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HARMLESS

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Project Acronym: HARMLESS

Deliverable 2.4

Open access repository of mRNA single cell response

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Level of Dissemination: Public

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Acknowledgement

HMGU acknowledge the support provided by colleagues from the *Institute of Lung Health and Immunity,* particularly Dr. Herbert Schiller and his team, for their contribution to generate and analyse the dataset. Furthermore, the first study has been conducted at the end of the SmartNanoTox EU project (Smart Tools for Gauging Nano Hazards (SmartNanoTox), EU, H2020-EU.2.1.2., ID: 686098, 2016-2020). General acknowledgement goes to all WP2 members, and the HARMLESS consortia for discussions around.

Description of Task

Introduction

Depending on the material, particle inhalation can cause severe health effects, including chronic inflammation, fibrosis or even cancer. Because of their small size, combustion derived or engineered nanomaterials (NM), deposit effectively in the fragile, alveolar region of the lungs. The AOP concepts for all respiratory adverse outcomes generated by particle inhalation coincide in a common chain of events, starting from particle cell interaction over local inflammatory and injury responses to the respective pathology like pulmonary fibrosis or cancer. Therefore, the question which lung cell types interact with the inhaled material and initiate for example the inflammatory cascade underlies the AOP concept and its translation to cell based in vitro assays for predictive toxicology. According to recent transcriptomic studies, our lungs consist of over 50 different cell types (Travaglini, et al. *Nature* 2020), so to depict which cells interact with the respective particles or initiate specific AOP key events is not trivial.

There is scientific consensus, that tissue resident, but motile phagocytes, so-called alveolar macrophages, rapidly engulf inhaled and pulmonary deposited particles of all kinds, and thereby represent the first line of defence. Further pulmonary inflammation is a generic response to inhaled NMs but the fate of inflammation and subsequent injury is dependent on NM dose and shape. Whether alveolar macrophages however initiate the inflammatory response, as known for many pathogens, is uncertain for sterile and pyrogen-free particles and other lung cell types such as alveolar epithelial cell might be much more relevant (Chen et al., Part Fibre Toxicol. 2016).

Task 2.4. Strategic toxicity studies (Lead: HMGU; NRCWE, DTU, INIA), duration month 1-31

To complement existing knowledge we based subtask **2.4A** (respiratory toxicology of NMs) on data from a previous project (SmartNanoTox) were single-cell transcriptomics of lungs from to selected NM-exposed mice was performed, to identify key cell types involved in initiation and continuation of specific cellular responses related to relevant Key Events (KE). To unveil the material dependent underlying cellular perturbation pattern, we exposed mice intratracheally to three different NMs, soot like carbon black (CNP), tangled double-walled (DWCNT) and rigid, fibrogenic multi-walled carbon nanotubes (MWCNT). LPS was used as inflammation benchmark control. All three NMs are in-depth investigated at various levels within the HARMLESS project. After 12h, 6d and 28d of exposure, lungs were analyzed by single cell RNA sequencing (scRNAseq), histology and bronchoalveolar lavage (BAL) to elucidate the cellular response pattern in the lungs after nanomaterial inhalation.

For reasons of practicability, the majority of NM exposure mouse studies described in the literature, and also those from partner NRCWE building the HARMLESS in vivo database, use intratracheal instillation but not inhalation as rote of exposure. Therefore in a second single-cell transcriptomics approach we aim to support the validity of the responses and the consensus of the cell specificity of toxicity pathways and KE, identified in the previously described single cell response profiles by comparing (intubated- ventilated) inhalation and instillation exposed mice for one selected NM (**D2.4**).

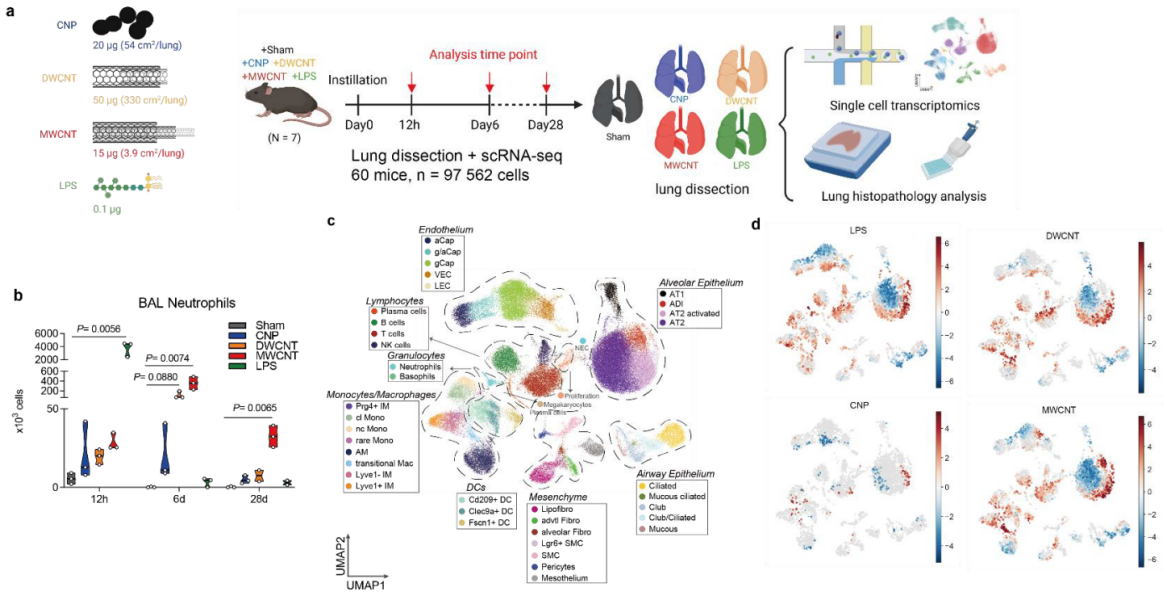
We have selected CNP (Printex90) for this comparative study because of two reasons:

1. The CNP material has been used for many at NRCWE conducted instillation studies as an internal benchmark and we have already time course data from CNP instillation study, informing us about the time points to be analyzed for the transcriptional study (Chen at al. 2015).

2. CNP was selected for technical reasons of its dispersion stability in aqueous solutions, suitable for aerosol the generation required for the intubated-ventilated inhalation exposure method (Yang et al. 2019).

Description of Action

The first aim of task 2.4 was to identify the material dependence of the key cell types involved in initiation of specific cellular responses related to AOP relevant key events. This information is not deducible from conventional bulk transcriptomics where all response profiles over the more than 50 different lung cell types get averaged. scRNAseq of NM exposed and control lungs (Fig.1a) in contrast allowed us to identify 40 different cell clusters which according to their expression pattern were mapped to 22 tissue resident cells types and 18 leukocyte types (Fig. 1c). Analysis of NM exposure related expression changes in each cell population allowed us to detect cell specific response pattern (Fig. 1d) and to identify cell types in which the expression of inflammatory mediatory (cytokines) was induced in a NM specific manner (Fig. 1e). A preprint manuscript describing the methodology and providing an amble overview over the results from the study is public available at https://www.biorxiv.org/content/10.1101/2024.02.10.579746v1.



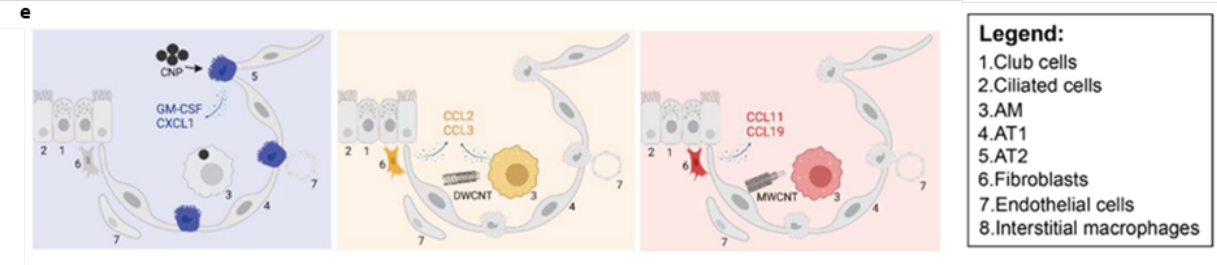


Fig. 1: Nanomaterial specific cellular response patterns in the lung

a. Experimental setup, mice (n = 7) were exposed to three different NMs (CNP: 20µg, 54cm2/lung; DWCNT: 50µg, 330 cm2/lung; MWCNT: 15ug, 3.9cm2/lung) and LPS (0.1µg). NM doses generated comparable acute (12h) lung inflammation. Subsequent analyses followed at 12h, d6 and d28 by single-cell transcriptomics (scRNAseq), FACS and histopathology (n=4) as well as bronchoalveolar lavage (BAL, n=3). b. Differential neutrophil counts in BAL. c. Visualization of dimension-reduced single cell transcriptomic data by Uniform Manifold Approximation and Projection (UMAP) reveals treatment specific cellular response patterns in the lungs within 41 annotated cell types, classified into 8 niches. d. Treatment-dependent differential cell abundance testing across all timepoints, highlighting NM-induced cell state shifts compared to sham analyzed by Milopy. e. Suggested NM-specific mode of action during the initiation of lung inflammation by cytokine expression and secretion. BAL neutrophils are shown as the mean ± standard error of the mean (SEM) of three mice (n = 3, except n = 2 for 28d DWCNT).

The second aim of task 2.4 was to compare CNP inhalation with instillation by scRNAseq of exposed mouse lungs, to test the translatability of the by instillation exposure generated single cell perturbation pattern. For the exposure, well studied carbon black like nanoparticles (CNP) were used to understand whether by instillation characterized material and cell specificity of toxicity pathways and KE, can be generalized for inhalation as well.

The methodology for CNP delivery by intratracheal instillation has been described most recently by Han et al. 2023. and the methodology for CNP delivery by ventilator-assisted aerosol delivery is described in Yang et al. 2019.

The overall comparability of particle deposition pattern generated by intratracheal instillation and inhalation has been shown before for fluorescent particles applied to mouse lungs (Yang et al. 2019).

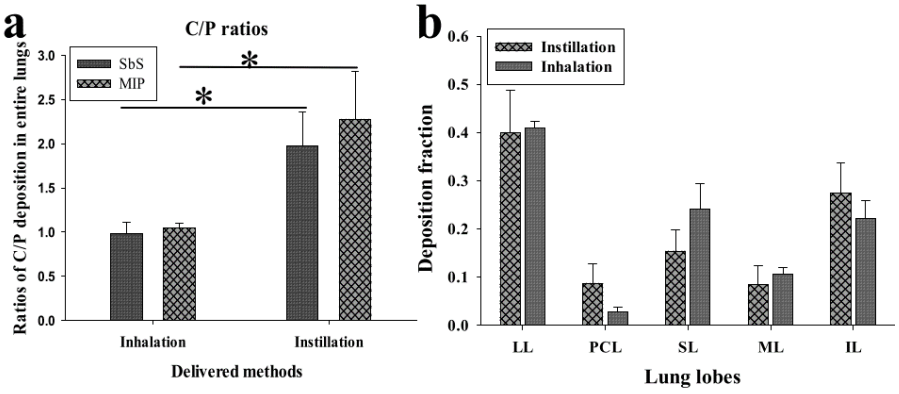


Figure 2. Quantitative analysis of spatial particle deposition in the lungs of mice after instillation and inhalation application routes. (a) Ratio of central to periphery (C/P) deposition analysis: C/P fluorescence intensity was normalized to the C/P area ratio. Lobewise deposition fraction (b, fractional MF dose in each lobe). Abbreviations: SBS: slice by slice analysis; MIP: maximum intensity projection analysis of a whole lung;

LL: left lung; PCL: postcaval lobe; SL: superior lobe; ML: middle lobe; IL: inferior lobe (Figure taken from Yang et al. 2019)

The experiments were initially planned to be started in 2022, but due to changes in the methodological set up and the personnel of the bioinformatics team at HMGU the experiment had to be postponed to early 2023. Noteworthy single cell analysis can only be performed from fresh tissue, taken immediately at the respective time point of investigation, and does not allow any storage by freezing. In March 2023 however the IT infrastructure of HMGU got inoperative because of a cyberattack, which made ordering of material and animals as well handling of large data impossible causing another serious delay. End of 2024 we performed the mouse exposure study and possessed the lungs for scRNAseq. After respective quality control scRNAseq data was processed for analysis of exposure route dependent expression and cellular perturbation pattern.

The scRNAseq data is available via GEO accession GSE267246, and a detailed manuscript discussing similarities and exposure mode dependent difference of cellular perturbation pattern is in preparation and will be submitted for publication before the end of the project.

Results

Summary of results: Cellular response patter to inhaled NMs

Cell type mapped gene expression revealed NM specific cellular perturbation patterns, which demonstrates material specific cellular responses of varying duration and in different cell niches, despite the similar level of acute lung inflammation (Fig. 1b). A major aim is to identify the inflammation initiating cell types, i.e. the cell types that produced the inflammatory mediators (i.e. cytokines) relevant for the neutrophil accumulation in the airspace of NM exposed lungs. *Inflammation*, at the level of 'pro-inflammatory mediator release' or 'recruitment of inflammatory cells' is accordingly a common key event (KE) in several NM triggered Adverse Outcome Pathways (AOPs) such as AOP173 (Pulmonary Fibrosis) or AOP303 & 409 (Lung Cancer), see D2.1 p21 – 23. Neutrophil influx is one of the most important early key events downstream of the molecular initiating event (MIE) triggered by particle-cell interaction in the lung. To this end, we analyzed the single cell expression data for the cell types that express the highest cytokine score at the early phase of 12h after NM exposure. CNPs and DWCNTs triggered highest cytokine expressions from alveolar epithelial type 2 (AT2) and adjacent bronchiolar cells, whereas for MWCNTs cytokine levels where highest for a specific fibroblast population. Of note, only the LPS control caused at 12h a high cytokine signature in lung macrophages, indicating a different pathway of inflammation initiation for pyrogen-free and sterile materials or by pattern recognition receptors sensed pathogens (See Fig.1e).

Our cell specific gene expression analysis suggests the following cell types and pro-inflammatory read outs to be used for *in vitro* tests to predict the acute inflammatory response on a material specific manner:

NM exposure	cell type	read out (mRNA)
CNP (carbon black)	epithelial cells (alveolar)	*Cxcl1, Csf2*
DWCNT (curly, flexible CNTs)	macrophages (alveolar, interstitial)	*Ccl3*
	fibroblasts (alveolar)	*Ccl2*
MWCNT (long, rigid MWCNTs)	fibroblasts (alveolar, adventitial)	*Ccl11, Ccl19*

The data has been made open access available at:

- Study 1, RNA expression data and study design and outcome are available at: https://www.biorxiv.org/content/10.1101/2024.02.10.579746v1.
- Study 2, RNA expression data via Gene Expression Omnibus with the GEO accession code GSE267246 at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi (token can be provided at request from tobias.stoeger@helmholtz-munich.de)

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