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Supplementary Materials for

Alveolar macrophages initiate the spatially targeted recruitment of neutrophils after nanoparticle inhalation

Qiongliang Liu *et al.*

Corresponding author: Qiongliang Liu, qiongliang.liu@shgh.cn; Markus Rehberg, markus.rehberg@helmholtz-munich.de

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Figs. S1 to S8

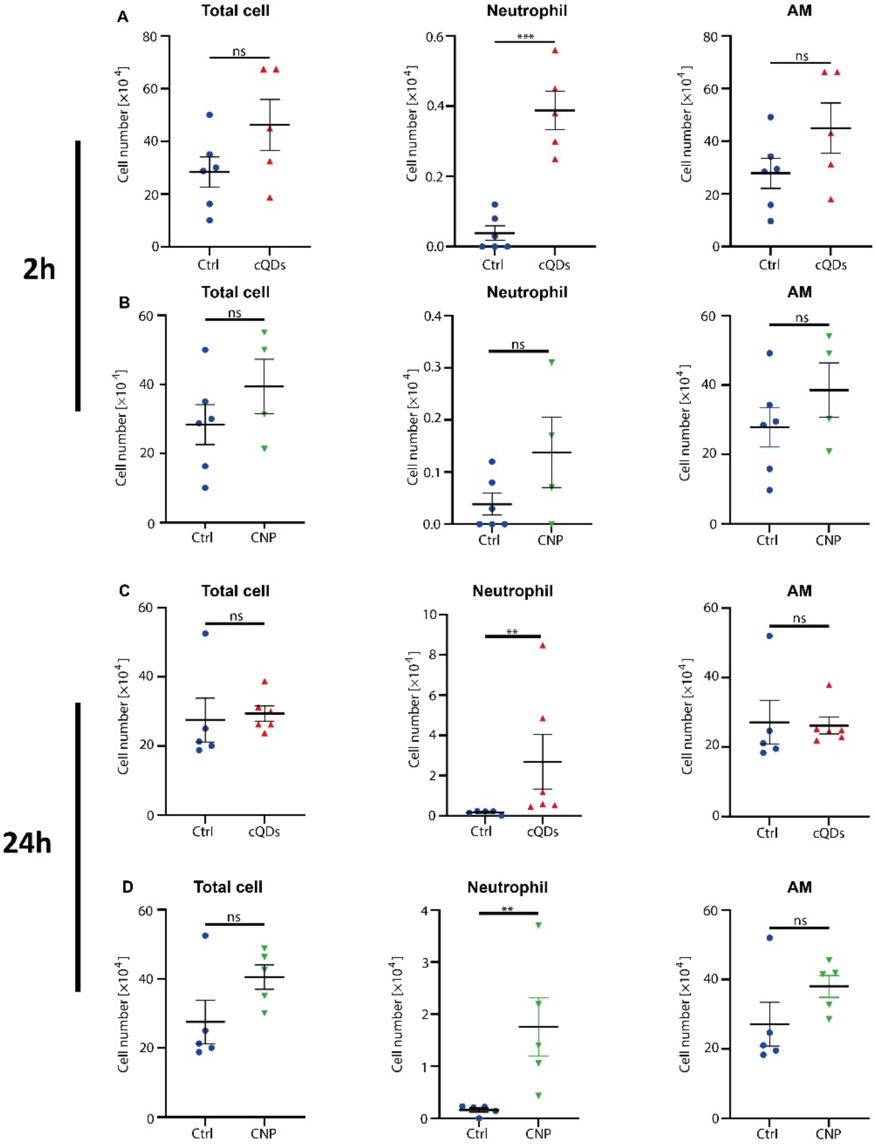

Fig. S1. Bronchiolar-alveolar-lavage cell numbers at 2 hours and 24 hours after cQDs/CNP inhalation. (**A**) and (**B**) 2 h and (**C**) and (**D**) 24 h after NP inhalation the numbers of total cells, AMs and neutrophils were determined in May Grunwald-stained-cytospin samples of cQD and CNP exposed mice (16 cm2/g cQDs; 170 cm2/g CNP), respectively, and compared to vehicle controls.

Data are presented as means ± SEM, *n*= 4-6 mice/group, ns: $p \geq 0.05$, *: $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$ by paired Student's t-test (parametric data) or Mann-Whitney rank-sum test (nonparametric data).

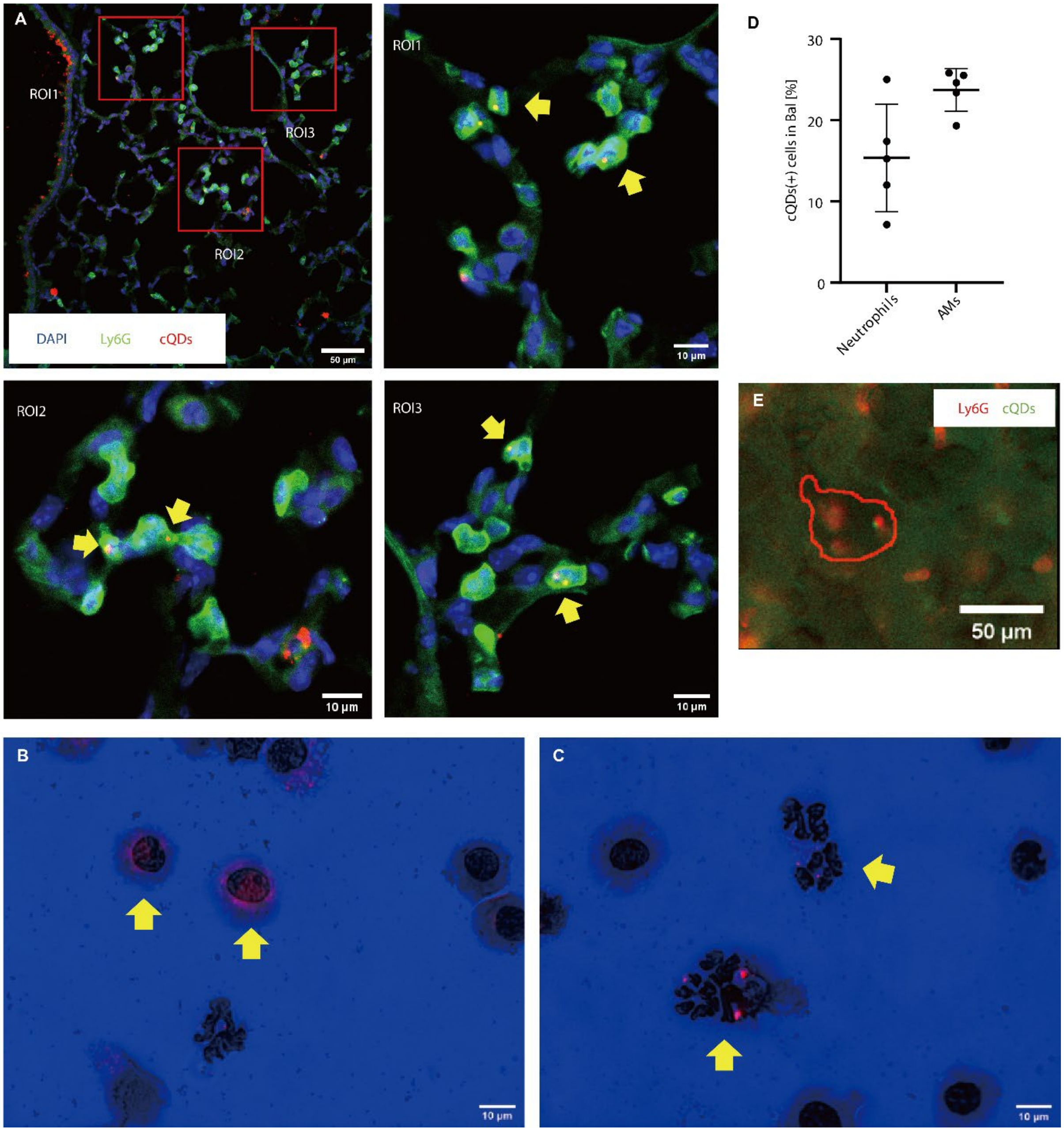

Fig. S2. Infiltrating neutrophils internalize nanoparticles in the alveoli.

A) Fluorescence confocal microscopy of WT mice lung slices obtained 90 min after cQDs (red) inhalation. Neutrophils labeled with anti-Ly6G mAb (green). Nuclei stained by DAPI (blue) (Scale bar: 50 µm). Frequently alveolar localized neutrophils are colocalized with cQD fluorescent spots, indicating cQDs cellular uptake. ROI1-ROI3: Magnifications highlight cQDs-laden neutrophils (arrows), scale bars: 10 µm.

(**B**) and (**C**) cQDs (red) can be detected in AMs (b, arrows) as well as in neutrophils (**C**, arrows) in May Grunwald-stained BAL cytospin-samples, collected at 24 h after cQD exposure, by combined epifluorescence and phase contrast imaging (Scale bars: 10 µm).

(**D**) Quantification of cQD positive AMs and neutrophils in BAL samples obtained 24 h after cQDs inhalation, *n*=4 mice.

(**E**) L-IVM image at 60 min after cQDs-inhalation shows cQD colocalization with alveolar localized neutrophils (cQDs: green, neutrophils labeled by anti-Ly6G mAbs (*i.v.*): red, scale bar: 50 µm).

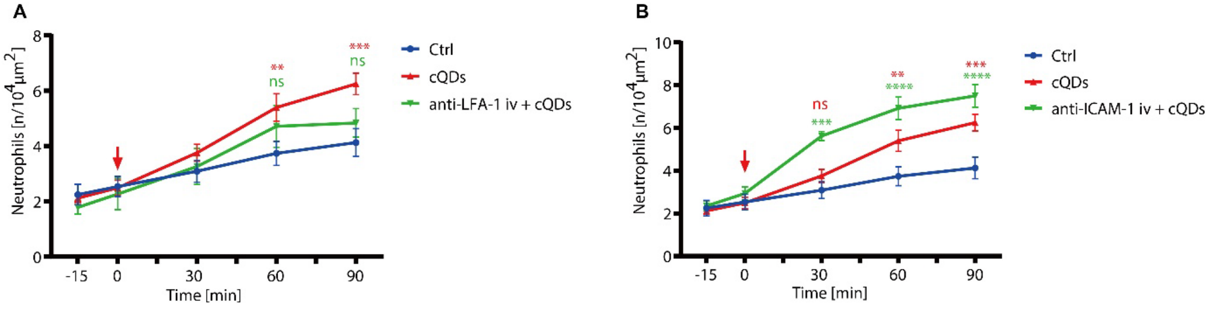

Fig. S3. Blockade of intravascular LFA-1 as well as ICAM-1 has no mitigating effect on cQDs-evoked recruitment of neutrophils.

(**A, B**) Alterations in neutrophil numbers in the alveolar region over 90 min were analyzed by L-IVM in mice receiving LFA-1 or ICAM-1 blocking mAbs via intravenous injection 30 min prior to inhalation of cQDs or vehicle-control, *n*=3 mice/group.

Data are presented as Means ± SEM. ns: $p \geq 0.05$, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ and ****: $p < 0.0001$.

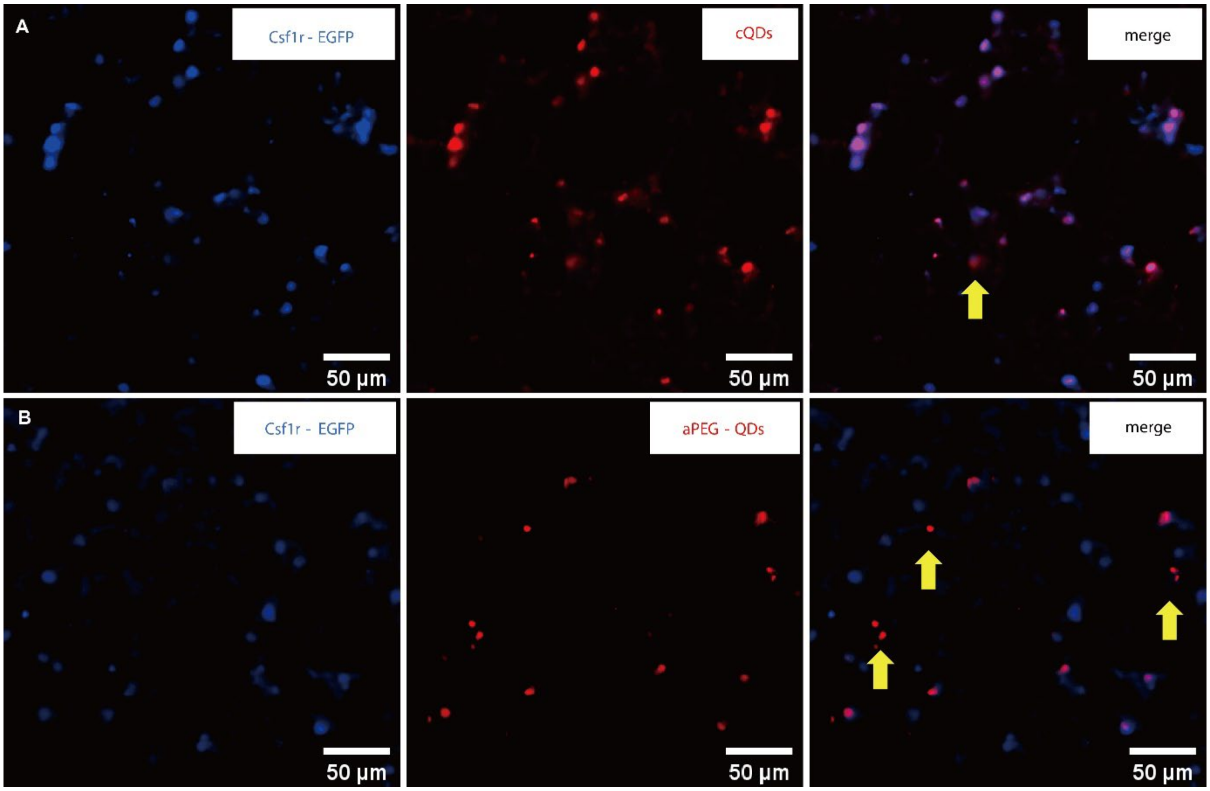

Fig. S4. PEGylation prevents QD NPs from being internalized by AMs.

(**A, B**) Tissue clearing was used to achieve co-imaging of AMs from Macgreen mice (Csf1r-EGFP is expressed selectively in macrophage and monocyte cell lineages) after transcranial perfusion for flushing out all Csf1r-EGFP labeled cells in the blood. 2D images from lightsheet fluorescence microscope images of cQDs and aPEG-QDs exposed mice (2 h). Csf1r-1(+) AMs are shown in blue, QDs are shown in red, AMs with ingested QDs ingested appear in purple. (panel A: cQDs, panel B: aPEG-QDs, Scale bar: 50 μm).

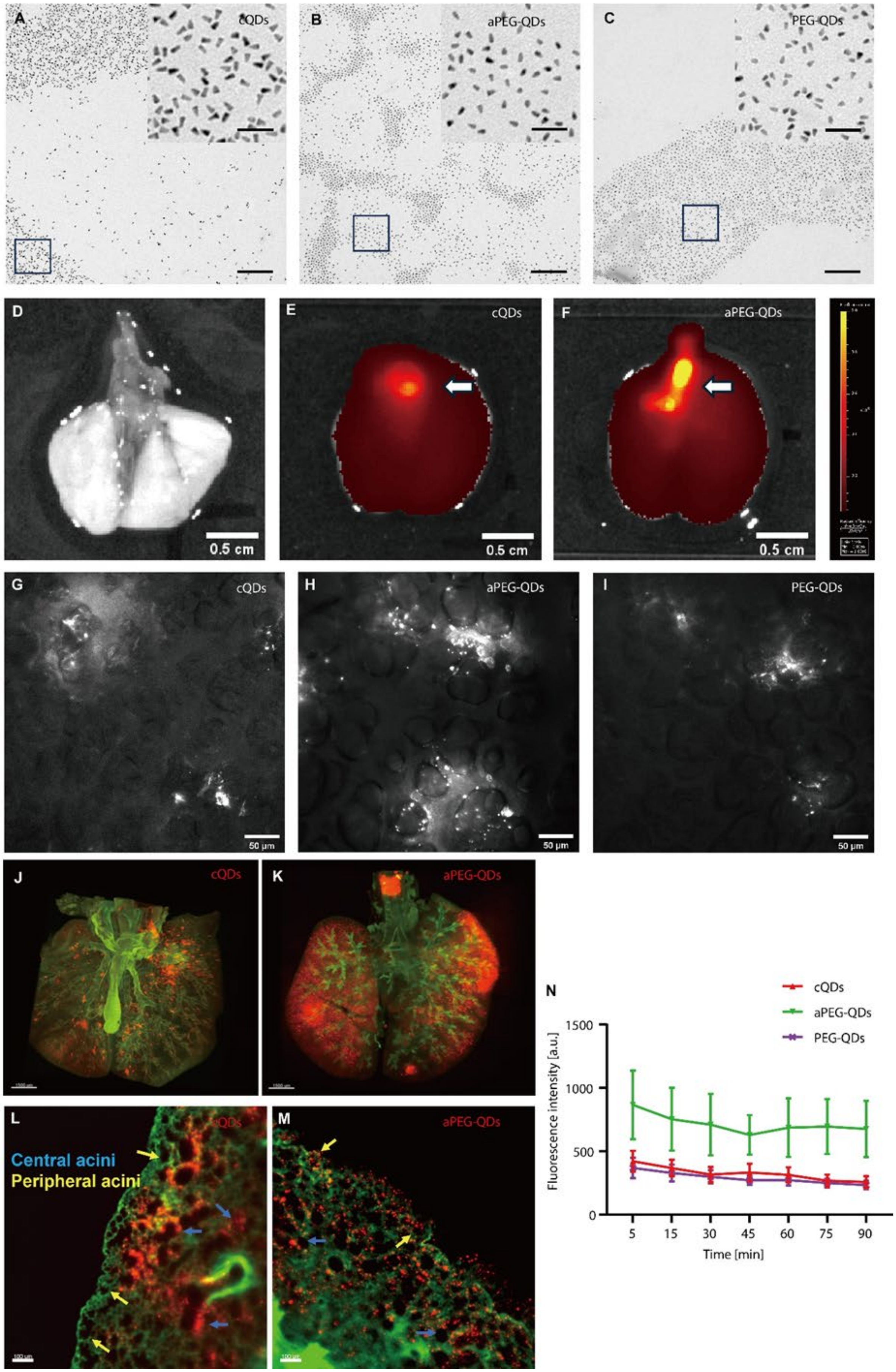
A
cQDs
B
aPEG-QDs
C
PEG-QDs
D
0.5 cm
E
cQDs
0.5 cm
F
aPEG-QDs
0.5 cm
G
cQDs
50 µm
H
aPEG-QDs
50 µm
I
PEG-QDs
50 µm
J
cQDs
K
aPEG-QDs
L
cQDs
Central acini
Peripheral acini
M
aPEG-QDs
N
cQDs
aPEG-QDs
PEG-QDs
Fluorescence intensity [a.u.]
1500
1000
500
0
5
15
30
45
60
75
90
Time [min]

Fig. S5. 3D/2D mapping of QD distribution in entire mouse lungs upon ventilator-assisted QD-NP aerosol inhalation.

(**A**-**C**) Electron microscopic images of the three different QD-species. QDs were diluted in distilled water (panel A: cQDs, panel B: aPEG-QDs and panel C: PEG-QDs, scale bars: 200 nm).

(**D**-**F**) Analysis of cQD and aPEG-QD distribution in whole lungs using epifluorescence imaging. Typical ex vivo lung images from mice at 2 h after receiving 16 cm2/g (geom-surface area of NPs / mass-lung) cQDs or aPEG-QDs via inhalation and vehicle control using the IVIS system, which is particularly sensitive to the most peripheral part of the lung which is the focus of L-IVM analysis (panel D: Ctrl, panel E: cQDs, panel F: aPEG-QDs).

(**G-I**) L-IVM images of the three types of QDs (G: cQDs; H: aPEG-QDs; I: PEG-QDs) showing the QD distribution pattern in the most peripheral alveolar part of the lungs 5 min after pulmonary NP inhalation. QDs are shown in white. Scale bars: 50 µm.

(**J-M**) The distribution of QDs (red) in the Z-stack images of entire cleared lungs obtained by light sheet microscopy (panel J and K: 3D MIP, L: cQDs and M: aPEG-QDs, scale bar: 1500 µm) and in single z-planes (panel L and M: 2D xy slice, L: cQDs and M: aPEG-QDs, scale bar: 200 µm) of the tissue structure (autofluorescence, green) 1h after inhalation of different types of (16 cm2/g) QDs.

(**N**) Average QD fluorescence intensities in L-IVM images at consecutive time points after inhalation of cQDs, aPEG-QDs, or PEG-QDs (Means ± SEM, *n*= 3 mice/group)

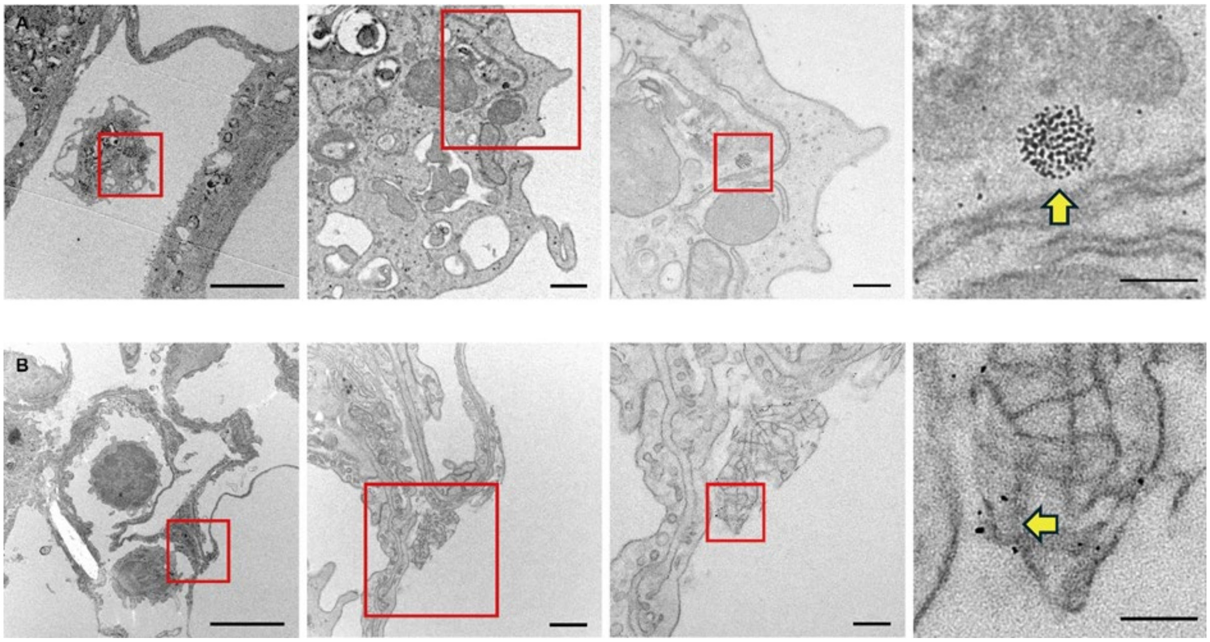

Fig. S6. cQDs accumulate in AMs and are attached to surfactant lattices.

Transmission electron microscopy of cQDs-exposed lungs (1 h).

(**A**) Aggregates of cQDs were found within the cytoplasm and endolysosomes of AMs.

(**B**) Non-cell associated “free” cQDs can also be found attached to surfactant components (tubular myelin sheaths) at the alveolar air-liquid interface. QDs are visible as distinct black points (yellow arrows) within the surfactant lattice. Overview and detailed higher magnification pictures of depicted areas with corresponding scale bars: 5 µm, 500 nm, 200 nm, 100 nm.

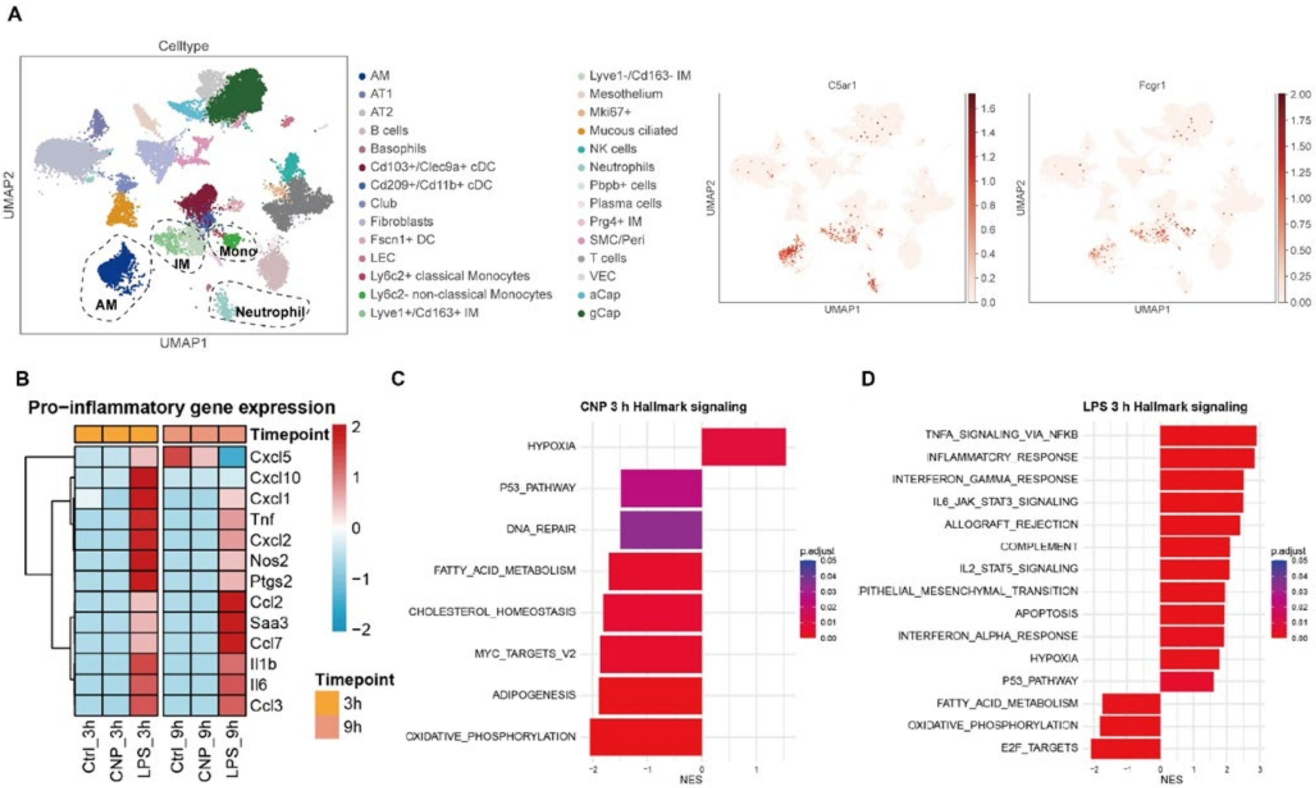

Fig. S7. Single-cell transcriptomic and pathway analysis reveals inflammatory and signaling responses to CNP and LPS treatment.

(**A**) Visualization of dimension-reduced single cell transcriptomic data by Uniform Manifold Approximation and Projection (UMAP) reveals different annotated cell types and the expression of genes (GSE185006).

(**B**) Heatmap visualization of classical pro-inflammatory genes induced in CNP and LPS (inflammation control) exposed Ana-1 macrophages after 3 and 9 h.

(**C, D**) Gene Set Enrichment Analysis (GSEA) of Hallmark signaling pathways revealed different pathways for CNP (**C**) and LPS (**D**) after 3 h exposure in Ana-1 macrophages. Result is visualized by bar plot; x-axis: the normalized enrichment score (NES); color code of the bar: the adjusted p-value.

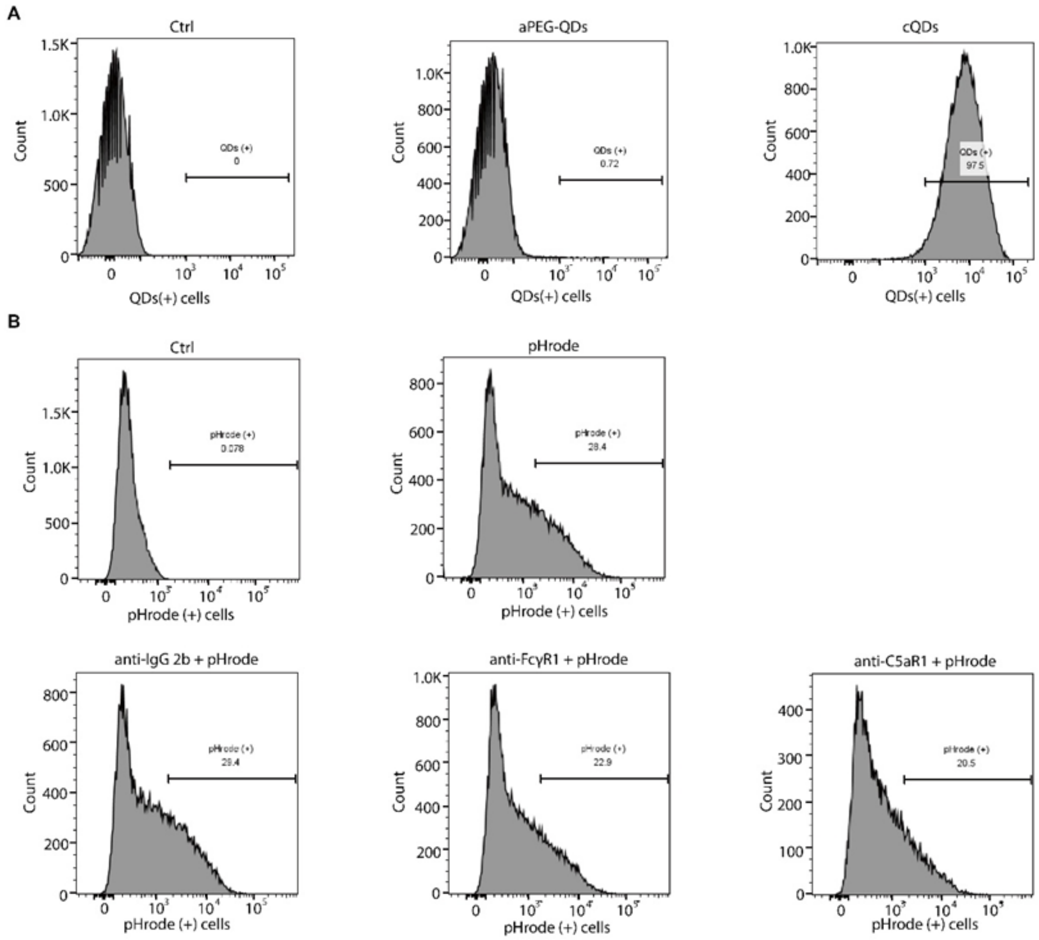

Fig. S8. Flow cytometry analysis of QD uptake and pHrodo-based phagocytic activity.
(**A**) Flow cytometry analysis of MH-S macrophage uptake of QDs. cQDs showed high uptake, while aPEG-QDs had minimal uptake.
(**B**) pHrodo-labeled particle uptake (2h) was inhibited by anti-FcγRI and anti-C5aR1 antibodies, indicating receptor involvement in phagocytosis.