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The antimicrobial peptide S100A8/A9 produced by airway epithelium functions as a potent and direct regulator of macrophage phenotype and function

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Abstract

Elevated counts of alveolar macrophages and attenuated phagocytic capacity are associated with COPD. Factors governing macrophage phagocytosis are poorly understood. In this study we aimed to compare the influence of airway epithelial cell secretions from COPD and nonCOPD subjects on macrophage phagocytic activity, and the role of antimicrobial peptides (AMPs).

Supernatants from nonCOPD and COPD small airway epithelial cell (SAEC) cultures exposed to *Haemophilus influenzae* (*NTHi*), were applied to human monocyte-derived macrophages (MDM) for assessment of their influence on phagocytosis. SAECs were analysed for changes in AMP expression by qRT-PCR and the influence of select AMPs on macrophage phenotype and function was assessed by flow cytometry and metabolic activity assay.

Secretions from the apical and basolateral surface of *NTHi*-exposed SAECs from nonCOPD donors elicited superior phagocytic capacity of MDMs. Moreover, *NTHi* exposure led to a rapid increase in expression of a range of AMPs by nonCOPD SAECs, but this response was delayed in COPD SAECs. We demonstrate that treatment with AMPs β -defensin 2 and S100A8/A9 improved the phagocytic capacity of MDMs. In depth analysis of S100A8/A9 influence of MDMs revealed a role of this AMP in macrophage phenotype and function. Furthermore, we show that the expression of S100A8 and S100A9 is directly regulated by WNT/ β -catenin signalling activity, a known deregulated pathway in COPD.

In conclusion, for the first time, we demonstrate that airway epithelium from COPD subjects has a reduced capacity to support the phagocytic function of macrophages in response to acute *NTHi* exposure, and we identify the WNT/ β -catenin signalling-modulated and epithelium-derived S100A8/A9 as a potent regulator of macrophage phenotype and function.

Introduction

Chronic obstructive pulmonary disease (COPD) is one of the leading causes of morbidity and mortality worldwide [1], characterized by destruction of the lung parenchyma (emphysema), chronic inflammation [2], and increased lung colonisation with pathogenic bacteria including *nontypeable Haemophilus influenzae* (NTHi) [3]. The airway epithelium represents a key barrier to microbes and microbial products, through its mucociliary clearance capacity and secretion of antimicrobial peptide and protein (AMP) [4, 5]. AMPs, also known as host defense peptides, are naturally occurring cationic small proteins that can directly inactivate pathogens [6]. The best studied groups of human AMPs are defensins and cathelicidins (LL-37) [7]. Levels of AMPs in sputum as well as serum are modulated during COPD exacerbations [8]. Furthermore, differences in bacterial killing activity of nonCOPD and COPD epithelium secretions, have previously been reported and linked to differences in AMP expression (BD2, LCN2 and S100A7) [9]. Pulmonary macrophages are also critical for effective clearance of bacteria in the airways. Elevated macrophage numbers have been reported in COPD, however their phagocytic capacity is impaired [10, 11]. As critical innate immune mechanisms, disease-related alterations in phagocytosis and AMP production could contribute to increased bacterial colonization in COPD exacerbations [11]. Interestingly, neutrophil-derived LL-37 and Human Neutrophil Peptide (HNP) 1-3 AMPs have been reported to exert immunomodulatory function on macrophages by improving their phagocytic capacity [12, 13]. However, the mechanisms underlying epithelial secretion-induced changes in macrophage phagocytic capacity and the role of AMPs in this crosstalk are currently unknown.

In this study we sought to understand the differences between the capacity of nonCOPD and COPD epithelial secretions to stimulate macrophage phagocytosis and investigated the involvement of airway epithelium-derived AMPs in this process.

Methods

Isolation of peripheral blood mononuclear cells (PBMCs), monocytes and generation of macrophages

Leukopack concentrates were obtained from healthy donors at DRK-Blutspendedienst Ulm and all donors provided written consents. PBMCs were isolated from leukopacks concentrates, obtained from healthy donor by standard density gradient centrifugation with Ficoll-Paque.

Monocytes were isolated using the EasyStep™ Human Monocyte Isolation Kit (#19359, StemCell) in combination with RoboSep (StemCell).

Macrophages were subsequently generated by seeding monocytes in Nunc™ plates with UpCell™ surface (ThermoFisher) in XVIVO10 media (with no phenol red and no transferrin, Lonza) with addition of M-CSF (MILT-130-096-489, Miltenyi Biotec) 100ng/ml.

Cell culture

Small airway epithelial cells (SAECs) from nonCOPD and COPD individuals (characterization of the patients in Table 1) were purchased from Lonza (CC-2547) and cultured in air-liquid interface (ALI) setting. Cells were grown to 80% confluence in Pneumacult-ex plus media with supplements, 1% hydrocortisone and 1% Penicillin-Streptomycin (all STEMCELL Technologies). Next, cells were trypsinized and seeded onto Bovine Collagen (#5005, Medical Industrie GmbH) coated transwell inserts (#3470 Corning). After cells reached confluence media from apical chamber was aspirated, so the cells were exposed to air and the media in basolateral chamber was exchanged to the Pneumacult ALI2 media with addition of maintenance supplements, 2% heparin, 5% hydrocortisone and 1% Penicillin-Streptomycin (all STEMCELL Technologies). Cells were cultured for 4 weeks, until fully differentiated; identified by reaching stable transepithelial electrical resistance of $1000 \Omega \cdot \text{cm}^2$ (formation of stable barrier), mucus production and cilia beating across the surface of the transwell culture.

For *NTHi* experiments, basolateral media was exchanged to media without antibiotics 24h before *NTHi* addition. The apical surface of SAEC was washed with warm PBS and 20µl of PBS or *NTHi* (10^7 bacteria) was added on top. Cells were incubated for 3h or 24h. Afterwards, basolateral media (basolateral SN) and apical surface washes (apical SN; washed two times

with PBS in total volume of 300µl). All SNs were centrifuged with 14 000g for 10 min to remove cell debris and any bacteria residues.

For WNT modulation, SAECs were exposed to FH535 (FH, 20µM, #4344/10, Tocris), WNT5A (RnD #645-WN, 100ng/ml) or Chir99021 (CT, 2µM) for 24h. Afterwards cells were washed one time with PBS and stored at -80°C until RNA isolation.

To assess influence of *NTHi* on WNT5A expression, M-CSF-generated MDMs were exposed to *NTHi* for 24h, washed with PBS and kept at -80°C before RNA isolation.

Phagocytosis assay

Macrophages were seeded in a 96-well plate (30 000/well) and rested for 2h. For experiments with SAEC SNs, cells were treated with SN mixed 1:1 with XVIVO10 media and co-exposed to pHrodo® *E.coli* particles (Essen BioScience). For the experiments with recombinant AMPs, cells were pre-treated for 1h with recombinant proteins: BD2 (#167300-49-B, Peprotech), SLPI (#1274-PI-100, R&D), S100A8 (#9876-S8-050, R&D), S100A9 (#9254-S9-050, R&D), S100A8/A9 (#8226-S8-050, R&D) (all 5µg/ml, based on the literature [14]) or vehicle as a control). Subsequently, cells were co-treated with pHrodo® *E.coli* or *S.aureus* particles and particle fluorescence (when localized in the acidic environment of the phagolysosome) measured by IncuCyte S3 Live-Cell Analysis System (Essen Bioscience) to monitor phagocytosis progression over time.

Statistical analyses

Statistical analyses were performed using GraphPad Prism (GraphPad Software). Data are expressed as mean or mean±sem. Means between two groups were compared using the Mann-Whitney test or Student's t-tests. Comparisons between more than two groups were performed by Kruskal-Wallis test followed by Dunn's post test or one way ANOVA followed by Sidak multiple comparison test. P values were considered to indicate a statistically significant difference when <0.05.

Additional material and methods can be found in the supplement

Results

Supernatants from *NTHi*-exposed COPD airway epithelial cells have diminished impact to support the phagocytic capacity of macrophages

To study the influence of nonCOPD and COPD epithelium-derived factors on the function of macrophages, small airway epithelial cells (SAEC) from nonCOPD and COPD individuals (donor information in Table 1) were exposed to PBS or *NTHi* at the apical surface. No differences were apparent between nonCOPD and COPD SAEC based on H&E staining of the SAECs, transepithelial electrical resistance (TEER), as well as expression analysis of epithelial and mesenchymal markers (Supplementary Fig. 1). Supernatants (SN) were collected from the apical surface and basolateral media of SAEC cultures and subsequently used to stimulate MDMs (scheme depicted in the Fig. 1a). SN from nonCOPD-obtained SAEC exposed to *NTHi* for 3h, relative to PBS-exposed SAEC SN, supported macrophage phagocytic function significantly better than COPD-obtained SN in the case of both apical and basolateral SN (Fig. 1b; 3h apical: mean $\pm$ sem, nonCOPD vs COPD: 2h 114 $\pm$ 6.4% vs 99.6 $\pm$ 3.1%, 4h 115 $\pm$ 6.1% vs 98.5 $\pm$ 2.1%, 6h 116 $\pm$ 5.3% vs 98 $\pm$ 2.6%; basolateral: mean $\pm$ sem, nonCOPD vs COPD: 2h 101 $\pm$ 2.5% vs 92 $\pm$ 2.5%, 4h 101 $\pm$ 2.2% vs 92 $\pm$ 2.2%, 6h 101 $\pm$ 2% vs 93 $\pm$ 1.8%). However, the differential effect of nonCOPD and COPD SNs on macrophage phagocytosis, was no longer apparent after longer exposure of epithelium to *NTHi* over 24h. No obvious differences were observed in the viability of macrophages exposed to nonCOPD and COPD SNs (Supplementary Fig. 2a) nor in surface marker expression profiles (Supplementary Fig. 2b). Overall, these results suggest a delayed secretory response from COPD epithelium to bacterial exposure.

COPD airway epithelial cells exert delayed AMP response to *NTHi*

While recent experimental evidence has suggested that neutrophil-derived AMPs can modulate the function of macrophages [12, 13], little is known about the potential influence of epithelial-derived AMPs on macrophage function.

Based on literature [9, 12] and our unpublished data we selected a number of known epithelial-derived AMPs to investigate. We assessed potentiation of AMP expression upon *NTHi* exposure (*IL6* mRNA expression used as a positive control for bacterial stimulation shown in Supplementary Fig. 3a; $\Delta\Delta C_p$ mean $\pm$ sem, 3h: nonCOPD 3.2 $\pm$ 0.38, COPD 2.7 $\pm$ 0.28, 24h: nonCOPD 1.7 $\pm$ 0.27, COPD 1.34 $\pm$ 0.37) in nonCOPD and COPD SAECs and identified several

differentially regulated AMPs; namely *DEFB4A*, *SLPI*, *S100A9* and *PI3* were more highly expressed in *NTHi*-exposed nonCOPD than COPD SAECs (Fig 2; $\Delta\Delta C_p$ mean $\pm$ sem; 3h nonCOPD vs COPD: *DEFB4A* 2.44 $\pm$ 0.3 vs 1.1 $\pm$ 0.38 *SLPI* 0.38 $\pm$ 0.12 vs -0.1 $\pm$ 0.1, *S100A9* 1.3 $\pm$ 0.19 vs 0.67 $\pm$ 0.19) and for *S100A8* there was a trend after 3h *NTHi* exposure (1.5 $\pm$ 0.27 vs 0.66 $\pm$ 0.35). However, these differences in epithelial AMP expression were not observed after 24h of *NTHi* exposure, consistent with a delayed response to bacterial infection from COPD epithelium.

S100A8/A9 heterodimer and BD2 modulate macrophage phagocytosis

Based on these findings, we next interrogated the ability of selected AMPs (BD2 – gene *DEFB4A*, *SLPI*, *S100A8*, *S100A9* and the *S100A8/A9* heterodimer) to directly influence macrophage phagocytosis. We used the bacterial particles in these assays, not live bacteria, to eliminate possible direct bactericidal effects of the AMPs.

We did not observe modulation of phagocytosis with *SLPI*, nor with *S100A8* or *S100A9* homodimers (Supplementary Fig. 4). However, we found that the *S100A8/A9* heterodimer and BD2 could enhance phagocytosis of *E.coli* (Gram-negative) particles (Fig 3a; mean $\pm$ sem, *S100A8/A9* relative to vehicle: 2h 105.3 $\pm$ 1.9%, 4h 102.8 $\pm$ 0.9%, 6h 103 $\pm$ 0.45%; BD2 relative to vehicle: 2h 104 $\pm$ 1.9%, 4h 105.7 $\pm$ 1.6%, 6h 106.5 $\pm$ 1.4%, 12h 105.2 $\pm$ 1.3%) as well as *S.aureus* (Gram positive) particles (Fig. 3b; mean $\pm$ sem, *S100A8/A9* relative to vehicle: 2h 120 $\pm$ 6.4%, 4h 98.8 $\pm$ 3.2%, 6h 98 $\pm$ 3%; BD2 relative to vehicle: 2h 136 $\pm$ 2.2%, 4h 122 $\pm$ 1.8%, 6h 120.8 $\pm$ 1.3%). Notably, in the case of *S.aureus* the positive effect was the most apparent at acute exposure. We therefore investigated the potential additive/synergistic effect at the early time point and found that there was a trend towards an additive effect on MDM phagocytosis when *S100A8/A9* and BD2 were applied together (Fig. 3c; mean $\pm$ sem, *S100A8/A9* 120 $\pm$ 6.4%, BD2 136 $\pm$ 2.2% vs *S100A8/A9*+BD2 145 $\pm$ 3.2%).

S100A8/A9 and BD2 proteins are differentially regulated by *NTHi* in nonCOPD and COPD SAEC secretions

As we observed that BD2 and *S100A8/A9* proteins could improve the phagocytic function of macrophages, we investigated the expression of these proteins in SAEC SN upon *NTHi* exposure. We found that 3h *NTHi* stimulation significantly increased the expression of BD2 protein in the apical SN of nonCOPD SAEC (mean $\pm$ sem; relative to control as 100%; 117 $\pm$ 6.4%),

but not COPD SAEC ($114 \pm 13\%$). In the case of S100A8/A9, *NTHi* did not increase the detectable levels of this AMP in apical SN of nonCOPD SAEC ($81 \pm 9\%$), but significantly decreased the expression in SN of COPD SAEC ($70 \pm 9\%$). *NTHi* stimulation did not, however, have an influence on the secretion of BD2 or S100A8/A9 to the basolateral compartment (Fig. 4 and Supplementary Fig. 5).

S100A8/A9 modulates macrophage phenotype and metabolism

Our findings related to BD2 support data published in a previous report showing its role in enhancing phagocytosis of bacteria by macrophages [16]. Therefore, we decided to further characterize the yet unknown role of S100A8/A9 in modulation of MDM phenotype and function.

Analysis of the downstream effects of S100A8/A9 on macrophages (evidence for binding of S100A8/A9 to MDM is presented in Supplementary Fig. 6) revealed enhanced phosphorylation of CREB, ERK1/2, HSP27, JNK 1/2/3, MSK 1/2, AKT 1/2/3, c-JUN, p70S6, RSK 1/2/3 and STAT3 (Fig. 5a and Supplementary Fig. 7). A number of these signalling molecules are known to be associated with phagocytosis [17-19]. Furthermore, we found that S100A8/A9 also significantly altered the expression of receptors known to play a role in phagocytosis, inducing an upregulation of *FCGR2A*, *MARCO* and *TLR2* mRNA and a downregulation of *FCGR1A* and *TLR4* (Fig. 5b). We were able to confirm the upregulation of CD32 (*FCGR2A*) and MARCO protein expression by FACS, and despite not changing at the mRNA level, FACS analysis also revealed increased expression of cell surface expression of CD64 (*FCGR1A*) protein upon S100A8/A9 exposure (Fig. 5c).

We investigated the expression of a spectrum of surface markers to characterize changes in macrophage phenotype upon S100A8/A9 and we found that that CD163, CD40, CD80 and CD38 were significantly upregulated, whereas CD206 and CD11C were not affected (Fig. 5c). Additionally, the secretion profile of macrophages was considerably modulated upon S100A8/A9 exposure, inducing multiple cytokines (Fig. 5d). These S100A8/A9-induced changes in macrophage phenotype also correlated with enhanced glycolysis (Fig. 5e and Supplementary Fig. 8). Taken together, this data show that S100A8/A9 is a potent modulator of macrophage phenotype and function.

WNT signalling is influenced by *NTHi* and can regulate expression of AMPs in airway epithelial cells

Next, we aimed to understand some of the upstream factors involved in the differential regulation of S100A8/A9 expression in COPD airway epithelium. We performed KEGG pathway analysis on the publicly available datasets from COPD vs nonCOPD small airway epithelial cells, which revealed that the WNT signalling pathway is one of the most prominently deregulated pathways in COPD (Supplementary Fig. 9a). Therefore, we focused our attention on whether WNT signalling contributes to the disease-dependent regulation of S100A8/A9. S100A8 and S100A9 promoter analysis revealed several binding sites (TCF/LEF) for β -catenin, a key player of canonical WNT/ β -catenin signalling (Supplementary Fig. 9b). Interestingly, we also found that *NTHi* exposure reduced WNT signalling in COPD-derived SAECs (as shown with *AXIN2* target gene expression), while in MDMs we found that it induced the expression of *WNT5A* (negative regulator of WNT signalling) (Supplementary Fig. 10). To assess the direct effect of WNT on S100A8 and S100A9 we exposed SAECs to WNT/ β -catenin modulators, WNT inducer Chir99021 (CT) and WNT inhibitors WNT5A or FH5353 (FH; reportedly also an antagonist of PPAR [20]). We confirmed that CT significantly increased, whereas WNT5A and FH significantly reduced the expression of *AXIN2* (WNT/ β -catenin target gene) in epithelial cells (Fig. 6a). Importantly, we found that both S100A8 and S100A9 were regulated along with the WNT signal activity; WNT inducer CT enhanced their expression whereas WNT inhibitors WNT5A and FH attenuated expression of these AMPs. (Fig. 6b).

Taken together, these results demonstrate that *NTHi* may attenuate WNT/ β -catenin signalling in epithelial cells, both as a direct effect on epithelium and indirectly by inducing expression of the negative WNT pathway regulator WNT5A in macrophages. Moreover, we demonstrate that WNT/ β -catenin signalling activity directly impacts expression of S100A8 and S100A9 in lung epithelial cells. Therefore, decreased WNT/ β -catenin signalling is likely contributing to impaired expression of S100A8 and S100A9 in lung epithelium.

Discussion

In this study we show for the first time that upon exposure to *NTHi*, SAECs from COPD patients elicit a delayed AMP response which is associated with impaired macrophage-mediated phagocytosis. We further identify BD2 and S100A8/A9, as airway epithelial-derived AMPs, that can enhance phagocytic capacity in human macrophages and show that S100A8/A9 is a potent modulator of macrophage phenotype and function. Our data point to aberrant WNT/ β -catenin signalling as a potential contributing mechanism of delayed AMPs expression by COPD airway epithelial cells upon *NTHi* exposure. Overall, our study identifies a role for AMPs in regulating the interplay between microbial factors, lung epithelium, and macrophage phagocytic activity.

Phagocytosis is the key mechanism used by macrophages to control microbial populations at barrier surfaces. Phagocytic uptake of *H.influenzae* and *S.pneumoniae* was shown to be impaired in alveolar macrophages and MDMs in COPD patients [15, 21] and macrophages from some COPD patients exerted further impairment in phagocytosis during COPD exacerbation [22]. While multiple factors can be attributed to altered macrophage phenotypes and impaired phagocytic capacity in COPD, aberrant cues from the diseased airway epithelium could play a prominent role given the importance of coordinated macrophage-epithelial crosstalk during bacterial exacerbations. Our data demonstrate that upon exposure to microbial products, secretions from COPD SAECs were inferior in their ability to support MDM phagocytosis. This was observed with secretions collected after 3 hours, but was diminished after 24 hours. These data suggest that healthy airway epithelium can rapidly respond to acute microbial exposure, but this process is delayed in COPD epithelium, resulting in sub-optimal support of macrophage phagocytosis. Of note, in our studies we utilized MDMs macrophages derived from healthy individuals. Due to the importance of infiltrating monocytes/macrophages from the periphery for respiratory bacterial clearance [23], and the physical proximity of macrophages with the epithelium, we concluded that MDMs would represent a relevant macrophage population for our studies. The possible effects of airway epithelium-derived factors on lung-resident alveolar macrophages would be of interest for future investigation.

Importantly, we decided to utilize the macrophages derived from healthy individuals. As our primary objective was the comparison of nonCOPD and COPD airway epithelium we intended to work with a primary macrophage population that was most likely to faithfully and

reproducibly respond to epithelium-derived factors. However, it would be an interesting future direction to also assess the influence of the epithelium on the macrophages obtained from the COPD patients as those patient-derived macrophages may elicit functional properties that are biased by the pre-exposure to stressors like smoke or *NTHi*.

Our data are consistent with previous mouse studies that indicated a role of mouse colonic epithelial cells and alveolar type II cell-derived SN in macrophage phagocytosis [24, 25] and also are in line with previous reports that expression of *NTHi*-stimulated bronchial epithelia AMPs, S100A7 and BD2, is reduced in COPD patient cells [9].

Furthermore, we showed not only that *NTHi*-induced AMP secretion was delayed in COPD small airway epithelium, but also that S100A8/A9 and BD2 could directly influence the phagocytic capacity of human MDMs. A study conducted with the THP-1 cell line reported that BD2 could enhance engulfment of *P.aeruginosa* as well as formation of the phagolysosome [16]. Interestingly, levels of BD2 are reduced in the sputum of COPD patients and are inversely correlated with exacerbation frequency [26]. Moreover, BD2 mRNA has been shown to be lower in central airways of COPD patients and positively correlated with FEV1/FVC ratios [8]. These reports, in combination with our data, suggest a role of BD2 in pathogenesis of COPD exacerbations.

We showed, for the first time, that the S100A8/A9 heterodimer can enhance phagocytosis in MDMs. A reciprocal relationship between S100A8/A9, microbial exposure and lung epithelium repair has previously been postulated, as deficiency in S100A8/A9 in mice promoted progression of pneumonia caused by *S.aureus* infection [27] and treatment with recombinant heterodimer reduced LPS-induced lung injury *in vivo* [28]. S100A8/A9 was reported to regulate migration of phagocytes into alveoli in experimental pneumonia [29] and to control polymerization of tubulin in phagocytes [30]. Even though both S100A8 and S100A9 were shown to be increased in bronchioalveolar lavage fluid of COPD patients, the heterodimer S100A8/A9 formation was not assessed [31].

We further demonstrated that S100A8/A9 has the capacity to alter macrophage phenotype and metabolism. To our knowledge, this is the first in-depth investigation of several aspects of S100A8/A9 influence on human macrophages including protein phosphorylation status, expression of surface markers, phagocytosis-associated receptors, secretion profile and metabolic activity. We show several proteins being phosphorylated upon S100A8/A9 exposure, including JNK [17], c-JUN [18], AKT and p70S6 [19], all signalling pathways previously linked to phagocytosis.

Multiple groups of receptors are engaged in phagocytosis, including opsonic receptors Fc (e.g. CD32 and CD64) and non-opsonic receptors, (eg. Scavenger receptor MARCO and TLR receptors) [32]. Here, we show that S100A8/A9 directly upregulates expression of CD32, CD64, MARCO and TLR2 receptors in MDMs. Interestingly, it has been shown that MARCO is downregulated in COPD, and its restoration upregulated phagocytosis in COPD macrophages [33]. Similarly, TLR2, which binds lipopeptides from *H.influenzae* and *S.pneumoniae* is decreased in COPD macrophages [34]. We could also show that S100A8/A9 altered the expression of surface markers, consistent with changed MDM polarization. In addition, we found that the secretion profile of S100A8/A9-exposed macrophages also changed, including a number of cytokines previously associated with phagocytosis e.g. IL-1 β , INF- γ and TNF- α [35] IL6, IL10 [36]. The secretions from S100A8/A9-treated MDMs could be attributed to both M1 and M2 polarization and thus exert both pro- and anti-inflammatory properties. Interestingly, we found that S100A8/A9 also enhanced the glycolytic activity in macrophages consistent with a metabolism preference that would be expected in the context of M1-biased polarization [37].

Analysis of upstream pathways potentially regulating S100A8/A9 expression revealed WNT signalling as one of the most deregulated pathways in COPD epithelial cells, confirming previous reports [38]. Furthermore, a link between WNT/ β -catenin activity and AMP expression has been established in other cell types [39]. Therefore, we explored the hypothesis that the WNT/ β -catenin pathway may regulate the expression of AMPs in airway epithelial cells. We found several binding sites for β -catenin in the promoter for S100A8 and S100A9. We could also show for the first time that WNT/ β -catenin signal activity directly influences AMP expression in primary human small airway epithelium. While literature evidence highlights the contribution of several signalling pathways and external factors, including cigarette smoke (CS), to the regulation of AMP expression [9] there are a number of

studies linking WNT signal activity to phagocytosis [40] and AMP production [39, 40]. Thus, it is tempting to speculate that therapeutic modulation of WNT/ β -catenin signalling could be explored as an approach for pharmacologic calibration of macrophage phagocytic activity.

In conclusion, we show that COPD small airway epithelium exhibits delayed AMPs induction response to *NTHi* compared to nonCOPD epithelium and this corresponds to a significantly lower impact of COPD epithelial secretions on macrophage phagocytosis. We identified epithelial AMPs, BD2 and S100A8/A9, to have a positive impact on MDM phagocytic capacity. Moreover, our comprehensive analysis of the influence of the S100A8/A9 heterodimer have revealed its role in regulating macrophage polarization and metabolism, as well its own regulation by WNT/ β -catenin signalling, a prominent deregulated pathway in COPD airway epithelium. Further studies addressing potential utility of S100A8/A9 to improve the function of macrophages in COPD as well as impact on other pathological features of the disease could therefore provide new therapeutic avenues for COPD.

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Author Contributions

W. S-W. and S.P. conceived the project. W.S-W., K.K., J.P.G. and S.P. were involved in the experimental design. W.S-W, S.D., H.Q.L. and V.S. performed the experiments. W.S.-W. analyzed the data. W.S-W. and S.P. drafted the manuscript. All authors participated in writing or editing the paper.

Disclosure

Authors declare no conflict of interest.

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Figure legends

Fig. 1

Figure 1

Supernatants from *NTHi*-exposed COPD SAEs have diminished impact on phagocytic capacity of macrophages.

(a) Schematic of experimental procedure. Small airway epithelial cells (SAEC) obtained from nonCOPD and COPD-diagnosed individuals were cultured in an air-liquid interface (ALI) setup to full differentiation. On the apical surface fully differentiated SAECs were exposed to vehicle (PBS) or heat-inactivated *Haemophilus influenzae* (*NTHi*) for 3h or 24h. Washings from the apical chamber or media from the basolateral chamber was used for subsequent incubation with human monocyte-derived macrophages (MDM) to investigate effects on MDM phagocytosis of the fluorescently-labelled pHrodo® *E.coli* particles (based on the

fluorescence emission when particles encounter the acidic environment of phagosome). (b) MDMs (3 healthy donors) were exposed to apical washings supernatants (SN, upper panel), or basolateral supernatants (lower panel), obtained from nonCOPD (black) and COPD (red) SAEC (n=9) exposed to PBS or heat-inactivated *NTHi* for 3h (left) or 24h (right) (each MDM donor + SAEC donor represents a unique pair). Impact of SN on phagocytosis of pHrodo® *E.coli* particles by MDMs was measured with IncuCyte S3 Live-Cell Analysis System over time (2h, 4h and 6h time point depicted). Data presented as a mean relative phagocytosis of MDMs exposed to SN obtained from *NTHi*-stimulated SAEC to corresponding PBS-exposed SAEC. Means were compared using Kruskal-Wallis test followed by Dunn's post test. *P<0.05.

Fig. 2

nonCOPD
COPD

Figure 2

COPD SAECs exert delayed AMP response to *NTHi*

mRNA expression of antimicrobial peptides (AMPs: *DEFB1*, *DEFB4A* (gene name for BD2 protein), *CAMP*, *SLPI*, *S100A7*, *S100A8*, *S100A9*, *PI3*) in nonCOPD-derived (nC, black) and COPD-derived (C, red) SAEC exposed to *NTHi* for 3h or 24h relative to PBS-exposed SAEC. Gene expression comparative crossing point change ($\Delta\Delta C_p$) is presented as a mean (n=11-18). Differences between *NTHi* and corresponding PBS-exposed SAEC was calculated with paired t-test; * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$. Differences between means of nonCOPD and COPD SAECs were calculated with a Mann-Whitney test. # $P<0.05$.

Fig. 3

a) *E.coli* (gram-negative)

b) *S.aureus* (gram-positive)

c) *S.aureus*

Figure 3

Antimicrobial peptides modulate phagocytosis in macrophages

Influence of recombinant AMPs: S100A8/A9 and BD2 (gene name *DEFB4A*) on phagocytic capacity of MDMs. Cells were pre-treated with indicated proteins or vehicle and progression of phagocytosis of (a) the pHrodo® *E.coli* particles, (b) the pHrodo® *S.aureus* particles was monitored with IncuCyte S3 Live-Cell Analysis System. For each time point, data is presented as a % of phagocytosis relative to respective vehicle control (100%, dotted horizontal line) (*E.coli* n=7, *S.aureus* n=4). The impact of the recombinant AMP on phagocytosis was calculated with paired t-test. *P<0.05, **P<0.01, ***P<0.001. (c) Comparison of BD2, S100A8/A9 (S8/9), and BD2 co-treatment with S8/9 on phagocytic capacity of MDMs of pHrodo® *S.aureus* particles at 2h time point. Impact of the recombinant AMP on phagocytosis was calculated with paired t-test. ***P<0.001. Differences between treatments were calculated with one-way ANOVA followed by Sidak multiple comparison test.

Fig. 4

Figure 4

S100A8/A9 and BD2 proteins are differentially regulated by *NTHi* in nonCOPD and COPD SAEC secretions

Expression of (a) BD2 and (b) S100A8/A9 proteins in supernatants obtained from SAEC ALI cultures from apical (left) and basolateral (right) compartment measured by ELISA. Data is presented as an expression of the protein in SN obtained from SAEC exposed to *NTHi*, relative to an expression of protein in SN obtained from SAEC exposed to PBS for 3h (PBS-exposed condition as 100%; BD2 n=9, S100A8/A9 apical nonCOPD SN n=11, apical COPD SN n=10, basolateral nonCOPD SN n=6, COPD SN n=4). Impact of *NTHi* on protein expression in nonCOPD and COPD SAEC SN was calculated with one-sample t-test. *P<0.05

Fig. 5

Figure 5

S100A8/A9 modulates macrophage phenotype and metabolism

Influence of S100A8/A9 on phosphorylation profile of signalling molecules, macrophage cell surface phenotype, cytokine secretion, and metabolic profile. (a) Phosphorylation status of signalling molecules upon 30 min S100A8/A9 (5µg/ml) treatment depicted as fold-difference relative to PBS-induced phosphorylation (n=3). Influence of S100A8/A9 on phosphorylation was calculated with one-sample t-test. (b) mRNA expression of *FCGR1A*, *FCGR2A*, *MARCO*, *TLR2*, *TLR4* in MDMs exposed to PBS or S100A8/A9 (5µg/ml) for 6h. Relative gene expression, expressed as crossing point change ($\Delta C_p = C_p \text{ for } GAPDH - C_p \text{ for gene of interest}$) is presented as a mean (n=11). (c) Expression of surface markers (CD64, CD332, MARCO, CD163, CD206, CD11C, CD40, CD80, CD38) depicted as Mean Fluorescence Intensity (MFI, geometric mean) on MDMs exposed for 24h to PBS or S100A8/A9, measured by Flow Cytometry and depicted as mean (n=5). (d) Expression of cytokines (pg/ml) in supernatants from MDMs exposed to PBS or S100A8/A9 for 24h and presented as a mean (n=5). (e) Glycolysis Stress Test: glycolysis, glycolytic capacity and glycolytic reserve in MDMs upon PBS or S100A8/A9 exposure for 24h, shown as a mean (n=3). Impact of S100A8/A9 calculated with paired t-test, *P<0.05, **P<0.01, ***P<0.001

Fig. 6

Figure 6

WNT signalling regulates expression of AMPs in small airway epithelial cells.

mRNA expression of (a) *AXIN2* and (b) *S100A8* and *S100A9* in small airway epithelial cells exposed to WNT inducer Chir99021 (CT) or WNT inhibitors; FH535 (FH) or WNT5A for 24h, relative to vehicle-exposed basal cells. Gene expression comparative $\Delta\Delta C_p$ is presented as a mean (n=9-14). The impact of CT, WNT5A and FH was calculated with paired t-test, *P<0.05, **P<0.01, ***P<0.001

	nonCOPD	COPD
n	9	9
female / male	5 / 4	6 / 3
age, years	53 ± 15	58 ± 8
smoking	3 yes, 6 no	8 yes, 1 no

Table1. Subject characterization

Supplement

Methods

Generation of heat-inactivated *NTHi*

NTHi bacteria (3198-R strain) were grown overnight at 37°C in MaxQ 4450 Benchtop Orbital Shaker (Thermo Scientific) in Mueller-Hinton broth medium. Fresh broth medium was inoculated with the overnight culture and grown to OD₄₉₀ of 0.11. Subsequently, bacteria were washed twice with 10ml PBS and adjusted to OD₄₉₀ of 0.11 in PBS (equivalent to approximately 10⁹ colony forming units (CFU)/ml). Next *NTHi* were heat-inactivated by incubation at 80°C for 45min.

Enzyme-linked immunosorbent assay (ELISA)

SAEC secretions from apical and basolateral compartment of ALI cultures were collected and S100A8/A9 and BD2 protein was measured according to the manufacturers' instructions (S100A8/A9, S-1011, BMA BIOMEDICALS; BD2, EK-072-37, Phoenix Pharmaceuticals).

RNA isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR, qPCR)

Total RNA was extracted using RNeasy Plus Mini kit (Qiagen) according to the manufacturer's protocol and cDNA was generated by reverse transcription using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher). Quantitative (q)RT-PCR was performed using fluorogenic SYBR Green and the Light Cycler 480 detection system (Roche). PCR was performed using the primers: Interleukin 6 (*IL6*), assay ID 144013; *AXIN2*, assay ID 102998, secretory leukocyte peptidase inhibitor (*SLPI*), assay ID 108143; S100 calcium binding protein A7 (*S100A7*), assay ID 115051, S100 calcium binding protein A8 (*S100A8*), assay ID 105537; S100 calcium binding protein A9 (*S100A9*), assay ID 113434; Elafin (*PI3*), assay ID 103876; human beta defensin 1 (*DEFB1*), assay ID 114433, human beta defensin 2 (*DEFB4A*), assay ID 148831, Macrophage Receptor With Collagenous Structure (MARCO), (CD163), assay ID 114683, Interleukin 10 (IL10), assay ID 137154, High Affinity Immunoglobulin Gamma Fc Receptor I (FCGR1A), assay ID 103507, Low Affinity Immunoglobulin Gamma Fc Region Receptor II-A (FCGR2A), assay ID

113119, Toll Like Receptor 4 (TLR4) assay ID 149447 and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), assay ID 141139 (Roche) and Antigen Peptide Transporter 1 (TAP1), TAP_1, Antigen Peptide Transporter 2 (TAP2), TAP2_1, Toll Like Receptor 2 (TLR2), TLR2_1 from Sigma Aldrich. *GAPDH* was used as a reference gene.

Relative gene expression is presented as ΔC_p value ($\Delta C_p = C_p \text{ GAPDH} - C_p \text{ gene of interest}$). Relative change in transcript level upon treatment is expressed as $\Delta\Delta C_p$ value ($\Delta\Delta C_p = \Delta C_p \text{ of treated sample} - \Delta C_p \text{ of control}$).

Immunohistochemistry

Transwell cultures were fixed for 10min with ice-cold acetone-methanol (1:1) and the whole membrane was cut out for further processing. Following standard procedures, inserts were dehydrated and embedded in paraffin. Next 3 μ m thick sections were prepared and inserts were rehydrated with descending series of ethanol. Staining with haematoxylin and eosin (H&E) and alcian blue/PAS staining were prepared according to standard protocols. Images were acquired with the use of AxioCam MR3 (Zeiss).

For fluorescence staining the sections were incubated for 1h with primary antibody (E-cadherin, BD 610181, 1:200), followed by secondary antibody (Invitrogen A-11029, 1:1000) for 2h and DAPI for 10 min to visualize cell nuclei. Next, the sections were mounted with Fluoromount-G™ (Invitrogen, 00495802). Images were acquired with the use of LSM 710 (Zeiss).

Flow Cytometry

For live-dead staining cells were incubated with Pacific Orange (1:3000) for 10min at RT. For surface staining, cells were incubated with antibodies in 0.2% BSA-PBS for 10 min at 4°C. Following antibodies were used: CD14 (BD 564054, concentration 1:50), CD16 (BD 565421, 1:50), CD40 (BD 565258, 1:50), CD80 (BD 565210, 1:50), CD163 (BD 562643, 1:50), CD206 (BD 550889, concentration 1:50), CD64 (Biolegend 305042, 1:50), CD32 (BD 564838, 1:50), MARCO (Invitrogen 25-5447-42, 2.5:50), CD163 (BD 562643, 1:50), CD206 (BD 550889, 1:50), CD11C (Biolegend 337220, 0.5:50), CD40 (BD 565258, 1:50), CD80 (BD 565210, 1:50), CD38

(BD 562325, 2:50) In order to avoid Fc-receptor binding, stainings were performed in the presence of 2µg/mL αhFcγ-receptor. Subsequently, cells were analyzed using flow cytometer LSRII (BD Bioscience).

MSD assay

For the assessment of the protein levels in apical and basolateral supernatants V-PLEX ELISA as used according to the manufacturer's protocol (Cytokine Panel 1 (K15050D), Proinflammatory Panel 1 (K15049D).

WST-1 assay

MDMs were seeded in 96-well plates and treated with apical or basolateral SAEC SN for 24h. Metabolic activity was monitored using the WST-1 assay kit according to the manufacturer's protocol (Roche, 11644807001). Media was aspirated and 100µl of 10% WST-1 solution was added to each well and cells were incubated for 2h. Absorbance (wavelength 450nm) was measured using microplate reader (EnVision, PerkinElmer).

Phospho-kinase array

MDMs were treated with 5µg/ml of S100A8/A9 or vehicle for 30min. Equal amounts of cell lysates (300µg) were used to simultaneously detect the relative levels of phosphorylation of kinases according to the manufacturer's protocol (dot blot array ARY003C; R&D Systems). Phosphorylation was visualized using an ImageQuant LAS 4000 (GE Healthcare). A pixel density module in ImageJ was used to quantify phosphorylation levels of the proteins.

Seahorse analysis

One hundred MDMs per well were seeded into a Seahorse XF96 plate and left for 30min at room temperature. Then cells were stimulated with 5 µg/ml S100A8/A9 (triplicates) for 24 h. For analysis of glycolysis parameters, Seahorse assays were performed according to the

manufacturer's instructions of the Seahorse Bioscience XF cell glycolysis stress test kit (#103020–100). During the assay cells were treated with 5mM Glucose, 1μM Oligomycin and 50mM 2-Deoxyglucose. Density of the cells at the end of experiment was assessed with the use of CyQUANT® Direct Cell Proliferation Assay (Thermo Fisher C35011) and the glycolysis parameters were normalized to the cell density.

Transepithelial electric resistance (TEER) measurement

TEER measurement was performed on ALI cultures at the end of experiment. 150μl of PBS was added to the insert and TEER was measure with the Voltohmmeter.

Binding assay

To assess the binding of S100A8/A9 recombinant protein to MDMs, S100A8/A9 and control protein (soluble Thy1) was labelled with RPE (RPE Conjugation Kit, LNK024RPE, Bio-Rad). MDMs were incubated on ice with labelled proteins for 30 min. Afterwards cells were washed with PBS and centrifuged 3 times and the fresh media was added on top. Cells were scanned with IncuCyte and the RPE signal was measured. Values were normalized to the cell density (brightfield image).

Promoter analysis

In order to perform the analysis of S100A8 and S100A9 promoter for TCF/LEF (β-catenin) tool provided by alggen.lsi.upc.es was used.

GEO analyses

To reveal the most deregulated pathways between nonCOPD and nonCOPD small airway epithelial cells the GSE19407 dataset was also used.

Supplementary Figure Legends

Supp. Fig. 1

Supplementary Figure 1

There are no differences in characteristics between nonCOPD and COPD epithelial cells

Representative images of (a) H&E staining, (b) Alcian Blue/PAS staining and (c) E-Cadherin IF staining of representative transwell cultures from nonCOPD and COPD SAEC exposed to PBS or *NTHi* for 3h or 24h. (d) Transepithelial electrical resistance (TEER) values across SAEC cells exposed to PBS or *NTHi* presented as a mean (n=9). Differences calculated with one-way ANOVA followed by Tukey's multiple comparison test. (e) mRNA expression of *TJP*, *SCGB1A1*, *VIM* in nonCOPD and COPD SAEC exposed to PBS or *NTHi* for 3h or 24h (each n=6). Relative gene expression, expressed as crossing point change ($\Delta C_p = C_p \text{ for } GAPDH - C_p \text{ for gene of interest}$) is presented as a mean. Differences calculated with one-way ANOVA followed by Tukey's multiple comparison test.

Supp. Fig. 2

Supplementary Figure 2

There are no differences in viability and surface marker expression between macrophages exposed to nonCOPD and COPD epithelial cells supernatants

(a) Viability of MDMs exposed to apical (left) and basolateral (right) SN from nonCOPD and COPD SAEC (each n=12) measured by Flow Cytometry. (b) Expression of surface markers (CD14, CD16, CD206) depicted as Mean Fluorescence Intensity (MFI, geometric mean) on MDMs (2 donors) exposed for 24h to apical (top) and basolateral (bottom) SN from

nonCOPD (nC; n=6) and COPD (C, n=6) SAEC (each MDM donor + SAEC donor represents a unique pair) measured by Flow Cytometry.

Supp. Fig. 3

Supplementary Figure 3

***NTHi* exposure increases expression of *IL6* in SAEs**

IL6 mRNA expression in nonCOPD- (black) and COPD-derived (red) SAEs exposed to *NTHi* for 3h or 24h relative to PBS-exposed SAEs. Gene expression comparative crossing point change ($\Delta\Delta C_p$) is presented as a mean (n=11-15). Impact of *NTHi* to corresponding PBS-exposed SAEs was calculated with paired t-test; ** $P < 0.01$, **** $P < 0.0001$.

Supp. Fig. 4

a

Supplementary Figure 4

SLPI, S100A8 and S100A9 antimicrobial peptides do not influence phagocytosis in macrophages

(a) Influence of recombinant AMPs: SLPI, S100A8 and S100A9 on phagocytic capacity of MDMs. Cells were pre-treated with indicated proteins or vehicle and progression of phagocytosis of the pHrodo® *E.coli* particles was monitored with IncuCyte S3 Live-Cell Analysis System. For each time point, data is presented as a % of phagocytosis to respective vehicle control (100%, dotted line) (SLPI n=8, S100A8 n=6, S100A9 n=8). (b) Viability of MDMs exposed to S100A8, S100A9, S100A8/A9, SLPI and BD2 (depicted as a % of vehicle control) measured by WST-1 assay, shown as a mean (n=8=13). Impact of the AMPs calculated with paired t-test, * $P < 0.05$.

Supp. Fig. 5.

BD2

S100A8/A9

Supplementary Figure 5

S100A8/A9 and BD2 proteins are differently regulated by *NTHi* in nonCOPD and COPD SAEC secretions

(a) BD2 and (b) S100A8/A9 protein expression in apical and basolateral SN from nonCOPD (left) and COPD (right) SAECs exposed to PBS or *NTHi* for 3h, measured by ELISA.

Supp. Fig. 6

a

b

Supplementary Figure 6

S100A8/A9 recombinant protein binds to macrophages

Binding of RPE-labelled control protein (soluble Thy1) and S100A8/A9 to MDMs: (a) pictures visualizing binding. (b) Quantification of the RPE signal relative to cell density in cells treated with control protein and S100A8/A9.

Supp. Fig. 7

Supplementary Figure 7

S100A8/A9 induces phosphorylation of different molecules in macrophages

Representative blot showing MDM phosphorylation status of signalling molecules upon 30 mins exposure to PBS (upper blot) or S100A8/A9 (lower blot). Affected signalling molecules marked.

Supp. Fig. 8

Supplementary Figure 8

S100A8/A9 impacts glycolysis in macrophages

Representative the Extracellular Acidification Rate (ECAR) profile plot (upper panel) and the Oxygen Consumption Rate (OCR; lower panel) upon PBS or S100A8/A9 treatment.

Supp. Fig. 9

a

COPD vs non smokers

COPD vs smokers

b

S100A8
LEF1 – 4
TCF4 - 1

S100A9
LEF1 – 3
TCF4 - 1

Supplementary Figure 9

WNT signalling pathway is one of the top deregulated pathways in COPD epithelium

(a) *Kyoto Encyclopedia of Genes and Genomes* (KEGG) analysis of most deregulated pathways in COPD compared to nonCOPD small airway epithelial cells (GSE19407 dataset). (b) Promoter analysis of S100A8 and S100A9 for the TCF4 and LEF1 (β -catenin binding sites).

Supp. Fig. 10

Supplementary Figure 10

***NTHi* modulates WNT pathway in macrophages and epithelial cells**

(a) mRNA expression of *WNT5A* in MDMs treated with PBS or exposed to *NTHi* for 24h (n=2).

(b) mRNA expression of *AXIN2* in nonCOPD and COPD SAEC exposed to *NTHi* for 3h, relative to PBS-exposed SAEC. Gene expression comparative $\Delta\Delta$ Cp is presented as a mean (n=7-10).

Impact of *NTHi* calculated with paired t-test.