Single-cell sequencing reveals spatio-temporal and material specific cellular perturbation patterns in the lungs of nanoparticle exposed mice

C. Voss[1,3], M. Ansari[2,4], I. Angelidis[2], C. Mayr[2], M. Strunz[2], H. B. Schiller[2], T. Stöger[1]*

[1]Dynamics of Pulmonary Inflammation, iLBD/CPC, Helmholtz Center Munich, Germany., [2]Systems Medicine of Chronic Lung Disease, iLBD/CPC, Helmholtz Center Munich, Germany. , [3]Mechanism of Neonatal Chronic Lung Disease, Ludwig-Maximilians-University, Munich, Germany. , [4]Institute of Computational Biology, Helmholtz Center Munich, Germany

Many rodent studies focus on the global pulmonary response to nanomaterial exposure. Cellular key players orchestrating specific downstream event initiation or leading to long-term consequences are still unknown. Recapitulation of these *in vivo* toxicological findings *in vitro* (IVIV) is a major challenge of the current nanotoxicology community. Mimicking adverse outcome pathways for chronic pulmonary effects in vitro is of great importance, especially in line with the 3R strategy and the development of NAMs (new approach methodologies) in nano-hazard assessment. In this study, we investigated lungs instilled with carbon nanoparticles (CNPs), double walled carbon nanotubes (DWCNTs) and multi-walled carbon nanotubes (MWCNTs) with single-cell RNA sequencing technology. Instillation doses of CNPs, DWCNTs and MWCNTs were chosen to result in a comparable level of inflammatory response measured by the neutrophil numbers in BAL fluid at 12h. Neutrophilia persisted over 6d for all NMs but not LPS. After 28d, neutrophil numbers were further reduced but did not reach base levels of sham controls. With scRNAseq we identified over 30 different pulmonary cell types represented in our dataset. Global differential gene expression patterns already indicate a strong involvement of the bronchial epithelium for all treatments and time points, however, the temporal patterns were surprising. CNPs and DWCNTs for instance elicit an increasing, time-dependent response in club/ciliated cells peaking at 28d. Also MWCNTs induce gene expression changes in the bronchial compartment at 12h which is mainly replaced at 6d by orchestrating monocytes and macrophage populations. This was also evident in cell communication network analyses showing functional interactions of bronchial epithelium with matrix- and lipofibroblasts at 12h and a remarkable role for classical monocytes to modulate the inflammatory response in later time points. Furthermore, all NMs and particularly MWCNTs cause alveolar epithelial type 2 cell injury marked by elevated Lipocalin 2 (Lcn2) release over 28d. Lcn2 is mainly regulated in activated AT2s for all treatments, in contrast neutrophils only express elevated Lcn2 levels after LPS and CNTs. Additionally, MWCNTs rapidly (12h) and efficiently induce an intermediate epithelial cell state recently identified for their important role in epithelial regeneration ($Krt8^+$ ADI cells [1]). These ADI however do not contribute to the inflammatory resolution as at 6d ADI cannot be detected, instead, cells expressing markers of (re-)activated AT2s highly appear, indicating a cycle of inflammatory stimuli by MWCNTs. Alveolar macrophage cell death was identified as key initiating event for DWCNTs and MWCNTs, not for CNPs, long term toxicity. Experiments are ongoing to further substantiate the hypothesis of lysosomal destabilization.[2] Giant monocytic/macrophage-like cells are already visible at d6 after instillation, especially for MWCNTs and persist up to day 28 indicating granuloma formation to remove CNTs efficiently. The major role of monocytes orchestrating the long term signaling events is therefore to be studied in detail. Further analysis of specific cell-cell communication is currently ongoing based on intricate connectome analysis. This comprehensive dataset will, for the first time, facilitate mimicking of acute and chronic inflammatory processes by identifying new AOP key events for *in vitro* testing strategies.

[1] Strunz, M., et al. Nature Communications, 2020. **11**(1): p. 3559.

[2] Zhu, W., et al. Proceedings of the National Academy of Sciences, 2016. **113**(44): p. 12374-12379.