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

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

ABSTRACT

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

INTRODUCTION

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

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

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

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

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

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

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

MATERIALS AND METHODS

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

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

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

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

swH1N1 (2 x $10^{6.25}$ $TCID_{50}$/ml) was then added to each well and incubated for 1 h at room temperature. Different wells represented a 2-fold dilutions of *S. suis*/swH1N1virus ratios, beginning at a ratio of 200 for the wild type encapsulated strain and 10 000 for the non-encapsulated B218 mutant. Afterward, 50 µl of a 0.5% suspension of whole rooster red blood cells (RBC) in PBS were added to each well and gently mixed. The HI was evaluated after incubating the plate at room temperature for 1 h. For this experiment, PBS was used as negative RBC control and serial dilutions of reference heat-inactivated anti-swH1N1 serum was used as a positive HI control. Under the conditions tested, capsulated and non-encapsulated *S. suis* strains did not induce any hemagglutination (results not shown).

***S. suis* CPS purification and CPS desialylation.** The CPS of *S. suis* serotype 2 reference strain S735 was prepared and purified as previously described (8). For quality controls, CPS was analyzed by nuclear magnetic resonance. Lack of protein and RNA/DNA contamination was verified by Lowry method and by spectrophotometry, respectively. CPS was also desialylated by mild acid hydrolysis. CPS (8 mg) was heated in 1 ml of HCl (70 mM) at 60 °C for 4 h, neutralized with NH_4OH (2 M), and purified on a Sephadex G10 column (1.5 x 10 cm). Presence (native CPS) or absence (desialylated CPS) of sialic acid was verified by gas chromatography after methanolysis and acetylation and by nuclear magnetic resonance as well as by a reaction with an enzyme linked-lectin assay as previously described (26).

Confocal and electron microscopy. For confocal microscopy analysis, cells were placed on coverslips and infected (or not) with swH1N1 and either *S. suis* strain 31533 or its non-encapsulated mutant strain (B218) as described above and further incubated for 2 h at 37°C in 5% CO_2. Coverslips were washed with PBS to remove non-associated bacteria and cells were fixed

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

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

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

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

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

RESULTS

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

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

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

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

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

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

Influence of epithelial cell swH1N1 pre-infection on adhesion/invasion abilities of *S. suis* serotype 2 was confirmed by microscopy. First, confocal microscopy revealed that very few encapsulated wild-type bacteria could be observed interacting with epithelial cells in the absence 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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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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 $P \leq 0.01$.

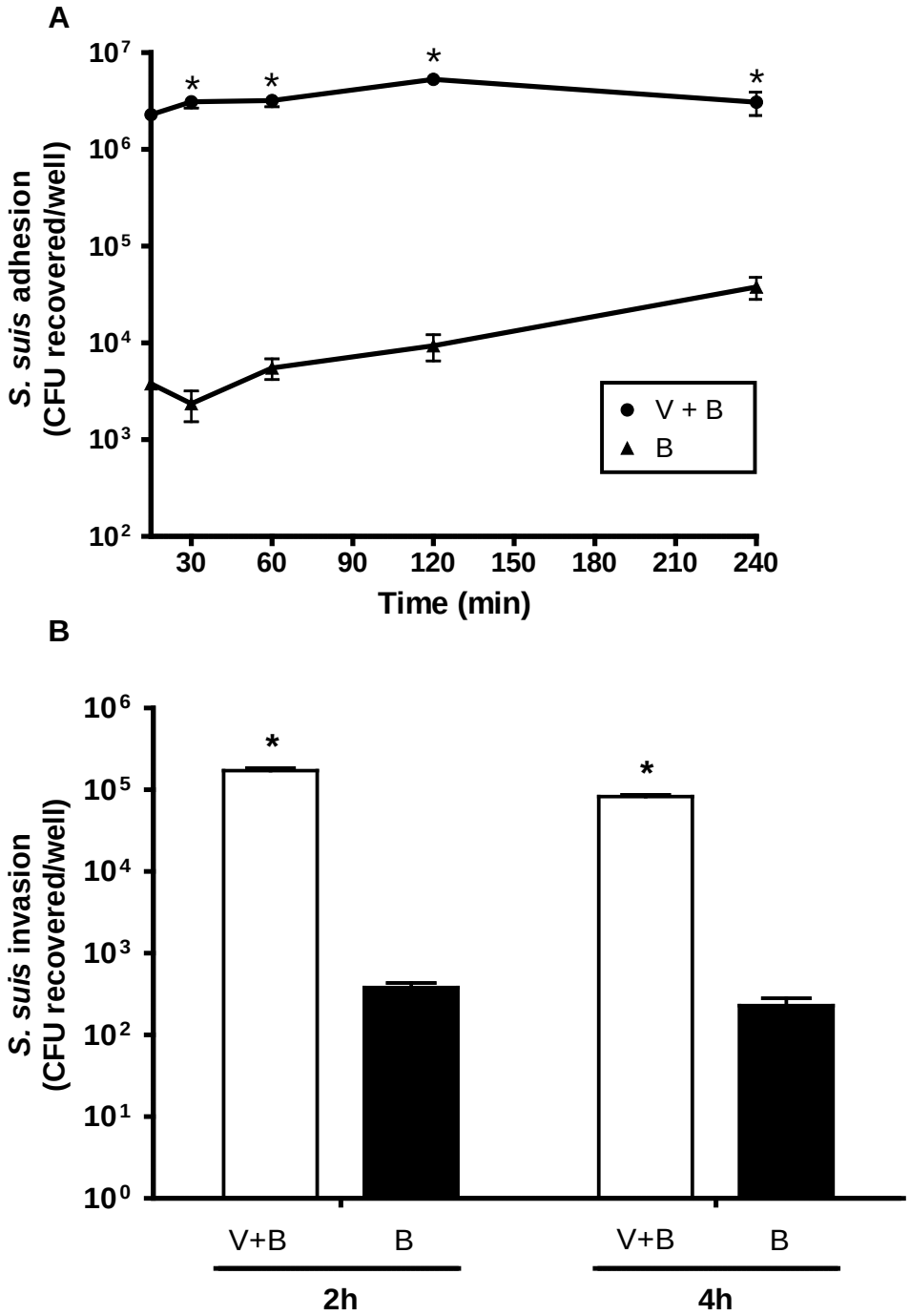

FIG 1 Adhesion to and invasion of virus-free or swH1N1-infected NPTr cells by *S. suis* serotype 2 strain 31533. NPTr cells were either pre-infected with swH1N1 for 12 h (MOI: 1) or not, and subsequently infected with *S. suis* serotype 2 strain 31533 (MOI: 10). (A) Kinetics of adhesion of *S. suis* 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 ($P < 0.01$).

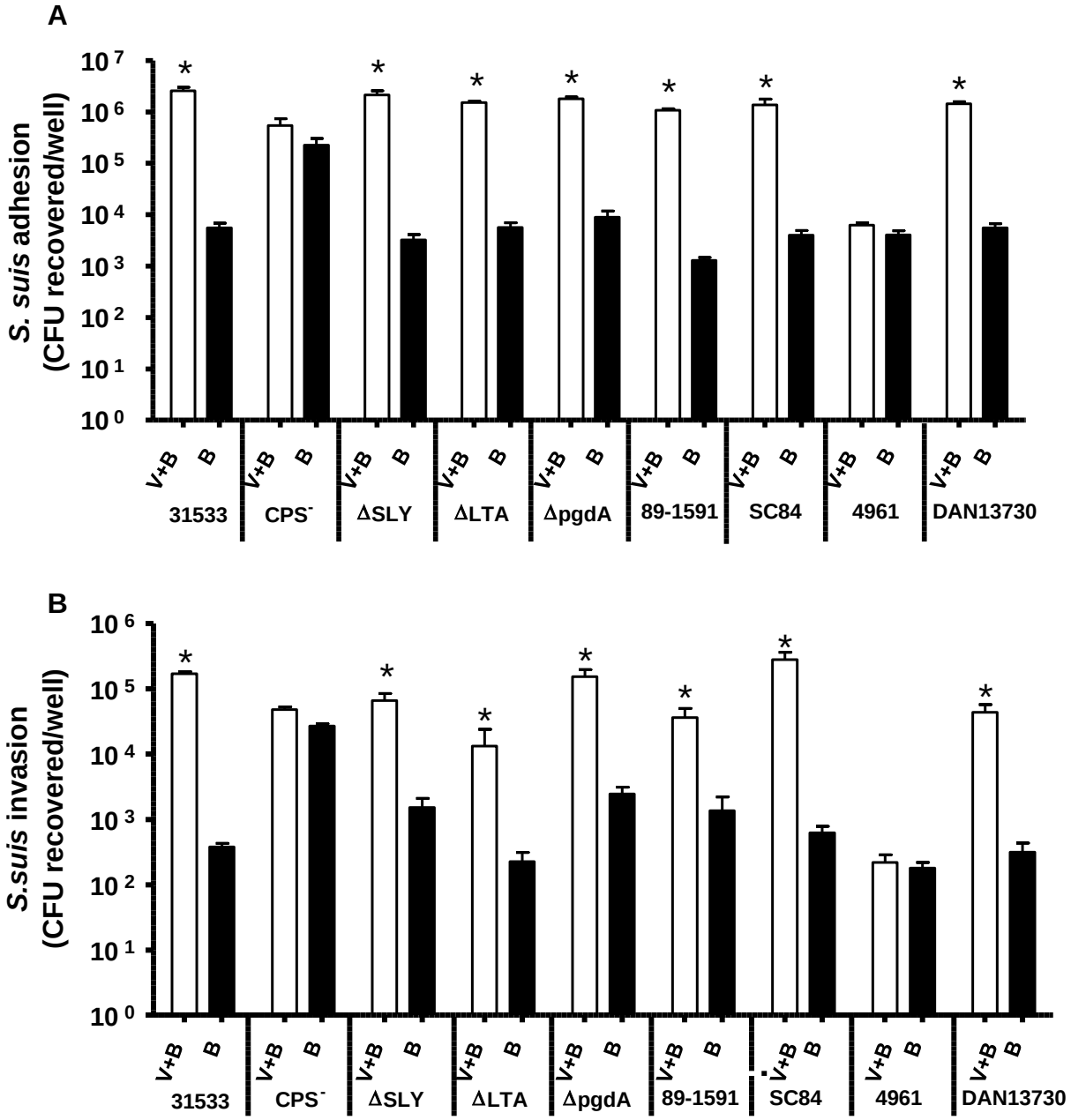

FIG 2 Adhesion to and invasion of virus free or swH1N1-infected NPTr cells by different strains of *S. suis*. NPTr cells were either pre-infected with swH1N1 for 12 h (MOI: 1) or not, and subsequently infected with *S. suis* strains (MOI: 10). (A) Adhesion (incubation time of 2 h) of different *S. suis* 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 ($P < 0.01$).

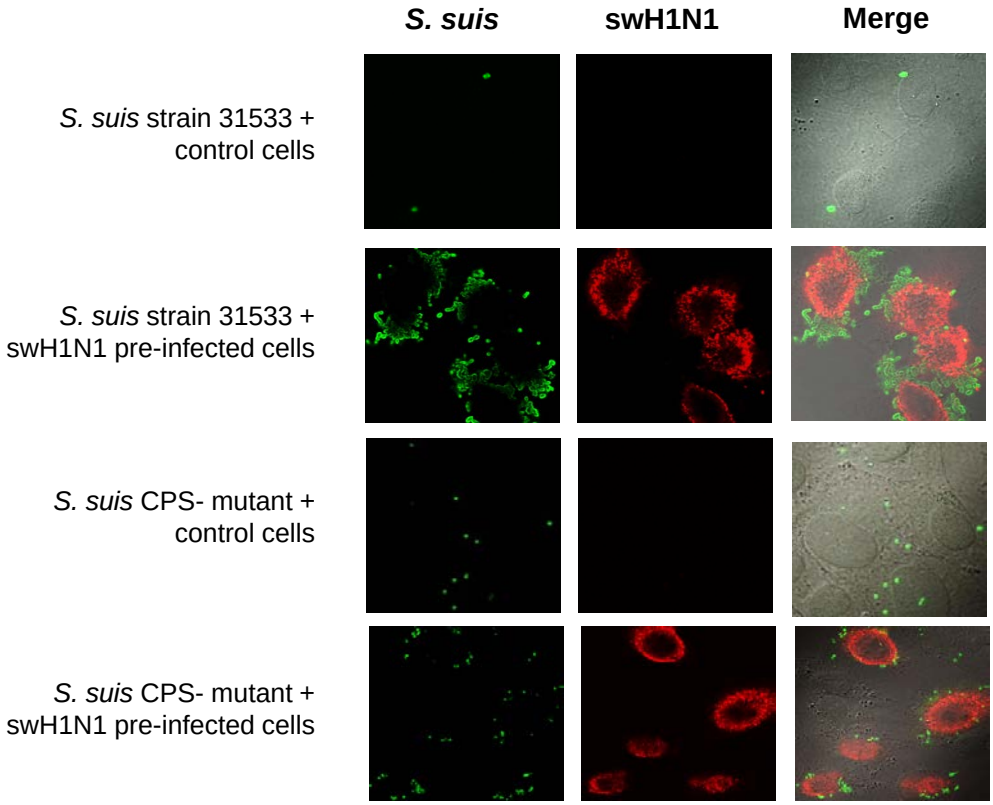

FIG 3 Increased interactions of *S. suis* serotype 2 with NPTr cells pre-infected with swH1N1 virus: Role of the capsular polysaccharide. Confocal microscopy showing association of wild-type encapsulated *S. suis* serotype 2 strain 31533 or its non-encapsulated mutant (CPS-) with virus free cells (control) 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**.**

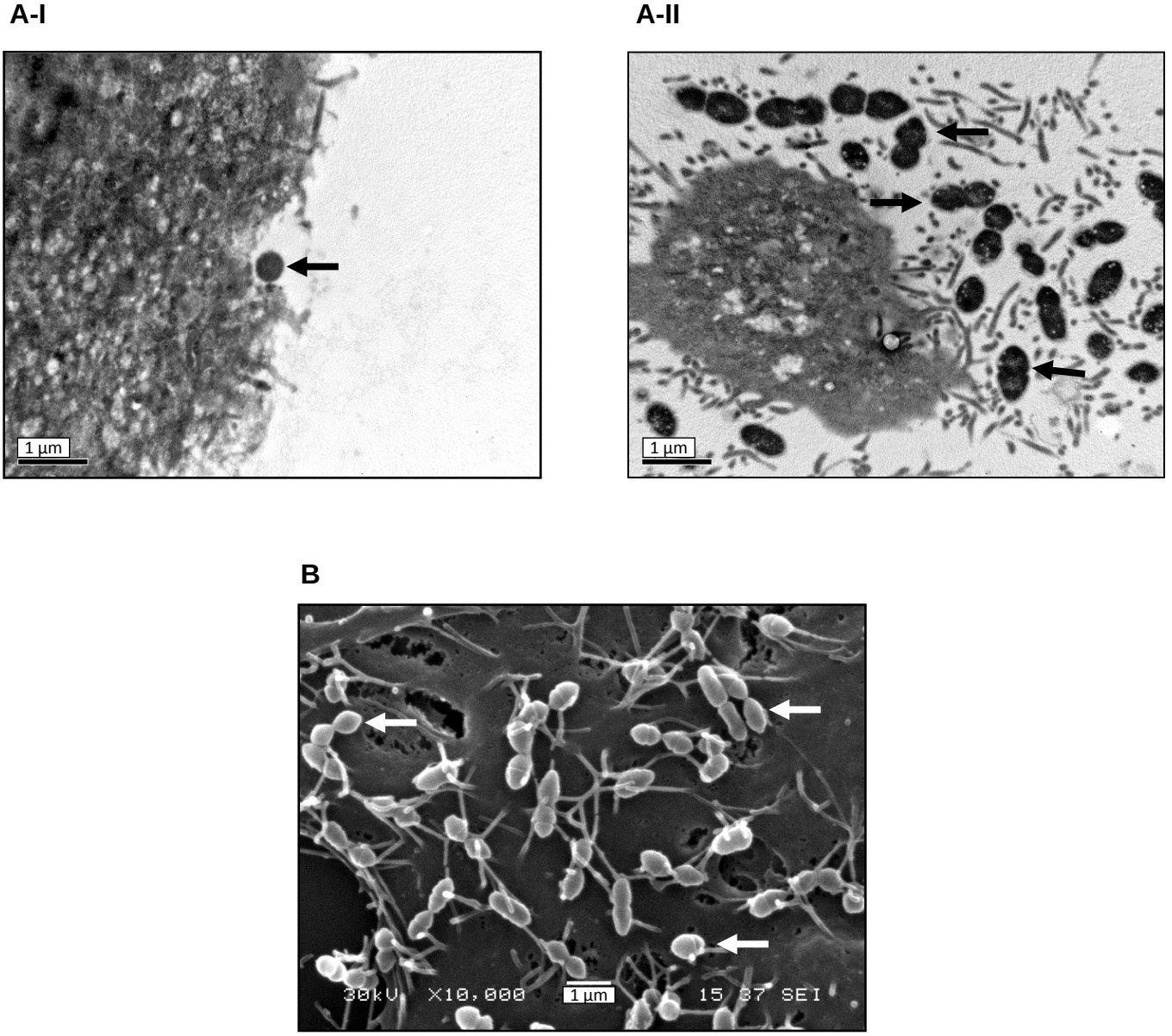

FIG 4 Transmission (TEM) and scanning (SEM) electron microscopy showing interactions between *S. suis* serotype 2 and NTPr cells. NPTr cells were either pre-infected with swH1N1 for 12 h (MOI: 1) or not, and subsequently infected with *S. suis* serotype 2 strain 31533 (MOI: 10). (A-I) TEM micrograph 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.

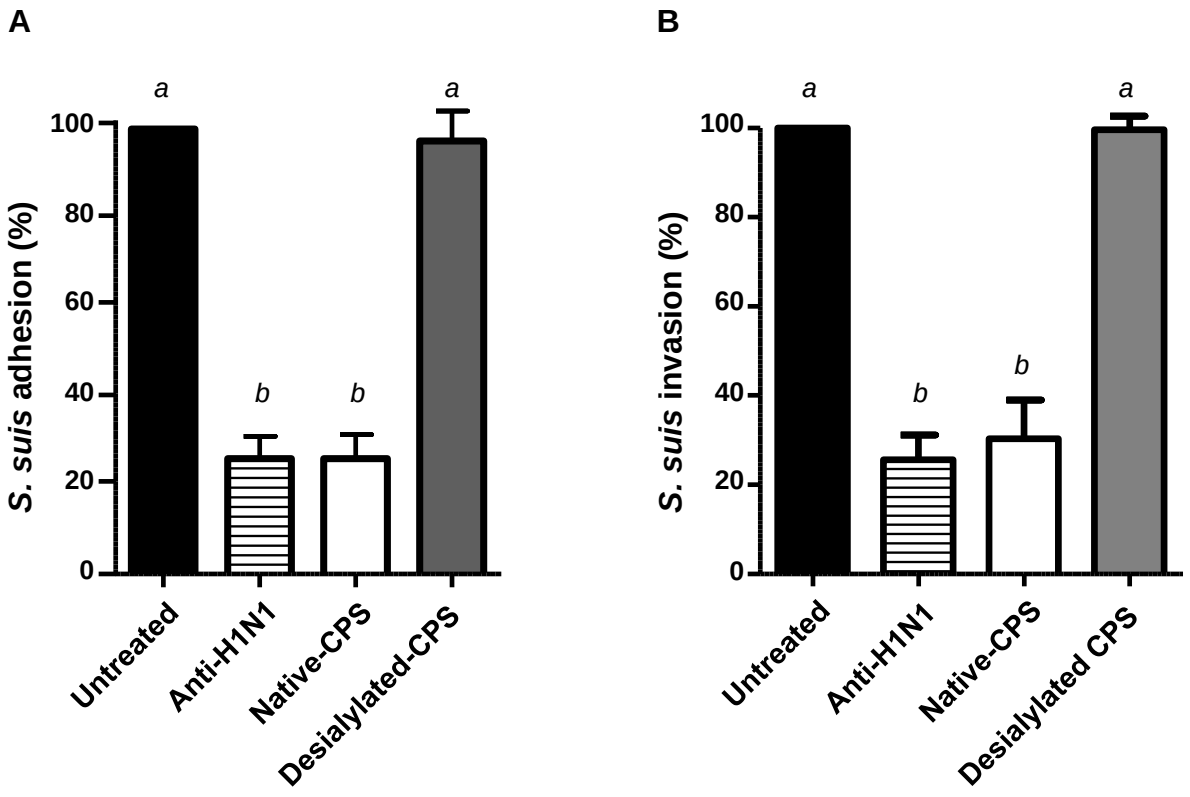

FIG 5 *S. suis* native, but not desialylated, capsular polysaccharide (CPS), inhibits *S. suis* adhesion to (A) and invasion of (B) of NPTr cells pre-infected with swH1N1. NPTr cells were infected with swH1N1 (MOI: 1) for 12 h and then incubated with the native CPS (100 μg/well) desialylated CPS (100 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 $P \leq 0.01$.

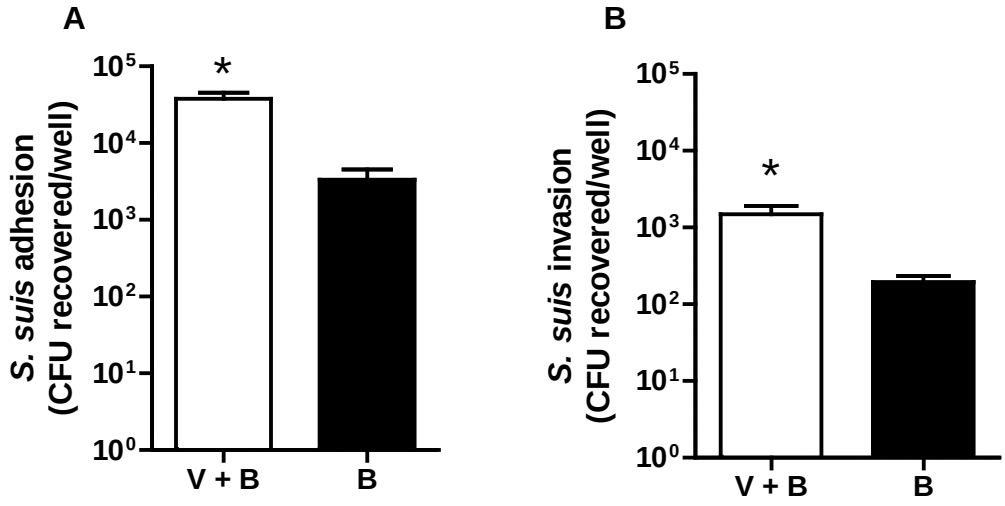

FIG 6 Pre-incubation of *S. suis* and swH1N1 significantly increases bacterial adhesion to and invasion of NPTr cells. swH1N1 and *S. suis* serotype 2 strain 31533 (1:1 ratio; TCID50: CFU) were pre-incubated for 1 h at 4° C. This mixture ("V + B") was then added to NPTr cells for an incubation time of 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 ($P < 0.01$).

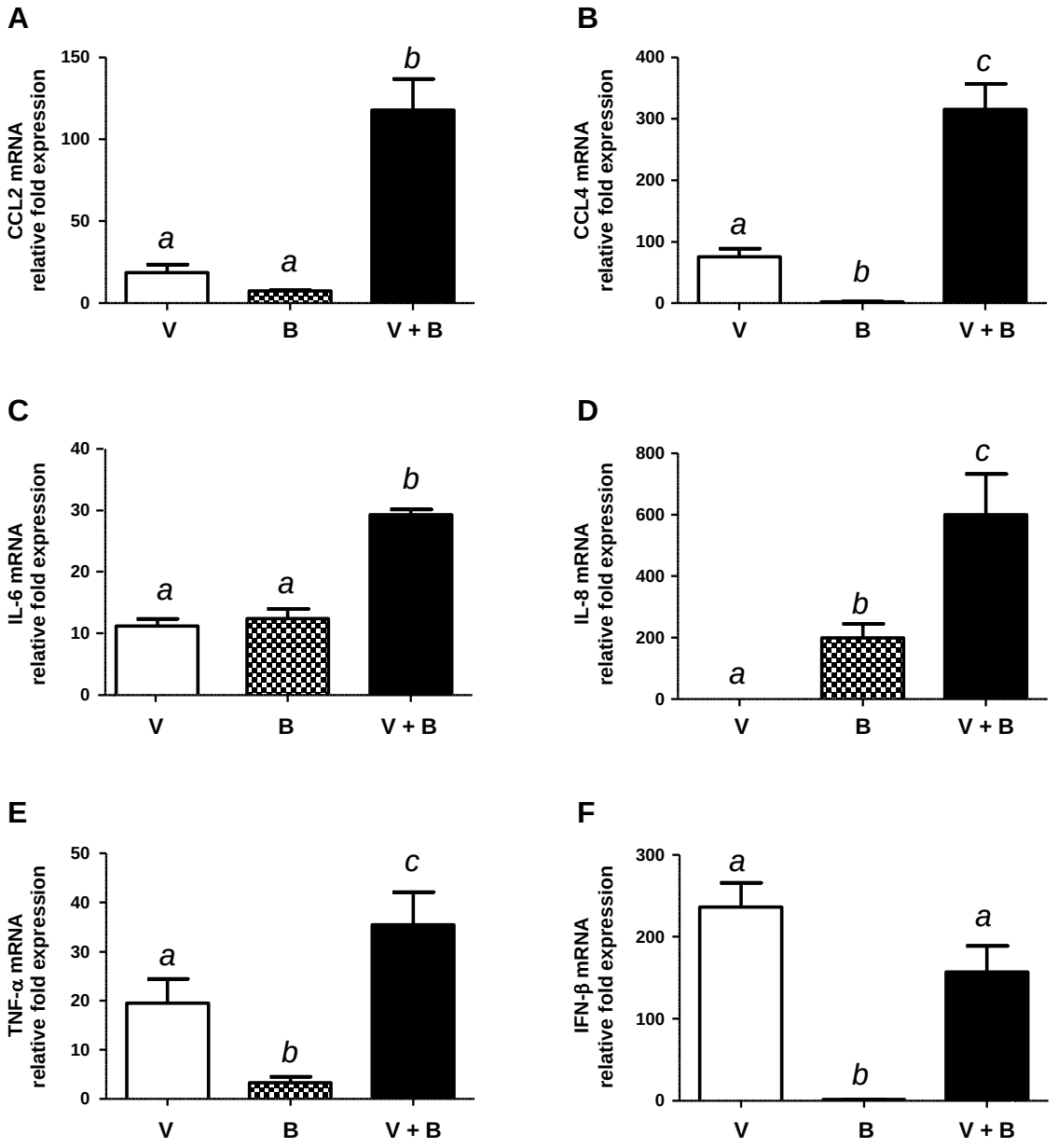

FIG 7 Gene expression of pro-inflammatory mediators by NPTr cells. NPTr cells were either pre-infected with swH1N1 for 12 h (MOI: 1) or not, and subsequently infected with *S. suis* serotype 2 strain 31533 (MOI: 10) for 24 h. Total RNA was extracted from *S. suis* and virus co-infected cells ("V+B"), 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 $\pm$ 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 $P \leq 0.01$.