Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

Sofie Altermark

Investigation of the inflammatory response in human monocytes and macrophages exposed to micro- and nanoplastics *in vitro*

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Supervisor: Berit Johansen & Martin Wagner
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Abstract

Humans are exposed to micro- and nanoplastics (MNPs) in increasing amounts through food and drink consumption, inhalation, and dermal contact. Nevertheless, the impact these particles can have on human health is still not fully understood. In this study, we set out to investigate if MNPs trigger the release of pro-inflammatory cytokines (IL-6 & TNFα) in primary human monocytes and monocyte-derived macrophages (MDMs) isolated from whole blood. Additionally, we investigating if the Monomac 6 (MM6) cell line be a functional model for representing the immune response after exposure to MNPs in primary human monocytes. To explore the cytotoxic effects and pro-inflammatory response of MNPs we exposed these cell models to three different polymers (polymethyl methacrylate (PMMA), Polystyrene (PS) and Polyvinyl chloride (PVC)) as irregular, secondary MNPs.

We assessed the cytotoxic effects of the MNPs with the use of viability assays, by exposing the MM6 cell line at concentrations in the range of 0.05 to 300 particles per cell and 1, 100, and 300 particles per cell for the primary human monocytes. Additionally, we assessed the inflammatory response of the NMP exposure in the models by exposing both the MM6 and the primary human monocytes and MDMs to 300 and 1 particle(s) per cell, and thereafter measuring the release of pro-inflammatory cytokines IL-6 and TNFα using ELISA.

In the primary human monocytes exposure to 300 particles per cell of PVC MNPs resulted in significant release of IL-6, and for MDMs exposure to this concentration of PVC caused significant release of TNFα and IL-6. Neither monocytes nor macrophages had a significant response after exposure to PMMA or PS particles. The findings in this study indicated that the pro-inflammatory release induced by MNPs is subject to large variation between cell donors. Furthermore, our study has revealed that exposure to PVC MNPs can cause a reduction in viability at concentrations above 1.2 particles per cell when exposed for 72 h, however, induces no significant release of pro-inflammatory cytokines in the MM6 cell line. The MM6 cell line had no significant reduction in viability nor pro-inflammatory release after exposure to PMMA or PS MNPs.

In summary, this thesis has found evidence that MNPs can induce release of IL-6 and TNFα in primary human monocytes and MDMs, however, the pro-inflammatory response is dependent on cell donors, polymer type and particle concentration. Furthermore, our study shows that MNPs did not trigger pro-inflammatory responses in the MM6 cell line, indicating that the MM6 monocytes are not a representative alternative model for primary human monocytes in the context of MNP exposure.

Sammendrag

Mennesker eksponeres for mikro- og nanoplast (MNP) i økende mengder gjennom inntak av mat og drikke, innånding og hudkontakt. Likevel er innvirkningen disse partiklene kan ha på menneskers helse fortsatt ikke fullt ut forstått. I denne studien forsøkte vi å undersøke om MNP-er utløser utskillelse av pro-inflammatoriske cytokiner (IL-6 & TNFα) i primære humane monocytter og monocyttavledede makrofager (MDM) isolert fra fullblod. I tillegg undersøkte vi om Monomac 6 (MM6) cellelinjen kan være en funksjonell modell for å representere immunresponsen etter eksponering for MNP i primære humane monocytter. For å utforske de cytotoksiske effektene og pro-inflammatoriske responsene til MNP-er eksponerte vi disse cellemodellene for tre forskjellige polymerer (polymetylmetakrylat (PMMA), polystyren (PS) og polyvinylklorid (PVC)) som uregelmessige, sekundære MNP-er.

Vi undersøkte de cytotoksiske effektene av MNP-ene ved bruk av viabilitetsanalyser, ved å eksponere MM6-cellelinjen i konsentrasjoner i området 0,05 til 300 partikler per celle og 1, 100 og 300 partikler per celle for de primære humane monocyttene. I tillegg vurderte vi den inflammatoriske responsen til NMP-eksponeringen i modellene ved å eksponere både MM6 og de primære humane monocyttene og MDM-ene for 300 og 1 partikkel(er) per celle, og deretter måle utskillelseen av pro-inflammatoriske cytokiner IL-6 og TNFα ved bruk av ELISA.

I de primære humane monocyttene resulterte eksponering for 300 partikler per celle av PVC MNP i signifikant utskillelse av IL-6, og for MDM forårsaket eksponering for denne konsentrasjonen av PVC signifikant utskillelse av TNFα og IL-6. Verken monocytter eller makrofager hadde en signifikant respons etter eksponering for PMMA- eller PS-partikler. Funnene i denne studien indikerte at den pro-inflammatoriske utskillelseen indusert av MNP-er er gjenstand for stor variasjon mellom celledonorer. Videre har vår studie avslørt at eksponering for PVC-MNP kan forårsake en reduksjon i levedyktighet ved konsentrasjoner over 1,2 partikler per celle når de eksponeres i 72 timer, men induserer ingen signifikant utskillelse av pro-inflammatoriske cytokiner i MM6-cellelinjen. MM6-cellelinjen hadde ingen signifikant reduksjon i levedyktighet eller pro-inflammatorisk utskillelse etter eksponering for PMMA eller PS MNP.

Oppsummert har denne oppgaven funnet bevis for at MNP-er kan indusere utskillelse av IL-6 og TNFα i primære humane monocytter og MDM-er, men den pro-inflammatoriske responsen er avhengig av celledonorer, polymertype og partikkelkonsentrasjon. Videre viser vår studie at MNP-er ikke utløste pro-inflammatoriske responser i MM6-cellelinjen, noe som indikerer at MM6-monocyttene ikke er en representativ alternativ modell for primære humane monocytter i sammenheng med MNP-eksponering.

Preface and Acknowledgements

This thesis concludes a five-year degree in Biotechnology at the Norwegian University of Science and Technology (NTNU). The research work of this thesis was conducted at the Department of Biology from the fall of 2021 to the spring of 2022 under the supervision of Prof. Berit Johansen via Coegin Pharma AS and NTNU.

First and foremost, I would like to thank my supervisors for all your guidance, support and encouragement throughout this project. Berit Johansen and Martin Wagner, for all your guidance, and input, and for giving me the opportunity to pursue this degree. Felicity Ashcroft, for all the encouragement and support when it was needed most.

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Lastly, I would like to thank my family and friends for supporting me through the years it took to complete this degree. Your encouragement, love, and support have been crucial for this thesis completion. A special thought goes out to all the humorous moments of long lunch breaks and encouraging chats over innumerable cups of coffee.

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Sofie Altermark

Abbreviations

ELISA	Enzyme-Linked Immunosorbent Assay
HI-FBS	Heat Inactivated Fetal bovine serum
IL-6	Interleukin 6
LPS	Lipopolysaccharides
M-CSF	macrophage colony-stimulating factor
MPs	Microplastics
MNPs	Micro- and Nanoplastics
MDMs	Monocyte-derived macrophages
MM6	MonoMac 6
NPs	Nanoplastics
NTA	Nanoparticle tracking analysis
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PBMCs	Peripheral blood mononuclear cells
PCR	polymerase chain reaction
PE	polyethylene
PET	polyethylene terephthalate
PP	polypropylene
PMA	phorbol 12-myristate 13-acetate
PMMA	Poly(methyl methacrylate)

PRRs Pattern recognition receptors

PS Polystyrene

PVC Polyvinylchloride

RT-qPCR Reverese Transcription quantitive polymerase chain reaction

TNF*a* Tumor necrotic factor alpha

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1 Introduction

1.1 The Plastic Predicament

Plastics are some of the most multifaceted materials used by humans, and their versatile properties have made them indispensable in many industries and society at large, from industrial devices, medical equipment, to food packaging. However, the widespread use of plastic has also resulted in a massive environmental predicament as plastic and plastic-containing products have become one of the most abundant waste products originating from humans. It has been estimated that 380.7 million metric tons of plastic have been manufactured as of 2021 [1], and only an estimated 9% of this plastic material is recycled [2].As the majority of plastic products are not biodegradable, they accumulate in landfills or the surrounding land or marine environment, becoming a global waste challenge [2–6].

1.1.1 Defining micro-and nanoplastics

The term "plastics" has been in use since the beginning of the twentieth century, and it refers to any material containing a synthetic, high molecular-weight polymer as a crucial component. Plastics comprise a number of polymers, such as polyethylene (PE), polyethylene terephthalate (PET), Poly(methyl methacrylate) (PMMA), Polystyrene (PS), polypropylene (PP), Polyvinylchloride (PVC),), and additional chemicals, such as additives (e.g., stabilizers, flame retardants, plasticizers, fillers, and pigments) and non-intentionally added substances [7]. The term Microplastics (MPs) lacks an internationally recognized, standardized definition. However, MPs are generally accepted to include particles with a diameter in the size range of 0.1–5000 µm and include fragments, fibers, spheroids, and pellets. A further distinction can be made by separating plastic particles into primary and secondary microplastics [3,4], where primary microplastics are manufactured for industrial or personal uses, for example, as glitter in fashion products or exfoliates in industrial cleaning agents [8]. Secondary microplastics started their life cycle as larger macroplastics and are the products of degradation by hydrolysis, photodegradation, or physical and mechanical corrosion [9] to become smaller forms of plastic. Secondary microplastics come from a large variety of products, including textiles, paint, single-use plastics, and packaging materials that have been released into the environment. The exact

percentage of MPs in the environment that are primary or secondary MPs is unknown, however, there have been estimates on the impact of primary MPs in bodies of water, which suggested around 30% of plastic released is from primary MPs [10].

Nanoplastics (NPs), likewise with MPs, lack a recognized standardized definition but are considered as a continuation of MPs in relation to shapes and material makeup. Some research groups set the size range at 1 to 1000 nm, while others at 1 to 100 nm. The latter is in line with the current ISO definition of nanomaterials but this standard only includes intentionally manufactured particles and therefore excludes secondary NPs. The European Food Safety Authority (EFSA) has defined nanoplastics as "a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range of 1 nm to 100 nm" [6, 11, 12].

Due to the lack of a recognized standard for the definition of MNPs, throughout this thesis, the term MNPs will be used to refer to plastic particles in the range of 1 nm to 5 µm.

1.1.2 Exposure and presence of MNPs in humans

MNPs have had an increased presence in both research and politics because of their ubiquitous presence and possible impact on humans. MNPs have been detected in the environment including in soil [13], in bodies of water [4], in fresh fallen snow [14] and as air borne particles [15], to consumable products such as food and drinking water [16, 17]. As the amount of plastic in the environment increase, the amount of plastic humans are exposed to increases with it. Plastic particles can enter the human body through multiple routes of exposure [18]. Ingestion of MNPs through consumable items has been found as a major route of exposure, as plastic particles have been found as contaminants in all parts of the food chain [18, 19] (Figure 1.1). Furthermore, inhalation of airborne particles, particularly secondary MPs from industrial byproducts or automobile tire degradation, has recently been discovered as a major exposure route. Lastly, dermal contact through personal care products or clothing fibre is a possible route, however, have been considered to be less prominent compared to ingestion and inhalation [20].

Thus, the majority of the scientific consensus is that exposure to MNPs leads to particles entering the human body [18–20], and it has been postulated, based on rodent studies, that once they have entered the body, the particles can circulate through the lymphatic system and to the liver, spleen, and kidneys. The hypothesis is supported by studies investigating the presence of plastic particles in humans, which have found MNPs to be present in human blood [21], in lung tissue [22], in breast milk, theplacenta [23, 24], testis, and semen [25]. However, calculations of how much MPs the average human

internalizes differ widely. At the low end, a study examining plastic uptake in relation to secretion calculated humans consume 4.1 µg of MPs per week [26]. At the high end, a recent study estimated that humans may consume 0.1 to 5 g of MPs per week. The latter study was based on an analysis of the MPs in various food items [19]. For NPs, there exists no quantification of the exposure to humans due to the nanoparticles small size making them hard to detect, however, the current assumption is that exposure to NPs could be in higher number of particles than MPs as the degradation of MPs leads to NPs. As research into the ubiquitous presence of MNPs continues to uncover particles everywhere researchers look, both in the environment and in the human body, the question of MNPs' potential adverse effects becomes increasingly urgent.

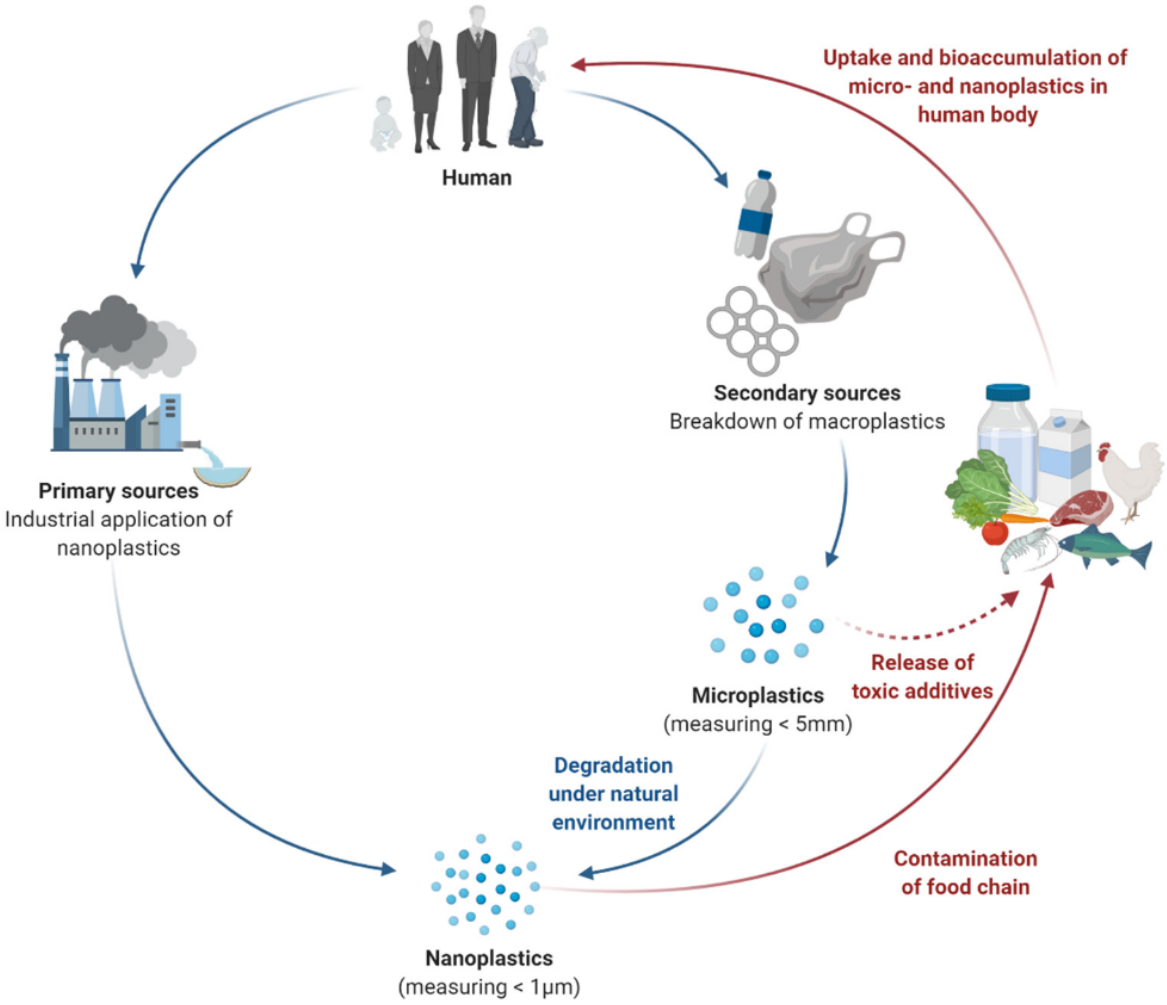

Figure 1.1: Sources and exposure routes of Micro- and nanoplastics. Micro- and nanoplastics are generated by both consumer goods and industrial manufacturing from primary and secondary sources. Larger plastic products can disintegrate into microplastics, eventually turning into nanoplastics, which can accumulate in the food chain and water supplies leading to human exposure. Figure adapted with permission from Yee et al [18]

1.1.3 Toxicity of MNPs in humans

The potential adverse effects of MNPs have been of interest since the late 1980s, although research on this topic has produced conflicting results. While studies on various animal and cell models have suggested that exposure to MNPs can induce oxidative stress, inflammation, immune dysfunction, neurotoxicity, and changes to hormone secretion and metabolism [5, 7], the actual effect these particles can have on human cells is still being researched.

There have been an increasing number of *in vitro* studies on the toxic and inflammatory effects from MNPs in cells from the innate immune system. There are proportionally more studies on the adverse effect using immortalized cell lines, but there has also been studies using primary human cells isolated from donors. The review study on cellular toxicity by Banerjee & Shelver have presented that multiple different plastics (PS, PP, PE and PVC) can have a toxic effect on cell lines when exposed. In review they found that the adverse effect of the particles were dependant on the particle shape, size and concentration as well as which cell line was used. While cell lines can give an indication of the adverse effects of MNPs, they are not fully representative models as they often lack certain *in vivo* characteristic [27]. Primary human cells are therefore believed to give a results more indivitive of the actual effect of MNP exposre. Studies that have used primary human cells gives a similar picture as the cell lines. There have been studies using PBMCs which has found that exposure of MNPs from the polymers PP, PE, and PS can cause release of pro-inflamamtory cytokines [5, 28, 29], however these studies only found the adverse effect from certain concentrations and sizes of MNPs. Again, this further indicates that while MNPs can be toxic in human cells, this is dependant on the size and concentration of particles.

While *in vitro* cell models have found evidence that plastic particles can have adverse effect on human cells, recreation of the inflammatory response in *in vivo* animal models has been shown to be less consistent [30, 31]. With rodent models studies have found inflammation after plastic exposure [7, 32], however at the same time there are studies finding no significant relationship [31]. While there are a good body of work the acute effects of MNPs, the effect from long term exposure and tissue resident particles are still understudies [33]. The overall implication of the findings in these studies are that the adverse effects in model animals seem to trend towards MNPs can induce a toxic effect.

As there is increasing evidence that humans are both exposed to and internalizing plastic particles, uncovering the possible adverse effect of these particles become increaseingly important. While there are studies indicating there is no relationship between plastic exposure and toxicity, there is an increasing amount of studies indicating the can have an toxic effect. One of the difficult aspects when considering the adverse effects of MNPs is that the shape, size and concentration of particles used between studies are often different,

which makes the comparison of adverse effects difficult. Nonetheless, the overall trend does seem to indicate that MNPs can have toxic effects in humans.

1.2 MNPs and inflamamtion

When foreign particles enter the human body, they can trigger a response in the immune system. The immune system is a complex network of cells, tissues, and organs that work together to protect the body from harmful pathogens and foreign substances. The immune system is divided into two main branches: the innate and adaptive immune system.

1.2.1 The innate and adaptive immune system

The innate immune system is the body's first line of defense against pathogens and is composed of physical barriers, such as the skin and mucous membranes, as well as the effector immune cells, such as the monocytic cells or natural killer cells. The innate immune system provides a general defense against pathogens, and its response is rapid and non-specific. Pattern recognition receptors (PRRs) are a collection of receptors found on the surface of innate immune cells that are involved in the detection of pathogens. PRRs recognize specific Pathogen-associated molecular patterns (PAMPs) on the surface of pathogens, such as bacterial Lipopolysaccharides (LPS), or fungal cell wall components. When a PAMP is recognized by a PRR, it triggers a signaling cascade that activates innate immune cells. The adaptive immune system is a specialized defense system that provides specific and long-lasting immunity against pathogens, and is composed of T- and B-lymphocytes which recognize specific pathogens and develop a memory of them after infections. The adaptive immune system takes time to develop a specific response to a particular pathogen, but once established, it provides long-lasting immunity, allowing the body to quickly respond to subsequent infections caused by the same pathogen.

The innate and adaptive immune systems operate both individually and together in response to infection. When PAMPs are recognized by PRRs, the signaling cascade activates the phagocytic, antigen-presenting cells of the innate immune system, monocytes, granulocytes, and dendritic cells. These cells engulf and break pathogens down into fragments, antigens, which are then presented to and recognized by the lymphocytes. Once activated, T-lymphocytes differentiate into effector cells that can attack cells infected with the pathogen, while B lymphocytes recognize the antigens and produce antibodies that can bind to and neutralize the pathogen. In addition to activating the adaptive immune system, the innate immune system also provides further signals that help coordinate the adaptive response, and cytokines released by innate immune cells can activate and recruit more immune cells to the site of infection, which he LPS to amplify the immune response. The interplay of the innate and adaptive immune systems can give rise to the

inflammatory response [34, 35].

1.2.2 The inflammatory response of the immune system

Inflammation is a multifaceted, biological response of the immune system. This reaction is characterized by tissue becoming red, swollen, painful, and heated, caused by alterations in local blood vessels, which become dilated and permeable leading to increased blood flow to the site of infection. Simultaneously, the endothelial cells that line the blood vessels are stimulated to express cell adhesion proteins, promoting the attachment and movement of immune cells of both the adaptive and innate immune systems. Inflammation can be acute or chronic and is and the innate immune response leads to upregulation of phagocytotic activity and cytokine secretion [35]. There are a multitude of cytokines commonly associated with inflammation. Tumor necrotic factor alpha (TNFα) is a primary inflammatory cytokine produced by immune cells in response to infection or tissue damage and stimulates the production of other cytokines, like IL-1β and Interleukin 6 (IL-6), and promotes the activation of endothelial cells and leukocytes, leading to increased vascular permeability. IL-6 and IL-1β are produced by immune cells in response to microbial infection or tissue damage and promote the recruitment of immune cells to the site of inflammation. IL-6 acts as a regulator of the immune response by promoting B-lymphocyte maturation and T-lymphocyte activation. Notably, IL-6 can have both pro-inflammatory and anti-inflammatory dependent on the context in which it is produced, while TNFα is primarily considered a pro-inflammatory cytokine. IL-10, on the other hand, is primarily an anti-inflammatory cytokine that is produced by immune cells as a feedback mechanism to dampen the inflammatory response and prevent tissue damage [34, 36].

1.2.3 Monocytes and macrophages in the inflammatory response

The effector cells that facilitate inflammation in the innate immune system through their phagocytotic abilities are monocytes, macrophages, dendritic cells, and neutrophils. As mentioned, these cells will engulf foreign objects and pathogens through the activation of their PRRs. Monocytes are leukocytes that originate in the bone marrow and circulate in the spleen and blood. When their phagocytotic abilities are activated, monocytes can initiate the singalong cascade of the inflammatory response, however, they can also migrate to tissue, where they can differentiate into both macrophages and dendritic cells. Macrophages are large, phagocytic cells that are responsible for scavenging and eliminating pathogens, damaged cells, and other debris from the body. Macrophages can both originate as monocyte or be found as tissue-resident macrophages in all tissues of the body, including the lymph nodes, spleen, liver, and lungs [34, 35]. Macrophages are considered essential in steady-state tissue homeostasis for growth factor production and clearance of apoptotic cells. Unlike monocytes, macrophages are terminally differenti-

ated cells but both cells have similar functions in amplifying the inflammatory response through phagocytosis and cytokine secretion [37].

Macrophages can be activated via two seperate pathways. The first pathway, known as the ”classical” or ”M1” activation, leads to a pro-inflammatory phenotype. In response to extracellular or intracellular pathogens, PRRs on the surface of M1 macrophages upregulate the release of various pro-inflammatory cytokines (eg. IL-6 and TNF α). Additionally, when M1 macrophages act as APC, they induce a Type-1 helper (Th1) T cell response through MHCII presentation which furthers the inflammatory response [38]. The second pathway is known as ”alternative” or ”M2” activation. M2 macrophages are associated with tissue repair and down-regulation of the initial inflammatory response. M2 activation leads to a Type-2 helper (Th2) T cell response, which is associated with the humoral immune response and B-lymphocyte maturation. It is worth noting that the polarization of macrophages into distinct activation states is not black and white, but rather exists on a spectrum with various stimuli eliciting a range of responses, and there exists a subset of the responses based on the specific case of stimuli the monocytic cells are exposed to [38, 39]. Both subsets of macrophages and monocytes are crucial players in the inflammatory response.

1.3 Models of inflammation

While the initiation and progression of inflammation in the human body is a complex multi-cellular process. Several different model systems can be utilized to predict the response to an inflammatory stimulus. Whole animal models, or primary immune cells isolated from blood are commonly used, along with immortalized cell lines, typically transformed hematopoietic cells with the capacity to respond to immunogenic stimuli. All models have advantages and disadvantages.

Animal models provide valuable insights into the study of inflammation, and rodent models are one of the most utilized in research. These models involve the use of rodents such as mice or rats to mimic inflammatory responses in humas. One of the key advantages of rodent models is their physiological and genetic similarity to humans, enabling researchers to study various aspects of inflammation in a living, complex organism. Rodent models allow for the investigation of interactions between different cell types, tissues, and organs involved in the inflammatory process [40]. While useful, rodent models are not completely representative for interaction in the human immune system, and thus cannot replace the use of human cell cultures and tissue entirely.

Cellular models offer a controlled environment for studying inflammation at a cellular level, usually either as primary cells or immortalized cell lines. Primary cell cultures originating from specific tissues or organs allow researchers to directly study the behavior and response of cells involved in inflammation. These models offer the advantage of more

closely matching the native characteristics and functionality of cells, as they can provide a more accurate representation of the *in vivo* environment. However, obtaining primary cells can be challenging, and their maintenance and propagation in the laboratory can be time-consuming and costly. One should also consider the ethical implications of using cells derived from humans, as researchers have to consider the well-being, privacy and dignity of the donor.

PBMCs are one primary cell model isolated from peripheral blood obtained from humans. They are characterized by their circular nucleus, and consist of lymphocytes, monocytes, and dendritic cells. PBMCs can be used as a model for molecular inflammation as the can both be used as a whole model consisting of multiple cells interacting together, or specific cells such as monocytic cells can be isolated for study or used to generate Monocyte-derived macrophages (MDMs) *in vitro* [37, 41]. Primary monocytes can be differentiation to MDMs *in vitro* with growth factors; either granulocyte-macrophage colony-stimulating factor (GM-CSF) or macrophage colony-stimulating factor (M-CSF). GM-CSF promotes the differentiation of pro-inflammatory monocytes into classical M1 macrophages, whereas M-CSF regulates the development of anti-inflammatory monocytes into M0 macrophages with M2-like phenotypic and functional features (Figure 1.2). The activation of the macrophages are dependant of the time frame the monocytes are exposed to the growth factors, as M-CSF will at the early stages lead to a M0-type macrophage which can thereafter be terminally activated through stimuli to become either M1 or M2 [35, 37].

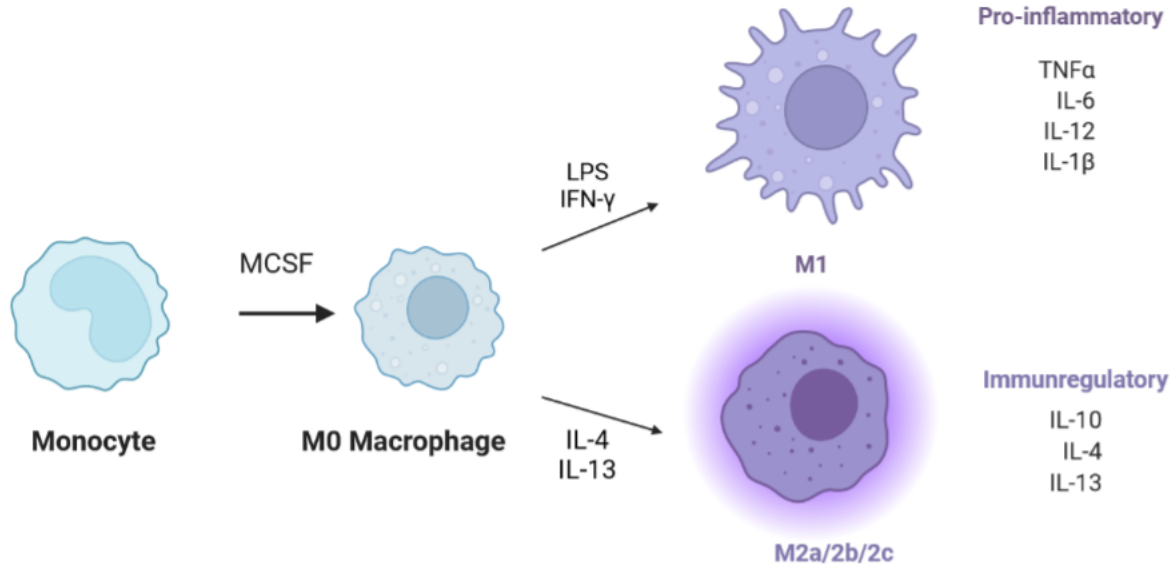

Figure 1.2: Differentiation and polerization of monocytes to macrophages *in vitro*. Macrophages can be differentiated from monocytic cells *in vitro* through chemical stimuli of growth factors. The differentiated M0 macrophgages can be polarized into M1 and M2 macrophages through further related stimuli [37]. Figure created using biorender.com

Immortalized cell lines are another type of cellular model commonly used in inflammation research. These cell lines offer the advantage of being readily available and easy to maintain compared to primary human cell models. They provide a standardized and reproducible system for studying specific cellular processes involved in inflammation. Cell lines are often derived from cancerous or transformed cells, which may exhibit altered characteristics compared to their primary cell counterparts [27]. This can affect the cell lines ability to accurately model the behavior of cells in an inflamed tissue or organ.

There exist multiple cell lines which can be used to model monocytes and the inflammatory response. The THP-1 and RAW264.7 are two commonly used monocytic cell lines. The THP-1 is a cell line orginating from acute monocytic leukemia. The THP-1 cell line retains many characteristics of primary monocytes, such as the ability to differentiate into macrophage-like cells in response to various stimuli [42]. On the other hand, RAW264.7 cells were derived from a tumor obtained from mouse. This cell line was established in 1977 and has become a widely used model for studying murine monocytes and macrophages [43]. In this thesis we investigated a less known monocytic cell line, the MM6 cell line. This is a monocytic cell model that exhibits monocyte-like characteristics such as enzyme expression and immune responses similar to primary monocytes, and the MM6 has mature monocyte characteristics such as erythrocyte phagocytosis. For the study of inflammation, MM6 is a cell line that exhibits the phenotype and functions of mature monocytes such as release of IL-6, TNFα, and IL-1β [44, 45].

In summary, rodent models allow for the study of inflammation in a whole organism, providing a comprehensive understanding of its effects on physiology. Primary cell cultures offer a cellular model which have preserved many *in vivo* characteristics but can be challenging to obtain and maintain. Lastly, Cell lines, while readily available and comparatively easier to use, may have limitations in accurately representing the complex nature of inflammation. Each model has its own advantages and limitations, and studies often use a combination of these models to gain a more comprehensive understanding of inflammatory processes.

2 | Rationale of the study

As it is known that humans are both exposed to and internalizing plastics, the interest of this study is concerning whether MNPs has the potential to affect the functionality of the human immune system. By using polydisperse samples, it was revealed by Weber et al that MNPs derived from PMMA, PS, and PVC can induce pro-inflammatory responses in primary human monocytes and dendritic cells [46]. Comparatively produced and characterized MNPs did not however induce any pro-inflammatory responses in the THP-1 cell line, as investigated by Harini Pechiappan in her masters thesis [47]. Given the discrepancy in findings between the THP-1 cell model and the primary human monocytes, along with the lack of clarity regarding the extent to which THP-1 cells are able to recapitulate the functions of primary human monocytes or macrophages, we suggest that THP-1 cell line may not be a suitable model to study the effects of NMPs on innate immune responses. Instead we propose the use of either primary human monocyte and MDMs, or a more differentiated monocyte cell line (MM6 cells) as alternative models.

Hence, this thesis sets out to:

1. Test whether earlier reported findings in Weber et al. study on human monocytes release pro-inflammatory cytokines following exposure to MNPs are reproducable.

2. Investigate the release of pro-inflammatory cytokines following MNPs exposure to primary human monocyte-derived macrophages.

3. To explore an alternative cell line model to for studying the potential impact of MNPs on inflammation.

3 | Materials and methods

3.1 Micro- and nanoplastics

MNPs made of the three polymer types PMMA, PS, and PVC were used for these experiments. The irregular, polydispersed plastic particles of green-fluorescent PMMA and orange-fluorescent PS were sourced from household materials that had been cryo-milled, while the irregular non-fluorescent PVC powder was provided by PyroPowders (Erfurt, Germany). The PVC powder provided had a size of $\leq$ 50 µm, with an average size of 13–17 µm. All plastic samples were provided by Prof. Martin Wagner, NTNU.

3.1.1 Particle preparation

A protocol for the preparation of the plastic particles was adapted from Weber et al's paper [46]. To isolate particles $\leq$ 5 µm, the particles were weighed and suspended in 1 mL of autoclaved ultrapure water in each of the 15 x 1.5 mL glass vials. Each glass vial contained 15-30 mg of MNPs . To limit contamination of the plastic, the weighing was performed in a sterile environment, and bottles were kept sealed and air-tight outside a sterile setting. The suspensions were sonicated at room temperature (27°C) for 1 h and left to settle. To calculate the settling times of the particles, Stokes' law was utilized. Stokes' law of particle settling (3.1), describes the behavior of small particles as they settle in a fluid. According to this law, the velocity at which a particle settles is proportional to the square of its radius and the density difference between the particle and the fluid. This means that larger particles or particles with a greater density difference will settle more quickly than smaller or less dense particles.

$$F_F = 6\pi\eta r v \tag{3.1}$$

This was utilized to ensure particles $>$ 5 µm would settle to the bottom of the glass vials. Using Stokes' law, the settling times of the different particles were calculated to be 24 min for PS and PMMA MNPs and 12 min for PVC MNP. 750 µL of the microplastic suspension was removed from each vial, pooled into three 10 mL glass vials, and frozen at a slated angle at -20°. The frozen suspensions were freeze-dried using a FreeZone Benchtop

Freeze Dryer (Labcono) for 36 h at a pressure of 0.038 mBar and temperature of -51°C. To reduce the possible contamination between the samples, the vials were covered with aluminum foil taped around the bottom, and punctured with a needle in the center to allow for vapor to escape. After freeze drying, the particles were resuspended in 1 mL sterile PBS for PMMA and PVC MNPs and PBS + 1:10,000 dilution Tween_{20} for PS MNPs . These suspensions were chosen to keep the plastic particles from agglomerating. The plastic suspensions were stored at 4°C in the dark for the duration of the experiments.

3.1.2 Characterization of MNPs

Particle size distribution and concentrations were determined by Nanoparticle tracking analysis (NTA) [48] using a Nanosight LM10. The NanoSight can assess particles within the parameters of 10^6-10^9 particles/mL, and to account for this differential the samples were assessed and thereafter diluted with $_{\text{di}}H_2O$. As the actual amount of plastic particles per mL was unknown, a 1:5 dilution was first tested, and based on this result the samples were further diluted to a final concentration of 1:100 (PMMA, PS) and 1:1000 (PVC). A blank control consisting of $_{\text{di}}H_2O$. was utilized, and for solvent controls, vials containing either PBS or PBS + Tween were prepared at the same dilutions as the samples. As the human cells to be exposed are kept in cell media, there was an interest in determining how cell media might affect the size distribution of the plastic particles. Suspensions at the same dilutions as the previous PBS & PBS+Tween_{20} solvents were prepared and the process was performed with identical settings as previously. A complete overview of samples for the NTA can be found in Table C.1.

The standard setting for video recording for the NanoSight is 60 s for solutions containing particles < 200 nm, and 180 s for particles > 200 nm. The measurements were taken at 120 s to account for particles in both ranges. A summary of settings used for the NTA can be found in table C.2. Replicates of the plastic solutions and corresponding blank and solvent controls were prepared in three Eppendorf tubes. Three captures were taken with the NanoSight per replica tube, and the results were averaged. To account for background particles, the concentration of particles found in the solvent controls was subtracted from their corresponding plastic sample. The size distributions of the particles were plotted, and curve fitted using GraphPad Prism (v. 9.5.1, California USA) by fitting the individual datasets for the plastic particles to a non-linear regression model (lognormal) and transforming the data to the relative abundance based on particle size for that plastic. The data was thereafter combined in one graph.

3.2 Monomac 6 cell line

3.2.1 Maintenance of the Monomac 6 cell line

The MM6 cell line is derived from acute monocytic leukemia [44]. The cells were maintained in RPMI-1640, with added L-Glutamine (200 mM), Gentamycin (5 mg/mL), NEAA (100 mM), Insulin (0.9 mg/mL), NaPyruvate (100 mM), and Oxaloacetic acid (0.25M). The growth medium was supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS). As maintaining the logarithmic growth phase are essential to keep the cells healthy and in a less stressful environment, different cell densities were tested for optimal growth. After excavating the cell model from liquid nitrogen, the cells were allowed to grow for one week after being thawed and thereafter plated at 100,00, 200,000, and 500,000 cells/mL. The growth was recorded daily, and the 200,000 starting density was found to be the most beneficial to maintain the cells in a healthy environment. To maintain this density, the media was changed every 4-5 d, and the cells were diluted to 200,000 cells/mL to keep them from reaching a stressful density for the remaining experiments.

3.2.2 Differentiation of monocytes to macrophages like monocytes

To investigate whether the MM6 cell model could be differentiated from a monocytic state to a macrophage-like state, 2 mL of cells were plated at 200,000 cell/mL in a 6-well plate. The effect of two stimuli known to induce differentiation to a macrophage-like state, phorbol 12-myristate 13-acetate (PMA), and calcitriol were tested. The cells were treated with PMA at concentrations of 10, 100, and 1000 nM. Simultaneously, three wells with solvent controls were prepared with DMSO. Calcitriol was used at 50, 100, and 1000 nM per well. Likewise, with the PMA, these wells were compared to the solvent control, ethanol. The cells were left to incubate for 24 h and 72 h and imaged at 24 h, 48 h, and 72 h to check for adherence, which is a sign of differentiation [37]. When the 24h and 72h time points were reached, the cell media was transferred to 2 mL Eppendorf tubes and spun down to separate the cell pellet from the supernatant. The supernatants were transferred to separate tubes and stored at –80°C for later determination of the levels of proinflammatory cytokines, while the cell pellets were processed for RNA extraction and qPCR (section 3.4).

3.3 Primary human monocytes

Whole blood from healthy donors received from St. Olavs hospital (section 3.3.5) was used to isolate PBMCs and subsequently monocytes for experiments. The protocol described

below was adapted by combining protocols from Sigma-Aldrich and ThermoFisher [49,50], and Nur Mohammed. Isolated monocytes were kept in RPMI-1640 supplemented with 10% HI-FBS, 20 mM L-glutamine and 1% gentamycin.

3.3.1 Isolation of PBMCs

Blood samples were collected in 3 x 6 mL blood collection tubes (VACUETTE) per donor, which were pooled together, and PBS was added at a ratio of 1:2 (blood:PBS), and inverted several times to ensure a uniform mixture of PBS and blood. SepMate tubes were prepared with the SepMate insert filled with preheated density gradient medium (LymfoPrep), and the blood:PBS mixture was transferred to SepMate tubes carefully to prevent mixing of LymphoPrep and blood:PBS. To separate the different components of whole blood (Serum, PBMCs, and red blood cells) the tubes were centrifuged at 1800 RPM for 30 min. Serum was pipetted from the tubes, and the uppermost layer of serum and PBMCs were decanted into a 50 mL centrifuge tube. The tubes were filled up with a PBS/2.5% HI-FBS mixture and equalized so that all tubes contained 45 mL of total liquid. These were centrifuged at 2000 RPM for a further 10 min, resulting in a pellet of the isolated cells. A wash step was performed to remove any erythrocytes still present in the PBMC mixture. This wash consisted of removing serum:PBS mixture from the 50 mL tubes, resuspending the cell pellet in 500µL PBS/2.5% HI-FBS mixture, and transferring it to a 2 mL Eppendorf tube. 1000 µL RBC lysis buffer (Roche) was added in two batches to the 50 mL tubes, washing the inside to ensure optimal cell harvesting. The lysis buffer was thereafter transferred to the Eppendorf and left to incubate on an inverted shaker for 10 minutes. The Eppendorf were spun down at 1500 RPM for 5 minutes to isolate the PMBCs while discarding the erythrocytes in the supernatant. This process was performed multiple times in the case of erythrocytes still being present after the first wash. The isolated PBMCs were resuspended in RPMI-1640 media supplemented with 10% HI-FBS, L-glutamine, and Gentamycin.

3.3.2 Optimization of monocyte isolation

Viable cell counts were determined using Trypan Blue exclusion and gating the measurements within the range of PBMCs (4-20µm). The Trypan Blue dye distinguishes between viable and dead cells as a result of the dye penetrating the membranes of the dead cells, staining them blue. The stained cells are counted using a TC20 Automated Cell Counter (Bio-Rad). To ensure adherence of monocytic cells, the isolated PBMCs were plated at a concentration of 8 x 10^6 cells/mL at 0.5 mL per well in PBMC media and left to adhere. As different protocols suggest a range of 1-24 hour incubation time for monocytic adhesion, the time point that results in the most uniform adherence from the monocytes were determined [49, 50]. Timepoints 45, 90, and 180 min were tested based on existing

protocols for monocytic adherence. An incubation for 180 min was found to result in the most uniform results of monocytic adherence and the highest reproducibility of the method. After incubation, the media was removed, and the cells were washed thrice to remove non-adherent PBMCs. All cells that were left in the wells were assumed to be adherent monocytes. The wells were filled with 500 µL of their preferred media, and the cells were ready for plastic exposure.

3.3.3 Characterization of PBMCs with Flow cytometry

Flow cytometry is an analytical technique that uses this laser to analyze a continuous stream of individual cells, based on the principles of light scattering and fluorescence emission. The scattered light is collected through mirrors and filters and detected by a photodetector in both the forward and side directions. Because larger cells scatter more light in the forward direction, forward scatter indicates cell size, whereas side scatter indicates the presence of intracellular membranous structures, reflecting the cell's granularity or intracellular complexity [51]. Flow cytometry was used to characterize the population of isolated cells from whole blood. The distribution of cell types in the PBMCs were analyzed using flow cytometry. 10 µL of the resuspended cells were mixed with 90 µL PBS and run on a NovoCyte 2000 Flow Cytometer (ACEA). Using a SSC-A/FSC-A gating, the populations were found. As can be seen on picture Figure 4.11, the circled population is assumed to be monocytes based on the size of the cells as the literature suggest these are larger than lymphocytes.

3.3.4 Differentiation of Monocytes to Macrophages

For the differentiation of monocytes to MDMs, a protocol was adapted based on existing literature [49,50]. The monocytes were differentiated into M0 macrophages in RPMI-1640 media supplemented with 50 ng/mL recombinant human M-CSF (Gibco), 10% HI-FBS at 37°. M-CSF can be used to differentiate monocytes into M0 and anti-inflammatory M2 macrophages based on the amount of time the M-CSF is allowed to interact with the monocytic cells, or which other supplements are added to the media [37]. The medium was changed at day 3 after plating, and after 5 days of incubation with the M-CSF-supplemented media, the M-CSF was removed to prevent the M0 macrophages from polarizing into M2 macrophages. At this stage, the cells left in the wells were assumed to be macrophages, and the procedure for MNP exposure was followed likewise as with the monocytic cells. After the plastic exposure, the media was removed and stored at -80°C for analysis of cytokine release by Enzyme-Linked Immunosorbent Assay (ELISA), and the cell pellets were harvested for RNA extraction and Reverese Transcription quantitive polymerase chain reaction (RT-qPCR).

3.3.5 Ethics Statement

Whole blood was drawn from anonymous blood donors who participate routinely in the blood donation program at St. Olavs Blood Bank with approval through rek-318656 Plast og immunsystemet. All donors were considered to be healthy at the time of sample collection. Prior to the blood being sampled, all participants were given a pamphlet with information describing the study, and thereafter the donors signed a written informed consent form. All donors were anonymized after this consent was given, except for their stated biological sex.

3.4 Cytotoxicity of MNPs

A resazurin assay was utilized to measure cytotoxicity. Resazurin is a dye that reduces in a living organism, leading to a redox reaction where the oxidized from of resazurin enters the cell and is reduced by the mitochondrial enzymes. These enzymes accept electrons from NADPH, FADH, FMNH, and NADH, leading to a reduction of resazurin which serves as a red fluorescent dye. The reduction rate is directly related to the number of viable cells and can therefore be used as a measurement for cell proliferation [40]. The resazurin agent was added to the cells two hours before the plates were assessed, and fluorescence was measured using a Cytation 5-cell imagine multi-mode reader at 544nm/590nm excitation/emission, respectively. The cumulative effect of cytotoxicity was measured and fitted to a dose-response curve.

3.4.1 Cytotoxic effects in the MM6 cell model

MM6 cells were plated in 96-well plates at a density of 2x104 cells/90 µL. To prevent edge effects from media evaporation, the outer wells of the plate were filled with 200 µL of PBS. The vials containing the MNP samples were vortexed to ensure that the particles were resuspended, then they were diluted in a series of 1:3 in either PBS (PVC & PMMA) or a 1:10,000 dilution of Tween_{20} in PBS (PS), and 10 µl was added to the well such that each well contained a total of 100 µL. The cells were then incubated at 37.2°C, 5% CO2 for either 24 h or 72 h before the resazurin assay was performed. Three technical replicates were performed per treatment. Particles per cell, particles per mL, and particles per mm2 of plates with respect to the dilution series can be found in Table 3.1.

Table 3.1: Concentrations of plastic particles used in cytotoxicity assays based on the number of micro- and nanoplastics determined by nanoparticle tracking analyses. The concentrations decrease with a 1:3 dilution. In the table, H indicated a High dose, M for medium, and L equals a low dose of particles.

Dilutions	Particles/cell	Particles/mL	Particles/mm2
1^{H}	300	60,000	187,500
2^{M}	100	20,000	62,500
3	33.3	6,666.6	20,833.3
4	11.1	2,222.2	6,944.4
5	3.7	740.1	2314.8
6^{L}	1.2	246,9	771.6
7	0.41	82.5	256.2
8	0.13	27.4	85.7
9	0.05	9.1	28.5

3.4.2 Cytotoxic effects in the primary monocytes

To explore the cytotoxic effects in the primary monocytes, a cytotoxicity assay was performed. Due to the limited number of primary monocytes, the cytotoxicity was investigated for a high, medium, and low dose of MNPs (Table 3.1) echoing the concentrations used for the follow-up experiments. After the washing process of the cells was complete the cells were exposed to the MNPs . The cells were plated in the center of a 24-well plate, and to prevent edge effects the outer wells were filled with 1 mL PBS. The plates were left to incubate for 18 h to mimic the exposure times of the inflammatory experiments, upon which the supernatant was transferred to eppendors and kept in -80° until analyzis.

3.5 Inflammatory response of MNPs

The inflammatory response to MNPs in the MM6 cell model and the isolated primary monocytes and macrophages were investigated. The experiment was performed with LPS as the positive control, solvent controls for PBS and PBS+Tween_{20}, and a blank sample consisting of only cells with no additives as the negative control. To determine the concentration of LPS for the sufficient inflammatory response in the positive controls, a dose-response curve was performed before plastic exposure, and EC50 was calculated for cytokine release. The cells were plated and exposed to different concentrations of MNPs for 18h with controls. After the 18 h exposure, the cell supernatant was removed for quantification of inflammatory cytokines using ELISA (section 3.4.3), while the cell pellet was harvested for RNA extraction. Both supernatant and cell pellet were kept at -80°until their respective analysis.

3.5.1 Inflammatory effect in the MM6 cell model

The MM6 cells were plated at 200,000 cells/well and exposed to a high dose of 300 particles per cell and a low dose of 1 particle per cell. The LPS concentration was chosen to be 500pg/mL. As stated, the cells were incubated with the MNPs for 18 h, and after the incubation the media was removed from the wells, transferred to 1.5mL Eppendorf tubes, and spun down for 5 min at 1500 RPM to collect the cell pellet. The suspension was carefully pipetted off into a new 1.5 mL tube while leaving the cell pellet. To ensure the collection of cell material, RNA lysis buffer, and β- mercaptoethanol were added to the plates, and incubated for 5 minutes. After the incubation, the mixture was transferred to new Eppendorfs'. The cell pellets and supernatant were stored at -80°C between use.

3.5.2 Inflammatory effect in the adherent monocytes

The inflammatory response from the adherent monocytes was investigated by exposing the adherent cells to MNPs . After the washing process, the media was supplemented with plastic and left for 18h to incubate at 37°C. Based on the LPS-dose response curve performed (figure 4.11), the positive LPS control dose was chosen to be 10 pg/mL.

The cell count differed widely between donors, and therefore there was an inconsistent number of wells available for MNP exposure. The average number of PBMCs isolated for each donor was 3.5 x 10^7 cells, and because the cells were plated at 0.5 mL of 0.8 x 10^7 cells/mL, the average cell donor provided enough cells for 8 wells. As there needed to be four controls per experiment, this resulted in most cell donors in this thesis being checked for inflammatory response from each of the MNP polymer types at a high dose, and one low dose of PVC. When possible, a high and low dose of all three MNPs was utilized, and for a few donors with higher number of isolated PBMCs either a high, medium, and low exposure was utilized or a high dose of plastic in tandem with a 10 pg/mL LPS exposure.

As there was a probability that the number of adherent cells in the wells was not consistent after the washing process, keeping the MNP exposure consistent proved difficult. Therefore, the number of MNPs the cells were exposed to was kept consistent. The dose of plastics was calculated based on the percentage of PBMCs that were assumed to be monocytic from the assessment of flow cytometry. Furthermore, as the solvents could induce a response from the cells, the MNP suspensions were diluted to the highest particle concentration per µL, and the solvent controls were matching the amount of µL used for the plastic samples.

3.6 Cytokine release and gene expression

3.6.1 Cytokine release by ELISA

To investigate the concentrations of TNFα and IL-6 in the supernatant, ELISA was performed according to the manufacturer's instructions (R&D system DuoSet; TNFα & IL-6). To account for the environment of the cells, the reagent dilute of the assay was replaced with an appropriate medium for the creation of standard solvents and the blocking of the plates. The resulting values were read at absorbance 450nm/-570nm with a Cyt5 imaging plate reader, and the online tool MyAssay was used for analysis [52].

3.6.2 RNA isolation and RT-qPCR

RNA isolation for the harvested cell pellets was performed according to the manufacturer's instructions using Qiagen's RNeasy Mini kit. The quantity and purity of total RNA in the samples were measured by NanoDrop One/OneC Microvolume UV-Vis Spectrophotometer (Thermo Scientific), and the RNA was stored at -80 °C.

Reverse transcription of RNA (0.5-1 µg) was performed using QuantiTect Reverse Transcription kit following the manufacturer protocol (Qiagen), and the cDNA was stored at -20°C. PCR analysis was performed as instructed by the manufacturer using LightCycler 480 SYBR Green I Master and LightCycler 96 Instrument (Roche). The program settings for the PCR and the list of primers used are shown in Appendix B. To ensure primer specificity, a melting temperature analysis was conducted using Lightcycler 96 Software (V. 1.1.0.1320, Roche Diagnostics International Ltd). Amplification curves were then utilized for each sample to determine PCR efficiencies using LinRegPCR (V. 2021.2, Ruijter J.M., Amsterdam)

To determine if macrophage surface markers CD14 and CD36 were upregulated in response to the differentiation stimuli on the MM6, RT-qPCR was utilized. The differentiation was assessed in relation to housekeeping reference genes: GADPH, ACTB and RPS18 [53, 54].

3.7 Data analysis

In this thesis, there are references to technical and biological replicates. A technical replicate indicates separate wells plated using cells from the same flask. A biological replicate is defined as an independent experiment in which the cells are seeded from different culture flasks. Furthermore, the term "cell donor" and "donors" will be used and refers to the cells isolated from the blood donors.

Statistical analysis from experiments was analyzed using GraphPad Prism (v. 9.5.1,

California USA). Statistical significance between samples and controls was determined by using One-Way ANOVA followed by Dunnett's multiple comparisons tests. Correlation analysis was performed using the Pearson correlation coefficient and a two-tailed p-value. Statistical significance was achieved with a p-value < 0.05.

The read values from the ELISA were analyzed using MyAssay were used to analyze the results with a Four Parameter Logistic (4PL) regression. The 4PL regression is a model that has been developed to describe the dose-response relationship in ELISAs. The model takes into account the maximum Effect level (Emax), the concentration of analyte producing a half-maximal effect (EC50), the Hill slope (steepness of the dose-response curve), and the baseline effect(background). The resulting data were plotted with GraphPad Prism, and the data were normalized on a scale of 0-100% with the positive LPS control set as 100% and the negative blank control set at 0%. All samples were set in relation to these two endpoints. All cytokine levels below the detection range of the R&D system DuoSet was set as half of the lowest value of the assay. This was 7.8 pg/mL for the TNFα cytokine release and 4.9 pg/mL for the IL-6.

4 Results

4.1 Particle characterization

As previously defined, the size of MNPs can range from 1 nm to 5000 µM (section 1.1.1), and there exists a previous indication that different sizes of plastic polymers can induce different responses in human cells [5]. Therefore, it was important to determine the size range of the MNPs particles. The particles were characterized in relation to size distribution and number of particles per mL of solvent using a Nanosight NTA (section 3.1). The concentration of suspended particles was 4.38 x 10^9 particles/mL for PS MNPs, 3.33 x 10^{10} particles/mL for PMMA MNPs, and 1.80 x 10^{11} particles/mL for PVC MNPs. The mean diameter of 10%, 50%, and 90% of particles was determined and the relative distribution of particles was fitted to a log-normal distribution based on three technical replicates (Figure 4.1, Table 4.1).

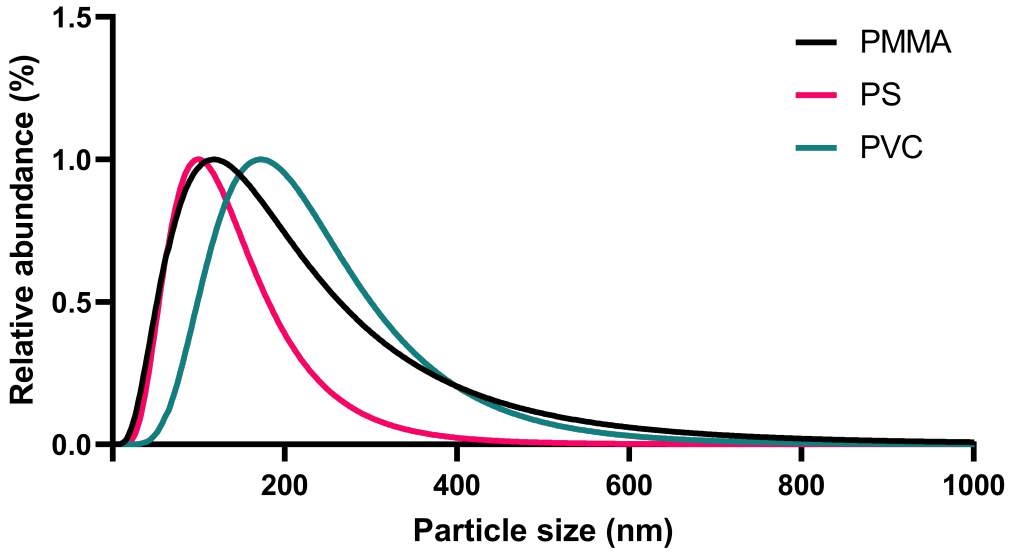

Figure 4.1: Particle size distribution of the micro- and nanoplastics used in this study. The figure presents the relative abundance of particles distributed by size collected from the Nanoparticle tracking analysis. Particle abundance was fitted using a lognormal regression model, and the relative abundance is given in % of total particles.

Notably, MNPs created with the original protocol from Weber et al. had a larger mean for PMMA and PVC, theirs being 193 nm and 203nm, respectively. While their PS particles were smaller, 117nm, than what was found in using our protocol [46], indicating that the same protocol can lead to slight variations in size distributions of the MNPs. As the settling times for all three polymers were determined based on Stokes' law, there was an expectation that the plastic particles were similar in size, which was confirmed as size distribution of the particles was found to be in a similar range.

Table 4.1: Size distributions of the micro- and nanoplastics used in this study. The table displays the mean particle size of the plastic particles, as well as the cut-off for the size of all particles of the smallest 10%, 50%, and 90% of the total distribution.

Plastics	**PMMA**	**PS**	**PVC**
Mean	156,03 nm	123,73 nm	197,97 nm
D10	68,93 nm	42,93 nm	107,80 nm
D50	141,00 nm	121,00 nm	202,83 nm
D90	299,6 nm	228,3 nm	349,8 nm

The settling time based on Stokes' law would ensure all particles were less than 5 µm. However, as the limits of detection of the NTA are 1000 nm [48], we could not confirm if there were an larger particles present in the suspensions. Furthermore, plastic particles can agglomeration over time, creating larger particles not detected. Visual inspection in light microscope of particles after exposure to cells suggested there were particles larger than 1000 nm in the suspension, and there was an attempt to utilize a Coulter Counter to account for particles $>$ 1000nm, however, due to technical difficulties with the instrument this yielded no results.

Additionally, as the cells were exposed to MNPs while in media, there was an attempt to characterize the particles in RPMI-1640 media supplemented with HI-FBS to determine if the media affects the size distributions (section 3.1). This experiment was unsuccessful as the background particle concentration found in RPMI-1640 and HI-FBS were too high, and the particles were large enough to be detected by the Nanosight, leading to no concrete separation of plastic particles and RPMI-1640:HI-FBS particles.

The total surface area and mass of the particles were calculated by assuming spherical particles and using the densities of the three polymers (PS = 1.05 g/cm^3, PMMA = 1.18 g/cm^3, PVC = 1.3 g/cm^3) to derive the mean mass and surface area of the particles (Table 4.2). Calculating the densities for the three polymers based on the particle concentrations in the dilution series from Table 3.1, with a starting concentration of 300 particles/cell. These calulations revealed that the PVC MNPs had high mass and surface area compared to PMMA and PS MNPs. At the same concentrations of exposure, the mass of the PVC MNPs were 2.3 and 5.1 times larger that PMMA and PS MNPs, and the PVC particles cover 1.5 and 2.5 more surface area compared to the PMMA and PS particles, respectively.

Table 4.2: Dose metrics of the micro- and nanoplastics used in this study. The table shows the calculated theoretical mass and surface area of the different polymers based on the 1:3 dilution series starting with 300 particles/cell. The surface area was calculated based on the mean particle size and assuming a spherical shape for the particles. In the table, H indicated a High dose, M for medium, and L equals a low dose of particles.

	PMMA		**PS**		**PVC**	
Dilutions	**Mass (µg/mL)**	**Surface area (mm^2)**	**Mass (µg/mL)**	**Surface area (mm^2)**	**Mass (µg/mL)**	**Surface area (mm^2)**
1^H	3.950	1569	1.750	961.9	9.010	2463
2^M	1.317	523	0.583	320.6	3.003	821
3	0.439	174.3	0.194	106.8	1.001	273.7
4	0.146	58.1	0.065	35.6	0.334	91.2
5	0.049	19.4	0.022	11.9	0.111	30.4
6^L	0.016	6.5	0.007	4.0	0.037	10.1
7	0.005	2.2	0.002	1.3	0.012	3.4
8	0.002	0.7	0.001	0.4	0.004	1.1
9	0.001	0.2	0.000	0.1	0.001	0.4

4.2 Monomac 6 cell model

In this thesis, we tested the hypothesis that the MM6 cell line could be used as a model for investigating the possible cytotoxic and inflammatory effects of MNPs. To investigate this, we first deterimnd the optimal range of densities for growing the cells, and a protocol for inducing macrophage-like markers in the monocytic cell model.

4.2.1 Characterizing growth conditions for the MM6 cell line

To reduce variety between biological replicates, cell lines should be in the same conditions between experiments, and therefore it is important to to know their specific growth characteristics. The growth characteristics and optimal starting density was determined by plating the MM6 cells at three different densities (100,000, 200,000 and 500,000 cells/mL), and recording daily growth for 8 days without changing the medium. The growth was fitted to a non-linear regression for logarithmic growth (section 3.2). We chose to use the starting density of 200,000 cells/mL, as the cell plated at this density were found to grow at a consistent rate, reaching the log-phase at day 4 and the death phase at day 7 (figure 4.2). For the duration of experiments the cells were seeded on day 4 of growth, and split back to the 200,000 cell/mL density to keep the cells from reaching a density that would cause stress.

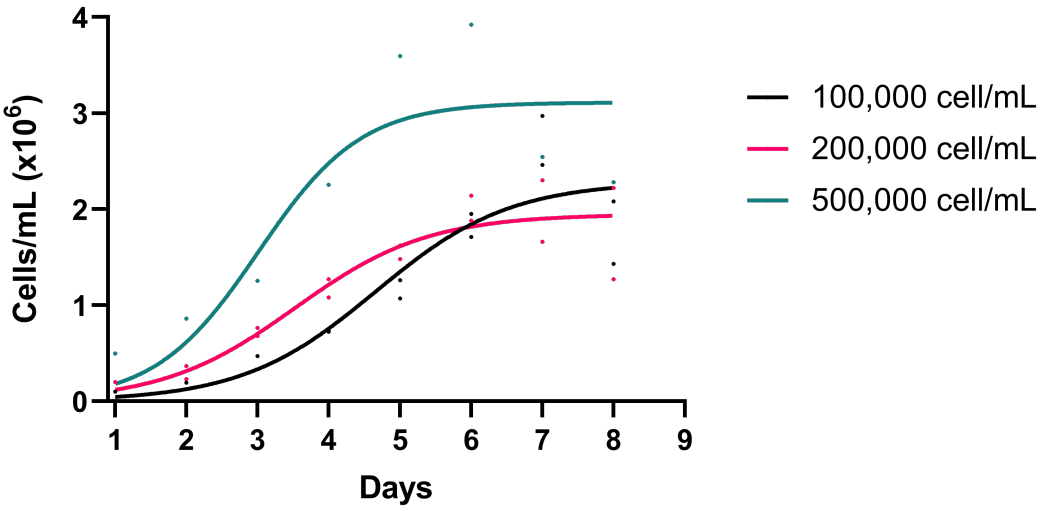

Figure 4.2: The graph shows the growth of the three different starting densities of the Monomac 6 cell line, fitted with a non-linear regression for logartimic growth. These desities were 100,000, 200,000, and 500,000 cells/mL.

4.2.2 Calcitriol induced macrophage-like markers in MM6

Macrophages are terminally differentiated cells, which can be derived by the in vitro differentiation of monocytes. The THP-1 monocytic cell line is often differentiated to a macrophage-like phenotype by treatment with either phorbol 12-myristate 13-acetate (PMA) or calcitriol [55] but no studies have reported the differentiation of the MM6 cell line using either stimulus. We, therefore, investigated whether the MM6 cell line could be induced to differentiate to a macrophage-like phenotype with either PMA or calcitriol by observing their adherence to the surface of the well (which is associated with differentiation of THP-1 cells) and using qPCR, to measure macrophage-associated biomarkers CD14 and CD36. Using 100nM of Calcitriol the MM6 cell line displayed increased release of CD14 compared to the control, however, there was found no significant change in CD36 after 72 h of stimulation (Figure 4.3a-b). The CD14-positive monocytes did not exhibit the typical adherent nature of macrophages and stayed suspended in media after stimulation of calcitriol, and the cells can therefore be suspected to not be fully differentiated into macrophages [37]. 50nM of PMA resulted in no significant difference in CD14 nor CD36 release compared to the control (Figure 4.3c-d) and did not exhibit the typical adherence found in macrophage-like cells. It was therefore concluded that PMA was not appropriate stimulation for inducing macrophage-like monocytes in the MM6 cells.

4.2.3 Cytotoxicity in the MM6 cell line

To investigate whether the MNPs caused cytotoxicity in the MM6 cell line before the inflammatory response was measured, viability assays were performed with use of serial

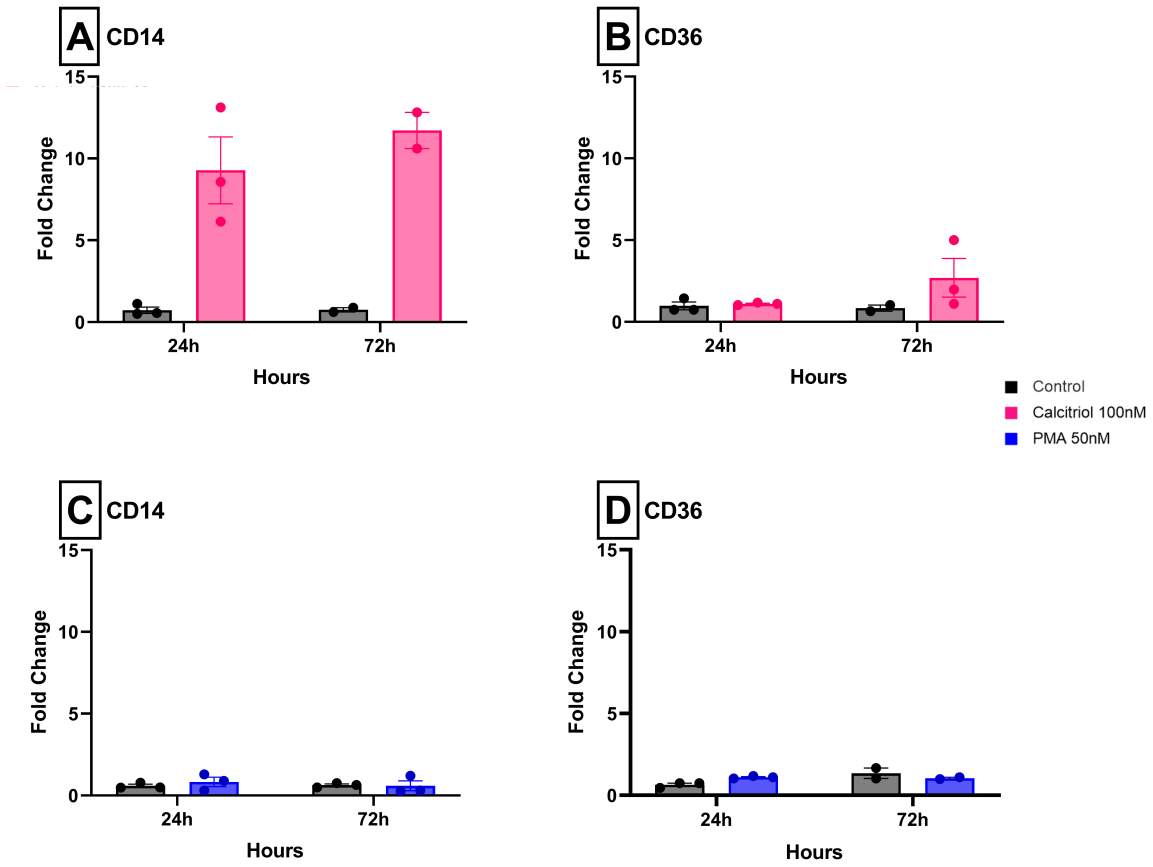

Figure 4.3: Macrophage differentiation as indicated by the biomarkers CD14 and CD36 compared to control for 24 and 72 h of stimulation. The figure shows the fold change in gene release measured by qPCR for CD14 and CD36 induced by 100nM calcitriol and 50nM PMA exposure to the MM6 cell line. Three biological replicated with three technical replicates. (a and c) shows CD14, while (b and d) show CD36. (a-b) shows Calcitriol 100nM, (c-d) shows PMA 50nM.

dilutions with exposure times of 24 h and 72 h (section 3.4). We conducted two experiments to investigate the cytotoxic effect of plastics, with the first experiment involving exposing MM6 to prepared MNPs from Pechiappans' exposure to the THP-1 cell line, which is reported in Appendix E, and the second experiment involving the particles prepared for this study. The cell viability was measured with a resazurin assay, and the data were normalized to the vehicle control. Exposure to PMMA or PS MNPs had no significant effect on cell viability at either 24 h or 72 h exposure (Figure 4.4a-d). Treatment with PVC for 24 h at the two highest concentrations decreased the cell viability in the MM6 by 33.2 and 29.7%, respectively (Figure 4.4c). When the exposure time was increased to 72 h, the cell viability was reduced by $> 25\%$ for concentrations of 1.2 particle per cell and higher (Figure 4.4f). From these results, we can conclude that PVC particles can have a cytotoxic effect on MM6 monocytes at concentrations of 1.2 particle per cell and higher, while the PS and PMMA MNPs do not induce cytotoxicity in these monocytes.

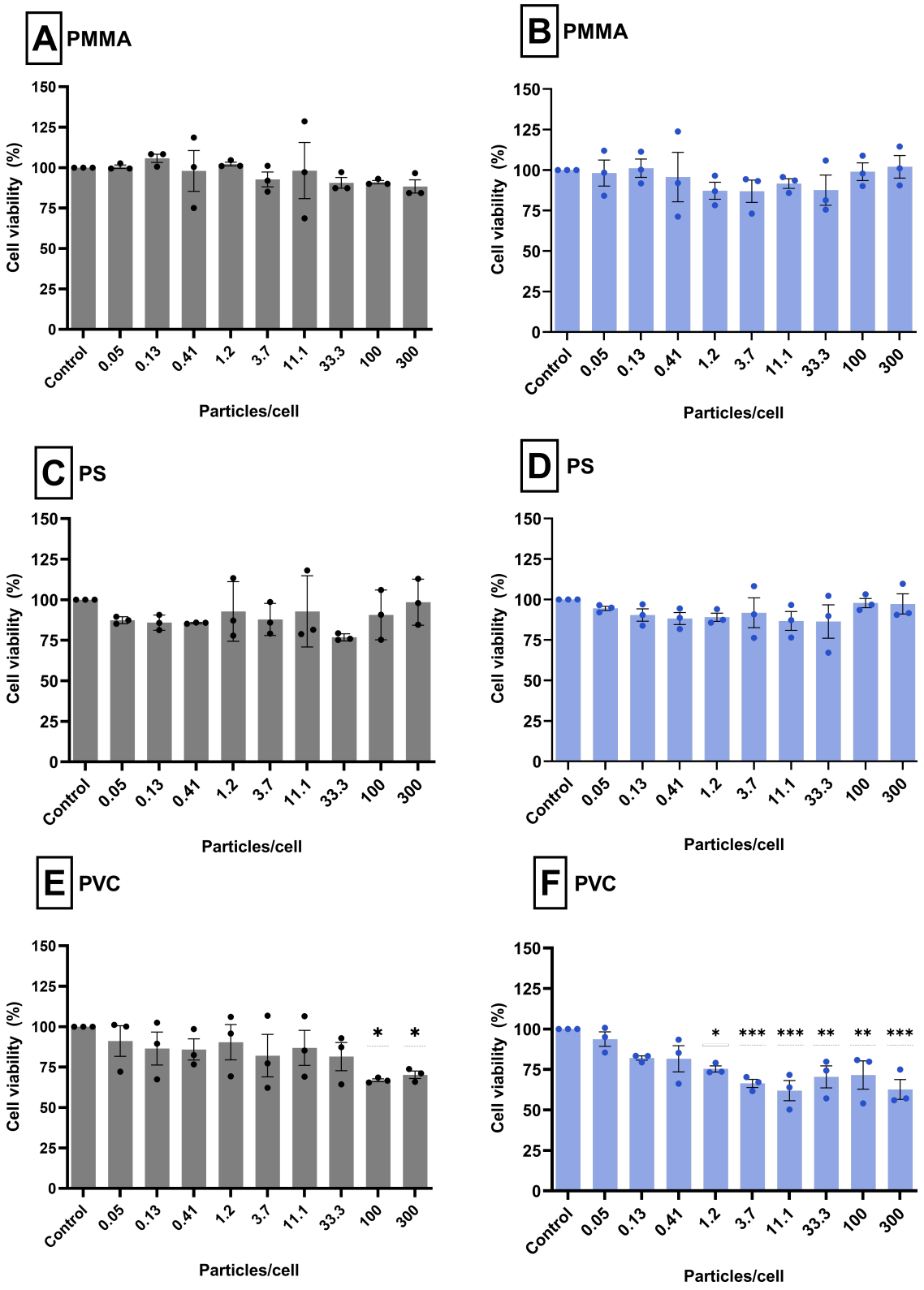

Figure 4.4: Cytotoxic effects of micro- and nanoplastics in Monomac 6 monocytes. The graph shows the percent of viable cells after 24 h (a, c, and e) and 72 h (b, d and f) of exposure (a-b: PMMA, c-d: PS, e-f: PVC). The control (no particle treatment) was set to 100% viability. Data shown in % viability are the mean ± SEM of 3 technical replicates from 3 independent experiments (n=3). * $p < 0.05$, ** $p < 0.01$.

4.2.4 Cytotoxicity in MM6 monocyte-like macrophages

Macrophages can have a different sensitivity to pathogens and foreign objects compared to monocytes, and therefore there was an interest if the cells treated with calcitriol to express more macrophage like characteristic would have a different response to plastic compared to the untreated MM6 monocytic cells. These macrophage-like monocytes did not exhibit the typical adherent quality expected of macrophages, and therefore a specific protocol had to be implemented to test this hypothesis. The cells were treated with calcitriol for 24 h in 6-well plates and thereafter collected and spun down to remove the media while collecting cells. The cells were resuspended in calcitriol-free media and exposed to MNPs per the method described (section 3.2). None of the MNP exposures was found to include significant reduction in cell viability at neither 24 h nor 72 h exposure (Figure 4.5). However, it is worth noting that there was higher reduction in cell viability at 72 h after exposure to all three MNPs compared to the monocytes (Figure 4.5b,d,f), nevertheless due to the variability between biological replicates none of the results can be considered significant.

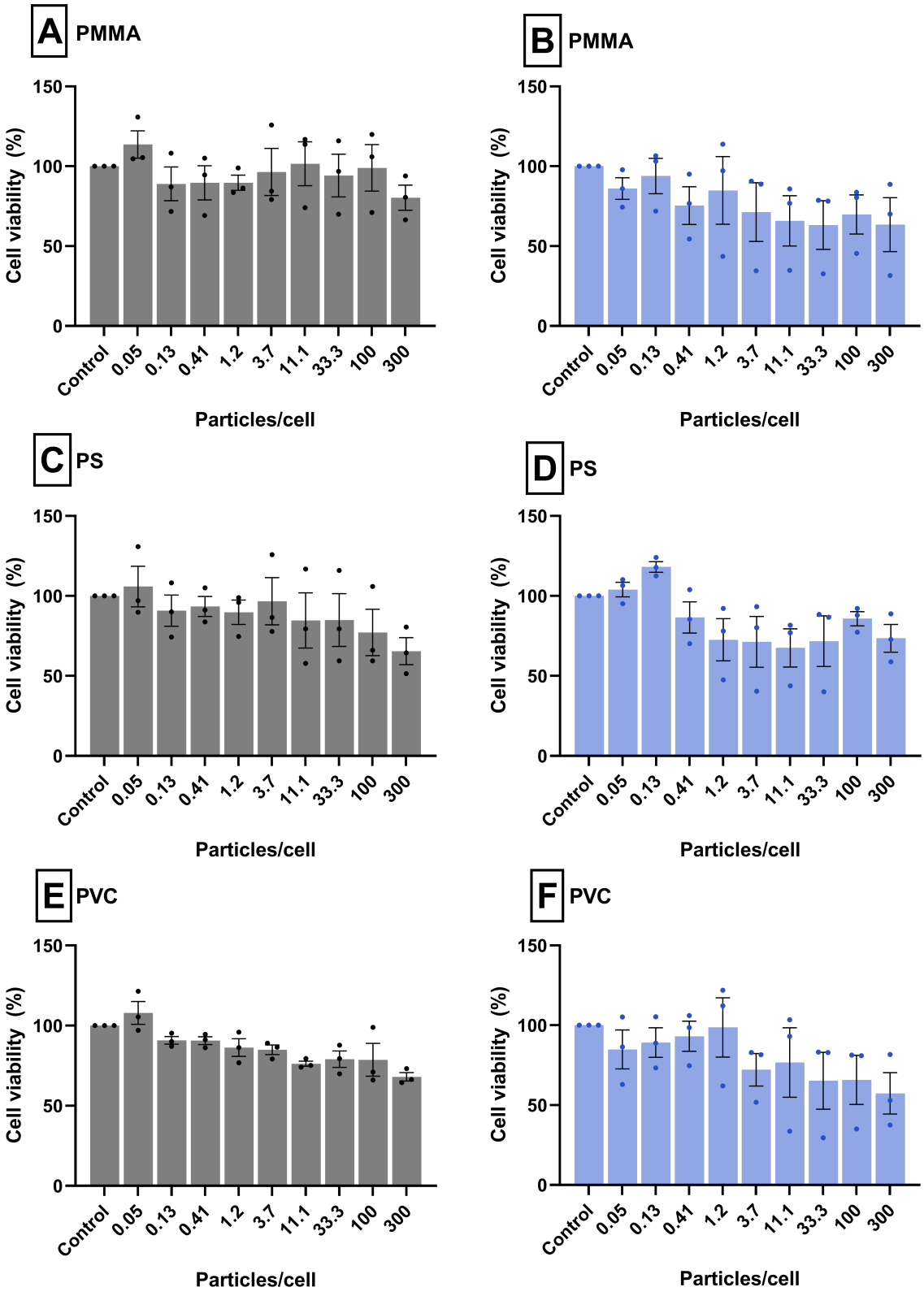

Figure 4.5: Cytotoxic effects of micro- and nanoplastics in Monomac 6 monocytes. The graph shows the percent of viable cells after 24 h (a, c, and e) and 72 h (b, e and f) of exposure (a-b: PMMA, c-d: PS, e-f: PVC). The control (no particle treatment) was set to 100% viability. Data shown in % viability are the mean ± SEM of 3 technical replicates from 3 independent experiments (n=3)

4.2.5 Pro-inflammatory release in MM6 after MNP exposure

Pro-inflammatory responses are an adverse effect that has been linked with exposure to MNPs in some model animals and cells [5, 7]. Our group have showed previously that MNPs did not induce inflammatory response in THP-1 monocytes. Here we investigate the inflammatory response in MM6 cells, which represent a more differentiated phentoype. We exposed the cells to a high and low concentration of MNPs for 18h. The supernatants were harvested after 18h, and ELISA was performed to quantify the release of the pro-inflammatory cytokines IL-6 and TNFα (Section 3.5).

LPS is known to cause pro-inflammatory release in monocytes [56], and we therefore exposed the cells to LPS to determine a LPS concentration as positive control. The MM6 cell line shows a detectable release of TNFα in response to exposure to 1pg/mL to 0.1 ug/mL. To find the concentration of LPS that induces 50% of maximal cytokine release, EC50 was calculated and found to be 474.5 pg/mL (Figure 4.6). This consentration was utilized to set the positive control for the MNPs at 500 pg/mL.

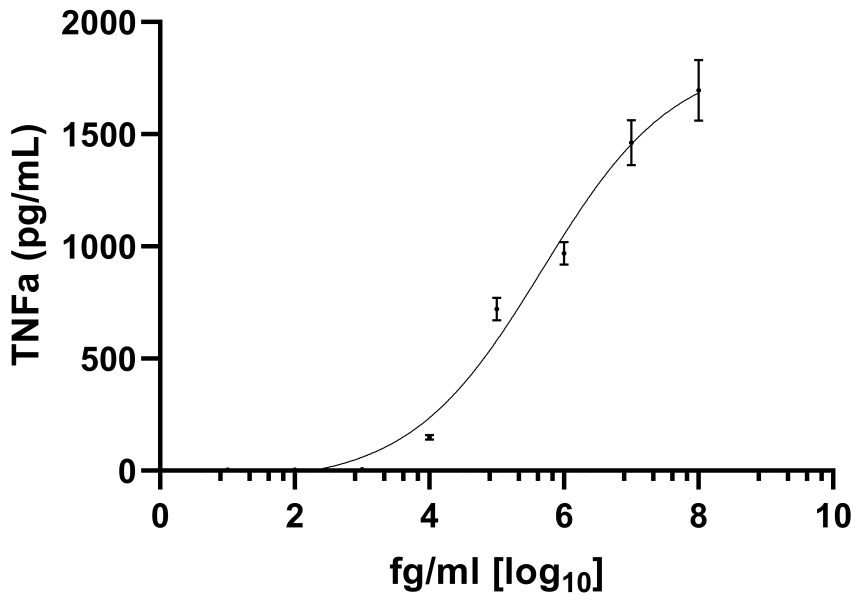

Figure 4.6: Dose-dependent TNFα release in MM6 cells exposed to LPS (0.01pg/mL – 0.1ug/mL) for 18h. The data was fitted with non-linear log(agonist) vs. response – 4PL slope regression. Data are the mean ± SEM of 2 biological replicates.

Our results indicate a slight increase in TNFα release after exposure to the high concentration of PVC MNPs. The PVC MNPs induced a mean release of 26.6 pg/mL (Figure 4.7a), and was significantly different from the negative control (p = 0.023), however, not to the corresponding solvent control. None of the other exposures of MNPs induced significant release of TNFα. The mean release of TNFα from the positive control was 852.1 pg/mL, while the negative blank control was outside the limits of detection of the TNFα ELISA (15.6-1000 pg/mL). No significant IL-6 cytokine release after exposure to MNPs was found. While not significant, the high consetration of PVC MNPs induced a mean release of 23.8 pg/mL of IL-6 (Figure 4.7b), while the low dose of PVC MNPs induces a mean release of 11.63 pg/mL. No other MNP exposure nor solvent induced detectable release of IL-6 in the cells they were exposed to. The cells exposed to LPS as positive control released 544 pg/mL of IL-6, while the negative control were below the limits of detection of the IL-6 ELISA (9.8-600 pg/mL).

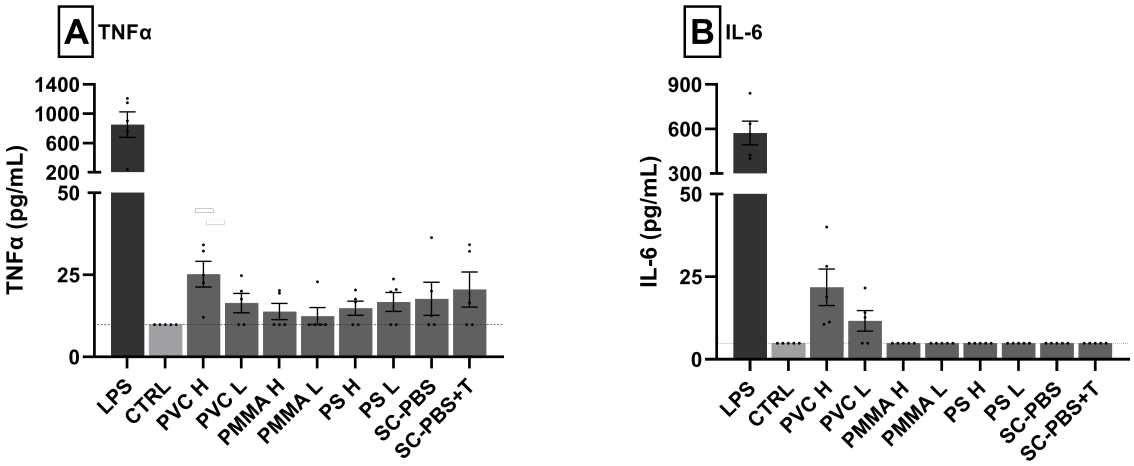

Figure 4.7: Release of the cytokines TNF (a) and IL-6 (b) in Monomac 6 cells exposed to micro- and nanoplastics. The figure shows the release of TNFα and IL-6 found in the supernatant of the Monomac 6 cells. Data from 5 biological replicates and two technical replicates. LPS = Positive control, CTRL = negative control, SC-PBS = solvent control PBS, SC-PBT+T = Solvent control PBT+Tween20, n.d = non-detectable.

The release of TNFα and IL-6 in response to high doses of PVC could suggest the MM6 cell line is more sensitive to plastic particles compared to previously established monocytic cell models such as the THP-1 [47]. However, the overall conclusion of this experiment is that the three types of MNPs does not induce the release of any significant pro-inflammatory cytokines.

4.3 Human derived monocytes

One of the main goals of this thesis was to investigate if primary monocytes and MDMs would have release of pro-inflammatory cytokines when exposed to MNPs. This hypothesis has been investigated previously in a paper by Weber et al [46] and building of their work the hypothesis was that exposure of PVC MNPs would lead to higher levels of pro-inflammatory cytokines compared to PS and PMMA MNPs. Furthermore, we hypothesised that the primary cell would have higher release of cytokines compared to the MM6 cell model.

4.3.1 Isolation of monocytic cells from whole blood

A reliable method for isolating PBMCs from whole blood and for isolating monocytic cells from the PBMCs was needed before exposing the cells to MNPs. We isolated PBMCs from whole blood as described in section 3.3.1.The expected range of PBMCs isolated were is 0.5–3 x 10^6 PBMCs/mL blood [41]. We found that nine donors were above the expected range, while the remaining donors were within (Figure 4.8)

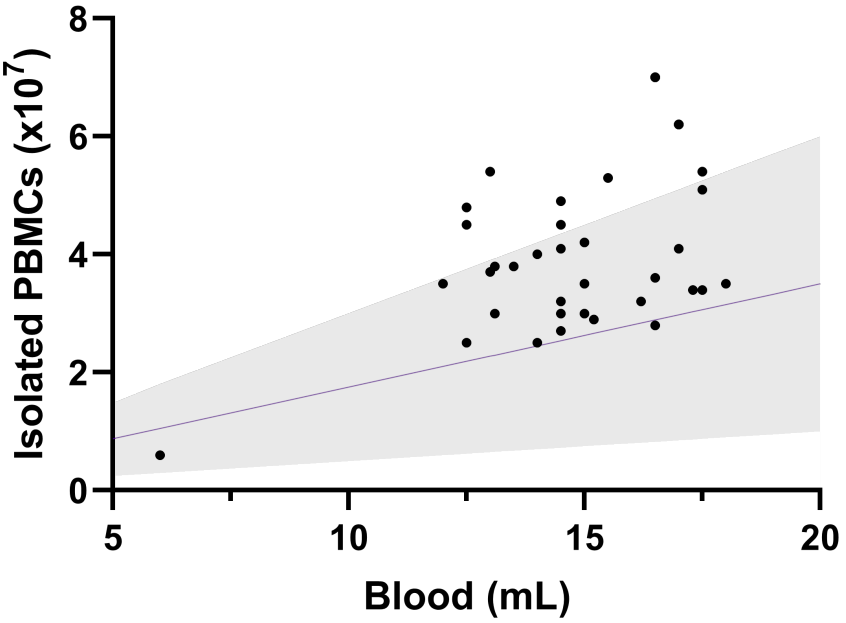

Figure 4.8: The figure shows the number of isolated PBMCs from whole blood samples compared to the expected range. The grey box shows the upper and lower bounds of this range, and the line shows the mean. Donors represented with black dots (n = 38).

The adherence of monocytes is dependent on the time frame they are allowed to settle to the bottom of plastic plates, and previous established protocols suggest that leaving them settle for too long can result in the monocytes differentiating to M0 macrophages

[49,50]. We tested adherence at 45, 90 and 180 minutes and the cells were imaged between each washing step visualise of the number of adherent cells (Figure 4.9).

As there was a limited number of donors, when a time point which resulted in consistently adherent monocytes were found, this time point was kept for the remainder of the experiments. Based on the image, 180 minutes was found to result in the most adherent monocytes after washing and all donors after donor 11 left to adhere for 3 hours.

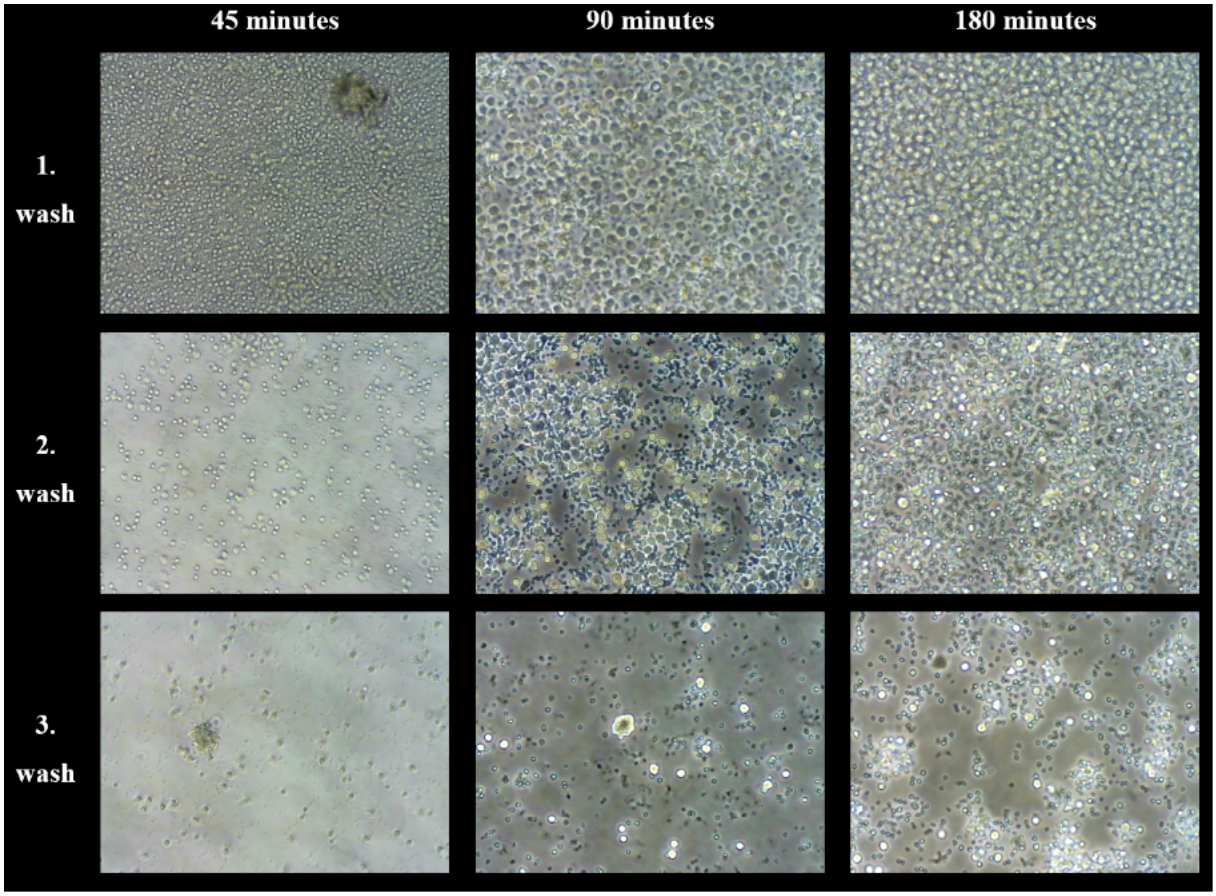

Figure 4.9: Optimizing of incubation times for monocyte adherence. The figure shows the results from the process of washing the PBMCS and removing all cells non-adherent cells. The images were taken at 10x with a light microscope using phase contrast.

4.3.2 PBMC characterization using flow cytometry

Flow cytometry can be used as an indicator of how large the population of monocytes was in the PBMCs. The analysis was performed by diluting 10µL of PBMCs in 90µL PBS. Based on existing protocols indicating that gating the flow cytometry to the SSC-A/FSC-A in the range of 40–60 x 10^4/80–120 x 10^4 would be the expected size range of monocytes (Figure 4.10) [57]. While the circled population is expected to be monocytes based on their size, we did not use antibodies to confirm this and therefore these findings should be considered an estimate.

Using this gating we found that a proportion of the population of the PBMCs from donors were in the range of 1.9% to 6.7%. The mean proportion of monocytes were 3.5% (Table D.2). Monocytes typically make up 5–10% of PBMCs [41], meaning that the range found in the isolated PBMCs is on the lower end of what could be expected of isolated PBMCs.

We used the data from the flow cytometry to calculate the consentration of MNPs to use for exposure. This was determined based on the mean of the first 4 donors analysed using the flow cytometer, which was 3%. We therefore assumed 3% of the plated PBMCs should be considered adherent monoctes, and this cell number was used for determining particles/cell for MNPs exposure.

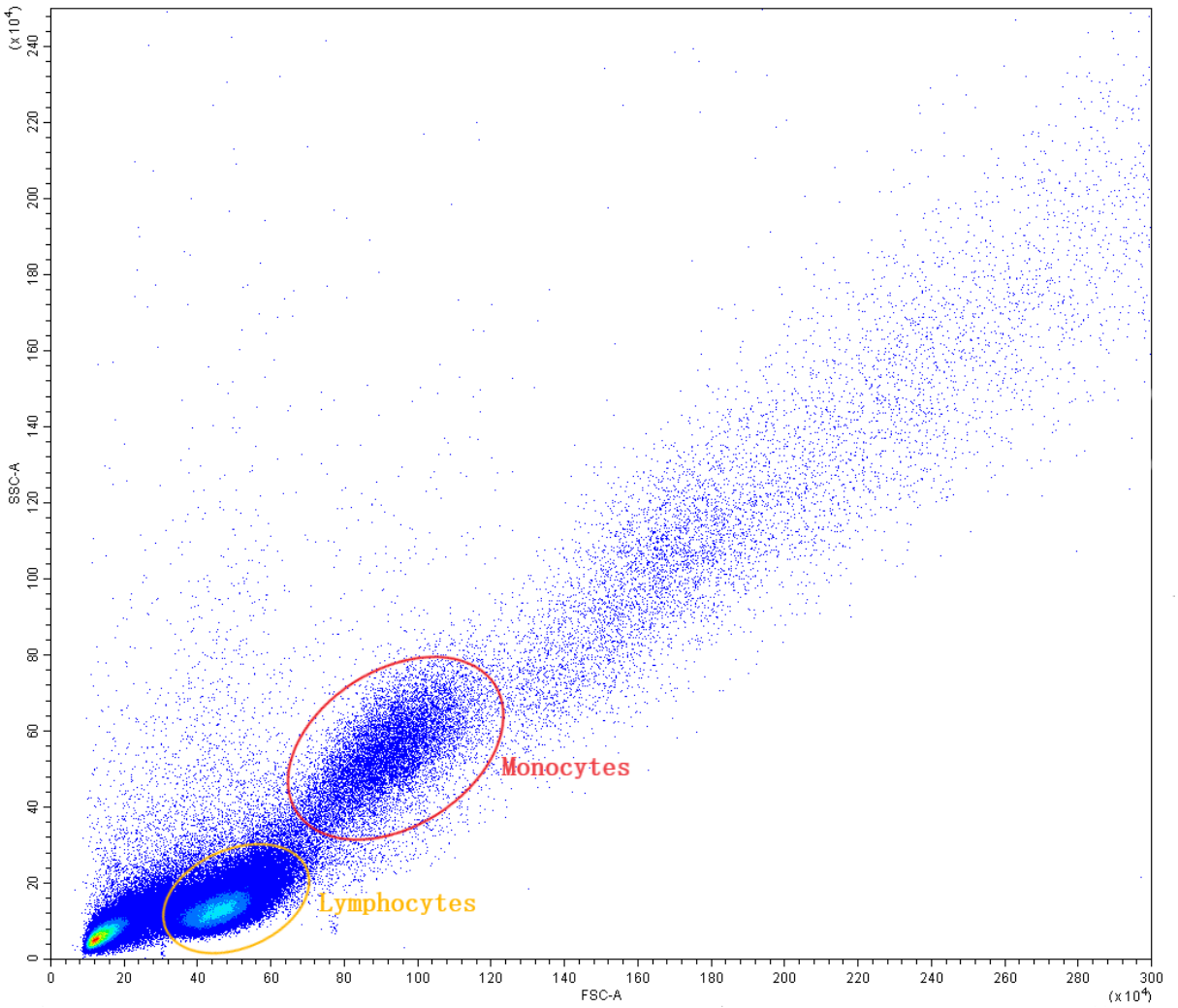

Figure 4.10: Populations of monocytes in peripheral blood mononuclear cells characterized by flow cytometry. The analysis was performed with SSC-A/FSC-A plots. The circled areas were considered the population of monocytes and lymphocytes found in the PBMCs.

4.3.3 Cytotoxicity of MNPs in primary human monocytes

To test whether the MNPs were cytotoxic to the primary monoctes, we performed a viability assay. We exposed the cells to a high, medium, and low dose of plastic or the appropriate solvent controls for 18 h (Table 3.1), and measured viability using a resazurin assay.

The assay showed that at 18h all doses of PVC and PMMA induced a reduction in cell viability compared to the control. The highest dose of 300 particles per cell reduced the viability by 36.7% (PVC) and 34.9% (PMMA) (Table 4.3). These results indicate that the MNPs used in this study can have some cytotoxic effect on monocytes. This data was collected from a single donor, donor 31, that had enough cells to plate different plastic concentrations. As we did not test further donors for reduction in viability, this reduction could be due to inter-donor variabilities.

Table 4.3: Reduction in cell viability in primary human monocytes exposed to micro- and nanoplastics. The table shows the reduction in cell viability in % obtained from donor 31.

Concentration	**PMMA**	**PS**	**PVC**
Control	0	0	0
Low	-1.30	6.26	-10.97
Medium	-13.27	0.455	-23.98
High	-34.98	-16.04	-36.70

4.3.4 Pro-inflammatory release of MNPs in primary monocytes

To investigate Whether MNPs cause a pro-inflamamtory response in primary human monocytes we exposed the monocytes to MNPs for 18h before the supernatant was collected and tested for TNFα and IL-6 cytokine release using ELISA.

Solvent controls were employed to account for any inflammatory response induced by the chemicals in the solvents, and LPS was used as the positive control. The possible number of plastic exposures was limited based on the total number of isolated PBMCs per donor, so typically MNPs were only used at the highest doses. Some of the samples had enough cells to test both high and low doses, as well as plastic in tandem with positive control. To determine an LPS concentration to use as a positive control, a dose-response experiment was performed with the primary monocytes exposed to LPS concentrations ranging from 0.01 pg/mL – 1 ng/mL. The cytokine release in response to the LPS stimulation was curve fitted by log transforming the LPS concentration and fitting it to a log(agonist) vs response 4PL curve (Figure 4.11). We found that a concentration of 10 pg/mL of LPS induced a strong inflammatory response in both TNFα and IL-6 and was therefore used as the concentration for the positive control in these experiments.

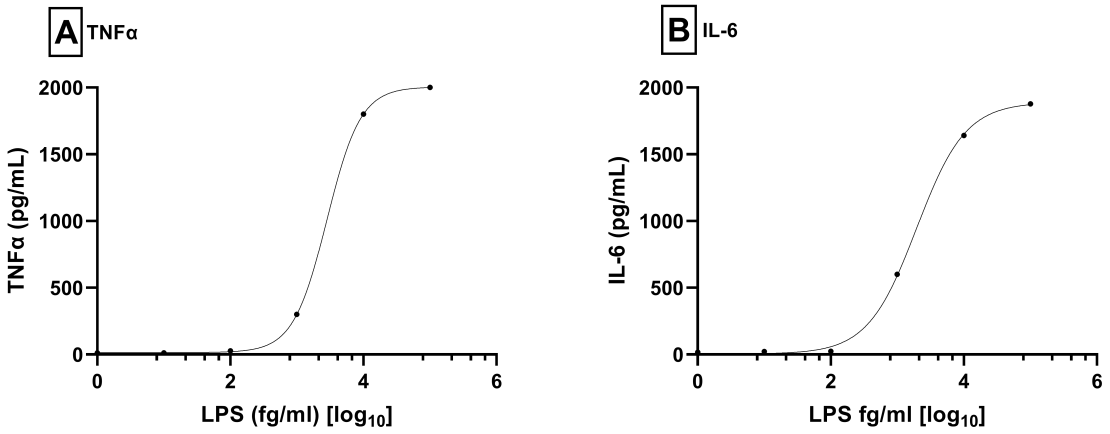

Figure 4.11: Dose-dependent TNFα and IL-6 cytokine release in primary human monocytes exposed to LPS (0.01 pg/mL – 0.1 ug/mL) for 18 h. The data was fitted with non-linear log(agonist) vs. response – 4PL slope regression. (a) shows TNFα and (b) shows IL-6. This data was collected from donor 6.

As there were variations between how the cell donors responded to the positive controls, a criteria had to be established to determine which donors to include in the datasets. In a case where a donor had an atypically low response to the positive control or inspection of the plated cells revealed a lack of adherence of the monocytes, the data from these donors were excluded from analyses. Atypically low responses to the LPS were considered below the limit of detection of the assay, 15.6 pg/mL (TNFα) and 9.8 pg/mL (IL-6). Cytokine release obtained from the cell donors that fit within these criteria can be found in

Table 4.4. The complete dataset of cytokine release, including donors that did not meet these criteria, can be found in appendix B.

We found large variability in both the donors' responses to MNPs and solvents. None of the MNP exposures resulted in significant mean TNFα cytokine release. The mean release of TNFα from the monocytes was 260 pg/mL for the positive control and 14.5 pg/mL for the negative control, while the mean release in response to the MNPs exposure was 20.5 pg/mL (PMMA), 21.7 pg/mL (PS) and 26.1 pg/mL (PVC) (Figure 4.12a). When normalized to the positive and negative controls, the increase in release was 2.1 % (PMMA), 6.9% (PS), and 7.9% (PVC) (Figure 4.12c).

Likewise, the IL-6 cytokine release resulting from exposure to MNPs, and solvents exhibited substantial inter-donor variability, as well as differences in sensitivity to LPS. The IL-6 mean cytokine release induced by exposure to PVC MNPs was 543.6 pg/mL and was significantly higher compared to the negative control ($p = 0.038$), but not the corresponding solvent control. The mean IL-6 cytokine release was 1339.2 pg/mL in the positive control and 62.4 pg/mL for the negative control. While not significant, the mean IL-6 release in response to the PMMA and PS MNPs exposure were 159.2 pg/mL (PMMA) and 128.7 pg/mL (PS) (Figure 4.12b). Upon normalization to the controls, the relative release of IL-6 cytokine was 6.8% (PMMA), 5.8% (PS), and 28.9% (PVC) (Figure 4.12d).

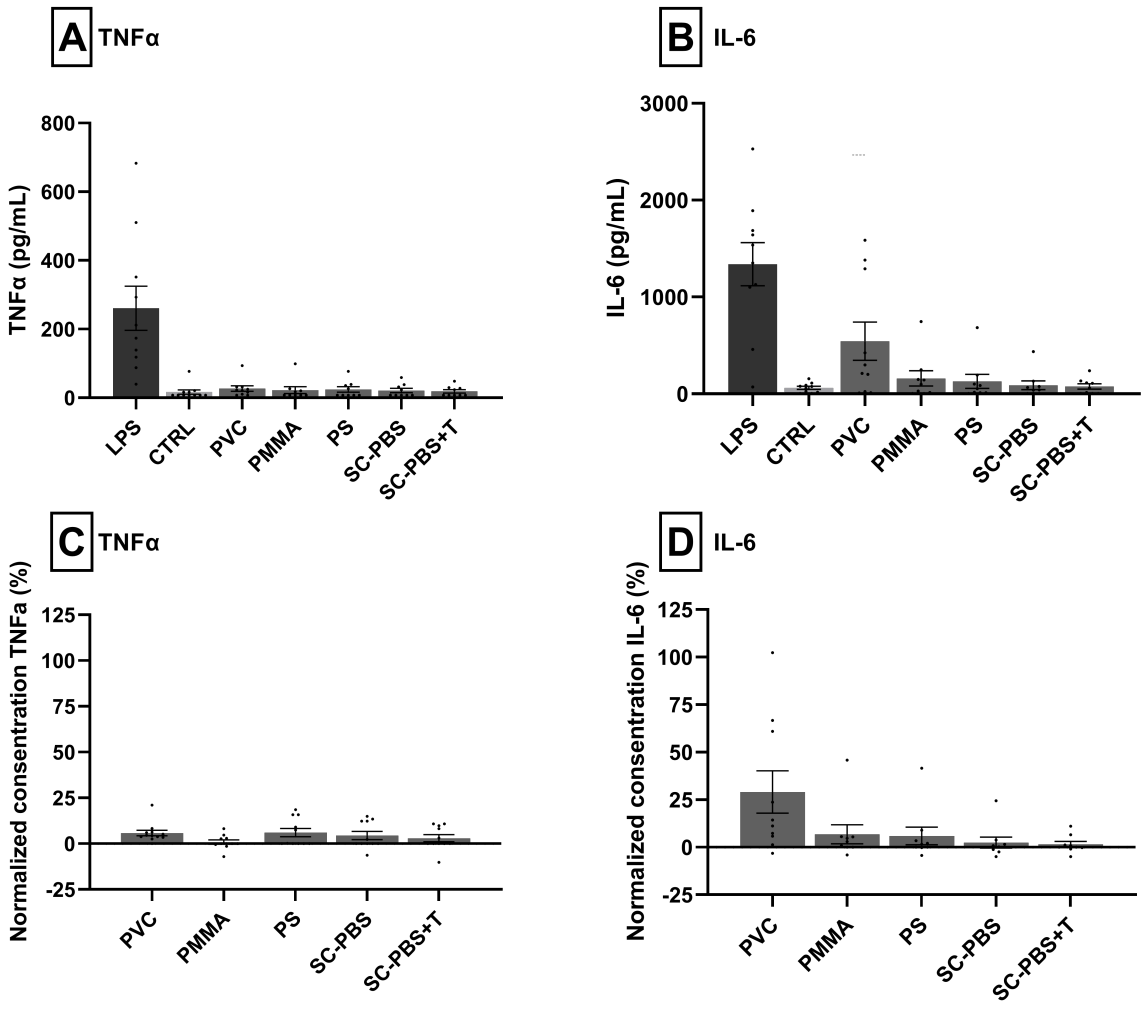

Figure 4.12: release of the TNFα and IL-6 cytokines from primary human monocytes. (a-b) shows the summarized data with the individual values of cytokine release, while (c-d) shows the data normalized on a scale where positive control (LPS) is set as 100, and negative control (CTRL). SC-PBS = solvent control PBS, SC-PBT+T = Solvent control PBT+Tween20. * $p < 0.05$

When exposed to plastic particles, monocytes released more IL-6 than TNFα, and this was also found for the LPS control. When the cytokine release was normalized to controls, the IL-6 cytokine release showed similar results between samples for PS, while PMMA and PVC exposed cells had an increased release.

There were large variabilities in cytokine release between donors both in response to the control as well as the plastic particles (Table 4.4). The trend observed with individual donors, which was summarized previously, is that PVC at the high dose of 300 particles per cell had overall higher cytokine release than the other plastic particles. This trend can be seen when comparing donor 36 and donor 14 (Figure 4.13). The figure shows how even when the TNFα levels in the positive control from both donors is similar, donor 14 shows only detectable cytokine release from PVC MNP exposure and this release is

doubled from donor 14 to donor 36 (22.7 vs 40.4 pg/mL). When considering the cytokine release of IL-6 we observe that donor 36 has more than 1000 pg/mL more release to the positive control when compared to donor 14, and this increase is similar for PVC MNPs but not observed with the other plastics or solvents.

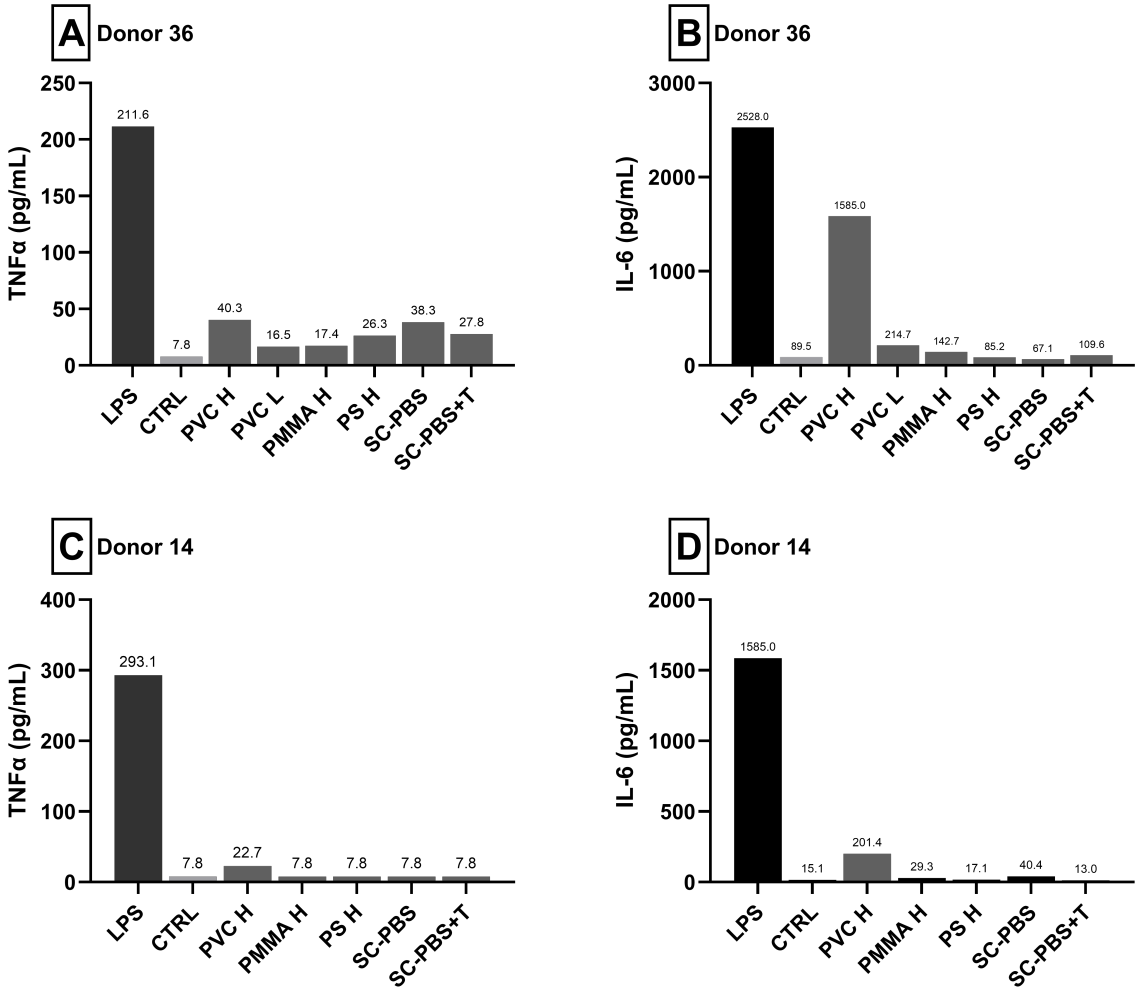

Figure 4.13: Cytokine release Donor 36 and 14. The figure shows the differences in TNFα and IL-6 cytokine release from two donors. (a) TNFα from Donor 36 (b) IL-6 from donor 36 (c) TNFα from donor 14 (d) IL-6 from donor 14. N.d = Non-detacatable from the ELISA assay. LPS = Positive control, CTRL = negative control, SC-PBS = solvent control PBS, SC-PBT+T = Solvent control PBT+Tween20

Table 4.4: Cytokine release of TNFα and IL-6 in primary monocytes. The tables shows an overview of cytokine release in the primary human monocytes after exposure to plastics and controls. † = Values was above the limits of detection for ELISA. N.D = Value was below limits of detection for ELISA. LPS = Positive control, CTRL = negative control, SC-PBS = solvent control PBS, SC-PBT+T = Solvent control PBT+Tween20

Cytokine release (pg/mL)								
Cytokine	**Donor**	**LPS**	**SC-PBS**	**SC-PBS+T**	**PMMA**	**PS**	**PVC**	**CTRL**
TNFα	7	173.5	N.D	N.D	N.D	N.D	N.D	N.D
	8	510.3	N.D	N.D	N.D	N.D	N.D	N.D
	11	39.8	NA	NA	N.D	N.D	10.45	N.D
	12	351.5	59.2	48.5	93.9	77.17	99.21	76.74
	13	87.8	NA	NA	NA	NA	31.3	16.27
	14	293.1	N.D	N.D	N.D	N.D	22.69	N.D
	15	682.8	0.47	14.6	26.05	N.D	31.37	N.D
	31	118.4	25.32	20.46	38.91	23.09	23.83	20.48
	36	211.6	38.27	27.84	17.38	26.32	40.32	11.38
	37	139	31.54	29.71	14.71	35.83	87.29	16.51
IL-6	7	459.7	N.D	N.D	N.D	N.D	10.610	N.D
	8	1128†	N.D	N.D	13.76	11.65	24.2	57.8
	11	71.9	N.D	N.D	N.D	10.87	N.D	N.D
	12	1535†	435	239.6	745.1	683.2	423	78.2
	13	1101†	NA	NA	NA	NA	212.3	157.2
	14	1585†	40.4	12.96	29.3	17.11	201.4	15.05
	15	1642†	37.8	61.7	149.2	100.8	299.9	76.2
	31	1351	76.29	113.1	97.55	53.3	1381	25.78
	36	2528	134.3	132.2	247.6	194.8	1585	89.5
	37	1890	67.06	109.6	142.7	85.19	1290	112.3

4.3.5 Pro-inflammatory release of MNPs in primary MDMs

To differentiate the monocytes to MDMs, the isolated monocytes were incubated with 50 ng/mL M-CSF for 5 days to produce m0 MDMs, and after the differentiation period was complete the cells were exposed to plastics in the same way as with the monocytes (section 3.4).

The mean release of TNFα when exposed to PVC MNPs was 104.9 pg/mL, while cells exposed to PMMA and PS had a cytokine release of 47.6 pg/mL and 42.7 pg/mL respectively. When exposed to PVC particles the mean release of IL-6 was 543.6 pg/mL, while the cytokine release after PMMA exposure was 159.2 and PS exposure equal to 128.8 pg/mL (Figure 4.14).

The mean cytokine release of both TNFα and IL-6 became lower for the LPS positive control compared to the monocytes, however, the mean response to the plastic particles, solvent controls, and negative control increased and were consistently higher. The mean release of cytokines in the negative control for the macrophages was 27.5 pg/mL (TNFα)

and 12.8 pg/mL (IL-6), while the mean release of cytokines found in the LPS positive control was 213.2 pg/mL (TNFα) and 622.7 pg/mL (IL-6). The change in sensitivity to the stimulants suggests that macrophages are more sensitive and prone to cytokine release when exposed to MNPs. As the macrophages are primary responders and phagocytes in the immune system in response to invading pathogens in tissue this is not unexpected, however, the size of the increase in release is quite large compared to the monocytes. The impact of the exposure of the MNPs to the MDMs suggested that the cells had been driven from M0 differentiation to M1 macrophages. After the monocytes had been exposed to M-CSF for the allotted time required to differentiate them to an M0 stage of macrophages, there was an expectation that the M0 macrophages could either be driven towards a pro-inflammatory M1 macrophage or the anti-inflammatory M2 subset of macrophages. The high releases of TNFα and IL-6 detected by ELISA suggest that the macrophages display more typical M1 characteristics, as the release of these cytokines is used to detect the maturation of the macrophages [58].

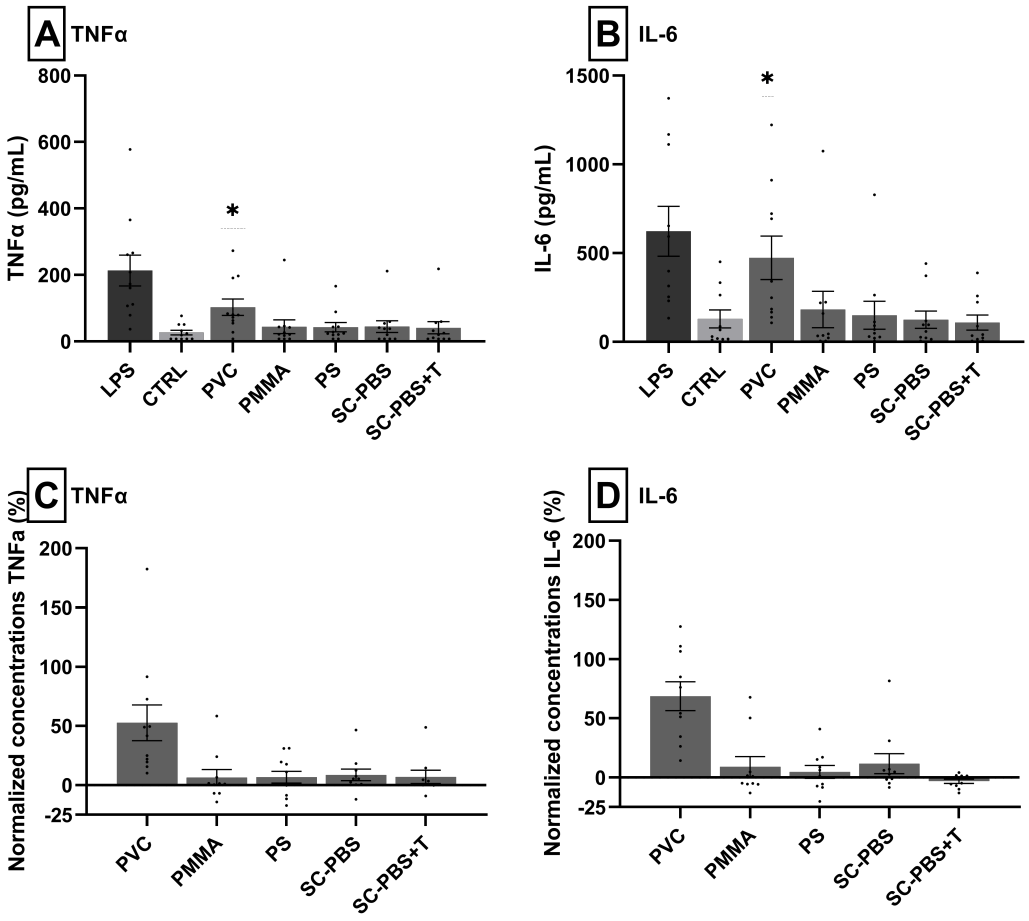

Figure 4.14: The figures shows release of the TNFα and IL-6 cytokines from primary human monocytes-derived macrophages. (a-b) shows the summarized data with the individual values of cytokine release, while (c-d) shows the data normalized on a scale where positive control (LPS) is set as 100, and negative control (CTRL). SC-PBS = solvent control PBS, SC-PBT+T = Solvent control PBT+Tween20. * $p < 0.05$

As we saw in the monocytes, there was high inter-donor variability in responsiveness to both controls and plastic particles, as well as differences in the release of cytokines to the same LPS stimulation (Table 4.5).

Donor 27 has one of the highest releases of TNFα (577.6 pg/mL) in the macrophage dataset when stimulated with LPS while being in the lower range of IL-6 cytokine release (592.9 pg/mL). This LPS sensitivity was not reflected in the donors' sensitivity to MNPs as the cytokine release for TNFα was consistent with donors with lower release following LPS stimulation, while the IL-6 cytokine release was on the lower end of the range found within the dataset (Figure 4.15). When considering the MNP exposure, the high dose of PVC MNPs resulted in the highest release of both IL-6 and TNFα compared to the other MNP exposures. Interestingly, for donor 27 we additionally tested the effects of exposing the cells to PVC in combination with LPS. Here, PVC+LPS resulted in a TNFα release lower than the positive LPS control (362 pg/mL), whereas for the IL-6, LPS+PVC

induced higher cytokine release than LPS alone (1166 pg/mL).

Donor 30 was highly expressive overall, even when considering the negative control (Figure 4.16). This donor had the highest overall cytokine release of the macrophage dataset, with a negative control having a cytokine release equal to 50.8 pg/mL (TNFα) and 450.9 pg/mL (IL-6). Both concentrations of PS and PMMA MNP exposure resulted in higher IL-6 release when exposed to the cells compared to both concentrations of PVC MNPs, which is not observed in the other donors in the macrophage dataset. This observation is not consistent for the TNFα release, whereas the PVC MNP exposure results in higher release than all other plastics except for PMMA L. PMMA L exposure resulted in the highest cytokine release for both TNFα (90 pg/mL) & IL-6 (1121 pg/mL), which for the IL-6 had a comparably high release to the PVC with LPS stimulation. Notably, when we tested the effects of PVC in combination with LPS for donors 30 we saw the opposite effect than donor 27. The PVC+LPS induced higher levels of TNFα compared to the LPS control (179.8 pg/mL), wheras for the IL-6, the LPS+PVC exposure resulted in lower cytokine levels compared to the control (1191.0 pg/mL)

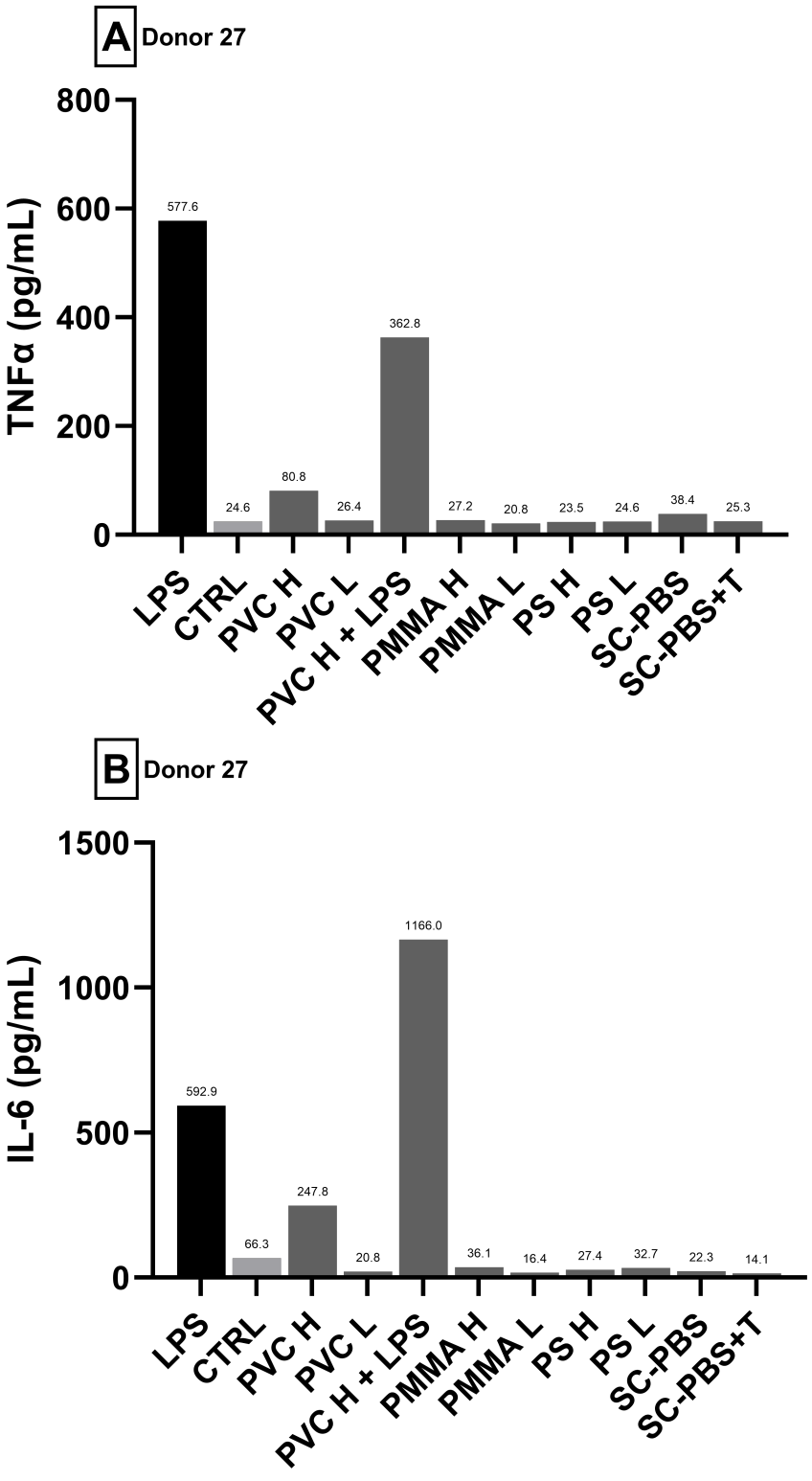

Figure 4.15: Cytokine release of TNFα and IL-6 found in Donor 27. The figure shows the differences in (a) TNFα and (b) IL-6 cytokine release from donor 27. LPS = Positive control, CTRL = negative control, SC-PBS = solvent control PBS, SC-PBT+T = Solvent control PBT+Tween20

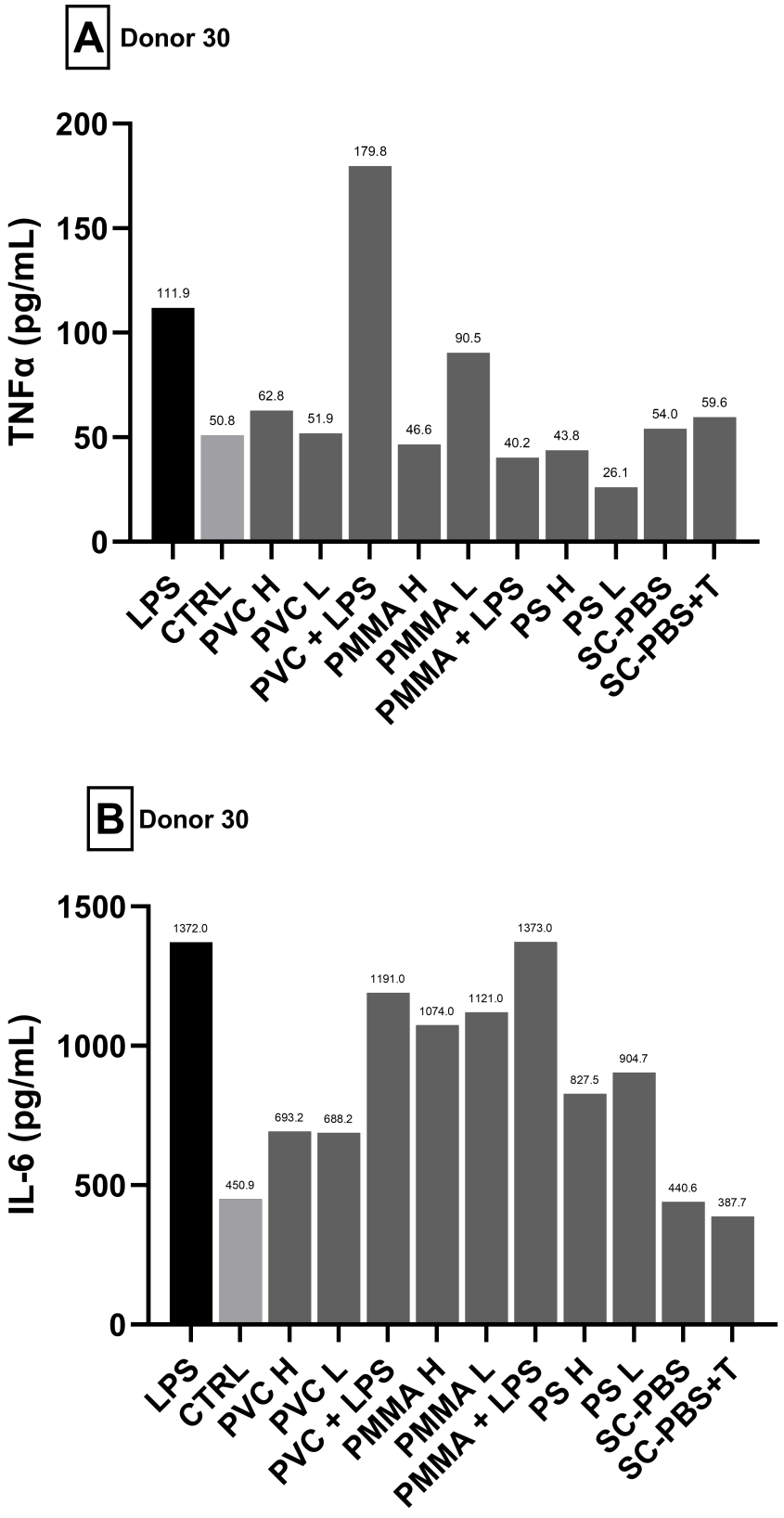

Figure 4.16: Cytokine release of TNFα and IL-6 found in Donor 30. The figure shows the differences in (a) TNFα and (b) IL-6 cytokine release from donor 30. LPS = Positive control, CTRL = negative control, SC-PBS = solvent control PBS, SC-PBT+T = Solvent control PBT+Tween20

The trend observed with the majority of donors was that exposure to a high dose of PVC MNPs induced the strongest release of both cytokines, as can be seen in donor 27, however, this was not consistent for all donors. Donor 30 displayed higher release of TNFα and IL-6 after exposure to both the high and low doses of PMMA MNPs compared to the highest dose of PVC MNPs (Figure 4.15). The variability of plastic sensitivity can be seen in further donors (Table 4.5). This could suggest sensitivity to types of MNPs differs between people. Additionally, there could be a connection between the basal inflammation levels in the donors which affect their sensitivity to both control stimulation and exposure to MNPs.

Table 4.5: Cytokine release of TNFα and IL-6 in primary monocytes-derived macrophages. The tables shows an overview of cytokine release in the primary human monocytes after exposure to plastics and controls. † = Values was above the limits of detection for ELISA. N.D = Value was below limits of detection for ELISA. LPS = Positive control, CTRL = negative control, SC-PBS = solvent control PBS, SC-PBT+T = Solvent control PBT+Tween20

		Cytokine release (pg/mL)						
Cytokine	**Donor**	**LPS**	**SC-PBS**	**SC-PBS+T**	**PMMA**	**PS**	**PVC**	**CTRL**
TNFα	25	260.9	20.09	20.09	21.59	20.84	79.77	19.33
	26	266.1	N.D	N.D	21.22	19.71	72.15	N.D
	27	577.6	38.41	25.32	27.16	23.46	80.81	24.58
	28	172.7	59.97	56.47	42.34	88.76	273.2	50.84
	29	78.73	38.05	32.28	43.41	28.26	55.06	32.28
	30	111.9	54.01	59.62	46.61	43.77	62.76	50.84
	32	208.6	41.34	10.88	N.D	44.01	191.3	N.D
	33	161.3	15.16	N.D	17.38	24.38	83.58	N.D
	34	36.94	N.D	N.D	N.D	8.328	27.4	N.D
	35	107.1	N.D	N.D	N.D	N.D	30.87	16.85
	38	365.7	211.4	218	245.2	166.5	197.3	77
IL-6	25	653†	58.13	41.1	44.21	69.04	721.7†	14.13
	26	397.6	26.13	21.39	21.79	20.68	340.5	16.06
	27	592.9	22.27	14.07	36.08	27.44	247.8	66.27
	28	1168†	372.1	223.3	223	262.7	1222†	332.8
	29	1112†	95.61	258.5	219.9	91.19	910.1†	262.9
	30	1372†	440.6	387.7	1074†	827.5†	693.2†	450.9
	32	314.9	117.9	34.68	32.57	47.65	184.5	29.89
	33	133.2	N.D	N.D	N.D	29.48	166.9	N.D
	34	252.7	14.05	N.D	N.D	N.D	138.1	18.04
	35	231	95.37	88.61	159.3	111.5	107.3	86.7
	38	653	58.13	41.1	44.21	69.04	721.7	14.13

4.3.6 Statistical analysis of cytokine release

As we saw inter-donor variabilities in the cytokine release to the same MNP exposures as well as the controls, there was an interest in if there were any correlation between the end-point when considering the pro-inflammatory cytokine release from the primary cells after exposure to the MNPs. As has been stated above, there were variances between how the different donors responded both to the control samples as well as the plastic exposure.

A Pearson R correlation matrix was performed to investigate the correlation between the multiple variables of cytokine release. From the matrix of the correlation for the monocytes, it can be observed that for the IL-6 cytokine a correlation of 0.81 is found between LPS and PVC (Figure 4.17a) Additionally, while neither PS nor PMMA has a positive correlation with LPS, they are correlated with each other at 1.0 for the correlation coefficient. As these showed little to no response to the MNP exposure, this correlation is most likely a result of both being normalized to the negative control. For the TNFα cytokine release from the monocytes, there is not a strong relationship between cytokine release in response to LPS and the plastics, however, there is an increased correlation between the cytokine release from the negative control with the responses as a result of the different plastic exposures (Figure 4.17b). Lastly, the correlation matrix for the TNFα release from the macrophages can imply a correlation between the release of TNFα after exposure to the different MNPs, while not having a positive correlation to the response from the LPS.

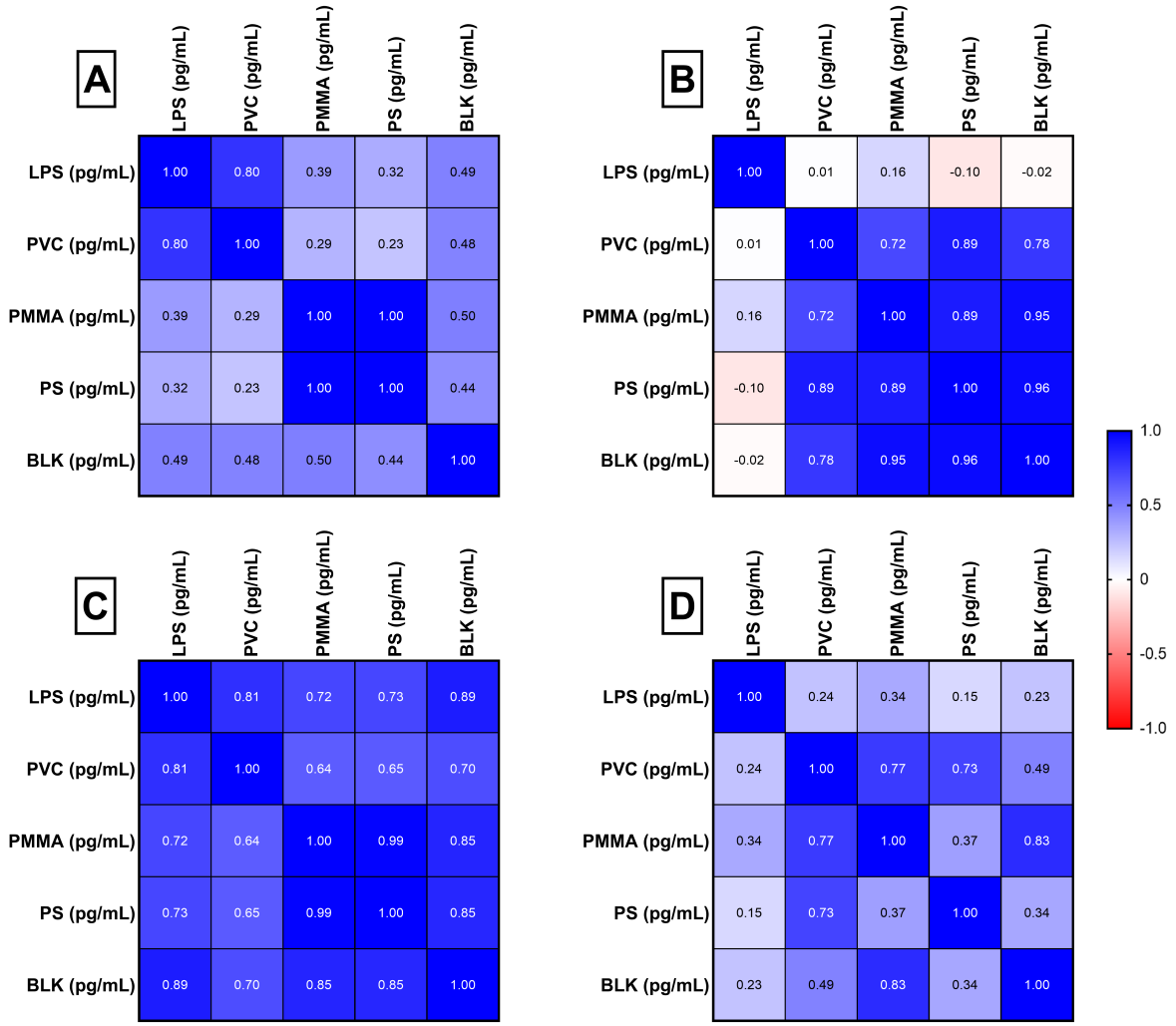

Figure 4.17: Pearson R Correlation matrix of cytokine release. The figure shows the correlation matrix of cytokine release between different end-points. (a-b) shows the correlation matrix for monocytes, while (c-d) shows for macrophages. (a-c) displays the cytokine release of IL-6 while (b-d) shows the TNFα. Correlation coefficient above 0.5 was considered significant. LPS = Positive control, BLK = negative control, SC-PBS = solvent control PBS, SC-PBT+T = Solvent control PBT+Tween20

5 Discussion

In this master's project, we have investigated the cytotoxic effects and release of pro-inflammatory cytokines from human monocytic cells after exposure to irregular plastic particles, PMMA, PS, and PVC. Our findings suggest that MNPs can induce pro-inflammatory responses in primary human monocytes and MDMs. However, the extent of the pro-inflammatory response varies depending on the cell donor, the type of polymer, and the concentration of particles. Additionally, our study demonstrates that MNPs do not elicit pro-inflammatory responses in the MM6 cell line.

5.1 Inflammatory response to MNP exposure

5.1.1 The inflammatory response in primary human monocytes

It has been established that MNPs are found in the human body, however, the extent of the adverse effect these particles can have is still a topic being researched. Studies on the inflammatory effect of MNPs on primary human immune cells are limited, but a previous study by Weber et al. reports that in primary human monocytes, irregular PVC MNPs induce release of pro-inflammatory cytokines; IL-6 and TNFα [46].

The results of our study also showed that exposure of PVC MNPs to primary human monocytes increased the release of IL-6, however, we observed no significant change in the levels of TNFα. Furthermore, our study did not find any significant pro-inflammatory response from exposure to the PS or the PMMA MNPs. These findings were not the absolute trend as we observed PS and PMMA MNP exposure eliciting pro-inflammatory response in certain donors. Large inter-donor variabilities in pro-inflammatory cytokine release have been observed in a previous study on plastic exposure using PBMCs [29]. Based on our study we suggest that pro-inflammatory responses in primary human monocytes are dependant on the plastic particles and cell donors, both topics which will be revisited in this discussion.

As we set out to reproduce the study by Weber et al. [46], the low pro-inflammatory response from exposure to PS and PMMA MNPs was anticipated. However, the low release of TNFα in the monocytes after PVC particle exposure was unexpected as the particle size distribution and concentration utilized in our study and Weber et al.'s were

comparably similar. This inconsistency suggests that the inflammatory response is affected by other factors than polymer type and shape, such as being dependent on the specific donor's response to MNPs which will be discussed below. Due to the difference in the TNFα release, it is difficult to conclude that the study was reproduced, nonetheless, this thesis has given additional insight into MNP exposure in primary human monocytes.

One prominent difference is that in our study we observed a large inter-donor variation in the cytokine release after exposure to the three MNP types (table 4.4). This was not observed as prominently in Weber et al. data. To assess the differences we consider the fold change from the controls in both datasets, as absolute cytokine release values are affected by the number of adherent cells. Of the six donors in their data, all six donors had a $\geq$ 5-fold difference in release of TNFα and IL-6 from the control for the monocytes exposed to PVC MNPs but not in cells exposed to PS or PMMA MNPs. In our study, we found that only two of the ten cell donors had a 5-fold difference between the release of TNFα from exposure to PVC MNPs compared to the negative control, suggesting that the remaining cell donors did not have a strong pro-inflammatory response to PVC MNPs. For the IL-6 release, 5 cell donors had an $\leq$ 5-fold difference in release after PVC exposure, while this was observed only for one donor for both PS and PMMA MNP exposure. This difference suggests that the inter-donor variability will become prominent when the number of donors increases. It can also suggest that our methods of exposure might not have been as consistent as Weber et al. as our protocols for exposure were developed during the process. Positive and negative controls were employed for the normalization of the inflammatory response in our study to be able to account for the inter-donor variation. We did still observe a variation between donors as the cytokine release from the controls did not have cahnge consistently with the change observed in the MNP exposures (figure 4.17), and the possible implications of this will be discussed in section 5.1.3.

5.1.2 The inflammatory response in primary human MDMs

To our knowledge, there have been no studies exploring the inflammatory effects caused by exposure to irregular MNPs in primary MDMs. In our research, we found that exposure of MDMs to PVC MNPs resulted in a significant increase in the release of IL-6 and TNFα compared to the control. Furthermore, the exposure of PS or PMMA MNPs did not elicit a similar response. Intriguingly, the mean release of TNFα was higher in MDMs exposed to PVC MNPs compared to primary monocytes, while the release of IL-6 was slightly lower (Figure 4.14). A noteworthy observation is that the release of IL-6 in MDMs following PVC exposure demonstrated less variability among different cell donors, leading to a lower standard deviation (Table D.1). This suggests that the response to PVC MNPs in terms of IL-6 production is more consistent across different individuals. On the other hand, the variability in TNFα release was higher, indicating that the individual donor

characteristics may play a more substantial role in the release of TNFα in response to PVC MNPs. These findings emphasize the differential effects of various types of MNPs on cytokine production in MDMs and highlight the importance of considering individual donor variability in future studies. The relatively consistent IL-6 response observed after PVC MNPs exposure suggests a specific activation of signalling pathways associated with IL-6 release in both cell types. Further investigations are needed to understand the underlying mechanisms behind these differential responses and to explore the potential implications for MDM-mediated inflammatory processes when it concerns MNP exposure.

For comparable analysis in another monocyte-derived immune cell exposed to plastic, we consider the monocyte-derived dendritic cells in Weber et al. These cells displayed a higher mean release of IL-6 cytokine compared to monocytes as well. Similarly, they also observed a large inter-donor variability in their findings. In our study with MDMs, four out of eleven cell donors demonstrated high IL-6 cytokine release after exposure to both controls and MNPs, which aligns with the observations made by Weber et al. Overall, our findings indicate that the inflammatory response to PVC MNPs in MDMs does suggest a trend that this these particles induce inflammation, but the variability observed in IL-6 cytokine release highlights the importance of assessing individual donor characteristics. These results provide valuable insights into the specific cellular responses induced by irregular MNPs and emphasize the need to consider cell donor variability when studying the inflammatory effects of MNPs on MDMs.

Both the monocytes and the MDMs had a higher release of IL-6 compared to TNFα after exposure to PVC MNPs. The release of TNFα and IL-6 are both associated with early acute phase inflammatory response in the innate immune system, however, they have specific roles and act on specific receptors. The relatively higher release of IL-6 in both monocytes and MDMs could indicate that PVC MNPs activate the signalling pathways that would induce IL-6 release, however not as strongly for TNFα [34, 36]. These observations highlight the potential differential effects of PVC MNPs on cytokine production and suggest that the underlying mechanisms of MNP-induced inflammation may involve specific signalling cascades that preferentially trigger IL-6 release. Further investigation is necessary to determine the molecular mechanisms involved in this differential cytokine response to PVC MNPs.

5.1.3 Variability in inter-donor inflammatory response

The inter-donor variability observed between the release of cytokines in response to MNPs does pose the question if different people have unique sensitivities to MNPs. While we observed an increased release of both pro-inflammatory cytokines after MNP exposure, this was not a consistent observation (Table 4.4 & 4.5). The variability between donors has previously been discussed as a constraint with the use of primary human cells by

Matthews et al. [29]. The authors found high variability in cytokine release in PBMCs between cell donors when exposing them to the same concentration of PE particles, and theorise this could be a result of genetic differences in the release of these cytokines. Another explanation for the high variation could be different basal levels of inflammation. In our study, we observed that cell donors with a high amount of pro-inflammatory cytokine release in the negative control also had a high correlation with the cytokine release after MNP exposure. The strongest correlation was between the TNFα release of the MNP-treated monocytes and the negative control, which had a correlation coefficient above 0.8 for all polymers (Figure 4.17c). Basal inflammation can modulate the ability of primary immune cells to recognize and engage with foreign particles, potentially impacting the signalling of pattern recognition receptors (PRRs) [35]. In the context of an ongoing inflammatory response, the presence of pro-inflammatory mediators may influence the cellular response to additional triggers, such as MNPs. There are multiple ways to address the issue of inter-donor variability in future studies, such as increasing the number of donors and performing a basal inflammation check before the collection of whole blood could help understand the variability in the effect of MNPs in the human immune system. Furthermore, one limitation of this study that could have revealed how the differentiation of monocytes to MDMs shapes the inflammatory response would be to use cells from the same donors for both experiments. Due to the limited number of cells in this experiment, this was not accounted for here, but should be considered for future prospects. Summarizing, one of the main findings in this study uncovered is the large inter-donor variability in inflammatory response both after exposure to controls and MNPs, which suggests there might be individual sensitives to plastic particles or that preexisting inflammation could affect the further response.

5.1.4 Effect of MNP in combination with LPS stimulation

LPS stimulation in combination with MNPs exposure resulted in a lower inflammatory response compared to the positive control for certain donors in our study. A similar observation was made by Harini Pechiappan in her master thesis with the THP-1 cell line [47]. This indicates that the plastic particles, as hydrophobic materials, attract the LPS. MNPs attracting LPS would mean that the concentration of LPS interacting with the cells decreases, thus resulting in lower stimulation of the toll-like receptor, decreasing the inflammatory signalling. Alternatively, this can indicate that plastic particles interacting with the PRRs hinder LPS in acting as ligand, which would decrease the bioavailability of LPS. It should be noted that these results were also subject to inter-donor variabilties, as was seen on the two donors in Figures 4.15 & 4.16. Furthermore, the LPS+MNP stimulation was only performed on 4 donors, making it difficult to observe a significant trend and make conclusive statements. Nevertheless, our finding does pose an interesting

question on how the inflammatory response is affected by both LPS and MNP stimulation and this should be tested more with additional donors.

5.1.5 Particle metric affects inflammatory response

The inflammatory response in relation to MNPs could depend on particle size, shape, and dose. To our knowledge, Weber et al. is the first published study on the topic of pro-inflammatory cytokine release after exposure to irregular MNPs in primary human monocytes. However, there have been studies investigating the effect of other polymer types, shapes and sizes on PBMCs and other subsets of immune cells.

Irregular particles have previously been found to induce stronger inflammatory responses than spherical ones. In our study, we found individual donors had a pro-inflammatory response to irregular MNPs, with the irregular PVC particles inducing the most consistently high response. This is supported by Gelb et al. [59], a study that discovered that subcutaneous implantation of irregular PMMA nanoparticles led to a higher concentration of TNFα tissue in their rodent model, in comparison to PMMA nanospheres. The exact mechanisms behind how MNPs can interact with cells are still under investigation, nevertheless, the implication of their study is that due to their irregular shape, the particles have a larger surface area relative to spherical particles of similar size. This larger area results in an increased surface area for cell-particle interactions of the PRRs, which thereafter can trigger phagocytosis and inflammatory signalling [35]. As we saw little response from the irregular PMMA and PS MNPs there is a possibility that solely an irregular surface is not the only factor determining inflammatory response. Thus, the overall implication is that irregular particles can have a pro-inflammatory effect, however, this effect could also be subject to other parameters than irregularity.

Particle size does seem to affect the release of inflammatory cytokines. A study by Hwang et al. with irregular PP particles found that particles < 20 µm induced an increased release of both TNFα and IL-6 in human PBMCs, which was not found for 25–200 µm particles [28]. Additionally, another study found that smaller ultra-high molecular weight PE particles (0.24, 0.45, and 1.7 µm) induce higher inflammatory cytokine release than larger particles (7,6 and 88 µm) in PBMCs [29]. In our study, we saw very low responses from exposure of PMMA and PS particles which had a mean size of 156 and 123.7 nm, respectively, while we did observe larger pro-inflammatory release from the PVC exposure (mean = 197.9 nm). These studies propose that larger particles induce less inflammatory cytokine release compared to smaller particles at the same concentrations, although it is still unknown how small particles could be while still inducing inflammation. Conclusively, we propose that there is a range of MNPs which can induce inflammation in human immune cells and that a future prospect could be to filter out irregular particles at different size intervals to determine how the size differentials in irregular particles affect the

inflammatory response.

Particle concentration could also affect the inflammatory response. Hwang et als study found significant pro-inflammatory response in PBMCS, and used a higher particle concentration (100-1000 µg/mL) compared to the metrics used in Weber et al. and in this master's project (<10 µg/mL for all polymers (Table 4.2)). Furthermore, we saw an overall larger mean pro-inflammatory response from cells exposed to 300 particles/cell compared to 1 particle/cell, which does imply that higher doses of plastic induce a more inflammatory response. This is supported by the Matthes et al. study mentioned above as they also found an inflammatory response from particle concentrations of 100 particles/-cell, indicating that although lower than used in our study, this concentration induces inflammation [29]. The range of particle concentration that would induce toxic effects is still unknown, and as our data found higher pro-inflammatory response for the lower doses of PVC MNPs compared to the highest doses of PMMA and PS, the number of particles required to induce response might be polymer dependent. Based on this, we conclude that higher particle concentrations have a more adverse effect on primary immune cells compared to lower concentrations and that this should be taken into consideration for future studies.

The densities of the particles could have effected the pro-inflammatory response. Another aspect of the MNP exposures to consider is that even at the same concentration of particles per cell, the three polymers used in this study had vastly different mass and surface area. PVC MNPs were found to induce the highest release of pro-inflammatory cytokines, and it also has the largest surface area and mass compared to PMMA and PS particles (Table 4.2). In this study we found that the low dose of PVC MNPs often induced higher release of pro-inflammatory cytokines compared to the highest doses of PMMA and PS. The low dose of PVC MNPs had lower mass and surface area compared to the high doses of PMMA and PS MNPs, which could indicate that it is pro-inflammatory response if not exclusively tied to particle mass or surface area. Conclusively, we believe the polymer density could have effect on the pro-inflammatory response, and that one of the aspect to consider with this is that it can be difficult to actually compare the adverse effects of different polymers as even when the particle number is similar, other metrics might differ.

Since the exact amount of plastic humans are consuming and internalizing is still unknown [19, 26], it is still under debate which particle dose is realistic to accurately model inflammation in human cells. The implication discussed above is that particle concentration and size do affect the pro-inflammatory response in the human immune system. Previous studies have used concentrations of > 1000 µg/mL [28] to investigate the inflammatory response, while in this study we have used 300 particles/cell (< 10ug/mL) as the highest concentration. Even our relatively lower concentration could also be unrealistic for the number of particles a circulating monocyte would interact within the human body,

as the current data on the presence of MPs in human blood is 1.6 µg/mL [21]. As of now, it is unknown if the particle concentration is cumulative, and therefore we should expect to see higher particle concentrations in blood in the future. The implication of studies on plastic particles in tissue [22–24] would suggest this could be the case. Nonetheless, we propose the importance of uncovering the particle metrics which can lead to an inflammatory response in the human body as the actual amount of internalized particles is unknown.

5.2 The MM6 cell line as a model for inflammation in response to MNPs

In this study, we investigated the inflammatory response after exposure to MNPs in two monocytic models, the MM6 cell line and primary human monocytic cells. Our results indicate that the MM6 cell line can have a slight increase in IL-6 and TNFα cytokine release after exposure to 300 PVC particles/cell, and no significant response from PS or PMMA MNPs nor the lower doses of PVC.

The release of TNFα in the MM6 was comparable to the release observed in primary human monocytes, however much lower compared to the IL-6 release. As we decided to not go forward with the differentiation of the MM6 to macrophage-like cells we cannot draw comparisons to the MDMs. This difference in the inflammatory response in the cell lines versus the primary human monocytic cells could be attributed to several factors, such as receptor release. While the MM6 cell line has been found to express several toll-like receptors related to the release of TNFα and IL-6 [45], the lack of release of these cytokines would suggest that MM6 lack the receptors which in primary cells which induce pro-inflammatory release. The MM6 cell line is found to express low levels of CD14 compared to primary monocytes [60], a receptor found to be associated with pro-inflammatory signalling [56]. Our findings, therefore, suggest that the MM6 cell line is not susceptible to pro-inflammatory responses when exposed to MNPs.

Interestingly, while the MM6 cell line did not express any inflammatory cytokines, it had a reduction in cell viability when exposed to high concentrations of MNPs. This implies that concentrations > 1 particle per cell have a negative effect on the cell viability after 72h and that above 100 particles per cell can result in reduced viability after 24 h. PVC had been classified as a hazardous material since 2016 [61], and it was proposed by Weber et al [46] that PVC could have a more toxic effect on cells due to the particle density. Higher particle density means that larger amounts of PVC particles will settle to the bottom of the plates and interact with the cells compared to PS and PMMA particles in the same time frame. Notably, the THP-1 cell model showed little to no reduction from exposure to irregular PVC particles in the master thesis of Harini Pechiappan [47],

which can imply the MM6 cell model might be more sensitive to PVC particles compared to the THP-1. This could be due to differential expression in receptors between the cell lines, such as the mechanism for breaking down internalized MNPs. As the mechanism for the breakdown of internalized MNPs is still unclear, the difference between the cell lines is difficult to speculate on. Furthermore, the PS and PMMA induce no significant reduction in cell viability in the MM6 cell line. This was unexpected, as PS particles have been shown to have a negative impact on cell viability in both PBMCs and in the THP-1 cell model in previous publications [5, 7, 62]. Conclusively, we have found that MNPs can induce cytotoxicity in the MM6 cell line, and this reduction in viability is dependent on polymer type and particle dose.

Another aspect to consider when establishing a cell line as a model for primary cells is that the inter-donor variability is not well represented by cell lines. While cell lines can have differences in response between biological replicates, they are still seeded from the same origin. Our results indicate different people have unique sensitivities to plastic particles, as well as pre-existing basal inflammatory levels, which could affect how responsive the monocytes and MDMs were to MNPs. As cell line represents the same models between replicates, they cannot show the whole picture of differences in the expressiveness of receptors found in studies using primary human cells [29].

Cell lines are useful tools to study molecular inflammation, however, we suggest that the monocytic cell line, MM6, is not a representative model to study the impact MNPs can have on the release of inflammatory cytokines. We suggest this based on the difference in the release of pro-inflammatory cytokines between the primary monocytes and the cell line. To expand the knowledge on the inflammatory response MNPs could have on the human immune system future studies should focus on using primary monocytes isolated from whole blood, which seems to be especially important due to inter-donor variabilities.

5.3 Technical limitation of the study

The MNPs used in this study were milled, irregular plastics characterized with an NTA, and from this NTA the resulting particles were found to be in the range of 10-1000 nm for all polymers. It should be noted that these results were most likely affected by the limits of detection of the NTA instrument, which is 1000 nm [48]. Visual inspection with a light microscope of the particles confirmed that there were particles larger than the monocytes, which typically fall in the size range of 16 to 22 µm, present in the MNP suspensions. If the particles were this size due to agglomeration or if this was the size of a single irregular particle is unknown, however, this technical limitation should be taken into account when assessing the results of the inflammatory and cytotoxic analysis in this study. As mentioned previously, there was an attempt to characterise the larger particles with a Coulter Counter, however, due to technical limitations this yielded no results.

Therefore all metrics used for calculations in the results and in this discussion were based on the data from the NTA. As the metrics of particles seem to be an important factor in cytotoxicity and inflammation, correct characterization was essential for the validity of the results in this thesis.

The methods and protocols for the isolation of monocytes from whole blood and exposure of MNPs were developed and optimised throughout the experiments. This could have affected the findings. As the methods developed, we had more consistent cell adherence later in the project, which could have affected the data from early cell donors. While the same number of PBMCs were plated in each well, there was a possibility that the number of adherent cells was not consistent and therefore resulted in different numbers of monocytes between wells. As the number of plastic particles the cells were exposed to were calculated based on a mean assumption of how many cells were adherent, there might have been inconsistent amounts of MNPs for the cells to interact with. This should be taken into account when assessing our findings.

Furthermore, we used LPS as a positive control to account for inter-donor variability in this study, however, this control was above the limits of detection for the ELISA for 13 of the donors tested in the primary human monocytes and MDMs. The supernatant from four of these cell donors was diluted 1:10 and analysed again, this was not performed for all due to limited biological materials and time constraints. Due to this limitation, we cannot confirm the absolute cytokine release in the positive control and PVC MNP exposure for certain donors, therefore affecting the normalisation of samples against the positive control, and possibly downplaying the inflammatory response from PVC.

6 | Conclusions and future prospects

In this master thesis, we set out to reproduce and expand on the finding in Weber et al's study on the inflammatory response after exposure to MNPs created from the polymers PMMA, PS, and PVC in primary human monocytes and MDMs. Additionally, we set out to investigate if the MM6 cell line could be established as a model for primary human monocytes in the context of MNP exposure.

While our findings suggest that PVC MNPs can induce an increased release of IL-6 in primary monocytes, we found no significant increase in TNFα levels. Our data suggest that MDMs also can have pro-inflammatory responses after PVC MNP exposure. However, for both cell types, we saw large variabilities in cytokine levels and high basal levels of inflammation. Our finding suggests that pro-inflammatory response in human primary monocytes and MDMs is dependent on polymer type, as well as related to the individual cell donors' sensitivities to plastic particles. For future prospects, the number of cell donors should be increased and the study should be performed with consistent methods throughout the experiment to better understand to impact of MNPs exposure on human health. Furthermore, to understand how the differentiation of monocytes to MDMs can shape the immune response, both cell types should originate from the same cell donors. While cell lines are valuable for investigating molecular inflammation, we propose that the MM6 monocytic cell line may not accurately represent the impact of MNPs on the release of inflammatory cytokines. To further our understanding of the inflammatory response induced by MNPs in the human immune system, it is recommended that future studies prioritize the use of primary monocytes isolated from whole blood or other models that better mimic the complexities of the immune system. This approach will provide more representative and reliable data in exploring the effects of MNPs on inflammatory processes.

As the topic of MNPs in the environment and exposure to humans becomes more prevalent the need to understand how these particles can affect humans becomes increasingly important. Understanding the risks of MNP exposure requires detailed knowledge of how these particles are recognized and interact with cells in the human body. Given the complexity of MNPs and the variety of immune cells and activation states, this poses a significant challenge that needs further research.

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A | Equipment and reagents

Table A.1: The tables contains the reagents and equipment used in this project.

Components	Supplier	Catalogue number
Cell cultivation		
RPMI-1640 medium	Sigma-Aldrich	R0883
Heat-inactivated FBS, Qualified	Gibco	10437028
L-glutamine	Lonza	17-605E
Gentamicin	Sigma-Aldrich	G1397
Non-Essential Amino Acid (NEAA)	Gibco	11140-035
Insulin solution, Human recombinant	Sigma-Aldrich	I9278-5ML
Sodium Pyruvate	Gibco	11360070
Trypan blue	Sigma-Aldrich	T8154
TC20™ Automated Cell Counter	Bio-Rad	145-0101
Resazurin assay		
Resazurin	R&D Systems	AR002
RNA isolation and reverse transcription		
RNeasy® Mini Kit	Qiagen	74106
QuantiTect® Reverse Transcription Kit	Qiagen	205313
β-mercaptoethanol	Sigma-Aldrich	M7522
LightCycler® 480 Multiwell Plate 96	Roche	04729692001
LightCycler® 480 SYBR® Green I Master	Roche	04887352001
LightCycler® 480 Sealing Foil	Roche	04729757001
ELISA		
Human TNFα DuoSet	R&D Systems	DY210-05
Human IL-6 DuoSet	R&D Systems	DY210-05
DuoSet ELISA Ancillary Reagent Kit 2	R&D Systems	DY008
PBMCs isolation		
SepMate™-50 (IVD)	STEMCELL	85450
Lymphoprep	STEMCELL	07801
Phosphate Buffered Saline Tablets	Sigma-Aldrich	P4417
Differentiation reagents		
Phorbol 12-myristate 13-acetate	Sigma-Aldrich	P8139-5MG
Calcitriol	Sigma-Aldrich	32222-06-3
Macrophage colony-stimulating factor	Thermo Fisher	PHC9504

B | Gene expression with qPCR

The qPCR program settings used for gene expression analysis of MM6 cell line is given in Table B.1, and a list of the primers, provided by Sigma-Aldrich and used in the gene expression assay, is given in Table B.2

Table B.1: The table shows the qPCR programs settings for gene expression analysis

Step	Temperature	Time	Cycles
Preincubation	95 °C	600 seconds	1
Denaturation	95 °C	10 seconds	
Annealing	55 °C	10 seconds	45
Extending	72 °C	10 seconds	
Melting	95 °C	5 seconds	
	65 °C	60 seconds	1
	97 °C	1 seconds	
Cooling	40 °C	10 seconds	1

Table B.2: The tables contains an overview of primers used in the RT-qPCR; reference and test genes' sequences

Gene	Type	**Sequence**
ACTB	Reference	forward 5´- AAGACCTCTATGCCAACAC -3´ reverse 5´- TGATCTTCATGGTGCTAGG -3´
ALOX5	Test	forward 5´-FIND-3´ reverse 5´-OUT-3´
CCL2	Test	forward 5´- AGACTAACCCAGAAACATCC -3´ reverse 5´- ATTGATTGCATCTGGCTG -3´
CD14	Test	forward 5´-CTCAGAATCTACCGACCA-3´ reverse 5´-ATAGATTGAGCGAGTTTAGC-3´
CD16	Test	forward 5´-FIND-3´ reverse 5´-OUT-3´
GADPH	Reference	forward 5´-ACAGTTGCCATGGTAGACC-3´ reverse 5´-TTTTTGGTTGAGCACAGG-3´
RPS18	Reference	forward 5´- CAGAAGGATGTAAAGGATGG-3´ reverse 5´-TATTTCTTCTTGGACACACC-3´

C | Additional data - Characterization of MNPs by NTA

This chapter contains the overview of the plastic particle weights (table C.1), and the setting for the Nanosight NTA (Table C.2).

Table C.1: The table shows the particle weights for the irregular plastic particles, the average of three weighings.

Sample	PVC (mg)	PS (mg)	PMMA (mg)
1	0.0201	0.2090	0.0301
2	0.0215	0,0189	0.0171
3	0.0225	0.0214	0.0225
4	0.0252	0.0209	0.0222
5	0.0231	0.0194	0.0266
6	0.0299	0.0181	0.0265
7	0.0189	0.0217	0.0301
8	0.0214	0.0171	0.0210
9	0.0233	0.0220	0.0292
10	0.0189	0.0184	0.158
11	0.0170	0.0188	0.0203
12	0.0282	0.0212	0.0169
13	0.0189	0.0243	0.0223
14	0.0273	0.0195	0.0222
15	0.0196	0.0234	0.0214

Table C.2: The table shows the settings and dilutions use for the Nanosight Nanoparticle tracking analysis.

Sample	Dilution factor	Replicates	Camera level	Measurment time	Light threshold	Detection threshold	Temperature
$_{di}H_2O$	1	1x3	16	120s	1	10	21.2
PMMA	1:100	3x3	16	120s	1	10	21.2
PMMA CTRL	1:100	3x3	16	120s	1	10	21.2
PS	1:100	3x3	16	120s	1	12	21.2
PS CTRL	1:100	3x3	16	120s	1	12	21.2
PVC	1:1000	3x3	16	120s	1	10	21.2
PVC CTRL	1:1000	3x3	16	120s	1	10	21.2

D | Supplementary material on donors

This section of the appendix contains additional information on the data collected from the cell donors in this masters' project. Table D.2 shows the complete overview of the donors, all information collected regarding blood collected, PBMCs isolated, % of monocytes identified with flow cytomery and if the donor were included or excluded. If the donor were excluded the reason is stated. While Table D.1 shows the collected information of the standard deviations in the values for the donors.

Table D.1: The tables shows the mean cytokine release of IL-6 and TNFα with the standard deviation in primary human monocytes and monocyte-derived macrophages.

		Cytokine release (pg/mL)						
Cell Type	**Cytokine**	**LPS**	**SC-PBS**	**SC-PBS+T**	**PMMA**	**PS**	**PVC**	**CTRL**
Monocytes	**TNFα**	260,7±203.7	19,4±22.7	17,6±17.5	22,0±30.0	20,1±24.8	35,4±32.9	16,1±24.1
	IL-6	1329,1±698.2	89,2±136.5	75,9±80.6	159,2±235.0	128,7±216.9	543,7±622.1	59,5±52.1
Macrophages	**TNFα**	213,4±146.5	45,8± 58.1	44,9±68.8	43,3±68.9	42,7±47.2	104,9±79.3	27,5±23.3
	IL-6	625,5± 421.4	196,4±292.1	101,2±129.4	169,5±311.1	141,9±238.1	495,8±375.5	118,4±155.8

Table D.2: overview of all donor mectrics. The table contains an overview of all donors blood was collected from in this study. In the table the donors have been noted with their metrics and if they were excluded from the result section, the reason in noted.

Donor	IL-6 (LPS)) (pg/mL)	TNFα (LPS) (pg/mL)	Biological sex	Blood (mL)	Cell count PBMCs (10^7)	% monocytes (Flow cytometry)	Note
1	NA	NA	Female	NA	1.5	2.01	Excluded: Plating experiment – no MNP exposure data
2	NA	NA	Male	NA	1.6	1.89	Excluded: Plating experiment – no MNP exposure data
3	NA	NA	Male	15.0	3.5	NA	Excluded: Plating experiment - no MNP exposure data
4	NA	NA	Male	16.2	3.2	NA	Excluded: Plating experiment - no MNP exposure data
5	NA	NA	Female	15.0	4.2	NA	Excluded: LPS Dose-response experiment - no MNP exposure data
6	NA	NA	Female	15.0	3,7	NA	Excluded: LPS Dose-response experiment - no MNP exposure data
7	459,7	173,5	Male	13.1	3.0	3.86	Included
8	1128	510,3	Female	14.5	4.1	3.41	Included
9	213,9	31,3	Female	13.5	3.8	5.78	Excluded: No adherent monocytes
10	213,9	24,9	Female	14.5	3.2	3.53	Excluded: No adherent monocytes
11	71,9	39,8	Male	12.5	2.5	6.77	Included
12	1535	351,5	Male	14.5	3.0	00	Included
13	1101	87,8	Female	6	0.6	2.97	Included
14	1685	293,1	Male	14.5	2.7	1.47	Included
15	1642	682,8	Female	13.0	5.4	2.59	Included
16	NA	NA	Female	14.5	4.5	2.15	Excluded: Cell dried in imaging process – no viable cells
17	NA	NA	Male	14	2.5	6.84	Excluded: Cell dried in imaging process – no viable cells
18	NA	NA	Male	15.0	3,2	9.14	Excluded: Cell dried in imaging process – no viable cells
19	NA	NA	Female	14.5	4.9	5.17	Excluded: Cell dried in imaging process – no viable cells
20	6,6	8,67	Male	12.0	3.5	4.71	Excluded: LPS destabilized – no positive control
21	5,8	7,47	Female	14.0	4.0	2.10	Excluded: LPS destabilized – no positive control
22	0	0	Female	12.5	4.8	2.81	Excluded: LPS destabilized – no positive control
23	6,1	7,92	Male	13.0	5.4	1.89	Excluded: LPS destabilized – no positive control
24	0	0	Female	15.5	5.3	4.6	Excluded: LPS destabilized – no positive control
25	653	260,9	Male	15.2	2.9	1.90	Included
26	397,6	266,1	Male	13.0	3.7	2.86	Included
27	592,9	577,6	Female	12.5	4.5	1.13	Included
28	1168	172,7	Male	17.0	4.1	2.16	Included
29	1112	78,73	Male	16.5	3.6	3.19	Included
30	1372	111,9	Female	17.5	5.1	3.05	Included
31	1351	31	Female	17.5	5.4	4.64	Included
32	314,9	208,6	Male	17.0	6.2	1.95	Included
33	133,2	161,3	Female	16.5	2.8	3.91	Included
34	252,7	36,94	Male	17.5	3.4	2.84	Included
35	231	107,1	Male	16.5	7.0	2.9	Included
36	2528	211,6	Female	17.3	3.4	3.2	Included
37	1890.5	139	Male	16.5	3.6	1.78	Included
38	653	365,7	Female	18	3.5	2.8	Included

TNFα

The following section contains the cytokine level of TNFα from all donors this was recorded for, including donors 7-15, and 25-38. The figures have been separated into monocytes (Figures D.1 & D.2) and MDMs (Figures D.3 & D.4).

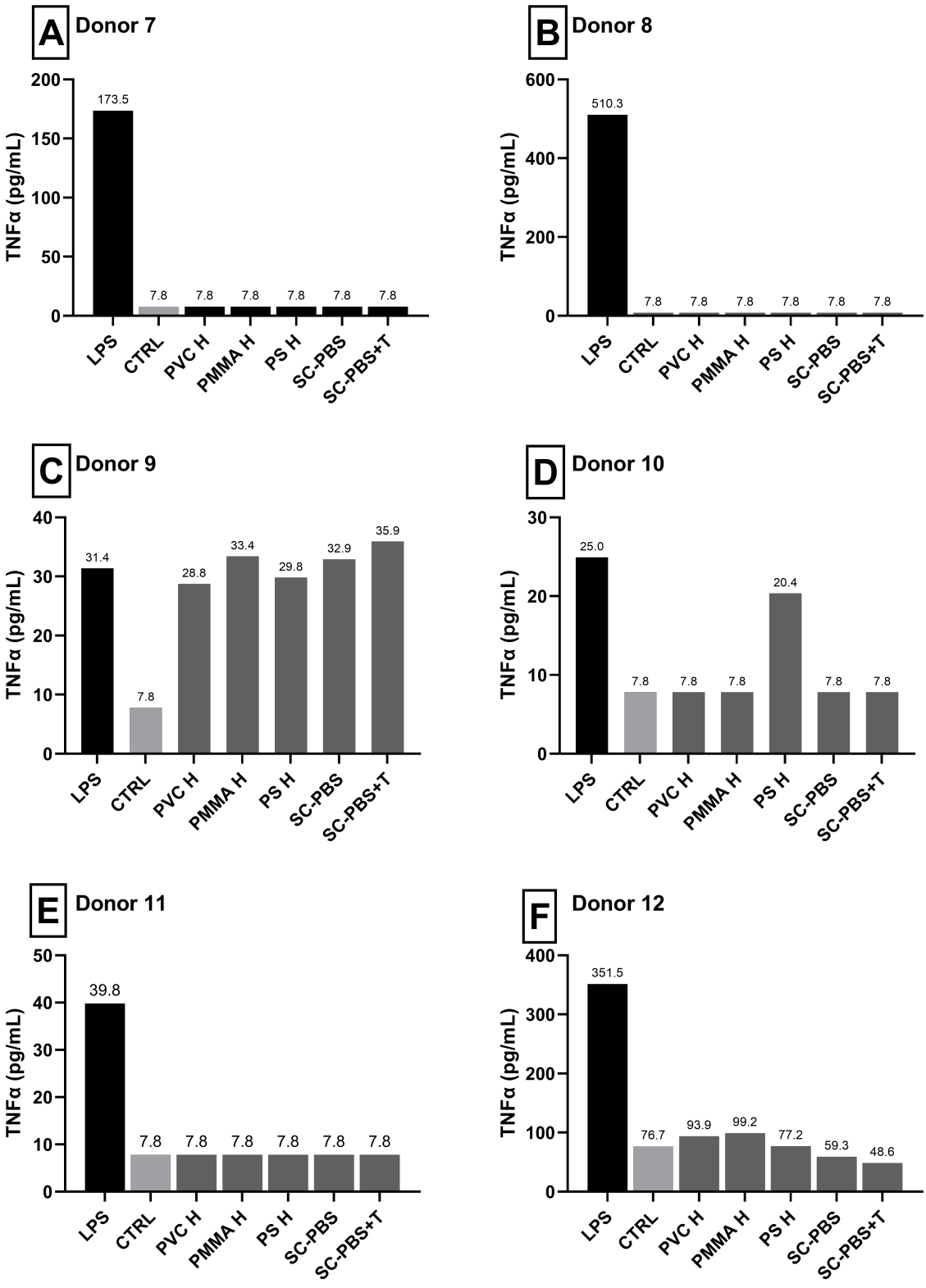


Figure D.1: The figure shows the TNFα cytokine level for donors 7-12. (a) 7 (b) 8 (c) 9 (d) 10 (e) 11 (f) 12. LPS = Positive control, CTRL = negative control, SC-PBS = solvent control PBS, SC-PBT+T = Solvent control PBT+Tween20.

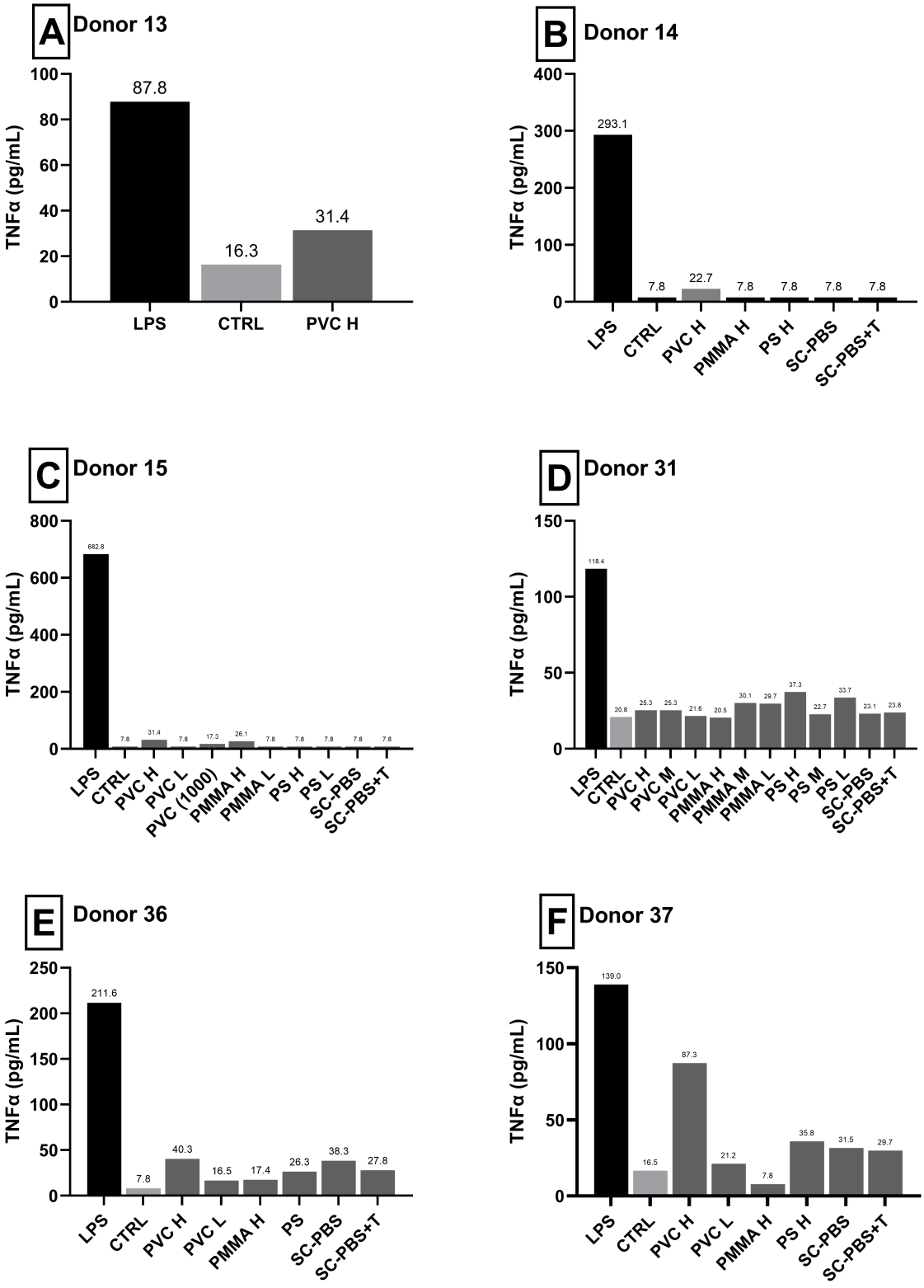


Figure D.2: The figure shows the TNFα cytokine level for donors 13-15, 31, and 36-37. (a) 13 (b) 14 (c) 15 (d) 31 (e) 36 (f) 37 LPS = Positive control, CTRL = negative control, SC-PBS = solvent control PBS, SC-PBT+T = Solvent control PBT+Tween20.

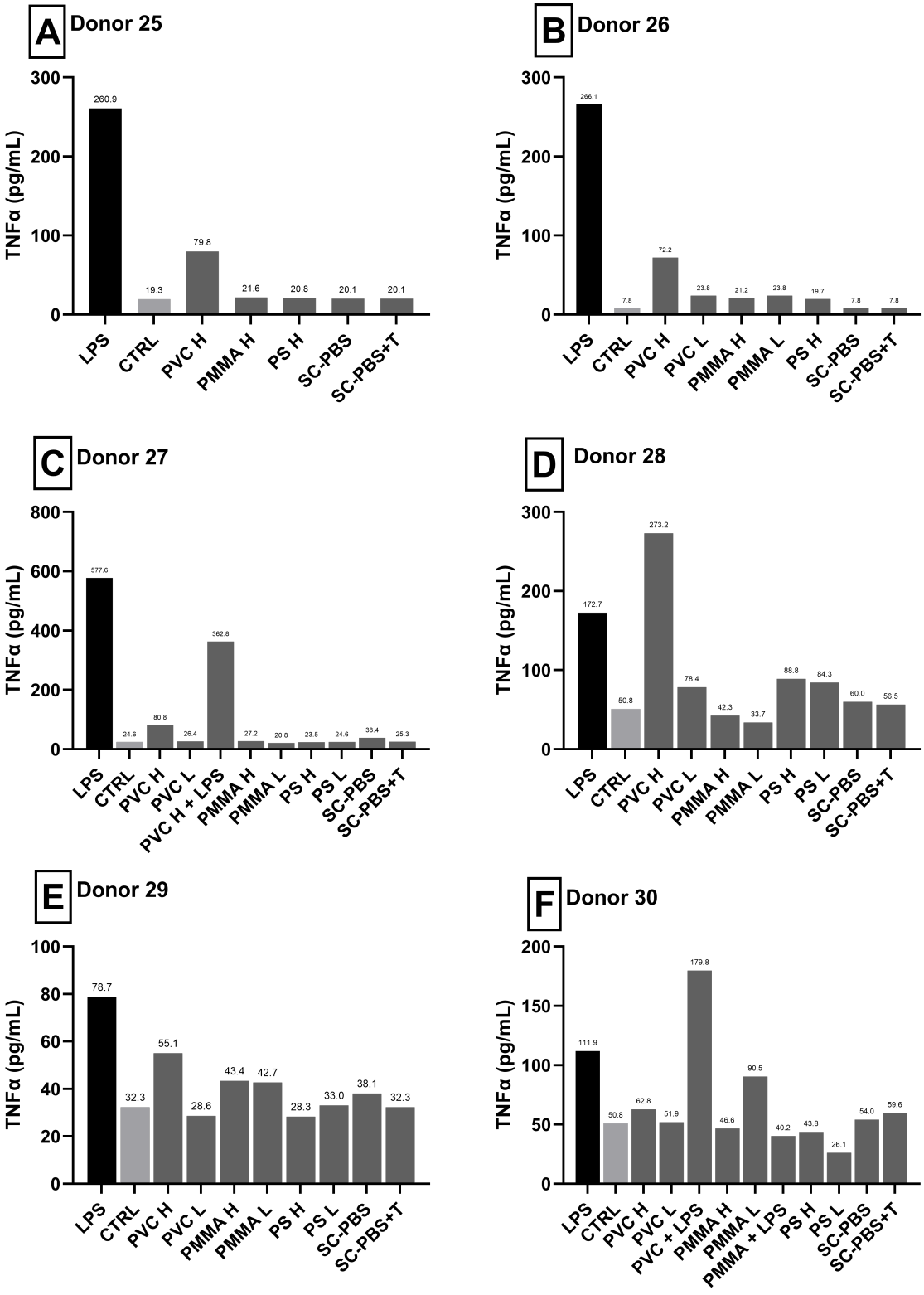


Figure D.3: The figure shows the TNFα cytokine level for donors 25-30. (a) 25 (b) 26 (c) 27 (d) 28 (e) 29 (f) 30. LPS = Positive control, CTRL = negative control, SC-PBS = solvent control PBS, SC-PBT+T = Solvent control PBT+Tween20.

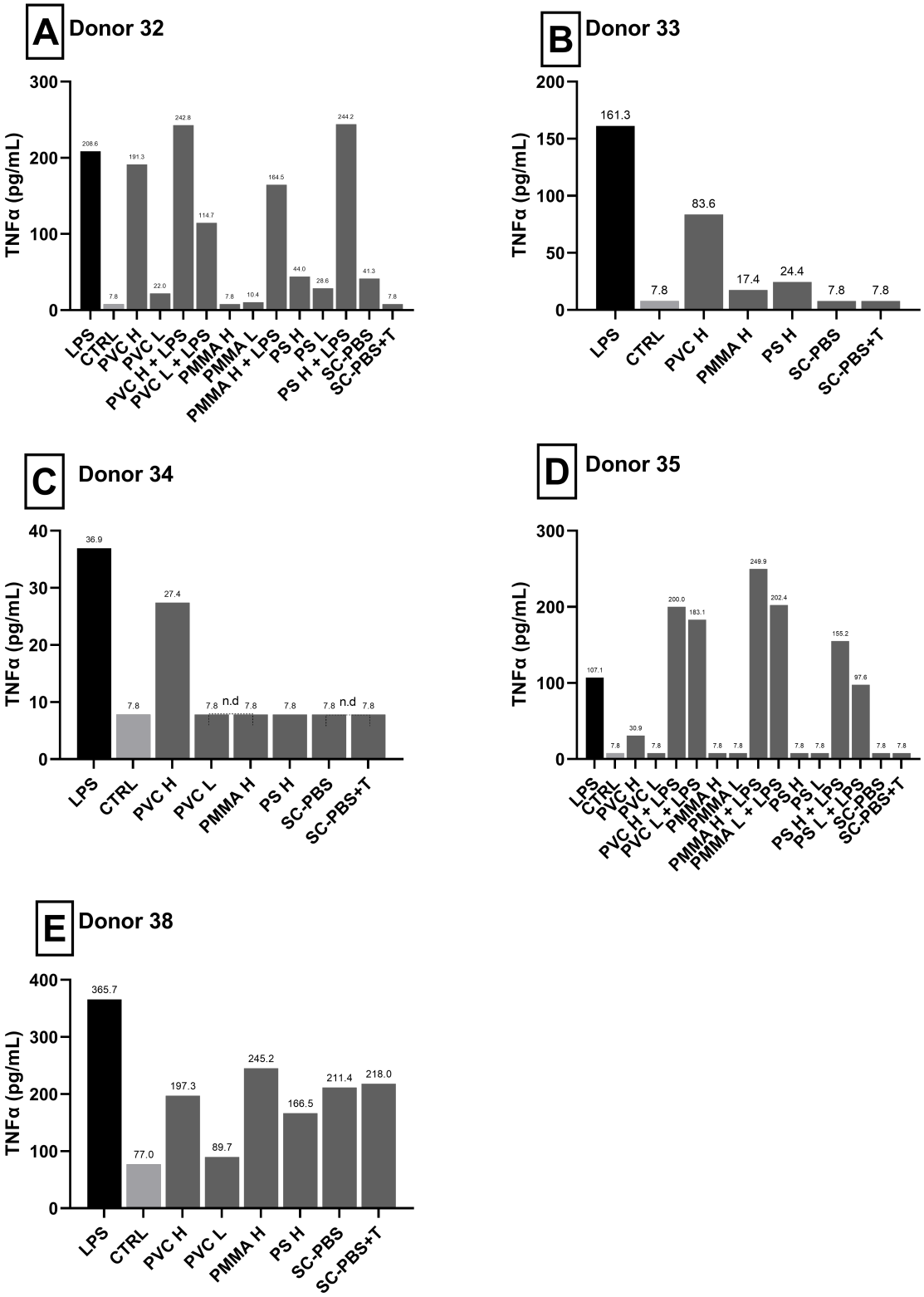


Figure D.4: The figure shows the TNFα cytokine level for donors 32-35 and 38. (a) 32 (b) 33 (c) 34 (d) 35 (e) 38. LPS = Positive control, CTRL = negative control, SC-PBS = solvent control PBS, SC-PBT+T = Solvent control PBT+Tween20.

IL-6

The following section contains the cytokine level of IL-6 from all donors this was recorded for, including donors 7-15, and 25-38. The figures have been separated into monocytes (Figures D.5 & D.6) and MDMs (Figures D.7 & D.8).

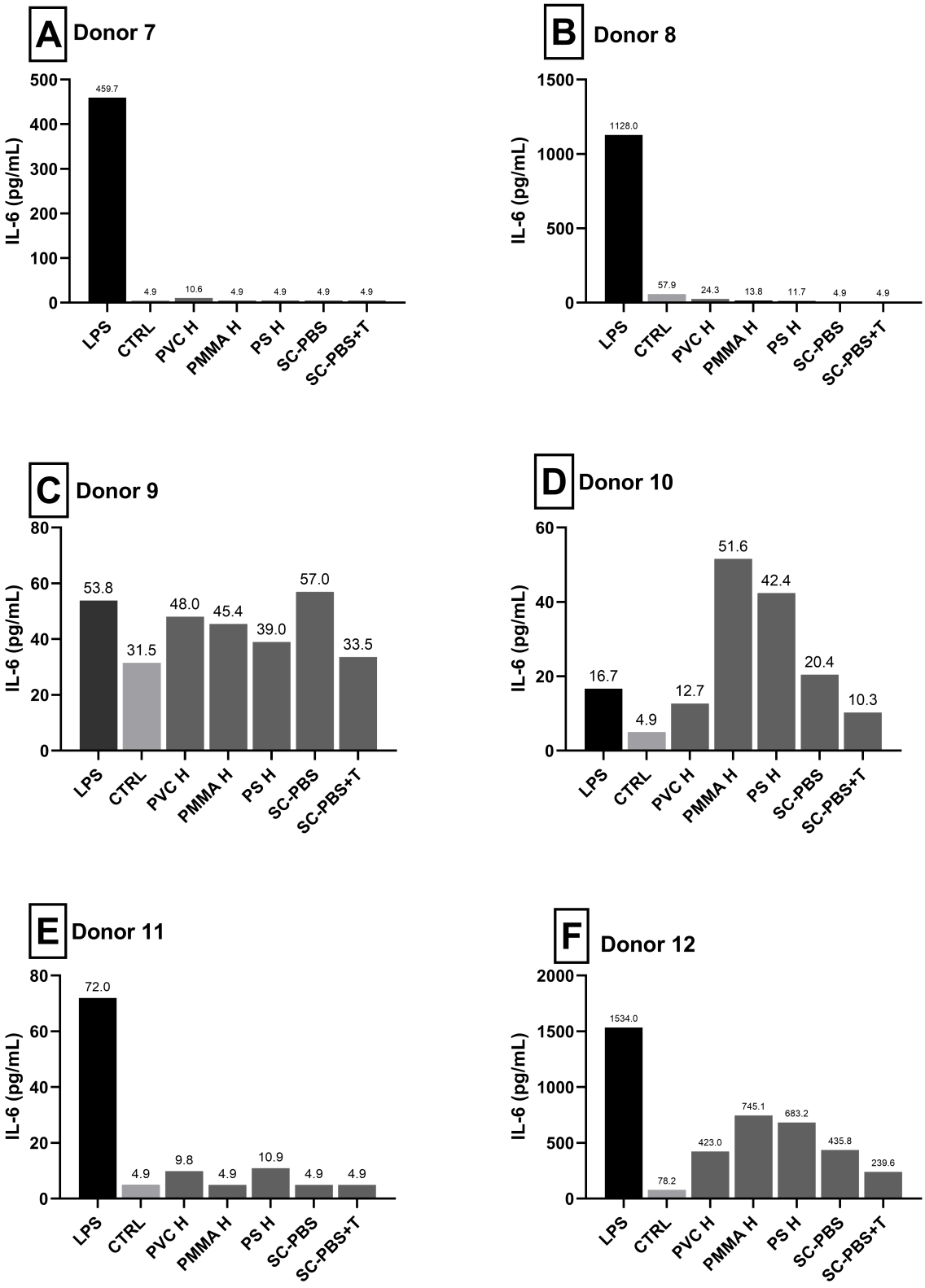


Figure D.5: The figure shows the IL-6 cytokine levels for donors (a) 7 (b) 8 (c) 9 (d) 10 (e) 11 (f) 12. LPS = Positive control, CTRL = negative control, SC-PBS = solvent control PBS, SC-PBT+T = Solvent control PBT+Tween20.

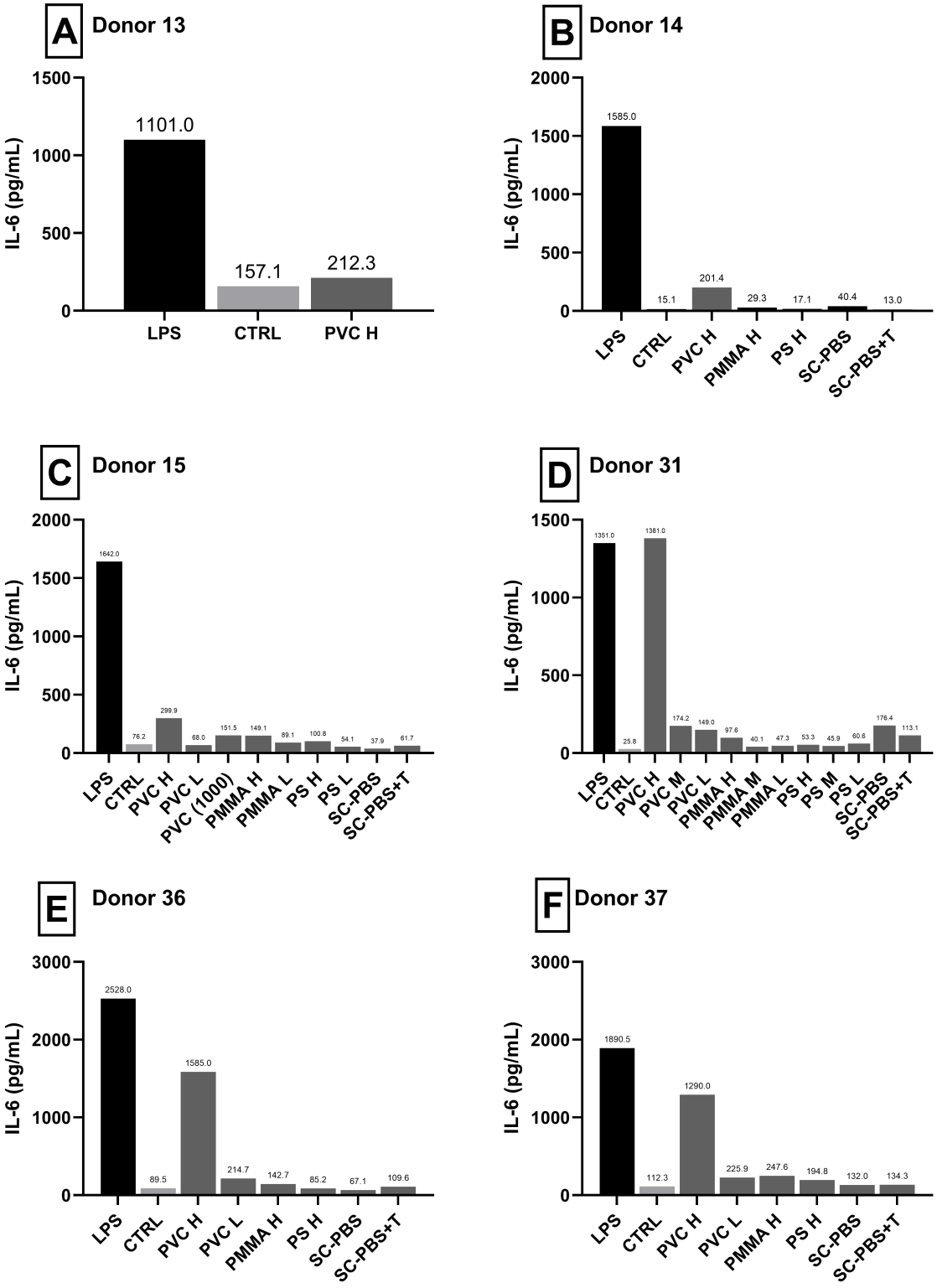


Figure D.6: The figure shows the IL-6 cytokine level for donors (a) 13 (b) 14 (c) 15 (d) 31 (e) 36 (f) 37. LPS = Positive control, CTRL = negative control, SC-PBS = solvent control PBS, SC-PBT+T = Solvent control PBT+Tween20.

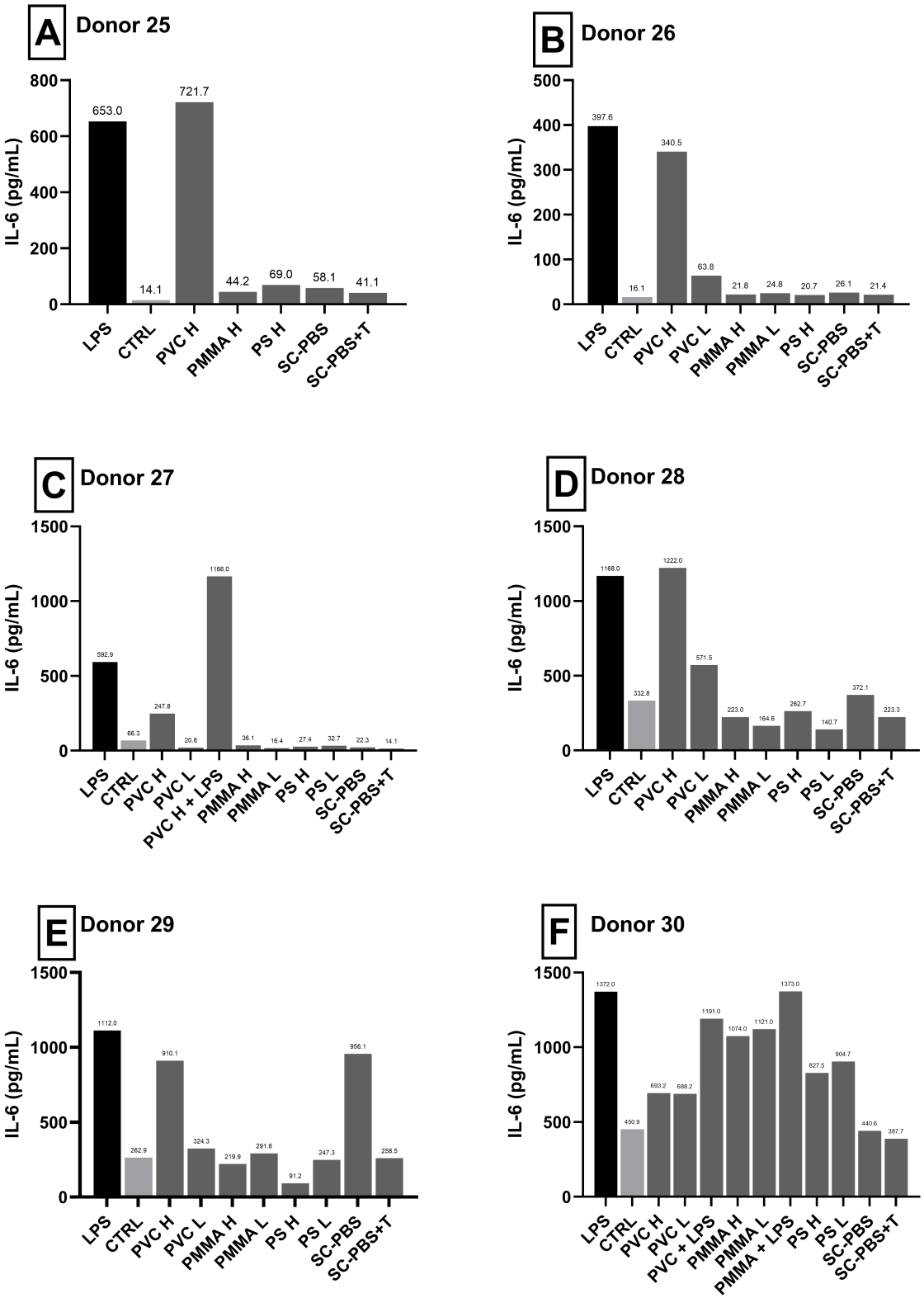


Figure D.7: The figure shows the IL-6 cytokine level for donors (a) 25 (b) 26 (c) 27 (d) 28 (e) 29 (f) 30. LPS = Positive control, CTRL = negative control, SC-PBS = solvent control PBS, SC-PBT+T = Solvent control PBT+Tween20.

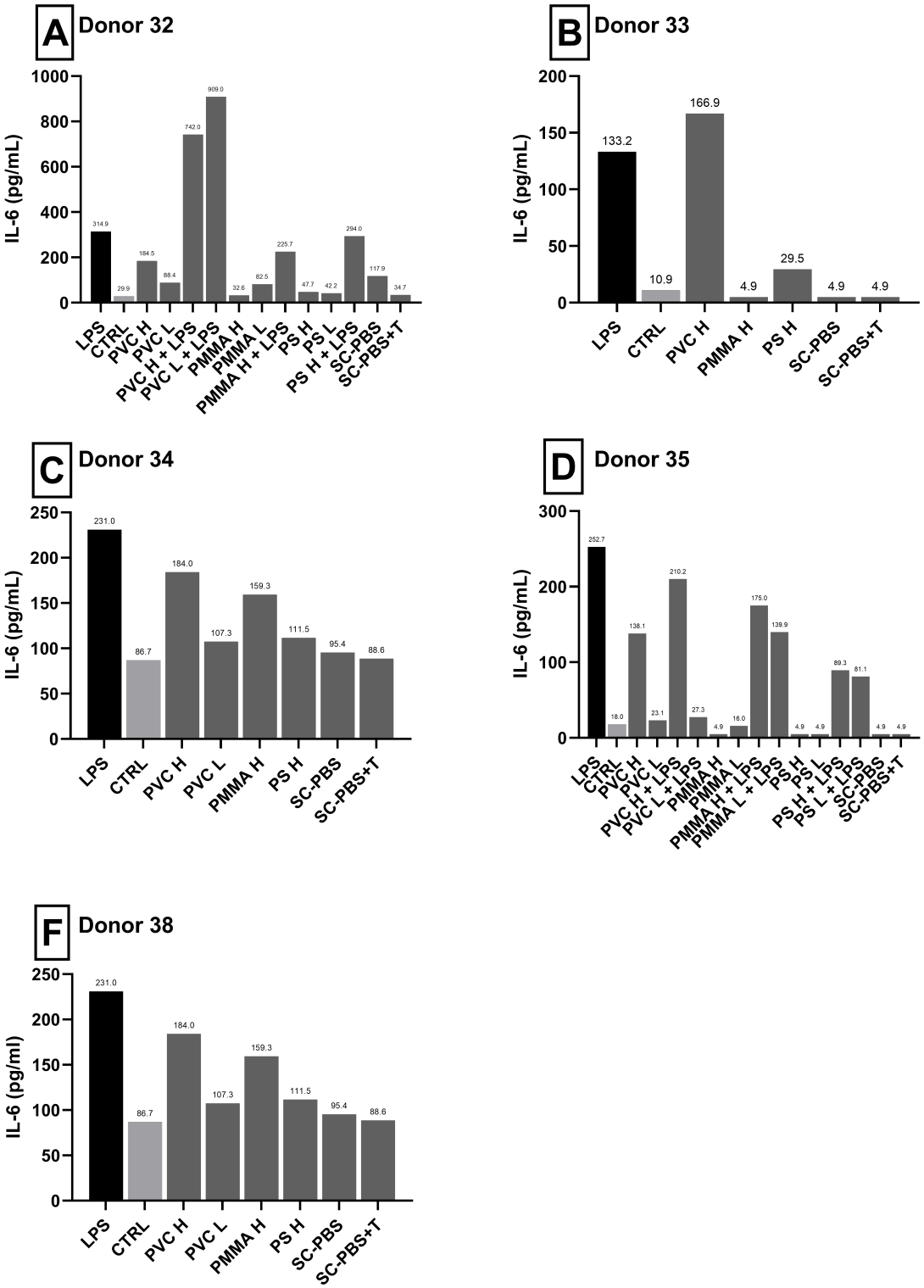


Figure D.8: The figure shows the IL-6 cytokine level for donors (a) 32 (b) 33 (c) 34 (d) 35 (e) 38. LPS = Positive control, CTRL = negative control, SC-PBS = solvent control PBS, SC-PBT+T = Solvent control PBT+Tween20.

E | Additional data - Cytotoxic effects of aged MNPs

There were used aged plastics from the master thesis of Harini Pechiappan for comparative analysis of the plastics that had been kept in solvents for over a year to the newly created plastics [47]. For the aged plastics, neither of the aged MNPs induced cytotoxicity after 24h nor 72h exposure. The experiment revealed a large variability between biological replicates, as cell had inconsistent reduction in viability in relation to the same plastic concentrations. Due to the high variability none of the plastic doses could be considered significant, despite a mean reduction of 22.7% and 25.2% in cell exposed for 72h with PMMA and PVC, respectively (Figure E.1). As particles are known to agglomerate and therefore become larger in size over time, the variability observed between biological replicates could be explained by these plastics kept for over a year and having agglomerated.

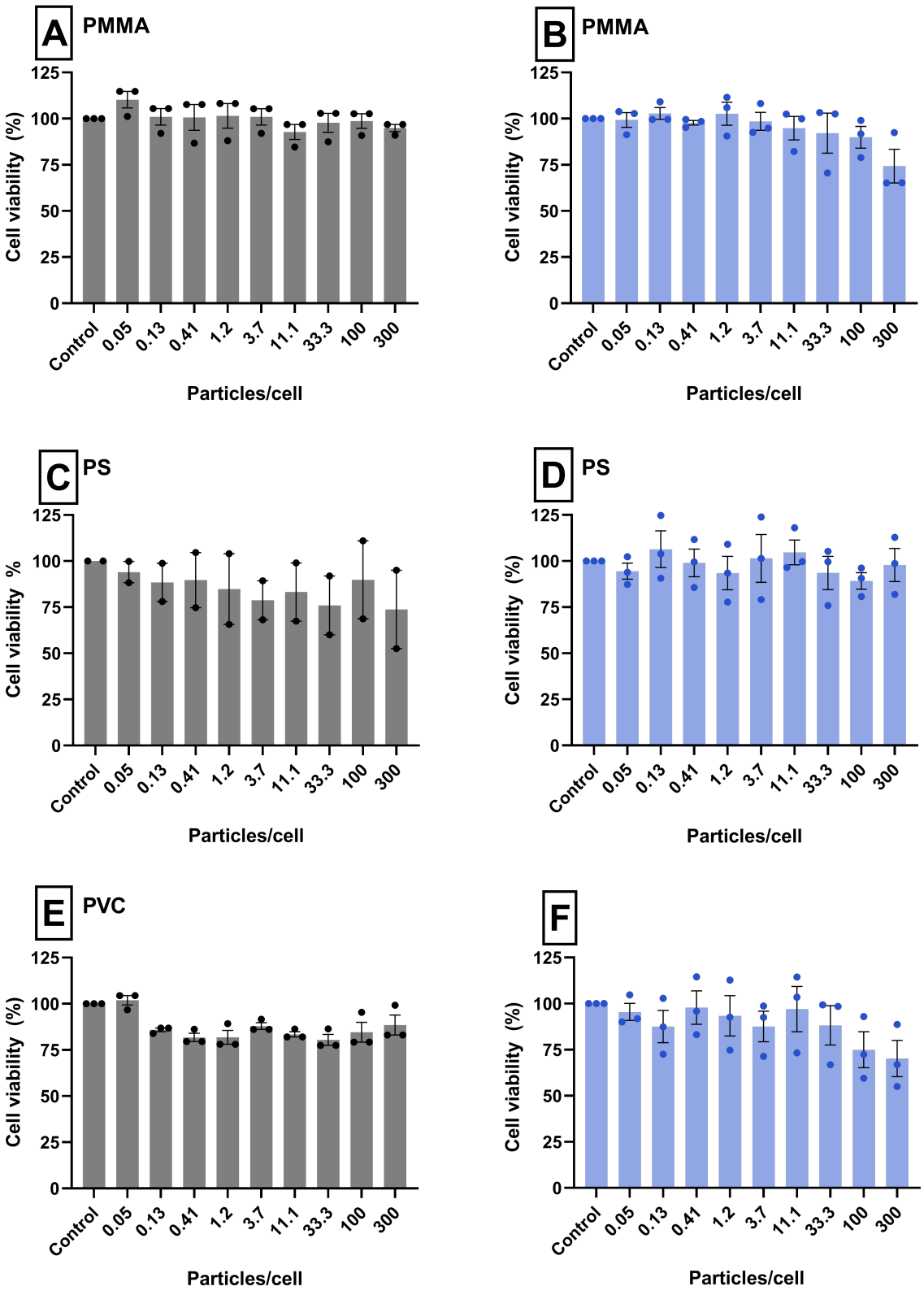
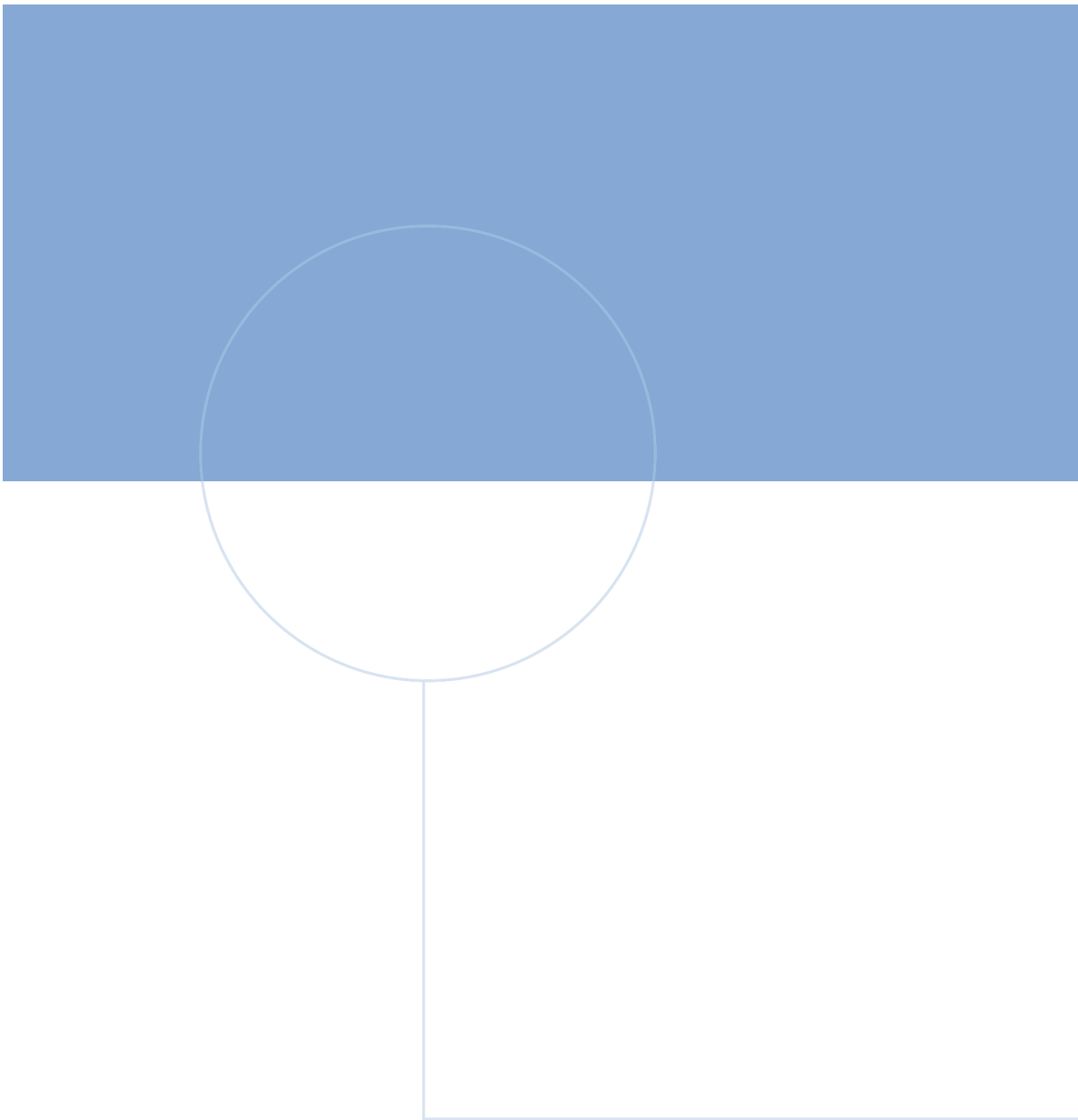


Figure E.1: Cytotoxic effects in the MM6 cell line after exposure to aged plastics The graph shows the percent of viability over the number of particles per cell. The control (no plastic treatment) was set to 100% viability. The graphs show reduction in cell viability after (a, c, and e) 24h exposure and (b, e, and f) 72h exposure. (a-b) displays PMMA (c-d) PS and (e-f) shows PVC. Data shown in % viability are the mean ± SEM of (a-b, d-f) 3 technical replicates from 3 independent experiments (n=3), (c) 2 independent experiments.



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