Title page:

Single-cell mass cytometry reveals cell type- and cluster-specific heterogeneity in silver nanoparticle responses in a 3D alveolar tetra-culture model

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Supplementary Methods

Silver nanoparicle characterization

AgNPs had a purity of 99.99%, as confirmed by the manufacturer's specification sheet (Lot No. SXV0061). The stock suspension, provided in aqueous solution at a silver mass concentration of 1.05 mg/mL, stabilized with 0.059% sodium citrate dihydrate, comprised over 99% water. Endotoxin content was confirmed to be <5 EU/mL using a Pyros kinetic-turbidimetric LAL assay. The AgNPs, identified as spherical silver nanoparticles, had a primary particle diameter of 9.9 ± 2.0 nm and a specific surface area of 53.4 m^2/g, both determined by transmission electron microscopy (TEM) according to the manufacturer's specifications.

To further evaluate their behavior under experimental conditions, dispersions of AgNPs were prepared in DI water and culture media (DMEM supplemented with 15% RPMI 1640, 10% IMDM, 1% FBS, and buffered with 25 mM HEPES) and vortexed to ensure uniform dispersion. The hydrodynamic size distribution and suruface charge of AgNPs were measured in a diposable cuvette at a concentration of 10 mg/mL, using a Zetasizer Nano (Malvern Instruments, UK). UV-vis spectra of the AgNPs were collected using NanoDrop One (Thermo Fisher Scientific, USA).

Supplementary Figures

Figure S1. Time-resolved DLS size distributions of AgNPs.

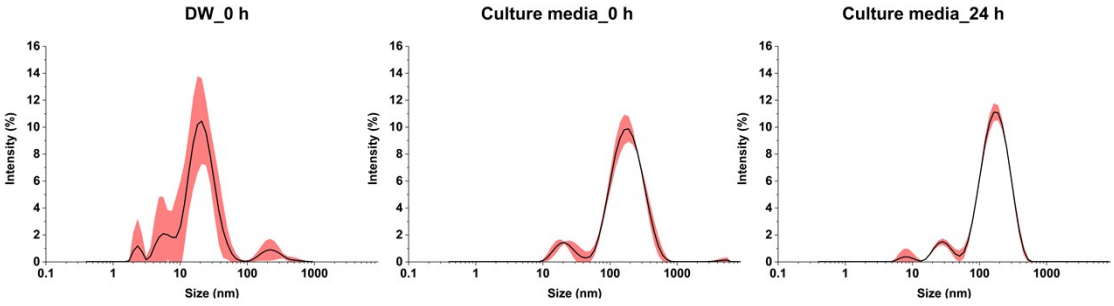


Figure S2. Time-resolved UV–visible absorption spectra of AgNPs.

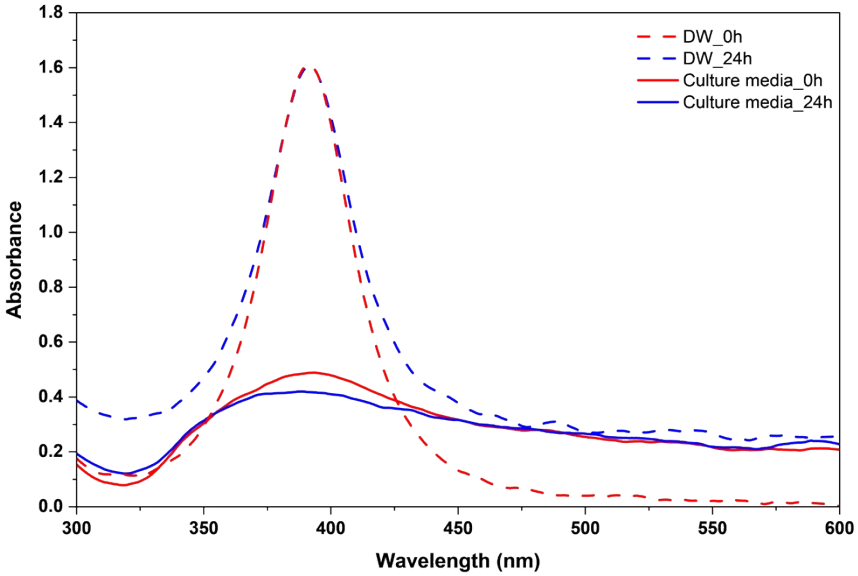


Figure S3. Manual gating strategy to distinguish cell types comprising the 3D alveolar model.

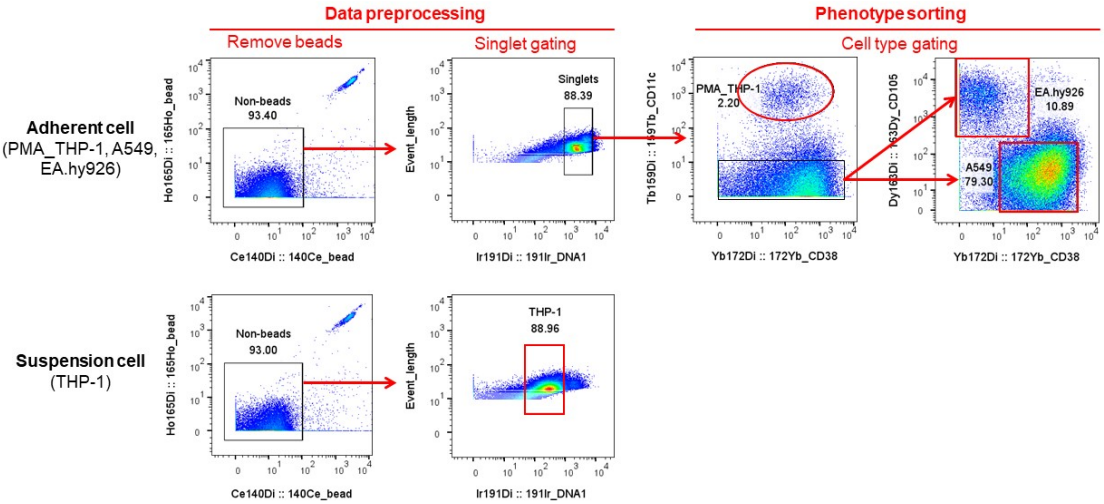


Figure S4. AgNP association and cytotoxicity across different cell types (repeat experiments of Fig. 2).

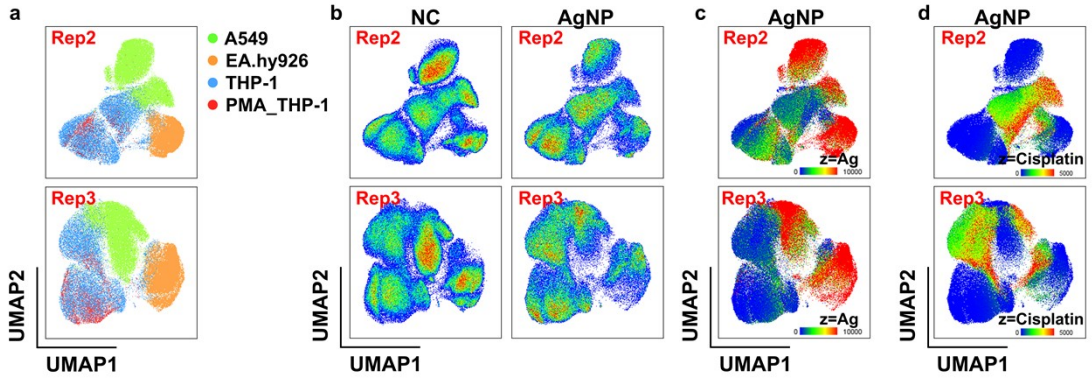


Figure S5. Consensus clustering-based estimation of optimal metacluster number for FlowSOM using delta area plot.

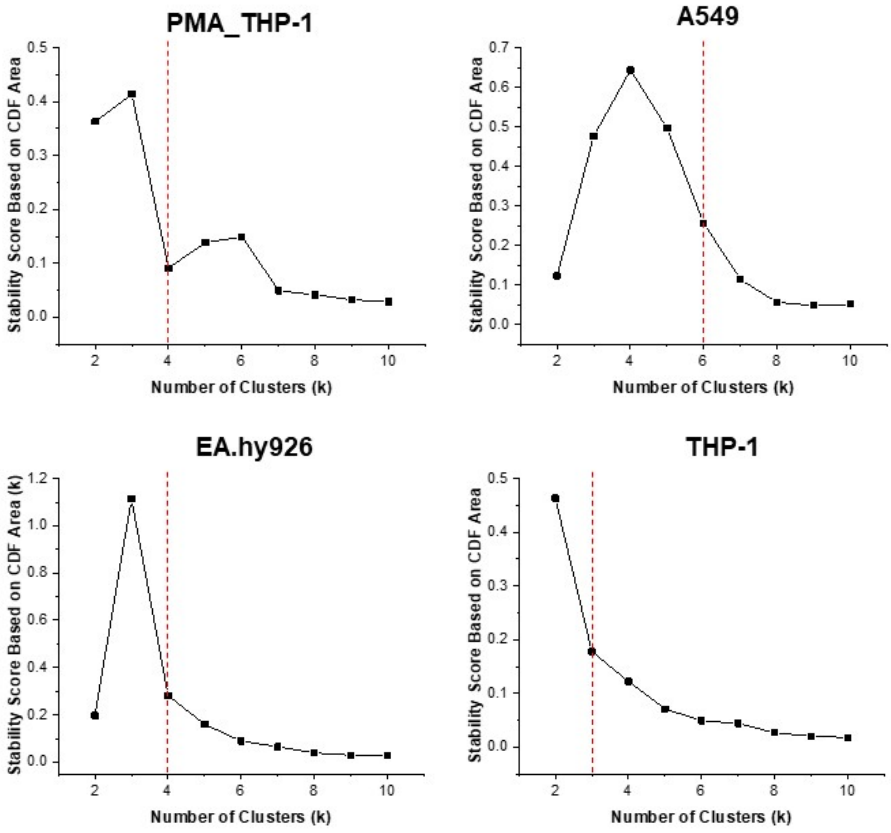


Figure S6. Visualization of FlowSOM metaclusters overlaid on the minimum spanning tree, illustrating the hierarchical relationships between subpopulations.

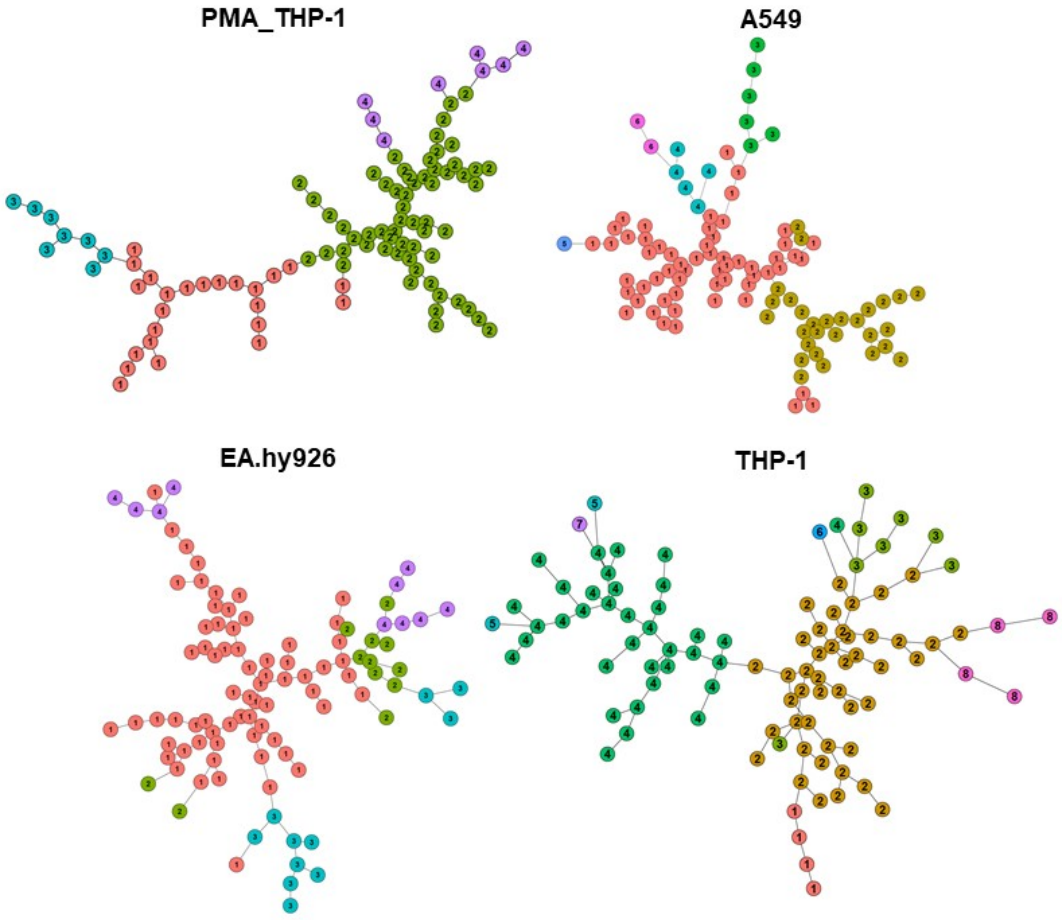


Figure S7. Pairwise Spearman's rank correlations of Z-scored marker means for FlowSOM metaclusters across three independent biological replicates of each cell type.

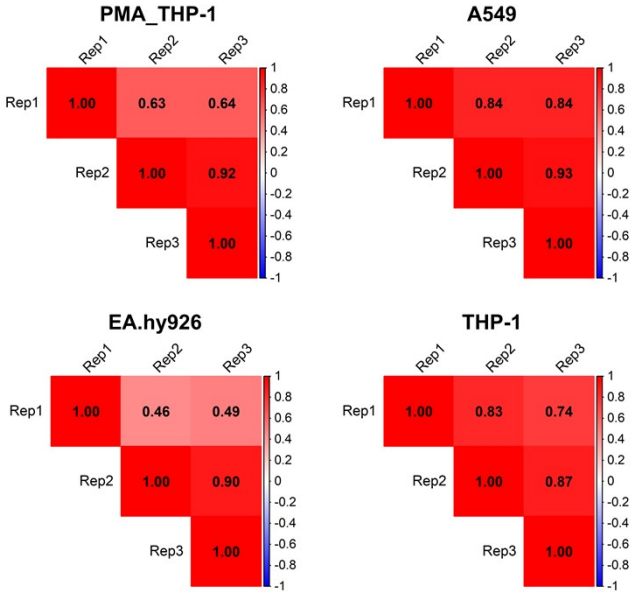


Figure S8. Minimum spanning tree showing subpopulation heterogeneity via intracellular expression of stress, inflammatory, and apoptotic markers.

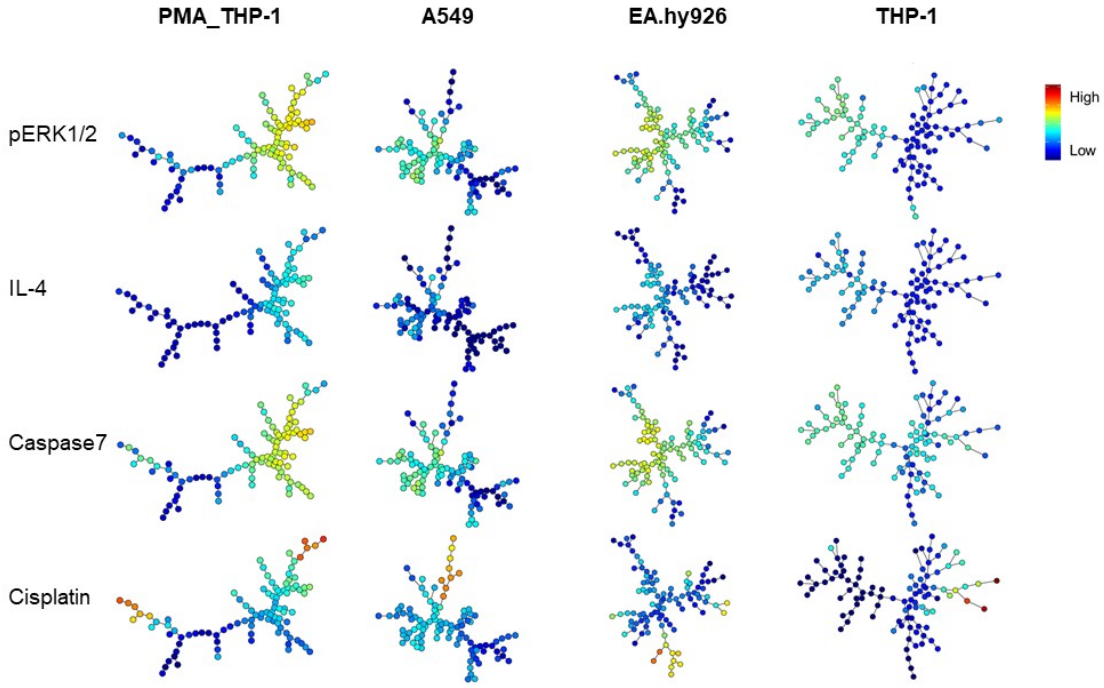
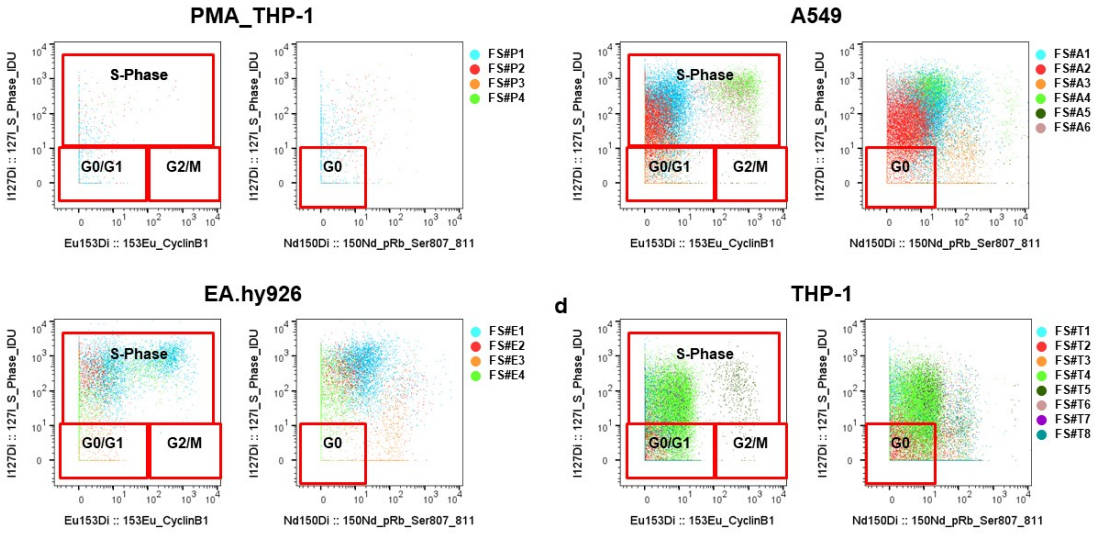


Figure S9. Cell cycle phase analysis across FlowSOM clusters using IdU, CyclinB1, and pRb expression.



Supplementary Tables

Table S1. Physicochemical properties of AgNPs.

Time point	Dispersant	Hydrodynamic size [1] (nm)	PDI [2]	Zeta potential [3] (mV)
0 h	DW	19.2 ± 2.6	0.381	-24.2 ± 5.8
0 h	Culture media	112.2 ± 2.6	0.507	-20.5 ± 0.7
24 h	Culture media	104.7 ± 0.9	0.519	-19.1 ± 0.6

[1] Confidence intervals indicate standard deviations of the size distribution.
[2] PDI, polydispersity index; a measure of the heterogeneity of the particle size distribution.
[3] Confidence intervals indicated standard deviations of 3 replicate measurements.

Table S2. Antibody panel used for CyTOF analysis.

Target	Metal	Cell type/Function
CD105/Endoglin	163Dy	Endothelial cell
CD11c	159Tb	Monocyte/Macrophage
CD16	148Nd	Monocyte(C/I/N)
CD14	160Gd	Monocyte(C/I/N)
CD38	172Yb	Macrophage activation
CD163	154Sm	Macrophage/M2
DNA1	191Ir	DNA
DNA2	193Ir	DNA
Cisplatin	195Pt	Viability
S-Phase (IdU)	127I	Cell cycle
CyclinB1	153Eu	Cell cycle
pRb [Ser807/811]	150Nd	Cell cycle
pERK1/2	167Er	ERK pathway
pBad	161Dy	Anti-apoptosis
IL-4	144Nd	Anti-inflammation
IL-6	156Gd	Pro/Anti-inflammation
TNF-α	175Lu	Pro-inflammation
IFN-γ	158Gd	Pro-inflammation
Cleaved caspase7	152Sm	Apoptosis

Table S3. Proportions of manually gated adherent cell types in negative control and AgNP-treated samples.

Condition	PMA_THP-1	A549	EA.hy926
NC1	1.36%	88.17%	10.46%
NC2	2.12%	91.91%	5.97%
NC3	2.73%%	80.27%	17.00%
Average	**2.07%**	**86.78%**	**11.14%**
AgNP1	2.72%	86.10%	10.18%
AgNP2	2.27%	91.91%	5.82%
AgNP3	1.08%	85.02%	13.90%
Average	**2.02%**	**87.68%**	**9.97%**

Table S4. GUIDEnano K-score evaluation: Assessment of study reliability based on test design and reporting considerations.

No	K score for *in vitro* toxicity studies	Score	Comments
1	Is the cell model or organism given?	1	Specified (A549, EA.hy926, THP-1, PMA-THP-1)
2	Is information given on the source/origin of the test system?	1	ATCC/KCLB catalog & origin
3	Are necessary information on test system properties, and on conditions of cultivation and maintenance given?	1	Media, supplements, differentiation protocol, 3D model setup
4	Is the method of administration given (see explanations for details)?	1	AgNP concentration, dispersion method
5	Are duration of exposure as well as time-points of observa tions explained?	1	24h exposure, post-exposure cell collection
6	Were negative and positive controls included (where and when needed)?	1	Particle-free negative control
7	Is the number of replicates (or complete repetitions of experiment) given?	1	n=3 biological replicates analyzed
8	Are the study endpoint(s) and their method(s) of determination clearly described?	1	^{107}Ag, cisplatin, intracellular markers
9	Have the results been analyzed using statistical methods?	1	Fisher's exact test, Spearman's rank correlation
	Total out of 9	9	
	GUIDEnano score (K)[1]	K1	

[1] As defined in the GUIDEnano framework: K1 = high reliability (8–9 "YES" including all red items), K2 = moderate reliability (7 "YES" with all red items), K3 = low reliability (any red item answered "NO").

Table S5. GUIDEnano S-score evaluation: Assessment of NP physicochemical properties and characterization in the exposure medium.

No	Substance (S) score for chemical substances and nanomaterials	Score	Comments
1	Was the test substance identified?	1	Silver nanoparticles
2	Is information on the source/origin of the substance given?	1	nanoComposix (USA), Lot SXV0061
3	Is purity (concentration) of the substance given?	1	99.99% purity, 1.05 mg/mL stock
4	Is endotoxin content of the substance given?	1	< 5 EU/mL (LAL assay)
5	Were impurities stated?	0	
6	Was the substance concentration measured in the exposure medium?	0	
7	When the substance is a nanoparticle (NP) were protocols of dispersion and characterization in the exposure medium identified? or, were protocols of preparation of exposure medium stated?	1	Sonication & vortexing protocol detailed
8	Was the stability of the substance concentration measured during the exposure period?	1	UV-VIS spectra over 24h
9	Are doses administered or concentrations in exposure media given?	1	2 µg/mL (0.44 µg/cm²) stated
10	Was the type of test medium or vehicle used stated?	1	Specific mixed culture medium detailed
11	For ecotoxicity studies, were mandatory exposure medium conditions measured?	n/a	Human *in vitro* study
12	For ecotoxicity studies, were any other exposure medium conditions measured?	n/a	Human *in vitro* study
13	Size	1	9.9 ± 2.0 nm (TEM)
14	Surface area	1	53.4 m²/g (TEM)
15	Surface charge	1	Implied (citrate stabilized)
16	Shape	1	Nanospheres
17	Other relevant information	0	
18	Size at the start or at the end of the exposure period	1	112.2 nm (0h), 104.7 nm (24h) in medium
19	Surface charge	1	-20.5 mV (0h), -19.1 mV (24h) in medium
20	Other relevant information (i.e. ion release, solubility, shape, etc.)	0	
	Total out of 18	**14**	
	GUIDEnano score (S)[1]	**S2**	

[1] As defined in the GUIDEnano framework: S1 = very good characterization (16–18 “YES” including all red items), S2 = acceptable characterization (11–15 “YES” with all red items), S3 = insufficient characterization (<11 “YES” or any red item answered “NO”).

References

M. L. Fernández-Cruz, D. Hernández-Moreno, J. Catalán, R. K. Cross, H. Stockmann-Juvala, J. Cabellos, V. R. Lopes, M. Matzke, N. Ferraz, J. J. Izquierdo, J. M. Navas, M. Park, C. Svendsen and G. Janer, Quality evaluation of human and environmental toxicity studies performed with nanomaterials – the GUIDEnano approach, *Environ. Sci.: Nano*, 2018, **5**, 381–397.

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