73. Update on neutrophil and macrophage function in COPD

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Identification of pathogenic macrophage subpopulations in lung disease

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Residential macrophages may exist in discreet subpopulations which subserve specific roles in the maintenance of tissue and immunological homeostasis. In lung, alveolar macrophages (AMΦs) comprise of >95% cells in the alveolar airspaces, where they act as the primary sentinels of pathogens. Increased alveolar macrophage numbers are observed in many animal models of COPD and also clinically in COPD patients. Currently, M2 macrophage polarisation is thought to be a contributor of lung disease, although this in vitro-derived macrophage paradigm may not completely explain the complex behaviour of AMΦs in vivo.
Using flow cytometry, we have developed an approach which shows that mouse AMΦs instead form distinct subpopulations during acute inflammation and in chronic inflammatory lung disease. During acute inflammation, AMΦ subpopulations are characterised by differential Mac-1 and CD11c integrin expression rather than M1 or M2 MΦ surface markers exclusively, and display differential gene signatures ex vivo. Resolution is characterised by restoration to a single population of Mac-$1^{low/neg}$ AMΦs mirroring lung homeostasis. In contrast, SHIP-$1^{-/-}$ mice which develop chronic inflammatory lung disease spontaneously have an additional subpopulation of Mac-1^{pos} macrophages which highly expresses MMP-12. This additional AMΦ subpopulation also tracks with the induction of lung disease using SHIP-$1^{-/-}$ chimeric mice.
Following this, we are now screening for markers of AMΦ subpopulations in patients with COPD. Our studies of both animal models and clinical samples may allow us to better understand the role of AMΦ subpopulations in homeostasis and disease.

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Lack of macrophage plasticity in COPD

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COPD is associated with increased numbers of highly-activated lung macrophages (mϕ) with reduced phagocytic ability. Mϕ may exist as M1-like (classically-activated) or M2-like (alternatively-activated) states. In COPD, M1-like mϕ may persist, and not respond to the environment. To test this, monocyte-derived mϕ from non-smokers (NS), smokers (S) and COPD patients were cultured in GM-CSF (G-mϕ, M1-like) or M-CSF (M-mϕ, M2-like) for 12d (n=6-12). Media was switched for 6d and mϕ stimulated with LPS. TNFα and IL-10 were measured by ELISA (n=2-3). Beads, *H.influenzae* (HI) or *S.pneumoniae* (SP) phagocytosis was measured fluorimetrically (n=10), CD163 and CD16 by FACS (n=3). All G-mϕ released similar levels of TNFα and IL-10. Switching to M-CSF, TNFα release decreased in NS and S, but not COPD whereas IL-10 increased in NS but not S and COPD (Table 1). M-mϕ from NS and S released less TNFα *vs* COPD but more IL-10. Switching to GM-CSF, IL-10 decreased in all mϕ (Table 1).

Table 1. LPS-stimulated cytokine release

ng/ml	GM-CSF→M-CSF					
	Day 0			Day 6		
TNFα	7.5±2.8	6.9±0.7	6.4±3.4	2.9±0.1	3.2±0.3	8.7±3.5
IL-10	0.1±0.01	0.1±0.07	0.1±0.02	3.6±0.1	1.1±0.2	1.3±0.2
	M-CSF→GM-CSF					
TNFα	2.0±0.3	3.2±0.4	7.7±4.9	4.4±1.6	6.5±0.8	19.7±4.8
IL-10	3.7±0.1	2.1±0.5	1.9±0.4	0.2±0.02	0.3±0.1	0.5±0.03

Bead phagocytosis was similar by all mϕ. G-mϕ phagocytosed less HI and SP *vs* M-mϕ in all groups (50%, $p<0.05$). All COPD mϕ phagocytosed ~45% less HI and SP *vs* NS, and expressed less CD163 and CD16. Culturing G-mϕ in M-CSF, increased phagocytosis of HI (400%) and SP (200%) in NS *vs* S and COPD. Culturing M-mϕ in GM-CSF, HI and SP phagocytosis was less in all mϕ. COPD mϕ appear "hyper-inflammatory" regardless of environment suggesting lack of plasticity. Altering COPD mϕ plasticity may be a therapeutic target.

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Chemokinome characterization in human lung macrophages following stimulation with Th2-type cytokines IL-4 or IL-13

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Background: Macrophages may acquire polarized phenotypes, the two extremes being the proinflammatory (M1) and immunoregulatory (M2) phenotypes characterized by surface markers and chemokinome profiles. Reprogramming of AM toward a partially M2-polarized phenotype has been suggested to contribute to COPD pathogenesis. We thus sought to characterize the phenotype of human lung macrophages (LM) following stimulation with the Th2-type cytokines IL-4 and IL-13.
Methods: LM were isolated from human resected lungs challenged for 24 or 48hrs with IL-4 or IL-13 (1-150 ng/mL). Cytokines transcript expression was assessed with RT-qPCR, whereas proteins of M1 (TNF-α, CCL3, CCL4 and CXCL8) and M2 cytokines (CCL13, CCL17 and CCL22) were quantified in supernatants.
Results: Unstimulated LM exhibited a rather undifferenciated phenotype, with weak M1/M2 cytokines expression. On the other hand, transcriptome analysis of 80 cytokines gene revealed that only four M2-type transcripts levels were increased (4- to 8-fold) following stimulation with IL-13 (CCL13, CCL17, CCL22 and CCL26). M2-type cytokine production at the protein level was also concentration-dependently increased (3- to 20-fold), whereas M1 cytokines were unaffected. CCL13 and CCL22 increase was maximal at 48hrs, while the maximum was reached at 24hrs for CCL17. The results obtained with IL-4 were similar, except that IL-4 potency was greater than IL-13 since the low 10 ng/mL concentration provided submaximal cytokines increases.
Conclusions: Our data demonstrate that IL-4 and IL-13 favours LM polarization toward the immunoregulatory M2 phenotype characterized by the expression of CCL13, CCL17, CCL22 and CCL26.

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Lipids in the lung: Respiratory inflammation in COPD

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Background: There is strong association between COPD and metabolic co-morbidities involving lipids. Abnormalities of lipid metabolism are associated with increased inflammatory responses. The aim of this study was to identify abnormalities of lipid content in the lungs of COPD patients, focusing on lung macrophages.
Methods: Resected lung tissue from patients undergoing surgery for cancer was used; isolated macrophages and macrophages within formalin fixed paraffin embedded (FFPE) tissue were examined. Comparisons were made between 5 subject groups, never smokers (NS), current smokers with normal lung function (S), ex-smokers with normal lung function (E), COPD current smokers (COPDS) and COPD ex-smokers (COPDE). The presence of lipid laden macrophages was identified using oil red o stain (ORO) where positive staining is expressed as % mean (SD).
Results: The number of ORO positive macrophages was increased in COPD patients and controls who were current smokers (COPDS and S) compared to the other groups of ex- or never smokers, in both isolated macrophages and FFPE tissue.
Discussion: We have shown an increase in lipid laden macrophages in the lungs of

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Percentage of ORO +ve lung macrophages

	% ORO +ve macrophages isolated from resected lung	% ORO +ve macrophages in FFPE lung
NS	18.0 (24.5) [N=2]	0.6 (1.4) [N=10]
E	11.1 (24.6) [N=10]	5.7 (14.0) [N=8]
S	87.0 (14.4) [N=8]	75.6 (26.4) [N=10]
COPDE	6.1 (10.9) [N=7]	18.8 (34.4) [N=10]
COPDS	81.6 (22.3) [N=10]	77.2 (13.5) [N=10]

S and COPDS. Current smoking appears to cause dysfunctional lipid metabolism within lung macrophages that may contribute to respiratory inflammation. Smoking cessation may have benefits in returning lipid metabolism to normal in these cells.

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Effect of neutrophil supernatants on *ex vivo* small airway contractility in COPD

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Airway neutrophilia is a significant feature of COPD. Neutrophils release a variety of cytotoxic products (e.g. proteases) that degrade components of extracellular matrix. This may result in destruction of the lung and impedance of airflow characteristic of COPD. Small airways are the main site of airflow obstruction in COPD. Our aim was to study the effects of products from activated neutrophils on the contractility of small airways. Rat precision cut lung slices (PCLS) were obtained and videomicroscopy used to assess contractility of small airways. Neutrophils were isolated from whole blood from non-smokers, smokers and COPD patients, stimulated with fMLP (100μM) and supernatants collected. PCLS were incubated overnight in neutrophil supernatants, and the contractility of small airways assessed by addition of increasing concentrations of carbachol. PCLS incubated in supernatants from COPD patients caused a significant leftward shift in $EC_{50,}$ compared with untreated controls (13±2 *vs* 34±8μM, n=6, $p<0.01$). Conversely, PCLS incubated in supernatants from smokers had no effect on EC_{50}, but significantly reduced maximal contraction compared with untreated controls (65±1% *vs* 88±3%, n=4, $p<0.05$). PCLS incubated in supernatants from non-smokers showed small but significant reductions in both EC_{50} (16±3 *vs* 32±8μM, n=6, $p<0.05$) and maximal contraction (79±4 *vs* 87±2%, n=6, $p<0.05$), compared with untreated controls. Elastase content of supernatants correlated with maximal contraction, but not with EC_{50}. We conclude that neutrophil supernatants from COPD patients increase the sensitivity of small airways to cholinergic stimulation, which may contribute to the airflow limitation characteristic of the disease.

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Reduced phagocytosis of pathogenic bacteria by neutrophils from COPD patients

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Acute exacerbations of COPD are the commonest cause of acute medical admissions in the UK, with ~50% associated with bacterial infection. An acute bacterial insult is usually associated with increased recruitment and activation of neutrophils. COPD is characterized by airway neutrophilia; however, despite increased numbers of these cells, bacterial colonization persists. This study examined whether neutrophil phagocytosis was altered in COPD. Neutrophils were obtained from COPD, smoking and healthy subjects and phagocytosis of fluorescently-labelled polystyrene beads, *Haemophilus influenzae* (HI) or *Streptococcus pneumoniae* (SP) measured by flow cytometry. Neutrophils from all subjects ingested beads similarly showing that all cells were capable of phagocytosis. Neutrophils from all subjects phagocytosed SP with a maximum response at 5 min, however COPD neutrophils ingested significantly less SP ($p<0.05$) than those isolated from non-smokers (NS) and smokers (S) (NS: 17.1±1.7 MFI, n=19 vs S: 14.8±1.2 MFI, n=19 vs COPD: 12.8±0.5 MFI, n=20). Approximately 95% of cells phagocytosed HI, but the capacity of COPD neutrophils to ingest HI was significantly attenuated ($p<0.01$) compared with control neutrophils for all time points up to 30 min. Neutrophils from S phagocytosed HI similarly to cells from NS initially (NS: 18.0±3.8 MFI, n=19 vs S: 19.3±4.8 MFI, n=20 vs COPD: 5.2±1.5 MFI, n=20). However, after 10 min this response became blunted. These effects were not associated with differences in expression of CD11b, TLR2 or TLR4 or in cell viability. Therefore, neutrophils from COPD patients exhibit reduced phagocytosis of pathogenic bacteria which could account for persistent airway colonization.

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Short term effects of alpha1-antitrypsin substitution therapy on the degranulation of neutrophil granulocytes

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Introduction: Alpha-1-antitrypsin-deficiency (AATD) is a hereditary, co-dominant condition which results in low levels of circulating alpha-1-antitrypsin (A1AT), unbalanced protease activity and frequent development of emphysema. The concept behind substitution therapy with human plasma A1AT is to normalize the concentration in blood and tissue and thereby protecting the lungs from progressive destruction by proteases.
Aims: To better understand the influence of weekly augmentation therapy on polymorphnuclear neutrophils (PMNs) function we examined direct effects on chemokine release and degranulation of matrix metallopeptidase-9 (MMP-9) and myeloperoxidase (MPO).
Methods: PMNs were isolated from peripheral blood of ten AATD-patients (PI-ZZ, mean age 55±8,7; FEV1[%] 38,1±14,33) pre and two hours post substitution infusion. The release of IL-8, MMP-9 and MPO was quantified in neutrophil supernatants via ELISA. Standard inflammation markers as well as MMP-9, MPO and the tissue inhibitor of metalloproteinase-1 (TIMP-1) were evaluated in patient sera.
Results: PMN-stimulation experiments displayed no differences on chemokine release or degranulation between pre and post substitution infusion. Two hours after substitution we measured increased MMP-9 ($p=0.002$) and MPO ($p=0.0034$) concentrations in sera of patients, whereas TIMP-1 decreased drastically ($p=0.002$). IL-8 levels in sera of these patients were within normal range and no changes were detectable due to substitution.
Conclusions: We conclude that elevated MMP-9 and MPO concentrations after substitution depend on rapid degranulation of PMNs whereas the decline in TIMP-1 concentration possibly depends on a feedback loop.

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Lung inflammation is significantly reduced by recovery of neutrophil apoptosis in vivo

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Introduction: Neutrophil apoptosis is considered to be a major regulator of neutrophil driven lung inflammation. The compatible solute ectoine has been described to act preventive against lung inflammation induced by environmental particles (Sydlik et al., Am J Respir Crit Care Med 2009). As a therapeutic approach, here, we investigated the influence of this compound on neutrophil apoptosis in the inflammatory microenvironment.
Methods: Human neutrophils were treated ex vivo with particles or inflammatory mediators in the presence or absence of ectoine in order to study apoptosis rates and pro-apoptotic signalling. Lung inflammation was induced in rats by intra-tracheal application of 2.5 mg/kg environmental particles and studied in lung lavages after control or ectoine intervention.
Results: Apoptosis rates of human neutrophils from COPD patients and volunteers, which were significantly reduced by the inflammatory stimuli, recovered significantly in the presence of ectoine. Mechanistic analyses demonstrated the preventive effect of ectoine of pro-apoptotic signalling events via Akt and Mcl-1. The in vivo relevance of the data was shown by significantly reduced neutrophil inflammation after ectoine intervention which correlated with restored neutrophil apoptosis rates in the lung.
Conclusions: The current studies demonstrate the effectivity to prevent anti-apoptotic signalling in neutrophils by the compatible solute ectoine. This effect led to recovered apoptosis rates and a reduction of environmentally induced lung inflammation. The relevance of ectoine treatment for humans is demonstrated by the EFECT study which will be presented on the same meeting.

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