Deleterious effect of various microplastics to the function of macrophage in innate immune response

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Deleterious effect of various microplastics to the function of macrophage in innate immune response

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2022년 7월, 인턴 생활을 시작으로 끝나지 않을 것 같던 2년간의 석사 과정을 마치며 그 동안 많은 도움과 힘을 주셨던 분들께 감사의 말을 전하고자 합니다.

가장 먼저, 조언을 아끼지 않으시고 학위 논문을 작성하기까지 2년의 시간 동안 연구지도해주신 지도교수 현영민 교수님께 감사의 말씀 올립니다. 교수님의 진심 어린 지도가 있었기에 학위 과정을 잘 마칠 수 있었습니다. 학위 논문 지도를 위해 시간을 내주시고 애정과 관심으로 조언해주신 김락균 교수님과 김창수 교수님께도 진심으로 감사드립니다. 또한, 논문 작성에 많은 도움을 주신 홍진기 교수님과 박경태 박사님께도 감사드립니다.

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2024년 6월

김기은 올림

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ABSTRACT

Deleterious effect of various microplastics to the function of macrophages in innate immune response

Plastic, a synthetic material valued for its durability and cost-effectiveness, plays an essential role in modern life. However, the environmental accumulation of microplastics raises significant concerns due to their potential uptake by organisms. Prior research has revealed the harmful effects of microplastics on various cell types, though these studies often used a single type of microplastics with uniform size and shape, which may not accurately represent those found in natural environments. However, this study focused on the distinct effects of three different microplastics - PET, ABS, and PP- of various sizes and shapes on the function of the innate immune system, specifically macrophages. It was verified that PET and ABS could induce bone marrow-derived macrophage (BMDM) recognition and phagocytosis as foreign substances, while PP did not directly interact with macrophages due to its hydrophobicity. Exposure to PET and ABS resulted in a significant decrease in the cell viability of BMDMs. Moreover, after recognition and phagocytosis, PET and ABS induced M1 macrophage polarization in a concentration-dependent manner and increased the secretion levels of pro-inflammatory cytokines. Furthermore, two-photon intravital imaging in mice demonstrated that microplastics accumulate in the intestine upon ingestion. Transmission electron microscopy (TEM) images revealed changes in mitochondrial morphology,

characterized by a round shape and less dense cristae. These findings suggest that microplastics can accumulate in mouse intestines and may cause deleterious effects on the innate immune system, particularly macrophage function, as demonstrated by *in vitro* experiments.

Key words: microplastics, bone marrow-derived macrophages, cell death, macrophage polarization, pro-inflammatory cytokine, intestine, mitochondria

1. Introduction

Plastic which is composed of polymers, is renowned for its versatility, durability, strength, and cost-effectiveness[1]. In modern society, plastic has become indispensable and can find application in diverse areas such as packaging, clothing, construction, and more[1-3]. Plastic debris, when exposed to environmental stressors like UV radiation, seawater, and marine organisms, undergoes fragmentation into microplastics[1,4-6]. The annual rise in plastic and microplastic particle accumulation highlights the urgency of the issue, as microplastics can enter the food chain and organisms, and even human can ingest or inhale these plastic particles easily from their natural surroundings[7-12]. These ingested and inhaled microplastics to organisms can lead to several damages such as abrasion and blockage of the digestive tract and result in starvation, and reduction of reproductive capacity[13,14]. Previous studies showed that ingestion of microplastics can obstruct the digestive tracts of aquatic organisms, decreasing their feeding drive and causing physical harm and oxidative stress[15,16].

Microplastics may also trigger cellular responses such as apoptosis or necrosis and inflammatory signaling upon internalization by epithelial cells or innate immune cells[10,17-19]. Among others, macrophages are crucial components of innate immune cells and can migrate to sites of pathogen invasion and phagocyte the pathogens, infected, debris, and dead cells[20,21]. In addition, derived from bone marrow progenitors, macrophages can differentiate into distinct sub-populations in response to various stimuli, including pro-inflammatory cytokines and microbial products [20,22]. The two major sub-populations, M1 (classically activated or inflammatory) and M2 (alternatively activated or anti-inflammatory) macrophages perform distinct functions and responses[22,23]. M1 macrophages, induced by Th1 cytokines or bacterial lipopolysaccharide (LPS)

recognition, produce elevated levels of pro-inflammatory cytokines and mediate anti-microbial responses but may also contribute to tissue damage. In contrast, M2 macrophages, activated by Th2 cytokines, play a role in tissue repair, wound healing, and anti-inflammatory responses[24,25]. Based on the roles performed by macrophages as innate immune cells and the plasticity of macrophages by various stimuli, prior studies have shown that upon exposure to microplastics, macrophages can engulf the plastic particles, leading to increased generation of NO and ROS[26,27]. Therefore, understanding the interaction between microplastics and macrophages in more detail can provide insights into their potential immunological effects and implications for human health.

Unfortunately, prior research has primarily focused on processed microplastics, which may not accurately represent those found in natural environments[28,29]. However, this study aims to investigate various types of plastics with different sizes and shapes, occurring naturally in the environment, rather than processed forms identical to each other. Among the distinct types of plastic polymers commonly found in marine environments, polyethylene terephthalate (PET), polypropylene (PP), polyamide (PA), polyurethane (PU), acrylonitrile butadiene styrene (ABS), polystyrene (PS) and rayon are the most prevalent[14]. Based on this investigation, this study focused on three different types of microplastics, PET and ABS, PP to discover the impacts of microplastics on the innate immune system. Ultimately, this study revealed the harmful effect of microplastics on macrophage functions and also the difference in the impact of three types of microplastics on the function of macrophages depending on their distinct properties.

2. MATERIALS AND METHODS

2.1. Mice

C57BL/6 mice were purchased from Orient Bio (Seongnam, Korea) and housed at the Avison Biomedical Research Center (ABMRC) of Yonsei University College of Medicine. In all experiments, 8-10 weeks old male mice were used. $CX3CR1^{GFP/+}$ (heterozygote mice) were used for visualizing monocyte and macrophage. All procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Yonsei University College of Medicine and listed on animal research proposal (IACUC No. 2022-0118).

2.2. Preparation and differentiation of bone marrow-derived macrophage (BMDM) cells

C57BL/6 mice, 8-10 weeks old, were euthanized by cervical dislocation. Bone marrow cells were isolated from femurs and tibia by flushing with FACS buffer [1X PBS with 2% FBS (Welgene, Gyeongsan, Korea) and 2 mM EDTA (Invitrogen, Carlsbad, California, USA)] under sterile conditions. Red blood cells in bone marrow were removed by ACK lysis buffer (Gibco, Waltham, Massachusetts, USA). Cells were seeded on 100 mm petri dishes (10 mL/dish) and *in vitro* cultured with 20 ng/mL of macrophage colony-stimulating factor (M-CSF) (93, Biolegend, San Diego, California, USA) which was used for differentiation to macrophage. Culture media was changed on day 3, and bone marrow-derived macrophages (BMDM) were matured and harvested on day 7.

2.3. Flow cytometry

Flow cytometry was performed to confirm the viability and differentiation population of M1 macrophage of microplastics treated-BMDMs. BMDMs were seeded the day before the experiment in 6 well plates at a density of 1×10^6 cells well in culture medium at 37°C. After 24 hours of incubation, BMDMs were treated with five different concentrations (0.05, 0.1, 0.25, 0.5, and 1 mg/mL) of three types of microplastics polyethylene terephthalate (PET), acrylonitrile butadiene styrene (ABS), and polypropylene (PP) (연세대 화학공학과 홍진기 교수님 제공). For the control group, 100 μl of 3' D.W was treated and for the positive control group, LPS (100 ng/mL) and IFN-γ (20 ng/mL) were treated to induce the polarization to M1 macrophage. After 24 hours of incubation, BMDMs were detached by 1X trypsin/EDTA for 3 minutes and 37°C. Single cells were blocked with 1 μg purified anti-FcR (CD16/32) antibody (Biolegend) for 10 minutes at 4°C. For flow cytometry analysis of single macrophage cells, BMDMs were stained with APC-Anti-CD11b and BV711-Anti-F4/80 for 30 minutes at 4°C with light blocked. The viability assay of BMDMs was conducted by FITC-Annexin V (Biolegend) and DAPI (Invitrogen). To validate the differentiation of M1 macrophage, PE-Anti-CD86, and BV785-Anti-CD206 were used. Stained samples were washed with Annexin V buffer twice. Flow cytometry was conducted by LSRII. RAW data of flow cytometry was analyzed by FlowJo v10.2 (BD Bioscience)

2.4. MTT assay for cell viability

BMDMs were seeded with DMEM the day before the experiment in 96 well plates at a density of 1×10^4 cells/well in culture medium at 37°C. After 24 hours of incubation, BMDMs were treated with different concentrations (0.05, 0.1, 0.5, and 1 mg/mL) of three types of microplastics (PET,

ABS, and PP). The positive control group was treated with 10% of DMSO. BMDMs were incubated at the same conditions for 24 hours. 20 μl of MTT dye was added to each well and incubated at 37°C for 3 hours with light blocked. Media was removed, and 100 μl of DMSO was added to each well to dissolve the formazan crystal pellets. Then incubated at 37°C for 15 minutes. The plate absorbance was measured with an ELISA reader at 570 nm.

2.5. *In vitro* cell migration assay

For visualizing the migration pattern of microplastics treated-BMDMs, 2×10^5 cells of BMDMs were seeded in confocal dishes with 20 ng/mL of macrophage colony-stimulating factor (M-CSF) (Biolegend) and incubated at 37°C for 24 hours. The confocal dish was coated with fibronectin (10 μg/mL) to attach BMDMs. Fibronectin was treated on a confocal dish at 37°C for 1 hour. After 24 hours, 0.5 mg/mL of three different microplastics (PET, ABS, and PP) were treated with BMDMs. For the control group, 100 μl of 3' distilled water was treated with BMDMs. The confocal dish was mounted on Nikon Ti2e microscope and imaged at 30-second intervals for 2 hours at 37°C with 5% CO_2 incubation.

2.6. RNA extraction and quantitative real-time PCR

1×10^6 cells of BMDMs were seeded the day before the experiment in non-treated 6 well plates and incubated at 37°C. After incubation, BMDMs were treated with 0.5 mg/mL of three types of microplastics (PET, ABS, and PP). 100 μl of 3’ distilled water was treated for the control group. After 24 hours of incubation, BMDMs were collected by using a scraper and centrifugated for RNA extraction. Supernatants were carefully removed, and cells were suspended with 1 mL of Trizol (Invitrogen). Samples were vortexed and incubated for 5 minutes at room temperature. 200

μl of chloroform was added and incubated for 3 minutes at room temperature. The aqueous phase of RNA was collected by centrifugation for 15 minutes at 12,000 g at 4°C. 400 μl of aquas phase of RNA and isopropanol were mixed at a 1:1 ratio. Samples were incubated overnight at 4°C. After overnight of incubation, the RNA pellet was collected with centrifugation for 20 minutes at 12,000g at 4°C. Pellets were washed with 75% ethanol and eluted with ultra-pure distilled water. RNA concentration of microplastic-treated BMDM was measured by nanodrop. Complement DNA (cDNA) was synthesized by AccuPower® CycleScript RT PreMix dT20 (Bioneer, Daejeon, Korea) with the manufacturer's protocol. TB green® Premix Ex Ta1 TM II (Takara, Kyoto, Japan), primers, and cDNA were mixed. The mixture was loaded to the MicroAmp TM Fast Optical 96 well reaction plate. Real-time PCR was conducted by Quantstudio3 Real-Time PR system (Applied Biosystems, Forster City, California, USA), and data was analyzed by ΔΔCT method. All primers used in real-time PCR were listed in Table 1.

2.7. Protein extraction and western blotting

1×10^6 cells of BMDMs were seeded the day before the experiment in non-treated 6 well plates and incubated at 37°C. After 24 hours of incubation, BMDMs were treated with 0.5 mg/mL of three types of microplastics (PET, ABS, and PP). For the control group, 100 μl of 3' distilled water was treated and for the positive control group, LPS (100 ng/mL) and IFN-γ (20 ng/mL) were treated to induce the polarization to M1 macrophage. After 24 hours of incubation, BMDMs were collected by using a scraper and incubated with RIPA lysis and extraction buffer (Thermo Scientific, Waltham, Massachusetts, USA) containing 1X Xpert protease inhibitor cocktail (GenDEPOT, Houston, Texas, USA) and 1X Xpert phosphatase inhibitor cocktail (GenDEPOT) on ice with vortexing. Supernatants were collected with centrifugation for 15 minutes at 13,000

rpm at 4°C. Concentration was measured by bicinchoninic acid (BCA) assay. Samples were diluted at 5X sample loading buffer containing DTT (Tech&Innovation, Chuncheon, Korea) and boiled for 5 minutes at 95°C heat block. 10% gel was used for western blotting. SDS-PAGE was conducted with 1X running buffer for 2 hours. 0.2 μm size PVDF membrane was activated with methanol before transfer. Transfer was performed for 1 hour on ice. Membrane was blocked for 1 hour at room temperature with 5% non-fat skim milk in 1X TBST (0.1% tween 20) and 5% BSA in 1X TBST. Mouse anti-mouse Akt mAb (1:1000), and mouse anti-mouse IKK mAb (1:1000) were diluted into antibody dilution buffer (5% skim milk, 0.02% sodium azide containing 1X TBST), Mouse anti-mouse p-Akt mAb (1:1000), mouse anti-mouse p-IKK mAb (1:1000) were diluted into antibody dilution buffer (5% BSA, 0.02% sodium azide containing 1X TBST). 1^{st} antibody was blotted with shaking incubation overnight at 4°C. Membrane was washed with 1X TBST and mouse-anti IgG Ab with HRP conjugate was used for 2^{nd} antibody blotting. After washing the membrane, Clarity Max ECL (Bio-Rad, Hercules, California, USA) (1:1) was used for low expressed protein development.

2.8. Two photon intravital imaging

Mice were deeply anesthetized with a Zoletil-Rompun mixture diluted (1:10) in PBS. Two-photon intravital microscopy was used to observe microplastics in intestine. Mice was fed with 10 mg/kg of mixed three types of microplastics for 2 weeks. Three types of microplastics were suspended in 3’ distilled water. The dosage was determined based on the estimated environmental accumulation and the amount of microplastics ingested or inhaled by organisms. To observe blood flow, Texas Red Dextran (70 kDa) was injected intravenously before anesthesia. Additionally, to visualize monocytes in C57BL/6 mice, $CX3CR1^{GFP/+}$ (heterozygote mice) were used. Hair of

abdomen was removed by using hair remover cream. After vertically incising the abdomen, the small intestine was exposed. Subsequently, a part of the small intestine intended for imaging was selected, and small incisions were made at both ends to gradually open the cylindrical shape of the intestine, allowing the interior to be exposed. Following this, feces and foreign materials within the intestine were removed using 1X PBS and waiting until bleeding subsided as much as possible. After all procedures were done, mice were set on the chamber for imaging. A 25X water immersed objective lens was used for all in vivo imaging. 4D imaging videos (512×512 pixels) were recorded with 1 μm slice for depth of 40 μm. Intravital imaging was conducted by FVMPE (Olympus, Tokyo, Japan)

2.9. Transmission Electron Microscopy (TEM)

Mice was fed with 10 mg/kg of mixed three types of microplastics for 2 weeks. Transmission Electron microscopy Specimens were fixed for 12 hours in 2% Glutaraldehyde-2% Paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and washed in 0.1 M phosphate buffer, post-fixed with 1% OsO4 in 0.1 M phosphate buffer for 2 hours and dehydrated with an ascending ethanol series (50, 60, 70, 80, 90, 95, 100, 100%) for 10 minutes each, and infiltrated with propylene oxide for 10 minutes. Specimens were embedded with a Poly/Bed 812 kit (Polysciences), polymerized in an electron microscope oven (TD-700, DOSAKA, Japan) at 65℃ for 12 hours. The block is equipped with a diamond knife in the Ultra-microtome (UC7, Leica Microsystems Ltd, Vienna, Austria), and is cut into 200 nm semi-thin section and stained toluidine blue for observation of optical microscope. The region of interest was then cut into 80 nm thin sections using the ultra-microtome, placed on copper grids, double stained with 5% uranyl acetate for 20 minutes and 3% Lead citrate for 7 minutes staining, and imaged with a transmission

electron microscopy (HT7800, HITACHI, Tokyo, Japan) at the acceleration voltage of 80 kV equipped with a RC camera.

2.10. Statistical analysis

Analysis of cell migration pattern of BMDMs was obtained through Volocity (PerkinElmer, Waltham, MA, USA). All statistical analysis were conducted by GraphPad Prism 7.00 (GraphPad Software, Inc, CA, USA) software. More than two independent groups were analyzed by one-way analysis of variance (ANOVA). Statistical significance was defined as a $p\text{-value}<0.05$. All data were expressed as the mean ± standard error of the mean unless specified otherwise. All experiments were conducted at least in triplicate.

Table 1. Primer sequences used for qRT-PCR in mouse

Gene	Primer sequence (5'→3')
Tbp	F: GAGTTGCTTGCTCTGTGCTG
	R: CTGGCTTGTGTGGGAAAGAT
IL-6	F: GTCCTTCCTACCCCAATTTCCA
	R: TAACGCACTAGGTTTGCCGA
IL-1β	F: GAAGAAGAGCCCATCCTCTGT
	R: TTGTCGTTGCTTGGTTCTCC
IL-10	F: ACAGCCGGGAAGACAATAAC
	R: GGCAACCCAAGTAACCCTTA
TNF-α	F: TCTACTGAACTTCGGGGTGA
	R: CACTTGGTGGTTTGCTACGA

3. RESULTS

3.1. Surface characteristics of PET, ABS and PP based on different fragmentation behavior

In this study, I explored the influence of the diverse chemical structures of polymeric materials on fragmentation behavior and the potential toxicity of microplastic fragmentation on immune cell function. Microplastics, pervasive pollutants in the environment, pose ingestion risks to various organisms, including humans. However, the effects of microplastic fragmentation on immune cell responses remain poorly understood. To address this gap, I utilized *in vitro* models to evaluate the impact of fragmented microplastics on immune cells, comparing their effects with intact microplastics.

This study focused on three representative microplastics: PET, ABS and PP. Given status of each plastic as one of the most produced and consumed plastics, understanding its fragmentation behavior and potential effects is crucial. Each polymer's distinct chemical structure requires an investigation into its physicochemical properties and fragmentation patterns to fully understand its potential toxicity to human health. To obtain microplastics, I employed a physical micronization process on polymeric materials, using the cryo-milling technique consistent with prior methodologies. Following fragmentation, I analyzed the chemical and physical attributes of each microplastic. I hypothesized that the chemical structure of each plastic would manifest in unique characteristics in the fragmented microplastics.

Initially, I examined the surface features of each plastic post-fragmentation. ABS displayed rough and globular surfaces, whereas PP and PET exhibited cylinder-like shapes with sharp edges

on both sides. Chemical characteristics were confirmed via Raman spectroscopy after micronization. Figures 1(B) revealed identical peak assignments in the Raman spectra for each plastic before and after fragmentation, affirming the consistency of their chemical structure post-fragmentation. Furthermore, the distinct fragmentation behaviors were influenced by the inherent chemical structures of the plastics.

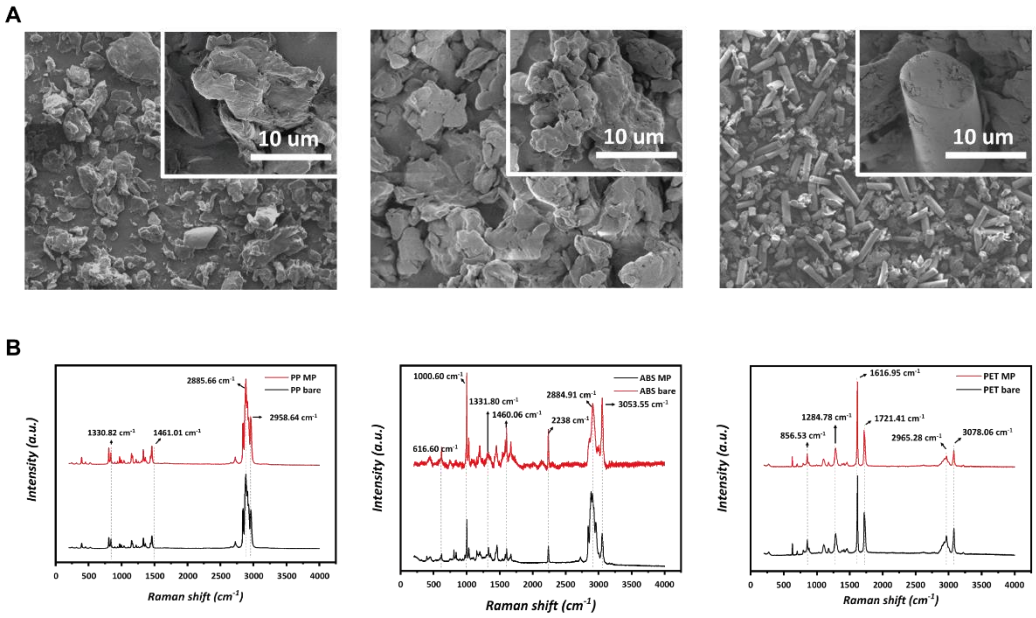


Figure 1. Surface characteristics of PET, ABS and PP based on different fragmentation behavior. (A) Scanning electron microscopy of PET, ABS, and PP. (B) Raman spectroscopy shows the chemical characteristics of PET, ABS, and PP. The upper spectra show the analysis after fragmentation, and the lower spectra shows the analysis before fragmentation.

3.2. Physical characteristics of PET, ABS and PP

To analyze the physical characteristics of three different microplastics, I conducted X-ray diffraction analysis (XRD). Figures 2(A) illustrate distinct peaks for each microplastic sample, reflecting their unique XRD patterns. PP exhibited the highest crystalline size post-fragmentation, aligning with its typically high crystallinity of around 50% and pronounced crystalline structure. This correlation is consistent with the rough surface characteristics observed in Figure 1(A). The high crystallinity of PP likely leads to its fragmentation into sharp edges during mechanical micronization processes. In contrast, ABS displayed broad XRD peaks, indicative of lower crystalline size and altered shape post-fragmentation. This corresponds to the globular and round surface characteristics of ABS microplastics observed in Figure 1(A).

Interestingly, PET demonstrated a different trend in both XRD and surface characteristics compared to the other plastics. Despite exhibiting low crystallinity post-fragmentation, PET microplastics displayed sharp edges and a cylindrical form. This disparity can be attributed to the manufacturing process. The production of bulk PET pellets involves extrusion in a one-way manner, resulting in the cylindrical structure observed in fragmented PET microplastics.

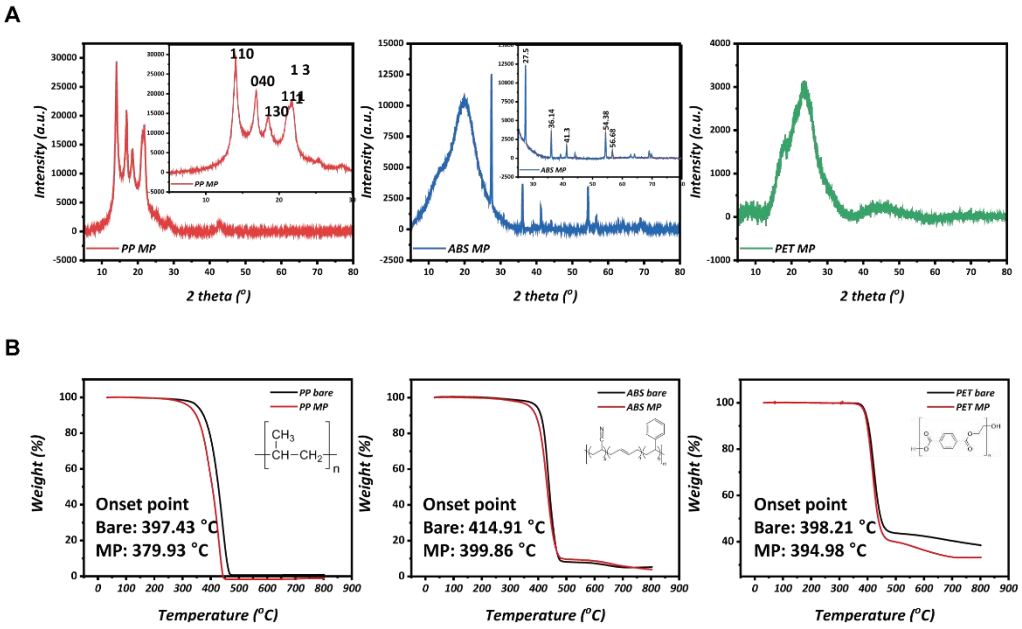


Figure 2. Physical characteristics of PET, ABS and PP. (A) X-ray diffraction analysis of PET, ABS, and PP. (B) Thermal gravimetric analysis of PET, ABS, and PP.

3.3. PET and ABS can be recognized as foreign substances by BMDMs

Macrophages are renowned as key players in the innate immune response, especially the function of recognizing and phagocyte pathogens[24]. Depending on their function of recognizing foreign substances, I tried to investigate the interaction between bone marrow-derived macrophages (BMDM) and three different microplastics (PET, ABS, and PP) by using Nikon Ti2e microscopy. To mimic the extracellular matrix (ECM) environment *in vitro*, fibronectin (10 μl/mL) was coated on confocal dishes, enabling BMDMs migration via integrin receptor binding. BMDMs were seeded at confocal dishes with M-CSF (20 ng/mL) and after 24 hours PET, ABS, and PP (0.5 mg/mL) were treated to BMDMs. Through *in vitro* imaging, it became clear that BMDMs can recognize PET and ABS as foreign substances and easily interact with and internalize them very actively. This finding suggests that even when microplastics are entered into the human body, the interaction between microplastics and macrophages is inevitable, and it may influence the innate immune system. However, in the case of PP, unlike PET and ABS, due to its property of remaining suspended above, BMDMs did not recognize or interact with them. It means the response to macrophages and the innate immune system may be different depending on the characteristics of the microplastics.

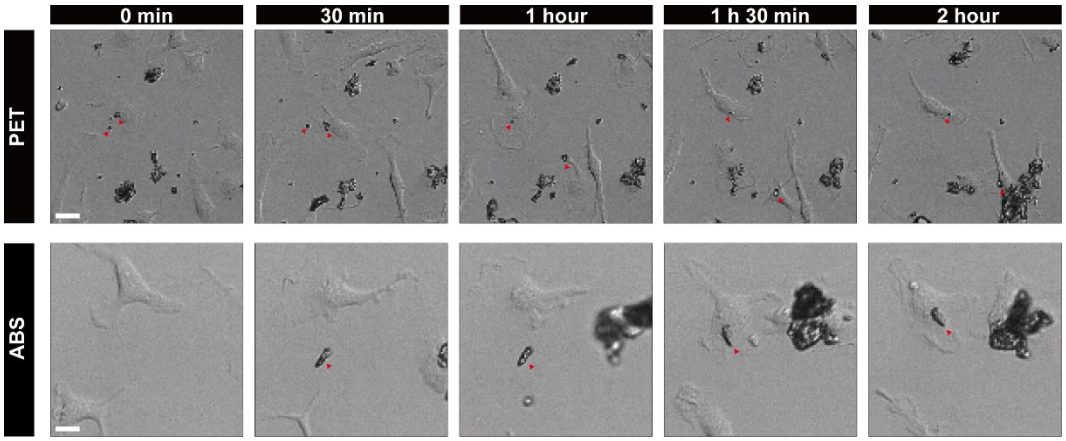


Figure 3. BMDMs recognized and uptook PET and ABS but do not interact with PP. Representative images of BMDMs treated with PET and ABS were shown. PET and ABS were treated to BMDMs 30 minutes before imaging. The movies were taken at 30-second intervals for 2 hours and were offered the image every 30 minutes. Scale bar: 10 μm.

3.4. Exposure of PET and ABS to BMDMS decreased cell viability but not by PP

To confirm whether exposure to microplastics can have an effect on the cell viability of BMDMs, PET, ABS, and PP were tested at four different concentrations ranging from 0.05 mg/mL to 1 mg/mL (0.05, 0.1, 0.5, 1 mg/mL). 100 μl of 3’ D.W was added to the control group, while the positive control group was treated with 10% of DMSO.

After the 24-hour incubation period, significant cytotoxicity is induced by PET and ABS on BMDMs, in a concentration-dependent manner. PET induced the most significant cell death among the three different microplastics tested, exhibiting only 40% cell viability even at the lowest concentration of 0.05 mg/mL. ABS also demonstrated a concentration-dependent decrease in cell viability, with cell viability dropping below 50% at the highest concentration of 1 mg/mL. This suggests the exposure of microplastics to BMDMs can lead to cell death via mitochondrial dysfunction and result in a harmful effect on the innate immune system. Conversely, PP did not exhibit a significant impact on BMDMs viability according to MTT assay analysis. Utilizing the concentrations resulting in less than 50% cell viability, the study proceeded to assess inflammatory cytokine and protein levels, with the 0.5 mg/mL concentration selected for further investigations.

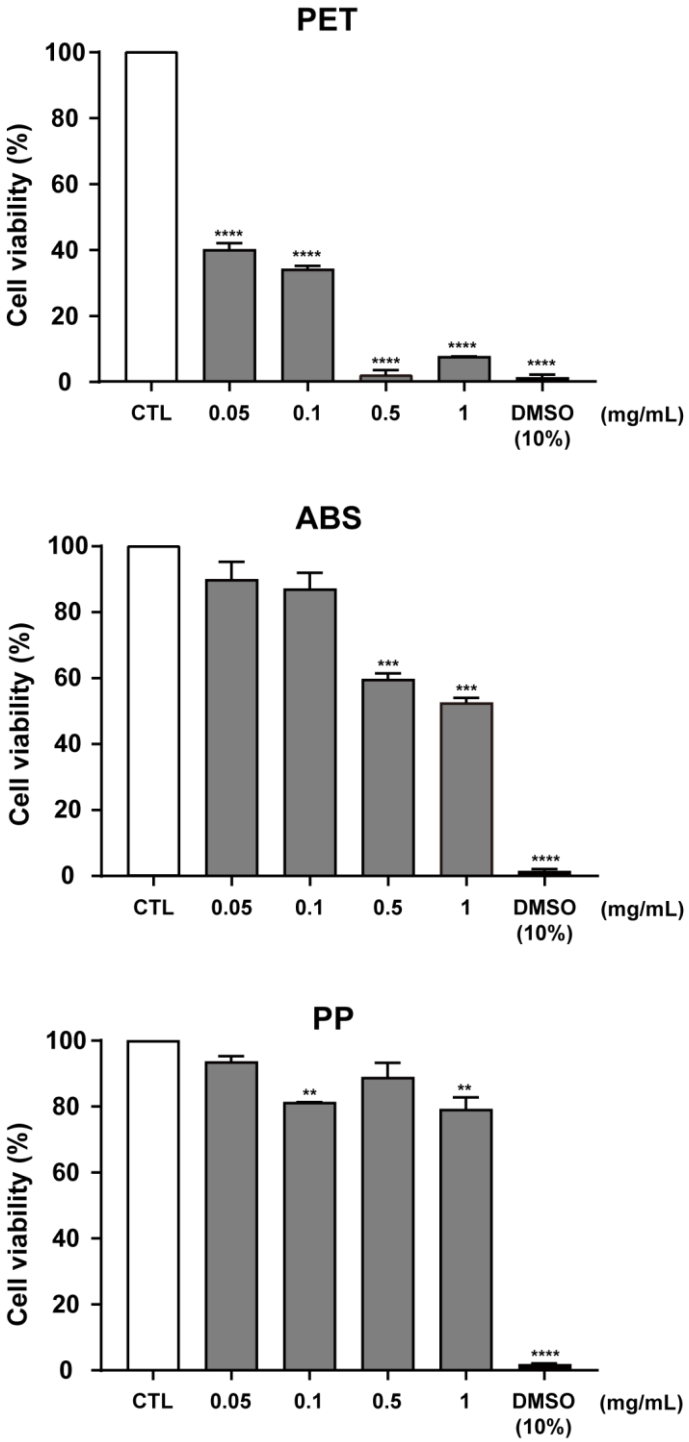


Figure 4. Cell viability of BMDM decreased after incubated with PET and ABS. Bar graph of cell viability of microplastic-treated BMDMs. Cell viability changes are assessed by MTT assay. Statistical analysis is performed by multiple t-test. Data were presented as the mean ± SEM. Statistical significance was indicated with asterisk. [*P*-value: 0.05> (*), 0.01> (**), 0.001> (***), 0.0001> (****)]

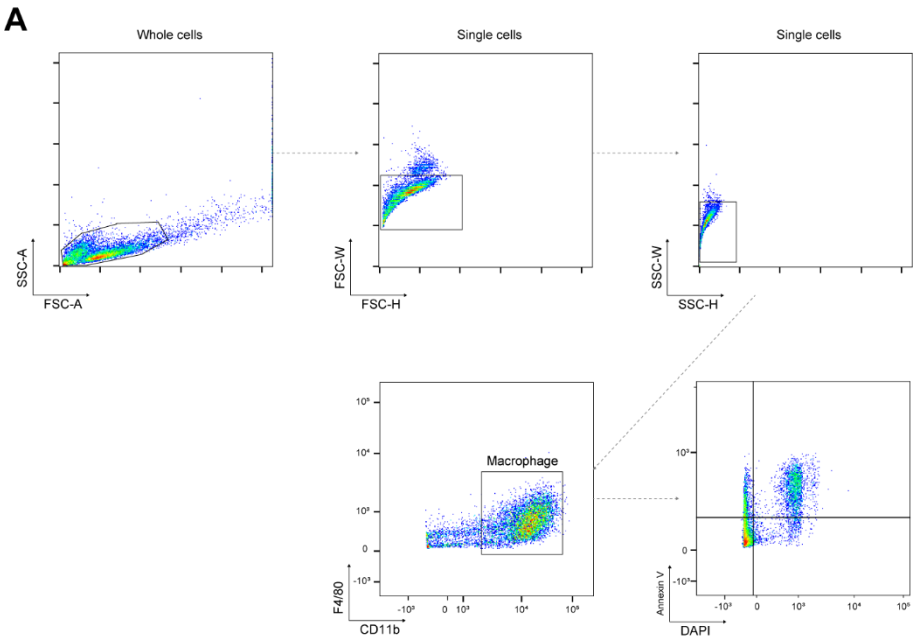
3.5. PET and ABS induced cell death, especially necrosis, to BMDMs

Apoptosis is a regulated form of cell death that plays crucial roles in development, aging, maintaining homeostasis, as well as in defending against cellular damage induced by disease or harmful agents[30,31]. In contrast, necrosis is an alternative form of cell death triggered by external injuries such as hypoxia or inflammation. Unlike apoptosis, necrosis is an uncontrolled and passive process and energy-independent cell death, occurring when cellular damage is severe and irreparable. Morphological changes characteristic of necrosis include cell swelling, cytoplasmic vacuole formation, disruption of organelle membranes, and ultimately cell membrane rupture, resulting in the release of cytoplasmic contents and the secretion of various chemokines and cytokines to recruit immune cells which involved in inflammatory reaction[32,33].

Depends on the effect of microplastics on BMDMs viability, flow cytometry was performed to determine the type of cell death in more detail. DAPI and Annexin V staining were utilized for this purpose. Cell populations were classified as live cell population if negative for both DAPI and Annexin V, apoptotic cell population if positive for DAPI alone, and necrotic cell population if positive for both DAPI and Annexin V. Experimental groups included a control group treated with 100 μl of 3' D.W and a positive control group treated with 10 μl of H_2O_2. The result showed that necrotic cell death population were increased when BMDMs exposed to PET and ABS in a concentration-dependent manner. On the other hand, the population of apoptotic cell death were only increased in the highest concentration, 1 mg/mL of PET and ABS were exposed to BMDMs. Conversely, PP did not show any significant change in the necrotic and apoptotic cell death population, consistent with the findings of cell viability tests.

Based on this result, exposure to microplastics can cause cellular membrane damage and necrotic cell death in BMDMs and lead to inflammation with the secretion of cytoplasmic contents

and the secretion of various chemokines and cytokines involved in inflammation.



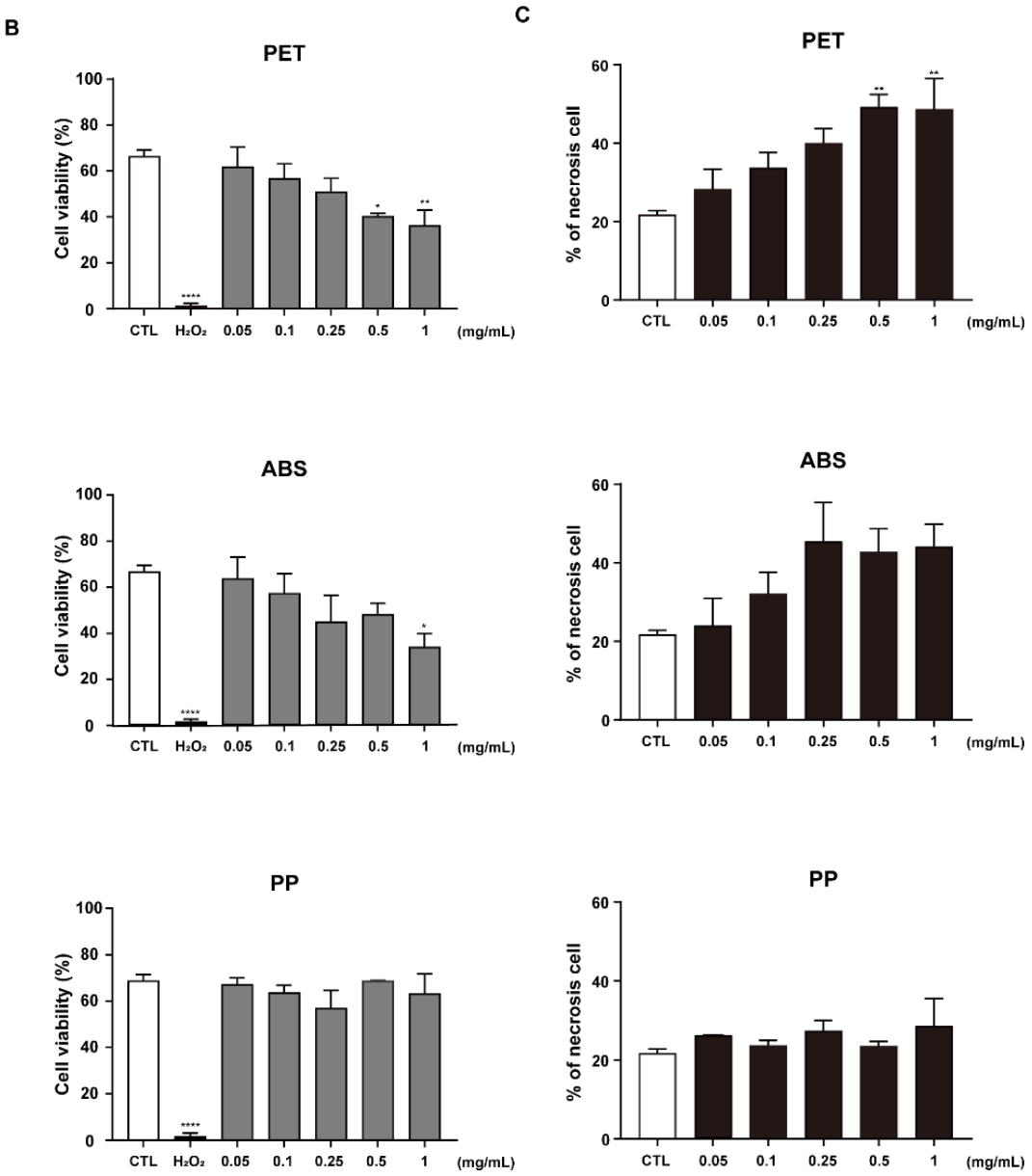


Figure 5. Necrotic cell death populations increased in BMDMs when exposed to PET and ABS.

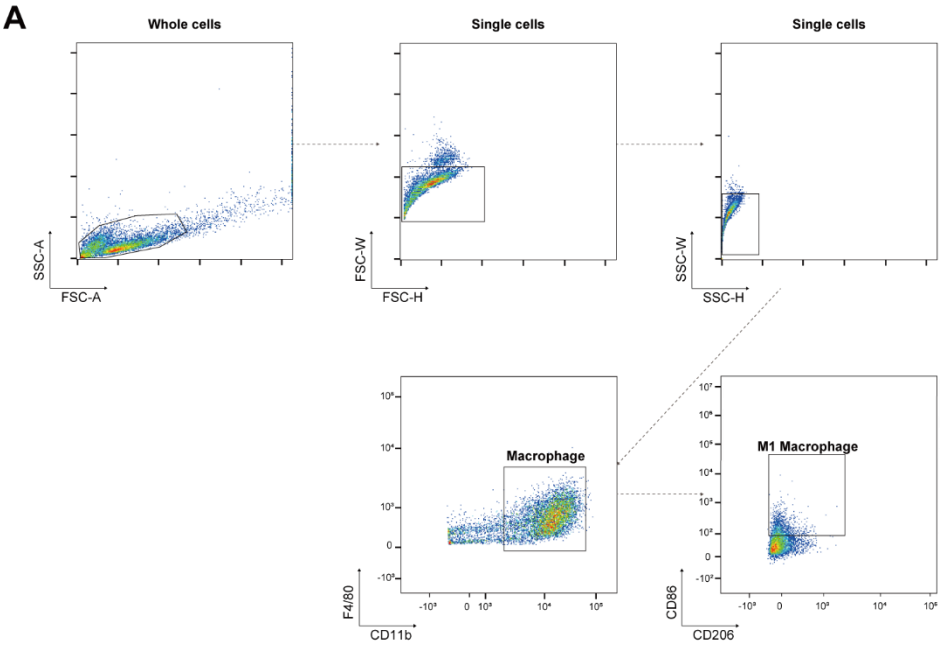
(A) Gating strategy and representative scatter plot to identify the population of live, apoptotic, and

necrotic cell populations of BMDMs treated with microplastics (B) Bar graph of cell viability of microplastic-treated BMDMs. Cell viability changes are assessed by Flow cytometry. (C) Bar graph of necrotic cell death population of microplastic-treated BMDMs. The change in necrotic cell populations was assessed by Flow cytometry. Statistical analysis is performed by multiple t-test. Data were presented as the mean ± SEM. Statistical significance was indicated with asterisk. [*P*-value: 0.05> (*), 0.01> (**), 0.0001> (****)]

3.6. Exposure to PET and ABS induced M1 macrophage polarization

Macrophage is known for its plasticity which can polarize to several subtypes of macrophage when exposed to several factors such as local growth factors, pro-inflammatory cytokines, and microbial products[34]. Two major subtypes of macrophage are classically activated or inflammatory (M1) macrophage and alternative activated or anti-inflammatory (M2) macrophage[20,23]. It is well known that when macrophages polarize to M1 macrophage which is related to pro-inflammatory signal, CD86 expression is increased in cell surface and usually secrete the pro-inflammatory cytokines such as TNF α -, IL-1 β, and IL-6[35,36].

To confirm whether exposure to microplastics can lead to the differentiation of BMDMs to M1 macrophage, I performed flow cytometry. For the control group, 100 μl of 3' D.W was treated and for the positive control group, LPS (100 ng/mL) and IFN-γ (20 ng/mL) were treated to induce the polarization to M1 macrophage. Five different concentrations of microplastics were used which is the same in previous experiments. The result showed that significant increase in the expression of CD86 in the cell surface which is known as the marker of M1 macrophage. The result was presented using both a bar graph and a histogram, which illustrate the increase in CD86 expression, indicated by a rightward shift. This result suggested that exposure of microplastics to BMDM induces the secretion of pro-inflammatory cytokines such as TNF-α and IFN-γ. By these inflammatory cytokines, the differentiation of BMDMs to M1 macrophages is induced and verified that the population of M1 macrophages increased depending on concentration when treated with PET and ABS. However, there is no significant increase in CD86 expression in BMDMs treated with PP.



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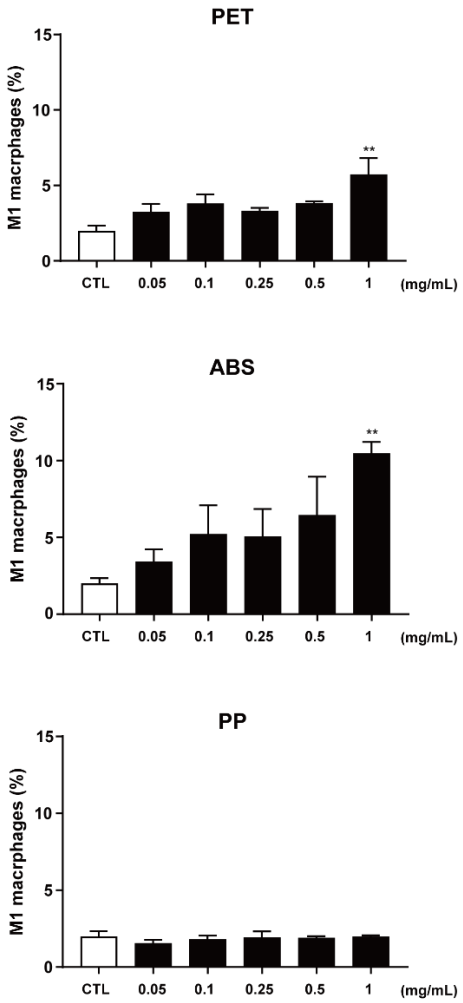

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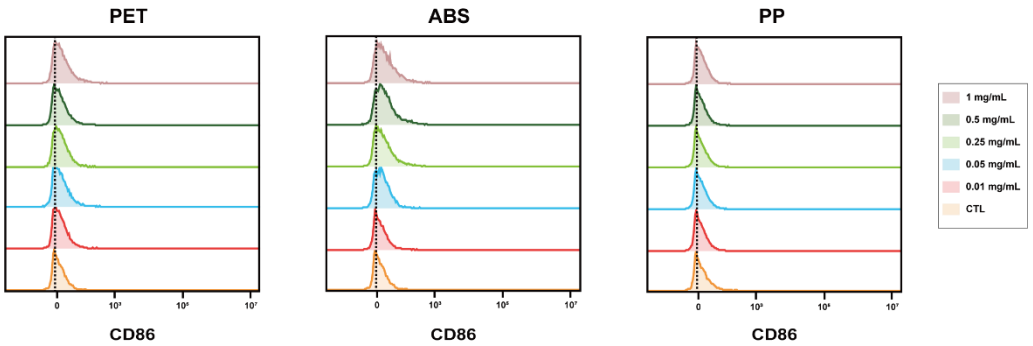

Figure 6. M1 polarization population increased after incubated with PET and ABS. (A) Gating strategy and representative scatter plot to identify the population of M1 macrophages when BMDMs were treated with microplastics (B) Bar graph of the population of M1 macrophages in microplastic-treated BMDMs. The population of M1 macrophage changes is assessed by Flow cytometry. Statistical analysis is performed by multiple t-tests. Data were presented as the mean ± SEM. Statistical significance was indicated with asterisk. [*P*-value: 0.01> (**)] (C) CD86 expression by BMDMs treated with microplastics was displayed by histogram. Data are representative of three independent experiments.

3.7. Pro-inflammatory cytokine levels increased in BMDMs exposed to microplastics

After confirming that the exposure of microplastics to BMDMs can induce differentiation to M1 macrophages, I conducted RT-qPCR to assess the elevation of inflammatory cytokine levels in microplastic-treated BMDMs. M1 macrophages produce inflammatory genes, including TNF-α, IL-6, and IL-1β[36]. M2 macrophages produce anti-inflammatory cytokines such as IL-10, IL-1ra, TCF-β[37,38]. BMDMs were collected after 24 hours of microplastic treatment at a concentration of 0.5 mg/mL. The expression levels of prominent inflammatory cytokines, including TNF-α, IL-6, and IL-1β, and the anti-inflammatory cytokine IL-10, were examined. For the control group, 100 μl of 3' D.W was treated.

While all pro-inflammatory cytokines showed increased expression, PET and ABS exhibited significant elevation, whereas PP showed a negligible effect. Notably, there was no detectable expression of IL-10, further confirming the lack of differentiation into M2 macrophages. These results suggest that when microplastics are recognized by BMDMs and secrete the pro-inflammatory cytokines and this means that sustained unnecessary inflammation can be induced within the body. This disruption in macrophage function could potentially lead to chronic inflammation which may have deleterious effects when other related complications occur in the future.

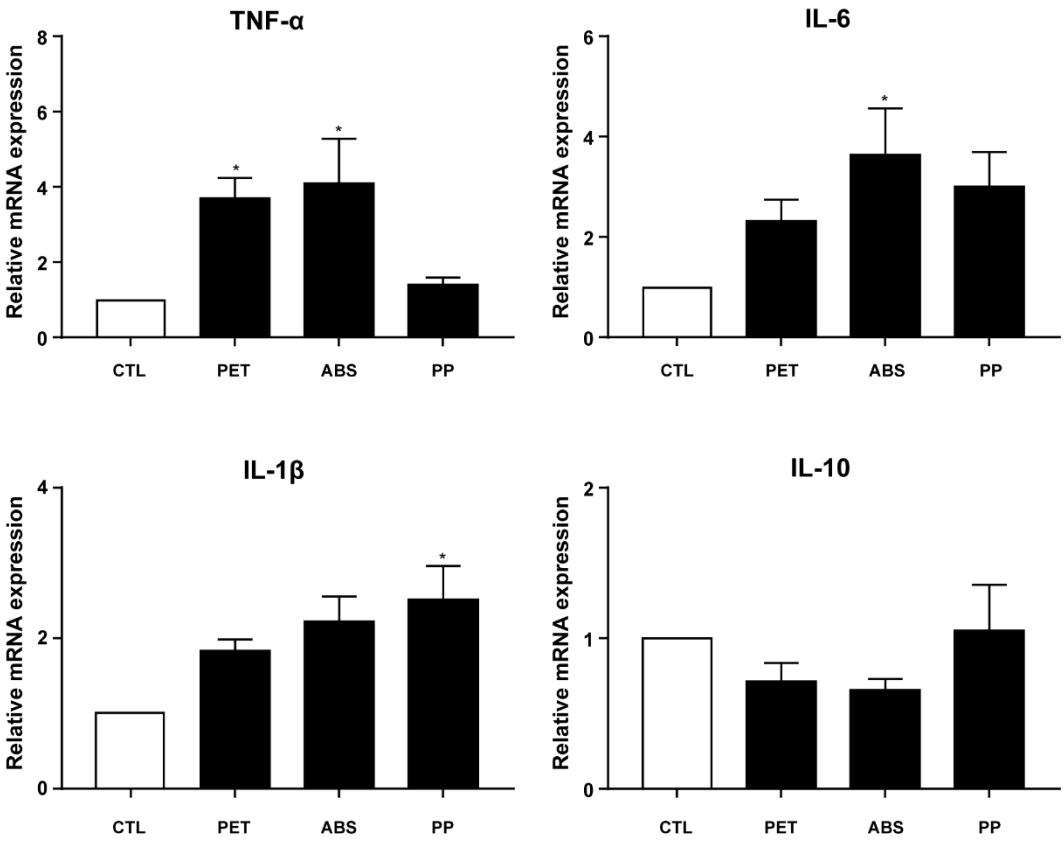

Figure 7. Pro-inflammatory cytokine levels of TNF-α, IL-6 and IL-1β were increased in microplastics-treated BMDMs. Bar graph of the increased or decreased level of cytokines in microplastic-treated BMDMs. The relative mRNA expression changes are assessed by RT-qPCR. Statistical analysis is performed by multiple t-tests. Data were presented as the mean ± SEM. Statistical significance was indicated with asterisk. [*P*-value: 0.05> (*)]

3.8. Microplastics regulate activation of Akt signaling pathway in BMDMs

Based on the results of RT-qPCR which indicate that BMDMs were activated by microplastics and secreted pro-inflammatory cytokines, I investigated the signaling pathway involved in BMDMs activation. For the control group, 100 μl of 3' D.W was treated, while the positive control group weas treated with LPS (100 ng/mL) and IFN-γ (20 ng/mL) to induce inflammation in BMDMs.

The Akt signaling pathway is well known to play a significant role in inflammation and can be activated through several mechanisms. In macrophages, when TRL4 is activated by LPS or saturated fatty acid (SFA), MyD88 is recruited, leading to the activation of PI3K. This activation results in the phosphorylation of AIK and IKK[39,40]. Western blot analysis demonstrated a significant increase in the expression of p-Akt and p-IKK. These findings suggest that the exposure of microplastics is associated with the Akt signal pathway which is involved in the inflammatory response.

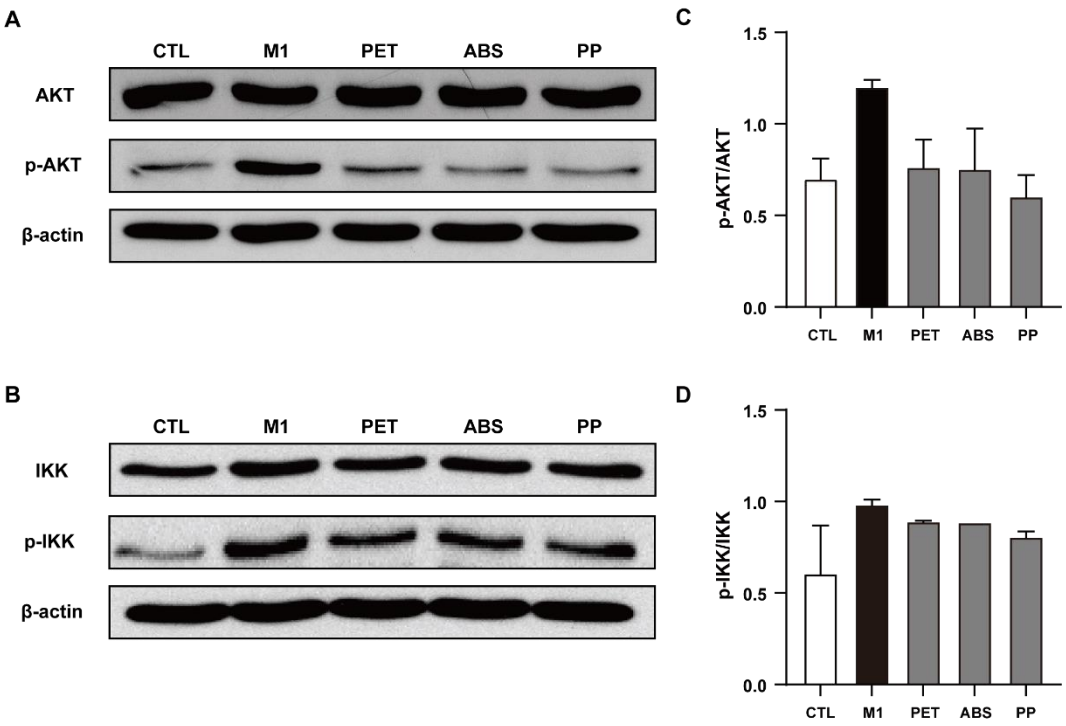

Figure 8. Exposure of microplastics to BMDMs mediates activation of the Akt signaling pathway. (A)(B) Western blotting data of Microplastics-treated BMDMs were shown in a snapshot. Results were obtained 3 times. Quantitative analysis of (C) p-Akt/Akt, (D) p-IKK/IKK production from triplicate western blots. Data were presented as the mean ± SEM.

3.9. The presence of microplastics and the change of mitochondrial morphology is observed in microplastics-fed mouse intestine

After obtaining several results indicating that microplastics can have deleterious effects on the function of BMDMs, I confirmed the presence of microplastics in mouse intestine after feeding microplastics for 2 weeks to CX3CR1$^{GFP/+}$ mouse and C57/BL6 mouse. The C57/BL6 mice were orally fed a mixed form of PET, ABS, and PP microplastics (10 mg/ kg) once daily for two weeks. Three types of microplastics were suspended in 3’ distilled water. The dosage was determined based on the estimated environmental accumulation and the amount of microplastics ingested or inhaled by organisms. According to the Worldwide Fund for Nature, humans ingest or inhale approximately 5 g of microplastics weekly. Based on this information, I calculated and adjusted the dosage for mice of 10 mg/kg [41,42].

Two-photon intravital imaging was employed to observe the presence of microplastics in the intestines of CX3CR1$^{GFP/+}$ mouse, with the microplastics tagged using Nile Red. Several microplastic particles were observed, suggesting that the deleterious effects of the mouse immune system observed *in vitro* with BMDMs can also occur in *in vivo*. Transmission electron microscopy (TEM) images of microplastics-fed C57/BL6 mice did not show direct evidence of the presence of microplastics but did reveal significant changes in the morphology of mitochondria in the intestines. Unlike the control group, where mitochondria were typically longish with dense cristae, the microplastics-fed group exhibited altered mitochondrial shapes which are round and reduced cristae density. These changes indicate that exposure to microplastics leads to mitochondrial dysfunction, potentially resulting in cell death through apoptosis and necrosis. This finding aligns with decreased cell viability and an increased population of necrotic cells among BMDMs exposed to microplastics.

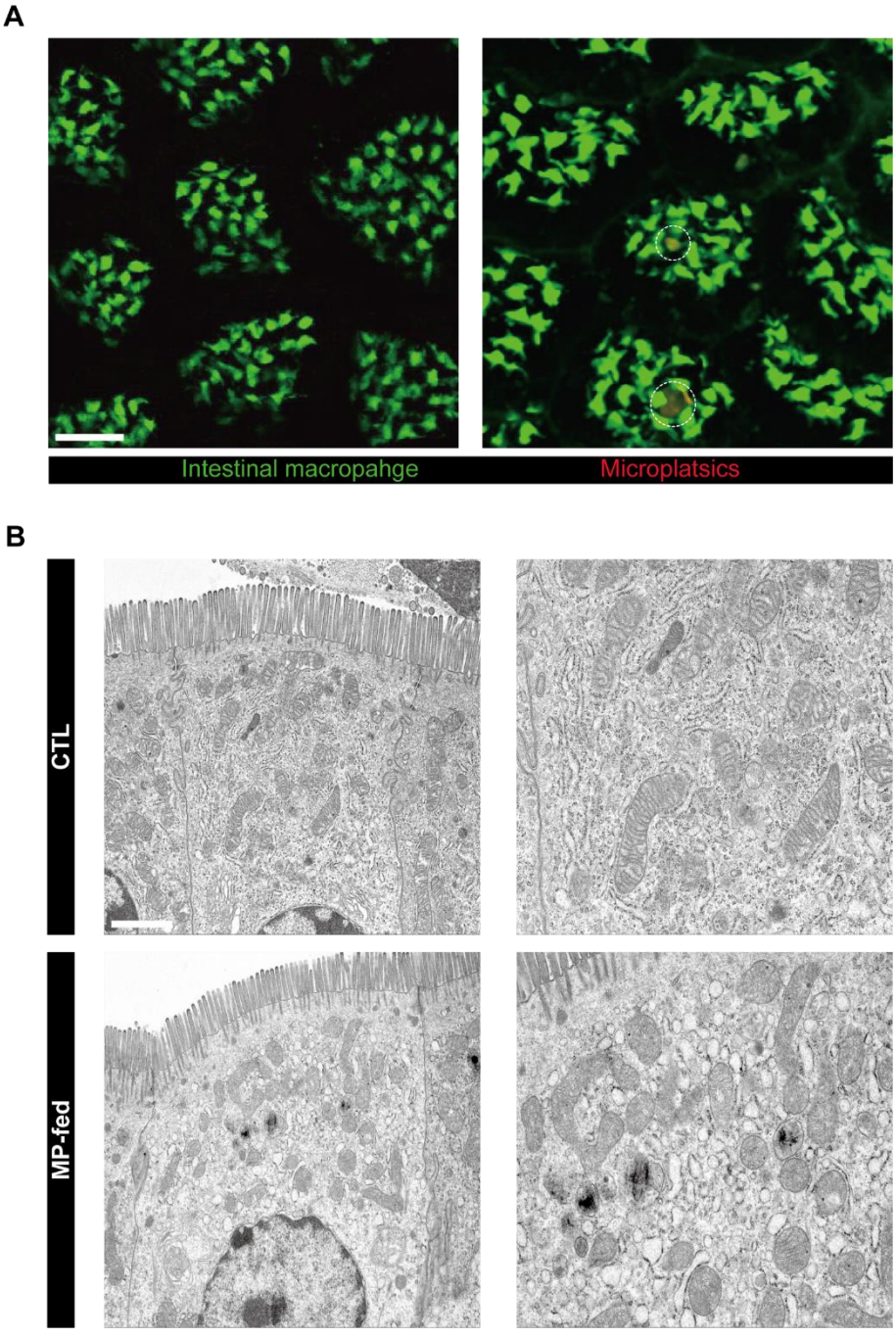

Figure 9. The presence of microplastics in mouse intestine and the change of mitochondria morphology. (A) Representative image of two-photon intravital imaging of CX3CR1$^{GFP/+}$ mice and microplastics-fed CX3CR1$^{GFP/+}$ mice intestine. Microplastics tagged with Nile Red were shown in red color. Scale Bar: 50 μm. (B) Representative image of transmission electron microscopy (TEM) image of C57/BL6 mice and microplastics-fed C57/BL6 mice intestine. Scale bar: 2 μm.

4. DISCUSSION

The accumulation of microplastics has recently emerged as a major social concern, and the effects of microplastic ingestion and inhalation on the human body have been elucidated. When microplastics enter the human body, they can be exposed to various organs and disrupt various cellular functions. Among these, macrophages, which are the first to recognize and respond to foreign substances as innate immune cells, were investigated for their response to microplastics. To this end, I studied the effects of three different types of plastics (PET, ABS, PP) on bone marrow-derived macrophages (BMDMs) obtained from mouse bone marrow. While previous studies have shed light on the correlation between microplastics and macrophages, most have been limited to the effects of only one type of plastic with the same size and shape. However, this study aimed to investigate the impact of microplastics more similar to the natural environment on the immune system by using three different types of plastics with varying sizes and shapes to assess their effects when exposed to BMDMs.

Results showed that among the three types of plastics, PET and ABS exposure led to a noticeable decrease in BMDM cell viability, suggesting that this could be attributed to damage to the cell membrane caused by the sharp edges of plastics or problems with mitochondrial function, leading to cell death. Additionally, differences in microplastic characteristics resulted in a minimal decrease in viability with PP exposure, indicating varying immune system responses depending on the type of plastic. Moreover, I expected that macrophages can recognize and phagocytose microplastics as external substances, and observed the extension of BMDM pseudopod, engulfing PET and ABS in the surrounding area and pulling them into the cytoplasm.

Based on these results, to differentiate cell death populations in more detail, flow cytometry was conducted. The results showed that microplastics-exposed BMDMs exhibited necrotic cell death

rather than apoptotic cell death, indicating damage to the cell membrane by microplastics. Furthermore, based on previous studies suggesting macrophage differentiation into M1 and M2 types in inflammatory conditions, I expect that macrophages would differentiate into M1 macrophages when inflammation is induced by microplastics. As expected, exposure to PET and ABS significantly increased the proportion of M1 macrophage differentiation and the expression of CD86, the maker of M1 macrophages, in cell surfaces, suggesting that microplastics entering the body may induce unnecessary inflammation, disrupting macrophage function and potentially adversely affecting the innate immune system. Moreover, expecting an increase in pro-inflammatory cytokine levels associated with differentiation into M1 macrophages, RT-qPCR was performed. The results showed increased expression of representative pro-inflammatory cytokines TNF-α, IL-6, and IL-1β, known to be primarily secreted by M1 macrophages, following exposure to PET, ABS, and PP. These results suggest that PET and ABS both increase BMDM cell death and M1 polarization, while PP may not induce BMDM cell death but can activate macrophages and lead to an increase in the expression of pro-inflammatory cytokines. At last, I conducted two-photon intravital imaging and transmission electron microscopy imaging using $CX3CR1^{GFP/+}$ and C57BL/6 mice to confirm the presence of microplastics in the mouse intestine. Microplastics were observed in the mouse intestine after feeding microplastics for 2 weeks and the morphological change in the mitochondria. This showed when microplastics were inhaled or ingested into body, they can be found in the intestine and have some effects on organs.

In this study, difficulties were met in accurately assessing the impact of PP on macrophage function due to its inability to directly interact with BMDMs. However, since microplastics entering the body can easily interact with macrophages through the digestive system or bloodstream, I believe that PET, ABS, and PP will all induce an increase in cell death, polarization into M1 macrophages, and expression of pro-inflammatory cytokines when introduced into the

body based in *in vitro* results. Also, still the limitation that the verification of microplastic contamination remained. Microplastics which used in experiments is always used after sterilization, but it still has question that any contamination is left and does not have any effect to demonstrate the effect of microplastics on BMDMs.

5. CONCLUSION

This study elucidated the impact of microplastics on the function of Bone Marrow-Derived Macrophages (BMDMs), with a particular focus on three distinct types of microplastics (PET, ABS, and PP). I revealed that exposure to PET and ABS resulted in a significant concentration-dependent decrease in BMDM cell viability, while PP showed no significant impact on cell viability. This suggests that PP does not directly contact with BMDMs, which may account for its lack of effect on cell viability. Furthermore, I investigated the interaction between BMDMs and microplastics, particularly PET and ABS, and unveiled that BMDMs recognize and try to phagocytose microplastics with their pseudopods. And it leads to subsequent cell death upon failed phagocytosis. Analysis of the type of cell death revealed that microplastics induce necrosis rather than apoptosis, specifically by PET and ABS but not by PP. Additionally, microplastic-treated BMDMs exhibited increased polarization toward M1 macrophages, characterized by elevated CD80 expression of the macrophage surface, particularly in response to PET and ABS exposure. Also, I assessed the levels of inflammatory cytokines in microplastics-treated BMDMs, proving that the mRNA levels of pro-inflammatory cytokines were significantly elevated by PET and ABS exposure, but not by PP. Finally, the presence of microplastics in the mouse intestine was observed and confirmed that it can cause a change in mitochondrial morphology change which can lead to the dysfunction of mitochondria. These findings underscore the potential inflammatory response triggered by microplastic exposure and highlight the distinct and harmful effects of various microplastic types on BMDM function and immune response.

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Abstract in Korean

미세플라스틱에 의한 대식세포 기능 교란이 선천면역반응에 미치는 영향

현대사회에서 단단하고 가소성이 높은 플라스틱은 유용한 재료로 널리 사용되고 있다. 플라스틱은 사용되어진 이후 바다나 자연 생태계에 버려지게 되고 이는 UV나 파도 등과 같은 자연 요인 때문에 크기가 작은 미세플라스틱으로 변하게 된다. 생태계에 축적된 미세플라스틱이 먹이 사슬을 통해 체내로 유입될 가능성이 선행연구에 의해 밝혀진 바에 있다. 이에 미세플라스틱이 호흡기나 소화기를 통해 체내로 유입되었을 때에, 선천 면역 그 중에서도 가장 먼저 외부 물질을 인지하는 역할을 수행하는 대식세포의 기능에 미칠 영향에 대해 조사하고자 하였다. 이를 위해 자연 생태계에 존재하는 미세플라스틱과 유사한 모양과 크기를 가지는 세 개의 다른 미세 플라스틱: PET, ABS 그리고 PP가 대식세포에 노출되었을 때 일어날 수 있는 기능교란에 대해 조사하고자 하였다. 미세플라스틱 세가지 종류 중 PET와 ABS가 대식세포에 노출되었을 때, 세포 독성을 가지며 대식세포가 미세플라스틱을 항원과 같이 인지하고 이후 대식 작용이 일어남을 확인하였다. 또한, 대식 작용이 일어난 이후 대식세포가 염증 반응에 관여하는 M1 대식 세포로의 분화가 일어남과 M1 대식

세포로의 분화가 일어난 이후 염증 관련 사이토카인의 발현도 증가함을 확인하였다. 이후, 마우스 소장 내에 마이크로 플라스틱의 존재를 확인하였으며, 미토콘드리아의 모양에도 이상이 생김을 확인하였다. 이러한 결과는 미세플라스틱이 체내에 유입될 경우, 대식 세포가 이를 인지하고 불필요한 염증 반응이 유발되는, 즉 선천 면역 기능에 교란이 발생할 수 있음을 시사한다.

핵심되는 말: 미세 플라스틱, 대식세포, 세포 독성, 대식세포 분화, 염증 관련 사이토카인, 소장, 미토콘드리아