Influenza A virus infection predisposes hosts to secondary infection with different Streptococcus pneumoniae serotypes with similar outcome but serotype-specific manifestation.

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Running title: Bacterial strain-dependency in the IAV-*S. pn.* synergism.

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Abstract

Influenza A virus (IAV) and *Streptococcus pneumoniae (S. pn.)* are major causes of respiratory tract infections, particularly during co-infection. The synergism between these two pathogens is characterized by a complex network of dysregulated immune responses, some of which last until recovery post IAV infection. Despite the high serotype-diversity of *S. pn.* and the serotype-replacement observed since the introduction of conjugate vaccines, little is known about pneumococcal strain-dependency in the enhanced susceptibility to severe secondary *S. pn.* infection following IAV infection. Thus we studied how pre-infection with IAV alters host susceptibility to different *S. pn.* strains with varying degrees of invasiveness using a highly invasive serotype 4, an invasive serotype 7F and a carrier serotype 19F strain. A murine model of pneumococcal co-infection during the acute phase of IAV infection showed a significantly increased degree of pneumonia and mortality for all tested pneumococcal strains at otherwise sublethal doses. The incidence and kinetics of systemic dissemination however remained bacterial strain-dependent. Furthermore we observed strain-specific alterations in the pulmonary levels of alveolar macrophages, neutrophils and inflammatory mediators ultimately affecting immunopathology. During the recovery phase following IAV infection, bacterial growth in the lungs and systemic dissemination were enhanced in a strain-dependent manner. Altogether, this study shows that acute IAV infection predisposes the host to lethal *S. pn.* infection irrespective of the pneumococcal serotype, while the long lasting synergism between IAV and *S. pn.* is bacterial strain-dependent. These results hold implications for developing tailored therapeutic treatment regimens for dual infections during future IAV outbreaks.

Introduction

Infection with secondary bacterial pathogens is attributed to be the major cause of excessive mortality during influenza A virus (IAV) outbreaks. This lethal synergism has been recognized as early as during the 1918/1919 IAV pandemic with an estimated global death toll of 50-100 million (1, 2). Retrospective studies disclosed that 71% of the fatal cases during this pandemic were positive for *Streptococcus pneumoniae* (*S. pn.* or the pneumococcus) providing the first epidemiological evidence for viral-bacterial co-infections (2). A clear pre-disposition to bacterial disease was also evident in all of the succeeding influenza pandemics including the more recent 2009 H1N1 outbreak with a 10-55% higher incidence of hospitalizations and mortality due to bacterial pneumonia (3). Pneumococcal colonization is transient and asymptomatic in immunocompetent individuals and most commonly occurs in early childhood (4). At the same time however, pneumococci are able to cause a variety of diseases ranging from mild sinusitis and otitis media to more severe infections like sepsis and meningitis. Even though the introduction of the polyvalent pneumococcal conjugate vaccines (PCV) has reduced the incidence of childhood carriage and disease for the vaccine serotypes (3-5), 1.6 million people die from pneumococcal infections annually with the majority being children under the age of 5 (6). The development of pneumococcal disease depends on both bacterial factors such as the pneumococcal capsular type or serotype and the host innate immune response (7, 8). *S. pn.* expresses a plethora of virulence factors of which the encapsulating polysaccharide layer is the most important and best-studied so far (9, 10). The capsule confers anti-phagocytic and anti-opsonophagocytic properties that determine the invasive pneumococcal disease (IPD) potential of different serotypes (11).

IPD is defined as the recovery of *S. pn.* from a normally sterile site such as the blood or brain (7, 12, 13). To date, 97 distinct serotypes have been described based on unique chemical and immunogenic properties of their capsule (10, 14) and these serotypes can be divided into invasive as well as non-invasive/carrier serotypes (15, 16). Otherwise non-invasive serotypes are able to lethally infect immunocompromised patients reflecting the impact of host-immunity on the IPD potential (8, 17). Once pneumococci enter the airways, innate immune responses are initiated by lung resident alveolar macrophages (AM) (18) that release pro-inflammatory cytokines and chemokines to recruit pro-inflammatory cells such as polymorphonuclear cells (PMN) and mononuclear phagocytes into the lung parenchyma and alveoli to contain the infection (19).

Several studies have illustrated a multifactorial nature of IAV-*S. pn.* co-pathogenesis with a plethora of underlying mechanisms (3, 20, 21). These include virus-mediated immune modulations such as aberrant inflammatory cell recruitment and function as well as increased cell death, often leading to changes in the anti-pneumococcal cytokine and chemokine responses (22, 23). Apart from the often devastating effect on anti-bacterial responses observed during acute influenza, some reports have demonstrated long-term immune defects. These include impaired neutrophil influx due to sustained desensitization of AM and the induction of an immune-suppressive state during recovery (3, 24, 25). However, the mechanisms underlying enhanced susceptibility to *S. pn.* following IAV infection are not fully understood and reports are at times contradictory. One major limitation of past studies is the use of singular *S. pn.* strains despite large differences in pathogenesis. Therefore it remains unclear if the identified mechanisms generally apply to strains of different pneumococcal serotypes. Importantly, blood cultures from living cases of the 1918 IAV pandemic revealed a higher prevalence of

less invasive serotypes in secondary pneumococcal infections than the common pathogenic serotypes found at that time (2, 26). This observation implicates a preference for otherwise colonizing strains to cause severe infections in IAV infected individuals and strongly supports the hypothesis that a pre-existing IAV infection influences the pathogenic effect of these strains.

In this study we used an *S. pn.*/IAV co-infection mouse model to address host susceptibility to selected *S. pn.* strains with varying IPD potential during the acute and recovery phase of IAV. Our data show that acute IAV infection leads to equally fatal outcomes for all the tested *S. pn.* strains at otherwise sublethal doses. At the same time however, we detected *S. pn.* strain-specific changes in the underlying innate immune responses that presumably contributed to lung immunopathology. Following recovery from IAV infection, there were no significant effects on the survival following co-infection with any of the tested *S. pn.* strains. Nevertheless, pneumococcal growth in the airways and systemic dissemination were enhanced in a strain-specific manner late after IAV infection.

Materials and Methods

Mice 7-8 weeks old C57BL/6JOlaHsd females were purchased from Harlan Winkelmann (Borchen, Germany) and Harlan Laboratories (Venray, Netherlands). Mice were housed in a specific pathogen free environment according to the guidelines of the regional animal care committees. All the experiments were approved and conducted in accordance to the guidelines set by the local animal welfare and ethics committees for the Helmholtz Centre for Infection Research (Niedersächsisches Landesamt für

Verbraucherschutz und Lebensmittelsicherheit) and the Karolinska Institute (KI; Stockholms Norra Djurförsöksetiska Nämnd).

Bacterial and viral preparation For the *S. pn.* infections the serotype 4 strain TIGR4 (ATCC BAA-334^{TM}), a serotype 19F strain (BHN100) and a 7F strain (BHN54) were used. All strains were obtained from the laboratory of B. Henriques-Normark (KI). The bacteria were grown to the mid-logarithmic growth phase in pre-warmed Todd-Hewitt Yeast (THY) medium (Todd-Hewitt broth (Sigma-Aldrich, Germany) supplemented with 1% yeast extract (Roth, Germany)) in a water bath at 37°C. Bacteria were harvested at an optical density of 0.35 to 0.50 at 620 nm (for T4 and 19F/7F respectively) by adding 10% v/v glycerol (Roth,Germany) and were frozen at -70°C. For animal infections, the frozen stocks were thawed, centrifuged at 6081xg at room temperature and washed once in 1 ml phosphate-buffered saline (PBS; Gibco, UK) before they were diluted to the desired concentration. The challenge dose was confirmed by plating 10-fold serial dilutions on blood agar plates (Columbia Agar with 5% sheep blood, BD Diagnostic Systems, Germany) that were incubated overnight at 37°C and 5% CO_2. At KI, the blood agar plates and THY medium were produced by the Karolinska Microbiology Laboratory (Solna, Sweden). For viral challenges, influenza A/PR/8/34 virus (H1N1; PR8) was produced in Madin-Darby canine kidney (MDCK) cells as described previously (27). The 50% tissue culture infectious dose ($TCID_{50}$) of the viral stock was determined by incubating 10-fold dilutions of virus stock solution on Madin-Darby canine kidney (MDCK) cells cultured in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Germany) supplemented with 0.0002% trypsin (Sigma-Aldrich, Germany) and 1% penicillin /streptomycin (ThermoFisher, USA). After 5 days, 0.5% chicken red blood cells

were added and the $TCID_{50}$ was calculated from the observed agglutination using the endpoint calculation by Reed and Muench (28).

Infection models Mice were weighed and anaesthetized through intraperitoneal administration of a mixture of ketamine and xylazine in PBS (100mg/kg ketamine, 10mg/kg xylazine). For bacterial challenges, mice were placed on their back on an intubation slope and the larynx was illuminated by an external cold-light source. A dose of $1x10^6$ CFU *S. pn.* in 25µl PBS was instilled into the laryngopharynx for aspiration to the lower respiratory tract. For IAV challenges, anaesthetized mice were held upright with the head titled back slightly and a dose of $0.31TCID_{50}$ in 25µl of PBS was administered dropwise to each nostril using a pipet. For all survival experiments following bacterial mono- or co-infection, mice were monitored three to six times per day and scored for the following parameters: body weight, movement, posture, pilo-erection, respiration, eye discharge, redness of the eye conjunctiva and response to stimulus. Moribund animals with severe symptoms of any one or a combination of the aforementioned parameters were euthanized and the infection was considered lethal. For all IAV mono-infections mice were monitored and weighed daily. The humane endpoint was set at 75% of the original body weight.

Assessment of the organ-wide bacterial burden To obtain nasopharyngeal lavage fluid, the trachea was exposed and the nasopharynx was flushed once with 1ml PBS from the trachea towards the nasal cavity. The lavage fluid was collected from the nostrils. For broncho-alveolar lavage fluid (BALF), the lungs were flushed once with 1ml PBS. For lung homogenates, the lungs were then aseptically excised and homogenized in 1ml PBS through a 100µm filter (Corning Inc., USA) using a syringe plunger. 5µl of

blood was collected from the tail vein and diluted in 45µl PBS for plating. Bacterial loads were determined by plating serial dilutions of the samples on blood agar plates. CFU were counted manually after incubating the plates for 16-18 hours at 37°C with 5% CO_2.

Quantitative RT-PCR for viral load Lungs were perfused with 10ml PBS through the heart, excised and stored at -70°C in RNA later ^{RM} solution (Ambion, USA). RNA was extracted using the RNAeasy mini kit (Qiagen, Germany) according to the manufacturer's protocol. 1µg RNA was transcribed into cDNA using the SuperScript® III First-Strand Synthesis System (Invitrogen, USA). Purity and concentration of cDNA was verified by performing a PCR for the housekeeping gene rps9. The quantitative RT-PCR performed to detect the absolute number of nucleoprotein (NP) copies in the samples was carried out on an ABI PRISM 7500 cycler (Applied Biosystems) using 35ng of cDNA/sample. A standard curve was prepared using a reference plasmid standard with known numbers of NP copies/sample ($3x10^1$ – $3x10^9$). The primers used were: *rps9* 5'CTGGACGAGGGCAAGATGAAGC and 3'TGACGTTGGCGGATGAGCACA; *np* 5'GAGGGGTGAGAATGGACGAAAAAC and 3'CAGGCAGGCAGGCAGGACTT (Eurofins MWG Operon, Germany).

Single cell preparation and staining for flow cytometry BALF was obtained as described above and the lungs were then perfused with PBS through the heart. The BALF was centrifuged at 4°C and 2000rpm in a table top centrifuge to pellet cells. The lungs were excised and manually minced on ice using scissors. Iscove's Modified Dulbecco's Medium (IMDM) with GlutaMAX-1 (Life Technologies, Germany) supplemented with 0.2mg/ml Collagenase D (Roche Diagnostics, Germany), 1mg/ml DNase (Sigma-Aldrich, Germany) and 5% fetal bovine serum (FBS Forte; Pan Biotech,

Germany) was freshly prepared for enzymatic digestion of the lung tissue. The minced lungs were suspended in 5ml of the digestion media for enzymatic digestion at 37°C for 45 minutes. The reaction was stopped by adding 5mM EDTA (working concentration) and the cell suspension was passed through a 100µm filter (Corning Inc., USA) followed by centrifugation at 420xg at 4°C. Erythrocyte lysis was performed and cells were resuspended for counting and staining. Single cell suspensions were incubated at room temperature in the dark in a mixture of the LIVE/DEAD® fixable blue stain (ThermoFisher, USA) for dead cell exclusion and anti-mouse CD16/CD32 antibody (clone 93, purified; BioLegend, USA) for Fc-receptor blocking. Cell surface staining was then performed using antibodies specific for mouse F4/80 (clone BM8, APC; BioLegend, USA), CD11b (clone M1/70, pacific blue; BioLegend, USA) and Ly6G (clone 1A8, PE-Cy7; BioLegend, USA). Samples were acquired on a BD LSR II Fortessa using the FACS Diva (BD) software and analysis was performed using the FlowJo software (Tree Star, USA). Tissue-resident alveolar macrophages (AM; $SSC^{high}FSC^{high}CD11b^{-}F4/80^{+}autoflourescence^{+}$), neutrophils (PMN; $SSC^{high}FSC^{high}CD11b^{+}Ly6G^{+}F4/80^{-}$) and infiltrating mononuclear-phagocytes (IMP; $CD11b^{+}F4/80^{-/low}Ly6G^{-}$) were identified through gating on the respective populations.

Histopathological analysis Lungs were fixed in 4% formalin and routinely embedded in paraffin. 5µm thick sections were cut, dewaxed and stained with hematoxylin and eosin. A blinded histological evaluation was performed by a veterinary pathologist certified by the European College of Veterinary Pathologists. The grade, extent and pattern of pneumonia were classified into broncho-interstitial pneumonia and broncho-pneumonia for IAV and *S. pn.* infection respectively. The scoring of lung

inflammation (grade 0 = not detected, grade 1 = minimal, grade 2 = mild; grade 3 = moderate and grade 4 = severe) was based on the number, kind (neutrophils, macrophages, lymphocytes) and location (interstitial, perivascular, intra-alveolar / -bronchial) of infiltrating cells.

Cytokine and chemokine detection For the detection of cytokines in lung homogenates, mouse ELISAMax kits (BioLegend, USA) were used according to the manufacturer's protocol. For the detection of chemokines in lung homogenates a mouse LEGENDplex™ pro-inflammatory chemokine kit (BioLegend, USA) was used according to the manufacturer's protocol. Samples were acquired on a BD LSR II Fortessa instrument and analyzed using the LEGENDplex™ v7.0 (Vigene Tech, USA) software.

Statistical analysis Graph Pad Prism 5.0 (Graph Pad software, USA) and RStudio (version 0.99.902, RStudio Inc., USA) were used to perform statistical analyses. The log-rank test was applied on the Kaplan Meier survival data. Otherwise, the one-way ANOVA with Bonferroni's multiple comparison test was used following log-transformation of the data to compare groups. The range of p values indicated is * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Results

IAV infection establishes a persisting and unresolved pneumonia even after complete viral clearance and recovery of the original body weight.

Before establishing suited co-infection models, we characterized the underlying sublethal PR8/34 (H1N1) IAV mono-infection. Following intranasal infection, IAV infected mice started losing weight by day 4-5 post infection (pi) with a maximum weight loss of

12-20% between days 7 and 9. By day 12 all mice recovered to 100% of the original body weight (Fig. 1a). High viral titers on day 7 and day 9 post IAV infection were consistent with the peak of weight loss (Fig. 1b) and viral clearance was observed by day 14 post IAV infection (Fig. 1b). Pulmonary histopathology was analyzed on days 7, 14 and 21 (Fig. 1c). Seven days post IAV infection inflammatory lesions were characterized by the accumulation of sloughed bronchial and alveolar epithelial cells and by an expansion of the alveolar septum, interstitium and bronchial lumen by neutrophils and numerous lymphocytes. At the later time-points, lesions were dominated by signs of advanced regeneration with severe hyperplasia of type II pneumocytes (Fig. 1c and supplementary Fig. S1b and c). Surprisingly, at the same time a wide-spread unresolved and partially active pneumonia was established by day 14 and day 21 post infection (Fig. 1c and supplementary Fig. S1a). Based on these findings, in our model the acute phase of IAV infection was represented by day 7 and the recovery phase persisted from day 14 until at least day 21 after IAV infection.

Acute IAV infection sensitizes the host to all tested *S. pn.* serotypes.

To assess the effect of IAV infection on the susceptibility to different *S. pn.* serotypes we selected three strains with known differences in nasopharyngeal colonization and IPD potential. First, we characterized the infection with these strains alone as a reference for co-infection. We used the highly invasive strain TIGR4 (T4) of serotype 4 (27), an invasive strain of serotype 7F and a carrier strain of serotype 19F (29). According to previous reports, serotype 4 and serotype 7F harbor a high invasive disease potential while serotype 19F is less invasive (30-32). To determine the clinical nature of infection with these strains *in vivo* in our mouse model, naïve mice were infected with sub-lethal

doses of *S. pn.* T4, 19F or 7F. At 18hpi with T4, nasopharyngeal colonization and pneumonia were detected in 83% and 58% of the animals respectively (Fig. 2a). Furthermore, 21% of all the T4 infected animals showed bacteremia (Fig. 2a). In contrast, 100% of the 19F infected mice carried bacteria in the nasopharynx and 91% showed high bacterial loads in the lungs (Fig. 2a). Importantly, despite the high frequency of pneumonia, 19F infection did not cause bacteremia in any of the mice (Fig. 2a). Following infection with the 7F strain, 96% of the animals were colonized in the nasopharynx at 18hpi (Fig. 2a). Bacteria were detected in the lungs of 34% of the 7F infected animals at comparably low titers but not in the blood (Fig. 2a). Taken together, results from the mono-infections with the three selected *S. pn.* strains reflected clear differences in pathogenesis in our mouse model since only the *S. pn.* strain T4 caused IPD, while the *S. pn.* strains 19F and 7F were restricted to the respiratory tract at 18hpi. To gain insight into bacterial strain-dependent effects on susceptibility to *S. pn.* following IAV infection, co-infections with all three *S. pn.* strains were performed during the acute phase of IAV infection (day 7 post IAV infection) (Fig. 2b). The progression of the bacterial disease was assessed by the quantification of the bacterial load in the respiratory tract and blood at 18hpi as well as the approximate mortality rates over 96hpi. A non-significant tendency for increased bacterial loads in the nasopharynx was evident for all three pneumococcal strains when comparing mono-infection to co-infection at 18hpi (Table S1). At the same time, co-infection with all three bacterial strains resulted in exceptionally high and significantly elevated bacterial loads in the BALF and lung tissue compared to the respective *S. pn.* mono-infection (Fig. 2c and d). These results clearly demonstrated IAV-dependent and bacterial strain-independent pneumococcal outgrowth following co-infection during acute IAV infection. Nevertheless,

we observed a bacterial strain-dependent increase in the incidence of systemic disease following co-infection compared to the respective mono- infection (Fig. 2e). Only the strains previously classified as potentially invasive strains (T4 and 7F) demonstrated systemic dissemination at 18h post co-infection while 19F was fully restricted to the respiratory tract at this time point (Fig. 2e). Ultimately however, the underlying IAV infection led to significantly elevated mortality rates for all the tested *S. pn.* strains (79%, 75% and 63% following T4, 19F and 7F co-infection, respectively) without significant strain-specific differences (Fig. 2f). The overall high lethality observed following co-infection correlated well with the consistently high lung bacterial loads detected at 18hpi (Fig. 2d) and at the time point severely ill mice had to be euthanized according to the predefined endpoint criteria (data not shown). Interestingly, at the time of death 90% of the euthanized T4 co-infected animals showed bacteremia (median bacterial titer: $5.9x10^6$ CFU/ml) (Fig. 2g and S2). In contrast, only 33% and 55% of the animals sacrificed following 19F and 7F co-infection showed bacteremia (Fig. 2g). Additionally, these bacteremic 19F and 7F co-infected mice yielded lower median bacterial titers than the bacteremic T4 co-infected mice (Fig. S2). Of note, survival studies for mono-infection with 19F and 7F using a 10-fold higher infection dose ($1x10^7$ CFU) than used for the co-infections still showed 100% survival without any bacteremia for all the mice (data not shown). Taken together, these results demonstrate acute IAV-infection to predispose the host to lethal secondary pneumococcal disease at low bacterial doses irrespective of the IPD potential of the *S. pn.* serotype. However, at the same time, the manifestation of disease was *S. pn.* strain-dependent regarding the incidence and kinetic of bacteremia.

The bacterial strain-dependent manifestation of secondary pneumococcal disease persists during the recovery phase of IAV infection.

To assess whether long-term IAV-mediated enhanced susceptibility to pneumococcal disease occurs in a strain- and time-dependent manner, co-infections were performed during the recovery phase of IAV infection either on day 14 or day 21. Bacterial loads were determined 18 hours later (Fig. 2b). In general, for co-infections performed on day 14 post IAV infection the overall bacterial loads in BALF, lung and blood were reduced compared to co-infections performed on day 7 post IAV infection (Fig. 2c-e and 3a-c). Regarding the bacterial load in the nasopharynx, again there were no significant differences between co-infected and *S. pn.* mono-infected mice irrespective of the pneumococcal strain (Table S1). In contrast, delayed clearance was evident in the LRT for the two invasive strains T4 and 7F. Bacterial loads were higher in the BALF and lung tissue of the T4 and 7F co-infected mice compared to the respective mono-infections (significant for the 7F co-infected lungs, Fig. 3a and 3b). In line with the increased bacterial burden in the LRT, systemic dissemination occurred at a higher incidence in T4 and 7F co-infected than mono-infected mice (significant for T4, Fig. 3c). Of note, the 33% mortality of T4 co-infected mice (Fig. 3d) was accompanied by strong pneumonia and bacteremia in all mice with lethal infection (data not shown). Taken together, even though there was no significant increase in mortality in any of the groups co-infected 14 days following IAV infection (Fig. 3d), bacterial clearance was markedly and significantly delayed for two of the three strains (T4 and 7F). Therefore these results show enhanced susceptibility to IPD during recovery from IAV infection to depend on the pneumococcal strain.

When co-infections were performed 21 days following IAV infection there was still a trend, however not significant, towards delayed bacterial clearance from the LRT in all the co-infected groups (Table S2). However, at this time point post IAV infection, the pneumococcal infection remained restricted to the lungs for the strain 7F and the sporadic systemic dissemination observed only for the highly invasive strain T4 was not dependent on a previous IAV infection (Table S2). Taken together, the kinetic of enhanced long-term susceptibility to *S. pn.* was strain-dependent.

IAV-mediated changes in immune cell recruitment following co-infection are bacterial strain-dependent particularly during the acute phase of IAV infection.

Of the different time points tested following IAV infection, the acute phase proved to be most detrimental to the host regarding susceptibility to secondary pneumococcal disease. The strong bacterial outgrowth in the LRT observed for all tested *S. pn.* strains pointed at defective innate immune responses. Therefore the recruitment of innate immune cells to the lung was assessed following co-infection with the three *S. pn.* strains on day 7 post IAV infection. Absolute numbers of i) alveolar macrophages (AM), ii) infiltrating mononuclear phagocytes (IMP), that are comprised of newly recruited monocytes, macrophages and dendritic cells, as well as iii) polymorphonuclear neutrophils (PMN) were determined in the lung. Strikingly, at 18h post co-infection the T4 and 19F co-infected groups showed significantly reduced numbers of AMs in the lung tissue compared to the *S. pn.* mono-infection (Fig. 4a). Of note, they were also marginally but not significantly reduced compared to the IAV mono-infection (Fig. 4a). In contrast, the number of IMPs was increased for all the co-infected groups compared to the respective *S. pn.* mono-infection at 18hpi (significantly for T4 and 7F co-infected

342 groups) (Fig. 4b). Importantly, the number of IMPs in all the co-infected groups remained
343 comparable to the IAV mono-infection (Fig. 4b). Interestingly, PMN recruitment to the
344 lung following bacterial mono-infection was significantly altered between the three
345 different *S. pn.* strains. Here, the strongest recruitment of PMNs was detected following
346 the 19F infection (Fig. 4c). Following co-infection, even though there were no significant
347 changes between the co- and mono-infected groups for any of the tested pneumococcal
348 strains, we observed a trend towards increased PMN numbers following co-infection for
349 the strains T4 and 7F (Fig. 4c).However, there was a decrease following 19F co-
350 infection when compared to the 19F mono-infection. Importantly, mean PMN numbers in
351 all co-infected animals exceeded those present in the lung after IAV mono-infection. Of
352 note, a similar pattern of differences was also observed for the BAL cells, except that the
353 reduction of PMN in 19F co-infected mice was not observed (Fig. 4d, e and f).
354 Importantly, the elevated PMN numbers observed for the invasive *S. pn.* strains T4 and
355 7F upon co-infection correlated well with the histopathological analysis performed 18h
356 following co-infection (Fig. 5). Here, 80% and 100% of the T4 and 7F co-infected mice,
357 respectively, showed the most severe grade of inflammation, while this was the case for
358 only 57% of the 19F co-infected mice (Fig. 5a and b). However, the percentage of the
359 lungs affected by bacterial broncho-pneumonia was comparable between all three
360 strains post co-infection during acute IAV infection (Fig. 5c). Taken together, severe
361 inflammatory lesions were observed for all the co-infected groups during the acute
362 phase of IAV infection.
363 Furthermore, the recruitment of innate immune cells to the lungs was determined
364 following bacterial co-infection during the recovery phase of IAV infection. Of note, the
365 number of AMs and IMPs detected in the lungs of co-infected mice were almost equal to

366 those in the *S. pn.* mono-infected groups at 18h following co-infection on day 14 post
367 IAV infection (Fig. 6a and b). However, there were still trends for bacterial strain-
368 dependent changes in the PMN numbers following co-infection (Fig. 6c). Here, the 19F
369 co-infected group still showed marginally, though not significantly, lower cell numbers
370 than the 19F mono-infected group whereas there was no difference in PMN numbers
371 between the co-infected and mono-infected T4 groups and a trend for increased PMN
372 numbers in the co-infected 7F group (Fig. 6c). By day 21 following IAV infection, AM and
373 PMN numbers were unchanged between the co-infected and mono-infected groups for
374 all bacterial strains (Fig. 6d). In contrast, the number of IMP remained marginally higher
375 for the T4 co-infected group compared to the bacterial mono-infection (Fig. 6e).
376 Altogether these data show that the recruitment of innate immune cells in response to *S.*
377 *pn.* is substantially affected by a preceding IAV infection if co-infection occurs during the
378 acute phase of the IAV infection. Most importantly, we identified both general, *S. pn.*
379 strain-independent changes such as the increased presence of infiltrating cells following
380 co-infection as well as strain-dependent changes such as altered AM and PMN
381 numbers.

382 **Changes in the cytokine and chemokine responses post co-infection during acute**
383 **IAV infection are pneumococcal strain-dependent.**
384 The local inflammatory responses following infection are majorly orchestrated by a
385 network of cytokines and chemokines. In order to obtain more insight into the
386 pneumococcal strain-dependent inflammatory processes taking place following co-
387 infection during acute IAV infection, we characterized and compared the lung
388 inflammatory profile following *S. pn.* mono- and co-infection for the three strains.

389 Interleukin (IL)-6 and tumor necrosis factor (TNF)-α are multifunctional pro-inflammatory
390 cytokines important for both local and systemic immune stimulation. For all three
391 pneumococcal strains, co-infection during acute IAV infection led to a clear increase in
392 the production of IL-6 compared to the respective bacterial mono-infection (Fig. 7a). Of
393 note, this excess production of IL-6 observed following co-infection was significant only
394 for the invasive strains T4 and 7F (Fig. 7a). Nevertheless we detected similarly high
395 concentrations of IL-6 in all co-infected groups at 18hpi that demonstrated a strong
396 inflammatory response irrespective of the co-infecting pneumococcal strain (Fig. 7a).
397 Also for TNF-α, all the co-infected groups revealed elevated cytokine levels in the lung
398 compared to the respective bacteria-only group (Fig. 7b). Of note, this increase was
399 again strongest and significant only for co-infections with the strains T4 and 7F (Fig. 7b).
400 Next, the lung protein concentrations of critical chemokines such as MCP-1 (monocyte
401 chemoattractant protein-1), MIP-1 (macrophage inflammatory protein), KC (keratinocyte
402 chemoattractant) and LIX (lipopolysaccharide-induced CXC chemokine), all of which are
403 implicated in driving immunopathology in IAV infected patients was elucidated. For the
404 bacterial mono-infection, we show that *S. pn.* strain 19F induced higher amounts of the
405 neutrophil chemoattractants IL-6, KC and LIX compared to T4 and 7F (Fig. 7a, c and d).
406 The least induction of IL-6, LIX and especially KC was detected after the 7F infection,
407 which correlated well with the PMN counts observed in the lung following bacterial
408 mono-infection (Fig. 7a, c and d). Following co-infection, KC levels were significantly
409 elevated independent of the pneumococcal strain when compared to the respective
410 bacterial mono-infection (Fig. 7c). Despite a trend for increased levels of LIX in the lungs
411 of mice co-infected with T4 and 7F, there were no significant changes between co-
412 infection and the bacterial mono-infection for any of the strains tested (Fig. 7d). In

contrast, both MCP-1, a potent monocytic chemoattractant, and MIP-1β, which is produced by macrophages to activate granulocytes, were significantly increased following co-infection compared to the respective bacterial mono-infection for all pneumococcal strains tested (Fig. 7e and f). Of note, in most cases, i.e. for TNF-α, KC, MCP-1and MIP-1β, the lowest levels were detected in the 19F co-infected group compared to the T4 and 7F co-infected groups (Fig 7b, c, e and f). In conclusion, when comparing between the mono- and co-infections, especially co-infections with T4 and 7F in contrast to co-infection with 19F showed a strongly synergistic rather than merely additive effect for nearly all the tested mediators. Furthermore, these data demonstrate that an underlying acute IAV infection indeed affects the local pro-inflammatory cytokine response towards *S. pn.* in a pneumococcal strain-dependent manner.

Discussion

Bacterial co-infections during IAV infection remain a significant cause of hospitalizations and mortality world-wide. Moreover, as broadly used pneumococcal conjugate vaccines influence capsular switch and serotype distribution, understanding serotype-specific differences in secondary pneumococcal infection following IAV infection is of great importance. In order to elucidate pneumococcal strain-dependent effects in the synergism between IAV and *S. pn.,* we determined the outcome and the host immune response following co-infection with three different pneumococcal serotypes. Our model differs from previous co-infection models as the three strains tested were exemplarily selected to represent strains of high, intermediate and low IPD potential. The data generated from this model shows clear strain-specific differences in the host immune

responses and implicates the use of different treatment strategies based on the co-infecting pneumococcal strain in the future.

Interestingly, despite clear differences in the manifestation of mono-infection with the different strains, all the three strains caused similarly severe pneumonia and mortality following co-infection during acute IAV infection. This clearly demonstrated that acute IAV infection has devastating effects on anti-pneumococcal host defenses that are independent of the IPD potential of the co-infecting pneumococcal strain. Strikingly, even the carrier strain 19F caused severe disease and mortality following co-infection at a dose that was non-lethal in the absence of IAV infection in our model as well as in previously described studies (29, 33). IAV infection has been shown to also support the development of otitis media by 19F (34, 35). The strongest colonization was observed following 19F mono-infection compared to T4 and 7F however without any apparent disease symptoms. Importantly, these findings highlight the requirement of a fully competent innate immune system to confine this strain to its asymptomatic carrier state. In line with this, infections with 19F are most commonly found in children and immune-compromised patients (36). Furthermore, our findings are consistent with reports that bacterial strain-specific differences are surpassed and mortality does not correlate with the incidence of bacteremia during the acute phase of IAV infection (37, 38). In fact, mortality was proportional to the uncontrolled bacterial outgrowth in the LRT and not bacteremia as the co-infected animals that succumbed to the infection showed high variability in the extent of bacteremia. These results imply that one of the major causes for mortality was severe pneumonia. The consistently high bacterial loads detected in the lungs of mice co-infected with any of the tested *S. pn.* strains may further increase the cytolytic activity of bacterial virulence factors such as the pore-forming pneumolysin

459 (39), which is a potent pro-inflammatory signal. This in turn most likely explains the high
460 levels of cytokines and chemokines in the lungs of all animals co-infected during acute
461 IAV infection. In fact, computational modelling data have demonstrated that the robust
462 local inflammatory responses induced by *S. pn.* were responsible for rapid mortality and
463 IPD during active IAV co-infection (37).
464 Next to the pneumococcal strain-independent effects mediated by IAV infection, we
465 observed distinct changes in the inflammatory response mounted towards *S. pn.* co-
466 infection that were dependent on the pneumococcal serotype. Depletion of AMs during
467 the acute phase of IAV infection has previously been shown to contribute to severe
468 secondary pneumococcal disease (40). Strikingly, in our model we did not observe a
469 significant reduction of AM numbers in the lung during IAV mono-infection. However, co-
470 infection during acute IAV infection led to a reduction in the mean AM counts that was
471 particularly clear and also significant for the strains T4 and 19F. For co-infections with T4
472 this decrease in AM numbers was associated with an increase in the late-apoptotic or
473 necrotic state of AMs (data not shown). Most likely, the loss in AMs in co-infection not
474 only has detrimental effects on bacterial clearance but also on the resolution of
475 inflammation as AM are the major effector cells to clear apoptotic PMN (41). Therefore,
476 such a clear loss of AMs is likely to tip the balance towards a prolonged pro-
477 inflammatory response culminating in immunopathology and morbidity (42, 43). For T4
478 co-infected mice, this scenario correlated well with the strong neutrophil influx and high
479 levels of inflammatory mediators. In contrast, AM reduction was accompanied by a
480 marginal decrease in lung neutrophil numbers following the 19F co-infection compared
481 to the 19F mono-infection. Of note, also, the local concentrations of TNF-α and MIP-1β
482 were marginally, but not significantly, lower for the 19F co-infected mice than for the T4

and 7F co-infected mice. This situation makes the AM decrease less consequential in the 19F anti-bacterial response when compared to the T4 co-infection. In our study, neutrophils emerged as the major cell type responsible for combating 19F infection, in line with the copious PMN influx observed during 19F mono-infection that was significantly elevated compared to T4 and 7F mono-infection. This is in accordance with a recent study that demonstrated complete abrogation of bacterial clearance in 19F pre-colonized animals upon neutrophil depletion (44). For co-infection with the strain 7F, cell numbers in the respiratory tract represented a more balanced state and the lethal disease outcome can most likely be attributed to local immunopathology, driven by the excessive inflammatory mediators and inflammatory monocytes observed in all 7F co-infected animals. For IAV infection alone, a strong TNF-α response has been shown to induce severe pathology (45) and thus neutralization protected against immunopathology mediated mortality (46, 47). Even though TNF-α neutralization failed to lower the disease severity following T4 co-infection (48), it still holds potential for a treatment strategy for the 7F co-infections and should be tested in the future.

Altogether our study has identified several characteristics in the host response towards different pneumococcal strains following co-infection during acute IAV infection. Nevertheless, the outcome was equally devastating for all tested strains. Effective treatment of secondary pneumococcal infection following IAV infection has been suggested to combine antibiotic measures with immune modulators (49, 50). Therefore, future studies will be needed to exploit our findings regarding the serotype-specific inflammatory responses following co-infection for treatment regimens tailored to the co-infecting pneumococcal strain.

506 Importantly, we have extended our study of the *S. pn.* strain-dependency in host
507 susceptibility and inflammatory responses following co-infection to the recovery phase of
508 IAV infection. Histological analyses performed until 21 days post IAV infection revealed
509 that pneumonia was established even after complete recovery of body weight and viral
510 clearance. In fact, the few long-term follow up studies with small patient cohorts that
511 survived acute H1N1 infection have revealed that structural abnormalities in the lung
512 parenchyma can last up to a year after respiratory disease (51, 52). When co-infections
513 were performed on day 14 following IAV infection, we indeed still observed impaired
514 clearance of *S. pn.*. While survival was unaffected, we detected increased bacterial
515 growth in the LRT and significantly increased incidence of systemic dissemination in a
516 strain-dependent manner. In fact, IAV-induced impairment of AM sensitivity is proposed
517 to persist until 100 days post IAV infection in mice (24). Furthermore, it has also been
518 reported that a state of immunosuppression marked by the upregulation of IL-10 (25) is
519 set up to promote repair and recovery post IAV infection, which may explain the
520 marginally delayed bacterial clearance in all the co-infected animals until day 21 post
521 IAV infection and the low levels of inflammation in the lungs compared to day 7 post IAV
522 infection. In line with previous reports, our results show that IAV infection affects
523 pneumococcal clearance even after elimination of the virus during recovery of the host.
524 Importantly, we show a pneumococcal strain-dependency which implies that strains with
525 higher IPD potential such as T4 to take advantage of the IAV pre-infected host for a
526 longer time than colonizers such as 19F. Despite equally low mortality following co-
527 infection with the different pneumococcal strains late following IAV infection in our
528 model, we believe this finding holds implications e.g. for the surveillance of survivors of

529 severe IAV infections during outbreaks of pneumococcal strains with moderate to high
530 IPD potential.
531 Altogether, the results of our study strongly highlight the need to identify the serotype
532 infecting the patient before administering standard treatment regimens such as e.g. the
533 increasingly used corticosteroids (45, 50, 53). Following co-infection during the acute
534 phase of IAV infection the invasive strains T4 and 7F caused stronger pro-inflammatory
535 responses compared to the 19F strain. Therefore immunosuppression along with
536 antiviral and antibacterial treatment may work better for these strains. In case of co-
537 infection with colonizing strains such as the 19F, a strong bacteriostatic antibiotic along
538 with antivirals may be more potent in preventing mortality. Certainly, these hypotheses
539 will need to be tested in future studies, for which we believe our results provide a
540 valuable first basis. Furthermore, our results imply the imperative need to strongly
541 consider the pneumococcal serotype when studying the mechanisms underlying severe
542 IAV-*S. pn.* co-infections in animal models.
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744 **Author contribution**

745 Experimental planning, execution and data analysis: NSC, SSK, VS, JV; Mouse
746 histology: OK, AG; Project consumables/animals and analysis tools: DB, BHN;
747 Manuscript writing: NSC, SSK, DB; Manuscript proof-reading: all; Project design and
748 supervision: SSK, BHN, DB.

749

750 **Competing financial interests**

751 No conflict of financial interest declared by the authors.

752

753 **Figure legends**

754 **Figure 1: Persisting unresolved pneumonia post IAV infection.** WT C57BL/6J mice
755 were intra-nasally infected with 0.31TCID$_{50}$ of the A/PR8/34 H1N1 strain of IAV or treated
756 with PBS as control on day 0. (**a**) Changes in body-weight are represented as
757 percentage relative to the starting weight. Data are shown as mean ± SEM. (**b**) Absolute
758 quantification of viral nucleoprotein (NP) copy numbers in the lungs of PBS-treated and
759 IAV-infected mice at the indicated number of days post IAV infection. Lines indicate the
760 median. Statistical analysis was performed using the one-way ANOVA with Bonferroni's
761 multiple comparison test with the asterisk * indicating significant differences between the
762 groups, *P<0.05; **P<0.01; ***P<0.001. Data are compiled of at least two independent
763 experiments with 3-5 mice in each group. (**c**) Representative example (one out of n=3)
764 of histopathological changes in the lungs of IAV infected or PBS treated mice at 7, 14
765 and 21 days post infection analyzed following H and E staining. Lungs of PBS treated
766 mice were unchanged. For day 7 after IAV infection the arrowheads indicate an
767 attenuation of the bronchial epithelium and the neutrophils and macrophages within the
768 alveoli are encircled; for day 14 after IAV infection the arrowheads indicate type II
769 hyperplasia and the neutrophils and macrophages in the bronchi (indicating bronchitis)
770 are encircled; for day 21 after IAV infection the arrowheads indicate type II pneumocyte
771 hyperplasia with increased numbers of lymphocytes in the surrounding interstitium
772 (encircled).

773 **Figure 2: Enhanced susceptibility to secondary *S. pn.* infection during the acute**
774 **phase of IAV infection.** Groups of 5-6 WT C57BL/6J mice were oro-pharyngeally
775 infected with 1x10^{6} CFU of *S. pn.* strain T4, 19F or 7F. (**a**) Incidence of colonization,

pneumonia and bacteremia at 18hpi with *S. pn.* strains T4, 19F or 7F according to the bacterial burden detected in the nasopharynx, post-lavage lungs and blood for all bacterial mono-infections performed in the study. Data are shown as mean ± SEM. (**b**) Schematic diagram for co-infections with *S. pn.* strains T4, 19F or 7F post IAV infection. Mice were infected with $1x10^6$ CFU of *S. pn.* strains T4, 19F or 7F on day 7 post infection with $0.31TCID_{50}$ of IAV or PBS treatment. **(c - e)** Bacterial burden in the (**c**) BALF, (**d**) post-lavage lungs and (**e**) blood at 18h after secondary infection with T4, 19F or 7F. Lines indicate the median. Statistical analysis was performed using the one-way ANOVA with Bonferroni's multiple comparison test with asterisks * indicating significant differences, *P<0.05; **P<0.01; ***P<0.001. (**f**) Survival rates of mice infected with *S. pn.* T4, 19F or 7F on day 7 post IAV infection or PBS treatment. The asterisk * indicates significant differences assessed by the Log-rank test on the Kaplan-Meier survival data for the co-infected groups compared to the *S. pn.* only groups, *P<0.05; **P<0.01. (**g**) Incidence of colonization, pneumonia and bacteremia in the co-infected and single *S. pn.* infected mice with lethal infection according to the bacterial burden detected in the nasopharynx, whole lung and blood respectively.

Figure 3: Strain-specific alterations in the course of secondary pneumococcal disease during recovery from IAV infection. Mice were infected with $1x10^6$ CFU of *S. pn.* strain T4, 19F or 7F on day 14 post infection with $0.31TCID_{50}$ of IAV or PBS treatment. (**a - c**) Bacterial burden in the (**a**) BALF, (**b**) post-lavage lungs and (**c**) blood at 18h post-secondary infection with T4, 19F or 7F. Lines indicate the median. Statistical analysis was performed using the one-way ANOVA with Bonferroni's multiple comparison test with the asterisk * indicative of significant differences between the co-

infected and *S. pn.* only groups, *P<0.05;**P<0.01;***P<0.001. (**d**) Survival rates of mice co-infected on day 14 post IAV infection or infected with *S. pn.* only.

Figure 4: Strain-dependent changes in the innate immune cell composition of the lungs of mice co-infected during the acute phase of IAV infection.

Mice were infected with $1x10^6$ CFU of *S. pn.* strain T4, 19F or 7F or treated with PBS on day 7 post infection with $0.31TCID_{50}$ of IAV or PBS treatment. Absolute numbers of (**a**) alveolar macrophages (#AM), (**b**) infiltrating mononuclear phagocytes (#IMP) and (**c**) polymorphonuclear cells (#PMN) in post-lavage lungs at 18h after co-infection. Absolute numbers of (**d**) alveolar macrophages (#AM), (**e**) infiltrating mononuclear phagocytes (#IMP) and (**f**) polymorphonuclear cells (#PMN) in BALF 18h after co-infection. Data are shown as Tukey box plots with lines indicating the median. Data are compiled of n>5 from at least two independent experiments with 2-3 WT C57BL/6J mice per group. Statistical analysis was performed using the one-way ANOVA with Bonferroni's multiple comparison test with the asterisk * indicative of significant differences, *P<0.05;**P<0.01.

Figure 5: Histopathological changes in the lung tissue of *S. pn.* mono-infected mice and mice co-infected during the acute phase of IAV infection. Mice were infected with $1x10^6$ CFU of *S. pn.* strain T4, 19F or 7F on day 7 post infection with $0.31TCID_{50}$ of IAV or PBS treatment. (**a**) Representative example (one out of n=3-7) of the histopathological changes examined by H and E staining of the lungs of co-infected and *S. pn.* mono-infected mice at 18h post infection with T4, 19F or 7F. Arrowheads indicate selected regions of broncho-pneumonia. (**b**) Histopathological scores for

inflammation observed in the lungs of co-infected and *S. pn.* mono-infected mice. Acute inflammation was characterized as perivascular and interstitial immune cell infiltration. (**c**) Percentage of the lung affected by broncho-pneumonia in the co-infected and *S. pn.* mono-infected mice. Histological analysis was performed twice with 2-4 mice per group. Lines indicate the median.

Figure 6: Inflammatory cell profiles in the lungs of mice *S. pn.* mono-infected or co-infected during the recovery phase of IAV infection. Mice were infected with $1x10^6$ CFU of *S. pn.* strain T4, 19F or 7F or treated with PBS on day 14 post infection with $0.31TCID_{50}$ of IAV or PBS treatment. Absolute numbers of (**a**) alveolar macrophages (#AM), (**b**) infiltrating mononuclear phagocytes (#IMP) and (**c**) polymorphonuclear cells (#PMN) in post-lavage lungs 18h after the bacterial infection or PBS treatment. Mice were infected with $1x10^6$ CFU of *S. pn.* strain T4, 19F or 7F or treated with PBS on day 21 post infection with $0.31TCID_{50}$ of IAV or PBS treatment. Absolute numbers of (**d**) alveolar macrophages (#AM), (**e**) infiltrating cells (#IMP) and (**f**) polymorphonuclear cells (#PMN) in post-lavage lungs 18h after the bacterial infection or PBS treatment. All data are shown as Tukey box plots with lines indicating the median. Data are compiled of at least two independent experiments with 3-4 WT mice per group.

Figure 7: Pro-inflammatory cytokine and chemokine profiles in the lungs of mice *S. pn.* mono-infected or co-infected during the acute phase of IAV infection. Mice were infected with $1x10^6$ CFU of *S. pn.* strain T4, 19F or 7F or treated with PBS on day 7 post infection with $0.31TCID_{50}$ of IAV or PBS treatment. Protein concentrations of (**a**) IL-6 and (**b**) TNF-α in the homogenates of post-lavage lungs at 18h after the secondary

bacterial infection or PBS treatment. Data are shown as mean ± SEM of the number of mice/group indicated in parentheses and are compiled from two independent infection experiments with 1-4 mice per group. Protein concentrations of (**c**) KC, (**d**) LIX, (**e**) MCP-1 and (**f**) MIP-1β in the homogenates of post-lavage lungs at 18h after the secondary bacterial infection or PBS treatment. Data are shown as mean ± SEM of the number of mice/group indicated in parentheses and are compiled from two independent infections with 2-5 mice per group. Statistical analysis was performed using the one-way ANOVA with Bonferroni's multiple comparison post-test. Asterisks * indicate significant differences, *P<0.05;**P<0.01;***P<0.001.