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3 Capsular sialic acid of *Streptococcus suis* serotype 2 binds to swine 4 influenza virus and enhances bacterial interactions with virus-infected 5 tracheal epithelial cells

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20 **Running title: *S. suis* serotype 2 capsule binds to influenza virus**

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23 **ABSTRACT**

24 *Streptococcus suis* serotype 2 is an important swine bacterial pathogen and it is also an emerging
25 zoonotic agent. It is unknown how *S. suis* virulent strains, which are usually found in low
26 quantities in pig tonsils, manage to cross the first host defense lines to initiate systemic disease.
27 Influenza virus produces a contagious infection in pigs which is frequently complicated by
28 bacterial co-infections leading to significant economic impacts. In this study, the effect of a
29 preceding swine influenza H1N1 virus (swH1N1) infection of swine tracheal epithelial cells
30 (NTPr) on the ability of *S. suis* serotype 2 to adhere, invade and activate these cells was
31 evaluated. Cells pre-infected with swH1N1, showed bacterial adhesion and invasion levels
32 increased more than 100 fold when compared to normal cells. Inhibition studies confirmed that
33 the capsular sialic acid moiety is responsible for the binding to virus-infected cell surface. Also,
34 pre-incubation of *S. suis* with swH1N1 significantly increased bacterial adhesion/invasion to
35 epithelial cells, suggesting that *S. suis* may also use swH1N1 as a vehicle to invade epithelial
36 cells when the two infections occur simultaneously. Influenza infection may facilitate the
37 transient passage of *S. suis* at the respiratory tract to reach the bloodstream and cause bacteremia
38 and septicemia. *S. suis* may also increase the local inflammation at the respiratory tract during
39 influenza infection, as suggested by an exacerbated expression of pro-inflammatory mediators in
40 co-infected cells. These results give a new insight in the elucidation of complex interactions
41 between influenza virus and *S. suis* in a co-infection model.

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47 **INTRODUCTION**

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49 *Streptococcus suis* is one of the most important post-weaning bacterial pathogens in swine and it
50 is also an emerging zoonotic agent (1). Among the 35 *S. suis* described serotypes, type 2 is the
51 most virulent one for both pigs and humans (2), although differences in virulence have been
52 described for this serotype (3). Pigs may acquire *S. suis* very early in life and some colonized
53 animals may never develop disease (carrier animals); on the other hand, some carrier piglets will
54 eventually develop bacteremia, septicemia and meningitis following dissemination of *S. suis* in
55 the bloodstream (1). Human infections with *S. suis* manifest mainly as meningitis, septicemia and
56 septic shock (4). It is believed that people can become infected through skin lesions, surface
57 mucosa and/or the oral route (5).

58 It is still unknown how low quantities of *S. suis* virulent serotype 2 strains present in
59 tonsils of pigs manages to cross the first natural line of the host defense to initiate disease. It is
60 believed that the pathogen would breach the mucosal epithelium at the upper respiratory tract (6).
61 Bacterial adhesion and invasion of epithelial cells are usually associated with the first steps of
62 colonization by mucosal pathogens; however, few data are available concerning the interaction
63 between *S. suis* and swine respiratory epithelial cells. Ferrando and colleagues described for the
64 first time *S. suis* adhesion (but not invasion) to porcine tracheal epithelial cells (7).

65 The *S. suis* capsular polysaccharide (CPS), which defines the serotype, is essential for the
66 virulence of this pathogen mainly due to its antiphagocytic activity (6). The analysis of the
67 serotype 2 CPS revealed the presence of different sugars including Neu5Ac or sialic acid.
68 Interestingly, sialic acid was found to be terminal [(2→6)- β -D-Galactose], and the CPS can be
69 quantitatively desialylated by mild acid hydrolysis (8). It has been previously shown that

70 expression of CPS interferes with adhesion and (if any) invasion of *S. suis* to epithelial cells (9,
71 10). So, classically, the role of this virulence factor has been suggested to be crucial once bacteria
72 reach the bloodstream (6). Among other suggested *S. suis* virulence factor, secreted proteins, such
73 as the hemolysin (suilysin), surface proteins and other cell wall components have been reported
74 (11).

75 Secondary bacterial infections associated to influenza virus infection in humans are a
76 leading cause of human morbidity and mortality worldwide (12). Swine influenza virus infections
77 in pigs also cause serious respiratory disease (13). Although this infection is typically self-limited
78 with high-morbidity but low mortality, secondary complications substantially increase illness and
79 death (14). In fact, influenza is a key contributor to the porcine respiratory disease complex
80 (PRDC), a multifactorial syndrome characterized by severe respiratory disease after infection
81 with two or more agents (15). Pathogens associated with PRDC include (among others)
82 *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis*, *Mycoplasma hyopneumoniae, S. suis*
83 and porcine reproductive and respiratory syndrome virus (15). Subtypes of swine influenza virus
84 that are most frequently identified in pigs include H1N1 (classical and pandemic), H1N2 and
85 H3N2 (13). Influenza virus strains uniformly recognize cell surface oligosaccharides with a
86 terminal sialic acid either 2,3 Neu5Ac–galactose or 2,6 Neu5Ac–galactose. However, their
87 receptor specificity varies according to host. Pigs are unique among influenza virus hosts in that
88 they are susceptible to infection with influenza viruses of human and avian origin as well as to
89 swine influenza virus, because their tracheal epithelium contains these two sialyloligosaccharides
90 (16).

91 In this study we demonstrated, for the first time, a novel mechanism used by a bacterial
92 species to facilitate the invasion of respiratory epithelial cells already infected with an influenza
93 virus. More specifically, we showed that the sialic acid moiety present in the CPS of *S. suis*

94 serotype 2 directly interacts with swine influenza virus leading to an increased bacterial adhesion,
95 invasion and activation of tracheal epithelial cells. This mechanism could explain, at least in part,
96 how secondary bacterial infection with virulent *S. suis* strain could be enhanced following
97 influenza infection.
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102 **MATERIALS AND METHODS**

103

104 **Bacterial strains, epithelial cells and influenza virus strain.** *S. suis* strains used in this study are
105 listed in Table 1. The well characterized *S. suis* serotype 2 virulent strain 31533 (10, 17) was used
106 throughout this study. Other previously well characterized isogenic mutants derived from this
107 strain and devoid of either CPS or suilysin production, or modified at the either peptidoglycan
108 (PG) or lipoteichoic acid (LTA) levels were also included (18-21). In addition, serotype 2 field
109 strains with lower (Canadian strain) or higher (epidemic strain isolated from a deadly *S. suis*
110 human outbreak in China) virulence potential (3), as well as reference strains of serotypes 3 and
111 14 were also included for comparison purposes (Table 1). A swine influenza virus H1N1
112 (swH1N1, strain A/swine/St-Hyacinthe/148/1990) isolated from a case of swine flu in Canada
113 was used (22).

114 Bacterial were cultured as previously reported (17). The number of CFU/ml in the final
115 suspension before each experiment was determined by plating samples onto THA using
116 Autoplate® 4000 Automated Spiral Plater (Spiral Biotech, Norwood, MA). The pig trachea
117 epithelial cell line (NPTr) was used for virus growth and co-infection studies as described (23).
118 For assays, cells were treated with 0.05% trypsin in 0.03% EDTA solution and diluted in culture
119 medium to obtain a final concentration of 10^5 cells/ml. Then, the cell suspension was distributed
120 into tissue culture plates and incubated until cell confluence was reached. Twenty-four hours
121 before the assays, culture medium was removed from the wells and replaced by fresh complete
122 medium without antibiotics. Virus was produced by replication in NPTr cells as previously
123 described (23). The titer of the viral production was $10^{7.25}$ $TCID_{50}$/ml.

124

125 **NPTr co-infection by swH1N1 and *S. suis*.** swH1N1 (MOI: 1) was inoculated onto NPTr cell
126 monolayers in 24-well culture plates and incubated with 2% FBS (as standardized in preliminary
127 experiments) and antibiotic free MEM for 1 h at 37°C in 5% CO_2. The virus-infected cells were
128 then washed twice with PBS and fresh media containing 10% FBS without antibiotic was added.
129 The increased serum concentration did not affect virus replication and kept cells healthy for the
130 whole experiment. Following a 12 h incubation time at 37°C in 5% CO_2, cells were infected with
131 *S. suis* (10^6 CFU/well, MOI:10). Plates were centrifuged at 800 x *g* for 10 min in order to bring
132 bacteria in close contact with the cells (24). Bacterial infected cells were then incubated at 37°C in
133 5% CO_2 for different incubation times (see below). Infectious viral load profile was determined in
134 cell cultures for virus infected cells and for virus-bacteria co-infected cells by virus titration
135 evaluation as described above. Cell cytotoxicity levels were determined using Cytotox 96 kit
136 (Promega, Madison, WI) from culture supernatants according to manufacturer's instruction. In
137 selected experiments, swH1N1 and *S. suis* were pre-incubated for 1 h at 4°C (10^6 *S. suis* CFU and
138 10^6 $TCID_{50}$ of swH1N1, respectively; final bacteria/virus ratio of 1). Afterwards, the virus-*S.*
139 *suis* mixture was washed twice with PBS and resuspended with complete medium, inoculated to
140 cells and incubated at 37°C in 5% CO_2 for bacterial adhesion and invasion assays, as described
141 below. Mock-treated bacteria were used as control.

142 The invasion assay was performed as previously described (17), with some modifications.
143 After 2 or 4 h of incubation with *S. suis*, the NPTr cells monolayers were washed twice with PBS,
144 and 1 ml of cell culture medium containing 100 µg of gentamicin and 5 µg of penicillin G
145 (Invitrogen, Burlington, ON, Canada) was added to each well. The plates were then further
146 incubated for 1 h at 37°C with 5% CO2 to kill extracellular and surface-adherent bacteria. After
147 washing, cells were disrupted with sterile ice-cold deionized water followed by cell scrapping
148 from the bottom of the well in order to liberate intracellular bacteria. Bacterial CFU numbers were

149 determined by plating serial dilutions as described above. Levels of invasion were expressed as
150 the total number of CFU recovered per well. An "adhesion assay" which in fact quantifies total
151 cell-associated bacteria (intracellular bacteria and surface-adherent bacteria) was performed
152 similarly to the invasion assay. However, the cells were vigorously washed five times to eliminate
153 nonspecific bacterial attachment and no antibiotic treatment to kill the extracellular bacteria was
154 used. At different incubation times (see results), the levels of "adhesion" (total associated
155 bacteria) were expressed as the total number of CFU recovered per well.

156 For the inhibition studies, and after removing the cell supernatant and washing twice the
157 wells with fresh PBS, 100 μg of purified native CPS or desialylated CPS (prepared as described
158 below) resuspended in cell culture medium were added to swH1N1-infected cells. Control cells
159 were treated similarly but without addition of CPS. After 1 h of incubation, cells were washed
160 twice with PBS and infected with *S. suis* as previously described. Bacterial adhesion and invasion
161 studies were performed as described above and compared to non-treated cells. Results were
162 expressed in percentage when compared to bacterial adhesion and invasion of untreated swH1N1
163 pre-infected cells (considered as 100%). Swine polyclonal antibody serum against the whole
164 swH1N1 virus strain (serum from a convalescent animal) was used a positive inhibition control.
165 Supernatants of swH1N1-infected cells were removed and the serum (diluted 1/40 in cell culture
166 medium) was added to the wells.

167

168 **Hemagglutination inhibition assay (HI).** HI test was carried out as previously described (25)
169 with the modification that swine sera were replaced by different concentrations of *S. suis*. Serial
170 dilutions of *S. suis* strains (wild-type 31533 strain or non-encapsulated B218 mutant strain) were
171 used for the HI assay. Briefly, 50 μl of bacterial suspensions (grown as described above) were
172 dispensed at different concentrations in triplicate in a 96-well round bottom plate. Fifty μl of

173 swH1N1 (2 x $10^{6.25}$ $TCID_{50}$/ml) was then added to each well and incubated for 1 h at room
174 temperature. Different wells represented a 2-fold dilutions of *S. suis*/swH1N1virus ratios,
175 beginning at a ratio of 200 for the wild type encapsulated strain and 10 000 for the non-
176 encapsulated B218 mutant. Afterward, 50 µl of a 0.5% suspension of whole rooster red blood
177 cells (RBC) in PBS were added to each well and gently mixed. The HI was evaluated after
178 incubating the plate at room temperature for 1 h. For this experiment, PBS was used as negative
179 RBC control and serial dilutions of reference heat-inactivated anti-swH1N1 serum was used as a
180 positive HI control. Under the conditions tested, capsulated and non-encapsulated *S. suis* strains
181 did not induce any hemagglutination (results not shown).
182
183 ***S. suis* CPS purification and CPS desialylation.** The CPS of *S. suis* serotype 2 reference strain
184 S735 was prepared and purified as previously described (8). For quality controls, CPS was
185 analyzed by nuclear magnetic resonance. Lack of protein and RNA/DNA contamination was
186 verified by Lowry method and by spectrophotometry, respectively. CPS was also desialylated by
187 mild acid hydrolysis. CPS (8 mg) was heated in 1 ml of HCl (70 mM) at 60 °C for 4 h, neutralized
188 with NH_4OH (2 M), and purified on a Sephadex G10 column (1.5 x 10 cm). Presence (native
189 CPS) or absence (desialylated CPS) of sialic acid was verified by gas chromatography after
190 methanolysis and acetylation and by nuclear magnetic resonance as well as by a reaction with an
191 enzyme linked-lectin assay as previously described (26).
192
193 **Confocal and electron microscopy.** For confocal microscopy analysis, cells were placed on
194 coverslips and infected (or not) with swH1N1 and either *S. suis* strain 31533 or its non-
195 encapsulated mutant strain (B218) as described above and further incubated for 2 h at 37°C in 5%
196 CO_2. Coverslips were washed with PBS to remove non-associated bacteria and cells were fixed

197 with 4% paraformaldehyde solution for 10 min. Cells were then washed and permeabilized with
198 PBS containing 0.2% Triton X-100 (Thermo Hyclone, Burlington, ON, Canada) for 2 min. The
199 coverslips were blocked for 10 min with PBS containing 2% bovine serum albumin and 0.2%
200 gelatin (Sigma-Aldrich, Oakville, ON, Canada). Coverslips were then incubated for 1 h with a
201 mouse monoclonal antibody against an epitope within influenza virus A nucleoprotein H1N1 (US
202 Biologica, Swampscott, MA l; 1/500 dilution) and a rabbit anti-*S. suis* serum against either wild-
203 type strain 31533 (1/5000) or its non-encapsulated B218 mutant strain (1/1000) (27). After
204 washing with PBS, coverslips were incubated with secondary antibodies Alex-Fluor 568 goat
205 anti-mouse IgG (for swH1N1) and Alex-Fluor 488 goat anti-rabbit IgG (for *S. suis*) (both from
206 Invitrogen) for 30 min. Coverslips were then washed and mounted on glass slides with moviol
207 containing DABCO.

208 For transmission electron microscopy (TEM) and scanning electron microscopy (SEM),
209 samples were fixed for 1 h at room temperature with 2% (vol/vol) glutaraldehyde in 0.1 M
210 cacodylate buffer (pH 7.3) and were then post-fixed for 45 min at room temperature with 2%
211 osmium tetroxide. Specimens for TEM were dehydrated in a graded series of ethanol solutions
212 and embedded with LR white resin. Thin sections were cut with a diamond knife and
213 were poststained with uranyl acetate and lead citrate. Samples were observed with an electron
214 microscope model JEM-1230 (JEOL, Tokyo, Japan). Samples for SEM were dehydrated in a
215 graded series of ethanol solutions and covered with gold after critical point drying and were
216 examined with a Hitachi S-3000N microscope.

217

218 **Quantitative RT-PCR (qRT-PCR) for cytokine and chemokine expression.** qRT-PCR assay
219 was performed as previously described (28). Primers (IDT DNA, Coralville, IA) used for
220 detection of genes were all verified to have PCR amplification efficiency ranked between 90-

221 110% using a CFX96 rapid thermal cycler system (Bio-Rad, Hercules, CA) (Table 2). The
222 GeNorm applet v.3.5 (http://medgen.ugent.be/~jvdesomp/genorm/) was used to initially determine
223 the two most stable reference genes from a set of six reference genes using random samples from
224 the cDNA panel generated for the qPCR analysis of cytokine/chemokine gene expression.
225 Therefore, normalization of data was done using the reference genes hypoxanthine
226 phosphoribosyltransferase 1 (*Hprt1*) and Peptidylprolyl isomerase A (*Ppia*). Fold-change of gene
227 expression was calculated using the normalized gene expression ($\Delta\Delta C_q$) calculation method of the
228 CFX software manager v.2.1 (Bio-Rad). Mock-infected samples were used as calibrator and
229 consequently, relative fold-differences were calculated for the rest of the samples compared to the
230 mean of the calibrator samples.

231

232 **Statistical analysis.** All data are expressed as mean ± SEM. Prism statistical software v.5
233 (Graphpad, San Diego, CA) was employed for data analysis. Data from the adhesion and
234 invasion assays were analyzed for significance using Student's unpaired *t* -test. Data from qPCR
235 assays were subjected to one way ANOVA analysis followed by Tukey's post hoc test. A *P* value
236 < 0.01 was used as threshold for statistical significance. Results reflect mean values of at least
237 three independent experiments.

238

239

240 **RESULTS**

241

242 ***S. suis* serotype 2 adhesion and invasion are significantly increased when cells are**
243 **previously infected by swH1N1, independently of the virulence of the *S. suis* strain.**

244 The kinetics of adhesion of the highly virulent *S. suis* serotype 2 strain 31533 to NPTr
245 cells was studied. As shown in Fig.1A, in the absence of virus infection, adhesion was time
246 dependent, increasing from 30 min to 4 h of incubation. After 4 h of incubation, a plateau was
247 reached (data not shown). Results of the kinetics and levels of adhesion are similar to those
248 previously obtained with porcine endothelial and other epithelial cells (10, 17). However, when
249 cells were pre-infected with swH1N1 for 12 h, the adhesion levels increased more than 100 folds
250 compared to those observed in the absence of virus (Fig.1A). In addition, adhesion levels
251 immediately reached a plateau (Fi.g 1A), even after 5 min of incubation (data not shown). When
252 strains of serotype 2 with lower or higher virulence potential than that of 31533 strain were tested
253 (intermediate virulence Canadian strain 1591 or epidemic strain SC84 from a Chinese human
254 outbreak) (3), bacterial adhesion levels were statistically similar to those obtained with the
255 virulent strain 31533, either in the absence or presence of swH1N1 infection (Fig. 2A).

256 Surprisingly, and different from what has been previously reported with other epithelial
257 cells of swine origin (10), encapsulated *S. suis* serotype 2 was able to clearly invade NPTr cells
258 (Fig. 1B). However, when cells were pre-infected with the swH1N1 strain, invasion rates also
259 increased more than 100 folds at both, 2 h and 4 h incubation times ($P < 0.01$) (Fig. 1B). Similar
260 to the adhesion results, invasion rates of the two additional *S. suis* serotype 2 strains were
261 statistically similar to those obtained with strain 31533 in the presence or absence of swH1N1
262 pre-infection (Fig. 2B). For all adhesion and invasion experiments, cells presented cytotoxicity

263 levels lower than 20% (data not shown). Interestingly, virus replication levels in NPTr cells were
264 similar in the presence or absence of bacterial infection (Supplemental Fig. S1).
265

266 **Critical role of the capsular polysaccharide (CPS) in the increased *S. suis* adhesion/invasion**
267 **to swH1N1 pre-infected NPTr cells.**

268 Isogenic mutants defective in suilysin production, *D*-alanylation of LTA or *N*-
269 deacetylation of PG behaved statistically similarly to the wild-type strain 31533 either in the
270 presence or absence of swH1N1 pre-infection. Only the non-encapsulated (CPS-) mutant
271 presented a different pattern. In the absence of virus infection, the adhesion and invasion levels of
272 the mutant strain were significantly higher ($P < 0.01$) than those of the wild-type strain (Fig. 2A
273 and 2B), confirming previous published results which indicated that the CPS interferes with *S.*
274 *suis*-host cell interactions (9, 10). However, these adhesion and invasion levels were unmodified
275 after a swH1N1 pre-infection. These data suggest that the CPS might play a role in the observed
276 increased levels of wild-type *S. suis* adhesion/invasion to virus infected cells (Fig. 2A and 2B).
277 Since the antigenic characteristics of the CPS define the serotype (1), two additional *S. suis*
278 serotypes (3 and 14) were tested. Although both strains are well encapsulated (29), only the
279 adhesion and invasion of *S. suis* serotype 14 reference strain (DAN13730), but not those of
280 serotype 3 (strain 4961), were significantly affected by a pre-infection with swH1N1 (Fig. 2A
281 and 2B). This would indicate that the CPS structure and/or composition directly influence the
282 interactions between *S. suis* and swH1N1 pre-infected cells.

283 Influence of epithelial cell swH1N1 pre-infection on adhesion/invasion abilities of *S. suis*
284 serotype 2 was confirmed by microscopy. First, confocal microscopy revealed that very few
285 encapsulated wild-type bacteria could be observed interacting with epithelial cells in the absence
286 of virus pre-infection (Fig. 3). However, after 12 h of swH1N1 pre-infection, levels of wild-type

287 encapsulated *S. suis* adhesion were clearly higher and grouped around the cells (in “grapes”),
288 especially where the red staining with anti-H1N1 monoclonal antibody was present, indicating a
289 possible co-localization of virus and bacteria. In the absence of virus infection, the non-
290 encapsulated mutant showed a higher level of adhesion than the wild-type strain, although
291 bacteria were randomly distributed on the cell surface (diffuse adhesion). A similar adhesion
292 pattern of the mutant strain was observed when cells were pre-infected with swH1N1. Electron
293 microscopy (TEM and SEM) confirmed the influence of a pre-infection with influenza virus on *S.*
294 *suis*-cell interactions (Fig. 4). In the absence of virus infection, very few cocci (if any) could be
295 observed interacting with cells (Fig. 4A-I). In the presence of a virus pre-infection, cells were
296 highly activated (clearly showing cilia at their surface) and high numbers of cocci were at the cell
297 surface (closely interacting with cilia) (Fig. 4AII and 4B) and, sometimes, inside the cells (Fig. 4
298 AIII).
299

300 **Bacterial capsular sialic acid is responsible for bacterial-virus interactions in infected cells.**

301 Since the CPS of *S. suis* serotype 2 was shown to be implicated in the increased bacterial-
302 cell interactions when cells were pre-infected with swH1N1, it was hypothesized that the sialic
303 acid moiety present in the CPS of this serotype may be involved through interactions with viral
304 hemagglutinin. In fact, the reference strain of serotype 14 CPS (which also interacted with
305 swH1N1 pre-infected cells) possesses an identical sialic acid-containing side chain (also with a
306 link 2,6 to the adjacent galactose) as serotype 2 CPS (30), whereas the reference strain of
307 serotype 3 lacks this sugar (31-33). To confirm such hypothesis, inhibition studies were
308 performed. Interestingly, when wells were simply washed before adding the bacterial suspension
309 and used as a control, no differences could be obtained with previous results with non-washed
310 wells, indicating that free virus were either not present at significant number or that they did not

311 significantly interfere with baceterial adhesion/invasion to epithelial cells. A pre-treatment of
312 swH1N1-NPTr pre-infected cells with purified native CPS inhibits >75% of adhesion and
313 invasion by *S. suis* serotype 2. This inhibition was similar to that obtained with a pre-treatment
314 with an anti-swH1N1 specific antibody (Fig. 5). When the same amount of desialylated CPS was
315 used, no inhibition of bacterial adhesion/invasion could be observed, confirming the involvement
316 of the CPS sialic acid in the interactions of *S. suis* with swH1N1 pre-infected cells (Fig. 5).
317

318 ***In vitro* binding of swH1N1 to *S. suis* enhances bacterial adhesion and invasion to epithelial**
319 **cells.**

320 To investigate if well encapsulated *S. suis* may directly interact with the swH1N1 strain, a
321 test of hemagglutination inhibition was performed. Results showed that a 1 h pre-incubation of *S.*
322 *suis* serotype 2 strain 31533 and swH1N1 virus (in a bacteria/virus ratio >50) resulted in the
323 complete inhibition of RBC hemagglutination (Supplemental figure 2). Lower concentrations of
324 bacteria did not present any visual inhibition. Interestingly, no inhibition of RBC
325 hemagglutination was observed when the non-encapsulated mutant was used, even in a
326 bacteria/virus ratio of 10 000) (Supplemental figure 2). Finally, a pre-incubation of *S. suis*
327 serotype 2 strain 31533 with the swH1N1 strain significantly increase the interaction between *S.*
328 *suis* and NPTr cells, since bacterial adhesion and invasion to epithelial cells presented up to 10
329 fold increase values when compared to those infected with *S. suis* without a pre-incubation with
330 swH1N1 (Fig. 6). These results suggest that *S. suis* may also use swH1N1 virus as a vehicle to
331 adhere and invade epithelial cells. The fact that some bacteria may aggregate with virus (forming
332 micro-clumps) enhancing somehow the total number of bacterial adhesion to cells cannot be
333 ruled out. No increase in bacteria-cell interactions was observed when the non-encapsulated
334 mutant was used (data not shown).

335

336 **Co-infected NPTr cells express higher levels of pro-inflammatory genes than single-infected**
337 **cells.**

338 Although a complete kinetics was studied (results not shown), results showed that 24 h
339 post-bacterial infection (36 h post virus infection) reflected optimal differences among groups.
340 NPTr cells infected with bacteria alone showed absence or low expression levels of CCL2 (MCP-
341 1), CCL4 (MIP-1β), IFN-β and TNF-α, intermediate expression levels of IL-6 and high levels of
342 IL-8 expression (Fig. 7). Virus-mediated NPTr cell activation at that incubation time showed
343 absence of IL-8 expression. On the other hand, the swH1N1 strain activated gene expression of
344 other mediators at similar levels (CCL2 and IL-6) or at significantly higher levels (CCL4, TNF-α
345 and IFN-β) than those obtained after activation with *S. suis* alone (Fig. 7). Interestingly,
346 swH1N1-*S. suis* co-infection significantly increased the expression of CCL2, CCL4, IL-6, IL-8
347 and TNF-α mRNA. In some cases, an additive effect seemed responsible for such differences (IL-
348 6 and TNF-α). However, the increase of mRNA expression of CCL2, CCL4 and IL-8 mRNA
349 expression was clearly ahead of a simple additive effect. Expression of IFN-β mRNA was
350 probably attributed solely to the effect of swH1N1 (Fig. 7).

351

352 **DISCUSSION**

353 The pathogenesis of the infection caused by *S. suis* is far from being completely
354 understood (6). In swine, *S. suis* is mainly transmitted by aerosols, and airborne transmission
355 among pigs has been clearly demonstrated (34). *S. suis* play a certain role in mixed respiratory
356 infections, although it is not considered a primary cause of swine pneumonia (1), indicating that
357 it may also use the respiratory tract as a transient passage before reaching the bloodstream and
358 causing bacteremia, which is essential for the pathogen to cause meningitis (35). The actual early
359 mechanisms used by this pathogen to interact with epithelial cells to further invade the
360 bloodstream are, in fact, poorly known.

361 *S. suis* clinical association with virus infections have been largely reported (36, 37). More
362 recently, several outbreaks in swine due to swine influenza virus with a significant level of
363 systemic co-infection due to *S. suis* have been reported in England (38). In humans, it is well
364 known that influenza cases are heavily complicated by bacterial infections (12). In fact, it has
365 been previously reported that influenza as well as other respiratory virus increase the
366 adhesion/invasion capacities of bacterial pathogens (including streptococci) to epithelial cells,
367 although mechanisms have not been fully elucidated (39). The goal of the present work was to
368 study interactions between *S. suis* and tracheal epithelial cells either pre-infected or not with
369 swH1N1.

370 Results showed that *S. suis* is able to not only to adhere to but also invade swine tracheal
371 epithelial cells. In the absence of virus infections, adhesins involved in such interactions seem to
372 be located in the bacterial cell wall, since they are hindered by the presence of the CPS, as
373 previously suggested (6, 9, 10). Indeed, significant higher levels of adhesion and, most important,
374 invasion rates were observed with a non-encapsulated *S. suis* mutant. Interestingly, results
375 obtained with isogenic mutants showed that alteration at the LTA and PG as well as the lack of

376 suilysin production did not influence the adhesion/invasion capacities of *S. suis*. Different *S. suis*
377 surface-exposed proteins have been described as bacterial adhesins to extracellular matrix
378 proteins present in host cells (6, 11). In fact, ApuA, a surface protein with bifunctional
379 amylopullulanase activity, was described to play an important role in such adhesion to tracheal
380 epithelial cells (7). No differences could also be observed between strains of serotype 2 of
381 difference virulence potential or strains belonging to other serotypes showing that those adhesins
382 are probably common to most strains of *S. suis*, independently of their virulence/serotype.

383 In the presence of a prior swH1N1 infection, more than 100 fold increases in *S. suis*
384 adhesion and invasion could be observed. This increased interaction was confirmed by confocal,
385 TEM and SEM. Increased cell susceptibility to *S. suis* adhesion and invasion following a virus
386 infection may have different explanations. One of the most well-known interactions is that
387 between influenza virus and *Streptococcus pneumoniae* (40). *In vivo* increased susceptibility has
388 been attributed to an alteration of anti-bacterial phagocyte functions through a diminished
389 bactericidal activity and/or damage to the respiratory epithelium resulting in defective
390 mucociliary clearance mechanisms, which in turn leads to an increased numbers of bacteria that
391 remains in the respiratory tract (41). *In vitro* studies suggested damage to the respiratory
392 epithelium by exposing surface molecules and cell receptors to which pneumococci more readily
393 adhere and invade cells. This effect would be mainly done by the viral neuraminidase (42),
394 although a certain synergistic role of neuraminidase produced by *S. pneumoniae* cannot be rule
395 out (43).

396 Results from the present study indicate that interactions between influenza virus and *S.*
397 *suis* are clearly different from those with *S. pneumoniae*. In fact, no neuraminidase activities have
398 been so far demonstrated for *S. suis*. On the other hand, a clear role of the surface exposed CPS in
399 the *S. suis* interactions with swH1N1-infected cells could be established. Similar results were

400 previously obtained with Group A *Streptococcus* (GAS) and A549 epithelial cells (44). Although
401 a certain direct binding between GAS and influenza virus could be observed, molecules involved
402 in such interactions have so far not been elucidated (45). Interestingly, GAS lacks sialic-acid in
403 its surface. In the present study, the main serotypes of *S. suis* containing sialic acid (serotypes 2
404 and 14) clearly interact with swH1N1-infected cells, whereas interactions of a serotype lacking
405 this sugar (serotype 3) were not affected by a virus pre-infection. In addition, serotype 2 strains of
406 different virulence potential behaved similarly, due to the fact that capsular composition of the
407 three strains is most probably identical. In an inhibition assay using highly purified native and
408 desialylated CPS purified from the reference strain of serotype 2, it was clearly showed that the
409 bacterial sialic acid moiety was responsible for the virus-bacterial interactions. It was then
410 hypothesized that the *S. suis* sialic acid binds to the hemagglutinin of the swH1N1. This was
411 further demonstrated by the fact that well-encapsulated *S. suis* (but not its non-encapsulated
412 mutant) incubated with the swH1N1 strain was able to inhibit the RBC hemagglutination activity
413 of the virus. The binding of *S. suis* CPS to influenza hemagglutinin was not exclusive of the
414 H1N1 strain used. Another swine influenza field strain (H3N2) used in parallel studies offered
415 identical results than those obtained with the H1N1 strain (unpublished observations).
416 Interestingly, direct binding of Group B *Streptococcus* (GBS) to influenza virus has also been
417 described previously (46). It was hypothesized that the sialyl-galactose linkage in GBS was
418 responsible for binding to the virus (46). We suggest that GBS would behave similarly to *S. suis*
419 since the structures of the CPS of both pathogens are similar (8). Interestingly, not all bacterial
420 pathogens possessing capsular sialic acid use a similar mechanism. For example, it has been
421 proposed that a direct interaction between the neuraminidase of influenza virus and the CPS of
422 *Neisseria meningitidis* enhances bacterial adhesion to cultured epithelial cells, most likely
423 through cleavage of capsular sialic acid-containing bacterial polysaccharides (47).

424 Although a typical pre-infection with influenza virus is believed to be followed by a
425 bacterial complication, a simultaneous infection with both pathogens cannot be disregarded. In
426 pigs, for example, both pathogens may infect animals at the same age range (1). In this study, a
427 binding between free *S. suis* serotype 2 to free swH1N1 promotes enhanced bacterial adhesion
428 and invasion to swine epithelial cells, similarly to what has been shown for GAS (45). Similarly,
429 previous *in vitro* binding of non-identified surface exposed proteins of *Staphylococcus aureus* to
430 the viral hemoagglutinin enhances bacterial invasion to virus-uninfected cells (48). Hence,
431 influenza infection may promote adhesion and internalization of *S. suis* not only by binding of
432 bacteria to the membrane-associated hemagglutinin but also by binding of bacteria to free virions
433 followed by internalization of virus-coated bacteria into non-infected epithelial cells. Therefore, a
434 possible synergy between the two pathogens cannot be ruled out. However, further studies on the
435 exact mechanisms involved should be performed.

436 Influenza virus is able to stimulate epithelial cells and induce the over-production of
437 different inflammatory mediators. In addition, it may directly or indirectly interfere with the
438 balance of cytokine/chemokine production (49). In co-infection studies, activation of epithelial
439 cells by influenza virus enhances the induction of cytokine and chemokine gene transcripts by *S.*
440 *pneumoniae* (50). Inflammation has been reported to be highly important in *S. suis* infections
441 (51). So far, the inflammatory response of respiratory epithelial cells generated by *S. suis* has not
442 been addressed. In the present study, *S. suis* was shown to strongly up-regulate gene expression
443 of mainly IL-6 and IL-8, similar to that observed with epithelial cells of the choroid plexus (52).
444 Differently from what was described with these cells, relatively low levels of TNF-α expression
445 were observed with *S. suis*-activated NPTr cells, even at shorter incubation times (data not
446 shown), indicating some differences between the two cell types. When NPTr cells were pre-
447 infected with swH1N1, the significant increase of IL-8 expression that was observed may be

448 explained by a higher number of bacteria interacting with influenza-infected cells. It has been
449 shown that IL-8 expression by *S. suis*-activated endothelial cells is bacterial-concentration
450 dependent (53). In the case of IL-6 and TNF-α, the increased expression observed under the co-
451 infection conditions may be also explained by an additive effect of swH1N1 and *S. suis*. On the
452 other hand, *S. suis* alone did not produce significant levels of CCL2 and CCL4. However, when
453 cells were pre-infected with swH1N1, between 100 and 300 fold increases in mRNA expression
454 of these mediators were detected. Influenza virus replicates in the respiratory epithelium and
455 induces an inflammatory infiltrate comprised of mononuclear cells and neutrophils (54), to which
456 *S. suis* possesses anti-phagocytic capacities (6). Since *S. suis* is not a primary pulmonary
457 pathogen, an exacerbated production of pro-inflammatory mediators during a co-infection with
458 influenza virus may be important in the pathogenesis of the influenza infection.

459 In conclusion, a new role of *S. suis* CPS, other than that of anti-phagocytic factor (55), has
460 been demonstrated in the present study. Although it was previously reported that the presence of
461 sialic acid in *S. suis* could not be directly related to virulence (56), we demonstrated that its
462 presence plays a major role in the interactions with respiratory epithelial cells previously infected
463 by swine influenza virus, acting as a bacterial receptor for the virus. Simultaneous co-infections
464 with both pathogens may also be mutually beneficial due to direct bacterial-virus interaction.
465 Binding of bacteria to influenza virus-infected cells or directly to influenza virus could play an
466 important role allowing bacteria to move towards the lower airways, initiating the systemic
467 invasion that characterizes the pathogenesis of the infection caused by *S. suis*. The increased
468 production of local pro-inflammatory mediators in the presence of both pathogens may also play
469 an important role in the pathogenesis of the pneumonia caused by swine influenza.

470

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476

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637

638

TABLE 1 List of *Streptococcus suis* strains used in this study

Strains	Relevant phenotype and/or description	References
31533	Serotype 2 highly pathogenic European strain isolated from a disease pig	(10)
SC84	Serotype 2 epidemic virulent strain isolated from a human outbreak in China	(3)
89-1591	Serotype 2 iintermediate virulent strain isolated from a disease pig in Canada	(3)
CPS$^-$	Non-encapsulated B218 mutant strain derived from strain 31533	(19)
ΔSly	Suilysin negative SX911 mutant strain derived from strain 31533	(18)
ΔdLTA	*D*-alanylation of LTA mutant strain derived from strain 31533	(20)
ΔpgdA	*N*-deacetylation of peptidoglycan mutant strain derived from strain 31533	(21)
4961	Reference strain, serotype 3, isolated from a diseased pig	(57)
DAN13730	Reference strain, serotype 14 isolated from a diseased human	(57)
S735	Reference strain, serotype 2, isolated from a diseased pig	(8)

TABLE 2 Sequences of porcine-specific real-time PCR primers

Gene	Genebank ID	Amplicon size	Forward Sequence	Reverse Sequence	Efficiency (qPCR)
Hprt1	NM_001032376	142 bp	GCAGCCCCAGCGTCGTGATT	CGAGCAAGCCGTTCAGTCCTGT	99
Ppia	NM_214353	133 bp	TGCAGACAAAGTTCCAAAGACAG	GCCACCAGTGCCATTATGG	97
Ccl2	NM_214214	169 bp	CAGGTCCTTGCCCAGCCAGATG	CACAGATCTCCTTGCCCGCGA	90
Ccl4	NM_213779	125 bp	TCCCACCTCCTGCTGCTTCACAT	GCCTGCCCTTTTTGGTCTGGAA	100
Il6	NM_214399	105 bp	ACTCCCTCTCCACAAGCGCCTT	TGGCATCTTCTTCCAGGCGTCCC	97
Il8	NW_003300390	80 bp	TGTGAGGCTGCAGTTCTGGCAAG	GGGTGGAAAGGTGTGGAATGCGT	95
Ifnβ	NM_001003923.1	150 bp	TGCAACCACCACAATTCCAGAAGG	TCTGCCCATCAAGTTCCACAAGGA	96
Tnf	NM_214022	112 bp	GCCACCACGCTCTTCTGCCTA	ACGATGATCTGAGTCCTTGGGCCA	91

FIGURE LEGENDS

FIG 1. **Adhesion to and invasion of virus-free or swH1N1-infected NPTr cells by *S. suis* serotype 2 strain 31533**. NPTr cells were either pre-infected with swH1N1 for 12 h (MOI: 1) or not, and subsequently infected with *S. suis* serotype 2 strain 31533 (MOI: 10). (A) Kinetics of adhesion of *S. suis* to virus-infected ("V+B") or control ("B") NPTr cells. After *S. suis* infection cells were extensively washed to remove non-adherent bacteria and then lysed to determine *S. suis* viable counts. (B) *S. suis* invasion of swH1N1-infected ("V+B") or control ("B") NPTr cells at 2 and 4 h bacterial incubation times. Results were determined as described above except that after washing, cells were exposed to antibiotics to kill extracellular bacteria. Data are expressed as mean ± SEM of at least four independent experiments, each done in triplicate. * indicates significant difference between samples infected with bacteria alone and those co-infected with virus and bacteria ($P < 0.01$).

FIG 2. Adhesion to and invasion of virus free or swH1N1-infected NPTr cells by different strains of *S. suis*. NPTr cells were either pre-infected with swH1N1 for 12 h (MOI: 1) or not, and subsequently infected with *S. suis* strains (MOI: 10). (A) Adhesion (incubation time of 2 h) of different *S. suis* strains to NPTr cells. Results were determined after exposure of swH1N1-infected ("V+B") or control ("B") NPTr cells to *S. suis*, followed by extensive washing of non-adherent bacteria and cell lysis to obtain *S. suis* viable counts. (B) Invasion (incubation time of 1 h) of swH1N1-infected ("V+B") or control ("B") NPTr cells by different *S. suis* strains. Results were determined as described above except that after washing, cells were exposed to antibiotics to kill extracellular bacteria. See Table 1 for strain description. Data are expressed as mean ± SEM of at least four independent experiments, each done in triplicate. * indicates significant difference between samples infected with bacteria alone and those co-infected with virus and bacteria ($P < 0.01$).

FIG 3. **Confocal microscopy showing association of wild-type encapsulated *S. suis* serotype 2 strain 31533 and its non-encapsulated mutant (CPS-) with virus free cells (control) or swH1N1 pre-infected NPTr cells.** Cells were non-infected (control) or virus-infected for 12 h

with swH1N1 (MOI: 1) and then infected with either *S. suis* wild-type strain or the CPS- mutant strain (MOI: 10) for 2 h. Samples were labeled using polyclonal antibodies conjugated with Alexa Fluor 488 against *S. suis* (green) and a mouse monoclonal antibody against influenza virus A nucleoprotein H1N1 conjugated with Alexa Fluor 568 against swH1N1 (red). (A) Wild type *S. suis* strain 31533 shows high level of interactions with cells only when pre-infected with swH1N1. Non-encapsulated *S. suis*/cell interaction is not altered by a pre-infection with influenza virus. (B) High adhesion/invasion of *S. suis* strain 31533 to swH1N1 pre-infected cells. Scale bar, 10 µm Original magnification 100X**.**

FIG 4. **Transmission (TEM) and scanning (SEM) electron microscopy showing interactions between *S. suis* serotype 2 and NTPr cells**. (A-I) TEM micrograph of *S. suis* serotype 2 strain 31533 infection of virus free (control) NPTr cells showing very few cocci at the cell surface. (A-II and A-III) TEM micrographs of *S. suis* strain 31533 infection of swH1N1 pre-infected NPTr cells showing high numbers of cocci interacting with epithelial cells (A-II) and also intracellular bacteria (A-III). Scale bar, 1 µm. Original magnification, 5000x. (B) SEM micrograph of *S. suis* serotype 2 strain 31533 infection of swH1N1 pre-infected NPTr cells showing high numbers of cocci intimately interacting with cell cilia. Scale bar, 1 µm. Original magnification, 10 000x. No bacteria could be found in all observed SEM fields of control NPTr cells infected with *S. suis* strain 31533 only (data not shown). Black arrows show bacterial cells and arrow heads show cilia. CM: cell membrane.

FIG 5. ***S. suis* native, but not desialylated, capsular polysaccharide (CPS), inhibits *S. suis* adhesion to (A) and invasion of (B) of NPTr cells pre-infected with swH1N1**. NPTr cells were infected with swH1N1 (MOI: 1) for 12 h and then incubated with the native CPS (100 µg/well) desialylated CPS (100 µg/well), or a polyclonal antibody serum against SIV H1N1 (1/40 dilution, positive control) for 1 h at 37°C. *S. suis* strain 31533 (MOI: 10) was then added to pre-treated NPTr cells. Two hours post-infection, adhesion and invasion of *S. suis* was assessed as described in Materials and Methods. Results are expressed in percentage when compared to bacterial adhesion and invasion of untreated swH1N1 pre-infected cells (considered as 100%). Data are

expressed as mean ± SEM of at least three independent experiments. Groups that are significantly different from each other are indicated by different letters (*a* and *b*), as determined by One-way ANOVA with $P \leq 0.01$.

FIG 6. **Pre-incubation of *S. suis* and swH1N1 significantly increases bacterial adhesion to and invasion of NPTr cells**. swH1N1 and *S. suis* serotype 2 strain 31533 (1:1 ratio; TCID50: CFU) were pre-incubated for 1 h at 4° C. This mixture ("V+B") was was then added to NPTr cells for an incubation time of 1 h or 2 h for adhesion/invasion assays, respectively, as described in Materials and Methods. Mock-treated bacteria were used as control ("B"). Data are expressed as mean ± SEM of at least three independent experiments. * indicates significant differences ($P <$ 0.01).

FIG 7. **Gene expression of pro-inflammatory mediators by NPTr cells. NPTr cells were either pre-infected with swH1N1 for 12 h (MOI: 1) or not, and subsequently infected with *S. suis* serotype 2 strain 31533 (MOI: 10) for 24 h**. Total RNA was extracted from *S. suis* and virus co-infected cells ("V+B"), virus single-infected cells ("V") or bacteria single-infected cells ("B") and quantitative PCR analysis of selected genes was performed. Normalization of the data was done using the reference genes *Hprt1* and *Ppia*. Mock non-infected samples were used as calibrator and consequently, relative fold-differences were calculated for the rest of the samples compared to the mean of the calibrator samples. Data represent mean values ± SEM of relative fold expression. Groups that are significantly different from each other are indicated by different letters (*a, b,* and *c*), as determined by One-way ANOVA with $P \leq 0.01$.

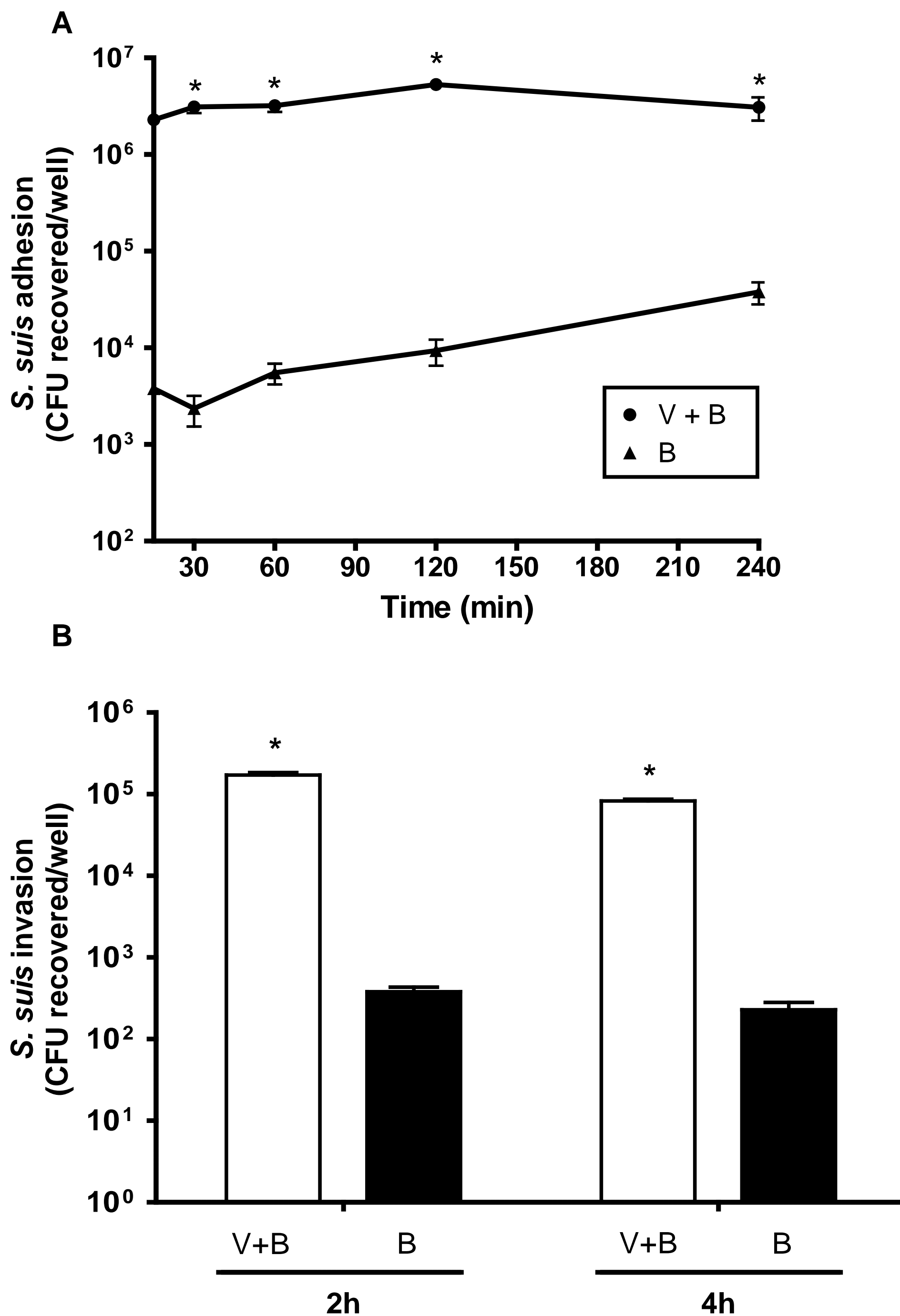


FIG 1 Adhesion to and invasion of virus-free or swH1N1-infected NPTr cells by *S. suis* serotype 2 strain 31533. NPTr cells were either pre-infected with swH1N1 for 12 h (MOI: 1) or not, and subsequently infected with *S. suis* serotype 2 strain 31533 (MOI: 10). (A) Kinetics of adhesion of *S. suis* to virus-infected (“V+B”) or control (“B”) NPTr cells. After *S. suis* infection cells were extensively washed to remove non-adherent bacteria and then lysed to determine *S. suis* viable counts. (B) *S. suis* invasion of swH1N1-infected (“V+B”) or control (“B”) NPTr cells at 2 and 4 h bacterial incubation times. Results were determined as described above except that after washing, cells were exposed to antibiotics to kill extracellular bacteria. Data are expressed as mean ± SEM of at least four independent experiments, each done in triplicate. * indicates significant difference between samples infected with bacteria alone and those co-infected with virus and bacteria ($P < 0.01$).

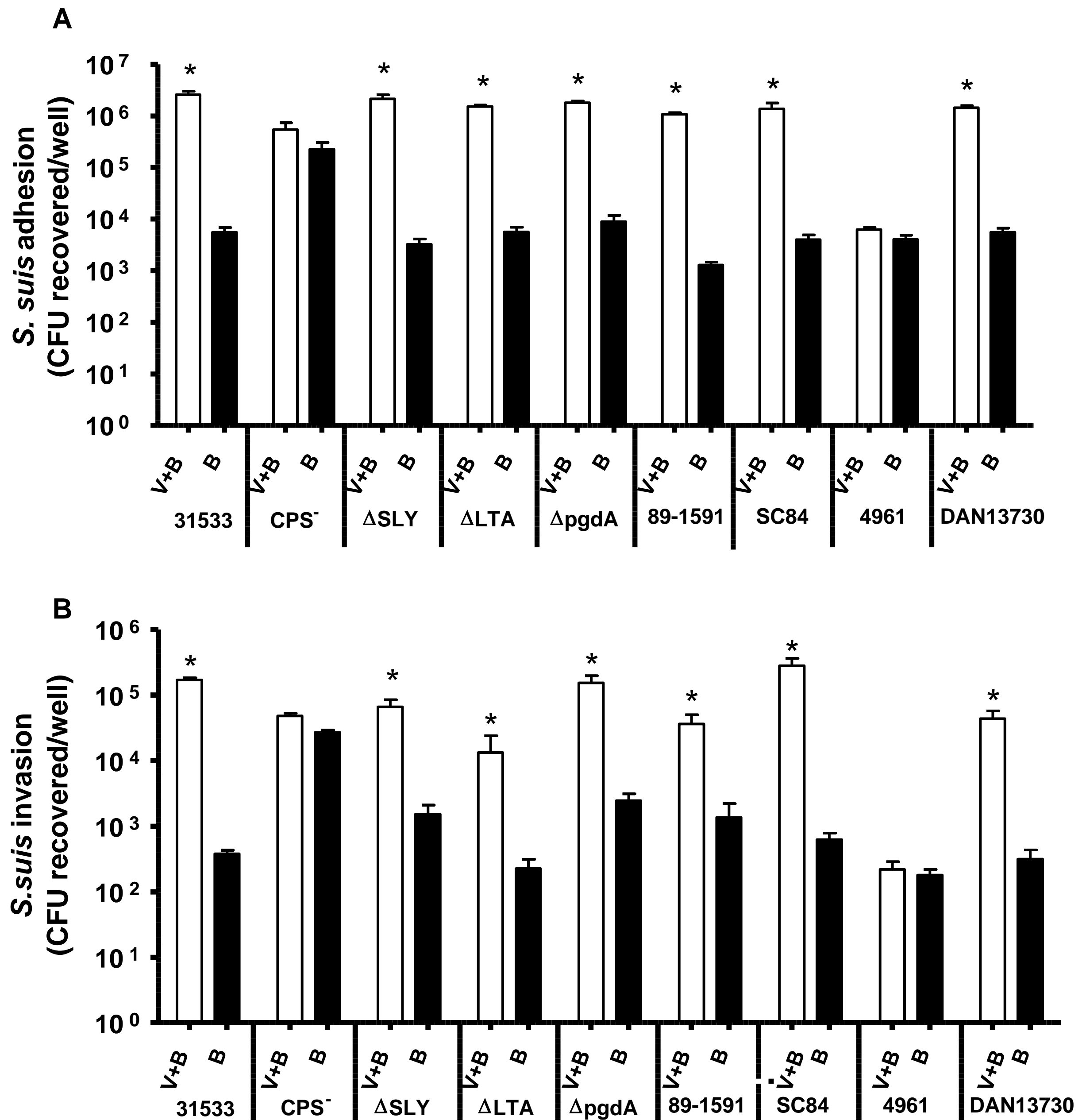


FIG 2 Adhesion to and invasion of virus free or swH1N1-infected NPTr cells by different strains of *S. suis*. NPTr cells were either pre-infected with swH1N1 for 12 h (MOI: 1) or not, and subsequently infected with *S. suis* strains (MOI: 10). (A) Adhesion (incubation time of 2 h) of different *S. suis* strains to NPTr cells. Results were determined after exposure of swH1N1-infected ("V+B") or control ("B") NPTr cells to *S. suis*, followed by extensive washing of non-adherent bacteria and cell lysis to obtain *S. suis* viable counts. (B) Invasion (incubation time of 1h) of swH1N1-infected ("V+B") or control ("B") NPTr cells by different *S. suis* strains. Results were determined as described above except that after washing, cells were exposed to antibiotics to kill extracellular bacteria. See Table 1 for strain description. Data are expressed as mean ± SEM of at least four independent experiments, each done in triplicate. * indicates significant difference between samples infected with bacteria alone and those co-infected with virus and bacteria ($P < 0.01$).

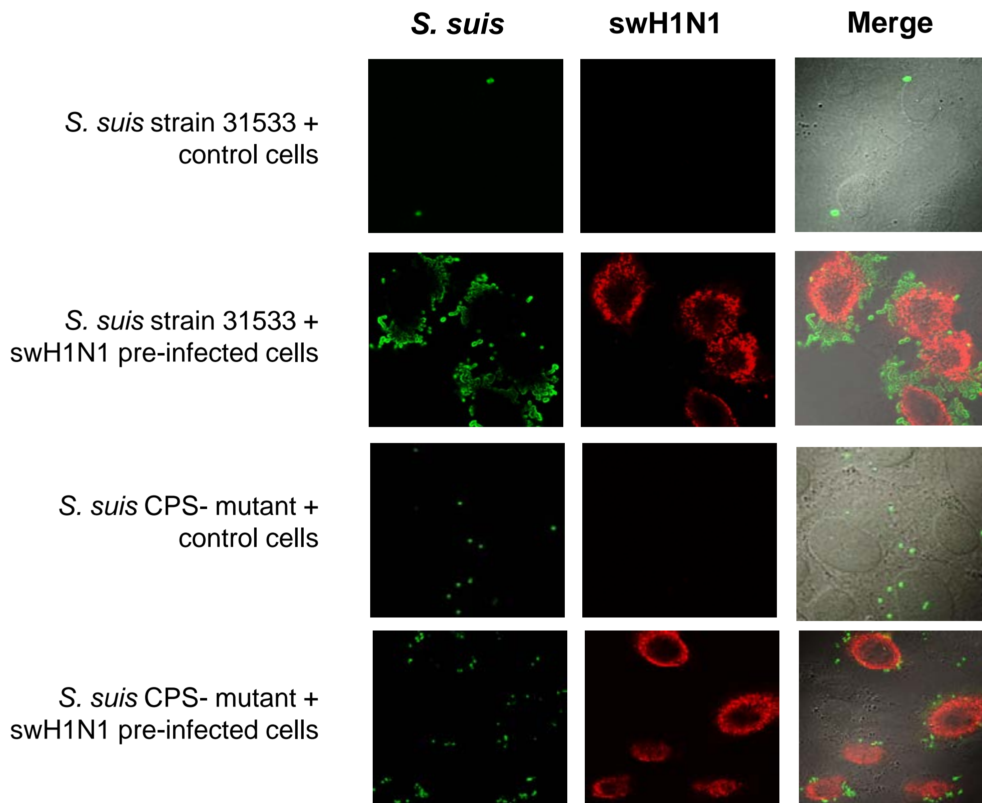
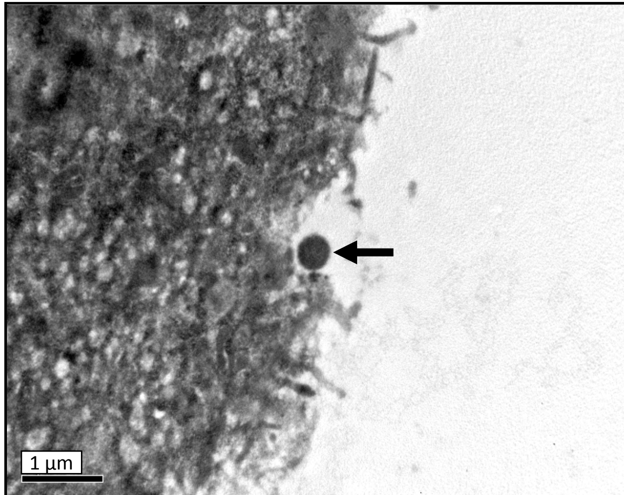
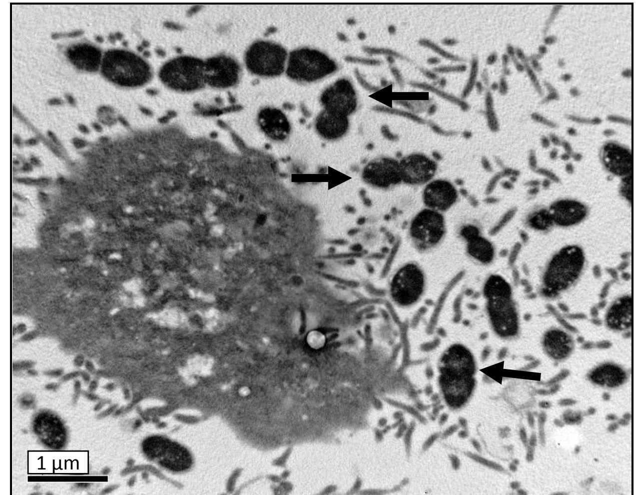


FIG 3 Increased interactions of *S. suis* serotype 2 with NPTr cells pre-infected with swH1N1 virus: Role of the capsular polysaccharide. Confocal microscopy showing association of wild-type encapsulated *S. suis* serotype 2 strain 31533 or its non-encapsulated mutant (CPS-) with virus free cells (control) or swH1N1 pre-infected NPTr cells. Cells were non-infected (control) or virus-infected for 12 h with swH1N1 (MOI: 1) and then infected with either *S. suis* wild-type strain or the CPS- mutant strain (MOI: 10) for 2 h. Samples were labeled using antibodies conjugated with Alexa Fluor 488 against *S. suis* (green) and antibodies conjugated with Alexa Fluor 568 against swH1N1 (red). Original magnification 100X.

A-I



A-II



B

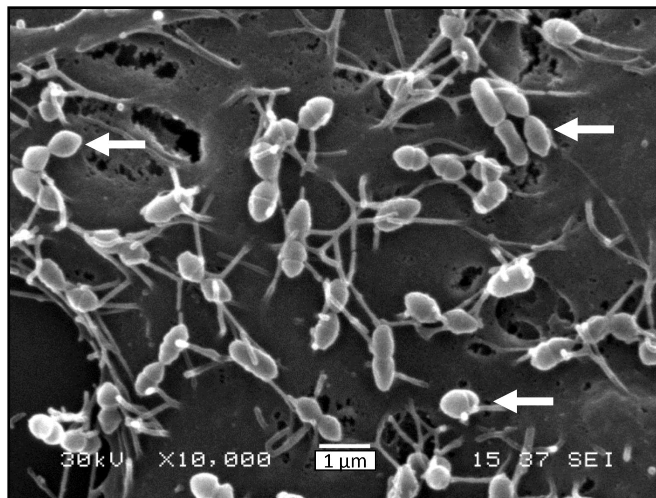


FIG 4 Transmission (TEM) and scanning (SEM) electron microscopy showing interactions between *S. suis* serotype 2 and NTPr cells. NPTr cells were either pre-infected with swH1N1 for 12 h (MOI: 1) or not, and subsequently infected with *S. suis* serotype 2 strain 31533 (MOI: 10). (A-I) TEM micrograph of *S. suis* serotype 2 strain 31533 infection (incubation time of 1h) of virus free (control) NPTr cells showing very few cocci at the cell surface. Scale bar, 1 µm. Original magnification, 5000x. (A-II) TEM micrograph of *S. suis* strain 31533 infection of swH1N1 pre-infected NPTr cells showing high numbers of cocci interacting with epithelial cells. Scale bar, 1 µm. Original magnification, 5000x. (B) SEM micrograph of *S. suis* serotype 2 strain 31533 infection (incubation time of 1h) of swH1N1 pre-infected NPTr cells showing high numbers of cocci intimately interacting with cell cilia. Scale bar, 1 µm. Original magnification, 10 000x. No bacteria could be found in all observed SEM fields of control NPTr cells infected with *S. suis* strain 31533 only (data not shown). Arrows show bacterial cells.

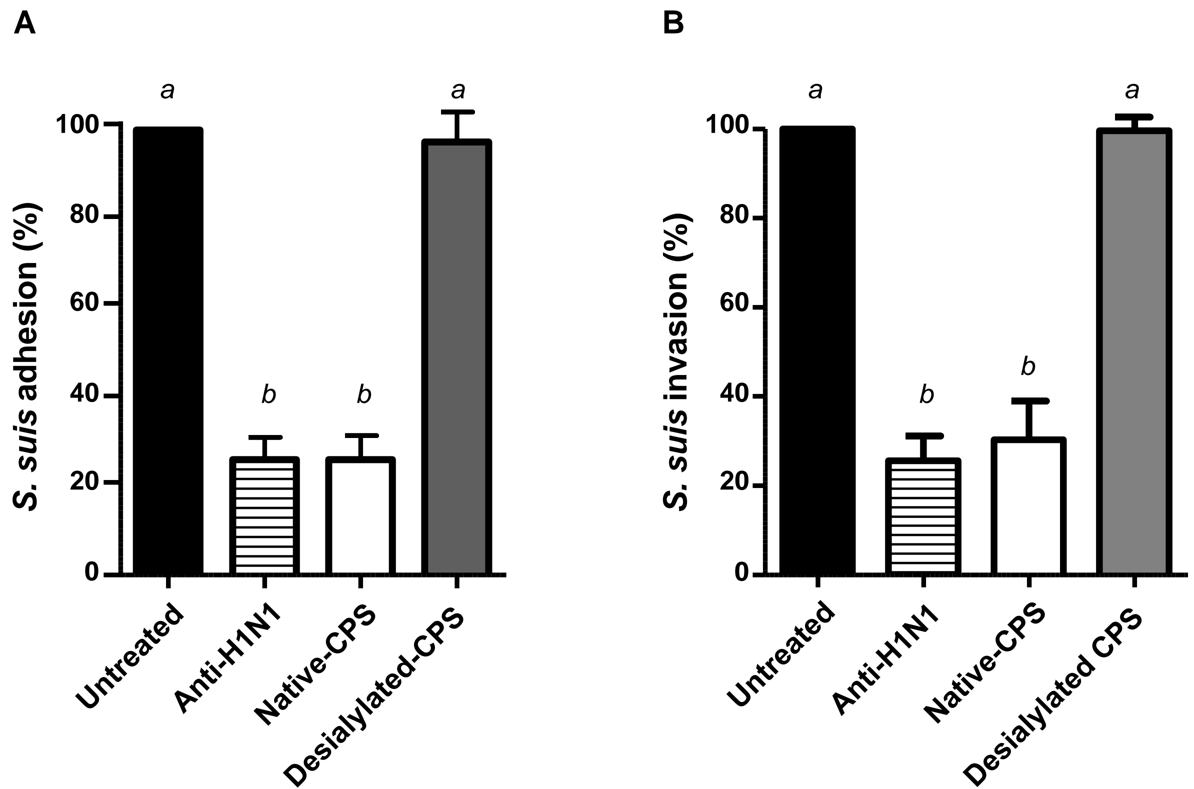


FIG 5 *S. suis* native, but not desialylated, capsular polysaccharide (CPS), inhibits *S. suis* adhesion to (A) and invasion of (B) of NPTr cells pre-infected with swH1N1. NPTr cells were infected with swH1N1 (MOI: 1) for 12 h and then incubated with the native CPS (100 µg/well) desialylated CPS (100 µg/well), or a polyclonal antibody serum against SIV H1N1 (1/40 dilution, positive control) for 1 h at 37° C. *S. suis* strain 31533 (MOI: 10) was then added to pre-treated NPTr cells. Two hours post-infection, adhesion and invasion of *S. suis* was assessed as described in Materials and Methods. Results are expressed in percentage when compared to bacterial adhesion and invasion of untreated swH1N1 pre-infected cells (considered as 100%). Data are expressed as mean ± SEM of at least three independent experiments. Groups that are significantly different are indicated by letters (*a* and *b*), as determined by One-way ANOVA with $P \leq 0.01$.

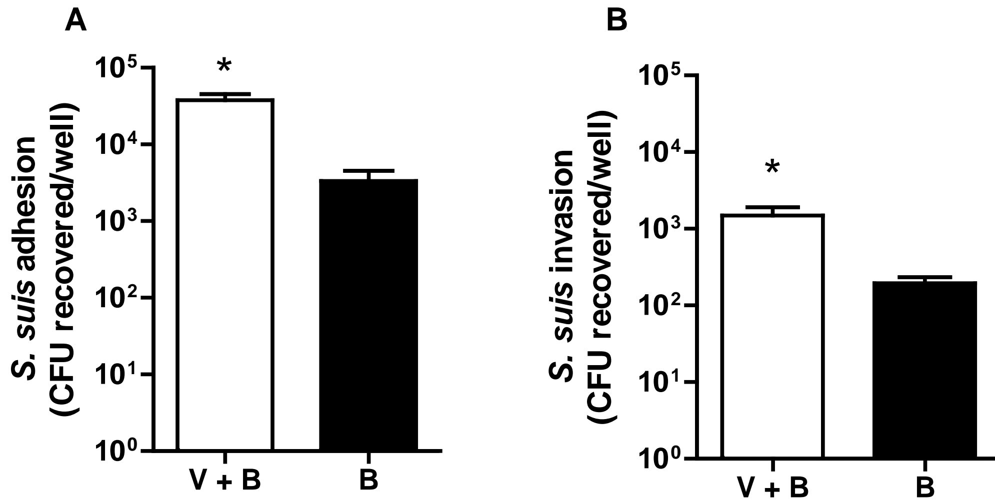


FIG 6 Pre-incubation of *S. suis* and swH1N1 significantly increases bacterial adhesion to and invasion of NPTr cells. swH1N1 and *S. suis* serotype 2 strain 31533 (1:1 ratio; TCID50: CFU) were pre-incubated for 1 h at 4° C. This mixture ("V + B") was then added to NPTr cells for an incubation time of 1 h or 2 h for adhesion/invasion assays, respectively, as described in Materials and Methods. Mock-treated bacteria were used as control ("B"). Data are expressed as mean ± SEM of at least three independent experiments. * indicates significant differences ($P < 0.01$).

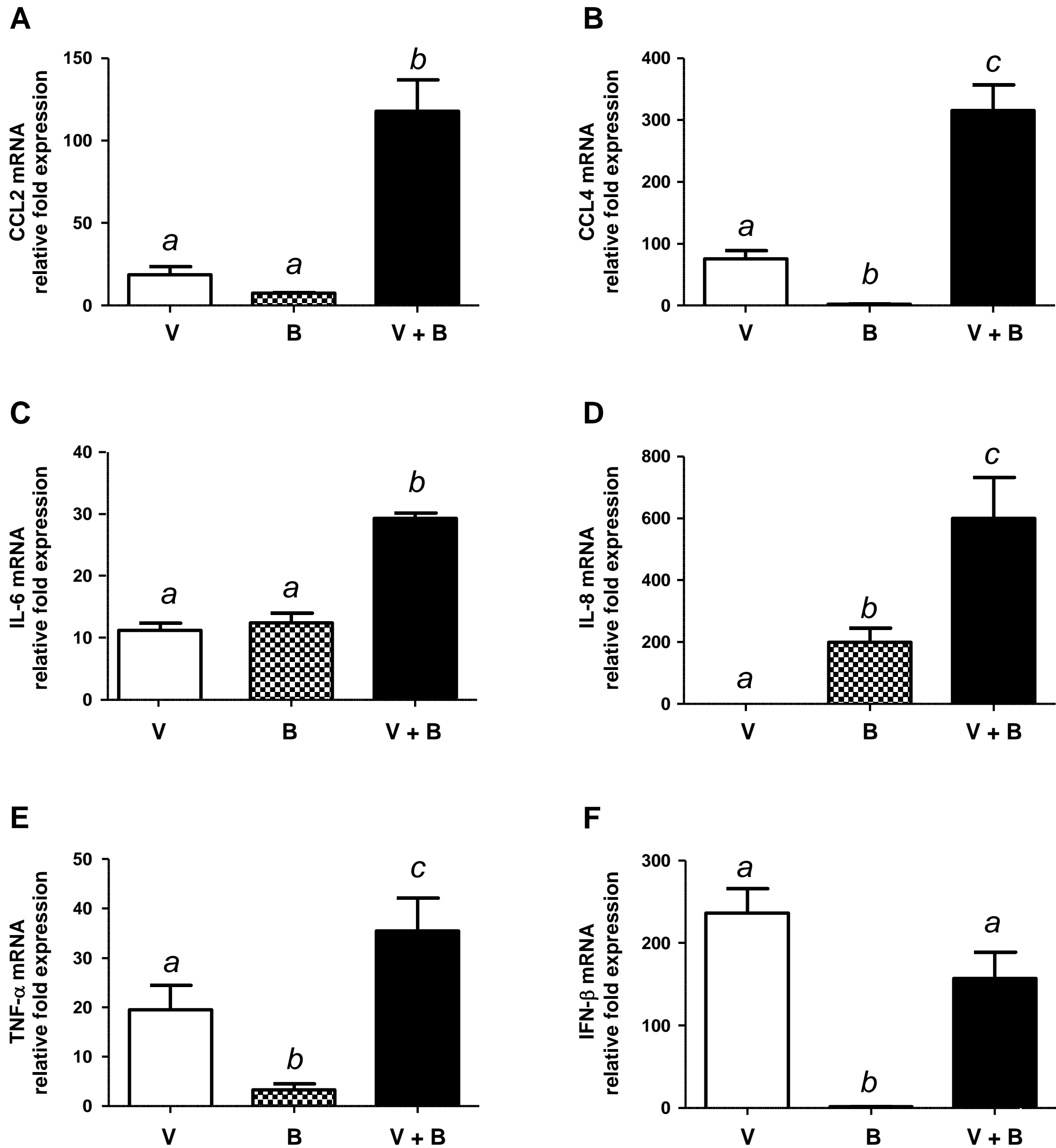


FIG 7 Gene expression of pro-inflammatory mediators by NPTr cells. NPTr cells were either pre-infected with swH1N1 for 12 h (MOI: 1) or not, and subsequently infected with *S. suis* serotype 2 strain 31533 (MOI: 10) for 24 h. Total RNA was extracted from *S. suis* and virus co-infected cells ("V+B"), virus single-infected cells ("V") or bacteria single-infected cells ("B"), and quantitative PCR analysis of selected genes was performed. Normalization of the data was done using the reference genes *Hprt1* and *Ppia*. Mock non-infected samples were used as calibrator and consequently, relative fold-differences were calculated for the rest of the samples compared to the mean of the calibrator samples. Data represent mean values ± SEM of relative fold expression. Groups that are significantly different are indicated by letters (*a, b,* and *c*), as determined by One-way ANOVA with $P \leq 0.01$.