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Abstract

Nontypeable *Haemophilus influenzae* (NTHi) is commonly isolated from airways patients suffering from chronic respiratory diseases, such as chronic obstructive pulmonary disease (COPD) or cystic fibrosis (CF). However, to what extent NTHi long-term infection contributes to the lung inflammatory burden during chronic airway disease is still controversial.

Here, we exploited human respiratory samples from a small cohort of CF patients and found that patients chronically infected by NTHi had significantly higher levels of IL-8 and CXCL1 than those who were not infected. To better define the impact of chronic NTHi infection in fuelling inflammatory response in chronic lung diseases, we developed a new mouse model using both laboratory and clinical strains. Chronic NTHi infection was associated with chronic inflammation of the lung, characterized by recruitment of neutrophils and cytokine release (KC, MIP-2, G-CFS, IL-6, IL-17A and IL-17F) at 2 and 14 days post-infection. An increased burden of T cell mediated response (CD4$^+$ and γδ cells) and higher levels of matrix metalloproteinase 9, known to be associated with tissue remodelling, were observed at 14 days post-infection. Of note we found that both CD4$^+$IL-17$^+$ cells and levels of IL-17 cytokines were enriched in mice at advanced stage of NTHi chronic infection. Moreover, by immunohistochemistry we found CD3$^+$, B220$^+$ and CXCL-13$^+$ cells localized in bronchus-associated lymphoid tissue-like structures at day 14.

Our results demonstrate that chronic NTHi infection exerts a pro-inflammatory activity in the human and murine lung, and could therefore contribute to the exaggerated burden of lung inflammation in patients at risk.

Introduction

Recurrent or chronic infections by several opportunistic pathogens are common in patients affected by different chronic respiratory diseases, including chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) [1–3]. Infections by *Nontypeable Haemophilus influenzae* (NTHi), a non-capsulated Gram-negative bacterium, are associated with worse clinical prognosis in several chronic respiratory diseases, such as COPD [4, 5], while its pathogenic potential in CF disease is still controversial [1].

Vicious cycles of chronic inflammation and infections, characterized by exaggerated and continuous recruitment of neutrophils in the airways via powerful inflammatory signals, such as Interleukin (IL)-8 or IL-6 [6, 7], have been often associated with immunopathology and decline in pulmonary function among patients suffering from chronic respiratory diseases [8, 9]. In this context, the IL-17-mediated host response is an emerging immunological determinant influencing pulmonary defense and function in several pathologies, including COPD or CF lung disease [6, 10, 11]. For example, IL-17-mediated immunity has been shown to modulate host defense against opportunistic pathogens (e.g. *Pseudomonas aeruginosa*), as well as the exaggerated neutrophil recruitment generated during bacterial chronic infection [12–14] .

It has been reported that the presence of NTHi can be associated with acute exacerbation episodes in CF [15]. Similar observations have been made in COPD patients, although in this case the NTHi infection was also associated with a worse clinical prognosis [16, 17]. However, there are no observations on the effect of chronic NTHi infection in the progression of CF chronic airway disease, due to the presence of several clinical and microbiological cofounding factors, such as concomitant infections [15, 19] and lack of available human cohorts. Nonetheless, it has been observed that patients with COPD and chronically colonized with *Haemophilus influenzae* during stable disease phase display sustained airway inflammation [18, 19]. So far, to what extent NTHi long-term infection contributes to the lung inflammatory burden during chronic airway disease is controversial and animal models could be instrumental to clarify its role in fuelling inflammation. Previous experimental studies on NTHi were mainly focused on the acute virulence of genotypically and phenotypically different bacterial isolates [20–23] rather than on the pathogenic potential of chronic NTHi infection in the airways.

However, at the time of this writing no experimental studies have developed a model of long-term NTHi infection in murine airways that strictly mimics human infectious processes. Exaggerated inflammation, immunopathology, pulmonary damage and remodelling are difficult to be reproduced in mice using the available NTHi transient infection models. As a consequence, the cascade of events mediated by chronic NTHi infection has been difficult to address.

Here, to validate our hypothesis that chronic NTHi infection contributes to fuelling the inflammatory burden in CF lung, we measured the concentrations of a few pro-inflammatory cytokines, related to neutrophil recruitment, in respiratory samples from CF patients infected by NTHi alone or not infected by any CF opportunistic pathogens. In addition, NTHi laboratory and clinical

strains were used to test their pathogenic potential i) in a new mouse model for chronic infection mirroring clinical chronic NTHi lung infection and ii) in *in vitro* host-pathogen experiments. Overall, our data demonstrate that chronic NTHi infection, independent from NTHi phenotypic diversity, may contribute to fuelling the exaggerated inflammatory burden in the airways of patients suffering from chronic respiratory disease.

Methods

Ethical committee

Animal studies were performed according to the protocols set forth by the Italian Ministry of Health guidelines for the use and care of experimental animals (IACUC protocols #920) and approved by the San Raffaele Scientific Institute (Milan, Italy), Institutional Animal Care and Use Committee (IACUC). Human samples and the bacteria from CF patients were collected at the Cystic Fibrosis Center of Milan, Italy. The study was approved by the Ethical Committees of San Raffaele Scientific Institute and of Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico, Milan, Italy. Written informed consent was obtained from patients enrolled, or their parents, according to the Ethical Committee rules, in accordance with the laws of the Italian Ministero della Salute (approvals #1874/12 and 1084/14).

Human respiratory samples and lung function

Nasopharyngeal aspirates from 19 clinically stable CF patients with variable genotypes were collected from the Regional CF Center at Milan's Ospedale Maggiore Policlinico during routine care visits as described in Supplementary methods 1. Seven patients (five females, 13-20 years old) were negative for CF opportunistic pathogens, 12 patients (five females, 4-9 years old) were infected by NTHi but negative for other opportunistic pathogens. Further details are provided in the supplementary files (Supplementary methods and Supplementary table S1).

Bacterial Strains

ATCC 49766 is a reference NTHi laboratory strain. The eight NTHi clinical strains tested in *in vivo* or *in vitro* were isolated from CF patients and identified by CF Microbiology Laboratory, IRCCS Ca' Granda, Ospedale Maggiore Policlinico. Further details are provided in the supplementary files (Supplementary table S2).

Mouse model of chronic NTHi lung infection

Persistent airway infection was obtained by intratracheal injection of agar beads containing ~1×10^7 colony forming units (CFU) of NTHi (ATCC 49766 or NTHi 50, a CF clinical strain) or empty beads (as sham control) in 20-22g C57BL6/NCrl male mice (8-10 weeks, Charles River). Agar beads were produced by refining a previously described protocol [13, 14, 24, 25] as described in Supplementary methods. Sham infected mice were used as control mice. Animals were euthanized after 2, 7 and 14 days by CO_2

administration and lungs were recovered. Sample processing was performed as described in Supplementary methods.

Flow cytometry and intracellular cytokines staining
After euthanasia, lungs were harvested and mechanically dissociated in GentleMACS C tubes (Miltenyi Biotec) using gentleMACS dissociators, followed by straining through a 70-µm filter. Antibody staining was performed as previously described [24, 26]. Briefly, lung cell suspensions (1.5×10^6 cells) were incubated with blocking buffer (5% rat serum and 95% culture supernatant of 24G2 anti-FcR mAb-producing hybridoma cells) for 10 min at 4 °C. Then, cells were stained for 20 min at 4 °C in the darkness with different combinations of antibodies (supplementary table S3, S4 and S5). For intracellular cytokine staining, $1–3 \times 10^6$ cells were stimulated with PMA (3 ng/mL) and Ionomycin (1 µg/mL) for 4 h at 37 °C (the last 2 h with Brefeldin A (5 µg/mL). Cells were surface stained, fixed (paraformaldehyde 2% for 10 min), permeabilized (2% FBS, 0.2% NaN_3, 2% rat serum, 0.5% saponin in DPBS) and then stained for intracellular cytokines for 30 min at RT in darkness. Acquisition and analyses were performed using fluorescence-activated cell sorting FACSCanto cytometer (BD Biosciences) and FlowJo Software (Tree Star), as previously described [14, 26]. Further details are provided in the supplementary files.

Histological analysis
Lungs were removed and fixed in 4% paraformaldehyde. After paraffin embedding, consecutive 5 µm sections from the middle of the five lung lobes were used for histological and immunohistochemical examination. Sections were stained with Hematoxylin and Eosin (H&E) or immunostained as previously described with anti-CD3, anti-B220, anti CXCL-13, anti F4/80 antibodies [27]. A description of the primary antibodies, together with dilutions and unmasking techniques, is provided in the Supplementary table S6. Images were acquired using the Leica Biosystems Aperio CS2 scanner and analysed with the Aperio ImageScope software.

Morphometric analysis
A quantitative analysis was performed on tissue slides by using the Aperio ImageScope software. Lymphoid aggregates were defined as cell clusters positive for the CD3 molecule and were quantified as number of clusters per cm^2 of lung.

ELISA
Concentration of cytokines/chemokines in lung homogenates was assessed by ELISA using DuoSet kits (R&D Systems, Minneapolis, MN) for growth-regulated alpha protein/ keratinocyte-derived chemokine (CXCL1/KC), C-X-C motif chemokine 2/ macrophage inflammatory protein 2 (CXCL2/MIP-2), granulocyte colony-stimulating factor (G-CSF), IL-6, IL-17A, IL-17F and pro-matrix metalloproteinase 9 (Pro-MMP9) accordingly to manufacturer's instructions.

NTHi infection in A549 cell line

Human alveolar epithelial A549 cells were cultured and challenged with NTHi strains as described in the supplementary Methods. Cell viability was assessed by measuring the lactate dehydrogenase released in the supernatant with CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) following manufacturer's instruction.

Statistical Analysis

Statistics were performed with GraphPad Prism for statistical computing. Data at a specific time-point were compared through a nonparametric two/one-tailed Mann-Whitney test. Incidences of mortality and chronic infection were compared using Fisher exact test. Statistical analyses were considered significant at $p < 0.05$.

Results

Impact of NTHi colonization on chemokines associated with neutrophil recruitment in CF lung

We evaluated the levels of CF pro-inflammatory cytokines (IL-8 and CXCL1) in respiratory samples of 19 clinically stable CF patients with NTHi infection or not infected by NTHi (See Material and methods and Supplementary table S1). We found that CF patients infected by NTHi had significantly higher levels of both IL-8 ($p<0.05$) and CXCL1 ($p<0.05$) than those uninfected (Figure 1 a, b). These data suggest that chronic NTHi infection may contribute to increase the levels of chemokines recruiting neutrophils in CF lung.

Development of a new murine model to mirror chronic NTHi infection as observed in human pathology

To investigate whether NTHi infection can induce a sustained inflammatory response, we developed a new murine model that mirrors chronic NTHi infection as observed in human CF pathology.

We adapted a murine model, previously developed for chronic bacterial lung infections [13, 14], to study chronic NTHi infection in lower-middle airways. We developed chronic airways infection by NTHi with the 49766 reference strain up to 14 days with bacterial loads of ≈10^6 CFU/lung (Figure 2 a) and with a high incidence of chronic infection (80 %) at 14 days post-infection (Figure 2 b). No CFUs were detected in the spleens of infected mice, indicating that NTHi was confined to the lung compartment and did not spread systemically.

Focusing on the early (2 days) and advanced (14 days) stages of infection, we evaluated histopathology and the levels of pro-inflammatory cytokines/chemokines related to neutrophil recruitment into the lungs. H&E staining on day 2 and 14 days post-infection localized the agar beads to bronchial lumens. In term of inflammation, the early phase was mainly

characterized by the acute neutrophil and macrophage recruitment (Figure 2 c), while the chronic NTHi infection was featured also by adaptive immune response including the formation of organized macro structure of lymphocytes (Figure 2 d) similar to those observed for chronic *P. aeruginosa* infection [13, 14, 28].

The levels of KC, MIP-2 (both murine functional homologues of human IL-8 [29]) and IL-6 were significantly increased in comparison to sham infected (control mice) at 2 or 14 days post-infection (KC/MIP-2/IL-6: Control vs 2 day $p < 0.05$, Control vs 14 day $p < 0.05$), reflecting the higher recruitment of innate immune cells described in the histopathological analysis (Figure 1 e,f,h). Moreover, the production of IL-17A, IL-17F and G-CSF were also significantly higher (IL-17A/IL-17F/G-CSF: Control vs 2 day $p < 0.05$, Control vs 14 day $p < 0.01$) than those found in sham infected at 2 and 14 days (Figure 1 g,i,l). Of note, IL-17A levels increased by day 2 and remained high over the course of NTHi infection, suggesting the involvement of type 17 immunity. Overall, our new murine model can mirror the acute/early and advanced stages of chronic lung infection observed during the course of human airway diseases.

Chronic NTHi infection in the mouse lung promotes sustained type 17 immunity and remodeling processes

In order to dissect the lung infiltrating cells, FACS analysis was performed. The number of infiltrating leukocytes was significantly higher in samples recovered from infected mice at both day 2 ($p < 0.001$) and 14 ($p < 0.001$) post-infection when compared to control mice (Figure 3 a). Neutrophils and monocytes were recruited in infected lungs at both time points (Figure 3 b, c), while alveolar macrophages and dendritic cells (DC) were increased only at 14 days ($p < 0.001$) post infection (Figure 3 d, e). On the other hand, neutrophil and monocyte counts decreased on day 14 post-infection, although they remained higher than in control mice. Additionally, T cells were significantly ($p < 0.05$) more represented among infected mice compared to control ones (Figure 3 f). While both $\gamma\delta^+$ and CD4$^+$ T cell subsets were significantly enriched both at day 2 and day 14 post-infection (Figure 3 g, h), CD8$^+$ T cell subsets only increased at day 14 (Figure 3 i).

We also found a selective enrichment of CD4$^+$ IL-17A$^+$ T cells ($p < 0.05$), but not of CD4$^+$ IL-4$^+$, CD4$^+$ IFN-γ^+ or CD4+ IL-13$^+$ T cells (Figure 3 j). We quantified the levels of pro-MMP9 as a marker associated with tissue remodeling processes [30]. Levels of pro-MMP-9 were significantly upregulated ($p < 0.05$) during chronic NTHi infection in the lung (Figure 3 k), confirming that chronic NTHi infection is associated with sustained tissue remodeling processes. Thus, all together these data indicate that chronic infection by NTHi in the lower airways induces local Type 17 immune manifestations and sustained tissue remodeling processes.

Pathogenicity of a CF clinical isolate during chronic NTHi infection in murine airways

We tested whether a CF NTHi isolate may display a worse pathogenic potential in comparison to the reference laboratory strain during chronic lung

infection. We exploited our new model of chronic NTHi infection in order to compare bacterial burdens and host response between the reference laboratory strain and a CF isolate, named "NTHi50". As shown (Figure 4 a, b), NTHi50 did not differ from the 49766 reference strain in terms of bacterial burden and incidence of chronic infection in C57BL/6N mice 14 days post-challenge. We then investigated whether the inflammatory response in terms of quality and burden could be different during chronic infection by those NTHi strains. We evaluated both histological lesions by the analysis of lung tissue slices stained with H&E (Figure 4 c, d, e) and lung infiltrating cells by FACS analysis in the lung. Both analyses revealed that NTHi50 induced a host response similar to that due to the 49766 strain. As a matter of fact, the sustained immune response was characterized by T cells, comprising both $\gamma\delta^+$ and $CD4^+$ subsets, and associated with sustained neutrophil recruitment (Figure 4 f-m). In addition, by immunohistochemistry, we found that $CD3^+$ cells were localized in lymphocyte aggregates (Figure 5 and supplementary figure S2) at the advanced stage of chronic infection with both strains. Moreover, we determined that $CD3^+$ aggregates were characterized by the strong co-localization of B220 and CXCL13-expressing cells, demonstrating the presence of bronchus-associated lymphoid tissue (BALT)-like structures at day 14.

To test whether the bacterial pathogenic contribution may differ among CF NTHi isolates, we infected the A549 alveolar epithelial cells with clinical CF isolates (n=8), including NTHi50 and seven other CF clinical strains (supplementary table S2) and evaluated cytotoxicity by analyzing cell viability 6 hours after infection. We observed a similar cytotoxic potential of NTHi clinical strains. As shown in (supplementary figure S1), only NTHi32, NTHi33 and NTHi34 isolates induced a modest (although statistically significant, $p < 0.05$) increase in cell toxicity in comparison to the NTHi reference strain. Overall, our data highlight that NTHi persistence, rather than NTHi intrinsic virulence, fuels the inflammatory burden in the lungs promoting chronic inflammation characterized by the sustained recruitment of neutrophils and T cells.

Discussion

The pathogenic potential of chronic NTHi infection in lung disease is still controversial [1, 15]. Our research question was whether chronic NTHi infection could be considered as a potential risk factor for lung disease progression by fuelling the inflammatory burden. Our results demonstrate that chronic NTHi infection is associated with a sustained inflammatory burden, as also indicated by respiratory sample analysis in a small cohort of CF patients. The development of a new murine model was instrumental in mirroring chronic NTHi infection as observed in clinical settings and, consequently, in overtaking the apparent limited NTHi virulence observed in *in vitro* experiments with bacterial variants. In particular, we found out that chronic NTHi infection is associated with sustained recruitment of neutrophils in the

lung. This pro-inflammatory response seems to be mainly mediated by type 17 immunity and by the presence of BALT in the sub-mucosal lung tissue.

Epidemiological data on *H. influenzae* colonization and disease risk were mainly collected from patients with non-CF bronchiectasis [19, 31, 32]. In the context of CF patients with NTHi colonization/infection, only few studies reported on associations with clinical characteristics, morbidity and mortality. *H. influenzae* is more frequently observed in CF patients without a positive history of *P. aeruginosa* lung infection, rather than in those who had been previously infected at least once [33, 34] . Our data indicate that chronic infection by NTHi may account for a higher pro-inflammatory burden.

Interestingly, *H. influenzae* detection on respiratory tract culture was identified as a risk factor for substantial annual FEV_1 decline in 4680 adolescents and young adults with CF from US and Canada within the Epidemiologic Study of CF [35]. Although we recruited clinically stable CF patients with similar clinical conditions in term of Forced Expiratory Volume 1 (FEV_1), a limitation of our human results, due to the limited number of patients, is represented by the missing evaluation of the contribution of other confounding factors, such as genotype, age or gender. To further support our observations in CF patients, prospective observational clinical studies with a higher number of patients are required, possibly by setting up a multicenter study to increase availability of subjects with comparable clinical and demographic characteristics. In this context, the development of biological clinical samples banks (e.g. blood, respiratory samples or clinical strains) associated with a large clinical CF database may be useful to definitively address the potential of early NTHi infection as a risk factor for lung disease progression in CF.

As for clinical and basic science studies, the NTHi literature fails to deeply and fully discriminate colonization from acute/intermittent or chronic infection/colonization episodes of infection. The novelty of our work also relies in the dissection of the pro-inflammatory potential during chronic NTHi infection in the airways of mice. To date, animal models have been developed to mimic the acute phase of NTHi infection rather than the chronic phase, limiting steps forward in the field. Airway chronic infection by NTHi is common in CF disease and COPD, but also in other lung diseases with different aetiology, such as lung cancer [36–38]. In this context, *Croasdell A. et al.* exploited an acute infection model in which different amounts of NTHi were inoculated by oropharyngeal aspiration in C57BL/6J mice (up to 10^8 CFU) and 24 hours post-infection they found >90% of bacteria had been cleared, independently from the inoculum dose [39]. To date, mouse models based on the injection of NTHi planktonic bacterial cells in immunocompetent mice are useful for studying the acute NTHi infection phase rather than the chronic one. The new mouse model, developed in this manuscript, may reproduce the vicious cycles of infection, lung inflammation and tissue damage as observed in chronic respiratory disease mediated by NTHi infections. In the present study, we were able to evaluate the pro-inflammatory burden caused by chronic NTHi infection, associated with constitutive recruitment of neutrophils, as well as with increased levels of both $\gamma\delta^+$ and $CD4^+$ T cell subsets in the advanced phase of lung infection. Previous studies identified a protective role of type 17 immunity (intended as IL-17 secreting cells) during transient and acute NTHi infection in mice [23, 36, 40]. Here, we found that chronic inflammation due to NTHi is associated with $CD3^+$ $CD4^+$ IL-17$^+$ cells. As

previously demonstrated for *P. aeruginosa*, we could speculate that type 17 immunity may play a double-edged sword activity during NTHi infection: it could be protective by increasing host resistance in the early phase of infection, while being detrimental during the advanced phase of chronic infection by increasing the immunopathology [11, 14]. Further studies using transgenic mice or inhibitory drugs could address this hypothesis.

Our data suggest that chronic NTHi infection in mice can induce an inflammatory response similar to those mediated by other well know opportunistic pathogens (e.g. *P. aeruginosa* or *Staphylococcus aureus*) associated with chronic respiratory diseases. Here the NTHi-driven peribronchial lymphoid tissue structures, associated with sustained neutrophils recruitment, mimics pathological features similar to those observed for long-term *S. aureus* or *P. aeruginosa* infection in mice or humans with advanced diseases [14, 28]. In this context, the question whether NTHi-inflammatory response may promote the infection of the airways by other pathogens such as *P. aeruginosa* or *S. aureus* as occurs in CF patients, remains to be addressed.

Interestingly, our results with laboratory and clinical strains in mice suggest that the pro-inflammatory burden is mainly mediated by the immunopathological responses to chronic NTHi infection rather than by the specific pathogenic virulence of different bacterial strains. The in vitro results, obtained when different clinical isolates were tested, further support a similar pathogenicity. Of course, a study considering a higher number of clinical isolates is required to determine whether specific phenotypic traits can affect the pathogenic potential of different NTHi variants.

It is desirable that future studies would not exclusively contrast NTHi bacterial growth (e.g. with antimicrobial compounds), but also target the NTHi-induced immunopathology (e.g. with anti-inflammatory drugs) in chronic lung disease. These approaches will help to better explore the contribution of long-term NTHi infection on disease progression, pulmonary exacerbations or super infection events, such as the "predisposition" to *P. aeruginosa* infection. As already mentioned, NTHi infections are commonly associated with several chronic respiratory diseases with different aetiology [3, 4, 19]. Therefore, the murine model developed in this study will be exploitable to determine the contribution of NTHi chronic infections and new therapeutic opportunity in a broader spectrum of chronic lung diseases.

In conclusion, our results demonstrate that chronic NTHi infection, common during early lung disease, contributes to the exaggerated lung inflammatory burden and may contribute in declining lung function.

Author contributions

Conceiving and designing the experiments: N.I.L., C.Ci.; performing experiments: F.S., G.R., D.G., S.R. B.S. and N.I.L.; analyzing data: F.S., G.R., L.C., V.D., B.S., C.Ci. and N.I.L.; performing statistical analysis: F.S., G.R., V.D. and N.I.L.; interpretation of the experiment results: F.S., G.R., A.B., L.C., D.M.C., C.Co., V.D., C.Ci. and N.I.L.; preparing figures: F.S., G.R. and N.I.L.; writing the manuscript: F.S., G.R., A.B., L.C., D.M.C., V.D., C.Ci. and N.I.L.;

FIGURE LEGENDS

FIGURE 1 Contribution of NTHi colonization to lung inflammatory burden in CF patients.
Levels of proinflammatory chemokines (a) CXCL1 and (b) IL-8 were evaluated by ELISA in respiratory samples from a retrospective study in CF patients infected exclusively by NTHi (n=12) or uninfected (n=7) by any recognized CF pathogen. *p<0.05 compared to control (Mann-Whitney t test).

FIGURE 2 Bacterial burdens, histopathology and pro-inflammatory cytokines during NTHi infection in murine airways.
C57BL/6N mice were infected intratracheally with NTHi embedded agar beads. Bacterial loads in the lung (a) and the incidence of chronic infection (b) were measured at 2, 7 and 14 days post infection. The data of Fig 2a and 2b are pooled from at least three independent experiments, n=14 (day 2), n=5 (day 7), n=22 (day 14). H&E staining of lung tissue sections was performed at 2 (c) and 14 day d) post infection. Stars indicate the presence of agar beads in bronchial lumens, arrowheads indicate the presence of neutrophils and macrophages in bronchial lumens and arrows indicate the presence of lymphocyte aggregates. Representative images are shown from the lower i) to the higher iii) magnification. i) scale bar=2 mm; ii) scale bar=500 µm; iii) scale bar=200 µm. KC (e), MIP-2 (f), G-CSF (g), IL-6 (h), IL-17A (i) and IL-17F (j) concentration in the murine lungs was assessed by ELISA at both 2 and 14 days post infection. *p<0.05, **p<0.01, ***p<0.001 compared to control (Mann-Whitney t test).

FIGURE 3 Immune response during the development of chronic NTHi infection in mice.
Mice were infected intratracheally with NTHi embedded agar beads and lungs were dissociated at 2 and 14 days post infection. Flow cytometry was performed to quantify the total number of (a) leukocytes, (b) neutrophils, (c) monocytes/macrophages, (d) alveolar macrophages, (e) myeloid dendritic cells, (f) T cells, (g) T cells, (h) CD4$^+$ T cells and (i) CD8$^+$ T cells. Intracellular staining was performed (j) to identify IFN-γ, IL-4, IL-13 or IL-17 producing CD4$^+$ cells. (k) Pro-MMP9 was quantified by ELISA at 14 days post-infection. The data are pooled from at least two independent experiments (n=3-9). ns not significant, *p<0.05, **p<0.01, ***p<0.001 compared to control (Mann-Whitney t test).

FIGURE 4 Bacterial burdens, histopathology and immune response in murine airways during chronic infection by clinical NTHi strain.
Mice were infected intratracheally with NTHi embedded agar beads. Bacterial loads in the lung (a) and the incidence of chronic infection (b) were measured at 14 days post infection. H&E staining of lung tissue sections were performed for control mice (c) ATCC 49766 (d) and NTHi 50 (e) infected mice. Stars indicate the presence of agar beads in bronchial lumens, arrowheads indicate the presence of neutrophils and macrophages in bronchial lumens and arrows indicate the presence of lymphocytes aggregates. Representative images are

shown from the i) lower (scale bar=2 mm) to the ii) higher magnification (scale bar=200 μm). Recruitment of (f) leukocytes, (g) neutrophils, (h) monocytes/macrophages, (i) T cells, (l) γδ T cells and (m) CD3$^+$ CD4$^+$ T cells was measured by flow cytometric analysis in cell suspensions of murine lungs at 14 days post infection. The data are pooled from at least two independent experiments n=7 (ATCC49766), n=9 (NTHi 50). ns not significant, *p<0.05 (Mann-Whitney t test).

FIGURE 5 Chronic NTHi infection promotes the formation of peribronchial lymphoid tissue structures.
Mice were infected intratracheally with NTHi embedded agar beads and lung immunohistochemistry for the CD3 (a) B220 (b) CXCL13 (c) and F4/80 molecules was performed on lung tissue sections at 14 days post infection for control mice (i), ATCC 49766 (ii) and NTHi 50 (iii) infected mice (scale bar=80 μm). (e) Morphometric analysis of CD3$^+$ aggregates in murine lungs. Arrows indicate the presence of peribronchial lymphoid tissue structures. Horizontal bars represent median values. The data are pooled from at least two independent experiments (3 mice/groups).

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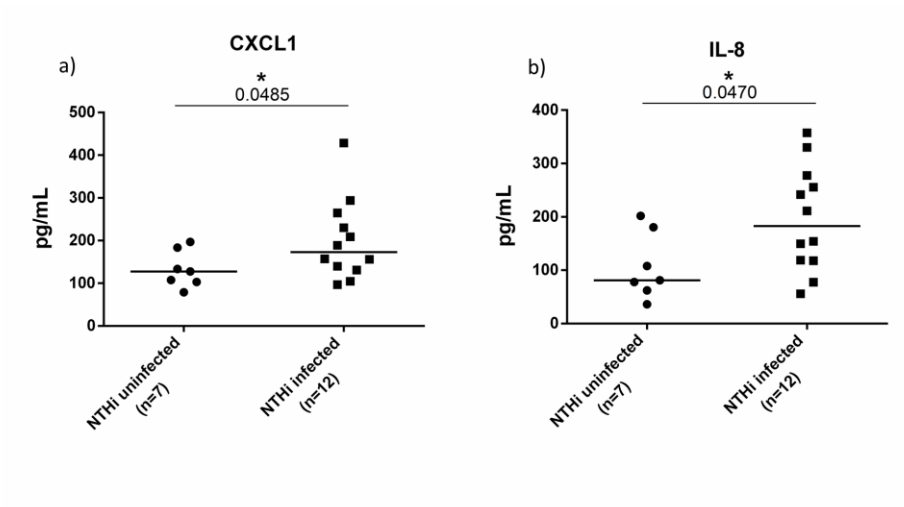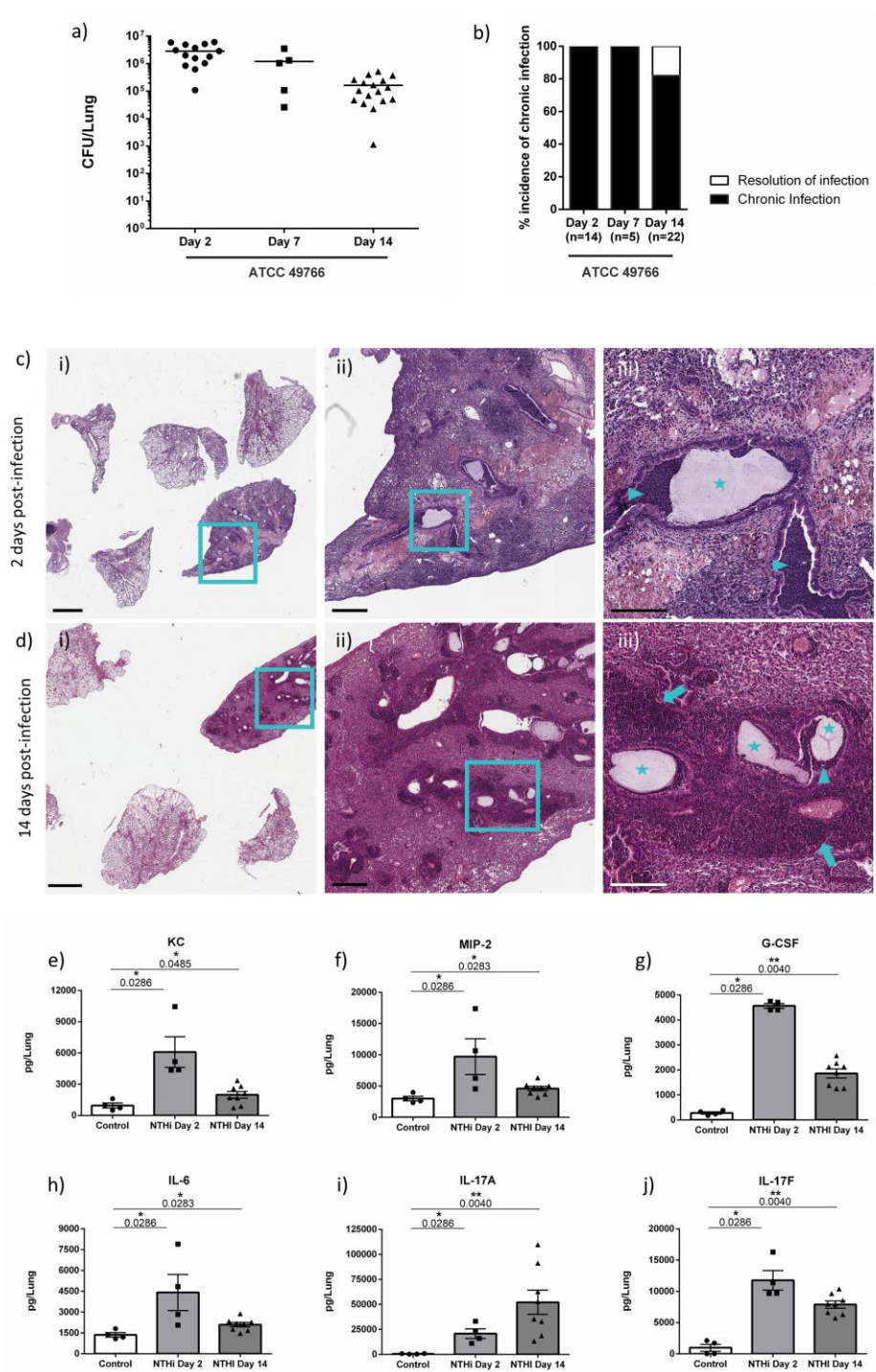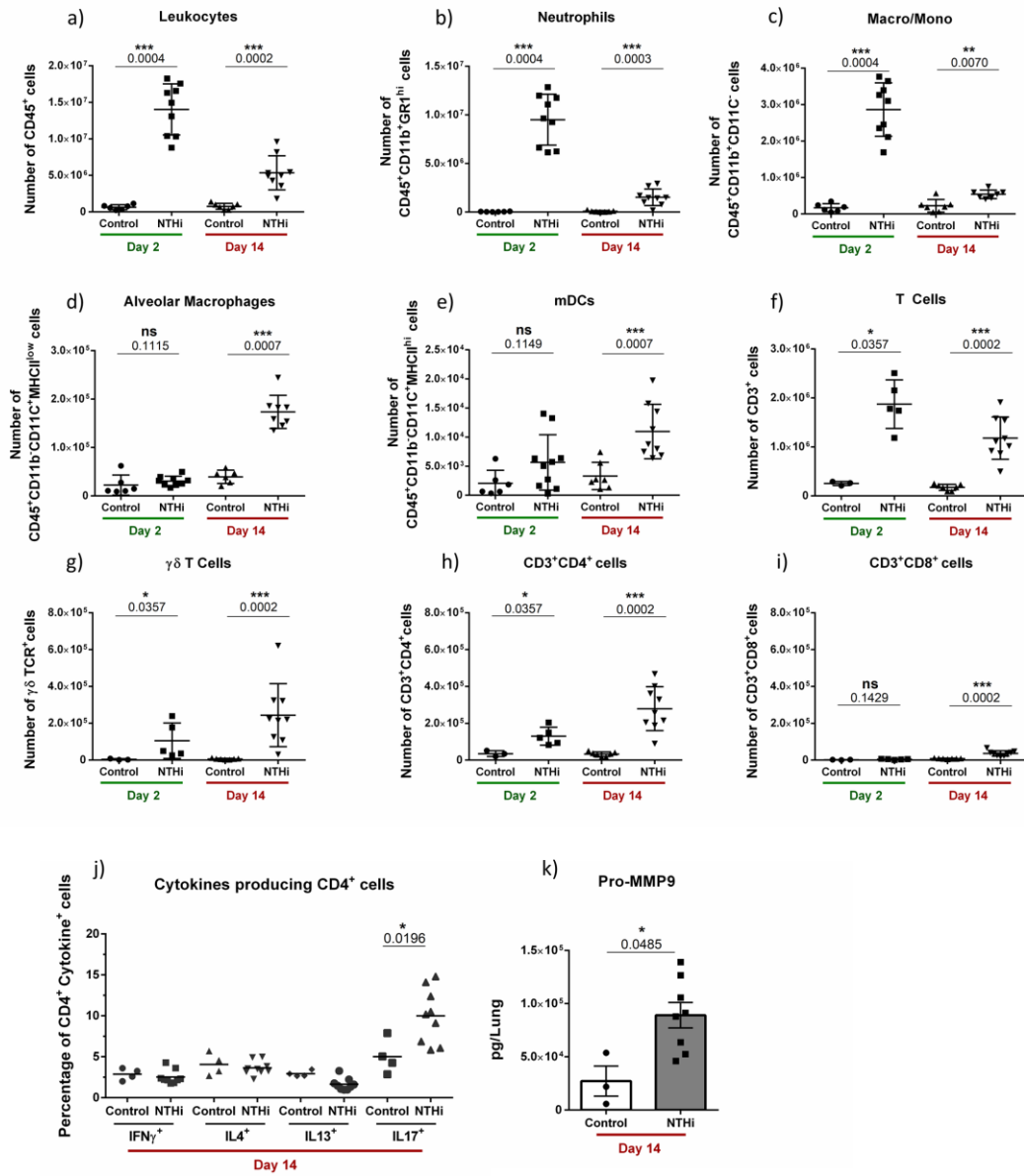
FIGURE 1

FIGURE 2
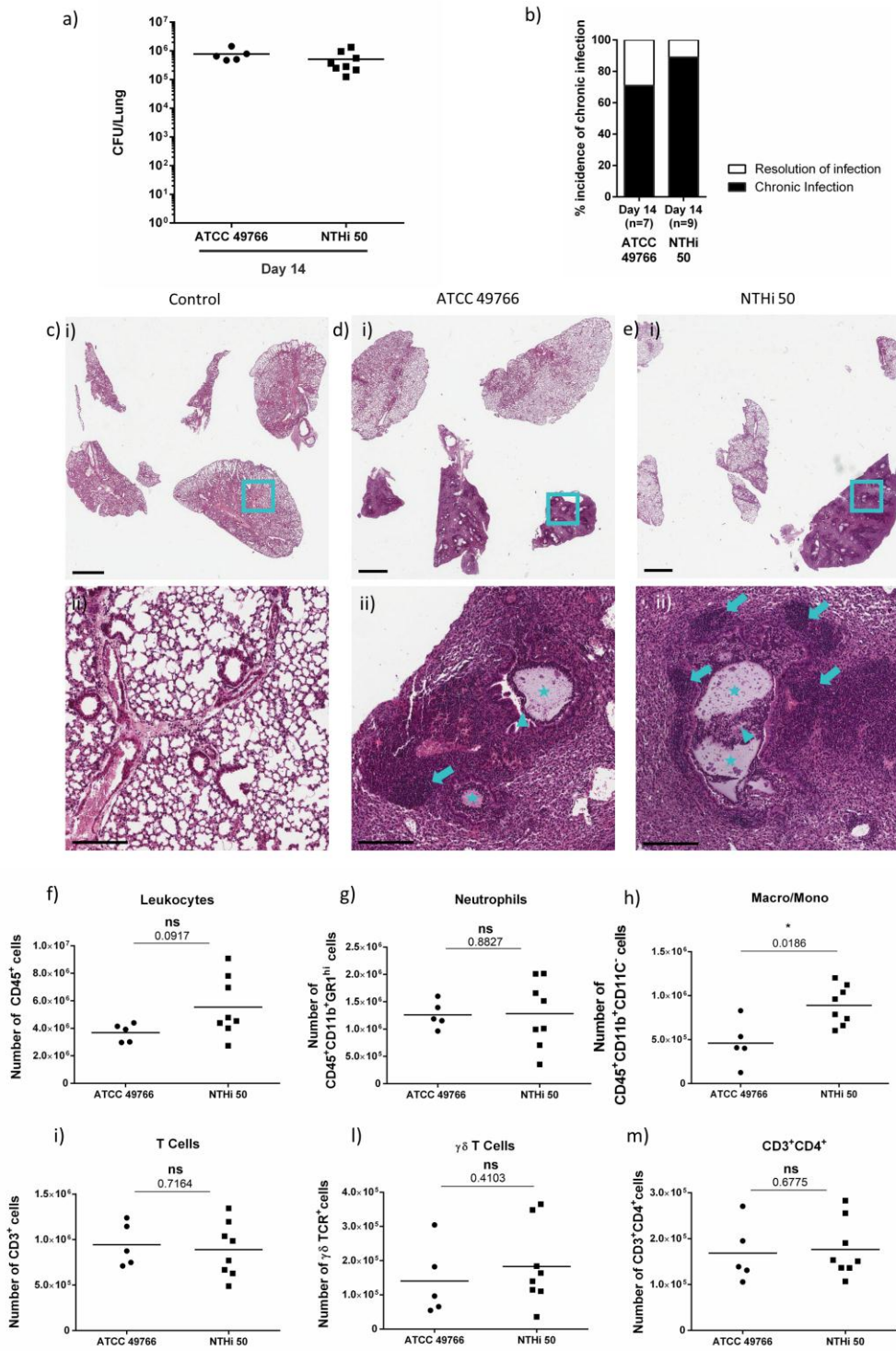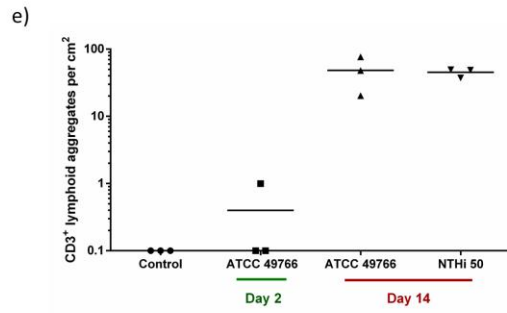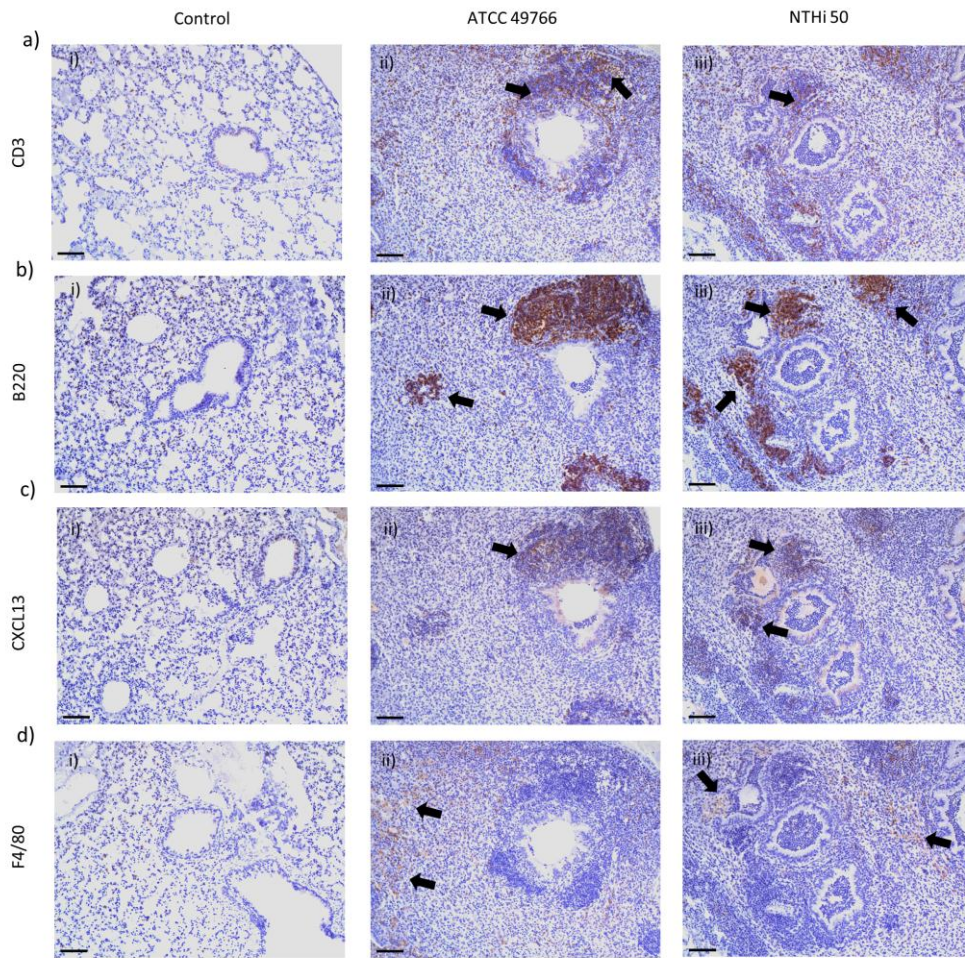
FIGURE 3

FIGURE 4

FIGURE 5

Supplementary Files

Chronic infection by Nontypeable *Haemophilus influenzae* fuels airway inflammation

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This article has Supplementary Information.

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Supplementary methods

Inclusion and exclusion criteria for CF patients

Nasopharyngeal aspirates from 19 clinically stable CF patients with variable genotypes were collected from the Regional CF Center at Milan's Ospedale Maggiore Policlinico Inclusion criteria were: a) diagnosis after neonatal screening or after onset of symptoms and followed since birth in the centre; b) complete clinical history available in the computerized database; c) at least two visits yearly since diagnosis; d) availability of at least four yearly sputum cultures for microbiological ascertainment in order to accesses NTHi infection; e) at least one CT every year; f) at least one respiratory function test/year after the age of 4.8 years; g) similar range of clinical condition in term of Forced Expiratory Volume 1 (FEV 1). %FEV$_1$ measurements were performed in routine care visits and data were collected accordingly with the local ethics committee. Exclusion criteria were the presence of acute pulmonary exacerbations (APE). APE was determined by the presence of at least four of the following criteria: 10% or greater decrease in baseline FEV1, increased cough or sputum production, change in sputum character, dyspnea, tachypnea, fever, weight loss, 5% or greater decrease in O2 saturation, new or worsening crackles on lung auscultation, or findings on chest X-ray consistent with pneumonia. The NTHi infection status was confirmed by microbiological analysis following national guidelines. The characteristics of the CF patients are summarized in the Table S1.

Agar beads production and NTHi mouse lung infection

NTHi strains were cultured in brain heart infusion (BHI, BD) supplemented with 10 µg haemin mL^{-1} and 5 µg NAD mL^{-1}(sBHI) overnight at 37°C, adjusted to a starting OD^{600} of 0.1, grown to mid-log phase (3h) at 37°C 200 RPM, to allow agar bead preparation. Then, the bacteria were harvested by centrifugation and resuspended in 1 mL of phosphate-buffered saline (PBS; pH 7.4). Bacteria were added to 9 ml of sBHI agar, previously warmed to 45°C. This mixture was pipetted forcefully into 150 ml of heavy mineral oil at 45°C and stirred rapidly with a magnetic stirring bar for 6 min at room temperature, followed by cooling at 4°C for 35 min with a slow and continuous stirring. The oil-agar mixture was centrifuged at 4,000 rpm for 15 min to sediment the beads, which were washed six times in PBS. The number of NTHi colony forming units (CFU) in the beads was determined by plating serial dilutions of the homogenized bacteria-bead suspension on sBHI agar plates. The inoculum was prepared by diluting the beads suspension with PBS to 1×10^7 CFU per inoculum. Immunocompetent C57BL/6NCrlBR male mice (8-10 weeks of age) were purchased from Charles River (Calco, Italy), shipped in protective, filtered containers, transported in climate-controlled trucks, and allowed to acclimatize for at least two days in the animal house prior to use. Mice were maintained in the biosafety level 3 (BSL-3) facility at

San Raffaele Scientific Institute (Milano, Italia) where 3-5 mice per cage were housed. Mice were maintained in sterile ventilated cages. Mice were fed with standard rodent autoclaved chow (VRFI, Special Diets Services, UK) and autoclaved tap water. Fluorescent lights were cycled 12h on, 12h off, and ambient temperature (23±1°C) and relative humidity (40-60%) were regulated. For infection experiments, mice were anesthetized by an intraperitoneal injection of a solution of ketamine (100 mg/kg) and xylazinein (10 mg/kg) in 0.9% NaCl and administered at a volume of 0.015 ml/g body weight. Mice were placed in a supine position. The trachea was directly visualised by ventral midline, exposed and intubated with a sterile, flexible 22-g cannula attached to a 1 ml syringe. An inoculum of 50 µl of agar-bead suspension was implanted via the cannula into the lung. After inoculation, all incisions were closed by suture. Mice were monitored daily for coat quality, posture, attitude, ambulation, hydration status and body weight. Mice that lost >20% body weight and had evidence of severe clinical disease, such as scruffy coat, inactivity, loss of appetite, poor locomotion, or painful posture, were sacrificed before the termination of the experiments with an overdose of carbon dioxide. Gross lung pathology was noted. Lungs were excised aseptically and homogenized in 2 ml PBS added with protease inhibitors using the homogenizer gentleMACS^{TM} Octo Dissociator. One-hundred µl of the homogenates and 10-fold serial dilutions were spotted onto sBHI. CFU were determined after overnight growth at 37°C.

Infections, treatments and sacrifices in the chronic infection models were all performed in the late morning. In addition, in all the experiments, mice had been subdivided according to the body weight to have similar mean in all the groups of treatment. Animal studies were conducted according to protocols approved by San Raffaele Scientific Institute (Milan, Italy) Institutional Animal Care and Use Committee (IACUC #920) and adhered strictly to the Italian Ministry of Health guidelines for the use and care of experimental animals.

NTHi infection in A549 cell line

Human alveolar epithelial A549 cells were cultured in Dulbecco Modified Eagle Medium (DMEM, Lonza) supplemented with 10% FBS, 2 nM L-glutamine and 100 U/ml penicillin and 100 µg/µl streptomycin. Cells were seeded in flat-bottom 96-well plates at a density of $2x10^4$ cells per well in 100 µL of DMEM. NTHi was grown on brain heart infusion (BHI, BD) agar plates supplemented with 10 µg haemin mL^{-1} and 5 µg NAD mL^{-1} (sBHI) at 37 °C in 5% CO2 overnight, then harvested and incubated overnight in sBHI broth. Next, bacteria were subcultured to log phase growth (3h) and resuspended in PBS to the desired CFUs. Then, 10 µL of bacteria suspension per well were inoculated onto the cells at the MOI of 100. Infected cells were incubated for 6 hours.

Supplementary Tables

TABLE S1 Characteristics of CF patients enrolled for cytokines quantification. Data are presented as median (interquartile range)

SUPPLEMENTARY TABLE 1 Subjects demographics	NTHi uninfected patientes [#] (n=7)	NTHi infected patients [$] (n=12)
Sex (M-F)	(2-5)	(7-5)
CFTR genotype n		
• **F508 del heterozygous**	4	5
• **F508 del homozygous**	0	2
• **Others**	3	5
FEV$_1$%	108 (104,6-121)	115,6 (101,5-119,1)
Age (years)	14 (13-20)	6,75 (4,875-9)
Diagnosis at screening n (age)	4 (0,1)	9 (0,1)
Pancreatic insufficiency n	1	4
Sweat chloride concentrations mEq/L (mean)	66,3 (40,8-91)	83 (44,7-124)
Patients with symptoms n*	3	3
Patients treated with antibiotics n[&]	3	3
Data are presented as median (interquartile range) when non differently indicated * Symptoms: rhinitis, cough, pharyngitis or nasopharyngitis & Antibiotics: amoxicillin/clavulanic acid	# Patients negative for CF opportunistic pathogens	$ Patients Negative for CF opportunistic pathogens and positive for NTHi colonization

TABLE S2 Characteristics of the origin of NTHi clinical.

SUPPLEMENTARY TABLE 2 NTHi clinical strains			
Strain name	**Age**	**Bacterial load**	**Other infections**
NTHi 31	5	1.00E+05	na
NTHi 32	9	1.00E+05	#
NTHi 33	39	1.00E+06	#
NTHi 34	5	1.00E+06	§
NTHi 39	9	1.00E+05	na
NTHi 46	6	1.00E+05	na
NTHi 47	40	1.00E+06	#
NTHi 50	4	1.00E+05	na
# Staphylococcus aureus $ Streptococcus pneumoniae			

SUPPLEMENTARY TABLE 3 APC staining		
Antibodies	**Manufacturer**	**Identifier**
PerCP anti-mouse/human CD45R/B220 Antibody	Biolegend	Cat #103234
FITC anti-mouse Ly-6G/Ly-6C (Gr-1) Antibody	Biolegend	Cat #108406
APC anti-mouse/human CD11b Antibody	Biolegend	Cat #101212
Pacific Blue™ anti-mouse CD45 Antibody	Biolegend	Cat #103126
PE/Cy7 anti-mouse CD11c Antibody	Biolegend	Cat #117318
PE Rat Anti-Mouse I-A/I-E Antibody	BD Biosciences	Cat #557000

SUPPLEMENTARY TABLE 4 T cell subsets staining		
Antibodies	**Manufacturer**	**Identifier**
FITC anti-mouse CD45 Antibody	Biolegend	Cat #103108
Pacific Blue™ anti-mouse CD3ε Antibody	Biolegend	Cat #100334
PE/Cy7 anti-mouse CD4 Antibody	Biolegend	Cat #100422
Brilliant Violet 510™ anti-mouse CD8a Antibody	Biolegend	Cat #100752
PE anti-mouse NK-1.1 Antibody	Biolegend	Cat #108708
APC Anti-mouse TCR γ/δ Antibody	Biolegend	Cat #118116

SUPPLEMENTARY TABLE 5 T cell surface and intracellular staining		
Antibodies	**Manufacturer**	**Identifier**
PE/Cy5 anti-mouse CD45 Antibody	Biolegend	Cat #103110
Pacific Blue™ anti-mouse CD3ε Antibody	Biolegend	Cat #100334
PE/Cy7 anti-mouse CD4 Antibody	Biolegend	Cat #100422
Brilliant Violet 510™ anti-mouse CD8a Antibody	Biolegend	Cat #100752
FITC anti-mouse IFN-γ Antibody	Biolegend	Cat #505806
APC anti-mouse IL-4 Antibody	Biolegend	Cat #504106
PE anti-mouse IL-17A Antibody	Biolegend	Cat #506904
APC-eFluor 780 anti-mouse IL-13 Antibody	eBioscience	Cat #47-7133-80

SUPPLEMENTARY TABLE 6
List of antibodies used in immunohistochemistry on mouse lung tissue slides

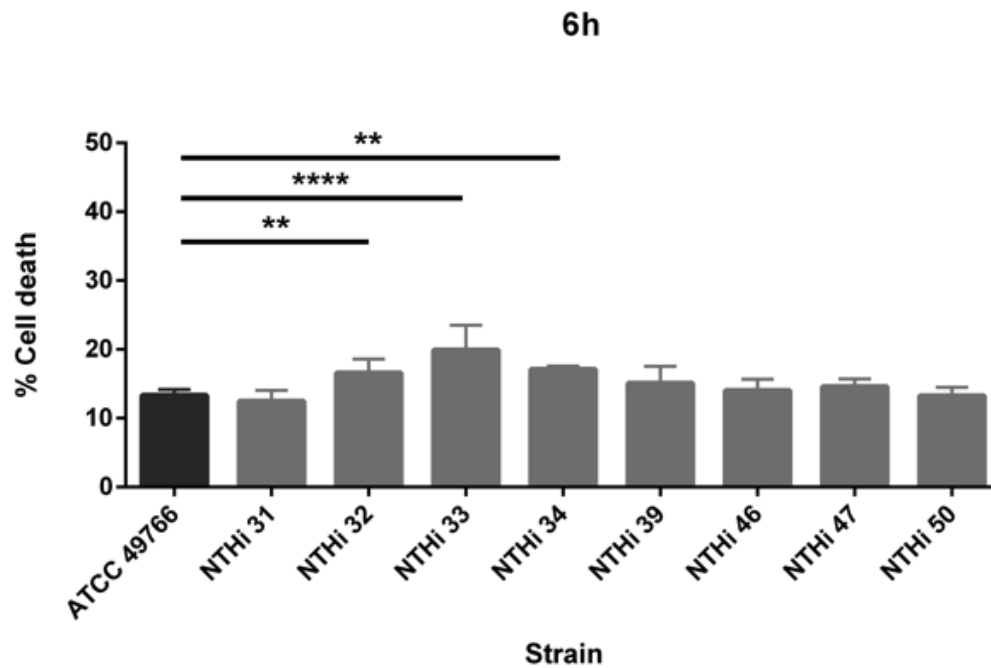| Antigen	Source	Manufacturer	Identifier	Dilution	Unmasking
CD3	Rabbit monoclonal	abcam	ab1666	1:100	EDTA
CD45R	Rat monoclonal	BD	550286	1:200	Citrate
CXCL13	Goat polyclonal	R&D	AF470	1:20	Citrate
F4/80	Rat monoclonal	AbD Serotec	MCA497G	1:200	Citrate

Supplementary Figures

FIGURE S1 A549 cells were infected with eight different clinical strains of NTHi plus the reference strain ATCC49766 at a MOI of 100. Percentage of cell death was assessed at 6 hours post infection by LDH assay. Data are presented as mean ± SD. Ordinary 2-way ANOVA and Bonferroni multiple comparisons test versus ATCC 49766 has been performed as statistical analysis. The data are pooled from at least three independent experiments. ** = p<0.01; **** = p<0.0001.
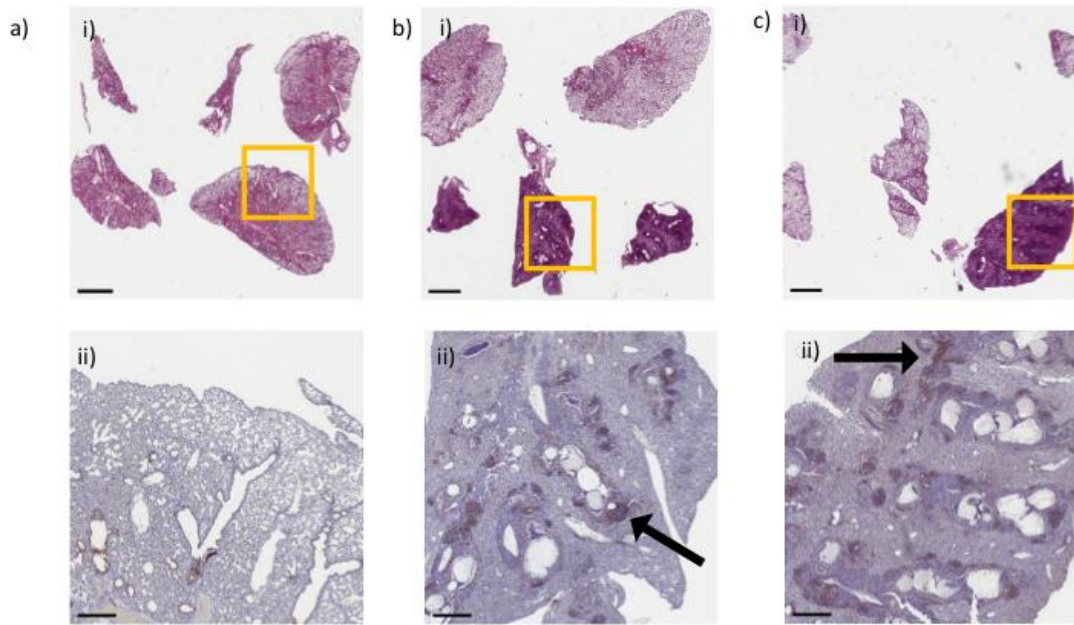
FIGURE S2 NTHi chronic infection promotes the formation of lymphoid aggregates. Mice were infected intratracheally with NTHi embedded agar beads. H&E stainings (i) and immunohistochemistry for the CD3 molecule (ii) of lung tissue sections were performed at 14 days post infection for control mice (a) ATCC 49766 (b) and NTHi 50 (c) infected mice. Representative images are shown from the lower i) to the higher (H&E, scale bar=2 mm); ii) magnification (CD3+, scale bar=200 µm). Some BALT-like structures are indicated by arrows. The data are pooled from at least two independent experiments (n=1-2)