The development of a secondary infection model in mice with pathogens influenza and *Streptococcus pneumoniae*.

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Abstract

A secondary infection is an infection by a microorganism that follows after an initial infection by another kind of microorganism. Secondary infections are responsible for high rates of morbidity and mortality. A *Streptococcus pneumoniae* secondary infection is often seen after an influenza infection. Despite vaccination for both pathogens, secondary infections still affect humans. We want to try if combi-vaccination (one vaccine against two pathogens) can be used as an important strategy to prevent secondary infections. Therefore, a mouse model had to be created for secondary infection with pathogens influenza and *Streptococcus pneumoniae*. We wanted to create a model in which separate infection with the two pathogens would not lead to disease, but the combination of the two pathogens would be lethal. In this study, we set up a secondary infection model for influenza (PR8) and *S. pneumoniae* (TIGR4). First, we selected doses of both pathogens which did not cause disease symptoms, but did induce seroconversion. The dose used for PR8, resulted in weight loss and an IgG titer against PR8. For the TIGR4, measuring of antibodies failed, and so we used three doses in which animals would not get symptoms of disease. The doses were combined in a model of secondary infection. Results of secondary infected mice with TIGR4 show severe weight loss and bacteremia in most secondary infected mice, what indicates the secondary infection model causes dead in most of the mice. 26 out of 41 secondary infected mice showed bacteremia after secondary infection. Our results show that mice are more vulnerable for *S. pneumoniae* strain TIGR4, when challenged 7 days before with influenza strain PR8. With this model, the road is open for research about combi-vaccination against influenza and *S. pneumoniae*. When an effective combi-vaccine can be developed, it can reduce both morbidity and mortality caused by secondary infections.

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Master report

Introduction

Seasonal outbreaks of influenza virus in autumn and winter result in three to five million cases of severe illness per year, and are responsible for about 250.000 to 500.00 yearly deaths in the developed world alone[1]. The elderly, children below the age of 2 and immunocompromised seem to be the most vulnerable for influenza infection. In the last decades, it has become clear that the mortality in seasonal epidemics and pandemics is not only caused by the influenza virus, but also by secondary bacterial infections[2]. Secondary infections are most often caused by *Streptococcus pneumoniae*, but can also be caused by other bacteria as *Streptococcus pyogenes* or *Staphylococcus aureus*[3,6]. Both influenza virus and *S. pneumoniae* rank as the two most important pathogens infecting humans nowadays, and the possible ability of the pathogens to work together is a great threat to world health[2].

Influenza viruses are members of the family *Orthomyxoviridae,* and are subdivided into three types: type A, B and C[2]. Where influenza B and C are predominantly human pathogens, type A influenza is also isolated from many animal species[4]. Furthermore, type A is the most virulent type, and is responsible for the occurrence of pandemics[2]. Type A influenza viruses are subtyped based on difference in both hemagglutinin (HA) and neuraminidase (NA), which are surface glycoproteins[4]. To date, 17 HA types and 9 NA types which circulate in the wild have been classified[4]. The function of HA is to mediate early steps in the viral replication cycle, membrane fusion and receptor binding, by binding to sialic acid on the receptors of epithelial cells[4,5]. Protection against viruses is mediated by neutralizing antibodies against the HA. Seasonal variation in the surface of the HA can result in antigenic drift: rapid changes in HA structure which prevent antibody binding to the virus, and this results in viruses with the potential to cause new epidemics[5]. A more threatening process is the occurrence of antigenic shift, in which the influenza virus can undergo rapid, radical change by acquiring a new HA, which can cause pandemics[2]. Influenza can cause death in three ways. The influenza infection might be so virulent that it can cause death by itself, the virus may increase the physiological load in persons with metabolic, lung and heart diseases in such a way that it causes death, and death can be the result of a secondary bacterial infection after the infection with influenza virus[2]. In most influenza infections, the virus only causes pulmonary symptoms without death.

S. pneumoniae is an encapsulated bacteria, lancet-shaped gram positive diplococcus commonly present in the microbial flora of the upper respiratory tract. It is present in approximately 20-50% of healthy children and 8-30% of healthy adults[6]. Colonization of *S. pneumoniae* in the naso-oropharynx seems to be asymptomatic. When *S. pneumoniae* gains access to the normally sterile parts of the airways a rapid inflammatory response ensues that results in disease[7]. S*. pneumoniae* is responsible for a considerable rate of mortality and morbidity, what makes it one the most important pathogens which negatively affects humans[2]. Annually, infections with *S. pneumoniae* results in 1.6 million deaths worldwide, of which 0.7-1 million are children below the age of 5[8,9]. *S. pneumoniae* can cause a variety of diseases, as otitis media, meningitis, pneumonia and sepsis, depending on the invasion site[10,11]. None of these diseases, promote pneumococcal transmission. Spreading of *S. pneumoniae* is thought to occur with the secretions of colonized individuals. The pathogen can be carried for weeks to months before its eventual clearance[7]. Protection against infections with *S. pneumoniae* is mediated by opsonin-dependent phagocytosis. The major immune mechanism thought to protect against pneumococcal infections is the antibody-initiated complement dependent opsonisation, which activates the classic complement pathway[12]. The capsule of *S. pneumoniae* is composed of polysaccharides, which determine the serotype specificity. Over 90 serotypes have been recognized[13,14]. *S. pneumoniae* contains more than 100 surface proteins, which are thought to play a role in virulence and pathogenicity[15]. Little is known about expression patterns of these proteins in different serotypes, and if differences in structure and expression patterns account for differences in invasiveness or virulence between strains of *S. pneumoniae*[2].

It has been shown that during the 1918 pandemic, secondary bacterial infections were the most prevalent cause of death[2]. An epidemic is an outbreak of a contagious agent restricted to one location, and in the case of an epidemic, more people are infected than usually is the case. A

pandemic is an epidemic which occurrence is worldwide. During the influenza pandemics in 1918 and 1957, which resulted together in about 40-50 million deaths, studies showed that the incidence of secondary bacterial pneumonia was the most common cause of mortality during influenza pandemics[16,17]. It is well known that after an upper respiratory tract viral infection, the road is cleared for a secondary bacterial infection in the lung. Certain combinations of organisms are thought to complement each other better than other pairings[18]. An infection with *S. pneumoniae* is frequently seen after an infection with influenza virus, however there is no obvious evidence for specific interactions between them[2]. Susceptibility to a secondary bacterial infection with *S. pneumoniae* is the highest one week after influenza infection, but is also high during infection and when infection is cleared[17,19]. It is still unclear what the synergistic mechanisms are between influenza and *S. pneumoniae*, although several mechanisms have been proposed, like viral destruction of the epithelium of the lungs, upregulation of bacterial adhesion molecules after influenza infection, suppression of neutrophils function after influenza infection, impaired phagocytosis after influenza infection and desensitization to toll like receptors ligands after influenza infection[2].

Seasonal vaccination against influenza has been proven to be safe and effective[20]. Vaccination against influenza has been used for 50 years, and is the most cost effective method for the prevention of influenza[21]. Several vaccines are licensed, like inactivated influenza virus in whole inactivated virus (WIV), subunit (SU) and split-virus (SV), which are all subcutaneously or intramuscularly injected[22]. WIV vaccines consist of killed whole influenza virions, SV contains degraded virions, and SU contains the purifies HA and NA[23]. Another vaccination strategy is the intranasal administration of live attenuated influenza vaccines (LAIV). This vaccine consists of a virus which has genetic mutations, resulting in a virus that is cold adapted and temperature sensitive, and thus is attenuated. This results in the fact that the virus only can replicate in a limited way in the upper airways[24]. Virosomal influenza vaccines are another type of vaccine, which is administered via intramuscular injection. They contain a membrane with associated HA and NA proteins, but lack the viral genome[21]. Vaccination is required to elicit a humoral response, because, as already mentioned, antibodies neutralize the virus particles. A cellular response is required to clear infected cells in the body[25]. All of the produced vaccines nowadays contain two influenza A strains and one influenza B strain, which are selected by the World Health Organization (WHO)[26].

The treatment for *S. pneumoniae* nowadays is limited by the fact that bacteria are developing resistance against antibiotics[26-28]. Because of antibiotic resistance and cost effective reasons, vaccines are considered as an important preventive strategy[28]. Vaccines against *S. Pneumonia* targets the polysaccharides of the bacteria capsule. The disadvantage of these vaccines is that not all serotypes are covered[27]. One example is the 23-valent pneumococcal polysaccharide vaccine (PV23), which only contain purified bacterial polysaccharides[29]. Despite the advantage that this vaccine is effective against 90% of the disease causing serotypes, there are several disadvantages, like lack of memory and poor immunogenicity in infants[6]. Conjugated vaccines do not have these drawbacks, and are effective in infants as well[30]. Examples are the 7/10/13 valent pneumococcal vaccine, which consists out of polysaccharides of 7/10/13 serotypes and are conjugated with a non-toxic diphtheria toxoid protein, which is capable of inducing a TH2 helper response[31]. An advantage of the conjugate vaccine is the activation of the T cell response, because the protein conjugate added can induce a T-helper response, what is not the case without adjuvant[31]. The whole cell pneumococcal vaccine is another vaccine, in which a large number of pneumococcal antigens are presented at once[32]. In vivo, it was shown that a whole cell pneumococcal vaccine with aluminium-hydroxide parenterally administered, reduced pneumonia and sepsis[33]. Advantaged of this vaccine are the low cost of production and serotype independent protection.

In this study, we set up a model for secondary infection, so we can investigate whether simultaneous vaccination against both influenza and *S. pneumoniae* is effective to prevent pneumococcal secondary infection after influenza infection. So before we can test this, an in vivo secondary infection model has to be created with the specific pathogens. Mouse models for separate infection for both influenza (PR8) and *S. pneumoniae* (TIGR4) already have been described in literature[34,35]. In vivo studies have successfully developed secondary infection models for *Haemophilus influenzae, S.*

pneumoniae and *S. aureus*[17,36-38]. We want to make a model for secondary infection in which neither the primary nor the secondary infection is lethal for the mice when challenged separately. In a study performed by McCullers et al. about secondary infection with influenza and *S. pneumoniae*, it was shown that 7 days after a challenge with influenza strain PR8, mice were the most susceptible for *S. pneumoniae* infection[17].
In the first part of the study, groups of mice were infected with different doses of either influenza or *S. pneumoniae*. The dose in which animals did not show symptoms of disease after a challenge with influenza or *S. pneumoniae*, but did develop an antibody response against the pathogen was selected. These two doses were selected for use in the next experiment. In the second part of the study, groups of mice were infected with the selected dose of influenza, and 7 days later infected with the selected doses of *S. pneumoniae*. Our hypothesis is that the doses selected for influenza and *S. pneumoniae*, which separately do not give disease symptoms, now will be lethal for the mice.

Material and methods

Ethical Statement

Animal experiments were evaluated and approved by the Committee for Animal Experimentation (DEC) of the University Medical Center Groningen, according to the guidelines provided by the Dutch Animal Protection Act (permit number DEC 6336A). Challenges were conducted under isoflurane anesthesia, and every effort was made to minimize suffering.

Infectious agents

The mouse-adapted influenza virus A/Puerto Rico/8 (PR8) (H1N1), was kindly provided by the molecular virology department of the UMCG. The virus was diluted to specific amounts of plaque forming units (PFU). The *S. pneumoniae* strain TIGR4 (TIGR4), was obtained from the molecular bacteriology department. TIGR4 was grown in brain heart infusion broth (BHI) to an optical density (OD) of 0,4, and stored -80°C till usage. Bacteria were plated on blood agar to obtain colony forming units (CFU) per ml. TIGR4 was diluted to specific amounts of CFU for challenge.

Mice

Female BALB/cOLaHsD and C57BL/6 mice (6-10 weeks old; Harlan, Zeist, The Netherlands) were maintained in a bio safety level 2 facility in the CDL at Groningen. All experimental procedures were done while mice were under general anesthesia with inhaled isoflurane 2,5-5% in O_2.

Infectious model

Infectious agents were diluted in sterile phosphate buffered saline (PBS) (9,0g NaCl per L, 1,2g KCl per L, 16,2g Na_2HPO_4 per L, PH 7,4) and administered intranasally in a volume of 40 µl in the PR8 challenge (20 µl/nostril), or 50 µl (TIGR4) (25 µl/nostril) in the TIGR 4 challenge to anesthetized mice held in upright position. Mice were monitored daily for illness (body weight) and mortality in the first week, and after the first week each 2 days. Mice found to be moribund were euthanized and considered to have died on that day.
For the setup of the secondary infection model, all mice were challenged with 6 PFU of PR8. 7 days after this challenge, 3 groups of 15 mice were randomly created. The first group was challenged with $5x10^3$ CFU TIGR4, the second group was challenged with $1x10^4$ CFU TIGR4, and the third group was challenged with $5x10^4$ CFU TIGR4. 5 mice were sacrificed 1,2 and 3 days after being secondary challenged with TIGR4, or earlier when the mice reached their human endpoint. As control, 5 mice were challenged with 6 PFU PR8, and 7 days later with PBS, and 4 mice were challenged with PBS, and 7 days later with the highest dose of TIGR4 ($5x10^4$ CFU).

Blood and lung collection

Blood was drawn by hart punction, and stored 1 night at 4 °C. Blood was centrifuged 10 minutes at 2200g and the supernatant was collected. Directly after hart punction, lungs were collected.

Lung titers of PR8 and CFU of TIGR4 in lungs and blood

Lungs were removed under sterile conditions, and placed in 1 ml of sterile PBS. For the quantification of *S. pneumoniae* presence in the lungs, lungs were homogenized in 1 ml of PBS. For the quantification of both pneumococcal colonies in the blood and pneumococcal colonies in the lungs 10-fold serial dilutions were made in sterile PBS and plated on blood agar. Identification of pneumococcal colonies was done by visual inspection and recognition of standard colony morphology and characteristic hemolysis on blood agar. To determine the lung titers of PR8, lungs were removed under sterile conditions, and homogenized in 1 ml of PBS. Homogenated lungs were centrifuged 5 min at 1400g and supernatant was used for the determination of virus titers. Virus titer was measured by hemagglutination assay. 96-wells plates were seeded with 100 µl of a solution which contained $1x10^5$ MDCK cells/ml (in Episerd medium 1/100 pen strep, 1/100 sodium bicarbonate and 1/40 Hepes), and were incubated overnight in a CO_2 incubator (37°C, 5% CO_2). The medium of the 96-wells was then removed, and twofold dilutions of lung samples were added in duplo, and incubated for 1 hour in a CO_2 incubator (37°C, 5% CO_2). Plated were washed with PBS, and 100 µl medium with trypsin (5 µg/ml) per well was added, and were incubated 72 hours in the CO_2 incubator(37°C, 5% CO_2). Supernatants were collected and transferred to a round bottom 96-wells plate, and 50 µl 0,5% (vol/vol) washed guinea pig erythrocytes were added. Plated were incubated 2 hours at room temperature, and hemagglutination was read.

ELISA

Enzyme-linked immunosorbent assays (ELISAs) were used to measure levels of serum IgG and IgM antibodies. Lysates of bacteria were used for coating ELISA plates. Lysates were made by growing bacteria till OD 0,4, and centrifuging bacteria at 1000g for 10 minutes. The cell pellet was dissolved in 1 ml PBS and glass beads were added, and 5 times 1 minute shaken in the bead beater. Then, lysates were centrifuged 2 minutes at 1000g, and supernatant was taken and boiled 15 min at 96°C. 96-wells plates were coated overnight with TIGR4 lysate or lactis lysate (negative control) (0,1 µg/ml,100 µl per well or whole influenza virus (10 µg/ml, 50 µl per well) and the next day blocked for 1 hour with 2,5% milk, both in coating buffer (1,89g Na_2C0_3 per L, 2,70g $NaHCO_3$ per L, PH 9,6-9,8). Individual sera from immunized mice were tested in duplicate. Serum samples (100 µl) were added and serially diluted in PBS containing 0.05% Tween-20 (PBST). After 1,5 h incubation at 37°C, plates were washed three times with PBST and titers of IgG were determined by addition of 100 µl of HRP conjugated goat anti-mouse IgG or goat anti-mouse IgM (Southern biotech), following incubation of 1 hour at 37 ◦C. Plates were washed again 3 times with PBST, and once with PBS, and then incubated with 100 µl o-phenylene-diamine (OPD) (Sigma Alderich) staining solution (2 tablets OPD dissolved in 25,7ml 0,2M dibasic sodium phosphate, 24,3 ml 0,1M citric acid and 50 ml water (PH 5,0)). 40 µl H_2O_2 per 100 ml staining solution was added prior to application to the plate. The ELISA was allowed to react for 30 minutes and stopped with 50 µl 2M H2SO4. Plates were read out at OD 492.

Results

PR8 Titration

Body weights and symptoms PR8 challenged mice

To test which dose of PR8 did not give symptoms of disease in BALB/cOLaHsD, but did induce an antibody response against PR8 in mice, BALB/cOLaHsD mice were challenged with different doses of PR8. Mice challenged with 100 and 50 PFU exhibited weight loss 2 days after challenge (Figure 1A). After 5 days this resulted in a body weight less than 85% of the weight before the challenge, and mice had to be sacrificed. Mice challenged with 25 PFU did not all show the same extent of body weight loss. 1 mice had to be sacrificed after 5 days, and 2 mice after 8 days because they had lost too much weight (Figure 1A). 1 mice however, did not show any weight loss, and was sacrificed after

9 days, what explains the increase of body weight on day 9 in the graph, because it was the last mouse left without any weight loss (Figure 1A). Mice challenged with 12,5 PFU, all had less than 85% of the weight before challenging after 8 days, and were sacrificed on day 8 (Figure 1A). Because the virus was more virulent than expected, the titration had to be repeated with 2 additional doses (6 PFU and 3 PFU) ,however, these challenges were performed in C57BL/6 mice. In the mice challenged with 6 PFU, 2 showed a decrease in body weight starting at day 6 after challenge, and 2 did not show weight loss (Figure 1B). Mice challenged with 3 PFU all showed a little drop in weight starting from day 4 after challenge (Figure 1B). Both groups of mice were sacrificed 28 days after challenge. Overall, this data shows that the PR8 strain used in this experiment is extremely virulent, or the mice used are extremely susceptible for PR8.

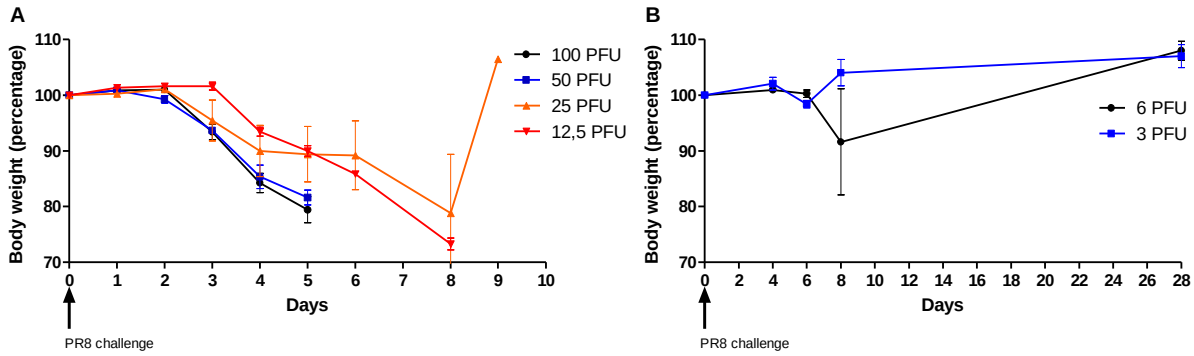

Figure 1. Average body weights of PR8 challenged mice. Groups of mice (n=4) were challenged with different amounts of PFU PR8. Body weight of mice challenge with 100, 50, 12,5 and 6 PFU (A) and body weight of mice challenged with 6 and 3 PFU (B). Mice challenged with 25 PFU were sacrificed on different days, what explains the increase of body weight on day 9 (1 healthy mouse left). Data is plotted as mean, error bars indicate SEM

IgG titers and virus titers in the lungs of in mice challenged with PR8.

To confirm that PR8 challenged mice induced seroconversion, IgG antibodies were measured. Our hypothesis is that when antibodies are present in the mice, the lungs are primed for a secondary infection. The antibody response against many viruses is dominated by IgG antibodies, which promote phagocytosis of microbes[39]. Because groups of mice were not all sacrificed on the same day, IgG titers were measured on different days after challenge per group. Mice challenged with 25 PFU were not sacrificed on the same day, what explains the different time points of IgG measurement in this group. As seen in figure 2, only 2 of the mice that were sacrificed after 5 days in the 100 and 50 PFU groups, showed an antibody IgG response (Figure 2). In the mice challenged with 25 PFU, IgG titers from 2 were measured mice after 8 days, and both from 1 mice 5 and 9 days after challenge. IgG titers of mice challenged with 12,5 PFU were measured 8 days after challenge. All the mice challenged with both 25 and 12,5 PFU did show an IgG titer in the blood. Also, the mice challenged with both 6 and 3 PFU showed an IgG titer in the blood 28 days after challenge (Figure 2).

To check whether the PR8 infection in the lungs was cleared after 28 days in the groups infected with 3 and 6 PFU, hemagglutination assay was performed. Both groups did not show any virus left in the lungs (data not shown). These results show that mice challenged with 25, 12,5, 6 and 3 PFU developed an IgG immune response against PR8, and their immune system responded against the virus with an IgG response. In mice challenged with both 6 and 3 PFU, which all survived the period of infection, the virus was cleared from the lungs after 28 days. This data shows that that IgG antibodies are produced against PR8, and that a lower dose of PR8 gives the mice more time to respond against the virus, so the virus can be cleared.

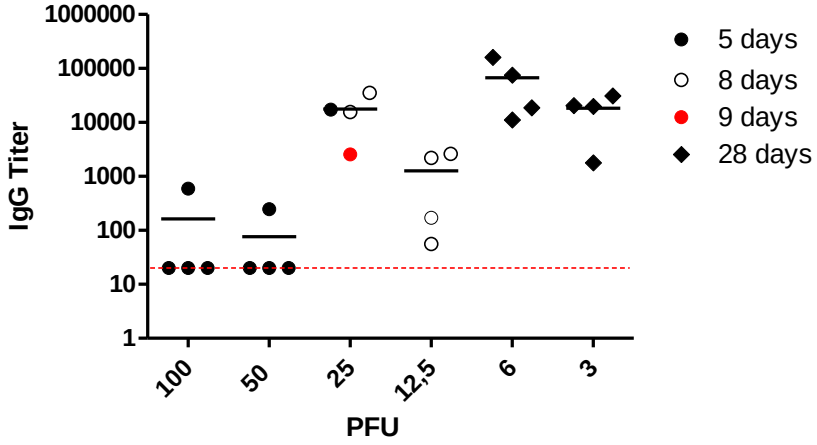

Figure 2. IgG titers of PR8 challenged mice. The red line represents the detection limit

TIGR4 Titration

Body weights and symptoms PR8 challenged mice

To test which dose of TIGR4 did not give symptoms of disease in BALB/cOLaHsD mice, but did result in an antibody response against TIGR4, mice were challenged intranasally with different doses CFU in PBS. Groups of 4/5 mice were challenged with $5x10^4$, $1x10^4$, $5x10^3$ or $1x10^3$ CFU. In all the groups, no clear effect of infection with TIGR4 could be observed on body weight (Figure 3), and no symptoms of disease were seen in challenged animals.

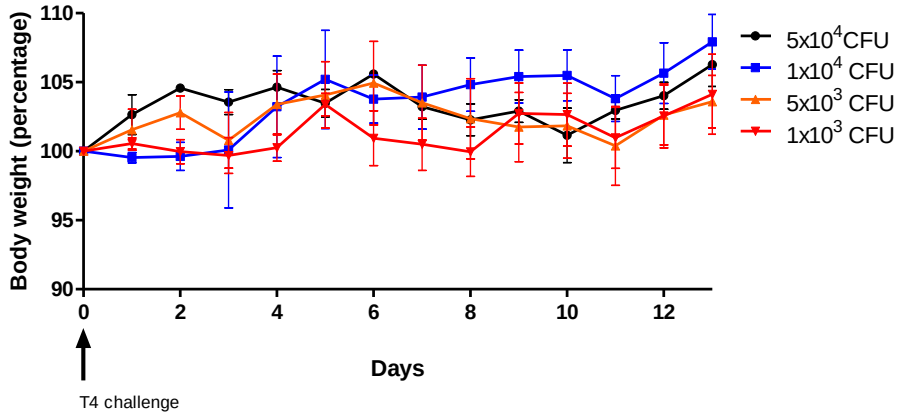

Figure 3. Average Body weights of TIGR4 challenged mice. Groups of mice (n=4) ($5x10^4$ & $1x10^4$) (n=5) ($5x10^3$ & $1x10^3$) were challenged with different amounts of CFU TIGR4. None of the groups showed weight loss. Data is plotted as mean, error bars indicate SEM

IgG titers and CFU in the blood and lungs of TIGR4 challenged mice

No IgG response was observed in *S. pneumoniae* challenged mice (data not shown). To confirm mice were not sick, bacteria in the blood were analyzed. None of the mice challenged with TIGR4 showed bacteria in the blood (data not shown). This result was expected, since the mice did not show any weight loss. To test whether TIGR4 was still present in the lungs 14 days after challenge, bacterial titers were examined in the lungs of TIGR4 challenged mice. Interestingly, all mice except 1 did show a TIGR4 titer in the lung (Figure 4). This result shows that the challenge was successful in minimal 17 out of 18 mice. The presence of TIGR4 is the lungs indicates that TIGR4 can be present in the lungs, without giving signs of disease.

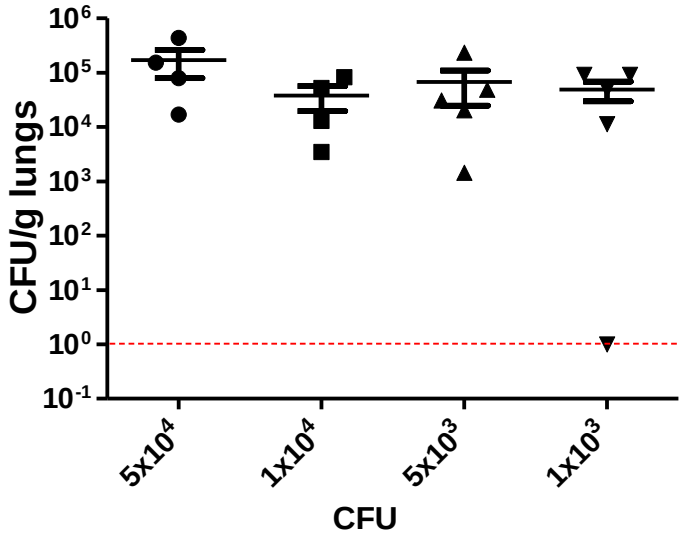

Figure 4. CFU/g TIGR4 in lungs of TIGR4 challenged mice. The red line represents the detection limit

Secondary infection

Body weight of secondary Infected mice

For the setup of the secondary infection model, we decided not to pick the lowest dose of influenza virus (3 PFU) which resulted in a little bit of loss weight, but we choose 6 PFU, what resulted in a large drop in body weight in half of the mice. For the TIGR4, we picked the 3 highest doses we tested in the titration setup ($5x10^3$, $1x10^4$ and $5x10^4$), because not one of the doses gave symptoms of disease. TIGR4 challenge was performed 7 days after influenza challenge. All mice survived the initial 7 days between infections.

In this experiment there were 2 control groups: 1 group challenged with PR8 and 7 days later with PBS, and 1 group challenged with PBS and 7 days later with TIGR5. In the control group challenged with PR8 - PBS, weight loss was observed (Figure 5A). This was expected because mice in the titration experiment challenged with 6 PFU also showed weight loss. In the other control group, PBS - TIGR, no effects on body weight could be observed (Figure 5B). In the groups infected with $5x10^3$ CFU TIGR4, body weight was decreasing till 2 days after challenge, compared with control group PR8 - PBS (Figure 5C). After 2 days of TIGR4 challenge, mice stopped losing more weight. Surprisingly, no obvious effect on body weight could be observed in mice challenged with $1x10^4$ CFU TIGR4 (Figure 5D). Mice challenged with the highest dose of TIGR, $5x10^4$ CFU (n=15) showed the biggest drop in weight, in comparison with control group PR8 - PBS (Figure 5E).

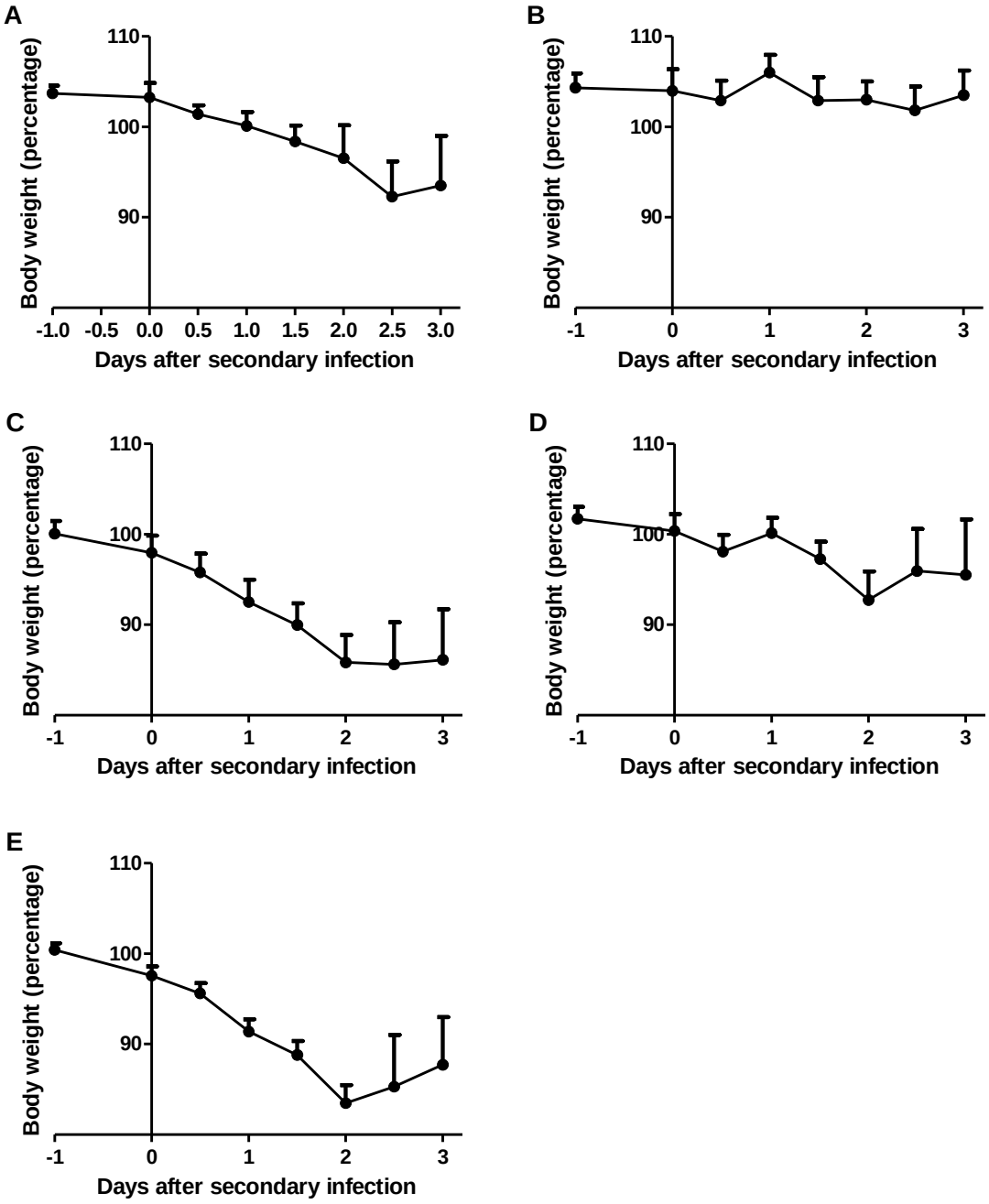

Figure 5. Body weight of and control groups (A&B n=5, 4) and secondary challenged mice (C, D, E) n=11, 15,15). 5 mice were sacrificed after 1, 2 and 3 days. Data is plotted as mean, error bars indicate SEM

IgG titers and CFU in the blood and the lungs of secondary infected mice

To confirm all mice responded against PR8, IgG titers were measured. Not all mice did show an IgG titer (Figure 6A). 8 of the 41 secondary challenged mice did not show an IgG titer against PR8. As expected, control mice that were challenged with PBS- TIGR4 did not show an IgG titer. No correlation could be observed between IgG titer and body weight. Time point of sacrifice did not correlate with IgG titer.

To check whether mice secondary challenged showed bacteremia, CFU of TIGR4 in the blood were analyzed. 26 out of 41 secondary challenged mice showed CFU TIGR4 in the blood (Figure 6B), which indicated that mice have bacteremia. Both control groups did not show bacteria in the blood. 15 of

the 41 secondary challenged mice did not have CFU in the blood. Interestingly, the 8 mice which did not show an IgG titer, all did not show CFU in the blood, but no significant correlation could be observed. Time point of sacrifice did not correlate with CFU TIGR 4 in the blood

Since the mice that were challenged with only TIGR4 in the titration experiment already showed CFU in the lungs, it was expected that all secondary infected animals showed a lung titer. All secondary infected mice indeed showed presence of bacteria in the lungs (Figure 6C). Surprisingly, PR8 - PBS controls did show different kind of strains bacteria than TIGR4 in the lungs, but diagnostics could not identify which bacteria strains were present in these samples. Time point of sacrifice did not correlate with CFU TIGR 4 in the lungs. Furthermore, less TIGR4 seemed to be present in the lungs of TIGR4 - PBS control group than in the secondary infected groups of mice, but no significant difference could be observed.

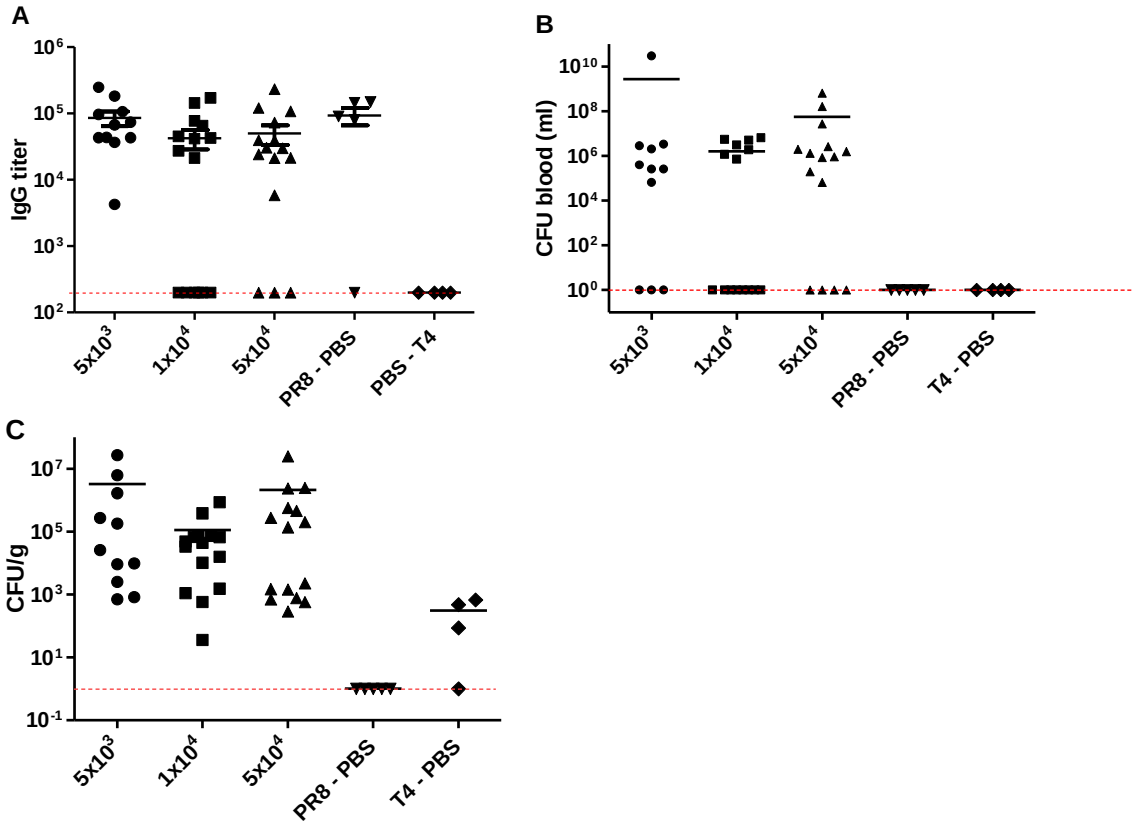

Figure 6. IgG titers of secondary infected mice (A), CFU per ml blood of secondary infected mice (B), and CFU/g in lung of secondary infected mice (C).

Discussion

Secondary infections with *S. pneumoniae* are responsible for a great number of hospitalization and mortality each year, despite the vaccination against both influenza and *S. pneumoniae*[2]. In the future, we want to investigate whether combi-vaccination against both influenza and *S. pneumoniae* can be an important strategy against secondary infections. Before we can test this hypothesis, a model for a secondary infection with PR8 and TIGR4 had to be set up. We hypothesized that a non lethal doses of PR8 and TIGR4, which does not give symptoms when challenged separately, will be lethal for the mice when they are combined.

In this study, we developed a model for secondary infection with influenza strain PR8 and *S. pneumoniae* strain TIGR4. In this model, a high mortality was seen after secondary infection, which is also seen in epidemiological studies. In accordance with studies about secondary infection by McCullers and Iverson[17,37], who developed a secondary for both *S. pneumoniae* and *S. aureus*, our model showed an increase in both morbidity and mortality after secondary infection. The difference

with these studies is that in the model of secondary infection created by Mccullers and Iverson, the primary infection could also be lethal for the mouse. In this study, we set up a model in which a combination of 2 non lethal doses would lead to lethality.

The titration of PR8 resulted in some unexpected results. The 4 highest doses of PR8 (100, 50, 25, 12,5 PFU) all led to severe weight loss. This result suggests that the virus used, PR8, is very virulent, or the mice used are extremely susceptible to the virus. We had to add 2 more groups (3 and 6 PFU), and even in the 6 PFU groups, half of the mice showed a big drop in weight. In the 3 PFU PR8 challenged group, a small drop of body weight was observed. Because it might be unlikely that a challenge with only 6 PFU leads to severe symptoms of disease, I suggest to recheck the amount of PFU in the virus batch that is used for challenge. If the amount of PFU is higher than assumed, results are more reliable, and it might be better to use the 3 PFU group. If the amount of PFU is right, I suggest to use another type of virus which is less virulent, and so can be inoculated in a more reliable and higher dose of PFU, without causing symptoms of disease. For example, in the study about secondary infection done by Lee et al. with PR8 and *H. influenzae*, 30 PFU of PR8 were used to infect mice. The dose of 30 PFU PR8 in this study was not lethal when mice were challenged with 30 PFU alone.

To be sure that the mice had been infected by the virus IgG antibodies were measured in the serum of PR8 challenged mice. Mice challenged with both 3 and 6 PFU PR8, were sacrificed after 28 days. Both groups showed an IgG response against PR8, and thus were challenged correctly. Furthermore, we checked whether the virus was still present in the mice after 28 days. After 28 days, no virus was present in the lungs of the challenged mice, which indicated that mice cleared the infection. Unfortunately, virus titers in the lungs of the mice challenged with 100, 50 , 25 and 12,5 PFU could not be measured. I expect that these mice show a very high titer of influenza in the lungs, because mice had to be sacrificed because they had reached their human endpoint after 5 and 8 days.

In the titration experiment of TIGR4 mice were challenged with 4 doses of CFU TIGR4 ($5x10^4$, $1x10^4$, $5x10^3$ and $1x10^3$). None of the mice did show a decrease in body weight or symptoms of disease, what was expected. No IgG titers were observed, probably because the TIGR4 has a capsule which consist of polysaccharides. MHC class 2 cannot present polysaccharides, since it can only present proteins, and that is why antibodies against TIGR4 are made without help from helper T cells. The T cell independent antibody response consists mainly of IgM, with limited isotype switching to some IgG subtypes, and the antibodies produced normally have a low affinity[39,40]. Some T-cell independent non-protein antigens do induce Ig isotypes other than IgM. In humans, the polysaccharides of *S. pneumoniae* can induce an IgG2 response in humans, (which resembles IgG3 in mouse)[39]. It might be that the mice showed a very low IgG3 titer below 20, which was below the detection limit of the IgG ELISA against TIGR4. Unfortunately, IgM responses of mice challenged with TIGR4 could not be measured, so we could not be certain that the mice made an antibody response against the virus. With these results we could not be sure mice were correctly challenged. The presence of bacteria in the lungs of these mice shows that mice were challenged correctly. Only 1 mice did not show presence of bacteria in the lungs. Since mice did not show symptoms of disease, I conclude that mice do not have to be sick when bacteria are present in the lungs. *S. pneumoniae* is a mostly commensal present in the upper respiratory tract of lungs in human, without causing complications. When the bacteria colonize beyond their normal niche, they infect the sterile parts of the respiratory tract or the blood and this can have serious complications[41]. Furthermore, all the mice challenged with TIGR4 did not show CFU in the blood. Taken together, this data of the titration of the TIGR4 challenge indicates that mice do not get symptoms of disease after intranasal challenge with the used doses of CFU TIGR4. Because mice did not show any symptoms of disease, we decided to pick the 3 highest doses of CFU TIGR4 for the secondary infection model.

We choose to secondary infect the mice with TIGR4, 7 days after PR8 challenge, because in literature it was described that mice were the most susceptible after 7 days for a secondary infection[17]. Most of the mice which were secondary challenged showed severe weight loss. Surprisingly, the mice secondary challenged with the middle dose ($1x10^4$) of TIGR4 showed the least weight loss of all groups. The groups of mice challenged with the lowest and the highest dose of TIGR4 showed a big

drop in weight 1 and 2 days after secondary infection. None of the mice in the control groups, infected with either PR8 or TIGR4, did reach their humane endpoint in 3 days. The mice challenged with only PR8 did show a decrease in body weight, what was expected because this pattern was already seen in the titration experiment of PR8. As expected, mice challenged with only TIGR4 did not show any symptoms of disease. The drop in body weight of the secondary challenged mice groups challenged with $5x10^3$ and $5x10^4$ CFU TIGR4 in comparison with the control groups indicates that in most of these mice the secondary infection model works.

Surprisingly, 8 out of 41 secondary challenged mice, did not show an IgG response against PR8. This suggests that these mice, were not correctly challenged with PR8, or mice did not respond against the virus. This might explain the fact that mice challenged with the middle dose of TIGR4 ($1x10^4$) did not show a clear drop in body weight as the other 2 secondary infected groups, since the immune system of these mice might not be primed for a secondary infection. All the 8 mice which did not show IgG in the blood, did not have bacteria in the blood. I speculate that the PR8 did not clear the road for the TIGR4 in the mice which did not show body weight loss, what means the immune system was not desensitized for TIGR4 to colonize behind their normal niche. Of the 8 mice which did not show an IgG response, 6 mice were in the group challenged with $1x10^4$ CFU TIGR4, the group in which the least decrease in body weight was seen. On the other hand, there were also mice which produced IgG, without bacteria in the blood. It might be that in these mice the IgG production was sufficient to prevent that bacteria could enter the bloodstream.

As expected, most of the mice (26 out of 41) showed CFU of TIGR4 in the blood, which indicated mice are very sick. The data of the CFU TIGR4 in the lungs, indicated that the mice were correctly challenged with TIGR4, since all the mice secondary infected show CFU TIGR4 in the lungs. Surprisingly, a remarkable result was seen in the lungs of mice only challenged with PR8. These mice did show CFU in the lungs, but strangely enough these were not TIGR4. Also, the weight of these mice did not decrease as much as secondary infected mice, what suggest that it might be contamination. Since mice might be more vulnerable for other (harmless) bacteria strain after a primary infection with influenza, it might be a different species of bacteria that colonized in the lung. In our model of secondary infection, TIGR4 was not already present as a commensal in the lungs, but it might be that in human the bacteria that induce a secondary infection is already present in the lungs for a longer time. When mice are primary infected with *S. pneumoniae* and then by influenza, it does not have such a devastating effect on the mice then when influenza is the primary infection[17]. This might indicate that a secondary infection is not caused by bacteria that are already present in the lungs when a primary infection occurs.

With this model of secondary infection, we want to test whether combi-vaccination against influenza and *S. pneumoniae* can be an important strategy to prevent secondary infections. Several studies already concluded that vaccination against influenza in humans, decrease the risk for infection by a secondary infection, what seems to be legitimate, thinking of the proposed synergy between influenza and *S. pneumoniae*. To our knowledge, no combi-vaccination study against influenza and *S. pneumoniae* has been done in mice, and our plans are to use different formulations of combi-vaccines.

In conclusion, our results show that mice are more vulnerable for *S. pneumoniae* strain TIGR4, when challenged 7 days before with influenza strain PR8. This shows that our secondary infection model works, so the road is open for testing different types of combi-vaccination. When an effective combi-vaccine can be developed, it can reduce both morbidity and mortality caused by secondary infections. Furthermore, this model can be used for investigating the mechanisms of synergism between influenza *and S. pneumoniae,* what might help in understanding the mechanisms of secondary infection and so might help in prevention of secondary infections. When more is known about the mechanisms in which influenza clears the road for a secondary infection, it might be possible to design new therapeutic treatment strategies to reduce the impact of secondary infections. In the end, this project will hopefully contribute to the development of alternative prevention strategies for the occurrence of lethal secondary bacterial infections after influenza infection.

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