Non-typeable Haemophilus influenzae directly and through TNF-α production enhances polymorphonuclear leukocytes adherence to bronchial epithelial cells and activation

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ABSTRACT

Rationale. Non-typeable Haemophilus influenzae (NTHi), the leading cause of localized upper respiratory tract infection in children, can be the causative agents of lower respiratory tract disorders and chronic lung disorder exacerbations. Infection of bronchial epithelial cells by NTHi is characterized by a sustained neutrophilic inflammation that is thought to play a key pathogenetic role in lung parenchyma damage. Methods. To characterize the mechanisms involved in BEC activation in response to NTHi, a human cell line (BEAS-2B) was stimulated with NTHi lysates. The production of TNF-α, and the expression of TLR2, the microbial ligand that recognizes NTHi molecular patterns, and of ICAM-1, an adhesion molecule required for neutrophil adhesion, were evaluated. The respective role of TNF-α and ICAM-1 in neutrophil adhesion to BEAS-2B cells was then evaluated by inhibition of their activity by specific monoclonal antibodies (mAbs). Results. A time- and dose-dependent induction of TNF-α synthesis and release by BEAS-2B cells was detected after 24-hour exposure to NTHi lysates (0.4 to 1.6 mg/ml). TNF-α, but also directly NTHi lysates, significantly amplified ICAM-1 and TLR2 expression and synthesis by BEAS-2B cells. Stimulation of BEAS-2B cells with NTHi lysates or with TNF-α induced a dose-dependent increase neutrophil adhesion, stronger after NTHi lysates exposure, associated with MPO production. Finally, the NTHi lysates-induced neutrophil adhesion to BEAS-2B cells was significantly inhibited by anti-TNF-α and anti-ICAM-1 mAbs. Conclusion. Exposure to NTHi lysates induced functional and structural changes in BEAS-2B cells leading to neutrophil recruitment, adhesion, and activation. The observation that all these BEAS-2B cell changes were also induced by TNF-α can at least partially explain the sustained inflammation seen in NTHi infections.
INTRODUCTION

*Haemophilus influenzae* (*H. influenzae*) is a pleomorphic Gram-negative coccobacillus that frequently colonizes the human mucosa of the upper respiratory tract but is also a common cause of invasive and non-invasive bacterial infections (1, 2). Isolates of *H. influenzae* can be subdivided into encapsulated and non-encapsulated forms. Encapsulated strains express one of their six capsular polysaccharides and are designated "a" through "f" subtypes, based on the capsule type (2, 3). Non-encapsulated strains are referred to as non-typable *H. influenzae* (NTHi) (4). Before the anti-*H. influenzae* type b (Hib) conjugate
vaccines became available in 1988, Hib was the leading cause of bacterial meningitis and a major cause of serious invasive diseases among children aged <5 years (5-6). The anti-Hib vaccination altered the epidemiology of the infection and the striking decrease in Hib incidence was associated with strain replacement with serotype “f” and NTHi strains (7-10). NTHi is present in the nasopharynx in approximately 50% of young healthy children, with colonization rates ranging from 14%, in those aged <6 months, to 32% in those aged 19-26 months (7-11). In young children, the level of carriage varies according to several factors that can have repercussions on resistance to antibiotics and on efficacy of the immune response to infection (11-14). These factors include the child chronological age, the presence, the number, and the age of siblings in the family, the climate of the geographic area and the size and location of the day care centers the children are attending (13). An elevated carriage of strains resistant to antibiotics is a source of a concern because NTHi strains can be highly prevalent and pathogenic in a variety of acute and recurrent/chronic lower respiratory tract conditions (14-17). The pathogenesis of acute infections due to NTHi begins with colonization of the mucosal surface, followed by contiguous spread to adjacent areas, usually consequence of abnormalities in either non-specific, or specific host defenses (18). Airway epithelial cells play a dominant role in innate defenses, being a mechanical barrier to microbial entry, detecting pathogens by pattern recognition receptors, recruiting, and activating leukocytes and directly killing microbes through the up-regulation antimicrobial peptides (19). A damaged airway epithelium facilitates bacteria surface contact thus promoting respiratory tract colonization and a subsequent sustained inflammatory reaction (18-20). Recruitment and activation of high numbers of neutrophils promote the release of toxic oxygen species and of harmful proteases, with further disruption of airway mucosal integrity and increased pathogen growth, creating a dangerous vicious circle (19-22). The pathogenetic mechanisms explaining why NTHi, a common commensal microbe can become an important respiratory mucosal pathogen are complex and not completely understood (23-25). To understand how NTHi can cause acute and/or chronic infection, a critical factor is the assessment of the initial interaction with the airway epithelium. With this background a study was designed to analyze in vitro the response of a human bronchial epithelial cell line to NTHi bacterial lysate exposure. The release of cytokines and the expression of surface adhesion molecules involved in neutrophil recruitment, adhesion and activation were evaluated.
MATERIALS AND METHODS

Cell cultures

The human bronchial epithelial cells line (BEAS-2B) was used in all the experiments (26). These cells, which retain electron microscopic features of epithelial cells and show positive staining with antibodies to cytokeratin, were grown as monolayer in a 1:1 mixture of Laboratory of Human Carcinogenesis (or LHC)-9 medium (Invitrogen SRL, Milan, Italy) and RPMI 1640 medium (EuroClone, Milan, Italy).

NTHi bacterial lysate

NTHi strain, originally a clinical isolate from a CF patient, was used in this study. A NTHi single colony was harvested from a chocolate agar plate, inoculated into 30 ml of brain heart infusion broth (BHI, BD Laboratories, Franklin Lakes, NY, USA) supplemented with hemoglobin B and incubated at 37°C in 5% CO₂ overnight (27). The supernatant was discarded after centrifugation at 10,000 × g for 10 min, the pellet resuspended in 10 ml of phosphate-buffered solution (PBS) and sonicated to lyse the bacteria. The lysate was then centrifuged at 10,000 × g for 10 min and the supernatant collected. The NTHi lysate protein concentration was determined using the Bicinchoninic (BCA) protein assay (Abbexa Ltd. 20 Cambridge, UK) and was in the range of 0.2 mg/ml. Stock solutions of 3 to 8 mg/ml were aliquoted by 1 mL and stored at -20°C (27).

BEAS-B2 cell cultures

In preliminary experiments, BEAS-B2 cells were cultured in bronchial epithelial cell growth medium (BEGM, Lonza Biologics, Basel, Switzerland), in Petri culture dishes to 90% confluence and then stimulated for 12, 24 or 48 h with different concentrations of NTHi lysates (0.4, 0.8 and 1.6 mg/ml). Cells were counted in a Neubauer chamber and viable cells detected by trypan blue dye exclusion test (EuroClone) (26). Cell viability in all cultures was ≥90% after 24 h incubation and decreased after 48 h incubation. Based on this observation, all other experimental cultures were not carried out beyond 24 hours.

TNF-α release

To evaluate TNF-α release, 60,000 cells/well were plated into 24-well plates and treated with 100 ng/mL LPS or with NTHi lysates (0.4-0.8-1.6 mg/ml) for 6, 12 and 24h. Culture medium supernatants
were then collected after each incubation time point and analyzed by enzyme-linked immunosorbent assay (ELISA), according to manufacturer instructions (Biosource, Camarillo, CA, USA) (28). After each incubation time point, the culture medium was collected centrifuged and stored at -20°C until measurement.

**Evaluation of ICAM-1 and TLR2 expression on BEAS-B2 cell surface**

To evaluate ICAM-1 and TLR2 expression, 100 μl of the cell suspensions were placed into round bottom 96-well plates and stimulated for 24 h with different concentrations of NTHi lysates (0.4, 0.8 and 1.6 mg/ml) or with TNF-α (10.0 ng/ml). BEAS-B2 cells were then incubated for 30 min and stained: a) for ICAM-1, with a fluorescein isothiocyanate (FITC) conjugated monoclonal antibody (mAb) anti-human CD54 (Caltag Laboratories, Burlingame, CA) and b) for TLR2, with a FIT conjugated ah-TLR2 Antibody (Santa Cruz Biotechnology Inc, Dallas, Texas, USA.). Cells were then fixed with 0.5% paraformaldehyde and analyzed by fluorescence-activated cell sorting (FACS) (Becton Dickinson, Milan, Italy) (-29). ICAM-1 and TLR2 expression was read on 10,000 acquired events and expressed as mean fluorescence channel (mfc) (29).

**Western blot analysis**

Analysis of TNF-α, ICAM-1 and TLR-2 expression was performed by Western blot analysis, as previously described (30). Briefly, BEAS-B2 cells were resuspended in lysis buffer and equal amounts of total proteins were loaded to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto a PVDF membrane (Immobilon-P, Millipore, Bedford, MA, USA). The membranes were blocked. The blots incubated with monoclonal antibodies against anti-human TNF-α, ICAM-1 and TLR-2 monoclonal antibodies (Santa Cruz Biotechnology) and visualized using the enhanced chemiluminescence system (Pierce Biotechnology Inc., Rockford, IL, USA). Blots were re-probed with anti-b-actin antibodies (clone C4, Boehringer Mannheim Inc., Mannheim, Germany). The relevant band intensities were quantified using a Versadoc Imaging System model 3000 (Biorad Laboratories Inc., Hercules, CA, USA). Densitometric analysis of the immunoblot was performed, normalized to b-actin, and plotted as means +SEM.
Purification of polymorphonucleated cells

Human polymorphonucleated cells (PMNs) were purified from normal blood donors using density gradient centrifugation (31). Neutrophils were isolated from the resulting cell suspension using Ficol-Histopaque (Healthcare Bio-Sciences AB, Uppsala, Sweden) density centrifugation, and resuspended in RPMI 1640 medium (EuroClone), supplemented with 10% fetal bovine serum (EuroClone), L-glutamine, and penicillin/streptomycin, at a concentration of 10,000,000 cells/ml, and were kept on ice until needed. Neutrophils were counted and stained with Diff-Quick® (Medion Diagnostics AG, Switzerland) to assess purity (>97%) and with Trypan Blue test (EuroClone) to verify viability (>98%) (31).

Neutrophil adherence to BEAS-2B cells and activation

Isolated neutrophils were resuspended in Heps/Hanks’ balanced salt solution (HBSS), labelled with 1mM Calcein-AM (Sigma-Aldrich, Gillingham, Dorset, UK) for 30 min in the dark, then washed, resuspended and added to wells containing BEAS-2B cells stimulated with different concentrations of NTHi bacterial lysate or TNF-α. After incubation for 30 min at 37°C, non-adherent cells were removed by carefully washing two times with Heps/HBSS solution (Sigma-Aldrich) (32). Adhesion of neutrophils to BEAS-2B cells was then assayed by measuring the fluorescence of the wells with a fluorescence photometer (485 nm) (32). Purified neutrophil samples were then fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, blocked with 5% BSA (Sigma-Aldrich), and stained with polyclonal rabbit anti-human MPO antibodies (DakoCytomation, Glostrup, Denmark) and, after 1 hr incubation, with a secondary antibody (1:200 - Molecular Probe). Slides were cover-slipped using the Vectashield fluoromount (Vector Laboratories Inc., Burlingame, CA, USA) and confocal images were obtained using a confocal fluorescence microscope (TCS SL microscope, Leica, Mannheim, Germany) and 40 × objective lenses (32). To evaluate the functional role of TNF-α and ICAM-1 in inducing neutrophil adherence, anti-human TNF-α or ICAM-1 mAbs (5 μg/ml) or mouse IgG, as isotype control (10 μg/mL), were added to the stimulated BEAS-2B cell cultures for 30 min at 37°C, prior to the adhesion assay.

Statistical analysis

Statistical evaluation was performed using the statistical software package GraphPadPrism3.02 (GraphPad Software, SanDiego, CA, USA). Data are expressed as arithmetic mean ± SEM. Kruskall-Wallis
test, followed by post hoc test (Dunn’s test), was used for multiple comparisons. The level of statistically significance was set at $p < 0.05$.

**RESULTS**

**NTHi-induced TNF-α release and expression**

Enzyme-linked immunosorbent assay analysis of supernatants of BEAS-2B cell cultures, exposed to NTHi lysates supernatants for two time periods (12 and 24 h) showed a significant increase in TNF-α release only after 24 h incubation (**Figure 1A**). The increase was highly significant ($p < 0.001$; each comparison) for all the NTHi lysate concentration tested (0.4, 0.8 and 1.6 mg/ml). Densitometric analysis of the immunoblots, normalized to β-actin, confirmed the remarkable increase of the TNF-α protein production after 24 h stimulation with each the NTHi lysate concentrations tested ($p < 0.001$; each comparison) (**Figure 1B**).

**NTHi lysate and TNF-α-induced ICAM-1 expression**

By FACS analysis a significant increase in ICAM-1 expression on BEAS-B2 cell surface was detected after exposure of the cell cultures to NTHi lysates (1.6 mg/ml) for 24 h, the increase after 12 h incubation was not statistically significant (**Figure 2A**). Densitometric analysis of the immunoblots, normalized to β-actin, showed that the exposure of BEAS-2B cells over 24 h to NTHi lysates also induced a significant increase in ICAM-1 protein production, lower but still significant ($p < 0.05$) at the lowest NTHi lysates concentration tested (0.4 mg/ml) (**Figure 2B**). A time-dependent induction of ICAM-1 protein production by TNF-α (10 ng/ml) was also demonstrated, already significant after 12 h incubation ($p < 0.05$) (**Figure 3A**). A highly significant increase in ICAM-1 expression on BEAS-B2 cell surface was also detected when BEAS-B2 cells were co-cultured with TNF-α (10 ng/ml) for 24 h ($p < 0.001$), an increase similar to that found after NTHi lysates (1.6 mg/ml) (**Figure 3B**).

**NTHi- and TNF-α-induced TLR2 expression and production**

A significant, but weak, increase in TLR2 expression on BEAS-B2 cell surface was seen after 24 h incubation with TNF-α (10 ng/ml) ($p < 0.05$) (**Figure 4A**). A more significant increase in TLR2 expression was observed when, in the same culture conditions, the cells were exposed to NTHi lysates (1.6 mg/ml) ($p < 0.001$). Densitometric analysis of the immunoblots confirmed the results observed in
TLR2 expression, significant production of TLR2 protein, weak after TNF-α exposure (p < 0.05) and stronger after NTHi-stimulation (p < 0.001) (Figure 4B).

**Neutrophil adherence to the epithelium and activation**

Both pre-exposure of BEAS-2B cells to NHTi (0.4, 0.8 and 1.6 mg/ml) and to TNF-α (0.05, 0.10, 1.00 and 10.00 ng/ml) induced a dose-dependent enhancement of neutrophil adhesion to the BEAS-2B cell surface, significant for all the concentration tested (Figure 5A and B). However, the magnitude of neutrophil adhesion was more striking higher after NTHi than after TNF-α exposure (p < 0.01 and p < 0.05, respectively, each comparison). The increase adhesion to the BEAS-2B cell surface was associated with neutrophil activation, as shown by confocal images demonstrating a strong staining with the anti-human MPO antibodies, stronger after NTHi exposure (Figures 5C, D and E). As expected, the TNF-α-induced enhancement of neutrophil adhesion was completely blocked by anti TNF-α-mAbs (Figure 6A), whilst the NTHi lysate-induced neutrophil adhesion was partially inhibited by the anti-TNF-α and the anti-ICAM-1 mAbs, the effect being more effective with the anti-TNF-α than with the anti-ICAM-1 mAbs (p < 0.01 and p < 0.05, respectively) (Figure 6B).

**DISCUSSION**

NTHi is a Gram-negative human pathogen which accounts for acute, recurrent, and chronic respiratory infections. The host response to NTHi is characterized by airway neutrophil recruitment and activation due to early induction of proinflammatory mediators in airway epithelial cells (33). Using BEAS-B2 cells we have shown that exposure to NTHi lysates induced a time- and concentration-dependent TNF-α production and release, and that exposure to NTHi lysates, but also to TNF-α, significantly increased ICAM-1 and TLR2 expression and production.

Moreover, exposure of BEAS-2B cells to NTHi lysate and to TNF-α induced a dose-dependent enhancement of neutrophil adhesion to BEAS-2B cells and that adhesion was associated with neutrophil activation with a sustained MPO production. Airway epithelial cells express TLRs, a class of pattern recognition receptors which, responding to specific microbial ligands, initiate downstream cascades able to activate both the innate and the adaptive immunity (34). TLR2 activation by microbial ligands induces the expression of NF-kB, the intracellular signalling factor most strongly associated with NTHi
infection. Moghaddam SJ, et al., demonstrated that administration of NTHi lysate by aerosol to mice induced a rapid NF-kB activation in airway cells, followed by TNF-α release and neutrophil recruitment (35). The inflammatory response to NTHi can be enhanced by co-secreted cytokines, such as TNF-α. Using human epithelial cell line and primary human bronchial epithelial cell cultures, Watanabe T, et al. demonstrated that NTHi and TNF-α synergistically induced a strong NF-kB activation via two distinct signaling pathways, involved in the induction of proinflammatory cytokines (36). TNF-α has been identified as a major cytokine involved in the pathogenesis of inflammatory and autoimmune diseases, characterized by neutrophil activation (37-39). Many of the proinflammatory effects of TNF-α are mediated by the ability to regulate neutrophil-vascular endothelium interactions (40). Neutrophils express adhesive glycoproteins of the CD11/CD18 family, the counter-receptors for ICAM-1 expressed by vascular endothelial cells (31, 41). In addition to guide neutrophil trans-endothelial migration, the ICAM-1-CD11/CD18 interaction promotes neutrophil-mediated cytotoxicity through toxic oxygen specie generation and protease release (42, 43). ICAM-1 is also expressed by airway epithelial cells, primarily on the apical surface (31, 42). Given its apical localization, ICAM-1 can promote the retention of the transmigrated neutrophils at the luminal airway surface (44). Neutrophil migration into tissues can function as double-edged swords because, in addition to the first line of defense against invading pathogens, has simultaneously the potential to cause substantial local tissue injury (44, 45). Neutrophil adhesion to the epithelium appears to be prerequisite for a large oxidative burst in response to proinflammatory cytokine (31, 33). Indeed, the results of our study showed that neutrophil adhesion to BEAS was associated with a sustained MPO release (31, 33). MPO is a peroxidase enzyme, catalyzing the oxidation of chloride by $H_2O_2$ to form the strong oxidant HOCl (46). The primary defensive function of neutrophils is phagocytosis and destruction of microorganisms. A rapid microbicidal effect follows the release of MPO and $H_2O_2$ into neutrophil phagosomes containing ingested microorganisms. However, MPO and $H_2O_2$ can also be released outside the cells where HOCl formation can induce extensive damage to adjacent tissues (46). Moreover, a persistent neutrophil activation can contribute to the formation of NETs. NETs are networks of extracellular fibers, primarily composed by neutrophil DNA and cytosolic and granule proteins assembled on a scaffold of decondensed chromatin (47). NETs can neutralize and kill bacteria, fungi, viruses, and parasites. However, over time NETs have the tendency to become
ineffectual in killing and clearing pathogens, but do not lose the ability to incite a vigorous long-lasting proinflammatory stimuli in the airways (48). In NETs, NTHi can persist forming biofilms which protect them from antibiotics and extracellular and phagocytic killing by neutrophils (33, 48, 49).

CONCLUSION
The BEAS-2B cells inflammatory response to NTHi lysates can be further promoted by co-secreted proinflammatory cytokines, such TNF-α (Figure 7). NTHi lysate- and TNF-α-enhanced neutrophil adhesion to the BEAS-2B cell surface, favored by the increase ICAM1 expression, was associated with neutrophil activation and MPO release, with further enhancement of the inflammatory reaction and the parenchymal tissue damage. The vicious circle, fostered by the increased TLR2 and ICAM-1 expression and by the NET and NTHi biofilm formation, can explains why the sustained neutrophilic inflammation that can become recurrent and chronic. To prevent this vicious circle, NTHi infections should be treated with a high dosage antibiotic to which NTHi is susceptible and for a suitable period (50). As shown for beta-lactam antibiotics in an in vitro study, exposure to subinhibitory antibiotic concentrations (i.e., amounts that partially inhibit bacterial growth) can act as a signaling molecule that promotes transformation of NTHi into the biofilm phenotype. Biofilms, act as a reservoir of viable bacteria once antibiotic exposure has ended, favoring antibiotic resistance and increased susceptibility to reinfection after treatment (51).

CONFLICT OF INTEREST
The authors have no conflicts of interest to disclose.

AUTHORS CONTRIBUTION
Made substantial contributions to the conception and design of the work: MS and GAR; performed the in vitro experiments, and made substantial contributions acquisition, analysis or interpretation of data for the work: MS and LP; drafted the work: GAR and OS; revised the work critically for important intellectual content: MS, GAR and OS. All the authors provide approval for publication of the content.

REFERENCES


**Figure 1.A.** Concentration- and time-dependent TNF-α release and **Figure 1.B.** Concentration-dependent TNF-α protein expression induced by NTHi bacterial lysate in BEAS-2B cells. The data are presented as mean +SEM from three independent experiments. ***: p <0.001 versus unstimulated cells.

**Figure 2.A.** Time dependent ICAM-1 expression on BEAS-B2 cell surface induced by NTHi bacterial lysate. **Figure 2.B.** NTHi bacterial lysate concentration-dependent increase of ICAM-1 protein expression induced in BEAS-2B cells. The data are presented as mean ±SEM from three independent experiments. *: p <0.05; ***: p <0.001 versus unstimulated cells.
Figure 3. Time-dependent increase of ICAM-1 protein expression induced in BEAS-2B cells by TNF-α (10 ng/ml). B. ICAM-1 expression on BEAS-B2 cell surface induced by NTHi bacterial lysate (1.6 mg/ml) or TNF-α (10 ng/ml), after 24 h incubation. The data are presented as mean ± SEM from three independent experiments. *: p <0.05; **: p <0.01; ***: p <0.001 versus unstimulated cells.

Figure 4.A. Cell surface TLR2 expression by BEAS-B2 cells after 24 h incubation with TNF-α (10 ng/ml) or with NTHi lysates (1.6 mg/ml). Figure 4.B. TLR2 protein expression, after TNF-α exposure or NTHi-stimulation for 24 h (Figure 4.B.). The data are presented as mean ±SEM from three independent experiments. *: p <0.05; ***: p <0.001 versus unstimulated cells.
Figure 5.A. Neutrophil adhesion to the BEAS-2B cell surface after pre-exposure to different concentrations of NHTi lysate (0.4, 0.8 and 1.6 mg/ml). Figure 5.B. Neutrophil adhesion to the BEAS-2B cell surface after pre-exposure of BEAS-2B cells to different concentrations of TNF-α (0.05, 0.10, 1.00 and 10.00 ng/ml). The data are presented as mean ±SEM from three independent experiments. *: p <0.05; **: p <0.01; ***: p <0.001 versus unstimulated cells. Figure 5.C., Figure 5.D., Figure 5.E. Confocal fluorescence microscope images showing neutrophils stained with the anti-human MPO, adherent to BEAS-2B cells pre-exposed to balanced salt solution (CTR), NHTi lysate (1.6 mg/ml) or TNF-α (10 ng/ml).
Figure 6. A. Inhibition of neutrophil adhesion to the BEAS-2B cells stimulated with TNF-α (10 ng/ml) by the addition of anti-human TNF-α mAb (5 μg/ml) or mouse IgG, as isotype control (10 μg/mL), prior to the adhesion assay. Figure 6.B. Inhibition of neutrophil adhesion to the BEAS-2B cells stimulated with NTHi (1.6 mg/ml) by the addition of anti-human TNF-α or anti ICAM-1 mAbs (5 μg/ml), or mouse IgG, as isotype control (10 μg/mL), prior to the adhesion assay. The data are presented as mean ±SEM from three independent experiments. *: p <0.05; **: p <0.01; ***: p <0.001 versus cells exposed to isotype control.

Figure 7. Interacting with TLR2, NTHi lysates induced TNF-α production and release and, together with TNF-α, significant increased ICAM-1 and TLR2 expression. Pre-exposure of BEAS-2B cells to NHTi lysate and to TNF-α also induced an enhancement of neutrophil adhesion to the BEAS-2B cell surface.