The Nanomaterial-Induced Bystander Effects Reprogrammed Macrophage Immune Function

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Research

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Abstract

Background: In the case of most popularly engineered nanomaterials (e.g., transition-metal dichalcogenide (TMDCs) nanosheets), direct exposure toxicities are investigated extensively to understand the effect on specific cells and organs; however, there has been limited focus on their potential bystander effects. Bystander effects in biological systems are the responses shown by nontargeted neighboring cells or tissues, and critical to the bio-nano interface interactions. In addition to direct effects, bystander effects also determine the design, applications and safety of nanomaterials, although the related information of bystander effects remain largely unknown.

Results: A coculture system of A549 and THP-1 was established to mimic the lung microenvironment to study the bystander effects of WS₂ nanosheets (representative TMDCs nanosheets) on microenvironment macrophages during the inhalation exposure or the nanomaterial biomedical application in the lung. Lung cells exposed to WS₂ nanosheet resulted in an increase in reactive oxygen species and the depolarization of mitochondrial membrane potential in neighboring macrophages. Bystander exposure also induced macrophage polarization toward the anti-inflammatory M2 phenotype, which is adverse to disease therapy. Metabolomics showed that WS₂ nanosheets disturbed the energy metabolism and amino acid metabolism of macrophages, consistent with the metabolic characteristics of M2 macrophages. Nitric oxide-transforming growth factor- β 1 played an important mediator in the bystander effects. Importantly, WS₂ nanosheet bystander exposure affected macrophage phagocytosis and migration and altered the macrophage immune response to environmental endotoxin.

Conclusion: This study demonstrated that WS₂ exposed lung cells would elicit bystander effects on surrounding macrophage cells, which weaken the immune response of surrounding macrophage cells. This study improves the current understanding of bio-nano interactions and highlights the importance of bystander effects and neighboring cell responses, allowing us to use the maximum benefits of nanomaterials while limiting their adverse bystander effects.

Background

The bystander effect in a biological system refers to a unique process in which factors released by cells or tissues exposed to stressors exert effects on other parts of the cells or tissues not exposed to stressors, causing stress responses, genomic instability and altered apoptosis or cell proliferation[1, 2]. To date, bystander effect studies have focused on post-irradiation effects[1, 2], but there is growing evidence that other environmental stressors also induce damage in bystander populations, including heat stresses, photodynamic therapy, genotoxic chemicals, heavy metals, and other multiple stressors[3, 4, 5, 6, 7]. The use of nanoparticles in biology continues to increase and release to the environment are inevitable[8, 9, 10]. Given the inherent interactions between targeted cells and neighboring cells, it is important to understand the nontargeted effects of nanoparticles[9, 11, 12, 13]. In addition to affecting the normal physiology of directly exposed cells, the exposure and internalization of nanoparticles have harmful effects on neighboring cells in a manner similar to the radiation-induced bystander effect[13, 14, 15, 16,

17]. The treatment of differentiated human colorectal adenocarcinoma cells with polystyrene nanoparticles induced apoptosis in bystander cells[14]. The direct exposure of small airway epithelial cells to gold nanoparticles induced bystander effects in unexposed lung fibroblasts by altering protein expression[15]. Current studies have shown that nanomaterials can profoundly affect host immune responses[18, 19, 20, 21], but the bystander effect of nanomaterials on immune function is largely unknown.

Recently, emerging two-dimensional (2D) transition-metal dichalcogenide (TMDC) nanosheets, such as WS₂ nanosheets, have shown tremendous potential for use in many fields, such as intelligent manufacturing, biomedicine and environmental protection[22, 23, 24, 25, 26, 27, 28]. The use of TMDCs, especially in biology, requires a thorough understanding of their potential interactions with the immune system. Despite the abundance of studies on cellular exposure to nanosheets, little is known about the bystander effects induced by TMDCs on neighboring cells that are not exposed to these nanosheets. The direct effects mainly depend on the properties of nanomaterials, while the bystander effects also depend on the features of interaction between targeted cells and neighboring cells. Therefore, a fundamental understanding of bystander effects is critical for their design and applications, ultimately maximizing the benefits of these nanomaterials while limiting their adverse bystander effects[12].

Coculture method *in vitro* has been frequently used in many experiments to study bystander responses. In this approach, both targeted and non-targeted cells are either physically connected or are separated by a physical barrier[2, 3, 15]. Transwell insert coculture system is a widely used no-physical contact coculture system to study bystander effects and its mediators *in vitro*[13, 15, 16]. As human nonsmall cell lung cancer cell line A549 and human monocyte THP-1 coculture represent a widely established *in vitro* lung cell culture model[29, 30], in this study, a coculture system of A549 and THP-1 was used to mimic the lung microenvironment to study the bystander effects of WS₂ nanosheets on microenvironment macrophages during the inhalation exposure or the nanomaterial application in the lung. The study may provide a perspective into the noncontact risks of nanomaterials.

Materials And Methods

Characterization of WS₂ nanosheets

WS₂ nanosheets (XF143) prepared by lithium intercalation were purchased from Nanjing XFNANO Materials Tech Co., Ltd., China. To remove small WS₂ nanosheets, the WS₂ nanosheet dispersion was centrifuged at 1500 × g for 5 min, and then the supernatant was removed. The pellet was used in subsequent experiments. WS₂ nanosheet morphology was examined using atomic force microscopy (AFM, Dimension Icon, Bruker, USA) and high-resolution transmission electron microscopy (HRTEM, JEM-2800, JEOL, Japan). X-ray photoelectron spectroscopy (XPS) was performed on a Thermo Scientific ESCALAB 250Xi spectrometer equipped with a monochromatic Al-Kα X-ray source (1486.6 eV). UV – vis absorption spectra were recorded on a T90 spectrophotometer (Purkinje General, China). The

hydrodynamic size and surface charge of WS₂ nanosheet dispersions in deionized water, phosphate-buffered saline (PBS) and cell culture medium were measured using a ZetaSizer Nano-ZS instrument (Malvern Instruments, UK).

Cell Culture And Coculture System

A549 and THP-1 cells were obtained from the Shanghai Cell Bank of Type Culture Collection of China. Both cell lines were grown in Roswell Park Memorial Institute medium (RPMI 1640, Genview, China) supplemented with 10% fetal bovine serum (FBS, AusGeneX, Australia) and 100 units/mL penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO₂. THP-1 monocytes were differentiated into macrophages by incubation with 160 nM phorbol 12-myristate 13-acetate (PMA, MedChem Express, China) for 24 h.

To study the factors in WS₂-treated A549 cells that were involved in the bystander effect in untreated THP-1 cells, a modified transwell insert coculture system was designed and utilized[31] (Fig. 1a). Transwell inserts were ligated to dialysis membranes (with an MWCO of 1000 kDa, approximately 100 nm) and placed in a 24-well plate or 6-well plate. WS₂ nanosheet-pretreated A549 cells were placed in the upper chamber, and untreated THP-1 cells were seeded in the lower chamber. The insert had a porous membrane with a pore size of 0.4 µm to allow the passage of molecules but not cells. Dialysis membranes between the chambers were applied to prevent the diffusion of WS₂ nanosheets from the upper chamber to the lower chamber.

For the coculture assay, A549 and THP-1 cells were seeded and cultured on 24-well (6-well) Millicell hanging cell culture inserts (Millipore, Germany) and 24-well (6-well) plates, respectively. After 24 h of incubation, the THP-1 cell medium containing PMA was replaced with fresh medium, and the A549 cell medium was replaced with fresh medium containing WS₂ nanosheets or other inhibitors. Then, cell culture inserts with A549 cells were transferred to plates containing differentiated THP-1 macrophages. After 24 h of coincubation, the upper chamber was removed, and the cells in the lower chamber were used for further analysis.

Medium Transfer Assay

A medium transfer assay was further used to study bystander effects (Fig. 1b). A549 cells were seeded in 24-well plates overnight and then exposed to WS₂ nanosheets for 24 h. After exposure, the medium of A549 cells was harvested and filtered through a sterile 0.1-µm filter (Millipore, Ireland) to ensure that no cells and no nanosheets remained in the medium. Then, the conditioned medium was transferred to 24-well plates where differentiated THP-1 macrophages were previously seeded. THP-1 cells were treated with the conditioned medium for another 24 h and then analyzed.

Cell viability, cellular reactive oxygen species and mitochondrial membrane potential assay

THP-1 monocytes were seeded at 1.6×10^5 cells/well in 24-well plates and cocultured with A549 cells or cultured with conditioned medium as described above. After incubation, the cell viability of differentiated THP-1 macrophages was determined using a cell counting kit-8 (CCK-8, Bimake, USA). The optical density (OD) at 450 nm was measured by a microplate reader (Synergy H4, Bio-Tek, USA). The cell viability of the treatment group was expressed as the percentage of live cells in the treatment group relative to that of live cells in the control group.

The generation of cellular ROS was determined by the change in fluorescence of an ROS probe, 2',7'-dichlorofluorescein diacetate (DCFH-DA, MedChem Express, China). The fluorescence intensities of DCFH-DA were measured by a microplate reader (Synergy H4, Bio-Tek, USA). The excitation/emission wavelengths for DCFH-DA was 485/530. The fluorescence of DCFH-DA was normalized to the cell viability of macrophages and presented as a percentage relative to the control group.

Mitochondrial membrane potential was analyzed by using the probe 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolo carbocyanine iodide (JC-1, MedChem Express, China). After staining, the red fluorescence (excitation/emission wavelengths, 530/590 nm) and green fluorescence (excitation/emission wavelengths, 485/530 nm) of macrophages were analyzed by a microplate reader (Synergy H4, Bio-Tek, USA). Mitochondrial membrane potential was determined by the ratio of the red to green fluorescence intensity and presented as a percentage relative to the control group. A decrease in the ratio indicated mitochondrial depolarization.

Micronucleus Assay

A cytokinesis-blocked micronucleus assay was employed to detect genotoxicity in bystander effects. When THP-1 cells were cocultured with WS₂ nanosheet-pretreated A549 cells or were exposed to the medium derived from WS₂ nanosheet-pretreated A549 cells, the culture medium contained 1 µg/mL cytochalasin-B (Meilune Biological Technology, China), an inhibitor of cytokinesis. After culturing for 24 h, the cells were fixed with methanol:acetic acid (3:1, v/v) for 30 min. After air-drying, the cells were stained with 4'-6-diamidino-2-phenylindole (DAPI, Beyotime Biotechnology, China). The nuclei with micronuclei were counted using fluorescence microscopy (IX71, Olympus, Japan).

Intracellular And Extracellular Nitric Oxide Detection

A549 cells were seeded onto 96-well plates at 1.0×10^4 cells per well overnight. WS₂ nanosheets and related inhibitors were resuspended in culture media and then added to 96-well plates. Intracellular NO was analyzed using the NO-specific fluorescent probe 3-amino,4-aminomethyl-2',7'-difluorescein,

diacetate (DAF-FM DA, Beyotime Biotechnology, China). After exposure to WS₂ nanosheets for 24 h, the medium was removed, and the cells were incubated with DAF-FM DA (5 μM) for 20 min. The fluorescence intensities of DAF-FM were measured by a microplate reader (Synergy H4, Bio-Tek, USA). The excitation/emission wavelengths used for DAF-FM were 495/515 nm. The fluorescence intensities of DAF-FM were presented as a percentage relative to the control group. The NO levels in the culture medium supernatant were measured by a Griess reagent kit (Beyotime Biotechnology, China). After exposure, 50 μL of culture medium supernatant was mixed with 50 μL of Griess Reagent I and 50 μL of Griess Reagent II. After incubation for 10 min at room temperature, the absorbance at 540 nm was measured by a microplate reader (Synergy H4, Bio-Tek, USA). The NO levels of each sample were calculated using a standard curve.

Cytokine Determination

To analyze transforming growth factor (TGF)-β1 secretion, A549 cells were seeded onto 96-well plates at 1.0×10^4 cells per well overnight. WS₂ nanosheets and related inhibitors were resuspended in culture media and then added to each well. After incubation for 24 h, the supernatant was collected, and the concentrations of TGF-β1 in culture medium were assayed with ELISA kits, according to the manufacturer's instructions (Dakewe, China). To analyze TNF-α and IL-6 secretion, THP-1 cells were seeded onto 96-well plates at 4.0×10^4 cells per well and differentiated into macrophages by incubation with PMA for 24 h. THP-1 cells were treated with conditioned medium derived from WS₂ nanosheet-pretreated A549 cells with or without inhibitors for another 24 h. Then, the conditioned medium was removed, and the cells were washed with PBS and cultured for 6 h in fresh medium with or without lipopolysaccharide (LPS) (200 ng/mL). The supernatants were collected, and cytokines were analyzed with ELISA kits according to the manufacturer's instructions (LiankeBio, China).

Wound-healing Assay

A wound-healing assay was performed to analyze the migration of differentiated THP-1 macrophages. THP-1 cells were seeded onto 24-well plates at 1.0×10^6 cells per well and differentiated into macrophages by incubation with PMA for 24 h. A scratch wound in the monolayer cells was made by a 200 μL pipette tip. Cells were washed three times with warm PBS and exposed to conditioned medium derived from WS₂ nanosheet-pretreated A549 cells. The cell migration into the scratch area at 0 h, 24 h, and 48 h was photographed using microscopy (IX71, Olympus, Japan).

Quantitative Real-time Polymerase Chain Reaction (qPCR)

The genes related to the polarization state of differentiated THP-1 macrophages modulated by paracrine factors produced by A549 cells exposed to WS₂ nanosheets were quantitatively analyzed by qPCR. For

the M1 macrophage control, THP-1 cells were treated with 200 ng/mL LPS and 50 ng/mL recombinant interferon-gamma (IFN- γ) for 24 h. For the M2 macrophage control, THP-1 cells were treated with 100 ng/mL IL-4 for 24 h. After the macrophages were cocultured with A549 cells exposed to WS₂ nanosheets for 24 h, the total RNA of macrophages was extracted using a total RNA Extraction Kit (Solarbio, China), and cDNA was generated using All-in-One cDNA Synthesis SuperMix (Bimake, USA) by the S1000 Thermal Cycler system (Bio-Rad, USA). qPCR was performed using the Bio-Rad IQ5 system (Bio-Rad, USA) with 2 $\times$ SYBR Green qPCR Master Mix (Bimake, USA). The PCR conditions were as follows: 95 °C for 10 min, then 45 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. The sequences of specific primers are shown in Table S1. To quantify gene expression changes, the 2^{- $\Delta\Delta$ CT} method was used with glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) as a housekeeping gene. The expression level of each gene in the treatment groups was subsequently normalized to that in the control group and presented as relative fold expression.

Immunofluorescent Staining

The polarization of differentiated THP-1 macrophages was further analyzed by immunofluorescent staining. After coculture with A549 cells exposed to WS₂ nanosheets for 24 h, the differentiated THP-1 macrophages were fixed with 4% (w/v) paraformaldehyde for 20 min at room temperature and thoroughly washed with PBS. The treated macrophages were permeabilized with 0.5% (v/v) Triton X-100 for 30 min at room temperature. Then, the permeabilized macrophages were incubated in a blocking buffer containing 5% (w/v) goat serum (Beyotime Biotechnology, China) for 40 min at room temperature. The treated macrophages were incubated in blocking buffer containing primary antibodies against iNOS (M1-specific indicator, 1:200, Santa Cruz, USA) and Arg-1 (M2-specific indicator, 1:200, Bioss, China) at 4 °C for 16 h and thoroughly washed with PBS containing 0.1% Tween 20. The cells were then incubated in blocking buffer containing secondary antibodies (goat anti-rabbit IgG/Alexa Fluor 555 and goat anti-mouse IgG/Alexa Fluor 488, 1:350, Bioss, China) for 2 h at room temperature. The cells were subsequently washed with PBS containing 0.1% Tween 20. The nuclei of macrophages were stained with DAPI. Images were observed with CLSM (Leica, Germany).

Phagocytosis Assay

When THP-1 cells were cocultured with WS₂ nanosheet-pretreated A549 cells, the culture medium contained 10 μ g/mL green fluorescent polystyrene microspheres (Aladdin, China). After culturing for 24 h, the cells were washed three times with PBS and collected. The fluorescence emission was measured by using flow cytometry (Accuri C6, Becton-Dickinson, USA).

Cytoskeleton Staining

Differentiated THP-1 macrophages were seeded on cover slips in 24-well plates. After coculture with A549 cells exposed to WS₂ nanosheets for 24 h, the THP-1 cells were fixed with 4% (w/v) paraformaldehyde for 15 min on ice and thoroughly washed with PBS. The cells were then permeabilized using 0.5% (v/v) Triton X-100 for 10 min at room temperature. YF555-phalloidin (US Everbright Inc., China) and DAPI were used to stain actin and cell nuclei, respectively. Images were acquired using confocal laser scanning microscopy (CLSM, Leica, Germany).

Western Blotting

THP-1 cells were seeded onto 6-well plates at 8.0×10^5 cells per well and differentiated into macrophages by incubation with PMA for 24 h. THP-1 cells were treated with conditioned medium derived from WS₂ nanosheet-pretreated A549 cells for another 24 h. Then, the conditioned medium was removed, and the cells were washed with PBS and cultured for 6 h in fresh medium with or without LPS (200 ng/mL). At the end of the exposure period, the cells were washed with PBS. Total protein, cytoplasmic protein and nuclear protein were extracted using radioimmunoprecipitation assay (RIPA) lysate buffer and nucleoprotein extraction kit (Solarbio, China), respectively. The protein concentrations were measured using a BCA kit (Beyotime Biotechnology, China). Equal amounts of proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Anti-IkB α , anti-NF κ B p65, anti- β -actin and anti-poly (ADPribose) polymerase (PARP) antibodies were used according to the manufacturer's instructions (Bioss, China). β -Actin was used as a loading control for total proteins and cytoplasmic protein, and PARP was used as a loading control for nuclear proteins. The density of bands was quantified using ImageJ software (National Institutes of Health, USA).

Metabolomics Analysis

After coculture with A549 cells exposed to WS₂ nanosheets for 24 h, the cell culture medium of differentiated THP-1 macrophages was removed, and the cells were washed twice with 1 mL of prewarmed PBS. Then, 1 mL of ice-cold 80:20 (v/v) methanol/water was immediately added, and the cells were scraped and collected in a 2 mL Eppendorf tube. The wells were washed again with an additional 1 mL of a methanol/water solution and combined with the previous solution. The metabolites were extracted using ice bath ultrasound (400 W, 30 min) followed by centrifugation (12000 $\times$ g, 5 min, 4 °C). The supernatant was filtered through a 0.22 μ m organic membrane filter, removed via nitrogen blowing, and lyophilized. Methoxamine hydrochloride (20 mg/mL, 50 μ L) and N-methyl-N-(trimethylsilyl) trifluoroacetamide (80 μ L) were added as derivatives. After derivatization, the samples (1 μ L) were injected and analyzed using gas chromatography-mass spectrometry (GC-MS, 6890N/5973, Agilent, USA). The metabolites were identified using full-scan monitoring with a detection slope of m/z 50–650, based on the National Institute of Standards and Technology (NIST) Mass Spectral Library in ChemStation software.

Statistical analysis

Statistical analysis was performed using SPSS 22.0 software (IBM, USA). Differences between two groups were analyzed using Student's t-test, and differences among three or more groups were analyzed using one-way analysis of variance (ANOVA) with Tukey's test. Before ANOVA, the Kolmogorov – Smirnov (KS) test (a significance level of 0.05) was performed to assess the normal distribution. *P* values less than 0.05 were considered statistically significant. Metabolic pathway analysis was performed using MetaboAnalyst version 4.0 (<http://www.metaboanalyst.ca>)[32].

Results And Discussion

Characteristics of WS₂ nanosheets

The TEM and AFM images showed that the average lateral size of the WS₂ nanosheets was on the micron scale (1~4 μm) (Figs. 2a and b). The thicknesses of the WS₂ nanosheets were approximately 3–4 nm, indicating that the WS₂ nanosheets were 3–4 layers (Fig. 2b). The XPS signals at ~33 (32) eV and ~35 (34) eV were attributed to W4 + 4f_{7/2} and W4 + 4f_{5/2} in the 2H WS₂ (1T WS₂) phase (Fig. 2c), respectively[33]. 1T was the main phase of the nanosheets, accounting for ~93%. The UV-vis spectrum at ~260 nm (Fig. 2d) was consistent with the feature reported for the 1T phase[34]. The hydrodynamic sizes of the nanosheets were 1523 $\pm$ 255 nm, 1990 $\pm$ 782 nm and 825 $\pm$ 330 nm in deionized water, PBS and cell culture media (RPMI 1640 + 10% FBS), respectively (Table S2). The increase in hydrodynamic sizes in PBS was due to the agglomeration of nanosheets stimulated by the high ionic strength in PBS[35], while the decrease in hydrodynamic size in the culture media was due to the presence of a large amount of serum albumin in the medium, resulting in the formation of a protein corona[36].

Ws Nanosheet-induced Bystander Effects Involve No And Tgf- β 1

WS₂ nanosheets pre-exposed to A549 were subsequently cocultured with differentiated THP-1 macrophages to mimic bystander effects during the inhalation exposure or the nanomaterial application in the lung (Fig. 1a). WS₂ nanosheets had no detectable cytotoxic effects on the viability of bystander cells (Fig. 3a). The level of reactive oxygen species (ROS) in the bystander cells increased by 10%, and the mitochondrial membrane potential depolarized by nearly 50% (Fig. 3b and c), showing the bystander effect on neighboring macrophages cells. As shown in Fig. 1f, no significant micronucleus formation was observed in WS₂ nanosheet-treated bystander cells compared to the control cells, suggesting that no serious genotoxicity occurred in the bystander effect.

The medium transfer assay is another important method to further study bystander responses[2], as shown in Fig. 1b. There were no detectable changes in cell viability (Fig. 3a) or micronucleus formation

(Fig. 3e). The trend in ROS content and mitochondrial membrane potential (Fig. 3b and c) was consistent with the coculture experiment. The above results indicate that mitochondrial toxicity is the bystander effect caused by WS₂ nanosheets.

Studies on bystander responses suggest that bystander signals may be transferred to neighboring cells either by gap junctional intercellular communication or by the production of exosomes or soluble extracellular signaling factors released from stressed targeted cells[3, 13, 16, 17]. Soluble extracellular signaling factors such as ROS, nitric oxide (NO), extracellular DNA, and cytokines including TGF- β have been found to play an important role in multiple stressor-induced bystander effects[37, 38, 39, 40]. Cell viability was the main cell response regulated by the bystander effect verified by the inhibitors (Fig. 3f). As shown in Fig. 1i, c-PTIO (an NO-specific scavenger) or SB431542 (a potent and selective inhibitor of TGF- β 1 receptor kinases) restored mitochondrial membrane potential depolarization to 25%. However, NAC (ROS scavenger) and DNase I (nucleic acid scavenger) had no corresponding effect. Therefore, both NO and TGF- β 1 may be signaling factors involved in WS₂ nanosheet-induced bystander responses. It should be noted that c-PTIO or SB431542 only partially restored the mitochondrial membrane potential (Fig. 3g), suggesting that other factors, such as exosomes, may also mediate the bystander effects. In this study, we mainly focus on the NO and TGF- β 1.

NO is the upstream regulatory signal of TGF- β in WS₂ nanosheets induced bystander effects

To further confirm the involvement of TGF- β 1 and NO in WS₂ nanosheets induced bystander effects, the TGF- β 1 level and NO content in the medium were detected. When A549 cells were exposed to 50 and 100 μ g/mL WS₂ nanosheets, the TGF- β 1 levels were approximately 1.5 times and 2 times as high as those in the control group, respectively (Fig. 4a). There was no detectable NO in the culture medium (detection limit, 1 μ M), but the fluorescence probe assay showed an approximately 1-3-fold increase in intracellular NO (Fig. 4b). WS₂ nanosheets induced cells to produce intracellular NO without releasing it to the extracellular space, which was consistent with the fact that NO radicals have a very short half-life and a very short diffusion distance[39]. To verify the hypothesis that NO may be involved in the bystander effect by regulating TGF- β 1, A549 cells were treated with an NO-specific scavenger (c-PTIO), and TGF- β 1 levels were measured in the medium. The TGF- β 1 level recovered to the control level after NO removal (Fig. 4c). After treatment with an inhibitor of TGF- β 1 receptor kinases (SB431542), no significant changes in intracellular NO were observed (Fig. 4d). These results indicated that A549 produced NO after exposure to WS₂ nanosheets and subsequently regulated the production of TGF- β 1, affecting neighboring cells.

WS₂ nanosheet bystander exposure induced THP-1 polarization into anti-inflammatory M2 macrophages

When macrophages migrate to the desired tissue and are activated, they can be polarized into the proinflammatory type (classically activated M1 phenotypes) or the anti-inflammatory and pro-regenerative type (alternatively activated M2 phenotypes)[41, 42]. The balance between M1 and M2 macrophage populations is believed to have a significant influence on the outcome of immune systems, disease progression, and wound healing. The above results showed that WS₂ nanosheets exposure makes A549 cells to produce large amounts of TGF-β1 and release it into the surrounding environment (Fig. 4a). Recent studies have revealed that TGF-β1 skews macrophage polarization towards an M2-like phenotype[43], and blocking TGFβ-induced signalling in the tumour microenvironment enhances antitumour immunity and may be beneficial for cancer therapy[44]. Subsequently, the polarization of THP-1 macrophages coculture with A549 cells exposed to WS₂ nanosheets was explored. qPCR analysis revealed that the expression levels of polarization marker genes in THP-1 cells cocultured with A549 cells were not significantly changed compared to those in cells cultured alone (Figure S1), suggesting that macrophages were not significantly polarized. WS₂ nanosheet bystander exposure also did not significantly affect the mRNA expression levels of M1 markers, including iNOS, CD86, and TNF-α (Fig. 5a). However, WS₂ nanosheet bystander treatment increased the mRNA expression levels of M2 markers, including Arg-1, CD206 and IL-10 (Fig. 5b). Moreover, NO scavenging or the inhibition of the TGF-β1 signaling pathway partially inhibited the increase in M2 marker gene expression levels (Fig. 5b). Immunostaining of THP-1 macrophages confirmed that WS₂ nanosheet bystander treatment yielded a considerably high Arg-1 fluorescence signal in most macrophage cells, similar to the tendency in gene expression (Fig. 5c). Taken together, the results suggest that WS₂ nanosheet exposure to A549 cells led to the polarization of cocultured macrophages toward the anti-inflammatory M2 phenotype and that TGF-β1 played an important role in this process. M1 macrophages have a tumor-suppressing phenotype, and M2 macrophages have a tumor-promoting phenotype[41, 44]. The transformation of macrophages to the M2 phenotype in WS₂ nanosheet bystander exposure is not beneficial for its application in tumor therapy and imaging. Song *et al.* reported that hyaluronic acid can transform the tumor-associated macrophages phenotype from pro-tumoral M2 phenotype to antitumor M1 phenotype, thereby enhancing the anti-tumor effect of hyaluronic acid-modified manganese dioxide nanoparticles[45]. Therefore, similar modifications to WS₂ nanosheets may limit their adverse bystander effects and enhance its anti-tumor applications.

The Bystander Effect Affects Macrophage Phagocytosis And Migration

The phagocytosis of macrophages plays an essential role in tumor progression and innate immunity by eliminating tumor cells and pathogens and priming the adaptive immune response[46, 47]. The influence of WS₂ nanosheets bystander exposure on macrophage phagocytosis function was further explored. WS₂ nanosheet bystander exposure reduced macrophage phagocytosis by approximately 50% (Fig. 6a). However, SB431542 and c-PTIO did not restore phagocytic function, suggesting that TGF-β1 was not the factor that mediated the decrease in phagocytic function. The dynamic actin cytoskeleton in cells plays multiple roles in regulating cellular morphology, motility, vesicle trafficking and phagocytosis by exerting

mechanical forces[47, 48]. Actin filaments were evenly and orderly distributed in the cytoplasm (control in Fig. 6b), but actin filaments became irregularly agglomerated after WS₂ nanosheet treatment (denoted by green arrows in Fig. 6b). Quantitative results showed that the irregular aggregation of actin filaments increased by ~ 20% in WS₂ nanosheet bystander exposed macrophage (Fig. 6c). The observed decrease in macrophage phagocytosis was linked to phagocytic dysfunction and cytoskeletal destruction (denoted by green arrows in Fig. 6b). Another key function of macrophages is migration to tumors or infection sites[49]. Stress fibers, as the bundles of contractile actin filaments, are the basis for tail contraction and cell shape changes during cell migration[49, 50]. As shown in Fig. 6d, WS₂ nanosheet bystander exposure led to a slight decrease in macrophage migration. Collectively, WS₂ nanosheet exposure to A549 cells elicited cytoskeletal disorder of neighboring macrophages and affected their migration and phagocytosis, which are two important aspects of macrophage immune function. However, the potential bystander effects mediators NO and TGF-β1 previously detected did not mediate those above disturbance, indicating that there were other factors involved in the WS₂ nanosheets induced bystander effects.

WS₂ nanosheet bystander exposure disrupts the immune response of macrophages

The main participants in the innate immune response are on phagocytes[21]. The polarization of macrophages and phagocytosis function changed upon bystander exposure to WS₂ nanosheets (Figs. 5 and 6). Further investigations into normal immune responses to the foreign material were performed. The nuclear factor-κB (NF-κB) pathway is a key regulator of immune responses and regulates both innate and adaptive immunity[51, 52]. The bacterial endotoxin LPS is known to induce the release of inflammatory factors and the activation of the NF-κB pathway in macrophages[19]. As shown in Fig. 7a, LPS treatment induced the release of TNF-α and IL-6, while WS₂ nanosheet bystander exposure reduced the LPS-induced increase in cytokine release. Furthermore, WS₂ nanosheet bystander exposure attenuated LPS-induced IκBα degradation and NF-κB/p65 translocation from the cytoplasm to the nucleus (Fig. 7b). Previous reports also showed that direct exposure to nanoparticles can weaken immune cells' response to bacterial infections or LPS stimulation[20, 21, 53, 54]. The present work reveals that bystander exposure to nanomaterials affects the macrophage immune response to LPS. Despite the low cytotoxicity, the indirect presence of nanosheets alters the natural response of cells to foreign materials or pathogens, which deserves further attention in the future.

Reprogramming of macrophage metabolic function supports the observed immune response.

After the development of the genomics and proteomics technologies, metabolomics has more recently been developed and used as an analytical tool for nanotoxicology research[55, 56]. We further used non-targeted metabolomics to investigate the effect of WS₂ nanosheets bystander exposure on the metabolism of surrounding macrophages. The two-dimensional score plots of both the principal

component analysis (PCA) (Figure S2a) and partial least-squares-discriminant analysis (PLS-DA) (Figure S2b) models demonstrated that WS₂ nanosheets induced conspicuous perturbation of the intracellular metabolic profile. The relative levels of the metabolites are presented using heat maps in Figure S3. Compared to the control group, both hierarchical clustering (HCL) analysis (Figure S3) and volcano plot screening (Figure S4) suggested that WS₂ nanosheets induced obvious metabolite alterations, which were partially alleviated by SB431542 and c-PTIO treatment. This result suggested that TGF- β 1 contributed to metabolism disruption. Based on the KEGG pathway database, the metabolic functions significantly changed by bystander exposure to WS₂ nanosheets were highly associated with the perturbation of energy metabolism (e.g., pyruvate metabolism, glycolysis or gluconeogenesis, glyoxylate and dicarboxylate metabolism, glycerolipid metabolism) and amino acid metabolism (e.g., arginine and proline metabolism, alanine, aspartate and glutamate metabolism, cysteine and methionine metabolism, taurine and hypotaurine metabolism) (Figure S5).

Based on the metabolomics analysis, energy metabolism disruption was observed in WS₂ nanosheet bystander-exposed THP-1 cells (Fig. 8). After WS₂ nanosheet bystander exposure, pyruvate and lactate levels presented slightly increasing tendencies, suggestive of the upregulation of glycolysis in the cytoplasm[57]. M1 macrophage metabolism is characterized by enhanced glycolysis, which converts glucose into pyruvate and lactate to support pyruvate influx into the tricarboxylic acid (TCA) cycle[58, 59, 60, 61]. M2 macrophages mainly produce adenosine triphosphate (ATP) through an oxidative TCA cycle coupled to oxidative phosphorylation. To fuel the TCA cycle, M2 macrophages rely on fatty acid β -oxidation and glutamine metabolism[58, 59, 60, 61]. This is contradictory to the observation that WS₂ nanosheet bystander exposure induced macrophage polarization toward the anti-inflammatory M2 phenotype (Fig. 5). On the one hand, WS₂ nanosheet bystander exposure caused mitochondrial membrane potential depolarization (Fig. 3c), which further led to insufficient energy supply by oxidative phosphorylation[59]. On the other hand, macrophages enable glycolysis to quickly replenish energy under WS₂ nanosheet stress[59]. The above mechanisms resulted in the slight upregulation of glycolysis in M2 macrophages. Two key metabolites in the TCA cycle, citric acid and succinate, were upregulated and not significantly changed, respectively (Fig. 8). In particular, increased lactic acid further induced macrophage polarization toward the M2 phenotype[62]. In M2 macrophages, the TCA cycle is intact and participates in oxidative phosphorylation, while in M1 macrophages, the TCA cycle is disrupted in two places – after citrate and after succinate[59, 61]. Succinate (succinic acid) did not change significantly, consistent with the TCA cycle characteristics of M2 macrophages. However, the upregulation of citric acid may be caused by the β -oxidation of fatty acids, as evidenced by the significant upregulation of palmitic acid and octadecanoic acid. The β -oxidation of fatty acids is also a metabolic feature of M2 macrophages[59].

In addition to the differences in energy metabolism, M1 macrophages and M2 macrophages exhibited opposite arginine metabolism, which is closely related to their functional polarization[59, 61]. M1 macrophages catalyze L-arginine to L-citrulline and the signaling molecule NO by upregulating iNOS[61]. M2 macrophages promote the production of urea and L-ornithine from L-arginine by upregulating Arg-1[61]. L-ornithine is further metabolized to produce L-proline for collagen synthesis, thus contributing to

wound repair, which is a key function of M2 macrophages[61]. qPCR showed that there was no significant change in iNOS after WS₂ nanosheet bystander exposure, but Arg-1 was upregulated. Consistent with the qPCR results, in the metabolomics analysis, urea, L-ornithine and L-proline were all upregulated, supporting the M2 profile in amino acid metabolism after WS₂ nanosheet bystander exposure.

Conclusions

A comprehensive understanding of the biological responses of nanomaterials is crucial for their design and applications. Currently, most evaluations of nanomaterial biological responses focus on direct effects. The potential indirect effects (bystander effects) of nanomaterials are poorly understood. The present study clearly uncovers the previously unknown detrimental bystander effects. We found that WS₂ nanosheet bystander exposure affected the phagocytosis and migration of macrophages, induced macrophage polarization toward the anti-inflammatory M2 phenotype, and more importantly, weakened the immune response of macrophages to endotoxin. The bystander effect is important for understanding the biological responses of nanomaterials, especially for the immune system. The information presented here provides a comprehensive understanding of bio-nano interactions. If this is a general feature of all nanomaterials, it offers new insight into nanotoxicity to limit potential adverse effects.

Abbreviations

TMDCs

Transition-metal dichalcogenide. WS₂:Tungsten disulfide. AFM:Atomic force microscopy. HRTEM:High-resolution transmission electron microscopy. XPS:X-ray photoelectron spectroscopy. DCFH-DA:2',7'-dichlorofluorescein diacetate. ROS:Reactive oxygen species. PBS:Phosphate buffer saline. JC-1:5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolo carbocyanine iodide. Arg-1:arginase. iNOS:inducible nitric oxide synthase. G6P:glucose-6-phosphate. F6P:fructose-6-phosphate. F1,6P:fructose-1,6-diphosphate. G3P:glucose-3-phosphate. α-KG:α-ketoglutarate. LPS:lipopolysaccharide. qPCR:Quantitative real-time polymerase chain reaction. NO:Nitric oxide.

Declarations

Authors' contributions

PY and XH designed research; PY and ZB performed research; PY and XH analyzed data; PY and XH wrote the paper; XH and QZ supervised the study and revised

the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing financial interest.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Figures

Figure 1

Schematic illustration of the Bystander effects assay. (a) coculture assay. (b) medium transfer assay.

Figure 2

Characteristics of WS₂ Nanosheets. (a) TEM images of WS₂ nanosheets. Scale bars, 2 μm . (b) AFM images of WS₂ nanosheets. Scale bars, 2 μm . (c) XPS spectra and deconvolution analysis of WS₂ nanosheets. (d) UV-Vis absorbance spectra of WS₂ nanosheets dispersion in H₂O.

Figure 3

Bystander effects induced by WS₂ nanosheets. (a) Cell viability, (b) ROS content and (c) mitochondrial membrane potential of THP-1 cells cocultured with WS₂ nanosheet-pretreated A549 cells or upon exposure to the conditioned medium derived from WS₂ nanosheet-pretreated A549 cells. Micronucleus analysis of THP-1 cells (d) cocultured with WS₂ nanosheet-pretreated A549 cells or (e) exposed to conditioned medium derived from WS₂ nanosheet-pretreated A549 cells. Scale bars, 20 μ m. (f) Cell

viability and (i) mitochondrial membrane potential of THP-1 cells cocultured with WS2 nanosheet-pretreated A549 cells with/without SB431542, c-PTIO, NAC, and DNase I. SB431542, a potent and selective inhibitor of TGF- β 1 receptor kinases. c-PTIO, an NO-specific scavenger. NAC, an ROS scavenger. DNase I, a nucleic acid scavenger. “*” represents statistical significance versus the control at $p < 0.05$.

Figure 4

The NO-TGF- β 1 signaling pathway is involved in the bystander effect. (a) The TGF- β 1 level in the cell medium of A549 cells exposed to WS2 nanosheets. Intracellular NO content was measured by (b) the DAF-FM DA fluorescent probe. (c) The relative TGF- β 1 level in the cell medium of A549 cells exposed to WS2 nanosheets with/without c-PTIO (an NO-specific scavenger). (d) Intracellular NO content of A549 cells exposed to WS2 nanosheets with/without SB431542 (a potent and selective inhibitor of TGF- β 1 receptor kinases). “*” represents statistical significance versus the control at $p < 0.05$.

Figure 5

The polarization of THP-1 macrophages cocultured with A549 cells exposed to WS2 nanosheets. The expression levels of (a) M1-specific indicators (iNOS, CD86 and TNF- α) and (c) M2-specific indicators (Arg-1, CD206, and IL-10). (c) Fluorescence images of THP-1 macrophages immunostained for iNOS (in green), Arg-1 (in red), and nuclei (in blue) after 24 h of coculture with A549 cells exposed to WS2 nanosheets. Scale bars, 20 μ m. “*” represents statistical significance versus control at $p < 0.05$.

Figure 6

WS2 nanosheet bystander exposure affects macrophage phagocytosis and migration. (a) Flow cytometry histograms of the phagocytosis of green fluorescent polystyrene microspheres by THP-1 cells cocultured with WS2 nanosheet-pretreated A549 cells. (b) The cytoskeleton of THP-1 cells cocultured with WS2 nanosheet-pretreated A549 cells. Actin filament staining and cell nuclei staining are shown by red fluorescence and blue fluorescence, respectively. Scale bars, 20 μ m. (c) Quantitative analysis of irregularly agglomerated actin filaments. (n>50). (d) The migration of THP-1 cells exposed to the conditioned medium derived from WS2 nanosheet-pretreated A549 cells was assessed by wound-healing assay. The mean percentage (%) of cell migration was calculated by analyzing the width of 6 scratches in 3 independent wells.

Figure 7

Disturbance of metabolic pathways of THP-1 cells cocultured with WS2 nanosheet-pretreated A549 cells. (a) Metabolic signaling pathways affected by WS2 bystander exposure. The colored metabolites are the metabolites identified in this study. The metabolites colored blue, red and green were unchanged, upregulated and downregulated, respectively. The main characteristic metabolic pathways of M1 and M2 macrophage polarization are labeled with pink arrows and yellow arrows, respectively. Arg-1, arginase; iNOS, inducible nitric oxide synthase; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F1,6P, fructose-1,6-diphosphate; G3P, glucose-3-phosphate; α -KG, α -ketoglutarate. (b) Simplified schematic of the changed metabolic pathways.

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