IAV-infected
 302 A549 cells. Altogether, these results suggest that VisRH mediates the synergistic mechanism
 303 between IAV and *S. pneumoniae* and that this phenomenon occurs only in autophagy-proficient
 304 cells.

305 Discussion

306 Although *S. pneumoniae* is a common extracellular colonizer of the human nasopharynx, it is
 307 known to cause otitis, sinusitis and invasive infections such as pneumonia, bacteremia, and
 308 meningitis. Bacterial pneumonia caused by *S. pneumoniae* in patients infected with influenza A
 309 has significant relevance in human health during seasonal and pandemic influenza. IAV
 310 infections cause physical and physiological changes in the respiratory epithelium that facilitate
 311 secondary bacterial infections [10]. Recent reports suggest that such infections are associated
 312 with the pneumococcal ability to survive intracellularly. In this regard, Ogawa described
 313 intracellular fates of *S. pneumoniae* and found that it is entrapped in specific autophagic vesicles
 314 in MEFs [14], which is consistent with our pneumocyte infection model [16]. In the present work,
 315 we expanded these studies and found that intracellular pneumococcal survival is clearly improved
 316 in IAV-infected pneumocytes.

317 Many bacterial TCSs, have been involved in intracellular survival mechanisms in eukaryotic cells,
 318 such as EvgSA in *Shigella flexneri* [49]; ArcAB [50], PhoPQ [51] and EnvZ/OmpR [52] in
 319 *Salmonella typhimurium*; SrrAB [53]; GraSR [54] in *Staphylococcus aureus*; PhoPQ in
 320 *Escherichia coli* [55]; BvrSR in *Brucella abortus* [56]; and PrrAB in *Mycobacterium tuberculosis*
 321 [57], among others. In *S. pneumoniae*, most of the TCSs are required for full virulence in animal
 322 models of infection [58,59] and we have shown that two of these systems, StkP/ComE and
 323 CiaRH [15,16], are important in response to acidic and/or oxidative stress. The poorly studied
 324 VisRH system (TCS01) has been previously involved in mediating virulence in intranasally-
 325 infected mice [18,19], and a rabbit endocarditis model [20]; however, the effects on intracellular
 326 pneumococcal survival were not explored. Here, we show that the intracellular pneumococcal
 327 survival of the $\Delta visR$ mutant is similar to the *wt* in A549 pneumocytes, suggesting that this system
 328 may not be directly linked to virulence in animal models.

329 A key finding of our work was that VisR, as well as ClpL and PsaB, are involved in the stress
 330 response induced by *S. pneumoniae* and are necessary for the increased pneumococcal survival
 331 in IAV-infected cells. ClpL was formerly described as a heat-shock chaperone induced in
 332 pneumococcal cells when incubated at 45°C [34]. The $\Delta clpL$ mutant is temperature sensitive (to

333 43°C) but virulence remains unaffected in a murine intraperitoneal model [60]. We observed that
 334 ClpL is mostly induced at pH 5.9 in the *wt* strain. In contrast, the $\Delta clpL$ mutant does tolerate an
 335 acidic pH of ≥ 4.8 in bacterial culture media. A similar phenotype was reported for *Streptococcus*
 336 *mutans* where ClpL was also induced at pH 5.0 and it was essential for the acid tolerance
 337 response [61]. We also found that the $\Delta clpL$ mutant displayed susceptibility to hydrogen peroxide,
 338 indicating that ClpL is likely a chaperone involved not only in thermal but also acidic and oxidative
 339 stress responses. Previous reports showed that ClpL's activity is Mn^{+2} -dependent [62], further
 340 adding to its potential relevance of these proteins in the general stress response of *S.*
 341 *pneumoniae*. Our studies suggest that that ClpL is a key chaperone related to the general stress
 342 response of *S. pneumoniae* and essential for bacterial intracellular survival in IAV-infected cells.

343 PsaB was previously described as an ATP-binding protein that belongs to the ABC-type
 344 manganese permease [63]. Mutations on the genes that constitute the *psaBCA* operon result in
 345 growth limitations in culture media with low Mn^{+2} concentration, and attenuation in four different
 346 animal models of infection [64]. The PsaBCA complex is indeed a Mn^{+2} transporter and its protein
 347 components are involved in virulence, resistance to hydrogen peroxide and superoxides [29]. The
 348 *psaBC* mutant shows hypersusceptibility to hydrogen peroxide and superoxides [65]. We
 349 observed the same phenotype in our $\Delta psaB$ mutant, confirming that this strain is more
 350 susceptible to exogenous hydrogen peroxide than the *wt* strain. Since the *S. pneumoniae* $\Delta visR$,
 351 $\Delta clpL$ and $\Delta psaB$ mutants showed alterations to both acidic and oxidative stress conditions, it
 352 suggests a common strategy to general stress adaptation that involves, at least, a TCS, a
 353 chaperone and a Mn^{+2} transporter. Such cross-response mechanisms are not unique to *S.*
 354 *pneumoniae*. In *Streptococcus mutans*, a cross-response effect between acidic and oxidative
 355 stress was reported for a mutant of the oxidative stress regulator SpxA. Similar to the *S.*
 356 *pneumoniae* $\Delta visR$ mutant, the *spxA* mutation impairs *S. mutans*' ability to grow under acidic and
 357 oxidative conditions [66].

358 We observed that VisR controls transcription of the *clpL* and *psaB* genes by unknown
 359 mechanisms. The VisR response regulator modulates the acidic/oxidative stress response of *S.*
 360 *pneumoniae* to improve intracellular survival in influenza-infected cells. However, transcription of

the *clpL* and *psaB* genes could be co-regulated by other regulators [62]. For example, the conserved repressor CtsR regulates the *clpL* expression in many streptococci and lactococci, and these bacteria present CtsR box elements in the *clpL* promoter region [62]. In *S. pneumoniae*, CtsR-binding sites were located upstream from the *clpL* gene [67], however, its regulation has not been yet elucidated. Based on the qPCR assays, we suggest that the *clpL* expression is induced by acidic pH and controlled by VisR, but we cannot discard the possibility that other regulators such as CtsR modulate ClpL stress response in *S. pneumoniae*. Equally complex appears to be the regulation of the *psaB* gene. Expression of the *psaBCA* operon is controlled by the PsaR regulator in a Mn^{+2} -dependent manner [68]. In addition, RR04, which belongs to the TCS04, is necessary for the activation of the *psaBCA* locus [27]. Here, we demonstrate that VisR is also essential for the transcriptional activation of the *psaB* gene, adding to complexity of this regulation.

Regarding the increased intracellular survival of *S. pneumoniae* in IAV-infected A549 cells, it is clear that VisR is necessary in IAV-infected cells but not in non-infected cells. It is likely that IAV infection produces stress conditions in pneumocytes that *S. pneumoniae* overcomes in a VisRH-dependent manner, to improve its capacity to survive intracellularly. Based on the bacterial survival assay in acidified media, where the $\Delta visR$, $\Delta clpL$ and $\Delta psaB$ cells showed impaired acidic tolerance, we suggest that *S. pneumoniae* needs an adaptive process to survive under acidic conditions, such in acidic vesicles in IAV-infected pneumocytes. Taking into account that ClpL expression is induced at pH 5.9 in the *wt* strain and that the $\Delta visR$ cells show decreased ClpL and PsaB expression, VisRH is likely sensing acidic stress and modulating adaptation to such condition (Fig 7).

Related to the putative role of oxidative stress in the synergistic mechanism between IAV and *S. pneumoniae*, it is known that IAV infection increases ROS production in A549 cells [69]. It was reported that *S. pneumoniae* induces an oxidative stress response to survive under oxidative conditions [70]. Analyzing the list of VisR-regulated genes, we focused our attention on the *psaB* gene that encodes for a Mn^{+2} transporter involved in oxidative stress response in *S. pneumoniae* [29,71]. It was described that the $\Delta psaB$ mutant was very sensitive to hydrogen peroxide, and this

probably occurs due to a low Mn^{+2} level that affects the SodA activity [71]. In view of our results, VisR controls, directly or indirectly, *psaB* transcription affecting the oxidative stress tolerance apparently supported by SodA. To confirm this hypothesis, we tested the $\Delta sodA$ mutant and we found the same phenotype that the $\Delta visR$ and $\Delta psaB$ mutants, which showed increased susceptibility to hydrogen peroxide. Curiously, the $\Delta clpL$ mutant also displayed an impaired hydrogen peroxide tolerance, suggesting that ClpL is essential for general stress response. Regarding a putative cross stress response, it is important to highlight that ClpL is a Mn^{+2} -dependent chaperone [33], in consequence, there is a direct association with the PsaB Mn^{+2} transporter. Probably, in the $\Delta psaB$ mutant, the observed decreased tolerance to acidic pH that corresponds to diminished activity of ClpL is also due to a low Mn^{+2} level.

The importance of oxidative stress response in the intracellular survival mechanism of *S. pneumoniae* was revealed when ROS production was inhibited in A549 cells by a NAC treatment during bacterial infection. Under these conditions, we clearly observed that the *wt* strain increased its survival, as described for A549 cells [43], indicating that *S. pneumoniae* must overcome this type of stress to survive intracellularly and that this pathogen is susceptible to changes in ROS levels. In the intracellular context of IAV-infected pneumocytes, this virus is able to increase ROS production [31] and, to achieve synergism, *S. pneumoniae* should be also able to overcome oxidative stress.

Our results indicate that the lack of PsaB impaired the intracellular survival of *S. pneumoniae*, even in non-IAV infected cells, probably because Mn^{+2} is needed in for many bacterial processes [72]. In contrast, VisR and ClpL were not essential for this survival mechanism but VisR, ClpL, and PsaB were found to be necessary for the synergism detected in *S. pneumoniae* in IAV-infected A549 cells. We propose that *S. pneumoniae* needs to induce a VisR-controlled adaptive process during superinfection to express chaperones, such as ClpL, to refold proteins denatured by acidic stress and by the IAV-induced ROS production [31], and Mn^{+2} transporter to provide this metal that is essential for chaperone activity, among other cellular processes (Fig 7).

As mentioned before, Gannage *et al* [26] reported that IAV infection produced accumulation of autophagosome by IAV M2-induced blockage of fusion with lysosomes. On the other hand,

Ogawa *et al.* [14] reported that *S. pneumoniae* is able to survive in autophagic vesicles. Based on these findings, we hypothesized that the IAV-*S. pneumoniae* synergism may depend on the autophagic process. This putative autophagy-dependence was confirmed using the MEF cell line, with which we reproduced the same synergism found in A549 cells, but not in the MEF *atg5*-KO cells that are deficient in autophagy, indicating that this synergistic mechanism occurs only in autophagy proficient cells.

Pneumococcal pathogenesis has been studied extensively in the last decades. Although *S. pneumoniae* is considered a typical extracellular pathogen, particular attention has been given to the intracellular survival mechanism in the last years, as mentioned before [15,16] [13,14]. In this work, we report for the first time that the intracellular survival of *S. pneumoniae* is enhanced in IAV-infected cells, and this synergism occurs in autophagic-proficient cells. For this survival, *S. pneumoniae* needs a physiological adaptation to IAV-induced conditions, and we propose that the VisRH TCS probably senses these changes at intracellular level and controls the expression of ClpL and PsaB, which are needed to tolerate the acidic pH found in intracellular vesicles, as well as the increased ROS level produced by influenza A.

We consider that our results contribute to the knowledge of the intracellular survival mechanism of *S. pneumoniae* in the context of eukaryotic cells infected with influenza A, with a consequent relevance for the management of secondary infections in influenza-infected patients. We propose that intracellular antibiotics should be also considered for the treatment of pneumococcal infections during an epidemic or pandemic influenza A. Many works have described this particular viral-bacterial synergism [1,8-11], and here we provide experimental evidence on how influenza A infections enhance the intracellular survival of *S. pneumoniae*.

Materials and methods

Bacterial and viral strains, plasmids, cell lines, and growth conditions

All bacterial strains, oligonucleotides and plasmids used in this study, as well as cloning and mutagenesis procedures, are listed in the supplementary material (Table S1). Oligonucleotide synthesis and DNA sequencing service were performed in Macrogen Inc. (Seoul, South Korea).

444 The growth conditions and stock preparation for the pneumococcal and *Escherichia coli* strains
445 have been reported elsewhere [23], and the transformation assays have been previously
446 described [23,73]. The influenza virus A/Puerto Rico/8/1934 (H1N1) (IAV) strain was used for
447 superinfection assays. Viruses were grown in embryonated chicken eggs, and the allantoic fluid
448 was collected, aliquoted, titrated in Madin-Darby canine kidney cells (50% tissue culture infective
449 doses [TCID₅₀]) and eggs (50% egg infective dose [EID₅₀]), and stored at –80°C until used [74].

450 **Cell lines and culture conditions**

451 The A549 cell line (human lung epithelial carcinoma, pneumocytes type II; ATCC® CCL-185™)
452 was cultured at 37°C, 5% CO₂ in Dulbecco's modified Eagle medium (DMEM) with 4.5 g/l of
453 glucose and 10% of heat-inactivated fetal bovine serum (FBS) (Gibco BRL, Gaithersburg, Md.).
454 Fully confluent A549 cells were split once every two or three days via trypsin/EDTA treatment and
455 diluted in fresh media before being cultivated in Filter cap cell flasks of 75 cm² (Greiner Bio-one
456 no. 658175) until passage 6, as described [16]. A549 cells were transfected/co-transfected with
457 pIRES2-EGFP and pIRES2-M2 using JetPRIME® (Polyplus-transfection, Illkirch, France)
458 following the manufacturer's instructions in serum-free DMEM (Invitrogen) supplemented with 5%
459 of Fetal Bovine Serum (FBS). The MEF (Mouse Embryonic Fibroblast) and the autophagy-
460 deficient MEF *atg5-KO* cell lines were generously provided by Dr. Noboru Mizushima [48]. These
461 cell lines were cultured under the same conditions as described for A549 cells. The mKate2-LC3
462 plasmids [47] was obtained from Addgene.

463 **Intracellular survival assays**

464 The intracellular survival assays of pneumococci were performed as reported previously [15,16]
465 with modifications. Briefly, approximately 1.5×10^5 of eukaryotic cells (A549, MEF, MEF *atg5-KO*,
466 or HeLa cell lines) per well were seeded in 12 well plates and cultured in DMEM (with 5% FBS)
467 and incubated at 37°C for 24 h. Pneumococci were grown in THYE to the mid-log phase (OD_{600nm}
468 0.3) and resuspended in DMEM (with 5% FBS). Infection of cell monolayers was carried out
469 using a multiplicity of infection (MOI) 30:1. A549 cells were incubated 3 h with pneumococcal
470 strains and cells were washed three times with phosphate-buffered saline (PBS) and it was
471 added fresh DMEM (with 5% FBS) containing 150 µg/ml gentamicin sulfate (US Biological

472 G2030). After a 30 min period, cells were washed three times with PBS. The eukaryotic cells
 473 were trypsinized and the occurrence of apoptosis/necrosis caused by pneumococcal infection
 474 was quantified by flow cytometry (Annexin V/propidium iodide labeling kit; Invitrogen) giving 5%
 475 approximately for all time points analyzed. To determine intracellular survival, cells were lysed by
 476 centrifugation for 5 min at 10,000 rpm and the bacterial pellet was resuspended in THYE
 477 medium. The number of internalized bacteria at different time points was quantified after serial
 478 dilutions and plating on BHI 5% sheep blood agar plates with incubation for 16 h at 37°C. The
 479 time scale referred to the time after elimination of the extracellular bacteria by antibiotic
 480 treatment. A 100% survival was defined after 30 min of antibiotic treatment (FigS1), and all the
 481 samples were referred to this point to calculate the respective percentages.

482 For intracellular survival determinations in the viral-bacterial superinfection assays, approximately
 483 1.5×10^5 of eukaryotic cells (A549, MEF wt, MEF *atg5-KO*, and Hela cell lines) per well were
 484 seeded in 6 well plates, cultured in DMEM (with 5% FBS) and incubated for 24 h. Posteriorly,
 485 DMEM was removed from plates, cells were washed three times with PBS and cultured with
 486 DMEM containing 1 µg/mL TPCK-treated trypsin for 1 h, and cells were infected with IAV at a
 487 viral MOI of 10 at 37°C for 24 h. In parallel, the occurrence of apoptosis/necrosis produced by IAV
 488 infection was determined by flow cytometry (Annexin V/propidium iodide labeling kit; Invitrogen)
 489 and it was approximately 5%. To perform survival assays in cells previously infected with IAV and
 490 treated with amantadine, we carried out the same protocol described above, but we added 50 µM
 491 of amantadine (Sigma) at the same time that gentamycin. An analysis was carried out using a
 492 confocal laser-scanning microscope (OlympusFV300) with a 100× oil immersion lens, as
 493 described [15].

494

495 **Susceptibility to acidic and oxidative stress**

496 To determine susceptibility to acidic pH, bacterial cells were grown in Brain Heart Infusion (BHI;
 497 pH 7.2) at 37°C until $OD_{600nm} \sim 0.3$, centrifuged at 10,000 g for 5 min, resuspended in Todd
 498 Hewitt-Yeast Extract (THYE; pH 4.8) and incubated for 1 h at 37°C. To measure susceptibility to
 499 oxidative stress, bacterial cells were grown in BHI at 37°C until $OD_{600nm} \sim 0.3$, and 20 mM H₂O₂
 500 was added to the cultures for 1 h at 37°C. To determine the survival percentage in these assays

(acidic and oxidative conditions), serial dilutions were made in THYE (pH 7.8) and plated onto 5% of sheep blood tryptic-soy agar (TSA) plates. After 24 h of incubation at 37°C, colonies were counted to determine the number of survivors. The percentages were calculated by dividing the number of survivors, at pH 4.8 or 20 mM H₂O₂, by the number of total cells at time zero before incubation at stressful conditions. Data were expressed as the mean percentage ± standard deviation (SD) of independent experiments performed in triplicate.

In-gel tryptic digestion and amino acid sequencing of protein bands separated by SDS-PAGE The protein band of 78 kDa, separated by SDS-PAGE and stained by Coomassie Blue, was cut and the gel slice was incubated in 100 mM ammonium bicarbonate (pH 8.3) containing 45 mM dithiothreitol at 60°C for 30 min. The sample was cooled at RT, and 100 mM iodoacetamide was added followed by incubation at RT in the dark for 30 min. The gel was then washed in 50% acetonitrile-100 mM ammonium bicarbonate with shaking for 1 h, cut in pieces, and transferred to a small plastic tube. Acetonitrile was added to shrink the gel slices and dried in a rotatory evaporator. Then, the gel pieces were treated with 100 mM ammonium bicarbonate (pH 8.3) containing trypsin at a 10:1 ratio (w/w, substrate: enzyme). The sample was incubated at 37 °C for 16 h, and digestion products were extracted twice from the gel with 0.1% trifluoroacetic acid for 20 min. Extractions were loaded into a C18 high-pressure liquid chromatography column (220 × 1 mm), and peptides were eluted with 80% acetonitrile-0.08% trifluoroacetic acid. Selected peaks were applied to a 477A protein-peptide sequencer equipped with a 140 HPLC (Applied Biosystems) and subjected to Edman degradation sequence analysis at the Laboratorio Nacional de Investigacion y Servicios en Péptidos y Proteínas facility (CONICET)[75].

Mass Spectrometry Analysis

Protein digestion and Mass Spectrometry analysis were performed at the Proteomics Core Facility CEQUIBIEM, at the University of Buenos Aires/ CONICET (National Research Council) as follows. Protein samples were reduced with dithiothreitol (DTT) in 50 mM of ammonium bicarbonate at a final concentration of 10 mM (45 min, 56°C) and alkylated with iodoacetamide in the same solvent at a final concentration of 30 mM (40 min, RT, in darkness). Proteins were digested with trypsin (Promega V5111). After that, the peptides were purified and desalted with

529 ZipTip C18 columns (Millipore). The digests were analyzed by nano-LC-MS/MS in a Q-Exactive
 530 Mass Spectrometer (Thermo Scientific) coupled to a nano-HPLC EASY-nLC 1000 (Thermo
 531 Scientific). For the LC-MS/MS analysis, approximately 1 µg of peptides were loaded onto the
 532 column and eluted for 120 minutes using a reverse phase column (C18, 2 µm, 100A, 50 µm x
 533 150 mm) Easy-Spray Column PepMap RSLC (P/N ES801) suitable for separating protein
 534 complexes with a high degree of resolution. The flow rate used for the nano-column was 300 nL
 535 min⁻¹ and the solvent range from 7% B (5 min) to 35% (120 min). Solvent A was 0.1% formic
 536 acid in water whereas B was 0.1% formic acid in acetonitrile. The injection volume was 2 µL. The
 537 MS equipment has a high collision dissociation cell (HCD) for fragmentation and a Q-Exactive
 538 Orbitrap analyzer (Thermo Scientific). A voltage of 3.5 kV was used for Electro Spray Ionization
 539 (Easy-Spray; Thermo Scientific,). XCalibur 3.0.63 (Thermo Scientific) software was used for data
 540 acquisition and equipment configuration that allows peptide identification at the same time of their
 541 chromatographic separation. Full-scan mass spectra were acquired in the Orbitrap analyzer. The
 542 scanned mass range was 400-1800 m/z, at a resolution of 70000 at 400 m/z and the twelve most
 543 intense ions in each cycle were sequentially isolated, fragmented by HCD and measured in the
 544 Orbitrap analyzer. Peptides with a charge of +1 or with unassigned charge state were excluded
 545 from fragmentation for MS2.

546 **Analysis of MS data**

547 Q-Exactive raw data was processed using Proteome Discoverer software (version 2.1.1.21
 548 Thermo Scientific) and searched against *Streptococcus pneumoniae* (strain ATCC BAA-255 R6)
 549 UP000000586 protein sequences database with trypsin specificity and a maximum of one missed
 550 cleavage per peptide. Proteome Discoverer searches were performed with a precursor mass
 551 tolerance of 10 ppm and a product ion tolerance to 0.05 Da. Static modifications were set to
 552 carbamidomethylation of Cys, and dynamic modifications were set to oxidation of Met and N-
 553 terminal acetylation. Protein hits were filtered for high confidence peptide matches with a
 554 maximum protein and peptide false discovery rate of 1% calculated by employing a reverse
 555 database strategy.

556 Proteome Discoverer calculates an area for each protein in each condition. To do this it uses the
 557 area under the curve of the 3 most intense peptides for a protein. Areas were calculated for each
 558 of the three triplicates and normalized. The data obtained for the area for each protein were
 559 processed with the Perseus program (Max Planck Institute of Biochemistry, 1.5.5.3 version,
 560 available for free) [76] that allows a deeper statistical analysis. Different scatter plots were done
 561 according to the compared samples. For each couple of samples, we plotted log p -value (-log
 562 Student T-test p -value A_B) on the y-axis versus Student T-test Difference A_B in the x-axis.
 563 Proteins that appear in the volcano plot with a fold change greater than 2 (less than -1 or greater
 564 than 1 on the x-axis of the graph) and a p -value < 0.05 (above 1.3 on the y-axis of the graph)
 565 were considered as differentially expressed.

566 **RNAseq analysis**

567 Cells were initially grown in THYE medium at pH 7.8 until OD_{600nm} ~0.3 (log phase), centrifuged at
 568 14,000 g for 10 min at 4°C, resuspended in the same volume in ABM at pH 5.9 [23] and
 569 incubated a 37°C for 1h. Then, cells were centrifuged at 14,000 x g for 10 min at 4°C,
 570 resuspended in a 1/10 vol of lysis buffer (DOC 1% in 0.9% Na Cl) and incubated 3 min a 37°C
 571 until complete lysis. Total RNA from three biological replicates for *wt* and the $\Delta visR$ mutant were
 572 purified by TRIzol reagent according to the manufacturer's instructions (Fisher Scientific). The
 573 RNA for RNAseq assays was obtained as described [16]. Data analysis was performed as
 574 reported [62].

575 **Differential gene expression.**

576 The aligned reads were assembled by Cufflinks (version-2.2.1), and then the differentially
 577 expressed genes were detected and quantified by Cuffdiff, which is included in the Cufflinks
 578 package, using a rigorous sophisticated statistical analysis. The expression of the genes was
 579 calculated in terms of FPKM (fragment per kilobase per million mapped reads). Differential gene
 580 expression analysis was carried out between *wt* and the $\Delta visR$ samples.

581 **Protein analysis by western blots**

582 The A549 cells were lysed and protease inhibitor cocktail added to obtain the whole protein to be
 583 quantified. The lysates with protein loading buffer were boiled for 5 min. The supernatants were

collected and 40 µg of each sample were loaded onto 15% SDS-PAGE gels and electrophoresed for protein resolution at RT using Tris-Glycine-SDS running buffer at a constant electric field of 100 V cm⁻¹. Posteriorly, proteins were electroblotted onto PVDF membranes, which were blocked for 2 h at room temperature and incubated overnight at 4°C with primary antibodies diluted at 1:1,000 in PBS with 5% bovine serum albumin buffer. After washing 3 times with Tris-buffered saline (TBS) with 0.5% (v/v) Tween, the membranes were incubated for 2 h at room temperature with Alexa-conjugated secondary antibody (1:1,000 dilution) to detect LC3-II and p62. The membranes were imaged under fluorescence mode in an Odyssey CLx Imaging System (LI-COR), and bands were quantified with Image Studio software (LI-COR). Rabbit monoclonal antibody against LC3A/B (D3U4C) XP(R) (12741P) was obtained from Cell Signaling Technology. Rapamycin (R8781; Rapa, mTOR inhibitor), Bafilomycin A 1 from *Streptomyces* (B1793) and Mouse monoclonal anti-beta-actin antibody (A2228) were obtained from Sigma Life Science. Mouse monoclonal antibody against Influenza A M2 protein [14C2] (ab5416) was obtained from Abcam. Recombinant Rabbit monoclonal antibody against SQSTM1/p62 (701510) was purchased to Invitrogen.

qRT-PCR

cDNA was synthesized from 2 µg RNA using the ProtoScript II First Strand cDNA Synthesis Kit (NEB) following the manufacturer's protocol, and cDNA was cleaned using the QIAquick PCR Purification Kit (Qiagen). Genes were amplified using the oligonucleotides listed in the Table S4 and PowerUp SYBR Green Master Mix (Applied Biosystem) following the manufacturer's protocol. Expression was determined relative to AU0158 normalized by *gyrA* (*spr1099*) expression using the $\Delta\Delta C_t$ method [77]. The *gyrA* had a similar expression by RNA-Seq for *wt* and the $\Delta visR$ mutant, and this had been used to normalize the expression in *S. pneumoniae* in other studies [78].

ROS detection

To assess ROS production, we used 2',7'-Dichlorodihydrofluorescein diacetate dye (H₂DCF-DA; Molecular Probes) following the manufacturer's instructions. Briefly, we infected A549 cells with IAV at MOI 10 as it was indicated above, 24 h post-infection the cells were trypsinized and washed twice with PBS, resuspended with PBS containing H₂DCF-DA (10 µM) and incubated for

613 30 min at 37°C. Then, cells were washed and resuspended with PBS and we measured the
614 intensity of fluorescence of the DCF by cytometry.

615 Accession numbers

616 The RNA-Seq data generated from this study are deposited at the NCBI SRA under the
617 accession numbers SAMN08473835 (*wt* strain) and SAMN08473837 ($\Delta visR$ strain). This data
618 corresponds to the Bioproject PRJNA433281, and the SRA IDs are SRR6679010 and
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625 discussions.

626 Figure legends

627 **Fig 1.** Enhancement of pneumococcal intracellular survival by Influenza A infection is mediated
628 by the VisRH two-component system.

629 **Fig 2.** VisRH controls the acidic and oxidative stress response of *S. pneumoniae* in both culture
630 media and pneumocytes.

631 **Fig 3.** VisR controls gene expression of the stress response in *S. pneumoniae*.

632 **Fig 4.** Comparative proteomic analysis of differentially expressed proteins in the $\Delta visR$ and *wt*
633 strains.

634 **Fig 5.** ClpL and PsaB are involved in the pneumococcal stress response needed for the viral-
635 bacterial synergism.

636 **Fig 6.** The viral-bacterial synergism is dependent on autophagic-proficient cells.

637 **Fig 7.** Proposed model for the synergistic mechanism that exists between influenza A and *S.*
638 *pneumoniae* in pneumocytes.

639

640 **Supporting Information**

641 **S1 fig.** Determination of apoptosis and necrosis levels in A549 cells infected with IAV and/or *S.*
642 *pneumoniae*.

643 **S2 fig.** Identification of histidine kinase (hk) mutants of *S. pneumoniae* displaying normal
644 intracellular survival in pneumocytes.

645 **S3 fig.** Confirmation of the IAV-induced ROS production in A549 cells.

646 **S4 fig.** VisR is a global regulator that controls gene expression during the stress response.

647 **S5 fig.** Comparative proteomic analysis between the *wt* and the $\Delta visR$ strains.

648 **S6 fig.** Identification of the pneumococcal 78-kDa ClpL chaperone expressed under acidic
649 conditions.

650 **S7 fig.** IAV and *S. pneumoniae* infection and superinfection induce autophagy in A549 cells.

651 **S1 table.** Plasmids and strains used in this work

652 **S2 table.** List of VisR-regulated genes as determined by RNAseq analysis

653 **S3 table.** List of differentially expressed proteins in the $\Delta visR$ mutant compared with *wt*

654 **S4 table.** Primers used in this work

655

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857

858 **Figure legends**

859 **Fig 1. Enhancement of pneumococcal intracellular survival by Influenza A infection is**
860 **mediated by the VisRH two-component system.** (A) The IAV-*S. pneumoniae* synergism is
861 independent of the cell line. The A549, MEF and HeLa cells were treated for 24 h with a viral
862 MOI of 10 and posteriorly infected with the pneumococcal wt strain using a bacterial MOI of 30.
863 Bacterial survival progression was monitored using a typical protection assay. Survival
864 percentages were calculated by considering the total amount of internalized bacteria after 30 min
865 of extracellular antibiotic treatment as representing 100% for each strain. After antibiotic
866 treatment, samples were taken at 4 hours, and pneumocytes were lysed to release pneumococci.
867 Samples were diluted in BHI, spread on BHI-blood-agar plates and incubated at 37°C for 16 h.
868 IAV-infected cells are indicated with green bars, IAV-infected cells with amantadine are indicated
869 with blue bars, and non-virus infected cells with white bars. (B) The synergism between IAV and
870 *S. pneumoniae* is mediated by the VisRH two-component system. A549 cells were previously
871 infected with a viral MOI of 10 for 24 h, and then coinfecting by the *wt*, $\Delta visH$, *hk01::ery* (or
872 *visH::ery*) and $\Delta visR$ strains, and the revertant of the $\Delta visR$ mutant (*wr visR⁺*). Intracellular
873 survival rates were determined as described in panel A. IAV-infected cells are indicated with
874 green bars and non-virus infected cells with white bars. For all panels, data are representative of
875 at least three independent experiments and statistically significant differences are indicated as
876 $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***).

877

878 **Fig 2. VisRH controls the acidic and oxidative stress response of *S. pneumoniae* in both**
879 **culture media and pneumocytes.** (A) The $\Delta visR$ mutant is susceptible to acidified media. The
880 $\Delta visR$, *wr visR⁺*, *atpC^{A49T}* and *wt* cells were grown in BHI until an OD_{620nm} 0.3 and then incubated

881 in ABM at pH 4.8 for 1 h. Viable cells were assessed by spreading dilutions in BHI-blood-agar
 882 plates and incubating these at 37°C for 16 h. (B) Bafilomycin-A1-induced lysosomal neutralization
 883 does not affect the impaired intracellular survival of the $\Delta visR$ mutant in IAV-infected cells. A549
 884 cells were infected with the $\Delta visR$, $atpC^{A49T}$ and wt cells and intracellular survival was determined
 885 as described in the Fig 1. White bars correspond to non-virus infected cells, green bars to IAV-
 886 infected cells and blue bars to Bafilomycin-A1-treated cells. (C) The $\Delta visR$ mutant is sensitive to
 887 H_2O_2 . The $\Delta visR$, $wr visR^+$, $\Delta sodA$ and wt cells were grown in BHI and then exposed at BHI
 888 medium containing 20 mM H_2O_2 for 2 h. After that, viable cells were determined by spreading
 889 dilutions in BHI-blood-agar plates and incubating these at 37°C for 16 h. (D) Inhibition of ROS
 890 production does not affect the intracellular survival of the $\Delta visR$ mutant. A549 cells were infected
 891 with the $\Delta visR$, $\Delta sodA$ and wt cells and intracellular survival was determined as described in the
 892 Fig 1 legend. White bars correspond to non-virus infected cells, green bars to IAV-infected cells
 893 and blue bars to NAC-treated cells. For all panels, data are representative of at least three
 894 independent experiments and statistically significant differences are indicated as $p < 0.05$ (*) or
 895 $p < 0.001$ (**).

896

897 **Fig 3. VisR controls gene expression of the stress response in *S. pneumoniae*.** (A) RNA-
 898 seq heatmap shows gene expression of the comparison between the $\Delta visR$ and wt strains
 899 incubated in ABM with relative gene expression in $\log_2$ fold change demonstrating increased
 900 expression in green and decreased expression in red. Gene expressions higher than 2 fold and p
 901 values < 0.05 were considered significant. (B) Categories of VisR-regulated genes obtained from
 902 an RNAseq analysis. An RNAseq generated distribution in functional categories of genes that are
 903 regulated in the $\Delta visR$ mutant relative to strain wt . (C) Putative VisR-regulated genes expressed
 904 in the $\Delta visR$ mutant relative to strain wt . Gene expression determined by RNAseq was confirmed
 905 by qPCR. The $\Delta visR$ and wt strains were grown in BHI to the mid-exponential phase in triplicate
 906 and then incubated in ABM for 1h. The fold change in gene expression was measured by RT-
 907 qPCR and calculated using the $2^{-\Delta\Delta CT}$ method. The *gyrA* gene was used as internal control.

908

909 **Fig 4. Comparative proteomic analysis of differentially expressed proteins in the $\Delta visR$**
 910 **and *wt* strains.** (A) Heat map of proteins expressed in the $\Delta visR$ mutant and referred to *wt*.
 911 Proteins with a fold change greater than 2 (less than -1 or greater than 1 on the x-axis of the
 912 graph) and a *p*-value < 0.05 were considered as differentially expressed. Higher expression in the
 913 *wt* is displayed in shades of green, and higher expression in the $\Delta visR$ mutant (compared to *wt*) is
 914 showed in shades of blue. (B) Comparison between log₂ folds change ($\Delta visR/wt$) obtained by
 915 both RNAseq (green bars) and proteomic (blue bars) analysis.

916

917 **Fig 5. ClpL and PsaB are involved in the pneumococcal stress response needed for the**
 918 **viral-bacterial synergism.** (A) Transcription levels of the *clpL* gene increased in cells exposed to
 919 acidic pH. The *wt* cells were grown in BHI/pH 7.8 to the mid-exponential phase and resuspended
 920 in ABM/pH 5.9, and total RNA was extracted at 1 h. The fold change in gene expression was
 921 measured by quantitative real-time PCR and calculated using the 2^{-ΔΔCT} method. The *gyrA*
 922 gene was used as the internal control. Error bars indicate the standard deviation of the mean.
 923 INSTAT software was used to perform Dunnet's statistical comparison test for each strain.
 924 References: ***p* < 0.01; ****p* < 0.001. (B) The $\Delta clpL$ and $\Delta psaB$ mutants are susceptible to acidified
 925 media. The $\Delta clpL$, $\Delta psaB$ and *wt* cells were grown in BHI until an OD_{620nm} 0.3 and then incubated
 926 in ABM medium at pH 4.8 for 1 h. After that, viable cells were assessed as described in the Fig 2
 927 legend. (C) The intracellular survival of the $\Delta clpL$ and $\Delta psaB$ mutant is decreased compared with
 928 *wt*. A549 cells were infected with the $\Delta clpL$, $\Delta psaB$ and *wt* cells and intracellular survival was
 929 determined as described in the Fig 1 legend. White bars correspond to non-virus infected cells,
 930 green bars to IAV-infected cells and blue bars to Bafilomycin-A1-treated cells. (D) The $\Delta clpL$ and
 931 $\Delta psaB$ mutants are susceptible to H₂O₂. The $\Delta visR$, $\Delta sodA$ and *wt* cells were grown in BHI until
 932 an OD_{620nm} 0.3 and then exposed at BHI medium containing 20 mM H₂O₂ for 2 h. After that,
 933 viable cells were determined as described in the Fig 2 legend. (E) Inhibition of ROS production
 934 does not affect the impaired intracellular survival of the $\Delta clpL$ and $\Delta psaB$ mutants. A549 cells
 935 were infected with the $\Delta visR$, $\Delta sodA$ and *wt* cells and intracellular survival was determined as
 936 described in the Fig 1 legend. White bars correspond to non-virus infected cells, green bars to
 937 IAV-infected cells and blue bars to NAC-treated cells. For all panels, data are representative of at

938 least three independent experiments and statistically significant differences are indicated as
939 $p < 0.01$ (**) or $p < 0.001$ (***).

940

941 **Fig 6. The viral-bacterial synergism is dependent on autophagic-proficient cells.** IAV-
942 infected and non-virus MEF (A) and MEF *atg5* KO (B) cells were incubated with the wt and the
943 $\Delta visR$ strains for 4 h, and bacterial intracellular survival was assessed as described in the Fig.
944 1A legend. White bars indicate bacterial infection and green bars indicates superinfection. Data
945 are representative of at least three independent experiments and statistically significant
946 differences are indicated as $p < 0.05$ (*) or $p < 0.01$ (**).

947

948 **Fig. 7. Proposed model for the synergistic mechanism that exists between influenza A and**
949 ***S. pneumoniae* in pneumocytes.**

950

951 **Supporting information**

952 **S1 fig. Determination of apoptosis and necrosis levels in A549 cells infected with IAV**
953 **and/or *S. pneumoniae*.** (A) A549 cells were infected with different MOI of IAV for 24 h and
954 coinfectd with a bacterial MOI of 30. Apoptosis/necrosis was measured at the single-cell level by
955 labeling cells with annexin-V-APC and counterstaining with propidium iodide (PI). Representative
956 data are shown and percentage of cells are indicated in each quadrant (lower left: APC⁻/PI⁻, intact
957 cells; lower right: APC⁺/PI⁻, apoptotic cells; upper left: APC⁻/PI⁺, necrotic cells; upper right:
958 APC⁺/PI⁺, late apoptotic or necrotic cells). (B) The bar chart describes the percentual distribution
959 of necrotic, apoptotic and viable cells after infection with different MOI of IAV or with
960 superinfection with *S. pneumoniae*.

961

962 **S2 fig. Identification of histidine kinase (*hk*) mutants of *S. pneumoniae* displaying normal**
963 **intracellular survival in pneumocytes.** A549 cells were infected with different *hk* mutants and
964 its intracellular survival capacity was determined as described for non-virus infected cells in the
965 Fig 1 legend, and these results were compared with those obtained for the *wt* strain. Green bars
966 and blue bars correspond to 2 h and 4 h of incubation after antibiotic treatment, respectively.

967

968 **S3 fig. Confirmation of the IAV-induced ROS production in A549 cells.** (A) Representative
969 flow cytometry histogram showing results of H₂DCF-DA staining (a measurement of ROS levels)
970 of IAV-infected A549 cells or mock-A549 cells. (B) Bar graph depicting results of IAV-infected
971 A549 cells compared with non-infected cells. Data are representative of at least three
972 independent experiments.

973

974 **S4 fig. VisR is a global regulator that controls gene expression during the stress**
975 **response.** (A) Gene expression scatter plot in samples obtained from the *wt* strain and the $\Delta visR$
976 mutants, with the *x*-axis representing the gene expression values for the control condition (*wt*)
977 and the *y*-axis representing those for the treated condition ($\Delta visR$). Each black dot represents a
978 significant single transcript, with the vertical position of each gene representing its expression
979 level in the experimental conditions and the horizontal one representing its control strength. Thus,
980 genes that fall above the diagonal are over-expressed whereas genes that fall below the diagonal
981 are under-expressed as compared to their median expression levels in the experimental groups.
982 (B) Volcano plot of gene expression in *wt* vs $\Delta visR$ samples measured by RNAseq. The *y*-axis
983 represents the mean expression value of the log₁₀ (*p*-value), while the *x*-axis displays the log₂ fold
984 change value. Black dots represent genes with an expression 2-fold higher in the $\Delta visR$ mutant
985 relative to strain *wt* with a *p*-value < 0.05, with red dots signifying genes with an expression 2-fold
986 lower in the $\Delta visR$ mutant, which are relative to strain *wt* with a *p* < 0.05.

987

988 **S5 fig. Comparative proteomic analysis between the *wt* and the $\Delta visR$ strains.** Volcano plot
989 reflecting the results from the statistical analysis of differentially expressed proteins quantified
990 among the proteome of the *wt* strain and the $\Delta visR$ mutant. Statistical analysis was performed by
991 Student t-test and statistical significance was considered for *p*-values < 0.05. Significant values
992 are represented as red dots.

993

994 **S6 fig. Identification of the pneumococcal 78-kDa ClpL chaperone expressed under acidic**
995 **conditions.** (A) SDS-PAGE analysis of protein extracts obtained from the *wt* cells grew at slightly

alkaline (pH 7.8) or acidic (pH 5.9) culture media. The protein band subjected to N-terminal sequencing is indicated by an arrow. (B) The N-terminal sequence obtained by Edman degradation were analyzed by tryptic digestion and HPLC-protein sequencer. The m/z values of ions matching peptides derived from the 78-kDa protein band are indicated by numbers. The amino acids sequences corresponding to pick 8 (11 amino acids) and pick 12 (14 amino acids) corresponded to the ClpL chaperone, according to the R6 pneumococcal genome (<https://www.uniprot.org/ proteomes/UP000000586>).

S7 fig. IAV and *S. pneumoniae* infection and superinfection induce autophagy in A549 cells. (A) LC3-II levels are induced by superinfection with IAV and *S. pneumoniae*. A549 cells were infected with IAV (MOI 10), *S. pneumoniae* (MOI 30) and coinfectd as described in the Fig.1A legend. As controls, A549 cells were also treated with inducers (rapamycin) and inhibitors (bafilomycin A1) of the autophagy process. Cell lysates were subjected to Western blot analysis using anti-LC3-II, anti-beta-actin antibodies with data being representative of at least three independent experiments. (B) Quantification of the LC3-II level in western blot: bar graphs represents LC3-II relative intensity (LC3-II/ β -actin) with data being representative of at least three independent experiments. (B) Formation of the puncta of mKate2-hLC3 indicating autophagy induction during IAV, *S. pneumoniae* and superinfection. The A549 cells were transfected with the mKate2-hLC3 plasmids for 24 hours, and followed by an IAV, *S. pneumoniae* or superinfection for 4 h. The far-red (mKate2) fluorescence in the cells were monitored using an Olympus FluoView FV1000 confocal laser scanning microscope.

A **B**

A **B** **C** **D**

A

B

C

A **B**

B

C

D

E

A**MEF *wt*** **B****MEF *atg5* KO**