Towards predictive nanotoxicology: from roundabout of molecular events to chronic inflammation prediction

Authors:

Hana Majaron*, Boštjan Kokot*, Aleksandar Sebastijanović*, Carola Voss, Rok Podlipec, Patrycja Zawilska, Trine Berthing, Carolina Ballester, Pernille Høgh Danielsen, Claudia Contini, Mikhail Ivanov, Ana Krišelj, Petra Čotar, Qiaoxia Zhou, Jessica Ponti, Vadim Zhernovkov, Matthew Schneemilch, Mojca Pušnik, Polona Umek, Stane Pajk, Olivier Joubert, Otmar Schmid, Iztok Urbančič, Martin Irmler, Johannes Beckers, Vladimir Lobaskin, Sabina Halappanavar, Nicholas Quirke, Alexander Lyubartsev, Ulla Vogel, Tilen Koklič**, Tobias Stöger**, Janez Štrancar**

Abstract

Many chronic diseases manifest themselves in prolonged inflammation and often ignored dysregulated lipid metabolism, both also associated with inhalation of certain nanomaterials. Limited knowledge of involved molecular events and their causal connections prevents reliable prediction of outcomes by efficient testing strategies. To unravel how acute nanomaterial exposure leads to chronic conditions, we employed advanced microscopy and omics *in vitro*, *in vivo* and *in silico*. For selected metal-oxide nanomaterials, we show that epithelial cells survive the exposure by excreting internalized nanomaterials and passivating them on the surface, employing elevated lipid synthesis. Macrophages, on the contrary, attack the defending epithelium but die degrading passivized complexes, releasing nanomaterial, which is reuptaken by epithelial cells. Constant proinflammatory signalling recruits new phagocytes that feed the vicious cycle of events resulting in a long-lasting response to a single exposure. The discovered mechanism predicts the nanomaterial-associated *in vivo* chronic outcomes based on simple *in vitro* measurements and potentially enlightens other chronic diseases.

Graphical abstract
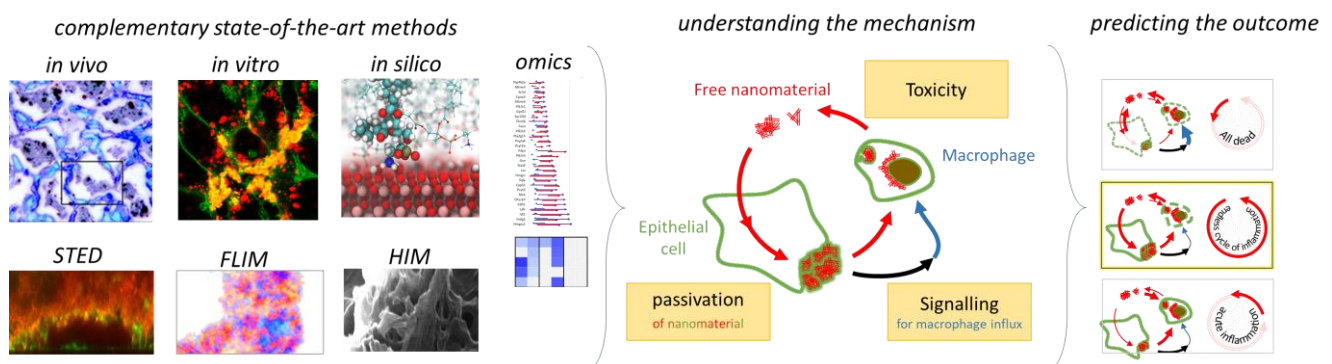
Introduction - Mechanism of persistent inflammation unknown

Today, chronic diseases such as asthma, lung cancer, heart disease, and brain damage with accelerated cognitive decline, are considered to be some of the most significant causes of death [1–3]. Despite the lack of understanding how these adverse outcomes evolve, they are known to be associated with air pollution and inhalation of particulate matter and nanoparticles [4]. According to the OECD and WHO, inhaled particulate matter kills four

35 million people globally every year [5,6]. In addition, the ever-increasing production of nanomaterials, as
36 consequence of the rapidly developing and extremely promising nanotechnology industry, generates concerns
37 about potential human exposure and health impacts. Decision-makers around the world (OECD, US EPA, NIH,
38 EC, JRC, etc.) recognized the need for elucidating molecular mechanisms involved in possible adverse outcome
39 pathways (AOPs) [7]. The later has emerged as the most promising conceptual construct towards predictive
40 toxicology elucidating the key events of respective toxicity pathways to improve the prediction of the apical
41 endpoints with alternative testing strategies [8].

42 Despite some important advances using multiple cell-line *in vitro* test systems [9] in the field of nanotoxicology,
43 the desired mechanism-based *in vitro* assays and *in silico* predictive tools have not yet reached the required level
44 of maturity and reliability [10]. This is especially true for alternative testing strategies addressing chronic outcomes
45 [11], that are inherently associated with a long-term development of pathophysiological changes. In this case, the
46 complicacy of long-term in-vitro exposures, together with the lack of understanding of the underlying
47 mechanisms and the associated molecular events behind the AOPs, completely precludes the prediction of
48 chronic outcomes.

49 Upon pulmonary exposure, some nanomaterials have been shown to induce exceptionally long lasting chronic
50 inflammatory responses, which is reflected in prolonged accumulation of infiltrated leukocytes in the lungs
51 following a single nanomaterial exposure event [12–17] or chronic inhalation of relatively low nanomaterial
52 concentrations [18–20]. The insolubility and biopersistence of the particles combined with continuous release of
53 pro-inflammatory mediators from irritated resident cells or dying immune cells could explain a perpetuation of
54 inflammation in the above-mentioned chronic response, which is frequently co-observed with chronic
55 dysregulated lipid metabolism [21–26].

56 Here we show that a minimal combination of *in vitro* and *in silico* tests can explain and reproduce chronically
57 dysregulated lipid metabolism accompanying chronic inflammation, which originates in nanomaterial cycling
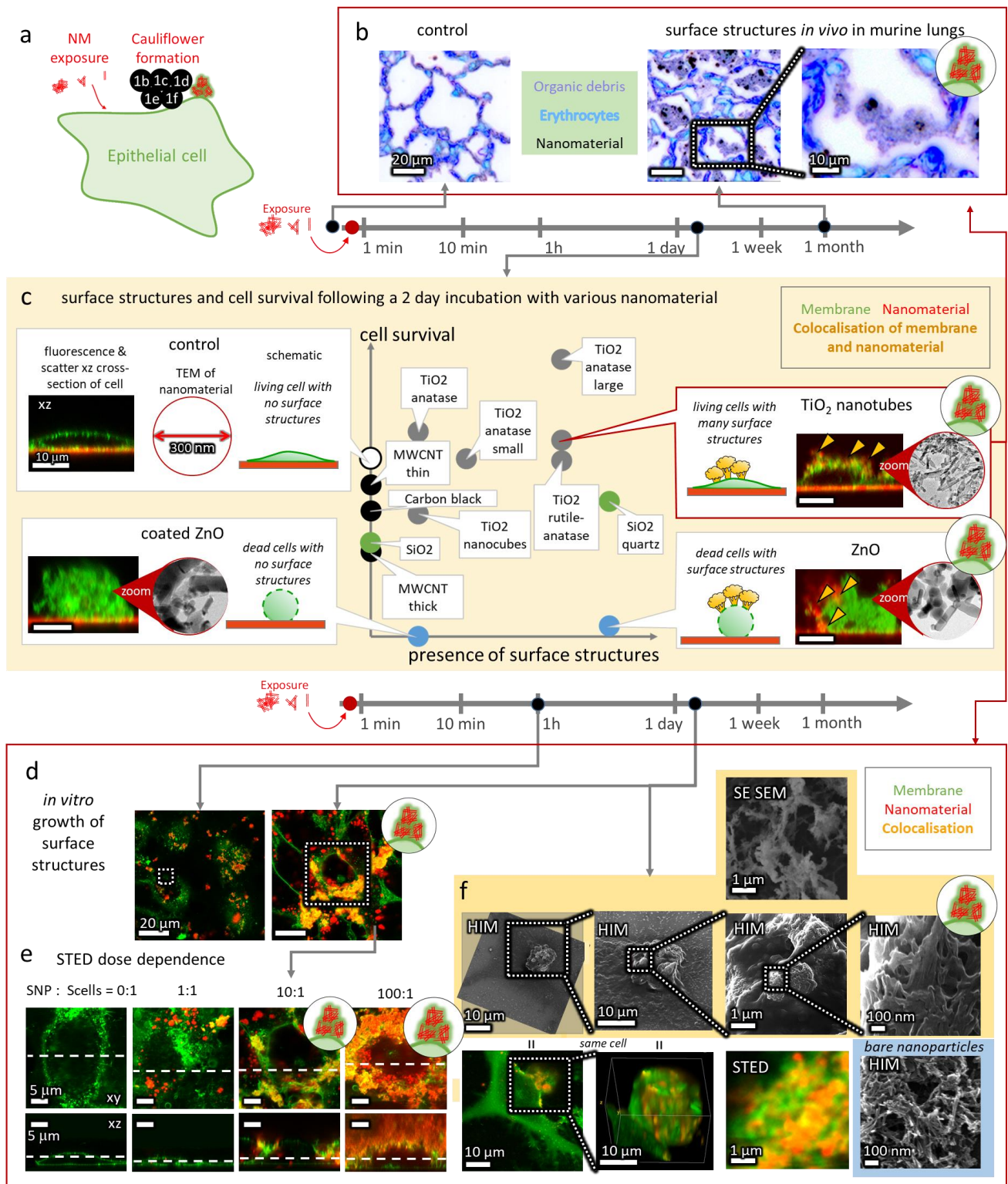58 between passivated form on epithelial cells and bare form released from dying immune cells.

59

Results and discussion
61

62   1.   Passivation of nanomaterials

Fig. 1: Formation of cauliflowers (bio-nano agglomerates) on epithelial cell surface. a *A general scheme of events shown in this figure.* **b** *Cytoviva dark-field scattering micrographs of bio-nano agglomerates observed on alveolar walls after instillation of mice with TiO₂ nanotubes (black and violet intermixed). In fluorescence micrographs* **c-f** *membranes are green and nanoparticles red.* **c** *Presence of surface structures, cell survival and cross-sections of alveolar epithelial (LA-4) cells after a 2-day exposure to several nanomaterials at nanomaterial-to-cell surface ratio of 10:1 (nanoparticles observed in backscatter). Inserts show 300 nm-large TEM micrographs of nanoparticles used.* **d** *Time-dependent cauliflower formation by LA-4 exposed to TiO₂ nanotubes at surface ratio of 10:1.* **e** *Super-resolved STED xy and xz cross-sections of dose-dependent cauliflower growth reveal that cauliflowers are located on the outer surface of cells after 2 days. The surface-*

74　*to-surface ratios are 0:1, 1:1, 10:1 and 100:1. f ❑ High-resolution correlative STED, SE SEM and HIM images*
75　*reveal the detailed structures of cauliflowers (arrowheads) at a surface dose of 10:1. For associated data see*
76　*Supp. info S1.*

77

78　To uncover the causal relationships of events leading from pulmonary nanomaterial exposure to chronic
79　inflammation, we applied a complex set of *in vivo*, *in vitro* and *in silico* experiments employing state-of-the-art
80　microscopy, spectroscopy, omics and modelling approaches. TiO_2 nanotubes were selected as the model
81　nanomaterial, as they induce very high and long-lasting chronic inflammatory responses *in vivo* accompanied
82　by markedly disturbed alveolar integrity of the lungs [12] with visible large bio-nano agglomerates on the alveolar
83　walls (Fig. 1b, purple structures). Importantly, this nanomaterial induces similar large bio-nano agglomerate
84　structures on the surface of the epithelial cells *in vitro* which remain viable for longer period (Fig. 1c), crucial
85　for elucidating the mechanism *in vitro*. Note, that similar structures were observed both *in vivo* and *in vitro* after
86　exposure to crystalline quartz (DQ12) [12], but not carbon nanotubes (CNTs) [26].

87　We have previously observed that TiO_2 nanotubes can wrap in parts of epithelial plasma membranes and relocate
88　them efficiently across the epithelial layer [27] already at lower concentration of nanotubes (surface-of-
89　nanomaterial-to-cell-surface dose 1:1) due to their high affinity for lipids. Thus, it is expected that at higher
90　surface doses, these nanoparticles should completely disrupt the epithelial cell membranes. Surprisingly, our
91　current experiments show that the epithelial cells survive exposures to surface doses as high as 100:1 (Fig. 1e,
92　supplement section S0c and S0g). A few days after exposure, the majority of the nanoparticles are found in huge
93　bio-nano agglomerates on the epithelial cell surface, consisting of at least nanoparticles and lipids, which we
94　term cauliflowers due to their shape and yellow colour in our fluorescence micrographs (Fig. 1d, Fig. 1e, yellow
95　colour).

96　Because cauliflowers are observed exclusively on the surface of epithelial cells, not inside (Fig. 1e, Fig. 1f),
97　they might be driven solely by physical interactions between nanoparticles and lipids as in the case of lipid
98　wrapping. However, the cauliflowers need one to two days to form, which suggests some involvement of active
99　biological response.

100

101　2.　The role of lipids
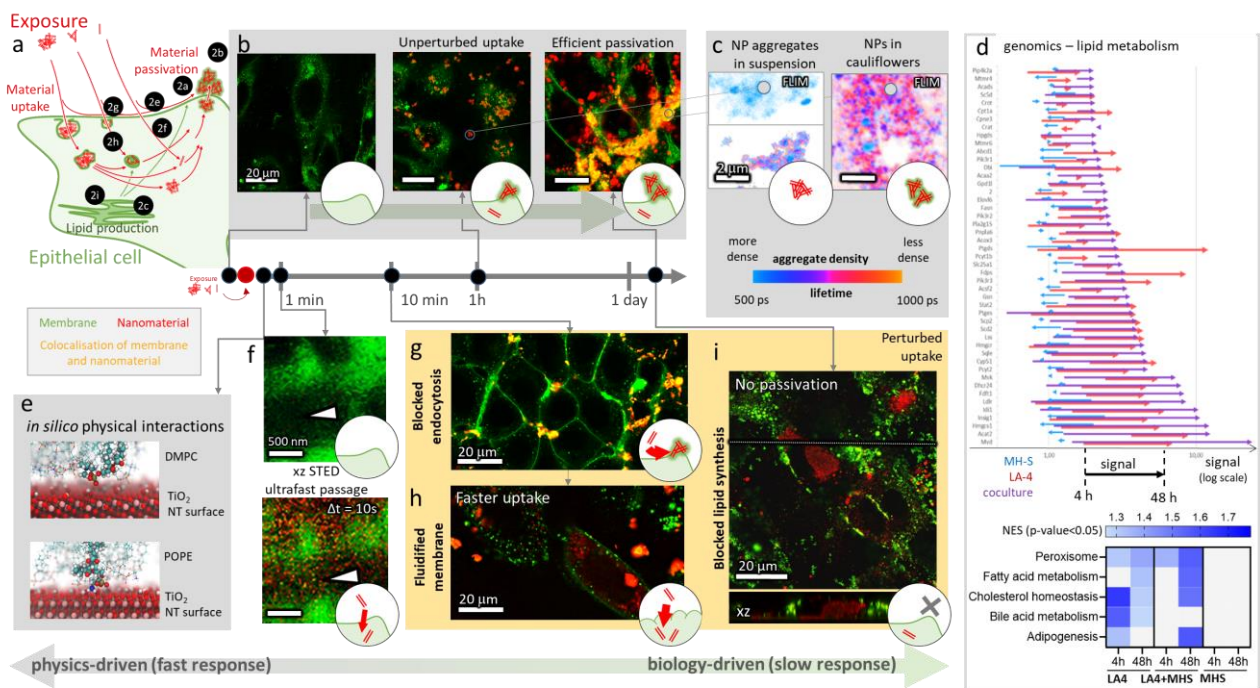
102

103 **Fig. 2: *Role of lipids in cauliflower formation.* a** *A general scheme of events shown in this figure. In*
104 *fluorescence micrographs, cell membranes are green and TiO₂ nanotubes red, surface dose was 10:1 (except*
105 ***f***). ***b*** *Unperturbed uptake of TiO₂ nanotubes after 0, 1 h and 2 days by lung epithelial LA-4 cells, same as Fig.*
106 *1d.* ***c*** *Increased fluorescence lifetime (FLIM) of Alexa Fluor 647 on TiO₂ nanotubes in cauliflowers (right)*
107 *compared to agglomerates in suspension (left) corresponds to increased distance between fluorophores on the*
108 *nanotubes (e.g. separation due to lipid interspacing).* ***d*** *Transcriptomics analysis of lipid metabolism on the*
109 *gene level (top) and pathway level (bottom) for MH-S macrophages (blue), LA-4 epithelial cells(red) and their*
110 *co-culture (purple) after 4 hours (beginning of arrow) and 48 hours (end of arrow) of nanomaterial exposure.*
111 ***e*** *Final state of full-atom in silico simulation confirms strong interaction between disordered lipids and the TiO₂*
112 *nanotubes.* ***f*** 🎥 *Cross-sections immediately before (above) and 10 s after (below) instant delivery of TiO₂*
113 *nanotubes onto cells by nebulisation (1:1 surface dose) show ultrafast membrane passage of the nanotubes*
114 *through the cell plasma membrane into the cell (arrowhead), driven by pure physical interactions.*
115 *Pharmaceutical-perturbed uptakes (to compare with **b**):* ***g*** 🎥 ☐ *chlorpromazine-blocked clathrin-mediated*
116 *endocytosis,* ***h*** ☐ *fluidified cell plasma membrane induced by cholesterol depletion (beta-methyl-cyclodextrin)*
117 ***i*** ☐ *inhibited fatty acid synthesis (resveratrol-blocked fatty-acid synthase). For associated data see Supp. info*
118 *S2.*

119

120 Coinciding with the formation of the lipid-rich bio-nano agglomerates (Fig. 2b), i.e. two days after the
121 nanomaterial exposure, a strong upregulation of membrane lipid metabolism-related genes is observed (Fig.
122 2d). Further modulation of the lipid synthesis pathway by blocking fatty acid synthase (FAS) with resveratrol
123 precludes the formation of large cauliflowers (Fig. 2i), confirming that epithelial cells usually respond to
124 nanomaterial exposure by an increased lipid synthesis, which is in turn required for cauliflower formation.

125 Because internalization of nanoparticles usually precedes cauliflower formation, we investigate the causality
126 between the two phenomena by blocking an important route of nanoparticle uptake, i.e. clathrin-mediated
127 endocytosis (supplement S0e), using chlorpromazine. Interestingly, small "proto" cauliflowers are formed soon
128 after exposure (15 min time scale) (Fig. 2g), indicating an additional mechanism of formation that requires no
129 intracellular processing. In this case, formation of cauliflowers presumably relies on the strong physical affinity
130 between nanoparticles and lipids, also supported by *in silico* simulations (Fig. 2e) and *in vitro* experiments on
131 model lipid membranes (S0d). However, these "proto" cauliflowers are rarely seen under normal conditions,
132 which lead us to conclude that this additional mechanism of formation is usually less probable, likely due to the
133 efficient particle uptake that displaces nanomaterial away from the plasma membrane, preventing their further
134 interaction.

135 Under unperturbed exposure (Fig. 2b), the basic physical interaction might therefore initiate the formation of
136 cauliflowers by driving nanoparticles and membrane lipids into small agglomerates anchored to the membrane.
137 The depletion of the functional lipid bilayer may trigger additional lipid synthesis, which later enables
138 passivation of even higher doses of nanoparticles in large agglomerates on the cellular surface (Fig. 1e).
139 Noteworthy, nanoparticles in these cauliflowers are effectively dissolved by interspaced lipids making them
140 more loosely packed compared to agglomerates of pure nanoparticles, as seen by increased fluorescence lifetime
141 (Fig. 2c).

142 Interestingly, cholesterol depletion from the plasma membrane by beta-methyl-cyclodextrin (a cell membrane
143 fluidifying agent which also inhibits endocytosis) leads to strong suppression of fast (membrane-lipid-drain
144 only) cauliflower formation (Fig. 2h). This indicates an important interaction between nanoparticles and
145 cholesterol which is reflected also in strongly upregulated cholesterol synthesis pathways (Fig. 2d heatmap,
146 supplement S0), which is also seen *in vivo* [12]. In the case of cholesterol-depleted plasma membranes, the majority
147 of nanoparticles pass the plasma membranes on a minute timescale, resulting in a fine distribution of particles
148 inside the cell. Interestingly, domination of such a passage can be observed also when nanoparticles are
149 delivered in a highly dispersed form through aerosol directly to the epithelial cell membranes and pass through
150 them in a matter of seconds (Fig. 2f, movie in supplement S0 🎥).

151 For the lungs, the lipid-synthesis-driven formation of bio-nano agglomerates thus seems to be an active response
152 of alveolar epithelial cells, enabling their survival after exposure to nanomaterial even at higher doses. As such,

153 this process can be seen as passivation of nanomaterial, a kind of protection mechanism (S0f). The remaining
154 question is the identification of the cellular mechanisms that can facilitate the export of the internalised material.

155

156   3.   The role of actin

157
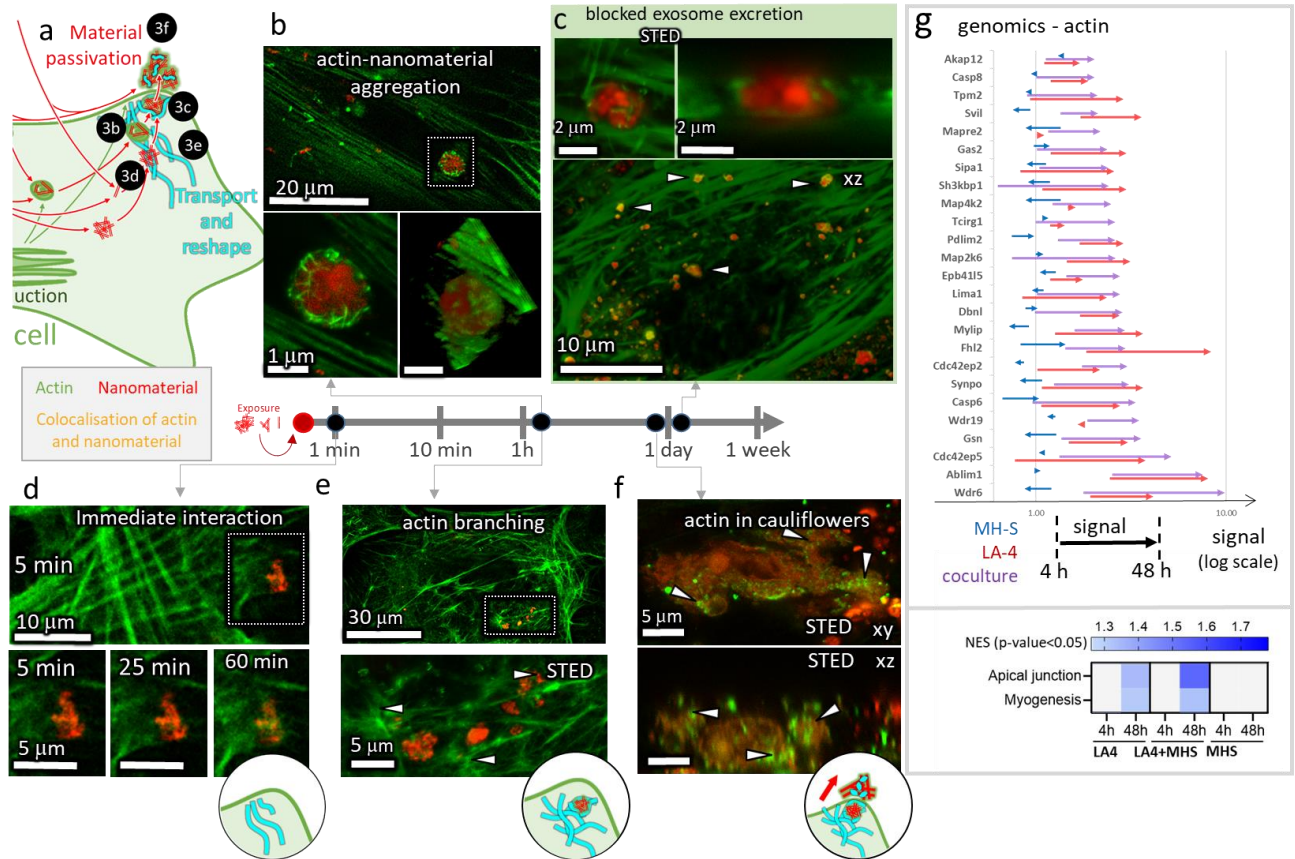
*Fig. 3: Role of actin in cauliflower formation. **a** A general scheme of events shown in this figure. Fluorescence micrographs of the actin network of LA-4 cells (green) after exposure to TiO_2 nanotubes (red) at a 10:1 surface dose. **d** ☛ Soon after exposure, actin interacts with uptaken nanoparticles, **b** ⬜ leading to formation of actin-nanoparticle agglomerates after a few hours. **e** Synchronously, the actin network branches (arrowheads), indicating changes in internal processes and reshaping of the cell. **c** Blocking the final stage of exocytosis with jasplakinolide traps nanoparticles in actin rings, prepared for exocytosis (arrowheads and zoom-ins). **f** ⬜ After a few days, actin fragments are observed in cauliflowers (arrowheads). **g** Transcriptomics analysis of actin-network on the gene level (top) and pathway level (bottom) for LA-4 (red), macrophages (blue), and their co-cultures (purple) after 4 hours (beginning of arrow) and 48 hours (end of arrow) of nanomaterial exposure. For associated data see Supp. info S3.*

168

169 Because exocytosis mechanisms involve cytoskeletal actin remodelling, the relevance of actin was investigated
170 next. Almost simultaneously with nanoparticle uptake and far before cauliflowers can form, many nanoparticles
171 evidently interact with actin fibres (Fig. 3d, movie in supplement S3d ☛), forming nanoparticle-actin 3D
172 agglomerates resembling Faberge eggs (Fig. 3b, 3D in supplement S0b ⬜). Hours after exposure the same
173 interaction causes actin network transformations from native to branched (Fig. 3e), indicating increased cell
174 motility [28], internal vesicular trafficking [29,30] and nanoparticles exocytosis [31,32].

175 By blocking actin fibre dynamics (polymerization and depolymerisation) with jasplakinolide, excretion of
176 exocytotic vesicles can be stopped, enabling their visualisation and identification of their content. Namely, after
177 uptake of nanoparticles and lipid synthesis, nanoparticles are trapped in exocytotic vesicles (actin rings),

178   prepared for exocytosis by the cell (Fig. 3c). Because lots of actin can be identified outside cells in cauliflowers
179   (Fig. 3f ⬚), excretion of nanoparticles is seemingly more destructive to the actin network than normal
180   exocytosis, where actin is retained inside cells. Actin adherence is also reflected in the coronome analysis of the
181   mobile fraction of nanoparticles after exposure in which we have previously found abundant actin proteins [27].
182   This clearly coincides with upregulation of the actin synthesis pathway (Fig. 3g). Up to now, the appearance of
183   actin in the nanoparticle corona outside of the cells could not be explained.

184   Creation of cauliflowers on the cell surface thus involves both membrane lipids and actin (Fig. 2d heatmap, Fig.
185   3c) that clearly interact with the nanoparticle surface directly. Due to strong binding of amines and phosphates
186   identified with *in silico* simulations (Fig. 2e) it is reasonable to expect that various biomolecules can strongly
187   bind to the same surface, from lipids and proteins to nucleic acids. Moreover, multiple binding sites on
188   nanomaterial and biomolecules directly lead to crosslinking and formation of large bio-nano agglomerates, such
189   as the observed cauliflowers. This implies that any strong interaction identified within *in silico* modelling of
190   biomolecule - nanomaterial surface pairs, is highly predictive of bio-nano agglomerates formation.

191   Ability to supply enough biomolecules to crosslink and thereby passivate the received dose of nanomaterial
192   explains epithelial cell survival even at large local dose of nanomaterial seen *in vivo* (Fig.1). This, however,
193   seems to be contradictory to the coinciding chronic inflammation, raising the question about the role of
194   surrounding cells, especially macrophages, which are responsible for the immune defence within the alveoli. To
195   address this, we expose a co-culture of LA-4 epithelial cells and MH-S macrophages in the same way as we did
196   with the epithelial monoculture.

197

198   4.   MH-S action against LA-4 defence

199
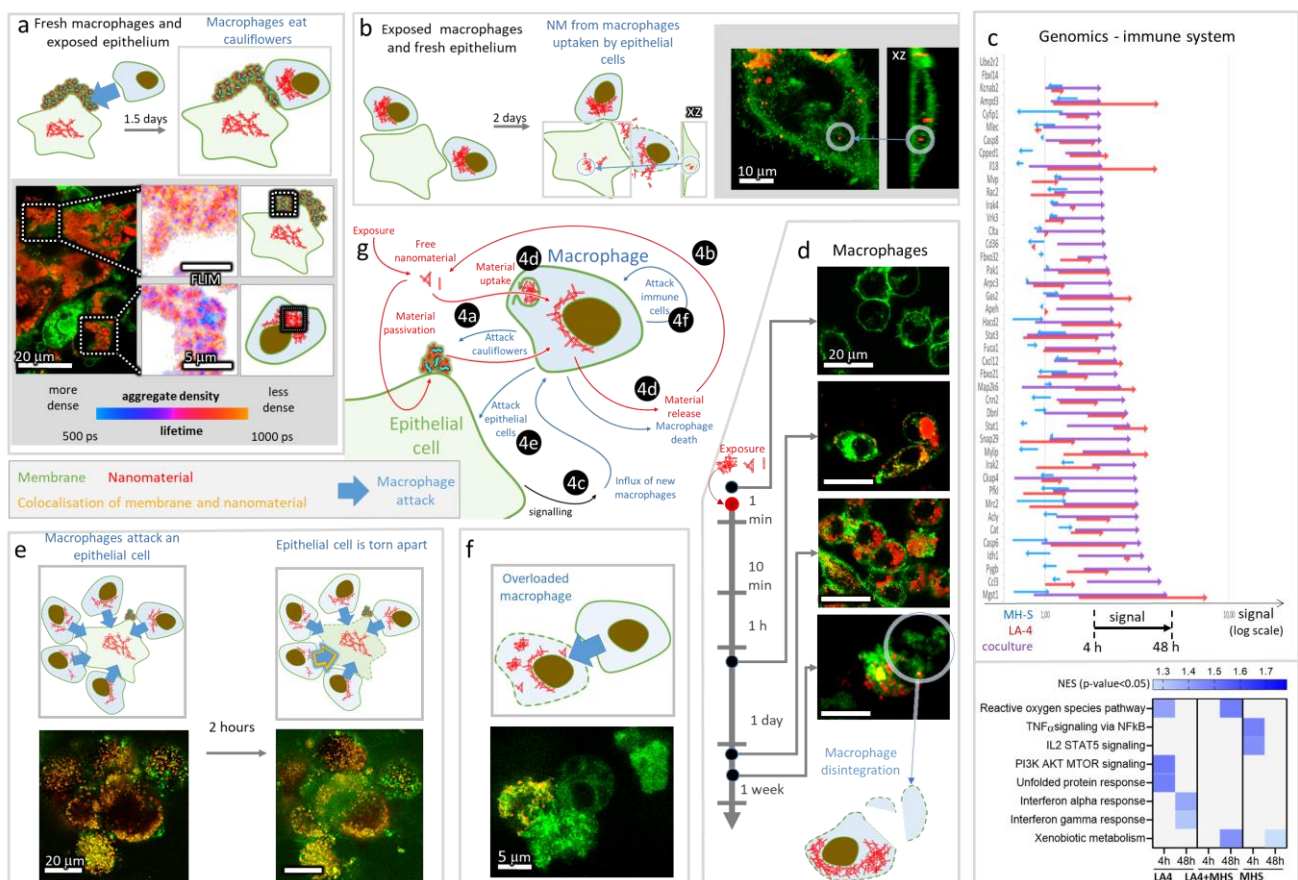
Fig. 4: The cycle of uptake, passivation and release in nanomaterial-exposed co-culture. *In all fluorescence micrographs, cell membranes are green and TiO₂ nanotubes red, and the surface dose of nanoparticles is 10:1.*

203 ***a** Unexposed macrophages (MH-S) were added to washed LA-4 with cauliflowers. In 1.5 days, MH-S phagocyte*
204 *the cauliflowers from LA-4, and degrade the organic (lipid) part, compacting the nanoparticles (fluorescence-*
205 *lifetime-maps FLIM, right). **b** Washed nanomaterial-laden MH-S were added to unexposed LA-4. After 2 days,*
206 *nanomaterial is found in LA-4 (encircled). **c** Transcriptomics analysis of innate and adaptive immune system*
207 *on a gene level (top) and pathway level (bottom) for LA-4 (red), MH-S (blue) and their co-culture (purple) after*
208 *4 hours (beginning of arrow) and 48 hours (end of arrow) of nanomaterial exposure. **d** Nanoparticle uptake by*
209 *MH-S followed by their disintegration after a few days (encircled): ▱🎥 (control) ▱🎥 (2 h) ▱🎥 (2 days) ▱*
210 *(4 days, MH-S disintegration) **e** 🎥 Time-lapse of MH-S attacking and tearing apart a nanomaterial-laden LA-*
211 *4 cell. **f** ▱ MH-S observed attacking another nanomaterial-laden MH-S. **g** A general scheme of events shown*
212 *in this figure. For associated data see Supp. info S4.*

213 With a co-culture MH-S macrophages and LA-4 epithelial cells we aimed to mimic the cell populations of the
214 lung alveoli, where approximately 3-5% of the alveolar surface is populated by alveolar macrophages spread
215 over nearly confluent alveolar epithelium[33]. When our co-culture is exposed to TiO_2 nanotubes, macrophages
216 internalize them, but cannot entirely prevent them from reaching epithelial cells (movie in supplement S0i 🎥)
217 due to their slow rate of cleaning nanoparticles from the epithelial surface. Aside from that, macrophages also
218 slow down considerably after having taken up large amounts of nanoparticles (graph in supplement S4e),
219 making them even less efficient. Thus, the exposed epithelium unavoidably produces cauliflowers also in our
220 co-culture (supplement S0), reproducing bio-nano agglomerates observed *in vivo* [12].

221 Although the nanoparticles are passivated in cauliflowers on the surface of LA-4 enabling their survival, the
222 same structures trigger the attack of macrophages, as seen in the experiment when unexposed macrophages
223 were added to pre-exposed epithelium with cauliflowers (Fig. 4a). After internalisation of the agglomerates,
224 macrophages are able to digest their organic part as revealed by decreased lifetime of probes on the
225 nanoparticles, indicating denser packing of nanoparticles in macrophages compared to cauliflowers (FLIM maps
226 in Fig. 4a insets). Unwrapping the passivated nanoparticles exposes the macrophage interior to their bare
227 surface, leading to the same end-state as after nanoparticle uptake by macrophages in monoculture. Such a
228 situation evidently leads to macrophage death and disintegration (Fig. 4d (4 days), 3D in supplement S4d ▱),
229 likely due to the lack of additional lipid synthesis, as supported by genomics (Fig. 2c). A similar fate of
230 macrophages is observed also after they have attacked a whole epithelial cell (Fig. 4e, movie in supplement S4e
231 🎥) or a contaminated macrophage (Fig. 4f, 3D in supplement S4e ▱). When nanomaterial-exposed
232 macrophages die, they release bare nanomaterial, which is later (re)uptaken by epithelial cells. This can be
233 observed experimentally: after nanomaterial-laden macrophages were added to the unexposed epithelial layer,
234 nanoparticles could be seen to enter epithelial cells (Fig. 4b).

235 Such reuptake would lead to fully passivated nanomaterial on the self-protected epithelial cells. *In vivo* however,
236 dead macrophages are replaced by the influx of new monocyte-derived macrophages, attracted to the site by
237 chemokines such as C-C motif ligand 3 (CCL3, aka macrophage inflammatory protein 1-alpha, MIP-1-alpha)
238 [12,26]. The macrophage influx brings the entire system to conditions very similar to the initial exposure, while
239 reuptake of nanomaterial by epithelium closes the chain of events, together forming a vicious cycle of endless
240 inflammation (Fig. 4g, Fig. 5a), which has never been shown before.

241 Strikingly, the same chemokine expressions can be detected both *in vivo* and *in vitro,* but exclusively in the co-
242 culture of LA-4 and MH-S cells (Fig. 4c, purple arrows) and not in either of the monocultures of LA-4 (Fig. 4c,
243 red arrows) or MH-S (Fig. 4c, blue arrows). This would imply that the *in vitro* co-culture can reproduce the cell
244 states under *in vivo* chronic inflammation conditions. Can we predict such an *in vivo* outcome by measuring
245 states of simple *in vitro* tests?

246

247   5.   Towards predictive toxicology
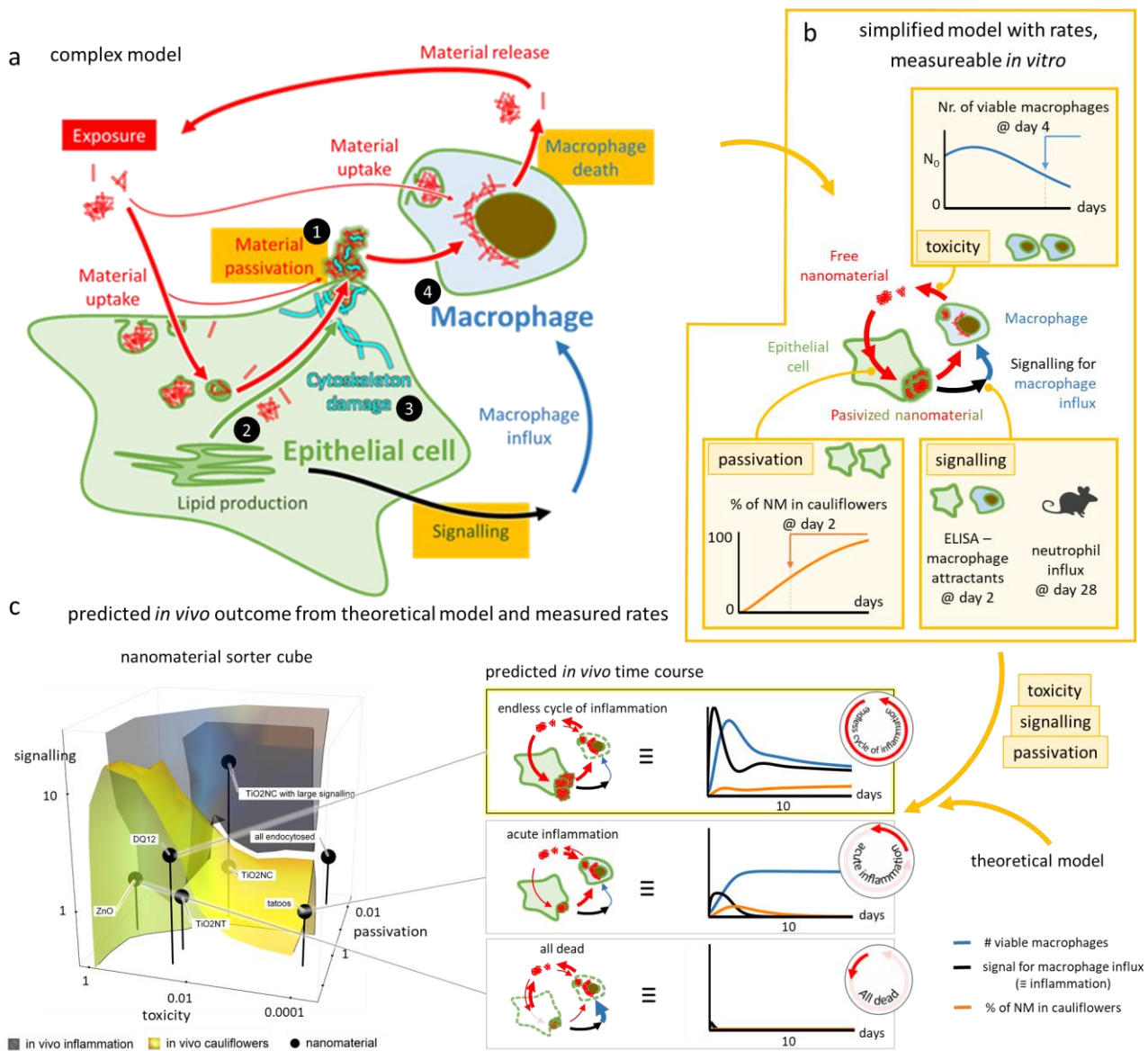
Fig. 5: Cycle of uptake, passivation and release of nanomaterial between epithelial cells and macrophages in co-cultures. a *A grand scheme connecting all inter- and intracellular events from Figures 1 – 4, simplified to* **b** *a theoretical model, defined by rates of passivation, toxicity and signalling. These rates can be measured in vitro or in vivo at a single time-point.* **c** *By combining the measured rates and the simple model, the time course (right) is determined and nanoparticles are sorted according to their predicted outcome (left). Chronic inflammation is defined as elevated macrophage influx for longer than 10 days (area above the black contour, black line in time-courses, right). Presence of cauliflowers after 10 days is observed below the yellow contour (orange line in time-courses, right). For associated data see Supp. info S5.*

The discovered complex pathway (Fig. 5a) describing a causal relationship between an acute exposure to nanoparticles and chronic inflammation conditions allows us to construct a simplified cyclical theoretical model defined with three descriptors, measurable in appropriate *in vitro* setups for each nanomaterial of interest (Fig. 5b):

1) capacity of epithelial cells to passivate nanomaterial is measured via the fraction of nanomaterial in cauliflowers in LA-4 monoculture after 2 days (Fig. 5b, passivation);

264   2) efficiency of signaling and monocyte influx replacing the dying macrophages is measured either via
265   macrophage attractants in *in vitro* co-culture of LA-4 and MH-S after 2 days or via polymorphonuclear cell
266   influx *in vivo* after 28 days (Fig. 5b, signalling);

267   3) toxicity of the nanomaterials to individual cells is measured via the number of viable macrophages in MH-
268   S monoculture after 4 days (Fig. 5b, toxicity).

269   Whether the cycle stops or goes on indefinitely, heavily depends on the rates of the associated processes,
270   calculated from the measured descriptors as described in (supplement S5). Using these rates, the model can
271   simulate the *in vivo* time courses of nanomaterial passivated in cauliflowers, signaling for macrophage influx,
272   as well as of the total macrophage number, and accordingly predict the nanomaterial-specific acute-to-chronic
273   inflammation outcome (Fig. 5c - time traces). For example, a very toxic nanomaterial such as ZnO, exhibits a
274   rapid decline in the number of all cells, preventing passivation as well as influx of new macrophages, resulting
275   in destruction of the alveolar layer [34]. A material similar to TiO_2 nanocubes with intermediate toxicity and
276   passivation rate, shows transient inflammation only, with all nanomaterial ending up in cells, as observed *in*
277   *vivo* [12]. Finally, for a material such as TiO_2 nanotubes with intermediate toxicity and high passivation rate,
278   persistently high inflammation and large cauliflowers are predicted (Fig. 1b) in line with previous studies [12]. In
279   this three-dimensional space of nanomaterial descriptors (Fig. 5c - 3D plot), we can now delineate regions
280   eliciting similar outcomes, thus sorting nanomaterials into several classes according to their mode-of-action.

281   This approach holds significant predictive value for long-term *in vivo* behavior based on outcomes of simple
282   high-throughput *in vitro* measurements. The nonlinear understanding of adverse outcome pathway initiation
283   which is crucial for understanding nanomaterial-induced chronic inflammation may also underlie cancer,
284   fibrosis, and other chronic diseases.

285

Methods

Materials

288   Alexa Fluor 647 NHS ester (Termo Fisher), Star 520 SXP NHS ester (Abberior), ATTO 594 NHS ester (Atto-
289   tec), CellMask Orange (Invitrogen), SiR Actin (Cytoskeleton), Star Red-DPPE (Abberior), 4-(8,9-Dimethyl-
290   6,8-dinonyl-2-oxo-8,9-dihydro-2H-pyrano[3,2-g]quinolin-3-yl)-1-(3-(trimethylammonio)   propyl)pyridin-1-
291   ium dibromide(SHE-2N), 3-(Benzo[d]thiazol-2-yl)-6,8,8,9-tetramethyl-2-oxo-8,9-dihydro-2H-pyrano[3,2-
292   g]quinoline-4-carbonitrile (SAG-38), LCIS-Live Cell Imaging Solution (Invitrogen), PBS-phosphate buffer
293   saline (Gibco), 100x dcb: 100-times diluted bicarbonate buffer (pH 10, osmolarity 5 miliosmolar, mixed in-
294   house), F-12K cell culture medium (Gibco), RPMI 1640 cell culture medium (Gibco), Trypsin (Sigma),
295   Penicillin-Streptomycin (Sigma), Non-essential amino acids (Gibco), Beta mercaptoethanol (Gibco), glucose
296   (Kemika), BSA-bovine serum albumin (Sigma), Hydrogen peroxide (Merck), Chlorpromazine (Alfa Aesar),
297   MBCD-Metyl-Beta-Cyclodextran (Acros organics), Resveratrol (Sigma), #1.5H □-dishes (Ibidi,) #1.5H □-
298   Slide 8-well (Ibidi), Limulus Amebocyte Lysate Assay (Lonza, Walkersville, MD, USA), 10% neutral buffered
299   formalin (CellPath Ltd, UK), haematoxylin and eosin (H&E), Pelcotec™ SFG12 Finder Grid Substrate- Si
300   wafers (Ted Pella), Aeroneb®Pro nebulizer (from VITROCELL® Cloud 6 system), GeneChip® WT PLUS
301   Reagent Kit (Thermo Fisher/Affymetrix)

302   Nanomaterials used in this study
303   Synthesized in-house by P. Umek:
304   name: TiO_2 nanotubes and TiO_2 nanocubes;
305   Official ID: PU-nTOX-01-03, PU-nTOX-01-21

306
307   Kind gift from U. Vogel:
308   name: Carbon black, MKNA015, MKNA100 and SiO_2 DQ12;
309   JRC ID: Printex 90, MKN- TiO_2 -A015, MKN- TiO_2 -A100, NA .

310
311   Kind gift from JRC:

312 JRC ID: NM101 TiO_2 anatase, NM105 TiO_2 rutil-anatase, NM200 Silica, NM402 MWCNT, NM401 MWCNT,
313 NM110 ZnO and NM 111 ZnO;
314 JRC ID: JRCNM01001a, JRCNM01005a, JRCNM02000a, JRCNM04002a, JRCNM04001a, JRCNM01101a,
315 JRCNM62101a

Cell culture

319 Murine epithelial lung tissue cell line (LA- 4; cat. no. ATCC CCL-196) and murine alveolar lung macrophage
320 (MH-S; cat. No. CRL2019) cell line were purchased from and cultured according to American Type Culture
321 Collection (ATCC) instructions. Cells were cultured in TPP cell culture flasks at 37 °C in a 5% CO_2 humidified
322 atmosphere until monolayers reached desired confluency. All experiments were performed with cells before the
323 twentieth passage. For long–term live cell experiments we used a homemade stage top incubator which
324 maintains a humidified atmosphere with a 5% CO_2 heated on 37 °C.

325 Medium used for culturing of the epithelial LA-4 cells is Ham's F-12K medium (Gibco) supplemented with
326 15% FCS (ATCC), 1% P/S (Sigma), 1% NEAA (Gibco), 2 mM L-gln.

327 For alveolar macrophages, MH-S, cell line we used RPMI 1640 (Gibco) medium supplemented with 10% FCS
328 (ATCC), 1% P/S (Sigma), 2 mM L-gln, and 0.05 mM beta mercapthoethanol (Gibco).

Nanomaterial synthesis and labelling

332 The TiO_2 anatase nanotubes used in this paper were synthesized, functionalized with AEAPMS, and labelled
333 with STED-compatible fluorescent probes via a covalent reaction between the AEAPMS and ester functional
334 group on the probe. All this was done in-house as described in [27]. Labelled TiO_2 was then stored in 100x diluted
335 bicarbonate buffer. For the multi-NM exposure experiments we used other NMs as well. All the NMs were
336 suspended in PBS and sonicated in ice bath using a tip sonicator (Sonicator 4000, Misonix, with 419 Microtip
337 probe) for 15 min with 5s ON/ 5s OFF steps.

338 The average hydrodynamic particle size of the TiO_2 tube in suspension (3.24 mg/ ml) was determined by
339 Dynamic Light Scattering (DLS). The TiO_2 tube suspension had a bimodal size distribution with a major peak
340 at 60 nm and a narrow peak at 21 nm (Danielsen 2019 TAAP). The intensity-based z-average size was 168.7
341 nm and the polydispersity index (PI) was 0.586, indicating some polydispersity in the suspensions. Endotoxin
342 levels were measured using the Limulus Amebocyte Lysate Assay. The level of endotoxins was low in TiO_2
343 nanotube suspensions (0.095 endotoxin units (EU)/mL), and in nanopure water with 2 % mouse serum (0.112
344 EU/ml, self-extracted).

In vitro sample preparation and exposure of MH-S&LA-4 to TiO_2

348 LA-4 and MH-S cells were seeded in Ibidi 1.5H dishes of various surface area, depending on the experiment.
349 After 24 h NM (c=1mg/mL) was added in a 1:1, 10:1 and 100:1 ($NM_{surface}$: $Cell_{surface}$) ratios, according to the
350 experiment needs. Before exposure, NM suspension was sonicated for 10s in an ultrasonic bath (Bransonic
351 ultrasonic cleaner, Branson 2510EMT). Cells were then incubated at 37°C and 5% CO2 atmosphere with the
352 NM for the following 24 h, 48 h or longer in order to observe the cells at the post-exposure time points of
353 interest. If the experiment required monoculture of either cell line, sample were prepared as described above, if
354 however, we experimented with the co-cultures, sample preparation differed slightly. For co-cultures, we grew
355 LA-4 and MH-S in separate dishes up to desired confluency (lower than for monocultures) and then mixed them
356 together by adding MH-S in the LA-4 dish (1 : 40). Co-cultures were then incubated for 24 h more, exposed to
357 NM as described above and incubated for additional desired amount of time. Growth medium for co-cultures
358 was mixture of equal volumes of F12K and RPMI 1640. Cells were then labelled with fluorescent dyes

359 according to the manufacturers recommendations. Unbound fluorescent label was washed and medium was
360 exchanged for LCIS.
361
362 In some experiments we used different chemicals for modulation of the metabolism. For blocking the CME,
363 cells were treated with 100 μm Chlorpromazine for 15 min. Membrane cholesterol was extracted with 24 h
364 incubation with 0.5 - 1 mM MBCD. FAS was inhibited with overnight 100 μM Resveratrol incubation. Finally,
365 for actin stabilization, we used higher concentration (≥1mM) of Sir-Actin Label based on Jasplankinolide. All
366 the chemical modulators were added before exposure to NM and incubated with the NM for abovementioned
367 time periods.
368
369 For the reuptake experiments different cell lines were grown separately and aspirate of one cell culture was
370 added in the other and then observed.
371
372 HIM, SEM
373 Samples were prepared as usual but we grew them on Si-wafers. After reaching desired confluency samples
374 were freeze-dried with metal mirror freezing technique.
375
376 Transcriptomics
377 Samples were prepared as described. Cells were exposed to TiO_2 and MWCNT for 4 h and 48 h. From exposed
378 and control samples (control at 0 h and 48 h) growth medium was removed from the wells and frozen at -70°C
379 with the 6-well plates containing cells only. RNA samples for the whole transcriptome expression were prepared
380 with the GeneChip® WT PLUS Reagent Kit (Thermo Fisher/Affymetrix) and analysed with the
381 GeneChip™Whole Transcript (WT) Expression Arrays according to the manufacturers guidelines.
382
383 Statistical analysis for all probe sets includes limma t-test and Benjamini-Hochberg multiple testing correction.
384 Significant genes were determined with a False Discovery Rate (FDR)<10%. Also, the p-values of the limma t-
385 test was used to define sets of regulated genes (p<0.01/0.05). p-values were used to exclude background signals:
386 significant genes were filtered for p<0.05 in more than half of the samples in at least one group (indicated by
387 "dabg", data above background).
388
389 In the arrow graphs, only genes which were up- or down-regulated more than two times compared to non-
390 exposed cells are shown. The signal (x axis) is drawn in logarithmic scale. Expression is normalized to
391 expression of control samples.
392
393 Detailed protocols are available in supplement material.
394
395 Imaging *in vitro*
396
397 STED
398 Super-resolution and confocal fluorescence micrographs were acquired using custom build STED microscope
399 from Abberior with an Olympus IX83 microscope and two avalanche photodiodes as detectors (APDs). Images
400 have been acquired using Imspector (version 16.2.8282-metadata-win64-BASE) software also provided by
401 Abberior. Microscope is equipped with two 120 picosecond pulsed laser sources (Abberior) with excitation
402 wavelengths 561 and 640 nm and maximal power of 50 μW in the sample plane. Pulse repetition frequency for
403 experiments was 40 - 80 MHz, depending on the experiment. STED depletion laser wavelength is 775 nm with
404 same repetition frequency as excitation lasers, pulse length of 1.2 ns and maximal power of 170 mW in the
405 sample plane. Filter sets used for detection have been 605–625 nm (green channel), 650–720 nm (red channel).
406 All the microscope settings that have been tuned separately for maximal resolution during each of the
407 experiments have been recorded and given with the experiment images in Supplement. The combinations of
408 excitation and filter sets have also been optimized for each experiment if necessary.
409
406 FLIM
407 Fluorescence lifetime images (FLIM) were obtained on the same custom-built STED microscope (Abberior
408 instruments) as confocal and STED fluorescence images in this study. The sample was excited by pulsed laser
409 sources with wavelengths 561 nm and 640 nm and the emitted fluorescence was detected using PMT detectors

359 according to the manufacturers recommendations. Unbound fluorescent label was washed and medium was
360 exchanged for LCIS.
361
362 In some experiments we used different chemicals for modulation of the metabolism. For blocking the CME,
363 cells were treated with 100 μm Chlorpromazine for 15 min. Membrane cholesterol was extracted with 24 h
364 incubation with 0.5 - 1 mM MBCD. FAS was inhibited with overnight 100 μM Resveratrol incubation. Finally,
365 for actin stabilization, we used higher concentration (≥1mM) of Sir-Actin Label based on Jasplankinolide. All
366 the chemical modulators were added before exposure to NM and incubated with the NM for abovementioned
367 time periods.
368
369 For the reuptake experiments different cell lines were grown separately and aspirate of one cell culture was
370 added in the other and then observed.
371
372 HIM, SEM
373 Samples were prepared as usual but we grew them on Si-wafers. After reaching desired confluency samples
374 were freeze-dried with metal mirror freezing technique.
375
376 Transcriptomics
377 Samples were prepared as described. Cells were exposed to TiO_2 and MWCNT for 4 h and 48 h. From exposed
378 and control samples (control at 0 h and 48 h) growth medium was removed from the wells and frozen at -70°C
379 with the 6-well plates containing cells only. RNA samples for the whole transcriptome expression were prepared
380 with the GeneChip® WT PLUS Reagent Kit (Thermo Fisher/Affymetrix) and analysed with the
381 GeneChip™Whole Transcript (WT) Expression Arrays according to the manufacturers guidelines.
382
383 Statistical analysis for all probe sets includes limma t-test and Benjamini-Hochberg multiple testing correction.
384 Significant genes were determined with a False Discovery Rate (FDR)<10%. Also, the p-values of the limma t-
385 test was used to define sets of regulated genes (p<0.01/0.05). p-values were used to exclude background signals:
386 significant genes were filtered for p<0.05 in more than half of the samples in at least one group (indicated by
387 "dabg", data above background).
388
389 In the arrow graphs, only genes which were up- or down-regulated more than two times compared to non-
390 exposed cells are shown. The signal (x axis) is drawn in logarithmic scale. Expression is normalized to
391 expression of control samples.
392
393 Detailed protocols are available in supplement material.
394
395 Imaging *in vitro*
396
397 STED
398 Super-resolution and confocal fluorescence micrographs were acquired using custom build STED microscope
399 from Abberior with an Olympus IX83 microscope and two avalanche photodiodes as detectors (APDs). Images
400 have been acquired using Imspector (version 16.2.8282-metadata-win64-BASE) software also provided by
401 Abberior. Microscope is equipped with two 120 picosecond pulsed laser sources (Abberior) with excitation
402 wavelengths 561 and 640 nm and maximal power of 50 μW in the sample plane. Pulse repetition frequency for
403 experiments was 40 - 80 MHz, depending on the experiment. STED depletion laser wavelength is 775 nm with
404 same repetition frequency as excitation lasers, pulse length of 1.2 ns and maximal power of 170 mW in the
405 sample plane. Filter sets used for detection have been 605–625 nm (green channel), 650–720 nm (red channel).
406 All the microscope settings that have been tuned separately for maximal resolution during each of the
407 experiments have been recorded and given with the experiment images in Supplement. The combinations of
408 excitation and filter sets have also been optimized for each experiment if necessary.
409
410 FLIM
411 Fluorescence lifetime images (FLIM) were obtained on the same custom-built STED microscope (Abberior
412 instruments) as confocal and STED fluorescence images in this study. The sample was excited by pulsed laser
413 sources with wavelengths 561 nm and 640 nm and the emitted fluorescence was detected using PMT detectors

410 and TCSPC technology developed by Becker & Hickl. 16-channel GaASP PMT detectors attached to a
411 spectrograph with diffraction grating 600 l/mm were used to measure fluorescence lifetime of emitted photons
412 with wavelengths ranging from 560 to 760 nm. Spectral information was discarded and the lifetimes were
413 gathered in Imspector 16.2 (Abberior Instruments).

415 The fluorescence lifetime data was analysed with SPCImage 7.3 (Becker & Hickl) software, where the Decay
416 matrix was calculated from the brightest pixel in the image (monoexponential fitting), binning was set to 3 and
417 threshold to 5. The rainbow LUT was rescaled to range from 500 ps to 1000 ps and intensity and contrast of the
418 lifetime-coded image were adjusted for easier comparison between experiments.

420 Imaging of nanomaterial in backscatter mode:
421 Simultaneously with measuring fluorescence from CellMask Orange in the cell membrane (as described in
422 STED section), backscattered light was detected as well to locate the nanomaterial in the sample. A tunable
423 Chameleon Discovery laser (Coherent) with 100 fs long pulses, pulse repetition frequency 80 MHz, and
424 maximal average power of 1.7 W at 850 nm was used as the scattering light. The pre-attenuated laser light with
425 a wavelength of 750 nm first passed through a 785 nm built-in dichroic where a fraction of the power was
426 directed onto the sample through the same 60x WI objective (NA 1.2) as the excitation light. The backscattered
427 light then went back through the same objective and dichroic, now mostly passing through the dichroic towards
428 the detectors. After passing through a pinhole (0.63 A.U.), the backscattered light was spectrally separated from
429 the fluorescence by short-pass 725 nm dichroic, afterwards being detected on the same PMT, as described in
430 the FLIM section, this time set to collect light with wavelengths above 725nm.

431 Due to the large coherence of the laser, the backscattered light exhibited a strong speckle pattern, which was
432 diminished by a 100-nm-wide Gaussian blur on the scattering image, thus decreasing false negative
433 colocalisation of NM on account of spatial resolution.

435 SEM
436 SEM imaging has been performed on MIRA3 Flexible FE-SEM produced by TESCAN, by detection of
437 secondary electrons. Beam powers used have been between 5.0 kV and 15 kV with variable field of view 1.8
438 μm to 180 μm. All samples have been measured under high pressure vacuum (HiVac). All analysis has been
439 performed in Tescan developed software.

441 HIM
442 Super-resolution imaging on the nanoscale was carried out using Helium Ion Microscope (Orion NanoFab,
443 Zeiss) available at IBC at the Helmholtz-Zentrum Dresden - Rossendorf e. V., a member of the Helmholtz
444 Association. Microscope equipped with GFIS injection system and additional in-situ backscatter spectrometry
445 and secondary ion mass spectrometry can achieve 0.5 nm lateral resolution imaging using 10-35 keV He ion
446 beams. Measurements of secondary electrons (Se) emitted from the first few nm of the sample were done by He
447 ion acceleration of 30 keV, current of 1.7 pA and were acquired under high vacuum inside the sample chamber
448 (3x10-7 mBar). Field-of-view was varied from 60 μm x 60 μm down to 1 μm x 1 μm, with pixel steps small as
449 2nm. Imaging was performed on non-tilted and tilted sample stage (45 degrees) for better 3-D visualization.

450 *In vivo* data – U. Vogel group

452 The materials and methods used for intratracheal instillation of mice with TiO_2 tube are described in detail by
453 Danielsen et. al [12] and included here in an abbreviated version.

454 Preparation and characterization of TiO_2 tube suspensions TiO_2 tubes were characterization in
455 Urbančič et. al [27].

457   TiO_2 tubes were suspended in nanopure water with 2 % v/v mouse serum (prepared in-house) to a final
458   concentration of 3.24 mg/ml. The suspension was probe sonicated on ice for 16 min with 10 % amplitude. 3.24
459   mg/ml corresponds to a dose of 162 μg TiO_2 tube per 50 μl instillation volume per mice. The vehicle of nanopure
460   water with 2 % v/v mouse serum was probe sonicated using the same protocol. The dose of 162 μg/mouse (3:1
461   $NM_{surface}$: $Cell_{surface}$ in vitro) is equivalent to 15 working days at the 8-h time-weighted average occupational
462   exposure limit for TiO_2 by Danish Regulations (6.0 mg/m^3 TiO_2).

463

464   Animal handling and exposure
465   Seven-week-old female C57BL/6jBomtac mice (Taconic, Ejby, Denmark) were randomized in groups for TiO_2
466   tube exposure (N=5 mice/group for histology) and vehicle controls (N = 2-4 mice/group). At 8 weeks of age the
467   mice were anaesthetized and exposed to 0 μg or 162 μg TiO_2 tube in 50 μl vehicle by single intratracheal
468   instillation. In brief, the mice were intubated in the trachea using a catheter. The 50 μl suspension was instilled
469   followed by 200 μL air. The mouse was transferred to a vertical hanging position with the head up. This ensures
470   that the administered material is maintained in the lung. Animal experiments were performed according to EC
471   Directive 2010/63/UE in compliance with the handling guidelines established by the Danish government and
472   permits from the Experimental Animal Inspectorate (no. 2015-15-0201-00465). Prior to the study, the
473   experimental protocols were approved by the local Animal Ethics Council.
474   More details regarding the animal study can be found in Danielsen et al.[12].

475

476   Histology and enhanced darkfield imaging
477   At 28, 90 or 180 days post-exposure mice were weighed and anesthetized. Lungs were filled slowly with 4%
478   formalin under 30 cm water column pressure. A knot was made on the trachea to secure formaldehyde in lungs
479   to fixate tissue in "inflated state". Lungs were then removed and placed in 4% neutral buffered formaldehyde
480   for 24 hours. After fixation the samples were trimmed, dehydrated and embedded in paraffin. 3 μm thin sections
481   were cut and stained with haematoxylin and eosin (H&E). Cytoviva enhanced darkfield hyperspectral system
482   (Auburn, AL, USA) was used to image particles and organic debris in the histological sections of mouse lungs.
483   Enhanced darkfield images were acquired at 100x on an Olympus BX 43 microscope with a Qimaging
484   Retiga4000R camera.

485

486   Modelling
487   *In silico* data – atomistic molecular dynamics simulation

488   System composition
489   Atomistic molecular dynamics simulations have been carried out for DMPC and POPE lipids near anatase (101)
490   TiO_2 surface in water environment. Anatase slab (71.8 x 68.2 x 30.5 Å) with (101) surface normal to the z axis
491   is used as a model of a nanoparticle surface. The slab contains 4536 Ti atoms of which 504 are five-fold
492   coordinated atoms on the surface. (101) anatase surface was chosen as a surface of the lowest energy. At neutral
493   pH TiO_2 surface is covered by hydroxyl groups and is negatively charged. In our model we bind hydroxyl groups
494   to 5-coordinated surface Ti atoms so that the surface charge density is close to the experimental value at neutral
495   pH. Thus we add 151 hydroxyl groups to randomly picked Ti surface atoms (which constitutes 30% of their
496   total amount) which results in a surface charge density of -0.62 electrons/nm^2, which is in line with the
497   experimental results[35].

498

499   The TiO_2 slab is then placed in the middle of the simulation box with 3D periodic boundary conditions. The box
500   size in X and Y directions is defined by the slab length and width so that the slab is periodic in those directions.
501   The height of the box is set to 130 Å to accommodate the TiO_2 slab (thickness of 30.5 Å), eventual formed lipid
502   bilayer on the both sides (2 x 40 Å) as well as their hydration layers (2 x 10 Å). 82 lipid molecules (POPE or
503   DMPC) are inserted at random unoccupied positions in the box in random orientations, after that the box is
504   filled with water molecules (about 12000). Then, a small number of water molecules are picked at random and

505 are substituted with Na^+ and Cl^- ions to balance the negative surface charge of the slab and provide NaCl
506 concentration of 0.15 M in the water phase of the simulated system.

Simulation protocol

508 First, energy minimization of the simulated systems using the steepest gradient descent method is performed,
509 followed by a short 100 ps pre-equilibration run at constant volume and temperature. After that, the pressure in
510 the system is equilibrated to 1 bar using anisotropic Berendsen barostat[36] with relaxation time of 5 ps during 10
511 ns, which is finally followed by 1 µs production run in the NVT ensemble. Leap-frog algorithm with time step
512 1 fs is used to integrate the equations of motion. Center-of-mass motion is removed every 100 steps. Verlet cut-
513 off scheme[37] with the buffer tolerance of 0.005 kJ x mol^{-1} x ps^{-1} per atom is used to generate the pair lists.
514 Minimum cut-off of 1.4 nm is used for both short ranged electrostatic and VdW interactions. Long range
515 electrostatics are calculated using PME[38] with the grid spacing of 0.12 nm and cubic interpolation. Long range
516 dispersion corrections are applied to both energy and pressure. Velocity rescaling thermostat[39] is used to control
517 the temperature, which is set to 303 K with the relaxation time of 1 ps. All bonds with hydrogen atoms are
518 constrained using the LINCS algorithm[40]. Atom coordinates and energies are saved every 5 ps. All simulations
519 were performed by the Gromacs 2019 software package[41]. Visualization of the simulations is done by VMD[42].

Models used

521 Lipids are described by the Slipids force field[43]. For TiO_2, we use parameters optimized to fit results on charge
522 density distributions and water-TiO_2 surface coordination obtained in *ab-initio* simulations of TiO_2-water
523 interface[44]. These parameters are listed in tables in supplement S5b, S5c and S5d. Water molecules are
524 represented by the TIP3P model[45], and for Na+ and Cl^- ions Yoo and Aksimentiev ion parameters is used[46].
525 Lorentz-Berthelot rules are applied to determine Lennard-Jones parameters for cross-interactions.

Model of chronic inflammation following NM exposure

527 The theoretical model of chronic inflammation following NM exposure is described by a series of differential
528 equations, describing the events observed in *in vitro* and *in vivo* experiments in this work. This minimal-
529 complexity *in vivo* model consists of 6 variables (surface of NM in epithelial cells, in cauliflowers, in
530 macrophages and freely-floating NM, surface of macrophages and surface of epithelial cells), 4 locked
531 parameters (endocytosis rate, rate of cauliflower endocytosis, delay, and epithelial cell replication rate) and 3
532 NM-associated parameters (cauliflower formation rate, signalling efficiency, and toxicity), which change from
533 nanomaterial to nanomaterial. Separate *in vitro* models were obtained from the *in vivo* model by swapping the
534 macrophage influx with macrophage replication and leaving out non-existent cells for monocultures.

535 The system of equations was solved numerically using Wolfram Mathematica 12.0, licence L5063-5112 to
536 obtain the time evolution and final state of the model. The same software was also used for visualization of the
537 results.

538 The phase space was scanned by calculating the time evolution of the appropriate system of equations from
539 chapter S5b for a set of nanomaterials with appropriately interspaced parameters: toxicity (*tox*), cauliflower
540 formation (*cff*) and signalling efficiency (*signalEff*). For each parameter, 30 logarithmically-equally-spaced
541 values in a sensible range were chosen – the total amount of values in the grid was thus 30 x 30 x 30 = 27.000.

542 More information can be found in S5b, S5c and S5d.

Software

544 Imspector (version 16.2.8282-metadata-win64-BASE) software provided by Abberior

545 SPCImage 7.3 (Becker & Hickl)

546 Fiji, ImageJ 1.52p (NIH)

547 syGlass (http://www.syglass.io/, RRID:SCR_017961)

548 Mathematica 12.0, licence L5063-5112 (Wolfram)

549 genomics software: GSEA by Broad Institute

550 modelling: GROMACS (calculation), VMD (visualisation)

551

552 Data availability

553 Source data is available online at http://lbfnanobiodatabase.ijs.si/file/data/cauliflowerpaper/ with all 3Ds and
554 movies as a part of a database develop for H2020 Smart Nano Tox project.

555 References

556 1. Netea, M. G. *et al.* A guiding map for inflammation. *Nat. Immunol.* **18**, 826–831 (2017).

557 2. Furman, D. *et al.* Chronic inflammation in the etiology of disease across the life span. *Nat. Med.* **25**,
558 1822–1832 (2019).

559 3. Roth, G. A. *et al.* Global, regional, and national age-sex-specific mortality for 282 causes of death in 195
560 countries and territories, 1980–2017: a systematic analysis for the Global Burden of Disease Study 2017.
561 *The Lancet* **392**, 1736–1788 (2018).

562 4. Underwood, E. The polluted brain. *Science* **355**, 342–345 (2017).

563 5. OECD. OECD Environmental Outlook to 2050. doi:http://dx.doi.org/10.1787/9789264122246-en.

564 6. WHO. Air pollution. https://www.who.int/westernpacific/health-topics/air-pollution.

565 7. EPA/600/R-12/056F Provisional Assessment of Recent Studies on Health Effects of Particulate Matter
566 Exposure. (2012).

567 8. Rohr, J. R., Salice, C. J. & Nisbet, R. M. Chemical safety must extend to ecosystems. *Science* **356**, 917–
568 917 (2017).

569 9. Huh, D. *et al.* Reconstituting Organ-Level Lung Functions on a Chip. *Science* **328**, 1662–1668 (2010).

570 10. Maynard, A. D. & Aitken, R. J. 'Safe handling of nanotechnology' ten years on. *Nat. Nanotechnol.* **11**,
571 998–1000 (2016).

572 11. Nel, A. E. & Malloy, T. F. Policy reforms to update chemical safety testing. *Science* **355**, 1016–1018
573 (2017).

574 12. Danielsen, P. H. *et al.* Effects of physicochemical properties of TiO2 nanomaterials for pulmonary
575 inflammation, acute phase response and alveolar proteinosis in intratracheally exposed mice. *Toxicol.*
576 *Appl. Pharmacol.* **386**, 114830 (2020).

577   13. Fujita, K. *et al.* Intratracheal instillation of single-wall carbon nanotubes in the rat lung induces time-
578   dependent changes in gene expression. *Nanotoxicology* **9**, 290–301 (2015).

579   14. Cho, W.-S. *et al.* NiO and Co3O4 nanoparticles induce lung DTH-like responses and alveolar
580   lipoproteinosis. *Eur. Respir. J.* **39**, 546–557 (2012).

581   15. van den Brule, S. *et al.* Nanometer-long Ge-imogolite nanotubes cause sustained lung inflammation and
582   fibrosis in rats. *Part. Fibre Toxicol.* **11**, 67 (2014).

583   16. Tian, F. *et al.* Pulmonary DWCNT exposure causes sustained local and low-level systemic inflammatory
584   changes in mice. *Eur. J. Pharm. Biopharm.* **84**, 412–420 (2013).

585   17. Kim, S.-H. *et al.* The early onset and persistent worsening pulmonary alveolar proteinosis in rats by
586   indium oxide nanoparticles. *Nanotoxicology* **0**, 1–11 (2019).

587   18. Kasai, T. *et al.* Lung carcinogenicity of inhaled multi-walled carbon nanotube in rats. *Part. Fibre Toxicol.*
588   **13**, 53 (2016).

589   19. Kasai, T. *et al.* Thirteen-week study of toxicity of fiber-like multi-walled carbon nanotubes with whole-
590   body inhalation exposure in rats. *Nanotoxicology* **9**, 413–422 (2015).

591   20. Pauluhn, J. Subchronic 13-week inhalation exposure of rats to multiwalled carbon nanotubes: toxic effects
592   are determined by density of agglomerate structures, not fibrillar structures. *Toxicol. Sci. Off. J. Soc.*
593   *Toxicol.* **113**, 226–242 (2010).

594   21. Hotamisligil, G. S. Inflammation and metabolic disorders. *Nature* **444**, 860–867 (2006).

595   22. Röhrig, F. & Schulze, A. The multifaceted roles of fatty acid synthesis in cancer. *Nat. Rev. Cancer* **16**,
596   732–749 (2016).

597   23. Peck, B. & Schulze, A. Lipid Metabolism at the Nexus of Diet and Tumor Microenvironment. *Trends*
598   *Cancer* **5**, 693–703 (2019).

599   24. Qiao, Y. *et al.* FABP4 contributes to renal interstitial fibrosis via mediating inflammation and lipid
600   metabolism. *Cell Death Dis.* **10**, 382 (2019).

601   25. Bourdon, J. A. *et al.* Hepatic and pulmonary toxicogenomic profiles in mice intratracheally instilled with
602   carbon black nanoparticles reveal pulmonary inflammation, acute phase response, and alterations in lipid
603   homeostasis. *Toxicol. Sci. Off. J. Soc. Toxicol.* **127**, 474–484 (2012).

26. Poulsen, S. S. *et al.* Changes in cholesterol homeostasis and acute phase response link pulmonary exposure to multi-walled carbon nanotubes to risk of cardiovascular disease. *Toxicol. Appl. Pharmacol.* **283**, 210–222 (2015).

27. Urbančič, I. *et al.* Nanoparticles Can Wrap Epithelial Cell Membranes and Relocate Them Across the Epithelial Cell Layer. *Nano Lett.* **18**, 5294–5305 (2018).

28. Pollard, T. D. & Cooper, J. A. Actin, a Central Player in Cell Shape and Movement. *Science* **326**, 1208–1212 (2009).

29. Tran, D. T., Masedunskas, A., Weigert, R. & Hagen, K. G. T. Arp2/3-mediated F-actin formation controls regulated exocytosis in vivo. *Nat. Commun.* **6**, 1–10 (2015).

30. Khaitlina, S. Y. Intracellular transport based on actin polymerization. *Biochem. Biokhimiia* **79**, 917–927 (2014).

31. Li, P., Bademosi, A. T., Luo, J. & Meunier, F. A. Actin Remodeling in Regulated Exocytosis: Toward a Mesoscopic View. *Trends Cell Biol.* **28**, 685–697 (2018).

32. Tran, D. T. & Ten Hagen, K. G. Real-time insights into regulated exocytosis. *J. Cell Sci.* **130**, 1355–1363 (2017).

33. Laskin, D. L., Malaviya, R. & Laskin, J. D. Chapter 32 - Pulmonary Macrophages. in *Comparative Biology of the Normal Lung (Second Edition)* (ed. Parent, R. A.) 629–649 (Academic Press, 2015). doi:10.1016/B978-0-12-404577-4.00032-1.

34. Gosens, I. *et al.* Comparative Hazard Identification by a Single Dose Lung Exposure of Zinc Oxide and Silver Nanomaterials in Mice. *PLoS ONE* **10**, (2015).

35. Akratopulu, K. C., Vordonis, L. & Lycourghiotis, A. Effect of temperature on the point of zero charge and surface dissociation constants of aqueous suspensions of γ-Al2O3. *J. Chem. Soc. Faraday Trans. 1 Phys. Chem. Condens. Phases* **82**, 3697–3708 (1986).

36. Berendsen, H. J. C., Postma, J. P. M., van Gunsteren, W. F., DiNola, A. & Haak, J. R. Molecular dynamics with coupling to an external bath. *J. Chem. Phys.* **81**, 3684–3690 (1984).

37. Páll, S. & Hess, B. A flexible algorithm for calculating pair interactions on SIMD architectures. *Comput. Phys. Commun.* **184**, 2641–2650 (2013).

631 38. Darden, T., York, D. & Pedersen, L. Particle mesh Ewald: An N·log(N) method for Ewald sums in large

632   systems. *J. Chem. Phys.* **98**, 10089–10092 (1993).

633 39. Bussi, G., Donadio, D. & Parrinello, M. Canonical sampling through velocity rescaling. *J. Chem. Phys.*

634   **126**, 014101 (2007).

635 40. Hess, B. P-LINCS: A Parallel Linear Constraint Solver for Molecular Simulation. *J. Chem. Theory*

636   *Comput.* **4**, 116–122 (2008).

637 41. Abraham, M. J. *et al.* GROMACS: High performance molecular simulations through multi-level

638   parallelism from laptops to supercomputers. *SoftwareX* **1–2**, 19–25 (2015).

639 42. Humphrey, W., Dalke, A. & Schulten, K. VMD: visual molecular dynamics. *J. Mol. Graph.* **14**, 33–38,

640   27–28 (1996).

641 43. Jämbeck, J. P. M. & Lyubartsev, A. P. Derivation and Systematic Validation of a Refined All-Atom Force

642   Field for Phosphatidylcholine Lipids. *J. Phys. Chem. B* **116**, 3164–3179 (2012).

643 44. Agosta, L., Brandt, E. G. & Lyubartsev, A. P. Diffusion and reaction pathways of water near fully

644   hydrated TiO2 surfaces from ab initio molecular dynamics. *J. Chem. Phys.* **147**, 024704 (2017).

645 45. Jorgensen, W. L., Chandrasekhar, J., Madura, J. D., Impey, R. W. & Klein, M. L. Comparison of simple

646   potential functions for simulating liquid water. *J. Chem. Phys.* **79**, 926–935 (1983).

647 46. Yoo, J. & Aksimentiev, A. Improved Parametrization of Li+, Na+, K+, and Mg2+ Ions for All-Atom

648   Molecular Dynamics Simulations of Nucleic Acid Systems. *J. Phys. Chem. Lett.* **3**, 45–50 (2012).

Acknowledgements

Author contributions

656 These authors have contributed equally: Hana Majaron, Boštjan Kokot, Aleksandar Sebastijanović.

657

Affiliations

659 Department of Condensed Matter Physics, Jožef Stefan Institute, Ljubljana, Slovenia

660 Hana Majaron, Boštjan Kokot, Aleksandar Sebastijanović, Rok Podlipec, Patrycja Zawilska, Ana Krišelj, Mojca
661 Pušnik, Petra Čotar, Polona Umek, Stane Pajk, Iztok Urbančič, Tilen Koklič, Janez Štrancar

662

663   Jožef Stefan International Postgraduate School, Jamova cesta 39, 1000 Ljubljana, Slovenia

664   Hana Majaron, Aleksandar Sebastijanović

665

666   Faculty of Natural sciences and Mathematics, University of Maribor, Maribor, Slovenia

667   Boštjan Kokot

668

669   Institute of Lung Biology and Disease, Helmholtz Zentrum München, 85764 Neuherberg, Germany

670   Carola Voss, Carolina Ballester, Qiaoxia Zhou, Otmar Schmid, Martin Irmler, Johannes Beckers, Tobias
671   Stoeger

672

673   National Research Centre for the Working Environment, Copenhagen Ø, Denmark

674   Trine Berthing, Pernille H. Danielsen, Ulla B. Vogel

675

676   Faculty of Pharmacy, University of Ljubljana, Ljubljana, Slovenia

677   Stane Pajk, Mojca Pušnik

678

679   Faculty of Mathematics and Physics, University of Ljubljana, Ljubljana, Slovenia

680   Petra Čotar

681

682   Department of Chemistry, Imperial College London, London, United Kingdom

683   Claudia Contini, Matthew Schneemilch, Nicholas Quirke

684

685   Institut Jean Lamour, CNRS-Université de Lorraine, Nancy, France

686   Olivier Joubert

687

688   School of Physics, University College Dublin, Belfield, Dublin 4, Ireland

689   Vladimir Lobaskin, Vadim Zhernovkov

690

691   Health Canada

692   Sabina Halappanavar

693

694   Department of Materials and Environmental Chemistry, Stockholm University, SE-10691 Stockholm, Sweden

695   Alexander Lyubartsev, Mikhail Ivanov

696

697   Joint Research Centre

698   Jessica Ponti

699   Corresponding authors
700   Correspondence to Janez Štrancar, Tilen Koklič and Tobias Stoeger.

Materials & Correspondence

702   Materials and correspondence should be addressed to H.M, B.K. or A.S.

Ethics declarations

704   Competing interests
705   The authors declare no competing interests.

Supplementary information

707   <u>Supplementary information</u>

708   This file contains the Supplementary Discussion, Supplementary References and a full guide for
709   Supplementary.

Source data

711   Is currently available upon request.