PHiD-CV induces anti-Protein D antibodies but does not augment pulmonary clearance of nontypeable *Haemophilus influenzae in mice*

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Abstract

Background

A recently-licensed 10-valent pneumococcal conjugate vaccine (PHiD-CV; Synflorix, GSK) uses Protein D from *Haemophilus influenzae* as a carrier protein. PHiD-CV therefore has the potential to provide additional protection against nontypeable *H. influenzae* (NTHi). NTHi frequently causes respiratory tract infections and is associated with significant morbidity and mortality worldwide and there is currently no vaccine.

Methods

We developed mouse models of NTHi infection and influenza/NTHi superinfection. Mice were immunized with PHiD-CV, heat-killed NTHi, or a 13-valent pneumococcal conjugate vaccine that did not contain Protein D (PCV13; Prevenar, Pfizer) and then infected intranasally with NTHi.

Results

Infection with NTHi resulted in weight loss, inflammation and airway neutrophilia. In a superinfection model, prior infection with pandemic H1N1 influenza virus (strain A/England/195/2009) augmented NTHi infection severity, even with a lower bacterial challenge dose. Immunization with PHiD-CV produced high levels of antibodies that were specific against Protein D, but not heat-killed NTHi. Immunization with PHiD-CV led to a slight reduction in bacterial load, but no change in disease outcome.

Conclusions

PHiD-CV induced high levels of Protein D-specific antibodies, but did not augment pulmonary clearance of NTHi. We found no evidence to suggest that PHiD-CV will offer added benefit by preventing NTHi lung infection.

Introduction

Nontypeable *Haemophilus influenzae* (NTHi) is a common coloniser of the human nasopharynx [1], and frequently causes respiratory tract infections, including otitis media and sinusitis [2]. Occasionally, NTHi can cause serious invasive infections, including septicaemia and meningitis, particularly in individuals with underlying co-morbidities, and is associated with significant case-fatality [3, 4]. However, the main burden of disease with NTHi is pneumonia [5] and although data on the aetiology of non-bacteraemic pneumonia are limited, numerous studies have identified NTHi in lung aspirates and sputum of patients with pneumonia [6, 7]. Depending on geographical area, NTHi is either the second [8] or third [9] most common cause of community acquired pneumonia after *Streptococcus pneumoniae* and *Mycoplasma pneumoniae*. NTHi disease is most frequently associated with viral infection [10] presenting as secondary or superinfections which increase disease severity and lead to increased mortality [11].

NTHi also plays a major role in exacerbations of chronic respiratory conditions, including chronic suppurative lung disease (CSLD) and bronchiectasis in children [12, 13] and chronic obstructive pulmonary disease (COPD) in the elderly [14]. Treatments that reduce the load of bacteria, including NTHi, can improve symptoms and reduce long-term lung damage [15]. However, in the absence of a licensed vaccine for NTHi [16], this is currently only possible through undesirable, prolonged multiple courses of broad-spectrum antibiotics, which are associated with significant adverse effects, including the development of antibiotic resistance [17].

The recently licensed 10-valent pneumococcal conjugate vaccine (Synflorix, GSK Biologicals) (PHiD-CV) uses *H. influenzae* Protein D as its carrier protein for 8 of the 10 pneumococcal serotypes and therefore offers a potential advantage in that it might also

prevent NTHi infections. NTHi pneumonia is the most severe manifestation of the respiratory tract infection spectrum of NTHi and much more common than invasive NTHi, therefore prevention of NTHi pneumonia should be a key goal of any NTHi vaccine. The difficulties in identifying the aetiology of non-bacteraemic pneumonia in patients mean that animal modelling is useful in determining vaccine efficacy against NTHi lung infection. If PHiD-CV was efficacious against NTHi pneumonia, it could protect millions of children and would also become an attractive alternative to the pneumococcal polysaccharide vaccine (Pneumovax 23, Merck Vaccines) (PPV23). PPV23 is routinely offered to older adults (≥65- year olds) and high-risk individuals in many countries, despite having limited protective efficacy [18]. An NTHi vaccine may also benefit older adults, who are at increased risk of NTHi infections, through establishing herd immunity, as observed following the introduction of the *Haemophilus influenzae B* (HiB) vaccine [19].

Protein D is a highly-conserved, surface-exposed 42 kDa outer membrane lipoprotein found in all *H. influenzae* strains, making it a promising vaccine candidate antigen [20]. Studies of the protective efficacy of PHiD-CV against NTHi have thus far focused on otitis media, with research into lung infections being somewhat neglected. Rats immunised with Protein D produced high titres of serum antibody against Protein D, clearing NTHi after middle ear bacterial challenge [20] and chinchillas vaccinated with Protein D showed significant protection against NTHi otitis media [21]. Passive injection of anti-Protein D antibody reduced the incidence of middle ear effusion in the chinchilla model [20]. Vaccination of human volunteers with PHiD-CV induces antibodies specific to Protein D [22, 23] and the 11-valent precursor vaccine was estimated to prevent 35% of NTHi acute otitis media episodes [24].

The aim of the current study was to determine whether the Protein D in PHiD-CV can protect against NTHi lung infection. To test this, we developed two mouse models of NTHi lung infection (with and without prior influenza infection). We immunized mice with PHiD-CV, PCV13 (which does not contain Protein D) or heat-killed (HK) NTHi, and subsequently infected with NTHi alone or with H1N1 influenza virus followed by NTHi. We observed high levels of antibody against Protein D in mice immunized with PHiD-CV, but did not increase clearance of pulmonary NTHi.

Materials and Methods

NTHi

NTHi strain 162 was isolated by the Finnish Otitis Media Study Group [25] and encodes Protein D in its genome. Strain 162 is a representative of a major clade of NTHI causing otitis media, and in the Chinchilla model of infection causes purulent middle ear disease with a high density of microorganisms that persists for 3 weeks [26]. Bacteria were grown at 37°C to OD_{600} 0.5 in brain heart infusion (BHI) broth (Difco, BD, Oxford, UK) (3.7%, w/v) supplemented with nicotinamide adenine dinucleotide (NAD; 10 μg/mL) and hemin (10 μg/mL) and washed in PBS prior to use. For enumeration, NTHi was grown on BHI agar (Difco) supplemented with 5% heat-inactivated defibrinated horse blood (Oxoid, Basingstoke, UK) in 5% CO_2 at 37°C. Enumeration of NTHi from mouse samples was performed by serial dilution in 5% BHI in PBS and plating on BHI-blood agar as above.

Influenza

H1N1 Influenza (strain A/England/195/2009) was grown in Madin-Darby Canine Kidney (MDCK) cells, in serum free DMEM supplemented with 1 μg/mL trypsin. H1N1 Influenza strain (A/England/195/2009) was isolated by Public Health England, in the UK in April 2009 [27]. The virus was harvested 3 days after inoculation and stored at -80°C. Viral titre and lung viral load determined by plaque assay as previously described [28].

Mice immunizations and infection

6–8 week old female BALB/c mice were obtained from Harlan UK Ltd (Speke Hall, Liverpool, UK) and kept in specific-pathogen-free conditions in accordance with the United Kingdom's Home Office guidelines and all work was approved by the Ethical Review Board at Imperial College London. Mice were immunized intramuscularly (i.m.)

with 50 µL of PHiD-CV (Lot ASPNA189CG, Synflorix, GSK, Rixart, Belgium), PCV-13 (Lot F4133, Prevenar 13, Merck, UK), 10^7 CFU of heat-killed (72°C for 1 hour) NTHi 162, or PBS. This volume is 1/10^{th} of the human dose. A boost immunization was performed at day 21. For infections**,** mice were anesthetized using isoflurane and infected intranasally (i.n.) with 10^7 CFU of log phase NTHi 162 in a volume of 100 µL 5% BHI in PBS (used to maximize viability of NTHi). For superinfection experiments, mice were first infected with 5 x 10^4 or 10^5 PFU of influenza A H1N1 followed 7 days later by 10^5 or 10^7 CFU of log phase NTHi 162. Mock infection was performed using 5% BHI in PBS or PBS alone.

Tissue and cell recovery and isolation

Mice were culled using 100 µL intraperitoneal pentobarbitone (20 mg dose, Pentoject, Animalcare Ltd. UK) and tissues collected. Blood was collected from carotid vessels and sera isolated after clotting by centrifugation. Bronchoalveolar lavage (BAL) was obtained by inflating the lungs via an intratracheal cannula with 5% BHI in PBS. Lungs were removed and homogenized by passage through 100 µm cell strainers and centrifugation at 200 x *g* for 5 minutes. Aliquots were removed and serially diluted for bacterial enumeration and the remainder treated with red blood cell lysis buffer (ACK; 0.15 M ammonium chloride, 1 M potassium hydrogen carbonate, and 0.01 mM EDTA, pH 7.2) before centrifugation at 200 x *g* for 5 minutes. The remaining cells were resuspended in RPMI 1640 medium with 10% fetal calf serum and viable cell numbers determined by trypan blue exclusion. For differential cell counts, 100 µL of cells from BAL and the lung homogenate were centrifuged onto glass slides. Samples were air dried, and fixed in methanol, before staining of with haematoxylin and eosin.

Cytokine ELISA

CXCL1/KC and IL-6 were assessed in BAL by ELISA (R&D systems) following manufacturer's instructions.

Semi-quantitative antigen specific ELISA

Serum antibodies specific to Protein D or whole-cell NTHi were measured using a standardized ELISA. MaxiSorp 96-well plates (Nunc) were coated with 100 µL of formaldehyde-fixed whole-cell NTHi in PBS (50 µg/mL), or recombinant Protein D in PBS (1 µg/mL) (GSK Biologicals, Rixart, Belgium), or a combination of anti-murine lambda and kappa light chain specific antibodies (AbDSerotec, Oxford, UK) and incubated overnight at 4°C. Plates were blocked with 1% BSA in PBS. Bound IgG was detected with HRP-conjugated goat anti-mouse IgG (AbD Serotec). A dilution series of recombinant murine IgG was used as a standard to quantify specific antibodies. TMB with H_2SO_4 stop was used to detect the response and optical densities read at 450 nm.

Statistical Analysis

Comparisons of two groups were performed using Student's *t* tests. Comparisons of multiple groups were performed using one- or two-way ANOVA with appropriate post-tests. All statistical tests were performed using GraphPad Prism version 6.01 for Windows (GraphPad Software, San Diego California USA).

Results

Characterization of NTHi infection

BALB/c mice were intranasally infected with 10^7 CFU of NTHi strain 162 in 100 µL. Weight loss was apparent after 24 hours and infected mice showed a significant reduction in weight compared to controls up to 72 hours after infection (p<0.01, Fig.1A). At two hours after infection, a bacterial load of ~5 x10^6 CFU was recoverable from both the BAL and lungs (Fig.1B and C). After 24 hours, the concentration of viable bacteria had fallen ~100-fold in the BAL and ~50-fold in the lungs. By 48 hours, ~5 x 10^2 CFU were remaining in both the BAL and lungs, no viable NTHi were detected by 72 hours. Airway cellular infiltration was observed after 24 hours and peaked at 48 hours (p<0.001, Fig.1D); at 72 hours, cell numbers had begun to decrease. In the lungs, cellular infiltration occurred more rapidly and persisted for longer than in BAL, with a significant increase in cell counts from 2 hours which was maintained for the entire time course of 72 hours (p<0.001, Fig.1E). Differential cell counts from BAL identified a prominent airway neutrophilia, though increases in lymphocytes and macrophages were also observed (Fig.1F). In addition to the cellular markers of inflammation the pro-inflammatory cytokine interleukin-6 (IL-6) (Fig.1G) and the neutrophil chemoattractant CXCL1 were detected (Fig.1H) in the BAL of infected animals. Levels of IL-6 and CXCL1 in the BAL correlate well with CFU load and are rapidly reduced to levels below detection upon clearance of bacteria, with an acute peak of both mediators at 2 hours after infection and there were no detectable cytokines in the airway by 72 hours after infection.

Antibody responses to immunization

BALB/c mice were intramuscularly immunised with PHiD-CV, PCV13, or 10^7 CFU of heat-killed NTHi and boosted with the same immunogen at day 21. IgG responses in

sera collected at day 35 to recombinant Protein D or whole-cell NTHi were determined by ELISA. Immunization with PHiD-CV resulted in a high titre of Protein D specific IgG (p<0.001, Fig.2A). No other immunizations induced detectable antibodies against Protein D. Sera from mice immunized with heat-killed NTHi, but no other groups, had high titres of IgG against whole-cell NTHi (p<0.05, Fig.2B).

PHiD-CV does not augment pulmonary NTHi clearance

BALB/c mice were intramuscularly immunised with PHiD-CV, PCV13, or 10^7 CFU of heat-killed NTHi and boosted with the same immunogen at day 21 and infected with 10^7 CFU NTHi. To screen for augmented clearance we measured NTHi in BAL at 24 and 48 hours after infection. No difference was seen between the immunized, control immunized and non-immunized groups (Fig.3A). We wished to determine whether there was an effect on disease rather than only bacterial clearance and so focussed on 48 hours after infection, the peak of weight loss and cellular infiltration. 48 hours after infection, lower bacterial loads were observed for all the immunized groups compared to the non-immunized control group in the BAL (p<0.05, Fig.3B) and in the lung (p<0.001, Fig.3C), but there was no significant difference between the PHiD-CV and the PCV13 or HK-NTHi immunized groups. In the lung, both the PCV13 and HK-NTHi groups had a lower bacterial load than the non-immunized groups. However, these changes in bacterial load did not lead to a significant change in disease outcome. There was no significant difference in weight loss between the infected groups at either 24 or 48 hours after infection (Fig.3D). Cellular infiltration in the BAL was similar for all infected groups (Fig.3E), but the HK-NTHi immunized group had a significantly lower lung cell count at 48 hours than the PCV13 or non-immunized groups (Fig.3F). Differential counts indicated prominent airway neutrophilia in all infected groups (Fig.3G), levels of IL-6 (p<0.01, Fig.3H) and CXCL1 (p<0.01, Fig.3I) were significantly increased compared to

uninfected controls. Immunization with PHiD-CV therefore does little to augment bacterial clearance or reduce disease following infection with NTHi alone.

Development of a viral-bacterial lung superinfection model

Since most NTHi infections occur after viral infection, we developed a viral-bacterial superinfection model to assess the protective effect of PHiD-CV in the context of prior viral infection. Seven days after primary infection with influenza A H1N1 virus (strain A/England/195/2009), mice were infected with NTHi. Infection with either a high (10^5 PFU) or low (5×10^4 PFU) dose of influenza resulted in weight loss from day 3 after infection (Fig.4A). Secondary infection with either a high (10^7) or low (10^5) CFU of NTHi led to further weight loss compared with influenza infection alone ($p<0.05$). The group that received the high dose influenza followed by the high dose NTHi was culled at 24 hours after NTHi infection because the animals were approaching the humane endpoint and as such bacterial load or cell data was not collected. NTHi superinfection led to a significantly increased cellular recruitment to lungs compared to influenza infection alone ($p<0.001$, Fig.4B). Prior infection with influenza led to a significantly greater recovery of bacteria ($p<0.05$) compared to NTHi infection alone. Strikingly, influenza infection enabled NTHi infection with a 100 fold lower dose of bacteria and led to a significantly greater bacterial load than NTHi alone even when the higher dose of NTHi was used ($p<0.05$). Infection with 10^5 CFU NTHi alone caused a very mild illness, compared to infection with the same bacterial inoculum after influenza infection. Mice infected with 10^5 CFU NTHi showed a 5-fold lower airway cellular infiltration and a 10-fold lower bacterial load than groups infected with influenza and NTHi ($p<0.05$). Whilst influenza infection alone results in inflammation, superinfection with NTHi exasperates overall disease and prior influenza infection diminishes the ability of mice to clear NTHi.

253 **Immunization fails to protect against NTHi superinfection**

254 A superinfection protocol using 5 x 10^4 PFU influenza and 10^5 CFU NTHi was selected
255 for assessment of protection by PHiD-CV. These doses were chosen as they model a
256 typical opportunistic NTHi infection in which immunity must be compromised to establish
257 disease, and, importantly, use a low dose for bacterial infection. No significant
258 differences in weight change after NTHi-infection were observed between the differently
259 immunized groups (Fig.5A). Airway bacterial loads (Fig.5B and C) and cell infiltration
260 (Fig.5E and F) at 48 hours after NTHi infection demonstrated no evidence of protection.
261 Airway neutrophilia was present and did not significantly differ between groups (Fig.5F),
262 neither did IL-6 (Fig.5G) or CXCL1 (Fig.5H) levels in the BAL.

Discussion

This study aimed to determine whether immunization with PHiD-CV cross-protects against NTHi lung infection. While immunization with PHiD-CV induced high levels of Protein D specific antibodies, the small reduction in bacterial load in the airways and lungs was not sufficient to affect disease outcome. Furthermore, the reduced bacterial load compared to PBS-immunized mice must be interpreted with caution, because the bacterial load in the PHiD-CV immunized mice was not significantly different to immunization with PCV13, which contains no NTHi antigens. PHiD-CV immunization did not protect against disease or reduce bacterial load in the influenza A-NTHi superinfection model. One limitation is that we only tested one dose regime of the vaccine ($1/10^{th}$ human dose), other doses may have had different efficacy, though the dose used is a larger bolus of vaccine per muscle mass than used in humans. Only one strain of NTHi was tested in this study, 162, and it may be that PHiD-CV is protective against other strains, The significant strain heterogeneity is exactly the rationale for assessing PD as a vaccine candidate, as it is highly conserved across NTHi. Therefore, if an effect was observed it would be theoretically be expected for the majority of NTHi strains. NTHi strain 162 is an upper respiratory tract pathogen isolated from a child in Finland [25], it was chosen on account of good growth in media and virulence in mice upon i.n. infection into the lung in our preliminary studies and virulence in other models [26]. Based on the immunisation and challenge regime used in this study we conclude that PHiD-CV immunisation does not reduce bacterial load or disease after NTHi lung infection or superinfection.

Protection against lung infection by PHiD-CV has not been previously investigated. Previous research has focused on the protective efficacy of Protein D against NTHi-induced otitis media. Whilst enhanced pulmonary clearance of NTHi has been reported

with other NTHi immunogens in mice [29], these studies assessed bacterial load at very acute time points after infection (≤6 hours) compared to the 24 and 48 hour timepoints in our study. Longer timepoints have the advantage of allowing assessment of disease itself. As observed in our study, reduced bacterial load had no impact on disease outcome in previous studies [29, 30].

The minimal protection observed against disease in our study reflects the recent clinical trial experience. Although prevention of 35% of NTHi acute otitis media episodes was observed after immunisation with the 11-valent precursor vaccine [24], a recent study observed only a trend towards reduction but no significant effect on NTHi otitis media [31]. Other studies have reported an inconsistent effect on NTHi carriage, despite the induction of high levels of Protein D antibodies [32-34]. Lack of an effect on NTHi carriage is significant as nasopharyngeal colonization is considered a prerequisite to lung infection [35, 36]. The protective role of Protein D antibodies has also been called into question by recent studies reporting that children with a history of recurrent acute otitis media have significantly higher serum IgG concentrations against several NTHi proteins, including Protein D, compared with healthy controls [37].

Why do antibodies against Protein D not protect against disease, in spite of bactericidal activity against NTHi? Firstly, published bactericidal activity was demonstrated in vitro using serum which may not be representative of antibody and complement profiles in the airway [38]. Secondly, antibody raised against the Protein D present in PHiD-CV may not be active against the whole organism [38]. In our assays, antibodies against Protein D did not bind whole-cell NTHi. This lack of efficacy against the whole organism may be due to differences in structure between the vaccine Protein D (a delipidated recombinant protein) and naturally expressed Protein D, which occurs in NTHi as a lipoprotein [39].

Alternatively, antibodies raised by Protein D in PHiD-CV may not be specific for surface-exposed Protein D epitopes on whole NTHi. Though Protein D is a surface exposed protein, it is highly conserved among *H. influenzae* suggesting that it is under little immune pressure [40]. The lack of protection observed may be influenced by the challenge dose used, which though similar to other studies [41, 42], may overwhelm local antibody responses, particularly as pneumonia is probably caused by micro-aspiration of the nasopharyngeal microflora seeding the lung with a relatively small amount of bacteria. However, no protection was observed in our superinfection model which used a two-log lower challenge dose. Furthermore, the infection dose was similar to the reported concentration of NTHi in the nasopharynx of children [34]. It was surprising that the heat killed NTHi failed to protect against subsequent lung infection and this may reflect limitations of the mouse model for lung bacterial infections. The route of immunisation may also have had an impact on the protection; intramuscular immunization, although it generates high levels of antibodies in serum, may not induce sufficiently strong local mucosal responses. As NTHi is a mucosal pathogen, mucosal immunization might be a more effective route for protection [43]. Finally, as observed with other bacterial lung infection models in mice [42, 44], the bacteria were cleared rapidly from the lungs indicating a strong and rapid protective innate response, which may mask any adaptive immune contribution.

Irrespective of the presence or absence of Protein D antibodies, we observed rapid clearance of NTHi from the airways of mice within 72 hours, associated with a prominent neutrophilia. This is consistent with previous literature showing the importance of neutrophils in the clearance of NTHi from the lungs of mice [45]. Innate recognition of NTHi-derived lipooligosaccharide by TLR4 on alveolar macrophages and epithelial cells leads to rapid production of pro-inflammatory cytokines sufficient for clearance of NTHi

from the mouse respiratory tract [46]. There was no difference in disease severity between immunized and non-immunized mice and only a limited difference in clearance of NTHi, suggesting that the innate inflammatory response was the major factor in eliminating NTHi. Greater inflammation was associated with accelerated bacterial clearance in a model of cigarette smoke exposure and bacterial challenge [47] and COPD patients have an impaired innate response, which may contribute to their susceptibility to bacterial lung infection [48] supporting the importance of the innate response in controlling NTHi.

The precise mechanisms by which colonisation leads to disease are not well-understood, but intercurrent or prior viral infection has been postulated to promote local invasion [2]. Previous studies have demonstrated that prior infection with either rhinovirus (RV) [49], or respiratory syncytial virus (RSV) [50], increases NTHi bacterial load in the lungs and upper airways. Here, we show for the first time that infection with influenza increases bacterial load and disease severity following NTHi infection. Severe viral infection is hypothesized to dampen the innate response and facilitate bacterial infection and we observed impaired NTHi clearance after viral infection. If the innate response is a major requirement for clearing NTHi lung infection, then the induction of a strong antibody response would likely have little additional protective effect. It is possible that antibody-dependent bacterial killing may be more important for preventing invasive disease, while prevention of pneumonia predominantly involves antibody-independent mechanisms.

Overall, the results indicate that PHiD-CV provides minimal protection against NTHi infection in an experimental mouse model, despite induction of high levels of antibodies against Protein D. There are limits translating these findings from the mouse model to

367 humans, but, in combination with the lack of a clear protective effect against carriage in
368 recent clinical trials, we believe that PHiD-CV is unlikely to offer any additional benefit
369 against NTHi pneumonia.

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Competing interests

The authors either do not have a commercial or other association that might pose a conflict of interest. GSK made no financial contribution to this study.

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543

Figure Legends

Figure 1. Timecourse of NTHi lung infection. Mice were infected i.n. with 100µL volume of 10^7 CFU NTHi (filled circles) or mock-infected with 5% BHI in PBS (open circles) and weights measured at indicated timepoints (A). CFU/mL counts were determined from BAL (B) and the lungs (C). Cellular infiltration was measured in BAL (D) and the lungs (E). Differential counts identified numbers of lymphocytes (filled squares), macrophages (filled diamonds), and neutrophils (filled triangles) in BAL. IL-6 (G) and CXCL1 (H) in BAL measured by ELISA. Points represent individual mice. Lines represent mean values. ** $p<0.01$, *** $p<0.001$, calculated using two-way ANOVA and Sidak's test (A), or one-way ANOVA and Tukey's test (B-E). This was a single experiment.

Figure 2. Serum antibody titres after immunization. Mice were immunized i.m. with a 50 µL volume of PHiD-CV (filled circles), PCV-13 (filled triangles), 10^7 CFU of heat-killed NTHi (filled squares), or PBS (inverted open triangles) and boosted at day 21. IgG titres against recombinant Protein D (A) or whole-cell NTHi (B) were determined at day 35. Points represent individual mice. Lines represent mean values. * $p<0.05$, *** $p<0.001$, calculated using one-way ANOVA and Tukey's test. This is pooled data from 2 experiments.

Figure 3. Effect of immunization on NTHi infection of lungs.
Mice were immunized i.m. with a 50 µL volume of PHiD-CV (filled circles), PCV-13 (filled triangles), 10^7 CFU of heat-killed NTHi (filled squares), or PBS (inverted open triangles) and boosted at day 21 and infected i.n. with 10^7 CFU NTHi on day 35. Uninfected controls received mock-immunization with PBS and mock-infection with 5% BHI in PBS (open circles). CFU/mL counts in BAL (A) were measured 24 and 48 hours after

infection. Mice were immunised under the same regime and CFU/mL counts in BAL (B) and the lungs (C) measured 48 hours after infection. Weight change was monitored (D) and cellular infiltration in BAL (E) and lungs (F) and neutrophil influx in BAL (G), IL-6 (H) and CXCL1 (I) in BAL measured by ELISA at 48 hours after infection. Points represent individual mice. Lines represent mean values. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, calculated using one-way ANOVA and Tukey's test. Panel A is a separate repeat to the data presented in the other panels. This figure shows the data from 2 studies.

Figure 4. Influenza-NTHi superinfection model. Mice were infected i.n. with 100µL volume of 10^5 (red icons) or 5 x 10^4 PFU of Eng/195 H1N1 influenza (blue icons) or mock infected with PBS (black icons), 7 days later mice were infected with 10^7 (circles) or 10^5 CFU of NTHi (inverted triangles) or mock-infected with 5% BHI in PBS (open circles) and weights measured at indicated timepoints (A). Cellular infiltration (B) and CFU/mL counts (C) were determined from BAL 48 hours after infection with NTHi. Points represent individual mice. Lines represent mean values. In panel A ** represents $p<0.01$ between Low flu/High NTHi and Low Flu/No NTHi and # represents $p<0.05$ between Low flu/Low NTHi and Low Flu/No NTHi. In panels B, C, * $p<0.05$, *** $p<0.001$, calculated using one-way ANOVA and Tukey's test. This was a single experiment.

Figure 5. Effect of immunization on Influenza-NTHi superinfection. Mice were immunized i.m. with a 50 µL volume of PHiD-CV (filled circles), PCV-13 (filled triangles), 10^7 CFU of heat-killed NTHi (filled squares), or PBS (inverted open triangles) and boosted at day 21 and infected i.n. with 5 x 10^4 PFU of H1N1 influenza at day 35. 7 days after influenza infection, mice were infected i.n. with 10^7 CFU/mL NTHi. Flu-infected only controls were mock-infected with 5% BHI in PBS at the NTHi infection step (open circles). Weight change was monitored (A). CFU/mL counts in BAL (B) and lungs (C),

596 cellular infiltration in BAL (D) and lungs (E) neutrophil influx in BAL (F) and IL-6 (G) and
597 CXCL1 (H) in BAL were measured 48 hours after infection with NTHi. Points represent
598 individual mice. Lines represent mean values. * $p<0.05$ calculated using one-way
599 ANOVA and Tukey's test. This was a single experiment.

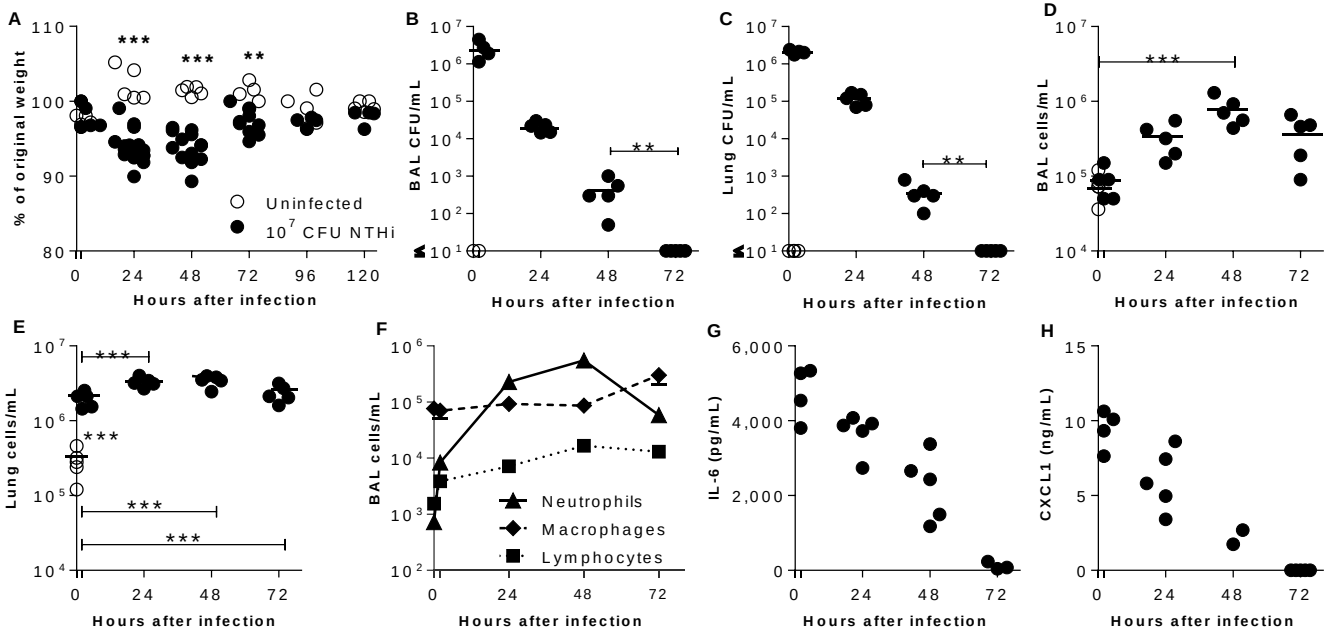

A
% of original weight
Uninfected
10^7 CFU NTHi
Hours after infection
B
BAL CFU/mL
Hours after infection
C
Lung CFU/mL
Hours after infection
D
BAL cells/mL
Hours after infection
E
Lung cells/mL
Hours after infection
F
BAL cells/mL
Neutrophils
Macrophages
Lymphocytes
Hours after infection
G
IL-6 (pg/mL)
Hours after infection
H
CXCL1 (ng/mL)
Hours after infection

Figure 1

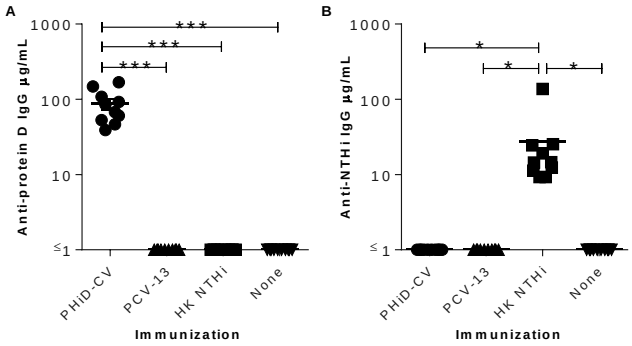

A
1000
100
10
≤1
Anti-protein D IgG µg/mL
***
***
***
PHiD-CV
PCV-13
HK NTHi
None
Immunization
B
1000
100
10
≤1
Anti-NTHi IgG µg/mL
*
*
*
PHiD-CV
PCV-13
HK NTHi
None
Immunization

Figure 2

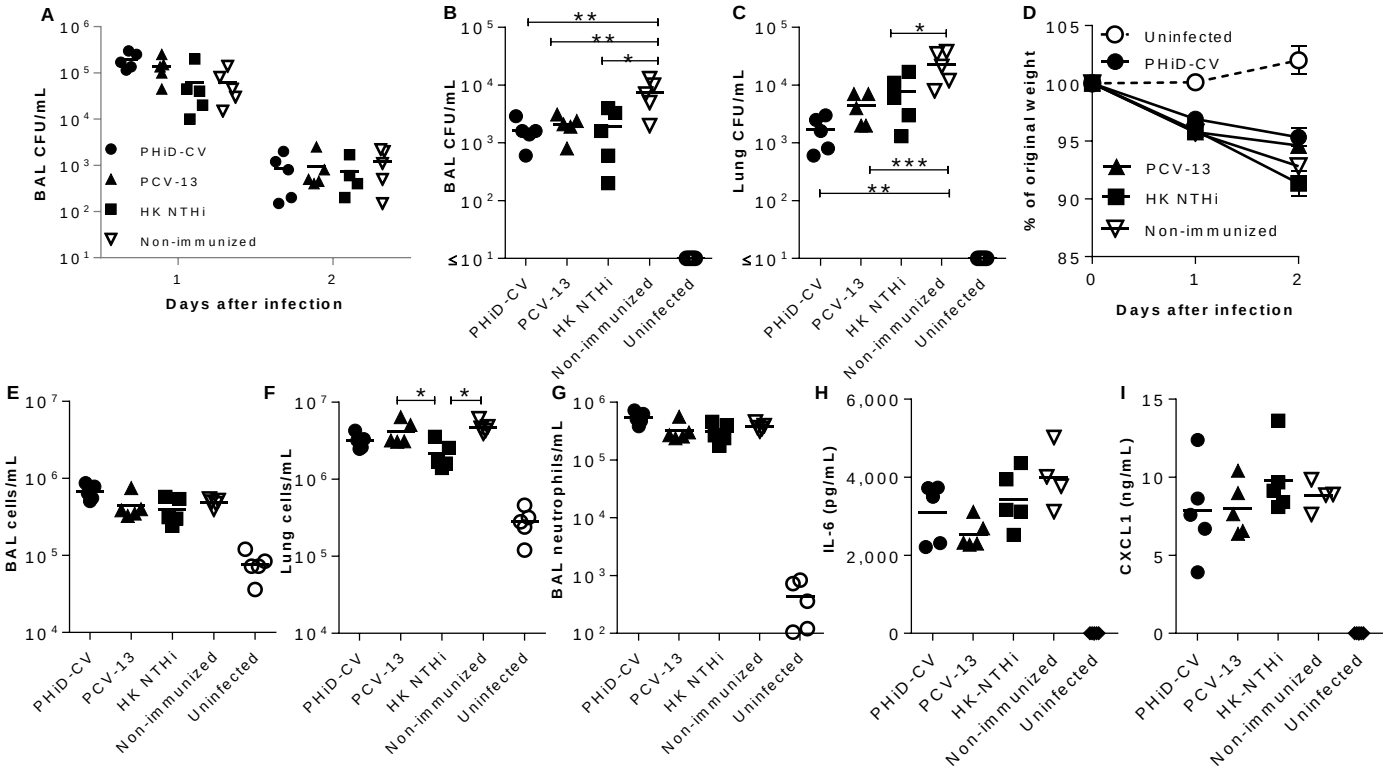

A
BAL CFU/mL
PHiD-CV
PCV-13
HK NTHi
Non-immunized
Days after infection
B
BAL CFU/mL
PHiD-CV
PCV-13
HK NTHi
Non-immunized
Uninfected
C
Lung CFU/mL
D
% of original weight
Uninfected
PHiD-CV
PCV-13
HK NTHi
Non-immunized
Days after infection
E
BAL cells/mL
F
Lung cells/mL
G
BAL neutrophils/mL
H
IL-6 (pg/mL)
I
CXCL1 (ng/mL)

Figure 3

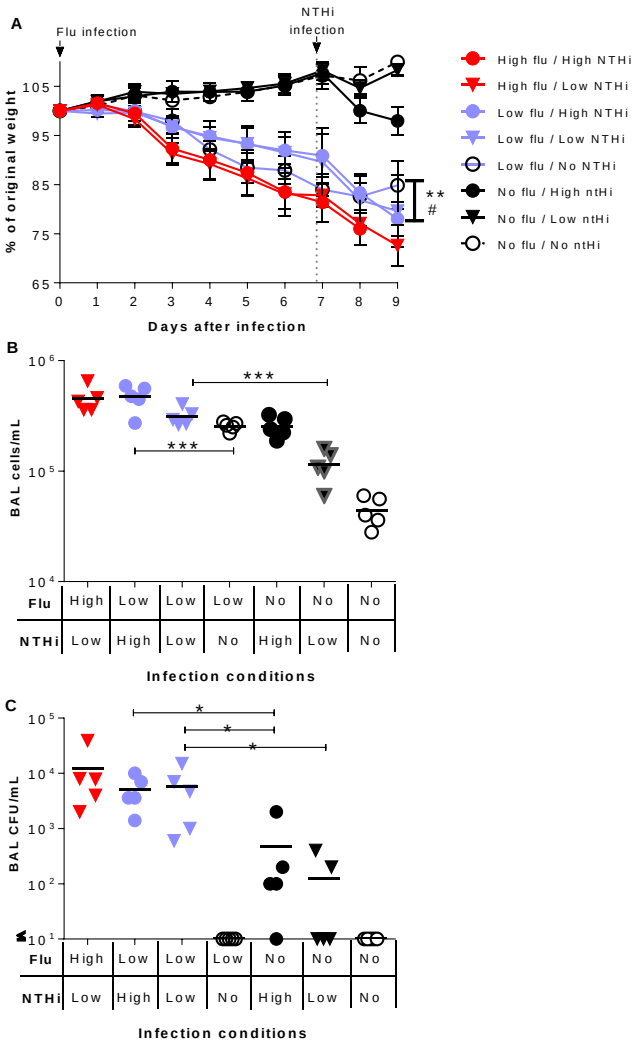

A
Flu infection
NTHi infection
% of original weight
105
95
85
75
65
0 1 2 3 4 5 6 7 8 9
Days after infection
**
#
High flu / High NTHi
High flu / Low NTHi
Low flu / High NTHi
Low flu / Low NTHi
Low flu / No NTHi
No flu / High ntHi
No flu / Low ntHi
No flu / No ntHi
B
BAL cells/mL
10^6
10^5
10^4
***
***
Flu High Low Low Low No No No
NTHi Low High Low No High Low No
Infection conditions
C
BAL CFU/mL
10^5
10^4
10^3
10^2
10^1
*
*
*
Flu High Low Low Low No No No
NTHi Low High Low No High Low No
Infection conditions

Figure 4

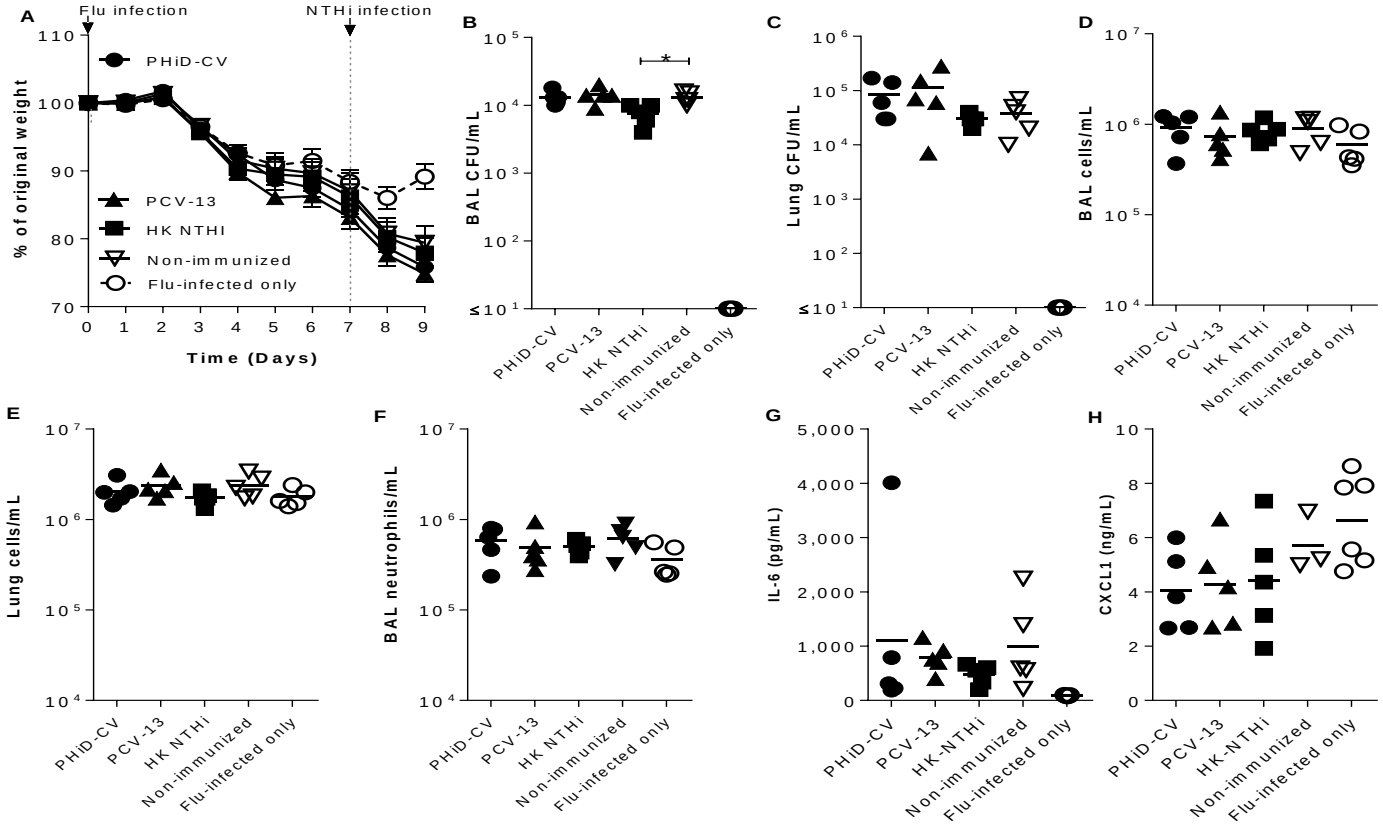

A
Flu infection
NTHi infection
PHiD-CV
PCV-13
HK NTHI
Non-immunized
Flu-infected only
% of original weight
Time (Days)
B
BAL CFU/mL
C
Lung CFU/mL
D
BAL cells/mL
E
Lung cells/mL
F
BAL neutrophils/mL
G
IL-6 (pg/mL)
H
CXCL1 (ng/mL)
PHiD-CV
PCV-13
HK NTHi
HK-NTHI
Non-immunized
Flu-infected only

Figure 5