| | | **Inventaris Wob-verzoek W17-08** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | **wordt verstrekt** | | | | **weigeringsgronden** | | | |
| **nr.** | | **document NTS 2016714** | **reeds openbaar** | **niet** | **geheel** | **deels** | **10.1.c** | **10.2.e** | **10.2.g** | **11.1** |
| 1 | | Origineel aanvraagformulier | | | | x | | x | x | |
| 2 | | Origineel NTS | | | x | | | | | |
| 3 | | Origineel projectvoorstel | | | | x | x | | x | |
| 4 | | Originele bijlage | | | | x | x | | x | |
| 5 | | Ontvangstbevestiging en factuur | | | | x | | x | x | |
| 6 | | Verzoek om aanvullende informatie | | | | x | | x | x | |
| 7 | | Antwoord op verzoek om aanvullende informatie | | | | x | | x | x | |
| 8 | | Projectvoorstel versie 2 | | | | x | x | | x | |
| 9 | | Bijlage 1 versie 2 | | | x | | | | | |
| 10 | | Bijlage 2 versie 2 | | | | x | x | | x | |
| 11 | | Bijlage 3 versie 2 | | | | x | x | | x | |
| 12 | | Bijlage 4 versie 2 | | | | x | x | | x | |
| 13 | | DEC advies | | | | x | | x | x | |
| 14 | | NTS versie 2 | | | x | | | | | |
| 15 | | Projectvoorstel definitief | | | | x | x | | x | |
| 16 | | Bijlage 1 definitief | | | x | | | | | |
| 17 | | Bijlage 2 definitief | | | | x | x | | x | |
| 18 | | Bijlage 3 definitief | | | | x | x | | x | |
| 19 | | Bijlage 4 definitief | | | | x | x | | x | |
| 20 | | NTS definitief | x | | | | | | | |
| 21 | | Advies CCD | | x | | | | | | x |
| 22 | | Reactie op voornemen afwijzing CCD | | | | x | | x | x | |
| 23 | | Aanvullend advies DEC | | | | x | | x | x | |
| 24 | | Beschikking en vergunning | | | | 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 bel met 0900-2800028 (10 ct/min).

## 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* 11500
☐ Nee > *U kunt geen aanvraag doen*

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

| | |
|---|---|
| Naam instelling of organisatie | UMC Utrecht |
| Naam van de portefeuillehouder of diens gemachtigde | [redacted] |
| KvK-nummer | 30244197 |

1.3 Vul de gegevens van het postadres in.
*Alle correspondentie van de CCD gaat naar de portefeuillehouder of diens gemachtigde en de verantwoordelijke onderzoeker.*

| | |
|---|---|
| Straat en huisnummer | Instantie voor Dierenwelzijn Utrecht |
| Postbus | 12007 |
| Postcode en plaats | 3501AA Utrecht |
| IBAN | NL27INGB0000425267 |
| Tenaamstelling van het rekeningnummer | Universiteit Utrecht |

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

| | |
|---|---|
| (Titel) Naam en voorletters | [redacted] ☐ Dhr. ☒ Mw. |
| Functie | Assistant Professor |
| Afdeling | [redacted] |
| Telefoonnummer | [redacted] |
| E-mailadres | [redacted] |

1.5 *(Optioneel)* Vul hier de gegevens in van de plaatsvervangende verantwoordelijke onderzoeker.

| | |
|---|---|
| (Titel) Naam en voorletters | [redacted] ☐ Dhr. ☒ Mw. |
| Functie | OIO |
| Afdeling | [redacted] |
| Telefoonnummer | [redacted] |
| E-mailadres | [redacted] |

| | | | |
|---|---|---|---|
| 1.6 | *(Optioneel)* Vul hier de gegevens in van de persoon die er verantwoordelijk voor is dat de uitvoering van het project in overeenstemming is met de projectvergunning. | (Titel) Naam en voorletters | ☐ Dhr. ☐ Mw. |
| | | Functie | |
| | | Afdeling | |
| | | Telefoonnummer | |
| | | E-mailadres | |
| 1.7 | 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 | Wat voor aanvraag doet u? | ☒ Nieuwe aanvraag > Ga verder met vraag 3<br>☐ Wijziging op (verleende) vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn<br>Vul uw vergunde projectnummer in en ga verder met vraag 2.2<br>☐ Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn<br>Vul uw vergunde projectnummer in en ga verder met vraag 2.3 |
| 2.2 | Is dit een *wijziging* voor een project of dierproef waar al een vergunning voor verleend is? | ☐ Ja > Beantwoord dan in het projectplan en de niet-technische samenvatting alleen de vragen waarop de wijziging betrekking heeft en onderteken het aanvraagformulier<br>☐ Nee > Ga verder met vraag 3 |
| 2.3 | Is dit een *melding* voor een project of dierproef waar al een vergunning voor is verleend? | ☐ Nee > Ga verder met vraag 3<br>☐ Ja > Geef hier onder een toelichting en ga verder met vraag 6 |

## 3 Over uw project

| | | | |
|---|---|---|---|
| 3.1 | Wat is de geplande start- en einddatum van het project? | Startdatum | 1 - 11 - 2016 |
| | | Einddatum | 31 - 10 - 2021 |
| 3.2 | Wat is de titel van het project? | Neutrophil subsets in health and disease | |
| 3.3 | Wat is de titel van de niet-technische samenvatting? | Neutrofielen subtypes in gezondheid en ziekte | |
| 3.4 | Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt? | Naam DEC | DEC Utrecht |
| | | Postadres | Postbus 85500 3508 GA Utrecht |
| | | E-mailadres | dec-utrecht@umcutrecht.nl |

# 4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

☒ Nieuwe aanvraag Projectvergunning € 935,- Lege

☐ Wijziging € Lege

4.2 Op welke wijze wilt u dit bedrag aan de CCD voldoen.
*Bij een eenmalige incasso geeft u toestemming aan de CCD om eenmalig het bij 4.1 genoemde bedrag af te schrijven van het bij 1.2 opgegeven rekeningnummer.*

☐ Via een eenmalige incasso

☒ Na ontvangst van de factuur

# 5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht

☒ Projectvoorstel

☒ 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 of per post naar:

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

Ondertekening door de instellingsvergunninghouder of gemachtigde (zie 1.7). 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 F 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 [redacted]

Functie [redacted]

Plaats Utrecht

Datum 11-10-2016

Handtekening [redacted]

Centrale Commissie Dierproeven

2.

# Format
## Niet-technische samenvatting

- Dit format gebruikt u om uw niet-technische samenvatting te schrijven
- Meer informatie over de niet-technische samenvatting vindt u op de website www.centralecommissiedierproeven.nl.
- Of neem telefonisch contact op. (0900-2800028).

## 1 Algemene gegevens

| | | |
|---|---|---|
| 1.1 | Titel van het project | Neutrofielen subtypes in gezondheid en ziekte |
| 1.2 | Looptijd van het project | 5 jaar |
| 1.3 | Trefwoorden (maximaal 5) | Neutrofiel, inflammatie, kanker, microplastics |

## 2 Categorie van het project

2.1 In welke categorie valt het project.

*U kunt meerdere mogelijkheden kiezen.*

- ☒ Fundamenteel onderzoek
- ☐ Translationeel of toegepast onderzoek
- ☐ Wettelijk vereist onderzoek of routinematige productie
- ☐ Onderzoek ter bescherming van het milieu in het belang van de gezondheid
- ☐ Onderzoek gericht op het behoud van de diersoort
- ☐ Hoger onderwijs of opleiding
- ☐ Forensisch onderzoek
- ☐ Instandhouding van kolonies van genetisch gemodificeerde dieren, niet gebruikt in andere dierproeven

## 3 Projectbeschrijving

3.1 Beschrijf de doelstellingen van het project (bv de wetenschappelijke vraagstelling of het wetenschappelijk en/of maatschappelijke belang)

Het afweersysteem is van levensbelang om ziekteverwekkers, kanker en ander niet-lichaamseigen materiaal te bedwingen. Bij een infectie moeten afweercellen (witte bloedcellen) opgeroepen en aangezet worden om ziekteverwekkers te bestrijden en zo het lichaam te beschermen. Neutrofielen zijn de meest voorkomende afweercellen in het bloed en zijn als eerste ter plekke bij de infectie of ontsteking. Maar soms schieten de neutrofielen door. Ze reageren dan te hevig op de infectie, met als gevolg dat de neutrofielen gezond weefsel beschadigen. Zo kunnen de neutrofielen zelfs meer schade aanrichten dan de ziekteverwekker zelf. Het is dus van groot belang dat neutrofielen uitgezet worden wanneer ze niet meer nodig zijn. Recentelijk zijn er twee extra subtypen neutrofielen gevonden. Eentje die beter bacteriën kunnen doden dan de 'gewone' in het bloed en een andere die de afweer juist kunnen onderdrukken. Als we begrijpen wanneer deze

| | | |
|---|---|---|
| | | neutrofielen opgeroepen worden, waar ze vandaan komen en hoe lang ze leven kunnen we dit in de toekomst gebruiken voor therapie. Dan kunnen we bijvoorbeeld de onderdrukkers sturen als de schade uit de hand dreigt te lopen of de goede bacteriedoders inzetten als er een systemische infectie (sepsis) is.<br>Ook willen we de rol van neutrofielen (subtypen) bij uitzaaiingen van kanker bestuderen. Er is namelijk een positief verband tussen het aantal neutrofielen en tumorprogressie.<br>Een andere deelvraag is hoe neutrofielen reageren op microplastics. Dit zijn microscopisch kleine stukjes plastic (kleiner dan 1 micrometer) die tegenwoordig veelvuldig in het milieu en onze voedselketen terechtkomen. Er wordt nog erg weinig onderzoek gedaan naar de invloed van microplastics op de gezondheid en neutrofielen zijn waarschijnlijk de afweercellen die deze plastics als eerste tegenkomen in ons lichaam. |
| 3.2 | Welke opbrengsten worden van dit project verwacht en hoe dragen deze bij aan het wetenschappelijke en/of maatschappelijke belang? | Met dit project willen wij het grondwerk leggen voor de ontwikkeling van nieuwe therapieën om doorgeslagen afweerreacties te dempen of juist een zwak afweersysteem te helpen bacteriën te overwinnen. Tevens willen we bestuderen of en hoe neutrofielen tumorprogressie bewerkstelligen. Tenslotte willen we vaststellen of microplastics, die steeds meer in het milieu en onze voedselketen terechtkomen, een negatief effect hebben op de functie van de neutrofiel. |
| 3.3 | Welke diersoorten en geschatte aantallen zullen worden gebruikt? | In dit project zullen muizen als proefdier gebruikt worden.<br>Aantallen: max. 3000 muizen gedurende 5 jaar |
| 3.4 | Wat zijn bij dit project de verwachte negatieve gevolgen voor het welzijn van de proefdieren? | Kortdurend licht tot matig ongerief als gevolg van het toedienen van stoffen, bijvoorbeeld via injecties (niet door de gevolgen hiervan) en in een aantal gevallen matig ongerief door operaties. Gegeven het doel van het onderzoek krijgen de muizen tumoren, infecties, ontstekingen of microplastics toegediend om de neutrofielen in deze situaties te kunnen bestuderen. |
| 3.5 | Hoe worden de dierproeven in het project ingedeeld naar de verwachte ernst? | Een groot deel (±90%) van de proefdieren zal matig ongerief te verdragen krijgen en de rest licht ongerief |
| 3.6 | Wat is de bestemming van de dieren na afloop? | Na afloop van de experimenten worden de muizen gedood om de organen en cellen van de muizen tot in detail te kunnen bestuderen. |

## **4** Drie V's

| | | |
|---|---|---|
| 4.1 | **Vervanging**<br>Geef aan waarom het gebruik van dieren nodig is voor de beschreven doelstelling en waarom proefdiervrije alternatieven niet gebruikt kunnen worden. | Voordat we besluiten over te gaan tot proefdierstudies, doen we eerst experimenten met humane cellen uit het bloed van zowel gezonde als zieke individuen. De resultaten uit deze experimenten bepalen uiteindelijk het besluit om een dierexperiment te gaan doen. Gegevens uit klinische studies bij patiënten kunnen ook aanleiding zijn voor het uitvoeren van dierproeven. De afweerbalans in het lichaam is uitermate complex. Het samenspel tussen meerdere organen speelt hierbij een cruciale rol die we in de mens onvoldoende kunnen bestuderen omdat we de complexiteit van een menselijk lichaam nog niet in een reageerbuisje kunnen nabootsen. Ook |

| | | |
|---|---|---|
| | | kunnen we bij mensen niet zomaar van alle weefsels monsters nemen. |
| 4.2 | **Vermindering**<br>Leg uit hoe kan worden verzekerd dat een zo gering mogelijk aantal dieren wordt gebruikt. | Door een goede statistische onderbouwing gekoppeld aan jarenlange ervaring kunnen we wetenschappelijk verantwoorde studies uitvoeren met een minimum aantal muizen. Afhankelijk van de uitkomsten van de eerste experimenten wordt telkens tussentijds kritisch bekeken of de hypotheses aangepast moeten worden en de uitvoering van de experimenten nog steeds essentieel is. Uiteraard worden alleen door de Instantie voor Dierenwelzijn goedgekeurde experimenten uitgevoerd. |
| 4.3 | **Verfijning**<br>Verklaar de keuze voor de diersoort(en). Verklaar waarom de gekozen diermodel(len) de meest verfijnde zijn, gelet op de doelstellingen van het project. | In dit project maken we gebruik van muizen. We weten dat het afweersysteem van de muis lijkt op dat van de mens en dat menselijke ziektes nagebootst kunnen worden in de muis. Er is al veel informatie beschikbaar over het afweersysteem van de muis, wat ons zal helpen in ons onderzoek. Veel van de voor ons onderzoek benodigde hulpmiddelen zijn alleen beschikbaar voor muizen. |
| | Vermeld welke algemene maatregelen genomen worden om de negatieve (schadelijke) gevolgen voor het welzijn van de proefdieren zo beperkt mogelijk te houden. | Gedurende het verloop van de ziektes zullen we de dieren frequent beoordelen op welbevinden en gewicht. Bij onverwacht of overmatig ongerief zullen de muizen worden gedood. |

## **5** In te vullen door de CCD

| | |
|---|---|
| Publicatie datum | |
| Beoordeling achteraf | |
| Andere opmerkingen | |

Centrale Commissie Dierproeven

3.

# 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 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'. | 11500 |
| 1.2 | Provide the name of the licenced establishment. | UMC Utrecht |
| 1.3 | Provide the title of the project. | Neutrophil subsets in health and disease |

## 2 Categories

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

X Basic research

☐ Translational or applied research

☐ Regulatory use or routine production

☐ Research into environmental protection in the interest of human or

☐ 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.

- For legally required animal procedures, indicate which statutory or regulatory requirements apply (with respect to the intended use and market authorisation).
- For routine production, describe what will be produced and for which uses.
- For higher education or training, explain why this project is part of the educational program and describe the learning targets.

The neutrophilic granulocyte is the first line of defense against invaders such as bacteria, fungi, foreign particles and aberrant cells. As the most abundant white blood cell, it continuously circulates the blood until a signal causes it to extravasate towards the tissue. In the tissue upon phagocytosis the neutrophil will exploit the content of its granules to degrade the foreign invader or cell debris. Besides pathogens

and cell debris, we will also further zoom in on one type of foreign particles that neutrophils can encounter in the human body, microplastics. These ≤1µm plastic particles are very prevalent in our environment (eg in our drinking water and aquatic animals via erosion from plastic litter), but research into health effects in mammals is virtually non-existent.
Neutrophils have always been described as a homogeneous population of short-lived cells. However, recent publications of our research group and others challenge this view. Our group described that neutrophils have a longer lifespan than previously thought. And whereas the population in blood in homeostasis might be homogeneous, various stimuli disturbing homeostasis lead to heterogeneity in phenotype and function. Our group showed that if healthy humans receive an LPS injection, immature, banded neutrophils appear in the blood, which are much better at killing bacteria than mature neutrophils (Leliefeld *et al.*, manuscript in preparation).
Recently, neutrophils have been described to play a role in the pathogenesis of various human diseases such as cardiovascular disease, cancer, auto-immune diseases and allergy[1-4]. Linked to these conditions, several neutrophil subsets have been newly described such as low-density granulocytes, granulocytic myeloid-derived suppressor cells (G-MDSC), tumor-associated neutrophils (TAN), and hypersegmented neutrophils[5]. Although we know that homeostatic neutrophils in humans and mice behave very similar, the similarity of neutrophil subsets in humans and mice is poorly described.
In disease, neutrophils can play a beneficial but also often a detrimental role. For example, neutrophils have been described to both promote and inhibit tumor progression and metastasis[6]. They can be both pro- and anti-inflammatory[5]. At the wrong time or the wrong place, neutrophil activation and degranulation will result in overwhelming inflammation and major tissue damage. Neutrophils present in a tumor are correlated with a worse prognosis, but the mechanisms behind this correlation are unclear[6]. The ability to steer neutrophil function, activation or localization from the outside would be beneficial for many diseases. The treatment of cancer would likely benefit from targeting only the pro-tumor neutrophil subset as opposed to all neutrophils. In contrast, the treatment of asthma would greatly benefit from targeting only the pro-inflammatory neutrophil subset.
But the basic knowledge on the timing, localization or even origin of neutrophil subsets is lacking, because we cannot easily sample human tissues. We know neutrophils develop in the bone marrow, but the time they need to develop is uncertain. We know neutrophils are able to migrate to lymph nodes via lymph vessels, but the signal driving them as well as their function in the lymph node are unclear. We know different subtypes of neutrophils have different functions, but when and where the differentiation of these subsets starts to diverge is again unclear.
To answer these types of questions, we need to gain a better understanding of the complex interplay of the entities that can guide the neutrophil: most importantly the bone marrow, spleen, endothelium, inflamed tissue, tumor cells, foreign particles, other immune cells and the neutrophil itself. As well, we think it is important to understand whether neutrophil subsets observed upon inflammation are the same throughout the range of inflammatory disorders. If they have a different phenotype and/or function for different types of disorders, that would be highly relevant for designing new therapies.
Understanding how and where the different neutrophil subsets arise in inflammatory conditions and what mechanisms underlie their function is essential to determine their role in disease and to establish potential treatment options. Redirection of immune responses has an enormous societal and scientific relevance as inflammation-associated diseases (including cardiovascular disease, cancer, auto-immunity and allergy) are associated with major pathology and morbidity.
In this research project we will combine data of healthy mice, mice with acute inflammation and mice with chronic inflammation, to help us understand why and when different neutrophil subsets are developed and recruited. Even if murine neutrophil subsets do not resemble those in human, this data will together show whether the response of the neutrophil to inflammation is generic, or tailored to the stimulus. Examples of inflammatory stimuli are pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs) and tumor antigens. At the same time, we will have a better idea whether inhibiting or stimulating neutrophils in these specific conditions would be beneficial or detrimental to the disease progression.

### 3.2 Purpose

Describe the project's main objective and explain why this objective is achievable.

- If the project is focussed on one or more research objectives, which research questions should be addressed during this project?
- If the main objective is not a research objective, which specific need(s) does this project respond to?

The objective of this project is to gain more fundamental knowledge on origin, timing, localization and

function of neutrophils in inflammatory conditions. These research questions will therefore be addressed:

- Is the neutrophil population heterogeneous in morphology?
- Is the neutrophil population heterogeneous in function?
- Does the migration & distribution of neutrophils throughout the body depend on the type of inflammatory stimulus?
- Does the differentiation of neutrophils depend on the type of inflammatory stimulus or organ?
- Does the function of neutrophils depend on the type of inflammatory stimulus?
- Does the life-span of neutrophils depend on the type of inflammatory stimulus?
- Does the interaction of neutrophils differ per type of inflammatory stimulus?
- Does the phagocytosis of microplastics influence neutrophil function?

From human data, we know that heterogeneous neutrophil subsets appear in the blood upon strong immune stimulation such as LPS injection, cancer, trauma or viral infection. However, these different conditions result in a different timing of the presence of subsets. Our preliminary data shows neutrophil heterogeneity is also induced in mice upon trauma (acute inflammation) or solid tumor growth (chronic inflammation).
In healthy mice, mice with acute inflammation and mice with chronic inflammation, we will analyze the phenotype and function of the neutrophils *ex vivo*, crucially supported by analysis of the kinetics of murine neutrophils *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., etc.). In the kinetics studies we will study the distribution of neutrophils 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 [7, 8].
There are several other reasons why we are confident that we can achieve our aims: Our group is embedded in the Laboratory of Translational Immunology (LTI), which is a center-of-excellence on fundamental and translational immunological research. 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. The LTI 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 TA-COAST consortium. 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[9-15]. 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.

### 3.3 Relevance

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

The enormous burden of inflammation-associated diseases (including cardiovascular disease, cancer, auto-immunity and allergy) worldwide is aggravated by lack of adequate treatment options. This is due to insufficient knowledge about the common player in virtually all inflammatory processes: the neutrophil. The burden of neutrophil-mediated pathology is high, but no specific therapy is currently available. The estimated costs to society (EU) are now above 250 billion euros annually, which will only rise in the future[16]. Recent insights show that the neutrophil can be both pro- and anti-inflammatory[5]. Inhibition of pro-inflammatory neutrophils and activation of anti-inflammatory neutrophils will be beneficial for patients in pro-inflammatory states, whereas patients with a suppressed immune system such as in cancer or after acute severe inflammation may profit from a reverse approach. The lack of knowledge regarding this emerging concept has to date precluded the development and translation of the manipulation of neutrophils into a clinical application. Successful manipulation of the different neutrophil subsets will be widely applicable to a range of different inflammatory diseases.
Pollution of the water environment with microplastics by erosion of plastic litter is a pressing problem that has received a lot of attention in the last few years. It has been shown that microplastics end up in

our food chain, but their effects on human health are currently understudied. To assess their effects on human health we look at neutrophils, since they are specialized in taking up foreign particles. Our preliminary human *in vitro* data shows that neutrophils are also able to engulf microplastics. These microplastics are likely not degraded by the neutrophils and might have an effect on their survival and their capacity to subsequently kill pathogens. If we demonstrate that microplastics adversely affect human health, evidence-based regulatory measures can be formulated.

### 3.4 Research strategy

3.4.1 Provide an overview of the overall design of the project (strategy).

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, tumor size).

The methods for obtaining these readout parameters will be the same for all experiments in this proposal, but the inflammatory stimulus will differ per experiment. We think the combination of several inflammatory stimuli is the strength of this project, because currently it is unknown whether neutrophils respond the same to diverse inflammatory signals (e.g. PAMPs, DAMPs, tumor antigens).
Supported by our own research on human material, pigs and mice, we have a number of hypotheses that we will start with. However, since our research is mostly fundamental and 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.

3.4.2 Provide a basic outline of the different components of the project and the type(s) of animal procedures that will be performed.

Firstly, we want to investigate whether the morphology and function of murine neutrophil subsets is similar to the subsets we described in humans ([17], Tak *et al.* submitted, Leliefeld *et al.* manuscript in preparation). Therefore we will start with an experiment of LPS injection in mice similar to our experiments with LPS injection in humans. But to extend our findings to more physiological inflammatory stimuli, we want to use bacterial infection, viral infection, plastic particles or sterile injury. To look at stimuli specific for chronic inflammation, we want to use animals with solid tumor growth or airway allergy, two relevant diseases with a significant but opposite role for neutrophils. Homeostatic neutrophils will be analyzed *ex vivo* and *in vivo* and compared to the neutrophils arising in an inflammatory state.
As described in 3.1, neutrophil behavior is determined by a complex interplay of the bone marrow, spleen, endothelium, inflamed tissue, tumor cells, foreign particles, other immune cells and the neutrophil itself. To study this system, we want to modify these entities one by one. Therefore we will perform one or more of the following interventions in these mice:

- Administration of drugs/antibodies/inhibitors
  We might be able to rescue or mimic the phenotypes of the neutrophil subsets and therefore further identify the function of these cells *in vivo*. If possible and/or relevant, we will always test these compounds first *in vitro* and in case relevant effects are observed shift to in the *in vivo* experiments.
- *In vivo* labeling
  *In vivo* antibody administration, injection of fluorescent compounds for short term labeling of blood vessels, transfer of labelled erythrocytes to long-term label blood vessels, injection of propidium iodide to monitor cell death, injection of Hoechst to stain nuclei, administration of compounds which incorporate into DNA to measure cell life span and proliferation such as deuterated water, deuterated glucose, EdU or BrdU.
- Adoptive transfer
  To study human cells *in vivo* or to, for instance, compare wildtype neutrophils with neutrophils that have a mutation we need to adoptively transfer donor neutrophils into recipient mice. Irradiation of recipient mice to deplete their endogenous immune system might be required. Also (fluorescently) labeled cells might be transferred for tracking purposes.
- Bone marrow scaffold
  To study neutrophil progenitors and differentiation. Intravital imaging of the bone marrow compartment is hard due to the solid thick nature of the surrounding bone. In a pilot study we have successfully placed an imaging window on a subcutaneous bone marrow scaffold [Groen Blood 2012] allowing the visualization of cells in the bone marrow.

- Splenectomy
  To study [redacted] and [redacted]. Literature as well as our preliminary data suggests that some [redacted] may specifically [redacted]. Additionally, [redacted] with other [redacted], may [redacted]. To study how these subtypes or cell-cell interactions affect the immune system as a whole, we are interested in applying our inflammatory stimuli in splenectomized animals.
- Neutrophil depletion
  Administration of anti-Ly6G-antibody or use of Ly6G-Cre conditional knockout to investigate the role of neutrophils.

3.4.3 Describe the coherence between the different components and the different steps of the project. If applicable, describe the milestones and selection points.

For most experiments we first consider *ex vivo* experiments (mild discomfort), before we consider intravital imaging experiments (mild to moderate discomfort). In some experiments we first consider 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 (can be up to a reduction of 20x); multiple time points can be measured in one individual, and there is no inter-mice variation. After completion of the intravital imaging experiments, cells, tissues and organs will be isolated and analyzed to reduce the number of mice required. For using the different inflammatory stimuli mentioned under 3.4.2, the experiments for optimization and set-up will be the same.
Milestones:

- Phenotypic definition of neutrophil subsets (surface marker expression, nuclear morphology, etc.)
- Functional definition of neutrophil subsets (better/worse in killing, pro- or anti-inflammatory, etc.)

*Go/no-go moment: When we do not find distinct subsets in the mouse but a homogenous neutrophil population, this milestone will not be pursued:

- To know where [redacted]

But these milestones below are independent of the go/no-go moment:

- To know whether phenotype and function of neutrophils is the same throughout a range of inflammatory disorders
- To know whether the interaction between the neutrophils and the inflammatory stimulus is the same throughout a range of inflammatory disorders
- To know the distribution pattern of neutrophils/neutrophil subsets after leaving the bone marrow, in homeostasis vs. inflammatory conditions
- To know the life-span of murine neutrophils/neutrophil subsets, in homeostasis vs. inflammatory conditions
- To confirm whether neutrophils/neutrophil subsets continue migrating towards other organs after phagocytosis of pathogens in infected tissue
- To confirm whether [redacted]
- To know whether inhibition of neutrophils/neutrophil subsets can relieve symptoms in allergic airway disease
- To know whether inhibition of neutrophils/neutrophil subsets has an effect on tumor growth

These milestones can be reached simultaneously and do not depend on each other. When we do not reach the first three milestones, i.e. when we do not find distinct subsets in the mouse but a homogenous neutrophil population, then the latter milestones remain valuable for this homogenous neutrophil population.

***References***

*1. 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).*
*2. Moses, K. & Brandau, S. Human neutrophils: Their role in cancer and relation to myeloid-derived suppressor cells. Semin. Immunol.* ***28****, 187-196 (2016).*
*3. Kaplan, M. J. Role of neutrophils in systemic autoimmune diseases. Arthritis Res. Ther.* ***15****, 219 (2013).*
*4. Bruijnzeel, P. L., Uddin, M. & Koenderman, L. Targeting neutrophilic inflammation in severe neutrophilic asthma: can we target the disease-relevant neutrophil phenotype? J. Leukoc. Biol.* ***98****, 549-556 (2015).*
*5. Pillay, J., Tak, T., Kamp, V. M. & Koenderman, L. Immune suppression by neutrophils and granulocytic myeloid-derived suppressor cells: similarities and differences. Cell Mol. Life Sci.* ***70****, 3813-3827 (2013).*
*6. Uribe-Querol, E. & Rosales, C. Neutrophils in Cancer: Two Sides of the Same Coin. J. Immunol. Res.* ***2015****, 983698 (2015).*
*7. Hasenberg, A. et al. Catchup: a mouse model for imaging-based tracking and modulation of neutrophil granulocytes. Nat. Methods* ***12****, 445-452 (2015).*
*8. 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).*
*9. Beerling, E., Ritsma, L., Vrisekoop, N., Derksen, P. W. & van Rheenen, J. Intravital microscopy: new insights into metastasis of tumors. J. Cell. Sci. **124**, 299-310 (2011).*
*10. Ritsma, L., Vrisekoop, N. & van Rheenen, J. In vivo imaging and histochemistry are combined in the cryosection labelling and intravital microscopy technique. Nat. Commun. **4**, 2366 (2013).*
*11. Torabi-Parizi, P. et al. Pathogen-related differences in the abundance of presented antigen are reflected in CD4+ T cell dynamic behavior and effector function in the lung. J. Immunol. **192**, 1651-1660 (2014).*
*12. van Golen, R. F. et al. The mechanisms and physiological relevance of glycocalyx degradation in hepatic ischemia/reperfusion injury. Antioxid. Redox Signal. **21**, 1098-1118 (2014).*
*13. Ritsma, L. et al. Intravital microscopy through an abdominal imaging window reveals a pre-micrometastasis stage during liver metastasis. Sci. Transl. Med. **4**, 158ra145 (2012).*
*14. Zomer, A. et al. Intravital imaging of cancer stem cell plasticity in mammary tumors. Stem Cells **31**, 602-606 (2013).*
*15. Beerling, E. et al. Plasticity between Epithelial and Mesenchymal States Unlinks EMT from Metastasis-Enhancing Stem Cell Capacity. Cell. Rep. **14**, 2281-2288 (2016).*
*16. Gibson, G. J., Loddenkemper, R., Lundback, B. & Sibille, Y. Respiratory health and disease in Europe: the new European Lung White Book. Eur. Respir. J. 42, 559-563 (2013).*
*17. Pillay, J. et al. A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. J. Clin. Invest. 122, 327-336 (2012).*

3.4.4 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 | Analysis of the development and function of neutrophils |
| 2 | |
| 3 | |
| 4 | |
| 5 | |
| 6 | |
| 7 | |
| 8 | |
| 9 | |
| 10 | |

Centrale Commissie Dierproeven

4.

# 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, see 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'. | 11500 |
| 1.2 | Provide the name of the licenced establishment. | UMC Utrecht |
| 1.3 | List the serial number and type of animal procedure.<br><br>*Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.* | Serial number: 1<br>Type of animal procedure: Analysis of the development and function of neutrophils |

## 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.

The objective of this project is to gain more fundamental knowledge on origin, timing, localization and function of neutrophils in diverse inflammatory conditions. In order to study the origin and kinetics of the neutrophil populations we need to investigate the different relevant organs. Neutrophils develop in the bone marrow and occasionally the spleen and subsequently migrate through the blood to the inflammatory site. In addition, neutrophils are thought to reside in the lungs and liver (marginated pool). All these organs are therefore relevant for our studies. Whenever we will perform animal experiments we will therefore harvest multiple organs for our analysis. 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, tumor size).

The methods for obtaining these readout parameters will be the same for all experiments in this proposal, but the inflammatory stimulus will differ per experiment. The purpose of our experiments is to compare the neutrophil populations between all the different inflammatory conditions.

*Ex vivo analysis:*
After the different interventions, 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 (e.g. survival assay, bacterial killing capacity, T cell suppression capacity).
This *ex vivo* analysis will allow us to link surface markers between human and mouse neutrophils.

Surface marker expression and nuclear morphology allow us to distinguish different neutrophil subsets in mice, in multiple organs. We can see whether the different inflammatory conditions recruit the subsets with similar dynamics. Lastly, using functional *in vitro* assays we can investigate whether the subsets differ in their capacity e.g. to kill microbes and suppress T cell proliferation like we found in humans.

*Intravital imaging:*
Two different strategies will be used for the imaging experiments: (1) Imaging in an acute experiment under anesthesia and (2) implanting an imaging window (such as on the breast, skin, abdomen and skull) and repeatedly image through that window. 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 intracutaneous 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. 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.
*In vivo* imaging will allow us to study 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 timepoints, greatly reducing the amount of animals needed. Additionally, this provides paired data, introducing less variation and therefore fewer animals 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.

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

The objective of this project is to gain more fundamental knowledge on origin, timing, localization and function of neutrophils in diverse inflammatory conditions. Below the experimental approach per inflammatory stimulus and per intervention will be described. The choices about mice strains and numbers are further explained in B.

1. 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.
*Description:* Mice receive a single bolus of E. coli lipopolysaccharide intravenously (i.v.).
*Mice:* wildtype or intravital imaging strains
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours-days
*Level of discomfort:* mild or moderate

2. Inflammatory stimulus: Bacterial infection

*Justification:* Bacterial infection is a more complex, but more physiological relevant model for acute inflammation with which we have a lot of experience in vitro in humans.
*Description:* Mice are infected with (fluorescent) staphylococci via intradermal or intraperitoneal injection as described before[1, 2].
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to

analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – 2 weeks
*Level of discomfort:* moderate

3. Inflammatory stimulus: Viral infection

*Justification:* Viral infection is a complex physiological relevant model for inflammation with which we have some experience in vitro in humans.
*Description:* Mice are infected with influenza virus or rhinovirus 16 via intranasal administration, as performed before (Suzanne Bal, personal communication).
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – 8 weeks
*Level of discomfort:* moderate

4. Inflammatory stimulus: Plastic particles

*Justification:* The effects of microplastics on human health are currently understudied. We have found neutrophils can engulf microplastics and now want to investigate their effect on neutrophil survival and function.
*Description:* Microplastics (plastic particles ≤ 1 µm) are administered via subcutaneous injection, via addition to the drinking water, or via i.v. injection to mimic exposure routes in humans.
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – one year
*Level of discomfort:* mild or moderate

5. Inflammatory stimulus: Sterile injury

*Justification:* Sterile injury is known to recruit neutrophils without a pathogenic stimulus. Therefore this can act as a control for pathogen infection or as a model for damage-associated molecular pattern (DAMP) release.
*Description:* Under anesthesia, sterile injury is inflicted in the skin by needle insertion or laser damage
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours - days
*Level of discomfort:* mild or moderate

6. Inflammatory stimulus: Airway allergy

*Justification:* Allergy is a complex physiological relevant model for chronic inflammation with which we have a lot of experience in humans.
*Description:* Mice are sensitized and challenged with house dust mite by intranasal administration, as performed before (Suzanne Bal, personal communication).
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* 2 – 8 weeks
*Level of discomfort:* mild or moderate

7. Inflammatory stimulus: Solid tumor growth

*Justification:* Cancer is a complex physiological relevant model for chronic inflammation. The suppressive neutrophil has been described in this model.
*Description:* We use genetic mouse models for breast or colorectal cancer, or transfer tumor cells/pieces from these mouse models to healthy mice of the same background to increase reproducibility or for intravital imaging.
*Mice:* Spontaneous tumor models or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – months
*Level of discomfort:* mild or moderate

The final level of discomfort also depends on the choice of the readout parameter. Harvesting of organs for *ex vivo* analysis or single session intravital imaging will result only in mild discomfort, but repeated intravital imaging will probably result in moderate discomfort.

To pinpoint the role of separate components of the inflammatory reaction, we will perform in these mice one or more of the following interventions:

1. Intervention: Administration of drugs/antibodies/inhibitors

*Description:* Drugs, antibodies, small molecules or chemicals are administered to mice via the appropriate route as described in literature (i.v., i.p., diet, etc).
*Rationale:* inhibit, stimulate, deplete or mimic components of the inflammatory reaction. E.g. by deleting neutrophils with anti-Ly6G antibodies we can delineate the role neutrophils play in disease progression or behavior of other cell types. The specific nature of these compounds will follow from our experimental results in the next five years.
*For Inflammatory stimulus:* 1-7
*Extra experimental groups:* treated vs. not treated.
*Number of mice per group:* as described per inflammatory stimulus.
*Duration of intervention:* seconds – minutes, possibly repeatedly
*Level of discomfort:* mild or moderate

2. Intervention: *In vivo* labeling

*Description:* Cells or structures in a living mouse are labelled by administration of: an antibody, fluorescent compounds for short term labeling of blood vessels, labelled erythrocytes to long-term label blood vessels, 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.
*Rationale:* During intravital imaging different cells and structures should be distinguished.
*For Inflammatory stimulus:* 1-7
*Extra experimental groups:* none.
*Number of mice per group:* as described per inflammatory stimulus.
*Duration of intervention:* seconds – months, possibly repeatedly
*Level of discomfort:* mild

3. Intervention: Adoptive transfer

*Description:* a single cell suspension of donor cells is administered i.v. to recipient mice.
*Rationale:* Firstly, (fluorescently) labeled cells will be transferred for tracking migration and function throughout the body. Cells harbouring a specific gene knockout will be transferred to study the role of this gene in specific organs/diseases. Human cells will be transferred to investigate the behavior of human neutrophils in an *in vivo*-like situation.
*For Inflammatory stimulus:* 1-7
*Extra experimental groups:* Donor mice & recipient mice.
*Number of mice per group:* ratio donor:recipient mostly 1:1, sometimes 2:1 or 3:1
*Donor mice:* wt mice, intravital imaging strains, knockout strains.
*Recipient mice:* wt mice, intravital imaging strains, knockout strains, immunodeficient mice. Irradiation of recipient mice to deplete their endogenous immune system might be required.
*Duration of intervention:* seconds – minutes
*Level of discomfort:* mild

4. Intervention: Bone marrow scaffold

*Description:* Biphasic calcium phosphate particles loaded with human MSCs will be implanted subcutaneously in immunodeficient mice to create a niche for hematopoiesis.
*Rationale:* To study neutrophil progenitors and differentiation. Intravital imaging of the bone marrow compartment is hard due to the solid thick nature of the surrounding bone. In a pilot study we have successfully placed an imaging window on a subcutaneous bone marrow scaffold allowing the visualization of cells in the bone marrow[3].
*For Inflammatory stimulus:* 1-3,5,6
*Extra experimental groups:* With vs. without bone marrow scaffold.
*Number of mice per group:* as described per inflammatory stimulus
*Duration of intervention:* days - months
*Level of discomfort:* moderate during surgery, mild thereafter

5. Intervention: Splenectomy

*Description:* Surgery to remove the spleen is performed as described before[4].
*Rationale:* Since we hypothesize that the spleen [redacted], we will study the effect of removing the spleen. Data from [redacted] suggest that the number of [redacted] is decreased.
*For Inflammatory stimulus:* 1-3,5,6
*Extra experimental groups:* Splenectomized vs. non-splenectomized
*Number of mice per group:* as described per inflammatory stimulus.
*Duration of intervention:* hours
*Level of discomfort:* moderate during surgery, mild thereafter

Now that all models and interventions have been described we will describe some examples of how these experimental procedures can help us to achieve some of our milestones:
Milestones:

i. Phenotypic definition of neutrophil subsets (surface marker expression, nuclear morphology, etc.)
ii. Functional definition of neutrophil subsets (better/worse in killing, pro- or anti-inflammatory, etc.)
iii. To know whether phenotype and function of neutrophils is the same throughout a range of inflammatory disorders
iv. To know the distribution pattern of neutrophils/neutrophil subsets after leaving the bone marrow, in homeostasis vs. inflammatory conditions

Milestones i-iv can be achieved by comparing the morphology and cell surface expression of neutrophils in bone marrow, spleen, blood, liver, lung and inflammatory site after the different inflammatory stimuli compared to controls. Next, subsets found will be tested in vitro functional

assays. Subsequently, the in vivo functionality can be tested by intravital microscopy in combination with adoptive transfer (intervention 2 and 3) of the different subset and/or depletion of the different subsets (intervention 1).

v. To know the life-span of murine neutrophils/neutrophil subsets, in homeostasis vs. inflammatory conditions

vi. To know where [redacted]
Combine the phenotypic and functional neutrophil subsets with in vivo labeling (intervention 2) in order to determine their life span. This will also reveal whether the subsets all have the same age or if one is younger than the other, giving clues if one subset is likely to differentiate from the other.

vii. To confirm whether [redacted] is a [redacted] and neutrophil [redacted]
Combine the phenotypic and functional neutrophil subsets with splenectomy (intervention 5) in order to determine the role of the spleen [redacted].

viii. To know whether inhibition of neutrophils/neutrophil subsets has an effect on tumor growth
Combine tumor models with neutrophil depletion (intervention 1).

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. 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 (most of the animal procedures described in this appendix): 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, estimated numbers, and life stages. Provide justifications for these choices.

Species: Mus musculus
Origin: Common breeding facility (Gemeenschappelijk Dierenlaboratorium) at Utrecht University/ Hubrecht institute/external licensed breeders
Estimated numbers: max. 3000
Life stages: adults

Species
All animal studies in this project will exclusively involve mice. Up to today, there are no alternative methods to fully understand neutrophil development, homeostasis and response to inflammation in the context of the whole organism. For the proposed studies, the mouse is the most appropriate animal model because: (1) physiology is more extensively characterized than other animal models; (2) a large number of relevant transgenic and knock out lines is already available; (3) a large number of relevant antibodies and other compounds is already available; (4) our research shows murine neutrophils are representative for human neutrophils; (5) many procedures for intravital imaging have already been described. The zebrafish is also an animal widely used for intravital imaging, but the differentiation of zebrafish neutrophils is much less similar to human neutrophils. Since mice are mammals too, they are closer to the human.
Mice strains used are amongst others:

- Wildtype BL/6
- Wildtype Balb/c
- Wildtype FVB
- LysM-GFP mice; for visualization of fluorescent neutrophils in intravital imaging. We are already working with this strain.
- Catchup$^{IVM}$ mice (Ly6G-Cre + Rosa-Td-Tomato); for visualization of fluorescent neutrophils in intravital imaging and for conditional knockouts in neutrophils[5]. This strain was very recently

created, we want to import this strain. If the results will be superior to LysM-GFP mice, we will switch to this strain.
- MRP/S100A8-Tg-EGFP-Cre; for conditional knockouts in neutrophil progenitors[6]. We want to import this strain. As opposed to the Catchup$^{IVM}$ mice, Cre recombinase is expressed in granulocyte-macrophage progenitors in the bone marrow.
- Knockout mice (e.g. CD11b-knockout mice); to investigate the role of a specific gene or protein
- Tumor models such as MMTV-PyMT, MMTV-Wnt1 or C26 colorectal cancer mice. In our currently running animal experiments we are comparing different tumor models with each other, to optimize for future experiments. Therefore we have not completely decided which tumor models we will use in the next five years, but it will be a model that is similar in execution and discomfort as the here mentioned examples.
- Humanized mice[7] or immunocompromised mice (e.g. NSG mice); to investigate the behavior of human neutrophils in an *in vivo*-like situation.

If the required genetically modified mouse is not available, we will obtain this model by generating, importing or by crossing existing models. Other strains will be used when in the next five years, novel strains are described or created that are very relevant to our hypotheses.

Estimated numbers
Our experiments can be divided into:
- Descriptive analysis (5-15 animals x max 5 time points = max 75 animals)
- Functional assays (5-15 animals x max 5 time points = max 75 animals)
- Intravital imaging (5-10 animals)

This totals to 55-160 animals
However, we have 7 inflammatory stimuli, 5 interventions and no intervention per stimuli = 42 conditions.
So for the total we have to multiply the 55-160 animals with 42 = 2310-6720 animals

However, these numbers are overestimations because:
- 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
- Successful pilot studies may be included in the dataset

Therefore we do not anticipate we will need more than 2500 mice for the experiments.

Additionally, a number of ca. 500 mice is needed for:
- Maintenance of breeding
- Creation of new knockout models
- Pilot studies for validation of models/techniques
- Training of new personnel or new techniques
- Rederivation of imported strains via embryo transfer
- To compensate for unforeseen loss of animals (max 10%, e.g. due to location not suitable for imaging, problems with the window)

Mice that become surplus during breeding, will be used for pilot studies and training purposes.

Together we therefore anticipate we need a grand total of 3000 mice.

Sex
In general, we will try to use both male and female mice. However, it is clear that the immune response of males and females can greatly differ[8]. Therefore, we will perform pilot studies to ensure that the sex of the animal does not pose a bias in answering the particular research question. These pilots may prove it necessary to use only one sex within one animal experiment, to be able to reliably compare experimental groups.
One clear exception is the MMTV-PyMT breast cancer model; in this model only female mice consistently develop breast tumors.

**C. Re-use**

Will the animals be re-used?

X No, continue with question D.

☐ 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.

**D. 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:*
Immune responses are highly complex and multifaceted. Multiple compartments such as the bone marrow, spleen, endothelial wall and inflamed tissue interact. Certain aspects of the immune response can be separately simulated *in vitro*, but it is not yet possible to study the full course of infection and the elicited immune response *in vitro*. This requires animal models. Similarly, the use of experimental interventions to test their potential beneficial impact on the course of disease can only be assessed in animal models. Clearly, such experimental interventions cannot be performed in patients, while *in vitro* studies with isolated human cells do not capture the full complexity of infection. However, before turning to mice, we will first study human cells *in vitro* to ensure we only perform the most relevant experiments in mice.

*Reduction:*
Intravital imaging using imaging windows allows us to image the same mouse repeatedly over time as opposed to analyzing separate mice at different timepoints greatly reducing the amount of animals needed. Additionally, this provides paired data, introducing less variation and therefore fewer animals are needed to get statistical significant results. Statistical power calculation based on our own previous experience and literature will be used to determine the optimal number of mice for each experimental group. Moreover, experiments will be performed sequentially, based on the described go/no-go decisions, via which we ensure that we will use the minimum number of mice per group that will be informative. Whenever possible, we will perform pilot studies with the minimum number of animals possible.

*Refinement:*
Except for mice with windows, where co-housing can give problems, mice will be housed in groups in cages. Nesting opportunities will always be provided. Mice with windows will be monitored at least twice a week, mice after bacterial or viral infection will be monitored at least every other day, more if necessary, for clinical signs. If unexpectedly severe suffering is observed, mice will be euthanized.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

As described under refinement, mice welfare will be regularly monitored. Mice reaching the humane endpoints or otherwise suffering from severe discomfort will be euthanized. New personnel will be trained to recognize discomfort in mice according to current guidelines (e.g. [9]). Surgical exposure will be performed under aseptic conditions using sterile instruments. Heating devices will be used to ensure a stable body temperature of the mouse during anaesthesia. Personnel conducting the surgeries is first properly trained, starting with training on cadavers.
Surgical exposure will be performed under adequate anesthesia and analgesia. Pre- and post-procedural analgesia will be used when indicated or when pain-related behavior is observed. However, common analgesics such as opioids and non-steriodal anti-inflammatory drugs (NSAIDs) have been shown to significantly alter the immune response[10-12]. Since this is our main research topic, we might sometimes refrain from using analgesia. It may be necessary to do pilot studies to prove whether analgesics pose a bias in answering our particular research question. From literature, it seems buprenorphine may be a reasonable candidate[13].
There are no expected adverse effects on the environment.

## Repetition and duplication

**E. Repetition**

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

## Accommodation and care

**F. Accommodation and care**

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☐ No

X Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Mice with an imaging window will be housed individually to prevent other mice from biting or damaging the window.

**G. Location where the animals procedures are performed**

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

X No > Continue with question H.

☐ 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.

## Classification of discomfort/humane endpoints

**H. Pain and pain relief**

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

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

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

Surgical exposure will be performed under adequate anesthesia and analgesia. Pre- and post-procedural analgesia will be used when indicated or when pain-related behavior is observed. However, common analgesics such as opioids and non-steriodal anti-inflammatory drugs (NSAIDs) have been shown to significantly alter the immune response[10-12]. Since this is our main research topic, we might sometimes refrain from using analgesia. However, pain can also influence the immune system. Therefore, it may be necessary to do pilot studies to prove whether analgesics pose a bias in answering our particular research question.

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

Surgical exposure will be performed under aseptic conditions using sterile instruments. Heating devices will be used to ensure a stable body temperature of the mouse during anaesthesia. Personnel conducting the surgeries is first properly trained, starting with training on cadavers. Before, during and after surgery adequate anesthesia and analgesia (e.g. isoflurane and buprenorphine) will be used, according to experience as well as published protocols. New personnel will be trained to recognize discomfort in mice according to current guidelines (e.g. [9]).

**I. Other aspects compromising the welfare of the animals**

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

Other than in terminally anaesthetized animals dosing and sampling procedures will consist of a combination of volumes, routes and frequencies that will result in no more than moderate discomfort. Animals bearing tumors will never reach end-stage clinical effects. All relevant scientific data can and will be gathered *before* the cancer, infection or allergy studies reach a level of severe discomfort. If unexpectedly severe suffering is observed, mice will be euthanized.
It is expected that no animals (0%) will be experiencing more than mild discomfort due to the genetic

modification. However, it is impossible to predict the nature or severity of any potential genetic defect. So, in all cases, animals will be careful monitored for possible side effects. Animals exhibiting any unexpected harmful phenotypes will be killed within a day and analyzed when possible.
Animals may lose their window (<5%). In the vast majority of these animals (>95%), we notice signs of detachment of the window far before it becomes lose, and the mice will be sacrificed. In the remaining animals, the window loss will be noticed within 24hrs and the mouse will be immediately sacrificed.

Explain why these effects may emerge.

Tumor development, inflicted inflammation, genetic alterations and administration of compounds/ drugs/ chemicals/ toxins.
It is unclear why some animals may lose their windows.

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

Mice with windows will be monitored at least twice a week, mice after bacterial or viral infection will be monitored at least every other day, more if necessary, for clinical signs. If unexpectedly severe suffering is observed, mice will be euthanized.

**J. 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 K.

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

As described in D, F, H and I, the experiments will be set-up in such a way that the incidence of reaching the humane end-point is kept to a minimum.
Weight loss (>20% in comparison with control or original weight or 15% weight loss in two days), reaching a score of 4 on the illness scale (specified below), severe shortness of breath, or loss of window is set as an humane end point. For all tumor models the guidelines of the Code of Practice Animals in Cancer research will strictly be followed, taking into account e.g. the size, location and ulceration of the tumor(s).

Next to model-specific parameters, 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

Indicate the likely incidence.

Expected <5% and <1day.

**K. Classification of severity of procedures**

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

The cumulative level of discomfort is dependent on the intervention, the read-out and the animal model. The discomfort of these three range from mild to moderate. Therefore combinations of these result in moderate discomfort for the majority (~90%) of animals.

**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. Groen, R. W. *et al*. Reconstructing the human hematopoietic niche in immunodeficient mice: opportunities for studying primary multiple myeloma. *Blood* **120**, e9-e16 (2012).
4. Levy, L. *et al*. Splenectomy inhibits non-small cell lung cancer growth by modulating anti-tumor adaptive and innate immune response. *Oncoimmunology* **4**, e998469 (2015).
5. Hasenberg, A. *et al*. Catchup: a mouse model for imaging-based tracking and modulation of neutrophil granulocytes. *Nat. Methods* **12**, 445-452 (2015).
6. Lagasse, E. & Weissman, I. L. Bcl-2 Inhibits Apoptosis of Neutrophils but Not their Engulfment by Macrophages. *J.*

*Exp. Med.* **179**, 1047-1052 (1994).
7. Coughlan, A. M., Freeley, S. J. & Robson, M. G. Animal models of anti-neutrophil cytoplasmic antibody-associated vasculitis. *Clin. Exp. Immunol.* **169**, 229-237 (2012).
8. Gubbels Bupp, M. R. Sex, the aging immune system, and chronic disease. *Cell. Immunol.* **294**, 102-110 (2015).
9. Burkholder, T., Foltz, C., Karlsson, E., Linton, C. G. & Smith, J. M. Health Evaluation of Experimental Laboratory Mice. *Curr. Protoc. Mouse Biol.* **2**, 145-165 (2012).
10. Salimi, V. *et al*. Opioid receptors control viral replication in the airways. *Crit. Care Med.* **41**, 205-214 (2013).
11. Sacerdote, P. Opioids and the immune system. *Palliat. Med.* **20 Suppl 1**, s9-15 (2006).
12. Hish, G. A., Diaz, J. A., Hawley, A. E., Myers, D. D. & Lester, P. A. Effects of Analgesic Use on Inflammation and Hematology in a Murine Model of Venous Thrombosis. *J. Am. Assoc. Lab. Anim. Sci.* **53**, 485-493 (2014).
13. Mundt, S., Groettrup, M. & Basler, M. Analgesia in mice with experimental meningitis reduces pain without altering immune parameters. *ALTEX* **32**, 183-189 (2015).

## End of experiment

**L. Method of killing**

Will the animals be killed during or after the procedures?

☐ No

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

In order to obtain organs and isolated cells for *ex vivo* analysis the mice must first be killed.

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.

X Yes

Centrale Commissie Dierproeven

5.

> Retouradres Postbus 20401 2500 EK Den Haag

UMC Utrecht
[redacted]
Postbus 12007
3501 AA UTRECHT

**Centrale Commissie Dierproeven**
Postbus 20401
2500 EK Den Haag
centralecommissiedierproeven.nl
0900 28 000 28 (10 ct/min)
info@zbo-ccd.nl

**Onze referentie**
Aanvraagnummer
AVD115002016714

**Bijlagen**
2

Datum 13 januari 2017
Betreft Ontvangstbevestiging aanvraag projectvergunning Dierproeven

Geachte [redacted]

Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 13 oktober 2016. Het gaat om uw project "Neutrophil subsets en health and disease". Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD115002016714. Gebruik dit nummer wanneer u contact met de CCD opneemt.

**Wacht met de uitvoering van uw project**
Als wij nog informatie van u nodig hebben dan ontvangt u daarover bericht. Uw aanvraag is in ieder geval niet compleet als de leges niet zijn bijgeschreven op de rekening van de CCD. U ontvangt binnen veertig werkdagen een beslissing op uw aanvraag. Als wij nog informatie van u nodig hebben, wordt deze termijn opgeschort. In geval van een complexe aanvraag kan deze termijn met maximaal vijftien werkdagen verlengd worden. U krijgt bericht als de beslisperiode van uw aanvraag vanwege complexiteit wordt verlengd. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

**Factuur**
Bijgaand treft u de factuur aan voor de betaling van de leges. Wij verzoeken u de leges zo spoedig mogelijk te voldoen, zodat we uw aanvraag in behandeling kunnen nemen. Is uw betaling niet binnen dertig dagen ontvangen, dan kan uw aanvraag buiten behandeling worden gesteld. Dit betekent dat uw aanvraag niet beoordeeld wordt en u uw project niet mag starten.

**Meer informatie**
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

**Datum:**
13 januari 2017
**Aanvraagnummer:**
AVD115002016714

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlagen:
- Gegevens aanvraagformulier
- Factuur

**Datum:**
13 januari 2017
**Aanvraagnummer:**
AVD115002016714

## Gegevens aanvrager

Uw gegevens

| | |
|---|---|
| Deelnemersnummer NVWA: | 11500 |
| Naam instelling of organisatie: | UMC Utrecht |
| Naam portefeuillehouder of diens gemachtigde: | [redacted] |
| KvK-nummer: | 30244197 |
| Postbus: | 12007 |
| Postcode en plaats: | 3501 AA UTRECHT |
| IBAN: | NL27INGB0000425267 |
| Tenaamstelling van het rekeningnummer: | Universiteit Utrecht |

Gegevens verantwoordelijke onderzoeker

| | |
|---|---|
| Naam: | [redacted] |
| Functie: | Assistant Professor |
| Afdeling: | [redacted] |
| Telefoonnummer: | [redacted] |
| E-mailadres: | [redacted] |

**Datum:**
13 januari 2017
**Aanvraagnummer:**
AVD115002016714

Gegevens plaatsvervangende verantwoordelijke onderzoeker

| | |
|---|---|
| Naam: | [redacted] |
| Functie: | OIO |
| Afdeling: | [redacted] |
| Telefoonnummer: | [redacted] |
| E-mailadres: | [redacted] |

**Over uw aanvraag**

| | |
|---|---|
| Wat voor aanvraag doet u? | [x] Nieuwe aanvraag<br>[ ] Wijziging op een (verleende) vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn<br>[ ] Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn |

**Over uw project**

| | |
|---|---|
| Geplande startdatum: | 1 november 2016 |
| Geplande einddatum: | 31 oktober 2021 |
| Titel project: | Neutrophil subsets en health and disease |
| Titel niet-technische samenvatting: | Neutrofielen sybtypes in gezondheid en ziekte |
| Naam DEC: | DEC Utrecht |
| Postadres DEC: | Postbus 85500 3508 GA Utrecht |
| E-mailadres DEC: | dec-utrecht@umcutrecht.nl |

**Betaalgegevens**

| | |
|---|---|
| De leges bedragen: | € 1.584,- |
| De leges voldoet u: | na ontvangst van de factuur |

**Checklist bijlagen**

| | |
|---|---|
| Verplichte bijlagen: | [x] Projectvoorstel<br>[x] Beschrijving Dierproeven<br>[x] Niet-technische samenvatting |
| Overige bijlagen: | [x] DEC-advies |

**Ondertekening**

Naam: [redacted]

Functie: [redacted]

Plaats: Utrecht

Datum: 11 oktober 2016

**Datum:**
13 januari 2017
**Aanvraagnummer:**
AVD115002016714

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

UU-ASC
Postbus 80011
3508 TA UTRECHT

**Centrale Commissie Dierproeven**
Postbus 20401
2500 EK Den Haag
centralecommissiedierproeven.nl
0900 28 000 28 (10 ct/min)
info@zbo-ccd.nl

**Onze referentie**
Aanvraagnummer
AVD115002016714

**Bijlagen**
2

Datum 13 januari 2017
Betreft Factuur aanvraag projectvergunning Dierproeven

**Factuur**
Factuurdatum: 13 januari 2017
Vervaldatum: 12 februari 2017
Factuurnummer: 16700714
Ordernummer: CB.841910.3.01.011

| **Omschrijving** | **Bedrag** |
|---|---|
| Betaling leges projectvergunning dierproeven<br>Betreft aanvraag AVD115002016714 | € 1.584,00 |

Wij verzoeken u het totaalbedrag vóór de gestelde vervaldatum over te maken op rekening NL29INGB 070.500.1512 onder vermelding van het factuurnummer en aanvraagnummer, ten name van Centrale Commissie Dierproeven, Postbus 93144, 2509 AC te 's Gravenhage.

Centrale Commissie Dierproeven

6.

> Retouradres Postbus 20401 2500 EK Den Haag

UMC Utrecht
[redacted]
Postbus 12007
3501 AA UTRECHT

**Centrale Commissie Dierproeven**
Postbus 20401
2500 EK Den Haag
centralecommissiedierproeven.nl
0900 28 000 28 (10 ct/min)
info@zbo-ccd.nl

**Onze referentie**
Aanvraagnummer
AVD115002016714

Datum 13 januari 2017
Betreft Aanvulling aanvraag projectvergunning Dierproeven

Geachte Prof. [redacted]

Op 13 oktober 2016 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Neutrophil subsets en health and disease" met aanvraagnummer AVD115002016714. In uw aanvraag zitten voor ons nog enkele onduidelijkheden. In deze brief leest u wat wij nog nodig hebben en wanneer u een beslissing kunt verwachten.

**Welke informatie nog nodig**
Wij hebben de volgende informatie van u nodig om uw aanvraag verder te kunnen beoordelen:

**Onduidelijkheden**
1) U beschrijft meerdere modellen in elke bijlage dierproeven. Kunt u per bijlage dierproeven, per handeling, voor elk model/inflammatoire stimulus aangeven wat de exacte handelingen zijn met bijbehorende ongerief per handeling en het cumulatief ongerief per model voor de verschillende dieren, inclusief dieraantallen. U kunt dit bijvoorbeeld in tabelvorm doen.

2) De onderbouwing van de dieraantallen zijn gebaseerd op 5-15 dieren per groep. Daarnaast geeft u aan veel ervaring te hebben met de te gebruiken diermodellen, waardoor het ons inziens mogelijk zou moeten zijn deze aantallen nauwkeuriger in te schatten. Graag een betere inschatting van het aantal dieren weergeven.

3) Wordt intranasale toediening van de virussen en huisstofmijt onder anesthesie uitgevoerd?

4) U beschrijft verschillende inflammatoire stimuli in uw bijlagen. Kunt u voor

alle stimuli weergeven wat de gevolgen zijn van deze stimuli voor de dieren? Welke symptomen kunnen zij hierbij krijgen?

5) U beschrijft een extra 500 muizen voor o.a. onderhoud fok, maken van knock-out modellen, pilot studies etc. Kunt u voor elk van deze doelen de geschatte aantallen geven en de handelingen die deze dieren zullen ondergaan. Voor fok en creatie van nieuwe muizenlijnen is een aparte bijlage wenselijk.

6) Kunt u een betere beschrijving geven van de handelingen aan en het ongerief voor de dieren voor implanteren van Bone marrow scaffolds?

7) Kunt u een betere beschrijving geven van de handelingen aan en het ongerief voor de dieren voor implanteren van imaging windows?

**Leges**
De leges die u verschuldigd bent zijn nog niet door ons ontvangen of de betaling is nog niet verwerkt. Uw aanvraag is niet compleet als de leges niet zijn ontvangen.
Zonder deze aanvullende informatie kan de beslissing nadelig voor u uitvallen omdat de gegevens onvolledig of onduidelijk zijn.

**Opsturen binnen veertien dagen**
Stuur de ontbrekende informatie binnen veertien dagen na de datum van deze brief op. U kunt dit aanleveren via NetFTP. Stuurt u het per post op, gebruik dan het formulier dat u bij deze brief krijgt.

**Wanneer een beslissing**
De behandeling van uw aanvraag wordt opgeschort tot het moment dat wij de aanvullende informatie hebben ontvangen. Uw aanvraag is in ieder geval niet compleet als de leges niet zijn ontvangen. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

**Meer informatie**
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

7.

Geachte CCD,

Hierbij stuur ik u de extra gevraagde informatie toe met betrekking tot de aanvraag "Neutrophil subsets in health and disease" met aanvraagnummer AVD115002016714. Hieronder kunt u een korte toelichting vinden op uw vragen. Tevens kunt u gedetailleerde aanpassingen in rode tekst vinden in de betreffende bijlagen.

1) **U beschrijft meerdere modellen in elke bijlage dierproeven. Kunt u per bijlage dierproeven, per handeling, voor elk model/inflammatoire stimulus aangeven wat de exacte handelingen zijn met bijbehorende ongerief per handeling en het cumulatief ongerief per model voor de verschillende dieren, inclusief dieraantallen. U kunt dit bijvoorbeeld in tabelvorm doen.**
Zoals gevraagd heb ik voor elk model/ inflammatoire stimulus gedetailleerder de exacte handelingen met bijbehorende symptomen en ongerief in rode tekst geïncludeerd. Dit project omschrijft onderzoek naar neutrofielen subsets in de diverse inflammatoire situaties. Het is een exploratief onderzoek wat we initieel breed inzetten. Wellicht zullen we de subsets maar in een aantal van de modellen vinden en zullen we juist die modellen gebruiken in vervolg experimenten. Gezien het fundamentele karakter van dit onderzoek is het onmogelijk vooraf aan te geven hoeveel dieren precies per model gebruikt zullen gaan worden. Uiteraard zullen er meerdere go/ no go momenten worden opgenomen in de werkprotocollen. Een uitgewerkt voorbeeld van deze aanpak kunt u vinden in Scheme 2 in ons project proposal.

2) **De onderbouwing van de dieraantallen zijn gebaseerd op 5-15 dieren per groep. Daarnaast geeft u aan veel ervaring te hebben met de te gebruiken diermodellen, waardoor het ons inziens mogelijk zou moeten zijn deze aantallen nauwkeuriger in te schatten. Graag een betere inschatting van het aantal dieren weergeven.**
Ik begrijp de verwarring. We hebben zeker ervaring met de verschillende diermodellen (bijv viral infections: Torabi-Parizi P JI 2014 & Mandl JN Immunity 2013/ tumorgroei en intravital imaging: Ritsma L STM 2012 en Zomer A Stem Cells 2013), echter we weten nog niet of de verschillende neutrofielen subsets in de diverse inflammatoire situaties te vinden zijn, in welke mate en wat de variatie daarin is. In een gezonde muis zijn niet veel neutrofielen in het bloed aanwezig. Het zou dus kunnen dat we voor een complete beschrijvende analyse aan 1 muis niet genoeg hebben. We verwachten wel een toename van neutrofielen na een inflammatoire stimulus. We geven in sectie 2B van de bijlages ook aan dat deze aantallen waarschijnlijk een overschatting zijn. We kunnen helaas pas een betere inschatting geven van het aantal dieren als we meer informatie hebben over de neutrofielen subsets na iedere inflammatoire stimulus. Uiteraard zullen de individuele werkprotocollen na voortschrijdend inzicht wel een betere inschatting bevatten.

3) **Wordt intranasale toediening van de virussen en huisstofmijt onder anesthesie uitgevoerd?**
De intranasale toediening zal onder anesthesie uitgevoerd worden. Dit is ook in rode tekst toegevoegd aan de bijlagen.

4) **U beschrijft verschillende inflammatoire stimuli in uw bijlagen. Kunt u voor alle stimuli weergeven wat de gevolgen zijn van deze stimuli voor de dieren?**
Zoals gevraagd heb ik voor elk model/ inflammatoire stimulus de gevolgen van de stimulus in rode tekst geïncludeerd.

5) **Welke symptomen kunnen zij hierbij krijgen?**
Zoals gevraagd heb ik voor elk model/ inflammatoire stimulus de symptomen van de stimulus in rode tekst geïncludeerd.

6) **U beschrijft een extra 500 muizen voor o.a. onderhoud fok, maken van knock-out modellen, pilot studies etc. Kunt u voor elk van deze doelen de geschatte aantallen geven en de handelingen die deze dieren zullen ondergaan. Voor fok en creatie van nieuwe muizenlijnen is een aparte bijlage wenselijk.**
De fok betreft bestaande genetische tumormodellen (beschreven onder stimulus 7) en bestaande knock-out modellen. Deze diermodellen zijn reeds aanwezig of zullen geimporteerd worden. We

hebben het maken van knock-out modellen geincludeerd omdat we de mogelijkheid wilden hebben om eventuele verschillen in subtype neutrofielen in meer detail te bestuderen. Het is in deze fase van het onderzoek echter nog niet bekend om welke knock-out modellen het gaat. Mijn voorstel is om dit deel er op dit moment uit te laten en een amendement in te sturen zodra dit relevant wordt. Ik heb de overige muizen beter getracht te specificeren in rode tekst, maar ik wil benadrukken dat dit schattingen zijn omdat nog niet duidelijk is hoeveel pilot experimenten nodig zijn, hoeveel onderzoekers op het project ingewerkt moeten worden en of we tumormodellen zullen gaan fokken (of dat we in plaats daarvan tumor stukjes zullen gaan implanteren in gezonde muizen). De muizen zullen geen andere handelingen ondergaan dan beschreven in de project aanvraag.

7) **Kunt u een betere beschrijving geven van de handelingen aan en het ongerief voor de dieren voor implanteren van Bone marrow scaffolds?**
   Zie rode tekst in bijlage 4.3 sectie 2A.

8) **Kunt u een betere beschrijving geven van de handelingen aan en het ongerief voor de dieren voor implanteren van imaging windows?**
   Zie rode tekst in bijlages 4.1/ 4.2/ 4.3 en 4.4 sectie 2A.

Ik hoop u hierbij voldoende te hebben geinformeerd. Mochten er nog vragen zijn dan hoor ik het graag.

Vriendelijke groet,
[redacted]

Centrale Commissie Dierproeven

8.

# 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 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'. | 11500 |
| 1.2 | Provide the name of the licenced establishment. | UMC Utrecht |
| 1.3 | Provide the title of the project. | Neutrophil subsets in health and disease |

## 2 Categories

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

X Basic research

☐ Translational or applied research

☐ Regulatory use or routine production

☐ Research into environmental protection in the interest of human or

☐ 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.

- For legally required animal procedures, indicate which statutory or regulatory requirements apply (with respect to the intended use and market authorisation).
- For routine production, describe what will be produced and for which uses.
- For higher education or training, explain why this project is part of the educational program and describe the learning targets.

The neutrophilic granulocyte is the first line of defense against invaders such as bacteria, fungi, foreign particles and aberrant cells. As the most abundant white blood cell, it continuously circulates the blood until a signal causes it to extravasate towards the tissue. In the tissue upon phagocytosis the neutrophil will exploit the content of its granules to degrade the foreign invader or cell debris. Besides pathogens

and cell debris, we will also further zoom in on one type of foreign particles that neutrophils can encounter in the human body, microplastics. These ≤1µm plastic particles are very prevalent in our environment (eg in our drinking water and aquatic animals via erosion from plastic litter), but research into health effects in mammals is virtually non-existent.
Neutrophils have always been described as a homogeneous population of short-lived cells. However, recent publications of our research group and others challenge this view. Our group described that neutrophils have a longer lifespan than previously thought. And whereas the population in blood in homeostasis might be homogeneous, various stimuli disturbing homeostasis lead to heterogeneity in phenotype and function. Our group showed that if healthy humans receive an LPS injection, immature, banded neutrophils appear in the blood, which are much better at killing bacteria than mature neutrophils (Leliefeld *et al.*, manuscript in preparation).
Recently, neutrophils have been described to play a role in the pathogenesis of various human diseases such as cardiovascular disease, cancer, auto-immune diseases and allergy[1-4]. Linked to these conditions, several neutrophil subsets have been newly described such as low-density granulocytes, granulocytic myeloid-derived suppressor cells (G-MDSC), tumor-associated neutrophils (TAN), and hypersegmented neutrophils[5]. Although we know that homeostatic neutrophils in humans and mice behave very similar, the similarity of neutrophil subsets in humans and mice is poorly described.
In disease, neutrophils can play a beneficial but also often a detrimental role. For example, neutrophils have been described to both promote and inhibit tumor progression and metastasis[6]. They can be both pro- and anti-inflammatory[5]. At the wrong time or the wrong place, neutrophil activation and degranulation will result in overwhelming inflammation and major tissue damage. Neutrophils present in a tumor are correlated with a worse prognosis, but the mechanisms behind this correlation are unclear[6]. The ability to steer neutrophil function, activation or localization from the outside would be beneficial for many diseases. The treatment of cancer would likely benefit from targeting only the pro-tumor neutrophil subset as opposed to all neutrophils. In contrast, the treatment of asthma would greatly benefit from targeting only the pro-inflammatory neutrophil subset.
But the basic knowledge on the timing, localization or even origin of neutrophil subsets is lacking, because we cannot easily sample human tissues. We know neutrophils develop in the bone marrow, but the time they need to develop is uncertain. We know neutrophils are able to migrate to lymph nodes via lymph vessels, but the signal driving them as well as their function in the lymph node are unclear. We know different subtypes of neutrophils have different functions, but when and where the differentiation of these subsets starts to diverge is again unclear.
To answer these types of questions, we need to gain a better understanding of the complex interplay of the entities that can guide the neutrophil: most importantly the bone marrow, spleen, endothelium, inflamed tissue, tumor cells, foreign particles, other immune cells and the neutrophil itself. As well, we think it is important to understand whether neutrophil subsets observed upon inflammation are the same throughout the range of inflammatory disorders. If they have a different phenotype and/or function for different types of disorders, that would be highly relevant for designing new therapies.
Understanding how and where the different neutrophil subsets arise in inflammatory conditions and what mechanisms underlie their function is essential to determine their role in disease and to establish potential treatment options. Redirection of immune responses has an enormous societal and scientific relevance as inflammation-associated diseases (including cardiovascular disease, cancer, auto-immunity and allergy) are associated with major pathology and morbidity.
In this research project we will combine data of healthy mice, mice with acute inflammation and mice with chronic inflammation, to help us understand why and when different neutrophil subsets are developed and recruited. Even if murine neutrophil subsets do not resemble those in human, this data will together show whether the response of the neutrophil to inflammation is generic, or tailored to the stimulus. Examples of inflammatory stimuli are pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs) and tumor antigens. At the same time, we will have a better idea whether inhibiting or stimulating neutrophils in these specific conditions would be beneficial or detrimental to the disease progression.

### 3.2 Purpose

Describe the project's main objective and explain why this objective is achievable.

- If the project is focussed on one or more research objectives, which research questions should be addressed during this project?
- If the main objective is not a research objective, which specific need(s) does this project respond to?

The objective of this project is to gain more fundamental knowledge on origin, timing, localization and

function of neutrophils in inflammatory conditions. These research questions will therefore be addressed:

- Is the neutrophil population heterogeneous in morphology?
- Is the neutrophil population heterogeneous in function?
- Does the migration & distribution of neutrophils throughout the body depend on the type of inflammatory stimulus?
- Does the differentiation of neutrophils depend on the type of inflammatory stimulus or organ?
- Does the function of neutrophils depend on the type of inflammatory stimulus?
- Does the life-span of neutrophils depend on the type of inflammatory stimulus?
- Does the interaction of neutrophils differ per type of inflammatory stimulus?
- Does the phagocytosis of microplastics influence neutrophil function?

From human data, we know that heterogeneous neutrophil subsets appear in the blood upon strong immune stimulation such as LPS injection, cancer, trauma or viral infection. However, these different conditions make the subsets appear in the circulation at a different timing. Our preliminary data shows neutrophil heterogeneity is also induced in mice upon trauma (acute inflammation) or solid tumor growth (chronic inflammation).
In healthy mice, mice with acute inflammation and mice with chronic inflammation, we will analyze the phenotype and function of the neutrophils *ex vivo*, crucially supported by analysis of the kinetics of murine neutrophils *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., etc.). In the kinetics studies we will study the distribution of neutrophils 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 [7, 8].
There are several other reasons why we are confident that we can achieve our aims: Our group is embedded in the Laboratory of Translational Immunology (LTI), which is a center-of-excellence on fundamental and translational immunological research. 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. The LTI 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 TA-COAST consortium. 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[9-15]. 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.

### 3.3 Relevance

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

The enormous burden of inflammation-associated diseases (including cardiovascular disease, cancer, auto-immunity and allergy) worldwide is aggravated by lack of adequate treatment options. This is due to insufficient knowledge about the common player in virtually all inflammatory processes: the neutrophil. The burden of neutrophil-mediated pathology is high, but no specific therapy is currently available. The estimated costs to society (EU) are now above 250 billion euros annually, which will only rise in the future[16]. Recent insights show that the neutrophil can be both pro- and anti-inflammatory[5]. Inhibition of pro-inflammatory neutrophils and activation of anti-inflammatory neutrophils will be beneficial for patients in pro-inflammatory states, whereas patients with a suppressed immune system such as in cancer or after acute severe inflammation may profit from a reverse approach. The lack of knowledge regarding this emerging concept has to date precluded the development and translation of the manipulation of neutrophils into a clinical application. Successful manipulation of the different neutrophil subsets will be widely applicable to a range of different inflammatory diseases.
Pollution of the water environment with microplastics by erosion of plastic litter is a pressing problem that has received a lot of attention in the last few years. It has been shown that microplastics end up in

our food chain, but their effects on human health are currently understudied. To assess their effects on human health we look at neutrophils, since they are specialized in taking up foreign particles. Our preliminary human *in vitro* data shows that neutrophils are also able to engulf microplastics. These microplastics are likely not degraded by the neutrophils and might have an effect on their survival and their capacity to subsequently kill pathogens. If we demonstrate that microplastics adversely affect human health, evidence-based regulatory measures can be formulated.

### 3.4 Research strategy

3.4.1 Provide an overview of the overall design of the project (strategy).

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, tumor size).

The methods for obtaining these readout parameters will be the same for all experiments in this proposal, but the inflammatory stimulus will differ per experiment. We think the combination of several inflammatory stimuli is the strength of this project, because currently it is unknown whether neutrophils respond the same to diverse inflammatory signals (e.g. PAMPs, DAMPs, tumor antigens).
Supported by our own research on human material, pigs and mice, we have a number of hypotheses that we will start with. However, since our research is mostly fundamental and 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.

3.4.2 Provide a basic outline of the different components of the project and the type(s) of animal procedures that will be performed.

Firstly, we want to investigate whether the morphology and function of murine neutrophil subsets is similar to the subsets we described in humans ([17], Tak *et al.* submitted, Leliefeld *et al.* manuscript in preparation). Therefore we will start with an experiment of LPS injection in mice similar to our experiments with LPS injection in humans. But to extend our findings to more physiological inflammatory stimuli, we want to use bacterial infection, viral infection, plastic particles or sterile injury. To look at stimuli specific for chronic inflammation, we want to use animals with solid tumor growth or airway allergy, two relevant diseases with a significant but opposite role for neutrophils. Homeostatic neutrophils will be analyzed *ex vivo* and *in vivo* and compared to the neutrophils arising in an inflammatory state.
As described in 3.1, neutrophil behavior is determined by a complex interplay of the bone marrow, spleen, endothelium, inflamed tissue, tumor cells, foreign particles, other immune cells and the neutrophil itself. To study this system, we want to modify these entities one by one. Therefore we will perform one or more of the following interventions in these mice:

- **A:** Administration of drugs/antibodies/inhibitors/labels
  We might be able to rescue or mimic the phenotypes of the neutrophil subsets and therefore further identify the function of these cells *in vivo*. If possible and/or relevant, we will always test these compounds first *in vitro* and in case relevant effects are observed shift to in the *in vivo* experiments. Other examples include *in vivo* antibody administration, injection of fluorescent compounds for short term labeling of blood vessels, transfer of labelled erythrocytes to long-term label blood vessels, injection of propidium iodide to monitor cell death, injection of Hoechst to stain nuclei, administration of compounds which incorporate into DNA to measure cell life span and proliferation such as deuterated water, deuterated glucose, EdU or BrdU.
- **B:** Adoptive transfer
  To study human cells *in vivo* or to, for instance, compare wildtype neutrophils with neutrophils that have a mutation we need to adoptively transfer donor neutrophils into recipient mice. Irradiation of recipient mice to deplete their endogenous immune system might be required. Also (fluorescently) labeled cells might be transferred for tracking purposes.
- **C:** Bone marrow scaffold
  To study neutrophil progenitors and differentiation. Intravital imaging of the bone marrow compartment is hard due to the solid thick nature of the surrounding bone. In a pilot study we have successfully placed an imaging window on a subcutaneous bone marrow scaffold [Groen Blood 2012] allowing the visualization of cells in the bone marrow.
- **D:** Splenectomy
  To study neutrophil [redacted] and [redacted] in the spleen. Literature as well as our

preliminary data suggests that some [redacted]
Additionally, [redacted] with [redacted], especially [redacted], may [redacted] To study how these subtypes or cell-cell interactions affect the immune system as a whole, we are interested in applying our inflammatory stimuli in splenectomized animals.

3.4.3 Describe the coherence between the different components and the different steps of the project. If applicable, describe the milestones and selection points.

For most experiments we first consider *ex vivo* experiments (mild discomfort), before we consider intravital imaging experiments (mild to moderate discomfort). In some experiments we first consider 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 (can be up to a reduction of 20x); multiple time points can be measured in one individual, and there is no inter-mice variation. After completion of the intravital imaging experiments, cells, tissues and organs will be isolated and analyzed to reduce the number of mice required. For using the different inflammatory stimuli mentioned under 3.4.2, the experiments for optimization and set-up will be the same.
Milestones:

i. Phenotypic definition of neutrophil subsets (surface marker expression, nuclear morphology, etc.)
ii. Functional definition of neutrophil subsets (better/worse in killing, pro- or anti-inflammatory, etc.)

*Go/no-go moment: When we do not find distinct subsets in the mouse but a homogenous neutrophil population, this milestone will not be pursued:

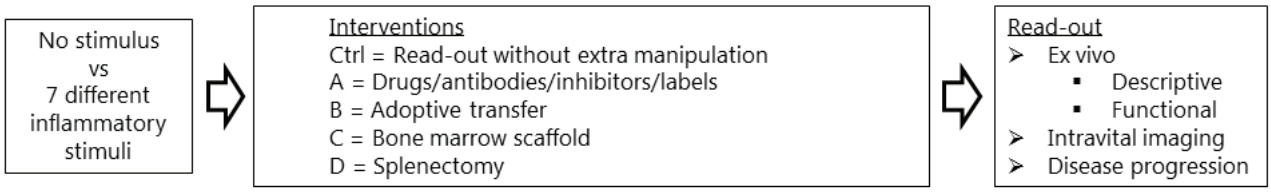
iii. To know where [redacted]

But these milestones below are independent of the go/no-go moment:

iv. To know whether phenotype and function of neutrophils is the same throughout a range of inflammatory disorders
v. To know whether the interaction between the neutrophils and the inflammatory stimulus is the same throughout a range of inflammatory disorders
vi. To know the distribution pattern of neutrophils/neutrophil subsets after leaving the bone marrow, in homeostasis vs. inflammatory conditions
vii. To know the life-span of murine neutrophils/neutrophil subsets, in homeostasis vs. inflammatory conditions
viii. To confirm whether neutrophils/neutrophil subsets continue migrating towards other organs after phagocytosis of pathogens in infected tissue
ix. To confirm whether the [redacted]
x. To know whether inhibition of neutrophils/neutrophil subsets can relieve symptoms in allergic airway disease
xi. To know whether inhibition of neutrophils/neutrophil subsets has an effect on tumor growth

These milestones can be reached simultaneously and do not depend on each other. When we do not reach the first three milestones, i.e. when we do not find distinct subsets in the mouse but a homogenous neutrophil population, then the latter milestones remain valuable for this homogenous neutrophil population.
To further clarify how different inflammatory stimuli will be combined with the interventions described in 3.4.2 to reach these milestones, we provided Scheme 1.

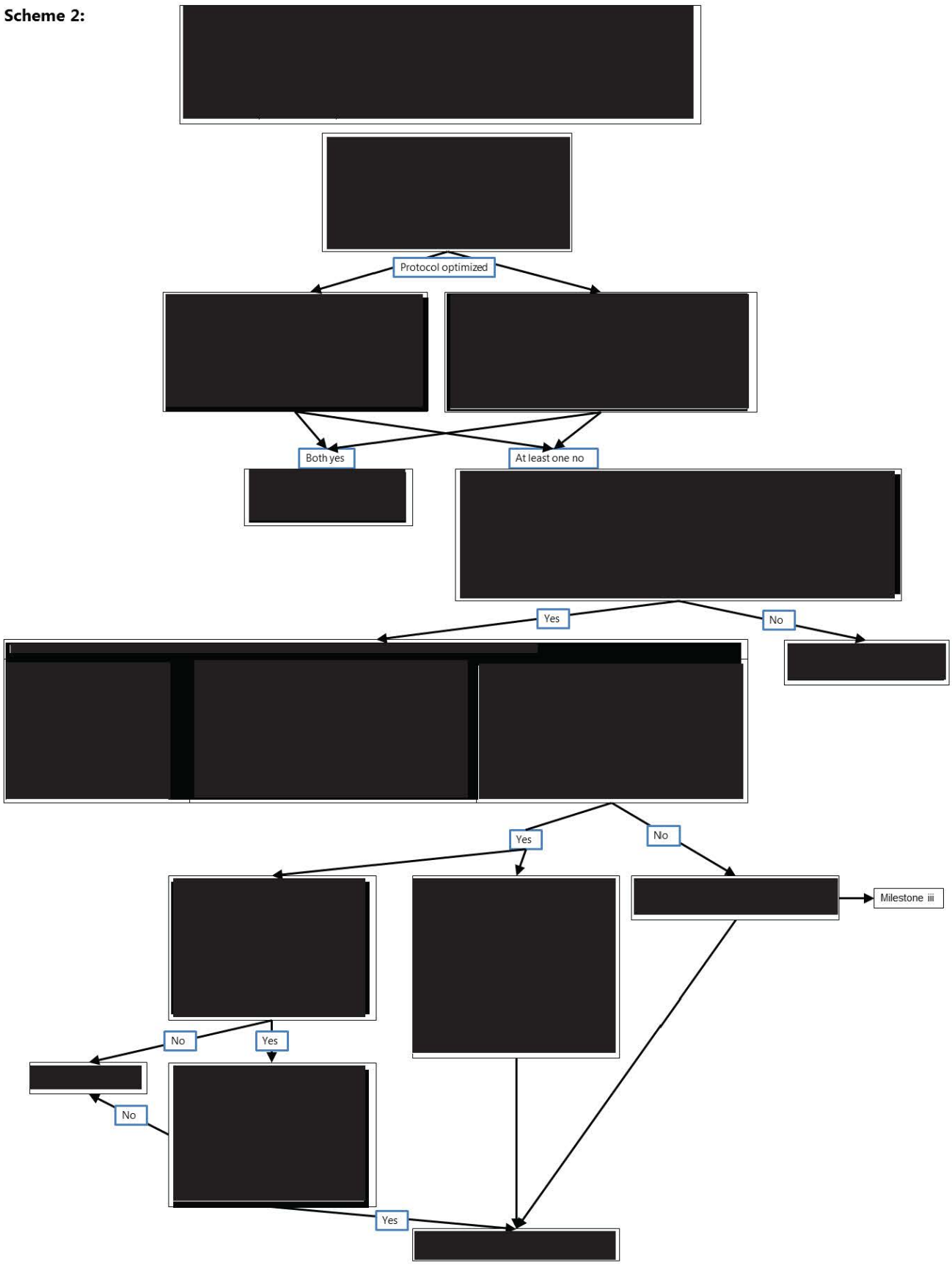
**Scheme 1:**
**Combinations of inflammatory stimuli – interventions – milestones**




| Ctrl | A | B | C | D | Milestone | Discomfort |
|---|---|---|---|---|---|---|
| √ | | | | | i-xi | Mild/Mod |
| | √ | | | | i-xi | Mild/Mod |
| | | √ | | | ii-xi | Mild/Mod |
| | | | √ | | iii-vi, viii | Moderate |
| | | | | √ | ix | Moderate |
| | √ | √ | | | ii-xi | Moderate |
| | √ | | √ | | iii-vi, viii, ix | Moderate |
| | | √ | √ | | iii-vi, viii | Moderate |
| | √ | √ | √ | | iii-vi, viii | Moderate |
| | √ | | | √ | ix | Moderate |
| | | √ | | √ | ix | Moderate |
| | √ | √ | | √ | ix | Moderate |
| | | | √ | √ | ix | Moderate |

For more technical details on the procedures, we refer to the appendices. To further clarify how typical experiments within this project would be performed, we have chosen to elaborate on the approach of milestone ix in detail. In Scheme 2 we listed the experiments that will be performed to reach this milestone.

**Scheme 2:**


Protocol optimized
Both yes
At least one no
Yes
No
Yes
No
Milestone iii
No
Yes
No
Yes

***References***
*1. 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).*
*2. Moses, K. & Brandau, S. Human neutrophils: Their role in cancer and relation to myeloid-derived suppressor cells. Semin. Immunol.* ***28****, 187-196 (2016).*
*3. Kaplan, M. J. Role of neutrophils in systemic autoimmune diseases. Arthritis Res. Ther.* ***15****, 219 (2013).*
*4. Bruijnzeel, P. L., Uddin, M. & Koenderman, L. Targeting neutrophilic inflammation in severe neutrophilic asthma: can we target the disease-relevant neutrophil phenotype? J. Leukoc. Biol.* ***98****, 549-556 (2015).*
*5. Pillay, J., Tak, T., Kamp, V. M. & Koenderman, L. Immune suppression by neutrophils and granulocytic myeloid-derived suppressor cells: similarities and differences. Cell Mol. Life Sci.* ***70****, 3813-3827 (2013).*
*6. Uribe-Querol, E. & Rosales, C. Neutrophils in Cancer: Two Sides of the Same Coin. J. Immunol. Res.* ***2015****, 983698 (2015).*
*7. Hasenberg, A. et al. Catchup: a mouse model for imaging-based tracking and modulation of neutrophil granulocytes. Nat. Methods* ***12****, 445-452 (2015).*
*8. 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).*
*9. Beerling, E., Ritsma, L., Vrisekoop, N., Derksen, P. W. & van Rheenen, J. Intravital microscopy: new insights into metastasis of tumors. J. Cell. Sci.* ***124****, 299-310 (2011).*
*10. Ritsma, L., Vrisekoop, N. & van Rheenen, J. In vivo imaging and histochemistry are combined in the cryosection labelling and intravital microscopy technique. Nat. Commun.* ***4****, 2366 (2013).*
*11. Torabi-Parizi, P. et al. Pathogen-related differences in the abundance of presented antigen are reflected in CD4+ T cell dynamic behavior and effector function in the lung. J. Immunol.* ***192****, 1651-1660 (2014).*
*12. van Golen, R. F. et al. The mechanisms and physiological relevance of glycocalyx degradation in hepatic ischemia/reperfusion injury. Antioxid. Redox Signal.* ***21****, 1098-1118 (2014).*
*13. Ritsma, L. et al. Intravital microscopy through an abdominal imaging window reveals a pre-micrometastasis stage during liver metastasis. Sci. Transl. Med.* ***4****, 158ra145 (2012).*
*14. Zomer, A. et al. Intravital imaging of cancer stem cell plasticity in mammary tumors. Stem Cells* ***31****, 602-606 (2013).*
*15. Beerling, E. et al. Plasticity between Epithelial and Mesenchymal States Unlinks EMT from Metastasis-Enhancing Stem Cell Capacity. Cell. Rep.* ***14****, 2281-2288 (2016).*
*16. Gibson, G. J., Loddenkemper, R., Lundback, B. & Sibille, Y. Respiratory health and disease in Europe: the new European Lung White Book. Eur. Respir. J. 42, 559-563 (2013).*
*17. Pillay, J. et al. A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. J. Clin. Invest. 122, 327-336 (2012).*

3.4.4 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 | Intervention A: Compound administration |
| 2 | Intervention B: Adoptive transfer |
| 3 | Intervention C: Bone marrow scaffold |
| 4 | Intervention D: Splenectomy |
| 5 | |
| 6 | |
| 7 | |
| 8 | |
| 9 | |
| 10 | |

Centrale Commissie Dierproeven

9.

# 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, see 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'. | 11500 |
| 1.2 | Provide the name of the licenced establishment. | UMC Utrecht |
| 1.3 | List the serial number and type of animal procedure.<br><br>*Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.* | Serial number: 1<br>Type of animal procedure: Compound administration |

## 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.

The objective of this project is to gain more fundamental knowledge on origin, timing, localization and function of neutrophils in diverse inflammatory conditions. To achieve this, we need to administer compounds to

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

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, tumor size).

The methods for obtaining these readout parameters will be the same for all experiments, but the inflammatory stimulus will differ per experiment. The purpose of our experiments is to compare the neutrophil populations between all the different inflammatory conditions.

*Ex vivo analysis:*
In order to study the origin and kinetics of the neutrophil populations we need to investigate the different relevant organs. Neutrophils develop in the bone marrow and occasionally the spleen and subsequently migrate through the blood to the inflammatory site. In addition, neutrophils are thought to reside in the lungs and liver (marginated pool). All these organs are therefore relevant for our studies. Whenever we will perform animal experiments we will therefore harvest multiple organs for our analysis.
After the different interventions, 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 (e.g. survival assay, bacterial killing capacity, T cell suppression capacity).
This *ex vivo* analysis will allow us to link surface markers between human and mouse neutrophils. Surface marker expression and nuclear morphology allow us to distinguish different neutrophil subsets in mice, in multiple organs. We can see whether the different inflammatory conditions recruit the subsets with similar dynamics. Lastly, using functional *in vitro* assays we can investigate whether the subsets differ in their capacity e.g. to kill microbes and suppress T cell proliferation like we found in humans.

*Intravital imaging:*
Two different strategies will be used for the imaging experiments: (1) Imaging in an acute experiment under anesthesia and (2) implanting an imaging window (such as on the breast, skin, abdomen and skull) and repeatedly image through that window. 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 intracutaneous 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. 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.
*In vivo* imaging will allow us to study 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 timepoints, greatly reducing the amount of animals needed. Additionally, this provides paired data, introducing less variation and therefore fewer animals 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.

*Procedures:* 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.[14, 15] 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 intracutaneaous 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.[15] 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 during surgery, mild thereafter for intracutaneous windows, moderate thereafter for abdominal and skull windows.

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

Below the experimental approach per inflammatory stimulus and the intervention will be described. The choices about mice strains and numbers are further explained in B.

1. 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.
*Description:* Mice receive a single bolus of E. coli lipopolysaccharide intravenously (i.v.).
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.

*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours-days
*Procedures:* 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:* mild or moderate

2. Inflammatory stimulus: Bacterial infection

*Justification:* Bacterial infection is a more complex, but more physiological relevant model for acute inflammation with which we have a lot of experience in vitro in humans.
*Description:* Mice are infected with (fluorescent) staphylococci via intradermal, i.v. or intraperitoneal injection as described before[1, 2].
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – 2 weeks
*Procedures:* Intraperitoneal and i.v. vein injections will be performed without anesthesia, whereas intradermal or retro-orbital injections will be performed under anesthesia.
*Symptoms:* Intradermal injections of bacteria are well tolerated and do not lead to noticeable symptoms. Intraperitoneal or i.v. injection 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

3. Inflammatory stimulus: Viral infection

*Justification:* Viral infection is a complex physiological relevant model for inflammation with which we have some experience in vitro in humans.
*Description:* Mice are infected with influenza virus or rhinovirus 16 via intranasal administration, as performed before (Suzanne Bal, personal communication).
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – 8 weeks
*Procedures:* Mice will be lightly anesthetized and drops of saline containing virus will be delivered intranasally.
*Symptoms:* Viral infection 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

4. Inflammatory stimulus: Plastic particles

*Justification:* The effects of microplastics on human health are currently understudied. We have found neutrophils can engulf microplastics and now want to investigate their effect on neutrophil survival and function.
*Description:* Microplastics (plastic particles ≤ 1 µm) are administered via subcutaneous/intradermal injection, via addition to the drinking water, or via i.v. injection to mimic exposure routes in humans.
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – one year
*Procedures:* i.v. vein injections will be performed without anesthesia, whereas subcutaneous/intradermal or retro-orbital injections will be performed under anesthesia. Microplastics will also be added to the drinking water.
*Symptoms:* Intradermal injections of microplastics are well tolerated and do not lead to noticeable symptoms. The effects of i.v. microplastics injection and administration in the drinking water have not been described before, but as these are inert materials they are not expected to cause symptoms. However, weight loss and behavioral changes as described in section J will be tightly monitored.
*Level of discomfort:* mild or moderate

5. Inflammatory stimulus: Sterile injury

*Justification:* Sterile injury is known to recruit neutrophils without a pathogenic stimulus. Therefore this can act as a control for pathogen infection or as a model for damage-associated molecular pattern (DAMP) release.
*Description:* Under anesthesia, sterile injury is inflicted in the skin by needle insertion or laser damage
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours - days
*Procedures:* 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. However, behavioral changes as described in section J will be tightly monitored.
*Level of discomfort:* mild or moderate

6. Inflammatory stimulus: Airway allergy

*Justification:* Allergy is a complex physiological relevant model for chronic inflammation with which we have a lot of experience in humans.
*Description:* Mice are sensitized and challenged with house dust mite by intranasal administration, as performed before (Suzanne Bal, personal communication).
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.

We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* 2 – 8 weeks
*Procedures:* Mice will be lightly anesthetized and drops of saline containing allergens (eg house dust mite) will be delivered intranasally 5 days a week for 5 consecutive weeks.
*Symptoms:* Allergen administration is generally well tolerated and does not lead to noticeable symptoms. However, repetitive anesthesia will cause some discomfort. Behavioral changes as described in section J will be tightly monitored.
*Level of discomfort:* mild or moderate

7. Inflammatory stimulus: Solid tumor growth

*Justification:* Cancer is a complex physiological relevant model for chronic inflammation. The suppressive neutrophil has been described in this model.
*Description:* We use genetic mouse models for breast or colorectal cancer, or transfer tumor cells/pieces from these mouse models to healthy mice of the same background to increase reproducibility or for intravital imaging.
*Mice:* Spontaneous tumor models or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – months
*Procedures:* Genetic mouse models will spontaneously develop tumors or will require i.p. tamoxifen administration for tumor initiation. I.p. injections will not be performed under anesthesia. Tumor cells can be injected or tumor pieces can be positioned at different locations (e.g. mammary gland, liver, intestine, brain, mesenteric vein) under anesthesia.
*Symptoms:* Mice can experience post-operative pain for 1 day after surgery. Small tumors are generally well tolerated and do not lead to noticeable symptoms. Animals bearing tumors will never reach end-stage clinical effects. For all tumor models the guidelines of the Code of Practice Animals in Cancer research will strictly be followed, taking into account e.g. the size, location and ulceration of the tumor(s). Behavioral changes as described in section J will be tightly monitored.
*Level of discomfort:* mild or moderate

These different inflammatory stimuli will be combined with the intervention described here and/or with interventions B/C/D, as described in the project proposal.

Intervention A: Administration of drugs/antibodies/inhibitors/labels
*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).
*Rationale:*

- 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* to help achieve milestones i-iv, vi, viii and ix.
- Measure cell life span and proliferation
  E.g. by deuterium incorporation into DNA of proliferating cells we can determine the lifespan of neutrophils in homeostasis vs inflammatory conditions as described in milestone iii, vii and ix.
- Inhibit, stimulate, deplete or mimic components of the inflammatory reaction.
  E.g. by deleting neutrophils with anti-Ly6G antibodies we can delineate the role neutrophils play in disease progression or behavior of other cell types to accomplish milestone x and xi. The specific nature of these compounds will follow from our experimental results in the next five years.

*Extra experimental groups:* treated vs. not treated.
*Number of mice per group:* as described per inflammatory stimulus.
*Duration of intervention:* seconds – months, possibly repeatedly

*Level of discomfort:* mild or moderate

The final level of discomfort depends on the inflammatory stimulus, whether the intervention is used in combination with other interventions and the choice of the readout parameter. Harvesting of organs for *ex vivo* analysis or single session intravital imaging will result only in mild discomfort, but repeated intravital imaging will probably result in moderate discomfort.

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.

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. 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 (most of the animal procedures described in this appendix): 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, estimated numbers, and life stages. Provide justifications for these choices.

Since for our experiments the different interventions described in the different appendices will be combined, it is difficult to pinpoint the estimated numbers per intervention. Therefore here we describe the grand total of estimated numbers for our whole project.

Species: Mus musculus
Origin: Common breeding facility (Gemeenschappelijk Dierenlaboratorium) at Utrecht University/ Hubrecht institute/external licensed breeders
Estimated numbers: max. 2850
Life stages: adults

Species
All animal studies in this project will exclusively involve mice. Up to today, there are no alternative methods to fully understand neutrophil development, homeostasis and response to inflammation in the context of the whole organism. For the proposed studies, the mouse is the most appropriate animal model because: (1) physiology is more extensively characterized than other animal models; (2) a large number of relevant transgenic and knock out lines is already available; (3) a large number of relevant antibodies and other compounds is already available; (4) our research shows murine neutrophils are representative for human neutrophils; (5) many procedures for intravital imaging have already been described. The zebrafish is also an animal widely used for intravital imaging, but the differentiation of zebrafish neutrophils is much less similar to human neutrophils. Since mice are mammals too, they are closer to the human.
Mice strains used are amongst others:

- Wildtype BL/6
- Wildtype Balb/c
- Wildtype FVB
- LysM-GFP mice; for visualization of fluorescent neutrophils in intravital imaging. We are already working with this strain.
- Catchup$^{IVM}$ mice (Ly6G-Cre + Rosa-Td-Tomato); for visualization of fluorescent neutrophils in intravital imaging and for conditional knockouts in neutrophils[5]. This strain was very recently created, we want to import this strain. If the results will be superior to LysM-GFP mice, we will switch to this strain.
- MRP/S100A8-Tg-EGFP-Cre; for conditional knockouts in neutrophil progenitors[6]. We want to import this strain. As opposed to the Catchup$^{IVM}$ mice, Cre recombinase is expressed in

granulocyte-macrophage progenitors in the bone marrow.
- Knockout mice (e.g. CD11b-knockout mice); to investigate the role of a specific gene or protein
- Tumor models such as MMTV-PyMT, MMTV-Wnt1 or C26 colorectal cancer mice. In our currently running animal experiments we are comparing different tumor models with each other, to optimize for future experiments. Therefore we have not completely decided which tumor models we will use in the next five years, but it will be a model that is similar in execution and discomfort as the here mentioned examples.
- Humanized mice[7] or immunocompromised mice (e.g. NSG mice); to investigate the behavior of human neutrophils in an *in vivo*-like situation.

If the required genetically modified mouse is not available, we will obtain this model by generating, importing or by crossing existing models. Other strains will be used when in the next five years, novel strains are described or created that are very relevant to our hypotheses.

Estimated numbers

Our experiments can be divided into:
- Descriptive analysis (5-15 animals x max 5 time points = max 75 animals)
- Functional assays (5-15 animals x max 5 time points = max 75 animals)
- Intravital imaging (5-10 animals)

This totals to 55-160 animals
However, we have 7 inflammatory stimuli, 4 interventions and no intervention per stimuli = 35 conditions.
So for the total we have to multiply the 55-160 animals with 35 = 1925-5600 animals

However, these numbers are overestimations because:
- 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
- Successful pilot studies may be included in the dataset

Therefore we do not anticipate we will need more than 2500 mice for the experiments.

Additionally, a number of ca. 350 mice is needed for:

| | Estimated nr |
|---|---|
| Maintenance of breeding | 100 |
| Pilot studies for validation of models/techniques# | 50 |
| Training of new personnel or new techniques | 50 |
| Rederivation of imported strains via embryo transfer | 100 |
| To compensate for unforeseen loss of animals* | 50 |

*(max 10%, e.g. due to location not suitable for imaging, problems with the window)
#Only procedures mentioned in this project proposal
Mice that become surplus during breeding, will be used for pilot studies and training purposes.

Together we therefore anticipate we need a grand total of 2850 mice.

Sex

In general, we will try to use both male and female mice. However, it is clear that the immune response of males and females can greatly differ[8]. Therefore, we will perform pilot studies to ensure that the sex of the animal does not pose a bias in answering the particular research question. These pilots may prove it necessary to use only one sex within one animal experiment, to be able to reliably compare experimental groups.
One clear exception is the MMTV-PyMT breast cancer model; in this model only female mice consistently develop breast tumors.

**C. Re-use**

Will the animals be re-used?

X No, continue with question D.

☐ 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.

**D. 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:*
Immune responses are highly complex and multifaceted. Multiple compartments such as the bone marrow, spleen, endothelial wall and inflamed tissue interact. Certain aspects of the immune response can be separately simulated *in vitro*, but it is not yet possible to study the full course of infection and the elicited immune response *in vitro*. This requires animal models. Similarly, the use of experimental interventions to test their potential beneficial impact on the course of disease can only be assessed in animal models. Clearly, such experimental interventions cannot be performed in patients, while *in vitro* studies with isolated human cells do not capture the full complexity of infection. However, before turning to mice, we will first study human cells *in vitro* to ensure we only perform the most relevant experiments in mice.

*Reduction:*
Intravital imaging using imaging windows allows us to image the same mouse repeatedly over time as opposed to analyzing separate mice at different timepoints greatly reducing the amount of animals needed. Additionally, this provides paired data, introducing less variation and therefore fewer animals are needed to get statistical significant results. Statistical power calculation based on our own previous experience and literature will be used to determine the optimal number of mice for each experimental group. Moreover, experiments will be performed sequentially, based on the described go/no-go decisions, via which we ensure that we will use the minimum number of mice per group that will be informative. Whenever possible, we will perform pilot studies with the minimum number of animals possible.

*Refinement:*
Except for mice with windows, where co-housing can give problems, mice will be housed in groups in cages. Nesting opportunities will always be provided. Mice with windows will be monitored at least twice a week, mice after bacterial or viral infection will be monitored at least every other day, more if necessary, for clinical signs. If unexpectedly severe suffering is observed, mice will be euthanized.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

As described under refinement, mice welfare will be regularly monitored. Mice reaching the humane endpoints or otherwise suffering from severe discomfort will be euthanized. New personnel will be trained to recognize discomfort in mice according to current guidelines (e.g. [9]). Surgical exposure will be performed under aseptic conditions using sterile instruments. Heating devices will be used to ensure a stable body temperature of the mouse during anaesthesia. Personnel conducting the surgeries is first properly trained, starting with training on cadavers.
Surgical exposure will be performed under adequate anesthesia and analgesia. Pre- and post-procedural analgesia will be used when indicated or when pain-related behavior is observed. However, common analgesics such as opioids and non-steriodal anti-inflammatory drugs (NSAIDs) have been shown to significantly alter the immune response[10-12]. Since this is our main research topic, we might sometimes refrain from using analgesia. It may be necessary to do pilot studies to prove whether analgesics pose a bias in answering our particular research question. From literature, it seems buprenorphine may be a reasonable candidate[13].
There are no expected adverse effects on the environment.

## Repetition and duplication

**E. Repetition**

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

## Accommodation and care

**F. Accommodation and care**

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☐ No

X Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Mice with an imaging window will be housed individually to prevent other mice from biting or damaging the window.

**G. Location where the animals procedures are performed**

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

X No > Continue with question H.

☐ 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.

## Classification of discomfort/humane endpoints

**H. Pain and pain relief**

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

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

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

Surgical exposure will be performed under adequate anesthesia and analgesia. Pre- and post-procedural analgesia will be used when indicated or when pain-related behavior is observed. However, common analgesics such as opioids and non-steriodal anti-inflammatory drugs (NSAIDs) have been shown to significantly alter the immune response[10-12]. Since this is our main research topic, we might sometimes refrain from using analgesia. However, pain can also influence the immune system. Therefore, it may be necessary to do pilot studies to prove whether analgesics pose a bias in answering our particular research question.

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

Surgical exposure will be performed under aseptic conditions using sterile instruments. Heating devices will be used to ensure a stable body temperature of the mouse during anaesthesia. Personnel conducting the surgeries is first properly trained, starting with training on cadavers. Before, during and after surgery adequate anesthesia and analgesia (e.g. isoflurane and buprenorphine) will be used, according to experience as well as published protocols. New personnel will be trained to recognize discomfort in mice according to current guidelines (e.g. [9]).

**I. Other aspects compromising the welfare of the animals**

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

Other than in terminally anaesthetized animals dosing and sampling procedures will consist of a combination of volumes, routes and frequencies that will result in no more than moderate discomfort. Animals bearing tumors will never reach end-stage clinical effects. All relevant scientific data can and will be gathered *before* the cancer, infection or allergy studies reach a level of severe discomfort. If unexpectedly severe suffering is observed, mice will be euthanized.
It is expected that no animals (0%) will be experiencing more than mild discomfort due to the genetic modification. However, it is impossible to predict the nature or severity of any potential genetic defect.

So, in all cases, animals will be careful monitored for possible side effects. Animals exhibiting any unexpected harmful phenotypes will be killed within a day and analyzed when possible.
Animals may lose their window (<5%). In the vast majority of these animals (>95%), we notice signs of detachment of the window far before it becomes lose, and the mice will be sacrificed. In the remaining animals, the window loss will be noticed within 24hrs and the mouse will be immediately sacrificed.

Explain why these effects may emerge.

Tumor development, inflicted inflammation, genetic alterations and administration of compounds/ drugs/ chemicals/ toxins.
It is unclear why some animals may lose their windows.

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

Mice with windows will be monitored at least twice a week, mice after bacterial or viral infection will be monitored at least every other day, more if necessary, for clinical signs. If unexpectedly severe suffering is observed, mice will be euthanized.

**J. 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 K.

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

As described in D, F, H and I, the experiments will be set-up in such a way that the incidence of reaching the humane end-point is kept to a minimum.
Weight loss (>20% in comparison with control or original weight or 15% weight loss in two days), reaching a score of 4 on the illness scale (specified below), severe shortness of breath, or loss of window is set as an humane end point. For all tumor models the guidelines of the Code of Practice Animals in Cancer research will strictly be followed, taking into account e.g. the size, location and ulceration of the tumor(s).

Next to model-specific parameters, 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

Indicate the likely incidence.

Expected <5% and <1day.

**K. Classification of severity of procedures**

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

The cumulative level of discomfort is dependent on the intervention, the read-out and the animal model. The discomfort of these three range from mild to moderate. Therefore combinations of these result in moderate discomfort for the majority (~90%) of animals.

**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. Groen, R. W. *et al*. Reconstructing the human hematopoietic niche in immunodeficient mice: opportunities for studying primary multiple myeloma. *Blood* **120**, e9-e16 (2012).
4. Levy, L. *et al*. Splenectomy inhibits non-small cell lung cancer growth by modulating anti-tumor adaptive and innate immune response. *Oncoimmunology* **4**, e998469 (2015).
5. Hasenberg, A. *et al*. Catchup: a mouse model for imaging-based tracking and modulation of neutrophil granulocytes. *Nat. Methods* **12**, 445-452 (2015).
6. Lagasse, E. & Weissman, I. L. Bcl-2 Inhibits Apoptosis of Neutrophils but Not their Engulfment by Macrophages. *J. Exp. Med.* **179**, 1047-1052 (1994).

7. Coughlan, A. M., Freeley, S. J. & Robson, M. G. Animal models of anti-neutrophil cytoplasmic antibody-associated vasculitis. *Clin. Exp. Immunol.* **169**, 229-237 (2012).
8. Gubbels Bupp, M. R. Sex, the aging immune system, and chronic disease. *Cell. Immunol.* **294**, 102-110 (2015).
9. Burkholder, T., Foltz, C., Karlsson, E., Linton, C. G. & Smith, J. M. Health Evaluation of Experimental Laboratory Mice. *Curr. Protoc. Mouse Biol.* **2**, 145-165 (2012).
10. Salimi, V. *et al*. Opioid receptors control viral replication in the airways. *Crit. Care Med.* **41**, 205-214 (2013).
11. Sacerdote, P. Opioids and the immune system. *Palliat. Med.* **20 Suppl 1**, s9-15 (2006).
12. Hish, G. A., Diaz, J. A., Hawley, A. E., Myers, D. D. & Lester, P. A. Effects of Analgesic Use on Inflammation and Hematology in a Murine Model of Venous Thrombosis. *J. Am. Assoc. Lab. Anim. Sci.* **53**, 485-493 (2014).
13. Mundt, S., Groettrup, M. & Basler, M. Analgesia in mice with experimental meningitis reduces pain without altering immune parameters. *ALTEX* **32**, 183-189 (2015).
14. Ritsma L, Steller EJA, Beerling E, et al. (2012) Intravital Microscopy Through an Abdominal Imaging Window Reveals a Pre-Micrometastasis Stage During Liver Metastasis. Sci Transl Med 4:158ra145–158ra145.
15. Ritsma L, Steller EJ a, Ellenbroek SIJ, et al. (2013) Surgical implantation of an abdominal imaging window for intravital microscopy. Nat Protoc 8:583–94. doi: 10.1038/nprot.2013.026

## End of experiment

**L. Method of killing**

Will the animals be killed during or after the procedures?

☐ No

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

In order to obtain organs and isolated cells for *ex vivo* analysis the mice must first be killed.

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.

X Yes

Centrale Commissie Dierproeven

10.

# 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, see 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'. | 11500 |
| 1.2 | Provide the name of the licenced establishment. | UMC Utrecht |
| 1.3 | List the serial number and type of animal procedure.<br><br>*Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.* | Serial number: 2<br>Type of animal procedure: Adoptive transfer |

## 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.

The objective of this project is to gain more fundamental knowledge on origin, timing, localization and function of neutrophils in diverse inflammatory conditions. To achieve this we need to adoptively transfer cells to:

- Visualize the migratory behavior of neutrophils and different neutrophil subsets
- Investigate whether [redacted] can [redacted] or rather that [redacted]
- Determine whether different neutrophil subsets have different functions (including bacterial killing and the inhibition of immune responses)

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, tumor size).

The methods for obtaining these readout parameters will be the same for all experiments in this proposal, but the inflammatory stimulus will differ per experiment. The purpose of our experiments is to compare the neutrophil populations between all the different inflammatory conditions.

*Ex vivo analysis:*
In order to study the origin and kinetics of the neutrophil populations we need to investigate the different relevant organs. Neutrophils develop in the bone marrow and occasionally the spleen and subsequently migrate through the blood to the inflammatory site. In addition, neutrophils are thought to reside in the lungs and liver (marginated pool). All these organs are therefore relevant for our studies. Whenever we will perform animal experiments we will therefore harvest multiple organs for our analysis.

After the different interventions, 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 (e.g. survival assay, bacterial killing capacity, T cell suppression capacity).
This *ex vivo* analysis will allow us to link surface markers between human and mouse neutrophils. Surface marker expression and nuclear morphology allow us to distinguish different neutrophil subsets in mice, in multiple organs. We can see whether the different inflammatory conditions recruit the subsets with similar dynamics. Lastly, using functional *in vitro* assays we can investigate whether the subsets differ in their capacity e.g. to kill microbes and suppress T cell proliferation like we found in humans.

*Intravital imaging:*
Two different strategies will be used for the imaging experiments: (1) Imaging in an acute experiment under anesthesia and (2) implanting an imaging window (such as on the breast, skin, abdomen and skull) and repeatedly image through that window. 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 intracutaneous 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. 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.
*In vivo* imaging will allow us to study 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 timepoints, greatly reducing the amount of animals needed. Additionally, this provides paired data, introducing less variation and therefore fewer animals 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.

*Procedures:* 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.[14, 15] 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 intracutaneaous 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.[15] 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 during surgery, mild thereafter for intracutaneous windows, moderate thereafter for abdominal and skull windows.

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

The objective of this project is to gain more fundamental knowledge on origin, timing, localization and function of neutrophils in diverse inflammatory conditions. Below the experimental approach per inflammatory stimulus and the intervention will be described. The choices about mice strains and numbers are further explained in B.

1. 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.

*Description:* Mice receive a single bolus of E. coli lipopolysaccharide intravenously (i.v.).
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours-days
*Procedures:* 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:* mild or moderate

2. Inflammatory stimulus: Bacterial infection

*Justification:* Bacterial infection is a more complex, but more physiological relevant model for acute inflammation with which we have a lot of experience in vitro in humans.
*Description:* Mice are infected with (fluorescent) staphylococci via intradermal or intraperitoneal injection as described before[1, 2].
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – 2 weeks
*Procedures:* Intraperitoneal and i.v. vein injections will be performed without anesthesia, whereas intradermal or retro-orbital injections will be performed under anesthesia.
*Symptoms:* Intradermal injections of bacteria are well tolerated and do not lead to noticeable symptoms. Intraperitoneal or i.v. injection 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

3. Inflammatory stimulus: Viral infection

*Justification:* Viral infection is a complex physiological relevant model for inflammation with which we have some experience in vitro in humans.
*Description:* Mice are infected with influenza virus or rhinovirus 16 via intranasal administration, as performed before (Suzanne Bal, personal communication).
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – 8 weeks
*Procedures:* Mice will be lightly anesthetized and drops of saline containing virus will be delivered intranasally.
*Symptoms:* Viral infection 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

4. Inflammatory stimulus: Plastic particles

*Justification:* The effects of microplastics on human health are currently understudied. We have found neutrophils can engulf microplastics and now want to investigate their effect on neutrophil survival and function.
*Description:* Microplastics (plastic particles ≤ 1 µm) are administered via subcutaneous injection, via addition to the drinking water, or via i.v. injection to mimic exposure routes in humans.
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – one year
*Procedures:* i.v. vein injections will be performed without anesthesia, whereas subcutaneous/intradermal or retro-orbital injections will be performed under anesthesia. Microplastics will also be added to the drinking water.
*Symptoms:* Intradermal injections of microplastics are well tolerated and do not lead to noticeable symptoms. The effects of i.v. microplastics injection and administration in the drinking water have not been described before, but as these are inert materials they are not expected to cause symptoms. However, weight loss and behavioral changes as described in section J will be tightly monitored.
*Level of discomfort:* mild or moderate

5. Inflammatory stimulus: Sterile injury

*Justification:* Sterile injury is known to recruit neutrophils without a pathogenic stimulus. Therefore this can act as a control for pathogen infection or as a model for damage-associated molecular pattern (DAMP) release.
*Description:* Under anesthesia, sterile injury is inflicted in the skin by needle insertion or laser damage
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours - days
*Procedures:* 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. However, behavioral changes as described in section J will be tightly monitored.
*Level of discomfort:* mild or moderate

6. Inflammatory stimulus: Airway allergy

*Justification:* Allergy is a complex physiological relevant model for chronic inflammation with which we have a lot of experience in humans.
*Description:* Mice are sensitized and challenged with house dust mite by intranasal administration, as performed before (Suzanne Bal, personal communication).
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein

expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* 2 – 8 weeks
*Procedures:* Mice will be lightly anesthetized and drops of saline containing allergens (eg house dust mite) will be delivered intranasally 5 days a week for 5 consecutive weeks.
*Symptoms:* Allergen administration is generally well tolerated and does not lead to noticeable symptoms. However, repetitive anesthesia will cause some discomfort. Behavioral changes as described in section J will be tightly monitored.
*Level of discomfort:* mild or moderate

7. Inflammatory stimulus: Solid tumor growth

*Justification:* Cancer is a complex physiological relevant model for chronic inflammation. The suppressive neutrophil has been described in this model.
*Description:* We use genetic mouse models for breast or colorectal cancer, or transfer tumor cells/pieces from these mouse models to healthy mice of the same background to increase reproducibility or for intravital imaging.
*Mice:* Spontaneous tumor models or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – months
*Procedures:* Genetic mouse models will spontaneously develop tumors or will require i.p. tamoxifen administration for tumor initiation. I.p. injections will not be performed under anesthesia. Tumor cells can be injected or tumor pieces can be positioned at different locations (e.g. mammary gland, liver, intestine, brain, mesenteric vein) under anesthesia.
*Symptoms:* Mice can experience post-operative pain for 1 day after surgery. Small tumors are generally well tolerated and do not lead to noticeable symptoms. Animals bearing tumors will never reach end-stage clinical effects. For all tumor models the guidelines of the Code of Practice Animals in Cancer research will strictly be followed, taking into account e.g. the size, location and ulceration of the tumor(s). Behavioral changes as described in section J will be tightly monitored.
*Level of discomfort:* mild or moderate

These different inflammatory stimuli will be combined with the intervention described here and/or with interventions A/C/D, as described in the project proposal.

Intervention B: Adoptive transfer
*Description:* a single cell suspension of donor cells is administered i.v. to recipient mice.
*Rationale:* Firstly, (fluorescently) labeled cells will be transferred for tracking migration and function throughout the body. Cells harbouring a specific gene knockout will be transferred to study the role of this gene in specific organs/diseases. Human cells will be transferred to investigate the behavior of human neutrophils in an *in vivo*-like situation.
E.g. different neutrophil subsets might be isolated from a donor mouse and adoptively transferred to a recipient mouse to visualize their difference in functionality to accomplish milestone ii or to follow their distribution to achieve milestone vi.
*Extra experimental groups:* Donor mice & recipient mice.
*Number of mice per group:* ratio donor:recipient mostly 1:1, sometimes 2:1 or 3:1
*Donor mice:* wt mice, intravital imaging strains, knockout strains.
*Recipient mice:* wt mice, intravital imaging strains, knockout strains, immunodeficient mice. Irradiation of recipient mice to deplete their endogenous immune system might be required.
*Duration of intervention:* seconds – minutes
*Level of discomfort:* mild

The final level of discomfort depends on the inflammatory stimulus, whether the intervention is used in combination with other interventions and the choice of the readout parameter. Harvesting of organs for *ex vivo* analysis or single session intravital imaging will result only in mild discomfort, but repeated intravital imaging will probably result in moderate discomfort.

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.

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. 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 (most of the animal procedures described in this appendix): 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, estimated numbers, and life stages. Provide justifications for these choices.

Since for our experiments the different interventions described in the different appendices will be combined, it is difficult to pinpoint the estimated numbers per intervention. Therefore here we describe the grand total of estimated numbers for our whole project.

Species: Mus musculus
Origin: Common breeding facility (Gemeenschappelijk Dierenlaboratorium) at Utrecht University/ Hubrecht institute/external licensed breeders
Estimated numbers: max. 2850
Life stages: adults

Species
All animal studies in this project will exclusively involve mice. Up to today, there are no alternative methods to fully understand neutrophil development, homeostasis and response to inflammation in the context of the whole organism. For the proposed studies, the mouse is the most appropriate animal model because: (1) physiology is more extensively characterized than other animal models; (2) a large number of relevant transgenic and knock out lines is already available; (3) a large number of relevant antibodies and other compounds is already available; (4) our research shows murine neutrophils are representative for human neutrophils; (5) many procedures for intravital imaging have already been described. The zebrafish is also an animal widely used for intravital imaging, but the differentiation of zebrafish neutrophils is much less similar to human neutrophils. Since mice are mammals too, they are closer to the human.
Mice strains used are amongst others:

- Wildtype BL/6
- Wildtype Balb/c
- Wildtype FVB
- LysM-GFP mice; for visualization of fluorescent neutrophils in intravital imaging. We are already working with this strain.
- Catchup$^{IVM}$ mice [redacted] Cre + Rosa-Td-Tomato); for visualization of fluorescent neutrophils in intravital imaging and for conditional knockouts in neutrophils[5]. This strain was very recently created, we want to import this strain. If the results will be superior to LysM-GFP mice, we will switch to this strain.
- MRP/S100A8-Tg-EGFP-Cre; for conditional knockouts in neutrophil progenitors[6]. We want to import this strain. As opposed to the Catchup$^{IVM}$ mice, Cre recombinase is expressed in granulocyte-macrophage progenitors in the bone marrow.

- Knockout mice (e.g. CD11b-knockout mice); to investigate the role of a specific gene or protein
- Tumor models such as MMTV-PyMT, MMTV-Wnt1 or C26 colorectal cancer mice. In our currently running animal experiments we are comparing different tumor models with each other, to optimize for future experiments. Therefore we have not completely decided which tumor models we will use in the next five years, but it will be a model that is similar in execution and discomfort as the here mentioned examples.
- Humanized mice[7] or immunocompromised mice (e.g. NSG mice); to investigate the behavior of human neutrophils in an *in vivo*-like situation.

If the required genetically modified mouse is not available, we will obtain this model by generating, importing or by crossing existing models. Other strains will be used when in the next five years, novel strains are described or created that are very relevant to our hypotheses.

Estimated numbers

Our experiments can be divided into:
- Descriptive analysis (5-15 animals x max 5 time points = max 75 animals)
- Functional assays (5-15 animals x max 5 time points = max 75 animals)
- Intravital imaging (5-10 animals)

This totals to 55-160 animals
However, we have 7 inflammatory stimuli, 4 interventions and no intervention per stimuli = 35 conditions.
So for the total we have to multiply the 55-160 animals with 35 = 1925-5600 animals

However, these numbers are overestimations because:
- 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
- Successful pilot studies may be included in the dataset

Therefore we do not anticipate we will need more than 2500 mice for the experiments.

Additionally, a number of ca. 350 mice is needed for:

| | Estimated nr |
|---|---|
| Maintenance of breeding | 100 |
| Pilot studies for validation of models/techniques# | 50 |
| Training of new personnel or new techniques | 50 |
| Rederivation of imported strains via embryo transfer | 100 |
| To compensate for unforeseen loss of animals* | 50 |

*(max 10%, e.g. due to location not suitable for imaging, problems with the window)
#Only procedures mentioned in this project proposal
Mice that become surplus during breeding, will be used for pilot studies and training purposes.

Together we therefore anticipate we need a grand total of 2850 mice.

Sex

In general, we will try to use both male and female mice. However, it is clear that the immune response of males and females can greatly differ[8]. Therefore, we will perform pilot studies to ensure that the sex of the animal does not pose a bias in answering the particular research question. These pilots may prove it necessary to use only one sex within one animal experiment, to be able to reliably compare experimental groups.
One clear exception is the MMTV-PyMT breast cancer model; in this model only female mice consistently develop breast tumors.

**C. Re-use**

Will the animals be re-used?

X No, continue with question D.

☐ 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.

**D. 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:*
Immune responses are highly complex and multifaceted. Multiple compartments such as the bone marrow, spleen, endothelial wall and inflamed tissue interact. Certain aspects of the immune response can be separately simulated *in vitro*, but it is not yet possible to study the full course of infection and the elicited immune response *in vitro*. This requires animal models. Similarly, the use of experimental interventions to test their potential beneficial impact on the course of disease can only be assessed in animal models. Clearly, such experimental interventions cannot be performed in patients, while *in vitro* studies with isolated human cells do not capture the full complexity of infection. However, before turning to mice, we will first study human cells *in vitro* to ensure we only perform the most relevant experiments in mice.

*Reduction:*
Intravital imaging using imaging windows allows us to image the same mouse repeatedly over time as opposed to analyzing separate mice at different timepoints greatly reducing the amount of animals needed. Additionally, this provides paired data, introducing less variation and therefore fewer animals are needed to get statistical significant results. Statistical power calculation based on our own previous experience and literature will be used to determine the optimal number of mice for each experimental group. Moreover, experiments will be performed sequentially, based on the described go/no-go decisions, via which we ensure that we will use the minimum number of mice per group that will be informative. Whenever possible, we will perform pilot studies with the minimum number of animals possible.

*Refinement:*
Except for mice with windows, where co-housing can give problems, mice will be housed in groups in cages. Nesting opportunities will always be provided. Mice with windows will be monitored at least twice a week, mice after bacterial or viral infection will be monitored at least every other day, more if necessary, for clinical signs. If unexpectedly severe suffering is observed, mice will be euthanized.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

As described under refinement, mice welfare will be regularly monitored. Mice reaching the humane endpoints or otherwise suffering from severe discomfort will be euthanized. New personnel will be trained to recognize discomfort in mice according to current guidelines (e.g. [9]). Surgical exposure will be performed under aseptic conditions using sterile instruments. Heating devices will be used to ensure a stable body temperature of the mouse during anaesthesia. Personnel conducting the surgeries is first properly trained, starting with training on cadavers.
Surgical exposure will be performed under adequate anesthesia and analgesia. Pre- and post-procedural analgesia will be used when indicated or when pain-related behavior is observed. However, common analgesics such as opioids and non-steriodal anti-inflammatory drugs (NSAIDs) have been shown to significantly alter the immune response[10-12]. Since this is our main research topic, we might sometimes refrain from using analgesia. It may be necessary to do pilot studies to prove whether analgesics pose a bias in answering our particular research question. From literature, it seems buprenorphine may be a reasonable candidate[13].
There are no expected adverse effects on the environment.

## Repetition and duplication

**E. Repetition**

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

## Accommodation and care

**F. Accommodation and care**

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☐ No

X Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Mice with an imaging window will be housed individually to prevent other mice from biting or damaging the window.

**G. Location where the animals procedures are performed**

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

X No > Continue with question H.

☐ 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.

## Classification of discomfort/humane endpoints

**H. Pain and pain relief**

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

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

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

Surgical exposure will be performed under adequate anesthesia and analgesia. Pre- and post-procedural analgesia will be used when indicated or when pain-related behavior is observed. However, common analgesics such as opioids and non-steriodal anti-inflammatory drugs (NSAIDs) have been shown to significantly alter the immune response[10-12]. Since this is our main research topic, we might sometimes refrain from using analgesia. However, pain can also influence the immune system. Therefore, it may be necessary to do pilot studies to prove whether analgesics pose a bias in answering our particular research question.

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

Surgical exposure will be performed under aseptic conditions using sterile instruments. Heating devices will be used to ensure a stable body temperature of the mouse during anaesthesia. Personnel conducting the surgeries is first properly trained, starting with training on cadavers. Before, during and after surgery adequate anesthesia and analgesia (e.g. isoflurane and buprenorphine) will be used, according to experience as well as published protocols. New personnel will be trained to recognize discomfort in mice according to current guidelines (e.g. [9]).

**I. Other aspects compromising the welfare of the animals**

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

Other than in terminally anaesthetized animals dosing and sampling procedures will consist of a combination of volumes, routes and frequencies that will result in no more than moderate discomfort. Animals bearing tumors will never reach end-stage clinical effects. All relevant scientific data can and will be gathered *before* the cancer, infection or allergy studies reach a level of severe discomfort. If unexpectedly severe suffering is observed, mice will be euthanized.
It is expected that no animals (0%) will be experiencing more than mild discomfort due to the genetic modification. However, it is impossible to predict the nature or severity of any potential genetic defect.

So, in all cases, animals will be careful monitored for possible side effects. Animals exhibiting any unexpected harmful phenotypes will be killed within a day and analyzed when possible.
Animals may lose their window (<5%). In the vast majority of these animals (>95%), we notice signs of detachment of the window far before it becomes lose, and the mice will be sacrificed. In the remaining animals, the window loss will be noticed within 24hrs and the mouse will be immediately sacrificed.

Explain why these effects may emerge.

Tumor development, inflicted inflammation, genetic alterations and administration of compounds/ drugs/ chemicals/ toxins.
It is unclear why some animals may lose their windows.

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

Mice with windows will be monitored at least twice a week, mice after bacterial or viral infection will be monitored at least every other day, more if necessary, for clinical signs. If unexpectedly severe suffering is observed, mice will be euthanized.

**J. 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 K.

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

As described in D, F, H and I, the experiments will be set-up in such a way that the incidence of reaching the humane end-point is kept to a minimum.
Weight loss (>20% in comparison with control or original weight or 15% weight loss in two days), reaching a score of 4 on the illness scale (specified below), severe shortness of breath, or loss of window is set as an humane end point. For all tumor models the guidelines of the Code of Practice Animals in Cancer research will strictly be followed, taking into account e.g. the size, location and ulceration of the tumor(s).

Next to model-specific parameters, 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

Indicate the likely incidence.

Expected <5% and <1day.

**K. Classification of severity of procedures**

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

The cumulative level of discomfort is dependent on the intervention, the read-out and the animal model. The discomfort of these three range from mild to moderate. Therefore combinations of these result in moderate discomfort for the majority (~90%) of animals.

**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. Groen, R. W. *et al*. Reconstructing the human hematopoietic niche in immunodeficient mice: opportunities for studying primary multiple myeloma. *Blood* **120**, e9-e16 (2012).
4. Levy, L. *et al*. Splenectomy inhibits non-small cell lung cancer growth by modulating anti-tumor adaptive and innate immune response. *Oncoimmunology* **4**, e998469 (2015).
5. Hasenberg, A. *et al*. Catchup: a mouse model for imaging-based tracking and modulation of neutrophil granulocytes. *Nat. Methods* **12**, 445-452 (2015).
6. Lagasse, E. & Weissman, I. L. Bcl-2 Inhibits Apoptosis of Neutrophils but Not their Engulfment by Macrophages. *J. Exp. Med.* **179**, 1047-1052 (1994).

7. Coughlan, A. M., Freeley, S. J. & Robson, M. G. Animal models of anti-neutrophil cytoplasmic antibody-associated vasculitis. *Clin. Exp. Immunol.* **169**, 229-237 (2012).
8. Gubbels Bupp, M. R. Sex, the aging immune system, and chronic disease. *Cell. Immunol.* **294**, 102-110 (2015).
9. Burkholder, T., Foltz, C., Karlsson, E., Linton, C. G. & Smith, J. M. Health Evaluation of Experimental Laboratory Mice. *Curr. Protoc. Mouse Biol.* **2**, 145-165 (2012).
10. Salimi, V. *et al*. Opioid receptors control viral replication in the airways. *Crit. Care Med.* **41**, 205-214 (2013).
11. Sacerdote, P. Opioids and the immune system. *Palliat. Med.* **20 Suppl 1**, s9-15 (2006).
12. Hish, G. A., Diaz, J. A., Hawley, A. E., Myers, D. D. & Lester, P. A. Effects of Analgesic Use on Inflammation and Hematology in a Murine Model of Venous Thrombosis. *J. Am. Assoc. Lab. Anim. Sci.* **53**, 485-493 (2014).
13. Mundt, S., Groettrup, M. & Basler, M. Analgesia in mice with experimental meningitis reduces pain without altering immune parameters. *ALTEX* **32**, 183-189 (2015).
14. Ritsma L, Steller EJA, Beerling E, et al. (2012) Intravital Microscopy Through an Abdominal Imaging Window Reveals a Pre-Micrometastasis Stage During Liver Metastasis. Sci Transl Med 4:158ra145–158ra145.
15. Ritsma L, Steller EJ a, Ellenbroek SIJ, et al. (2013) Surgical implantation of an abdominal imaging window for intravital microscopy. Nat Protoc 8:583–94. doi: 10.1038/nprot.2013.026

## End of experiment

**L. Method of killing**

Will the animals be killed during or after the procedures?

☐ No

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

In order to obtain organs and isolated cells for *ex vivo* analysis the mice must first be killed.

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.

X Yes

Centrale Commissie Dierproeven

11.

# 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, see 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'. | 11500 |
| 1.2 | Provide the name of the licenced establishment. | UMC Utrecht |
| 1.3 | List the serial number and type of animal procedure.<br><br>*Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.* | Serial number: 3<br>Type of animal procedure: Bone marrow scaffold |

## 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.

The objective of this project is to gain more fundamental knowledge on origin, timing, localization and function of neutrophils in diverse inflammatory conditions. In order to achieve this we need to implant a bone marrow scaffold to:

- Visualize the migratory behavior of neutrophils and different neutrophil subsets in the bone marrow. More specifically, we can monitor the recruitment of young banded neutrophils to the circulation upon inflammatory stimuli and establish whether [redacted] through the [redacted] (as our own data in humans predict) or only [redacted] (as has been reported in literature).
- Investigate whether [redacted] can [redacted] or rather that [redacted]

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, tumor size).

The methods for obtaining these readout parameters will be the same for all experiments in this proposal, but the inflammatory stimulus will differ per experiment. The purpose of our experiments is to compare the neutrophil populations between all the different inflammatory conditions.

*Ex vivo analysis:*
In order to study the origin and kinetics of the neutrophil populations we need to investigate the different relevant organs. Neutrophils develop in the bone marrow and occasionally the spleen and subsequently migrate through the blood to the inflammatory site. In addition, neutrophils are thought to reside in the

lungs and liver (marginated pool). All these organs are therefore relevant for our studies. Whenever we will perform animal experiments we will therefore harvest multiple organs for our analysis.
After the different interventions, 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 (e.g. survival assay, bacterial killing capacity, T cell suppression capacity).
This *ex vivo* analysis will allow us to link surface markers between human and mouse neutrophils. Surface marker expression and nuclear morphology allow us to distinguish different neutrophil subsets in mice, in multiple organs. We can see whether the different inflammatory conditions recruit the subsets with similar dynamics. Lastly, using functional *in vitro* assays we can investigate whether the subsets differ in their capacity e.g. to kill microbes and suppress T cell proliferation like we found in humans.

*Intravital imaging:*
Two different strategies will be used for the imaging experiments: (1) Imaging in an acute experiment under anesthesia and (2) implanting an imaging window (such as on the breast, skin, abdomen and skull) and repeatedly image through that window. 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 intracutaneous 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. 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.
*In vivo* imaging will allow us to study 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 timepoints, greatly reducing the amount of animals needed. Additionally, this provides paired data, introducing less variation and therefore fewer animals 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.

*Procedures:* 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.[14, 15] 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 intracutaneaous 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.[15] 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 during surgery, mild thereafter for intracutaneous windows, moderate thereafter for abdominal and skull windows.

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

The objective of this project is to gain more fundamental knowledge on origin, timing, localization and function of neutrophils in diverse inflammatory conditions. Below the experimental approach per inflammatory stimulus and the intervention will be described. The choices about mice strains and numbers are further explained in B.

1. 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.
*Description:* Mice receive a single bolus of E. coli lipopolysaccharide intravenously (i.v.).
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours-days
*Procedures:* 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:* mild or moderate

2. Inflammatory stimulus: Bacterial infection

*Justification:* Bacterial infection is a more complex, but more physiological relevant model for acute inflammation with which we have a lot of experience in vitro in humans.
*Description:* Mice are infected with (fluorescent) staphylococci via intradermal or intraperitoneal injection as described before[1, 2].
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – 2 weeks
*Procedures:* Intraperitoneal and i.v. vein injections will be performed without anesthesia, whereas intradermal or retro-orbital injections will be performed under anesthesia.
*Symptoms:* Intradermal injections of bacteria are well tolerated and do not lead to noticeable symptoms. Intraperitoneal or i.v. injection 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

3. Inflammatory stimulus: Viral infection

*Justification:* Viral infection is a complex physiological relevant model for inflammation with which we have some experience in vitro in humans.
*Description:* Mice are infected with influenza virus or rhinovirus 16 via intranasal administration, as performed before (Suzanne Bal, personal communication).
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – 8 weeks
*Procedures:* Mice will be lightly anesthetized and drops of saline containing virus will be delivered

intranasally.
*Symptoms:* Viral infection 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

4. Inflammatory stimulus: Plastic particles

*Justification:* The effects of microplastics on human health are currently understudied. We have found neutrophils can engulf microplastics and now want to investigate their effect on neutrophil survival and function.
*Description:* Microplastics (plastic particles ≤ 1 µm) are administered via subcutaneous injection, via addition to the drinking water, or via i.v. injection to mimic exposure routes in humans.
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – one year
*Procedures:* i.v. vein injections will be performed without anesthesia, whereas subcutaneous/intradermal or retro-orbital injections will be performed under anesthesia. Microplastics will also be added to the drinking water.
*Symptoms:* Intradermal injections of microplastics are well tolerated and do not lead to noticeable symptoms. The effects of i.v. microplastics injection and administration in the drinking water have not been described before, but as these are inert materials they are not expected to cause symptoms. However, weight loss and behavioral changes as described in section J will be tightly monitored.
*Level of discomfort:* mild or moderate

5. Inflammatory stimulus: Sterile injury

*Justification:* Sterile injury is known to recruit neutrophils without a pathogenic stimulus. Therefore this can act as a control for pathogen infection or as a model for damage-associated molecular pattern (DAMP) release.
*Description:* Under anesthesia, sterile injury is inflicted in the skin by needle insertion or laser damage
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours - days
*Procedures:* 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. However, behavioral changes as described in section J will be tightly monitored.
*Level of discomfort:* mild or moderate

6. Inflammatory stimulus: Airway allergy

*Justification:* Allergy is a complex physiological relevant model for chronic inflammation with which we have a lot of experience in humans.
*Description:* Mice are sensitized and challenged with house dust mite by intranasal administration, as performed before (Suzanne Bal, personal communication).
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.

Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* 2 – 8 weeks
*Procedures:* Mice will be lightly anesthetized and drops of saline containing allergens (eg house dust mite) will be delivered intranasally 5 days a week for 5 consecutive weeks.
*Symptoms:* Allergen administration is generally well tolerated and does not lead to noticeable symptoms. However, repetitive anesthesia will cause some discomfort. Behavioral changes as described in section J will be tightly monitored.
*Level of discomfort:* mild to moderate

7. Inflammatory stimulus: Solid tumor growth

*Justification:* Cancer is a complex physiological relevant model for chronic inflammation. The suppressive neutrophil has been described in this model.
*Description:* We use genetic mouse models for breast or colorectal cancer, or transfer tumor cells/pieces from these mouse models to healthy mice of the same background to increase reproducibility or for intravital imaging.
*Mice:* Spontaneous tumor models or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – months
*Procedures:* Genetic mouse models will spontaneously develop tumors or will require i.p. tamoxifen administration for tumor initiation. I.p. injections will not be performed under anesthesia. Tumor cells can be injected or tumor pieces can be positioned at different locations (e.g. mammary gland, liver, intestine, brain, mesenteric vein) under anesthesia.
*Symptoms:* Mice can experience post-operative pain for 1 day after surgery. Small tumors are generally well tolerated and do not lead to noticeable symptoms. Animals bearing tumors will never reach end-stage clinical effects. For all tumor models the guidelines of the Code of Practice Animals in Cancer research will strictly be followed, taking into account e.g. the size, location and ulceration of the tumor(s). Behavioral changes as described in section J will be tightly monitored.
*Level of discomfort:* mild or moderate

These different inflammatory stimuli will be combined with the intervention described here and/or with interventions A/B/D, as described in the project proposal.

Intervention C: Bone marrow scaffold
*Description:* Biphasic calcium phosphate particles loaded with human MSCs will be implanted subcutaneously in immunodeficient mice to create a niche for hematopoiesis.
*Rationale:* To study neutrophil progenitors and differentiation. Intravital imaging of the bone marrow compartment is hard due to the solid thick nature of the surrounding bone. In a pilot study we have successfully placed an imaging window on a subcutaneous bone marrow scaffold allowing the visualization of cells in the bone marrow[3].
E.g. we will be able to visualize neutrophils exiting (milestone vi) or re-entering (milestone viii) the bone marrow
*Extra experimental groups:* With vs. without bone marrow scaffold.
*Number of mice per group:* as described per inflammatory stimulus
*Duration of intervention:* days - months
*Procedures:* Mice will be anesthetized, positioned on a heat pad for proper temperature control and shaved before making a small incision in the skin. An in vitro generated bone marrow scaffold will be

transplanted subcutaneously and the small incision will be closed with an absorbable suture.
*Symptoms:* None (besides mild symptoms possibly caused by the anesthesia).
*Level of discomfort:* moderate during surgery, mild thereafter

The final level of discomfort depends on the inflammatory stimulus, whether the intervention is used in combination with other interventions and the choice of the readout parameter. Harvesting of organs for *ex vivo* analysis or single session intravital imaging will result only in mild discomfort, but repeated intravital imaging will probably result in moderate discomfort.

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.

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. 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 (most of the animal procedures described in this appendix): 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, estimated numbers, and life stages. Provide justifications for these choices.

Since for our experiments the different interventions described in the different appendices will be combined, it is difficult to pinpoint the estimated numbers per intervention. Therefore here we describe the grand total of estimated numbers for our whole project.

Species: Mus musculus
Origin: Common breeding facility (Gemeenschappelijk Dierenlaboratorium) at Utrecht University/ Hubrecht institute/external licensed breeders
Estimated numbers: max. 2850
Life stages: adults

Species
All animal studies in this project will exclusively involve mice. Up to today, there are no alternative methods to fully understand neutrophil development, homeostasis and response to inflammation in the context of the whole organism. For the proposed studies, the mouse is the most appropriate animal model because: (1) physiology is more extensively characterized than other animal models; (2) a large number of relevant transgenic and knock out lines is already available; (3) a large number of relevant antibodies and other compounds is already available; (4) our research shows murine neutrophils are representative for human neutrophils; (5) many procedures for intravital imaging have already been described. The zebrafish is also an animal widely used for intravital imaging, but the differentiation of zebrafish neutrophils is much less similar to human neutrophils. Since mice are mammals too, they are closer to the human.
Mice strains used are amongst others:

- Wildtype BL/6
- Wildtype Balb/c
- Wildtype FVB
- LysM-GFP mice; for visualization of fluorescent neutrophils in intravital imaging. We are already working with this strain.
- Catchup$^{IVM}$ mice (Ly6G-Cre + Rosa-Td-Tomato); for visualization of fluorescent neutrophils in intravital imaging and for conditional knockouts in neutrophils[5]. This strain was very recently created, we want to import this strain. If the results will be superior to LysM-GFP mice, we will switch to this strain.

- MRP/S100A8-Tg-EGFP-Cre; for conditional knockouts in neutrophil progenitors[6]. We want to import this strain. As opposed to the Catchup$^{IVM}$ mice, Cre recombinase is expressed in granulocyte-macrophage progenitors in the bone marrow.
- Knockout mice (e.g. CD11b-knockout mice); to investigate the role of a specific gene or protein
- Tumor models such as MMTV-PyMT, MMTV-Wnt1 or C26 colorectal cancer mice. In our currently running animal experiments we are comparing different tumor models with each other, to optimize for future experiments. Therefore we have not completely decided which tumor models we will use in the next five years, but it will be a model that is similar in execution and discomfort as the here mentioned examples.
- Humanized mice[7] or immunocompromised mice (e.g. NSG mice); to investigate the behavior of human neutrophils in an *in vivo*-like situation.

If the required genetically modified mouse is not available, we will obtain this model by generating, importing or by crossing existing models. Other strains will be used when in the next five years, novel strains are described or created that are very relevant to our hypotheses.

Estimated numbers

Our experiments can be divided into:
- Descriptive analysis (5-15 animals x max 5 time points = max 75 animals)
- Functional assays (5-15 animals x max 5 time points = max 75 animals)
- Intravital imaging (5-10 animals)

This totals to 55-160 animals
However, we have 7 inflammatory stimuli, 4 interventions and no intervention per stimuli = 35 conditions.
So for the total we have to multiply the 55-160 animals with 35 = 1925-5600 animals

However, these numbers are overestimations because:
- 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
- Successful pilot studies may be included in the dataset

Therefore we do not anticipate we will need more than 2500 mice for the experiments.

Additionally, a number of ca. 350 mice is needed for:

| | Estimated nr |
|---|---|
| Maintenance of breeding | 100 |
| Pilot studies for validation of models/techniques# | 50 |
| Training of new personnel or new techniques | 50 |
| Rederivation of imported strains via embryo transfer | 100 |
| To compensate for unforeseen loss of animals* | 50 |

*(max 10%, e.g. due to location not suitable for imaging, problems with the window)
#Only procedures mentioned in this project proposal
Mice that become surplus during breeding, will be used for pilot studies and training purposes.

Together we therefore anticipate we need a grand total of 2850 mice.

Sex

In general, we will try to use both male and female mice. However, it is clear that the immune response of males and females can greatly differ[8]. Therefore, we will perform pilot studies to ensure that the sex of the animal does not pose a bias in answering the particular research question. These pilots may prove it necessary to use only one sex within one animal experiment, to be able to reliably compare experimental groups.
One clear exception is the MMTV-PyMT breast cancer model; in this model only female mice consistently develop breast tumors.

**C. Re-use**

Will the animals be re-used?

X No, continue with question D.

☐ 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.

**D. 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:*
Immune responses are highly complex and multifaceted. Multiple compartments such as the bone marrow, spleen, endothelial wall and inflamed tissue interact. Certain aspects of the immune response can be separately simulated *in vitro*, but it is not yet possible to study the full course of infection and the elicited immune response *in vitro*. This requires animal models. Similarly, the use of experimental interventions to test their potential beneficial impact on the course of disease can only be assessed in animal models. Clearly, such experimental interventions cannot be performed in patients, while *in vitro* studies with isolated human cells do not capture the full complexity of infection. However, before turning to mice, we will first study human cells *in vitro* to ensure we only perform the most relevant experiments in mice.

*Reduction:*
Intravital imaging using imaging windows allows us to image the same mouse repeatedly over time as opposed to analyzing separate mice at different timepoints greatly reducing the amount of animals needed. Additionally, this provides paired data, introducing less variation and therefore fewer animals are needed to get statistical significant results. Statistical power calculation based on our own previous experience and literature will be used to determine the optimal number of mice for each experimental group. Moreover, experiments will be performed sequentially, based on the described go/no-go decisions, via which we ensure that we will use the minimum number of mice per group that will be informative. Whenever possible, we will perform pilot studies with the minimum number of animals possible.

*Refinement:*
Except for mice with windows, where co-housing can give problems, mice will be housed in groups in cages. Nesting opportunities will always be provided. Mice with windows will be monitored at least twice a week, mice after bacterial or viral infection will be monitored at least every other day, more if necessary, for clinical signs. If unexpectedly severe suffering is observed, mice will be euthanized.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

As described under refinement, mice welfare will be regularly monitored. Mice reaching the humane endpoints or otherwise suffering from severe discomfort will be euthanized. New personnel will be trained to recognize discomfort in mice according to current guidelines (e.g. [9]). Surgical exposure will be performed under aseptic conditions using sterile instruments. Heating devices will be used to ensure a stable body temperature of the mouse during anaesthesia. Personnel conducting the surgeries is first properly trained, starting with training on cadavers.
Surgical exposure will be performed under adequate anesthesia and analgesia. Pre- and post-procedural analgesia will be used when indicated or when pain-related behavior is observed. However, common analgesics such as opioids and non-steriodal anti-inflammatory drugs (NSAIDs) have been shown to significantly alter the immune response[10-12]. Since this is our main research topic, we might sometimes refrain from using analgesia. It may be necessary to do pilot studies to prove whether analgesics pose a bias in answering our particular research question. From literature, it seems buprenorphine may be a reasonable candidate[13].
There are no expected adverse effects on the environment.

## Repetition and duplication

**E. Repetition**

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

## Accommodation and care

**F. Accommodation and care**

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☐ No

X Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Mice with an imaging window will be housed individually to prevent other mice from biting or damaging the window.

**G. Location where the animals procedures are performed**

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

X No > Continue with question H.

☐ 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.

## Classification of discomfort/humane endpoints

**H. Pain and pain relief**

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

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

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

Surgical exposure will be performed under adequate anesthesia and analgesia. Pre- and post-procedural analgesia will be used when indicated or when pain-related behavior is observed. However, common analgesics such as opioids and non-steriodal anti-inflammatory drugs (NSAIDs) have been shown to significantly alter the immune response[10-12]. Since this is our main research topic, we might sometimes refrain from using analgesia. However, pain can also influence the immune system. Therefore, it may be necessary to do pilot studies to prove whether analgesics pose a bias in answering our particular research question.

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

Surgical exposure will be performed under aseptic conditions using sterile instruments. Heating devices will be used to ensure a stable body temperature of the mouse during anaesthesia. Personnel conducting the surgeries is first properly trained, starting with training on cadavers. Before, during and after surgery adequate anesthesia and analgesia (e.g. isoflurane and buprenorphine) will be used, according to experience as well as published protocols. New personnel will be trained to recognize discomfort in mice according to current guidelines (e.g. [9]).

**I. Other aspects compromising the welfare of the animals**

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

Other than in terminally anaesthetized animals dosing and sampling procedures will consist of a combination of volumes, routes and frequencies that will result in no more than moderate discomfort. Animals bearing tumors will never reach end-stage clinical effects. All relevant scientific data can and will be gathered *before* the cancer, infection or allergy studies reach a level of severe discomfort. If unexpectedly severe suffering is observed, mice will be euthanized.
It is expected that no animals (0%) will be experiencing more than mild discomfort due to the genetic

modification. However, it is impossible to predict the nature or severity of any potential genetic defect. So, in all cases, animals will be careful monitored for possible side effects. Animals exhibiting any unexpected harmful phenotypes will be killed within a day and analyzed when possible.
Animals may lose their window (<5%). In the vast majority of these animals (>95%), we notice signs of detachment of the window far before it becomes lose, and the mice will be sacrificed. In the remaining animals, the window loss will be noticed within 24hrs and the mouse will be immediately sacrificed.

Explain why these effects may emerge.

Tumor development, inflicted inflammation, genetic alterations and administration of compounds/ drugs/ chemicals/ toxins.
It is unclear why some animals may lose their windows.

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

Mice with windows will be monitored at least twice a week, mice after bacterial or viral infection will be monitored at least every other day, more if necessary, for clinical signs. If unexpectedly severe suffering is observed, mice will be euthanized.

**J. 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 K.

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

As described in D, F, H and I, the experiments will be set-up in such a way that the incidence of reaching the humane end-point is kept to a minimum.
Weight loss (>20% in comparison with control or original weight or 15% weight loss in two days), reaching a score of 4 on the illness scale (specified below), severe shortness of breath, or loss of window is set as an humane end point. For all tumor models the guidelines of the Code of Practice Animals in Cancer research will strictly be followed, taking into account e.g. the size, location and ulceration of the tumor(s).

Next to model-specific parameters, 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

Indicate the likely incidence.

Expected <5% and <1day.

**K. Classification of severity of procedures**

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

The cumulative level of discomfort is dependent on the intervention, the read-out and the animal model. The discomfort of these three range from mild to moderate. Therefore combinations of these result in moderate discomfort for the majority (~90%) of animals.

**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. Groen, R. W. *et al*. Reconstructing the human hematopoietic niche in immunodeficient mice: opportunities for studying primary multiple myeloma. *Blood* **120**, e9-e16 (2012).
4. Levy, L. *et al*. Splenectomy inhibits non-small cell lung cancer growth by modulating anti-tumor adaptive and innate immune response. *Oncoimmunology* **4**, e998469 (2015).
5. Hasenberg, A. *et al*. Catchup: a mouse model for imaging-based tracking and modulation of neutrophil granulocytes. *Nat. Methods* **12**, 445-452 (2015).
6. Lagasse, E. & Weissman, I. L. Bcl-2 Inhibits Apoptosis of Neutrophils but Not their Engulfment by Macrophages. *J.*

*Exp. Med.* **179**, 1047-1052 (1994).
7. Coughlan, A. M., Freeley, S. J. & Robson, M. G. Animal models of anti-neutrophil cytoplasmic antibody-associated vasculitis. *Clin. Exp. Immunol.* **169**, 229-237 (2012).
8. Gubbels Bupp, M. R. Sex, the aging immune system, and chronic disease. *Cell. Immunol.* **294**, 102-110 (2015).
9. Burkholder, T., Foltz, C., Karlsson, E., Linton, C. G. & Smith, J. M. Health Evaluation of Experimental Laboratory Mice. *Curr. Protoc. Mouse Biol.* **2**, 145-165 (2012).
10. Salimi, V. *et al*. Opioid receptors control viral replication in the airways. *Crit. Care Med.* **41**, 205-214 (2013).
11. Sacerdote, P. Opioids and the immune system. *Palliat. Med.* **20 Suppl 1**, s9-15 (2006).
12. Hish, G. A., Diaz, J. A., Hawley, A. E., Myers, D. D. & Lester, P. A. Effects of Analgesic Use on Inflammation and Hematology in a Murine Model of Venous Thrombosis. *J. Am. Assoc. Lab. Anim. Sci.* **53**, 485-493 (2014).
13. Mundt, S., Groettrup, M. & Basler, M. Analgesia in mice with experimental meningitis reduces pain without altering immune parameters. *ALTEX* **32**, 183-189 (2015).
14. Ritsma L, Steller EJA, Beerling E, et al. (2012) Intravital Microscopy Through an Abdominal Imaging Window Reveals a Pre-Micrometastasis Stage During Liver Metastasis. Sci Transl Med 4:158ra145–158ra145.
15. Ritsma L, Steller EJ a, Ellenbroek SIJ, et al. (2013) Surgical implantation of an abdominal imaging window for intravital microscopy. Nat Protoc 8:583–94. doi: 10.1038/nprot.2013.026

## End of experiment

**L. Method of killing**

Will the animals be killed during or after the procedures?

☐ No

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

In order to obtain organs and isolated cells for *ex vivo* analysis the mice must first be killed.

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.

X Yes

Centrale Commissie Dierproeven

12.

# 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, see 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'. | 11500 |
| 1.2 | Provide the name of the licenced establishment. | UMC Utrecht |
| 1.3 | List the serial number and type of animal procedure.<br><br>*Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.* | Serial number: 4<br>Type of animal procedure: Splenectomy |

## 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.

The objective of this project is to gain more fundamental knowledge on origin, timing, localization and function of neutrophils in diverse inflammatory conditions. We hypothesize that the [redacted] in the [redacted] (milestone ix). Although the dogma is that these cell are [redacted], our preliminary data [redacted] are [redacted], suggesting these cells either come directly from the spleen and not the [redacted] or alternatively [redacted]. To confirm this we need to perform splenectomy in mice.

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, tumor size).

The methods for obtaining these readout parameters will be the same for all experiments in this proposal, but the inflammatory stimulus will differ per experiment. The purpose of our experiments is to compare the neutrophil populations between all the different inflammatory conditions.

*Ex vivo analysis:*
In order to study the origin and kinetics of the neutrophil populations we need to investigate the different relevant organs. Neutrophils develop in the bone marrow and occasionally the spleen and subsequently migrate through the blood to the inflammatory site. In addition, neutrophils are thought to reside in the lungs and liver (marginated pool). All these organs are therefore relevant for our studies. Whenever we will perform animal experiments we will therefore harvest multiple organs for our analysis.
After the different interventions, 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 (e.g. survival assay, bacterial killing capacity, T cell suppression capacity).
This *ex vivo* analysis will allow us to link surface markers between human and mouse neutrophils. Surface marker expression and nuclear morphology allow us to distinguish different neutrophil subsets in mice, in multiple organs. We can see whether the different inflammatory conditions recruit the subsets with similar dynamics. Lastly, using functional *in vitro* assays we can investigate whether the subsets differ in their capacity e.g. to kill microbes and suppress T cell proliferation like we found in humans.

*Intravital imaging:*
Two different strategies will be used for the imaging experiments: (1) Imaging in an acute experiment under anesthesia and (2) implanting an imaging window (such as on the breast, skin, abdomen and skull) and repeatedly image through that window. 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 intracutaneous 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. 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.
*In vivo* imaging will allow us to study 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 timepoints, greatly reducing the amount of animals needed. Additionally, this provides paired data, introducing less variation and therefore fewer animals 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.

*Procedures:* 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.[14, 15] 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 intracutaneaous 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.[15] 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 during surgery, mild thereafter for intracutaneous windows, moderate thereafter for abdominal and skull windows.

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

The objective of this project is to gain more fundamental knowledge on origin, timing, localization and function of neutrophils in diverse inflammatory conditions. Below the experimental approach per inflammatory stimulus and the intervention will be described. The choices about mice strains and numbers are further explained in B.

1. 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.

*Description:* Mice receive a single bolus of E. coli lipopolysaccharide intravenously (i.v.).
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours-days
*Procedures:* 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:* mild or moderate

2. Inflammatory stimulus: Bacterial infection

*Justification:* Bacterial infection is a more complex, but more physiological relevant model for acute inflammation with which we have a lot of experience in vitro in humans.
*Description:* Mice are infected with (fluorescent) staphylococci via intradermal or intraperitoneal injection as described before[1, 2].
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – 2 weeks
*Procedures:* Intraperitoneal and i.v. vein injections will be performed without anesthesia, whereas intradermal or retro-orbital injections will be performed under anesthesia.
*Symptoms:* Intradermal injections of bacteria are well tolerated and do not lead to noticeable symptoms. Intraperitoneal or i.v. injection 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

3. Inflammatory stimulus: Viral infection

*Justification:* Viral infection is a complex physiological relevant model for inflammation with which we have some experience in vitro in humans.
*Description:* Mice are infected with influenza virus or rhinovirus 16 via intranasal administration, as performed before (Suzanne Bal, personal communication).
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – 8 weeks
*Procedures:* Mice will be lightly anesthetized and drops of saline containing virus will be delivered intranasally.
*Symptoms:* Viral infection 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

4. Inflammatory stimulus: Plastic particles

*Justification:* The effects of microplastics on human health are currently understudied. We have found neutrophils can engulf microplastics and now want to investigate their effect on neutrophil survival and function.
*Description:* Microplastics (plastic particles ≤ 1 µm) are administered via subcutaneous injection, via addition to the drinking water, or via i.v. injection to mimic exposure routes in humans.
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – one year
*Procedures:* i.v. vein injections will be performed without anesthesia, whereas subcutaneous/intradermal or retro-orbital injections will be performed under anesthesia. Microplastics will also be added to the drinking water.
*Symptoms:* Intradermal injections of microplastics are well tolerated and do not lead to noticeable symptoms. The effects of i.v. microplastics injection and administration in the drinking water have not been described before, but as these are inert materials they are not expected to cause symptoms. However, weight loss and behavioral changes as described in section J will be tightly monitored.
*Level of discomfort:* mild or moderate

5. Inflammatory stimulus: Sterile injury

*Justification:* Sterile injury is known to recruit neutrophils without a pathogenic stimulus. Therefore this can act as a control for pathogen infection or as a model for damage-associated molecular pattern (DAMP) release.
*Description:* Under anesthesia, sterile injury is inflicted in the skin by needle insertion or laser damage
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours - days
*Procedures:* 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. However, behavioral changes as described in section J will be tightly monitored.
*Level of discomfort:* mild or moderate

6. Inflammatory stimulus: Airway allergy

*Justification:* Allergy is a complex physiological relevant model for chronic inflammation with which we have a lot of experience in humans.
*Description:* Mice are sensitized and challenged with house dust mite by intranasal administration, as performed before (Suzanne Bal, personal communication).
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein

expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* 2 – 8 weeks
*Procedures:* Mice will be lightly anesthetized and drops of saline containing allergens (eg house dust mite) will be delivered intranasally 5 days a week for 5 consecutive weeks.
*Symptoms:* Allergen administration is generally well tolerated and does not lead to noticeable symptoms. However, repetitive anesthesia will cause some discomfort. Behavioral changes as described in section J will be tightly monitored.
*Level of discomfort:* mild or moderate

7. Inflammatory stimulus: Solid tumor growth

*Justification:* Cancer is a complex physiological relevant model for chronic inflammation. The suppressive neutrophil has been described in this model.
*Description:* We use genetic mouse models for breast or colorectal cancer, or transfer tumor cells/pieces from these mouse models to healthy mice of the same background to increase reproducibility or for intravital imaging.
*Mice:* Spontaneous tumor models or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – months
*Procedures:* Genetic mouse models will spontaneously develop tumors or will require i.p. tamoxifen administration for tumor initiation. I.p. injections will not be performed under anesthesia. Tumor cells can be injected or tumor pieces can be positioned at different locations (e.g. mammary gland, liver, intestine, brain, mesenteric vein) under anesthesia.
*Symptoms:* Mice can experience post-operative pain for 1 day after surgery. Small tumors are generally well tolerated and do not lead to noticeable symptoms. Animals bearing tumors will never reach end-stage clinical effects. For all tumor models the guidelines of the Code of Practice Animals in Cancer research will strictly be followed, taking into account e.g. the size, location and ulceration of the tumor(s). Behavioral changes as described in section J will be tightly monitored.
*Level of discomfort:* mild or moderate

These different inflammatory stimuli will be combined with the intervention described here and/or with interventions A/B/C, as described in the project proposal.

Intervention D: Splenectomy
*Description:* Surgery to remove the spleen is performed as described before[4].
*Rationale:* Since we hypothesize that the [redacted] and neutrophil [redacted] (milestone ix), we will study the effect of [redacted]. Data from [redacted] suggest that the [redacted] to the [redacted].
*Extra experimental groups:* Splenectomized vs. non-splenectomized
*Number of mice per group:* as described per inflammatory stimulus.
*Duration of intervention:* hours
*Level of discomfort:* moderate during surgery, mild thereafter

The final level of discomfort depends on the inflammatory stimulus, whether the intervention is used in combination with other interventions and the choice of the readout parameter. Harvesting of organs for *ex vivo* analysis or single session intravital imaging will result only in mild discomfort, but repeated intravital imaging will probably result in moderate discomfort.

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.

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. 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 (most of the animal procedures described in this appendix): 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, estimated numbers, and life stages. Provide justifications for these choices.

Since for our experiments the different interventions described in the different appendices will be combined, it is difficult to pinpoint the estimated numbers per intervention. Therefore here we describe the grand total of estimated numbers for our whole project.

Species: Mus musculus
Origin: Common breeding facility (Gemeenschappelijk Dierenlaboratorium) at Utrecht University/ Hubrecht institute/external licensed breeders
Estimated numbers: max. 2850
Life stages: adults

Species
All animal studies in this project will exclusively involve mice. Up to today, there are no alternative methods to fully understand neutrophil development, homeostasis and response to inflammation in the context of the whole organism. For the proposed studies, the mouse is the most appropriate animal model because: (1) physiology is more extensively characterized than other animal models; (2) a large number of relevant transgenic and knock out lines is already available; (3) a large number of relevant antibodies and other compounds is already available; (4) our research shows murine neutrophils are representative for human neutrophils; (5) many procedures for intravital imaging have already been described. The zebrafish is also an animal widely used for intravital imaging, but the differentiation of zebrafish neutrophils is much less similar to human neutrophils. Since mice are mammals too, they are closer to the human.
Mice strains used are amongst others:

- Wildtype BL/6
- Wildtype Balb/c
- Wildtype FVB
- LysM-GFP mice; for visualization of fluorescent neutrophils in intravital imaging. We are already working with this strain.
- Catchup$^{IVM}$ mice (Ly6G-Cre + Rosa-Td-Tomato); for visualization of fluorescent neutrophils in intravital imaging and for conditional knockouts in neutrophils[5]. This strain was very recently created, we want to import this strain. If the results will be superior to LysM-GFP mice, we will switch to this strain.
- MRP/S100A8-Tg-EGFP-Cre; for conditional knockouts in neutrophil progenitors[6]. We want to import this strain. As opposed to the Catchup$^{IVM}$ mice, Cre recombinase is expressed in granulocyte-macrophage progenitors in the bone marrow.
- Knockout mice (e.g. CD11b-knockout mice); to investigate the role of a specific gene or protein
- Tumor models such as MMTV-PyMT, MMTV-Wnt1 or C26 colorectal cancer mice. In our currently running animal experiments we are comparing different tumor models with each other, to optimize for future experiments. Therefore we have not completely decided which tumor models we will use in the next five years, but it will be a model that is similar in execution and discomfort as the here mentioned examples.

- Humanized mice[7] or immunocompromised mice (e.g. NSG mice); to investigate the behavior of human neutrophils in an *in vivo*-like situation.

If the required genetically modified mouse is not available, we will obtain this model by generating, importing or by crossing existing models. Other strains will be used when in the next five years, novel strains are described or created that are very relevant to our hypotheses.

Estimated numbers

Our experiments can be divided into:

- Descriptive analysis (5-15 animals x max 5 time points = max 75 animals)
- Functional assays (5-15 animals x max 5 time points = max 75 animals)
- Intravital imaging (5-10 animals)

This totals to 55-160 animals

However, we have 7 inflammatory stimuli, 4 interventions and no intervention per stimuli = 35 conditions.

So for the total we have to multiply the 55-160 animals with 35 = 1925-5600 animals

However, these numbers are overestimations because:

- 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
- Successful pilot studies may be included in the dataset

Therefore we do not anticipate we will need more than 2500 mice for the experiments.

Additionally, a number of ca. 350 mice is needed for:

| | Estimated nr |
|---|---|
| Maintenance of breeding | 100 |
| Pilot studies for validation of models/techniques# | 50 |
| Training of new personnel or new techniques | 50 |
| Rederivation of imported strains via embryo transfer | 100 |
| To compensate for unforeseen loss of animals* | 50 |

*(max 10%, e.g. due to location not suitable for imaging, problems with the window)

#Only procedures mentioned in this project proposal

Mice that become surplus during breeding, will be used for pilot studies and training purposes.

Together we therefore anticipate we need a grand total of 2850 mice.

Sex

In general, we will try to use both male and female mice. However, it is clear that the immune response of males and females can greatly differ[8]. Therefore, we will perform pilot studies to ensure that the sex of the animal does not pose a bias in answering the particular research question. These pilots may prove it necessary to use only one sex within one animal experiment, to be able to reliably compare experimental groups.

One clear exception is the MMTV-PyMT breast cancer model; in this model only female mice consistently develop breast tumors.

**C. Re-use**

Will the animals be re-used?

X No, continue with question D.

☐ 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.

**D. 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:*
Immune responses are highly complex and multifaceted. Multiple compartments such as the bone marrow, spleen, endothelial wall and inflamed tissue interact. Certain aspects of the immune response can be separately simulated *in vitro*, but it is not yet possible to study the full course of infection and the elicited immune response *in vitro*. This requires animal models. Similarly, the use of experimental interventions to test their potential beneficial impact on the course of disease can only be assessed in animal models. Clearly, such experimental interventions cannot be performed in patients, while *in vitro* studies with isolated human cells do not capture the full complexity of infection. However, before turning to mice, we will first study human cells *in vitro* to ensure we only perform the most relevant experiments in mice.

*Reduction:*
Intravital imaging using imaging windows allows us to image the same mouse repeatedly over time as opposed to analyzing separate mice at different timepoints greatly reducing the amount of animals needed. Additionally, this provides paired data, introducing less variation and therefore fewer animals are needed to get statistical significant results. Statistical power calculation based on our own previous experience and literature will be used to determine the optimal number of mice for each experimental group. Moreover, experiments will be performed sequentially, based on the described go/no-go decisions, via which we ensure that we will use the minimum number of mice per group that will be informative. Whenever possible, we will perform pilot studies with the minimum number of animals possible.

*Refinement:*
Except for mice with windows, where co-housing can give problems, mice will be housed in groups in cages. Nesting opportunities will always be provided. Mice with windows will be monitored at least twice a week, mice after bacterial or viral infection will be monitored at least every other day, more if necessary, for clinical signs. If unexpectedly severe suffering is observed, mice will be euthanized.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

As described under refinement, mice welfare will be regularly monitored. Mice reaching the humane endpoints or otherwise suffering from severe discomfort will be euthanized. New personnel will be trained to recognize discomfort in mice according to current guidelines (e.g. [9]). Surgical exposure will be performed under aseptic conditions using sterile instruments. Heating devices will be used to ensure a stable body temperature of the mouse during anaesthesia. Personnel conducting the surgeries is first properly trained, starting with training on cadavers.
Surgical exposure will be performed under adequate anesthesia and analgesia. Pre- and post-procedural analgesia will be used when indicated or when pain-related behavior is observed. However, common analgesics such as opioids and non-steriodal anti-inflammatory drugs (NSAIDs) have been shown to significantly alter the immune response[10-12]. Since this is our main research topic, we might sometimes refrain from using analgesia. It may be necessary to do pilot studies to prove whether analgesics pose a bias in answering our particular research question. From literature, it seems buprenorphine may be a reasonable candidate[13].
There are no expected adverse effects on the environment.

## Repetition and duplication

**E. Repetition**

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

## Accommodation and care

**F. Accommodation and care**

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☐ No

X Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Mice with an imaging window will be housed individually to prevent other mice from biting or damaging the window.

**G. Location where the animals procedures are performed**

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

X No > Continue with question H.

☐ 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.

## Classification of discomfort/humane endpoints

**H. Pain and pain relief**

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

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

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

Surgical exposure will be performed under adequate anesthesia and analgesia. Pre- and post-procedural analgesia will be used when indicated or when pain-related behavior is observed. However, common analgesics such as opioids and non-steriodal anti-inflammatory drugs (NSAIDs) have been shown to significantly alter the immune response[10-12]. Since this is our main research topic, we might sometimes refrain from using analgesia. However, pain can also influence the immune system. Therefore, it may be necessary to do pilot studies to prove whether analgesics pose a bias in answering our particular research question.

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

Surgical exposure will be performed under aseptic conditions using sterile instruments. Heating devices will be used to ensure a stable body temperature of the mouse during anaesthesia. Personnel conducting the surgeries is first properly trained, starting with training on cadavers. Before, during and after surgery adequate anesthesia and analgesia (e.g. isoflurane and buprenorphine) will be used, according to experience as well as published protocols. New personnel will be trained to recognize discomfort in mice according to current guidelines (e.g. [9]).

**I. Other aspects compromising the welfare of the animals**

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

Other than in terminally anaesthetized animals dosing and sampling procedures will consist of a combination of volumes, routes and frequencies that will result in no more than moderate discomfort. Animals bearing tumors will never reach end-stage clinical effects. All relevant scientific data can and will be gathered *before* the cancer, infection or allergy studies reach a level of severe discomfort. If unexpectedly severe suffering is observed, mice will be euthanized.
It is expected that no animals (0%) will be experiencing more than mild discomfort due to the genetic modification. However, it is impossible to predict the nature or severity of any potential genetic defect. So, in all cases, animals will be careful monitored for possible side effects. Animals exhibiting any unexpected harmful phenotypes will be killed within a day and analyzed when possible.
Animals may lose their window (<5%). In the vast majority of these animals (>95%), we notice signs of detachment of the window far before it becomes lose, and the mice will be sacrificed. In the remaining animals, the window loss will be noticed within 24hrs and the mouse will be immediately sacrificed.

Explain why these effects may emerge.

Tumor development, inflicted inflammation, genetic alterations and administration of compounds/ drugs/ chemicals/ toxins.
It is unclear why some animals may lose their windows.

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

Mice with windows will be monitored at least twice a week, mice after bacterial or viral infection will be monitored at least every other day, more if necessary, for clinical signs. If unexpectedly severe suffering is observed, mice will be euthanized.

**J. 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 K.

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

As described in D, F, H and I, the experiments will be set-up in such a way that the incidence of reaching the humane end-point is kept to a minimum.
Weight loss (>20% in comparison with control or original weight or 15% weight loss in two days), reaching a score of 4 on the illness scale (specified below), severe shortness of breath, or loss of window is set as an humane end point. For all tumor models the guidelines of the Code of Practice Animals in Cancer research will strictly be followed, taking into account e.g. the size, location and ulceration of the tumor(s).

Next to model-specific parameters, 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

Indicate the likely incidence.

Expected <5% and <1day.

**K. Classification of severity of procedures**

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

The cumulative level of discomfort is dependent on the intervention, the read-out and the animal model. The discomfort of these three range from mild to moderate. Therefore combinations of these result in moderate discomfort for the majority (~90%) of animals.

**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. Groen, R. W. *et al*. Reconstructing the human hematopoietic niche in immunodeficient mice: opportunities for studying primary multiple myeloma. *Blood* **120**, e9-e16 (2012).
4. Levy, L. *et al*. Splenectomy inhibits non-small cell lung cancer growth by modulating anti-tumor adaptive and innate immune response. *Oncoimmunology* **4**, e998469 (2015).
5. Hasenberg, A. *et al*. Catchup: a mouse model for imaging-based tracking and modulation of neutrophil granulocytes. *Nat. Methods* **12**, 445-452 (2015).
6. Lagasse, E. & Weissman, I. L. Bcl-2 Inhibits Apoptosis of Neutrophils but Not their Engulfment by Macrophages. *J. Exp. Med.* **179**, 1047-1052 (1994).
7. Coughlan, A. M., Freeley, S. J. & Robson, M. G. Animal models of anti-neutrophil cytoplasmic antibody-associated vasculitis. *Clin. Exp. Immunol.* **169**, 229-237 (2012).
8. Gubbels Bupp, M. R. Sex, the aging immune system, and chronic disease. *Cell. Immunol.* **294**, 102-110 (2015).
9. Burkholder, T., Foltz, C., Karlsson, E., Linton, C. G. & Smith, J. M. Health Evaluation of Experimental Laboratory Mice. *Curr. Protoc. Mouse Biol.* **2**, 145-165 (2012).
10. Salimi, V. *et al*. Opioid receptors control viral replication in the airways. *Crit. Care Med.* **41**, 205-214 (2013).
11. Sacerdote, P. Opioids and the immune system. *Palliat. Med.* **20 Suppl 1**, s9-15 (2006).
12. Hish, G. A., Diaz, J. A., Hawley, A. E., Myers, D. D. & Lester, P. A. Effects of Analgesic Use on Inflammation and

Hematology in a Murine Model of Venous Thrombosis. *J. Am. Assoc. Lab. Anim. Sci.* **53**, 485-493 (2014).
13. Mundt, S., Groettrup, M. & Basler, M. Analgesia in mice with experimental meningitis reduces pain without altering immune parameters. *ALTEX* **32**, 183-189 (2015).
14. Ritsma L, Steller EJA, Beerling E, et al. (2012) Intravital Microscopy Through an Abdominal Imaging Window Reveals a Pre-Micrometastasis Stage During Liver Metastasis. Sci Transl Med 4:158ra145–158ra145.
15. Ritsma L, Steller EJ a, Ellenbroek SIJ, et al. (2013) Surgical implantation of an abdominal imaging window for intravital microscopy. Nat Protoc 8:583–94. doi: 10.1038/nprot.2013.026

## End of experiment

**L. Method of killing**

Will the animals be killed during or after the procedures?

☐ No

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

In order to obtain organs and isolated cells for *ex vivo* analysis the mice must first be killed.

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.

X Yes

13.

**A. Algemene gegevens over de procedure**

1. Aanvraagnummer : 2016.II.524.017
2. Titel van het project : Neutrophil subsets in health and disease
3. Titel van de NTS : Neutrofielen subtypes in gezondheid en ziekte

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

5. Contactgegevens DEC

| | |
|---|---|
| Naam DEC | : DEC Utrecht |
| Telefoonnummer contactpersoon | : 088 – 75 59 247 |
| Emailadres contactpersoon | : dec-utrecht@umcutrecht.nl |

6. Adviestraject (data dd-mm-jjjj):
   - ☒ ontvangen door DEC: 11-08-2016
   - ☐ aanvraag compleet:
   - ☒ in vergadering besproken: 24-08-2016 en 21-09-2016
   - ☐ anderszins behandeld:
   - ☒ termijnonderbreking(en) van / tot : 29-08-2016/18-09-2016
   - ☐ besluit van CCD tot verlenging van de totale adviestermijn met max. 15 werkdagen:
   - ☐ aanpassing aanvraag:
   - ☒ advies aan CCD: 05-10-2016

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

8. Eventueel horen van aanvrager
   - Datum:
   - Plaats:
   - Aantal aanwezige DEC-leden:
   - Aanwezige (namens) aanvrager:
   - Gestelde vragen en verstrekte antwoorden:
   - Het horen van de aanvrager heeft geleid tot aanpassing van de aanvraag.

9. Correspondentie met de aanvrager
   - Datum vragen: 29-08-2016
   - Datum antwoord: 18-09-2016
   - Gestelde vragen en antwoorden:

Projectvoorstel

- 3.2 Doel: De DEC zou graag iets meer informatie zien over het onderzoek naar microplastics en de relevantie hiervan. Graag toevoegen.
  *In het projectvoorstel is meer informatie toegevoegd over de relevantie van microplastics.*

Bijlage 1

- Experimentele aanpak en primaire uitkomstparameters, interventions: De DEC verzoekt u de modellen en de interventies -met name punt c, d en e- gedetailleerder te beschrijven. Wanneer kiest u voor welk model en waarom gebruikt u het auto-immuunmodel? De bijlage is nu te algemeen beschreven, waardoor het voor de DEC nu niet mogelijk om een oordeel te geven over de haalbaarheid van de studie en derhalve een advies te geven. Graag aanpassen.
  *Het auto-immuunmodel en de punten c, d en e staan niet beschreven in de juiste versie van de appendix. Aangezien we de neutrofielen in de verschillende modellen willen vergelijken is er geen sprake van een keuze tussen de modellen. Waarom de keuze voor deze modellen (7) zijn gemaakt hebben we nu beter beschreven in blauwe tekst. Tevens was de rationale van de interventies (5) al beter onderbouwd in de juiste versie na tips van de IVD. Om de haalbaarheid van de studie beter te kunnen beoordelen hebben we ook een aantal voorbeelden gegeven hoe de inflammatoire modellen en interventies kunnen leiden tot de beoogde milestones genoemd in ons projectvoorstel (groene tekst).*
- B. De dieren: U spreekt over geselecteerde condities en organen, maar beschrijft niet hoe deze geselecteerd worden. Graag alsnog doen.
  *In de bijlage is bij sectie 2A (eerste alinea) beter beschreven hoe de condities en organen geselecteerd worden.*
- B. De dieren: De berekening van het aantal dieren is voor de DEC niet navolgbaar. De DEC verzoekt u dit explicieter uit te schrijven.
  *Dit is gewijzigd in de aanvraag.*

- De antwoorden hebben geleid tot aanpassing van de aanvraag.

10. Eventuele adviezen door experts (niet lid van de DEC)
    - Aard expertise:
    - Deskundigheid expert:
    - Datum verzoek:
    - Strekking van het verzoek:
    - Datum expert advies:
    - Advies expert:

**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.

4. Er zijn geen DEC-leden betrokken bij het betreffende project.

**C. Beoordeling (inhoud):**

1. De aanvraag is toetsbaar en heeft voldoende samenhang. Het project richt zich op het verkrijgen van meer fundamentele kennis over de oorsprong, timing, lokalisatie en functie van neutrofielen bij inflammatoire aandoeningen. Neutrofielen zijn de meest voorkomende afweercellen in het bloed en daarmee belangrijke bestrijders van ziekteverwekkers. Het komt echter voor dat neutrofielen te heftig reageren op een ontsteking waardoor gezond weefsel beschadigd wordt. Het is daarom van belang dat neutrofielen geremd worden wanneer dit het geval is. Recentelijk zijn er twee nieuwe subtypen neutrofielen ontdekt. Eén subtype kan bacteriën beter doden dan 'gewone' in het bloed voorkomende neutrofielen en één subtype kan het afweersysteem juist onderdrukken. Onbekend is echter wanneer en waarom deze neutrofielen opgeroepen worden, waar ze vandaan komen en hoe lang ze leven. Meer kennis over deze subtypen neutrofielen kan in de toekomst ten gunste komen van therapieën voor ontstekingsgerelateerde ziekten, kanker, allergie, cardiovasculaire aandoeningen en bepaalde auto-immuumziekten. Daarnaast wordt ingezoomd op het effect van microplastics op neutrofielen. Microplastics komen voor in de gehele voedselketen en belanden zo in het menselijk lichaam. Neutrofielen komen de microplastics als eerste tegen in het menselijk lichaam, maar onbekend is of microplastics van invloed zijn op de neutrofielen en de gezondheid van de mens. Het geformuleerde doel is opgedeeld in 8 onderzoeksvragen en 11 'mijlpalen/subdoelen'. De strategie is erop gericht om ex vivo humane experimenten te combineren met in en ex vivo experimenten uitgevoerd in muizen. De uitleesparameters zijn helder en de werkwijze voor het verkrijgen van deze parameters zijn hetzelfde, maar de inflammatoire stimulus zal per experiment verschillen. Op deze manier kunnen de oorsprong, timing, lokalisatie en functie van neutrofielen tussen de verschillende inflammatoire aandoeningen vergeleken worden. De relatie tussen het hoofddoel en de subdoelen is daarmee helder en vergelijkbaar met voorbeeld 4B uit de 'Handreiking Invulling Definitie Project'.

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

3. De in de aanvraag aangekruiste doelcategorie sluit aan bij de hoofddoelstelling.

*Belangen en waarden*

4. Het directe doel van het project is het verkrijgen van meer fundamentele kennis over de oorsprong, timing, lokalisatie en functie van neutrofielen bij inflammatoire aandoeningen. Naast effecten van bacteriële componenten op neutrofielen zomen de onderzoekers ook in op microplastics. Het uiteindelijke doel van het project is het verbeteren van therapieën bij ontstekingsgerelateerde ziekten. Om een juiste therapie te ontwikkelen of te verbeteren is het van belang de werking van het afweersysteem zo goed mogelijk te doorgronden en te begrijpen. In dit project betreft het fundamenteel onderzoek naar neutrofielen, de meest

voorkomende afweercellen in het bloed, die een cruciale rol spelen bij inflammatoire aandoeningen. De DEC is daarom van mening dat er in voldoende mate een relatie is tussen het directe doel en het uiteindelijke doel.

5. De belangrijkste belanghebbenden in dit fundamentele onderzoeksproject naar het verkrijgen van meer kennis over neutrofielen zijn: de proefdieren, de patiënten met inflammatoire aandoeningen, de volksgezondheid, en onderzoekers. De morele waarden die voor de proefdieren in het geding zijn: gezondheid, stress, natuurlijk gedrag, intrinsieke waarde en integriteit. De morele waarden die voor patiënten met inflammatoire aandoeningen worden bevorderd zijn: kwaliteit van leven en de beschikbaarheid van een juiste therapie. De morele waarden die voor de volksgezondheid bevorderd worden: meer duidelijkheid over het eventuele gevaar van microplastics voor de gezondheid en bevordering van de algehele gezondheid/gezondere voedselketen. En tenslotte de morele waarden die voor onderzoekers worden bevorderd zijn: wetenschappelijke ontwikkelingen.

6. Er is geen sprake van substantiële milieueffecten.

*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 DEC is van mening dat het projectvoorstel aansluit bij recente inzichten en dat het geen belangrijke hiaten bevat die de bruikbaarheid van de resultaten beperken. De onderzoeksgroep heeft in de afgelopen jaren veel expertise opgebouwd op het gebied van in vivo beeldvormingstechnieken. Daarnaast werkt de groep samen met andere onderzoeksgroepen, deskundigen binnen het TA-COAST consortium en de kliniek. Tevens is de onderzoeksgroep opgenomen in het Laboratory for Translational Immunology, waardoor de groep kan beschikken over high-end technieken. Dit alles draagt bij aan de haalbaarheid van het project.

8. Het project is goed opgezet, de voorgestelde experimentele opzet en uitkomstparameters sluiten logisch en helder aan bij de aangegeven doelstellingen en de gekozen strategie en experimentele aanpak kan leiden tot het behalen van de doelstelling binnen het kader van het project. In dit project zullen humane in vitro studies gecombineerd worden met in vivo en ex vivo muizenstudies. Om de subdoelen te behalen zullen acute en chronische inflammatie modellen gebruikt worden. Deze modellen zijn helder uiteengezet, waarbij per model is aangegeven: de wijze waarop de inflammatie wordt aangebracht, de read out parameter, de experimentele groepen het aantal benodigde dieren, het te verwachten ongerief, de duur, etc. En ook de mogelijke interventies, die nodig zijn om de rol van de afzonderlijke componenten van de inflammatie te lokaliseren, zijn helder weergegeven.

*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 richtlijn)
   - ☐ Zwerfdieren (10h)
   - ☐ Hergebruik (1e lid 2)
   - ☐ Locatie: buit instelling vergunninghouder (10g)
   - ☐ Geen toepassing verdoving/pijnbestrijding (13)
   - ☐ Dodingsmethode niet volgens bijlage IV richtlijn (13c lid 3)

10. De dieren worden niet gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van de richtlijn. De muizen met een *imaging window* worden individueel gehuisvest om te voorkomen dat andere muizen bijten of de *imaging window* beschadigen. Met het oog op toenemend ongerief als gevolg van bijten en 'verspilling' van dieren als gevolg van een beschadigde *imaging window* is de DEC het eens met de solitaire huisvesting.

11. Het cumulatieve ongerief als gevolg van de dierproeven is realistisch ingeschat en geclassificeerd. Het ongerief is afhankelijk van het te gebruiken diermodel, de uitleesparameter en de interventie. Het cumulatieve ongerief wordt door de onderzoeker voor ca. 10% als licht ingeschat en voor ca. 90% als matig.

12. De integriteit van de dieren wordt fysiek, mentaal en gedragsmatig aangetast. Door het toebrengen van een ontsteking zullen de dieren pijn, stress en/of ziekte ervaren (fysieke aantasting) en bij een aantal dieren wordt onder anesthesie (mentale aantasting) een *imaging window* aangebracht (fysieke aantasting). De dieren met een *imaging window* worden bovendien solitair gehuisvest. Hierdoor wordt de dieren de mogelijkheid ontnomen op bepaalde aspecten van hun natuurlijk gedrag uit te oefenen (gedragsmatige aantasting).

13. De humane eindpunten is voor de bijlage dierproeven goed gedefinieerd en het percentage dieren dat naar verwachting een humaan eindpunt bereikt is goed ingeschat. De experimenten zijn zodanig opgezet dat verwacht wordt dat het aantal dieren dat een humaan eindpunt bereikt tot een minimum beperkt zal blijven. Alle benodigde gegevens zullen verkregen worden voordat de dieren met tumor, een infectie of een allergie meer ongerief ondervinden. Hiertoe zullen alle dieren nauwkeurig gemonitord worden. Naast de model-specifieke parameters wordt een beoordelingsschema gebruikt om op basis van de klinische kenmerken de mate van ziekte te

scoren. De DEC is derhalve van mening dat de onderzoeker tijdig kan ingrijpen indien onverwacht toch een humaan eindpunt bereikt wordt.

*3V's*

14. De aanvrager heeft voldoende aannemelijk gemaakt dat er geen geschikte vervangingsalternatieven zijn. Voorafgaand aan het onderzoek zal in vitro onderzoek met humane cellen plaatsvinden. Het is echter niet mogelijk om met alleen in vitro onderzoek de doelstellingen van het project te behalen. Het immuunsysteem is zeer complex en veelzijdig: het beenberg, de milt, de endotheelwand en het ontstoken weefsel zijn allemaal van invloed op het immuunsysteem. Los van elkaar kan het effect in vitro onderzocht worden, maar het is niet mogelijk om de interactie te bestuderen. Hiervoor zijn diermodellen noodzakelijk. Afhankelijk van de uitkomsten van de in vitro experimenten wordt bepaald welke dierproeven zullen 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 zo min mogelijk dieren wordt gewerkt waarmee een betrouwbaar resultaat kan worden verkregen. Met behulp van gegevens uit eerder uitgevoerd vergelijkbaar onderzoek en op basis van de literatuur is bepaald welke groepsgrootte nodig is om statistisch significante verschillen tussen groepen te kunnen detecteren. Het onderzoek wordt blind en gerandomiseerd uitgevoerd om bias zo goed mogelijk te voorkomen. Bij een aantal dieren wordt een *imaging window* aangebracht, waardoor het dier meerdere keren ge-imaged kan worden op verschillende tijdspunten. Dit heeft als voordeel dat niet voor elk tijdspunt nieuwe muizen nodig zijn en dat de variatie tussen de verschillende metingen kleiner is. Daardoor wordt het aantal benodigde muizen sterk gereduceerd.' De experimenten worden sequentieel uitgevoerd met heldere go/no go momenten. Deze go/no go momenten zorgen ervoor dat het minimum aantal dieren per groep gebruikt zal worden.

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 dieren worden goed gemonitord om ongerief tijdig te kunnen vaststellen en onnodig lijden te voorkomen. De experimenten worden uitgevoerd door goed getrainde biomedici/onderzoekers en er wordt adequate pre- en post operatieve pijnstilling toegepast. De DEC is van mening dat de aanvrager al het mogelijke heeft gedaan om het mogelijke ongerief te identificeren, te verminderen en waar mogelijk te voorkomen.

17. Er is geen sprake van wettelijk vereist onderzoek.

*Dieren in voorraad gedood en bestemming dieren na afloop proef*

18. In principe zullen dieren van beide geslachten in gelijke mate worden ingezet. De immuunrespons bij mannen en vrouwen kan echter sterkt verschillen. Om een bias te voorkomen zullen vooraf pilots worden uitgevoerd om te zien of dit ook in de toe te passen

modellen in muizen het geval is. Indien er te sterke verschillen optreden gaat de voorkeur uit naar het gebruik van één sekse, zodat betrouwbare vergelijkingen kunnen worden gemaakt tussen experimentele groepen. Daarnaast worden voor het spontane borstkanker model, logischerwijs, alleen vrouwelijke dieren ingezet.

19. De dieren worden in het kader van het project gedood, omdat de doelstelling van het project alleen behaald kan worden met behulp van uitgebreide analyses van de organen en cellen. De dieren worden volgens een, bijlage IV van de richtlijn, passende methode gedood.

20. Omdat in het projectvoorstel muizen worden aangevraagd is de vraag over herplaatsing/hergebruik niet van toepassing.

*NTS*

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

**D. Ethische afweging**

1. De centrale morele vraag luidt: rechtvaardigt het directe doel van het project (het verkrijgen van meer fundamentele kennis over de oorsprong, timing, lokalisatie en functie van neutrofielen bij inflammatoire aandoeningen) en het uiteindelijke doel (het verbeteren van therapieën voor ontstekingsgerelateerde ziekten, kanker, allergie en cardiovasculaire aandoeningen en bepaalde auto-immuumziekten), gezien de hoge waarschijnlijkheid dat de directe doelstellingen behaald worden, het lichte tot matige ongerief dat de dieren wordt aangedaan in het voorliggende project?

2. Waarden die voor proefdieren in het geding zijn: matig nadeel. Waarden die voor de samenleving wordt bevorderd: matig voordeel. Waarden die voor patiënten worden bevorderd: matig voordeel bij het directe doel, maar veel voordeel bij het uiteindelijke doel. Waarden die voor de onderzoekers worden bevorderd: gering voordeel. De DEC is van mening dat de belangen van de patiënten en de volksgezondheid in dit project zwaarder wegen dan de belangen/waarden van de proefdieren. Het feit dat de waarden voor de onderzoekers door dit project worden bevorderd speelde voor de DEC bij het maken van de ethische afweging geen rol van betekenis. Indien de hierboven genoemde doelstellingen behaald worden, dan zal dit project bijdragen aan de kennis omtrent de rol en het gedrag van neutrofielen bij inflammatoire aandoeningen en het effect van microplastics op neutrofielen. De patiënt zou erbij gebaat zijn wanneer met behulp van deze kennis nieuwe/betere therapieën ontwikkeld kunnen worden. De volksgezondheid zou erbij gebaat zijn als duidelijk wordt of microplastics in onze voedselketen effect hebben op de neutrofielen. Als blijkt dat deze een schadelijk effect hebben, dan nemen de bevorderende waarden toe naar 'veel voordeel'. Het is aannemelijk dat de doelstellingen

behaald zullen worden. Daarvoor is de inzet van proefdieren noodzakelijk, maar de onderzoekers doen er alles aan om het ongerief voor de dieren tot een minimum te beperken.

3. De DEC is overtuigd van het belang van de doelstellingen: het verkrijgen van meer fundamentele kennis over de oorsprong, timing, lokalisatie en functie van neutrofielen bij inflammatoire aandoeningen, met als uiteindelijke doel het verbeteren van therapieën voor ontstekingsgerelateerde ziekten.
   De DEC is van mening dat de waarden die voor de doelgroep bevorderd kunnen worden zwaarder wegen dan de waarden die voor de proefdieren in het geding zijn. De DEC is van mening dat de voorgestelde experimentele opzet en uitkomstparameters logisch en helder aansluiten bij de aangegeven doelstellingen en dat de gekozen strategie en experimentele aanpak kunnen leiden tot het behalen van de doelstellingen binnen het kader van het project. De DEC is er verder van overtuigd dat de aanvrager voldoende kennis en kunde heeft om de doelstellingen te behalen, om te kunnen voldoen aan de 3V-beginselen en om te kunnen voorkomen dat mens, dier en milieu negatieve effecten ondervinden als gevolg van de dierproeven. De DEC is ook van mening dat de aanvrager voldoende maatregelen treft om het ongerief en het aantal dieren tot een minimum te beperken. Met name het toepassen van de *imaging window* en het toepassen van go/no go momenten zijn van belang voor het verminderen van het aantal benodigde dieren. Dit alles brengt de DEC tot het oordeel dat het belang van de doelstellingen opweegt tegen het lichte tot matige ongerief dat de dieren zullen ondervinden, en dat de doelstellingen het gebruik van proefdieren rechtvaardigen.

**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.

   ☐ De DEC adviseert de vergunning niet te verlenen vanwege:

2. Het uitgebrachte advies is gebaseerd op consensus.

3. Er zijn geen knelpunten/dilemma's naar voren gekomen tijdens het beoordelen van de aanvraag en het opstellen van het advies.

Centrale Commissie Dierproeven

14.

# Format
## Niet-technische samenvatting

- Dit format gebruikt u om uw niet-technische samenvatting te schrijven
- Meer informatie over de niet-technische samenvatting vindt u op de website www.centralecommissiedierproeven.nl.
- Of neem telefonisch contact op. (0900-2800028).

## 1 Algemene gegevens

| | | |
|---|---|---|
| 1.1 | Titel van het project | Neutrofielen subtypes in gezondheid en ziekte |
| 1.2 | Looptijd van het project | 5 jaar |
| 1.3 | Trefwoorden (maximaal 5) | Neutrofiel, inflammatie, kanker, microplastics |

## 2 Categorie van het project

2.1 In welke categorie valt het project.

*U kunt meerdere mogelijkheden kiezen.*

- ☒ Fundamenteel onderzoek
- ☐ Translationeel of toegepast onderzoek
- ☐ Wettelijk vereist onderzoek of routinematige productie
- ☐ Onderzoek ter bescherming van het milieu in het belang van de gezondheid
- ☐ Onderzoek gericht op het behoud van de diersoort
- ☐ Hoger onderwijs of opleiding
- ☐ Forensisch onderzoek
- ☐ Instandhouding van kolonies van genetisch gemodificeerde dieren, niet gebruikt in andere dierproeven

## 3 Projectbeschrijving

3.1 Beschrijf de doelstellingen van het project (bv de wetenschappelijke vraagstelling of het wetenschappelijk en/of maatschappelijke belang)

Het afweersysteem is van levensbelang om ziekteverwekkers, kanker en ander niet-lichaamseigen materiaal te bedwingen. Bij een infectie moeten afweercellen (witte bloedcellen) opgeroepen en aangezet worden om ziekteverwekkers te bestrijden en zo het lichaam te beschermen. Neutrofielen zijn de meest voorkomende afweercellen in het bloed en zijn als eerste ter plekke bij de infectie of ontsteking. Maar soms schieten de neutrofielen door. Ze reageren dan te hevig op de infectie, met als gevolg dat de neutrofielen gezond weefsel beschadigen. Zo kunnen de neutrofielen zelfs meer schade aanrichten dan de ziekteverwekker zelf. Het is dus van groot belang dat neutrofielen uitgezet worden wanneer ze niet meer nodig zijn. Recentelijk zijn er twee extra subtypen neutrofielen gevonden. Eentje die beter bacteriën kunnen doden dan de 'gewone' in het bloed en een andere die de afweer juist kunnen onderdrukken. Als we begrijpen wanneer deze

| | | |
|---|---|---|
| | | neutrofielen opgeroepen worden, waar ze vandaan komen en hoe lang ze leven kunnen we dit in de toekomst gebruiken voor therapie. Dan kunnen we bijvoorbeeld de onderdrukkers sturen als de schade uit de hand dreigt te lopen of de goede bacteriedoders inzetten als er een systemische infectie (sepsis) is.<br>Ook willen we de rol van neutrofielen (subtypen) bij uitzaaiingen van kanker bestuderen. Er is namelijk een positief verband tussen het aantal neutrofielen en tumorprogressie.<br>Een andere deelvraag is hoe neutrofielen reageren op microplastics. Dit zijn microscopisch kleine stukjes plastic (kleiner dan 1 micrometer) die tegenwoordig veelvuldig in het milieu en onze voedselketen terechtkomen. Er wordt nog erg weinig onderzoek gedaan naar de invloed van microplastics op de gezondheid en neutrofielen zijn waarschijnlijk de afweercellen die deze plastics als eerste tegenkomen in ons lichaam. |
| 3.2 | Welke opbrengsten worden van dit project verwacht en hoe dragen deze bij aan het wetenschappelijke en/of maatschappelijke belang? | Met dit project willen wij het grondwerk leggen voor de ontwikkeling van nieuwe therapieën om doorgeslagen afweerreacties te dempen of juist een zwak afweersysteem te helpen bacteriën te overwinnen. Tevens willen we bestuderen of en hoe neutrofielen tumorprogressie bewerkstelligen. Tenslotte willen we vaststellen of microplastics, die steeds meer in het milieu en onze voedselketen terechtkomen, een negatief effect hebben op de functie van de neutrofiel. |
| 3.3 | Welke diersoorten en geschatte aantallen zullen worden gebruikt? | In dit project zullen muizen als proefdier gebruikt worden.<br>Aantallen: max. 2850 muizen gedurende 5 jaar |
| 3.4 | Wat zijn bij dit project de verwachte negatieve gevolgen voor het welzijn van de proefdieren? | Kortdurend licht tot matig ongerief als gevolg van het toedienen van stoffen, bijvoorbeeld via injecties (niet door de gevolgen hiervan) en in een aantal gevallen matig ongerief door operaties. Gegeven het doel van het onderzoek krijgen de muizen tumoren, infecties, ontstekingen of microplastics toegediend om de neutrofielen in deze situaties te kunnen bestuderen. |
| 3.5 | Hoe worden de dierproeven in het project ingedeeld naar de verwachte ernst? | Een groot deel (±90%) van de proefdieren zal matig ongerief te verdragen krijgen en de rest licht ongerief |
| 3.6 | Wat is de bestemming van de dieren na afloop? | Na afloop van de experimenten worden de muizen gedood om de organen en cellen van de muizen tot in detail te kunnen bestuderen. |

## **4** Drie V's

| | | |
|---|---|---|
| 4.1 | **Vervanging**<br>Geef aan waarom het gebruik van dieren nodig is voor de beschreven doelstelling en waarom proefdiervrije alternatieven niet gebruikt kunnen worden. | Voordat we besluiten over te gaan tot proefdierstudies, doen we eerst experimenten met humane cellen uit het bloed van zowel gezonde als zieke individuen. De resultaten uit deze experimenten bepalen uiteindelijk het besluit om een dierexperiment te gaan doen. Gegevens uit klinische studies bij patiënten kunnen ook aanleiding zijn voor het uitvoeren van dierproeven. De afweerbalans in het lichaam is uitermate complex. Het samenspel tussen meerdere organen speelt hierbij een cruciale rol die we in de mens onvoldoende kunnen bestuderen omdat we de complexiteit van een menselijk lichaam nog niet in een reageerbuisje kunnen nabootsen. Ook |

| | | |
|---|---|---|
| | | kunnen we bij mensen niet zomaar van alle weefsels monsters nemen. |
| 4.2 | **Vermindering**<br>Leg uit hoe kan worden verzekerd dat een zo gering mogelijk aantal dieren wordt gebruikt. | Door een goede statistische onderbouwing gekoppeld aan jarenlange ervaring kunnen we wetenschappelijk verantwoorde studies uitvoeren met een minimum aantal muizen. Afhankelijk van de uitkomsten van de eerste experimenten wordt telkens tussentijds kritisch bekeken of de hypotheses aangepast moeten worden en de uitvoering van de experimenten nog steeds essentieel is. Uiteraard worden alleen door de Instantie voor Dierenwelzijn goedgekeurde experimenten uitgevoerd. |
| 4.3 | **Verfijning**<br>Verklaar de keuze voor de diersoort(en). Verklaar waarom de gekozen diermodel(len) de meest verfijnde zijn, gelet op de doelstellingen van het project. | In dit project maken we gebruik van muizen. We weten dat het afweersysteem van de muis lijkt op dat van de mens en dat menselijke ziektes nagebootst kunnen worden in de muis. Er is al veel informatie beschikbaar over het afweersysteem van de muis, wat ons zal helpen in ons onderzoek. Veel van de voor ons onderzoek benodigde hulpmiddelen zijn alleen beschikbaar voor muizen. |
| | Vermeld welke algemene maatregelen genomen worden om de negatieve (schadelijke) gevolgen voor het welzijn van de proefdieren zo beperkt mogelijk te houden. | Gedurende het verloop van de ziektes zullen we de dieren frequent beoordelen op welbevinden en gewicht. Bij onverwacht of overmatig ongerief zullen de muizen worden gedood. |

## **5** In te vullen door de CCD

| | |
|---|---|
| Publicatie datum | |
| Beoordeling achteraf | |
| Andere opmerkingen | |

Centrale Commissie Dierproeven

15.

# 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 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'. | 11500 |
| 1.2 | Provide the name of the licenced establishment. | UMC Utrecht |
| 1.3 | Provide the title of the project. | Neutrophil subsets in health and disease |

## 2 Categories

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

X Basic research

☐ Translational or applied research

☐ Regulatory use or routine production

☐ Research into environmental protection in the interest of human or

☐ 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.

- For legally required animal procedures, indicate which statutory or regulatory requirements apply (with respect to the intended use and market authorisation).
- For routine production, describe what will be produced and for which uses.
- For higher education or training, explain why this project is part of the educational program and describe the learning targets.

The neutrophilic granulocyte is the first line of defense against invaders such as bacteria, fungi, foreign particles and aberrant cells. As the most abundant white blood cell, it continuously circulates the blood until a signal causes it to extravasate towards the tissue. In the tissue upon phagocytosis the neutrophil will exploit the content of its granules to degrade the foreign invader or cell debris. Besides pathogens

and cell debris, we will also further zoom in on one type of foreign particles that neutrophils can encounter in the human body, microplastics. These ≤1µm plastic particles are very prevalent in our environment (eg in our drinking water and aquatic animals via erosion from plastic litter), but research into health effects in mammals is virtually non-existent.
Neutrophils have always been described as a homogeneous population of short-lived cells. However, recent publications of our research group and others challenge this view. Our group described that neutrophils have a longer lifespan than previously thought. And whereas the population in blood in homeostasis might be homogeneous, various stimuli disturbing homeostasis lead to heterogeneity in phenotype and function. Our group showed that if healthy humans receive an LPS injection, immature, banded neutrophils appear in the blood, which are much better at killing bacteria than mature neutrophils (Leliefeld *et al.*, manuscript in preparation).
Recently, neutrophils have been described to play a role in the pathogenesis of various human diseases such as cardiovascular disease, cancer, auto-immune diseases and allergy[1-4]. Linked to these conditions, several neutrophil subsets have been newly described such as low-density granulocytes, granulocytic myeloid-derived suppressor cells (G-MDSC), tumor-associated neutrophils (TAN), and hypersegmented neutrophils[5]. Although we know that homeostatic neutrophils in humans and mice behave very similar, the similarity of neutrophil subsets in humans and mice is poorly described.
In disease, neutrophils can play a beneficial but also often a detrimental role. For example, neutrophils have been described to both promote and inhibit tumor progression and metastasis[6]. They can be both pro- and anti-inflammatory[5]. At the wrong time or the wrong place, neutrophil activation and degranulation will result in overwhelming inflammation and major tissue damage. Neutrophils present in a tumor are correlated with a worse prognosis, but the mechanisms behind this correlation are unclear[6]. The ability to steer neutrophil function, activation or localization from the outside would be beneficial for many diseases. The treatment of cancer would likely benefit from targeting only the pro-tumor neutrophil subset as opposed to all neutrophils. In contrast, the treatment of asthma would greatly benefit from targeting only the pro-inflammatory neutrophil subset.
But the basic knowledge on the timing, localization or even origin of neutrophil subsets is lacking, because we cannot easily sample human tissues. We know neutrophils develop in the bone marrow, but the time they need to develop is uncertain. We know neutrophils are able to migrate to lymph nodes via lymph vessels, but the signal driving them as well as their function in the lymph node are unclear. We know different subtypes of neutrophils have different functions, but when and where the differentiation of these subsets starts to diverge is again unclear.
To answer these types of questions, we need to gain a better understanding of the complex interplay of the entities that can guide the neutrophil: most importantly the bone marrow, spleen, endothelium, inflamed tissue, tumor cells, foreign particles, other immune cells and the neutrophil itself. As well, we think it is important to understand whether neutrophil subsets observed upon inflammation are the same throughout the range of inflammatory disorders. If they have a different phenotype and/or function for different types of disorders, that would be highly relevant for designing new therapies.
Understanding how and where the different neutrophil subsets arise in inflammatory conditions and what mechanisms underlie their function is essential to determine their role in disease and to establish potential treatment options. Redirection of immune responses has an enormous societal and scientific relevance as inflammation-associated diseases (including cardiovascular disease, cancer, auto-immunity and allergy) are associated with major pathology and morbidity.
In this research project we will combine data of healthy mice, mice with acute inflammation and mice with chronic inflammation, to help us understand why and when different neutrophil subsets are developed and recruited. Even if murine neutrophil subsets do not resemble those in human, this data will together show whether the response of the neutrophil to inflammation is generic, or tailored to the stimulus. Examples of inflammatory stimuli are pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs) and tumor antigens. At the same time, we will have a better idea whether inhibiting or stimulating neutrophils in these specific conditions would be beneficial or detrimental to the disease progression.

### 3.2 Purpose

Describe the project's main objective and explain why this objective is achievable.

- If the project is focussed on one or more research objectives, which research questions should be addressed during this project?
- If the main objective is not a research objective, which specific need(s) does this project respond to?

The objective of this project is to gain more fundamental knowledge on origin, timing, localization and

function of neutrophils in inflammatory conditions. These research questions will therefore be addressed:

- Is the neutrophil population heterogeneous in morphology?
- Is the neutrophil population heterogeneous in function?
- Does the migration & distribution of neutrophils throughout the body depend on the type of inflammatory stimulus?
- Does the differentiation of neutrophils depend on the type of inflammatory stimulus or organ?
- Does the function of neutrophils depend on the type of inflammatory stimulus?
- Does the life-span of neutrophils depend on the type of inflammatory stimulus?
- Does the interaction of neutrophils differ per type of inflammatory stimulus?
- Does the phagocytosis of microplastics influence neutrophil function?

From human data, we know that heterogeneous neutrophil subsets appear in the blood upon strong immune stimulation such as LPS injection, cancer, trauma or viral infection. However, these different conditions make the subsets appear in the circulation at a different timing. Our preliminary data shows neutrophil heterogeneity is also induced in mice upon trauma (acute inflammation) or solid tumor growth (chronic inflammation).
In healthy mice, mice with acute inflammation and mice with chronic inflammation, we will analyze the phenotype and function of the neutrophils *ex vivo*, crucially supported by analysis of the kinetics of murine neutrophils *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., etc.). In the kinetics studies we will study the distribution of neutrophils 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 [7, 8].
There are several other reasons why we are confident that we can achieve our aims: Our group is embedded in the Laboratory of Translational Immunology (LTI), which is a center-of-excellence on fundamental and translational immunological research. 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. The LTI 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 TA-COAST consortium. 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[9-15]. 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.

### 3.3 Relevance

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

The enormous burden of inflammation-associated diseases (including cardiovascular disease, cancer, auto-immunity and allergy) worldwide is aggravated by lack of adequate treatment options. This is due to insufficient knowledge about the common player in virtually all inflammatory processes: the neutrophil. The burden of neutrophil-mediated pathology is high, but no specific therapy is currently available. The estimated costs to society (EU) are now above 250 billion euros annually, which will only rise in the future[16]. Recent insights show that the neutrophil can be both pro- and anti-inflammatory[5]. Inhibition of pro-inflammatory neutrophils and activation of anti-inflammatory neutrophils will be beneficial for patients in pro-inflammatory states, whereas patients with a suppressed immune system such as in cancer or after acute severe inflammation may profit from a reverse approach. The lack of knowledge regarding this emerging concept has to date precluded the development and translation of the manipulation of neutrophils into a clinical application. Successful manipulation of the different neutrophil subsets will be widely applicable to a range of different inflammatory diseases.
Pollution of the water environment with microplastics by erosion of plastic litter is a pressing problem that has received a lot of attention in the last few years. It has been shown that microplastics end up in

our food chain, but their effects on human health are currently understudied. To assess their effects on human health we look at neutrophils, since they are specialized in taking up foreign particles. Our preliminary human *in vitro* data shows that neutrophils are also able to engulf microplastics. These microplastics are likely not degraded by the neutrophils and might have an effect on their survival and their capacity to subsequently kill pathogens. If we demonstrate that microplastics adversely affect human health, evidence-based regulatory measures can be formulated.

### 3.4 Research strategy

3.4.1 Provide an overview of the overall design of the project (strategy).

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, tumor size).

The methods for obtaining these readout parameters will be the same for all experiments in this proposal, but the inflammatory stimulus will differ per experiment. We think the combination of several inflammatory stimuli is the strength of this project, because currently it is unknown whether neutrophils respond the same to diverse inflammatory signals (e.g. PAMPs, DAMPs, tumor antigens).
Supported by our own research on human material, pigs and mice, we have a number of hypotheses that we will start with. However, since our research is mostly fundamental and 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.

3.4.2 Provide a basic outline of the different components of the project and the type(s) of animal procedures that will be performed.

Firstly, we want to investigate whether the morphology and function of murine neutrophil subsets is similar to the subsets we described in humans ([17], Tak *et al.* submitted, Leliefeld *et al.* manuscript in preparation). Therefore we will start with an experiment of LPS injection in mice similar to our experiments with LPS injection in humans. But to extend our findings to more physiological inflammatory stimuli, we want to use bacterial infection, viral infection, plastic particles or sterile injury. To look at stimuli specific for chronic inflammation, we want to use animals with solid tumor growth or airway allergy, two relevant diseases with a significant but opposite role for neutrophils. Homeostatic neutrophils will be analyzed *ex vivo* and *in vivo* and compared to the neutrophils arising in an inflammatory state.
As described in 3.1, neutrophil behavior is determined by a complex interplay of the bone marrow, spleen, endothelium, inflamed tissue, tumor cells, foreign particles, other immune cells and the neutrophil itself. To study this system, we want to modify these entities one by one. Therefore we will perform one or more of the following interventions in these mice:

- **A:** Administration of drugs/antibodies/inhibitors/labels
  We might be able to rescue or mimic the phenotypes of the neutrophil subsets and therefore further identify the function of these cells *in vivo*. If possible and/or relevant, we will always test these compounds first *in vitro* and in case relevant effects are observed shift to in the *in vivo* experiments. Other examples include *in vivo* antibody administration, injection of fluorescent compounds for short term labeling of blood vessels, transfer of labelled erythrocytes to long-term label blood vessels, injection of propidium iodide to monitor cell death, injection of Hoechst to stain nuclei, administration of compounds which incorporate into DNA to measure cell life span and proliferation such as deuterated water, deuterated glucose, EdU or BrdU.
- **B:** Adoptive transfer
  To study human cells *in vivo* or to, for instance, compare wildtype neutrophils with neutrophils that have a mutation we need to adoptively transfer donor neutrophils into recipient mice. Irradiation of recipient mice to deplete their endogenous immune system might be required. Also (fluorescently) labeled cells might be transferred for tracking purposes.
- **C:** Bone marrow scaffold
  To study neutrophil progenitors and differentiation. Intravital imaging of the bone marrow compartment is hard due to the solid thick nature of the surrounding bone. In a pilot study we have successfully placed an imaging window on a subcutaneous bone marrow scaffold [Groen Blood 2012] allowing the visualization of cells in the bone marrow.
- **D:** Splenectomy
  To study neutrophil [redacted] and [redacted] in the spleen. Literature as well as our

preliminary data suggests that [redacted]. Additionally, [redacted] with [redacted], especially [redacted], may [redacted]. To study how these subtypes or cell-cell interactions affect the immune system as a whole, we are interested in applying our inflammatory stimuli in splenectomized animals.

3.4.3 Describe the coherence between the different components and the different steps of the project. If applicable, describe the milestones and selection points.

For most experiments we first consider *ex vivo* experiments (mild discomfort), before we consider intravital imaging experiments (mild to moderate discomfort). In some experiments we first consider 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 (can be up to a reduction of 20x); multiple time points can be measured in one individual, and there is no inter-mice variation. After completion of the intravital imaging experiments, cells, tissues and organs will be isolated and analyzed to reduce the number of mice required. For using the different inflammatory stimuli mentioned under 3.4.2, the experiments for optimization and set-up will be the same.
Milestones:

i. Phenotypic definition of neutrophil subsets (surface marker expression, nuclear morphology, etc.)
ii. Functional definition of neutrophil subsets (better/worse in killing, pro- or anti-inflammatory, etc.)

*Go/no-go moment: When we do not find distinct subsets in the mouse but a homogenous neutrophil population, this milestone will not be pursued:

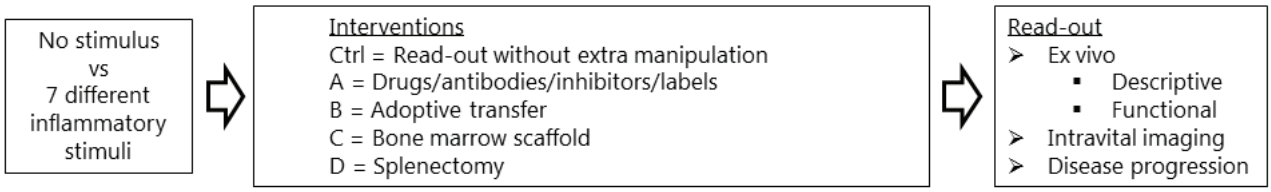
iii. To know where [redacted]

But these milestones below are independent of the go/no-go moment:

iv. To know whether phenotype and function of neutrophils is the same throughout a range of inflammatory disorders
v. To know whether the interaction between the neutrophils and the inflammatory stimulus is the same throughout a range of inflammatory disorders
vi. To know the distribution pattern of neutrophils/neutrophil subsets after leaving the bone marrow, in homeostasis vs. inflammatory conditions
vii. To know the life-span of murine neutrophils/neutrophil subsets, in homeostasis vs. inflammatory conditions
viii. To confirm whether neutrophils/neutrophil subsets continue migrating towards other organs after phagocytosis of pathogens in infected tissue
ix. To confirm whether the [redacted]
x. To know whether inhibition of neutrophils/neutrophil subsets can relieve symptoms in allergic airway disease
xi. To know whether inhibition of neutrophils/neutrophil subsets has an effect on tumor growth

These milestones can be reached simultaneously and do not depend on each other. When we do not reach the first three milestones, i.e. when we do not find distinct subsets in the mouse but a homogenous neutrophil population, then the latter milestones remain valuable for this homogenous neutrophil population.
To further clarify how different inflammatory stimuli will be combined with the interventions described in 3.4.2 to reach these milestones, we provided Scheme 1.

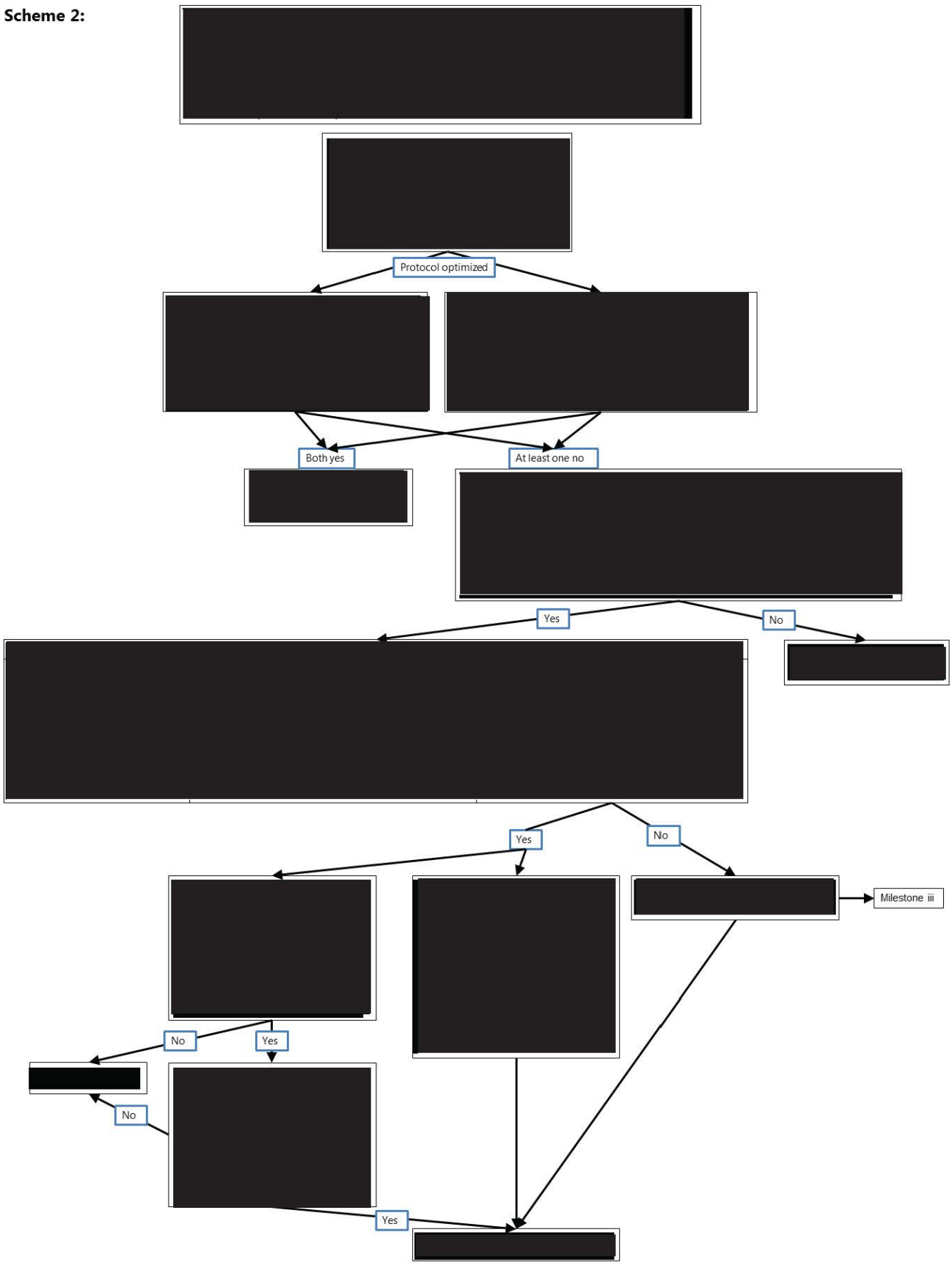
**Scheme 1:**
**Combinations of inflammatory stimuli – interventions – milestones**




| Ctrl | A | B | C | D | Milestone | Discomfort |
|---|---|---|---|---|---|---|
| √ | | | | | i-xi | Mild/Mod |
| | √ | | | | i-xi | Mild/Mod |
| | | √ | | | ii-xi | Mild/Mod |
| | | | √ | | iii-vi, viii | Moderate |
| | | | | √ | ix | Moderate |
| | √ | √ | | | ii-xi | Moderate |
| | √ | | √ | | iii-vi, viii, ix | Moderate |
| | | √ | √ | | iii-vi, viii | Moderate |
| | √ | √ | √ | | iii-vi, viii | Moderate |
| | √ | | | √ | ix | Moderate |
| | | √ | | √ | ix | Moderate |
| | √ | √ | | √ | ix | Moderate |
| | | | √ | √ | ix | Moderate |

For more technical details on the procedures, we refer to the appendices. To further clarify how typical experiments within this project would be performed, we have chosen to elaborate on the approach of milestone ix in detail. In Scheme 2 we listed the experiments that will be performed to reach this milestone.

**Scheme 2:**


Protocol optimized
Both yes
At least one no
Yes
No
Yes
No
Milestone iii
No
Yes
No
Yes

***References***
*1. 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).*
*2. Moses, K. & Brandau, S. Human neutrophils: Their role in cancer and relation to myeloid-derived suppressor cells. Semin. Immunol.* ***28****, 187-196 (2016).*
*3. Kaplan, M. J. Role of neutrophils in systemic autoimmune diseases. Arthritis Res. Ther.* ***15****, 219 (2013).*
*4. Bruijnzeel, P. L., Uddin, M. & Koenderman, L. Targeting neutrophilic inflammation in severe neutrophilic asthma: can we target the disease-relevant neutrophil phenotype? J. Leukoc. Biol.* ***98****, 549-556 (2015).*
*5. Pillay, J., Tak, T., Kamp, V. M. & Koenderman, L. Immune suppression by neutrophils and granulocytic myeloid-derived suppressor cells: similarities and differences. Cell Mol. Life Sci.* ***70****, 3813-3827 (2013).*
*6. Uribe-Querol, E. & Rosales, C. Neutrophils in Cancer: Two Sides of the Same Coin. J. Immunol. Res.* ***2015****, 983698 (2015).*
*7. Hasenberg, A. et al. Catchup: a mouse model for imaging-based tracking and modulation of neutrophil granulocytes. Nat. Methods* ***12****, 445-452 (2015).*
*8. 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).*
*9. Beerling, E., Ritsma, L., Vrisekoop, N., Derksen, P. W. & van Rheenen, J. Intravital microscopy: new insights into metastasis of tumors. J. Cell. Sci.* ***124****, 299-310 (2011).*
*10. Ritsma, L., Vrisekoop, N. & van Rheenen, J. In vivo imaging and histochemistry are combined in the cryosection labelling and intravital microscopy technique. Nat. Commun.* ***4****, 2366 (2013).*
*11. Torabi-Parizi, P. et al. Pathogen-related differences in the abundance of presented antigen are reflected in CD4+ T cell dynamic behavior and effector function in the lung. J. Immunol.* ***192****, 1651-1660 (2014).*
*12. van Golen, R. F. et al. The mechanisms and physiological relevance of glycocalyx degradation in hepatic ischemia/reperfusion injury. Antioxid. Redox Signal.* ***21****, 1098-1118 (2014).*
*13. Ritsma, L. et al. Intravital microscopy through an abdominal imaging window reveals a pre-micrometastasis stage during liver metastasis. Sci. Transl. Med.* ***4****, 158ra145 (2012).*
*14. Zomer, A. et al. Intravital imaging of cancer stem cell plasticity in mammary tumors. Stem Cells* ***31****, 602-606 (2013).*
*15. Beerling, E. et al. Plasticity between Epithelial and Mesenchymal States Unlinks EMT from Metastasis-Enhancing Stem Cell Capacity. Cell. Rep.* ***14****, 2281-2288 (2016).*
*16. Gibson, G. J., Loddenkemper, R., Lundback, B. & Sibille, Y. Respiratory health and disease in Europe: the new European Lung White Book. Eur. Respir. J. 42, 559-563 (2013).*
*17. Pillay, J. et al. A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. J. Clin. Invest. 122, 327-336 (2012).*

3.4.4 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 | Intervention A: Compound administration |
| 2 | Intervention B: Adoptive transfer |
| 3 | Intervention C: Bone marrow scaffold |
| 4 | Intervention D: Splenectomy |
| 5 | |
| 6 | |
| 7 | |
| 8 | |
| 9 | |
| 10 | |

Centrale Commissie Dierproeven

16.

# 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, see 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'. | 11500 |
| 1.2 | Provide the name of the licenced establishment. | UMC Utrecht |
| 1.3 | List the serial number and type of animal procedure.<br><br>*Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.* | Serial number: 1<br>Type of animal procedure: Compound administration |

## 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.

The objective of this project is to gain more fundamental knowledge on origin, timing, localization and function of neutrophils in diverse inflammatory conditions. To achieve this, we need to administer compounds to

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

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, tumor size).

The methods for obtaining these readout parameters will be the same for all experiments, but the inflammatory stimulus will differ per experiment. The purpose of our experiments is to compare the neutrophil populations between all the different inflammatory conditions.

*Ex vivo analysis:*
In order to study the origin and kinetics of the neutrophil populations we need to investigate the different relevant organs. Neutrophils develop in the bone marrow and occasionally the spleen and subsequently migrate through the blood to the inflammatory site. In addition, neutrophils are thought to reside in the lungs and liver (marginated pool). All these organs are therefore relevant for our studies. Whenever we will perform animal experiments we will therefore harvest multiple organs for our analysis.
After the different interventions, 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 (e.g. survival assay, bacterial killing capacity, T cell suppression capacity).
This *ex vivo* analysis will allow us to link surface markers between human and mouse neutrophils. Surface marker expression and nuclear morphology allow us to distinguish different neutrophil subsets in mice, in multiple organs. We can see whether the different inflammatory conditions recruit the subsets with similar dynamics. Lastly, using functional *in vitro* assays we can investigate whether the subsets differ in their capacity e.g. to kill microbes and suppress T cell proliferation like we found in humans.

*Intravital imaging:*
Two different strategies will be used for the imaging experiments: (1) Imaging in an acute experiment under anesthesia and (2) implanting an imaging window (such as on the breast, skin, abdomen and skull) and repeatedly image through that window. 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 intracutaneous 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. 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.
*In vivo* imaging will allow us to study 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 timepoints, greatly reducing the amount of animals needed. Additionally, this provides paired data, introducing less variation and therefore fewer animals 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.

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

Below the experimental approach per inflammatory stimulus and the intervention will be described. The choices about mice strains and numbers are further explained in B.

1. 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.
*Description:* Mice receive a single bolus of E. coli lipopolysaccharide intravenously (i.v.).
*Mice:* wildtype or intravital imaging strains
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours-days
*Level of discomfort:* mild or moderate

2. Inflammatory stimulus: Bacterial infection

*Justification:* Bacterial infection is a more complex, but more physiological relevant model for acute inflammation with which we have a lot of experience in vitro in humans.
*Description:* Mice are infected with (fluorescent) staphylococci via intradermal or intraperitoneal injection as described before[1, 2].
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.

*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – 2 weeks
*Level of discomfort:* moderate

3. Inflammatory stimulus: Viral infection

*Justification:* Viral infection is a complex physiological relevant model for inflammation with which we have some experience in vitro in humans.
*Description:* Mice are infected with influenza virus or rhinovirus 16 via intranasal administration, as performed before (Suzanne Bal, personal communication).
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – 8 weeks
*Level of discomfort:* moderate

4. Inflammatory stimulus: Plastic particles

*Justification:* The effects of microplastics on human health are currently understudied. We have found neutrophils can engulf microplastics and now want to investigate their effect on neutrophil survival and function.
*Description:* Microplastics (plastic particles ≤ 1 μm) are administered via subcutaneous injection, via addition to the drinking water, or via i.v. injection to mimic exposure routes in humans.
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – one year
*Level of discomfort:* mild or moderate

5. Inflammatory stimulus: Sterile injury

*Justification:* Sterile injury is known to recruit neutrophils without a pathogenic stimulus. Therefore this can act as a control for pathogen infection or as a model for damage-associated molecular pattern (DAMP) release.
*Description:* Under anesthesia, sterile injury is inflicted in the skin by needle insertion or laser damage
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours - days

*Level of discomfort:* mild or moderate

6. Inflammatory stimulus: Airway allergy

*Justification:* Allergy is a complex physiological relevant model for chronic inflammation with which we have a lot of experience in humans.
*Description:* Mice are sensitized and challenged with house dust mite by intranasal administration, as performed before (Suzanne Bal, personal communication).
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* 2 – 8 weeks
*Level of discomfort:* mild or moderate

7. Inflammatory stimulus: Solid tumor growth

*Justification:* Cancer is a complex physiological relevant model for chronic inflammation. The suppressive neutrophil has been described in this model.
*Description:* We use genetic mouse models for breast or colorectal cancer, or transfer tumor cells/pieces from these mouse models to healthy mice of the same background to increase reproducibility or for intravital imaging.
*Mice:* Spontaneous tumor models or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – months
*Level of discomfort:* mild or moderate

These different inflammatory stimuli will be combined with the intervention described here and/or with interventions B/C/D, as described in the project proposal.

Intervention A: Administration of drugs/antibodies/inhibitors/labels

*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).
*Rationale:*

- 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* to help achieve milestones i-iv, vi, viii and ix.
- Measure cell life span and proliferation
  E.g. by deuterium incorporation into DNA of proliferating cells we can determine the lifespan of neutrophils in homeostasis vs inflammatory conditions as described in milestone iii, vii and ix.
- Inhibit, stimulate, deplete or mimic components of the inflammatory reaction.
  E.g. by deleting neutrophils with anti-Ly6G antibodies we can delineate the role neutrophils play in disease progression or behavior of other cell types to accomplish milestone x and xi. The specific nature of these compounds will follow from our experimental results in the next five years.

*Extra experimental groups:* treated vs. not treated.
*Number of mice per group:* as described per inflammatory stimulus.

*Duration of intervention:* seconds – months, possibly repeatedly
*Level of discomfort:* mild or moderate

The final level of discomfort depends on the inflammatory stimulus, whether the intervention is used in combination with other interventions and the choice of the readout parameter. Harvesting of organs for *ex vivo* analysis or single session intravital imaging will result only in mild discomfort, but repeated intravital imaging will probably result in moderate discomfort.

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.

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. 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 (most of the animal procedures described in this appendix): 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, estimated numbers, and life stages. Provide justifications for these choices.

Since for our experiments the different interventions described in the different appendices will be combined, it is difficult to pinpoint the estimated numbers per intervention. Therefore here we describe the grand total of estimated numbers for our whole project.

Species: Mus musculus
Origin: Common breeding facility (Gemeenschappelijk Dierenlaboratorium) at Utrecht University/ Hubrecht institute/external licensed breeders
Estimated numbers: max. 3000
Life stages: adults

<u>Species</u>
All animal studies in this project will exclusively involve mice. Up to today, there are no alternative methods to fully understand neutrophil development, homeostasis and response to inflammation in the context of the whole organism. For the proposed studies, the mouse is the most appropriate animal model because: (1) physiology is more extensively characterized than other animal models; (2) a large number of relevant transgenic and knock out lines is already available; (3) a large number of relevant antibodies and other compounds is already available; (4) our research shows murine neutrophils are representative for human neutrophils; (5) many procedures for intravital imaging have already been described. The zebrafish is also an animal widely used for intravital imaging, but the differentiation of zebrafish neutrophils is much less similar to human neutrophils. Since mice are mammals too, they are closer to the human.
Mice strains used are amongst others:

- Wildtype BL/6
- Wildtype Balb/c
- Wildtype FVB
- LysM-GFP mice; for visualization of fluorescent neutrophils in intravital imaging. We are already working with this strain.
- Catchup$^{IVM}$ mice (Ly6G-Cre + Rosa-Td-Tomato); for visualization of fluorescent neutrophils in intravital imaging and for conditional knockouts in neutrophils[5]. This strain was very recently created, we want to import this strain. If the results will be superior to LysM-GFP mice, we will switch to this strain.
- MRP/S100A8-Tg-EGFP-Cre; for conditional knockouts in neutrophil progenitors[6]. We want to

import this strain. As opposed to the Catchup$^{IVM}$ mice, Cre recombinase is expressed in granulocyte-macrophage progenitors in the bone marrow.
- Knockout mice (e.g. CD11b-knockout mice); to investigate the role of a specific gene or protein
- Tumor models such as MMTV-PyMT, MMTV-Wnt1 or C26 colorectal cancer mice. In our currently running animal experiments we are comparing different tumor models with each other, to optimize for future experiments. Therefore we have not completely decided which tumor models we will use in the next five years, but it will be a model that is similar in execution and discomfort as the here mentioned examples.
- Humanized mice[7] or immunocompromised mice (e.g. NSG mice); to investigate the behavior of human neutrophils in an *in vivo*-like situation.

If the required genetically modified mouse is not available, we will obtain this model by generating, importing or by crossing existing models. Other strains will be used when in the next five years, novel strains are described or created that are very relevant to our hypotheses.

Estimated numbers

Our experiments can be divided into:
- Descriptive analysis (5-15 animals x max 5 time points = max 75 animals)
- Functional assays (5-15 animals x max 5 time points = max 75 animals)
- Intravital imaging (5-10 animals)

This totals to 55-160 animals
However, we have 7 inflammatory stimuli, 4 interventions and no intervention per stimuli = 35 conditions.
So for the total we have to multiply the 55-160 animals with 35 = 1925-5600 animals

However, these numbers are overestimations because:
- 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
- Successful pilot studies may be included in the dataset

Therefore we do not anticipate we will need more than 2500 mice for the experiments.

Additionally, a number of ca. 500 mice is needed for:
- Maintenance of breeding
- Creation of new knockout models
- Pilot studies for validation of models/techniques
- Training of new personnel or new techniques
- Rederivation of imported strains via embryo transfer
- To compensate for unforeseen loss of animals (max 10%, e.g. due to location not suitable for imaging, problems with the window)

Mice that become surplus during breeding, will be used for pilot studies and training purposes.

Together we therefore anticipate we need a grand total of 3000 mice.

Sex

In general, we will try to use both male and female mice. However, it is clear that the immune response of males and females can greatly differ[8]. Therefore, we will perform pilot studies to ensure that the sex of the animal does not pose a bias in answering the particular research question. These pilots may prove it necessary to use only one sex within one animal experiment, to be able to reliably compare experimental groups.
One clear exception is the MMTV-PyMT breast cancer model; in this model only female mice consistently develop breast tumors.

**C. Re-use**

Will the animals be re-used?

X No, continue with question D.

☐ 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.

**D. 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:*
Immune responses are highly complex and multifaceted. Multiple compartments such as the bone marrow, spleen, endothelial wall and inflamed tissue interact. Certain aspects of the immune response can be separately simulated *in vitro*, but it is not yet possible to study the full course of infection and the elicited immune response *in vitro*. This requires animal models. Similarly, the use of experimental interventions to test their potential beneficial impact on the course of disease can only be assessed in animal models. Clearly, such experimental interventions cannot be performed in patients, while *in vitro* studies with isolated human cells do not capture the full complexity of infection. However, before turning to mice, we will first study human cells *in vitro* to ensure we only perform the most relevant experiments in mice.

*Reduction:*
Intravital imaging using imaging windows allows us to image the same mouse repeatedly over time as opposed to analyzing separate mice at different timepoints greatly reducing the amount of animals needed. Additionally, this provides paired data, introducing less variation and therefore fewer animals are needed to get statistical significant results. Statistical power calculation based on our own previous experience and literature will be used to determine the optimal number of mice for each experimental group. Moreover, experiments will be performed sequentially, based on the described go/no-go decisions, via which we ensure that we will use the minimum number of mice per group that will be informative. Whenever possible, we will perform pilot studies with the minimum number of animals possible.

*Refinement:*
Except for mice with windows, where co-housing can give problems, mice will be housed in groups in cages. Nesting opportunities will always be provided. Mice with windows will be monitored at least twice a week, mice after bacterial or viral infection will be monitored at least every other day, more if necessary, for clinical signs. If unexpectedly severe suffering is observed, mice will be euthanized.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

As described under refinement, mice welfare will be regularly monitored. Mice reaching the humane endpoints or otherwise suffering from severe discomfort will be euthanized. New personnel will be trained to recognize discomfort in mice according to current guidelines (e.g. [9]). Surgical exposure will be performed under aseptic conditions using sterile instruments. Heating devices will be used to ensure a stable body temperature of the mouse during anaesthesia. Personnel conducting the surgeries is first properly trained, starting with training on cadavers.
Surgical exposure will be performed under adequate anesthesia and analgesia. Pre- and post-procedural analgesia will be used when indicated or when pain-related behavior is observed. However, common analgesics such as opioids and non-steriodal anti-inflammatory drugs (NSAIDs) have been shown to significantly alter the immune response[10-12]. Since this is our main research topic, we might sometimes refrain from using analgesia. It may be necessary to do pilot studies to prove whether analgesics pose a bias in answering our particular research question. From literature, it seems buprenorphine may be a reasonable candidate[13].
There are no expected adverse effects on the environment.

## Repetition and duplication

**E. Repetition**

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

## Accommodation and care

**F. Accommodation and care**

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☐ No

X Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Mice with an imaging window will be housed individually to prevent other mice from biting or damaging the window.

**G. Location where the animals procedures are performed**

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

X No > Continue with question H.

☐ 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.

## Classification of discomfort/humane endpoints

**H. Pain and pain relief**

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

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

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

Surgical exposure will be performed under adequate anesthesia and analgesia. Pre- and post-procedural analgesia will be used when indicated or when pain-related behavior is observed. However, common analgesics such as opioids and non-steriodal anti-inflammatory drugs (NSAIDs) have been shown to significantly alter the immune response[10-12]. Since this is our main research topic, we might sometimes refrain from using analgesia. However, pain can also influence the immune system. Therefore, it may be necessary to do pilot studies to prove whether analgesics pose a bias in answering our particular research question.

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

Surgical exposure will be performed under aseptic conditions using sterile instruments. Heating devices will be used to ensure a stable body temperature of the mouse during anaesthesia. Personnel conducting the surgeries is first properly trained, starting with training on cadavers. Before, during and after surgery adequate anesthesia and analgesia (e.g. isoflurane and buprenorphine) will be used, according to experience as well as published protocols. New personnel will be trained to recognize discomfort in mice according to current guidelines (e.g. [9]).

**I. Other aspects compromising the welfare of the animals**

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

Other than in terminally anaesthetized animals dosing and sampling procedures will consist of a combination of volumes, routes and frequencies that will result in no more than moderate discomfort. Animals bearing tumors will never reach end-stage clinical effects. All relevant scientific data can and will be gathered *before* the cancer, infection or allergy studies reach a level of severe discomfort. If unexpectedly severe suffering is observed, mice will be euthanized.
It is expected that no animals (0%) will be experiencing more than mild discomfort due to the genetic modification. However, it is impossible to predict the nature or severity of any potential genetic defect.

So, in all cases, animals will be careful monitored for possible side effects. Animals exhibiting any unexpected harmful phenotypes will be killed within a day and analyzed when possible.
Animals may lose their window (<5%). In the vast majority of these animals (>95%), we notice signs of detachment of the window far before it becomes lose, and the mice will be sacrificed. In the remaining animals, the window loss will be noticed within 24hrs and the mouse will be immediately sacrificed.

Explain why these effects may emerge.

Tumor development, inflicted inflammation, genetic alterations and administration of compounds/ drugs/ chemicals/ toxins.
It is unclear why some animals may lose their windows.

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

Mice with windows will be monitored at least twice a week, mice after bacterial or viral infection will be monitored at least every other day, more if necessary, for clinical signs. If unexpectedly severe suffering is observed, mice will be euthanized.

**J. 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 K.

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

As described in D, F, H and I, the experiments will be set-up in such a way that the incidence of reaching the humane end-point is kept to a minimum.
Weight loss (>20% in comparison with control or original weight or 15% weight loss in two days), reaching a score of 4 on the illness scale (specified below), severe shortness of breath, or loss of window is set as an humane end point. For all tumor models the guidelines of the Code of Practice Animals in Cancer research will strictly be followed, taking into account e.g. the size, location and ulceration of the tumor(s).

Next to model-specific parameters, 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

Indicate the likely incidence.

Expected <5% and <1day.

**K. Classification of severity of procedures**

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

The cumulative level of discomfort is dependent on the intervention, the read-out and the animal model. The discomfort of these three range from mild to moderate. Therefore combinations of these result in moderate discomfort for the majority (~90%) of animals.

**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. Groen, R. W. *et al*. Reconstructing the human hematopoietic niche in immunodeficient mice: opportunities for studying primary multiple myeloma. *Blood* **120**, e9-e16 (2012).
4. Levy, L. *et al*. Splenectomy inhibits non-small cell lung cancer growth by modulating anti-tumor adaptive and innate immune response. *Oncoimmunology* **4**, e998469 (2015).
5. Hasenberg, A. *et al*. Catchup: a mouse model for imaging-based tracking and modulation of neutrophil granulocytes. *Nat. Methods* **12**, 445-452 (2015).
6. Lagasse, E. & Weissman, I. L. Bcl-2 Inhibits Apoptosis of Neutrophils but Not their Engulfment by Macrophages. *J. Exp. Med.* **179**, 1047-1052 (1994).

7. Coughlan, A. M., Freeley, S. J. & Robson, M. G. Animal models of anti-neutrophil cytoplasmic antibody-associated vasculitis. *Clin. Exp. Immunol.* **169**, 229-237 (2012).
8. Gubbels Bupp, M. R. Sex, the aging immune system, and chronic disease. *Cell. Immunol.* **294**, 102-110 (2015).
9. Burkholder, T., Foltz, C., Karlsson, E., Linton, C. G. & Smith, J. M. Health Evaluation of Experimental Laboratory Mice. *Curr. Protoc. Mouse Biol.* **2**, 145-165 (2012).
10. Salimi, V. *et al*. Opioid receptors control viral replication in the airways. *Crit. Care Med.* **41**, 205-214 (2013).
11. Sacerdote, P. Opioids and the immune system. *Palliat. Med.* **20 Suppl 1**, s9-15 (2006).
12. Hish, G. A., Diaz, J. A., Hawley, A. E., Myers, D. D. & Lester, P. A. Effects of Analgesic Use on Inflammation and Hematology in a Murine Model of Venous Thrombosis. *J. Am. Assoc. Lab. Anim. Sci.* **53**, 485-493 (2014).
13. Mundt, S., Groettrup, M. & Basler, M. Analgesia in mice with experimental meningitis reduces pain without altering immune parameters. *ALTEX* **32**, 183-189 (2015).

## End of experiment

**L. Method of killing**

Will the animals be killed during or after the procedures?

☐ No

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

In order to obtain organs and isolated cells for *ex vivo* analysis the mice must first be killed.

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.

X Yes

Centrale Commissie Dierproeven

17.

# 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, see 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'. | 11500 |
| 1.2 | Provide the name of the licenced establishment. | UMC Utrecht |
| 1.3 | List the serial number and type of animal procedure.<br><br>*Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.* | Serial number: 2<br>Type of animal procedure: Adoptive transfer |

## 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.

The objective of this project is to gain more fundamental knowledge on origin, timing, localization and function of neutrophils in diverse inflammatory conditions. To achieve this we need to adoptively transfer cells to:

- Visualize the migratory behavior of neutrophils and different neutrophil subsets
- Investigate whether [redacted] can [redacted] or rather that [redacted]
- Determine whether different neutrophil subsets have different functions (including bacterial killing and the inhibition of immune responses)

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, tumor size).

The methods for obtaining these readout parameters will be the same for all experiments in this proposal, but the inflammatory stimulus will differ per experiment. The purpose of our experiments is to compare the neutrophil populations between all the different inflammatory conditions.

*Ex vivo analysis:*
In order to study the origin and kinetics of the neutrophil populations we need to investigate the different relevant organs. Neutrophils develop in the bone marrow and occasionally the spleen and subsequently migrate through the blood to the inflammatory site. In addition, neutrophils are thought to reside in the lungs and liver (marginated pool). All these organs are therefore relevant for our studies. Whenever we will perform animal experiments we will therefore harvest multiple organs for our analysis.

After the different interventions, 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 (e.g. survival assay, bacterial killing capacity, T cell suppression capacity).
This *ex vivo* analysis will allow us to link surface markers between human and mouse neutrophils. Surface marker expression and nuclear morphology allow us to distinguish different neutrophil subsets in mice, in multiple organs. We can see whether the different inflammatory conditions recruit the subsets with similar dynamics. Lastly, using functional *in vitro* assays we can investigate whether the subsets differ in their capacity e.g. to kill microbes and suppress T cell proliferation like we found in humans.

*Intravital imaging:*
Two different strategies will be used for the imaging experiments: (1) Imaging in an acute experiment under anesthesia and (2) implanting an imaging window (such as on the breast, skin, abdomen and skull) and repeatedly image through that window. 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 intracutaneous 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. 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.
*In vivo* imaging will allow us to study 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 timepoints, greatly reducing the amount of animals needed. Additionally, this provides paired data, introducing less variation and therefore fewer animals 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.

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

The objective of this project is to gain more fundamental knowledge on origin, timing, localization and function of neutrophils in diverse inflammatory conditions. Below the experimental approach per inflammatory stimulus and the intervention will be described. The choices about mice strains and numbers are further explained in B.

1. 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.
*Description:* Mice receive a single bolus of E. coli lipopolysaccharide intravenously (i.v.).
*Mice:* wildtype or intravital imaging strains
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours-days
*Level of discomfort:* mild or moderate

2. Inflammatory stimulus: Bacterial infection

*Justification:* Bacterial infection is a more complex, but more physiological relevant model for acute inflammation with which we have a lot of experience in vitro in humans.
*Description:* Mice are infected with (fluorescent) staphylococci via intradermal or intraperitoneal injection as described before[1, 2].
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.

Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – 2 weeks
*Level of discomfort:* moderate

3. Inflammatory stimulus: Viral infection

*Justification:* Viral infection is a complex physiological relevant model for inflammation with which we have some experience in vitro in humans.
*Description:* Mice are infected with influenza virus or rhinovirus 16 via intranasal administration, as performed before (Suzanne Bal, personal communication).
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – 8 weeks
*Level of discomfort:* moderate

4. Inflammatory stimulus: Plastic particles

*Justification:* The effects of microplastics on human health are currently understudied. We have found neutrophils can engulf microplastics and now want to investigate their effect on neutrophil survival and function.
*Description:* Microplastics (plastic particles ≤ 1 μm) are administered via subcutaneous injection, via addition to the drinking water, or via i.v. injection to mimic exposure routes in humans.
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – one year
*Level of discomfort:* mild or moderate

5. Inflammatory stimulus: Sterile injury

*Justification:* Sterile injury is known to recruit neutrophils without a pathogenic stimulus. Therefore this can act as a control for pathogen infection or as a model for damage-associated molecular pattern (DAMP) release.
*Description:* Under anesthesia, sterile injury is inflicted in the skin by needle insertion or laser damage
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.

We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours - days
*Level of discomfort:* mild or moderate

6. Inflammatory stimulus: Airway allergy

*Justification:* Allergy is a complex physiological relevant model for chronic inflammation with which we have a lot of experience in humans.
*Description:* Mice are sensitized and challenged with house dust mite by intranasal administration, as performed before (Suzanne Bal, personal communication).
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* 2 – 8 weeks
*Level of discomfort:* mild or moderate

7. Inflammatory stimulus: Solid tumor growth

*Justification:* Cancer is a complex physiological relevant model for chronic inflammation. The suppressive neutrophil has been described in this model.
*Description:* We use genetic mouse models for breast or colorectal cancer, or transfer tumor cells/pieces from these mouse models to healthy mice of the same background to increase reproducibility or for intravital imaging.
*Mice:* Spontaneous tumor models or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – months
*Level of discomfort:* mild or moderate

These different inflammatory stimuli will be combined with the intervention described here and/or with interventions A/C/D, as described in the project proposal.

Intervention B: Adoptive transfer
*Description:* a single cell suspension of donor cells is administered i.v. to recipient mice.
*Rationale:* Firstly, (fluorescently) labeled cells will be transferred for tracking migration and function throughout the body. Cells harbouring a specific gene knockout will be transferred to study the role of this gene in specific organs/diseases. Human cells will be transferred to investigate the behavior of human neutrophils in an *in vivo*-like situation.
E.g. different neutrophil subsets might be isolated from a donor mouse and adoptively transferred to a recipient mouse to visualize their difference in functionality to accomplish milestone ii or to follow their distribution to achieve milestone vi.
*Extra experimental groups:* Donor mice & recipient mice.
*Number of mice per group:* ratio donor:recipient mostly 1:1, sometimes 2:1 or 3:1
*Donor mice:* wt mice, intravital imaging strains, knockout strains.
*Recipient mice:* wt mice, intravital imaging strains, knockout strains, immunodeficient mice. Irradiation of recipient mice to deplete their endogenous immune system might be required.
*Duration of intervention:* seconds – minutes
*Level of discomfort:* mild

The final level of discomfort depends on the inflammatory stimulus, whether the intervention is used in combination with other interventions and the choice of the readout parameter. Harvesting of organs for *ex vivo* analysis or single session intravital imaging will result only in mild discomfort, but repeated intravital imaging will probably result in moderate discomfort.

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.

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. 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 (most of the animal procedures described in this appendix): 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, estimated numbers, and life stages. Provide justifications for these choices.

Since for our experiments the different interventions described in the different appendices will be combined, it is difficult to pinpoint the estimated numbers per intervention. Therefore here we describe the grand total of estimated numbers for our whole project.

Species: Mus musculus
Origin: Common breeding facility (Gemeenschappelijk Dierenlaboratorium) at Utrecht University/ Hubrecht institute/external licensed breeders
Estimated numbers: max. 3000
Life stages: adults

Species
All animal studies in this project will exclusively involve mice. Up to today, there are no alternative methods to fully understand neutrophil development, homeostasis and response to inflammation in the context of the whole organism. For the proposed studies, the mouse is the most appropriate animal model because: (1) physiology is more extensively characterized than other animal models; (2) a large number of relevant transgenic and knock out lines is already available; (3) a large number of relevant antibodies and other compounds is already available; (4) our research shows murine neutrophils are representative for human neutrophils; (5) many procedures for intravital imaging have already been described. The zebrafish is also an animal widely used for intravital imaging, but the differentiation of zebrafish neutrophils is much less similar to human neutrophils. Since mice are mammals too, they are closer to the human.
Mice strains used are amongst others:

- Wildtype BL/6
- Wildtype Balb/c
- Wildtype FVB
- LysM-GFP mice; for visualization of fluorescent neutrophils in intravital imaging. We are already working with this strain.
- Catchup$^{IVM}$ mice (Ly6G-Cre + Rosa-Td-Tomato); for visualization of fluorescent neutrophils in intravital imaging and for conditional knockouts in neutrophils[5]. This strain was very recently created, we want to import this strain. If the results will be superior to LysM-GFP mice, we will switch to this strain.
- MRP/S100A8-Tg-EGFP-Cre; for conditional knockouts in neutrophil progenitors[6]. We want to import this strain. As opposed to the Catchup$^{IVM}$ mice, Cre recombinase is expressed in granulocyte-macrophage progenitors in the bone marrow.

- Knockout mice (e.g. CD11b-knockout mice); to investigate the role of a specific gene or protein
- Tumor models such as MMTV-PyMT, MMTV-Wnt1 or C26 colorectal cancer mice. In our currently running animal experiments we are comparing different tumor models with each other, to optimize for future experiments. Therefore we have not completely decided which tumor models we will use in the next five years, but it will be a model that is similar in execution and discomfort as the here mentioned examples.
- Humanized mice[7] or immunocompromised mice (e.g. NSG mice); to investigate the behavior of human neutrophils in an *in vivo*-like situation.

If the required genetically modified mouse is not available, we will obtain this model by generating, importing or by crossing existing models. Other strains will be used when in the next five years, novel strains are described or created that are very relevant to our hypotheses.

Estimated numbers

Our experiments can be divided into:
- Descriptive analysis (5-15 animals x max 5 time points = max 75 animals)
- Functional assays (5-15 animals x max 5 time points = max 75 animals)
- Intravital imaging (5-10 animals)

This totals to 55-160 animals
However, we have 7 inflammatory stimuli, 4 interventions and no intervention per stimuli = 35 conditions.
So for the total we have to multiply the 55-160 animals with 35 = 1925-5600 animals

However, these numbers are overestimations because:
- 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
- Successful pilot studies may be included in the dataset

Therefore we do not anticipate we will need more than 2500 mice for the experiments.

Additionally, a number of ca. 500 mice is needed for:
- Maintenance of breeding
- Creation of new knockout models
- Pilot studies for validation of models/techniques
- Training of new personnel or new techniques
- Rederivation of imported strains via embryo transfer
- To compensate for unforeseen loss of animals (max 10%, e.g. due to location not suitable for imaging, problems with the window)

Mice that become surplus during breeding, will be used for pilot studies and training purposes.

Together we therefore anticipate we need a grand total of 3000 mice.

Sex

In general, we will try to use both male and female mice. However, it is clear that the immune response of males and females can greatly differ[8]. Therefore, we will perform pilot studies to ensure that the sex of the animal does not pose a bias in answering the particular research question. These pilots may prove it necessary to use only one sex within one animal experiment, to be able to reliably compare experimental groups.
One clear exception is the MMTV-PyMT breast cancer model; in this model only female mice consistently develop breast tumors.

**C. Re-use**

Will the animals be re-used?

X No, continue with question D.

☐ 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.

**D. 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:*
Immune responses are highly complex and multifaceted. Multiple compartments such as the bone marrow, spleen, endothelial wall and inflamed tissue interact. Certain aspects of the immune response can be separately simulated *in vitro*, but it is not yet possible to study the full course of infection and the elicited immune response *in vitro*. This requires animal models. Similarly, the use of experimental interventions to test their potential beneficial impact on the course of disease can only be assessed in animal models. Clearly, such experimental interventions cannot be performed in patients, while *in vitro* studies with isolated human cells do not capture the full complexity of infection. However, before turning to mice, we will first study human cells *in vitro* to ensure we only perform the most relevant experiments in mice.

*Reduction:*
Intravital imaging using imaging windows allows us to image the same mouse repeatedly over time as opposed to analyzing separate mice at different timepoints greatly reducing the amount of animals needed. Additionally, this provides paired data, introducing less variation and therefore fewer animals are needed to get statistical significant results. Statistical power calculation based on our own previous experience and literature will be used to determine the optimal number of mice for each experimental group. Moreover, experiments will be performed sequentially, based on the described go/no-go decisions, via which we ensure that we will use the minimum number of mice per group that will be informative. Whenever possible, we will perform pilot studies with the minimum number of animals possible.

*Refinement:*
Except for mice with windows, where co-housing can give problems, mice will be housed in groups in cages. Nesting opportunities will always be provided. Mice with windows will be monitored at least twice a week, mice after bacterial or viral infection will be monitored at least every other day, more if necessary, for clinical signs. If unexpectedly severe suffering is observed, mice will be euthanized.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

As described under refinement, mice welfare will be regularly monitored. Mice reaching the humane endpoints or otherwise suffering from severe discomfort will be euthanized. New personnel will be trained to recognize discomfort in mice according to current guidelines (e.g. [9]). Surgical exposure will be performed under aseptic conditions using sterile instruments. Heating devices will be used to ensure a stable body temperature of the mouse during anaesthesia. Personnel conducting the surgeries is first properly trained, starting with training on cadavers.
Surgical exposure will be performed under adequate anesthesia and analgesia. Pre- and post-procedural analgesia will be used when indicated or when pain-related behavior is observed. However, common analgesics such as opioids and non-steriodal anti-inflammatory drugs (NSAIDs) have been shown to significantly alter the immune response[10-12]. Since this is our main research topic, we might sometimes refrain from using analgesia. It may be necessary to do pilot studies to prove whether analgesics pose a bias in answering our particular research question. From literature, it seems buprenorphine may be a reasonable candidate[13].
There are no expected adverse effects on the environment.

## Repetition and duplication

**E. Repetition**

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

## Accommodation and care

**F. Accommodation and care**

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☐ No

X Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Mice with an imaging window will be housed individually to prevent other mice from biting or damaging the window.

**G. Location where the animals procedures are performed**

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

X No > Continue with question H.

☐ 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.

## Classification of discomfort/humane endpoints

**H. Pain and pain relief**

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

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

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

Surgical exposure will be performed under adequate anesthesia and analgesia. Pre- and post-procedural analgesia will be used when indicated or when pain-related behavior is observed. However, common analgesics such as opioids and non-steriodal anti-inflammatory drugs (NSAIDs) have been shown to significantly alter the immune response[10-12]. Since this is our main research topic, we might sometimes refrain from using analgesia. However, pain can also influence the immune system. Therefore, it may be necessary to do pilot studies to prove whether analgesics pose a bias in answering our particular research question.

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

Surgical exposure will be performed under aseptic conditions using sterile instruments. Heating devices will be used to ensure a stable body temperature of the mouse during anaesthesia. Personnel conducting the surgeries is first properly trained, starting with training on cadavers. Before, during and after surgery adequate anesthesia and analgesia (e.g. isoflurane and buprenorphine) will be used, according to experience as well as published protocols. New personnel will be trained to recognize discomfort in mice according to current guidelines (e.g. [9]).

**I. Other aspects compromising the welfare of the animals**

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

Other than in terminally anaesthetized animals dosing and sampling procedures will consist of a combination of volumes, routes and frequencies that will result in no more than moderate discomfort. Animals bearing tumors will never reach end-stage clinical effects. All relevant scientific data can and will be gathered *before* the cancer, infection or allergy studies reach a level of severe discomfort. If unexpectedly severe suffering is observed, mice will be euthanized.
It is expected that no animals (0%) will be experiencing more than mild discomfort due to the genetic modification. However, it is impossible to predict the nature or severity of any potential genetic defect. So, in all cases, animals will be careful monitored for possible side effects. Animals exhibiting any unexpected harmful phenotypes will be killed within a day and analyzed when possible.
Animals may lose their window (<5%). In the vast majority of these animals (>95%), we notice signs of

detachment of the window far before it becomes lose, and the mice will be sacrificed. In the remaining animals, the window loss will be noticed within 24hrs and the mouse will be immediately sacrificed.

Explain why these effects may emerge.

Tumor development, inflicted inflammation, genetic alterations and administration of compounds/ drugs/ chemicals/ toxins.
It is unclear why some animals may lose their windows.

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

Mice with windows will be monitored at least twice a week, mice after bacterial or viral infection will be monitored at least every other day, more if necessary, for clinical signs. If unexpectedly severe suffering is observed, mice will be euthanized.

**J. 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 K.

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

As described in D, F, H and I, the experiments will be set-up in such a way that the incidence of reaching the humane end-point is kept to a minimum.
Weight loss (>20% in comparison with control or original weight or 15% weight loss in two days), reaching a score of 4 on the illness scale (specified below), severe shortness of breath, or loss of window is set as an humane end point. For all tumor models the guidelines of the Code of Practice Animals in Cancer research will strictly be followed, taking into account e.g. the size, location and ulceration of the tumor(s).

Next to model-specific parameters, 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

Indicate the likely incidence.

Expected <5% and <1day.

**K. Classification of severity of procedures**

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

The cumulative level of discomfort is dependent on the intervention, the read-out and the animal model. The discomfort of these three range from mild to moderate. Therefore combinations of these result in moderate discomfort for the majority (~90%) of animals.

**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. Groen, R. W. *et al*. Reconstructing the human hematopoietic niche in immunodeficient mice: opportunities for studying primary multiple myeloma. *Blood* **120**, e9-e16 (2012).
4. Levy, L. *et al*. Splenectomy inhibits non-small cell lung cancer growth by modulating anti-tumor adaptive and innate immune response. *Oncoimmunology* **4**, e998469 (2015).
5. Hasenberg, A. *et al*. Catchup: a mouse model for imaging-based tracking and modulation of neutrophil granulocytes. *Nat. Methods* **12**, 445-452 (2015).
6. Lagasse, E. & Weissman, I. L. Bcl-2 Inhibits Apoptosis of Neutrophils but Not their Engulfment by Macrophages. *J. Exp. Med.* **179**, 1047-1052 (1994).
7. Coughlan, A. M., Freeley, S. J. & Robson, M. G. Animal models of anti-neutrophil cytoplasmic antibody-associated vasculitis. *Clin. Exp. Immunol.* **169**, 229-237 (2012).
8. Gubbels Bupp, M. R. Sex, the aging immune system, and chronic disease. *Cell. Immunol.* **294**, 102-110 (2015).
9. Burkholder, T., Foltz, C., Karlsson, E., Linton, C. G. & Smith, J. M. Health Evaluation of Experimental Laboratory

Mice. *Curr. Protoc. Mouse Biol.* **2**, 145-165 (2012).
10. Salimi, V. *et al*. Opioid receptors control viral replication in the airways. *Crit. Care Med.* **41**, 205-214 (2013).
11. Sacerdote, P. Opioids and the immune system. *Palliat. Med.* **20 Suppl 1**, s9-15 (2006).
12. Hish, G. A., Diaz, J. A., Hawley, A. E., Myers, D. D. & Lester, P. A. Effects of Analgesic Use on Inflammation and Hematology in a Murine Model of Venous Thrombosis. *J. Am. Assoc. Lab. Anim. Sci.* **53**, 485-493 (2014).
13. Mundt, S., Groettrup, M. & Basler, M. Analgesia in mice with experimental meningitis reduces pain without altering immune parameters. *ALTEX* **32**, 183-189 (2015).

## End of experiment

**L. Method of killing**

Will the animals be killed during or after the procedures?

☐ No

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

In order to obtain organs and isolated cells for *ex vivo* analysis the mice must first be killed.

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.

X Yes

Centrale Commissie Dierproeven

3.

# 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, see 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'. | 11500 |
| 1.2 | Provide the name of the licenced establishment. | UMC Utrecht |
| 1.3 | List the serial number and type of animal procedure.<br><br>*Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.* | Serial number: 3<br>Type of animal procedure: Bone marrow scaffold |

## 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.

The objective of this project is to gain more fundamental knowledge on origin, timing, localization and function of neutrophils in diverse inflammatory conditions. In order to achieve this we need to implant a bone marrow scaffold to:

- Visualize the migratory behavior of neutrophils and different neutrophil subsets in the bone marrow. More specifically, we can monitor the recruitment of young banded neutrophils to the circulation upon inflammatory stimuli and establish whether [redacted] through the [redacted] (as our own data in humans predict) or only [redacted] (as has been reported in literature).
- Investigate whether [redacted] can [redacted] or rather that [redacted]

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, tumor size).

The methods for obtaining these readout parameters will be the same for all experiments in this proposal, but the inflammatory stimulus will differ per experiment. The purpose of our experiments is to compare the neutrophil populations between all the different inflammatory conditions.

*Ex vivo analysis:*
In order to study the origin and kinetics of the neutrophil populations we need to investigate the different relevant organs. Neutrophils develop in the bone marrow and occasionally the spleen and subsequently migrate through the blood to the inflammatory site. In addition, neutrophils are thought to reside in the

lungs and liver (marginated pool). All these organs are therefore relevant for our studies. Whenever we will perform animal experiments we will therefore harvest multiple organs for our analysis.
After the different interventions, 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 (e.g. survival assay, bacterial killing capacity, T cell suppression capacity).
This *ex vivo* analysis will allow us to link surface markers between human and mouse neutrophils. Surface marker expression and nuclear morphology allow us to distinguish different neutrophil subsets in mice, in multiple organs. We can see whether the different inflammatory conditions recruit the subsets with similar dynamics. Lastly, using functional *in vitro* assays we can investigate whether the subsets differ in their capacity e.g. to kill microbes and suppress T cell proliferation like we found in humans.

*Intravital imaging:*
Two different strategies will be used for the imaging experiments: (1) Imaging in an acute experiment under anesthesia and (2) implanting an imaging window (such as on the breast, skin, abdomen and skull) and repeatedly image through that window. 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 intracutaneous 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. 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.
*In vivo* imaging will allow us to study 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 timepoints, greatly reducing the amount of animals needed. Additionally, this provides paired data, introducing less variation and therefore fewer animals 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.

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

The objective of this project is to gain more fundamental knowledge on origin, timing, localization and function of neutrophils in diverse inflammatory conditions. Below the experimental approach per inflammatory stimulus and the intervention will be described. The choices about mice strains and numbers are further explained in B.

1. 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.
*Description:* Mice receive a single bolus of E. coli lipopolysaccharide intravenously (i.v.).
*Mice:* wildtype or intravital imaging strains
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours-days
*Level of discomfort:* mild or moderate

2. Inflammatory stimulus: Bacterial infection

*Justification:* Bacterial infection is a more complex, but more physiological relevant model for acute inflammation with which we have a lot of experience in vitro in humans.
*Description:* Mice are infected with (fluorescent) staphylococci via intradermal or intraperitoneal injection as described before[1, 2].

*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – 2 weeks
*Level of discomfort:* moderate

3. Inflammatory stimulus: Viral infection

*Justification:* Viral infection is a complex physiological relevant model for inflammation with which we have some experience in vitro in humans.
*Description:* Mice are infected with influenza virus or rhinovirus 16 via intranasal administration, as performed before (Suzanne Bal, personal communication).
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – 8 weeks
*Level of discomfort:* moderate

4. Inflammatory stimulus: Plastic particles

*Justification:* The effects of microplastics on human health are currently understudied. We have found neutrophils can engulf microplastics and now want to investigate their effect on neutrophil survival and function.
*Description:* Microplastics (plastic particles ≤ 1 μm) are administered via subcutaneous injection, via addition to the drinking water, or via i.v. injection to mimic exposure routes in humans.
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – one year
*Level of discomfort:* mild or moderate

5. Inflammatory stimulus: Sterile injury

*Justification:* Sterile injury is known to recruit neutrophils without a pathogenic stimulus. Therefore this can act as a control for pathogen infection or as a model for damage-associated molecular pattern (DAMP) release.
*Description:* Under anesthesia, sterile injury is inflicted in the skin by needle insertion or laser damage
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice

We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours - days
*Level of discomfort:* mild or moderate

6. Inflammatory stimulus: Airway allergy

*Justification:* Allergy is a complex physiological relevant model for chronic inflammation with which we have a lot of experience in humans.
*Description:* Mice are sensitized and challenged with house dust mite by intranasal administration, as performed before (Suzanne Bal, personal communication).
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* 2 – 8 weeks
*Level of discomfort:* mild or moderate

7. Inflammatory stimulus: Solid tumor growth

*Justification:* Cancer is a complex physiological relevant model for chronic inflammation. The suppressive neutrophil has been described in this model.
*Description:* We use genetic mouse models for breast or colorectal cancer, or transfer tumor cells/pieces from these mouse models to healthy mice of the same background to increase reproducibility or for intravital imaging.
*Mice:* Spontaneous tumor models or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – months
*Level of discomfort:* mild or moderate

These different inflammatory stimuli will be combined with the intervention described here and/or with interventions A/B/D, as described in the project proposal.

Intervention C: Bone marrow scaffold
*Description:* Biphasic calcium phosphate particles loaded with human MSCs will be implanted subcutaneously in immunodeficient mice to create a niche for hematopoiesis.
*Rationale:* To study neutrophil progenitors and differentiation. Intravital imaging of the bone marrow compartment is hard due to the solid thick nature of the surrounding bone. In a pilot study we have successfully placed an imaging window on a subcutaneous bone marrow scaffold allowing the visualization of cells in the bone marrow[3].
E.g. we will be able to visualize neutrophils exiting (milestone vi) or re-entering (milestone viii) the bone marrow
*Extra experimental groups:* With vs. without bone marrow scaffold.
*Number of mice per group:* as described per inflammatory stimulus
*Duration of intervention:* days - months
*Level of discomfort:* moderate during surgery, mild thereafter

The final level of discomfort depends on the inflammatory stimulus, whether the intervention is used in combination with other interventions and the choice of the readout parameter. Harvesting of organs for *ex vivo* analysis or single session intravital imaging will result only in mild discomfort, but repeated intravital imaging will probably result in moderate discomfort.

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.

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. 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 (most of the animal procedures described in this appendix): 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, estimated numbers, and life stages. Provide justifications for these choices.

Since for our experiments the different interventions described in the different appendices will be combined, it is difficult to pinpoint the estimated numbers per intervention. Therefore here we describe the grand total of estimated numbers for our whole project.

Species: Mus musculus
Origin: Common breeding facility (Gemeenschappelijk Dierenlaboratorium) at Utrecht University/ Hubrecht institute/external licensed breeders
Estimated numbers: max. 3000
Life stages: adults

Species
All animal studies in this project will exclusively involve mice. Up to today, there are no alternative methods to fully understand neutrophil development, homeostasis and response to inflammation in the context of the whole organism. For the proposed studies, the mouse is the most appropriate animal model because: (1) physiology is more extensively characterized than other animal models; (2) a large number of relevant transgenic and knock out lines is already available; (3) a large number of relevant antibodies and other compounds is already available; (4) our research shows murine neutrophils are representative for human neutrophils; (5) many procedures for intravital imaging have already been described. The zebrafish is also an animal widely used for intravital imaging, but the differentiation of zebrafish neutrophils is much less similar to human neutrophils. Since mice are mammals too, they are closer to the human.
Mice strains used are amongst others:

- Wildtype BL/6
- Wildtype Balb/c
- Wildtype FVB
- LysM-GFP mice; for visualization of fluorescent neutrophils in intravital imaging. We are already working with this strain.
- Catchup$^{IVM}$ mice (Ly6G-Cre + Rosa-Td-Tomato); for visualization of fluorescent neutrophils in intravital imaging and for conditional knockouts in neutrophils[5]. This strain was very recently created, we want to import this strain. If the results will be superior to LysM-GFP mice, we will switch to this strain.
- MRP/S100A8-Tg-EGFP-Cre; for conditional knockouts in neutrophil progenitors[6]. We want to import this strain. As opposed to the Catchup$^{IVM}$ mice, Cre recombinase is expressed in granulocyte-macrophage progenitors in the bone marrow.
- Knockout mice (e.g. CD11b-knockout mice); to investigate the role of a specific gene or protein

- Tumor models such as MMTV-PyMT, MMTV-Wnt1 or C26 colorectal cancer mice. In our currently running animal experiments we are comparing different tumor models with each other, to optimize for future experiments. Therefore we have not completely decided which tumor models we will use in the next five years, but it will be a model that is similar in execution and discomfort as the here mentioned examples.
- Humanized mice[7] or immunocompromised mice (e.g. NSG mice); to investigate the behavior of human neutrophils in an *in vivo*-like situation.

If the required genetically modified mouse is not available, we will obtain this model by generating, importing or by crossing existing models. Other strains will be used when in the next five years, novel strains are described or created that are very relevant to our hypotheses.

Estimated numbers

Our experiments can be divided into:

- Descriptive analysis (5-15 animals x max 5 time points = max 75 animals)
- Functional assays (5-15 animals x max 5 time points = max 75 animals)
- Intravital imaging (5-10 animals)

This totals to 55-160 animals

However, we have 7 inflammatory stimuli, 4 interventions and no intervention per stimuli = 35 conditions.

So for the total we have to multiply the 55-160 animals with 35 = 1925-5600 animals

However, these numbers are overestimations because:

- 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
- Successful pilot studies may be included in the dataset

Therefore we do not anticipate we will need more than 2500 mice for the experiments.

Additionally, a number of ca. 500 mice is needed for:

- Maintenance of breeding
- Creation of new knockout models
- Pilot studies for validation of models/techniques
- Training of new personnel or new techniques
- Rederivation of imported strains via embryo transfer
- To compensate for unforeseen loss of animals (max 10%, e.g. due to location not suitable for imaging, problems with the window)

Mice that become surplus during breeding, will be used for pilot studies and training purposes.

Together we therefore anticipate we need a grand total of 3000 mice.

Sex

In general, we will try to use both male and female mice. However, it is clear that the immune response of males and females can greatly differ[8]. Therefore, we will perform pilot studies to ensure that the sex of the animal does not pose a bias in answering the particular research question. These pilots may prove it necessary to use only one sex within one animal experiment, to be able to reliably compare experimental groups.

One clear exception is the MMTV-PyMT breast cancer model; in this model only female mice consistently develop breast tumors.

**C. Re-use**

Will the animals be re-used?

X No, continue with question D.

☐ 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.

**D. 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:*
Immune responses are highly complex and multifaceted. Multiple compartments such as the bone marrow, spleen, endothelial wall and inflamed tissue interact. Certain aspects of the immune response can be separately simulated *in vitro*, but it is not yet possible to study the full course of infection and the elicited immune response *in vitro*. This requires animal models. Similarly, the use of experimental interventions to test their potential beneficial impact on the course of disease can only be assessed in animal models. Clearly, such experimental interventions cannot be performed in patients, while *in vitro* studies with isolated human cells do not capture the full complexity of infection. However, before turning to mice, we will first study human cells *in vitro* to ensure we only perform the most relevant experiments in mice.

*Reduction:*
Intravital imaging using imaging windows allows us to image the same mouse repeatedly over time as opposed to analyzing separate mice at different timepoints greatly reducing the amount of animals needed. Additionally, this provides paired data, introducing less variation and therefore fewer animals are needed to get statistical significant results. Statistical power calculation based on our own previous experience and literature will be used to determine the optimal number of mice for each experimental group. Moreover, experiments will be performed sequentially, based on the described go/no-go decisions, via which we ensure that we will use the minimum number of mice per group that will be informative. Whenever possible, we will perform pilot studies with the minimum number of animals possible.

*Refinement:*
Except for mice with windows, where co-housing can give problems, mice will be housed in groups in cages. Nesting opportunities will always be provided. Mice with windows will be monitored at least twice a week, mice after bacterial or viral infection will be monitored at least every other day, more if necessary, for clinical signs. If unexpectedly severe suffering is observed, mice will be euthanized.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

As described under refinement, mice welfare will be regularly monitored. Mice reaching the humane endpoints or otherwise suffering from severe discomfort will be euthanized. New personnel will be trained to recognize discomfort in mice according to current guidelines (e.g. [9]). Surgical exposure will be performed under aseptic conditions using sterile instruments. Heating devices will be used to ensure a stable body temperature of the mouse during anaesthesia. Personnel conducting the surgeries is first properly trained, starting with training on cadavers.
Surgical exposure will be performed under adequate anesthesia and analgesia. Pre- and post-procedural analgesia will be used when indicated or when pain-related behavior is observed. However, common analgesics such as opioids and non-steriodal anti-inflammatory drugs (NSAIDs) have been shown to significantly alter the immune response[10-12]. Since this is our main research topic, we might sometimes refrain from using analgesia. It may be necessary to do pilot studies to prove whether analgesics pose a bias in answering our particular research question. From literature, it seems buprenorphine may be a reasonable candidate[13].
There are no expected adverse effects on the environment.

## Repetition and duplication

**E. Repetition**

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

## Accommodation and care

**F. Accommodation and care**

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☐ No

X Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Mice with an imaging window will be housed individually to prevent other mice from biting or damaging the window.

**G. Location where the animals procedures are performed**

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

X No > Continue with question H.

☐ 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.

## Classification of discomfort/humane endpoints

**H. Pain and pain relief**

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

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

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

Surgical exposure will be performed under adequate anesthesia and analgesia. Pre- and post-procedural analgesia will be used when indicated or when pain-related behavior is observed. However, common analgesics such as opioids and non-steriodal anti-inflammatory drugs (NSAIDs) have been shown to significantly alter the immune response[10-12]. Since this is our main research topic, we might sometimes refrain from using analgesia. However, pain can also influence the immune system. Therefore, it may be necessary to do pilot studies to prove whether analgesics pose a bias in answering our particular research question.

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

Surgical exposure will be performed under aseptic conditions using sterile instruments. Heating devices will be used to ensure a stable body temperature of the mouse during anaesthesia. Personnel conducting the surgeries is first properly trained, starting with training on cadavers. Before, during and after surgery adequate anesthesia and analgesia (e.g. isoflurane and buprenorphine) will be used, according to experience as well as published protocols. New personnel will be trained to recognize discomfort in mice according to current guidelines (e.g. [9]).

**I. Other aspects compromising the welfare of the animals**

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

Other than in terminally anaesthetized animals dosing and sampling procedures will consist of a combination of volumes, routes and frequencies that will result in no more than moderate discomfort. Animals bearing tumors will never reach end-stage clinical effects. All relevant scientific data can and will be gathered *before* the cancer, infection or allergy studies reach a level of severe discomfort. If unexpectedly severe suffering is observed, mice will be euthanized.
It is expected that no animals (0%) will be experiencing more than mild discomfort due to the genetic modification. However, it is impossible to predict the nature or severity of any potential genetic defect. So, in all cases, animals will be careful monitored for possible side effects. Animals exhibiting any unexpected harmful phenotypes will be killed within a day and analyzed when possible.
Animals may lose their window (<5%). In the vast majority of these animals (>95%), we notice signs of detachment of the window far before it becomes lose, and the mice will be sacrificed. In the remaining

animals, the window loss will be noticed within 24hrs and the mouse will be immediately sacrificed.

Explain why these effects may emerge.

Tumor development, inflicted inflammation, genetic alterations and administration of compounds/ drugs/ chemicals/ toxins.
It is unclear why some animals may lose their windows.

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

Mice with windows will be monitored at least twice a week, mice after bacterial or viral infection will be monitored at least every other day, more if necessary, for clinical signs. If unexpectedly severe suffering is observed, mice will be euthanized.

**J. 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 K.

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

As described in D, F, H and I, the experiments will be set-up in such a way that the incidence of reaching the humane end-point is kept to a minimum.
Weight loss (>20% in comparison with control or original weight or 15% weight loss in two days), reaching a score of 4 on the illness scale (specified below), severe shortness of breath, or loss of window is set as an humane end point. For all tumor models the guidelines of the Code of Practice Animals in Cancer research will strictly be followed, taking into account e.g. the size, location and ulceration of the tumor(s).

Next to model-specific parameters, 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

Indicate the likely incidence.

Expected <5% and <1day.

**K. Classification of severity of procedures**

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

The cumulative level of discomfort is dependent on the intervention, the read-out and the animal model. The discomfort of these three range from mild to moderate. Therefore combinations of these result in moderate discomfort for the majority (~90%) of animals.

**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. Groen, R. W. *et al*. Reconstructing the human hematopoietic niche in immunodeficient mice: opportunities for studying primary multiple myeloma. *Blood* **120**, e9-e16 (2012).
4. Levy, L. *et al*. Splenectomy inhibits non-small cell lung cancer growth by modulating anti-tumor adaptive and innate immune response. *Oncoimmunology* **4**, e998469 (2015).
5. Hasenberg, A. *et al*. Catchup: a mouse model for imaging-based tracking and modulation of neutrophil granulocytes. *Nat. Methods* **12**, 445-452 (2015).
6. Lagasse, E. & Weissman, I. L. Bcl-2 Inhibits Apoptosis of Neutrophils but Not their Engulfment by Macrophages. *J. Exp. Med.* **179**, 1047-1052 (1994).
7. Coughlan, A. M., Freeley, S. J. & Robson, M. G. Animal models of anti-neutrophil cytoplasmic antibody-associated vasculitis. *Clin. Exp. Immunol.* **169**, 229-237 (2012).
8. Gubbels Bupp, M. R. Sex, the aging immune system, and chronic disease. *Cell. Immunol.* **294**, 102-110 (2015).
9. Burkholder, T., Foltz, C., Karlsson, E., Linton, C. G. & Smith, J. M. Health Evaluation of Experimental Laboratory Mice. *Curr. Protoc. Mouse Biol.* **2**, 145-165 (2012).

10. Salimi, V. *et al*. Opioid receptors control viral replication in the airways. *Crit. Care Med.* **41**, 205-214 (2013).
11. Sacerdote, P. Opioids and the immune system. *Palliat. Med.* **20 Suppl 1**, s9-15 (2006).
12. Hish, G. A., Diaz, J. A., Hawley, A. E., Myers, D. D. & Lester, P. A. Effects of Analgesic Use on Inflammation and Hematology in a Murine Model of Venous Thrombosis. *J. Am. Assoc. Lab. Anim. Sci.* **53**, 485-493 (2014).
13. Mundt, S., Groettrup, M. & Basler, M. Analgesia in mice with experimental meningitis reduces pain without altering immune parameters. *ALTEX* **32**, 183-189 (2015).

## End of experiment

**L. Method of killing**

Will the animals be killed during or after the procedures?

☐ No

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

In order to obtain organs and isolated cells for *ex vivo* analysis the mice must first be killed.

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.

X Yes

Centrale Commissie Dierproeven

19.

# 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, see 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'. | 11500 |
| 1.2 | Provide the name of the licenced establishment. | UMC Utrecht |
| 1.3 | List the serial number and type of animal procedure.<br><br>*Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.* | Serial number: 4<br>Type of animal procedure: Splenectomy |

## 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.

The objective of this project is to gain more fundamental knowledge on origin, timing, localization and function of neutrophils in diverse inflammatory conditions. We hypothesize that the [redacted] in the [redacted] (milestone ix). Although the dogma is that these cell are [redacted], our preliminary data [redacted] are [redacted], suggesting these cells either [redacted] not the [redacted] or alternatively [redacted]. To confirm this we need to perform splenectomy in mice.

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, tumor size).

The methods for obtaining these readout parameters will be the same for all experiments in this proposal, but the inflammatory stimulus will differ per experiment. The purpose of our experiments is to compare the neutrophil populations between all the different inflammatory conditions.

*Ex vivo analysis:*
In order to study the origin and kinetics of the neutrophil populations we need to investigate the different relevant organs. Neutrophils develop in the bone marrow and occasionally the spleen and subsequently migrate through the blood to the inflammatory site. In addition, neutrophils are thought to reside in the lungs and liver (marginated pool). All these organs are therefore relevant for our studies. Whenever we will perform animal experiments we will therefore harvest multiple organs for our analysis.
After the different interventions, 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 (e.g. survival assay, bacterial killing capacity, T cell suppression capacity).
This *ex vivo* analysis will allow us to link surface markers between human and mouse neutrophils. Surface marker expression and nuclear morphology allow us to distinguish different neutrophil subsets in mice, in multiple organs. We can see whether the different inflammatory conditions recruit the subsets with similar dynamics. Lastly, using functional *in vitro* assays we can investigate whether the subsets differ in their capacity e.g. to kill microbes and suppress T cell proliferation like we found in humans.

*Intravital imaging:*
Two different strategies will be used for the imaging experiments: (1) Imaging in an acute experiment under anesthesia and (2) implanting an imaging window (such as on the breast, skin, abdomen and skull) and repeatedly image through that window. 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 intracutaneous 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. 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.
*In vivo* imaging will allow us to study 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 timepoints, greatly reducing the amount of animals needed. Additionally, this provides paired data, introducing less variation and therefore fewer animals 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.

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

The objective of this project is to gain more fundamental knowledge on origin, timing, localization and function of neutrophils in diverse inflammatory conditions. Below the experimental approach per inflammatory stimulus and the intervention will be described. The choices about mice strains and numbers are further explained in B.

1. 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.
*Description:* Mice receive a single bolus of E. coli lipopolysaccharide intravenously (i.v.).
*Mice:* wildtype or intravital imaging strains
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours-days
*Level of discomfort:* mild or moderate

2. Inflammatory stimulus: Bacterial infection

*Justification:* Bacterial infection is a more complex, but more physiological relevant model for acute inflammation with which we have a lot of experience in vitro in humans.
*Description:* Mice are infected with (fluorescent) staphylococci via intradermal or intraperitoneal injection as described before[1, 2].
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.

*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – 2 weeks
*Level of discomfort:* moderate

3. Inflammatory stimulus: Viral infection

*Justification:* Viral infection is a complex physiological relevant model for inflammation with which we have some experience in vitro in humans.
*Description:* Mice are infected with influenza virus or rhinovirus 16 via intranasal administration, as performed before (Suzanne Bal, personal communication).
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – 8 weeks
*Level of discomfort:* moderate

4. Inflammatory stimulus: Plastic particles

*Justification:* The effects of microplastics on human health are currently understudied. We have found neutrophils can engulf microplastics and now want to investigate their effect on neutrophil survival and function.
*Description:* Microplastics (plastic particles ≤ 1 μm) are administered via subcutaneous injection, via addition to the drinking water, or via i.v. injection to mimic exposure routes in humans.
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – one year
*Level of discomfort:* mild or moderate

5. Inflammatory stimulus: Sterile injury

*Justification:* Sterile injury is known to recruit neutrophils without a pathogenic stimulus. Therefore this can act as a control for pathogen infection or as a model for damage-associated molecular pattern (DAMP) release.
*Description:* Under anesthesia, sterile injury is inflicted in the skin by needle insertion or laser damage
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on

general patterns of neutrophil behavior *in vivo*.
*Duration:* hours - days
*Level of discomfort:* mild or moderate

6. Inflammatory stimulus: Airway allergy

*Justification:* Allergy is a complex physiological relevant model for chronic inflammation with which we have a lot of experience in humans.
*Description:* Mice are sensitized and challenged with house dust mite by intranasal administration, as performed before (Suzanne Bal, personal communication).
*Mice:* wildtype or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* 2 – 8 weeks
*Level of discomfort:* mild or moderate

7. Inflammatory stimulus: Solid tumor growth

*Justification:* Cancer is a complex physiological relevant model for chronic inflammation. The suppressive neutrophil has been described in this model.
*Description:* We use genetic mouse models for breast or colorectal cancer, or transfer tumor cells/pieces from these mouse models to healthy mice of the same background to increase reproducibility or for intravital imaging.
*Mice:* Spontaneous tumor models or intravital imaging strains or knockout strains.
*Experimental groups:* control + 5 different timepoints.
Neutrophil dynamics change over time, so multiple time-points need to be measured.
*Readout parameters:* descriptive analysis of neutrophils (e.g. flow cytometry, microscopy, protein expression, etc.) and/or *in vitro* functional assays and/or intravital imaging.
*Number of mice per group:* 5-15 mice
We know from experience that for *ex vivo* characterization & *in vitro* functional assays we need to analyze 5-15 mice to quantitatively determine differences.
We know from experience that we need to image 5-10 mice to capture enough footage for conclusions on general patterns of neutrophil behavior *in vivo*.
*Duration:* hours – months
*Level of discomfort:* mild or moderate

These different inflammatory stimuli will be combined with the intervention described here and/or with interventions A/B/C, as described in the project proposal.

Intervention D: Splenectomy
*Description:* Surgery to remove the spleen is performed as described before[4].
*Rationale:* Since we hypothesize that the [redacted] and neutrophil [redacted] (milestone ix), we will study the effect of [redacted]. Data from [redacted] suggest that the [redacted] to the [redacted].
*Extra experimental groups:* Splenectomized vs. non-splenectomized
*Number of mice per group:* as described per inflammatory stimulus.
*Duration of intervention:* hours
*Level of discomfort:* moderate during surgery, mild thereafter

The final level of discomfort depends on the inflammatory stimulus, whether the intervention is used in combination with other interventions and the choice of the readout parameter. Harvesting of organs for *ex vivo* analysis or single session intravital imaging will result only in mild discomfort, but repeated intravital imaging will probably result in moderate discomfort.

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.

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. 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 (most of the animal procedures described in this appendix): 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, estimated numbers, and life stages. Provide justifications for these choices.

Since for our experiments the different interventions described in the different appendices will be combined, it is difficult to pinpoint the estimated numbers per intervention. Therefore here we describe the grand total of estimated numbers for our whole project.

Species: Mus musculus
Origin: Common breeding facility (Gemeenschappelijk Dierenlaboratorium) at Utrecht University/ Hubrecht institute/external licensed breeders
Estimated numbers: max. 3000
Life stages: adults

Species
All animal studies in this project will exclusively involve mice. Up to today, there are no alternative methods to fully understand neutrophil development, homeostasis and response to inflammation in the context of the whole organism. For the proposed studies, the mouse is the most appropriate animal model because: (1) physiology is more extensively characterized than other animal models; (2) a large number of relevant transgenic and knock out lines is already available; (3) a large number of relevant antibodies and other compounds is already available; (4) our research shows murine neutrophils are representative for human neutrophils; (5) many procedures for intravital imaging have already been described. The zebrafish is also an animal widely used for intravital imaging, but the differentiation of zebrafish neutrophils is much less similar to human neutrophils. Since mice are mammals too, they are closer to the human.
Mice strains used are amongst others:

- Wildtype BL/6
- Wildtype Balb/c
- Wildtype FVB
- LysM-GFP mice; for visualization of fluorescent neutrophils in intravital imaging. We are already working with this strain.
- Catchup$^{IVM}$ mice (Ly6G-Cre + Rosa-Td-Tomato); for visualization of fluorescent neutrophils in intravital imaging and for conditional knockouts in neutrophils[5]. This strain was very recently created, we want to import this strain. If the results will be superior to LysM-GFP mice, we will switch to this strain.
- MRP/S100A8-Tg-EGFP-Cre; for conditional knockouts in neutrophil progenitors[6]. We want to import this strain. As opposed to the Catchup$^{IVM}$ mice, Cre recombinase is expressed in granulocyte-macrophage progenitors in the bone marrow.
- Knockout mice (e.g. CD11b-knockout mice); to investigate the role of a specific gene or protein
- Tumor models such as MMTV-PyMT, MMTV-Wnt1 or C26 colorectal cancer mice. In our currently running animal experiments we are comparing different tumor models with each other, to optimize for future experiments. Therefore we have not completely decided which tumor models we will use in the next five years, but it will be a model that is similar in execution and discomfort as the here mentioned examples.
- Humanized mice[7] or immunocompromised mice (e.g. NSG mice); to investigate the behavior of human neutrophils in an *in vivo*-like situation.

If the required genetically modified mouse is not available, we will obtain this model by generating, importing or by crossing existing models. Other strains will be used when in the next five years, novel strains are described or created that are very relevant to our hypotheses.

Estimated numbers
Our experiments can be divided into:
- Descriptive analysis (5-15 animals x max 5 time points = max 75 animals)
- Functional assays (5-15 animals x max 5 time points = max 75 animals)
- Intravital imaging (5-10 animals)

This totals to 55-160 animals
However, we have 7 inflammatory stimuli, 4 interventions and no intervention per stimuli = 35 conditions.
So for the total we have to multiply the 55-160 animals with 35 = 1925-5600 animals

However, these numbers are overestimations because:
- 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
- Successful pilot studies may be included in the dataset

Therefore we do not anticipate we will need more than 2500 mice for the experiments.

Additionally, a number of ca. 500 mice is needed for:
- Maintenance of breeding
- Creation of new knockout models
- Pilot studies for validation of models/techniques
- Training of new personnel or new techniques
- Rederivation of imported strains via embryo transfer
- To compensate for unforeseen loss of animals (max 10%, e.g. due to location not suitable for imaging, problems with the window)

Mice that become surplus during breeding, will be used for pilot studies and training purposes.

Together we therefore anticipate we need a grand total of 3000 mice.

Sex
In general, we will try to use both male and female mice. However, it is clear that the immune response of males and females can greatly differ[8]. Therefore, we will perform pilot studies to ensure that the sex of the animal does not pose a bias in answering the particular research question. These pilots may prove it necessary to use only one sex within one animal experiment, to be able to reliably compare experimental groups.
One clear exception is the MMTV-PyMT breast cancer model; in this model only female mice consistently develop breast tumors.

**C. Re-use**

Will the animals be re-used?

X No, continue with question D.

☐ 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.

**D. 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:*
Immune responses are highly complex and multifaceted. Multiple compartments such as the bone

marrow, spleen, endothelial wall and inflamed tissue interact. Certain aspects of the immune response can be separately simulated *in vitro*, but it is not yet possible to study the full course of infection and the elicited immune response *in vitro*. This requires animal models. Similarly, the use of experimental interventions to test their potential beneficial impact on the course of disease can only be assessed in animal models. Clearly, such experimental interventions cannot be performed in patients, while *in vitro* studies with isolated human cells do not capture the full complexity of infection. However, before turning to mice, we will first study human cells *in vitro* to ensure we only perform the most relevant experiments in mice.

*Reduction:*
Intravital imaging using imaging windows allows us to image the same mouse repeatedly over time as opposed to analyzing separate mice at different timepoints greatly reducing the amount of animals needed. Additionally, this provides paired data, introducing less variation and therefore fewer animals are needed to get statistical significant results. Statistical power calculation based on our own previous experience and literature will be used to determine the optimal number of mice for each experimental group. Moreover, experiments will be performed sequentially, based on the described go/no-go decisions, via which we ensure that we will use the minimum number of mice per group that will be informative. Whenever possible, we will perform pilot studies with the minimum number of animals possible.

*Refinement:*
Except for mice with windows, where co-housing can give problems, mice will be housed in groups in cages. Nesting opportunities will always be provided. Mice with windows will be monitored at least twice a week, mice after bacterial or viral infection will be monitored at least every other day, more if necessary, for clinical signs. If unexpectedly severe suffering is observed, mice will be euthanized.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

As described under refinement, mice welfare will be regularly monitored. Mice reaching the humane endpoints or otherwise suffering from severe discomfort will be euthanized. New personnel will be trained to recognize discomfort in mice according to current guidelines (e.g. [9]). Surgical exposure will be performed under aseptic conditions using sterile instruments. Heating devices will be used to ensure a stable body temperature of the mouse during anaesthesia. Personnel conducting the surgeries is first properly trained, starting with training on cadavers.
Surgical exposure will be performed under adequate anesthesia and analgesia. Pre- and post-procedural analgesia will be used when indicated or when pain-related behavior is observed. However, common analgesics such as opioids and non-steriodal anti-inflammatory drugs (NSAIDs) have been shown to significantly alter the immune response[10-12]. Since this is our main research topic, we might sometimes refrain from using analgesia. It may be necessary to do pilot studies to prove whether analgesics pose a bias in answering our particular research question. From literature, it seems buprenorphine may be a reasonable candidate[13].
There are no expected adverse effects on the environment.

## Repetition and duplication

**E. Repetition**

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

## Accommodation and care

**F. Accommodation and care**

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☐ No

X Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

Mice with an imaging window will be housed individually to prevent other mice from biting or damaging the window.

**G. Location where the animals procedures are performed**

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

X No > Continue with question H.

☐ 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.

## Classification of discomfort/humane endpoints

**H. Pain and pain relief**

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

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

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

Surgical exposure will be performed under adequate anesthesia and analgesia. Pre- and post-procedural analgesia will be used when indicated or when pain-related behavior is observed. However, common analgesics such as opioids and non-steriodal anti-inflammatory drugs (NSAIDs) have been shown to significantly alter the immune response[10-12]. Since this is our main research topic, we might sometimes refrain from using analgesia. However, pain can also influence the immune system. Therefore, it may be necessary to do pilot studies to prove whether analgesics pose a bias in answering our particular research question.

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

Surgical exposure will be performed under aseptic conditions using sterile instruments. Heating devices will be used to ensure a stable body temperature of the mouse during anaesthesia. Personnel conducting the surgeries is first properly trained, starting with training on cadavers. Before, during and after surgery adequate anesthesia and analgesia (e.g. isoflurane and buprenorphine) will be used, according to experience as well as published protocols. New personnel will be trained to recognize discomfort in mice according to current guidelines (e.g. [9]).

**I. Other aspects compromising the welfare of the animals**

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

Other than in terminally anaesthetized animals dosing and sampling procedures will consist of a combination of volumes, routes and frequencies that will result in no more than moderate discomfort. Animals bearing tumors will never reach end-stage clinical effects. All relevant scientific data can and will be gathered *before* the cancer, infection or allergy studies reach a level of severe discomfort. If unexpectedly severe suffering is observed, mice will be euthanized.
It is expected that no animals (0%) will be experiencing more than mild discomfort due to the genetic modification. However, it is impossible to predict the nature or severity of any potential genetic defect. So, in all cases, animals will be careful monitored for possible side effects. Animals exhibiting any unexpected harmful phenotypes will be killed within a day and analyzed when possible.
Animals may lose their window (<5%). In the vast majority of these animals (>95%), we notice signs of detachment of the window far before it becomes lose, and the mice will be sacrificed. In the remaining animals, the window loss will be noticed within 24hrs and the mouse will be immediately sacrificed.

Explain why these effects may emerge.

Tumor development, inflicted inflammation, genetic alterations and administration of compounds/ drugs/ chemicals/ toxins.
It is unclear why some animals may lose their windows.

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

Mice with windows will be monitored at least twice a week, mice after bacterial or viral infection will be monitored at least every other day, more if necessary, for clinical signs. If unexpectedly severe suffering is observed, mice will be euthanized.

**J. 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 K.

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

As described in D, F, H and I, the experiments will be set-up in such a way that the incidence of reaching the humane end-point is kept to a minimum.
Weight loss (>20% in comparison with control or original weight or 15% weight loss in two days), reaching a score of 4 on the illness scale (specified below), severe shortness of breath, or loss of window is set as an humane end point. For all tumor models the guidelines of the Code of Practice Animals in Cancer research will strictly be followed, taking into account e.g. the size, location and ulceration of the tumor(s).

Next to model-specific parameters, 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

Indicate the likely incidence.

Expected <5% and <1day.

**K. Classification of severity of procedures**

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

The cumulative level of discomfort is dependent on the intervention, the read-out and the animal model. The discomfort of these three range from mild to moderate. Therefore combinations of these result in moderate discomfort for the majority (~90%) of animals.

**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. Groen, R. W. *et al*. Reconstructing the human hematopoietic niche in immunodeficient mice: opportunities for studying primary multiple myeloma. *Blood* **120**, e9-e16 (2012).
4. Levy, L. *et al*. Splenectomy inhibits non-small cell lung cancer growth by modulating anti-tumor adaptive and innate immune response. *Oncoimmunology* **4**, e998469 (2015).
5. Hasenberg, A. *et al*. Catchup: a mouse model for imaging-based tracking and modulation of neutrophil granulocytes. *Nat. Methods* **12**, 445-452 (2015).
6. Lagasse, E. & Weissman, I. L. Bcl-2 Inhibits Apoptosis of Neutrophils but Not their Engulfment by Macrophages. *J. Exp. Med.* **179**, 1047-1052 (1994).
7. Coughlan, A. M., Freeley, S. J. & Robson, M. G. Animal models of anti-neutrophil cytoplasmic antibody-associated vasculitis. *Clin. Exp. Immunol.* **169**, 229-237 (2012).
8. Gubbels Bupp, M. R. Sex, the aging immune system, and chronic disease. *Cell. Immunol.* **294**, 102-110 (2015).
9. Burkholder, T., Foltz, C., Karlsson, E., Linton, C. G. & Smith, J. M. Health Evaluation of Experimental Laboratory Mice. *Curr. Protoc. Mouse Biol.* **2**, 145-165 (2012).
10. Salimi, V. *et al*. Opioid receptors control viral replication in the airways. *Crit. Care Med.* **41**, 205-214 (2013).
11. Sacerdote, P. Opioids and the immune system. *Palliat. Med.* **20 Suppl 1**, s9-15 (2006).
12. Hish, G. A., Diaz, J. A., Hawley, A. E., Myers, D. D. & Lester, P. A. Effects of Analgesic Use on Inflammation and Hematology in a Murine Model of Venous Thrombosis. *J. Am. Assoc. Lab. Anim. Sci.* **53**, 485-493 (2014).
13. Mundt, S., Groettrup, M. & Basler, M. Analgesia in mice with experimental meningitis reduces pain without altering immune parameters. *ALTEX* **32**, 183-189 (2015).


## End of experiment

**L. Method of killing**

Will the animals be killed during or after the procedures?

☐ No

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

In order to obtain organs and isolated cells for *ex vivo* analysis the mice must first be killed.

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.

X Yes

22.

Beste CDD,

Het voornemen van de CCD om de aanvraag met nummer AVD115002016714 af te wijzen heeft mij compleet verrast. Mijns inziens heb ik de vragen van de CCD goed kunnen beantwoorden en ik ben erg teleurgesteld dat deze mening niet door de CCD gedeeld wordt.
Door de opgegeven redenen van afwijzing moet ik concluderen dat een en ander nog niet helder genoeg is overgebracht en dat er dus nog een verbeterslag nodig is in de projectaanvraag. U vindt de aanvraag erg globaal beschreven met een groot aantal subdoelstellingen, waarvoor elk subdoel meerdere experimenten nodig zijn. Ik wil benadrukken dat de onderzoeksvraag niet breed is, maar behoorlijk afgekaderd. Namelijk, neutrofielen dynamiek in homeostase en ziekte. Deze vraag kan echter wel in gelinkte milestones beantwoord worden, zoals: wat is de levensduur, zijn er meerdere subpopulaties met meerdere functies, komen de subpopulaties voort uit dezelfde voorloper en zijn de subpopulaties en hun functie hetzelfde tussen verschillende ziektebeelden. Aangezien we aan het begin staan van een fundamentele onderzoeksvraag is de aanpak wel breed. Belangrijk is dat meerdere milestones beantwoord kunnen worden met gedeelde experimenten. Bijvoorbeeld, ex vivo analyse van neutrofielen na de diverse inflammatoire stimuli zal ons informatie geven over milestone i,ii, iv en v. Ook zullen go/no go momenten van één milestone de beslisboom beïnvloeden van andere milestones (zie voorbeeld in onderstaande alinea). Door de directe links tussen de milestones en de gedeelde experimentele aanpak is het daarom wenselijk om deze in één projectaanvraag te combineren. Het klopt inderdaad dat voor elk subdoel meerdere type experimenten nodig zijn. Dit maakt ook dat het opdelen in meerdere aanvragen niet tot een substantiële vernauwing van de experimentele aanpak zal leiden.

We bevinden ons momenteel aan het begin van de fundamentele zoektocht en dit vraagt een initieel brede experimentele aanpak. Het voorbeeld in scheme 2 van de projectaanvraag laat zien dat we voor het beantwoorden van milestone ix acht verschillende experimenten kunnen uitvoeren. Er zijn echter wel een aantal go-no go momenten ingebouwd. Dit schema laat zien dat we niet alle experimenten met alle stimuli willen uitvoeren, maar een heel gerichte beslisboom afwerken. We leggen eerst een basis zonder stimulus (exp 1 en 2), vervolgens kijken we naar de stimulus waar we aan de hand van preliminaire data het meest vertrouwen in hebben (exp 3) en pas dan gaan we kijken of we dit fenomeen ook zien bij andere meer fysiologisch relevante stimuli (exp 4 en 5b). De vervolg experimenten voeren we uit met stimuli die afhangen van exp 5b (exp 5c en 7), weer zonder stimulus (exp 6) of de initiele bekende stimulus (exp 8). Bovendien zullen de bevindingen van exp 4 en 5b van milestone ix directe conseqeunties hebben voor de beslisboom van bijvoorbeeld milestone xi.  We hebben met dit schema gehoopt aan te geven dat de aanpak breed is, maar toch maar 8 verschillende experimenten omvat en dat we zeker niet alle stimuli zullen inzetten voor elk experiment.

Van andere geaccepteerde aanvragen, de IvD en lokale DEC had ik begrepen dat het uitwerken van 1 milestone als voorbeeld voldoende was. Maar als de brede aanpak onzekerheid geeft dan zouden we eventueel extra milestones en hun interactie kunnen uitwerken. Ook is het mogelijk om de initiele werkprotocollen en go/no go momenten bijvoorbeeld niet alleen naar de IvD, maar ook naar de CCD terug te rapporteren totdat de vervolg experimenten en dieraantallen beter gekarakteriseerd kunnen worden. Wij staan uiteraard ook open voor andere oplossingen.

Ik heb de coherentie tussen de milestones en de afkadering van experimenten duidelijker (in onderstreepte tekst) getracht te includeren in de projectaanvraag. Mochten er nog altijd vragen en onzekerheden zijn omtrent de projectaanvraag ben ik zeker ook bereid om het project persoonlijk te komen toelichten en bespreken.

Vriendelijke groet,
[redacted]

23.

[redacted]

**Van:** [redacted] namens dec-utrecht <dec-utrecht@umcutrecht.nl>
**Verzonden:** woensdag 22 februari 2017 9:26
**Aan:** 'Info-zbo'
**Onderwerp:** RE: AVD115002016714

**Categorieën:** Dossier: [redacted]

Geachte leden van de CCD, geachte [redacted]

Bij e-mail van 16 februari jl. heeft u ons verzocht nader te adviseren op bovengenoemd project op basis van de reactie van de aanvrager d.d. 14 februari jl.

Wij hechten eraan om nog eens te herhalen dat het naar het oordeel van de DEC hier gaat om een fundamenteel onderzoek naar het voorkomen, het ontstaan, de functie en de betekenis van subsets van granulocyten. Het onderzoek is gebaseerd op bevindingen bij de mens. Uitbreiding van het onderzoek in muizen is nodig omdat het nu eenmaal zo is dat onvoldoende cellen verkregen kunnen worden bij mensen en stimulatie proeven, zoals voorgesteld in het project, om de generatie en lokalisatie van de cellen te bestuderen, niet in mensen kunnen worden uitgevoerd. De kracht van het project is dat resultaten steeds vergeleken worden met die van in vitro onderzoek bij de mens.

In een eerdere reactie van ons (zie e-mail d.d. 23 januari jl.) hebben wij opgemerkt dat het primaire doel van het onderzoek beperkt is en dat de aanpak breed moet zijn omdat de onderzoekers staan aan het begin van een exploratief project met een groot aantal deelvragen die veelal met elkaar samenhangen en logisch op elkaar volgen. De gebruikte methoden zijn adequaat om de deelvragen te beantwoorden.

Met betrekking tot de beantwoording van uw vragen is ons oordeel dat de onderzoeker uw vragen 1, 3, 4, 5, 6 en 7 goed beantwoord heeft en meer dan voldoende heeft verwerkt in de aanvraag. De experimenten zijn navolgbaar, het ongerief per handeling is ingeschat en de fok en creatie van nieuwe muizenlijnen zijn verwijderd uit de aanvraag.
Voor wat betreft de berekening/schatting van de aantallen dieren (vraag 2 van uw reactie) wijst de DEC u op het feit dat door zorgvuldig gebruik van verkregen materiaal en overlap van experimenten het benodigde aantal dieren met de helft kon worden teruggebracht.
De DEC heeft wel begrip voor uw opmerking dat de onderbouwing van de groepsgrootte aandacht behoeft en stelt daarom voor als voorwaarde op te nemen dat de IvD er op toeziet dat, nadat de eerste experimenten zijn uitgevoerd, de vervolgexperimenten onderbouwd worden met passende statistische methoden.

De DEC beoordeelt dit project als van substantieel belang en bevestigt bij deze het eerder afgegeven positief advies.

Met vriendelijke groeten,

[redacted],[redacted]
[redacted],[redacted]

UMC Utrecht

Secretaris DEC Utrecht | Raad van Bestuur, Kwaliteit en Patient Veiligheid, Bureau Toetsing van Onderzoek
Universitair Medisch Centrum Utrecht | Kamernummer [redacted] | Huispostnummer D01.343 | Postbus 85500 | 3508 GA UTRECHT
T: +31 88 75 592 47 | www.umcutrecht.nl | Werkdagen: ma, di, woe, do

**Van:** Info-zbo [mailto:info@zbo-ccd.nl]
**Verzonden:** donderdag 16 februari 2017 12:07
**Aan:** dec-utrecht
**Onderwerp:** AVD115002016714

Geachte DEC,

Wij hebben de reactie van aanvrager ontvangen over het voorgenomen besluit van de CCD over het project 'Neutrophil subsets in health and disease' met aanvraagnummer AVD115002016714.

Aangezien u heeft aangegeven ons aanvullend te willen adviseren over dit voorgenomen besluit (zoals telefonisch besproken met [redacted]), sturen wij u bij deze de reactie van de aanvrager toe (zie bijlagen). Voor de volledigheid stuur ik u ook de eerder gestelde vragen door de CCD en de door de aanvrager gegeven antwoorden hierop.
Wij ontvangen uw aanvullend advies graag uiterlijk 26 februari 2017, zodat de reacties van zowel u als de aanvrager tijdens de komende CCD vergadering besproken kunnen worden.
Bij voorbaat hartelijk dank,

Met vriendelijke groet,

[redacted]

**Centrale Commissie Dierproeven** **www.centralecommissiedierproeven.nl**

.....
Postbus 20401 | 2500 EK | Den Haag
.....
**T: 0900 2800028**
**E:** info@zbo-ccd.nl

Centrale Commissie Dierproeven

24.

> Retouradres Postbus 20401 2500 EK Den Haag

UMC Utrecht
[redacted]
Postbus 12007
3501 AA UTRECHT

**Centrale Commissie Dierproeven**
Postbus 20401
2500 EK Den Haag
centralecommissiedierproeven.nl
0900 28 000 28 (10 ct/min)
info@zbo-ccd.nl

**Onze referentie**
Aanvraagnummer
AVD115002016714
**Bijlagen**
1

Datum 14 maart 2017
Betreft Beslissing aanvraag projectvergunning Dierproeven

Geachte [redacted],

Op 13 oktober 2016 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Neutrophil subsets en health and disease" met aanvraagnummer AVD115002016714. Wij hebben uw aanvraag beoordeeld.

Op 27 januari 2017 en 15 februari 2017 heeft u uw aanvraag aangevuld. Op ons verzoek heeft u in uw bijlagen per handeling voor elk model aangegeven wat de exacte handelingen zijn en het bijbehorende ongerief en gevolgen voor de dieren per model beschreven, heeft u verklaard waarom de inschatting van het aantal dieren op dit moment niet nauwkeuriger gedaan kan worden en heeft u de fok van knock-out muizen uit dit project verwijderd.

Op basis van het aan u gecommuniceerde voorgenomen besluit heeft u nader onderbouwd waarom u vindt dat het project beschreven in deze aanvraag binnen één project valt en waarom dit project breed wordt ingestoken.

De CCD heeft deze informatie meegenomen in de uiteindelijke afweging.

**Beslissing**
Wij keuren uw aanvraag goed op grond van artikel 10a van de Wet op de Dierproeven (hierna: de wet). Hierbij gelden de voorwaarden zoals genoemd in de vergunning.
De algemene voorwaarde(n) zijn opgenomen op grond van artikel 1d lid 4, artikel 10a1 lid 2, artikel 10 lid 2 en/of artikel 10a3 van de wet.

U kunt met uw project "Neutrophil subsets en health and disease" starten. De vergunning wordt afgegeven van 14 maart 2017 tot en met 31 oktober 2021.

Overige wettelijke bepalingen blijven van kracht.

**Procedure**
Bij uw aanvraag heeft u een advies van de Dierexperimentencommissie DEC Utrecht gevoegd. Dit advies is opgesteld op 5 oktober 2016. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a, lid 3 van de wet. Wij hebben de DEC om aanvullende informatie gevraagd. Op 23 januari 2017 en 22 februari 2017 heeft de DEC gereageerd op onze vragen. De DEC heeft haar advies aangevuld met informatie over de inzet van pijnbestrijding in de dierproeven. Daarnaast heeft de DEC aanvullend geadviseerd over de brede aanpak van het ingeperkte primaire doel van het onderzoek. De DEC heeft geadviseerd dat het gaat om een exploratief onderzoek met een groot aantal deelvragen die veelal met elkaar samenhangen en logisch op elkaar volgen.
Wij kunnen ons vinden in de inhoud van het advies van de Dierexperimentencommissie. Dit advies van de commissie nemen wij over, inclusief de daaraan ten grondslag liggende motivering. Er worden aanvullende algemene voorwaarde(n) gesteld.
Het DEC-advies en de in de bijlage opgenomen beschrijving van de artikelen van de wet- en regelgeving zijn de grondslag van dit besluit.

**Bezwaar**
Als u het niet eens bent met deze beslissing, kunt u binnen zes weken na verzending van deze brief schriftelijk een bezwaarschrift indienen.
Een bezwaarschrift kunt u sturen naar Centrale Commissie Dierproeven, afdeling Juridische Zaken, postbus 20401, 2500 EK Den Haag.

Bij het indienen van een bezwaarschrift vragen we u in ieder geval de datum van de beslissing waartegen u bezwaar maakt en het aanvraagnummer te vermelden. U vindt deze nummers in de rechter kantlijn in deze brief.

Bezwaar schorst niet de werking van het besluit waar u het niet mee eens bent. Dat betekent dat dat besluit wel in werking treedt en geldig is. U kunt tijdens deze procedure een voorlopige voorziening vragen bij de Voorzieningenrechter van de rechtbank in de woonplaats van de aanvrager. U moet dan wel kunnen aantonen dat er sprake is van een spoedeisend belang.

Voor de behandeling van een voorlopige voorziening is griffierecht verschuldigd. Op http://www.rechtspraak.nl/Organisatie/Rechtbanken/Pages/default.aspx kunt u zien onder welke rechtbank de vestigingsplaats van de aanvrager valt.

**Meer informatie**
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

**Datum:**
14 maart 2017
**Aanvraagnummer:**
AVD115002016714

Centrale Commissie Dierproeven
namens deze:

[redacted]

ir. G. de Peuter
Algemeen Secretaris

Bijlagen:
- Vergunning
  Hiervan deel uitmakend:
  - DEC-advies
  - Weergave wet- en regelgeving

Centrale Commissie Dierproeven

# Projectvergunning

**gelet op artikel 10a van de Wet op de Dierproeven**

Verleent de Centrale Commissie Dierproeven aan

| | |
|---|---|
| Naam: | UMC Utrecht |
| Adres: | Postbus 12007 |
| Postcode en plaats: | 3501 AA UTRECHT |
| Deelnemersnummer: | 11500 |

deze projectvergunning voor het tijdvak 14 maart 2017 tot en met 31 oktober 2021, voor het project "Neutrophil subsets en health and disease" met aanvraagnummer AVD115002016714, volgens advies van Dierexperimentencommissie DEC Utrecht. Er worden aanvullende algemene voorwaarde(n) gesteld.
De functie van de verantwoordelijk onderzoeker is Assistant Professor.
De aanvraag omvat de volgende bescheiden:

1 een aanvraagformulier projectvergunning dierproeven, ontvangen op 13 oktober 2016
2 de bij het aanvraagformulier behorende bijlagen:
a Projectvoorstel, zoals ontvangen per digitale indiening op 15 februari 2017;
b Niet-technische Samenvatting van het project, zoals ontvangen per digitale indiening op 15 februari 2017;
c Advies van dierexperimentencommissie d.d. 5 oktober 2016, ontvangen op 13 oktober 2016.
d De aanvullingen op uw aanvraag, ontvangen op 27 januari 2017 en 15 februari 2017

**Aanvraagnummer:**
AVD115002016714

# Weergave wet- en regelgeving

**Dit project en wijzigingen**
Volgens artikel 10c van de Wet op de Dierproeven (hierna de wet) is het verboden om andere dierproeven uit te voeren dan waar de vergunning voor is verleend. De dierproeven mogen slechts worden verricht in het kader van een project, volgens artikel 10g. Uit artikel 10b volgt dat de dierproeven zijn ingedeeld in de categorieën terminaal, licht, matig of ernstig. Als er wijzigingen in een dierproef plaatsvinden, moeten deze gemeld worden aan de Centrale Commissie Dierproeven. Hebben de wijzigingen negatieve gevolgen voor het dierenwelzijn, dan moet volgens artikel 10a5 de wijziging eerst voorgelegd worden en mag deze pas doorgevoerd worden na goedkeuren door de Centrale Commissie Dierproeven.
Artikel 10b schrijft voor dat het verboden is een dierproef te verrichten die leidt tot ernstige mate van pijn, lijden, angst of blijvende schade die waarschijnlijk langdurig zal zijn en niet kan worden verzacht, tenzij hiervoor door de Minister een ontheffing is verleend.

**Verzorging**
De fokker, leverancier en gebruiker moeten volgens artikel 13f van de wet over voldoende personeel beschikken en ervoor zorgen dat de dieren behoorlijk worden verzorgd, behandeld en gehuisvest. Er moeten ook personen zijn die toezicht houden op het welzijn en de verzorging van de dieren in de inrichting, personeel dat met de dieren omgaat moet toegang hebben tot informatie over de in de inrichting gehuisveste soorten en personeel moet voldoende geschoold en bekwaam zijn. Ook moeten er personen zijn die een eind kunnen maken aan onnodige pijn, lijden, angst of blijvende schade die tijdens een dierproef bij een dier wordt veroorzaakt. Daarnaast zijn er personen die zorgen dat een project volgens deze vergunning wordt uitgevoerd en als dat niet mogelijk is zorgen dat er passende maatregelen worden getroffen.
In artikel 9 staat dat de persoon die het project en de dierproef opzet deskundig en bekwaam moet zijn. In artikel 8 van het Dierproevenbesluit 2014 staat dat personen die dierproeven verrichten, de dieren verzorgen of de dieren doden, hiervoor een opleiding moeten hebben afgerond.
Voordat een dierproef die onderdeel uitmaakt van dit project start, moet volgens artikel 10a3 van de wet de uitvoering afgestemd worden met de instantie voor dierenwelzijn.

**Pijnbestrijding en verdoving**
In artikel 13 van de wet staat dat een dierproef onder algehele of plaatselijke verdoving wordt uitgevoerd tenzij dat niet mogelijk is, dan wel bij het verrichten van een dierproef worden pijnstillers toegediend of andere goede methoden gebruikt die de pijn, het lijden, de angst of de blijvende schade bij het dier tot een minimum beperken. Een dierproef die bij het dier gepaard gaat met zwaar letsel dat hevige pijn kan veroorzaken, wordt niet zonder verdoving uitgevoerd. Hierbij wordt afgewogen of het toedienen van verdoving voor het dier traumatischer is dan de dierproef zelf en het toedienen van verdoving onverenigbaar is met het doel van de dierproef. Bij een dier wordt geen stof toegediend waardoor het dier niet meer of slechts in verminderde mate in staat is pijn te tonen, wanneer het dier niet tegelijkertijd voldoende verdoving of pijnstilling krijgt toegediend, tenzij wetenschappelijk gemotiveerd. Dieren die pijn

kunnen lijden als de verdoving eenmaal is uitgewerkt, moeten preventief en postoperatief behandeld worden met pijnstillers of andere geschikte pijnbestrijdingsmethoden, mits die verenigbaar zijn met het doel van de dierproef. Zodra het doel van de dierproef is bereikt, moeten passende maatregelen worden genomen om het lijden van het dier tot een minimum te beperken.

**Einde van een dierproef**
Artikel 13a van de wet bepaalt dat een dierproef is afgelopen wanneer voor die dierproef geen verdere waarnemingen hoeven te worden verricht of, voor wat betreft nieuwe genetisch gemodificeerde dierenlijnen, wanneer bij de nakomelingen niet evenveel of meer, pijn, lijden, angst, of blijvende schade wordt waargenomen of verwacht dan bij het inbrengen van een naald. Er wordt dan door een dierenarts of een andere ter zake deskundige beslist of het dier in leven zal worden gehouden. Een dier wordt gedood als aannemelijk is dat het een matige of ernstige vorm van pijn, lijden, angst of blijven schade zal blijven ondervinden. Als een dier in leven wordt gehouden, krijgt het de verzorging en huisvesting die past bij zijn gezondheidstoestand.
Volgens artikel 13b moet de dood als eindpunt van een dierproef zoveel mogelijk worden vermeden en vervangen door in een vroege fase vaststelbare, humane eindpunten. Als de dood als eindpunt onvermijdelijk is, moeten er zo weinig mogelijk dieren sterven en het lijden zo veel mogelijk beperkt blijven.
Uit artikel 13d volgt dat het doden van dieren door een deskundig persoon moet worden gedaan, wat zo min mogelijk pijn, lijden en angst met zich meebrengt. De methode om te doden is vastgesteld in de Europese richtlijn artikel 6.
In artikel 13c is vastgesteld dat proefdieren geadopteerd kunnen worden, teruggeplaatst in hun habitat of in een geschikt dierhouderijsysteem, als de gezondheidstoestand van het dier het toelaat, er geen gevaar is voor volksgezondheid, diergezondheid of milieu en er passende maatregelen zijn genomen om het welzijn van het dier te waarborgen.
De Minister heeft vrijstelling ontheffing verleend volgens artikel 13c, die de afwijkende methode van doden op basis van wetenschappelijke motivering ten minste even humaan acht als de in de richtlijn opgenomen passende methoden.