Sialic acid-dependent interaction of group B streptococci with influenza virus-infected cells reveals a novel adherence and invasion mechanism

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Abstract

Group B streptococci (GBS) contain a capsular polysaccharide with side chains terminating in \( \alpha_{2,3} \)-linked sialic acids. Because of this linkage type, the sialic acids of GBS are recognised by lectins of immune cells. This interaction results in a dampening of the host immune response and thus promotes immune evasion. As several influenza A viruses (IAV) use \( \alpha_{2,3} \)-linked sialic acid as a receptor determinant for binding to host cells, we analysed whether GBS and influenza viruses can interact with each other and how this interaction affects viral replication and bacterial adherence to and invasion of host cells. A co-sedimentation assay revealed that viruses with a preference for \( \alpha_{2,3} \)-linked sialic acids bind to GBS in a sialic acid-dependent manner. There is, however, a large variation in the efficiency of binding among avian influenza viruses of different subtypes as shown by a hemagglutination-inhibition assay. A delay in the growth curve of IAV indicated that GBS has an inhibitory effect on virus replication. On the other hand, both the adherence and invasion efficiency of GBS were enhanced when the cells were pre-infected by IAV with appropriate receptor specificity. Our results suggest that GBS infection may result in a more severe disease when patients are co-infected by influenza viruses. This co-infection mechanism may have relevance also to other human diseases, as there are more bacterial pathogens with \( \alpha_{2,3} \)-linked sialic acids and human viruses binding to this linkage type.

KEYWORDS
capsular polysaccharide, group B streptococci, influenza virus, invasion, sialic acid, viral–bacterial co-infection

1 INTRODUCTION

Group B streptococci (GBS; *Streptococcus agalactiae*) have been recognised as frequent causes of invasive diseases in newborns and a subset of adults (Edwards & Baker, 2005). Since 1940, invasive infections caused by GBS were also reported in non-pregnant adults especially in diabetes patients and elderly people (Edwards, Rench, Palazzi, & Baker, 2005; Eickhoff, Klein, Daly, Ingall, & Finland, 1964; Farley, 2001). Less commonly appreciated is that the burden of GBS infections among non-pregnant adults is considerable and that the number of cases is still increasing (Skoff et al., 2009). A common presentation of GBS disease in adults is pneumonia (Le Doare & Heath, 2013). GBS is able to invade respiratory epithelial cells (Rubens, Smith, Hulse, Chi, & van Belle, 1992; Valentin-Weigand, Jungnitz, Zock, Rohde, & Chhatwal, 1997), and several virulence factors may contribute to the adherence to and invasion of cells (Doran, Chang, Benoit, Eckmann, & Nizet, 2002a; Doran, Liu, & Nizet, 2003; Maisey, Hensler, Nizet, & Doran, 2007; Tazi et al., 2010). A major virulence factor of GBS is the capsular polysaccharide with side chains terminating in \( \alpha_{2,3} \)-linked sialic acid (Wessels, Rubens, Benedi, & Kasper, 1989). These sugar residues are considered to be important for evasion from the hosts’ immune response (Landwehr-Kenzel & Henneke, 2014). They can suppress complement activation, and, by

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molecular mimicry, they are able to interact with inhibitory sialic acid-specific lectins (Siglecs) on leukocytes. In this way, they impair the innate immune response, which may promote the survival of the pathogen (Chang et al., 2014).

Similar capsular polysaccharides are present on several serotypes of a porcine streptococcal pathogen, *Streptococcus suis* (Charland, Kobisch, MartineauDoize, Jacques, & Gottschalk, 1996; Van Calsteren, Gagnon, Lacouture, Fittipaldi, & Gottschalk, 2010). A structural difference between the two streptococci is that *S. suis* contains α2,6-linked sialic acids, which are recognised by the hemagglutinins of human and swine influenza viruses. Because of this interaction, adherence to and invasion of airway epithelial cells is enhanced, when *S. suis* encounters influenza virus-infected cells (Meng et al., 2015; Wang et al., 2013; Wu, Meng, Seitz, Valentin-Weigand, & Herrler, 2015). As several influenza viruses recognise α2,3-linked sialic acids, we were interested whether there is an interaction between influenza viruses and streptococci, which is dependent on α2,3-linked sialic acid, and how this affects bacteria adherence and invasion.

In this study, we show that influenza viruses, which recognise α2,3-linked sialic acid, efficiently interact with GBS. The binding of the viral hemagglutinins to the capsular polysaccharide of GBS had a negative effect on the virus as it delayed virus replication. On the other hand, both the adherence and invasion efficiencies of GBS were enhanced when the cells were pre-infected by influenza A viruses (IAV) with appropriate receptor specificity. Our results have relevance for the disease potential of IAV that are transmitted from avian to mammalian hosts.

### RESULTS

#### 2.1 Sialic acid-dependent interaction between influenza viruses and GBS

We were interested to know whether α2,3-linked sialic acid in the capsular polysaccharide can mediate binding of IAV to GBS. To confirm the presence of this type of sialic acid in the GBS strain used for this study, we performed lectin staining applying *Maackia amurensis* agglutinin II (MAA II), which recognises α2,3-linked sialic acid, and SNA, which has a preference for the α2,6 glycan-linkage type. As shown in Figure 1a, GBS (green signals) interacted with MAA II (red signals) but not with SNA (Figure 1b). By contrast, *S. suis* was recognised by SNA but not by MAA II (Figure 1c,d). Enzymatic desialylation of streptococci abolished the binding of both lectins to the bacteria (Figure 1e, f). This result confirms that the NEM316 strain of GBS used in our study expresses α2,3-linked sialic acid in the capsule as described for GBS serotype III (Gonzalez-Outeirino, Kadirvelraj, & Woods, 2005).

To determine whether the sialic acid residues on the capsular polysaccharide of GBS are recognised by influenza viruses, we performed a co-sedimentation assay with the recombinant influenza viruses R1 (recognising α2,6-linked sialic acid) and R2 (recognising α2,3-linked sialic acid). After co-incubation for 1 hr at 4 °C, HA titration revealed that almost all R1 viruses had disappeared from the supernatant of the sample incubated with *S. suis*, suggesting that most virions had bound to the streptococci (Figure 2a). In the samples incubated with GBS, only R2 but not R1 showed a significant reduction of the HA titre, though the reduction was not as pronounced as the case of...
the R1 with $S.\ suis$ sample. Both the co-sedimentation of R1 with $S.\ suis$ and of R2 with GBS were mediated by a sialic acid-dependent interaction because both viruses remained in the supernatant when the respective streptococci were pretreated with neuraminidase (NA; Figure 2a). The NA pretreatment has been confirmed not to affect the bacteria viability (data not showed), which is consistent with previous reports (Butler, Baker, & Edwards, 1987; Pan, Schmitt, Sanford, & Elbein, 1979). A similar result was obtained when the supernatants were analysed by an infectivity assay (Figure 2b). From these data, we conclude R2 binds to GBS, which is consistent with the sialic acid binding activity of the virus and with the linkage type that connects sialic acid to the capsular polysaccharide of GBS. To confirm that virus had actually bound to bacteria in the co-sedimentation assay, the sediments were resuspended and subjected to a plaque assay on Madin-Darby canine kidney (MDCK) cells. As shown in Figure 2c, a significant amount of R1 but not of R2 was found in the $S.\ suis$ sediment. By contrast, a marked amount of R2 but not of R1 was detected in the GBS pellet. The plaques induced by the two viruses were somewhat smaller in the co-sedimented samples compared to the control samples (Figure 2d), probably because of the binding to bacteria. These results indicate that an influenza virus that has a binding preference for $\alpha 2,3$-linked sialic acids can attach to encapsulated GBS.

2.2 | GBS inhibit the hemagglutinating activity of avian influenza viruses from different subtypes

The co-sedimentation assays have revealed that an influenza virus with avian-like receptor specificity is able to interact with GBS. To find out whether this type of interaction is also occurring in avian influenza viruses from different subtypes, a comparative hemagglutination-inhibition (HI) assay was carried out using the following avian viruses: A/Mallard/Germany/1488/09(H10N7), A/chicken/Emirates/R66/2002(H9N2), A/Wild duck/Germany/R30/06(H1N1), A/Mallard/Germany/R2379/09(H3N8), and A/Mallard/Germany/R726/08(H4N6). As shown in Figure 3, among the tested viruses, the H10 virus was affected most by GBS. HI titres determined for the H1, H3, and H9 viruses were somewhat lower and similar to that of the R2 virus. The lowest HI-activity of GBS was detected in the case of the H4 virus. The hemagglutinating activity of two swine viruses was affected by $S.\ suis$ but not by GBS. The results shown in Figure 3 confirm that avian influenza viruses are able to interact with

![FIGURE 2](image-url)  
**FIGURE 2** HA activity and infectivity of R1 or R2 viruses co-sedimented with group B streptococci (GBS) or *Streptococcus suis*. After incubation of virus with bacteria, the bacteria were sedimented by low-speed centrifugation. The supernatants were analysed for HA activity (a) by HA assay or for infectivity and (b) by plaque titration on Madin–Darby canine kidney (MDCK) cells. Neuraminidase treatment was performed to demonstrate the sialic acid dependence. The pellet (c) fractions were analysed for the presence of infectious viruses by plaque titration on MDCK cells. Neuraminidase treatment was performed to demonstrate the sialic acid dependence. In (d), the smaller size of the virus plaques in the co-sedimented samples (R2/GBS and R1/$S.\ suis$) is shown as compared to the plaque size of control virus. Statistical relevance was determined with unpaired Student’s t-test, ***$p < .001$.

![FIGURE 3](image-url)  
**FIGURE 3** Inhibition of the hemagglutinating activity of influenza viruses by group B streptococci (GBS) and *Streptococcus suis*. GBS and S. suis were analysed for their ability to inhibit the hemagglutinating activity of influenza viruses from different host species and different subtypes. IAV = influenza A viruses.
GBS, but there appear to be differences in the binding affinities among the different viruses.

### 2.3 Co-infection by GBS delays the replication of IAV

Next, we determined how the viral–bacterial interaction affects the infection by these microorganisms. In human laryngeal carcinoma epithelial (HEp-2) cells co-infected with GBS, R2 only reached a value of $8.9 \times 10^5$ TCID₅₀/ml at 24 hr post infection (hpi), whereas the titre determined for the R2 mono-infected sample or for cells co-infected with $S.\ suis$ was about 40-fold higher (Figure 4a). At 48 hpi, the values for the two co-infected samples did not differ significantly. Infection by R1 was delayed by $S.\ suis$ but not by GBS co-infection (Figure 4b). Comparable results were obtained when the experiments were performed with MDCK cells (Figure 4c,d). Therefore, we conclude that replication of R2 virus is delayed by co-infection with GBS.

### 2.4 Effects of IAV infection on adherence of streptococci

To find out how viral infection affects the subsequent infection by streptococci, we first investigated the adherence of GBS to virus-infected HEp-2 cells by immunofluorescence microscopy. For virus infection, a low multiplicity of infection (MOI) was applied, because a preferential binding to virus-infected cells is more evident when the number of virus-infected cells is low (Wu et al., 2015). At 16 hr post-virus infection, cells were incubated with streptococci for 1 hr at low temperature (4 °C or room temperature). Under these conditions, the viral NA will not interfere with the binding assay by releasing sialic acid from the streptococcal capsular polysaccharide. As shown in Figure 5a, many adherent GBS (stained in red) were found, and most of them were associated with R2 virus-infected cells (stained in green; Figure 5a), whereas hardly any GBS was detected on R1-infected HEp-2 cells. A similar association of GBS with R2-infected cells was observed when MDCK cells were analysed (Figure 5a). In contrast to GBS, $S.\ suis$ was predominantly detected adherent on R1 but not on R2-infected cells (Figure 5a). Adherence of GBS or $S.\ suis$ to cells is usually analysed at 37 °C. Under the low temperature conditions of experiments shown in Figure 5, streptococcal binding to mock-infected cells appears to be less efficient than binding to virus-infected cells; therefore, few streptococci are detected on cells not infected by influenza viruses. This result indicates that infection of cells by R2 enhances the adherence of GBS to the cell surface. This interaction was mediated by a sialic acid-dependent interaction because pretreatment of the streptococci with NA abolished the adherence of GBS (Figure 5b). From these results, we conclude that the hemagglutinins of R2 virus displayed on the surface of virus-infected cells interact with the sialic acid residues of the GBS capsular polysaccharide and thus facilitate the binding of streptococci to the cells.

### 2.5 Infection by R2 enhances the invasion of cells by GBS

To evaluate whether the increased adherence of GBS is paralleled by an increased invasion of epithelial cells, virus- and mock-infected cells...
were incubated with streptococci for 6 h at 37 °C. To detect intracellular bacteria, we applied confocal immunofluorescence microscopy and analysed GBS for co-localisation with lysosome-associated membrane protein-1 (LAMP-1). As shown in Figure 6a, A, in R2-infected cells (green), numerous intracellular GBS (stained in red) were associated with cellular structures containing LAMP-1 (stained in blue). By contrast, in cells mono-infected by GBS, only few streptococci were detected in cellular compartments containing LAMP-1 protein (Figure 6a, B). Furthermore, the total number of cell-associated GBS was increased significantly; there was a twofold enhancement of adherent bacteria compared to GBS mono-infection cells (Figure 6a, C). In the case of S. suis, which was less efficient than GBS in adhering to HEp-2 cells, prior R1 infection resulted in a sevenfold increase of cell-associated streptococci. Inactivation of extracellular bacteria by antibiotic treatment and subsequent serial plating of cell lysates were applied to determine the number of intracellular streptococci. The ratio of intracellular bacteria to total cell-associated bacteria was determined as an indicator of the relative invasion. The result shown in Figure 6a, D indicates that GBS was substantially more invasive than S. suis. For both streptococci, prior infection with the appropriate influenza virus resulted in an enhanced invasion; the relative invasion by GBS was increased more than twofold by R2 infection and that of S. suis fourfold by R1 infection. The lower invasion efficiency of S. suis may be explained by the lower adherence efficiency of this pathogen (6a–C).

GBS are known to contain a pore-forming toxin (β-hemolysin/cytolysin), which can promote streptococcal uptake into different eukaryotic cells (Doran, Chang, Benoit, Eckmann, & Nizet, 2002b). To address the question whether pore-formation may contribute to the uptake of GBS in a co-infection scenario, we measured the release of the intracellular enzyme lactate dehydrogenase (LDH), which is a widely used method for measuring cytolytic activities of such toxins. As shown in Figure 6b, a nearly twofold increase of released LDH was detected in the supernatant of GBS-infected cells. The increase was independent of a prior influenza virus infection. Thus, the increased invasion efficiency of GBS in influenza virus-infected cells cannot be explained by the pore-forming activity of GBS.

2.6 Analysis by electron microscopy

To get further evidence for the interactions of GBS with influenza virus-infected cells, an analysis by electron microscopy was performed. After pre-infection with R2 for 2 h, HEp-2 cells were inoculated with GBS for another 6 h at 37 °C. Field emission scanning electron microscopy revealed that HEp-2 cells mono-infected with GBS only rarely exhibited a single attached bacterium on the host cell membrane. Very rarely an invading bacterium was detected, which enters the host cell via an invagination in the membrane (Figure 7a, A). In contrast, HEp-2 cells pre-infected with R2 showed adherence of a larger number of streptococci on the surface of most cells (Figure 7b,c, arrows). Consistent with the confocal images (Figure 5a), some single HEp-2 cells were densely covered with adherent GBS (Figure 7c,d). At higher magnifications, two morphologically different invasion processes could be distinguished: (a) GBS invading through a large invagination into the HEp-2 cell (Figure 7e) and (b) GBS being taken up via interaction with microvilli (Figure 7f). Interestingly, in the latter case, chains of GBS were taken up first in the middle of the chain. Furthermore, membrane ruffling on the
site of attachment was not detectable. Transmission electron microscopy of ultrathin sections of infected HEp-2 cells showed that internalised streptococci were more readily detected in co-infected than in mono-infected cells. GBS were taken up into mono-infected HEp-2 cells (Figure 7g), and, rarely, intracellular GBS were detectable in the ultrathin sections residing in a membrane-bound compartment. In R2 pre-infected host cells, GBS were also detected in membrane-bound compartments with a large white hollow around the bacteria (Figure 7h) most likely representing a phagolysosome-like vacuole as suggested from the LAMP1-positive fluorescence staining of those vacuoles (see Figure 6a). In addition, GBS were detected, which seemed to “escape” out of these large membrane-bound compartments (Figure 7i). At this time-point of infection, no free residing GBS in the cytoplasm of HEp-2 cells were found.

FIGURE 5 Continued.

3 | DISCUSSION

Secondary bacterial pneumonia is a major cause of influenza-related deaths (McCullers, 2006; Morens, Taubenberger, & Fauci, 2008; Robertson, Caley, & Moore, 1958). Several streptococci are known to cause more severe disease in patients infected by influenza viruses (Loo & Gale, 2007; Luk, Gross, & Thompson, 2001). The mechanism of synergism between virus and bacteria may vary among different streptococci. In the case of Streptococcus pneumoniae, the action of the viral NA releases sialic acid from the cell surface and thus not only uncovers binding sites for bacterial adherence but also provides a source of nutrients for the bacterial metabolism (Li et al., 2015; Siegel, Roche, & Weiser, 2014). By contrast, group A streptococci directly interact with influenza viruses or virus-infected cells in a process that is capsule-dependent (Okamoto et al., 2003; Okamoto et al., 2004). As group A streptococci is not sialylated, the capsular ligand is different from sialic acid. The porcine pathogen S. suis, on the other hand, contains a capsular polysaccharide with terminal α2,6-linked sialic acids. The hemagglutinin of human and porcine influenza viruses recognises the capsular sialic acid and thus enhances the adherence and invasion of S. suis (Meng et al., 2015). Here, we provide evidence that GBS resembles S. suis in the sialic acid-dependent interaction with influenza viruses, differing only by the sialic acid linkage type and by the virus species.

A unique feature of the capsular polysaccharide of GBS and S. suis is the presence of a rhamnose residue (Van Calsteren et al., 2010).
Such a sugar is not present on the oligosaccharides of mammalian cells and is also absent in the glycan arrays used to determine the binding specificity of influenza viruses. Therefore, it was interesting to know to what extent the glycan structure of the capsular polysaccharide of GBS affects the recognition of the terminal sialic acids by influenza viruses. Our results revealed that the $\alpha_{2,3}$-linked sialic acids of GBS are recognised by avian but not by human and porcine influenza viruses. However, the results of the HI assay indicated that there are large differences between viruses from different subtypes. Among five different viruses analysed, an avian virus of the H10 subtype was affected most by GBS in its hemagglutinating activity. On the other hand, GBS was hardly able to prevent an H4 virus from agglutinating chicken erythrocytes. Although we have shown that several avian influenza viruses can interact with GBS, it should be noted that there...
are also some human viruses that recognise α2,3-linked sialic acid, for example, parainfluenzoviruses such as human parainfluenza virus 3 and mumps virus (Kubota et al., 2016). In fact, the HA activity of influenza A virus and mumps virus has been shown previously to be inhibited by GBS, and the binding of influenza virus to singular GBS was observed more efficiently in scanning electron microscope.
pictures (Hosaka et al., 2000; Hosaka, Kuroda, Ikeura, Iwamoto, & Suzuki, 1998). On the bacterial side, there are also several human pathogens that are known to contain α2,3-linked sialic acid as part of their capsular polysaccharides or lipooligosaccharides, for example, members of the genus Haemophilus, and, thus, they may well interact with viruses that recognise α2,3-linked sialic acid. Therefore, the relevance of our finding reaches beyond GBS and influenza viruses.

The interaction between influenza viruses and GBS resulted in a delayed replication of influenza virus. The reduced virus titres in the initial phase of the infection may be due to a direct binding of viral hemagglutinins to capsular sialic acid residues. This interaction may occur on the surface of the infected cell. Streptococci adhering to hemagglutinin proteins may interfere with the budding process, which might result in a prolonged replication phase. Binding of influenza viruses to GBS is also possible in the cell supernatant when the virions have been released from the infected cells. On the basis of our results of hemagglutination and HI assays, we know that the viral NA can cleave sialic acid from the capsular polysaccharide and thus get released from the bacteria. Such an enzymatic release of viruses provides a possible explanation for the delayed replication.

With respect to GBS, co-infection with appropriate influenza viruses supported bacterial adherence to and invasion of virus-infected cells. Several components of GBS have been described to mediate the adherence to host factors (for a review, see Landwehr-Kenzel & Henneke, 2014). Most important for binding to and colonisation of epithelial cells appear to be the adhesins BsAB and HvgA, the α2C proteins, the serine-repeat proteins as well as the pili. Because in our study, we did not use the respective mutants, the question to what extent these proteins contributed to the binding of GBS to HEP-2 and MDCK cells remains to be addressed in future studies. Nevertheless, the high efficiency of the sialic acid-mediated streptococcal binding to virus-infected cells is explained by the fact that the bacterial capsule is the outermost bacterial component and contains many sialic acid residues, that is, many receptor determinants for viral hemagglutinins are exposed on the surface of GBS. Furthermore, in cells infected by influenza viruses, a large number of hemagglutinins are transported to the cell surface. Therefore, GBS and virus-infected cells may establish many contact sites between hemagglutinins proteins and sialic acid residues, and this multivalent interaction enables a strong binding.

GBS is a commensal bacterium that asymptomatically colonises mucosal surfaces not only of humans but also of vertebrate and non-vertebrate species. Under certain conditions that are not well characterised, it may turn into a life-threatening pathogen. To cause disease, GBS has to proceed from colonisation to invasion. To spread from the site of colonisation to the site of sepsis, GBS has to overcome several epithelial or endothelial barriers. There are reports that GBS may get across epithelial cell layers via the paracellular route or via transcytosis (Doran et al., 2005). In the case of transcytosis, vesicles containing the streptococci are transported from the apical to the basolateral plasma membrane where they release their content on the serosal side of the cells. Endocytic uptake occurred also in our cell systems as indicated by co-staining of bacterial antigen and the lysosomal marker protein LAMP-1. It will be interesting in future studies to apply our approach to air-liquid interface cultures of differentiated airway epithelial cells to further determine whether GBS crosses the barrier of respiratory epithelial cells by the paracellular route or via transcytosis or by both mechanisms.

The biological importance of capsular sialic acids of GBS has up to now mainly been attributed to the interaction with immune cells contributing to the bacterial strategy of immune evasion. Thus, binding of GBS to immunoglobulin-like lectins recognising α2,3-linked sialic acids (Siglecs) on leukocytes can prevent complement activation and counteract the bactericidal activity of the host cells. The novel finding of our report is that the capsular sialic acids of GBS may also be an adherence and invasion factor when the bacteria get in contact with influenza virus-infected cells. As mentioned above, the adherence is explained as binding of the viral hemagglutinin to the capsular sialic acid of GBS. The enhancing effect on the invasion suggests that the strength of the streptococcal interaction with the target cells determines the efficiency of invasion. Thus, co-infection with avian influenza virus may be a factor responsible for some of the cases of severe GBS-induced disease. The reason why virus-induced enhancement of disease has not been reported for GBS may be related to the fact that infections of humans by avian influenza viruses are not a frequent phenomenon. However, other respiratory viruses such as human parainfluenza viruses may have the same capacity, and it should be noted that for this co-infection effect, it is not necessary that the patients suffer from a virus-induced disease. Our study demonstrated the principle concept that contact of GBS with influenza virus-infected cells may enhance the invasiveness of the bacterial pathogen and thus result in a more severe disease.

### 4 | EXPERIMENTAL PROCEDURES

#### 4.1 | Cells, influenza viruses, and bacteria

HEp-2 cells were obtained from the American Type Culture Collection (ATCC® CCL-23™). MDCK cells were obtained from European Molecular Biology Laboratory (Kai Simons), Heidelberg, Germany. Both cell types were maintained as described previously (Benga, Goethe, Rohde, & Valentin-Weigand, 2004; Meng, Wu, Seitz, Herrler, & Valentin-Weigand, 2016; Wu et al., 2016).

The two human recombinant viruses, R1 and R2, were kindly provided by Mikhail Matrosovich (Phillips-University Marburg). These two viruses have the hemagglutinin and the NA from the pandemic human virus A/Hong Kong/1/68 (HK/68) and the other six segments from A/WSN/33. R1 contains the original hemagglutinin of the HK/68 virus; R2 differs from R1 only by two mutations at amino acids 226 (L/Q) and 228 (S/G) (Matrosovich, Matrosovich, Uhlendorff, Garten, & Klenk, 2007). SIV of H1N1 subtype, A/sw/Bad Griesbach/IDT5604/2006 (sw/Bad Griesbach/06), and of H3N2 subtype, A/sw/Herford/IDT5932/2007 (sw/Herford/07), have been described previously (Meng et al., 2013). Avian influenza viruses: A/Wild duck/Germany/R30/06 (H1N1), A/Mallard/Germany/R2379/09(H3N8), A/Mallard/Germany/R726/08(H4N6), A/chicken/Emirates/R66/2002(H9N2), and A/Mallard/Germany/1488/09 (H10N7) were kindly provided by Timm Harder (German National Reference Laboratory for avian influenza, Friedrich-Loeffler-Institute). Viruses were propagated in MDCK cells (SIV, R1, and R2) or 10-day-old specific pathogen-free
embryonated chicken eggs (avian influenza viruses) as described previously (Meng et al., 2013). The virulent \textit{S. suis} serotype 2 strain 10 was kindly provided by Hilde Smith (Smith et al., 1999). Group B streptococcus serotype III strain NEM316 was a clinical isolate from an infected neonate and was kindly provided by Marcus Fulde (Institute for Microbiology and Epizootics, FU Berlin). In all infection experiments, cryo-conserved bacterial stocks were used and prepared as previously reported (Willenborg, Willms, Bertram, Goethe, & Valentin-Weigand, 2014).

4.2 | NA treatment

For deisalvation, 200 mU and 500 mU NA type V from \textit{Clostridium perfringens} (Sigma/Aldrich, Munich) were used in in vitro experiments (lectin staining and co-sedimentation assays) and in vivo co-infection on \textit{HEp-2} cells, respectively.

4.3 | Lectin staining

Fluorescein isothiocyanate-labelled \textit{Sambucus nigra} agglutinin (SNA, Vector laboratories, Burlungame, USA) and biotinylated MAA II were visualised by fluorescence microscopy as previously described (Wu et al., 2016).

4.4 | Co-infection of \textit{HEp-2} and MDCK cells by IAV and streptococci

\textit{HEp-2} and MDCK cells were seeded on 24-well plates (1.5 $\times$ 10$^5$ cells per well). After overnight cultivation, cells were inoculated with R1 or R2 at an MOI of 0.5 for 1 hr at 37 °C, respectively. To analyse bacterial adherence, the medium was removed from virus-infected cells at 16 hpi, followed by infection with \textit{S. suis} (MOI = 100) at room temperature for 1 hr or GBS (MOI = 125) at 4 °C for 1 hr. In control samples, bacteria were pretreated with NA to determine the importance of sialic acids on the capsular polysaccharide. For evaluation of virus replication kinetics, at 2 hours post-virus infection, \textit{S. suis} (MOI = 100) or GBS (MOI = 125) were added to the cells and maintained at 37 °C for another 2 hr before being washed away by phosphate-buffered saline (PBS). At 4, 8, 24, 48, 72 hr post-virus infection, the supernatants were collected for virus titration on MDCK cells as previously described (Meng et al., 2013).

4.5 | Assay for cytolytic activity (LDH release assay)

Releasing of the intracellular enzyme LDH from \textit{HEp-2} cells was determined as previously described. Briefly, 50-μl supernatants from infected or mock cells were collected at 2, 4, and 6 hr after GBS infection to detect the LDH by using the Cytotox® 96 assay kit (Promega, USA). To quantify the relative cellular damage of \textit{HEp-2} cells, results were related to uninfected control samples and expressed as x-fold increase of mock-infected samples.

4.6 | Bacterial adherence and invasion assays

To determine bacterial adherence, \textit{HEp-2} and MDCK cells pre-infected by R1 or R2 were inoculated at 16 hpi with GBS (4 °C, 1 hr) or \textit{S. suis} (room temperature, 1 hr). This incubation temperature is lower than that usually chosen for GBS adherence assays (37 °C). The lower temperature takes into account that influenza viruses not only have an efficient sialic acid binding activity but also contain a NA that would release sialic acids and thus counteract the binding activity if incubation occurs at 37 °C. Bound bacteria (red on \textit{HEp-2} and green on MDCK) and nucleoprotein of influenza A virus (green on \textit{HEp-2} and red on MDCK) were immunostained with monoclonal antibodies, respectively.

Invasion efficiency was determined by analysing virus- or mock-infected cells for intracellular GBS by an antibiotic protection assay. At 16 hr after virus infection, cells were inoculated with GBS or \textit{S. suis} and incubated for 6 hr at 37 °C. The time period was chosen because of the low invasion efficiency of \textit{S. suis}. To remove non-adherent bacteria, the cells were washed three times with PBS, and serum-free Dulbecco’s modified Eagle’s medium containing 20-μg penicillin (Sigma-Aldrich, Germany) and 100-μg gentamicin (ROTH, Germany) were added for continued incubation at 37 °C for 2 hr to kill extracellular bacteria. Then, the cells were lysed, and serial dilutions of the lysates in PBS were plated on Columbia agar supplemented with 7% sheep blood to determine colony forming units indicating the number of intracellular bacteria. The total cell-associated bacteria were numbered without antibiotic treatment of the infected cells.

4.7 | Immunofluorescence analysis

The cells were subjected to immunostaining as previously described (Meng et al., 2016; Wu et al., 2016). Briefly, for the detection of influenza viruses, a monoclonal antibody against influenza A virus nucleoprotein (AbDSeroTec, mouse) was used followed by a secondary antibody, Alexa Fluor® 488 (MDCK cells) or Alexa Fluor® 568 (\textit{HEp-2} cells) anti-mouse IgG (H + L) antibody (Invitrogen), respectively. For visualisation of bacteria, rabbit anti-\textit{S. suis} and rabbit anti-\textit{GBS} antiserum (raised by immunizations with whole dead bacterial cells, respectively) were used as primary antibodies followed by Alexa Fluor® 568 (MDCK cells) or Alexa Fluor® 488 (\textit{HEp-2} cells) anti-rabbit IgG (H + L) antibody (Invitrogen), respectively. Incubation of cells with mouse anti-human LAMP-1 antibody (BD Pharmingen) was followed by secondary Alexa Fluor® 488 anti-mouse IgG (H + L) antibody (Invitrogen).

4.8 | Co-sedimentation of IAV and streptococci

Virus suspensions (1 $\times$ 10$^5$ TCID$_{50}$/ml) and bacteria (1 $\times$ 10$^5$ colony forming units; bacteria/virus ratio of 1,000) were gently mixed and rotated at 4 °C for 1 hr. Then, the mixtures were immediately subjected to low-speed centrifugation at 6,000 rpm for 10 min. The supernatants were applied to determine the HA activity and infectivity of viruses by performing TCID$_{50}$ assay on MDCK cells. The pellets were washed three times by cool PBS containing antibiotic (penicillin/streptomycin) before determining the infectivity by plaque assays on MDCK cells.

4.9 | Hemagglutination (HA) and HI assay

The assays for detection of the hemagglutinating activities of influenza viruses were performed according to an established protocol (World Health Organization, 2011).
4.10  |  Field emission scanning electron microscopy and transmission electron microscopy

Infected HEP-2 cells were washed three times with PBS followed by fixation with 5% formaldehyde and 2% glutaraldehyde in cacodylate buffer (0.1 M cacodylate, 0.01 M CaCl2, 0.01 M MgCl2, 0.09 M sucrose, pH 6.9). Samples were washed with Trista-EDTA buffer (20 mM TRIS, 2 mM EDTA, pH 7.0) and then dehydrated with a graded series of acetone (10%, 30%, 50%, 70%, 90%, and 100%) before critical-point dried with liquid CO2 (CPD 030, Balzers) and sputter coated with gold-palladium (SCD 500, Bal-Tec). Samples were examined in a field emission scanning electron microscope Merlin (Carl Zeiss, Oberkochen) at an acceleration voltage of 5 kV applying the Everhart Thornley SE-detector and the in-lens SE-detector in a ratio of 25:75. Embedding and ultrathin sectioning were performed as previously described (Benga et al., 2004). Samples were examined in a transmission electron microscope EM910 (Carl Zeiss, Oberkochen) at an acceleration voltage of 80 kV. Images were recorded digitally at calibrated magnifications with a SlowScan CCD-Camera (ProScan, 1024 × 1024, Scheuring, Germany) with ITEM Software (Olympus Soft Imaging Solutions, Muenster, Germany). Contrast and brightness were adjusted with Adobe Photoshop CS5.

4.11  |  Statistical analysis

All statistical analyses were done by two-tailed, unpaired t-test using Prism 5 software (Prism 5 for Windows; GraphPad Software, San Diego, CA, USA).

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