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Specific Peptide Surface Coating: A Hint to Tune the Inflammatory Response of Nano Crystals

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Abstract

We provided a comprehensive line of evidence indicating Lanthanide-based nanoparticles elicited NLRP3 dependent inflammasome activation *in vitro* and inflammatory response *in vivo*. A short synthetic peptide identified by our group, RE-1(ACTARSPWICG), could form a stable coating layer on the surface of Lanthanide-based nanoparticles (LNs) through specifically binding and effectively block their autophagy-inducing activity and liver toxicity. Recently, RE-1 coating were also demonstrated to significantly abrogate LN-elicited inflammasome activation without influencing cell uptake of nanocrystals in macrophage cells, and inflammatory response in peritoneal cavity. Furthermore, the mechanism of the inflammasome-inhibiting effect of RE-1 coating was investigated. RE-1 coating did not effectively reduce LN-elicited potassium efflux, while the potassium channel inhibitor glibenclamide did, indicating that potassium efflux was necessary but insufficient for LN-induced inflammasome activation. RE-1 did reduce lysosomal damage induced by LNs, However, the inhibitor of cathepsin B could not alter LN-elicited caspase-1 activation and IL-1β release, indicating that lysosomal damage was not critically important for LN-induced inflammasome activation. In contrast, RE-1 could dramatically inhibit LN-induced up-regulation of intracellular reactive oxygen species (ROS), greatly important for inflammasome activation. And, the reduction on NADPH oxidase-generated ROS was more critical for RE-1's inflammasome-inhibiting effect than the reduction on mitochondria-generated ROS. ROS generation further triggered Transient Receptor Potential M2 (TRPM2) regulated Ca^{2+} influx to induce inflammasome activation, which could be completely canceled by RE-1 coating. In conclusion, RE-1 primarily inhibited NADPH oxidase regulated ROS generation and subsequently curbed TRPM2-mediated Ca^{2+} influx to abrogate LN-induced inflammasome activation. Our study gives a new direction to modulate the inflammatory response of nanocrystals to realize immune escape via surface peptide coating, great value for *in vivo* applications of engineered nanomaterials.

Commentary

Many kinds of nanoparticles have also gained widespread use in biomedical fields owing to their unique phsy-chemical properties. Happening with this, the potential health hazards of these nano-materials upon entering human body gradually attract people's attention. Inflammation is one of major parts of toxicity for nanoparticles. Inflammation is an important mechanism to protect the human body from both exogenous and endogenous "danger signals" [1]. Recognized as a critical mediator of inflammation, the inflammasomes, a family of cytosolic multi-protein complexes, contain Nod-like receptor (NLR) and caspase-1 proteins. Upon cellular infection or some detrimental stimuli, Inflammasomes would be triggered to recruit and active pro-inflammatory caspase-1 and subsequently accelerate maturation and secretion of several pro-inflammatory cytokines such as IL-1β and IL-18 [2,3].

A large number of nanoparticles were reported to induce inflammatory response. Having been widely used as contrast agents and in thermal therapy for cancer, iron oxide nanoparticles may subchronic induce inflammatory responses via oxidative stress in mice by a single intratracheal instillation [4]. Deposition of carbon black nanoparticles in lung induces inflammatory and genotoxic effects in mouse lung that persist considerably after the initial exposure [5]. Four metal oxide nanoparticles (CeO_2, NiO, ZnO, and CuO) were inflammogenic to the lungs of rats at the high doses used with differential inflammatory footprint [6]. Zinc oxide nanoparticles are internalized through caveolae pathway and the inflammatory responses involve PI3K mediated MAPKs signaling cascade [7]. Tanatase TiO_2 nanoparticles Induced inflammatory responses compared with other type of TiO_2 particles [8]. The application of lanthanide nanoparticles including rare earth oxide nanoparticles and lanthanide upconversion nanoparticles (UCNs) in the theranostics of some diseases is increasing rapidly, because of their excellent optical Characteristics and facile functionalization strategies [9]. Li et al. reported that lanthanide nanoparticles including rare earth oxide nanoparticles and lanthanide upconversion nanoparticles are capable of activating the NLRP3 inflammasome and inducing IL-1β secretion

[10,11]. Consistently, we also found a comprehensive line of evidence indicating LN-elicited NLRP3-mediated inflammasome activation *in vitro* and inflammatory response *in vivo*.

As inflammation contributes to a major part of toxicity for nanoparticles, curbing nanoparticles induced inflammation is essential for their application in biomedical fields.

Several strategies were reported to subsidize nanoparticles-induced inflammatory response. A protein corona, which forms on engineered particles as soon as they are introduced into biological environments, is known to provide particles with a "biological identity". It were demonstrated that protein coronas generally reduce cytotoxicity and immunotoxicity [12]. Peptide coated gold nanoparticle gave a potent anti-inflammatory activity in phagocytic immune cells, indicating specific peptide decorated nanoparticles that may represent a novel class of anti-inflammatory therapeutics for human inflammatory diseases [13]. Cell penetrating anti-inflammatory peptide KAFAKLAARLYRKALARQLGVAA (KAFAK) has the ability to suppress pro-inflammatory cytokines TNF-α and IL-6 when released from degradable and non-degradable Poly (NIPAm-AMPS) nanoparticles, indicating that KAFAK modification should reduce nanoparticles elicited inflammatory response [14]. Restoration of miR-155 expression significantly decreased by manganese nanoparticles completely abrogated that induced inflammatory response, which demonstrated that unique miRNA expression profiles provide novel targets for manipulating gene and protein expression, and therefore provide the potential of modifying cellular responses to NP exposure [15].

Previous report from our group showed that a short synthetic peptide, RE-1(ACTARSPWICG), identified by phage display, could bind to lanthanide (LN) oxide and upconversion nanocrystals (UCN), form a stable coating layer on their surface, and effectively blocks their autophagy-inducing activity and liver toxicity [16]. Herein, after RE-1 coating the surface of three representative LNs(UCN, Nd2O3 and Y2O3) by a simple coating procedure, LNs-induced inflammasome activation could be significantly abrogated in mouse bone marrow derived macrophage (BMDM) cells, human THP-1 cells and mouse peritoneal macrophages indicated by the huge inhibition of Caspase-1 activation and IL-1β secretion. No significant change of IL-1β secretion or nigericin-induced IL-1β secretion happened after the treatment with RE-1 peptide itself. The same abrogation of inflammasome activation was induced by surface coated UCN either with or without being washed after the coating procedure. These results showed that RE-1 itself and some unknown factors in the peptide binding buffer did not show inflammasome-inhibiting effect. And no infuence on the amount and uptake pathway of UCN internalized into BMDM cells by RE-1 coating indicated that RE-1 reduced inflammasome activation was not due to decreasing cellular internalization and altering uptake pahway of UCN. In addition, the lower levels of IL-1β secretion and neutrophil recruitment after intraperitoneal injection of RE-1 coated LNs relative to naked nanocrystals further verified RE-1's capacity of reducing the inflammasome activating activity of LNs.

Cytosolic Potassium efflux, lysosomal damage, reactive oxygen species (ROS) generation and intracellular calcium elevation were reported to be essential for the activation of the NLRP3 mediated inflammasome, while cathepsin B release from damaged lysosomes still controversial. Nextly, we assessed the mechanism of the RE-1's inhibitory effect on LN-elicited inflammasome activiation. Potassium efflux or lysosomal damage was inessential for the inflammasome-

inhibiting effect induced by RE-1 coating. Glibenclamide, a potassium channel inhibitor, could significantly decrease UCN-elicited potassium efflux, underlying blocking LN-induced NLRP3 inflammasome activation, while RE-1 coating did not. Hence, RE-1 coating abrogated the inflammasome activation did not result from inhibiting potassium efflux, and potassium efflux was necessary but insufficient for UCN-elicited inflammasome activation. UCN did induce lysosome alkalization mainly resulting from lysosome damage, which could be partially curbed by RE-1 coating. However, cathepsin B release was not the cause for UCN elicited inflammasome activation, because caspase-1 activation and IL-1β release elicited by UCN could not be inhibited by the cathepsin B inhibitor CA-074-Me.

We discovered that RE-1 coating could abrogate UCN-elicited NLRP3 inflammasome activation via inhibiting ROS generation from NADPH oxidase. UCN could significantly rise ROS generation. The free radical scavenger N-acetyl cysteine (NAC) did reduce caspase-1 activation and IL-1β release elicited by UCN. Interestingly, RE-1 coating did the same thing with NAC. As Intracellular ROS generation mainly from NADPH oxidase and damaged mitochondria, we further assessed their respective influence in UCN-induced inflammasome activation. UCN really elevated mitochondria-generated ROS, while RE-1 coating only gave it a slight decrease. However, the intracellular overall ROS elevated by UCN was completely abrogated by DPI and VAS2870, two inhibitors of NADPH oxidase. MitoTEMPO, an specific inhibitor of ROS in mitochondria, induced 30% reduction of UCN-induced IL-1β release, while DPI and VAS2870 achieved 70%. In general, abrogation of intracellular ROS generation was the primary mechanism underlying the inflammasome-inhibiting effect of RE-1 coating, and inhibition of NADPH oxidase-generated ROS was the major contributor for the observed suppression of ROS production.

Interestingly, the abolishment of TRPM2-mediated Ca²⁺ influx elicited by UCN through RE-1 coating was also demonstrated to inhibit UCN-induced inflammasome activation. Confocal microscopy and FACS analysis revealed that naked UCN treatment dramatically increased the level of intracellular Ca²⁺. Elevation of intracellular Ca²⁺ is proposed to be one factor to trigger NLRP3 inflammasome activation. Here, we demonstrated that calcium influx triggered UCN induced caspase-1 activation and IL-1β secretion, because BAPTA-AM, a chelating agent of Ca²⁺, could abrogated UCN induced NLRP3 inflammasome activation by inhibiting the Ca²⁺ influx. Nextly, we would like to verify whether RE-1 coating could inhibit UCN elicited Ca²⁺ influx underlying inhibiting NLRP3 inflammasome activation. Our study showed that RE-1 coating really could significantly block Ca²⁺ influx to subsequently withdraw caspase-1 activiation and IL-1β secretion. It's also reported that NLRP3 inflammasome activation elicited by crystals could be regulated by a nonselective and redox-sensitive cation channel TRPM2. So we would like to query whether UCN elicited Ca²⁺ influx, and the subsequent NLRP3 inflammasome activation were regulated by TRPM2. Interestingly, both TRPM2 inhibition by the chemical inhibitor ACA or 3MFA and knockdown by TRPM2-targeting shRNA did suppress Ca²⁺ influx in UCN-stimulated macrophages, and further inhibit UCN-induced caspase-1 activation and IL-1β secretion. Taken together, these results demonstrated that the observed calcium influx was mediated by TRPM2 and was critical for UCN-elicited inflammasome activation, and that the whole process was effectively suppressed by RE-1 coating.

Further study clarified that UCN elicited ROS generation was upstream of TRPM2-mediated calcium influx. Both the ROS scavenger NAC and the NADPH oxidase inhibitor DPI significantly inhibited

UCN-induced Ca^{2+} influx, while the TRPM2 inhibitors ACA and 3MFA did not affect UCN elicited ROS generation. These results demonstrated that UCN firstly induced ROS generation and subsequently activated TRPM2-mediated calcium influx to elicit the inflammasome activation. Hence, RE-1 coating could dramatically abrogate LN-elicited NLRP3 inflammasome activation mainly through inhibiting NADPH oxidase induced ROS generation and the subsequent TRPM2-mediated calcium influx.

Besides that, we think two points are necessary to be verified in our following study to help us understand the molecular mechanism of RE-1 coating mediated inflammatory inhibition. Many reports have showed that the inflammatory responses of nanoparticles were related with the nanometer characterization. It's demonstrated that more Inflammation could be induced by smaller size silver nanoparticles in Lung Tissues [17]. Barbara Rothen-Rutishauser et al. clarified that particle size and material affected the cellular responses to particle exposure via changing the translocation and entering characteristics of nanoparticles [18]. It's also indicated that nanoparticles(NPs) induced pro-inflammatory responses are well correlated not only with the BET (Brunauer, Emmett and Teller) surface of the individual NPs but also with the internalized amount of NPs, and Differences of even few nanometers in primary particle size lead to significant changes in inflammatory response [19]. Mesoporous silica nanoparticles MPS nanoparticles induced lower expression of pro-inflammatory cytokines than colloidal silica, indicating that the pore architecture of silica nanoparticles greatly influenced cellular immune response [20]. Differential inflammatory response were elicited by hydroxyapatite nanoparticles with differing morphologies (long rods, short rods, sheets, fibres), giving a hint that particle morphology affects inflammation activiation [21]. We have showed that RE-1 coating absolutely changed the surface architecture of LNs and increased the zeta potentials and sizes of UCNs relative to naked UCNs. To study the possible relationship between the change of nanometer characterization and inflammatory inhibition by RE-1 coating will further help us to understand the molecular mechanism of RE-1 coating mediated inflammatory modulation. In addition, protein coronas, located on engineered particles as soon as they are introduced into biological environments, generally decrease nanoparticles elicited inflammatory response. And the formation and immunological response to NP-protein coronas could be mainly affected by the physiochemical surface properties of the NPs (ie, physical surface architecture and chemical functionality). To analyze the differences of protein corona on the surface of naked UCNs and RE-1 coated UCNs in the cell culture medium or Ascites would be benefit for us to further understand how RE-1 coating influence UCN induced inflammasome activiation.

Different from surface-modifying polymers such as PEG, peptides like RE-1 work through specific high-affinity interactions rather than through non-specific absorption or covalent modification [16]. This method brings out an advantage that is high consistency and ease in scaling up because of the simple add-and-mix coating procedure [16]. In addition, unlike covalent modification, it is no need to supply some other chemical agents during RE-1 coating. RE-1 coating could abrogate lanthanide nanoparticle induced inflammatory response and increase their biocompatibility when lanthanide nanoparticles are used for the theranostics of some diseases especially for cancer. Peptide variants basing on RE-1 with functional chemical groups are easily synthesized to tune the interaction between cells and nanoparticles for different uses. A bi-functional peptide RE-1-RGD (CRGDCGGACTARSPWICG) is combining the RE-1 sequence with the RGD motif via a short linker [16]. RE-1-RGD coated UCN are prone to be uptook by cancer cells to realize the imaging of cancer cells [16]. Our study gives a new perspective that specific surface peptide coating might be used to control the extent of inflammasome activation of nanocrystals especially for LNs, great value for *in vivo* applications of engineered nanomaterials.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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