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Lung Remodeling After Pulmonary Exposure of Mice to Cerium oxide Nanoparticles - Role of Autophagy

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INSTITUT MONDOR DE RECHERCHE BIOMÉDICALE

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Introduction

Lung Fibrosis: Airway walls and bronchial thickening, irregular scars composed of dense collagen fibers, fibroblastic proliferation and cystically remodeled airspaces (Araya et al. 2008, 2013)

NPs can cause lung fibrosis

- Carbon nanotubes (CNTs) could cause progressive fibrotic response in the alveolar tissues of mice lungs (Shvedova et al. 2008, Mercer et al. 2011)
- Nickel NPs are implicated in exaggerated lung and airway remodeling in mice (Glista-Baker et al. 2014)
- Crystalline silica NPs could cause silicotic nodules with collagen fibers and dust-laden macrophages surrounding the mature collagen (Fujimura, 2000)
- CeO_2 NPs would induce inflammation, air/blood barrier damage, and phospholipidosis with enlarged alveolar macrophages leading to lung fibrosis (Ma et al. 2011, 2012, 2014)

Unanswered questions:

- Where does fibrotic lung remodelling occur? (Bronchial and/or Alveolar)
- What are the underlying mechanisms?

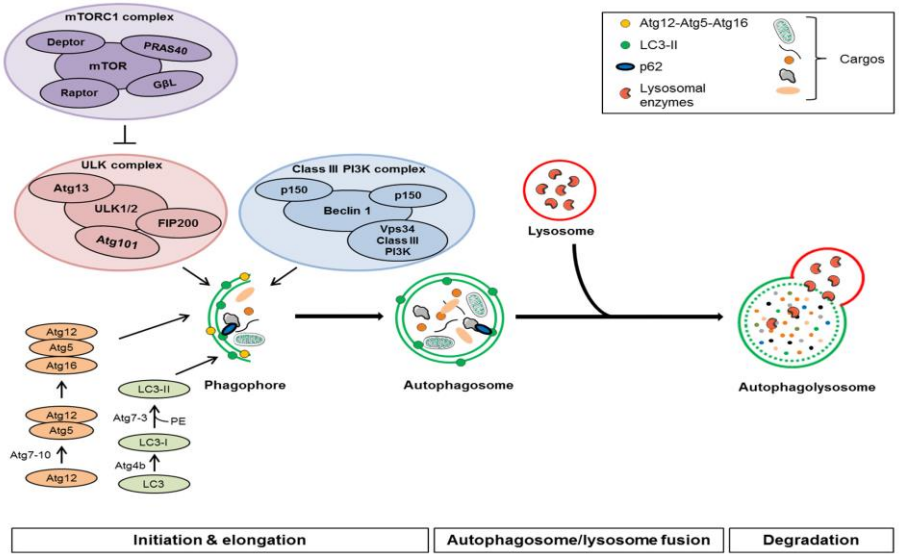
Defective Autophagy has a role to play in idiopathic pulmonary fibrosis

(Mi et al. 2011, Patel et al. 2012, Araya et al. 2013)

Autophagy: potential mechanism for fibrosis?

Autophagy: Turnover of unnecessary or dysfunctional cellular components

Induction, Autophagosome formation, Fusion and Degradation



Cohignac et al. 2014

Autophagy in fibrosis

Several factors (environmental agents, CS, ROS, ER stress) (Monick et al. 2012,Araya 2013)

→ **Defective or insufficient autophagy** (Monick et al. 2012,Araya 2013)

In lung cells

Macrophages
Secrete higher levels of ROS-induced IL1A and IL1B implicating in fibrosis development
(Lodder, J et al. 2015)

Epithelial cells
Increase apoptosis and accelerate senescence – could lead to abnormal epithelial-mesenchymal interactions
(Mi et al. 2011, Araya et al. 2013)

Fibroblasts (bronchial and parenchymal)
Excess production of extracellular matrix in fibroblasts, myofibroblasts differentiation
(Del Principe et al. 2011)

Lung fibrotic development

(Patel et al. 2012, Mi et al. 2011, Araya et al. 2013a,b, Del Principe et al. 2011)

Hypothesis

Pulmonary exposure to CeO_2NPs

Leads to lung fibrosis *via* defective autophagic pathway

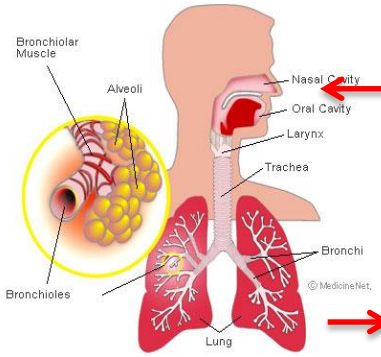
Objectives

1) To characterize the pulmonary fibrosis induced by exposure of mice to CeO_2NPs
2) To evaluate the role of autophagy in the fibrotic response to CeO_2NPs

Methods

Nanoparticles used: CeO_2NPs, (99.9% purity, Size range 15-30nm, spherical)

Diesel fuel catalysts to reduce the emission of particulate matter in diesel

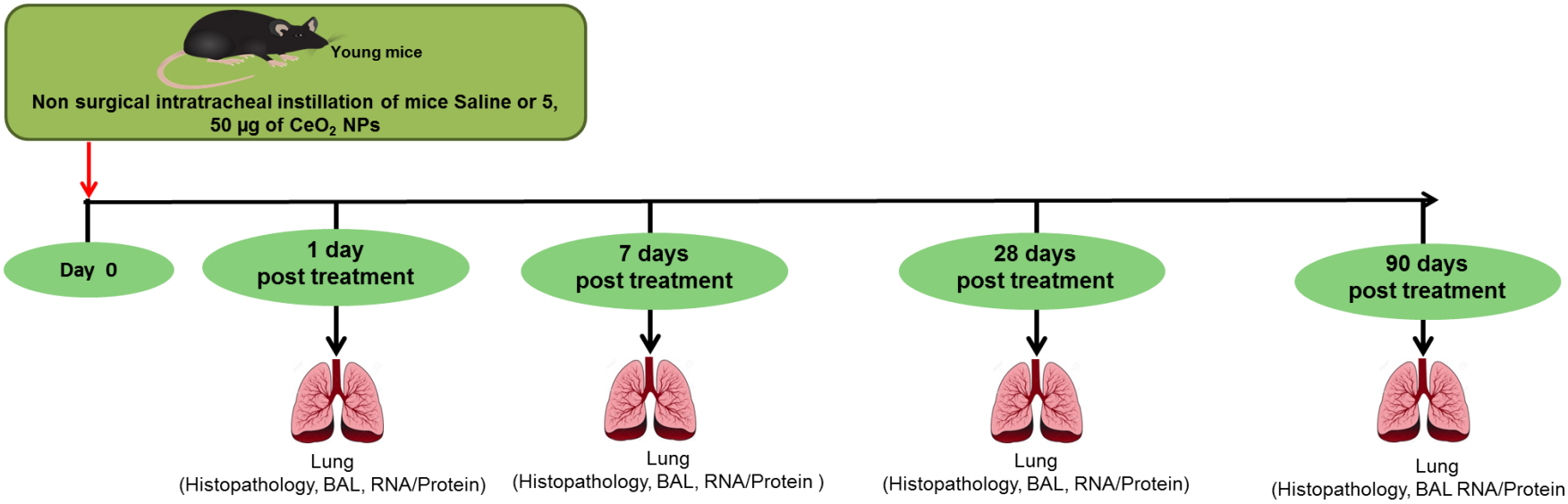


Inhaled DEP

Generates CeO_2 NPs (Hebb 1998)

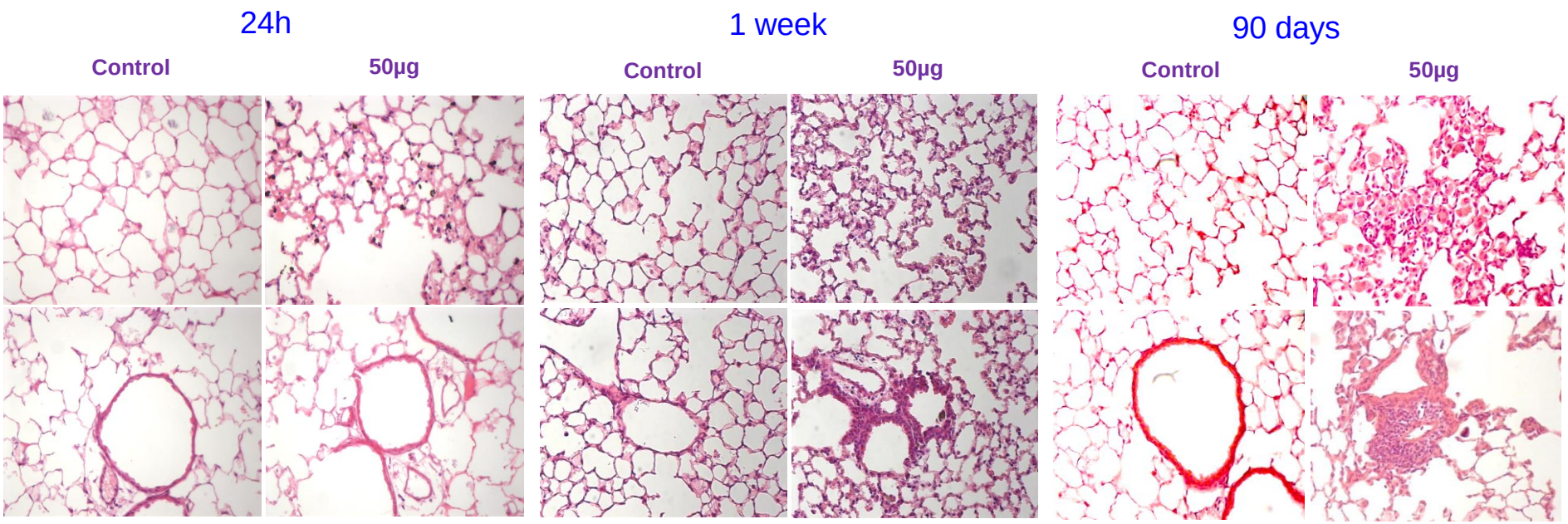
→ Causes lung diseases

Exposure Protocol:



Results:

CeO_2NPs induce lung fibrosis in mice

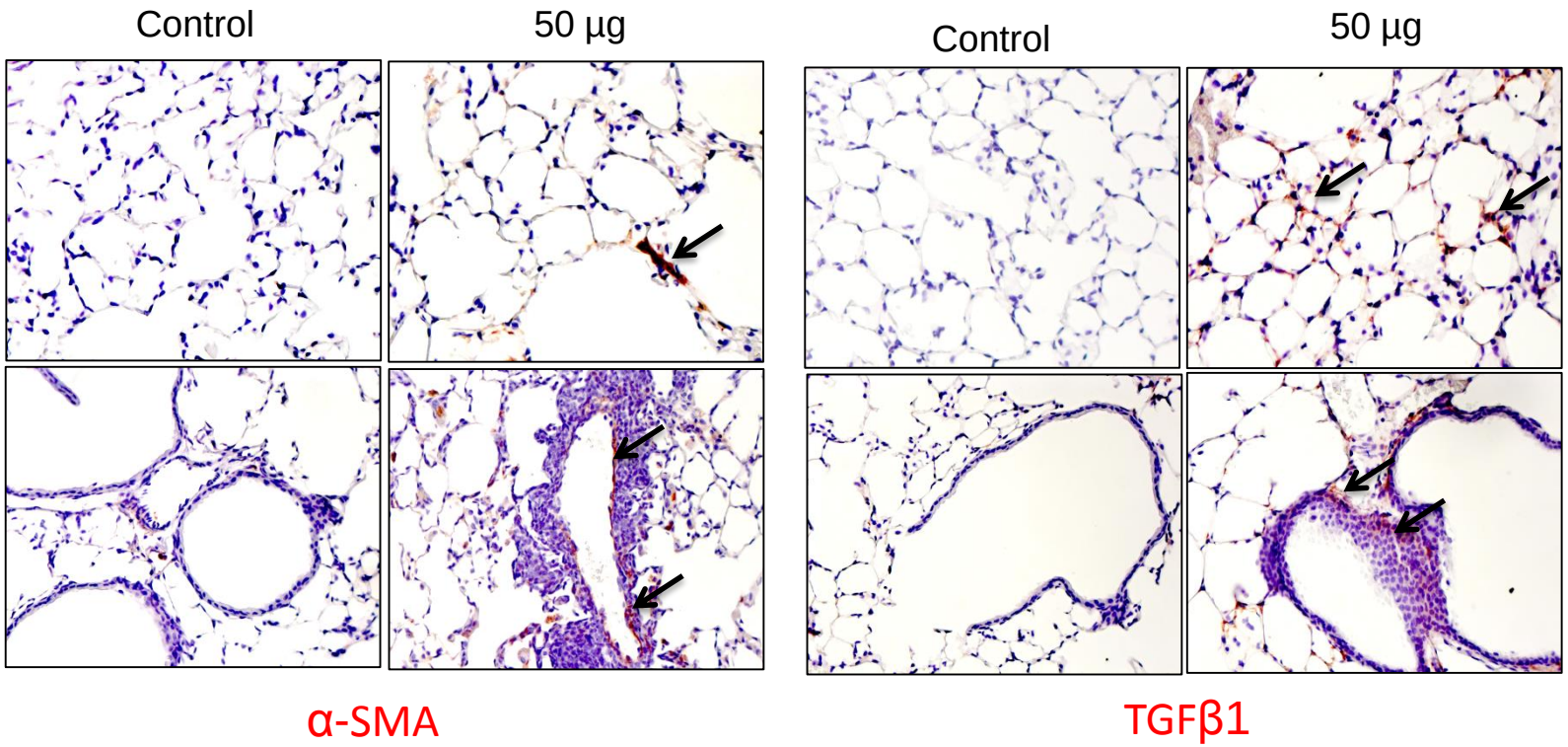


Alveolar and brocheolar thickening or inflammation observed in mice exposed to nanoceria after 1 week and 90days of exposure

(n=6)

CeO_2NPs induce lung fibrosis in mice

α-SMA and expression of TGF-β1in lung sections of mice exposed to CeO_2NPs



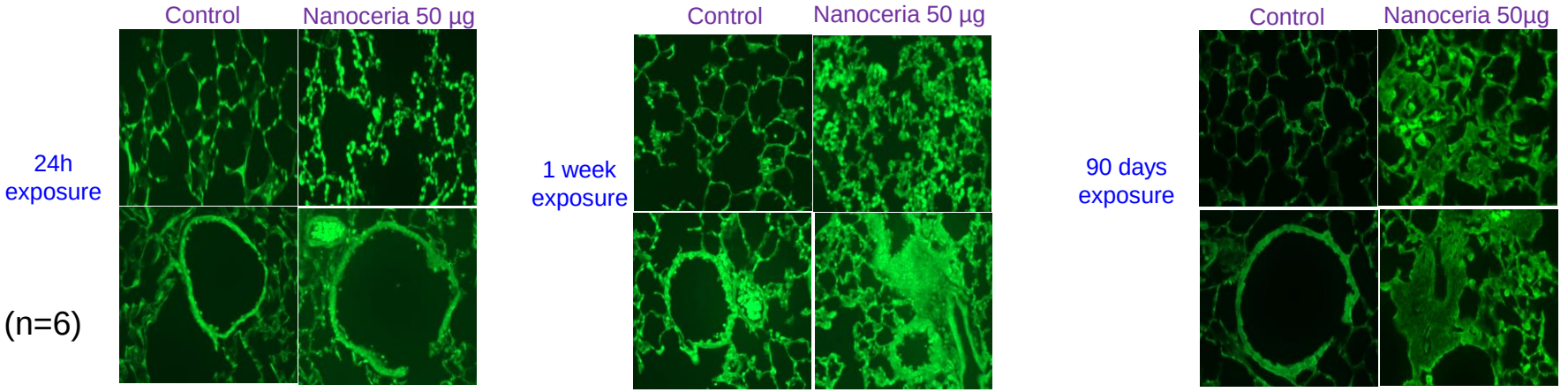
90 days exposure

IHC

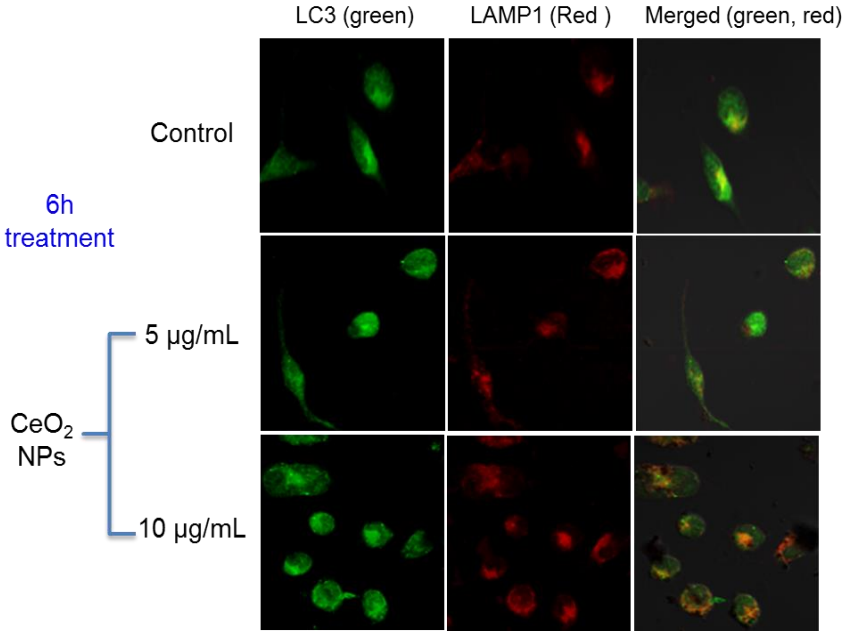
(n=6)

- An increase in α-SMA and TGF-β1expression expression observed

Induction of autophagy in GFP-LC3 mice exposed to CeO_2NPs



LC3 seems to be accumulated in macrophages *in vivo*



CeO_2NPs activate autophagy in macrophages a evidenced by co-localisation of LC3 and LAMP1

Role of autophagy in macropahes?

Atg5: an early marker of autophagy

What if Atg5 is floxed in macrophages?

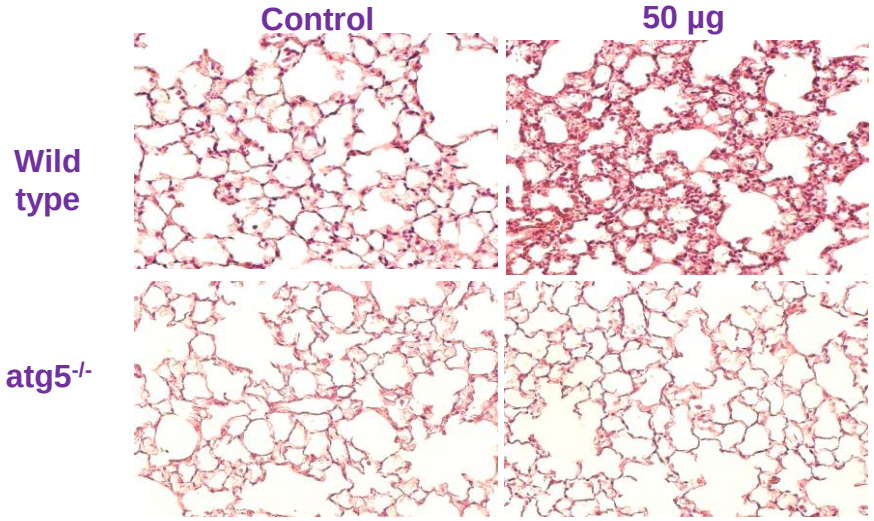
Conditional knockout of Atg5 gene in myeloid lineage

Lacks Atg5 activity in Macrophages

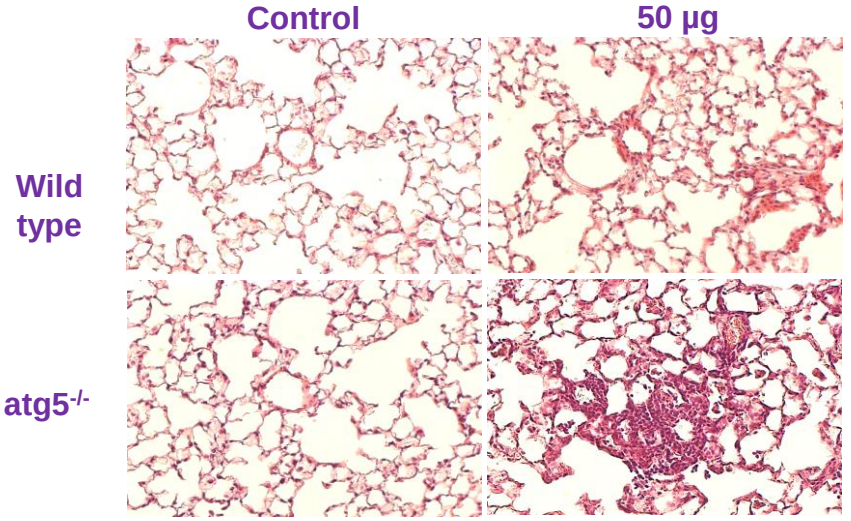
Defective autophagy in Macrophages

Implicated in CeO_2NPs-induced lung fibrosis?

Mice exposed to CeO_2NPs



- Alvelolar thickening or diffused inflammation in Wild type mice exposed to CeO_2NPs
- $Atg5^{-/-}$ mice are protected from CeO_2NPs induced alveloar thickening



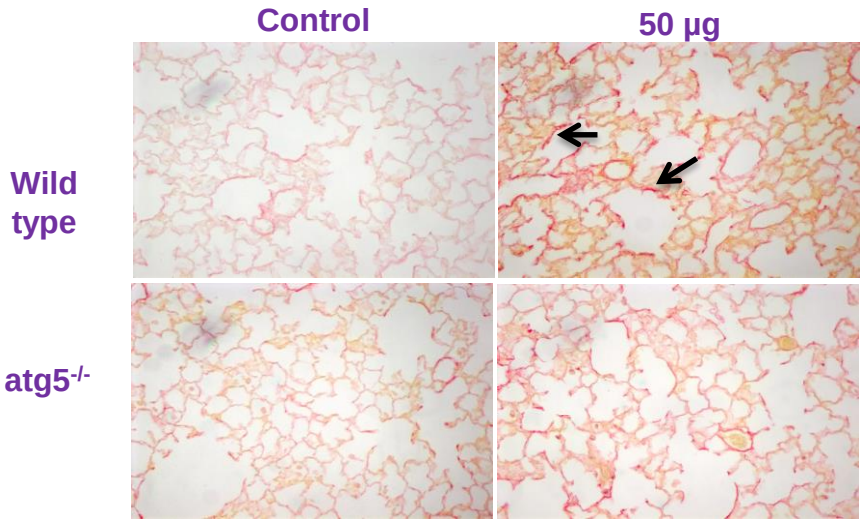
- Bronchial thickening in both wild type and $atg5^{-/-}$ mice exposed to CeO_2NPs
- Bronchial inflammation characterized by macrophages inflitration in $atg5^{-/-}$ mice

28 days exposure

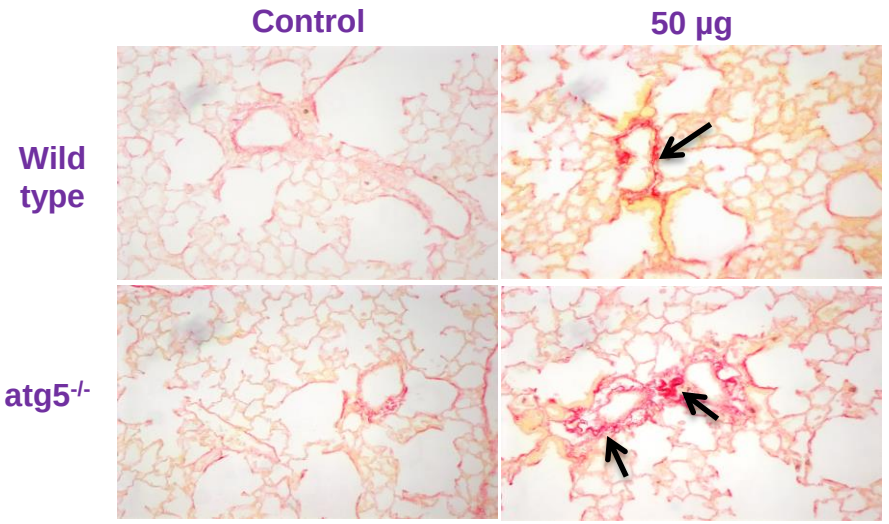
HE staining

(n=5)

Mice exposed to CeO_2NPs



- Type 1 collagen deposition in alveloli of wild type mice exposed to CeO_2NPs
- No Type 1 collagen deposition in alveoli occured in atg5$^{-/-}$ mice exposed to CeO_2 NPs



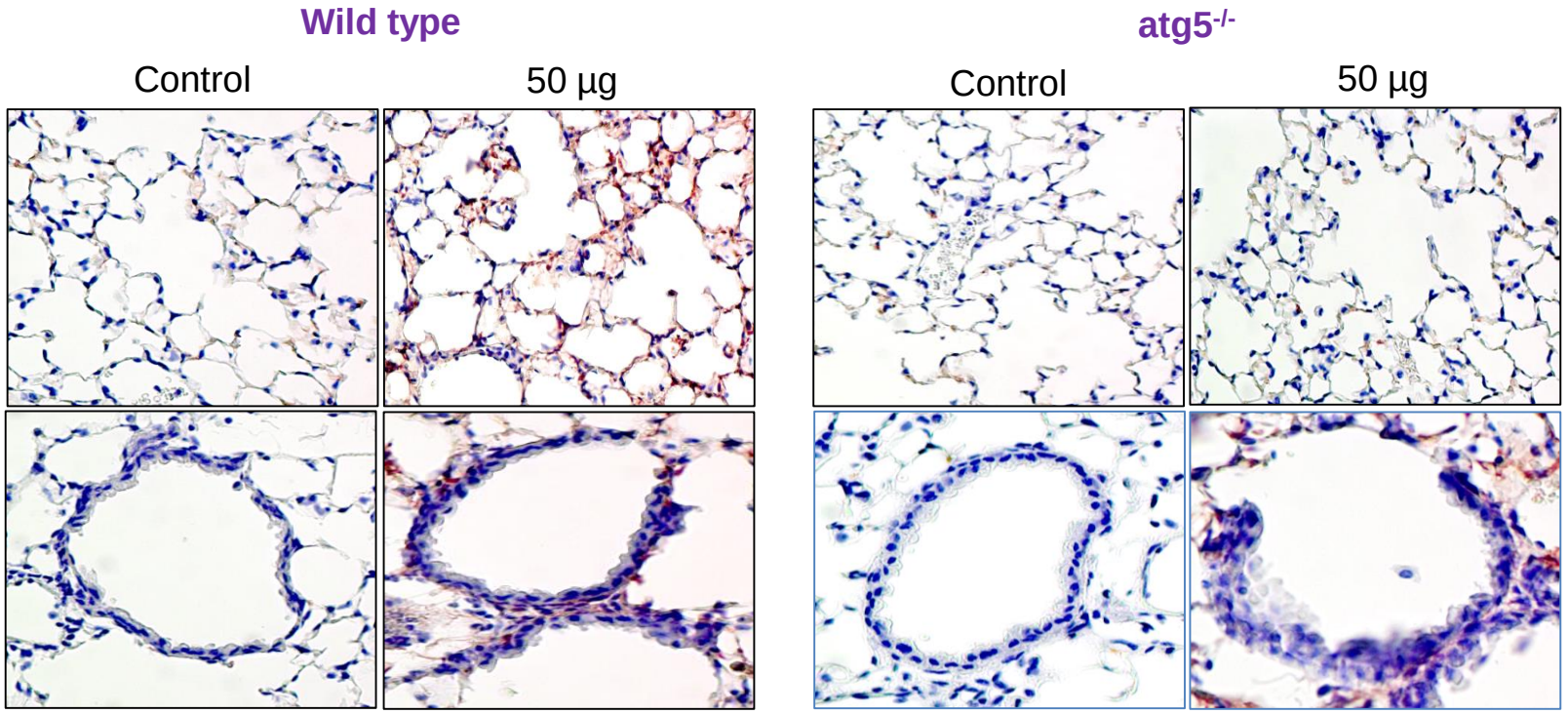
- Type 1 collagen deposition in bronchi of wild type mice treated with CeO_2NPs
- Type 1 collagen bundles in bronchi of atg5$^{-/-}$ treated with CeO_2NPs

28 days exposure

Picro sirius red staining

(n=5)

α-SMA expression in wild type and atg5$^{-/-}$ mice exposed to CeO_2NPs



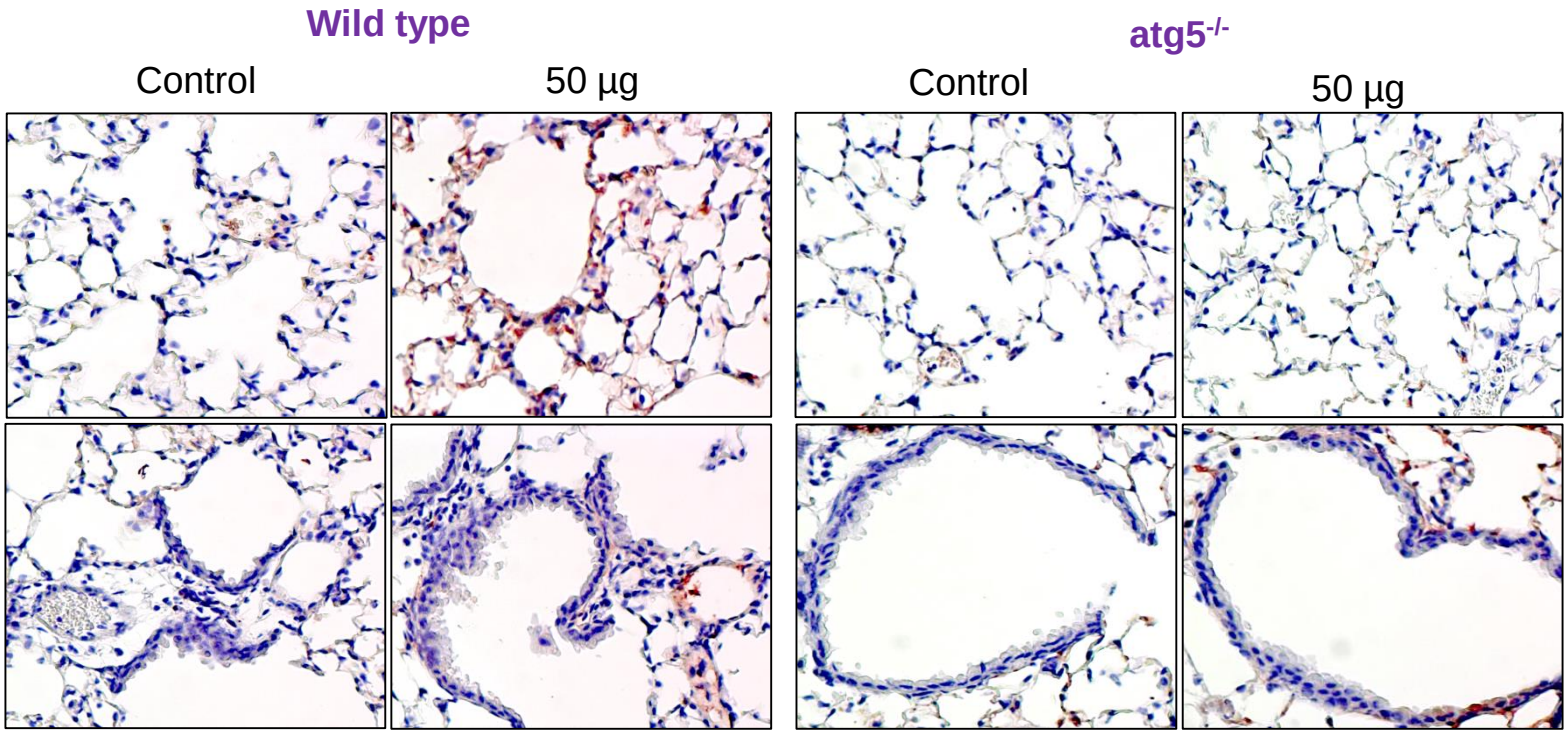
- Increased α-SMA in alveloli of wild type but not in alveloli of in atg5$^{-/-}$ mice
- Similar increase in α-SMA in bronchi of wild type and atg5$^{-/-}$ mice

28 days exposure

IHC: α-SMA

(n=5)

TGF-β1expression in Wild type and atg5$^{-/-}$ mice exposed to CeO_2NPs



- Expression of TGF-β1 in alveloli and bronchi in wild type mice noticed
- Atg5$^{-/-}$ mice are protected from CeO_2NPs-induced accumulation of TGF-β1 in alveoli but no protective effect in bronchi

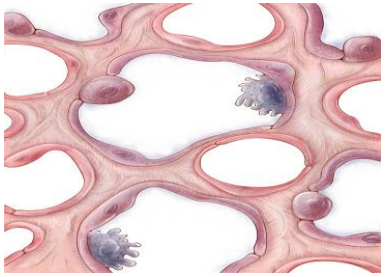
28 days exposure

IHC:TGF-β1

(n=5)

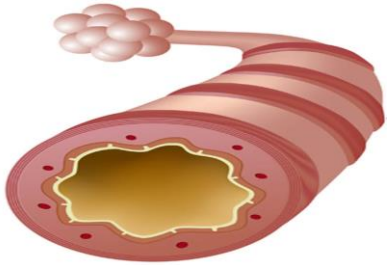
Summary

Alveoli



	Mice exposed to CeO_2NPs	
Fibrotic markers	**Wild type**	**atg5$^{-/-}$**
Thickening/ Inflammation	↑↑↑	↔
TypeI collagen	↑↑↑	↔
TGFβ1	↑↑↑	↔
αSMA	↑↑↑	↔

Bronchiole



	Mice exposed to CeO_2NPs	
Fibrotic markers	**Wild type**	**atg5$^{-/-}$**
Thickening/ Inflammation	↑↑	↑↑↑
TypeI collagen	↑↑↑	↑↑↑
TGFβ1	↑	↑
αSMA	↑↑↑	↑↑

Lack of ATG5 gene in myeloid lineage seems to be protective in alveoli but not in bronchi of atg5$^{-/-}$ over wild type mice

Autophagy may possibly play a dual role in CeO_2NPs-induced lung fibrosis

Thank you for your attention

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Future studies

1. Characterization of alveolar modifications:

- Quantification of histological modification and markers like Type collagen1, alpha SMA, TGF beta1, elastin,
- To study inflammatory infiltration by macrophages markers

2. Characterization of bronchial modifications:

- Quantification of histological modifications and expression of fibrotic markers

3. Luminex will be done on BALF samples of 24h, 1week and 90 days exposures

4. Mechanisms of pulmonary fibrosis *in vitro*:

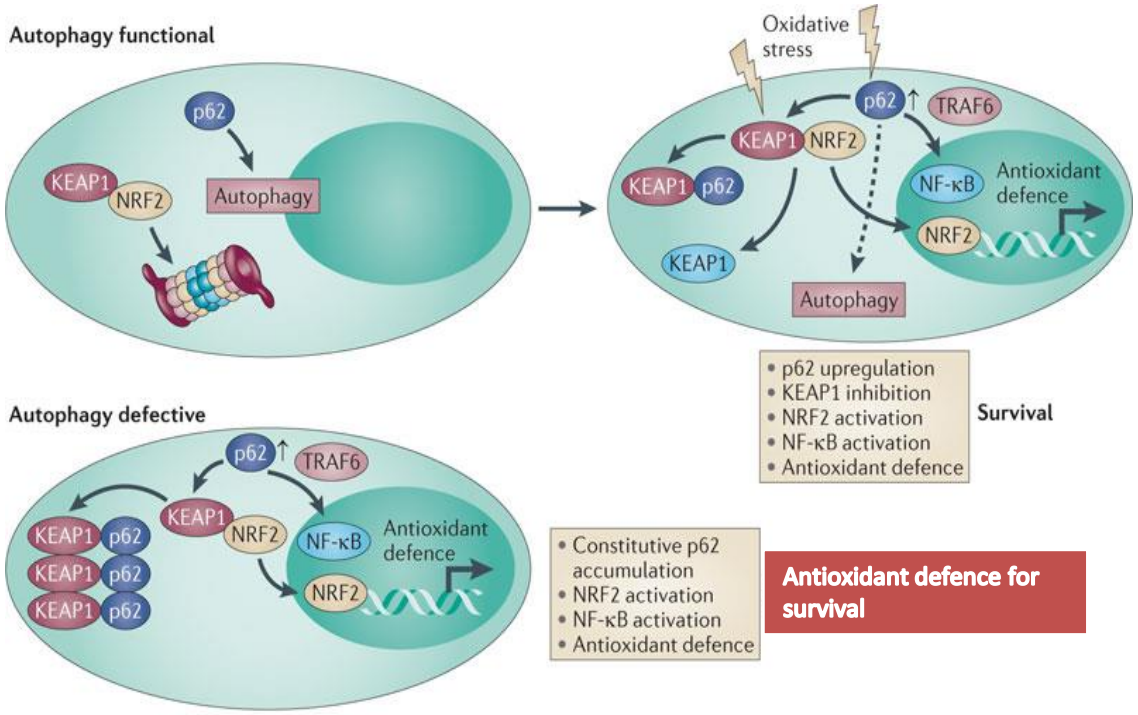
- Isolation of bronchial and parenchymal fibroblasts from mice lungs (in progress)
- Exposure to NPs
- Myofibroblasts analysis: α- Sma, collagen, migration and proliferation

5. Characterization and role of autophagy: *In vitro*

- Expression of LC3, p62 and LAMP1 in fibroblasts treated with nanoceria
- Exposing the fibroblasts with supernatants of macrophages treated with nanoceria
- Co-culture of the fibroblasts with marcophages, exposing to nanoceria

6. Analyses of lung sections from WT and atg5-/- mice exposed to nanoceria for 90 days (sections are ready)

- HES, IHC for alphaSMA, TGF beta1, collagen Type III, IV etc, Picro Sirius Red staining for Type 1 collagen etc

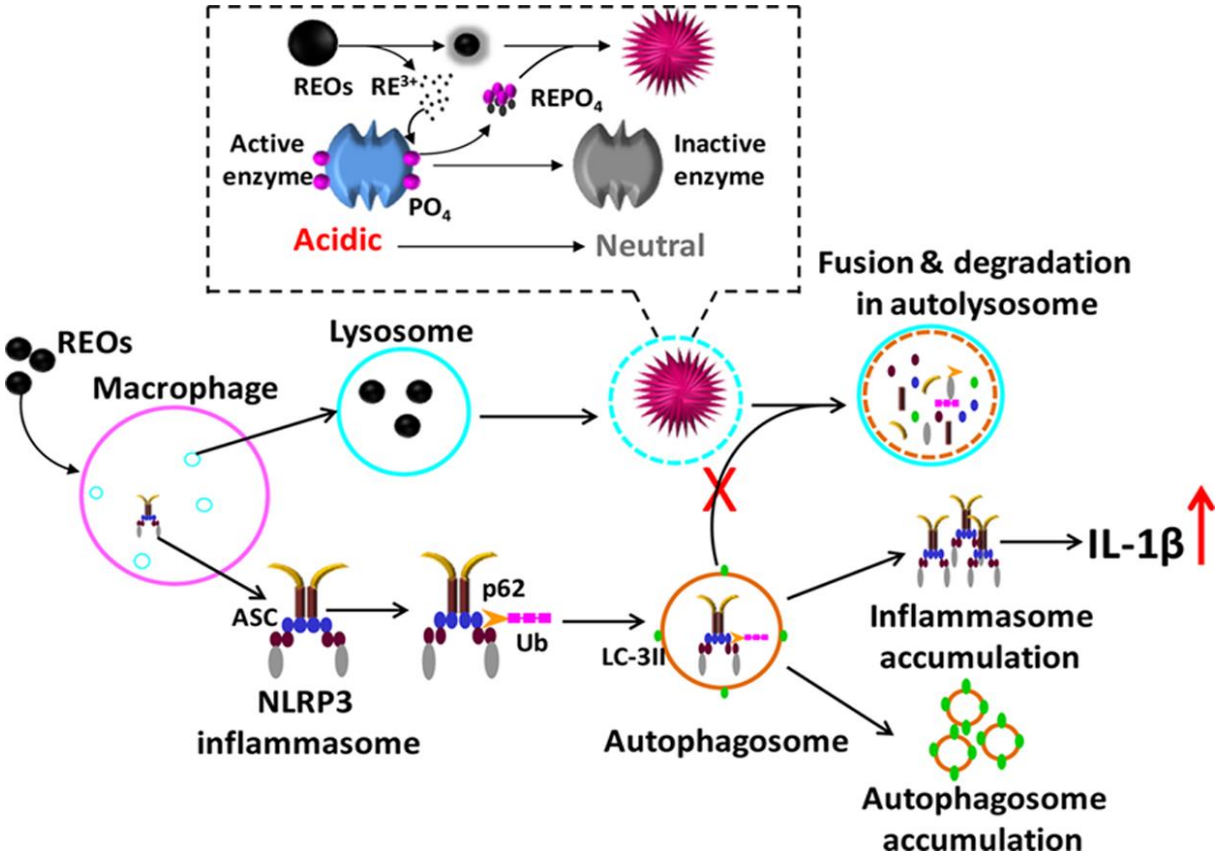


p62 is still subject to autophagy in cells experiencing cellular stress

Autophagy-defective cells and tissues, the autophagy substrate p62 is not degraded

Nature Reviews | Cancer

Nature Reviews Cancer **12**, 401-410 2012



LI ET AL. 2014, ACS Nano 8 (10) 10280–10292

Autophagy-defective (ATG5 gene knockout) in cells and tissues, the autophagy substrate p62 is not degraded

High levels of p62 Binds to Keap1 releasing NRF2

constitutive activation of NRF2 and antioxidant defence

Could counter NP induced oxidative stress

Thank you for your attention

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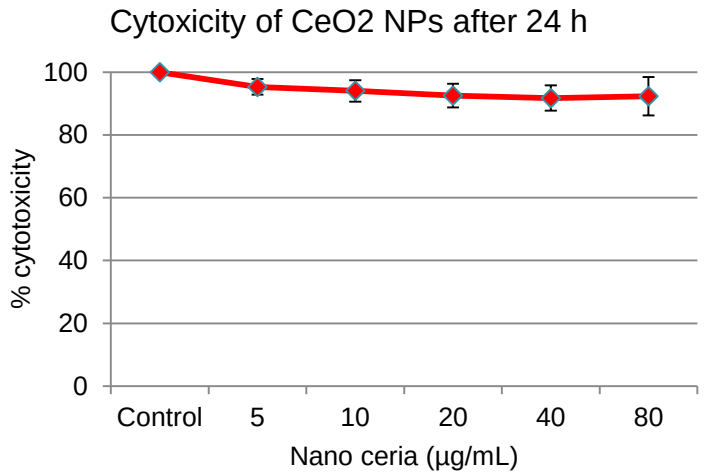
Results:

CeO_2 NPs are not cytotoxic in peritoneal macrophages

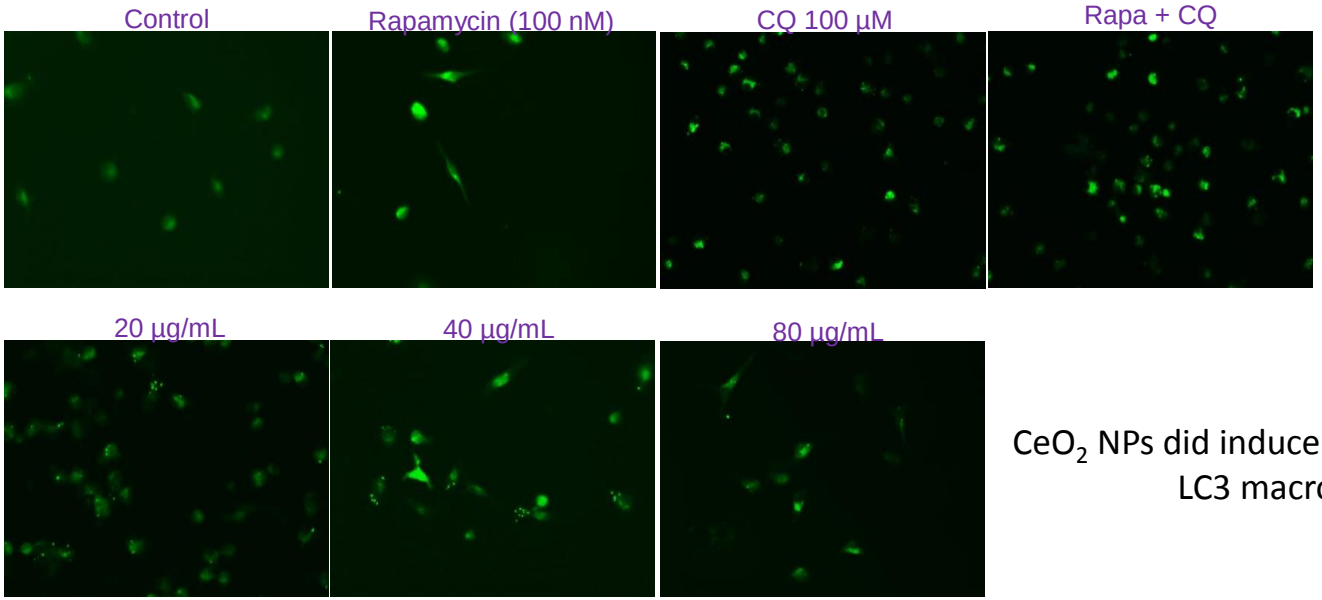
24h treatment



N=3

Induction of autophagy by CeO_2NPs in GFP-LC3 peritoneal macrophages

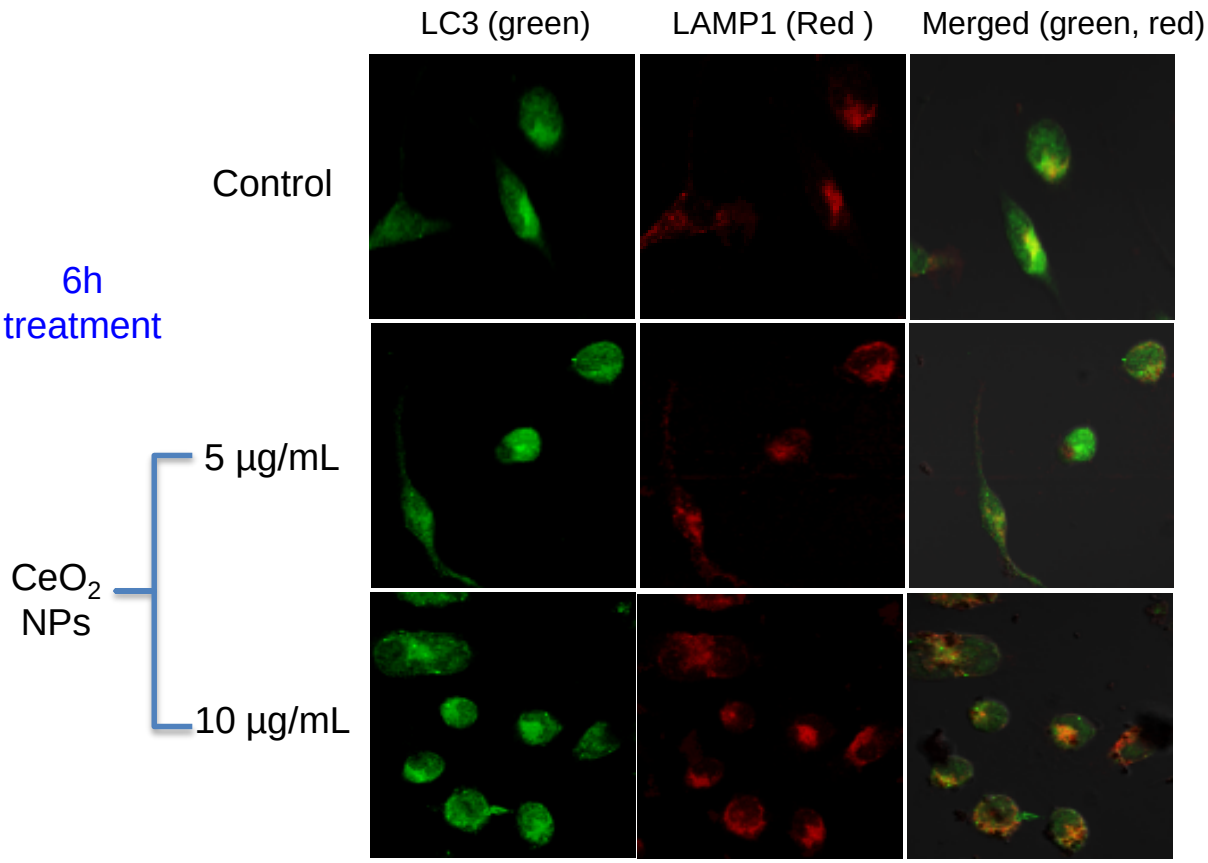
6h treatment



N=3

CeO_2 NPs did induce autophagy in GFP-LC3 macrophages

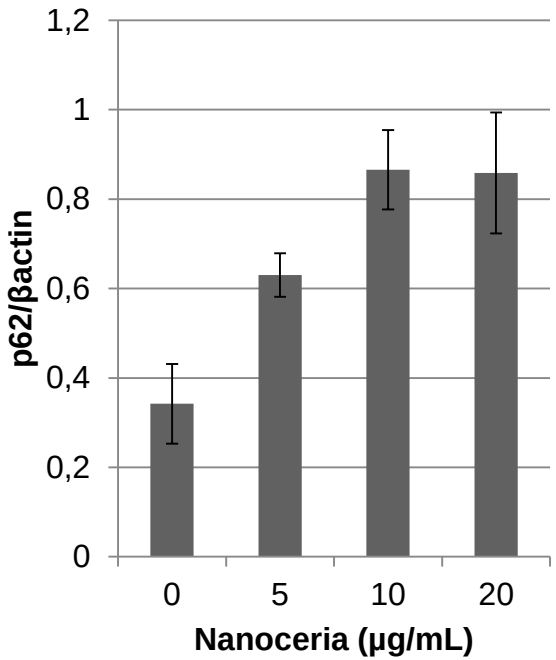
Our next idea was to see the co-localisation of GFP-LC3 and lysosomal associated membrane protein 1 (LAMP1)

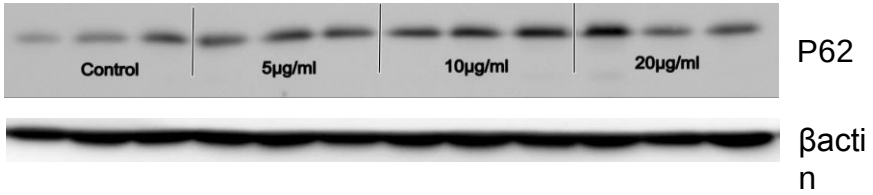

LC3 (green)
LAMP1 (Red)
Merged (green, red)
Control
6h treatment
5 µg/mL
CeO_2 NPs
10 µg/mL

N=1

Increased expression of P62 in macrophages (RAW 264.7) due to CeO_2NPs

24h treatment





Increased expression of P62 in macrophages could possibly indicate autophagy blockade due to CeO_2NPs

CeO_2NPs could possibly be involved in defective autophagy

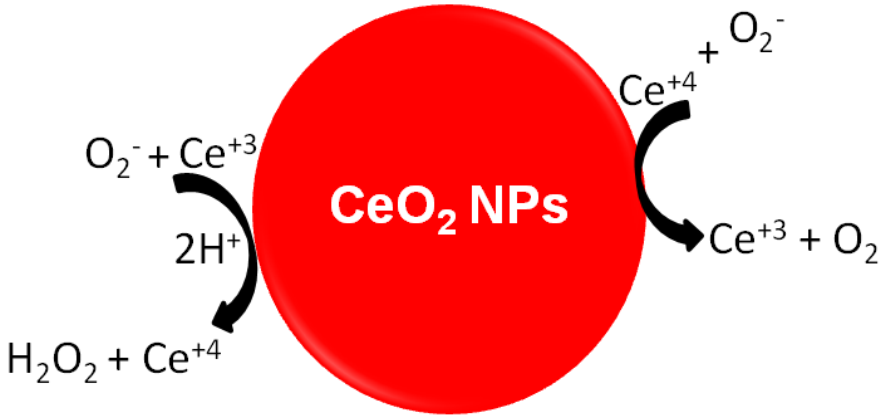
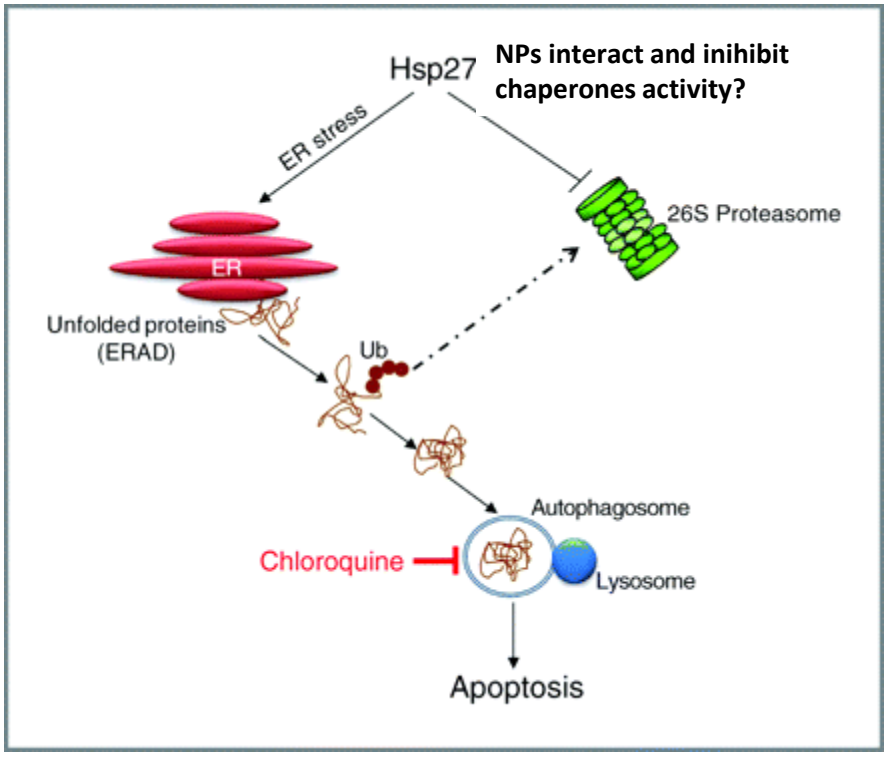


Fig. 2. Auto-regenerative red-ox cycle over CeO_2 NPs surface aids in scavenging oxygen free radicals.


Hsp27
NPs interact and inihibit chaperones activity?
ER stress
26S Proteasome
ER
Unfolded proteins (ERAD)
Ub
Autophagosome
Chloroquine
Lysosome
Apoptosis

The projected human pulmonary dose for inhalation of CeO2 in diesel exhaust from engines using a CeO2 fuel additive is 0.09 mg/kg body weight for 8 h (Health Effects Institute [HEI] 2001). CeO2 is insoluble particle, and studies have shown that the clearance of CeO2 from the lung may take 20 years or more (Pairon et al. 1994).
As a diesel exhaust product, it is likely that the potential exposure (occupational or environmental) to CeO2 is continuous and the lung burden is cumulative. Assuming a person has been exposed to the projected dose for 40 years with 8 h working day, the total lung burden of CeO2 will be 936 mg/kg (0.09 mg/kg.d 5 d/week 52 week/year 40 years = 936 mg/kg).

Usually, conversion from rodents to humans includes a safety factor of 10-fold.

Therefore, to assess the potential toxicological consequence of CeO2 NPs we used 50µg well with the range .