IL-6 trans-signalling: how *Haemophilus* surfs the NET to amplify inflammation in COPD

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Shareable abstract (@ERSpublications)
***Haemophilus* infection in COPD induces neutrophil extracellular traps which amplify inflammation through multiple mechanisms, including NET mediated IL-6 trans-signalling** https://bit.ly/3yFI2b7

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Background

COPD is characterised by neutrophilic inflammation in the majority of patients [1–3]. Neutrophils represent a key second line of defence against infection and are rapidly recruited to the airway if pathogens are able to evade first line defences, which include mucociliary clearance and resident immune cells such as macrophages [3]. Neutrophils typically clear infection through ingestion of invading microbes (phagocytosis) and through the internal generation of reactive oxygen species, serine proteases such as neutrophil elastase, cathepsin G and proteinase-3, and antimicrobial proteins [4]. These processes aim to clear infection without damage to host tissues, but in COPD there is a failure of these normal antimicrobial processes. Mucociliary clearance is impaired, macrophage phagocytosis is less effective, and pro-inflammatory cytokines signal recruitment and survival of neutrophils which fail to effectively kill bacteria, leading to chronic neutrophilic inflammation and chronic infection [3–6]. Recent data suggests that a distinct form of neutrophil behaviour in which neutrophils release a DNA scaffold decorated with granule proteins (such as proteases) and termed neutrophil extracellular trap (NET) formation is associated with worse symptoms, frequent exacerbations and lung infections in a subset of patients [7–9]. The precise drivers and implications of NETosis in COPD are, however, unclear.

Work in this issue of the *European Respiratory Journal* sheds new light on the harmful effects of NETs in COPD, identifying a novel mechanism by which NETs, particularly in the context of *Haemophilus influenzae* infection, amplifies inflammation through interleukin-6 (IL-6) trans-signalling [10].

What is IL-6 trans-signalling?

IL-6 is a powerful pro-inflammatory cytokine [11]. IL-6 exerts its effects through binding to its membrane bound receptor (referred to as classical signalling), but many cells have low expression of the IL-6 receptor and therefore have a limited response to stimulation with IL-6, including bronchial epithelial cells [10, 12, 13]. IL-6 trans-signalling (IL-6TS) is a mechanism that amplifies IL-6 dependent inflammatory responses by allowing IL-6 stimulation of cells that express low levels of IL-6 receptor. Binding of IL-6 to a soluble form of the receptor (sIL-6R) allows interactions of IL-6/sIL-6R with the signal transducer gp130. Dimerisation of gp130 leads to activation of Janus kinases that mediate phosphorylation of specific tyrosine residues on the gp130 cytoplasmic tail, which in turn function as docking sites for STAT3 (and STAT1), leading to STAT3 phosphorylation, dimerisation and nuclear translocation, where it exerts multiple pro-inflammatory effects (figure 1) [10, 13, 14].

What does the study show?

The authors used an impressive “multi-cohort, multi-omics” approach to addressing linked clinical questions [10]. As the study is relatively complex, the cohorts and findings are summarised in figure 2a. Initially, the authors studied bronchial biopsies of patients with COPD to identify if some patients had a gene transcriptional signature consistent with IL-6TS. The signature consists of eight genes found to be affected in IL-6TS-stimulated epithelial cells from prior experiments. This initial experiment found 12/38 patients had an

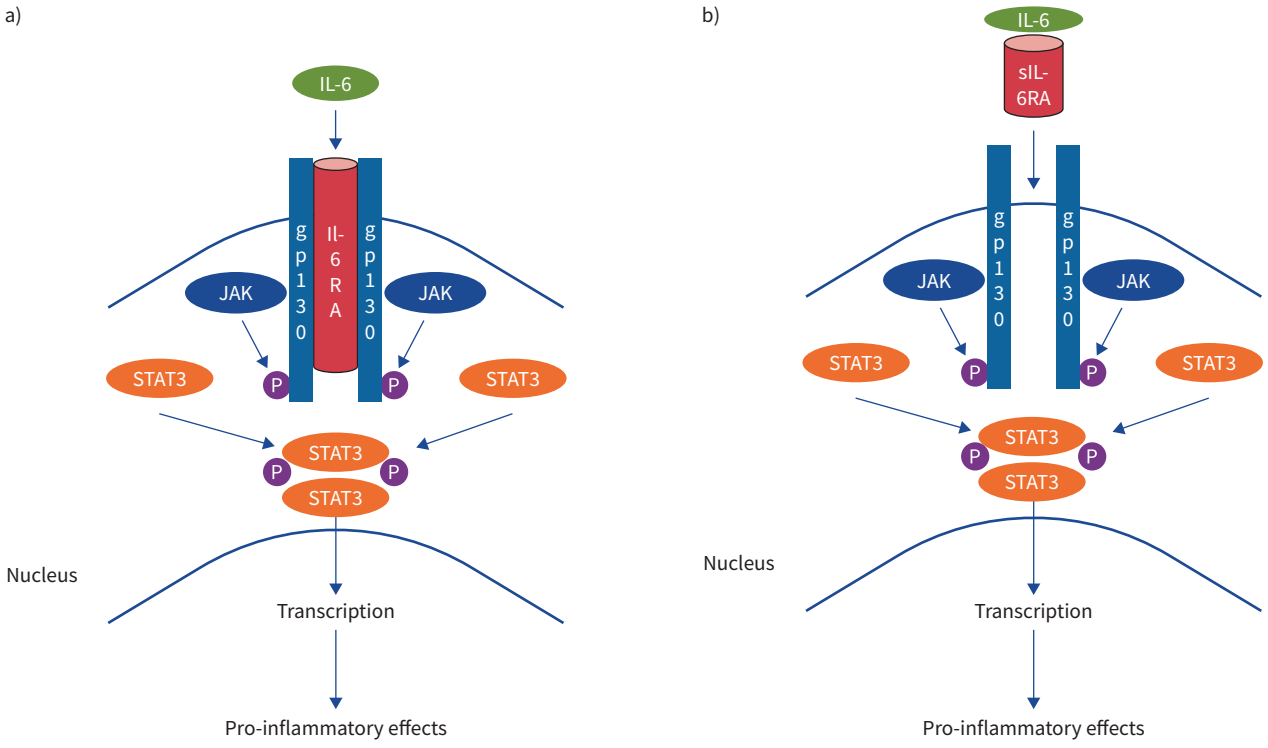

FIGURE 1 IL-6 classical signalling (a) and trans-signalling (b).

IL-6TS signature, with evidence of induction of genes downstream of STAT3. This first cohort establishes that IL-6TS is indeed occurring in a subset of COPD patients and provides the basis for the subsequent investigations into the underlying mechanisms. A signature of sputum proteins can also be used to demonstrate IL-6TS (IL-6, sIL-6R, MIP-1β, IL-1β and IL-8. The authors demonstrate in a multicentre cohort, BEAT-COPD, remarkably consistent results with 32.4% of patients having a signature consistent with IL-6TS. Importantly, the BEAT-COPD cohort demonstrates the likely clinical relevance of this by showing increased symptoms and increasing neutrophilic inflammation in this subset of patients. The BEAT-COPD study also included sputum microbiome as an endpoint and using this data obtained from sequencing of amplicons of the 16S rRNA gene [15], the authors show that patients with IL-6TS associated proteins have a higher relative abundance of proteobacteria, particularly *Haemophilus*. *Haemophilus* is a key pathogen in COPD [16]. In a study published in 2018, our group showed that *Haemophilus* was the key driver of NET formation in COPD and was associated with increased symptoms and exacerbations [7]. Extending this observation, integration of microbiome and proteomic data in a large cohort of 253 patients found *Haemophilus* dominance was associated with mortality and a neutrophil-dominated protein profile [17].

Haemophilus is resistant to killing by NETs and therefore inducing NETosis may be a survival mechanism for *Haemophilus* to allow it to persist in the airways [18]. The authors therefore extended their findings by investigating whether *Haemophilus* itself was a trigger of sIL-6R release from neutrophils. *In vitro*, they demonstrate that *Haemophilus* causes NET formation, and that neutrophils release sIL-6R during NETosis. Lastly, in a final cohort of patients, this time from Manchester, UK, they show positive associations between sIL-6R and surrogate markers of NETosis in bronchoalveolar lavage fluid, demonstrating that it is highly likely that the *in vitro* observations are relevant *in vivo*.

This is an impressive piece of work with important implications for the study and therapeutic targeting of neutrophilic inflammation in COPD. The key limitation of this work is that the data do not conclusively establish whether IL-6TS is “maker or marker” when it comes to the clinical manifestations of COPD. The fact that IL-6TS is occurring in the airway does not mean that it is contributing in a meaningful way to exacerbations of symptoms. This is not a criticism of the study as establishing causality in the complexity of airway inflammation is extremely challenging. *Haemophilus* infection and NET formation have multiple simultaneous damaging effects on the airway, including promoting goblet cell hyperplasia, mucus release,

a)

Cohort	Results	Implications/conclusions
Southampton (Bronchial biopsies, n=38)	IL-6TS high gene signature identified in 12 (31.6% of patients)	A subset of patients with evidence of IL-6 trans-signalling on bronchial epithelium is identified (validated in Manchester cohort, n=23)
BEAT-COPD cohort (sputum samples, n=74)	IL-6TS high sputum protein signature found in 32.4% IL-6TS high group have neutrophilic inflammation and worse symptoms	IL-6TS is related to neutrophilic inflammation and may be associated with a more severe phenotype
BEAT-COPD cohort (sputum microbiome, n=39)	Increase proteobactaria and particularly *Haemophilus* by 16s sequencing in patients in the IL-6TS high subset	*Haemophilus* infection is associated with IL-6TS
In vitro studies of neutrophils infected with *Haemophilus influenzae*	Neutrophils stimulated with *H. influenzae* form NETs and release soluble IL-6 receptor This is blocked with a PAD4 inhibitor that prevents NETosis	*Haemophilus* induced NET formation drives the release of sIL-6R, and therefore IL-6 trans-signalling Inhibition of NETosis can therefore reduce IL-6TS
Validation of the relationship between NETs and IL-6TS in COPD patients (Manchester cohort, n=58 COPD n=35 controls)	Significant relationships between surrogate markers of NETs in BAL fluid such as MPO and cell free DNA and sIL-6R	Confirms it is highly likely *in vitro* that NET formation is a significant contributor to IL-6TS

b)

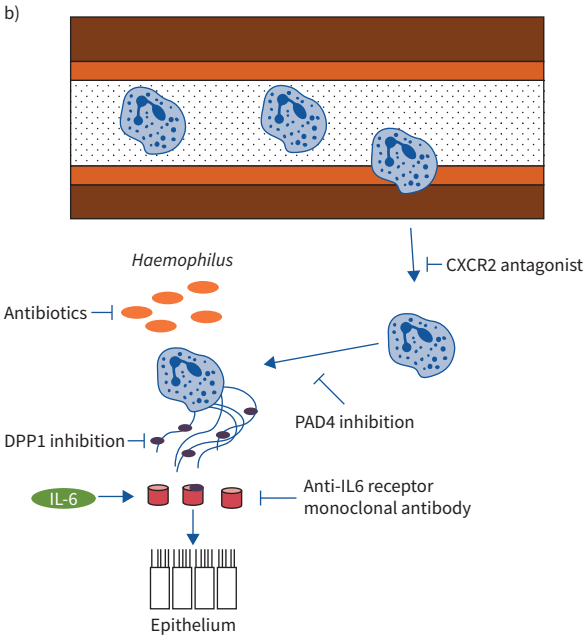

FIGURE 2 a) A summary of the key cohorts and findings from the study by Winslow *et al.* [10]. b) Neutrophilic inflammation in COPD and how this can be targeted. Neutrophils migrate from the circulation to the lung. CXCR2 antagonists reduce neutrophil migration as well as activation. Neutrophils are induced to form neutrophil extracellular traps (NETs), which can be blocked by peptidyl arginine deiminase 4 (PAD4) inhibition. *Haemophilus* is among a number of triggers of NET formation in the airway. NETs are released consisting of DNA and neutrophil granule products and particularly serine proteases. Serine proteases can be inhibited by dipeptidyl peptidase-1 (DPP1) inhibition. Soluble IL6-R is released *via* NETosis and can be blocked by monoclonal antibodies targeting the IL-6 receptor.

increased mucus viscosity through release of DNA, and protease-mediated airway damage [19–21]. This work cannot establish the extent to which targeting IL-6 or IL-6TS specifically would be beneficial. It nevertheless adds another piece of evidence to the centrality of *Haemophilus* infection and NETosis to the pathophysiology of COPD [7].

What does this tell us about potential treatments for neutrophilic COPD?

Most attempts to target neutrophilic inflammation in COPD and other neutrophilic airway diseases have focused on reducing neutrophil migration to the airways or blockade of specific proteases, such as neutrophil elastase [22, 23]. An example is antagonism of CXCR2, an approach which should lessen migration and neutrophil activation since CXCL8 (IL-8) is reported to be the dominant neutrophil chemoattractant/activator in COPD airway secretions [24, 25]. Reducing neutrophil migration to the airway, however, runs the risk of increasing infections since neutrophils are critical to host defence. A phase 2 study of a CXCR2 antagonist danirixin recently reported an increase in the frequency of exacerbations over 26 weeks with a higher frequency of pneumonia at high doses [24]. Our parallel mechanistic study examining the effect of CXCR2 antagonism on NETosis and neutrophil activation found no effect of CXCR2 antagonism on NET formation *in vivo* in COPD patients and a variable response *ex vivo* [25]. The work by Winslow *et al.* [10] illustrates one of the potential reasons why such upstream blockade of neutrophilic inflammation in the airway may be unsuccessful, since *Haemophilus* (and many other organisms) are able to induce NETosis independent of CXCL8.

If there are too many potential signals in the airway to block the induction of NETosis, what about blocking NETosis itself? Here, Winslow *et al.* [10] demonstrate that peptidyl arginine deiminase 4 (PAD4) inhibition may be an effective approach, since the citrullination of histones by PAD4 is a critical step in the induction of NETs [26]. The action of neutrophil elastase and myeloperoxidase are also reported to be essential for NET

formation and so blockade of these enzymes may also be a viable approach, among others [27, 28]. The question mark *in vivo* is whether NET blockade is desirable. NETs clearly form for a reason, likely to constrain infection by "trapping" bacteria and other pathogens which cannot be effectively killed. Even if NETs were found to be dispensable for bacterial control, NETs have been shown to be important for neutrophil responses to fungi, where hyphae are formed which are too large to be phagocytosed [29]. Care would therefore be required in ensuring that NET inhibitors did not increase the risk of infectious complications.

NETs are not only an issue in COPD, but are also elevated to an even greater extent in bronchiectasis and in cystic fibrosis [30]. We recently demonstrated a strong association between NETs and poor outcomes in bronchiectasis [30]. Importantly, using proteomics we demonstrated that effective bacterial clearance with antibiotics reduced NETs and NET-associated protein markers in sputum [30]. So if *Haemophilus* is a key driver of morbidity and mortality in COPD, should *Haemophilus* be directly targeted with antibiotics? The data of Winslow *et al.* [10] would suggest this would effectively reduce IL-6TS, in addition to other benefits [10]. We are unaware of any therapeutic trials of long-term antibiotics specifically in patients with *Haemophilus* infection, but long-term doxycycline trials in stable COPD cohorts have been completed (*e.g.* NCT02305940) and are awaited.

However, long-term antibiotics have their own problems, including antibiotic resistance, and are thus unlikely to be a sustainable solution; therefore, can we treat downstream? Blockade of IL-6 signalling and IL-6 trans-signalling is possible [31]. IL-6 receptor blocking monoclonal antibodies such as tocilizumab and sarilumab affect both classical and trans-signalling pathways and extensive clinical experience with their effects on lung infection is now being gained through the COVID-19 pandemic [31]. IL-6 receptor antagonist monoclonal antibodies have been shown to reduce mortality and the requirement for intensive care in hospitalised patients with COVID-19. The biomarkers identified by Winslow *et al.* [10], based on sputum markers or indeed the presence of *Haemophilus*, would seem to signal the optimal patient population in COPD. A final question with potential therapeutic relevance is: where does the IL-6R come from? In neutrophils, it is most likely cleaved from the neutrophil surface by the action of proteases. Among the three serine proteases released during NET formation [32, 33], cathepsin G has been shown to cleave the IL-6 receptor [34]. Therapeutic inhibition of dipeptidyl peptidase-1 (DPP1) in a phase 2 study of 256 patients with bronchiectasis achieved marked reductions in sputum levels of serine proteases, including cathepsin-G, that translated into a prolongation of the time to first exacerbation. It is therefore theoretically possible that DPP1 inhibition would both ameliorate the damaging effects of NETs while also preventing IL-6TS [35]. This should be explored in future studies. A summary of some of the potential ways in which neutrophil- and NET-associated inflammation in COPD could be targeted is shown in figure 2b.

The study by Winslow *et al.* [10] sheds new light on the interaction between *Haemophilus* infection and neutrophilic inflammation and COPD, highlighting multiple potential avenues for future research and therapeutic trials.

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