	Inventaris Wob-verzoek W23-03									
		wordt verstrekt				weigeringsgronden				
nr.	document NTS 202216420	reeds openbaar	niet	geheel	deels	5.1, lid 1c	5.1, lid 2e	5.1, lid 2f	5.1, lid 2h	5.2, lid 1
1	Aanvraag projectvergunning, d.d. 12-09-2022				x		x		x	
2	Projectvoorstel bij aanvraag				x	x	x	x	x	
3	Bijlage dierproeven_1 bij aanvraag				x	x	x	x	x	
4	NTS bij de aanvraag			x						
5	E-mail aan DEC om advies aanvraag projectvergunning, d.d. 23-9-2022				x		x		x	
6	Brief DEC aan CCD over aanvraag, d.d. 03-10-2022				x	x			x	
7	E-mail CCD aan DEC over aanvraag, d.d. 04-10-2022				x		x		x	
8	E-mail CCD aan vergunninghouder over aanvraag, d.d. 04-10-2022				x		x		x	
9	Aanpassingen nav DEC commentaar				x	x	x		x	
10	Projectvoorstel na niet toetsbare aanvraag				x	x	x	x	x	
11	Bijlage na niet toetsbare aanvraag				x	x	x	x	x	
12	Nieuwe NTS na niet toetsbare aanvraag			x						
13	DEC advies				x	x			x	
14	Projectvoorstel na DEC advies				x	x	x	x	x	
15	Bijlage dierproeven na DEC advies				x	x	x	x	x	
16	NTS na DEC advies			x						
17	AdviesNotaCCD, d.d. 26-04-2023_met opmerkingen				x		x		x	x
18	AdviesNotaCCD, d.d. 28-04-2023				x		x		x	x
19	E-mail CCD aan vergunninghouder over aanvraag, d.d. 28-4-2023				x		x		x	
20	Brief tbv antwoorden_na aanhouden 1, d.d. 12-05-2023				x		x		x	
21	Aanvraag na aanhouden 1				x		x		x	
22	Projectvoorstel na aanhouden 1				x	x	x	x	x	
23	AdviesNotaCCD, d.d. 12-05-2023				x		x		x	x
24	Beschikking concept, d.d. 12-05-2023				x		x		x	
25	Beschikking definitief, d.d. 15-05-2023				x		x		x	
26	E-mail CCD aan DEC, terugkoppeling over advies, d.d. 16-5-2023				x		x		x	

Centrale Commissie Dierproeven

1

Aanvraag
Projectvergunning Dierproeven
Administratieve gegevens

- U bent van plan om één of meerdere dierproeven uit te voeren.
- Met dit formulier vraagt u een vergunning aan voor het project dat u wilt uitvoeren. Of u geeft aan wat u in het vergunde project wilt wijzigen.
- Meer informatie over de voorwaarden vindt u op de website www.centralecommissiedierproeven.nl. of in de toelichting op de website.
- Of neem telefonisch contact op. (0900-2800028).

1 Gegevens aanvrager

1.1 Heeft u een deelnemernummer van de NVWA?
Neem voor meer informatie over het verkrijgen van een deelnemernummer contact op met de NVWA.

☒ Ja > *Vul uw deelnemernummer in*	5.1 lid2h
☐ Nee > *U kunt geen aanvraag doen*	

1.2 Wat voor aanvraag doet u?

☒ Nieuwe aanvraag > Ga verder met vraag 1.3

☐ Wijziging > Vul hiernaast het AVD nummer van uw vergunde project in en ga verder met vraag 2.1

☐ Melding > Vul hiernaast het AVD nummer van uw vergunde project in en ga verder met vraag 2.2

1.3 Vul de gegevens in van de instellingsvergunninghouder die de projectvergunning aanvraagt.

Naam instelling of organisatie	5.1 lid2h			
Titel, voorletters en achternaam van de portefeuillehouder	Titel 5.1 lid2e	Voorletters 5.1 lid2e	Achternaam 5.1 lid2e	☒ Dhr. ☐ Mw
E-mailadres contactpersoon	5.1 lid2h			
Titel, voorletters en achternaam van de diens gemachtigde (indien van toepassing)	Titel	Voorletters	Achternaam	☐ Dhr. ☐ Mw
E-mailadres gemachtigde				

Vul de gegevens van het postadres in.

Straat en huisnummer	5.1 lid2h
Postcode en plaats	
Postbus, postcode en plaats	

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	5.1 lid2e	☐ Dhr. ☒ Mw.
Functie	5.1 lid2e	
Afdeling	5.1 lid2h	
Telefoonnummer	5.1 lid2e	

	E-mailadres	5.1 lid2e
1.5 *(Indien van toepassing)* Vul hier de gegevens in van de plaatsvervangende verantwoordelijke onderzoeker.	(Titel) Naam en voorletters	☐ Dhr. ☐ Mw.
	Functie	
	Afdeling	
	Telefoonnummer	
	E-mailadres	
1.6 *(Indien van toepassing)* Vul hier de gegevens in van de persoon aan wie de portefeuillehouder de verantwoordelijkheid inzake de algemene uitvoering van het project en de overeenstemming daarvan met de projectvergunning heeft gedelegeerd.	(Titel) Naam en voorletters	☐ Dhr. ☐ Mw.
	Functie	
	Afdeling	
	Telefoonnummer	
	E-mailadres	
1.7 *(Optioneel)* Vul hier de gegevens in van de Instantie voor Dierenwelzijn	Telefoonnummer	5.1 lid2h
	E-mailadres	5.1 lid2h
1.8 Is er voor deze projectaanvraag een gemachtigde?	☐ Ja > *Stuur dan het ingevulde formulier Melding Machtiging mee met deze aanvraag*	
	☒ Nee	

2 Over uw aanvraag

2.1 Gaat uw aanvraag over een *wijziging* op een vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn?

☒ Nee > Ga verder met vraag 3

☐ Ja > Geef hier onder kort de wijziging en de onderbouwing daarvan weer. Geef in de originele formulieren (niet-technische samenvatting, projectvoorstel en bijlage dierproeven) duidelijk aan (bij voorbeeld in een andere kleur) waar de projectaanvraag wijzigt. Ga daarna verder met vraag 6.

2.2 Gaat uw aanvraag over een *melding* op een vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn?

☒ Nee > Ga verder met vraag 3

☐ Ja > Geef hier onder weer wat deze melding inhoudt en ga verder met vraag 6

3 Over uw project

3.1 Wat is de geplande start- en einddatum van het project?	Startdatum	1 - 10 - 2022
	Einddatum (t/m)	30 - 9 - 2027
3.2 Wat is de titel van het project?	Immunological health effects of microplastics	
3.3 Wat is de titel van de niet-technische samenvatting?	Schadelijkheid van microplastics	
3.4 Wat is de naam van de Dierexperimentencommissie (DEC) van voorkeur?	Naam DEC	5.1 lid2h
	Postadres	
	E-mailadres	

4 Factuurgegevens

4.1 (indien factuuradres afwijkt van de gegevens uit vraag 1.3) Vul de gegevens van het factuuradres in.

5.1 lid2h

4.2 (optioneel) Vul hier het ordernummer van de instelling in.

Ordernummer:
5.1 lid2h

5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht

☒ Projectvoorstel   Aantal bijlage(n) dierproeven 1

☒ Niet-technische samenvatting

Overige bijlagen, indien van toepassing

☐ Melding Machtiging

☐

6 Ondertekening

6.1 Print het formulier uit, onderteken het en stuur het inclusief bijlagen via de beveiligde e-mailverbinding naar de CCD en per post naar de Centrale Commissie Dierproeven (voor adresgegevens zie website)

Ondertekening door de portefeuillehouder namens de instellingsvergunninghouder of gemachtigde (zie 1.8). De ondergetekende verklaart:

- dat het projectvoorstel is afgestemd met de Instantie voor Dierenwelzijn.
- dat de personen die verantwoordelijk zijn voor de opzet van het project en de dierproef, de personen die de dieren verzorgen en/of doden en de personen die de dierproeven verrichten voldoen aan de wettelijke eisen gesteld aan deskundigheid en bekwaamheid.
- dat de dieren worden gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van richtlijn 2010/63/EU, behalve in het voorkomende geval de in onderdeel C van de bijlage bij het bij de aanvraag gevoegde projectvoorstel gemotiveerde uitzonderingen.
- dat door het ondertekenen van dit formulier de verplichting wordt aangegaan de leges te betalen voor de behandeling van de aanvraag.
- dat het formulier volledig en naar waarheid is ingevuld.

Naam: 5.1 lid2e

Functie:

Plaats: 5.1 lid2h

Datum: 12 09 - 2022

Handtekening: 5.1 lid2e

Centrale Commissie Dierproeven

Form
Project proposal

- This form should be used to write the project proposal for animal procedures.
- The appendix 'description animal procedures' is an appendix to this form. For each type of animal procedure, a separate appendix 'description animal procedures' should be enclosed.
- For more information on the project proposal, see the Guidelines to the project licence application form for animal procedures on our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1 Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	5.1 lid2h
1.2 Provide the name of the licenced establishment.	5.1 lid2h
1.3 Provide the title of the project.	Immunological health effects of microplastics

2 Categories

2.1 Please tick each of the following boxes that applies to your project.

- ☒ Basic research
- ☐ Translational or applied research
- ☐ Regulatory use or routine production
- ☒ Research into environmental protection in the interest of human or animal
- ☐ Research aimed at preserving the species subjected to procedures
- ☐ Higher education or training
- ☐ Forensic enquiries
- ☐ Maintenance of colonies of genetically altered animals not used in other animal procedures

3 General description of the project

3.1 Background

Describe the project (motivation, background and context) with respect to the categories selected in 2.1.

Microplastics are everywhere in our environment, in our food, our water and in our air (see https://youtu.be/YOEwRkbqm4A). We are exposed to them in more sterile form from sources like cosmetics and food packaging, but plastics in the environment are an attractive substrate for pathogens like bacteria to grow on and viruses to stick to. This biofilm of bacteria and viruses on the plastics can harbor antibiotic resistant bacteria[1,2]. These microplastics that are invading our environment will pose new challenges to our

immune system and immune cells. Immune cells protect our bodies against foreign invaders such as bacteria, fungi, aberrant cells and foreign particles. Research done by others has shown the presence of microplastics in our bloodstream[3]. In the bloodstream these plastics will most likely interact with our immune cells, but we have no information yet as to how they interact. Whether humans are able to clear the plastics after they cross into our bloodstream, or whether they accumulate in our organs, in and between our cells over our lifetime. Whether the plastics that remain in us result in chronic or only acute inflammation, act like asbestos fibers and link to cancer. Whether they worsen allergies and asthma upon inhalation, or whether they are inert and easy to deal with for our immune cells. Whether plastics made of different chemical compounds have different effects on our system, and what are the size limitations of plastic for crossing different epithelial barriers. These are questions that unfortunately cannot all be resolved with *in vitro* systems.

As the most abundant white blood cell, the most likely first cells to interact with microplastics that entered our bloodstream are neutrophils. Neutrophils play an important role in further activating the more specialized immune cells upon infection or the presence of foreign bodies, which is of vital importance to our survival. Despite their fundamental role in our immunity, many aspects of neutrophil biology are not yet or not fully understood .

Through the years more and more research has come out showing that neutrophil activity is linked to the development of multiple human diseases, and several neutrophil subsets have been described that were previously unknown: hypersegmented neutrophils, low-density granulocytes, granulocytic myeloid-derived suppressor cells (G-MDSCs), and tumor associated neutrophils (TANs)[4-8].

Neutrophil functioning depends on a network of signals and interactions. This network contains everything from the bone marrow, to the spleen as a potential neutrophil reservoir, blood vessel endothelium, sites of inflammation, as well as neutrophil interactions with foreign invaders and other immune cells. Since it is difficult to obtain human bone marrow, the production site of neutrophils, it is difficult to answers questions about for example the difference between homeostasis and inflammation, or the timing of development of neutrophils in homeostasis, or the aforementioned subsets recruited upon inflammation. In fact, we don't even know whether the subsets are developed in the bone marrow to begin with. To fully answer these questions we need a better understanding of the entire network of interactions that direct neutrophil differentiation and activation. Unfortunately we are not yet able to replicate the complexity of all these signals in an *in vitro* setting.

Neutrophils' main mode of action is through phagocytosis of the foreign invader or cell debris, after which the content of their granules degrade the phagocytosed material. However, microplastics in our bloodstream are a foreign invader that can't be degraded after phagocytosis by neutrophils unlike their natural targets. What will a neutrophil that has engulfed an undegradable plastic do: will it travel back to the bone marrow where dying neutrophils are cleaned up, or will it die on the spot due to cellular stress caused by the plastic? For now the hypothesis is that either case leads to activation of other immune cells and macrophages will get involved. Especially if the neutrophils die from cellular stress, the presence of the microplastics will lead to an acute and maybe long term inflammation reaction. We also don't know yet whether specific neutrophil subsets arise due to the presence of the plastic in our bodies. Given how recent the finding is of microplastics in the blood, the effects of phagocytosed microplastics on neutrophils and overall long term health are completely unknown.

In this project we aim to work on filling in the knowledge gaps of neutrophil biology mentioned above (immediate goal A), and determine the health effects of microplastics by investigating microplastics dynamics in a mammalian system and the response of the immune system to the plastics (immediate goal B). Combining the information from healthy mice, mice treated with plastic and mice with acute inflammation such as bacterial infection in this research project will help us to put the immune systems' reaction to microplastics in the right perspective.

References

1. Qiang L., Cheng J., Mirzoyan S., Kerkhof L., Häggblom M. Characterization of Microplastic-Associated Biofilm Development along a Freshwater-Estuarine Gradient. Environ Sci Technol. 2021 Dec 21

2. Guo X., Sun X., Chen Y., Hou L., Liu M., Yang Y. Antibiotic resistance genes in biofilms on plastic wastes in an estuarine environment. Sci Total Environ. 2020 Nov 25

3. Leslie H., Van Velzen M., Brandsma S., Vethaak A., Garcia-Vallejo J., Lamoree M. Discovery and quantification of plastic particle pollution in human blood. Environ Int. (2022)

4. Maskrey, B. H., Megson, I. L., Whitfield, P. D. & Rossi, A. G. Mechanisms of resolution of inflammation: a focus on cardiovascular disease. Arterioscler. Thromb. Vasc. Biol. ***31****, 1001-1006 (2011).*
5. Moses, K. & Brandau, S. Human neutrophils: Their role in cancer and relation to myeloid-derived suppressor cells. Semin. Immunol. ***28****, 187-196 (2016).*
6. Kaplan, M. J. Role of neutrophils in systemic autoimmune diseases. Arthritis Res. Ther. ***15****, 219 (2013).*
5.1 lid2e, 5.1 lid2h

3.2 Purpose

3.2.1 Describe the project's immediate and ultimate goals. Describe to which extent achieving the project's immediate goal will contribute to achieving the ultimate goal.

- If applicable, describe all subobjectives

Together with other research groups throughout the Netherlands a government funded consortium called 5.1 lid1c was set up. 5.1 lid1c has the goal to determine the health risk of the different types of plastics we are exposed to daily, looking into the effects of different materials/polymer types, sizes and shapes. This goal together with our expertise and interest in the immune system, slightly focused towards neutrophils, gives us the ultimate goal of this proposal: determine the health risk of plastics exposure, based on the plastics' potential to cross epithelial linings, their dispersion throughout the body, the immuno-toxicological effects, and their persistence in the body (fig 1).

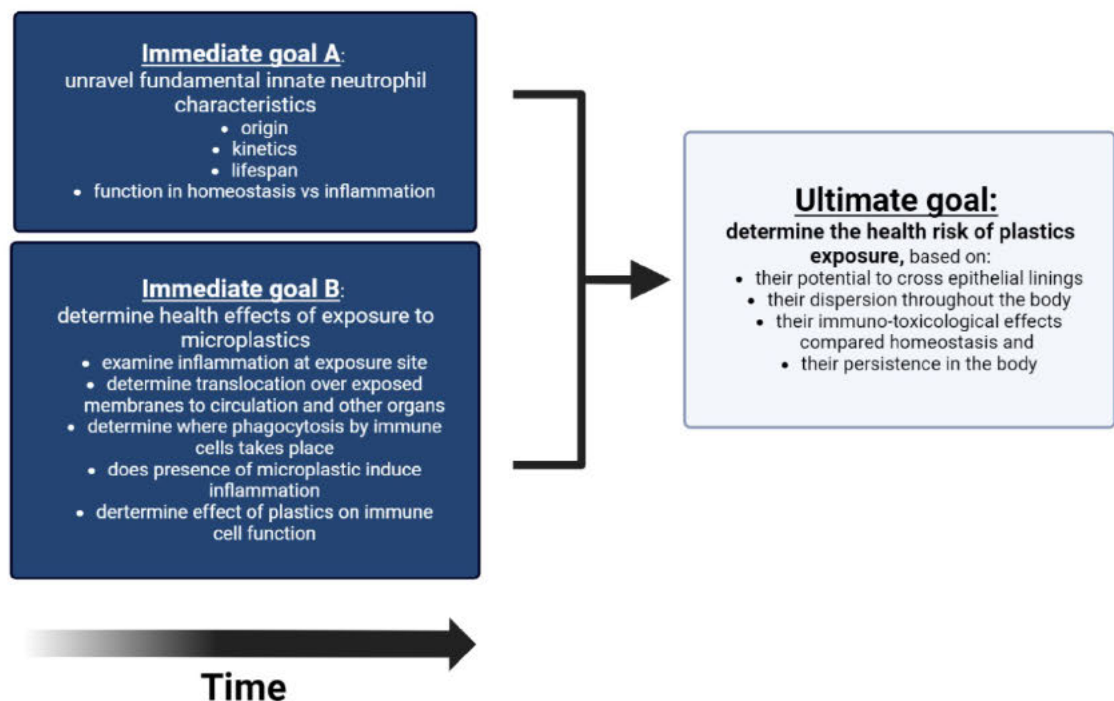

Figure 1. Schematic of the main and sub goals of this proposal. Sub goal A is a more fundamental question about neutrophil biology with specific aspects listed underneath it that we would like to address in this research. These basic aspects need to be understood to understand the full scope of the outcome of sub goal B. Sub goal B is a more translational question about the effects of plastics exposure with its relevant parameters that will be looked at underneath. The sub goals are not dependent on each other for the experiments that need to be performed. In most cases the two sub

goals can be worked on simultaneously, with experimental conditions for sub goal A also functioning as controls in experiments for sub goal B. (Created with Biorender.com)

In order to reach our main goal, we subdivided it in two sub goals (fig1). The first immediate goal (A) is: unravel the fundamental innate neutrophil characteristics, namely their origin, kinetics, lifespan, and their functioning in homeostasis versus acute or chronic inflammation. Neutrophils are the most abundant phagocytic cell in our blood, and the first and fastest responder to invasions. We hypothesize that the neutrophils will be the first to respond to the presence of plastic in our body, followed by other immune cells to continue the immune reaction. However, as described before, many basic biological concepts of neutrophils are still partially or completely unknown, because human material is limited and mouse experiments haven't been done extensively enough yet (fig 2). Understanding neutrophil biology better and how neutrophils respond to natural ques like infection and inflammation gives us a frame to understand neutrophils reaction to microplastics. This will also help determine the health risks of microplastics.

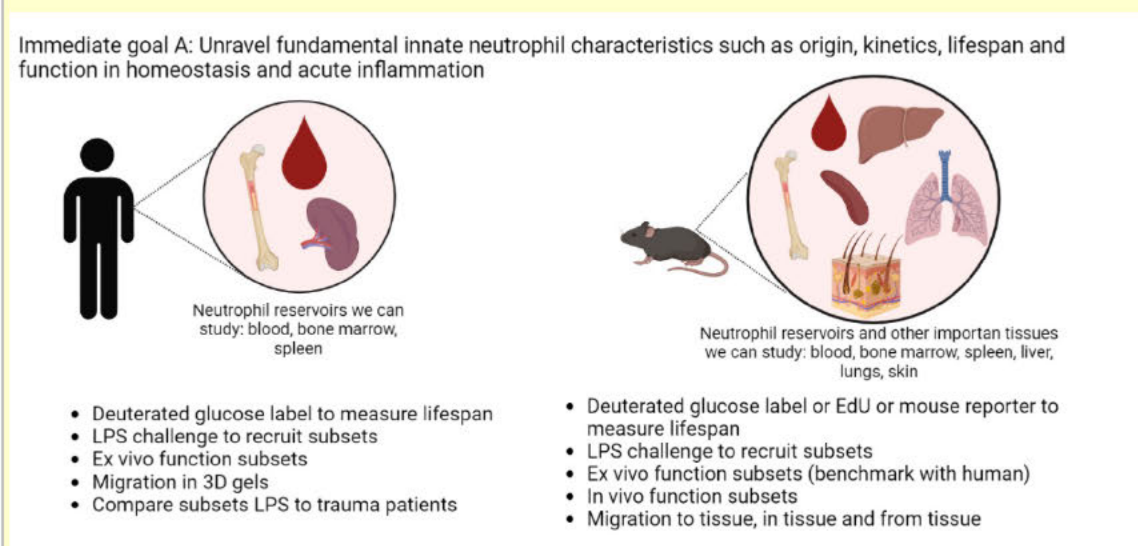

Figure 2. Schematic clarification of the experiments that can be or have been performed in human, and the experiments that can be or have been performed in mice. In mice we have easier access to all relevant tissues. These experiments will answer the questions posed for immediate goal A. (Created with Biorender.com)

Immediate goal A : Unravel fundamental innate neutrophil characteristics such as origin, kinetics, lifespan and function in homeostasis and acute inflammation

Sub questions for this immediate goal:

1. Determine the lifespan of neutrophil subsets by labeling the neutrophils with a label that shows their time spent in the body (e.g. deuterated glucose or EdU) and whether the production and lifespan changes upon an acute inflammation (induced by LPS).
2. Determine the origin of neutrophil subsets (local at the site of action, or distant like bone marrow or spleen) by i) harvesting neutrophil subsets from different organs during homeostasis or after an acute inflammation (LPS or injected bacteria such as but not limited to staphylococcus, epidermidis and pseudomonas*); and by ii) visualizing neutrophil migration with intravital microscopy. Determine the kinetics of different neutrophil subsets throughout the body by i) labeling the neutrophils present in the blood (e.g. using a CD45 antibody or neutrophil subset specific antibody) and following in time (by intravital microscopy and ex vivo analysis) when they leave the blood vessels and enter the tissues; and ii) by recruiting neutrophils to a tissue via a local sterile injury and subsequently monitor if these revert back to the blood vessel upon a systemic infection evoked by LPS (terminal experiment).
3. Establish the function of different neutrophil subsets by analyzing the response to injected bacteria such as but not limited to staphylococcus, epidermidis and pseudomonas*

*We have extensively used staphylococcus in human neutrophil in vitro assays but we have also experienced mouse neutrophils hardly kill this pathogen. Epidermidis are more easily killed by mouse neutrophils.

Currently 5.1 lid1c is conducting experiments to establish which pathogens are mainly found on plastics. A first candidate is pseudomonas, but other relevant bacteria might be added in the future.

This immediate goal doesn't have specific go/no go's, because these are fundamental questions which have been around for a long time, that still need to be answered, and are not connected to each other in caring out the experiments. Of course if we are uncertain about any aspect, experiments will not be started.

Immediate goal B focusses on the microplastics: determine the health effects of exposure to different microplastics (fig.3). In this part we will investigate the dynamics and effects of plastics. Most questions concerning plastic have not been answered yet or answered only with machine made, perfectly round and smooth polystyrene particles that are coated with specific chemicals. These polystyrene particles do not resemble the plastics we are exposed to. The microparticles we are about to test are made from a selection of the most used plastics to resemble our daily exposure. These will need to be tested in mice to answer when, where and how they get into our system, and the effects they will have on our health. For this goal the go/no go questions are clear:

- Only plastic polymer types found to have an effect *in vitro* on human neutrophils will be tested *in vivo*.
- The same plastics will be tested by consortium partners on other cell types, so if the majority finds no effect, they will not be tested in mice.
- By performing the endothelial exposure & translocation experiments first with (polystyrene) particles of well characterized sizes, we can determine which sizes ofthe 5.1 lid1c particles can be excluded, because only particles that cross the barrier are physiologically relevant for answering the other questions.

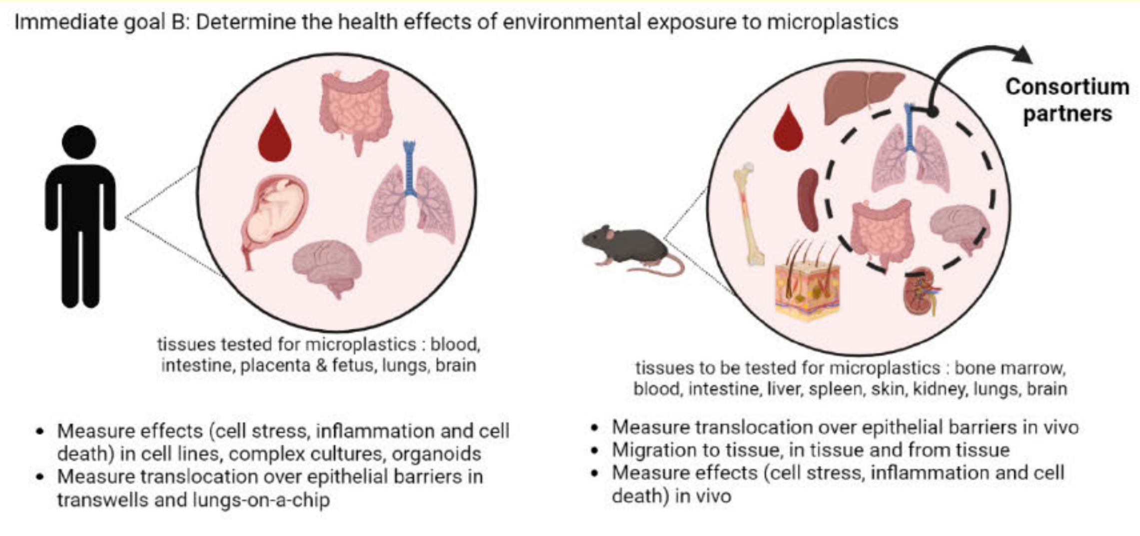

Figure 3. *Schematic clarification of the experiments that can and are performed in human, and the experiments we have been performing and intent to perform in mice. These experiments will answer the questions posed for immediate goal b. (Created with Biorender.com)*

Immediate goal B: Determine the health effects of environmental exposure to microplastics
Subquestions

1. Establish whether oral administration or inhalation of microplastics results in inflammation at the site of most-likely epithelial transfer (gut for oral, lung for inhalation)
2. Determine whether oral administration or inhalation of microplastics results in translocation to the circulation and to other organs like liver, spleen and kidneys
    a. Trafficking between organs in time
    b. Role of immune cells in transporting microplastics throughout the body
3. Determine if the presence of microplastics in blood or tissues induces inflammation

4. Determine if phagocytosis (engulfment of microplastic particles by immune cells) occurs in the bloodstream and/or in tissues
5. Determine the effect of (micro)plastics on immune cell function on:
    a. Survival
    b. Migration
    c. Bacterial killing of for example staphylococcus or pseudomonas family members
6. Compare the effects of microplastics to other inflammatory stimuli, in order to place the effect in the right perspective

Although the answers from immediate goal A will contribute to the answers of immediate goal B, experiments for both can be performed simultaneously, and a potential lag in answering the questions of A does not necessarily inhibit answering the questions of B. The health risk of microplastics doesn't solely depend on their effect on neutrophils, even though they are high in number and quick to respond. The health risk is not only depending on our neutrophils findings, we will also gather data on the other phagocytic and primary immune cells. These outcomes will be discussed with experts that are part of the 5.1 lid1c consortium to make the complete picture of exposure and risk.
Our project is partly observational and any of the evidence collected, positive or negative, will answer open toxicological questions for which answers are as of yet not available. A potential negative result (in the presence of positive controls) will not undermine our project but rather provide the public, government and companies with (reassuring) experimental evidence that is currently lacking.

3.2.2 Provide a justification for the project's feasibility.

In healthy mice, mice treated with plastic and mice with acute inflammation, we will analyze the phenotype and function of the immune cells *ex vivo*, crucially supported by analysis of the kinetics of immune cells *in vivo*. *Ex vivo* analysis is aided by our longstanding experience with flow cytometry and assays on neutrophil function (migration, phagocytosis, ROS formation, degranulation, etc.). In the kinetic studies we will examine the distribution of immune cells by *ex vivo* analysis of the neutrophils in different organs, as well as the migration of neutrophils by intravital imaging. In these intravital imaging experiments, neutrophil migration is easily tracked in mice that produce fluorescent neutrophils such as the LysM-GFP or the Catchup[IVM] mouse [9, 10].
Until now we and others in the microplastic field have used polystyrene perfect spheres of an exact size bought from a company. During this fabrication process a coating is formed on the plastic spheres that is not present on plastic we are exposed to daily. Currently we are testing the more environmentally relevant microplastics provided by our consortium partners *in vitro* with human immune cells. Real life plastics of 3 different plastic materials were milled to better reflect microplastics in the environment. Disadvantages are that the size ranges are broad and we don't have a lot of material compared to the fabricated polystyrene particles.
5.1 lid2f
These data demonstrate we have the experimental expertise to perform the project and also urge the research proposed in this project.
There are several other reasons why we are confident that we can achieve our aims: Our group is embedded in the 5.1 lid1c
Since the clinic is very close and our medical PhD students closely collaborate with clinical doctors, we can obtain patient material for research. The complementary use of patient material and well-defined animal models will ensure the successful completion of this project. 5.1 lid1c provides core facilities for various high-end techniques such as histology, fluorescent confocal imaging, intravital imaging and flow cytometry. Moreover, the animal facility offers dedicated staff providing the regular housing of the animals and support and counsels the scientist in their experiments. Within our group, only trained and experienced people perform experiments.
Our research and experiments are constantly evaluated within our group, and by various other groups within our institute and campus. To aid our research on microplastics, we collaborate with experts from different fields in the 5.1 lid1c and 5.1 lid1c consortia. Over the last few years, we have built up a repertoire of state-of-the-art *in vivo* imaging techniques to study immune cells in living mice. This has led to many new discoveries and breakthroughs published in scientific journals[11-17]. Our research is funded by major funding agencies. Our embedding in an excellent scientific environment, our unique techniques and approaches, and

our previous achievements make it very likely that with the experiments described in this project we will make large contributions to our main research questions.

References
9. Hasenberg, A. et al. Catchup: a mouse model for imaging-based tracking and modulation of neutrophil granulocytes. Nat. Methods ***12****, 445-452 (2015).*
10. Peters, N. C. et al. In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. Science ***321****, 970-974 (2008).*

5.1 lid2e, 5.1 lid2h

3.2.3 Are, for conducting this project, other laws and regulations applicable that may affect the welfare of the animals and/or the feasibility of the project?

☒No

☐Yes > Describe which laws and regulations apply en describe the effects on the welfare of the animals and the feasibility of the project.

Click or tap here to enter text.

3.3 Relevance

3.3.1 What is the scientific and/or social relevance of the objectives described above?

Complete understanding of neutrophil biology (immediate goal A) can help us not only understand the reaction to microplastics (immediate goal B), but also understand the neutrophil reaction to infection and inflammation, acute and chronic. This knowledge can help treat major trauma, cancer and asthma patients better. Understanding neutrophil biology will have both scientific and societal relevance.

Understanding the bodies reaction to exposure of particles like plastic (immediate goal B) has both scientific and societal importance. Preliminary results of research done by others has shown the presence of microplastics in our own bloodstream. From the bloodstream they end up in our organs. It is unlikely that these foreign particles will be ignored by our immune cells. To date there is no hard evidence for hazards of microplastics to our health. Thus, we have no clear indication whether exposure needs to prevented. This multi discipline study can be used as a guide for future research on particle exposure, hopefully reducing the experiments necessary for future questions. The societal relevance lies in the problem that we are constantly exposed to particles of which we have no idea what it does to our health. Giving conclusive evidence whether plastic polymer type, size, shape or amount matters in the negative health effects makes it easier for government officials to install laws to limit this, like already has been done for smoking.
We are part of a Dutch consortium called 5.1 lid1c which was set up with aid of the government because it was recognized how concerningly little is known about the health effects of microplastics. Within this consortium, plastics found in our environment, food and water will be tested in/on all important tissues: the lungs, the gut, the placenta, the brain, and of course the immune system. If these plastics are detrimental to our health, regulations should be put in place in regards to the production, usage, and recycling and disposal of those plastics.
We therefore aim to characterize if microplastics can induce inflammation and if so what important characteristics are and at what dose.
In addition to lack of hazard data, citizens generally do not see the microplastic pollution that is caused by their use of plastics, nor are they aware of the negative environmental and health effects of microplastics.

The result is that there is little incentive for citizens to use plastic-free products or to limit plastic waste through their consumption practices. Knowledge on plastic health effects leads to awareness, and is an essential step to empower the public to make changes in their households, demand changes from industry, and demand action from political representatives.
Our work will also have impact on science performed in the industrial setting. We collaborate with the textile industry (Inditex) who study machines that capture fibers from textiles before they leave the factory, thereby preventing shedding during daily use by citizens. In addition, they can design and test textiles that shed fewer fibers to begin with. In addition we work with air filter companies who might reduce microplastic concentrations in the household.

3.3.2 Who are the project's stakeholders? Describe their specific interests.

Mice are the obvious first stakeholders. They are undergoing scientific experiments beyond their own control. Their interests are the three Rs: Replacement, Reduction and Refinement (further defined in "Description animal procedures").

For immediate goal A the stakeholders are patients who need acute care like trauma patients at risk of developing life-threatening infections, chronic patients with for example asthma who need immune system related therapy their whole life, and also cancer patients that have neutrophils involved in the progression of their disease.

For immediate goal B in principle the whole human population is a stakeholder. We are all exposed to microplastics. The environment as a whole can also be considered a stakeholder, because if we find effects on our health and the health of mice, obviously other animals will be affected. The industry producing plastics and plastic alternatives are stakeholders too. The findings in our research can lead to legislation, potentially banning the use of certain plastic types for certain products or altogether. We perform unbiased and independent research, and report our progress and results to ZonMW as well as publish in internationally acknowledged journals

We fully intend to capitalize on the networks that we have through multiple consortia as needed to achieve our scientific objectives and to amplify our societal impact. These can also be considered stakeholders. For example, we collaborate with the 5.1 lid1c . Achieving societal changes based on our research findings will require the engagement and inclusion of the communities in which we conduct our research, patient organizations, industry players, local and national governing bodies, and non-governmental organizations (NGO). The consortia we take part in have representation of these communities and parts of society.
Longfonds is very interested in the outcome of our study as microplastics enter our body through inhalation. Their network and their outreach activities will help to spread awareness about microplastics and their lobbying network will help to involve government officials in our fight against microplastics.
The **Plastic Soup Foundation** is an international organization that focuses entirely on reducing plastic pollution. Their involvement will help to ensure that our findings are shared widely with the general public (including school children), as well as via their vast network of industrial, political and scientific stakeholders. They are also looking into practical solutions.
We are currently trying to acquire funding for a collaboration with the citizen network, **Onzelucht.nl**. This network consists of thousands of households to measure outdoor air quality, providing raw data to 5.1 lid1c working together with local governments, in schools and with both rural and urban communities.
Additional regulatory organizations, like the Dutch ministry of Infrastructure and Water Management, Science Advice for Policy by European Academies (SAPEA) and the World Health Organization (WHO) will also be important to reach out to as we expand our networks and disseminate our findings.

3.4 Strategy

3.4.1 Provide an overview of the overall design of the project (strategy). If applicable, describe the different phases in the project, the coherence, the milestones, selection points and decision criteria.

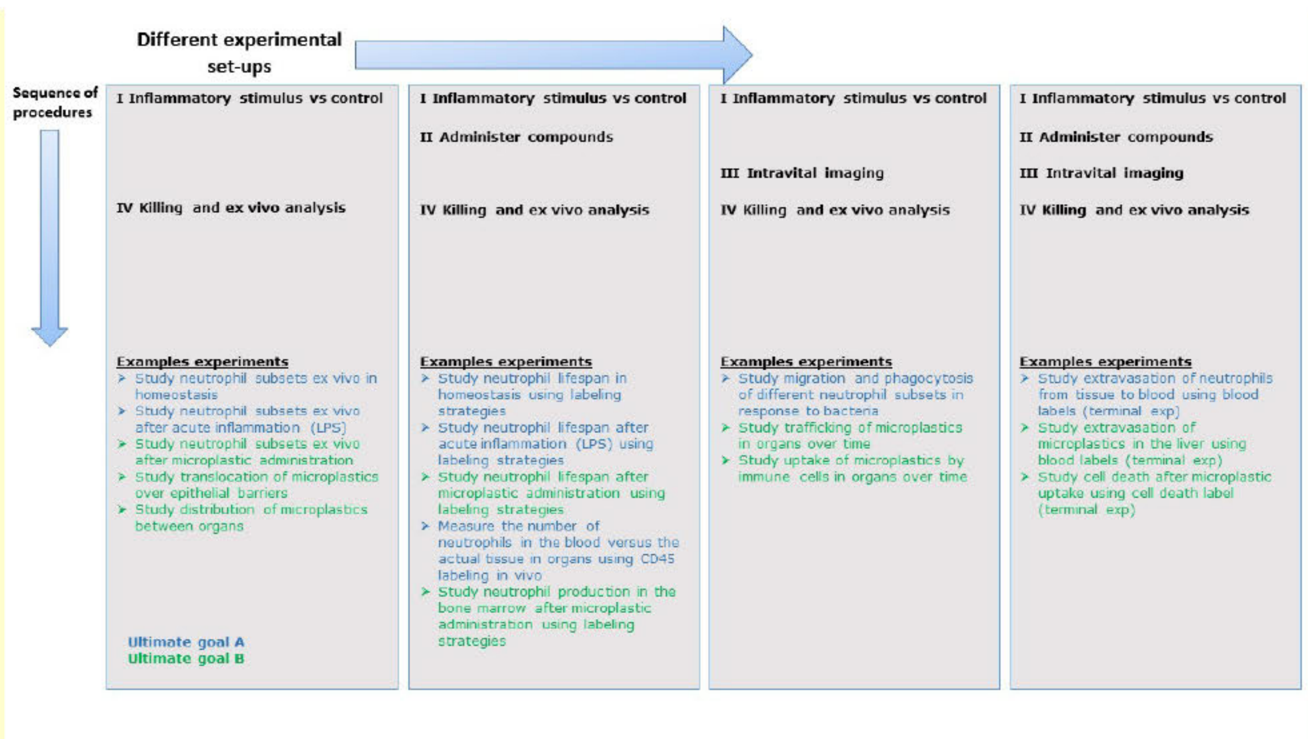

Figure 4. schematic overview of the general designs of the experiments that will answer our questions. They are ordered from left to right in level of complexity and intensity of the experiments. For step A the inflammatory stimulus can be either microplastics or administration-route relevant bacteria, or both in the same experiment. Under step B falls the administration of compounds like a CD45+ antibody or other labels. Step C of intravital imaging will be done for only a select number of experiments, due to the labour intensity on our end and the level of discomfort for the mice. These four different designs of experiments can be performed together in time but in different experiments. This also shows how the two immediate goals can be worked on in the same experimental set-up without interfering with each other.

The strategy of this project is to combine human *in vitro* experiments with murine *ex vivo* and *in vivo* experiments. The readout of each animal experiment will consist of some or all of the following:

- descriptive data on single cell level (flow cytometry, microscopy, protein expression, etc.)
- functional data (on specific functions of the neutrophil such as migration, phagocytosis, etc)
- intravital microscopy data
- descriptive data on disease progression (e.g. weight loss, microbial load).

The experimental set-ups and readout parameters will be the same for all experiments in this proposal (fig. 4), but the immune stimulating action or compound will differ per experiment (fig.5). Naturally, the different types of plastic particles will need to be tested by itself or in combination with another action or compound, as stated below. But also plastics with biofilm as explained earlier will need to be tested, because that is the status of plastics we would find in the environment.
One hypothesis is that characteristics like the surface texture, shape, and chemicals bound to the surface of the plastics will determine the response of our system to the plastics. As a control for these characteristics we will take along an equally non-degradable particle that is of a different material than plastic.
The act of injection makes a wound, which in and of itself is an activating signal for neutrophils. Therefore we need to take a sterile injection along as a control for injection experiments.

Immune stimulating action/compound	By itself or in combination	What will be determined:
Plastic particles	By itself (mainly)	- Effect on inflammation - Translocation - Distribution - Effect on immune cell function
	Bacterial infection	- Check if phagocytosis of microplastics prevents phagocytosis of bacteria (terminal exp)
	Sterile injury	- Establish if immobilized immune cells that have engulfed microplastics can be prompted to mobilize and distribute plastics upon local inflammation (terminal experiment)
	LPS injection	- Establish if immobilized immune cells that have engulfed microplastics can be prompted to mobilize and distribute plastics upon systemic inflammation (terminal experiment)
Plastic particles with biofilm	By itself (only)	- Establish whether a defective response to bacteria on plastic contributes to infections found in patients in vivo
Bacterial infection	By itself	- Compare the effects of microplastics to other inflammatory stimuli to place the effect in the right perspective - Unravel fundamental innate neutrophil characteristics such as origin, kinetics, lifespan and function in during acute inflammation
	Plastic particles	- (see above)
Non-plastic inert materials (injected particle reference)	By itself (mainly)	- Effect on inflammation - Translocation - Distribution - Effect on immune cell function
	Bacterial infection	- Check if phagocytosis of inert material prevents phagocytosis of bacteria (terminal experiment)
	Sterile injury	- Establish if immobilized immune cells that have engulfed inert material can be prompted to mobilize and distribute plastics upon local inflammation (terminal experiment)
	LPS injection	- Establish if immobilized immune cells that have engulfed inert material can be prompted to mobilize and distribute plastics upon inflammation (terminal experiment)
Sterile injury	By itself	- Compare the effects of microplastics to other inflammatory stimuli to place the effect in the right perspective - Unravel fundamental innate neutrophil characteristics such as origin, kinetics, lifespan and function in during acute inflammation
	Plastic particles	- (see above)
LPS injection	By itself	- Compare the effects of microplastics to other inflammatory stimuli to place the effect in the right perspective - Unravel fundamental innate neutrophil characteristics such as origin, kinetics, lifespan and function in during acute inflammation
	Plastic particles	- (see above)

Figure 5. Table giving an overview of the intended action to induce an immune response, whether other controls will/need to be taken along, and what will be determined from the experiment.

As already explained above, for most experiments we first consider *ex vivo* experiments (mild discomfort) and terminal experiments, before we consider repetitive intravital imaging experiments (moderate discomfort). In some experiments we first consider repetitive intravital imaging, either because some questions can only be answered by imaging the same tissue over multiple imaging sessions, or because it significantly reduces the number of required mice. After completion of the intravital imaging experiments, cells, tissues and organs will be isolated and analyzed to reduce the number of mice required. We have done intravital imaging in the past and have the set-up for most experiments already up and running.

3.4.2 Provide a justification for the strategy described above.

The set-up might seem a bit broad, but the research into the effects of microplastics is so new that there is little previous data or other work to base a more fine-tuned approach on. The methods for obtaining the readout parameters will be the same for all experiments in this proposal. We think that being able to compare the effects of microplastics to several inflammatory stimuli is the strength of this project. Supported by our own research on human material, pigs and mice, and the minimal amount of information we do have on the microplastics themselves and their effects, we have a number of hypotheses that we will start with. However, since our research is extremely novel, we cannot know whether these hypotheses will prove correct. In the next five years, human data will be combined with data from the animal experiments to adapt our hypotheses when necessary. Our data will be combined with data from our 5.1 lid1c colleagues,

population exposure data and exposure data from volunteers to help the setting up of plastics regulations by local and global government.

3.4.3 List the different types of animal procedures. Use a different appendix 'description animal procedures' for each type of animal procedure.

Serial number	Type of animal procedure
1	Microplastics exposure versus control
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9	Click or tap here to enter text.
10	Click or tap here to enter text.

Centrale Commissie Dierproeven

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information on the project proposal, see the Guidelines to the project licence application form for animal procedures on our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1 Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.

5.1 lid2h

1.2 Provide the name of the licenced establishment.

5.1 lid2h

1.3 List the serial number and type of animal procedure

Use the numbers provided at 3.4.3 of the project proposal.

Serial number	Type of animal procedure
1	Microplastic exposure versus controls

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

This project has two immediate goals that lead to the ultimate goal of determining the health risk of microplastics exposure. Immediate goal A aims to provide understanding of the aspects of neutrophils that we don't yet know, like the origin, timing, localization and function in certain situations. This helps understand the results of Immediate goal B, which is to investigate the effects of microplastics exposure on our health. In most experiments both immediate goals will be simultaneously addressed by comparing the response of immune cells to microplastics versus biological pathogens or sterile damage controls.

General design (see figure 4 of the project proposal):

I (every experiment)
- Administer plastics in different sizes (with and without different coatings) and compare these to controls, sterile damage or responses to biological pathogens

II (optional)
- Administer compounds to
  - Visualize our cells of interest
  - Measure cell death, proliferation and lifespan
  - Inhibit, stimulate, deplete or mimic components of the inflammatory reaction

III (optional)
- Intravital imaging

IV (every experiment)
- Killing of animal and perform ex vivo analysis

The readout of each animal experiment will consist of some or all of the following, most if not all performed postmortem (and therefor have no further impact on the quality of life):

- Descriptive data on single cell level (flow cytometry, microscopy, protein expression, etc.)
  Examples include: surface markers to distinguish different type of immune cells, activation markers on immune cells, proteins of different cell death mechanisms, measure the decrease of label over time to calculate lifespan.
- Functional data (on specific functions of the neutrophil such as migration, phagocytosis, etc)
  Examples include: *ex vivo* chemotaxis assay in 3D gels after phagocytosis of microplastics, *ex vivo* bacterial killing capacity of immune cells with and without microplastics, change in pH when plastics are engulfed by immune cells vs bacteria which are known to induce a lower pH.
- Intravital microscopy data
  Examples include: Are microplastic numbers in organs diminishing in time, is the location of microplastics in organs in time constant or changing, are microplastics engulfed by immune cells and how long are they present in the same immune cell.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

Here we will describe the general experimental set-up described above in more detail. Starting off with a schematic overview of the experimental design, details of the different parts of the experiment are explained in detail further down in this document.
First of we have a description of the experimental set-up used for the majority of the experiments (fig.1). These will consist of administration of compounds or a general immune stimulus. This administration will be in one of three ways per experiment: either IV injection, or oral administration, or intranasal administration. Mainly one compound will be administered (particle compared to control). In few specific cases a combination of maximum 2 compounds will be used (eg if we want to study whether the response to bacteria is altered in the presence of microplastics both need to be administered). All combinations are described in Fig 1 and 3. For oral and intranasal administration a pilot study will determine whether one administration is enough to find plastic back in the body of the mice, or whether more, but no more than 10, administrations are necessary to find the plastics back (we have previous data demonstrating plastics in the blood and liver after 10 days of oral exposure).
After the necessary administrations the mice will not need to be handled or undergo any procedures until near endpoint. For a few experiments, short before the endpoint a label will be administered via IV injection (details on labels described further down). Then at the endpoint, mice will be killed and organs harvested for analysis. The exact duration of the experiments is to be determined, but the amount of procedures the mice will need to undergo are minimal.

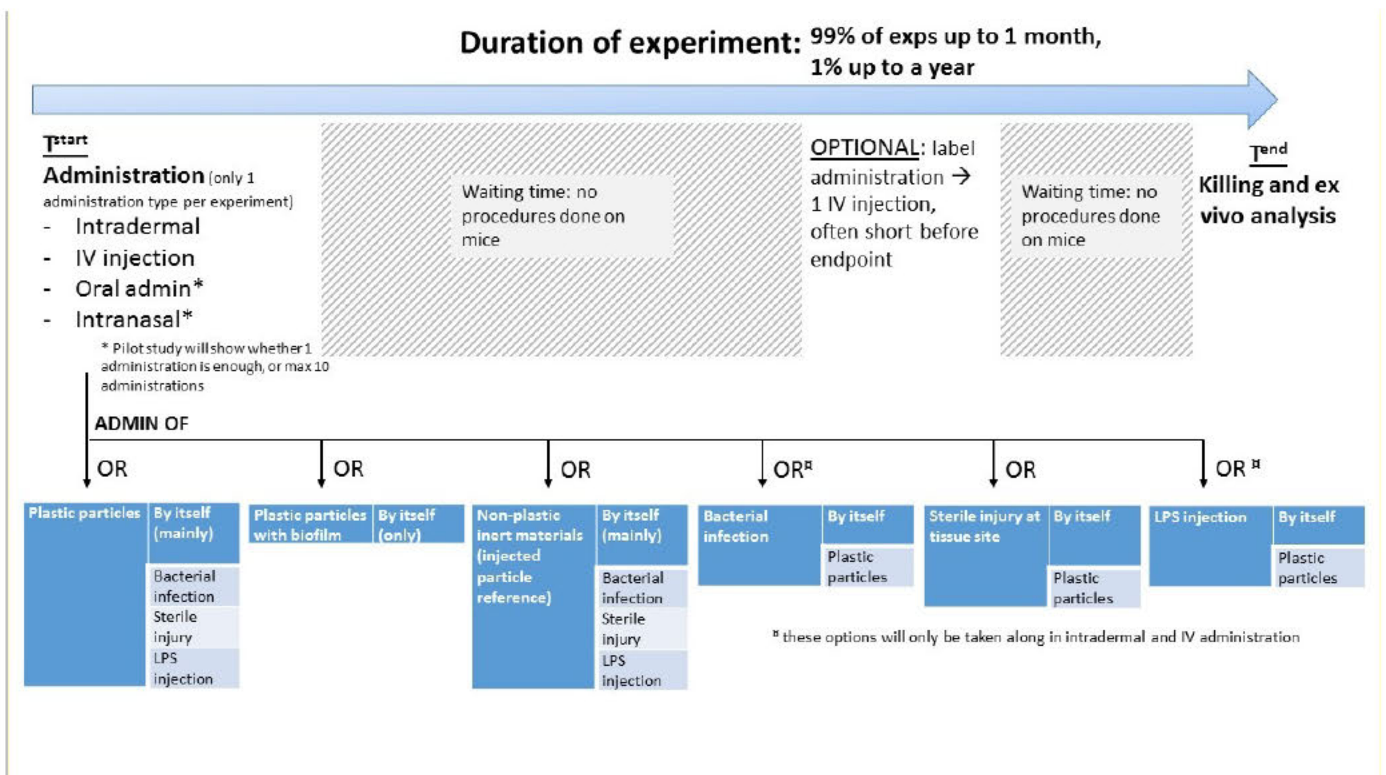

Figure 1. Schematic overview of the majority of the experiments. The duration is indicated at the top to show when certain procedures will take place during the experiment, starting on the left with the administration of the stimulus, the three different administration routes, the option to inject a label or not, and the killing of the animal at the end of the experiment. In-between these indicated procedures the mice will not undergo any procedures by us, represented by the gray blocks. Underneath the procedures are the options for combinations of controls and test material indicated. Only one these combinations will be administered per experiment. The three administration routes and the 5 combinations of material of stimuli lead to the majority of mice falling in this category of experiments.

A small part of the experiments will involve intravital microscopy. For these experiments the set-up is slightly different (fig.2). These experiments have two added options for administration: intradermal or subcutaneous. A big difference in these experiments compared to the other ones, is the repeated handling of the mice between administration and killing of the animals. Depending on the duration of the experiment, the mice will be brought at least two times to the microscope, anesthetized for at least an hour, allowed to recover, and brought back to the mouse house. Due to the intensity of these experiments, less experiments will be performed in this set-up, which results in less mice for these experiments.

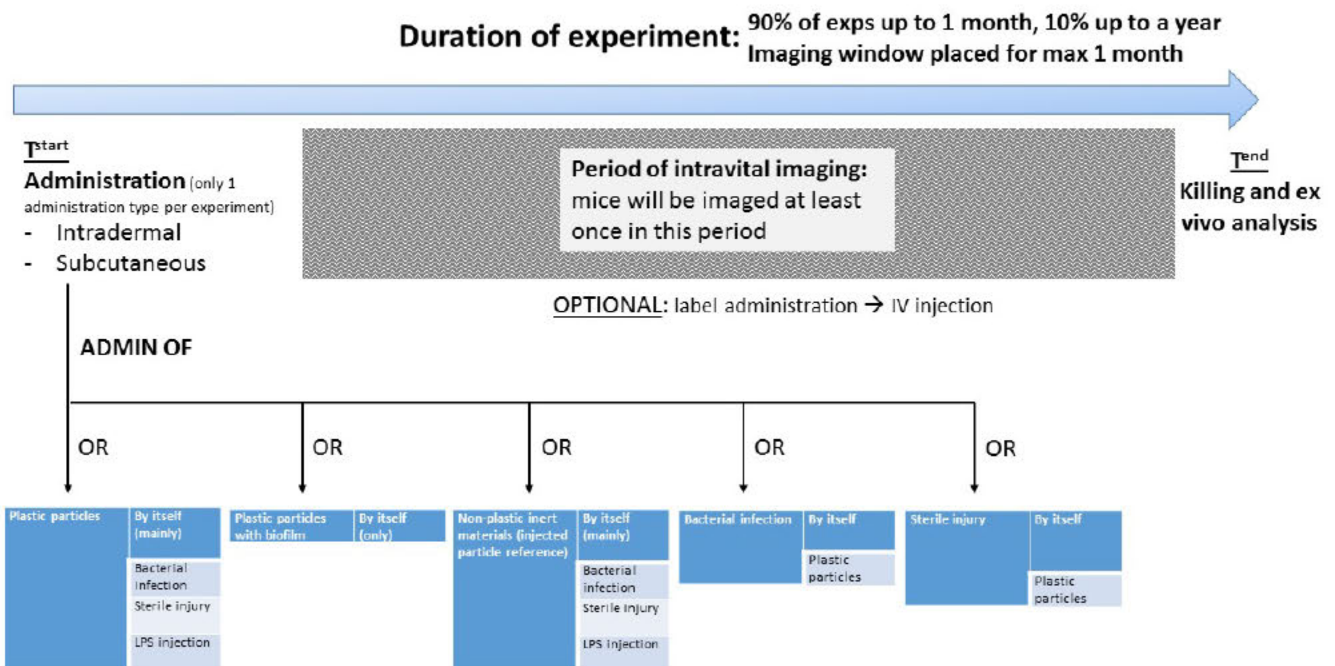

Figure 2. Schematic overview of the rest of the experiments. The duration is indicated at the top to show when certain procedures will take place during the experiment, starting on the left with the administration of the stimulus, the different administration routes, the option to inject a label or not, and the killing of the animal at the end of the experiment. In-between these indicated procedures the mice regularly be taken for anesthesia and intravital imaging, represented by the dark gray block. It will be experiment dependent how often the imaging will need to be done. Underneath the procedures are the options for combinations of controls and test material indicated. Only one of these combinations will be administered per experiment. Due to the intensity less experiments will be performed in this set-up.

The model

For these experiments we will use mice, since many fluorescent reporters exist, intravital imaging is optimized for this species, many required reagents to detect immune cells are readily available for this species. Wild type or intravital imaging (fluorescent reporter) strains or knockout strains will be used. Mice will never be kept alone in a cage.

The plastic polymers

The plastics that will be tested were decided on by the 5.1 lid1c consortium members. Real life plastics of 3 different plastic materials were milled to better reflect microplastics in the environment. Disadvantages are that the size ranges are broad and we don't have a lot of material compared to the fabricated polystyrene particles. The main plastic types selected are polypropylene (PP) which is used in medical supplies, polymethyl acrylate (PMA) which is used in some textiles, polyvinyl chloride (PVC), widely used in pipes. Nylon (synthetic textile fibers) might be added. These plastics are currently being tested in vitro first to determine if the different polymers have an effects on human neutrophils. Those that show no effect in vitro will not be tested in vivo, because particles that are not harmful to human cells don't need to be tested in mice. Also if multiple plastics give the same results in human neutrophils, it will not be necessary to test all of them in mice as well. One representative will suffice.

For some experiments like the translocation experiments fluorescent particles will be necessary to track them in the system. Commercially bought labeled polystyrene (PS) and polymethyl methacrylate (PMMA) will be compared to the same polymer particles labeled by our partners.

The particle sizes

The dogma has always been that 10um would already be too large a particle to be able to cross the barriers of the gut. However, our previous experiments showed that the 10um is able to cross the gut lining. At this moment we have no definitive answer as to what the size limit is for crossing barriers. Therefor multiple sizes will need to be tested per administration route to determine limits.
After this is determined, we can test only the relevant sizes in experiments with other administration routes like for example IV injections.

The coatings
Plastics are hardly ever in sterile form present, not in our environment, but also not in our body. In our environment the plastics pick up bacterial and viral components, which create a biofilm on the plastics. Bacterial coating/biofilm will be used for some experimental set-ups to mimic our exposure. 5.1 lid2f

In our bodies the plastics are exposed to many different solutions, many of which protein rich. We know from in vitro experiments that the commercially bought polystyrene particles and the particles made by 5.1 lid1c have a different effect on human neutrophils when the plastics have been incubated in human plasma or serum. We don't know yet whether substances like the acids in the stomach or the mucus in the gut or lungs have an effect on how our immune system responds to the plastics, whether they keep that coating as they cross epithelial barriers, of is it's easily exchanged. For most experiments we want to test uncoated plastics against plastics in relevant bodily fluids, and for some we want to test uncoated plastics against biofilm coated plastics. Only coatings relevant for that administration route will be used, and no unnecessary options will be considered. On average we compare 2-3 coatings per experiment.

I (every experiment)
Administer plastics in different sizes (with and without different coatings) and compare these to controls, sterile damage or responses to biological pathogens

Inflammatory stimulus: Plastic particles
Justification: We want to mimic different exposure routes in humans and determine the fate in the body. This condition is needed to reach answer immediate goal B. Until now we and others in the field have used polystyrene perfect spheres of an exact size bought from a company. During this fabrication process a coating is formed on the plastic spheres that is not present on normal every day plastic. In our previous experiments in mice and in our in vitro work with human immune cells we have found 1μm and 10μm have different effects on immune cell uptake and death. Both were translocated over the intestine.
Generally experiments in mice will first be performed with the commercial polystyrene particles with which we have a lot of experience. Selected experiments will be performed with the consortium plastics.
Description: In nature microplastics are present in many different sizes. Different sizes have different effects in our human in vitro studies and in our previous mouse studies. Therefore multiple sizes will be compared to fully understand the dynamics. We want to establish what the upper size limit is that is still translocated over the intestine and lung, therefore the size range is ≤100μm. However, the different sizes are all so small that they will cause no immediate extra response or discomfort for the mice.
Plastics are administered:

- Orally (gavage for controlled administration or via food, 1-10 days,≤200μm)
  Others have found microplastics accumulate to a plateau when orally administered for 10 days. We have confirmed before that 10 days of oral gavage leads to microplastics in the blood and in organs. We prefer to switch from oral gavage to food administration to reduce discomfort for the mice, but we need to perform comparative pilot studies before switching. During this pilot we will also determine if we can detect enough particles for our analysis at earlier time points. The aim is to administer via food and to perform the least number of administrations. Of note, microplastics can sediment or float, therefore administration via drinking water is not possible.
- Intravenously (retro-orbital under anesthesia or tail vein, once, ≤10μm to prevent clogging of veins)
  Microplastics have been detected in human blood. To mimic microplastic traffic in the blood we want to inject these microplastics directly. We have already performed experiments where we were able to detect microplastics in different organs up to day 30 after a single injection. Therefore a single injection has been proven sufficient.
- Intra-nasally (under anesthesia, 1-10 days, ≤200μm)
  These experiments have never been performed before. Based on the oral exposure data that is available we hypothesize 1-10 days of exposure might be necessary in order to detect the microplastics in organs. We will perform pilot experiments to determine the least number of exposures.
- Intra-dermally (under anesthesia, once, ≤100μm)

Our skin is exposed to microplastics from our clothing and we know very little about that way of exposure. The skin has resident immune cells that are capable of phagocytosing the microplastics, which might lead to further inflammation. Furthermore, the skin is easy accessible for injection and easy to image without any invasive procedures for the mice. Our experiments so far have shown that a single dose can still be detected 100 days later and is therefore sufficient.

- Subcutaneously (under anesthesia, once, ≤1cm).
  In healthcare many plastics products are used that stay long term or permanently in our bodies, like portal needles and catheters for IVs. These are large pieces of plastic, of which we don't know if or how the immune system responds to is. Especially if something goes wrong and it has bacteria on it. We collaborate with experts in bacteria where we tested a bacterial biofilm on a catheter of about 0.5cm. Placing these in the skin of the mice makes it easier to monitor the inflammation and infection, and we don't want to risk giving the mice sepsis, but keep the infection local.

For this last category also relatively big plastics will be used to mimic problems with catheters and prostheses.
Symptoms: Intradermal injections of microplastics, I.V. microplastics injection and oral microplastic administration are well tolerated and do not lead to noticeable symptoms. Intranasal administration of microplastics has not been described before, but are expected to be in line with the mild effects of the other administration routes. However, weight loss and behavioral changes as described in section J will be tightly monitored for the animals in this route. Subcutaneous administration will require a small surgical procedure with likely moderate discomfort as a result.
Level of discomfort: mild (oral via food, intranasal, I.V., intradermal) or moderate (oral gavage, intranasal when administered more than 2 times, subcutaneous)

Inflammatory stimulus: Plastic particles with biofilm
Justification: Plastics can acquire a biofilm formed by bacteria both in the environment as well as in vivo on e.g. catheters. We want to investigate the response of innate immune cells to this biofilm coated plastic compared to uncoated plastics. This condition is needed to answer subquestion 5 of immediate goal B. The coating of choice is a fluorescent S aureus with which we and our microbiology department has a lot of experience. Consortium partners are establishing which bacteria are found on plastics in the environment, establishing a protocol to grow these on plastics and then my own group will perform in vitro experiments with human immune cells to instruct the choice of other coatings.
Description: As described in "Inflammatory stimulus: Plastic particles" but with biofilm coated plastic.
Symptoms: Pathogens on the surface of the plastics will likely evoke symptoms associated with an acute inflammatory response such as hypothermia, weight loss and behavioral changes as described in section J.
Level of discomfort: moderate.

Inflammatory stimulus: Bacterial infection
Justification: For subquestion 4 of immediate goal A we want to investigate bacterial phagocytosis and killing of the different neutrophil subsets. For subquestion 6 of immediate goal B bacteria are degradable pathogens of around 1µm in size which are the natural targets of innate immune cells. These are the 'biological degradable' controls for our experiments.
Description: Mice are infected with live or dead (fluorescent) bacteria (eg staphylococci) via the same administration routes as mentioned in "Inflammatory stimulus: Plastic particles".[1,2]
Symptoms: Intradermal injections of dead bacteria are well tolerated and do not lead to noticeable symptoms. Intravenous injection of dead and live bacteria, intradermal injection of live bacteria and intranasal application of live and dead bacteria can lead to symptoms associated with an acute inflammatory response such as hypothermia, weight loss and behavioral changes as described in section J. For as many experiments as possible dead bacteria will be used to minimize the risk of a severe response of the mice.
Level of discomfort: moderate

Control inflammatory stimulus: Non-plastic inert materials
Justification: We need to understand if the effects we see are plastic dependent or rather true for all inert particles (subquestion 6 of immediate goal B). Adding this control makes the outcome of the experiments more useful and are the same size particles, so don't cause extra discomfort to the mice.
Description, symptoms and level of discomfort: As described in "Inflammatory stimulus: Plastic particles" but with other inert materials such as for instance silica, talc, metal, wool, cotton.

Control inflammatory stimulus: Sterile injury
Justification: Sterile injury is a quick and easy tool in intravital imaging to recruit and mobilize neutrophils without a pathogenic stimulus. This can be helpful to compare the migration of neutrophil subsets (subquestion 3 of immediate goal A) or to establish if immobilized immune cells that have engulfed microplastics can be prompted to mobilize and distribute plastics upon local inflammation (subquestion 5b of immediate goal B) or to establish if microplastic induced inflammation is more prolonged than sterile inflammation (subquestion 6 of

immediate goal B). This will only be performed in the skin and is only a needle prick or an unnoticeable small burn.
Description: The mice will be anesthetized and a sterile damage will be inflicted either by insertion of a sterile needle or by laser damage inflicted by the 2-photon microscope with a setting of much higher laser power and zoom than is used for imaging.
Symptoms: These local inflammations are well tolerated and do not lead to noticeable symptoms.
Level of discomfort: mild

Control inflammatory stimulus: LPS injection
Justification: LPS is a relatively simple model for acute inflammation with which we have a lot of experience in vivo in humans. It is in this model where we first described the different neutrophil subsets. This model can be helpful to compare the kinetics and function of neutrophil subsets (subquestion 1-4 of immediate goal A) or to establish if immobilized immune cells that have engulfed microplastics can be prompted to mobilize and distribute plastics upon systemic inflammation (subquestion 2 and 5b of immediate goal B). If the experiment allows, the mice will be kept under anesthesia while the LPS has an effect to reduce discomfort.
Description: Mice receive a single bolus of E. coli lipopolysaccharide intravenously (I.V.) in the tail vein (no anesthesia) or retro-orbitally (while under anesthesia).
Symptoms: Mice are expected to have transient (< 1 day) symptoms associated with an acute inflammatory response such as hypothermia, weight loss and behavioral changes as described in section J.
Level of discomfort: moderate

See also figure 5 of the proposal for a comprehensive Table describing the reasons and goals for using the different inflammatory stimuli.

II (optional)
Administer compounds to

- **Visualize our cells of interest**
- **Measure cell death, proliferation and lifespan**
- **Inhibit, stimulate, deplete or mimic components of the inflammatory reaction**

Justification:

- During intravital imaging different cells and structures should be distinguished.
  E.g. CD62L is a surface receptor that can distinguish different neutrophil subsets. By staining CD62L using a fluorescent antibody we can visualize these different subsets *in vivo*. Another example is to visualize if cells with microplastics are in the bloodstream or in the tissue by labeling the blood using fluorescent albumin or by labeling cells in blood with an anti-CD45 antibody.
- Measure cell life span and proliferation
  5.1 lid2f we can determine the lifespan of innate immune cells in homeostasis vs after plastic administration.
- Inhibit, stimulate, deplete or mimic components of the inflammatory reaction.
  E.g. adding therapeutic antibodies against biofilms might facilitate the biofilm clearance by neutrophils.

Description: Drugs, antibodies, small molecules, chemicals, fluorescent compounds, propidium iodide to monitor cell death, Hoechst to stain nuclei, or compounds which incorporate into DNA to measure cell life span and proliferation such as deuterated water, deuterated glucose, EdU, or BrdU are administered to mice via the appropriate route as described in literature (I.V., I.P., I.N., diet, etc).
Symptoms: Most compounds will not lead to noticeable symptoms.
Level of discomfort: mild or moderate

III (optional)
Intravital imaging

- **Non-survival**
- **Repetitive skin imaging**
- **Repetitive imaging after placing dermal imaging window**
- **Repetitive imaging after placing abdominal imaging window**

Justification: In vivo imaging will allow us to visualize dynamic processes that are missed in static analysis. Imaging windows allow us to image the same mouse repeatedly over time as opposed to analyzing separate mice at different time points, greatly reducing the amount of animals needed. Additionally, this provides paired data, introducing less variation and therefore fewer mice are needed to get statistically significant results.
At the end point of intravital microscopy, tissues will always be analyzed *ex vivo* to reduce the number of mice required.
Description: Different strategies will be used for the imaging experiments: (1) Imaging in an acute experiment under anesthesia (any organ), (2) repetitive skin imaging (after intradermal exposure), (3) repetitive imaging through a dermal imaging window (lymph node or subcutaneous plastics), (4) repetitive imaging through an

abdominal imaging window (such as on the liver, spleen or kidney). For long-term studies the animal needs to be imaged by multiple imaging sessions, and therefore imaging windows are required. From experience we know that the implantation of intra-cutaneous windows does not lead to post-operative discomfort.
For some research questions, the tissue needs to be visualized frequently for a relative short-period of time (up to max 2 days). It is ethically undesirable to anesthetize the animal multiple times a day, so the animal will be constantly anesthetized to prevent discomfort. We have a lot of experience in the lab with these procedures, and only well trained researchers will perform these experiments. We have observed homeostatic neutrophils respond normally to stimuli under the conditions of these experiments.
A detailed description of the window surgery, including postoperative assessment of behavior (largely normal scores) and weight (slight decreases 1 day post-surgery) is published by Ritsma *et al* in Sci Transl Med 2012 and Nature Protocols 2013.[3,4]These publications also describe subsequent intermittent intravital imaging. In short, mice will be anesthetized, positioned on a heat pad for proper temperature control and shaved. An incision is made in the skin for the intra-cutaneous window, while for the abdominal imaging window an incision is made in both skin and peritoneum. The incision is then sutured using a purse-string suture through both layers around the edges, creating four loops and leaving the suture untightened. Next, the window is placed within the incision. Different organs require different fixation techniques.[4] Ultimately, the skin (and peritoneum) are placed in the window groove, and the suture is tightened to fix the window.
For subsequent intravital imaging the mice will also be anesthetized and placed in a custom made imaging box. The microscope is covered by a climate chamber allowing proper temperature control. Saline will be administered subcutaneously (under anesthesia) for imaging sessions of more than an hour.
Symptoms: Mice can experience post-operative pain demonstrated by changed behavior as described in section J. Within 1 day post-surgery the majority of animals are found to behave normally. Intravital imaging sessions do not lead to noticeable symptoms.
Level of discomfort: moderate as a result of surgery, mild thereafter for intra-cutaneous windows, moderate thereafter for abdominal windows.

IV (every experiment)
Killing of the animal and perform ex vivo analysis
Justification: At the end of the experiment organs and cells will be harvested for ex vivo analysis.
Description: Depending on the type of experiment and the location of the experiment, mice will be killed by cervical dislocation while fully awake or while under anesthesia. Mice might also be killed by CO_2 asphyxiation followed by cervical dislocation if the necessary facilities are available. In many experiments we would like to harvest blood before sacrificing the mouse. In this case mice will be deeply anesthetized using isoflurane after which a heart puncture will be performed followed by cervical dislocation. Relevant organs will be harvested, both for us and consortium colleagues. Tissues can be isolated and analyzed with microscopy. In addition cells can be isolated from the different tissues for further descriptive analysis (e.g. FACS of cell surface expression) and/or for functional assays in vitro (e.g. survival assay, bacterial killing capacity, T cell suppression capacity). Our focus in the project is on immunotoxicology. We will look mostly at neutrophil, monocyte and macrophage numbers and activation status. We will look at blood, bone marrow, spleen, lymph nodes, kidney, liver and lungs. Brain, intestines and lungs if not needed by us will be shared with our other consortium members who have expertise with those organs.

The experimental design and the exact number of animals of each experiment is determined and described in detail in a work protocol according to the advice from the Animal Welfare Body and a statistician (figure 4 for example experiments).

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

When possible, blinding and randomization will be used to prevent bias as much as possible. For example, videos obtained with intravital imaging can be coded by an independent colleague before image analysis by the researcher.
Whenever possible we strive for a situation to express the outcome of our experiments in quantitative terms. This is possible for all FACS measurements and most imaging data. To estimate the number of animals to be used in an experiment, we use the effect size (if known, e.g., from data in the literature, from our own historical data or if unknown from small scale pilots) to estimate the sample size needed to achieve a certain power (usually around 0.8) with appropriate statistical test like the t test with a $p<0.05$. Qualitative analysis (descriptive analysis of some intravital imaging data): the number of animals (group size) is based on literature and/or years of experience with similar type of experiments. Moreover, these types of experiments will be performed sequentially via which we ensure that we will use the minimum number of mice per group that will be informative.

B. The animals

Specify the species, origin, life stages, estimated numbers, gender, genetic alterations and, if important for achieving the immediate goal, the strain.

Serial number	Species	Origin	Life stages	Number	Gender	Genetically altered	Strain
1	Mus musculus	Born in EU at registered breeder	No preference	5854	No preference	Yes, without harmful phenotype	Various

Provide justifications for these choices

Species	Our experience with mice and their immune system
Origin	We can use surplus mice from the breeding facility, and try to source our genetically altered more local to reduce impact on mice and the environment.
Life stages	We don't expect an age effect, so all mice can be used
Number	Estimated numbers **Immediate goal A: Unravel fundamental innate neutrophil characteristics such as origin, kinetics, lifespan and function in homeostasis and acute inflammation** Immediate goals 1. Determine the lifespan of neutrophil subsets 30 animals are needed to establish a labelling curve[6] Compare control versus acute inflammation with LPS (because we have comparative human data) Compare deuterium label (population based in humans) vs EdU (cell based, only possible in mice) **120 mice (60 mild, 60 moderate)** 2. Determine the origin of neutrophil subsets (local or distant like bone marrow or spleen) 3. Determine the kinetics of different neutrophil subsets throughout the body 4. Establish the function of different neutrophil subsets These are exploratory studies that will evolve during the project, numbers are estimates. Many times goal 2-4 can be addressed in the same experiments. Controls will be compared with LPS (because we have comparative human data). Typical work protocols are between 10 and 50 mice and typically 5 experiments are performed per year. (30 mice * 5 exp * 5 years =750) **Max 750 mice (400 mild, 350 moderate)** **Immediate goal B: Determine the health effects of environmental exposure to microplastics** Pilot studies ➢ Determine minimum number of oral administration + compare oral gavage with food uptake (5 mice, min 1 and max 10 administrations, 2 routes = **100 mice moderate due to gavage**) ➢ Determine minimum number of intranasal administration (5 mice, min 1 and max 10 administrations = **50 mice mild**) We have 1 reference polystyrene + 3 consortium plastics polymer types, of at most 3 different sizes. Not all sizes will be used for all administration routes. We can have up to 2 different coatings of the plastics in addition to the non-coated control: biofilm and relevant bodily fluid like mucus or serum. For each experiment we need at most two control groups. We are interested in long-term effects of the plastics to mimic human exposure, so multiple time point per administration route are needed, except when we can do intravital imaging. For our previous IV injection experiments we saw changing kinetics using 4h, 16h, 14 days and 30 days. In the future we would like to add a longer term time point as well. Oral exposures (ex vivo analysis): 4 plastics x 3 sizes per plastic type x 3 coatings + 2 controls x 7 animals x 5 time points)= **1330 mice mild or moderate depending on nr of administrations and administration route from pilot** Intranasal exposures (ex vivo analysis):

4 plastics x 3 sizes x 3 coatings + 2 controls x 7 animals x 5 time points)= **1330 mice mild or moderate depending on nr of administrations from pilot**

IV injection (ex vivo analysis):
4 plastics x 2 sizes (size restriction due to clotting) x 3 coatings + 2 controls x 7 animals average x 5 time points = **910 mice (120 mild without coating and 790 moderate)**

Intradermal (repetitive intravital imaging):
4 plastics x 3 sizes x 3 coatings + 2 controls x 7 animals = **266 mice moderate**

Subcutaneous (repetitive intravital imaging):
4 plastics x 1 size x 3 coatings + 2 controls x 7 animals= **98 mice moderate**

<u>Exploratory studies</u>
(See for examples Appendix C)
These are exploratory studies that will evolve during the project, numbers are estimates. Typical work protocols are between 10 and 50 mice and typically 5 experiments are performed per year. (30 mice * 5 exp * 5 years =750)
Max 750 mice (400 mild, 350 moderate)

However, these numbers are overestimations because:

- It is unlikely that all plastics will need to be tested *in vivo*. This will be determined before the animal experiments will be performed.
- For now we estimate to test two different coatings, but we may only need to test one. This will be determined in vitro before the animal experiments will be performed.
- Controls can be shared between experiments when these are performed simultaneously
- Descriptive single cell and functional assays will be combined when neutrophil numbers allow this
- When differences between groups turn out to be very striking, we do not need the maximum number of mice per group in consecutive studies.
- Successful pilot studies may be included in the dataset

<u>Additionally, a number of ca. 150 mice is needed for:</u>

	Estimated nr
Pilot studies for validation of models/techniques#	50
Training of new personnel or new techniques[x]	50
To compensate for unforeseen loss of animals*	50

#Only procedures mentioned in this project proposal
Mice that become surplus during breeding, will be used for pilot studies and training purposes.
[x]Intravital imaging is a procedure that requires proper training of surgical skills in order to prevent discomfort to the animal as much as possible
*(max 10%, e.g. due to location not suitable for imaging, problems with the window)

Together we therefore anticipate we need a grand total of **5854 mice (1030 mild and 4824 moderate).**

Gender	We don't expect a gender effect, so both female and male can be used
Genetic alterations	For some experiments we require intravital imaging strains or knock-out strains
Strain	Phagocytosis is such an evolutionary conserved mechanism that we expect differences between strains to be minimal. The genetically modified mice bearing green neutrophils are in the C57BL/6 background as are genetically modified mice bearing YFP dendritic cells, therefore we want to perform most if not all experiments in that strain. On top of that, by using this background we can also use surplus mice from standard breeding at the GDL facility, mice that would otherwise be discarded. Other genetically modified mice expressing a reporter for a fluorescent protein in different immune cells might be used if not available on the C57BL/6 background.

C. Accommodation and care

Is the housing and care of the animals used in experimental procedures in accordance with Annex III of the Directive 2010/63/EU?

☒ Yes

☐ No > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices

Click or tap here to enter text.

D. Pain and compromised animal welfare

Will the animals experience pain during or after the procedures?

☒ No

No pain (but mild discomfort) is expected to occur for:
- uptake via food or drinks
- oral gavage
- intravenous administration by tail vein
- intraperitoneal administration
- CO_2 asphyxiation
- cervical dislocation

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☒ No > Justify why pain relieving methods will not be used.

Some pain is expected to occur for:
- acute inflammatory response after LPS administration
- acute inflammatory response after bacterial administration

These acute inflammatory response will evoke fever, shivers and muscle ache. Anelgesia will influence the immune response that we are studying. We also perform these type of studies in human volunteers without anesthesia or anelgesia.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

The procedures requiring anesthesia and/or analgesia are listed below and detailed in section 2.
- retro-orbital intravenous injection under isoflurane anesthesia, no analgesia
- intranasal application under isoflurane anesthesia, no analgesia
- intradermal application under isoflurane anesthesia, no analgesia
- subcutaneous application under isoflurane anesthesia, no analgesia
- Sterile injury under isoflurane anesthesia, no analgesia
- Non-survival intravital imaging under isoflurane anesthesia, no analgesia
- Repetitive skin imaging under isoflurane anesthesia, no analgesia
- Repetitive imaging after placing dermal imaging window under isoflurane anesthesia, analgesia pre- and post-surgery[5]
- Repetitive imaging after placing abdominal imaging window under isoflurane anesthesia, analgesia pre- and post-surgery[3,4]
- Terminal heart puncture followed by cervical dislocation under isoflurane anesthesia, no analgesia

Describe which other adverse effects on the animals' welfare may be expected?

Inflammatory stimulus: Plastic particles/ Non-plastic inert materials

Symptoms: Intradermal injections of microplastics, I.V. microplastics injection and oral microplastic administration are well tolerated and do not lead to noticeable symptoms. Intranasal administration of microplastics has not been described before, but are expected to be in line with the mild effects of the other administration routes. However, weight loss and behavioral changes as described in section J will be tightly monitored for the animals in this route. Subcutaneous administration will require a small surgical procedure with likely moderate discomfort as a result.

Level of discomfort: mild (oral via food, intranasal, I.V., intradermal) or moderate (oral gavage, intranasal when administered more than 2 times, subcutaneous)

Inflammatory stimulus: Plastic particles with biofilm
Symptoms: Pathogens on the surface of the plastics will likely evoke symptoms associated with an acute inflammatory response such as hypothermia, weight loss and behavioral changes as described in section J.
Level of discomfort: moderate.

Inflammatory stimulus: Bacterial infection
Symptoms: Intradermal injections of dead bacteria are well tolerated and do not lead to noticeable symptoms. Intravenous injection of dead and live bacteria, intradermal injection of live bacteria and intranasal application of live and dead bacteria can lead to symptoms associated with an acute inflammatory response such as hypothermia, weight loss and behavioral changes as described in section J.
Level of discomfort: moderate

Control inflammatory stimulus: Sterile injury
Symptoms: These local inflammations are well tolerated and do not lead to noticeable symptoms.
Level of discomfort: mild

Control inflammatory stimulus: LPS injection
Symptoms: Mice are expected to have transient (< 1 day) symptoms associated with an acute inflammatory response such as hypothermia, weight loss and behavioral changes as described in section J.
Level of discomfort: moderate

Administer compounds
Symptoms: Most compounds will not lead to noticeable symptoms.
Level of discomfort: mild or moderate

Intravital imaging
Symptoms: Mice can experience post-operative pain demonstrated by changed behavior as described in section J. Within 1 day post-surgery the majority of animals are found to behave normally. Intravital imaging sessions do not lead to noticeable symptoms.
Level of discomfort: moderate as a result of surgery, mild thereafter for intra-cutaneous windows, moderate thereafter for abdominal windows.

Explain why these effects may emerge.

See above

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Only skilled personnel will perform the procedures. Due to our experience with injecting bacteria or LPS, we know the safe (concentration) range and the risk is minimal. Mice with windows will be monitored at least twice a week for clinical signs. If unexpectedly severe suffering is observed, mice will be euthanized. If the experimental set-up allows (e.g. end point is ≤3hours after I.V. LPS injection) the whole procedure will be done under anesthesia.

E. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question F

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

The experiments will be set-up in such a way that the incidence of reaching the humane end-point is kept to a minimum.
Reaching a score of 4 on the illness scale (specified below) or severe shortness of breath is set as an humane end point.

An illness grading scale will always be used to score a set of clinical features detected in mice with different degrees of illness:
0: healthy

1: barely ruffled fur
2: ruffled fur, but active
3: ruffled fur and inactive
4: ruffled fur, inactive, hunched, and gaunt
5: dead

Additional model specific parameters:
Plastic particles with biofilm/ Bacterial infection/ LPS injection
Weight loss of >20%

Intravital imaging
Loss of the intravital imaging window is set as a humane endpoint.
Infection of the skin around the intravital imaging window is additionally set as a humane endpoint (has never happened so far).

Indicate the likely incidence.

Expected to be <5% and <1 day

F. Classification of severity of procedures

Provide information on the experimental factors contributing to the discomfort of the animals and indicate to which category these factors are assigned ('non-recovery', 'mild', 'moderate', 'severe'). In addition, provide for each species and treatment group information on the expected levels of cumulative discomfort (in percentages).

Level of discomfort is indicated in section A for each injection and technique at the bottom of each paragraph. Also the category assignment is described extensively in the adverse effects section above.
We have a few experiments in the list which will cause more discomfort, but these will only be used for specific situations. Where possible we will be using anesthesia to reduce the discomfort of the mice. Unfortunately the usage of analgesics can have an effect on the immune cells, especially the neutrophils, and will therefore be prevented as much as possible. Overall, pain and discomfort should be short-lived, and we always aim to keep it as short an minimal as possible. The cumulative discomfort should be 17.6% of the number of animals (1030) concerning the mild procedures (oral administration via food, IV injection, intradermal injection), and 82.4% of the animals (4824) for the moderate procedures (intranasal administration, oral gavage, subcutaneous injection, window placement, inflammation models, intravital imaging).

G. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement	Initial testing for plastics of interest concerning their polymer composition, their size and their coating will be done in in vitro settings. Many experiments of the project will be performed using human primary cells, human and mouse cell lines, organoids and organs-on-a-chip by us and others. This will ultimately determine the number of animal experiments to be performed.
Reduction	By using techniques like intravital imaging, we reduce the number of mice used in longitudinal studies because the same mouse can be imaged over the entire span of the experiment. After completion of the intravital imaging experiments, cells, tissues and organs will be isolated and analyzed to reduce the number of mice required. Using genetically modified mice will give us more insight per mouse as to the effect of microplastics on neutrophils, and to a certain extend macrophages. With more information retrieved per mouse, less animals are necessary. By sharing organs, other institution no longer need to perform the experiments as well to obtain their tissues.
Refinement	Anesthesia will be used for most procedures and husbandry will kept at an optimum to reduce discomfort and stress. For most experiments we first consider ex vivo experiments (mild discomfort) or terminal experiments, before we consider repetitive intravital imaging experiments (moderate discomfort). In some experiments we first consider repetitive intravital imaging, either because some questions can only be answered by imaging the same tissue over multiple imaging sessions or because it reduces the number of mice (see above). In pilot experiments we will determine the least amount of oral and intranasal administrations

	needed to detect microplastics in the tissue and we will establish if we can switch from oral gavage to administration via food. Analgesia will be applied during the window placement. Mice will be placed on thermal plates during surgery. Windows are made of biocompatible titanium material and have a groove that fits and hides the suture. Saline will be administered i.p. during long intravital imaging sessions. Eye ointment will also be used to prevent dry eyes. We have over a decade of experience with this technique.

Are adverse environmental effects expected? Explain what measures will be taken to minimise these effects.

☒ No

☐ Yes > Describe the environmental effects and explain what measures will be taken to minimise these effects.

Click or tap here to enter text.

H. Re-use

Will animals be used that have already been used in other animal procedures ?

☒ No > Continue with question I.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☐ No

☐ Yes > Provide specific justifications for the re-use of these animals during the procedures.

Click or tap here to enter text.

I. Repetition

Explain for legally required animal procedures what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, describe why duplication is required.

Click or tap here to enter text.

J. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question K.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

We will remain with the animals at all times. We keep monitoring them throughout the procedure, and make sure they arrive back at the animal facility as soon as possible.

3. End of experiment

K. Destination of the animals

Will the animals be killed during or after the procedures?

☐ No > Provide information on the destination of the animals.

Click or tap here to enter text.

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

Full analysis of the organs exposed to plastics requires us to euthanize the mice

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

Click or tap here to enter text.

☒ Yes > Will a method of killing be used for which specific requirements apply?

☒ No > Describe the method of killing.

Most mice will be euthanized using cervical dislocation performed under anesthesia. Occasionally CO_2 asphyxiation followed by cervical dislocation or cervical dislocation without anesthesia will be performed.

☐ Yes > Describe the method of killing that will be used and provide justifications for this choice.

Click or tap here to enter text.

If animals are killed for non-scientific reasons, justify why it is not feasible to rehome the animals.

Not applicable

References for the whole appendix

1. Liese, J., Rooijakkers, S. H., van Strijp, J. A., Novick, R. P. & Dustin, M. L. Intravital two-photon microscopy of host-pathogen interactions in a mouse model of Staphylococcus aureus skin abscess formation. *Cell. Microbiol.* **15**, 891-909 (2013).
2. Spaan, A. N. *et al*. The staphylococcal toxins gamma-haemolysin AB and CB differentially target phagocytes by employing specific chemokine receptors. *Nat. Commun.* **5**, 5438 (2014).
3. Ritsma L, et al. (2012) Intravital Microscopy Through an Abdominal Imaging Window Reveals a Pre-Micrometastasis Stage During Liver Metastasis. Sci Transl Med 4:158ra145–158ra145.
4. Ritsma L, et al. (2013) Surgical implantation of an abdominal imaging window for intravital microscopy. Nat Protoc 8:583–94. doi: 10.1038/nprot.2013.026
5. Mourao L, et al (2022) Longitudinal intravital imaging using a mammary imaging window with replaceable lid.

5.1 lid2e, 5.1 lid2h

NIET-TECHNISCHE PROJECTSAMENVATTING

Naam van het project	Schadelijkheid van microplastics
NTS-identificatiecode	NTS-NL-627388 v.1
Nationale identificatiecode van de NTS *Veld wordt niet gepubliceerd.*	
Land	Nederland
Taal	nl
Indiening bij EU *Veld wordt niet gepubliceerd.*	ja
Duur van het project, uitgedrukt in maanden.	60
Trefwoorden	Microplastic Immuunsysteem Afweercellen
Doel(en) van het project	Fundamenteel onderzoek: Immuunstelsel Omzettinggericht en toegepast onderzoek: Niet op grond van regelgeving vereist toxicologisch en ecotoxicologisch onderzoek Bescherming van het milieu in het belang van de gezondheid of het welzijn van mens of dier

DOELSTELLINGEN EN VERWACHTE VOORDELEN VAN HET PROJECT

Beschrijf de doelstellingen van het project (bijvoorbeeld het aanpakken van bepaalde wetenschappelijke onduidelijkheden, of wetenschappelijke of klinische behoeften).	Dagelijks krijgen we microplastics binnen. De microplastics komen onder andere van flesjes water, voedselverpakking, en nylon kleding. Die komen via de lucht, ons eten en drinken ons lichaam binnen. Maar we weten nog niet wat er met ons gebeurt als plastic ons lichaam binnen komt, of hoe schadelijk het nu eigenlijk is. Daarnaast zwerft plastic in onze omgeving rond. Daar komt het in aanraking met bacteriën en virussen die ons ziek kunnen maken. Het zou zo kunnen zijn dat bacteriën via plastic ons lichaam binnen komen, maar dat weten we nog niet zeker. We hebben geld gekregen van de Nederlandse overheid om dit uit te zoeken, zodat zij de regels over plastic kunnen aanpassen. In ons lichaam zijn onze afweercellen altijd bezig om binnendringers op te ruimen. Normaal zijn dat bacteriën, parasieten en virussen. Deze drie indringers kunnen worden afgebroken en opgeruimd door de afweercellen. Plastic dat ons lichaam binnen komt kan niet worden afgebroken door afweercellen. Hierdoor gaan de afweercellen dood. Dit kan ontstekingen en andere slechte gevolgen hebben op korte termijn. We weten ook nog niet wat het gevolg is van dit soort irritatie van het afweersysteem als het de rest van ons leven aanhoudt. Met deze dierproeven willen wij uitzoeken hoe een plastic via de longen of de darmen ons lichaam binnen komt, en hoe schadelijk de meest voorkomende plastics zijn voor ons afweersysteem. We willen weten in welke organen de plastics terecht komen, en welke afweercellen erop reageren. We willen ook uitzoeken hoe lang een plastic op een bepaalde plek in ons lichaam blijft. Om een goede inschatting van het risico van plastics te maken, moeten we wel goed weten hoe ons afweersysteem reageert in meer bekende situaties, zoals een infectie of als je gewond raakt. Door in bekende situaties de verplaatsingen van onze afweercellen te volgen en dat te herhalen en vergelijken met plastics, kunnen we de gevolgen van plastics op onze afweer in een bredere context plaatsen. Zo willen we bepalen hoe schadelijk de plastics kunnen zijn, of dat het misschien wel mee valt.
Welke potentiële voordelen kan dit project opleveren? Leg uit hoe de wetenschap vooruit kan worden geholpen of mensen, dieren of het milieu uiteindelijk voordeel kunnen hebben bij het project. Maak, waar van	Door uit te zoeken wat plastic in ons lichaam met ons afweersysteem doet, kunnen we bepalen hoe schadelijk het is. Nu komen we elke dag met veel plastic in aanraking, omdat er geen regels over zijn. Dus we weten niet hoeveel schade we per dag oplopen door de plastics die we binnen krijgen. Op dit moment kunnen bedrijven zelf kiezen om minder plastic te gebruiken in hun verpakkingen, maar er zijn geen regels dat ze dat moeten doen. Als wij laten zien in hoeverre plastic schadelijk is voor onze gezondheid, kan de overheid regels maken die het plasticgebruik verminderen. Zo beschermen

toepassing, een onderscheid tussen voordelen op korte termijn (binnen de looptijd van het project) en voordelen op lange termijn (die mogelijk pas worden bereikt nadat het project is afgerond).	we niet alleen onze gezondheid, maar verminderen we ook de hoeveelheid plastic die in de natuur belandt vanuit ons afval.

VOORSPELDE SCHADE

<table>
<tr><td>In welke procedures worden de dieren gewoonlijk gebruikt (bijvoorbeeld injecties, chirurgische procedures)? Vermeld het aantal en de duur van deze procedures.</td><td>De muizen worden op natuurlijke of kunstmatige manier blootgesteld aan de plastics. We willen plastic via het eten en via de lucht toedienen om uit te zoeken hoeveel ze in hun bloed krijgen. We willen de plastic ook direct in het bloed spuiten om te zien waar in het lichaam plastic terecht komt. Daarnaast willen we plastic in de huid spuiten om met de microscoop te zien hoe afweercellen erop reageren. We gaan ook experimenten doen waarbij we een kijkvenster plaatsen op een orgaan zodat we over de tijd kunnen kijken of en hoe lang de plastics blijven zitten. Deze experimenten vergen een operatie en daar zullen we pijnstilling voor gebruiken.</td></tr>
<tr><td>Wat zijn de verwachte gevolgen/nadelige effecten voor de dieren, bijvoorbeeld pijn, gewichtsverlies, inactiviteit/verminderde mobiliteit, stress, abnormaal gedrag, en wat is de duur van die effecten?</td><td>Voor de meeste behandelingen worden de muizen in slaap gebracht zodat ze niks merken. We hebben eerder gezien dat de muizen geen zichtbare last hebben van de plastic als we tot een paar maanden kijken. De negatieve gevolgen zullen daarom klein zijn voor de muizen. Er zijn sommige procedures waarbij de muizen wel stress ervaren maar die bij mensen ook niet onder anesthesie worden uitgevoerd zoals bloedafname, orale toediening en het oproepen van een ontstekingsreactie. We gaan ook experimenten doen waarbij we een kijkvenster plaatsen op een orgaan zodat we over de tijd kunnen kijken of en hoe lang de plastics blijven zitten. Deze experimenten vergen een operatie en daar zullen we pijnstilling voor gebruiken.</td></tr>
<tr><td>Welke soorten en aantallen dieren zullen naar verwachting worden gebruikt? Wat zijn de verwachte ernstgraden en de aantallen dieren in elke ernstcategorie (per soort)?</td><td>
<table>
<tr><td rowspan="2">Soort:</td><td rowspan="2">Totaal aantal</td><td colspan="4">Geraamde aantallen naar ernstgraad</td></tr>
<tr><td>Terminaal</td><td>Licht</td><td>Matig</td><td>Ernstig</td></tr>
<tr><td>Muizen (Mus musculus)</td><td>5854</td><td>0</td><td>1030</td><td>4824</td><td>0</td></tr>
</table>
</td></tr>
<tr><td>Wat gebeurt er met de dieren die aan het einde van de procedure in leven worden gehouden?</td><td>
<table>
<tr><td rowspan="2">Soort:</td><td colspan="3">Geraamd aantal te hergebruiken, in het habitat-/houderijsysteem terug te plaatsen of voor adoptie vrij te geven dieren</td></tr>
<tr><td>Hergebruikt</td><td>Teruggeplaatst</td><td>Geadopteerd</td></tr>
</table>
</td></tr>
<tr><td>Geef de redenen voor het geplande lot van de dieren na de procedure.</td><td>We moeten weten wat er in het bloed en de organen gebeurt met de afweercellen door de plastic. Omdat we meerdere organen willen bekijken aan het einde van ieder experiment, is het niet mogelijk om de muizen te laten leven aan het einde.</td></tr>
</table>

TOEPASSING VAN DE DRIE V'S

1. Vervanging Beschrijf welke diervrije alternatieven op dit gebied voorhanden zijn en waarom zij niet voor het project kunnen worden gebruikt.	In ons consortium gebruiken we voornamelijk niet-dierlijke alternatieven. Verschillende consortiumpartners hebben verschillende orgaanexpertises. De plastics worden op gedoneerde menselijke cellen in het lab getest om te bepalen welke plastics mogelijk gevaarlijk zijn als we ze binnen krijgen. Hiervoor gebruiken we de cellen direct uit het lichaam, maar ook kweken we de cellen op tot meer geavanceerde mini-darmpjes en mini-longetjes. Met deze proeven in kweekschaaltjes kunnen we een inschatting maken van de toxiciteit. Wij zijn de enigen die ook dierexperimenten uitvoeren. Met deze dierproeven leveren we een cruciale link door de bewegingen van microplastics door het hele lichaam te volgen en door te bepalen hoe lang ze op een bepaalde plek blijven zitten. Ook zullen we een bepaalde hoeveelheid plastics aan een muis te eten geven en vervolgens bepalen hoeveel daarvan het lichaam binnendringt. Dit type proeven kunnen momenteel nog niet in kweekschaaltjes worden uitgevoerd.
2. Vermindering Leg uit hoe de aantallen dieren voor dit project zijn bepaald. Beschrijf de stappen die zijn genomen om het aantal te gebruiken dieren te verminderen en de beginselen die zijn gebruikt bij het opzetten van de studies. Beschrijf, waar van toepassing, de praktijken die gedurende het hele project zullen worden toegepast om het aantal dieren die in overeenstemming met de wetenschappelijke doelstellingen werden gebruikt, tot een minimum te beperken. Deze praktijken kunnen bijvoorbeeld bestaan uit proefprojecten, computermodellen, het delen van weefsel en hergebruik.	Door meerdere onderzoeken in dezelfde muis te doen, hebben we minder muizen nodig. Ook door in dezelfde muis op meerdere momenten live te kijken wat er met de plastics gebeurd, hebben we minder muizen nodig.
3. Verfijning Geef voorbeelden van de specifieke maatregelen (bv. verscherpte monitoring, postoperatieve behandeling, pijnbestrijding, training van dieren) die in verband met de procedures moeten worden genomen om de welzijnskosten (schade) voor de dieren tot een minimum te beperken. Beschrijf de mechanismen om gedurende de looptijd van het project nieuwe verfijningstechnieken in gebruik te nemen.	Voor de meeste handelingen die we met de muizen doen die pijn kunnen doen of stress geven, brengen we ze in slaap. Wanneer we een operatie uitvoeren waarna de muis wakker wordt geven we pijnstilling. Voor de orale toediening willen we vroeg in het project verschillende toedieningsmethodes vergelijken. We willen nagaan of de plastics ook de darmwand passeren als we ze in het voedsel geven of in een druppel gecondenseerde melk in plaats van via directe toediening in de slokdarm zoals we eerder gedaan hebben.
Licht de keuze van de soorten en de bijbehorende levensstadia toe	We hebben veel ervaring met muizen en hun afweersysteem. We weten hoe het op ons afweersysteem lijkt. Ook zijn de methoden om afweercellen te herkennen en te markeren in de muis het meest ontwikkeld. Daarom hebben we voor muizen gekozen. We gaan in muizen van verschillende leeftijden kijken omdat bij mensen ook jong en oud met plastic te maken heeft en omdat we eerder verschillende resultaten hebben gevonden bij muizen van verschillende leeftijden.

VOOR EEN BEOORDELING ACHTERAF GESELECTEERD PROJECT

Project geselecteerd voor BA?	nee
Termijn voor BA	
Reden voor de beoordeling achteraf	
Bevat ernstige procedures	
Maakt gebruik van niet-menselijke primaten	
Andere reden	
Toelichting van de andere reden voor de beoordeling achteraf	

AANVULLENDE VELDEN

Nationaal veld 1 *Veld wordt niet gepubliceerd.*	
Nationaal veld 2 *Veld wordt niet gepubliceerd.*	
Nationaal veld 3 *Veld wordt niet gepubliceerd.*	
Nationaal veld 4 *Veld wordt niet gepubliceerd.*	
Nationaal veld 5 *Veld wordt niet gepubliceerd.*	
Startdatum project *Veld wordt niet gepubliceerd.*	
Einddatum project *Veld wordt niet gepubliceerd.*	
Goedkeuringsdatum project *Veld wordt niet gepubliceerd.*	
ICD-code 1 *Veld wordt niet gepubliceerd.*	
ICD-code 2 *Veld wordt niet gepubliceerd.*	
ICD-code 3 *Veld wordt niet gepubliceerd.*	
Link naar de eerdere versie van de NTS buiten het EC-systeem	

Van: info@zbo-ccd.nl
Verzonden: vrijdag 23 september 2022 11:46
Aan: 5.1 lid2h
Onderwerp: Verzoek om advies over projectvergunningsaanvraag AVD5.1 lid2h 202216420
Bijlagen: 5.1 lid2h _2022_II_429 NTS_5.1 lid2e .xlsx; 5.1 lid2h _2022_II_429 Description_animal_procedures_5.1 lid2e 220909.pdf; 5.1 lid2h _2022_II_429 Project_proposal_5.1 lid2e 220909.pdf; 5.1 lid2h _2022_II_429 Aanvraag_Projectvergunning_NV2022.pdf

Geachte leden van 5.1 lid2h

De Centrale Commissie Dierproeven (hierna: CCD) verzoekt u in het kader van vergunningverlening advies te geven over het project met als titel: "Immunological health effects of microplastics" en aanvraagnummer: AVD5.1 lid2h 202216420.

Uw commissie wordt verzocht op grond van artikel 10.a.2 van de Wet op de dierproeven de aanvraag te beoordelen en een ethische toetsing uit te voeren waarbij wordt afgewogen of de doelstelling van het project, de verwachte voordelen voor mens, dier of milieu en de haalbaarheid van de doelstellingen, het gebruik van dieren en de schade die zal worden toegebracht aan de dieren in de vorm van lijden, pijn en angst kan rechtvaardigen.

Graag ontvangen wij van u bericht dat deze e-mail goed is ontvangen en wanneer u dit advies in de vergadering gaat bespreken.

Voor het in te dienen advies dient de DEC gebruik te maken van de meest actuele versie van het op de website van de CCD gepubliceerde Format DEC-advies en de toelichting daarbij. U dient deze aanvraag vertrouwelijk te behandelen. Voor de communicatie met de CCD dient u gebruik te maken van FileSecure.

De CCD verzoekt u uiterlijk binnen 20 werkdagen, na 23-09-2022, uw advies bij de CCD in te dienen. Indien de aanvraag door uw commissie niet in behandeling kan worden genomen, dient u dit per ommegaande per e-mail aan de CCD te melden.

Ingeval uw commissie tussentijds aanvullende informatie wil inwinnen bij de aanvrager wordt de termijn opgeschort en geeft u in uw advies aan wanneer dit is geweest. Opschorting van de adviestermijn vindt niet plaats ingeval u ten behoeve van uw advies een onafhankelijk extern expert raadpleegt. Mocht u verwachten door een andere reden dan opschorting uw advies later dan 20 werkdagen na 23-09-2022 bij de CCD in te dienen, dan verzoeken wij u dit direct aan de CCD te melden.

Mocht u vragen hebben, dan kunt u uiteraard contact met ons opnemen.

Met vriendelijke groet,
Centrale Commissie Dierproeven

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5.1 lid2h 6

Centrale Commissie Dierproeven
Postbus 93118
2509 AC DEN HAAG

Ons kenmerk FM/BA/22/0
Datum 03-10-2022
Betreft Advies inzake aanvraag AVD5.1 lid2h 202216420: '*Immunological health effects of microplastics*'

Geachte leden van de Centrale Commissie Dierproeven,

De commissie heeft tijdens de bespreking van de bovenvermelde vergunningaanvraag in de vergadering van 28 september jl. geconstateerd dat deze aanvraag in de huidige vorm meerdere onvolkomenheden bevat, waardoor de aanvraag niet toetsbaar is. De commissie kan zich het belang van het onderzoek naar microplastics wel voorstellen, maar de aanvraag bevat te veel onduidelijkheden om tot een afweging te kunnen komen. De DEC heeft de opties 'aanhouden en vragen stellen' besproken versus 'terugsturen naar de CCD' besproken. Omdat voorzien wordt dat de optie 'aanhouden en vragen stellen' een herschreven aanvraag in zal houden met een aanvullende vragenronde wil de DEC er de duidelijkheid voor kiezen om de aanvraag terug te sturen naar de CCD met de vragen die de DEC heeft bij deze aanvraag.

De voornaamste oorzaak voor het niet kunnen maken van de afweging is dat de onderzoekers niet duidelijk aangeven wat het voorgestelde onderzoek uiteindelijk oplevert. De aanvraag lijkt meer op een programma dan op een project. Het is de DEC wel duidelijk wat men wil bereiken, maar de aanvraag is onvoldoende uitgewerkt en er is geen duidelijke informatie waarom men denkt dat de voorgestelde benadering haalbaar is.
De DEC is van mening dat de aanvraag breed is beschreven en heeft gediscussieerd of er sprake is van een programma. Er mist cruciale informatie om de aanvraag te kunnen beoordelen: er ontbreken o.a. eigen referenties en een beschrijving van eerder onderzoek, een duidelijke afbakening van het onderzoeksgebied en het uiteindelijke doel, informatie over de takenverdeling binnen het consortium en een stroomdiagram met go/no-go's. Er worden macrofagen beschreven in de bijlagen, maar deze missen in de inleiding.
Er is relatief veel ruimte in de aanvraag voor eigen invulling. De IvD zal hierop monitoren.
De DEC heeft vragen bij de inzet van het humane materiaal en hoe dit verkregen wordt. Vanuit de IvD is vanwege de inzet van alternatieven voor proefdieren hiernaar gevraagd.

De onderzoeker heeft het volgende toegelicht (notulen DEC vergadering):
De DEC heeft de onderzoeker o.a. gehoord over het ontbreken van informatie, het uiteindelijke doel en de rollen en manieren van gegevensuitwisseling van de leden van het consortium: alleen in 5.1 lid2h *worden dierproeven uitgevoerd, andere centra voeren alleen in vitro onderzoek uit op bijvoorbeeld long- en darmepitheel. Resultaten hiervan kunnen invloed hebben op de huidige aanvraag, bijvoorbeeld over de beste*

5.1 lid2h

toedieningsmethode. De DEC ziet dit graag opgenomen in een flowchart met go/no-go's. Verder is niet duidelijk of er door deze groep ook onderzoek gedaan wordt naar monocyten, fagocyten en macrofagen.
Daarnaast is gesproken over de microplastics. Deze worden door 5.1 lid1c *geleverd en kunnen niet in 1 grootte gemaakt worden, waardoor karakterisatie moeilijk is. Verschillende groottes microplastics kunnen verschillende effecten hebben. Onderzoekers willen 'proof of principle' onderzoek doen. Bijvoorbeeld naar welke maximale grootte door het darmepitheel gaat.*
Onderzoeker heeft een toelichting gegeven op de keuze voor intradermale toediening, naast de orale en nasale toediening. Onderzoekers willen op deze manier de effecten van hogere hoeveelheden microplastics in het bloed onderzoeken. Verder is gesproken over de noodzaak van een betere onderbouwing van de aanvraag van muizen naast het door de VU uitgevoerde humane onderzoek. In de maatschappij is sprake van langdurige lage blootstelling aan wilde microplastics. In het experiment is sprake van korte hoge blootstelling van door 5.1 lid1c *gefabriceerde plastics. De DEC vraagt zich af of de resultaten wel te generaliseren zijn. Onderzoeker heeft toegelicht dat preliminair onderzoek laat zien dat microplastics zich in 10 dagen al opstapelen. Wilde plastics zijn zo moeilijk te maken dat deze niet beschikbaar zijn voor onderzoek. Aangetoonde effecten van het huidige onderzoek kunnen richting geven voor toekomstig onderzoek en zijn bedoeld als handvatten voor de politiek om maatregelen te kunnen nemen indien nodig.*
Uit het gesprek zijn onderstaande vragen voortgekomen. De DEC is van mening dat de antwoorden nieuwe vragen kunnen oproepen, waardoor meerdere behandelrondes noodzakelijk zullen zijn en zal daarom de aanvraag terugsturen naar de CCD.

Vragen die de DEC heeft:

Projectvoorstel
- 3.1 Achtergrond: Hoe zit het consortium in elkaar? Wie voert welk deel uit?
- 3.1: Achtergrond: De cruciale rol van macrofagen mist. Wilt u deze nog toevoegen?
- 3.2 Doel: Wat is het uiteindelijke doel van de aanvraag?
- 3.2 Doel: Waarom gebruikt u bij de muis een aantal extra organen ten opzichte van de mens?
- 3.2 Doel: immediate goal A: De DEC mist specifieke go/no-go's. Kunt u wel een richting aangeven waar u heen wilt?
- 3.2 Doel: Het is de DEC niet duidelijk hoe het humane deel van het onderzoek bij het verdere onderzoek past. Wilt u dit onderbouwen in de aanvraag? Daarnaast is het niet duidelijk waar het humane materiaal vandaan komt.
- 3.2 Doel: Wat is de reden dat microplastics niet terugkomen onder 'immediate goal A'? Waarom kiest u voor LPS? Daar is al veel over bekend vanuit eerder onderzoek.
- 3.2 Doel: 'Ultimate goal B' sluit naar mening van de DEC niet helemaal aan bij de bijlage. Hier staat intranasale en orale toediening, terwijl in de bijlagen ook intradermale toediening genoemd wordt. De DEC mist een stappenplan waaruit duidelijk wordt of toedieningen serieel of sequentieel worden toegepast. Wilt u dit nog toevoegen?
- 3.2 Doel: In hoeverre verwacht u dat er in de praktijk aanpassingen gedaan zullen worden als er effecten blijken te zijn bij bijvoorbeeld operaties? Kunt u de uitkomsten relateren aan het uiteindelijke doel?
- 3.2 Doel: Zijn de gebruikte muizen wel microplasticvrij? Microplastics kunnen ook in hun voer of drinkwater zitten. De muizen zijn dan eigenlijk al chronisch blootgesteld aan microplastics.
- 3.2 Doel: Is er een verschil bekend bij u tussen een acute grote toediening van microplastics en een kleinere toediening over de langere termijn?

5.1 lid2h

- 3.4 Strategie: De samenhang in figuur 4 is de DEC niet helemaal duidelijk: Is de uitvoering parallel of sequentieel? Hoe is de blootstelling? Hoe wordt de vertaling naar humaan gemaakt? En kunt u hier go/no-go's toevoegen?
- 3.4 Strategie: De DEC mist een rechtvaardiging voor de intervitale microscopie en ziet deze ook graag ingebed in het flowchart, met go/no-go's en daar bijbehorende criteria.
- 3.4.2: Op basis waarvan worden keuzes gemaakt en door wie worden deze genomen?

Bijlage 1

- A. Experimentele aanpak en primaire uitkomstparameters: Kunt u go/no-go's tussen de vaste en optionele stappen toevoegen?
- A. Experimentele aanpak en primaire uitkomstparameters: De DEC mist hier de 'alignment' met immediate goal A.

De DEC adviseert de CCD de aanvrager te verzoeken de aanvraag grondig te herschrijven met inachtneming van de vragen en opmerkingen hierboven. De DEC zal de onderzoeker hierover informeren.

Hoogachtend,

5.1 lid2h

5.1 lid2h

Van: Info-zbo
Verzonden: dinsdag 4 oktober 2022 07:57
Aan: 5.1 lid2h
Onderwerp: RE: Verzoek om advies over projectvergunningsaanvraag AVD5.1 lid2h202216420

Goedemorgen 5.1 lid2e

De vragen en opmerkingen zijn naar de aanvrager doorgezet. Op het moment dat de antwoorden binnen zijn worden deze doorgestuurd naar de DEC.

Heeft u nog vragen dan hoor ik dat graag,

Met vriendelijke groet,

5.1 lid2e
Aanwezig ma t/m do
Medewerker OBDA en CCD

Centrale Commissie Dierproeven www.centralecommissiedierproeven.nl
Nationaal Comité advies dierproevenbeleid www.ncadierproevenbeleid.nl
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T: **0800-7890789**
E: info@zbo-ccd.nl

Van: 5.1 lid2h
Verzonden: maandag 3 oktober 2022 18:36
Aan: info@zbo-ccd.nl
Onderwerp: [Waarschuwing : Mail kon niet gescand worden vanwege wachtwoord. Alleen openen als u de afzender vertrouwd.]RE: Verzoek om advies over projectvergunningsaanvraag AVD5.1 lid2h202216420

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Indien dit niet het geval is dient u deze e-mail direct te verwijderen.

DICTU Servicedesk

Geachte CCD,

De DEC heeft tijdens de bespreking van aanvraag AVD5.1 lid2h02216420 in de vergadering van 28 september jl. geconstateerd dat deze aanvraag in de huidige vorm meerdere onvolkomenheden bevat, waardoor de aanvraag niet toetsbaar is. Zie de brief in de bijlage voor een verdere toelichting.

Het wachtwoord stuur ik in een aparte mail.

Met vriendelijke groet,

5.1 lid2e

5.1 lid2h

5.1 lid2e Dierexperimentencommissie | 5.1 lid2h -
5.1 lid2h 5.1 lid2e | Postbus 5.1 lid2h

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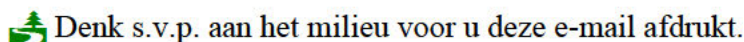
Denk s.v.p. aan het milieu voor u deze e-mail afdrukt.

Van: info@zbo-ccd.nl <info@zbo-ccd.nl>
Verzonden: vrijdag 23 september 2022 11:46
Aan: 5.1 lid2h
Onderwerp: Verzoek om advies over projectvergunningsaanvraag AVD 5.1 lid2h 202216420

Geachte leden van 5.1 lid2h

De Centrale Commissie Dierproeven (hierna: CCD) verzoekt u in het kader van vergunningverlening advies te geven over het project met als titel: "Immunological health effects of microplastics" en aanvraagnummer: AVD 5.1 lid2h 202216420.

Uw commissie wordt verzocht op grond van artikel 10.a.2 van de Wet op de dierproeven de aanvraag te beoordelen en een ethische toetsing uit te voeren waarbij wordt afgewogen of de doelstelling van het project, de verwachte voordelen voor mens, dier of milieu en de haalbaarheid van de doelstellingen, het gebruik van dieren en de schade die zal worden toegebracht aan de dieren in de vorm van lijden, pijn en angst kan rechtvaardigen.

Graag ontvangen wij van u bericht dat deze e-mail goed is ontvangen en wanneer u dit advies in de vergadering gaat bespreken.

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De CCD verzoekt u uiterlijk binnen 20 werkdagen, na 23-09-2022, uw advies bij de CCD in te dienen. Indien de aanvraag door uw commissie niet in behandeling kan worden genomen, dient u dit per ommegaande per e-mail aan de CCD te melden.

Ingeval uw commissie tussentijds aanvullende informatie wil inwinnen bij de aanvrager wordt de termijn opgeschort en geeft u in uw advies aan wanneer dit is geweest. Opschorting van de adviestermijn vindt niet

plaats ingeval u ten behoeve van uw advies een onafhankelijk extern expert raadpleegt. Mocht u verwachten door een andere reden dan opschorting uw advies later dan 20 werkdagen na 23-09-2022 bij de CCD in te dienen, dan verzoeken wij u dit direct aan de CCD te melden.

Mocht u vragen hebben, dan kunt u uiteraard contact met ons opnemen.

Met vriendelijke groet,
Centrale Commissie Dierproeven

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E: info@zbo-ccd.nlDit bericht kan informatie bevatten die niet voor u is bestemd. Indien u niet de geadresseerde bent of dit bericht abusievelijk aan u is gezonden, wordt u verzocht dat aan de afzender te melden en het bericht te verwijderen.
De Staat aanvaardt geen aansprakelijkheid voor schade, van welke aard ook, die verband houdt met risico's verbonden aan het elektronisch verzenden van berichten.

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Van: Info-zbo
Verzonden: dinsdag 4 oktober 2022 07:49
Aan: 5.1 lid2h
CC: 5.1 lid2e
Onderwerp: Aanhouden beoordelen DEC AVD5.1 lid2h 202216420

Geachte 5.1 lid2e ,

Op 23-09-2022 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Immunological health effects of microplastics" met aanvraagnummer AVD5.1 lid2h 202216420

Op 23-09-2022 is uw aanvraag aangeboden aan DEC Utrecht.

De DEC heeft ons laten weten dat in de voorliggende aanvraag informatie ontbreekt waardoor de DEC niet tot een advies aan de CCD kan komen, en de CCD niet kan komen tot de volledige beoordeling van uw aanvraag.

De DEC is van oordeel dat de volgende punten in de aanvraag ontbreken:

De voornaamste oorzaak voor het niet kunnen maken van de afweging is dat de onderzoekers niet duidelijk aangeven wat het voorgestelde onderzoek uiteindelijk oplevert. De aanvraag lijkt meer op een programma dan op een project. Het is de DEC wel duidelijk wat men wil bereiken, maar de aanvraag is onvoldoende uitgewerkt en er is geen duidelijke informatie waarom men denkt dat de voorgestelde benadering haalbaar is.
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De DEC heeft vragen bij de inzet van het humane materiaal en hoe dit verkregen wordt. Vanuit de IvD is vanwege de inzet van alternatieven voor proefdieren hiernaar gevraagd.
De onderzoeker heeft het volgende toegelicht (notulen DEC vergadering):
De DEC heeft de onderzoeker o.a. gehoord over het ontbreken van informatie, het uiteindelijke doel en de rollen en manieren van gegevensuitwisseling van de leden van het consortium: alleen in Utrecht worden dierproeven uitgevoerd, andere centra voeren alleen in vitro onderzoek uit op bijvoorbeeld long- en darmepitheel. Resultaten hiervan kunnen invloed hebben op de huidige aanvraag, bijvoorbeeld over de beste

toedieningsmethode. De DEC ziet dit graag opgenomen in een flowchart met go/no-go's. Verder is niet duidelijk of er door deze groep ook onderzoek gedaan wordt naar monocyten, fagocyten en macrofagen.
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Vragen die de DEC heeft:

Projectvoorstel

- 3.1 Achtergrond: Hoe zit het consortium in elkaar? Wie voert welk deel uit?
- 3.1: Achtergrond: De cruciale rol van macrofagen mist. Wilt u deze nog toevoegen?
- 3.2 Doel: Wat is het uiteindelijke doel van de aanvraag?
- 3.2 Doel: Waarom gebruikt u bij de muis een aantal extra organen ten opzichte van de mens?
- 3.2 Doel: immediate goal A: De DEC mist specifieke go/no-go's. Kunt u wel een richting aangeven waar u heen wilt?
- 3.2 Doel: Het is de DEC niet duidelijk hoe het humane deel van het onderzoek bij het verdere onderzoek past. Wilt u dit onderbouwen in de aanvraag? Daarnaast is het niet duidelijk waar het humane materiaal vandaan komt.
- 3.2 Doel: Wat is de reden dat microplastics niet terugkomen onder 'immediate goal A'? Waarom kiest u voor LPS? Daar is al veel over bekend vanuit eerder onderzoek.
- 3.2 Doel: 'Ultimate goal B' sluit naar mening van de DEC niet helemaal aan bij de bijlage. Hier staat intranasale en orale toediening, terwijl in de bijlagen ook intradermale toediening genoemd wordt. De DEC mist een stappenplan waaruit duidelijk wordt of toedieningen serieel of sequentieel worden toegepast. Wilt u dit nog toevoegen?
- 3.2 Doel: In hoeverre verwacht u dat er in de praktijk aanpassingen gedaan zullen worden als er effecten blijken te zijn bij bijvoorbeeld operaties? Kunt u de uitkomsten relateren aan het uiteindelijke doel?
- 3.2 Doel: Zijn de gebruikte muizen wel microplasticvrij? Microplastics kunnen ook in hun voer of drinkwater zitten. De muizen zijn dan eigenlijk al chronisch blootgesteld aan microplastics.
- 3.2 Doel: Is er een verschil bekend bij u tussen een acute grote toediening van microplastics en een kleinere toediening over de langere termijn?

- 3.4 Strategie: De samenhang in figuur 4 is de DEC niet helemaal duidelijk: Is de uitvoering parallel of sequentieel? Hoe is de blootstelling? Hoe wordt de vertaling naar humaan gemaakt? En kunt u hier go/no-go's toevoegen?
- 3.4 Strategie: De DEC mist een rechtvaardiging voor de intervitale microscopie en ziet deze ook graag ingebed in het flowchart, met go/no-go's en daar bijbehorende criteria.
- 3.4.2: Op basis waarvan worden keuzes gemaakt en door wie worden deze genomen?

Bijlage 1
- A. Experimentele aanpak en primaire uitkomstparameters: Kunt u go/no-go's tussen de vaste en optionele stappen toevoegen?
- A. Experimentele aanpak en primaire uitkomstparameters: De DEC mist hier de 'alignment' met immediate goal A.

De DEC adviseert de CCD de aanvrager te verzoeken de aanvraag grondig te herschrijven met inachtneming van de vragen en opmerkingen hierboven

Bij de besluitvorming van de CCD is het advies van de DEC zwaarwegend. Wij stellen u daarom in de gelegenheid om binnen 6 weken uw aanvraag op de door de DEC beschreven punten aan te passen. De beslistermijn wordt opgeschort tot de dag waarop wij van u de aanvulling hebben ontvangen. Uw aanvraag zal na aanvulling via de CCD opnieuw aan de DEC worden aangeboden.

Wanneer u van mening bent dat de behandeling van uw aanvraag in zijn huidige vorm doorgang moet vinden, zal deze met het oordeel van de DEC, op basis van de ingediende documenten aan de CCD ter besluitvorming worden voorgelegd.

Mocht u vragen hebben, dan kunt u uiteraard contact met ons opnemen.

Met vriendelijke groet,
Namens de Centrale Commissie Dierproeven

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Aanpassingen n.a.v. DEC commentaar: punt voor punt

3.1 Achtergrond: Hoe zit het consortium in elkaar? Wie voert welk deel uit?
Dit is toegevoegd aan het begin van de background en vervangt nu de samenvatting van wetenschappelijke vraagstukken en kennis (wat al bekend en nog niet bekend is over neutrofielen en microplastics). De introductie van de aanvraag is nu beter toegespitst op uitleggen binnen welke lijnen ons onderzoek en onze data bij gaan dragen aan het bepalen van gezondheidseffecten van microplastic blootstelling. Het Nederlandse consortium is multidisciplinair en we werken samen met experts in technieken, organen en model systemen waar wij geen expertise hebben. De samenwerking is nauw en contact is er minstens maandelijks.

3.1: Achtergrond: De cruciale rol van macrofagen mist. Wilt u deze nog toevoegen?
In de aanvraag staat nu duidelijker de rol van macrofagen benoemd, aan het einde van de een-na-laatste alinea van de background (pagina 3). We zijn ons bewust van de rol van macrofagen in ons vraagstuk, en we hopen door onze eigen kennis omtrent deze cellen te vergroten, en door samen te werken met experts in het macrofaag veld een volledig antwoord te geven op hoe het aangeboren afweersysteem op plastic reageert.

3.2 Doel: Wat is het uiteindelijke doel van de aanvraag?
Het doel van de aanvraag is duidelijker beschreven en aangeven aan het begin van sectie 3.2. het doel van de aanvraag is om onderzoek te kunnen doen naar de mogelijke schadelijkheid van microplastics.

3.2 Doel: Waarom gebruikt u bij de muis een aantal extra organen ten opzichte van de mens?
Naar aanleiding van deze vraag hebben we de figuur duidelijkere tekst gegeven (fig. 2&3). De organen voor de mens geven aan waar we in beperkte mate beschikking tot hebben om te proberen wetenschappelijke vragen te beantwoorden. Dit laat zien dat de muizen ons mogelijkheden bieden om het gevaar te analyseren die we mensen niet hebben. Maar waar we kunnen, zullen we altijd muis met mens vergelijken.

3.2 Doel: immediate goal A: De DEC mist specifieke go/no-go's. Kunt u wel een richting aangeven waar u heen wilt?
Het antwoord op de go/no go's staat duidelijker beschreven op pagina 7, verder is het belang van immediate goal A verduidelijkt op pagina 5 en 6. De richting die we op willen is begrijpen hoe neutrofielen reageren op natuurlijke binnendringers, en dat vergelijken met "onnatuurlijke" binnendringers zoals plastic.
Voor dit deel van de aanvraag kunnen we geen specifieke go/no-go's opstellen, anders dan als een andere onderzoeksgroep in de wereld de vraag al beantwoord heeft. Dit deel gaat over basis biologie van neutrofielen, vragen die onbeantwoord zijn en niet met elkaar in verbinding staan. Dat maakt het niet mogelijk om overkoepelende go/no-go's voor dit deel op te stellen. Uiteraard zal in de specifieke werkprotocollen voor deze experimenten wel geprobeerd worden go/no-go's (bijvoorbeeld op basis van tijdspunten) te implementeren. Dit zal wel doenbaar zijn omdat die experiment-specifiek van toepassing zijn.

3.2 Doel: Het is de DEC niet duidelijk hoe het humane deel van het onderzoek bij het verdere onderzoek past. Wilt u dit onderbouwen in de aanvraag? Daarnaast is het niet duidelijk waar het humane materiaal vandaan komt.
Deze vraag hangt samen met de verwarring rond figuren 2&3 (eerder beschreven). Op pagina 5 is nu toegevoegd: *We are only able to get donated human blood on a regular basis, but we need vital organ material to fill in the knowledge gaps. If we are lucky, we are occasionally able to get spleen and bone marrow material from surgeries. But this material is too limited to answer the questions that remain.* Om de relevantie van de muisexperimenten aan te tonen en te onderbouwen, willen we waar we kunnen vergelijkbare experimenten doen met menselijk materiaal. Helaas kunnen we dus alleen sporadisch en in zeer beperkte mate krijgen.

3.2 Doel: Wat is de reden dat microplastics niet terugkomen onder 'immediate goal A'? Waarom kiest u voor LPS? Daar is al veel over bekend vanuit eerder onderzoek.
Nog niet alles is bekend over de reactie van neutrofielen op LPS. Aan het einde van pagina 6 staat nu verdere uitleg over hoe LPS dient als model voor infectie, en hoe het als controle van natuurlijke infectie/invasie dient voor microplastics vragen.

3.2 Doel: 'Ultimate goal B' sluit naar mening van de DEC niet helemaal aan bij de bijlage. Hier staat intranasale en orale toediening, terwijl in de bijlagen ook intradermale toediening genoemd wordt. De DEC mist een stappenplan waaruit duidelijk wordt of toedieningen serieel of sequentieel worden toegepast. Wilt u dit nog toevoegen?
Naar aanleiding van deze vraag is figuur 4 toegevoegd (pagina 11). Wij hopen dat daaruit duidelijk wordt dat intranasale en orale toediening het belangrijkst zijn, omdat dat ook onze blootstelling is. De intradermale toediening wordt alleen gedaan als intravital imaging nodig is om een mechanisme goed te analyseren. Dat gaat nu eenmaal het makkelijkst in de huid: makkelijk toedienen, en vereist geen verdere operaties of zoiets dergelijks om te kunnen imagen met de microscoop.

3.2 Doel: In hoeverre verwacht u dat er in de praktijk aanpassingen gedaan zullen worden als er effecten blijken te zijn bij bijvoorbeeld operaties? Kunt u de uitkomsten relateren aan het uiteindelijke doel?
Wij waarderen deze vraag omdat we zelf ook als doel hebben om hier verder naar te kijken en toekomstige beurzen op te schrijven. Voor nu hebben we in deze aanvraag daar beperkte proeven voor in staan (animal procedures bovenaan pagina 7) om preliminaire data te genereren. Het grote merendeel van de experimenten in deze aanvraag is in het kader van ons 5.1 lid1c consortium om de effecten van microplastics uit de omgeving te bestuderen.
Onze insteek voor medisch relevante proeven zijn iets anders dan gesuggereerd in deze vraag. De effecten van grote stukken plastic zoals catheters of hartkleppen vinden we erg interessant. De data van onze consortium partners waar gekeken wordt naar de biofilm op plastics zijn hierbij relevant. We zijn beginnende samenwerkingen gestart met 5.1 lid2e om antilichamen te testen die neutrofielen kunnen aantrekken naar de biofilm op catheters. Maar de vraag is wat er gebeurt als de neutrofielen dan ook de plastic tegenkomen! Verder zijn we een samenwerking gestart met 5.1 lid2e om antibacteriële coatings te testen voor protheses. Ook hier is de vraag wat voor effect de protheses zelf hebben. Als de neutrofielen dood gaan als ze in

contact komen met hartkleppen of protheses kan dit ook een oorzaak zijn waarom bacteriën zo goed groeien op deze oppervlakten. We hebben ook beurzen geschreven om te onderzoeken of microplastics een verhogend inflammatoir effect hebben op chronisch inflammatoire ziekten zoals astma en IBD (niet in deze aanvraag). Tenslotte zijn we een beurs aan het schrijven om te kijken of er ook microplastics vrij komen in bloedtransfusie zakken en dialyse slangen. Wellicht zijn bepaalde materialen meer of minder activerend voor het immuun systeem en kunnen er gerichte keuzes gemaakt worden.

3.2 Doel: Zijn de gebruikte muizen wel microplasticvrij? Microplastics kunnen ook in hun voer of drinkwater zitten. De muizen zijn dan eigenlijk al chronisch blootgesteld aan microplastics.
Het klopt dat muizen hun hele leven doorbrengen in plastic, en dat dat een waarschijnlijke en onvermijdelijke contaminatie is. Dit is uiteraard ook het geval bij het te modelleren doeldier 'de mens'. Op pagina 7, bij de beschrijving van immediate goal B, wordt dit probleem en een redelijke oplossing besproken. Door het gebruik van speciaal gemaakte fluorescente partikels kunnen we deze (additioneel) toedienen en volgen in de cellen en in het lichaam, en kunnen we de reactie van het afweersysteem in de muizen wel analyseren.

3.2 Doel: Is er een verschil bekend bij u tussen een acute grote toediening van microplastics en een kleinere toediening over de langere termijn?
Nee dit is nog niet bekend en een van de dingen die we verder willen uitzoeken in dit project, vandaar dat deze vraag op pagina 2 al in de lijst staat met vragen die we willen beantwoorden.

3.4 Strategie: De samenhang in figuur 4 is de DEC niet helemaal duidelijk: Is de uitvoering parallel of sequentieel? Hoe is de blootstelling? Hoe wordt de vertaling naar humaan gemaakt? En kunt u hier go/nogo's toevoegen?
Figuur vier is aangepast om te laten zien hoe het merendeel van de experimenten parallel kan worden uitgevoerd, en alleen een klein deel sequentieel wordt uitgevoerd als daar noodzaak voor is. Ook de blootstelling en de mogelijke go/no-go's staan erin verwerkt.

3.4 Strategie: De DEC mist een rechtvaardiging voor de intervitale microscopie en ziet deze ook graag ingebed in het flowchart, met go/no-go's en daar bijbehorende criteria.
Ook dit hebben we in figuur 4 verwerkt.

3.4.2: Op basis waarvan worden keuzes gemaakt en door wie worden deze genomen?
Wel overwogen keuzes gebaseerd op onze uitkomsten en die van onze consortium partners worden gemaakt door 5.1 lid2e .

Bijlage 1
A. Experimentele aanpak en primaire uitkomstparameters: Kunt u go/no-go's tussen de vaste en optionele stappen toevoegen?
Figuur 1 is toegevoegd op pagina 2 om een duidelijker beeld te geven

A. Experimentele aanpak en primaire uitkomstparameters: De DEC mist hier de 'alignment' met immediate goal A.
Verder uitgeschreven op pagina 1 van de Handelingen bijlage.

Centrale Commissie Dierproeven

Form
Project proposal

- This form should be used to write the project proposal for animal procedures.
- The appendix 'description animal procedures' is an appendix to this form. For each type of animal procedure, a separate appendix 'description animal procedures' should be enclosed.
- For more information on the project proposal, see the Guidelines to the project licence application form for animal procedures on our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1 Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	5.1 lid2h
1.2 Provide the name of the licenced establishment.	5.1 lid2h
1.3 Provide the title of the project.	Immunological health effects of microplastics

2 Categories

2.1 Please tick each of the following boxes that applies to your project.

- ☒ Basic research
- ☐ Translational or applied research
- ☐ Regulatory use or routine production
- ☒ Research into environmental protection in the interest of human or animal
- ☐ Research aimed at preserving the species subjected to procedures
- ☐ Higher education or training
- ☐ Forensic enquiries
- ☐ Maintenance of colonies of genetically altered animals not used in other animal procedures

3 General description of the project

3.1 Background

Describe the project (motivation, background and context) with respect to the categories selected in 2.1.

The aim of this project is to understand the health risk posed by microplastics that we are exposed to on a daily basis. We work in both a Dutch and European consortium to look at all aspects of the risk of plastic exposure. The answers we find will guide the government in implementing stricter rules for plastic, based on how dangerous they are for our health.

Microplastics are everywhere in our environment, in our food, our water and in our air (see https://youtu.be/YOEwRkbgm4A). We are exposed to them in a more sterile form from sources like cosmetics and food packaging, but plastics in the environment are an attractive substrate for pathogens like bacteria to grow on and viruses to stick to. This biofilm of bacteria and viruses on the plastics can harbor antibiotic resistant bacteria[1,2]. These microplastics pose new challenges to our immune system and immune cells. Immune cells are the cells that deal with situations in our bodies like invasion of foreign intruders (e.g. bacteria, fungi, and all sort of foreign particles, probably including plastics), but also problematic cells. Researchers from Amsterdam have found microplastics in our bloodstream[3]. Unfortunately, they could not determine if the plastics in the bloodstream were free-floating, or were taken up by immune cells. We have done preliminary mouse work where we injected green fluorescent plastics, and found that the majority of plastics accumulated in a few specific organs. We saw that the plastics were taken up by immune cells, and we were also able to determine which immune cells take up the most plastics. So we know that immune cells indeed interact with the plastics, but we miss a lot of information on how exactly immune cells and plastics interact and what health effects this could have.

We need to determine:

- whether humans are able to clear the plastics after they cross into our bloodstream, or
- whether they accumulate in our organs, in and between our cells, over our lifetime.
- whether the plastics that remain in our bodies result in chronic or acute inflammation, act like asbestos fibers and link to cancer.
- whether plastics made of different chemical compounds have different effects on our body, and what the size limitations of plastics for crossing different epithelial barriers are.

The 5.1 lid1c consortium 5.1 lid1c funded by our government, connects Dutch research groups that are experts in their field to investigate plastics and their risk to our health. This large research question is divided in parts as indicated below (fig.1). We are the group that will perform all animal experiments in WP3 and WP4: exposure assessment and hazard assessment. The information from the experiments done by us and the others will contribute to each other's findings and into risk assessment and developing solutions. In WP3 and 4 we work together with other experts on innate immune cells, and experts in specific tissues like the lungs/brain barrier/placenta barrier/intestines. We are the experts concerning neutrophils. (all workpackage leaders and research partners can be found on the 5.1 lid1c website). Because we collaborate with experts in such diverse subjects, we get the most out of our mouse experiments. Together, we can analyze all parts of the mice from our experiments.

5.1 lid2f

Figure 1. Schematic representation of the structure of the 5.1 lid1c *consortium. The big question of the risk of plastic exposure is divided in smaller questions. For each sub question a group of experts is assembled to get a broad perspective. All parts communicate to come to a risk assessment and come up with solutions for plastics exposure. (source* 5.1 lid1c

All laboratories use plastics for all procedures and analyses, and so plastics contamination is everywhere. The researchers in Amsterdam built a lab specifically with only glass and metal to prevent this contamination in their experiments. Our solution to this problem of contamination from the environment is the use fluorescent particles provided by 5.1 lid1c consortium partners, because they are clearly distinguishable from contamination.

Since we are examining the active uptake of plastics in our body and the response of the immune cells, we need to work with live material. Dead human material will not answer the questions stated below. Therefor we need to perform mice experiments to determine the health risk. **The questions that need to be answered are questions that unfortunately cannot all be resolved with *in vitro* systems.** Because testing plastics on one type of immune cell at the time will not show the full immune response. And testing plastics on an artificial gut epithelial lining, we will miss important other cells that have an effect on plastic uptake, because the gut is a complex organ. Also in order to study the dynamics of plastics in the body in a way that is doable, we need to use fluorescent plastics for many of the questions, and just testing blood of human volunteers will not give us those answers.

So far, we have done what we can to investigate the effects of microplastics. We have isolated human immune cells from willing donors and exposed them to commercially bought/made microplastics. These are perfect spheres of one size and one material. This preliminary data showed that immune cells can eat the commercial plastics, but this can cause them to die. This seems to depend on the size and amount of plastics. Consortium partners have been doing comparable preliminary experiments for the other tissuesmentioned before. Real-life plastics can be of various materials and can be of very different shapes, influencing their effects on cells. We have access to unique plastics produced by 5.1 lid1c that resemble what we are exposed to daily, which are currently being tested. Results from us and our partners concerning these 5.1 lid1c plastics will determine what plastics need to be tested in mice. For example, if we and our partners don't find negative effects from a certain plastic *in vitro*, it will not need to be tested *in vivo*.

In the consortium we are the experts on neutrophils, and as the most abundant white blood cell, neutrophils are the most likely cells to interact with microplastics after they have entered our bloodstream. But even we don't know everything yet about neutrophil biology. For example, we identified different neutrophil subsets and have an idea of their role in different disseases[4-8]. But we still need to understand where and how neutrophils change into these subsets, and when they come into action. Neutrophil functioning depends on a network of signals and interactions. This network contains everything from the bone marrow, to the spleen as a potential neutrophil reservoir, blood vessel endothelium, sites of inflammation, as well as neutrophil interactions with foreign invaders and other immune cells. We are not able to get enough or even any human material at all to study these processes, and *in vitro* systems lack the complexity of all the interactions and signals. This is information needed to fully understand the effect of plastics. <u>Therefore, a small part of this project concerns expanding basic neutrophil knowledge</u>. <u>This is necessary to fully understand the impact microplastics exposure has on our system: do the neutrophils respond to plastics as they do to trauma, a bacterial infection, or maybe a chronic inflammation?</u>
Neutrophils respond much faster than other phagocytes, but they are not the most effective cleaning crew of our immune system. Those would be the macrophages. It takes time for monocytes to arrive at the site of the problem, and then differentiate into a functional monocyte-derived macrophage. Monocytes and macrophages are cell types that we only recently started working with in this project, because of how important they are. But we are not yet experts on these cell types, so this will be a learning opportunity for us to learn from our consortium partners that are experts on these cells. By combining our expertise in mouse experiments and neutrophils, with their expertise in other phagocytes, we hope to provide a complete picture of the immune system's response to microplastics.

In this project we aim to work on filling in the knowledge gaps of neutrophil biology (immediate goal A), and determine the health effects of microplastics by investigating microplastics dynamics in a mammalian system and the response of the immune system to the plastics (immediate goal B)(further explained in the next section). **Combining the information from healthy mice, mice treated with plastic and mice with acute inflammation such as bacterial infection in this research project will help us to put the immune systems' reaction to microplastics in the right perspective**.

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2. Guo X., Sun X., Chen Y., Hou L., Liu M., Yang Y. Antibiotic resistance genes in biofilms on plastic wastes in an estuarine environment. Sci Total Environ. 2020 Nov 25
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5.1 lid2e, 5.1 lid2h

3.2 Purpose

3.2.1 Describe the project's immediate and ultimate goals. Describe to which extent achieving the project's immediate goal will contribute to achieving the ultimate goal.

- If applicable, describe all subobjectives

As stated before, the goal of this project is to determine the health risk of microplastics exposure, this so the government can make informed decisions on plastic regulations. This is primarily aimed at single use packaging used in daily life. Future research will build from there and look more into specific situations of large amounts of plastic used, like in health care, where it might be more difficult to switch to plastic-free.

5.1 lid1c has the goal to determine the health risk of the different types of plastics we are exposed to daily, looking into the effects of different materials/polymer types, sizes and shapes. This goal together with our expertise and interest in the immune system gives us the **ultimate goal of this proposal**: **determine the health risk of plastics exposure, based on the plastics' potential to cross epithelial linings, their dispersion throughout the body, the immuno-toxicological effects, and their persistence in the body (fig 1).**

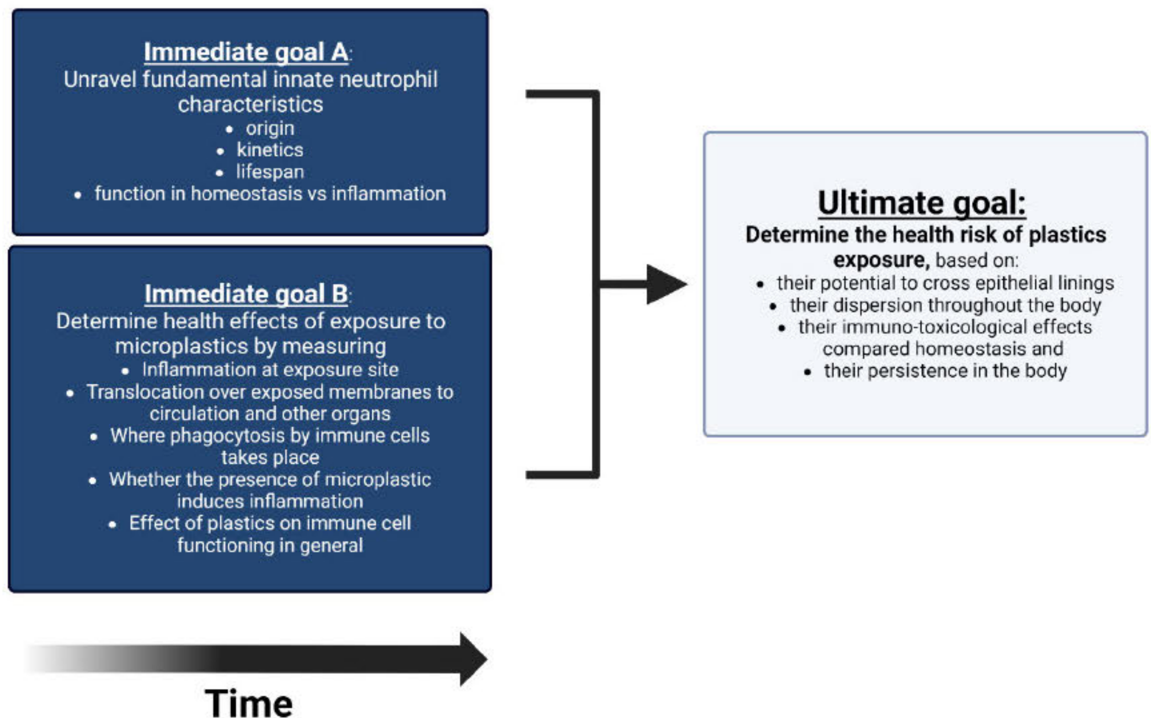

Figure 1. Schematic of the main and sub goals of this proposal. Sub goal A is a more fundamental question about neutrophil biology with specific aspects listed underneath it that we would like to address in this research. These basic aspects need to be understood to understand the full scope of the outcome of sub goal B. Sub goal B is a more translational question about the effects of plastics exposure with its relevant parameters that will be looked at underneath. The sub goals are not dependent on each other for the experiments that need to be performed. In most cases the two sub goals can be worked on simultaneously, with experimental conditions for sub goal A also functioning as controls in experiments for sub goal B. (Created with Biorender.com)

In order to reach our main goal, we subdivided it in two sub goals (fig1). Because in order to understand the effects of plastics on neutrophils, we first need to know what is normal for neutrophils. Therefore the **first immediate goal (A) is: fill in the gaps in fundamental neutrophil biology, namely their origin, kinetics, lifespan, and their functioning in homeostasis versus acute or chronic inflammation.** We hypothesize that the neutrophils will be the first to respond to the presence of plastic in our body, followed by other immune cells to continue the immune reaction. However, many basic biological concepts of neutrophils are still partially or completely unknown, because human material is limited and mouse experiments haven't been done extensively enough yet (fig 2). We are only able to get donated human blood on a regular basis, but we need vital organ material to fill in the knowledge gaps. If we are lucky, we are occasionally able to get spleen and bone marrow material from surgeries. But this material is too limited to answer the questions that remain.

In mice we are able to look at these important neutrophil compartments extensively enough. Understanding neutrophil biology better and how neutrophils respond to natural ques like infection and inflammation gives us a frame to understand neutrophils reaction to microplastics. This will also help determine the health risks of microplastics. This is basic knowledge we need to fully understand the toxicity of plastics.

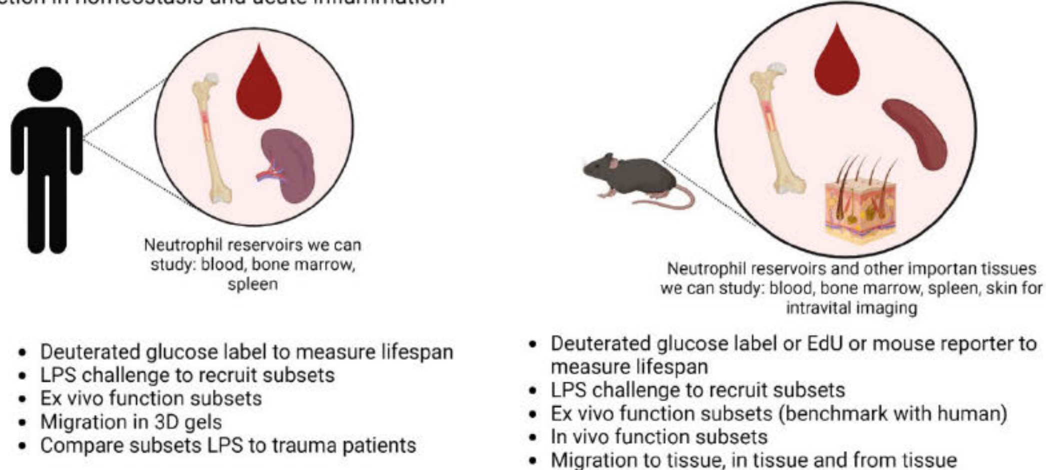

Figure 2. Schematic clarification of the experiments that can be or have been performed in human (left) , and the experiments that can be or have been performed in mice (right). In mice we have easier access to all relevant tissues. For humans only blood is easily available to us, in rare occasions we can investigate spleen and bone marrow material from operations. These experiments will answer the questions posed for immediate goal A. (Created with Biorender.com)

Immediate goal A : Unravel fundamental neutrophil characteristics such as origin, kinetics, lifespan and function in homeostasis and acute inflammation
Sub questions for this immediate goal:

1. Determine the lifespan of neutrophil subsets by labeling the neutrophils with a label that shows their time spent in the body (e.g. deuterated glucose or EdU) and whether the production and lifespan changes upon an acute inflammation (induced by LPS).
2. Determine the origin of neutrophil subsets (local at the site of action, or distant like bone marrow or spleen) by i) harvesting neutrophil subsets from different organs during homeostasis or after an acute inflammation (LPS or injected bacteria such as but not limited to staphylococcus, epidermidis and pseudomonas*); and by ii) visualizing neutrophil migration with intravital microscopy. Determine the kinetics of different neutrophil subsets throughout the body by i) labeling the neutrophils present in the blood (e.g. using a CD45 antibody or neutrophil subset specific antibody) and following in time (by intravital microscopy and ex vivo analysis) when they leave the blood vessels and enter the tissues; and ii) by recruiting neutrophils to a tissue via a local sterile injury and subsequently monitor if these revert back to the blood vessel upon a systemic infection evoked by LPS (terminal experiment).
3. Establish the function of different neutrophil subsets by analyzing the response to injected bacteria such as but not limited to staphylococcus, epidermidis and pseudomonas*

*We have extensively used staphylococcus in human neutrophil *in vitro* assays but we have also experienced mouse neutrophils hardly kill this pathogen. Epidermidis are more easily killed by mouse neutrophils. Currently 5.1 lid1c is conducting experiments to establish which pathogens are mainly found on plastics. A first candidate is pseudomonas, but other relevant bacteria might be added in the future.

Firstly, we use LPS in these questions to mimic a bacterial infection or sepsis, without actually injecting the bacteria. It has the same effect though, but it is more controlled than injecting bacteria. In order to answer the questions of immediate goal A, this condition of LPS injection (mimicking a bacterial infection) will be compared to homeostasis. This way we hope to find answers to the questions about basic neutrophil biology that are still unanswered.
Secondly, LPS administration will function as a positive control for the plastics administration. This way we can compare the two and determine if plastics induce the same immune response as bacteria. But all questions concerning microplastics are formulated in immediate goal B, because the structure for that

research is slightly different. Which controls and conditions are compared in each experiment will be explained and specified further down (fig.5) and in the "Description Animal Procedures" (fig 1&2).

Immediate goal A go/no go: another group answers one or more of these questions, then we don't have to try to answer those questions.

This immediate goal A doesn't have specific go/no go's, because these are independent fundamental questions. They are not connected to each other in carrying out the experiments, and so they do not dependent on answering one to continue to the other. Of course if we are uncertain about any aspect, experiments will not be started.

- -

Immediate goal B focusses on the microplastics: determine the health effects of exposure to different microplastics (fig.3). In this part we will investigate the dynamics and effects of plastics: which polymers are taken up, and by gut and/or lung epithelium, does size matter, does shape matter, does dosage matter, where do they go, and what effect do they have there. Most questions concerning the dynamics and risk of plastic exposure have not been answered yet, or are answered only with machine made, perfectly round and smooth polystyrene particles that are coated with specific chemicals. With these plastics, we have done oral exposure and saw that 10 days is necessary to have enough plastics in the mouse in order to find a handful back in the blood. From injecting the microplastics intravenously we know that they accumulate in the liver spleen, and a bit in the bone marrow.
The polystyrene particles do not resemble the plastics we are exposed to, not in size, shape, polymer or coating. The microplastics we are about to test are made from a selection of the most used plastics in our daily life, to resemble our daily exposure. They are also made fluorescently green, so we can find them back with relative ease anywhere in the mouse. This way we can distinguish the intentionally administered plastics from the standard daily exposure plastics in the mice (a contamination that is impossible to prevent, because everything in the mouse stables is plastic or packaged in plastic). These fluorescent microplastics administered to mice will show us when, where and how they get into our system, and the effects they will have on our health.

For this goal the go/no go questions are:

- Only plastic polymer types found to have an effect *in vitro* on human neutrophils (for example because they are taken up by them, they induce cell death or the release of inflammatory molecules) will be tested *in vivo*.
- If the majority of our consortium partners finds no effect of a certain plastic in their *in vitro* studies, they will not be tested in mice.
- Endothelial exposure & translocation experiments will be performed first with (polystyrene) particles of well characterized sizes. Size ranges of particles that don't cross the barriers will not be tested further and can be excluded, because only particles that cross the barrier are physiologically relevant for answering the other questions.

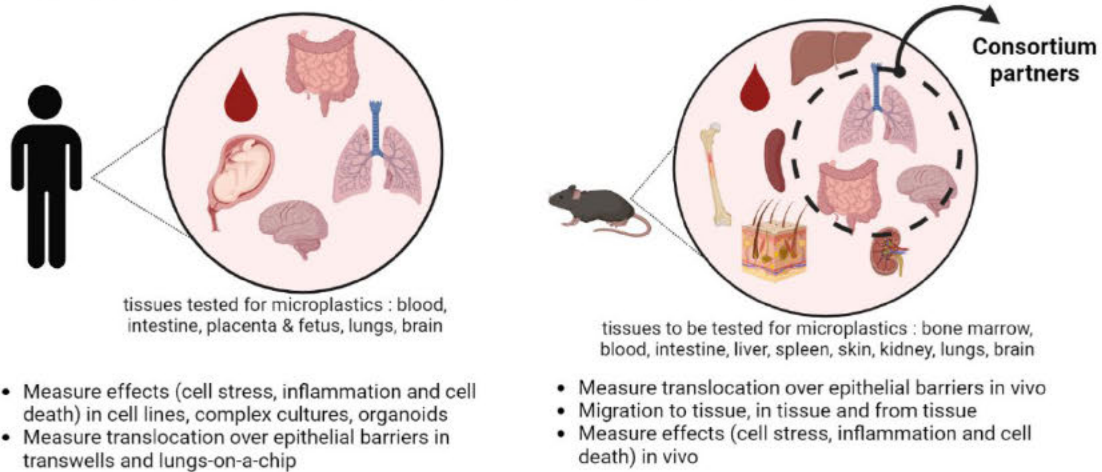

Figure 3. *Schematic clarification of the experiments that can and are performed in human (left), and the experiments we have been performing and intent to perform in mice (right). These experiments will answer the questions posed for immediate goal B. tissues within the inner circle will be offered to consortium partners to resolve their research questions within the consortium. (Created with Biorender.com)*

Immediate goal B: Determine the health effects of environmental exposure to microplastics
Subquestions

1. Establish whether oral administration or inhalation of microplastics results in inflammation at the site of most-likely epithelial transfer (gut for oral, lung for inhalation)
2. Determine whether oral administration or inhalation of microplastics results in translocation to the circulation and to other organs like liver, spleen and kidneys
    a. Trafficking between organs in time
    b. Role of immune cells in transporting microplastics throughout the body
3. Determine if the presence of microplastics in blood or tissues induces inflammation
4. Determine if phagocytosis (engulfment of microplastic particles by immune cells) occurs in the bloodstream and/or in tissues
5. Determine the effect of (micro)plastics on immune cell function on:
    a. Survival
    b. Migration
    c. Bacterial killing of for example staphylococcus or pseudomonas family members
6. Compare the effects of microplastics to other inflammatory stimuli, in order to place the effect in the right perspective

<u>Although the answers from immediate goal A will add context to the answers of immediate goal B, experiments for both can be performed simultaneously, and a potential delay in answering the questions of A does not necessarily inhibit answering the questions of B.</u> The health risk of microplastics doesn't solely depend on their effect on neutrophils, even though they are high in number and quick to respond. The health risk is not only depending on our neutrophils findings, we will also gather data on the other phagocytic and primary immune cells. These outcomes will be discussed with experts that are part of the 5.1 lid1c consortium to make the complete picture of exposure and risk.
Our project is partly observational and any of the evidence collected, positive or negative, will answer open toxicological questions for which answers are at the moment not available. A potential negative result (in the presence of positive controls) will not undermine our project but rather provide the public, government and companies with (reassuring) experimental evidence that is currently lacking.

3.2.2 Provide a justification for the project's feasibility.

In healthy mice, mice treated with plastic and mice with acute inflammation, we will analyze the phenotype and function of the immune cells *ex vivo*, crucially supported by analysis of the kinetics of immune cells *in vivo*. *Ex vivo* analysis is aided by our longstanding experience with flow cytometry and assays on neutrophil function (migration, phagocytosis, ROS formation, degranulation, etc.). In the kinetic studies we will examine

the distribution of immune cells by *ex vivo* analysis of the neutrophils in different organs, as well as the migration of neutrophils by intravital imaging. In these intravital imaging experiments, neutrophil migration is easily tracked in mice that produce fluorescent neutrophils such as the LysM-GFP or the Catchup^{IVM} mouse [9, 10].

Until now we and others in the microplastic field have used polystyrene perfect spheres of an exact size bought from a company. During this fabrication process a coating is formed on the plastic spheres that is not present on plastic we are exposed to daily. Currently we are testing the more environmentally relevant microplastics provided by our consortium partners *in vitro* with human immune cells. Real life plastics of 3 different plastic materials were milled to better reflect microplastics in the environment. Disadvantages are that the size ranges are broad and we don't have a lot of material compared to the fabricated polystyrene particles.

5.1 lid2f

These data demonstrate we have the experimental expertise to perform the project and also urge the research proposed in this project.

There are several other reasons why we are confident that we can achieve our aims: Our group is embedded in 5.1 lid1c . Since the clinic is very close and our medical PhD students closely collaborate with clinical doctors, we can obtain patient material for research. The complementary use of patient material and well-defined animal models will ensure the successful completion of this project. 5.1 lid1c provides core facilities for various high-end techniques such as histology, fluorescent confocal imaging, intravital imaging and flow cytometry. Moreover, the animal facility offers dedicated staff providing the regular housing of the animals and support and counsels the scientist in their experiments. Within our group, only trained and experienced people perform experiments.

Our research and experiments are constantly evaluated within our group, and by various other groups within our institute and campus. To aid our research on microplastics, we collaborate with experts from different fields in the 5.1 lid1c and 5.1 lid1c consortia. Over the last few years, we have built up a repertoire of state-of-the-art *in vivo* imaging techniques to study immune cells in living mice. This has led to many new discoveries and breakthroughs published in scientific journals[11-17]. Our research is funded by major funding agencies. Our embedding in an excellent scientific environment, our unique techniques and approaches, and our previous achievements make it very likely that with the experiments described in this project we will make large contributions to our main research questions.

References

9. *Hasenberg, A. et al. Catchup: a mouse model for imaging-based tracking and modulation of neutrophil granulocytes. Nat. Methods* ***12****, 445-452 (2015).*

10. *Peters, N. C. et al. In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. Science* ***321****, 970-974 (2008).*

5.1 lid2h, 5.1 lid2e

3.2.3 Are, for conducting this project, other laws and regulations applicable that may affect the welfare of the animals and/or the feasibility of the project?

☒No

☐Yes > Describe which laws and regulations apply en describe the effects on the welfare of the animals and the feasibility of the project.

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3.3 Relevance

3.3.1 What is the scientific and/or social relevance of the objectives described above?

Complete understanding of neutrophil biology (immediate goal A) can help us not only understand the reaction to microplastics (immediate goal B), but also understand the neutrophil reaction to infection and inflammation, acute and chronic. This knowledge can help treat major trauma, cancer and asthma patients better. Understanding neutrophil biology will have both scientific and societal relevance.

Understanding the bodies reaction to exposure of particles like plastic (immediate goal B) has both scientific and societal importance. Preliminary results of research done by others has shown the presence of microplastics in our own bloodstream. From the bloodstream they end up in our organs. It is unlikely that these foreign particles will be ignored by our immune cells. To date there is no hard evidence for hazards of microplastics to our health. Thus, we have no clear indication whether exposure needs to prevented. This multi disciplinary study can be used as a guide for future research on particle exposure, hopefully reducing the experiments necessary for future questions. The societal relevance lies in the problem that we are constantly exposed to particles of which we have no idea what it does to our health. **Giving conclusive evidence whether plastic polymer type, size, shape or amount matters in the negative health effects makes it easier for government officials to install laws to limit this**, like already has been done for smoking.
We are part of a Dutch consortium called 5.1 lid1c which was set up with aid of the government because it was recognized how **worryingly little is known about the health effects of microplastics**. Within this consortium, plastics found in our environment, food and water will be tested in/on all important tissues: the lungs, the gut, the placenta, the brain, and of course the immune system. **If these plastics are detrimental to our health, regulations should be put in place in regards to the production, usage, and recycling and disposal of those plastics**.
We therefore aim to characterize if microplastics can induce inflammation and if so what important characteristics are and at what dose.
In addition to lack of hazard data, citizens generally do not see the microplastic pollution that is caused by their use of plastics, nor are they aware of the negative environmental and health effects of microplastics. The result is that there is little incentive for citizens to use plastic-free products or to limit plastic waste through their consumption practices. **Knowledge on plastic health effects leads to awareness, and is an essential step to empower the public to make changes in their households, demand changes from industry, and demand action from political representatives**.
Our work will also have impact on science performed in the industrial setting. We collaborate with the textile industry (Inditex) who study machines that capture fibers from textiles before they leave the factory, thereby preventing shedding during daily use by citizens. In addition, they can design and test textiles that shed fewer fibers to begin with. In addition we work with air filter companies who might reduce microplastic concentrations in the household.

3.3.2 Who are the project's stakeholders? Describe their specific interests.

Mice are the obvious first stakeholders. They are undergoing scientific experiments beyond their own control. Their interests are the three Rs: Replacement, Reduction and Refinement (further defined in "Description animal procedures").

For immediate goal A the stakeholders are patients who need acute care like trauma patients at risk of developing life-threatening infections, chronic patients with for example asthma who need immune system related therapy their whole life, and also cancer patients that have neutrophils involved in the progression of their disease.

For immediate goal B in principle the whole human population is a stakeholder. We are all exposed to microplastics. The environment as a whole can also be considered a stakeholder, because if we find effects on our health and the health of mice, obviously other animals will be affected. The industry producing plastics and plastic alternatives are stakeholders too. **The findings in our research can lead to legislation,**

potentially banning the use of certain plastic types for certain products or altogether. We perform unbiased and independent research, and report our progress and results to ZonMW as well as publish in internationally acknowledged journals

We fully intend to capitalize on the networks that we have through multiple consortia as needed to achieve our scientific objectives and to amplify our societal impact. These can also be considered stakeholders. For example, we collaborate with the 5.1 lid1c . Achieving societal changes based on our research findings will require the engagement and inclusion of the communities in which we conduct our research, patient organizations, industry players, local and national governing bodies, and non-governmental organizations (NGO). The consortia we take part in have representation of these communities and parts of society.

Longfonds is very interested in the outcome of our study as microplastics enter our body through inhalation. Their network and their outreach activities will help to spread awareness about microplastics and their lobbying network will help to involve government officials in our fight against microplastics.

The **Plastic Soup Foundation** is an international organization that focuses entirely on reducing plastic pollution. Their involvement will help to ensure that our findings are shared widely with the general public (including school children), as well as via their vast network of industrial, political and scientific stakeholders. They are also looking into practical solutions.

We are currently trying to acquire funding for a collaboration with the citizen network, **Onzelucht.nl**. This network consists of thousands of households to measure outdoor air quality, providing raw data to 5.1 lid1c working together with local governments, in schools and with both rural and urban communities.

Additional regulatory organizations, like the Dutch ministry of Infrastructure and Water Management, Science Advice for Policy by European Academies (SAPEA) and the World Health Organization (WHO) will also be important to reach out to as we expand our networks and disseminate our findings.

3.4 Strategy

3.4.1 Provide an overview of the overall design of the project (strategy). If applicable, describe the different phases in the project, the coherence, the milestones, selection points and decision criteria.

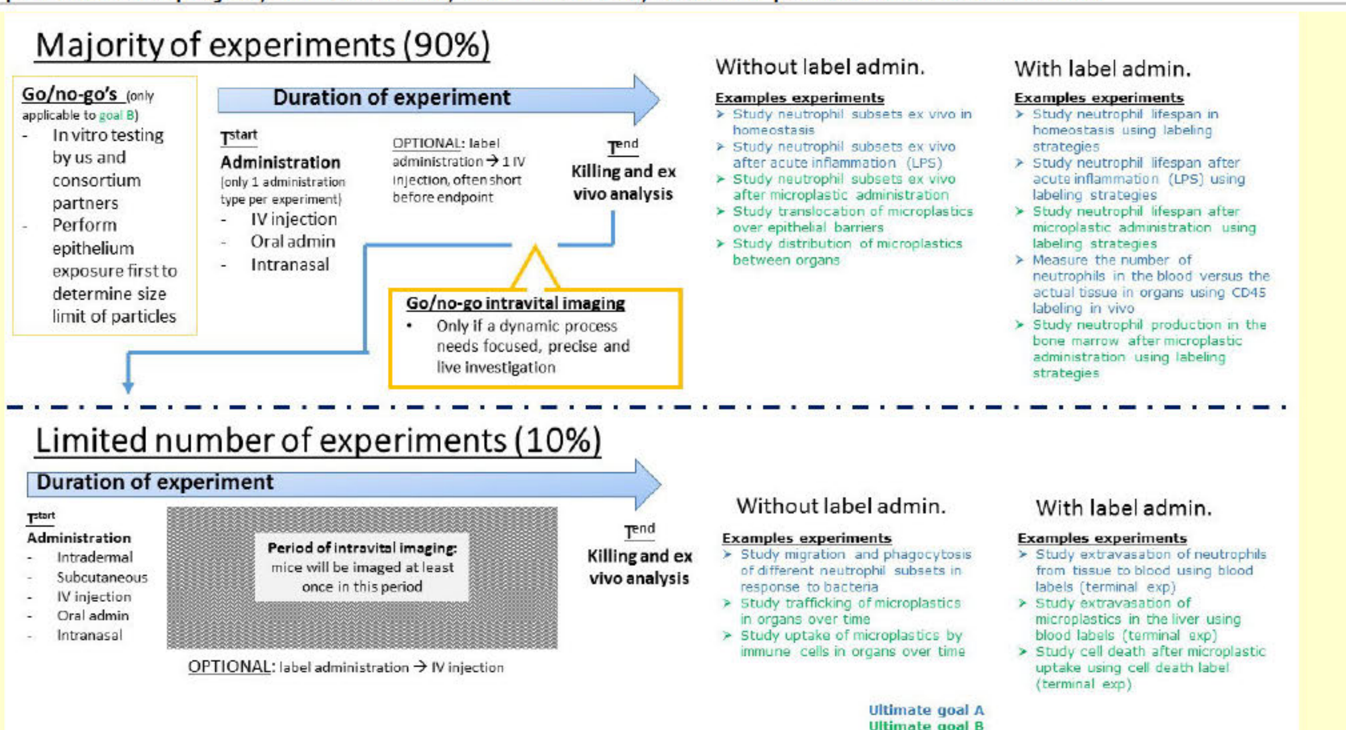

Figure 4. schematic overview of the general designs of the experiments and the questions they will answer. Questions are color-coded to indicate whether they relate to immediate goal A (blue) or B (green). Goal A has no initial go/no-go's, and so the go/no-go's in the top left are only applicable for immediate B. As indicated will the majority of the experiments be

performed in a simple set-up: one route of administration, with only the option of label administration (like a CD45 antibody). Only if the results from these experiments raises questions about specific details of the dynamics, will intravital imaging be performed. This also shows how the two immediate goals can be worked on in the same experimental set-up without interfering with each other.

The strategy of this project is to combine human *in vitro* experiments with murine *ex vivo* and *in vivo* experiments. The readout of each animal experiment will consist of some or all of the following:

- descriptive data on single cell level (flow cytometry, microscopy, protein expression, etc.)
- functional data (on specific functions of the neutrophil such as migration, phagocytosis, etc)
- intravital microscopy data
- descriptive data on disease progression (e.g. weight loss, microbial load).

The experimental set-ups and readout parameters will be the same for all experiments in this proposal (fig. 4), but the immune stimulating action or compound will differ per experiment (fig.5). Naturally, the different types of plastic particles will need to be tested by itself, or in combination with another action or compound, as stated below. But also plastics with biofilm as explained earlier will need to be tested, because that is the status of plastics we would find in the environment. In mice we are also able to do intravital imaging, where we can look in a live mouse and see the real time neutrophil response. But this is labor intensive, so it will only be performed if the results from the exposure require more elucidation.

One hypothesis is that characteristics like the surface texture, shape, and chemicals bound to the surface of the plastics will determine the response of our system to the plastics. As a control for these characteristics we will take along an equally non-degradable particle that is of a different material than plastic.
The act of injection makes a wound, which in and of itself is an activating signal for neutrophils. Therefore we need to take a sterile injection along as a control for injection experiments.

Immune stimulating action/compound	By itself or in combination	What will be determined:
Plastic particles	By itself (mainly)	- Effect on inflammation - Translocation - Distribution - Effect on immune cell function
	Bacterial infection	- Check if phagocytosis of microplastics prevents phagocytosis of bacteria (terminal exp)
	Sterile injury	- Establish if immobilized immune cells that have engulfed microplastics can be prompted to mobilize and distribute plastics upon local inflammation (terminal experiment)
	LPS injection	- Establish if immobilized immune cells that have engulfed microplastics can be prompted to mobilize and distribute plastics upon systemic inflammation (terminal experiment)
Plastic particles with biofilm	By itself (only)	- Establish whether a defective response to bacteria on plastic contributes to infections found in patients in vivo
Bacterial infection	By itself	- Compare the effects of microplastics to other inflammatory stimuli to place the effect in the right perspective - Unravel fundamental innate neutrophil characteristics such as origin, kinetics, lifespan and function in during acute inflammation
	Plastic particles	- (see above)
Non-plastic inert materials (injected particle reference)	By itself (mainly)	- Effect on inflammation - Translocation - Distribution - Effect on immune cell function
	Bacterial infection	- Check if phagocytosis of inert material prevents phagocytosis of bacteria (terminal experiment)
	Sterile injury	- Establish if immobilized immune cells that have engulfed inert material can be prompted to mobilize and distribute plastics upon local inflammation (terminal experiment)
	LPS injection	- Establish if immobilized immune cells that have engulfed inert material can be prompted to mobilize and distribute plastics upon inflammation (terminal experiment)
Sterile injury	By itself	- Compare the effects of microplastics to other inflammatory stimuli to place the effect in the right perspective - Unravel fundamental innate neutrophil characteristics such as origin, kinetics, lifespan and function in during acute inflammation
	Plastic particles	- (see above)
LPS injection	By itself	- Compare the effects of microplastics to other inflammatory stimuli to place the effect in the right perspective - Unravel fundamental innate neutrophil characteristics such as origin, kinetics, lifespan and function in during acute inflammation
	Plastic particles	- (see above)

Figure 5. Table giving an overview of the intended action to induce an immune response, whether other controls will/need to be taken along, and what will be determined from the experiment.

As already explained above, for most experiments we first consider *ex vivo* experiments (mild discomfort) and terminal experiments, before we consider repetitive intravital imaging experiments (moderate discomfort). In some experiments we first consider repetitive intravital imaging, either because some questions can only be answered by imaging the same tissue over multiple imaging sessions, or because it significantly reduces the number of required mice. After completion of the intravital imaging experiments, cells, tissues and organs will be isolated and analyzed to reduce the number of mice required. We have done intravital imaging in the past and have the set-up for most experiments already up and running.

3.4.2 Provide a justification for the strategy described above.

The set-up might seem a bit broad, but the research into the effects of microplastics is so new that there is little previous data or other work to base a more fine-tuned approach on. The methods for obtaining the readout parameters will be the same for all experiments in this proposal. We think that being able to compare the effects of microplastics to several inflammatory stimuli is the strength of this project. Supported by our own research on human material, pigs and mice, and the minimal amount of information we do have on the microplastics themselves and their effects, we have a number of hypotheses that we will start with. However, since our research is extremely novel, we cannot know whether these hypotheses will prove correct. In the next five years, human data will be combined with data from the animal experiments to adapt our hypotheses when necessary. Our data will be combined with data from our 5.1 lid1c colleagues,

population exposure data and exposure data from volunteers to help the setting up of plastics regulations by local and global government.

3.4.3 List the different types of animal procedures. Use a different appendix 'description animal procedures' for each type of animal procedure.

Serial number	Type of animal procedure
1	Microplastics exposure versus control
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10	Click or tap here to enter text.

Centrale Commissie Dierproeven

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information on the project proposal, see the Guidelines to the project licence application form for animal procedures on our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	5.1 lid2h
1.2	Provide the name of the licenced establishment.	5.1 lid2h

1.3 List the serial number and type of animal procedure

Use the numbers provided at 3.4.3 of the project proposal.

Serial number	Type of animal procedure
1	Microplastic exposure versus controls

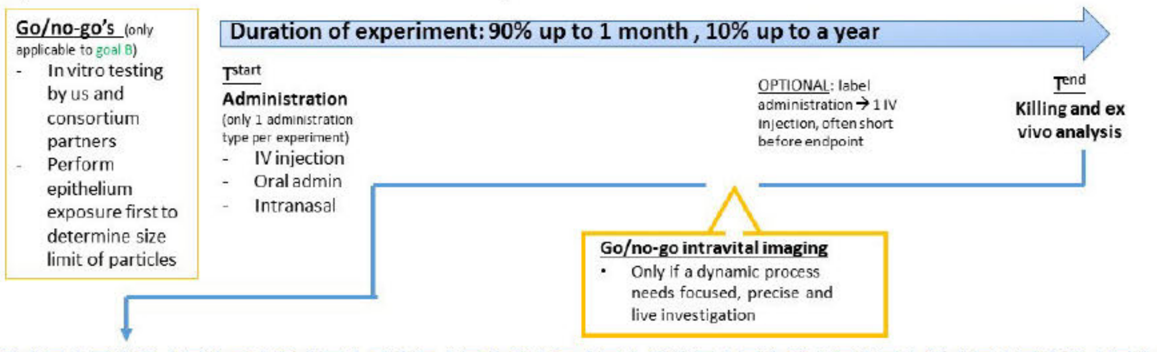
2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

This project has two immediate goals that lead to the ultimate goal of determining the health risk of microplastics exposure. Immediate goal A aims to provide understanding of the aspects of neutrophils that we don't yet know, like the origin, timing, localization and function in certain situations. **The answers to these questions explain the neutrophil response to foreign invaders. If we have this response mapped out, we can determine where microplastics fall in this response map (aim for immediate goal B).** And that determines the immunotoxicity of plastics.
In most experiments both immediate goals will be simultaneously addressed by comparing the response of immune cells to microplastics versus biological pathogens/LPS or sterile damage controls.

Majority of experiments (90%)



Limited number of experiments (10%)

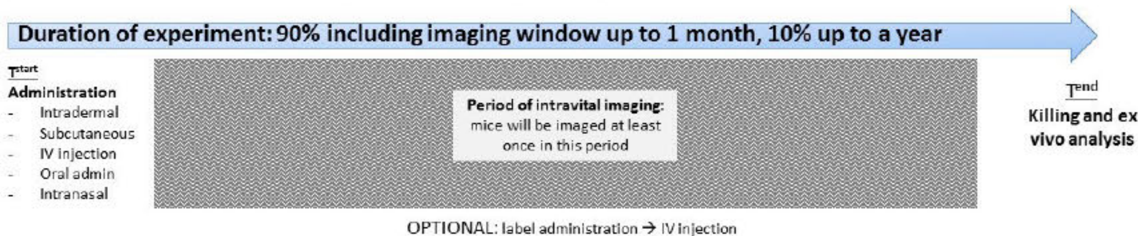


Figure 1. Schematic representation of the experiments with the go/no-go's indicated.

General design (see figure 4 of the project proposal):

I (every experiment)
- Administer plastics in different sizes (with and without different coatings), using various administration routes and compare these to placebo controls, sterile damage or responses to biological pathogens

II (optional)
- Administer compounds to
  - Visualize our cells of interest
  - Measure cell death, proliferation and lifespan
  - Inhibit, stimulate, deplete or mimic components of the inflammatory reaction

III (optional)
- Intravital imaging

IV (every experiment)
- Killing of animal and perform ex vivo analysis

The readout of each animal experiment will consist of some or all of the following, most if not all performed postmortem (and therefor have no further impact on the quality of life):
- Descriptive data on single cell level (flow cytometry, microscopy, protein expression, etc.)
  Examples include: surface markers to distinguish different type of immune cells, activation markers on immune cells, proteins of different cell death mechanisms, measure the decrease of label over time to calculate lifespan.
- Functional data (on specific functions of the neutrophil such as migration, phagocytosis, etc)
  Examples include: *ex vivo* chemotaxis assay in 3D gels after phagocytosis of microplastics, *ex vivo* bacterial killing capacity of immune cells with and without microplastics, change in pH when plastics are engulfed by immune cells vs bacteria which are known to induce a lower pH.
- Intravital microscopy data
  Examples include: Are microplastic numbers in organs diminishing in time, is the location of microplastics in organs in time constant or changing, are microplastics engulfed by immune cells and how long are they present in the same immune cell.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

Here we will describe the general experimental set-up described above in more detail. Starting off with a schematic overview of the experimental design, details of the different parts of the experiment are explained in detail further down in this document.

First of we have a description of the experimental set-up used for the majority of the experiments (fig.2). These will consist of administration of compounds or a general immune stimulus. This administration will be in one of three ways per experiment: either IV injection, or oral administration, or intranasal administration. Mainly one compound will be administered (particle compared to control). In few specific cases a combination of maximum 2 compounds will be used (eg if we want to study whether the response to bacteria is altered in the presence of microplastics both need to be administered). All combinations are described in Fig 2 and 3. For oral and intranasal administration a pilot study will determine whether one administration is enough to find plastic back in the body of the mice, or whether more, but no more than 10, administrations are necessary to find the plastics back (we have previous data demonstrating plastics in the blood and liver after 10 days of oral exposure).
After the necessary administrations the mice will not need to be handled or undergo any procedures until near endpoint. For a few experiments, short before the endpoint a label will be administered via IV injection (details on labels described further down). Then at the endpoint, mice will be killed and organs harvested for analysis. The exact duration of the experiments is to be determined, but the amount of procedures the mice will need to undergo are minimal.

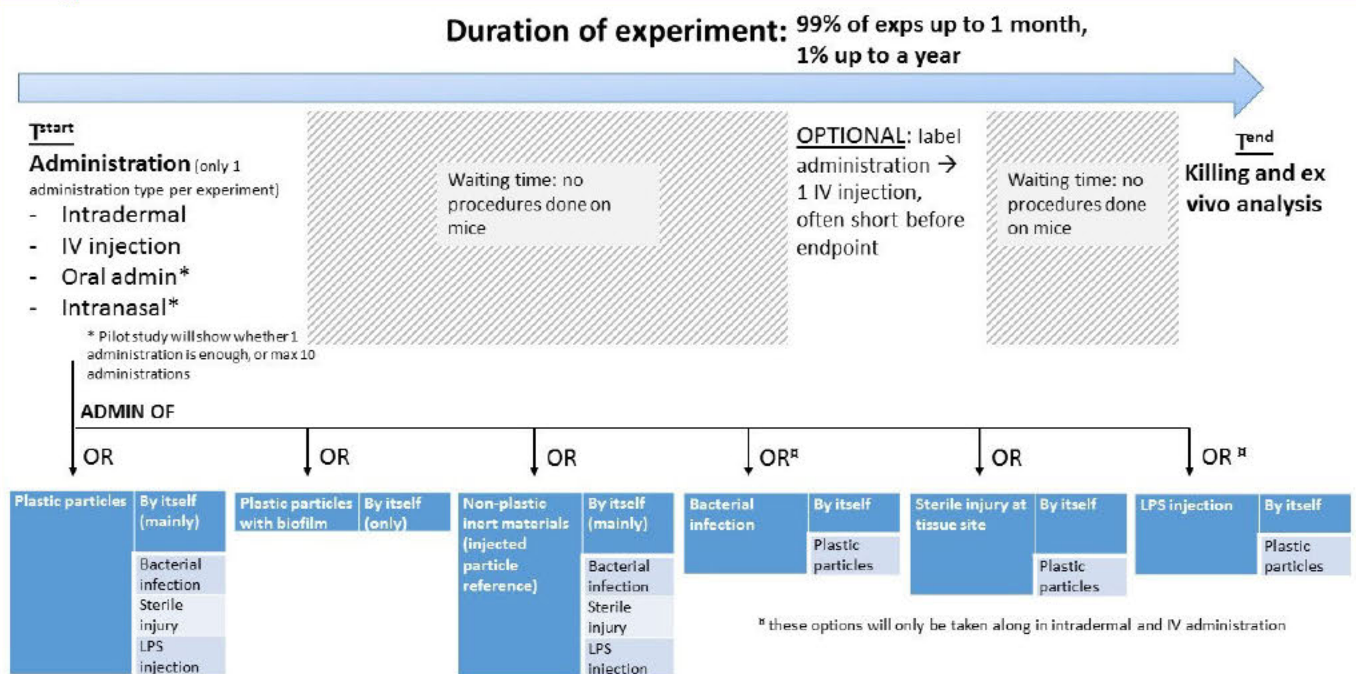


Figure 2. Schematic overview of the majority of the experiments. The duration is indicated at the top to show when certain procedures will take place during the experiment, starting on the left with the administration of the stimulus, the three different administration routes, the option to inject a label or not, and the killing of the animal at the end of the experiment. In-between these indicated procedures the mice will not undergo any procedures by us, represented by the gray blocks. Underneath the procedures are the options for combinations of controls and test material indicated. Only one of these combinations will be administered per experiment. The three administration routes and the 5 combinations of material of stimuli lead to the majority of mice falling in this category of experiments.

A small part of the experiments will involve intravital microscopy. For these experiments the set-up is slightly different (fig.3). These experiments have an added option for administration: subcutaneous. A big difference in these experiments compared to the other ones, is the repeated handling of the mice between administration and killing of the animals. Depending on the duration of the experiment, the mice will be brought at least once and maximally eight times to the microscope. At the microscope mice will be anesthetized for at least an hour and maximum four hours for experiments with repetitive imaging. After an imaging session mice are allowed to recover, and brought back to the mouse house. In terminal experiments a maximum of 12 hours of intravital

imaging will be performed. Due to the intensity of intravital microscopy experiments, less experiments will be performed in this set-up, which results in less mice for these experiments.

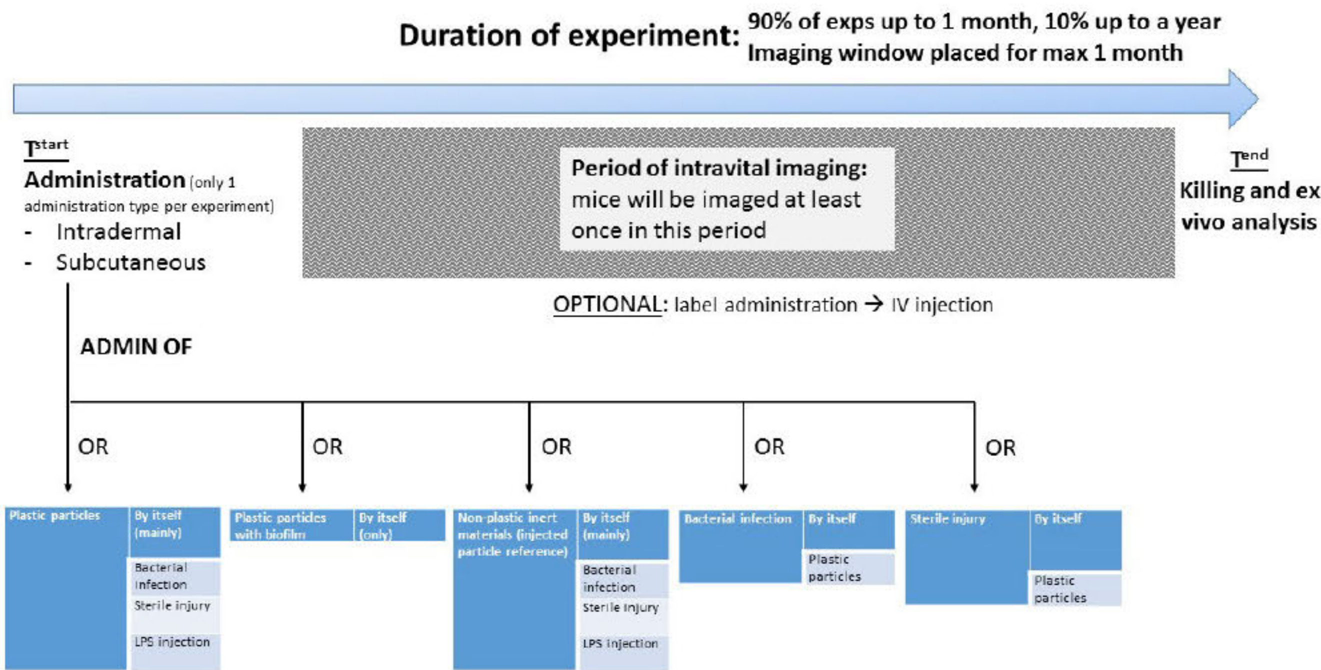


Figure 3. Schematic overview of the rest of the experiments. The duration is indicated at the top to show when certain procedures will take place during the experiment, starting on the left with the administration of the stimulus, the different administration routes, the option to inject a label or not, and the killing of the animal at the end of the experiment. In-between these indicated procedures the mice regularly be taken for anesthesia and intravital imaging, represented by the dark gray block. It will be experiment dependent how often the imaging will need to be done. Underneath the procedures are the options for combinations of controls and test material indicated. Only one of these combinations will be administered per experiment. Due to the intensity less experiments will be performed in this set-up.

The model

For these experiments we will use mice, since many fluorescent reporters exist, intravital imaging is optimized for this species, many required reagents to detect immune cells are readily available for this species. Wild type or intravital imaging (fluorescent reporter) strains or knockout strains will be used. The fact that mice are relatively cheap, easy to house and have shorter administration to response time due to the high metabolic rate, are additional reasons to use mice.

The plastic polymers

The plastics that will be tested were decided on by the 5.1 lid1c consortium members. Real life plastics of 3 different plastic materials were milled to better reflect microplastics in the environment. Disadvantages are that the size ranges are broad and we don't have a lot of material compared to the fabricated polystyrene particles. The main plastic types selected are polypropylene (PP) which is used in medical supplies, polymethyl acrylate (PMA) which is used in some textiles, polyvinyl chloride (PVC), widely used in pipes. Nylon (synthetic textile fibers) might be added. These plastics are currently being tested in vitro first to determine if the different polymers have an effects on human neutrophils and macrophages. Those that show no effect in vitro will not be tested in vivo, because particles that are not harmful to human cells don't need to be tested in mice. Also if multiple plastics give the same results in human neutrophils and macrophages, it will not be necessary to test all of them in mice as well. One representative will suffice.
For some experiments like the translocation experiments fluorescent particles will be necessary to track them in the system. Commercially bought labeled polystyrene (PS) and polymethyl methacrylate (PMMA) will be compared to the same polymer particles labeled by our partners.

The particle sizes
The dogma has always been that 10um would already be too large a particle to be able to cross the barriers of the gut. However, our previous experiments showed that the 10um is able to cross the gut lining. At this moment we have no definitive answer as to what the size limit is for crossing barriers. Therefor multiple sizes will need to be tested per administration route to determine limits.
After this is determined, we can test only the relevant sizes in experiments with other administration routes like for example IV injections.

The coatings
Plastics are hardly ever in sterile form present, not in our environment, but also not in our body. In our environment the plastics pick up bacterial and viral components, which create a biofilm on the plastics. Bacterial coating/biofilm will be used for some experimental set-ups to mimic our exposure. 5.1 lid2f

In our bodies the plastics are exposed to many different solutions, many of which protein rich. We know from in vitro experiments that the commercially bought polystyrene particles and the particles made by 5.1 lid1c have a different effect on human neutrophils when the plastics have been incubated in human plasma or serum. We don't know yet whether substances like the acids in the stomach or the mucus in the gut or lungs have an effect on how our immune system responds to the plastics, whether they keep that coating as they cross epithelial barriers, of is it's easily exchanged. For most experiments we want to test uncoated plastics against plastics in relevant bodily fluids, and for some we want to test uncoated plastics against biofilm coated plastics. Only coatings relevant for that administration route will be used, and no unnecessary options will be considered. On average we compare 2-3 coatings per experiment.

I (every experiment)
Administer plastics in different sizes (with and without different coatings) and compare these to controls, sterile damage or responses to biological pathogens

Inflammatory stimulus: Plastic particles
Justification: We want to mimic different exposure routes in humans and determine the fate in the body. This condition is needed to reach answer immediate goal B. Until now we and others in the field have used polystyrene perfect spheres of an exact size bought from a company. During this fabrication process a coating is formed on the plastic spheres that is not present on normal every day plastic. In our previous experiments in mice and in our in vitro work with human immune cells we have found 1µm and 10µm have different effects on immune cell uptake and death. Both were translocated over the intestine.
Generally experiments in mice will first be performed with the commercial polystyrene particles with which we have a lot of experience. Selected experiments will be performed with the consortium plastics.
Description: In nature microplastics are present in many different sizes. Different sizes have different effects in our human in vitro studies and in our previous mouse studies. Therefore multiple sizes will be compared to fully understand the dynamics. We want to establish what the upper size limit is that is still translocated over the intestine and lung, therefore the size range is ≤200µm. However, the different sizes are all so small that they will cause no immediate extra response or discomfort for the mice.
Plastics are administered:

- Orally (gavage for controlled administration or via food, 1-10 days,≤200µm)
  Others have found microplastics accumulate to a plateau when orally administered for 10 days. We have confirmed before that 10 days of oral gavage leads to microplastics in the blood and in organs. We prefer to switch from oral gavage to food administration to reduce discomfort for the mice, but we need to perform comparative pilot studies before switching. During this pilot we will also determine if we can detect enough particles for our analysis at earlier time points. The aim is to administer via food and to perform the least number of administrations. Of note, microplastics can sediment or float, therefore administration via drinking water is not possible.
- Intravenously (retro-orbital under anesthesia or tail vein, once, ≤10µm to prevent clogging of veins)
  Microplastics have been detected in human blood. To mimic microplastic traffic in the blood we want to inject these microplastics directly. We have already performed experiments where we were able to detect microplastics in different organs up to day 30 after a single injection. Therefore a single injection has been proven sufficient.
- Intra-nasally (under anesthesia, 1-10 days, ≤200µm)

These experiments have never been performed before. Based on the oral exposure data that is available we hypothesize 1-10 days of exposure might be necessary in order to detect the microplastics in organs. We will perform pilot experiments to determine the least number of exposures.

- Intra-dermally (under anesthesia, once, ≤100µm)
  Our skin is exposed to microplastics from our clothing and we know very little about that way of exposure. The skin has resident immune cells that are capable of phagocytosing the microplastics, which might lead to further inflammation. Furthermore, the skin is easy accessible for injection and easy to image without any invasive procedures for the mice. Our experiments so far have shown that a single dose can still be detected 100 days later and is therefore sufficient.
- Subcutaneously (under anesthesia, once, ≤1cm).
  In healthcare many plastics products are used that stay long term or permanently in our bodies, like portal needles and catheters for IVs. These are large pieces of plastic, of which we don't know if or how the immune system responds to is. Especially if something goes wrong and it has bacteria on it. We collaborate with experts in bacteria where we tested a bacterial biofilm on a catheter of about 0.5cm. Placing these in the skin of the mice makes it easier to monitor the inflammation and infection, and we don't want to risk giving the mice sepsis, but keep the infection local.

For this last category also relatively big plastics will be used to mimic problems with catheters and prostheses.
Symptoms: Intradermal injections of microplastics, I.V. microplastics injection and oral microplastic administration are well tolerated and do not lead to noticeable symptoms. Intranasal administration of microplastics has not been described before, but are expected to be in line with the mild effects of the other administration routes. However, weight loss and behavioral changes as described in section J will be tightly monitored for the animals in this route. Subcutaneous administration will require a small surgical procedure with likely moderate discomfort as a result.
Level of discomfort: mild (oral via food, intranasal, I.V., intradermal) or moderate (oral gavage, intranasal when administered more than 2 times, subcutaneous)

<u>Inflammatory stimulus: Plastic particles with biofilm</u>
Justification: Plastics can acquire a biofilm formed by bacteria both in the environment as well as in vivo on e.g. catheters. We want to investigate the response of innate immune cells to this biofilm coated plastic compared to uncoated plastics. This condition is needed to answer subquestion 5 of immediate goal B. The coating of choice is a fluorescent S aureus with which we and our microbiology department has a lot of experience. Consortium partners are establishing which bacteria are found on plastics in the environment, establishing a protocol to grow these on plastics and then my own group will perform in vitro experiments with human immune cells to instruct the choice of other coatings.
Description: As described in "Inflammatory stimulus: Plastic particles" but with biofilm coated plastic.
Symptoms: Pathogens on the surface of the plastics will likely evoke symptoms associated with an acute inflammatory response such as hypothermia, weight loss and behavioral changes as described in section J.
Level of discomfort: moderate.

<u>Inflammatory stimulus: Bacterial infection</u>
Justification: For subquestion 4 of immediate goal A we want to investigate bacterial phagocytosis and killing of the different neutrophil subsets. For subquestion 6 of immediate goal B bacteria are degradable pathogens of around 1µm in size which are the natural targets of innate immune cells. These are the 'biological degradable' controls for our experiments.
Description: Mice are infected with live or dead (fluorescent) bacteria (eg staphylococci) via the same administration routes as mentioned in "Inflammatory stimulus: Plastic particles".[1,2]
Symptoms: Intradermal injections of dead bacteria are well tolerated and do not lead to noticeable symptoms. Intravenous injection of dead and live bacteria, intradermal injection of live bacteria and intranasal application of live and dead bacteria can lead to symptoms associated with an acute inflammatory response such as hypothermia, weight loss and behavioral changes as described in section J. For as many experiments as possible dead bacteria will be used to minimize the risk of a severe response of the mice.
Level of discomfort: moderate

<u>Control inflammatory stimulus: Non-plastic inert materials</u>
Justification: We need to understand if the effects we see are plastic dependent or rather true for all inert particles (subquestion 6 of immediate goal B). Adding this control makes the outcome of the experiments more useful and are the same size particles, so don't cause extra discomfort to the mice.
Description, symptoms and level of discomfort: As described in "Inflammatory stimulus: Plastic particles" but with other inert materials such as for instance silica, talc, metal, wool, cotton.

<u>Control inflammatory stimulus: Sterile injury</u>

Justification: Sterile injury is a quick and easy tool in intravital imaging to recruit and mobilize neutrophils without a pathogenic stimulus. This can be helpful to compare the migration of neutrophil subsets (subquestion 3 of immediate goal A) or to establish if immobilized immune cells that have engulfed microplastics can be prompted to mobilize and distribute plastics upon local inflammation (subquestion 5b of immediate goal B) or to establish if microplastic induced inflammation is more prolonged than sterile inflammation (subquestion 6 of immediate goal B). This will only be performed in the skin and is only a needle prick or an unnoticeable small burn.
Description: The mice will be anesthetized and a sterile damage will be inflicted either by insertion of a sterile needle or by laser damage inflicted by the 2-photon microscope with a setting of much higher laser power and zoom than is used for imaging.
Symptoms: These local inflammations are well tolerated and do not lead to noticeable symptoms.
Level of discomfort: mild

<u>Control inflammatory stimulus: LPS injection</u>
Justification: LPS is a relatively simple model for acute inflammation with which we have a lot of experience in vivo in humans. It is in this model where we first described the different neutrophil subsets. This model can be helpful to compare the kinetics and function of neutrophil subsets (subquestion 1-4 of immediate goal A) or to establish if immobilized immune cells that have engulfed microplastics can be prompted to mobilize and distribute plastics upon systemic inflammation (subquestion 2 and 5b of immediate goal B). If the experiment allows, the mice will be kept under anesthesia while the LPS has an effect to reduce discomfort.
Description: Mice receive a single bolus of E. coli lipopolysaccharide intravenously (I.V.) in the tail vein (no anesthesia) or retro-orbitally (while under anesthesia).
Symptoms: Mice are expected to have transient (< 1 day) symptoms associated with an acute inflammatory response such as hypothermia, weight loss and behavioral changes as described in section J.
Level of discomfort: moderate

See also figure 5 of the proposal for a comprehensive Table describing the reasons and goals for using the different inflammatory stimuli.

II (optional)
Administer compounds to

- **Visualize our cells of interest**
- **Measure cell death, proliferation and lifespan**
- **Inhibit, stimulate, deplete or mimic components of the inflammatory reaction**

Justification:

- During intravital imaging different cells and structures should be distinguished.
  E.g. CD62L is a surface receptor that can distinguish different neutrophil subsets. By staining CD62L using a fluorescent antibody we can visualize these different subsets *in vivo*. Another example is to visualize if cells with microplastics are in the bloodstream or in the tissue by labeling the blood using fluorescent albumin or by labeling cells in blood with an anti-CD45 antibody.
- Measure cell life span and proliferation
  5.1 lid2f we can determine the lifespan of innate immune cells in homeostasis vs after plastic administration.
- Inhibit, stimulate, deplete or mimic components of the inflammatory reaction.
  E.g. adding therapeutic antibodies against biofilms might facilitate the biofilm clearance by neutrophils.

Description: Drugs, antibodies, small molecules, chemicals, fluorescent compounds, propidium iodide to monitor cell death, Hoechst to stain nuclei, or compounds which incorporate into DNA to measure cell life span and proliferation such as deuterated water, deuterated glucose, EdU, or BrdU are administered to mice via the appropriate route as described in literature (I.V., I.P., I.N., diet, etc).
Symptoms: Most compounds will not lead to noticeable symptoms.
Level of discomfort: mild or moderate

III (optional)
Intravital imaging

- **Non-survival**
- **Repetitive skin imaging**
- **Repetitive imaging after placing dermal imaging window**
- **Repetitive imaging after placing abdominal imaging window**

Justification: In vivo imaging will allow us to visualize dynamic processes that are missed in static analysis. Imaging windows allow us to image the same mouse repeatedly over time as opposed to analyzing separate mice at different time points, greatly reducing the amount of animals needed. Additionally, this provides paired data, introducing less variation and therefore fewer mice are needed to get statistically significant results.

At the end point of intravital microscopy, tissues will always be analyzed *ex vivo* to reduce the number of mice required.
Description: Different strategies will be used for the imaging experiments: (1) Imaging in an acute experiment under anesthesia (any organ), (2) repetitive skin imaging (after intradermal exposure), (3) repetitive imaging through a dermal imaging window (lymph node or subcutaneous plastics), (4) repetitive imaging through an abdominal imaging window (such as on the liver, spleen or kidney). For long-term studies the animal needs to be imaged by multiple imaging sessions, and therefore imaging windows are required. From experience we know that the implantation of intra-cutaneous windows does not lead to post-operative discomfort.
Depending on the duration of the experiment, the mice will be brought at least once and maximally eight times to the microscope. At the microscope mice will be anesthetized for at least an hour and maximum four hours for experiments with repetitive imaging. After an imaging session mice are allowed to recover, and brought back to the mouse house. In terminal experiments a maximum of 12 hours of intravital imaging will be performed.
We have a lot of experience in the lab with these procedures, and only well trained researchers will perform these experiments. We have observed homeostatic neutrophils respond normally to stimuli under the conditions of these experiments.
A detailed description of the window surgery, including postoperative assessment of behavior (largely normal scores) and weight (slight decreases 1 day post-surgery) is published by Ritsma *et al* in Sci Transl Med 2012 and Nature Protocols 2013.[3,4]These publications also describe subsequent intermittent intravital imaging. In short, mice will be anesthetized, positioned on a heat pad for proper temperature control and shaved. An incision is made in the skin for the intra-cutaneous window, while for the abdominal imaging window an incision is made in both skin and peritoneum. The incision is then sutured using a purse-string suture through both layers around the edges, creating four loops and leaving the suture untightened. Next, the window is placed within the incision. Different organs require different fixation techniques.[4] Ultimately, the skin (and peritoneum) are placed in the window groove, and the suture is tightened to fix the window.
For subsequent intravital imaging the mice will also be anesthetized and placed in a custom made imaging box. The microscope is covered by a climate chamber allowing proper temperature control. Saline will be administered subcutaneously (under anesthesia) for imaging sessions of more than an hour.
Symptoms: Mice can experience post-operative pain demonstrated by changed behavior as described in section J. Within 1 day post-surgery the majority of animals are found to behave normally. Intravital imaging sessions do not lead to noticeable symptoms.
Level of discomfort: moderate as a result of surgery, mild thereafter for intra-cutaneous windows, moderate thereafter for abdominal windows.

IV (every experiment)
Killing of the animal and perform ex vivo analysis
Justification: At the end of the experiment organs and cells will be harvested for ex vivo analysis.
Description: Depending on the type of experiment and the location of the experiment, mice will be killed by cervical dislocation while fully awake or while under anesthesia. Mice might also be killed by CO_2 asphyxiation followed by cervical dislocation if the necessary facilities are available. In many experiments we would like to harvest blood before sacrificing the mouse. In this case mice will be deeply anesthetized using isoflurane after which a heart puncture will be performed followed by cervical dislocation. Relevant organs will be harvested, both for us and consortium colleagues. Tissues can be isolated and analyzed with microscopy. In addition cells can be isolated from the different tissues for further descriptive analysis (e.g. FACS of cell surface expression) and/or for functional assays in vitro (e.g. survival assay, bacterial killing capacity, T cell suppression capacity). Our focus in the project is on immunotoxicology. We will look mostly at neutrophil, monocyte and macrophage numbers and activation status. We will look at blood, bone marrow, spleen, lymph nodes, kidney, liver and lungs. Brain, intestines and lungs if not needed by us will be shared with our other consortium members who have expertise with those organs.

The experimental design and the exact number of animals of each experiment is determined and described in detail in a work protocol according to the advice from the Animal Welfare Body and a statistician (figure 4 for example experiments).

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

When possible, blinding and randomization will be used to prevent bias as much as possible. For example, videos obtained with intravital imaging can be coded by an independent colleague before image analysis by the researcher.
Whenever possible we strive for a situation to express the outcome of our experiments in quantitative terms. This is possible for all FACS measurements and most imaging data. To estimate the number of animals to be used in an experiment, we use the effect size (if known, e.g., from data in the literature, from our own historical

data or if unknown from small scale pilots) to estimate the sample size needed to achieve a certain power (usually around 0.8) with appropriate statistical test like the t test with a $p<0.05$. Qualitative analysis (descriptive analysis of some intravital imaging data): the number of animals (group size) is based on literature and/or years of experience with similar type of experiments. Moreover, these types of experiments will be performed sequentially via which we ensure that we will use the minimum number of mice per group that will be informative.

B. The animals

Specify the species, origin, life stages, estimated numbers, gender, genetic alterations and, if important for achieving the immediate goal, the strain.

Serial number	Species	Origin	Life stages	Number	Gender	Genetically altered	Strain
1	Mus musculus	Born in EU at registered breeder	No preference	5854	No preference	Yes, without harmful phenotype	Various

Provide justifications for these choices

Species	Our experience with mice and their immune system
Origin	We can use surplus mice from the breeding facility, and try to source our genetically altered mice more local to reduce impact on mice and the environment.
Life stages	We don't expect an age effect, so all mice can be used
Number	Estimated numbers (see below)

Estimated numbers

Immediate goal A: Unravel fundamental innate neutrophil characteristics such as origin, kinetics, lifespan and function in homeostasis and acute inflammation

Immediate goals

1. Determine the lifespan of neutrophil subsets
   30 animals are needed to establish a labelling curve[6]
   Compare control versus acute inflammation with LPS (because we have comparative human data)
   Compare deuterium label (population based in humans) vs EdU (cell based, only possible in mice) **120 mice (60 mild, 60 moderate)**
2. Determine the origin of neutrophil subsets (local or distant like bone marrow or spleen)
3. Determine the kinetics of different neutrophil subsets throughout the body
4. Establish the function of different neutrophil subsets
   These are exploratory studies that will evolve during the project, numbers are estimates. Many times goal 2-4 can be addressed in the same experiments.
   Controls will be compared with LPS (because we have comparative human data).
   Typical work protocols are between 10 and 50 mice and typically 5 experiments are performed per year. (30 mice * 5 exp * 5 years =750)
   Max 750 mice (400 mild, 350 moderate)

Immediate goal B: Determine the health effects of environmental exposure to microplastics

Pilot studies

- Determine minimum number of oral administration + compare oral gavage with food uptake (5 mice, min 1 and max 10 administrations, 2 routes = **100 mice moderate due to gavage**)
- Determine minimum number of intranasal administration (5 mice, min 1 and max 10 administrations = **50 mice mild**)

We have 1 reference polystyrene + 3 consortium plastics polymer types, of at most 3 different sizes. Not all sizes will be used for all administration routes. We can have up to 2 different coatings of the plastics in addition to the non-coated control: biofilm and relevant bodily fluid like mucus or serum. For each experiment we need at most two control groups. We are interested in long-term effects of the plastics to mimic human exposure, so multiple time point per administration route are needed, except when we can do intravital imaging. For our previous IV injection experiments we saw changing kinetics using 4h, 16h, 14 days and 30 days. In the future we would like to add a longer term time point as well.

Oral exposures (ex vivo analysis):
4 plastics x 3 sizes per plastic type x 3 coatings + 2 controls x 7 animals x 5 time points)= **1330 mice mild or moderate depending on nr of administrations and administration route from pilot**

Intranasal exposures (ex vivo analysis):
4 plastics x 3 sizes x 3 coatings + 2 controls x 7 animals x 5 time points)= **1330 mice mild or moderate depending on nr of administrations from pilot**

IV injection (ex vivo analysis):
4 plastics x 2 sizes (size restriction due to clotting) x 3 coatings + 2 controls x 7 animals average x 5 time points = **910 mice (120 mild without coating and 790 moderate)**

Intradermal (repetitive intravital imaging):
4 plastics x 3 sizes x 3 coatings + 2 controls x 7 animals = **266 mice moderate**

Subcutaneous (repetitive intravital imaging):
4 plastics x 1 size x 3 coatings + 2 controls x 7 animals= **98 mice moderate**

Exploratory studies
(See for examples Appendix C)
These are exploratory studies that will evolve during the project, numbers are estimates. Typical work protocols are between 10 and 50 mice and typically 5 experiments are performed per year. (30 mice * 5 exp * 5 years =750)
Max 750 mice (400 mild, 350 moderate)

However, these numbers are overestimations because:

- It is unlikely that all plastics will need to be tested *in vivo*. This will be determined before the animal experiments will be performed.
- For now we estimate to test two different coatings, but we may only need to test one. This will be determined in vitro before the animal experiments will be performed.
- Controls can be shared between experiments when these are performed simultaneously
- Descriptive single cell and functional assays will be combined when neutrophil numbers allow this
- When differences between groups turn out to be very striking, we do not need the maximum number of mice per group in consecutive studies.
- Successful pilot studies may be included in the dataset

Additionally, a number of ca. 150 mice is needed for:

	Estimated nr
Pilot studies for validation of models/techniques#	50
Training of new personnel or new techniques[x]	50
To compensate for unforeseen loss of animals*	50

#Only procedures mentioned in this project proposal
Mice that become surplus during breeding, will be used for pilot studies and training purposes.
[x]Intravital imaging is a procedure that requires proper training of surgical skills in order to prevent discomfort to the animal as much as possible
*(max 10%, e.g. due to location not suitable for imaging, problems with the window)

Together we therefore anticipate we need a grand total of **5854 mice (1030 mild and 4824 moderate).**

Gender	We don't expect a gender effect, so both female and male can be used
Genetic alterations	For some experiments we require intravital imaging strains or knock-out strains
Strain	Phagocytosis is such an evolutionary conserved mechanism that we expect differences between strains to be minimal. The genetically modified mice bearing green neutrophils are

in the C57BL/6 background as are genetically modified mice bearing YFP dendritic cells, therefore we want to perform most if not all experiments in that strain. On top of that, by using this background we can also use surplus mice from standard breeding at the GDL facility, mice that would otherwise be discarded. Other genetically modified mice expressing a reporter for a fluorescent protein in different immune cells might be used if not available on the C57BL/6 background.

C. Accommodation and care

Is the housing and care of the animals used in experimental procedures in accordance with Annex III of the Directive 2010/63/EU?

☒ Yes

☐ No > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices

Click or tap here to enter text.

D. Pain and compromised animal welfare

Will the animals experience pain during or after the procedures?

☒ No

No pain (but mild discomfort) is expected to occur for:
- uptake via food or drinks
- oral gavage
- intravenous administration by tail vein
- intraperitoneal administration
- CO_2 asphyxiation
- cervical dislocation

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☒ No > Justify why pain relieving methods will not be used.

Some pain is expected to occur for:
- acute inflammatory response after LPS administration
- acute inflammatory response after bacterial administration

These acute inflammatory response will evoke fever, shivers and muscle ache. Anelgesia will influence the immune response that we are studying. We also perform these type of studies in human volunteers without anesthesia or anelgesia.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

The procedures requiring anesthesia and/or analgesia are listed below and detailed in section 2.
- retro-orbital intravenous injection under isoflurane anesthesia, no analgesia
- intranasal application under isoflurane anesthesia, no analgesia
- intradermal application under isoflurane anesthesia, no analgesia
- subcutaneous application under isoflurane anesthesia, no analgesia
- Sterile injury under isoflurane anesthesia, no analgesia
- Non-survival intravital imaging under isoflurane anesthesia, no analgesia
- Repetitive skin imaging under isoflurane anesthesia, no analgesia
- Repetitive imaging after placing dermal imaging window under isoflurane anesthesia, analgesia pre- and post-surgery[5]
- Repetitive imaging after placing abdominal imaging window under isoflurane anesthesia, analgesia pre- and post-surgery[3,4]
- Terminal heart puncture followed by cervical dislocation under isoflurane anesthesia, no analgesia

Describe which other adverse effects on the animals' welfare may be expected?

Inflammatory stimulus: Plastic particles/ Non-plastic inert materials

Symptoms: Intradermal injections of microplastics, I.V. microplastics injection and oral microplastic administration are well tolerated and do not lead to noticeable symptoms. Intranasal administration of microplastics has not been described before, but are expected to be in line with the mild effects of the other administration routes. However, weight loss and behavioral changes as described in section J will be tightly monitored for the animals in this route. Subcutaneous administration will require a small surgical procedure with likely moderate discomfort as a result.
Level of discomfort: mild (oral via food, intranasal, I.V., intradermal) or moderate (oral gavage, intranasal when administered more than 2 times, subcutaneous)

Inflammatory stimulus: Plastic particles with biofilm
Symptoms: Pathogens on the surface of the plastics will likely evoke symptoms associated with an acute inflammatory response such as hypothermia, weight loss and behavioral changes as described in section J.
Level of discomfort: moderate.

Inflammatory stimulus: Bacterial infection
Symptoms: Intradermal injections of dead bacteria are well tolerated and do not lead to noticeable symptoms. Intravenous injection of dead and live bacteria, intradermal injection of live bacteria and intranasal application of live and dead bacteria can lead to symptoms associated with an acute inflammatory response such as hypothermia, weight loss and behavioral changes as described in section J.
Level of discomfort: moderate

Control inflammatory stimulus: Sterile injury
Symptoms: These local inflammations are well tolerated and do not lead to noticeable symptoms.
Level of discomfort: mild

Control inflammatory stimulus: LPS injection
Symptoms: Mice are expected to have transient (< 1 day) symptoms associated with an acute inflammatory response such as hypothermia, weight loss and behavioral changes as described in section J.
Level of discomfort: moderate

Administer compounds
Symptoms: Most compounds will not lead to noticeable symptoms.
Level of discomfort: mild or moderate

Intravital imaging
Symptoms: Mice can experience post-operative pain demonstrated by changed behavior as described in section J. Within 1 day post-surgery the majority of animals are found to behave normally. Intravital imaging sessions do not lead to noticeable symptoms.
Level of discomfort: moderate as a result of surgery, mild thereafter for intra-cutaneous windows, moderate thereafter for abdominal windows.

Explain why these effects may emerge.

See above

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Only skilled personnel will perform the procedures. Due to our experience with injecting bacteria or LPS, we know the safe (concentration) range and the risk is minimal. Mice with windows will be monitored at least twice a week for clinical signs. If unexpectedly severe suffering is observed, mice will be euthanized. If the experimental set-up allows (e.g. end point is ≤3hours after I.V. LPS injection) the whole procedure will be done under anesthesia.

E. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question F

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

The experiments will be set-up in such a way that the incidence of reaching the humane end-point is kept to a minimum.

Reaching a score of 4 on the illness scale (specified below) or severe shortness of breath is set as an humane end point.

An illness grading scale will always be used to score a set of clinical features detected in mice with different degrees of illness:
0: healthy
1: barely ruffled fur
2: ruffled fur, but active
3: ruffled fur and inactive
4: ruffled fur, inactive, hunched, and gaunt
5: dead

Additional model specific parameters:
Plastic particles with biofilm/ Bacterial infection/ LPS injection
Weight loss of >20%

Intravital imaging
Loss of the intravital imaging window is set as a humane endpoint.
Infection of the skin around the intravital imaging window is additionally set as a humane endpoint (has never happened so far).

Indicate the likely incidence.

Expected to be <5% and <1 day

F. Classification of severity of procedures

Provide information on the experimental factors contributing to the discomfort of the animals and indicate to which category these factors are assigned ('non-recovery', 'mild', 'moderate', 'severe'). In addition, provide for each species and treatment group information on the expected levels of cumulative discomfort (in percentages).

Level of discomfort is indicated in section A for each injection and technique at the bottom of each paragraph. Also the category assignment is described extensively in the adverse effects section above.
We have a few experiments in the list which will cause more discomfort, but these will only be used for specific situations. Where possible we will be using anesthesia to reduce the discomfort of the mice. Unfortunately the usage of analgesics can have an effect on the immune cells, especially the neutrophils, and will therefore be prevented as much as possible. Overall, pain and discomfort should be short-lived, and we always aim to keep it as short an minimal as possible. The cumulative discomfort is mild for 17.6% of the number of animals (1030)(oral administration via food, IV injection, intradermal injection), and moderate for 82.4% of the animals (4824) (intranasal administration, oral gavage, subcutaneous injection, window placement, inflammation models, intravital imaging).

G. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement	Initial testing for plastics of interest concerning their polymer composition, their size and their coating will be done in in vitro settings. Many experiments of the project will be performed using human primary cells, human and mouse cell lines, organoids and organs-on-a-chip by us and others. This will ultimately determine the number of animal experiments to be performed.
Reduction	By using techniques like intravital imaging, we reduce the number of mice used in longitudinal studies because the same mouse can be imaged over the entire span of the experiment. After completion of the intravital imaging experiments, cells, tissues and organs will be isolated and analyzed to reduce the number of mice required. Using genetically modified mice will give us more insight per mouse as to the effect of microplastics on neutrophils, and to a certain extend macrophages. With more information retrieved per mouse, less animals are necessary. By sharing organs, other institution no longer need to perform the experiments as well to obtain their tissues.
Refinement	Anesthesia will be used for most procedures and husbandry will kept at an optimum to reduce discomfort and stress.

	For most experiments we first consider ex vivo experiments (mild discomfort) or terminal experiments, before we consider repetitive intravital imaging experiments (moderate discomfort). In some experiments we first consider repetitive intravital imaging, either because some questions can only be answered by imaging the same tissue over multiple imaging sessions or because it reduces the number of mice (see above). In pilot experiments we will determine the least amount of oral and intranasal administrations needed to detect microplastics in the tissue and we will establish if we can switch from oral gavage to administration via food. Analgesia will be applied during the window placement. Mice will be placed on thermal plates during surgery. Windows are made of biocompatible titanium material and have a groove that fits and hides the suture. Saline will be administered i.p. during long intravital imaging sessions. Eye ointment will also be used to prevent dry eyes. We have over a decade of experience with this technique.

Are adverse environmental effects expected? Explain what measures will be taken to minimise these effects.

☒ No

☐ Yes > Describe the environmental effects and explain what measures will be taken to minimise these effects.

Click or tap here to enter text.

H. Re-use

Will animals be used that have already been used in other animal procedures ?

☒ No > Continue with question I.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☐ No

☐ Yes > Provide specific justifications for the re-use of these animals during the procedures.

Click or tap here to enter text.

I. Repetition

Explain for legally required animal procedures what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, describe why duplication is required.

Click or tap here to enter text.

J. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question K.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Click or tap here to enter text.

3. End of experiment

K. Destination of the animals

Will the animals be killed during or after the procedures?

☐ No > Provide information on the destination of the animals.

Click or tap here to enter text.

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

Full analysis of the organs exposed to plastics requires us to euthanize the mice

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

Click or tap here to enter text.

☒ Yes > Will a method of killing be used for which specific requirements apply?

☒ No > Describe the method of killing.

Most mice will be euthanized using cervical dislocation performed under anesthesia. Occasionally CO_2 asphyxiation followed by cervical dislocation or cervical dislocation without anesthesia will be performed.

☐ Yes > Describe the method of killing that will be used and provide justifications for this choice.

Click or tap here to enter text.

If animals are killed for non-scientific reasons, justify why it is not feasible to rehome the animals.

Not applicable

References for the whole appendix

1. Liese, J., Rooijakkers, S. H., van Strijp, J. A., Novick, R. P. & Dustin, M. L. Intravital two-photon microscopy of host-pathogen interactions in a mouse model of Staphylococcus aureus skin abscess formation. *Cell. Microbiol.* **15**, 891-909 (2013).
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5.1 lid2h, 5.1 lid2e

NIET-TECHNISCHE PROJECTSAMENVATTING

Naam van het project	Wisselwerking tussen microplastics en ons aangeboren immuunsysteem
NTS-identificatiecode	NTS-NL-139994 v.1
Nationale identificatiecode van de NTS *Veld wordt niet gepubliceerd.*	
Land	Nederland
Taal	nl
Indiening bij EU *Veld wordt niet gepubliceerd.*	ja
Duur van het project, uitgedrukt in maanden.	60
Trefwoorden	Microplastic Immuunsysteem Afweercellen
Doel(en) van het project	Fundamenteel onderzoek: Immuunstelsel Omzettinggericht en toegepast onderzoek: Niet op grond van regelgeving vereist toxicologisch en ecotoxicologisch onderzoek Bescherming van het milieu in het belang van de gezondheid of het welzijn van mens of dier

DOELSTELLINGEN EN VERWACHTE VOORDELEN VAN HET PROJECT

Beschrijf de doelstellingen van het project (bijvoorbeeld het aanpakken van bepaalde wetenschappelijke onduidelijkheden, of wetenschappelijke of klinische behoeften).	Dagelijks krijgen we microplastics binnen. De microplastics komen onder andere van flesjes water, voedselverpakking, en nylon kleding. Die komen via de lucht, ons eten en drinken ons lichaam binnen. Maar we weten nog niet wat er met ons gebeurt als plastic ons lichaam binnen komt, of hoe schadelijk het nu eigenlijk is. Daarnaast zwerft plastic in onze omgeving rond. Daar komt het in aanraking met bacteriën en virussen die ons ziek kunnen maken. Het zou zo kunnen zijn dat bacteriën via plastic ons lichaam binnen komen, maar dat weten we nog niet zeker. We hebben geld gekregen van de Nederlandse overheid om dit uit te zoeken, zodat zij de regels over plastic kunnen aanpassen. In ons lichaam zijn onze afweercellen altijd bezig om binnendringers op te ruimen. Normaal zijn dat bacteriën, parasieten en virussen. Deze drie indringers kunnen worden afgebroken en opgeruimd door de afweercellen. Plastic dat ons lichaam binnen komt kan niet worden afgebroken door afweercellen. Hierdoor gaan de afweercellen dood. Dit kan ontstekingen en andere slechte gevolgen hebben op korte termijn. We weten ook nog niet wat het gevolg is van dit soort irritatie van het afweersysteem als het de rest van ons leven aanhoudt. Met deze dierproeven willen wij uitzoeken hoe een plastic via de longen of de darmen ons lichaam binnen komt, en hoe schadelijk de meest voorkomende plastics zijn voor ons afweersysteem. We willen weten in welke organen de plastics terecht komen, en welke afweercellen erop reageren. We willen ook uitzoeken hoe lang een plastic op een bepaalde plek in ons lichaam blijft. Zo willen we bepalen hoe schadelijk de plastics kunnen zijn, of dat het misschien wel mee valt.
Welke potentiële voordelen kan dit project opleveren? Leg uit hoe de wetenschap vooruit kan worden geholpen of mensen, dieren of het milieu uiteindelijk voordeel kunnen hebben bij het project. Maak, waar van toepassing, een onderscheid tussen voordelen op korte termijn (binnen de looptijd van het project) en voordelen op	Door uit te zoeken wat plastic in ons lichaam met ons afweersysteem doet, kunnen we bepalen hoe schadelijk het is. Nu komen we elke dag met veel plastic in aanraking, omdat er geen regels over zijn. Dus we weten niet hoeveel schade we per dag oplopen door de plastics die we binnen krijgen. Op dit moment kunnen bedrijven zelf kiezen om minder plastic te gebruiken in hun verpakkingen, maar er zijn geen regels dat ze dat moeten doen. Als wij laten zien in hoeverre plastic schadelijk is voor onze gezondheid, kan de overheid regels maken die het plasticgebruik verminderen. Zo beschermen we niet alleen onze gezondheid, maar verminderen we ook de hoeveelheid plastic die in de natuur belandt vanuit ons afval.

lange termijn (die mogelijk pas worden bereikt nadat het project is afgerond).	

VOORSPELDE SCHADE

<table>
<tr><td>In welke procedures worden de dieren gewoonlijk gebruikt (bijvoorbeeld injecties, chirurgische procedures)? Vermeld het aantal en de duur van deze procedures.</td><td>De muizen worden op natuurlijke of kunstmatige manier blootgesteld aan de plastics. We willen plastic via het eten en via de lucht toedienen om uit te zoeken hoeveel ze in hun bloed krijgen. We willen de plastic ook direct in het bloed spuiten om te zien waar in het lichaam plastic terecht komt. Daarnaast willen we plastic in de huid spuiten om met de microscoop te zien hoe afweercellen erop reageren. We gaan ook experimenten doen waarbij we een kijkvenster plaatsen op een orgaan zodat we over de tijd kunnen kijken of en hoe lang de plastics blijven zitten. Deze experimenten vergen een operatie en daar zullen we pijnstilling voor gebruiken.</td></tr>
<tr><td>Wat zijn de verwachte gevolgen/nadelige effecten voor de dieren, bijvoorbeeld pijn, gewichtsverlies, inactiviteit/verminderde mobiliteit, stress, abnormaal gedrag, en wat is de duur van die effecten?</td><td>Voor de meeste behandelingen worden de muizen in slaap gebracht zodat ze niks merken. We hebben eerder gezien dat de muizen geen zichtbare last hebben van de plastic als we tot een paar maanden kijken. De negatieve gevolgen zullen daarom klein zijn voor de muizen. Er zijn sommige procedures waarbij de muizen wel stress ervaren maar die bij mensen ook niet onder anesthesie worden uitgevoerd zoals bloedafname, orale toediening en het oproepen van een ontstekingsreactie. We gaan ook experimenten doen waarbij we een kijkvenster plaatsen op een orgaan zodat we over de tijd kunnen kijken of en hoe lang de plastics blijven zitten. Deze experimenten vergen een operatie en daar zullen we pijnstilling voor gebruiken.</td></tr>
<tr><td>Welke soorten en aantallen dieren zullen naar verwachting worden gebruikt? Wat zijn de verwachte ernstgraden en de aantallen dieren in elke ernstcategorie (per soort)?</td><td>
<table>
<tr><td rowspan="2">Soort:</td><td rowspan="2">Totaal aantal</td><td colspan="4">Geraamde aantallen naar ernstgraad</td></tr>
<tr><td>Terminaal</td><td>Licht</td><td>Matig</td><td>Ernstig</td></tr>
<tr><td>Muizen (Mus musculus)</td><td>4834</td><td>0</td><td>620</td><td>4214</td><td>0</td></tr>
</table>
</td></tr>
<tr><td>Wat gebeurt er met de dieren die aan het einde van de procedure in leven worden gehouden?</td><td>
<table>
<tr><td rowspan="2">Soort:</td><td colspan="3">Geraamd aantal te hergebruiken, in het habitat-/houderijsysteem terug te plaatsen of voor adoptie vrij te geven dieren</td></tr>
<tr><td>Hergebruikt</td><td>Teruggeplaatst</td><td>Geadopteerd</td></tr>
</table>
</td></tr>
<tr><td>Geef de redenen voor het geplande lot van de dieren na de procedure.</td><td>We moeten weten wat er in het bloed en de organen gebeurt met de afweercellen door de plastic. Omdat we meerdere organen willen bekijken aan het einde van ieder experiment, is het niet mogelijk om de muizen te laten leven aan het einde.</td></tr>
</table>

TOEPASSING VAN DE DRIE V'S

1. Vervanging Beschrijf welke diervrije alternatieven op dit gebied voorhanden zijn en waarom zij niet voor het project kunnen worden gebruikt.	In ons consortium gebruiken we voornamelijk niet-dierlijke alternatieven. Verschillende consortiumpartners hebben verschillende orgaanexpertises. De plastics worden op gedoneerde menselijke cellen in het lab getest om te bepalen welke plastics mogelijk gevaarlijk zijn als we ze binnen krijgen. Hiervoor gebruiken we de cellen direct uit het lichaam, maar ook kweken we de cellen op tot meer geavanceerde mini-darmpjes en mini-longetjes. Met deze proeven in kweekschaaltjes kunnen we een inschatting maken van de toxiciteit. Wij zijn de enigen die ook dierexperimenten uitvoeren. Met deze dierproeven leveren we een cruciale link door de bewegingen van microplastics door het hele lichaam te volgen en door te bepalen hoe lang ze op een bepaalde plek blijven zitten. Ook zullen we een bepaalde hoeveelheid plastics aan een muis te eten geven en vervolgens bepalen hoeveel daarvan het lichaam binnendringt. Dit type proeven kunnen momenteel nog niet in kweekschaaltjes worden uitgevoerd.
2. Vermindering Leg uit hoe de aantallen dieren voor dit project zijn bepaald. Beschrijf de stappen die zijn genomen om het aantal te gebruiken dieren te verminderen en de beginselen die zijn gebruikt bij het opzetten van de studies. Beschrijf, waar van toepassing, de praktijken die gedurende het hele project zullen worden toegepast om het aantal dieren die in overeenstemming met de wetenschappelijke doelstellingen werden gebruikt, tot een minimum te beperken. Deze praktijken kunnen bijvoorbeeld bestaan uit proefprojecten, computermodellen, het delen van weefsel en hergebruik.	Door meerdere onderzoeken in dezelfde muis te doen, hebben we minder muizen nodig. Ook door in dezelfde muis op meerdere momenten live te kijken wat er met de plastics gebeurd, hebben we minder muizen nodig.
3. Verfijning Geef voorbeelden van de specifieke maatregelen (bv. verscherpte monitoring, postoperatieve behandeling, pijnbestrijding, training van dieren) die in verband met de procedures moeten worden genomen om de welzijnskosten (schade) voor de dieren tot een minimum te beperken. Beschrijf de mechanismen om gedurende de looptijd van het project nieuwe verfijningstechnieken in gebruik te nemen.	Voor de meeste handelingen die we met de muizen doen die pijn kunnen doen of stress geven, brengen we ze in slaap. Wanneer we een operatie uitvoeren waarna de muis wakker wordt geven we pijnstilling. Voor de orale toediening willen we vroeg in het project verschillende toedieningsmethodes vergelijken. We willen nagaan of de plastics ook de darmwand passeren als we ze in het voedsel geven of in een druppel gecondenseerde melk in plaats van via directe toediening in de slokdarm zoals we eerder gedaan hebben.
Licht de keuze van de soorten en de bijbehorende levensstadia toe	We hebben veel ervaring met muizen en hun afweersysteem. We weten hoe het op ons afweersysteem lijkt. Ook zijn de methoden om afweercellen te herkennen en te markeren in de muis het meest ontwikkeld. Daarom hebben we voor muizen gekozen. We gaan in muizen van verschillende leeftijden kijken omdat bij mensen ook jong en oud met plastic te maken heeft en omdat we eerder verschillende resultaten hebben gevonden bij muizen van verschillende leeftijden.

VOOR EEN BEOORDELING ACHTERAF GESELECTEERD PROJECT

Project geselecteerd voor BA?	nee
Termijn voor BA	
Reden voor de beoordeling achteraf	
Bevat ernstige procedures	
Maakt gebruik van niet-menselijke primaten	
Andere reden	
Toelichting van de andere reden voor de beoordeling achteraf	

AANVULLENDE VELDEN

Nationaal veld 1 *Veld wordt niet gepubliceerd.*	
Nationaal veld 2 *Veld wordt niet gepubliceerd.*	
Nationaal veld 3 *Veld wordt niet gepubliceerd.*	
Nationaal veld 4 *Veld wordt niet gepubliceerd.*	
Nationaal veld 5 *Veld wordt niet gepubliceerd.*	
Startdatum project *Veld wordt niet gepubliceerd.*	
Einddatum project *Veld wordt niet gepubliceerd.*	
Goedkeuringsdatum project *Veld wordt niet gepubliceerd.*	
ICD-code 1 *Veld wordt niet gepubliceerd.*	
ICD-code 2 *Veld wordt niet gepubliceerd.*	
ICD-code 3 *Veld wordt niet gepubliceerd.*	
Link naar de eerdere versie van de NTS buiten het EC-systeem	