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RESEARCH LETTER – Pathogens & Pathogenicity

Effects of N-acetyl-L-cysteine on the membrane vesicle release and growth of respiratory pathogens

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One sentence summary: N-acetyl-L-cysteine enhances the membrane vesicle release but inhibits the bacterial growth and proinflammatory response to bacterial membrane vesicles of non-typeable Haemophilus influenzae, Moraxella catarrhalis, Streptococcus pneumoniae and Pseudomonas aeruginosa.

ABSTRACT

Bacterial infections contribute to the disease progression of chronic obstructive pulmonary disease e.g. by stimulating mucus production in the airways. This increased mucus production and other symptoms are often alleviated when patients are treated with mucolytics such as N-acetyl-L-cysteine (NAC). Moreover, NAC has been suggested to inhibit bacterial growth. Bacteria can release membrane vesicles (MVs) in response to stress, and recent studies report a role for these proinflammatory MVs in the pathogenesis of airways disease. Yet, until now it is not clear whether NAC also affects the release of these MVs. This study set out to determine whether NAC, at concentrations reached during high-dose nebulization, affects bacterial growth and MV release of the respiratory pathogens non-typeable Haemophilus influenzae (NTHi), Moraxella catarrhalis (Mrc), Streptococcus pneumoniae (Spn) and Pseudomonas aeruginosa (Psa). We observed that NAC exerted a strong bacteriostatic effect, but also induced the release of proinflammatory MVs by NTHi, Mrc and Psa, but not by Spn. Interestingly, NAC also markedly blunted the release of TNF-α by naive macrophages in response to MVs. This suggests that the application of NAC by nebulization at a high dosage may be beneficial for patients with airway conditions associated with bacterial infections.

Keywords: membrane vesicles; N-acetyl-L-cysteine; non-typeable Haemophilus influenzae; Moraxella catarrhalis; Streptococcus pneumoniae; Pseudomonas aeruginosa

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a respiratory condition that poses a huge burden on the society worldwide (Pauwels 2001; Lopez et al. 2006). Patients with COPD often show an increased mucus production, sputum purulence and cough (Hauber, Foley and Hamid 2006; Miravitlles 2011; Montes De Oca et al. 2012). To alleviate symptoms, mucolytics are frequently prescribed (van Overveld et al. 2005; Poole 2006, 2009; van Overveld et al. 2009; De Andrade et al. 2011; Herth et al. 2012). The mechanism of action of mucolytics is thought to be mainly due to their mucus reducing properties (Jonkman et al. 2012). Yet, some mucolytics like N-acetyl-L-cysteine (NAC) have been suggested to also possess antibacterial properties (Reis et al. 2008; Stanford et al. 2008; Goodarzi et al. 2009). In this report, we aimed to determine whether NAC at concentrations reached during high-dose nebulization affects the release of bacterial MVs and the subsequent growth of respiratory pathogens. NAC exerts a strong bacteriostatic effect, but also induced the release of proinflammatory MVs by non-typeable H. influenzae, M. catarrhalis and P. aeruginosa, but not by S. pneumoniae. This study suggests that the application of NAC by nebulization at a high dosage may be beneficial for patients with airway conditions associated with bacterial infections.

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Sadowska et al. 2006; Poole, Chong and Cates 2015). A commonly administered mucolytic is N-acetyl-L-cysteine (NAC): a small thiol-containing antioxidant that acts by disrupting disulfide bonds in mucus (Sheffner 1963; Poole 2006). Two recent meta-analyses concluded that patients with COPD experience less exacerbations after oral NAC administration (Cazzola et al. 2015; Poole, Chong and Cates 2015).

Bacterial respiratory infections are the main cause of exacerbations in patients with COPD and bacteria also have been shown to contribute the progression of stable disease (Sethi and Murphy 2008; Marin et al. 2012; King, MacDonald and Bardin 2013; Desai et al. 2014). A variety of studies have shown that NAC does not only have anti-oxidant or anti-inflammatory characteristics, but also bacteriostatic properties (Parry and Neu 1977; Rüse et al. 2000; Olofsson, Hermansson and Elwing 2003; Huynh et al. 2004; Zhao and Liu 2010). These bacteriostatic effects of NAC are thought to be mediated by the inhibition of cysteine utilization by bacteria (Parry and Neu 1977). It is now understood that cysteine deprivation, besides its bacteriostatic action, also induces a bacterial stress response which is characterized by the release of bacterial membrane vesicles (MVs) (van de Waterbeemd et al. 2013). MVs carry several bacterial virulence factors (reviewed in Brown et al. 2015; Schwechheimer and Kuehn 2015).

Therefore, in this study we examined the effect of NAC on both the MV release and growth of the common respiratory pathogens non-typeable Haemophilus influenzae, (NTHi), Moraxella catarrhalis (Mrc), Streptococcus pneumoniae (Spn) and Pseudomonas aeruginosa (Psa). Moreover, we determined if NAC affects the MV-induced proinflammatory responses.

MATERIALS AND METHODS

Reagents and antibodies

NAC (Sigma, St. Louis, MO, USA) was dissolved at 200 mM and the pH was corrected to 7.0–8.0. Antibodies against CD63 (unconjugated, mouse-anti-human clone H5C6) and CD81 (PE conjugated, mouse-anti-human clone JS-81) were obtained from BD (BD Bioscience, Franklin Lakes, NJ, USA). The anti-Haemophilus influenzae type b (μ-Hib; clone 1079/457) monoclonal antibody was obtained from Acris (Acris GmbH, Herford, Germany). The rabbit serum against Mrc (strain A 1.39N, isolated from children in a primary school in Nieuwegein, the Netherlands (1989)), was kindly provided by Dr J. Hays (Erasmus University, Rotterdam, the Netherlands). The antibody against Psa was from Antibodies online (Aviva Systems Biology, San Diego, CA, USA, neat raised in guinea pig). Antibodies were purified using the antibody serum purification kit based on protein A (Abcam, Cambridge, MA, USA). For flow cytometric analysis, detection antibodies were PE conjugated using PE-labeling kits from Abcam. One hundred microgram of each antibody was PE conjugated according to the manufacturers’ instructions.

Bacterial strains and culturing

The following bacterial strains were used: NTHi (ATCC-49247), Psa (ATCC-27853), Spn (ATCC-49619) and a Mrc clinical isolate (University Medical Centre Maastricht (MUMC+), the Netherlands). Mrc, Spn and Psa were pre-cultured on blood plates, and NTHi was cultured on vitalex-supplemented chocolate agar plates (Oxoid, Wesel, Germany) overnight at 5% CO2 and 37°C. After overnight pre-culture, bacterial suspensions with a density of 0.5 McFarland (1.5 × 10⁹ colony forming units (cfu)/ml) were prepared. These suspensions were then used to prepare bacterial cultures or for infection experiments. Bacteria were cultured at 5 × 10⁷ cfu/ml with or without NAC (5, 25 or 50 mM) for 6 h in 10 ml RPMI1640. NTHi and Mrc were cultured in medium supplied with 10 μg/ml hemin and nicotinamide adenine dinucleotide (both from Sigma). After 6 h, cultures were centrifuged at 1200 × g for 10 min, at room temperature. The pellets bacteria were washed, diluted in phosphate-buffered saline (PBS) and the optical density was determined at 600 nm in optical methacrylate disposable cuvettes (Sarstedt, Newton, NC, USA). The supernatant was again centrifuged at 1200 × g for 10 min to deplete possible remnant bacteria, followed by filtering the supernatants through 0.22 μm filters. To allow MV quantification by tunable resistive pulse sensing, supernatants were concentrated 20 times to a final volume of 500 μl by centrifugation at 4000 × g for 15 min using Amicon ultra-15 10-kDa centrifugal filter units (Millipore, Billerica, MA, USA).

Bacterial MVs for the stimulation experiments were obtained from bacteria that were cultured for 4 h in 30 ml complete vesicle-depleted medium (prepared as described in the cells and media section) at a density of 1 × 10⁶ cells/ml. Upon culture, supernatants were depleted from bacteria by two centrifugation steps at 1200 × g for 10 min and 0.22 μm filtration. Cleared supernatants were then further processed by ultrafiltration and size-exclusion chromatography (SEC), as described below.

Cells and media

Human monocytic THP-1 cells (ATCC-TIB202) were cultured at 5% CO2 and 37°C in RPMI1640 (Sigma) containing 10% fetal calf serum (FCS) (Lonza, Verviers, Belgium) with glucose (22.5%), sodium pyruvate (100 mM) and β-mercaptoethanol (25 mM). Monocytes, seeded at 0.5 × 10⁶ cells per well in 24-wells plates, 1 × 10⁴ per well in 96-wells plates and 1 × 10⁶ cells in 757-flasks, were differentiated using 200 mM phorbol 12-myristate 13-acetate (PMA; Sigma) for 72 h. Hereafter, the medium was replaced with vesicle-depleted culture medium containing 5% FCS containing glucose (22.5%), and sodium pyruvate (100 mM), hereafter referred to complete vesicle-depleted medium. Medium with 30% FCS was depleted from abundant and possibly confounding bovine MVs by combining FCS-free culture medium with vesicle-depleted medium with 30% FCS. Vesicle-depleted medium with 30% FCS was obtained by the overnight centrifugation at 100 000 × g using a 70Ti-rotor, x-factor 44 in an Optima L-90K ultracentrifuge (both Beckman Coulter, Fullerton, CA, USA).

Infection of macrophages for MV analysis

For macrophage infections in 24-wells plates, cells were washed three times with PBS whereafter the medium was replaced with complete vesicle-depleted culture medium. Hereupon, macrophages were infected with either one of the bacteria at a multiplicity of infection of 10. After 6 h of infection, the medium was harvested, processed by two centrifugation steps at 1200 × g for 10 min and 0.22 μm filtration, and used for bead-based flow cytometry.

MV concentration and purification from conditioned medium using ultrafiltration and SEC

MVVs were concentrated from bacteria-cleared supernatants by using ultrafiltration (Lobb et al. 2015) and separated from protein by SEC (Boëng et al. 2014). This method for vesicle...
isolation is considered one of the most appropriate methods for the isolation of verwijderen/mammalian extracellular vesicles as it provides with an efficient standardized method for the generation of pure intact MV populations (Boing et al. 2014; Lobb et al. 2015; Nordin et al. 2015). For ultrafiltration, the cleared supernatants were concentrated 60 times to 500 μl in two centrifugation runs at 4000 × g for 15 min at room temperature using Amicon ultra-15 10-kDa centrifugal filter units (Millipore). The method for MV purification by SEC was performed as previously described by Boing et al. (2014), with minor alterations. Briefly, a 15 ml-TELOS filtration column (Kinesis Scientific Experts, St. Neots, Cambridgeshire, UK) was stacked with 10 ml sepharose CL-2B (GE Healthcare, Uppsala, Sweden). Next, the concentrates were loaded onto the column and eluted using PBS. Twenty-four fractions of 500 μl were collected and the MV-containing fraction (based on five pooled fractions: 7–11, previously identified to be highly enriched for MVs and negative for free proteins) was stored at –80°C until further use.

MV analysis by tunable resistive pulse sensing

The MV concentration was determined by tunable resistive pulse sensing using the qNano Gold, the Izon Control Suite Software v3.2 and the reagent kit (type RK1) for EV analysis from Izon (Izon Science Ltd., Oxford, UK). Measurements were conducted using a NP150 pore with a fixed stretch of 47 mm, a transmembrane voltage of 0.48 V (led to a baseline current of ±100 nA) and a pressure of 6 mbar. Solution G was added (10%) to supernatants that were diluted in solution Q (1:1) and each sample was measured for 10 min and measurements were repeated when system instabilities occurred. The samples were calibrated using 114 nm polystyrene calibration beads (CPC100, Izon Science Ltd.) at a concentration of 1 × 10⁷ particles/ml diluted in culture medium.

Flow cytometry analysis of MVs using antibody-coated latex beads

Bead-based flow cytometry was used for the semiquantitative analysis of MVs released by macrophages and bacteria. This method was previously described (Ostrowski et al. 2010; Kapustin et al. 2015) and was adopted for bacterial vesicle quantification (Volgers et al. 2017). Briefly, 4–μm-sized aldehyde-sulfate beads were washed in MES buffer and coated with anti-CD63 for host-cell vesicles or with an antibody against one of the bacterial MVs. Antibody-coated beads were incubated overnight with 200 μl of cleared supernatant under constant agitation at 1000 rpm at room temperature. Next, the beads were washed twice with 0.22 μm filtered PBS with 2% (w/v) bovine serum albumin and incubated with a PE-conjugated detection antibody, for 90 min at room temperature under continuous agitation. Antibacterial antibodies were PE conjugated using a PE conjugation kit (Abcam) following the manufacturer’s instructions and for the host-cell MVs, the detection was based on anti-CD81-PE labeled detection antibodies. After incubation with the PE-labeled detection antibodies, the beads were washed and suspended in 30 μl PBS for flow cytometric analysis on a FACSCanto (BD Bioscience, Franklin Lakes, NJ, USA). The lower threshold for detection was set at 2% based on the percentage of PE-positive control beads (incubated with unconditioned culture medium). Analyses were performed using FACSDiva Software. The relative amount of MVs was calculated by the multiplication of the percentage of positive beads with the median fluorescence intensity and expressed relative to the control (as % of control).

Macrophage stimulation with MVs

Stimulation of macrophages with MVs was performed using macrophages seeded in 96-well plates. Prior to stimulation, macrophages in 96-well plates were washed three times with PBS after the medium was replaced with vesicle-depleted culture medium with 5% FCS. Then, cells were exposed to 20 μl of the MV-containing SEC fraction for 16 h, without or in the presence of NAC (5 μM, 5 mM or 25 mM). Hereafter, the culture supernatants were harvested and used for cytokine measurements.

Assessment of the bacterial growth and adhesion and internalization

To assess for bacterial growth during culture, the optical density at 600 nm was determined at a path length of 1 cm using a spectrophotometer (Novaspec II: Pharmacia Biotech, Uppsala, Sweden) (Sutton 2011). PMA-stimulated monocytes seeded in 24-well plates were washed three times with PBS after which vesicle-depleted culture medium was added. Next, the cells were infected with either one of the bacteria at an MOI of 10 for 4 h. After infection, the media were collected to assess bacterial survival (and for flow cytometric analysis of bacterial and host-cell MV release). To assess bacterial survival, bacteria were pelleted by centrifugation at 1200 × g, resuspended in 1 ml PBS, whereupon bacterial dilutions were made and plated. In addition to bacterial survival, bacterial adhesion and internalization were determined. After the supernatant was harvested, cells were washed three times with PBS and lysed by adding 0.025% saponin in distilled water for 10 min. After neutralization, by the addition of culture medium, bacterial adhesion and internalization were determined by bacterial plating. The number of adhered and internalized bacteria was then determined by counting.

Cytokine measurements

The human Ready-Set-Go TNF-α enzyme-linked immunosorbent assay kit (Affymetrix eBioscience, Santa Clara, CA, USA) was used to determine the TNF-α release according to the manufacturer’s instructions.

Statistical analyses

Statistical analysis was performed using Graph-Pad Prism 5 Software (Graph-Pad, San Diego, CA, USA). Statistical dispersion was determined by calculation of the standard error of the mean. A Mann-Whitney t-test was performed for the statistical analysis of the variance between the means of two groups. P-values were considered significant when <0.05.

RESULTS

The effect of NAC on bacterial growth

First, it was determined whether NAC affects the growth of the common respiratory pathogens NTHi, Mrc, Spn and Psa. We initially determined if the growth was affected by NAC concentrations of 5–50 mM, a concentration based on the estimated concentration of ~30 mM that may be achieved in the lower airway upon administration of 300 mg NAC by nebulization and which may therefore be physiologically relevant (Szklarek et al. 2004). As shown in Fig. 1, in the presence of 50 mM...
**Bacterial growth and MV release by respiratory pathogens during bacterial culture with or without NAC.** NTHi, Mrc, Spn and Psa were cultured for 6 h without or in the presence of NAC (5, 25 or 50 mM). Bacterial growth was determined with the optical density of the bacterial suspensions at 600 nm (A). Additionally, the MV release was determined by tunable resistive pulse sensing (B) and the MV release was adjusted for the number of bacteria (C). All values are presented here as percentage of the untreated control condition. (n = 3, *P* < 0.05).

NAC bacterial growth was reduced by 43%, 66%, and 80% of the Gram-negative bacteria NTHi, Mrc, and Psa, respectively. Finally, the bacterial growth of the Gram-positive Spn was also reduced by 47%.

**Bacterial MV release in response to NAC**

Next, the effect of NAC on the release of bacterial MVs was assessed by tunable resistive pulse sensing-based analysis. We observed that the addition of NAC at a concentration of 5 mM resulted in a 2-fold and 3-fold increase in the release of NTHi and Mrc MVs, respectively (Fig. 1B). This MV release was also increased after treatment with higher concentrations, albeit to a lesser extent. Yet, since NAC markedly inhibited bacterial growth (Fig. 1A), we adjusted the release of MVs for the number of bacteria present in each culture condition. After adjustment, the bacterial MV release was found to be increased 3-fold for NTHi and even 5-fold for Mrc (Fig. 1B). Although the bacterial MV release by Psa initially did not seem to be affected by NAC (Fig. 1A), when the percentage was adjusted for the number of bacteria the MV release was found to be increased 5-fold. Overall this suggests that although NAC significantly inhibited bacterial growth of these Gram-negative bacteria, the release of MVs per bacterium seemed to be increased in the presence of NAC. The release of MVs by Spn tended to decrease upon treatment and disappeared when adjustments for the total bacterial numbers were made suggesting that this decrease was the consequence of the reduced number of bacteria (Fig. 1A).

**The effects of NAC on bacterial growth during infection**

As bacterial infections of the airways are associated with prominent activation of alveolar macrophages, we examined the effects of NAC treatment on bacterial survival and MV release in an in vitro infection model (Martin and Frevert 2005). First, we determined bacterial growth and adhesion/internalization after macrophage infection with either one of the bacteria, and determined how this was affected by 25 mM NAC. NAC treatment almost completely prevented bacterial growth and adhesion/internalization of NTHi and Mrc (Fig. 2A and B). Treatment of Psa-infected macrophages had inconsistent effects on the bacterial growth and hardly affected adhesion/internalization (Fig. 2A and B). Finally, treatment of Spn led to a ~50% reduction of the bacterial growth but did not affect the adherence/internalization (Fig. 2A and B).

**The effect of NAC on the release of bacterial and host cell-derived MVs during macrophage infection**

Next, we studied if bacterial MV shedding during macrophage infection was affected by NAC. In the first set of experiments, tunable resistive pulse sensing was used to quantify MVs in a bacterial monoculture. However, during infection the MV population released consisted of both bacterial and host-cell MVs. Therefore, a bead-based flow-cytometry assay for the semiquantitative determination of MVs was applied here. Unfortunately, we failed to establish this platform for Spn so we could not quantify the vesicle release for this species in this setting.

At a concentration of 25 mM, NAC increased the MV release by NTHi and Mrc (Fig. 3A). When adjustments were made for the total number of bacteria, this increase was 5-fold for NTHi and 10-fold for Mrc (p ≤ 0.05 vs control, Fig. 3B). Regarding Psa, when adjustments were made, the MV release was found to increase on treatment with 25 mM NAC (Fig. 3B).

MV shedding during infection is a dynamic process that occurs by both bacteria and the host. As it has been shown previously that host cell-derived MVs also have immunostimulatory properties (Qazi et al. 2010; Kulshreshtha et al. 2013; Soni et al. 2016), we additionally determined the effect of NAC on the release of CD63+/CD81+ MVs by macrophages. Treatment with 25 mM NAC significantly reduced the release of host-derived vesicles by macrophages, irrespective of the bacteria they were infected with (Fig. 4).

**The proinflammatory response to MVs is reduced by NAC**

In earlier experiments, we demonstrated that bacterial MVs induce the release of proinflammatory cytokines by naïve
macrophages. Here, we determined if this proinflammatory re-
response induced by MVs from NTHi, Mrc, Spn and Psa was af-
fected by NAC treatment. As shown in Fig. 5A, all MV popu-
lations except those of Spn induced the release of TNF-α by naive
macrophages, albeit with different potencies. The response of
macrophages to MVs was dose dependently reduced in the pres-
ence of NAC for all conditions except for Spn. Importantly, we
determined that this decreased release of TNF-α was not the re-
sult of a treatment-induced cytotoxicity as NAC treatment was
not found to have cytotoxic effects (Fig. 5B). Interestingly, MVs
from NTHi and Mrc showed to have cytotoxic effects that could
be prevented by NAC treatment.

DISCUSSION
NAC is best known for its mucolytic properties, and because
of its additional antioxidant and anti-inflammatory properties,
it is an attractive drug in COPD therapy (van Overveld et al.
2005; Poole 2006; Sadowska et al. 2006; Cazzola et al. 2015; Poole,
Chong and Cates 2015). Moreover, bacteriostatic characteris-
tics have been assigned to NAC in several studies (Riise et al.
2000; Olofsson, Hermansson and Elwing 2003; Huynh et al. 2004;
Hafez et al. 2009; Zhao and Liu 2010). In this study, we con-
firmed this bacteriostatic effect as the growth of several respi-
ratory pathogens was markedly inhibited by NAC. Therefore, as
many exacerbations result from bacterial infections, the ther-
apapeutic effect of NAC may be beyond its mucolytic and anti-
inflammatory and anti-oxidative properties. Additionally, in this
study we demonstrate that NAC, despite an enhancement of the
release of proinflammatory MVs by the Gram-negative bacteria
used in this study, suppressed the release of inflammatory cy-
tokines by naive macrophages in response to these MVs.

There is a great demand for new treatments that relieve air-
way obstruction and inflammatory processes in patients with
COPD (Barnes 2003). Treatment with mucolytics, most promi-
nently NAC, has been shown to alleviate these symptoms (Caz-
zola et al. 2015; Poole, Chong and Cates 2015). Moreover, (part
of) these patients frequently suffer from severe exacerbations
which are mostly caused by respiratory infections. Recently,
Cazzola et al. (2015) concluded from their meta-analysis that pa-
tients with COPD or bronchitis who received oral NAC treatment
had a decreased risk of exacerbations, although higher doses
were required in patients with airway obstruction. There are in-
dications that this protective effect of NAC may not only rely on
its mucolytic, anti-oxidant or anti-inflammatory effects, but also
on its antibacterial activity. One study by Riise et al. (1994) shows
that COPD patients who received oral NAC therapy had a signifi-
cant reduction of the intrabronchial bacterial numbers. This an-
tibacterial activity has already been demonstrated in the 1970s
by Parry and Neu (1977), who demonstrated that NAC inhib-
ited the growth of both Gram-negative and Gram-positive bacte-
ria. Likewise, we also found that NAC caused a dose-dependent
growth reduction of all bacteria tested. Also, during infection of
naive macrophages, survival of NTHi and Mrc was significantly
reduced in the presence of NAC. Adhesion of these two Gram-
negative bacteria was markedly diminished by NAC. This data
suggest a possible role for NAC beyond its well-known effects.

The release of MVs, which have been shown to be im-
portant vehicles for the simultaneous delivery of many ef-
fector molecules to host cells, is a common feature for both
Gram-negative and Gram-positive bacteria (Brown et al. 2015; Schwechheimer and Kuehn 2015). MVs may influence the course of infection and the host immune response by presenting pathogen-associated molecular patterns and antigens to their respective host receptors. A potential involvement of bacterial MVs in the pathogenesis of lung disease is indicated by several studies that demonstrate that bacterial MVs are present in infected tissues (Bauman and Kuehn 2006; Perez Vidakovics et al. 2010; Ren et al. 2012). Moreover, it has been demonstrated that bacterial MVs are well able to activate immune cells and induce the release of proinflammatory cytokines. Additionally, bacterial MVs have been shown to facilitate replication of Legionella pneumophila in infected macrophages or to induce emphysema via IL-17A-mediated neutrophilic inflammation (Kim et al. 2015; Jung et al. 2016). Thus, inhibition of bacterial MV release might be beneficial for the course of pulmonary disease associated with infection (and other infection-related diseases). Here we examined the effects of NAC on the release of MVs by common respiratory pathogens. We provide evidence that treatment markedly enhanced the release of these vesicles by all Gram-negative bacteria when corrected for the number of bacteria. In contrast, no effects on the MV release by the Gram-positive Spn were observed.

The proposed mechanism by which NAC inhibits bacterial growth is by inhibiting the cysteine utilization by bacteria (Parry and Neu 1977). Additionally, this may also explain how NAC triggers the release of MVs as a recent study showed that the inhibition of cysteine utilization in Neisseria meningitis cultures had bacteriostatic effects and promoted the release of bacterial MVs (van de Waterbeemd et al. 2013). That the bacteriostatic effects of NAC are likely due to the inhibition of cysteine utilization might also explain the species-specific effects observed. Since the cysteine requirements differ among bacteria and can even vary between strains, these differences may affect the bacterial sensitivity to NAC (Guédon and Martin-Verstraete 2006).

We also showed that particularly MVs from the Gram-negative bacteria stimulated the release of TNF-α by naive macrophages. This is in accordance with earlier studies demonstrating a MV-induced release of proinflammatory cytokines and chemokines in vitro as well as in vivo (Bauman and Kuehn 2006; Schaar et al. 2011; Sharpe, Kuehn and Mason 2011; Lee et al. 2012; Park et al. 2013). Thus, in view of the enhanced release of MVs by our Gram-negative bacteria, the therapeutic potential of NAC may be limited as it may hereby enhance the pulmonary inflammation. However, when we stimulated naive macrophages with bacterial MVs, the release of TNF-α could be dose-dependently inhibited by increasing concentrations of NAC. This suggests that NAC might be able to counteract its proinflammatory effect due to the enhanced bacterial MV release by inhibiting the subsequent release of proinflammatory cytokines by activated immune cells. Additional studies are warranted to further examine the net effect of NAC in pulmonary bacterial infections.

Although several in vitro studies have shown that NAC has potent anti-oxidant and anti-inflammatory (as reviewed by Sadowska, Manuel-Y-Keenoy and De Backer 2007), as well as bacteriostatic properties (Parry and Neu 1977; Riise et al. 2000; Olofsson, Hermansson and Elwing 2003; Huynh et al. 2004; Hafez et al. 2009; Zhao and Liu 2010), this was mostly at concentrations which are hard to achieve by oral administration. For example, upon oral administration of 600 mg thrice daily for 5 days, NAC could not be detected in the epithelial lining fluid, bronchoalveolar lavage fluid or lung tissue (Cotgreave et al. 1987; Bridgeman et al. 1994). Yet, for certain respiratory conditions, such as idiopathic pulmonary fibrosis and cystic fibrosis, NAC is administered by nebulization (350 mg, twice daily), whereby a high local bioavailability is reached (Homma et al. 2012; Tam et al. 2013). In view of the present data, which suggest that COPD patients may also benefit from high local NAC concentrations, nebulization might form an attractive alternative in the treatment of these patients.

To summarize, in the current study we demonstrated that high NAC concentrations exert bacteriostatic effects on NTHi, Mrc, Spn and Psa cultures and on NTHi and Mrc during macrophage infection. On the other hand, this inhibition of the bacterial growth was accompanied by an increased MV release. However, as our results also show that NAC markedly reduces the proinflammatory response to MVs, it is likely that the increased MV release is of little consequence. This may implicate that NAC administration by nebulization may have a positive
treatment outcome during airway infections, such as bacterial exacerbations of COPD, but only when administered at a high concentration. Future research will be required to study these effects in vivo and to determine the long-term effects of NAC treatment on respiratory infections and exacerbations.

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