Polyhydroxylated Metallofullerenols Stimulate IL-1β Secretion of Macrophage through TLRs/MyD88/NF-κB Pathway and NLRP3 Inflamasome Activation

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Polyhydroxylated fullerenols especially gadolinium endohedral metallofullerenols (Gd@C$_{82}$(OH)$_{22}$) are shown as a promising agent for antitumor chemotherapeutics and good immunoregulatory effects with low toxicity. However, their underlying mechanism remains largely unclear. We found for the first time the persistent uptake and subcellular distribution of metallofullerenols in macrophages by taking advantages of synchrotron-based scanning transmission X-ray microscopy (STXM) with high spatial resolution of 30 nm. Gd@C$_{82}$(OH)$_{22}$ can significantly activate primary mouse macrophages to produce pro-inflammatory cytokines like IL-1β. Small interfering RNA (siRNA) knockdown shows that NLRP3 inflamasomes, but not NLRC4, participate in fullerenol-induced IL-1β production. Potassium efflux, activation of P2X$_7$ receptor and intracellular reactive oxygen species are also important factors required for fullerenols-induced IL-1β release. Stronger NF-κB signal triggered by Gd@C$_{82}$(OH)$_{22}$ is in agreement with higher pro-IL-1β expression than C$_{60}$(OH)$_{22}$. Interestingly, TLR4/MyD88 pathway but not TLR2 mediates IL-1β secretion in Gd@C$_{82}$(OH)$_{22}$ exposure confirmed by macrophages from MyD88$^{-/-}$/TLR4$^{-/-}$/TLR2$^{-/-}$ knockout mice, which is different from C$_{60}$(OH)$_{22}$. Our work demonstrated that fullerenols can greatly activate macrophage and promote IL-1β production via both TLRs/MyD88/NF-κB pathway and NLRP3 inflamasome activation, while Gd@C$_{82}$(OH)$_{22}$ had stronger ability C$_{60}$(OH)$_{22}$ due to the different electron affinity on the surface of carbon cage induced by the encaged gadolinium ion.

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1. Introduction

A number of functionalized fullerene derivatives have been widely investigated in biological applications after the main problem of poor solubility in the cellular environment was solved by chemical or supramolecular approaches. Due to their attractive physical, chemical, and biological properties and the availability of various methods for surface modification, fullerene derivatives were synthesized to achieve promising biomedical applications.\cite{1-3} For example, metallofullerenes show novel electron transfer properties and can protect the metal ion encapsulated from leakage and dissociation. Thus various Gd@C$_{n}$ (n = 60, 82) derivatives like Gd@C$_{60}$[C(COOH)$_{2}$]$_{10}$\cite{4} and Gd$_{2}$N@C$_{80}$ fullerenes\cite{5-8} have the potential for MRI application in clinical diagnosis. Some derivatives such as carboxyl fullerenes and polyhydroxyl fullerenes have been proposed as antioxidants and free radical scavengers for protection of neurodegenerative diseases, inhibition of apoptosis, etc.\cite{9-12} Cationic fullerene derivatives show promising anti-virus effect because of RNA-dependent polymerases inhibition.\cite{13,14} And besides, as well as fullerenes modified with PEG, pullulan etc, these derivatives possess interesting photo-physical properties and generate reactive oxygen species (ROS) by exposure to visible light. So they are developed to be potentially strong agents for photodynamic therapy, not only as antimicrobials and photosensitizers\cite{15,16} but also agents for tumor therapy.\cite{17,18} By exposure to low-intensity ($< 10^{3} \text{ W cm}^{-2}$) continuous-wave laser irradiation, certain functionalized fullerenes, especially polyhydroxyl fullerenes and carboxyl fullerenes were found to be heated to their ignition temperature recently. This heating property would be extremely advantageous for cancer photothermal therapy.\cite{19} In our previous studies, polyhydroxyl fullerenes (fullerenols) especially gadolinium endohedral metallofullerolen (Gd@C$_{62}$(OH)$_{22}$) exhibited an efficient antitumor activity with low toxicity.\cite{20} The possible mechanisms of fullerenols inhibiting tumor growth and metastasis in vivo are highly related to multiple ways, including oxidative stress modulation in tumor tissues,\cite{21-23} potent inhibition of tumor angiogenesis through reducing the formation of angiogenesis factors, tumor vessel density and the nutrient supply to tumor cells,\cite{24,25} and inhibiting the production of matrix metalloproteinase (MMP) enzymes.\cite{26} Notably, Gd@C$_{62}$(OH)$_{22}$ was found to have good regulatory effects on immune response, including activating antigen presenting cells, dendritic cells (DCs) and macrophages, promoting T cells proliferation, and polarizing towards T-helper cell type 1 (Th1) immune responses.\cite{27-30} All these results suggest that fullerenols affect not only various phases of the malignant process, such as carcinogenesis, tumor growth, and invasiveness, but also the interactions between malignant cells and host immune system.

Pathogen recognition by the innate immune system is mediated by a limited number of germline-encoded receptors, referred to as pattern-recognition receptors (PRRs). These receptors recognize conserved molecular patterns (pathogen-associated molecular patterns, PAMPs), they are expressed by many cell types including macrophages, monocytes, dendritic cells (DCs), neutrophils, and epithelial cells.\cite{31,32} Four kinds of PRRs families were found to be involved in the process, including Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-like helicases (RLHs), and C-type lectin receptors (CLRs).\cite{33} Nod-like receptors (NLRs) are cytoplasmic molecules thought to sense the presence of intracellular PAMPs, as well as the products of damaged tissues (danger-associated molecular patterns, DAMPs), thereby initiating host innate immune responses. To date, 22 members of the human NLR protein family have been reported.\cite{34} The best-characterized inflammasome consists of three main components, the Nod-like receptor (NLR)-family protein, NLRP3, procaspase-1, and the ASC (apoptosis speck-like protein containing a CARD) adapter, which bridges the interactions between the former proteins.\cite{35} Various bacterial compounds\cite{36,37} and ‘danger signals’ originate from vaccines such as aluminum salt (alum) adjuvant,\cite{38,39} from environmental pollutants including crystalline silica and asbestos,\cite{40,41} and from engineered nanoparticles such as long and needle-like carbon nanotubes, TiO$_{2}$ (20 and 80 nm) and silver nanoparticles\cite{35,42,43} can induce IL-1β secretion through activating NLRP3-inflammasome, and ultimately inducing an inflammatory response. However, most study of DAMPs in vitro showed that they cannot stimulate inflammation response independently. A two-signal model has been proposed to explain the regulation of IL-1β production. The synthesis of pro-IL-1β and NLRP3 is firstly triggered by transcriptional induction via ligands for Toll-like receptors (TLRs), whereas the second signal composed of inflammasome oligomerization, caspase-1 auto-activation leads to caspase-1-dependent cleavage of pro-IL-1β and the release of the biologically active, mature IL-1β.\cite{44,45}

So far, ten and twelve functional TLRs have been identified in humans and mice, respectively, with TLR1-TLR9 being conserved in both species.\cite{46} There is also a growing body of evidence to indicate that certain TLRs also sense other danger signals.\cite{47} Recently, a few reports suggested that TLRs are responsible for the induction of the immune response by engineered nanoparticles. Uto et al. reported that biodegradable poly(γ-glutamic acid) (γ-PGA) nanoparticles are able to induce potent innate and adaptive immune responses through TLR4/MyD88 pathways.\cite{48} We also found that pristine graphene induces the production of cytokines/chemokines by macrophages via TLR- and NF-κB-related signaling pathways.\cite{49} However, these studies are lack of comprehensive discussion for the process of immunoregulation. Previously, we found that metallofullerenols can stimulate IL-1β secretion from macrophages without any additional stimulus.\cite{24} The precise mechanisms of immunoregulation process induced by metallofullerenols remains unclear.

Herein, we investigated the ability of two fullerenols (C$_{60}$(OH)$_{22}$ and Gd@C$_{62}$(OH)$_{22}$), especially Gd@C$_{62}$(OH)$_{22}$, on activating macrophages. The underlying mechanism was carried out by cellular uptake and localization, western blotting analysis, immunofluorescence staining, small interfering RNA (siRNAs) assay and MyD88/TLR2/TLR4 knockout mouse experiments. Gd@C$_{62}$(OH)$_{22}$ can trigger profound IL-1β precursor production via TLRs/MyD88/NF-κB pathway and promote IL-1β release via NLRP3 inflammasome activation.
2. Results

2.1. Metallofullerenols Uptaken by Macrophages Keep Rising and Mainly Distribute in Cytoplasm

The morphology of fullerenols was found to be spherical/ellipsoidal in aqueous solution. Hydroxylated fullerene is actually a polyacidic molecule, and each proton of the hydroxyl groups (C–OH) can dissociate in aqueous solution, thus yielding a conjugated base C–O–. [10] Fullerenol and metallofullerenol are well dissolved in water and can form the polyanion nano-aggregates, whose size is mostly between 20 nm and 100 nm dependent on concentration, pH, temperature, ionic strength, and the presence of a relatively high molecular weight dissolved organic acid. The average diameter of Gd@C$_{82}$ (OH)$_{22}$ and C$_{60}$ (OH)$_{22}$ nanoparticles determined by DLS are 78 ± 5.3 and 123 ± 14.2 at 200 µM in PBS solution. [22,26,29] To make sure how fullerenols act on macrophages, we firstly observed uptake process of fullerenols by macrophages and their subcellular localization. For a long time, it is difficult to detect the biodistribution and subcellular localization of fullerene derivatives because of their carbon-structures. The spatial distribution of Gd at a high resolution of 30 nm can be obtained by the help of synchrotron-based scanning transmission X-ray microscopy (STXM) using the newly constructed BL08U1A STXM beamline at the Shanghai Synchrotron Radiation Facility (SSRF), a 3.5 GeV third-generation synchrotron source.

Near edge X-ray absorption fine structure (NEXAFS) spectrum from Gd@C$_{82}$ (OH)$_{22}$ near the M-edge of Gd atom (Figure 1A) was obtained and then, two absorption images of cells were scanned at two energies, E1 (1189 eV) and E2 (1185 eV) above and below the absorption edge of Gd, respectively. By calculating the signal ratio of each pair of the corresponding pixels’ optical density in the two absorption images, the cellular map of the spatial distribution of Gd demonstrated that the large amount of Gd@C$_{82}$ (OH)$_{22}$ had been uptaken by peritoneal macrophages after injected peritoneally into mice for nearly one week (Figure 1B).

We further investigated the internalization of Gd@C$_{82}$ (OH)$_{22}$ in vitro. Gd@C$_{82}$ (OH)$_{22}$ nanoparticles uptaken by primary mouse peritoneal macrophages and RAW264.7 were observed after 3 hours exposure, and kept increasing within 48 hours. The internalized Gd@C$_{82}$ (OH)$_{22}$ mainly distributed in cytoplasm, barely or not entered into nucleus (Figure 1C).

The internalized Gd@C$_{82}$ (OH)$_{22}$ in macrophages in vivo and in vitro was quantified by inductively coupled plasma mass
spectrometry (ICP-MS). Mean value of treated macrophages in vivo was 40.65 ppb, which means the average quantity of Gd@C_{82}(OH)_{22} was about 26 fmol in each macrophage, i.e. \(1.5 \times 10^9\) molecules per cell. Moreover, the amount of metallofullerenols in macrophages increased in the process of exposure in vitro with \(3 \times 10^{10}\) molecules per cell at 48 hours (Figure 1D), which was consistent with results of the Gd elemental mapping.

2.2. Fullerenols Stimulate the Secretion of IL-1β through NLRP3 Inflammasome

IL-1 is a pleiotropic cytokine. Except primary effect on inflammatory and immune responses, it also regulates other homeostatic functions of the body, involves in the pathogenesis of disease and malignant processes. As one of the most important pro-inflammatory cytokines of IL-1 family, IL-1β has been thoroughly studied. It is synthesized as precursors of 31 kDa and further processed by proteases caspase-1 to the mature secreted 17 kDa forms, which is always the result of NLRP3 inflammasome activation. It has been shown that RAW 264.7 cells lack ASC protein, which is an essential part of the inflammasome complex and therefore the cell line is unsuitable for NLRP3 inflammasome activation studies. Primary mouse peritoneal macrophages were therefore used to evaluate the essential role of NLRP3 inflammasome for IL-1β secretion during fullerenols exposure. We first detected IL-1β secretion of macrophages during fullerenols exposure for 6 hours after K⁺ efflux inhibition and P2X₇ receptor inhibition. *p<0.05 and **p<0.01 compared to the control group.

Figure 2. Essential role of NLRP3 inflammasome for IL-1β secretion during fullerenols exposure in primary mouse peritoneal macrophages. (A) Time dependence of IL-1β secretion by macrophages. (B) Expression level of intracellular NLRP3 inflammasomes and caspase-1 p10 subunit by western blotting. (C-D) IL-1β secretion of macrophages during fullerenols exposure for 6 hours after NLRP3/NLRC4 siRNA transfection. (E) IL-18 secretion of macrophages after NLRP3 siRNA transfection. (F-G) IL-1β secretion of macrophages during fullerenols exposure for 6 hours after K⁺ efflux inhibition and P2X₇ receptor inhibition. *p<0.05 and **p<0.01 compared to the control group.
fullerenols (Figure 2B). To clarify whether NLRP3 inflammasome is required for IL-1β secretion, we performed gene silencing with NLRP3 and NLRC4 targeting siRNA in macrophages. The NLRP3 siRNA treatment obviously decreased IL-1β secretion from macrophages after exposure to fullerenols, while NLRC4 siRNA had no influence on IL-1β secretion (Figure 2C, D). Similar results were also got for another pro-inflammatory cytokine IL-18 which belongs to the IL-1 family (Figure 2E). Confirmed with these results, NLRP3 inflammasome but not NLRC4 inflammasome is necessary for proinflammatory response during fullerenols exposure. K⁺ efflux, P2X7 receptor activation are known to be some important upstream activators of NLRP3 inflammasome. Here we utilized corresponding specific inhibitors: AZ11645373 for P2X7 receptor and KCl buffer for K⁺ efflux to determine whether these factors are required for NLRP3 inflammasome mediated IL-1β secretion during fullerenols exposures. Results showed that the inhibition of K⁺ efflux significantly blocked IL-1β secretion, while pre-treating with the P2X7 receptor inhibitor partly downregulated IL-1β secretion (Figure 2F and G).

2.3. Fullerenols Stimulate Macrophages to Produce IL-1β Without Additional Stimulus

Recently, some kinds of nanoparticles were found to promote pro-inflammatory cytokines IL-1β production. And a second stimulus was needed provided by additional stimulus such as LPS.[35,42] However, we found that mature IL-1β could be detected in the supernatant of macrophages very quickly (2 h) after exposure to fullerenols (Figure 2A), without the addition of co-stimulatory factors such as LPS (Figure 3A). To clarify whether fullerenols can stimulate macrophages to produce pro-IL-1β by itself, content of pro-IL-1β mRNA and intracellular protein were detected. Further results of real-time quantitative PCR (Figure 3B) and western blotting (Figure 3C) indicated that fullerenols could upregulate the expression of pro-IL-1β in macrophages. We also got the same result in fullerenols treated mouse macrophage cell line RAW264.7 (Figure 3D).

2.4. Fullerenols Promote the Expression of Pro-IL-1β through MyD88/NF-κB Pathway

It has been known that pathogen recognition by the innate immune system is mediated by TLRs. In the signaling pathway downstream of TLRs, myeloid differentiation factor 88 (MyD88) serves as a major adaptor protein which ultimately activates NF-κB pathways resulting in expression of pro-inflammatory genes.[46] In order to study whether NF-κB was activated after fullerenols exposure, NF-κB p65 immunofluorescence was done. We observed that p65 obviously traveled from the cytosol to the nucleus in macrophages at 0.5 hour exposure, and increased constantly in 1.5 hours, especially in
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Gd@C_{82}(OH)_{22} treated group (Figure 4A). The results above indicated that fullerenols initiated the activation and nuclear translocation of NF-κB rapidly. And more NF-κB p65 in cell nuclear triggered by Gd@C_{82}(OH)_{22} was consistent with more pro-IL-1β production. To certify whether NF-κB activation is required for pro-IL-1β production, we inhibited NF-κB activation using a specific inhibitor BAY 11–7082 in fullerenols-treated macrophages. Both of the IL-1β gene expression of cells and the IL-1β secretion of cell culture supernatant were obviously decreased (Figure 4B, C). NF-κB activation, which led to inflammatory responses, was crucial for IL-1β production in fullerenols-treated macrophages.

MyD88-dependent pathway, which was one of the major signals required for the NF-κB activation, was mediated by TLRs. In this work, we used MyD88, TLR2 and TLR4 knockout mice to investigate the crucial role of TLRs/MyD88 pathway for IL-1β production after fullerenols exposure. Primary macrophages from knockout mice and wild type mice were treated with fullerenols. In Gd@C_{82}(OH)_{22} exposure groups, IL-1β secretion were almost inhibited in MyD88−/− macrophages, which were partly impaired in TLR4−/− macrophages. And there was no difference between TLR2−/− and wild type macrophages. On the other hand, after C_{60}(OH)_{22} treatment, the level of IL-1β decreased when macrophages deficient in any of the three genes, compared with wild type ones (Figure 5). These results demonstrated that there was some differences between pathways triggered by Gd@C_{82}(OH)_{22} and C_{60}(OH)_{22}. Gd@C_{82}(OH)_{22} stimulated IL-1β secretion mainly via TLR4/MyD88 pathway, whereas C_{60}(OH)_{22} might act on multiple MyD88-dependent pathways or MyD88-independent pathways.

3. Discussion

The immune system distinguishes between “self” and danger (“non-self”) or “stranger” (“altered”-self). With the recognition of PAMPs and DAMPs by PPRs of phagocytic cells, immune responses are initiated to combat foreign intrusion or damage. Recently, studies have shown that engineered
nanomaterials (ENM) could be recognized and internalized by immune cells and influence the immune system, including initiating inflammatory responses. Previous works have demonstrated Gd@C82(OH)22 promoting T cell proliferation as a result of DCs and macrophages activation. However, the mechanism of Gd@C82(OH)22 acting on these immune cells and regulating immune responses is unclear. Notably, we observe the internalization and distribution of Gd@C82(OH)22 in macrophages by Soft-X-ray STXM. For X-rays, the energy of the X-ray photons is of the order of the binding energies of the atomic core electrons and this makes the X-ray spectroscopy element-specific. Thus, the X-ray absorption spectrum reveals the characteristic absorption edges of the elements and their chemical surroundings in the sample. STXM enables high-resolution NEXAFS spectroscopy and imaging, both at the 30-nm length scale with thin sample materials. And the element special distribution was analyzed with dual-energy contrast image. In the soft X-ray regime (E = 100–2000 eV), it is suitable to investigate the chemical composition and distribution of the organic specimens near the K-edge and the metallic element near L-edge. Here, for the most promising combination of soft X-ray absorption spectrum and imaging resolution, we attempted to obtain the information of intracellular Gd@C82(OH)22 by Soft-X-ray STXM near the Gd M-edge, and found it is also very suitable for high-resolution imaging of Gd element in cells with chemical sensitivity. The STXM results in vitro and in vivo revealed Gd@C82(OH)22 can be internalized by macrophages, which provide direct evidences of fullerenols acting on macrophages and initiating immune responses.

We further demonstrated Gd@C82(OH)22 can induce pro-inflammatory cytokine responses in macrophages like IL-1β and IL-18. IL-1β is a major agonistic protein of IL-1 family which primarily affects inflammatory and immune responses, and also has been extensively studied in the context of the malignant process especially cancer. Like tumor necrosis factor-α (TNF-α), IL-1β is defined as ‘an alarm cytokine’ that is secreted by macrophages and other nucleated cells. IL-1β initiates inflammatory responses which are necessary for the clearance of bacterial infection or foreign substances, including causing fever, increasing the level of circulating nitrogen oxide (NO), recruiting neutrophils, and costimulating T cell activation. IL-1β itself also has been shown to exhibit the adjuvant capacity to enhance humoral immunity. IL-18, which shares similar structure and functions with IL-1β, is also recognized as an important regulator of innate and adaptive immune responses. In addition, during anticancer chemotherapies, the production of IL-1β by DCs and the action of IL-1β on specific CD8+ T cells may contribute to therapeutically relevant anticancer immune responses. Like other reported nanoparticles, fullerenols triggered IL-1β secretion of primary macrophages depends on the NLRP3 inflammasome, but not NLRC4 inflammasome. Also, similar results of down-regulated IL-18 secretion after NLRP3 silencing during fullerenols exposure were obtained. These data further clarified the essential role of NLRP3 on fullerenols induced inflammatory responses. Various danger signals, PAMPs (pathogens or pathogens derived-molecules), DAMPs (extracellular ATP, Glucose, etc.) and environmental irritants (asbestos, silica, alum, etc.) activate the NLRP3 inflammasome. The mechanisms by which these structurally distinct molecules trigger NLRP3 oligimerization and inflammasome activation are varied and have been intensely debated in recent literature. All of the proposed models agree that cytoplasmic K+ concentration crucially affects inflammasome activation. In our work, higher K+ concentrations extracellular blocked IL-1β secretion of primary macrophage induced by fullerenols. This results revealed that, like other stimuli, K+ efflux during fullerenols exposure also may be the common trigger of NLRP3 inflammasome. K+ efflux from the cell should be factored into any proposed scheme for NLRP3 inflammasome activation. For example, some stimuli, e.g., ATP could activate endogenous K+ channels through activation of the P2X7 ATP-gated ion channel, which triggers rapid K+ efflux from the cell. Thus P2X7 inhibition more or less impaired IL-1β production of macrophages during fullerenols exposure presented here. However, the interplay between these pathways remains unclear.

IL-1β is highly regulated both transcriptionally and post-transcriptionally. The so called “rate-limiting” step of the IL-1β release appears to be the translation of the mRNA into the pro-IL-1β. It has been postulated that some nanoparticles promoting the release of active IL-1β usually need two signals: At first a signal for gene expression of pro-IL-1β, which is mediated by induction of cellular signaling pathways (e.g. NF-κB pathway) following PRR (e.g. TLRs) activation by PAMPs (e.g. LPS), a process sometimes described as “priming”; and a second signal through NLR signaling (e.g. Figure 5. Essential role of TLRs/MyD88 pathway for fullerenols induced IL-1β production in primary mouse peritoneal macrophages by using MyD88−/− (A), TLR4−/− (B) and TLR2−/− (C) knockout mice. *p<0.05 and **p<0.01 compared to the control group. *p<0.05 and **p<0.01 compared to the control group.
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NF-κB is an important transcription factor which regulates diverse cellular processes. One of the major functions is acting as a trans-activator of genes involved in proinflammatory cytokine production including IL-1β in macrophages, and thus regulates inflammatory response. So we hypothesized that the NF-κB signaling pathway might be responsible for the fullerenols-stimulated transcription and translation of pro-IL-1β. Results of NF-κB p65 immunofluorescence and inhibition of NF-κB from our work obviously showed that the activation of NF-κB plays a crucial role in the regulation of IL-1β production in fullerenols exposure. The activation of NF-κB is linked to both MyD88-dependent and -independent signaling pathways triggered by TLRs. TLRs are type-I integral membrane PRRs involved in induction of innate immune response. TLR4 is commonly expressed in innate immune cells such as neutrophils, macrophages, and dendritic cells. Activation of TLR4 triggers the innate immune response and leads to the induction of adaptive immunity, which contributes to antitumor therapy. TLR2 is another member of the family of TLRs for numerous bacterial products. Beside the potent inflammatory activities, TLR2 is also responsible for the adjuvant properties of bacterial lipoproteins. In our study, we decide to focus on TLR4 and TLR2 since these TLRs have been well studied for immune response. Macrophages deficient of MyD88, TLR4 apparently reduced IL-1β production triggered by Gd@C_{82}(OH)_{22} nanoparticles, which means Gd@C_{82}(OH)_{22} probably can be recognized by TLR4, and thus activates the NF-κB signaling pathway via MyD88. Whereas knockout macrophages in C_{60}(OH)_{22} showed multiple possible activation pathways, since TLR signaling pathways consist, at least, of a MyD88-dependent pathway that is common to all TLRs, and a MyD88-independent pathway that is peculiar to the TLR3 and TLR4 signaling pathways. In addition, Segovia J. et al found that activation of TLR2/MyD88 by RSV results in NF-κB activation and subsequent expression of NLRP3 genes. We suspect the result of upregulated NLRP3 protein expression is probably concerned with TLR/MyD88 pathway and NF-κB activation in fullerenols exposure. Nonetheless, further researches are needed to study the relationship between these two receptors.

Signals delivered from PRRs influence antigen presenting cells, with respect to the strength and type of immunity. Immunotherapy aims to assist the natural immune system in achieving control over immune responses, which are significant for cancer (including melanoma, leukemia, breast, and prostate cancer) and chronic infectious diseases (such as human immunodeficiency virus (HIV), cytomegalovirus (CMV), hepatitis B virus (HBV), and hepatitis C virus (HCV)). By the use of therapeutic vaccines and cytokines, for example, immune response especially antigen-specific T-cells response is promoted, while protective immunological memory is generated. Fullerenols have been found to take effect on macrophages, trigger PRRs signals-mediate proinflammatory cytokine responses. Since macrophage is one of the most important immune cells of innate immune system, our work may contribute to support that fullerenols link the innate immune system with adapted immune system.

In summary, we found the effect of fullerenols on IL-1β secretion of macrophages and demonstrated the mechanism of IL-1β secretion during fullerenol exposure in detail. On one hand, fullerenols prime macrophages to express pro-IL-1β via TLRs/MyD88 pathway subsequently activate NF-κB. On the other hand, fullerenols activate NLRP3 inflammasome under the assistance of relative factors including K+ efflux and P2X7 receptor activation in macrophages, which is required for processing pro-IL-1β into mature IL-1β. TLRs/MyD88/NF-κB pathway serves as the first signal for expression of pro-IL-1β. The second signal provided by NLRP3 inflammasome activation (Figure 6).

4. Conclusions

We have investigated the immunological impact of fullerenols nanoparticles on the macrophages. In this report, results focused on macrophages activation and multiple mechanisms of IL-1β release suggest that fullerenols especially Gd@C_{82}(OH)_{22} can activate macrophage via at least two signaling pathways, which would finally induce the proinflammatory cytokine-mediate immune responses. These data highlight the possible immunoregulatory mechanism in fullerenols exposure and also the possible role fullerenols takes in antitumor immune response. However, there still needs further research to confirm the immunoregulatory activity of fullerenols in vivo. Besides, the STXM technique we utilized to analyze the cell distribution of Gd@C_{60}(OH)_{22} may establish a method for assessing the cytological effects of metallofullerene nanoparticles, which are difficult to be visualized with traditional techniques.

5. Experimental Section

Cell Lines and Animals: Mouse macrophage cell line RAW264.7 was maintained in our lab. It was cultured in Dulbecco’s modified...
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Cellular Localization by STXM Analysis: In experiments in vivo, Gd@C_{82}(OH)_{22} nanoparticles were injected peritoneally into mice once a day for 6 times (2 μmol/kg), then peritoneal macrophages were separated and fixed on Si_{3}N_{4} membranes. In experiments in vitro, mouse peritoneal macrophages and RAW264.7 cell line were incubated and treated with Gd@C_{82}(OH)_{22} nanoparticles (50 μM) on 50 nm or 100 nm Si_{3}N_{4} membranes for 3 h, 24 h and 48 h. Cells were fixed in cold 70% ethanol for 40 min, and dehydrated in 85% ethanol for 15 min, 95% ethanol for 10 min and 100% ethanol for 10 min gradually. Then macrophages were scanned using Soft-X-ray STXM on beamline 08U in Shanghai Synchrotron Radiation Facility for Gd@C_{82}(OH)_{22} detection. We acquired images above-edge and before-edge, at 1,189 and 1,185 eV, the ratio of these two images provided a Gd distribution map.

Quantitative Determination by ICP-MS: After treated with Gd@C_{82}(OH)_{22} nanoparticles (50 μM) for 3 h, 24 h and 48 h, primary mouse peritoneal macrophages were collected and counted. 3 ml HNO_{3} was used to predigest cells. After mixed with 2 ml H_{2}O_{2}, samples were digested and heated at 150 °C–200 °C in open vessels on a hot plate. Remaining solution (about 1 ml) was cooled to room temperature and diluted to 3 ml with mixed acid solution containing 2% HNO_{3} and 1% HCl. Then samples were kept on being heated on a hot plate until the solution is 0.5 ml left.

Silencing of NLRP3 and NLRC4 with siRNAs Assay: Macrophages were isolated and purified as described above. After cultured in 24-well plate overnight, cells were transfected with 50 nM of three different NLRP3 siRNAs, three different NLRC4 siRNAs and negative control siRNA respectively, using Hiperfect Transfection Reagent (Qiagen) following the manufacturer’s instruction. 6 h after incubation, cell culture media were replaced with fresh media. Macrophages were treated with 50 μM of Gd@C_{82}(OH)_{22} or C_{60}(OH)_{22} 24 h post-transfection, and then supernatant was harvested 6 hours later. All the siRNA were synthesized by GenePharma (Shanghai, China).

Reverse Transcription PCR (RT-PCR) and Real-Time Quantitative PCR: Total RNA from cells were isolated by the methods of TRIzol reagent (Life Technology, CA, USA). 2 μg RNA was used to generate cDNA using 10 pmol of oligonucleotide primer and Moloney moloney murine leukemia virus reverse transcriptase (M-MLV; Promega, Madison, USA). Real-time quantitative PCR was performed using Master Mix (Promega, Madison, USA) and SYBR® Green I (Invitrogen, Paisley, UK) in a final reaction volume of 20 μL. The amplification cycle was performed by Realplex (Eppendorf) as follows: 95 °C for 2 min as initial denaturing step, followed by 40 cycles of denaturing (95 °C for 15 s), annealing (55°C for 15 s) and extending (72 °C for 15 s). PCR product was analyzed by detecting fluorescent value of SYBR® Green I. The primers used to detect the indicated genes by RT-PCR are shown below: Mouse pro-IL-1β Forward (5'-3') TGGGAAACAAACGTTGCTAGG,
Polyhydroxylated Metallofullerenols Stimulate IL-1β Secretion of Macrophage through TLRs/MyD88/NF-κB Pathway and NLRP3 Inflammasome Activation

Western Blotting Analysis: Expressions of intracellular pro-IL-1β, NLRP3 protein and caspase-1 p10 subunit were determined by western blotting. Protein lysates from cells were separated by 8% – 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Then the separated proteins were transferred to Immobilon-NC Transfer Membrane (Millipore, Billerica, MA, USA) using a Transblot semidy transfer cell (Bio-Rad) and blotted with antibodies specific for pro-IL-1β, NLRP3 and caspase-1 p10 subunit. After amplified with horseradish peroxidase (HRP)-conjugated secondary antibodies, the target protein bands were visualized by detection with SuperSignal West Pico Trial Kit (Thermo Scientific, USA). Anti-IL-1β, anti-NLRP3, anti-caspase-1 p10 subunit and anti-β-actin primary antibodies and secondary antibodies were all purchased from Santa Cruz Biotechnology (Santa Cruz, Dallas, TX, USA).

Enzyme-linked Immunosorbent Assay (ELISA): Cell culture supernatants were collected after fullerol exposure. IL-1β/IL-18 levels were analyzed by using mouse IL-1β/ IL-18 specific ELISA kit (eBioscience, San Diego, CA, USA) following the manufacturer’s instruction. Briefly, culture supernatants were collected. 96-multwell plates coated with the corresponding purified anti-human capture monoclonal antibody were used. Culture supernatants and serial dilutions of the standard were added to each well and incubated for 90 min at 37 °C. After four washes, bound samples were detected using the corresponding biotinylated anti-mouse antibody at 37 °C for 1 h. After another four washes, avidin-horseradish peroxidase solution was added, and plates were incubated at 37 °C for 30 min. After the final four washes, plates were kept at 37 °C for 20 min to react with the substrate solution. 100 ml of blocking solution was added to stop the reaction, and the absorbance at 450 nm was then recorded. Results were expressed in pg/mL, and three independent experiments were performed.

NF-κB Immunofluorescence Staining and Confocal Microscopy: NF-κB activity was performed with NF-κB activation-nuclear translocation assay kit (Beyotime, China). Briefly, macrophages isolated and purified as described above were seeded onto glass bottom dishes. After exposed to fullerol, cells were incubated with NF-κB p65 primary antibody at 4 °C overnight following cell fixation and blocking. Macrophages were incubated with Cy3-conjugated secondary antibody for 1 h at room temperature. Cells were stained with DAPI for 5 min at room temperature washed and then visualized by a confocal microscopy (Perkin Elmer Ultra View Vox system, USA).

Inhibition of Potassium Efflux and P2X7 Receptor: To inhibit potassium efflux, macrophages were cultured with a serum-free buffer containing 150 mM KCl with the following composition as recommended:[10] 10 mM HEPES, 5 mM NaH2PO4, 150 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 1% BSA, pH adjusted to 7.4 with KOH. For comparison, a buffer with 150 mM sodium chloride was used: 10 mM HEPES, 150 mM NaCl, 5 mM KH2PO4, 1 mM MgCl2, 1 mM CaCl2, 1% BSA, pH adjusted to 7.4 with NaOH. Gd@C82(OH)22 or Cu6(4OH)22 were added to the buffers 30 min later and cells were cultured for 6 h. To inhibit P2X7 receptor, 500 μl of 1 μM A211645373 were added to the buffer for 1 hour. subsequently, Gd@C82(OH)22 or Cu6(4OH)22 were added to the buffers and cells were cultured for 6 h. Finally, cell culture supernatants were harvested for ELISA analysis.

Statistical Analyses: The data were presented as mean ± SD. The statistical significance of differences between control and treatment groups was calculated using Student’s t-test and one-way analysis of variance (ANOVA). Statistical significance was determined by *p < 0.05 and **p < 0.01.

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References:
