ADVIES PROJECTVOORSTEL DIERPROEVEN

5.1 lid2h 13

A. Algemene gegevens over de procedure

1. Aanvraagnummer : AVD5.1 lid2h202216420
2. Titel van het project : *Characterizing the interplay between microplastics and the innate immune system* ***(let op aangepaste titel)***
3. Titel van de NTS : Wisselwerking tussen microplastics en ons aangeboren immuunsysteem

4. Type aanvraag:
   - ☒ nieuwe aanvraag projectvergunning
   - ☐ wijziging van vergunning met nummer :

5. Contactgegevens DEC

Naam DEC	: 5.1 lid2h
Telefoonnummer contactpersoon	: 5.1 lid2h
Emailadres contactpersoon	5.1 lid2h

6. Adviestraject (data dd-mm-jjjj):
   - ☒ ontvangen door DEC: 23-9-2022 (niet toetsbaar) en 16-1-2023
   - ☐ aanvraag compleet:
   - ☒ in vergadering besproken: 28-9-2022, 18-1-2023, 15-3-2023
   - ☐ anderszins behandeld:
   - ☒ termijnonderbreking(en) van / tot : 3-10-2022 opgeschort/ 16-1-2023; 25-1-2023/ 3-3-2023; 22-3-2023 / 12-4-2023

   ☒ besluit van CCD tot verlenging van de totale adviestermijn met max. 15 werkdagen: Door de CCD is een opschortbrief gericht aan de IvD Utrecht op 15-11-2022.
   - ☐ aanpassing aanvraag:
   - ☒ advies aan CCD:

7. De aanvraag is afgestemd met de IvD en deze is hiermee akkoord.

8a. Eventueel horen van aanvrager
- Datum: 28-9-2022
- Plaats: via Teams
- Aantal aanwezige DEC-leden: 7
- Aanwezige (namens) aanvrager: Verantwoordelijk onderzoeker
- Gestelde vragen en verstrekte antwoorden: De DEC heeft de onderzoekers o.a. gehoord en besloten, dat de aanvraag niet toetsbaar was.

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9a. Correspondentie met de aanvrager via de CCD

- De aanvraag was teruggestuurd naar de CCD omdat deze in die vorm niet beoordeelbaar was (datum 03-10-2022 met kenmerk FM/BA/22/0).

De DEC adviseert de CCD de aanvrager te verzoeken de aanvraag grondig te herschrijven met inachtneming van de vragen en opmerkingen zoals in de brief beschreven.

8b. Eventueel horen van aanvrager bij de herziene aanvraag:

- Datum: 18-1-2023
- Plaats: via Teams
- Aantal aanwezige DEC-leden: 8
- Aanwezige (namens) aanvrager: Verantwoordelijk onderzoeker en collega onderzoeker
- Gestelde vragen en verstrekte antwoorden die ook schriftelijk zijn gesteld:

9b. Correspondentie met de aanvrager

- Datum vragen: 25-1-2023
- Datum antwoord: 3-3-2023
- Gestelde vragen en antwoorden:

Projectvoorstel

3.1 Achtergrond:

- Kunt u de titel vernauwen tot het directe doel van dit dieronderzoek?
  Oude titel: Immunological health effects of microplastics.
  Nieuwe titel: Characterizing the interplay between microplastics and the innate immune system

- Kunt u de te testen microplastics nader specificeren in de mate van dispersie? (Dispersie-karakteristieken worden mede bepaald door het medium lucht, water, olie, etc).
  De dispersie karakteristieken in medium en PBS worden in het consortium bepaald door 5.1 lid1c *In de muizenstudies kunnen we de dispersie visualiseren met behulp van microscopie. We hebben er voor gekozen dit detail niveau niet te includeren in de aanvraag. Ons inziens heeft dit geen invloed op de uitvoer van de experimenten of het ongerief van de muizen.*

- Kunt u per type microplastic de relevantie verduidelijken en een prioritering geven?
- Kunt u beter beschrijven op basis van welke (klinische) aanknopingspunten u deze plastics hebt geselecteerd?
  5.1 lid1c *heeft bepaald wat de meest voorkomende plastics zijn in onze dagelijkse omgeving . Op basis hiervan zijn de plastics gekozen, met als vereiste dat ze produceerbaar moeten zijn op de manier waarop we er aan worden blootgesteld. Verdere prioritering staat in de go/no-go criteria. Doordat we in de looptijd van deze aanvraag al een groot gedeelte van de humane in vitro data hebben gerealiseerd kunnen we inmiddels een concreter voorbeeld geven (pagina 3 voorlaatste alinea en extra figuur bovenaan pagina 5).*

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- Kunt u toelichten waarom uw onderzoek zich voornamelijk op neutrofielen richt en de rol van macrofagen, ook in relatie tot neutrofielen, minder benoemd wordt?
  Er stond al beschreven dat wij experts zijn in neutrofielen en dat wij in samenwerking nu meer gaan leren over macrofagen (pagina 3, een-na-laatste alinea, QUOTE: 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.).

- U geeft aan, dat het niet mogelijk is om uw vragen *in vitro* te onderzoeken (op pagina 3). De DEC mist de argumentatie, waarom dat niet kan.
  Er staat inderdaad op pagina 3 vanaf regel 5 dat het niet mogelijk is, en ook waarom. We hebben deze tekst verder uitegwerkt. QUOTE: 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 can only verify the presence of microplastics, but it will not show uptake of plastics from food or inhalation, it will not show how and where plastics distribute in time, it will not have an immune response to plastics. 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, not even organoids will be complex enough****. Because testing plastics on one type of immune cell at the time will not show the full immune response nor the dynamics in time and location. And by testing plastics on an artificial epithelial linings, we will miss important other cells that have an effect on plastic uptake (like M cells in the gut and alveolar macrophages in the lung).*

- Kunt u duidelijker vermelden wanneer (tijd) en met welke parameters (bv 10%, ja/nee) u keuzes maakt op basis van resultaten uit *in vitro* experimenten?
  Wij maken deze keuzes zodra we de resultaten hebben, gebaseerd op significante effecten. We hebben een deel van de antwoorden, en een deel komt later als de relevante in vitro experimenten zijn gedaan. Doordat we in de looptijd van deze aanvraag al een groot gedeelte van de humane in vitro data hebben gerealiseerd kunnen we inmiddels een concreter voorbeeld geven (pagina 3 voorlaatste alinea en extra figuur bovenaan pagina 5).

- Moeten voor het Immediate goal A (neutrofiel biologie) extra groepen worden geïncludeerd ten opzichte van wat nodig is voor Immediate goal B? Zo ja, voor welke proeven geldt dit? Om hoeveel dieren gaat het?
  We hebben ingezien dat de combinatie van immediate goal A en B veel vragen opriep bij de IvD en de commissie en daarom besloten om voor de duidelijkheid immediate goal A uit deze aanvraag te verwijderen.

5.1 lid2h

3.2 Doel:

- Bij Immediate goal A ontbreken de go- no go momenten (kennis van de neutrofiel) om verder te kunnen naar ultimate goal B en te weten welke parameters bestudeerd moeten worden.
- Hoe zijn goals A en B met elkaar verweven?
  We hebben ingezien dat de combinatie van immediate goal A en B veel vragen opriep bij de IvD en de commissie en daarom besloten om voor de duidelijkheid immediate goal A uit deze aanvraag te verwijderen.

- Kunt u bij Immediate goal B de eerste 2 go- no go 'questions' vervangen door 'criteria', zodat voor de DEC duidelijk wordt op basis van welke overweging u deze beslissing maakt?
  Het woord is vervangen.

- Wat is de herkomst van het humaan materiaal, is er voldoende materiaal om de vragen te kunnen beantwoorden en hoe zeker is de toelevering van dit materiaal?
  De herkomst van het humane materiaal is niet van invloed op de muizen experimenten. Wij hebben constante toegang tot vers bloed. Ander materiaal is afhankelijk van operaties in het ziekenhuis, en dus een uitzonderlijke situatie. Maar hier worden geen go/no go beslissingen op gemaakt.

- Hoe spelen de resultaten uit humaan materiaal een rol bij het ontwerp van het *in vivo* onderzoek in muizen?
  Zoals al eerder vermeld hebben we nu een concreter voorbeeld kunnen geven.

- Hoe beïnvloeden resultaten uit dierproeven elkaar? Stel dat uit uw eerste modellen blijkt dat microplastics geen effect hebben op neutrofielen, hoe beïnvloedt dat het doorgaan van de andere modellen?
  De modellen beïnvloeden elkaar niet. Als we geen translocatie over de darmwand vinden na orale toediening willen we zeker nog intranasale toediening uitvoeren om te kijken of ze wel de longen over gaan. Omdat er waarschijnlijk weinig microplastics de barrière over gaan willen we ook zeker proof of principle experimenten uitvoeren na iv injectie. Er zijn immers al plastics gevonden in humaan bloed. Om onze in vitro data met humane neutrofiel kweken te extrapoleren naar in vivo zijn injecties in het oor en de respons van neutrofielen op microplastics in de weefsels belangrijk. Oftewel, de verschillende modellen beantwoorden verschillende vragen.

3.4 Strategie:

- De DEC interpreteert figuur 4 zo, dat proeven zowel doel A als doel B bedienen. Klopt dat? Zo niet, kunt u de figureren aanpassen om de volgorde van proeven inzichtelijk te maken?
  Dat klop, dat wordt hierboven ook nog genoemd.

5.1 lid2h

Bijlage 1

- A. Experimentele aanpak en primaire uitkomstparameters: Welke blootstelling wordt nagebootst met de intradermale route en wilt u dit beter toelichten in de bijlage?
  De intradermale toediening is niet bedoeld om een blootstelling na te bootsen. Deze toediening maakt het bestuderen van de interactie van immuun cellen met plastic d.m.v. intravitale microscopie mogelijk met minimaal ongerief voor de muizen. We hebben hier alleen hun oor voor nodig, hoeven geen buikholte o.i.d. open te maken, want het oor kunnen we relatief makkelijk onder de microscoop leggen. Meer uitleg toegevoegd op pagina 3.

- B. De dieren: Kunt u de berekening en aannames laten zien waarop u de 7 dieren per groep baseert? Bent u van plan deze berekening tussentijds bij te stellen op basis van uw eigen resultaten?
  We hebben een stuk tekst toegevoegd om dit te verduidelijken. QUOTE: Our studies are mainly descriptive and exploratory to determine the effects of microplastics. We do not yet know what their effects will be. We think 5 animals will be enough, but actually we can only determine the sample size after we performed the experiment on 3 mice and determine the variability between animals. To calculate the maximum number of mice needed for this proposal we overestimate the use by calculating with 7 animals per group.

- F. Classificatie van ongerief: Kunt u (bijv. in een tabel) voor de muizen alle verschillende ingrepen opsommen, het ongerief van elke ingreep classificeren, de frequentie of de duur van de ingrepen benoemen en het totale maximale cumulatieve ongerief van de ingrepen schatten?
  We hebben de gevraagde tabel toegevoegd.

- De antwoorden hebben geleid tot aanpassing van de aanvraag.

De aangepaste aanvraag is door de DEC in de vergadering van 15-3-2023 besproken. De onderzoekers hebben de aanvraag grondig herschreven en hebben besloten het fundamentele wetenschappelijke deel over de rol van neutrofielen weg te laten, daarmee is de aanvraag duidelijker en meer navolgbaar geworden. De IvD heeft de aanpassingen eerst gezien en na verdere aanpassingen goedgekeurd. Ook de herziene aanvraag heeft nog geleid tot aanvullende vragen.

9c. Correspondentie met de aanvrager

- Datum vragen: 22-3-2023
- Datum antwoord: 12-4-2023
- Schriftelijk gestelde vragen en antwoorden:

Projectvoorstel

3.4 Strategie

5.1 lid2h

- Wilt u de criteria in figuur 3 opnemen op basis waarvan u besluit tot een go of no-go? Bijvoorbeeld 'needs focused' is geen goed criterium.
  Figuur 1B toegevoegd om het go/no-go beslissingsproces te verduidelijken

Bijlage 1

A. Experimentele aanpak en primaire uitkomstparameters

- Wilt u de criteria in figuur 1 opnemen op basis waarvan u besluit tot een go of no-go? Bijvoorbeeld 'needs focused' is geen goed criterium.
- Verzamelt u voldoende informatie als u in 10% van de dieren intravitale microspcopie toepast?
- Wilt u de inzet van intravitale microscopie standaard opnemen in uw protocol en beschrijven onder welke omstandigheden u er niet voor kiest? Dan kan de DEC het maximale ongerief beter meenemen in de ethische afweging.
  Intravitale microscopie wordt in andere dieren gedaan en de experimenten hebben een ander doel. Namelijk, in de tijd kijken naar een effect dat we al hebben gevonden in vitro of in dieren. Omdat het hele gerichte proeven zijn hebben we hier minder dieren voor nodig.
  We hebben een nieuw figuur 1B toegevoegd om dit beter uit te leggen. Hier leggen we ook de go-no go beter uit.

B. De dieren

- Wilt u de aantallen dieren controleren en verbeteren, waar nodig? Wat is het maximale aantal muizen, waarop de DEC een besluit moet maken? Bv. 5854 of 4834 muizen?
  Gecorrigeerd

E. Humane eindpunten

- Wilt u bij gewichtsverlies ook een tijdspad noemen?
  Er is een uitgebreider beslissingstijdpad toegevoegd

F. Classificatie van ongerief

- Wilt u de aantallen dieren controleren en verbeteren, waar nodig? Wat is het maximale aantal muizen, waarop de DEC een besluit moet maken? Bv. wat bedoelt u met 87.2% of the animals (4834)?
  Gecorrigeerd

- De antwoorden hebben geleid tot aanpassing van de aanvraag (in groene highlights).

10. Eventuele adviezen door experts (niet lid van de DEC): n.v.t.

B. Beoordeling (adviesvraag en behandeling)

1. Het project is vergunningplichtig (dierproeven in de zin der wet).
2. De aanvraag betreft een nieuwe aanvraag.
3. De DEC is competent om hierover te adviseren.

5.1 lid2h

4. Vanwege betrokkenheid bij het betreffende project is één DEC-lid, met het oog op onafhankelijkheid en onpartijdigheid, niet betrokken bij de advisering.

C. Beoordeling (inhoud):

1. De aanvraag is toetsbaar en heeft voldoende samenhang. *De herschreven aanvraag is in de huidige vorm begrijpelijk opgesteld, navolgbaar in de onderzoeksvragen en hoe deze zijn vertaald in de diverse experimenten en daardoor toetsbaar geworden. De onderzoekers maken deel uit van een groter Nederlands consortium, dat gefinancierd wordt door de overheid. Het gehele onderzoek is verdeeld in verschillende delen per deelnemer van het consortium. De aanvragers zullen hun aandeel onderzoeken: wat de risico's zijn van blootstelling van microplastics bij de mens. Er zal door de aanvragers worden onderzocht wat de effecten in-vivo zijn, nadat andere partners de karakterisatie en de in-vitro blootstellingen hebben onderzocht.*
Het eerder beschreven fundamentele onderzoek naar specifieke rol van neutrofielen is er uit gehaald. Maar de onderzoekers zullen hun kennis van neutrofielen benutten om de in-vivo experimenten uit te voeren.
De onderzoekers zullen microplastics, die in vitro door andere partners effectief zijn bevonden, verder onderzoeken op schadelijke effecten op immuunsysteem en/ of andere organen. Er is een stapsgewijze aanpak beschreven met heldere beslismomenten. Voor een deel zijn het beschrijvende experimenten. Ook als geen effect wordt gevonden. Het is primair belangrijk voor de maatschappij, overheid en fabrikanten, dat informatie beschikbaar komt over blootstelling aan microplastics, omdat vertaald kan worden dat blootstelling schadelijk of (voor zover bekend) niet schadelijk zal zijn.

   De aanvraag komt het meest overeen met voorbeeld 1A uit de nieuwe Handreiking "Invulling Definitie Project".

2. Voor zover de DEC bekend, is er geen mogelijk tegenstrijdige wetgeving die het uitvoeren van de dierexperimenten in de weg zou kunnen staan.

3. De in de aanvraag aangekruiste doelcategorie(ën), te weten fundamenteel onderzoek en Onderzoek ter bescherming van het milieu in het belang van de gezondheid of het welzijn van mens of dier sluiten aan bij de hoofddoelstelling.

   Belangen en waarden

4. Het directe doel van het project is *het gezondheidsrisico van blootstelling aan microplastics te bepalen, zodat de overheid weloverwogen beslissingen kan nemen over plasticregelgeving als er risico's voor de gezondheid bestaan. Daarnaast is het fundamentele doel meer kennis te vergaren over de effecten van microplastics die epitheliaal weefsel kunnen passeren en een immunotoxisch effect kunnen veroorzaken en de rol daarin van neutrofielen. Het uiteindelijke doel is om overheden, publiek en fabrikanten te informeren over potentieel schadelijke microplastics en indien noodzakelijk regelgeving aan te passen.*
   De DEC is van mening dat er een duidelijke relatie is tussen het directe en het uiteindelijke doel, en dat het doel gerechtvaardigd is in de context van *het onderzoeksveld en de overheid* en de behoeften vanuit *de maatschappij en fabrikanten.*

5.1 lid2h

5. De belangrijkste belanghebbenden in dit onderzoeksproject zijn weergegeven on onderstaande tabel.

belanghebbenden	**Morele waarden die worden bevorderd**
onderzoekers	kennis vergaren, kunnen publiceren
maatschappij	eigen gezondheid, kennis omtrent mogelijke risico's
overheid	kennis over blootstelling en risico's kunnen communiceren naar maatschappij, indien noodzakelijk regelgeving instellen
omgeving/milieu	gezonde leefomgeving voor mens en dier
gezondheidsfondsen	publiek kunnen informeren over blootstelling en de mogelijke risico's
	Morele waarden die in het geding zijn
proefdieren	hebben belang dat er geen experimenten worden gedaan met hen

6. De aanvrager geeft niet aan nadelige effecten op het milieu te verwachten. De DEC ziet geen aanleiding om aan te nemen dat zich toch nadelige effecten zullen voordoen.

Proefopzet en haalbaarheid

7. De kennis en kunde van de onderzoeksgroep en andere betrokkenen bij de dierproeven zijn voldoende gewaarborgd en dragen eraan bij dat de doelstellingen behaald kunnen worden, dat aan de 3V-beginselen voldaan kan worden en dat voorkomen kan worden dat mens, dier en milieu negatieve effecten ondervinden als gevolg van de dierproeven. *De onderzoekers zijn partners in het consortium vanwege hun expertise in immunologische responsen in muizenstudies. De onderzoekers hebben al pilotexperimenten uitgevoerd om de haalbaarheid te onderzoeken. Hieruit is gebleken, dat aan het formaat van de microplastics, die gebruikt gaan worden in de experimenten, eisen moeten worden gesteld, maar waarmee het mogelijk is gebleken om immunologische effecten te kunnen meten.* 5.1 lid1c [redacted]

8. Het project is goed opgezet, de voorgestelde experimentele opzet en uitkomstparameters sluiten logisch en helder aan bij de aangegeven doelstellingen. De gekozen strategie en experimentele aanpak kunnen leiden tot het behalen van de doelstelling binnen het kader van het project. *De strategie van dit project is om menselijke in vitro experimenten te combineren met murine ex vivo en in vivo experimenten. De uitleesparameters van de in-vivo experimenten zijn helder beschreven en in principe gelijk, maar de immuunstimulerende werking of verbinding van het microplastic zal per experiment verschillen. De immunologische responsen tegen microplastics worden vergeleken met die van andere kunststoffen met biofilm en LPS om de risico-inschatting goed te kunnen maken. Een deel van het onderzoek zal plaatsvinden mbv intravitale microscopie,*

5.1 lid2h [redacted]

waardoor in een levende muis de real-time neutrofielenrespons zichtbaar gemaakt en gevolgd kan worden. De procedures worden uitgevoerd volgens de laatste best practices door bekwaam personeel.

Welzijn dieren

9. Er is geen sprake van de volgende bijzonderheden op het gebied van categorieën van dieren, omstandigheden of behandeling van de dieren:
   - ☐ Bedreigde diersoort(en) (10e lid 4)
   - ☐ Niet-menselijke primaten (10e)
   - ☐ Dieren in/uit het wild (10f)
   - ☐ Niet gefokt voor dierproeven (11, bijlage I EU richtlijn)
   - ☐ Zwerfdieren (10h)
   - ☐ Hergebruik (1e lid 2)
   - ☐ Locatie: buiten instelling vergunninghouder (10g)
   - ☐ Geen toepassing verdoving/pijnbestrijding (13)
   - ☐ Dodingsmethode niet volgens bijlage IV EU richtlijn (13c lid 3)

10. De dieren worden gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van de EU richtlijn.

11. Het cumulatieve ongerief als gevolg van de dierproeven is realistisch ingeschat en geclassificeerd. *Per uit te voeren handeling is het ingeschatte ongerief opgenomen en het cumulatieve ongerief per experiment op basis van het geheel van handelingen. De DEC kan zich vinden in de ongerief inschatting per handeling en het cumulatieve ongerief in een tabel met daarbij de grenzen van de frequentie en de tijdsduur. Per experiment kan immers pas de absolute frequentie en het tijdpad worden opgegeven als het finale experimentele ontwerp klaar is. De IvD heeft aangegeven om de grenzen die in de tabel in bijlage 1 onder F worden vermeld te gebruiken voor de navolgbaarheid.*

12. De integriteit van de dieren wordt *fysiek en gedragsmatig (bij de intravitale beeldvorming) aangetast door de uit te voeren handelingen en behoren bij de uitvoering van de proef.*

13. De humane eindpunten zijn in de bijlage dierproeven goed gedefinieerd en het percentage dieren dat naar verwachting een humaan eindpunt bereikt is goed ingeschat. *Er zijn algemene parameters opgegeven die verwacht kunnen worden en model specifieke humane eindpunten passend bij de gevolgen van LPS toediening of andere opwekking van de immuun responsen en worden per werkprotocol goed met de IvD afgestemd.*

3V's

14. De aanvrager heeft voldoende aannemelijk gemaakt dat er geen geschikte vervangingsalternatieven zijn. *"In ons consortium gebruiken we voornamelijk niet-dierlijke*

5.1 lid2h

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 worden directe cel assays gebruikt maar ook mini-darmpjes en mini-longetjes, waarmee een eerste inschatting van schadelijkheid kan worden vastgesteld." Met deze dierproeven leveren de onderzoekers een belangrijke bijdragen aan het onderzoek van het consortium om dat hiermee de bewegingen van microplastics door het hele lichaam te volgen zijn en hoe lang ze op een bepaalde plek blijven zitten. Dit type proeven kunnen momenteel nog niet met proefdiervrije methoden worden uitgevoerd.

15. Het aantal te gebruiken dieren is realistisch ingeschat en er is een heldere strategie om ervoor te zorgen dat tijdens het project met het kleinst mogelijke aantal dieren wordt gewerkt waarmee nog een betrouwbaar resultaat kan worden verkregen. *Door meerdere onderzoeken in dezelfde muis te doen, zijn er minder muizen nodig. Ook door in dezelfde muis op meerdere momenten live te kijken wat er met de plastics gebeurt, zijn minder dieren nodig.*

16. Het project is in overeenstemming met de vereiste van verfijning van dierproeven en het project is zodanig opgezet dat de dierproeven zo humaan mogelijk kunnen worden uitgevoerd. *De meeste handelingen worden onder anesthesie uitgevoerd. Perioperatief wordt pijnbestrijding toegepast. Er worden o.a. pilotexperimenten uitgevoerd om de dosering te bepalen voor orale en nasale toediening, waarmee microplastics in weefsels kunnen worden gedetecteerd. Indien mogelijk worden de microplastics via voer toegediend (en niet via orale sonde). In een beperkt aantal experimenten zal de biodistributie d.m.v. intravitale microscopie worden beoordeeld.*

17. Er is geen sprake van wettelijk vereist onderzoek.

Dieren in voorraad gedood en bestemming dieren na afloop proef

18. Dieren van beide geslachten zullen in gelijke mate worden ingezet.

19. De dieren worden in het kader van het project gedood, omdat na afloop van de experimenten in-vivo men bloed en organen wil uitnemen om verder te kunnen analyseren. De dieren worden op een passende wijze, in overeenstemming met bijlage IV van de EU richtlijn, gedood.

20. De vraag over hergebruik is niet van toepassing omdat de dieren gedood worden in het kader van het experiment.

NTS

21. De niet-technische samenvatting is een evenwichtige weergave van het project en begrijpelijk geformuleerd.

5.1 lid2h

D. Ethische afweging

1. De morele vraag die de DEC dient te beantwoorden is of het belang van dit onderzoek, namelijk *of het bepalen van het gezondheidsrisico aan blootstelling van microplastics met het uiteindelijke doel om overheden, publiek en fabrikanten te informeren over potentieel schadelijke microplastics en indien noodzakelijk regelgeving aan te passen en meer kennis te verzamelen over de effecten van microplastics, die epitheliaal weefsel kunnen passeren en een immunotoxisch effect kunnen veroorzaken en de rol daar in van neutrofielen,* de onvermijdelijke aantasting van het welzijn en de integriteit van de gebruikte proefdieren kan rechtvaardigen.

2. Er vindt een deels *beperkte en deels aanzienlijke* aantasting van welzijn en integriteit van de proefdieren plaats, met mild ongerief voor 620 dieren en voor 4214 met matig ongerief. Indien de hierboven genoemde doelstellingen behaald worden, dan zal dit project er toe bijdragen dat *er een goede risico inschatting gemaakt kan worden voor blootstelling aan microplastics in de mens en dat meer kennis verkregen zal worden over de rol van de neutrofielen.* Het is aannemelijk dat de doelstellingen voor fundamenteel onderzoek en onderzoek ter bescherming van het milieu in het belang van de gezondheid of het welzijn van mens of dier behaald zullen worden. Daarvoor is de inzet van proefdieren noodzakelijk, maar de onderzoekers doen al het mogelijke om het ongerief voor de dieren en het aantal dieren tot een minimum te beperken.

3. Op grond van het bovenstaande is de DEC van oordeel dat *het bepalen van het gezondheidsrisico aan blootstelling van microplastics met het uiteindelijke doel om overheden, publiek en fabrikanten te informeren over potentieel schadelijke microplastics en indien noodzakelijk regelgeving aan te passen en meer kennis op te doen over de effecten van microplastics die epitheliaal weefsel kunnen passeren en een immunotoxisch effect kunnen veroorzaken en de rol daar in van neutrofielen* een essentieel belang vertegenwoordigt en dat dit essentiële belang opweegt tegen de grotendeels aanzienlijke aantasting van het welzijn en de integriteit van de proefdieren. De relatie tussen het directe en het uiteindelijk doel is voldoende helder. Het is aannemelijk dat de directe doelstelling behaald zal worden. De commissie is overtuigd van de kwaliteit van het werk van de aanvrager. De aanvrager heeft voldoende aannemelijk gemaakt dat er geen geschikte vervangingsalternatieven zijn, dat het doel niet met minder dieren behaald kan worden, dat de gebruikte aanpak de meest verfijnde is en dat er geen sprake zal zijn van onbedoelde negatieve effecten voor mens, dier en milieu als gevolg van de dierproeven. Het gebruik van de proefdieren zoals beschreven in de aanvraag is daarmee gerechtvaardigd.

E. Advies

1. Advies aan de CCD

☒ De DEC adviseert de vergunning te verlenen.

☐ De DEC adviseert de vergunning te verlenen onder de volgende voorwaarden.

5.1 lid2h

☐ Op grond van het wettelijk vereiste dient de projectleider bij beëindiging van het project een beoordeling achteraf aan te leveren die is afgestemd met de IvD.
☐ Voor de uitvoering van dit project is tevens ministeriële ontheffing vereist
☐ Overige door de DEC aan de uitvoering verbonden voorwaarden, te weten...

☐ De DEC adviseert de vergunning niet te verlenen vanwege:
☐ De vaststelling dat het project niet vergunningplichtig is om de volgende redenen:...
☐ De volgende doorslaggevende ethische bezwaren:...
☐ De volgende tekortkomingen in de aanvraag:...

2. Het uitgebrachte advies is gebaseerd op een meerderheidsstandpunt, waarbij het quorum wel werd behaald. *Vanwege betrokkenheid bij het betreffende project is één DEC-lid, met het oog op onafhankelijkheid en onpartijdigheid, niet betrokken bij de advisering. Een ander DEC-lid onthoudt zich van stemming, omdat deze zich niet kan vinden in de focus op neutrofielen zonder de interactie met macrofagen hierbij te betrekken. De focus zou meer gericht moeten zijn op interactie macrofagen, welke als eerste in contact zullen komen met de microplastics en de activiteit van neutrofielen mede kunnen bepalen.*

3. De volgende knelpunten/dilemma's zijn naar voren gekomen tijdens het beoordelen van de aanvraag en het opstellen van het advies. Er is uitgebreid gediscussieerd, waarom de onderzoekers zich richten op de rol van de neutrofielen als de macrofagen als eerste betrokken zijn bij de immuunrespons? Samengevat ligt de focus van de aanvraag op het inschatten van het risico aan blootstelling van microplastics bij de mens, maar omdat de onderzoekers zelf veel onderzoek doen naar de rol van neutrofielen zijn die uitkomsten voor hen ook interessant. Het belang van de risico's voor mens en dier zijn zwaarwegend geweest bij de eindafweging voor de DEC.

5.1 lid2h

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.	Characterizing the interplay between microplastics and the innate immune system

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.

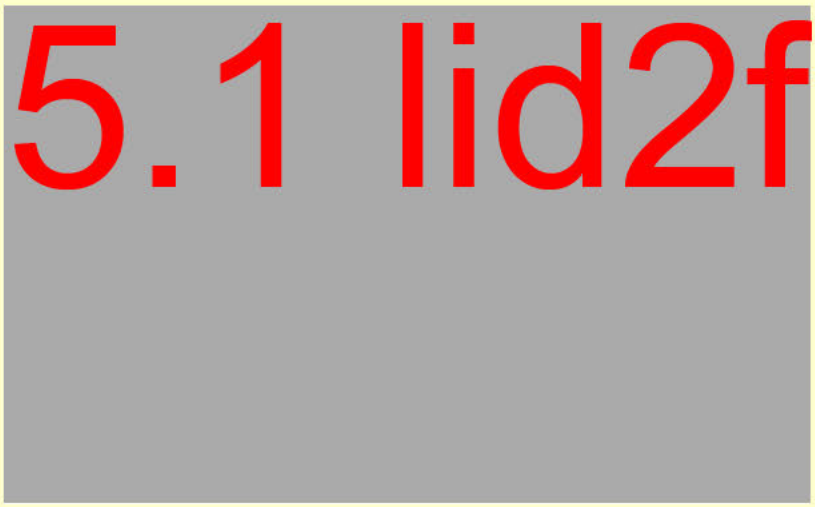


Figure 1. Schematic representation of the structure of the 5.1 lid1c *consortium. The big question of the risk of plastic exposure is divided in smaller questions. For each sub question a group of experts is assembled to get a broad perspective. All parts communicate to come to a risk assessment and come up with solutions for plastics exposure.* 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 can only verify the presence of microplastics, but it will not show uptake of plastics from food or inhalation, it will not show how and where plastics distribute in time, it will not have an immune response to plastics. 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, not even organoids will be complex enough.** Because testing plastics on one type of immune cell at the time will not show the full immune response nor the dynamics in time and location. And by testing plastics on an artificial epithelial linings, we will miss important other cells that have an effect on plastic uptake (like M cells in the gut and alveolar macrophages in the lung).

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 tissues mentioned 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, we have found toxic effects on human neutrophils in vitro of <1 µm and 1-5 µm PVC, but not of similar size nylon particles. Therefore it would be highly relevant to compare their in vivo effect on neutrophils.

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. Our lab has done numerous discoveries when it comes to neutrophil biology. For example, we identified different neutrophil subsets and have an idea of their role in different diseases[4-8]. We will combine our expertise on neutrophils with our experience in performing mouse experiments to study the innate immune system. 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.

By comparing the reaction of the immune system to foreign bodies like plastics to acute and chronic inflammation, we will be able to determine the health risks associated with our exposure to microplastics.

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 lid2h, 5.1 lid2e

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.

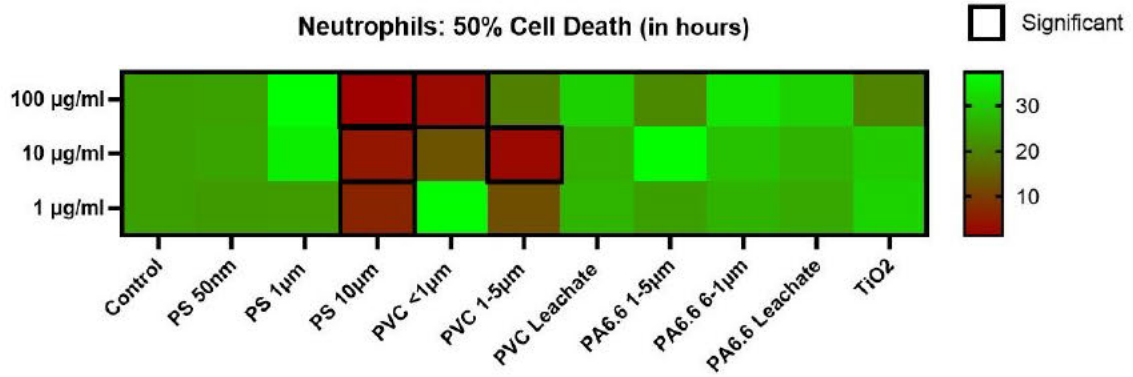
5.1 lid1c has the goal to determine the health risk of the different types of plastics we are exposed to daily, looking into the effects of different materials/polymer types, sizes and shapes. This goal, together with our expertise and interest in the immune system, gives us the **goal of this proposal**: **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.**

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. Our expertise in neutrophil biology and their response to natural ques like infection and inflammation gives us a frame to understand how neutrophils react to microplastics. This will also help determine the health risks of microplastics.

The focus of this proposal is on microplastics: determine the health effects of exposure to different and environmentally relevant microplastics. 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 chemicals, specific only for that production process. 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 and spleen, and a bit in the bone marrow. We also confirmed that it's both the neutrophils and different types of macrophages that take up these plastics.
The polystyrene particles do not resemble the plastics we are exposed to in real life, not in size, shape, polymer or coating. The microplastics we are about to test are made by 5.1 lid1c 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.

The go/no go criteria 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* but will be compared to a negative control particle that doesn't show toxicity.
- 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 unless when used as a negative control.



Example how in vitro results act as go/no go for in vivo experiments.
In the figure human neutrophil toxicity was tested in vitro for different MOMENTUM microplastics.
The positive control microplastics PS 10µm are toxic as are the PVC < 1µm and 1-5µm, other plastics do not show toxicity.
Based on this data it would be of interest to compare the in vivo neutrophil response of PVC 1-5µm to PA6.6 1-5µm in mice since these are similar sizes that show a very different toxicity in vitro.

- 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.

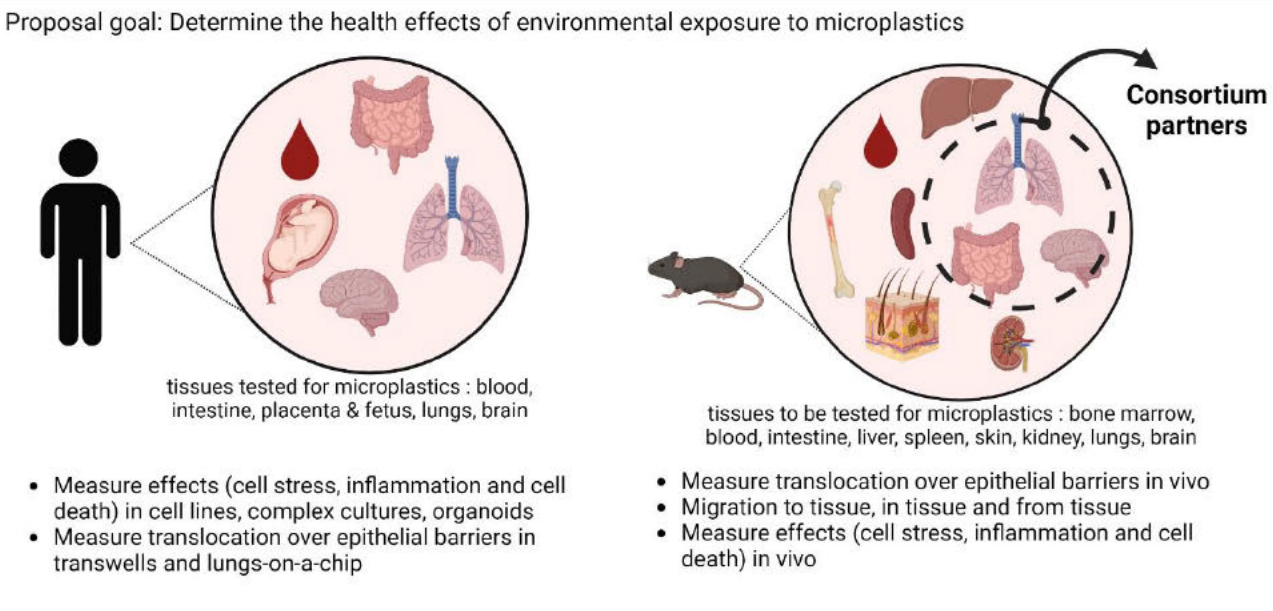


Figure 2. *Schematic clarification of the experiments that can and are performed in human (left), and the experiments we have been performing and intent to perform in mice (right). Tissues within the inner circle will be offered to consortium partners to resolve their research questions within the consortium. (Created with Biorender.com)*

Overall goal: 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

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, primarily monocytes and macrophages. 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. Determining the effect of microplastics on monocytes and macrophages can be done post mortem in most cases, and will be done in parallel to analyzing the effect on neutrophils in mice. The handful of experiments focused on macrophages will be performed in the same set-up as the neutrophil focused experiments and will not cause additional discomfort.

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 with acute inflammation and mice treated with plastic, 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 [redacted] 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 [redacted] 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.

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3.3 Relevance

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

Understanding the body's reaction to exposure of particles like plastic 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").

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 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.

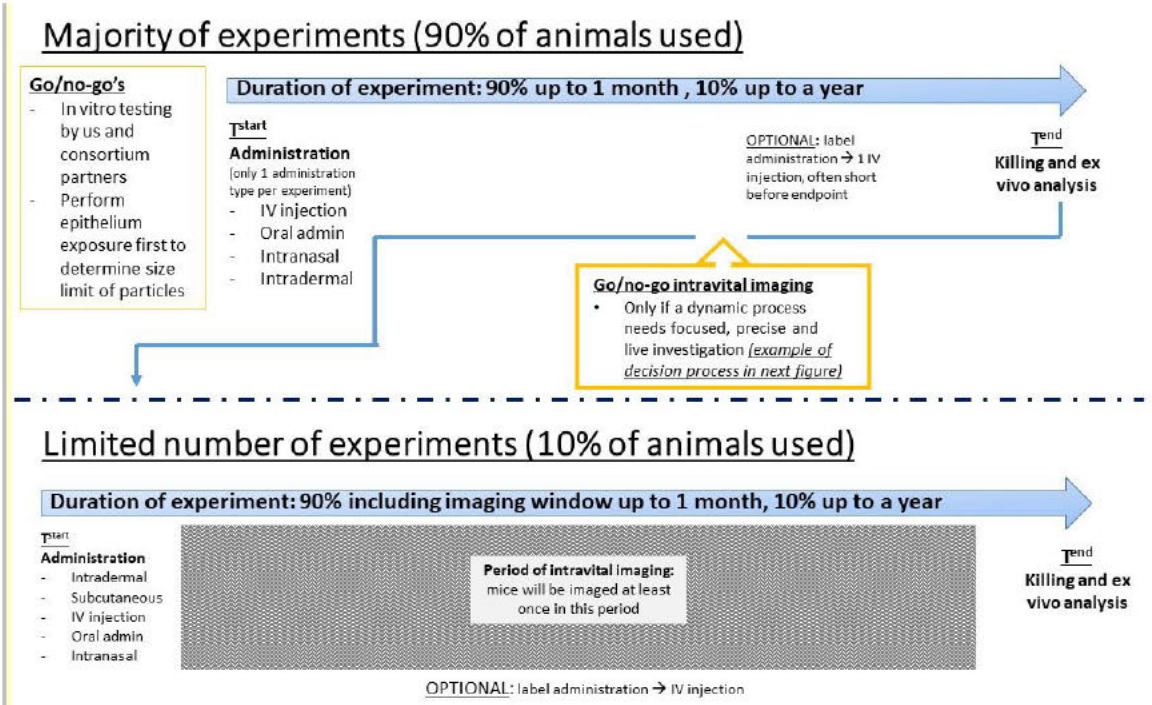

Figure 3. schematic overview of the general designs of the experiments and the questions they will answer. As indicated will the majority of the experiments be performed in a simple set-up: one route of administration, with only the option of label administration (like a CD45 antibody). Only if the results from these experiments raises questions about specific details of the dynamics, will intravital imaging be performed.

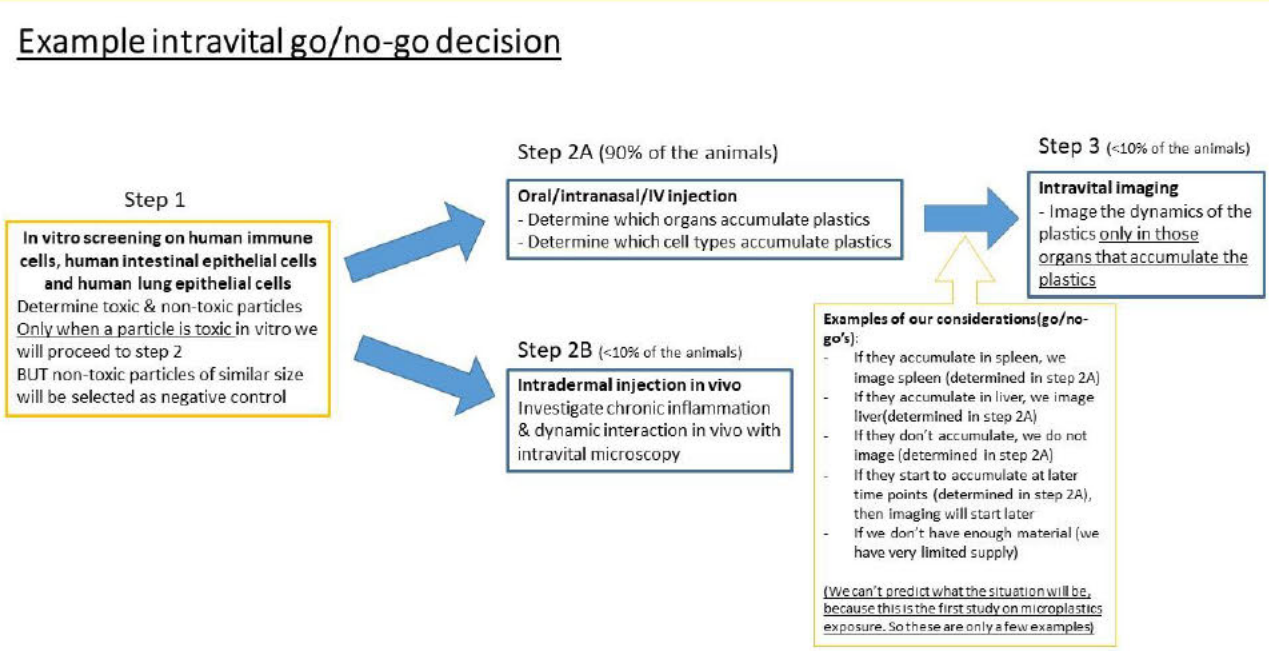

Figure 4. schematic overview of the go/no-go decision process for performing intravital imaging. Based on the in vitro findings we will do, we will expose mice to these plastics. The majority of animals will only receive oral/intranasal/IV plastics. These experiments will be used for in vivo toxicity confirmation, and also to determine which organs will be

imaged in intravital imaging and which cells to focus on. The plastics determined toxic in vitro will also be tested for long term immunological effects and easy-access dynamics with intradermal injection and imaging.

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 (decision making process in fig. 4)
- 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. 3), 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 (fig.5). But also plastics with biofilm as explained earlier will need to be tested, because that is the status of the 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 (fig. 4). Intravital imaging will also be performed in the way that gives least discomfort and most easy to perform. Intradermal and subcutaneous injection keeps the plastics within easy reach of imaging with the microscope, and are less invasive procedures to perform.

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 (positive control)	- Check if phagocytosis of microplastics prevents phagocytosis of bacteria (terminal exp) - Compare the effects of microplastics to other inflammatory stimuli to place the effect in the right perspective
	Sterile injury (negative control)	- Establish if immobilized immune cells that have engulfed microplastics can be prompted to mobilize and distribute plastics upon local inflammation (terminal experiment)
	LPS injection (positive control)	- Establish if immobilized immune cells that have engulfed microplastics can be prompted to mobilize and distribute plastics upon systemic inflammation (terminal experiment)
	Non-plastic inert materials (injected particle reference)	- Effect on inflammation - Translocation - Distribution - Effect on immune cell function
Plastic particles with biofilm	By itself (only)	- Establish whether a defective response to bacteria on plastic contributes to infections found in patients in vivo

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.

We use LPS in these questions to mimic a bacterial infection or sepsis, without actually injecting the bacteria. This will be one of the positive controls to compare the reaction of the neutrophils to: do they respond to

microplastics as they would to a systemic bacterial infection? It has the same effect as injecting bacteria, but it is more controlled. Where necessary we will be injecting bacteria as the positive control instead of LPS. This way we can compare the two and determine if plastics induce the same immune response as bacteria. In order to determine whether the microplastics are as inert as we always believed they would be, they will be compared to particles that have been proven to be inert.

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 by comparing the effects of microplastics to the proper controls (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 lid2h 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
2	Click or tap here to enter text.
3	Click or tap here to enter text.
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6	Click or tap here to enter text.
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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: 1 Type of animal procedure: 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 the ultimate goal of determining the health risk of microplastics exposure. In order to do so, microplastics exposure will be compared to bacterial exposure (in the form of bacteria or LPS), exposure to known inert materials, and/or sterile damage controls.

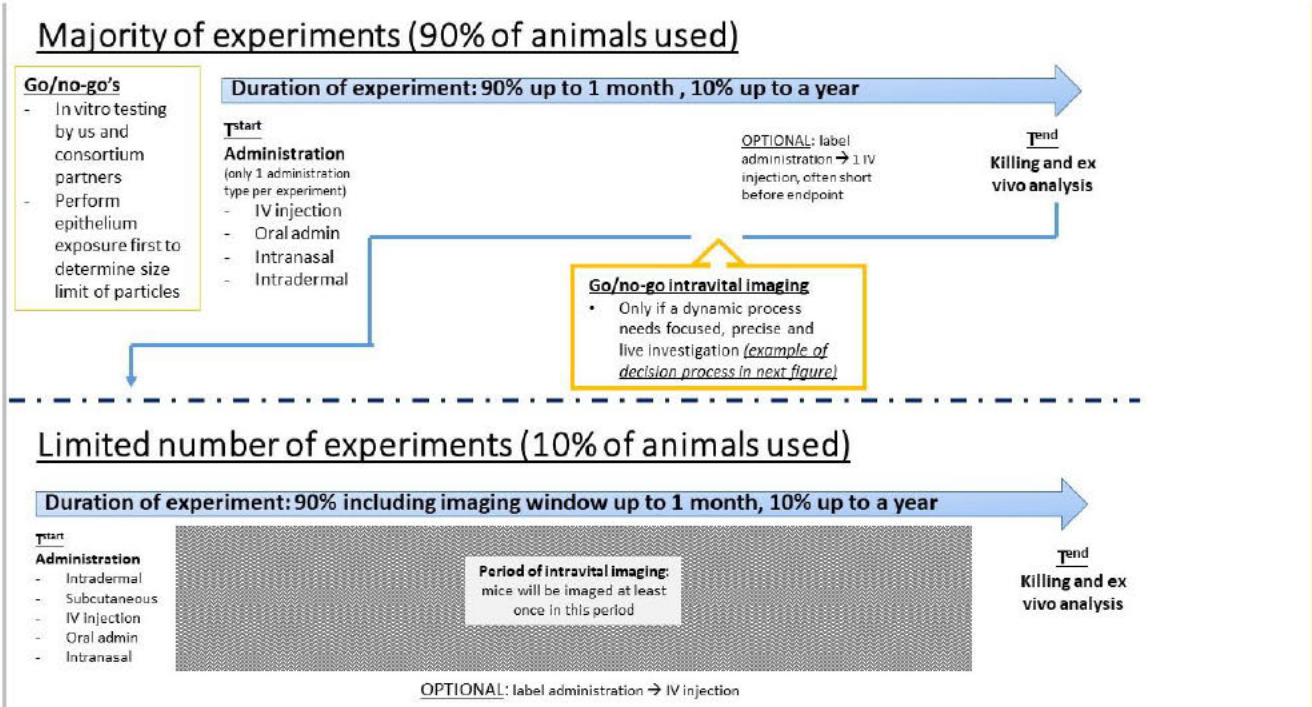

Figure 1A. Schematic representation of the experiments with the go/no-go's indicated.

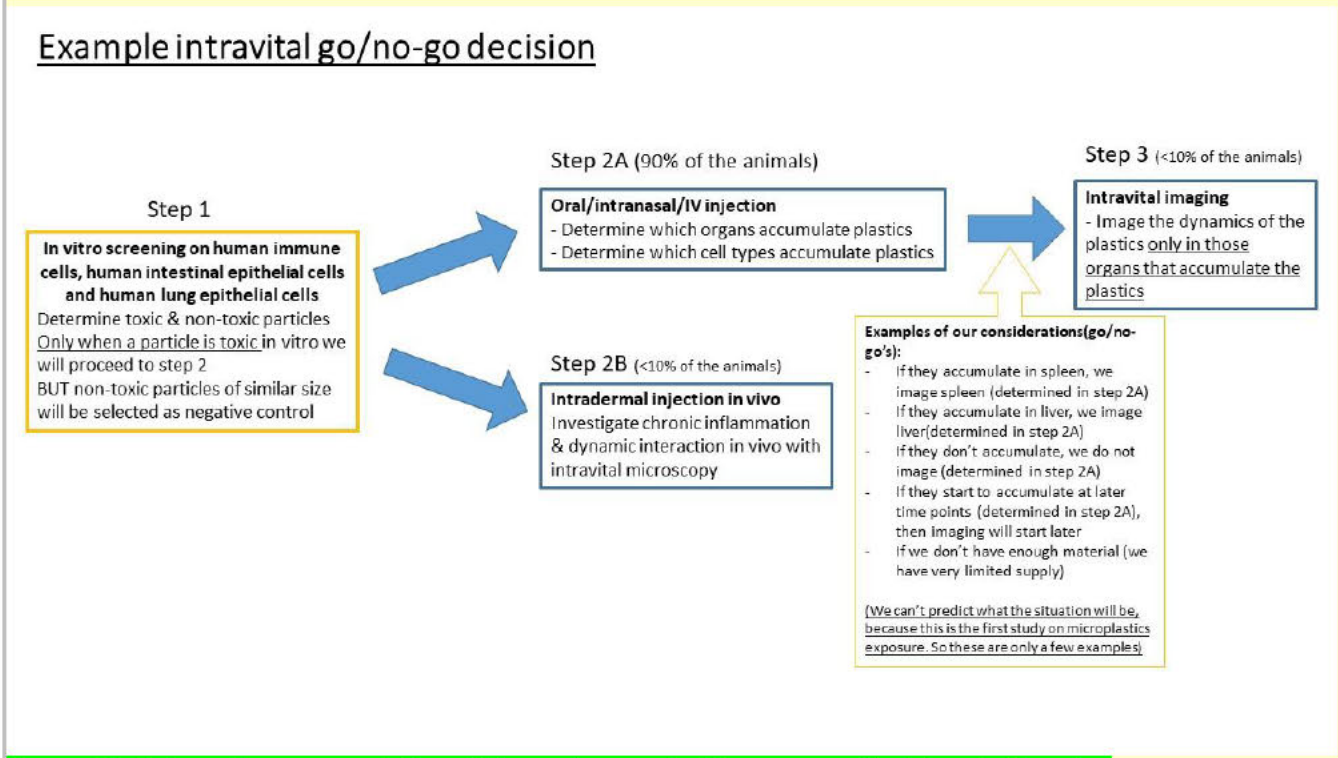

Figure 1B. Schematic representation of the go/no-go decision process for intravital imaging

General design (see figure 4 of the project proposal):
I (every experiment)
Adminstration of the following compounds via various administration routes
- plastics in different sizes (with and without different coatings)
- sterile damage (acute response to the procedure can happen)
- Non-plastics particle controls (biologically degradable non-immunogenic particles)

- biological pathogens like LPS or bacteria (dose and duration will determine acute or chronic inflammation)

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.
- 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.

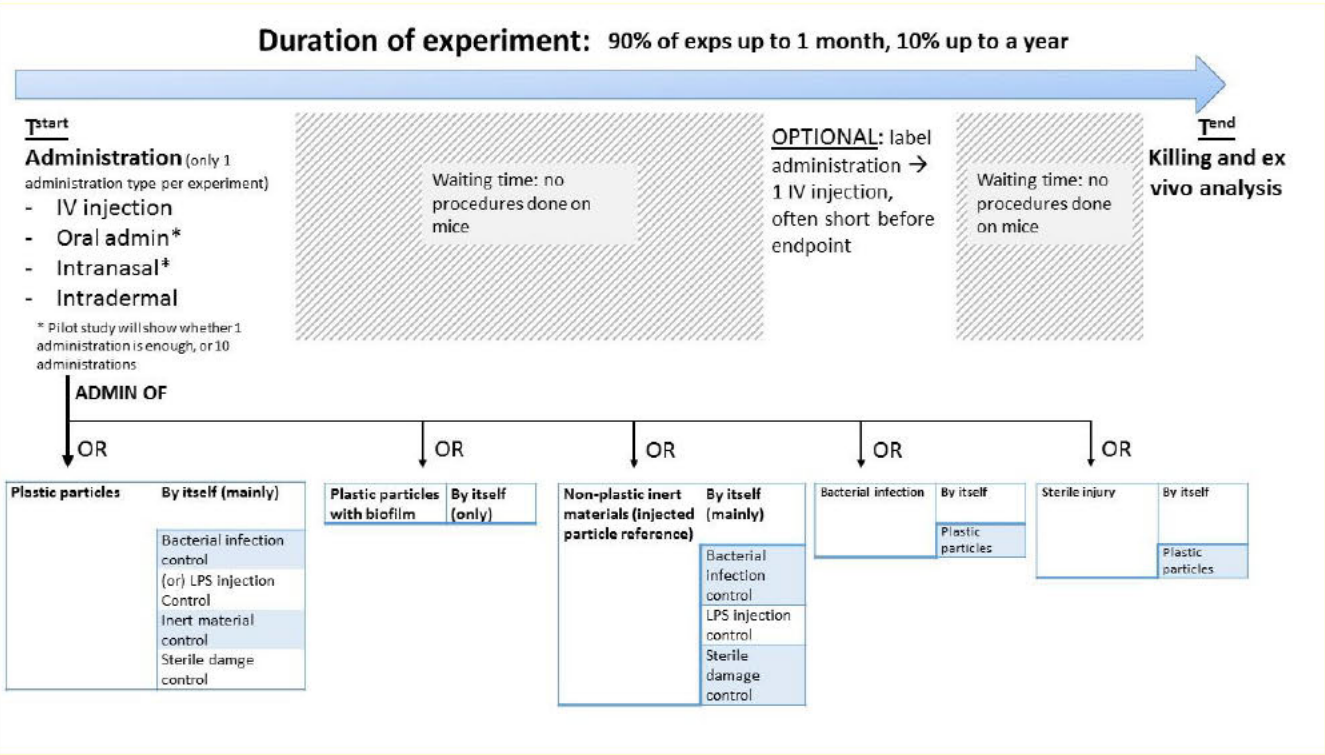

Figure 2. Schematic overview of the majority of the experiments. The duration is indicated at the top to show when certain procedures will take place during the experiment, starting on the left with the administration of the stimulus, the three different administration routes, the option to inject a 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: intradermal and subcutaneous. These two administration routes keep the plastics within easy, non-invasive, reach for repeated imaging with the 2-photon microscope. 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.

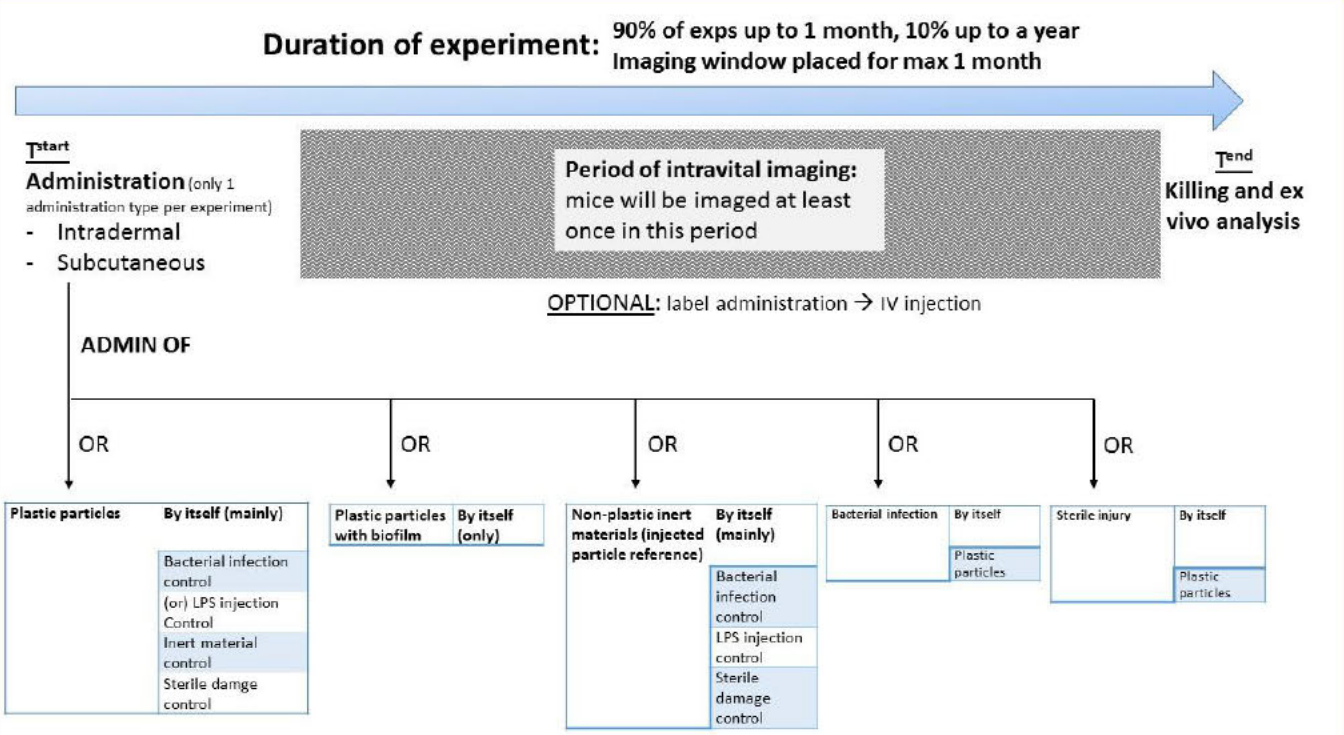

Figure 3. Schematic overview of the rest of the experiments. The duration is indicated at the top to show when certain procedures will take place during the experiment, starting on the left with the administration of the stimulus, the different administration routes, the option to inject a label or not, 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 first round 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 (PA6.6 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. 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. Most importantly, 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. 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.

Control Inflammatory stimulus: Bacterial infection
Justification: as a control for undegradable microplastics and as a naturally occurring infection, bacteria will be used as a control. 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. 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 establish if immobilized immune cells that have engulfed microplastics can be prompted to mobilize and distribute plastics upon local inflammation or to establish if microplastic induced inflammation is more prolonged than sterile. 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 establish if immobilized immune cells that have engulfed microplastics can be prompted to mobilize and distribute plastics upon systemic inflammation. 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 (eg to influence immune cell trafficking or prevent biofilm formation on plastics), antibodies (eg to visualize cells), 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