	Inventaris Wob-verzoek W16-09S								
		wordt verstrekt				**weigeringsgronden**			
nr.	**document**	**reeds openbaar**	**niet**	**geheel**	**deels**	**10.1.c**	**10.2.e**	**10.2.g**	**11.1**
	NTS2015322								
1	Aanvraagformulier				x		x	x	
2	Projectvoorstel oud				x		x	x	
3	Niet-technische samenvatting	x							
4	Bijlage beschrijving dierproeven 1				x		x	x	
5	Bijlage beschrijving dierproeven 2				x		x	x	
6	Bijlage beschrijving dierproeven 3				x		x	x	
7	Bijlage beschrijving dierproeven 4				x		x	x	
8	Bijlage beschrijving dierproeven 5				x		x	x	
9	Bijlage beschrijving dierproeven 6				x		x	x	
10	DEC-advies				x		x	x	
11	Ontvangstbevestiging				x		x	x	
12	Verzoek aanvulling				x		x	x	
13	Reactie verzoek aanvulling I				x		x	x	
14	Reactie verzoek aanvulling II				x		x	x	
15	Advies CCD		x						x
16	Beschikking en vergunning				x		x	x	

2 [illegible] 5

26 NOV. 2015

AVD 1150020155322

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.zbo-ccd.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	3 0 2 4 4 1 9 7

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	
Afdeling	
Telefoonnummer	
E-mailadres	

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

(Titel) Naam en voorletters	☐ Dhr. ☐ Mw.
Functie	
Afdeling	
Telefoonnummer	
E-mailadres	

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
		☐ Wijziging op (verleende) vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn Vul uw vergunde projectnummer in en ga verder met vraag 2.2
		☐ Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn 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
		☐ 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
		☐ 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	01-01-2016
		Einddatum	31-12-2020
3.2	Wat is de titel van het project?	Inhibitory immune receptors as therapeutic targets to dampen injurious immune respons	
3.3	Wat is de titel van de niet-technische samenvatting?	Nieuwe behandelmethoden voor schadelijke afweerreacties	
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?

- [x] Nieuwe aanvraag Projectvergunning € 741,00 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

- [x] Projectvoorstel
- [x] 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 1.6). 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 23-11-2015
Handtekening [redacted]

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.zbo-ccd.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.	Inhibitory immune receptors as therapeutic targets to dampen injurious immune responses

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 animal health or welfare
- ☐ 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 immune system is a double-edged sword. While the immune system provides protection from pathogens and cancerous cells, excessive immune responses can lead to auto-immunity or exacerbated tissue damage during infection. To ensure optimal immune function, stimulatory and inhibitory signals must be balanced.

Modulation of inhibitory immune signalling has recently been recognised for its tremendous potential in the treatment of cancer (Breakthrough of the Year 2013, Science). Antibody-mediated blocking of the inhibitory immune receptors PD-1 and CTLA-4 to stimulate the anti-tumour immune response enhances the survival of cancer patients (Pardoll, Nat. Immunol., 2012). This demonstrates the potency of inhibitory immune receptors for the beneficial modulation of immune responses in disease. Contrary to cancer, where disease results from an inadequate immune response that fails to clear cancerous cells, auto-immune diseases, such as systemic lupus erythematosus, and multiple severe viral respiratory infections, including respiratory syncytial virus-induced bronchiolitis and lower respiratory tract infections by influenza virus, are characterised by exaggerated immune responses that induce or exacerbate disease. Here too, inhibitory immune receptors represent promising therapeutic targets. However, rather than using antagonistic antibodies, agonistic agents could be employed to reduce excessive immune cell activity.

In this project, we will study the underlying biology of inhibitory immune receptors and apply the obtained knowledge to evaluate their potential as therapeutic targets in immune-mediated diseases to dampen injurious immune responses. Over the years, our work has focused on multiple inhibitory immune receptors, [redacted] Immune inhibitory receptors are differentially expressed among leukocytes, where expression is determined by both cell type and activation state, and employ varying modes of action to achieve suppression. Consequently, the role and therapeutic potential of inhibitory immune receptors varies per disease.

For instance, in severe respiratory infections such as respiratory syncytial virus infection-induced bronchiolitis, a massive neutrophil infiltration of the airways dominates the immune pathology in the lungs of patients. To suppress neutrophil-induced lung injury, an inhibitory immune receptor expressed by airway-infiltrated neutrophils is a prime target. [redacted] Hence, we will investigate the role of various inhibitory immune receptors in multiple immune-mediated disease settings.

Currently, the vast majority of research on the therapeutic capabilities of inhibitory immune receptors focusses on receptor blockade to increase anti-

tumour activity, whereas little to no research is published that addresses the potential of stimulating inhibitory receptors with agonists to prevent immune-induced pathology. We have shown that [redacted] (unpublished data). Thus, these inhibitory receptors dampen injurious lung inflammation. We therefore hypothesize that engaging inhibitory immune receptors, [redacted], with agonists may ameliorate immunopathology in viral respiratory diseases. Moreover, signaling by inhibitory immune receptors is thought to be critical to preventing autoimmunity and allergy. Indeed, mice deficient for inhibitory receptors are often prone to autoimmune diseases and inhibitory receptors can suppress allergic reactions in mice (Olde Nordkamp *et al.*, Clinical Immunology, 2013; Harvima *et al.*, J Allergy Clin Immunol, 2014). The capability of inhibitory immune receptors to regulate autoimmunity suggest that agonists of inhibitory receptors could alleviate the disease severity or possibly even stave off the progression of autoimmune diseases. However, experimental evidence that agonists of inhibitory receptors can mitigate disease is currently wanting.

In this project, we aim to provide proof-of-concept for agonists of inhibitory immune receptors as a therapy for immune-mediated diseases. To this end, we will use genetically modified mice to study the biological role of various inhibitory receptors, [redacted] in immune-mediated diseases. For those inhibitory immune receptors that we find to impact disease, we will develop agonists to test their efficacy in reducing immune pathology in these disease models. In particular, we will examine inhibitory receptors in the context of respiratory viral infections, including respiratory syncytial virus and influenza virus infection, house dust mite- and respiratory syncytial virus-induced asthma, and systemic lupus erythematosus. New inhibitory receptor agonists will be generated in collaboration with the in house antibody facility of the UMC Utrecht, i.e. UMab, which has the required ethical approval for the use of animals to generate new antibody clones.

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 main aim of this project is to improve our knowledge of inhibitory immune receptors and to apply this knowledge to obtain relevant preclinical evidence that inhibitory immune receptors represent promising therapeutic targets to dampen excessively injurious immune responses.
At the completion of the allotted project duration, we will have assessed the potential role of several inhibitory receptors in multiple disease models. To achieve this, our specific aims are to:

(i) Determine which inhibitory immune receptors play a role in multiple immune-mediated disease models (i.e. respiratory viral infection, house dust mite- and respiratory syncytial virus-induced asthma, and systemic lupus erythematosus).
(ii) Functionally (*in vitro*) and phenotypically characterize the genetically modified mice to be used in disease models (compared to wild-type mice), in particular relating to immune parameters.
(iii) Generate agonistic antibodies to inhibitory immune receptors that have been shown to limit disease *in vivo* and characterize these antibodies *in vivo*, for instance to determine whether they induce (unwanted) depletion effects.
(iv) Establish a novel experimental mouse model for the induction of asthma based on relevant environmental triggers and genetic susceptibility, namely house dust mite and respiratory syncytial virus in mice deficient for the human asthma susceptibility gene, [redacted]
(v) Test the inhibitory immune receptor agonists in the various disease models for amelioration of disease. Agonists for specific inhibitory receptors will be tested in those disease models for which an worsening of disease was observed when that particular receptor was genetically ablated.

The complementary use of patient material and well-defined animal models will ensure the successful completion of this project (Salimi *et al.*, Crit Care Med, 2013). Our research group has long-standing experience investigating inhibitory receptors [redacted] Relevant animal models, such as respiratory viral infection of mice, are up-and-running in our laboratory [redacted] Several promising agonistic and antagonistic antibodies [redacted] are already being developed in collaboration with the in-house antibody facility of the UMC Utrecht, UMab. Future agonistic antibodies against other inhibitory receptors will similarly be developed in collaboration with UMab, which has the required ethical approval for the use of animals to generate new antibody clones. Work relating to house dust mite- and respiratory syncytial virus-induced asthma will be performed as member of a consortium. Collaborators in the consortium will provide mice genetically susceptible to bronchial hyperresponsiveness. Combined, these factors guarantee practicability of the proposed project.

Thus, the overarching aim of the project is to obtain proof of concept for inhibitory immune receptors as therapeutic targets for immune-mediated diseases. To achieve this, established mouse models for viral respiratory infection and systemic lupus erythematosus will be used. Herein, mice are treated with newly developed and tested inhibitory receptor agonists after an biological role for the inhibitory receptor in the disease has been demonstrated in genetic knockout (or antagonist-treated) mice. In addition, a new asthma model based on relevant genetic susceptibility and environmental triggers will be developed (current models focus on ovalbumin as allergen, but this is not a relevant human allergen in asthma). Subsequently, inhibitory receptor agonist can also be tested in the new asthma model for alleviation of disease. (See also the schematic overview of the project in subsection 3.4.3 on page 9). For all disease models in this project, the final goal is to show amelioration of disease by treatment with inhibitory receptor agonists.

3.3 Relevance

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

Numerous prevalent diseases, including respiratory syncytial virus infection-induced bronchiolitis, asthma, and systemic lupus erythematosus, are characterised by excessive and injurious immune responses. These diseases place tremendous burden on individual patients and society at large. In this project, we will examine the role of inhibitory receptors in these diseases and their potential as therapeutic target.

Respiratory syncytial virus-induced bronchiolitis is the second most import cause of death during infancy, following malaria, in the developing world, claiming an estimated 250,000 lives annually. In the developed world, 1-2% of infants are admitted in hospital with respiratory syncytial virus bronchiolitis; therefore it is the foremost cause of infant hospitalization during the winter season. Severe bronchiolitis is linked to impaired lung function in adulthood and causally implicated in the onset of recurrent wheezing and asthma [redacted] Overly strong, injurious immune responses are also seen in other respiratory viral infections. For instance, exaggerated (innate) immune responses are a hallmark of highly pathogenic influenza virus infections. A number of antivirals for respiratory syncytial virus have entered clinical trials. However, bronchiolitis patients already have high, and declining, viral loads when they are admitted to the intensive care. Since immune-mediated injury to the lungs persist, by itself an antiviral may provide limited benefit. Similar to how oseltamivir must be administered within 36 to 48 hours after symptom onset to be effective against influenza. Targeting inhibitory immune receptors with agonists represents a novel therapeutic approach that could limit immunopathology in severe viral respiratory infections. To obtain proof-of-concept for this approach, will test newly developed agonists of inhibitory receptors in previously published, well-defined mouse models of viral respiratory infection [redacted]

Systemic lupus erythematosus (SLE) is a multisystem, autoimmune disorder with a wide range of clinical features, including involvement of the kidneys, heart, skin, joints, and nervous system. It has a prevalence of 20-70 per 100,000 persons and mainly affects young women of childbearing age. The survival

rate over 10 years is approximately 90% (5-year survival rate: >90%; 15 to 20-year survival rate: ca. 80%). Active lupus is commonly treated with the glucocorticoid prednisone, sustained usage of which carries notable risk of serious complications, both immunological and non-immunological, due to its broad effects. Inhibitory receptors could offer a better targeted and more efficient therapeutic approach to halt disease. For instance, neutrophils are thought to be instrumental to the pathogenic loop that underpins the disease by the formation of DNA-based neutrophil extracellular traps (NETs), which activate plasmacytoid dendritic cells. We found that [redacted] Other immune cell types thought to be crucial to disease pathogenesis, such as B cells and plasmacytoid dendritic cells, could also be targeted via other inhibitory receptors. To obtain proof-of-concept for the treatment of lupus by targeting inhibitory receptors, we will administer newly developed inhibitory receptor agonist to mice with tetramethylpentadecane (TMPD, or pristane)-induced lupus, which show key features of human disease including anti-double stranded DNA autoantibody production, an interferon signature, and immune complex deposition-induced nephritis (Reeves *et al.*, Trends Immunol, 2009).

Asthma is a serious chronic pulmonary inflammatory disease. During an asthma attack, unwarranted exaggerated inflammation causes the airways to narrow resulting in reduced airflow. Asthma is one of the most common chronic diseases world-wide. In Western Europe, asthma prevalence has doubled over the last ten years. Global mortality rates are estimated at 180,000 deaths per year. However, mortality due to asthma is not comparable in size to the day-to-day effects of the disease. Asthma imposes a severe burden on patients and society. The economic costs associated with asthma exceed those of tuberculosis and HIV/AIDS combined. The inception of asthma occurs in a susceptible child upon exposure to environmental triggers, typically respiratory viral infections and sensitization to aero-allergens, such as house dust mite. We propose to establish a novel experimental mouse model for the inception of asthma by house dust mite and respiratory syncytial virus exposure of mice genetically susceptible to asthma. [redacted] Once established, we may use this model to assess whether treatment with inhibitory immune receptor agonists could reduce symptoms or prevent the inception of asthma. Severe respiratory syncytial virus-induced bronchiolitis is a relevant environmental trigger for asthma inception and reducing the exaggerated pulmonary inflammatory response during infection by targeting inhibitory receptor could prevent subsequent asthma inception (Geerdink *et al.*, J [redacted] This would provide proof-of-concept for novel treatment and prevention strategies of asthma.

Current immune suppressive medication lacks specificity and fails to restrain potently damaging cells, such as neutrophils. Inhibitory immune receptors hold great promise as a novel treatment strategy to more specifically dampen injurious immune responses. In this project, we aim to provide proof-of-concept for the development of inhibitory immune receptor-based therapies to treat immune-mediated diseases.

3.4 Research strategy

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

In this project, we will make combined use of human material, from healthy controls and patients, and complementary animal models. Specific cellular functions related to inhibitory immune receptors will be studied using human cells. This will increase our knowledge on the specific functions of different inhibitory immune receptors in various immune cells. We will initially examine inhibitory receptor expression patterns on immune cells obtained from patients and compare this to healthy controls and/or other compartments in the patients. Subsequently, we will test the potential of expressed inhibitory receptors on relevant immune cells to suppress damaging effector mechanisms.

For example, in RSV bronchiolitis patients, excessive neutrophilic inflammation contributes to lung injury ([redacted]). Hence, we set out to identify inhibitory receptors that were expressed on airway-infiltrated neutrophils, which could be used to suppress damaging

inflammation (currently, no effective therapy is available to reduce neutrophilic inflammation). We found that [redacted]

Based on *in vitro* investigations such as these, we will select the most promising inhibitory immune receptors for further testing in animal models. Using mice deficient for (or, alternatively, treated with antagonist against) the inhibitory immune receptor of interest, or mice transgenetically altered to express human inhibitory immune receptors, we will be able to study the underlying biology of the receptor. *Ex vivo* analyses will pinpoint phenotypic and functional differences. By challenging the mice with respiratory viral infection, asthma-inducing stimuli, or by inducing autoimmunity a role for the receptor in preventing immune pathology can be assessed. Newly developed agonists for inhibitory receptors proven to limit immune-mediated injury will then be tested in these animal models to study their potential as therapeutic targets. Initially, these agonists will be tested for their potency to stimulate the targeted inhibitory receptor using *in vitro* reporter assays. Additionally, unwanted side effects of the inhibitory receptor agonists, such as immune cell depletion, will be assessed *in vivo* prior to administration of the agonists to mice during the disease models. The inhibitory receptor agonists will be developed in collaboration with the in house antibody facility of the UMC Utrecht, i.e. UMab, which has the required ethical approval for the use of animals to generate new antibody clones.

The biological role of a particular inhibitory immune receptor may vary in different diseases, due to cell-specific expression patterns of inhibitory receptors and the varying roles of immune cells in different diseases, as well as the different modes of inhibition by various inhibitory receptors, among other reasons. Therefore, we will examine multiple inhibitory receptors in several relevant immune-mediated mouse disease models, namely viral respiratory infection, house dust mite- and respiratory syncytial virus-induced asthma, and systemic lupus erythematosus.

While the course of disease of viral respiratory infections, by viruses such as respiratory syncytial virus and influenza virus, are not identical in mice and (wo)men, clinically relevant parameters such as cellular influx into the lungs, cytokine production, and lung pathology can still be assessed (Bern *et al.*, Am J Physiol Lung Cell Mol Physiol, 2011; Thangavel *et al.*, J Immunol Methods, 2014). Despite limitations, proof-of-concept for reduction of immune-mediated pathology can still be obtained. Previous studies in our group demonstrate that the limiting effect of an inhibitory receptor [redacted]) on pathology, pulmonary cell influx, and cytokine production during respiratory viral infection (influenza) can be successfully determined ([redacted]

The tetramethylpentadecane (TMPD)-induced lupus mouse model closely mimics human disease, including key features such as anti-double stranded DNA autoantibody production, an interferon signature, and immune complex deposition-induced nephritis (Reeves *et al.*, Trends Immunol, 2009). This makes it a relevant model to study the role and therapeutic potential of inhibitory immune receptors in systemic lupus erythematosus.

We will establish a novel house dust mite- and respiratory syncytial virus-induced asthma model using genetically susceptible mice. The inception of asthma occurs in a genetically susceptible child upon exposure to environmental triggers, typically respiratory viral infections and sensitization to aero-allergens, such as house dust mite. [redacted] was recently identified as a human asthma and bronchial hyperresponsiveness susceptibility gene and we will use [redacted]-deficient mice as a model of the genetically susceptible host [redacted]. Both house

dust mite aero-allergens and respiratory syncytial virus are involved in human asthma inception (Calderón *et al.*, J Allergy Clin Immunol, 2015; Geerdink *et al.*, J Allergy Clin Immunol, 2015). Clinical parameters, such as airway hyperresponsiveness, lung compliance, and eosinophilic airway inflammation will be measured. This will result in a relevant mouse model of asthma inception with clinically significant read-outs. In this model, the role of inhibitory receptors and their potential as therapeutic targets can be studied.

Currently, our studies focus mainly on four inhibitory receptors, [redacted] During this project, our investigative efforts, including mouse models, are expected to focus on these inhibitory receptors. However, we routinely screen patient material for expression (and function) of these and additional inhibitory receptors, [redacted] The possibility remains that we will unearth an inhibitory receptor with a promising expression profile and inhibitory capacity on immune cells of relevance to the disease under investigation. In which case, we could expand our studies to include further detailed investigation of this promising inhibitory immune receptor. The main aim of this project is, after all, to obtain proof of concept for inhibitory immune receptors as therapeutic targets in immune-mediated diseases.

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.

Inhibitory immune receptor biology
The role of inhibitory immune receptors in immune-mediated diseases will be examined using genetically modified mice that lack the receptor of interest. These mice and their wild-type counterparts will be challenged with viral respiratory infection, systemic lupus erythematosus, or house dust mite- and respiratory syncytial virus-induced asthma. If an inhibitory receptor plays a role in suppressing disease, then mice that lack this receptor should show exacerbated disease compared to wild-type mice. As an alternative to genetic ablation of an inhibitory receptor, mice may be treated with a receptor antagonist.

Specific effects (phenotypical and functional) of the genetic ablation will be assessed *ex vivo* with cells obtained from unchallenged mice. Several relevant immune-mediated mouse disease models will be studied, namely viral respiratory infection, house dust mite- and respiratory syncytial virus-induced asthma, and systemic lupus erythematosus. To obtain additional mechanistic data on the role of inhibitory receptors in lung inflammatory disease, we will induce controlled sterile pulmonary inflammation by intranasal administration of inflammatory agents. [redacted] Thus it is unclear whether the increased neutrophil migration is a neutrophil-intrinsic effect or induced via other immune cells that may produce more neutrophil-attracting chemokines. By intranasally instilling equal amounts of neutrophil-specific chemokine (e.g. KC/CXCL-1) in wild-type and knock-out mice we may learn whether [redacted] on neutrophils itself regulates neutrophil migration and whether possible additional lung pathology is due (in part) to released inhibition of the neutrophils. Such mechanism-oriented studies will only be performed when effects are observed in relevant disease models that would benefit from further detailed explanation.

Inhibitory immune receptor agonist generation
Agonistic antibodies against inhibitory receptors of interest will be generated in collaboration with the in-house antibody facility (UMab, UMC Utrecht). Following *in vitro* assessment of agonistic potential, agonistic antibodies will be characterized *in vivo* in unchallenged wild-type mice. Agonistic antibody-treated mice will be examined for unwanted effects such as immune cell depletion. Vetted antibodies will be used in experiments to obtain proof-of-concept for stimulatory inhibitory immune receptor therapy.

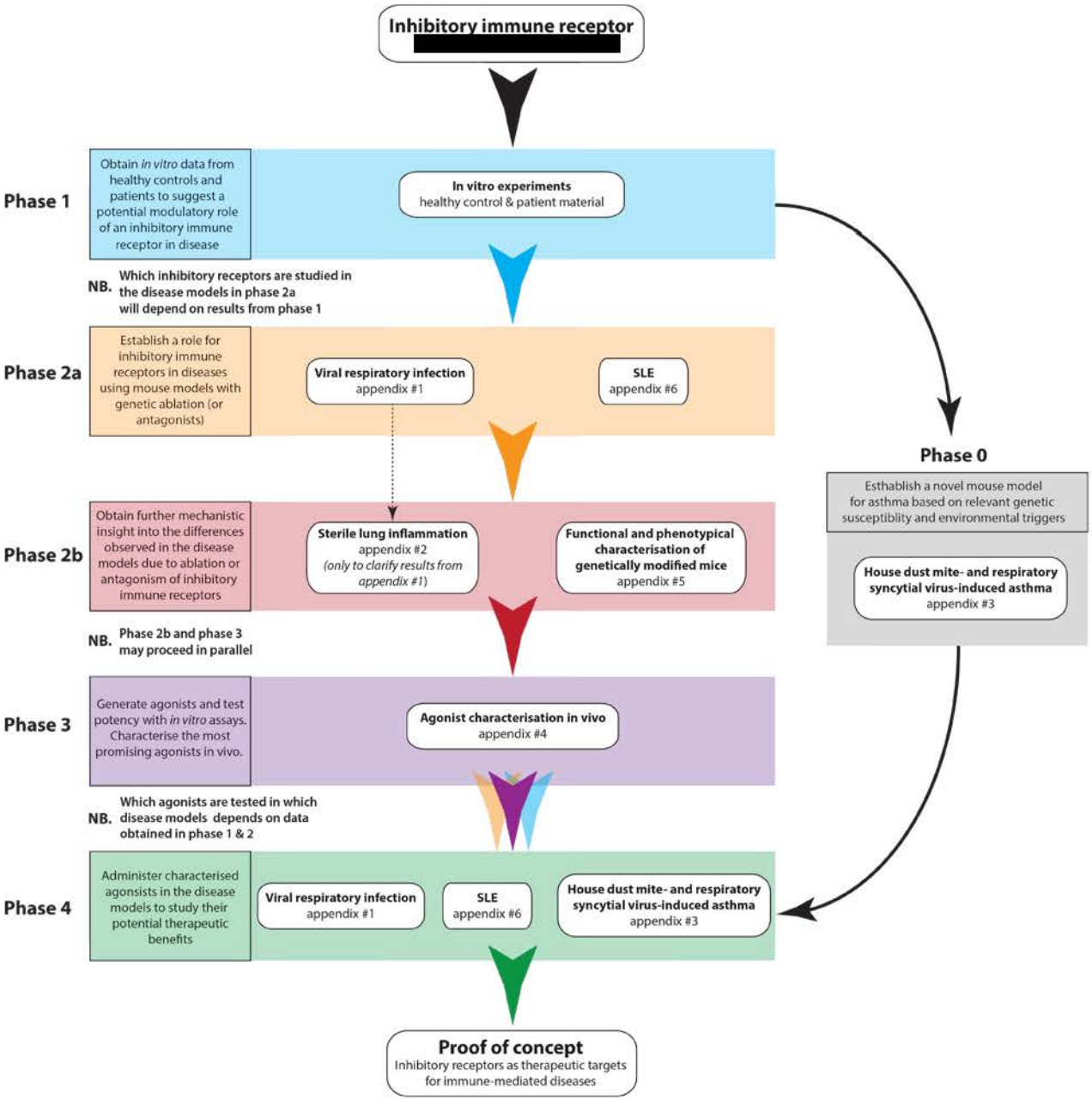
Therapeutic potential of inhibitory immune receptors
Wild-type mice will be challenged according to one of the disease models and will receive agonists for inhibitory immune receptors to examine whether

disease severity is ameliorated, disease inception can be prevented, or disease progression is halted. Agonists for specific inhibitory receptors will be tested in those disease models for which an worsening of disease was observed when that particular receptor was genetically ablated.

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.

Initially, we will study the expression and function of inhibitory immune receptors on immune cells derived from healthy controls and patients. In this setting, we can determine which inhibitory receptors can modulate immune cells thought to be important to the pathogenesis of several immune-mediated diseases, namely respiratory viral infections (appendix #1), asthma (appendix #3), and systemic lupus erythematosus (appendix #6). The biological role of the inhibitory receptors shown to modulate immune cells important to pathogenesis will then be examined using mice deficient for (or, if available, treated with antagonist against) the inhibitory receptor of interest in the corresponding disease model (appendix #1, #3, #6). Specific effects (phenotypical and functional) of the genetic ablation will be assessed *ex vivo* with cells obtained from unchallenged wild-type and genetically modified mice (appendix #5). For further mechanistic insight into the role of inhibitory receptors in lung inflammatory disease, once demonstrated that the inhibitory receptor affects disease, we will induce controlled sterile pulmonary inflammation by intranasal administration of inflammatory agents to wild-type and genetically modified mice (appendix #2). For inhibitory receptors that were shown to limit disease in the mouse models we will (in collaboration) develop agonists and their potency will be assessed *in vitro*. The most promising agonists will be characterized in wild-type mice and screened for unwanted side effects, such as immune cell depletion (appendix #4). Fully vetted agonists will be administered to wild-type mice in the appropriate disease models (i.e. the model for which the inhibitory immune receptor targeted by the agonist was shown to play a limiting role in disease severity/development) and the effects on disease severity/development will be studied (appendix #1, #3, #6).The effects of various inhibitory immune receptors, [redacted] will be examined in the different disease models, i.e. respiratory viral infections (appendix #1), asthma (appendix #3), and systemic lupus erythematosus (appendix #6), in parallel. Together, this will provide proof-of-concept for inhibitory immune receptors as therapeutic targets in immune-mediated diseases.

An schematic overview of the different components of the project can be found below. The scheme illustrates that the overarching aim common to all disease models is the amelioration of disease by treatment with inhibitory receptor agonists; and, in this way, to obtain proof of concept for inhibitory receptor agonists as therapeutic targets for immune-mediated diseases.


Inhibitory immune receptor
[redacted]
Phase 1
Obtain *in vitro* data from healthy controls and patients to suggest a potential modulatory role of an inhibitory immune receptor in disease
In vitro experiments
healthy control & patient material
NB. Which inhibitory receptors are studied in the disease models in phase 2a will depend on results from phase 1
Phase 2a
Establish a role for inhibitory immune receptors in diseases using mouse models with genetic ablation (or antagonists)
Viral respiratory infection
appendix #1
SLE
appendix #6
Phase 2b
Obtain further mechanistic insight into the differences observed in the disease models due to ablation or antagonism of inhibitory immune receptors
Sterile lung inflammation
appendix #2
(only to clarify results from appendix #1)
Functional and phenotypical characterisation of genetically modified mice
appendix #5
NB. Phase 2b and phase 3 may proceed in parallel
Phase 3
Generate agonists and test potency with *in vitro* assays. Characterise the most promising agonists in vivo.
Agonist characterisation in vivo
appendix #4
NB. Which agonists are tested in which disease models depends on data obtained in phase 1 & 2
Phase 4
Administer characterised agonsists in the disease models to study their potential therapeutic benefits
Viral respiratory infection
appendix #1
SLE
appendix #6
House dust mite- and respiratory syncytial virus-induced asthma
appendix #3
Phase 0
Esthablish a novel mouse model for asthma based on relevant genetic susceptiblity and environmental triggers
House dust mite- and respiratory syncytial virus-induced asthma
appendix #3
Proof of concept
Inhibitory receptors as therapeutic targets for immune-mediated diseases

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	Respiratory viral infections
2	Non-infectious pulmonary inflammation
3	House dust mite- and respiratory syncytial virus-induced asthma
4	Characterisation and testing of inhibitory immune receptor agonists
5	Functional and phenotypical characterisation of genetically modified mice
6	Induced auto-immune diseases
7	
8	
9	
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.zbo-ccd.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. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 1 — Type of animal procedure: Respiratory viral infections

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.

Mice, wild type and genetically modified, will be inoculated intranasally with respiratory virus, such as respiratory syncytial virus (RSV), influenza virus, or pneumonia virus of mice (PVM). Beforehand and/or during the infection mice may receive an agonist or antagonist of an inhibitory receptor of interest administered either intravenously, intraperitoneally, or nebulised. Novel inhibitory immune receptor agonists/antagonists will be generated in-house by UMab, the antibody production facility of the UMC Utrecht. Primary outcome parameters are: (i) clinical disease score, including weight (loss); (ii) lung function; (iii)

leukocyte infiltrate in bronchoalveolar lavage of whole lungs or histopathological and flow cytometric analysis of lung tissue; (iv) viral loads in the lungs. Inflammatory mediators may also be measured in the lung lavage fluid and blood obtained by cardiac puncture post-mortem. Viral replication can be studied using bioluminescence of luciferase-producing virus (Rameix-Welti *et al.*, Nat Commun, 2014; Karnam *et al.*, PLoS Pathog, 2012). Furthermore, macroscopic, microscopic and flow cytometric analysis of other (immunological) organs, including lymph nodes, spleen, and bone marrow, will be performed.

The chosen parameters will allow us to assess the immune- and/or virus-induced lung pathology and the impact thereon of the inhibitory receptor of interest. For respiratory infection of mice, intranasal administration of virus is the preferred method.

An illness grading scale will 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; mild to moderate dyspnea
4: ruffled fur, inactive, hunched, and gaunt; moderate to severe dyspnea
5: dead
Illness scores and body weight will be evaluated by a blinded observer. Scores of 4 are not expected to be reached and are considered a humane endpoint. If mice suffer from severe dyspnea (shortness of breath, laboured breathing) leading to excessive discomfort, this is also set as a humane end point.

Experimental groups
Comparisons between wild-type and genetically modified mice will include at least 4 groups:

1. mock-infected wild-type mice
2. mock-infected genetically modified mice
3. virus-infected wild-type mice
4. virus-infected genetically modified mice

NB. Mock-infected mice will be intranasally instilled with the same amount of sterile liquid also used to suspend the virus for inoculation, (e.g. phosphate-buffered saline with 10% [m/v] sucrose).
This set-up may be expanded to examine multiple doses of virus.

To study the effect of inhibitory receptor antagonism, at least the following groups will be included:

1. mock-infected wild-type mice treated with control
2. mock-infected wild-type mice treated with antagonist
3. virus-infected wild-type mice treated with control
4. virus-infected wild-type mice treated with antagonist

NB. In case of an antagonistic antibody the treatment control will be an isotype-matched control antibody; if, for instance, it is a competitively binding soluble version of the inhibitory receptor (in order to block access of the endogenous inhibitory receptor to its ligand), a control protein will be used.

The basic set-up for an experiment to study the effect of agonists of inhibitory receptors on disease severity will include the following groups:

1. mock-infected wild-type mice treated with control
2. mock-infected wild-type mice treated with agonist
3. virus-infected wild-type mice treated with control
4. virus-infected wild-type mice treated with agonist

NB. In case of an agonistic antibody the treatment control will be an isotype-matched control antibody.
This basic set-up could be expanded to include for instance different agonist/control doses, as well as varying frequencies and time points of agonist/control administration depending on the results from pilot experiments. Optimal conditions may vary for different inhibitory receptors and viruses. In such cases, treatment controls and mock infection controls will be taken along for the additional conditions.

Relevance
Viral respiratory infections of mice with, among others, respiratory syncytial virus and influenza virus are well-established models with which we have extensive previous experience ([redacted]. While the course of disease of viral respiratory infections, by viruses such as respiratory syncytial virus and influenza virus, are not identical in mice and (wo)men, relevant parameters such as cellular influx into the lungs, cytokine production, and lung pathology can still be assessed (Bern *et al.*, Am J Physiol Lung Cell Mol Physiol, 2011; Thangavel *et al.*, J Immunol Methods, 2014). Despite limitations, proof-of-concept for reduction of immune-mediated pathology can still be obtained. Previous studies in our group demonstrate that the limiting effect of an inhibitory receptor [redacted]) on pathology, pulmonary cell influx, and cytokine production during respiratory viral infection (influenza) can be successfully determined [redacted]

Relation to other experiments
For inhibitory receptors that were shown to limit disease severity during viral respiratory infection, (i.e. exacerbated disease in genetic knockout or antagonist treated mice), we will develop agonists and their potency will be assessed *in vitro*. The most promising agonists will be characterized in wild-type mice and screened for unwanted side effects, such as immune cell depletion (appendix #4). Fully vetted agonists will then be tested for amelioration of disease severity during respiratory viral infection as stipulated here. Specific effects (phenotypical and functional) of the genetic ablation will be assessed *ex vivo* with cells obtained from unchallenged wild-type and genetically modified mice (appendix #5). For further mechanistic insight into the role of inhibitory receptors in lung inflammatory disease, once demonstrated that the inhibitory receptor affects disease severity during respiratory viral infection, we will induce controlled sterile pulmonary inflammation by intranasal administration of inflammatory agents to wild-type and genetically modified mice (appendix #2). Investigation of inhibitory immune receptors in other mouse disease models, i.e. house dust mite- and respiratory syncytial virus-induced asthma (appendix #3), and systemic lupus erythematosus (appendix #6) will be studied in parallel. Prior to performing animal experiments, we will study the expression and function of inhibitory immune receptors on immune cells derived from healthy controls and patients. In this setting, we can determine which inhibitory receptors can modulate immune cells thought to be important to the pathogenesis of several immune-mediated diseases. Only inhibitory receptors thus shown to potentially limit disease will be tested *in vivo*.

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

Viral infection
Nature:
Intranasal infection with respiratory virus of mice will be performed by forced inhalation of approximately 50 µl PBS with viral inoculum under light isoflurane anaesthesia.

Frequency:
Single application or multiple interspersed application

Duration:
Inoculation with respiratory virus takes several minutes.

Mice will be terminated at several time points following inoculation to examine different aspects of the immune response. For instance, in the case of RSV infection, mice will be sacrificed 2 days after inoculation to study the innate immune response, and after 5 days to study the initial adaptive immune response. Experiments will last up to a maximum of 21 days, in order to, for instance, assess the development of cytotoxic T cell responses post-infection. At this point, mice will already have recovered from infection and will no longer suffer from infection-induced discomfort.

All measurements of outcome parameters, with the exception of weight loss, clinical scores, and lung function measurements, are performed post-mortem. Mice will be euthanized by intraperitoneal injection of pentobarbital.

Administration of agonist or antagonist
Nature:
Intraperitoneal, intravenous, or nebulised administration of inhibitory immune receptor agonist or antagonist. The exact doses are to be determined in future pilot experiments.

Frequency:
Variable. Frequency and time point(s) of agonist/antagonist administration are to be determined in future pilot experiments.

Bioluminescence
Mice will be anaesthetized with isoflurane and subsequently injected with 100 µl of luciferin dissolved in phosphate-buffered saline (25 mg/kg). Bioluminescent signals of anaesthetized mice are collected over a ca. 10 min interval. This only applies to mice specifically infected with luciferase-expressing virus.

Airway responsiveness in anaesthetized mice
Mice are intraperitoneally anaesthetized with ketamine 125 mg/kg and medetomidine 0.2 mg/kg. The animals are ventilated (O2/air (1:2)) at a frequency of 150 beats/min. The mice are prepared for the measurement of the following lung parameters: pulmonary resistance (RL) and tidal volume (TV). Increasing doses of methacholine (acetyl-β-methyl-choline chloride) are administered by aerosol generated in a nebulizer. After the first dose of methacholine, pulmonary resistance (RL) dynamic compliance (Cdyn) and tidal volume (TV) are measured for 3 min, and this procedure is repeated for all doses.

Invasive Right Ventricular Pressure Measurements
Open-chest right ventricular (RV) catheterization is performed before measuring lung function under general anesthesia. Under anesthesia, animals are intubated with a 19-gauge cannula into the trachea. Mice are attached to a mechanical microventilator, ensuring a breathing frequency of 150 breaths/min, inspiratory/expiratory ratio, 1:1, gas flow 1:1 O2/air mix. Right ventricular systolic pressure (RVSP), diastolic pressure (RVDP) and mean right ventricular pressure (RVMP) are recorded. Analyses are performed when steady state is reached over an interval of at least 3 minutes and averaged.

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

Power calculations based on previous experience with viral respiratory infection models indicate that a group size of 8 mice per group is sufficient to detect biologically relevant and statistically significant differences between wild-type and genetically modified mice [redacted]. A similar group size has proven to be sufficient to detect differences between control and LAIR-1 antagonist-treated mice (unpublished data). We expect that a group size in a similar range will prove sufficient to detect differences from agonist treatment, but will definitively determine group size based on future pilot experiments. Notably, since mock-infected, control-treated mice show little variance within their experimental group, a group size of 4 has been sufficient in the past and will thus be used.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Mice (*Mus musculus*) will be used as experimental animal. Respiratory infections in mice by, for instance, RSV and influenza virus are well-established disease models. While the course of disease of viral respiratory infections, by viruses such as respiratory syncytial virus and influenza virus, are not identical in mice and (wo)men, clinically relevant parameters such as cellular influx into the lungs, cytokine production, and lung pathology can still be assessed (Bern *et al.*, Am J Physiol Lung Cell Mol Physiol, 2011; Thangavel *et al.*, J Immunol Methods, 2014). Despite limitations, proof-of-concept for reduction of immune-mediated pathology can still be obtained. Previous studies in our group demonstrate that the limiting effect of an inhibitory receptor [redacted] on pathology, pulmonary cell influx, and cytokine production during respiratory viral infection (influenza) can be successfully determined [redacted] Many essential research tools, including genetic modifications and antibodies, are only available in the mouse system. The mice used in the proposed experiments, both wild-type and genetically modified strains, will be housed and bred at the common breeding facility (Gemeenschappelijk Dierenlaboratorium) at Utrecht University. Mice used in the experiments will be approximately 8 weeks of age, which conforms to previous experiments performed in our group and other published literature.

In general, we will only use female mice. The female immune response is stronger than that of males and we have shown that [redacted] Our research interest lies in preventing immune-mediated pathology. To this end, female mice are a more sensitive model.

In the five-year period of the project, we intend to study multiple inhibitory immune receptors, [redacted] in the context of several viral respiratory infections, such as respiratory syncytial virus and influenza virus. Depending on the efficiency by which potent agonists to inhibitory receptors are generated, various agonists may also be tested. Experiments will be repeated twice (n=3) to ensure reproducibility of results. Additionally, a number of pilot experiments will be required to determine optimal dosage, frequency, and timing of agonist delivery.

We also reserve a limited number of mice for training purposes and validation of new virus batches. Only (new) group members who will be involved in our research for more than one year will be eligible for training in intranasal inoculation, bronchoalveolar lavage, and other techniques related to viral respiratory infection. In our experience, there is variation between virus batches and new batches should first be validated, preferably in a direct comparison with a previous batch. Estimated numbers needed: 30 mice/year. For this estimate, we take into account that at least 4 mice need to be used per tested batch (old and new) and the average size of newly made batches of virus (issues of practicality in virus culture prohibit the generation of significantly larger batches). On occasion, a virus batch does not evoke a proper response *in vivo*, in which case a new batch needs to be made and tested again with new (immune naïve) mice.

Estimated total number of mice needed in the 5-year period: 1500 mice. This estimate is based on factors discussed in more detail above.
Experiments with KO (or antagonist-treated) mice (proposal Phase 2a):
- Mice per experiment: 24 (e.g. 4x mock WT, 4x mock KO, 8x virus WT, 8x virus KO)
- Estimated number of inhibitory receptors tested: 4
- Estimated number of viruses tested: 2
- Reproducibility of results: n = 3
- Total needed: 24 x 4 x 2 x 3 = 576 mice

Experiments with agonist treated mice (proposal Phase 4):
Mice per experiment: 24 (e.g. 4x mock & control WT, 4x mock & agonist WT, 8x virus & control WT, 8x virus & agonist WT)
Estimated number of inhibitory receptor agonists tested: 4
Estimated number of viruses tested: 2
Reproducibility of results: n = 3
Total needed: 24 x 4 x 2 x 3 = 576 mice

Testing of virus batches and technique training (training is only for new members that join the research group from more than one year):
Mice per experiment: 10 (e.g. for virus batch testing: 2x mock infected, 4x old batch, 4x new batch)
Estimated frequency: 3x per year
Total needed: 10 x 3 x 5 = 150 mice

Pilot experiments (e.g. agonist administration during disease model at different intervals or with different doses):
Mice per experiment: 10
Estimated number of inhibitory receptors tested: 4
Estimated number of viruses tested: 2
Different conditions tested: 3 (e.g. timing, dosing, administration route)
Total needed: 240 mice

The estimated grand total number of mice needed is therefore (576 + 576 + 150 + 240 = 1540) approximately 1500 mice.

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 to respiratory viral infections are highly complex and multifaceted. Interactions between multiple compartments such as bone marrow, circulation, lymphatic system, secondary lymphoid organs, and lungs are involved. 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 immune response it elicits *in vitro*. This requires animal models. Similarly, the use

of experimental agonist of inhibitory receptors to test their potential beneficial impact on the course of disease during viral infection 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 infections in mice, we will first study human cells *in vitro* to ensure we only perform the most relevant experiments in mice. Specifically, the expression and capacity of a particular inhibitory receptor to inhibit immune cells that are important to immune-mediated pathology can be tested using human healthy control and patient material. Only receptors that, based on these results, could potentially impact disease severity will be tested *in vivo* for their role in respiratory viral infection. Whether the expression pattern and functional capabilities of the inhibitory receptor are similar in steady-state mice can be assessed using unchallenged mice (as described in appendix #5).

Reduction:
To minimise the number of surplus mice and ensure the highest degree of comparability between wild-type and genetically modified mice, the modified mouse strains will be maintained as heterozygotes. This way, separately maintaining genetically modified and matched wild-type strains is not necessary. This is intended for maintaining the mouse strains, not for breeding mice for experiments. On the eve of experiments, when large numbers of homozygous mice (both genetically modified and wild type) are required, we will temporarily breed mice from homozygous parents to prevent that many heterozygous mice are lost as surplus. Differences due to genetic drift between wild-type and genetically modified mice will be kept to a minimum in this way. This ensures that experimental differences observed between the wild-type and genetically modified mice are due to the intended genetic modification.

For our experiments we generally employ only female mice, since we have shown there are significant differences between the two sexes in antiviral immune responses. The female immune response is stronger than that of males and we have shown [redacted]. Our research interest lies in preventing immune-mediated pathology. To this end, female mice are a more sensitive model [redacted] However, where possible we provide our male mice to collaborators, as we have done in the past, to prevent unnecessary losses.

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.

Refinement:
Mice will be housed in groups in cages with nesting opportunities. Virus-inoculated mice will be assessed at least once per day, more if necessary, for clinical signs of infection, including weight loss. 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.

Mice suffering from unexpectedly severe discomfort will be euthanized, this includes a clinical illness score of 4 and/or severe shortness-of-breath.

During inoculation with virus, mice will be anaesthetised with isoflurane. No pain medication will be administered during the course of infection, since this could interfere with immune regulation, as we have shown for opioids [redacted] but also analgesic nonsteriodal anti-inflammatory drugs interfere with immune signaling, e.g. via inhibition of cyclooxygenase enzymes that produce inflammatory lipid mediators. Moreover, discomfort experienced by experimental animals is likely to depend more on other factors such as shortness of breath or fever rather than pain. During invasive lung and heart function measurements, mice will be fully anaesthetised with ketamine and medetomidine.

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?

X No

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

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.

During inoculation with virus, mice will be anaesthetised with isoflurane. No pain medication will be administered during the course of infection, since this could interfere with immune regulation, as we have shown for opioids [redacted] but also analgesic nonsteriodal anti-inflammatory drugs interfere with immune signaling, e.g. via inhibition of cyclooxygenase enzymes that produce inflammatory lipid mediators. Moreover, discomfort experienced by experimental animals is likely to depend more on other factors such as shortness of breath or fever rather than pain. During invasive lung and heart function measurements, mice will be fully anaesthetised with ketamine and medetomidine.

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

I. Other aspects compromising the welfare of the animals

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

As a direct result of viral infection, mice may experience discomfort in the form of shortness of breath and fever.
Where applicable, handling of mice for bioluminescent imaging can result in transient mild discomfort.
Where applicable, the administration of agonist/antagonist can result in transient mild discomfort, resulting e.g. from intraperitoneal injection.
While invasive lung and heart function tests would result in severe discomfort, mice will be fully anaesthetised with ketamine and medetomidine during measurements and euthanized at completion.

Explain why these effects may emerge.

Viral respiratory infection induces discomfort due to pathological effects of the virus and immune response. The handling of mice, e.g. injecting agonist/antagonist or measuring bioluminescence, is necessary to test experimental parameters.

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

This discomfort is inherent to the model of viral respiratory infection and cannot be treated without compromising the model.

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.

Weight loss exceeding 20% of original weight, reaching a score of 4 on the illness scale (specified above), and severe shortness of breath is set as the humane end point.

Indicate the likely incidence.

Severity of disease depends on the dose of the viral inoculum. The dose will be chosen to avoid that mice reach the humane end point. However, we have previously shown that mice in which inhibitory receptors are genetically ablated present with more severe viral disease (Karnam *et al*., PLoS Pathog, 2012). For initial experiments with previously untested mouse strains it is therefore difficult to predict the proportion of mice that will reach the humane end point. Pilot experiments will be employed to determine the appropriate dose for new mouse strains.

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').

Cumulative levels of discomfort are expected to be moderate.
Pilot studies for (genetically modified) mouse strains not yet tested for infection with a particular respiratory virus will be undertaken to prevent occurrence of unexpectedly severe discomfort in subsequent experiments.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No > Continue with Section 3: ‘signatures’.

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

In order to obtain a whole lung lavage or lung tissue for histopathological analysis the mice must first killed, since the mice would not survive either of these procedures. Neither are invasive measurements of lung and heart function compatible with continued survival of the test subject

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

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.zbo-ccd.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. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 2 Type of animal procedure: Non-infectious pulmonary inflammation

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.

Mice, wild type and genetically modified, will receive intranasal or nebulised/gaseous administration of sterile inflammatory agent(s), such as CXCL-1 (KC) or C5a (Trujillo *et al*., J Immunol, 2013), to induce non-infectious pulmonary inflammation. This will allow us to more specifically (compared to viral respiratory infection) delineate the effects of inhibitory receptors during inflammation, e.g. increased immune cell recruitment in response to identical dose of inflammatory agent. Additionally, inhibitory immune receptor agonists or antagonists may be administered to wild-type mice. Agonists will be studied for the

mechanistic effects underlying their therapeutic potential (e.g. specifically inhibiting cell recruitment and/or function), whereas antagonists can be used to mimic genetic ablation of a receptor should a knock-out mice not be available (on an appropriate genetic background). Primary outcome parameters are: leukocyte infiltrate in bronchoalveolar lavage of whole lungs or histopathological and flow cytometric analysis of lung tissue. Inflammatory mediators may also be measured in the lung lavage fluid and blood obtained by cardiac puncture post-mortem. Furthermore, macroscopic, microscopic and flow cytometric analysis of other (immunological) organs, including lymph nodes, spleen, and bone marrow, will be performed.

Experimental groups
Comparisons between wild-type and genetically modified mice will include at least 4 groups:

1. mock-instilled wild-type mice
2. mock-instilled genetically modified mice
3. inflammatory mediator-instilled wild-type mice
4. inflammatory mediator-instilled genetically modified mice

NB. Mock-instilled mice will be intranasally instilled with the same amount of sterile liquid also used to suspend the virus for inoculation, (e.g. phosphate-buffered saline).
This set-up may be expanded to examine multiple doses of inflammatory agent.

The basic set-up for an experiment to study the effect of agonists of inhibitory receptors on pulmonary inflammation will include the following groups:

1. mock-instilled wild-type mice treated with control
2. mock-instilled wild-type mice treated with agonist
3. inflammatory mediator-instilled wild-type mice treated with control
4. inflammatory mediator-instilled wild-type mice treated with agonist

NB. In case of an agonistic antibody the treatment control will be an isotype-matched control antibody.

Relation to other experiments
Initially, we will study the expression and function of inhibitory immune receptors on immune cells derived from healthy controls and patients suffering from respiratory viral infection. Based hereupon, we will select inhibitory receptors to study in animal models of respiratory viral infections. If an inhibitory receptor limits pathology, we expect to see increased disease severity in mice deficient for this inhibitory receptor (appendix #1). To obtain additional mechanistic data on the role of inhibitory receptors in lung inflammatory disease, we will induce controlled sterile pulmonary inflammation by intranasal administration of inflammatory agents. [redacted] Thus it is unclear whether the increased neutrophil migration is a neutrophil-intrinsic effect or induced via other immune cells that may produce more neutrophil-attracting chemokines. By intranasally instilling equal amounts of neutrophil-specific chemokine (e.g. KC/CXCL-1) in wild-type and knock-out mice we may learn whether [redacted] on neutrophils itself regulates neutrophil migration and whether possible additional lung pathology is due (in part) to released inhibition of the neutrophils.

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

Administration inflammatory agent
Nature:
Intranasal administration will be performed by forced inhalation of 50 µl PBS with inflammatory agent under light isoflurane anaesthesia.

Frequency:

Single application

Duration:
Mice will be euthanized at several time points up to 48 h.

Measurements of outcome parameters are performed post-mortem. Mice will be euthanized by interperitoneal injection of pentobarbital.

Administration of agonist or antagonist
Nature:
Intraperitoneal, intravenous, or nebulised administration of inhibitory immune receptor agonist or antagonist. The exact timing and doses are to be determined in future experiments. Agonist/antagonist may be administered prior to or after instilling the inflammatory agent.

Frequency:
To be determined in future pilot experiments.

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

Power calculations based on literature indicate that a group size of 8 mice per group is sufficient to detect biologically relevant and statistically significant differences between wild-type and genetically modified mice (Trujillo *et al.*, J Immunol, 2013). This mirrors our experience with viral respiratory infections with a similar group size [redacted] We expect that a group size in a similar range will prove sufficient to detect differences from agonist treatment, but will definitively determine group size based on future pilot experiments. Notably, since mock-instilled, control-treated mice show little variance within their experimental group, a group size of 4 will be sufficient.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Mice (*Mus musculus*) will be used as experimental animal. This model of sterile lung inflammation is meant to provide additional mechanistic insight into the respiratory viral infection model (appendix #1). Respiratory infections in mice by, for instance, RSV and influenza virus are well-established disease models. While the course of disease of viral respiratory infections, by viruses such as respiratory syncytial virus and influenza virus, are not identical in mice and (wo)men, clinically relevant parameters such as cellular influx into the lungs, cytokine production, and lung pathology can still be assessed (Bern *et al.*, Am J Physiol Lung Cell Mol Physiol, 2011; Thangavel *et al.*, J Immunol Methods, 2014). Despite limitations, proof-of-concept for reduction of immune-mediated pathology can still be obtained. Previous studies in our group demonstrate that the limiting effect of an inhibitory receptor [redacted]) on pathology, pulmonary cell influx, and cytokine production during respiratory viral infection (influenza) can be successfully determined [redacted], [redacted]. Additionally, many essential research tools, including genetic modifications and antibodies, are only available in the mouse system. Therefore, in this sterile lung inflammation model we will also use mice. The mice used in the proposed experiments, both wild type and genetically modified strains, will be housed and bred at the common breeding facility (Gemeenschappelijk Dierenlaboratorium) at Utrecht University. Mice used in the experiments will be approximately 8-12 weeks of age.

In general, we will only use female mice. The female immune response is stronger than that of males and we have shown that [redacted] Our research interest lies in preventing immune-mediated pathology. To this end, female mice are a more sensitive model. The model of sterile lung inflammation

described here is used to obtain further mechanistic insights into the results obtained in the viral respiratory infection model (appendix #1). In the viral respiratory model, for reasons also described above, only female mice are used. Hence, for the further study of mechanistic aspects, we will also female mice.

In the five-year period of the project, we intend to study multiple inhibitory immune receptors, [redacted] Experiments will be repeated twice (n=3) to ensure reproducibility of results. Additionally, a number of pilot experiments will be required to determine optimal dosage, frequency, and timing of agonist delivery.

Experiments with inhibitory receptor KO mice
Mice per experiment: 24 (e.g. 4x mock WT, 4x mock KO, 8x treated WT, 8x treated KO)
Estimated number of inhibitory receptors tested: 4
Reproducibility of results: n = 3
Total needed: 24 x 4 x 3 = 288 mice

Mice per experiment: 24 (e.g. 4x mock & control WT, 4x mock & agonist WT, 8x treated & control WT, 8x treated & agonist WT)
Estimated number of inhibitory receptor agonists tested: 2 (not expected to be needed for each tested inhibitory receptor)
Estimated number of viruses tested: 2
Reproducibility of results: n = 3
Total needed: 24 x 2 x 3 = 144 mice

Pilot experiments (e.g. agonist administration during disease model at different intervals or with different doses):
Mice per experiment: 10
Estimated number of inhibitory receptors tested: 2
Different conditions tested: 3 (e.g. timing, dosing, administration route)
Total needed: 60 mice

The estimated grand total number of mice needed is therefore (288 + 144 + 60 = 492) approximately 500 mice.

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:
Pulmonary inflammation is a highly complex and multifaceted process. Interactions between multiple compartments such as bone marrow, circulation, and lungs are involved. Certain aspects of the inflammatory immune response can be separately simulated *in vitro*, but it is not yet possible to study lung inflammation *in vitro*. In this setting we can use of genetically modified animals and experimental agonists/antagonists to assess the role of inhibitory immune receptors in pulmonary inflammation. Clearly, such experimental interventions cannot be performed in patients, while *in vitro* studies with isolated human cells do not capture all aspects of inflammation. Mechanistic studies described here will only be performed once and effect has been found in a relevant respiratory viral infection model. Before turning to infections in mice, we will first study human cells *in vitro* to ensure we only perform the most relevant experiments in mice. Specifically, the expression and capacity of a particular inhibitory receptor to inhibit immune cells that are important to immune-mediated pathology can be tested using human healthy control and patient material. Only receptors that, based on these results, could potentially impact disease severity will be tested *in vivo* for their role in respiratory viral infection. Whether the expression pattern and functional capabilities of the inhibitory receptor are similar in steady-state mice can be assessed using unchallenged mice (as described in appendix #5).

Reduction:
To minimise the number of surplus mice and ensure the highest degree of comparability between wild type and genetically modified mice, the modified mouse strains will be maintained as heterozygotes. This way, separately maintaining genetically modified and matched wild-type strains is not necessary. This is intended for maintaining the mouse strains, not for breeding mice for experiments. On the eve of experiments, when large numbers of homozygous mice are required, we will temporarily breed mice from homozygous parents to prevent that many heterozygous mice are lost as surplus. Differences due to genetic drift between wild-type and genetically modified mice will be kept to a minimum in this way. This ensures that experimental differences observed between the wild-type and genetically modified mice are due to the intended genetic modification and not an 'off-target effect.'

For our experiments we generally employ only female mice, since we have shown there are significant differences between the two sexes in antiviral immune responses. The female immune response is stronger than that of males and we have shown that [redacted] Our research interest lies in preventing immune-mediated pathology. To this end, female mice are a more sensitive model. [redacted] However, where possible we provide our male mice to collaborators, as we have done in the past, to prevent unnecessary losses.

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.

Refinement:
Mice will be housed in groups in cages with nesting opportunities. Mice will be inspected for discomfort 4 h after administration of inflammatory mediator, the time point at which pulmonary inflammation is expected to reach its peak (Trujillo *et al.*, J Immunol, 2013), and inspected again the next day. If unexpectedly severe suffering is observed during the experiment, 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.

Mice suffering from unexpectedly severe discomfort will be euthanized.
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?

X No

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

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.

During administration of the inflammatory agent, mice will be anaesthetised with isoflurane. No pain medication will be administered during the course of infection, since this could interfere with immune regulation, as we have shown for opioids [redacted] but also analgesic nonsteriodal anti-inflammatory drugs interfere with immune signaling, e.g. via inhibition of cyclooxygenase enzymes that produce inflammatory lipid mediators. Moreover, discomfort experienced by experimental animals is likely to depend more on other factors such as shortness of breath or fever rather than pain.

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

I. Other aspects compromising the welfare of the animals

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

Mice may experience discomfort in the form of shortness of breath.
Where applicable, the administration of agonist/antagonist can result in transient mild discomfort, resulting e.g. from intraperitoneal injection.

Explain why these effects may emerge.

Shortness of breath may be induced by the induced pulmonary inflammation.

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

This discomfort is inherent to the model of pulmonary inflammation and cannot be treated without compromising the model.

J. Humane endpoints

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

X No > Continue with question K.

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

Indicate the likely incidence.

The induced pulmonary inflammation is not expected to be severe enough for mice to reach a humane endpoint. In fact, the dose of the inflammatory agent will be chosen to specifically avoid severe discomfort.

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').

Cumulative levels of discomfort are expected to be moderate.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No > Continue with Section 3: 'signatures'.

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

Whole lung lavage or excision of lung tissue for histopathological analysis is not compatible with continued survival of the mice.

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

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

☐ Yes

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.zbo-ccd.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. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3 — Type of animal procedure: House dust mite- and respiratory syncytial virus-induced asthma

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.

[redacted] is a recently identified asthma and bronchial hyperresponsiveness (BHR) susceptibility gene that is expressed in airway epithelium. Common polymorphisms in the gene encoding [redacted] are associated with increased susceptibility to BHR and asthma [redacted] Preliminary data suggest that [redacted])-deficient mice show exacerbated BHR in response to house dust mite allergen exposure and that [redacted]-deficient mice are more susceptible to RSV infection, both of which are associated with asthma

development. For these reasons, [redacted]-deficient and transgenic mice will be used as model for genetic susceptibility to asthma. To trigger the inception of asthma, mice will be exposed to aeroallergens (from house dust mite) and/or viral respiratory infection (by respiratory syncytial virus). The optimal exposure of these environmental triggers to induce asthma will be determined in initial pilot experiments. Once the asthma inception model has been optimized, inhibitory receptors can be targeted herein to assess their impact on asthma inception and their potential as therapeutic target. Primary outcome parameters are: (i) clinical symptoms; (ii) lung function; (iii) leukocyte infiltrate in bronchoalveolar lavage of whole lungs or histopathological and flow cytometric analysis of lung tissue; (iv) systemic antibody and inflammatory mediator responses. Additionally, viral replication can be assessed by bioluminescence in mice infected with luciferase-expressing virus (Rameix-Welti *et al*., Nat Commun, 2014); and epithelial gene expression signatures will be examined to study the effect of asthma-inducing stimuli and inhibitory immune receptor stimulation on airway epithelium.

The experimental set-up for the initial experiments to optimize the asthma inception model are described below. An example pilot experiment on the induction of asthma inception by RSV infection will include the following groups:

1. mock-infected wild-type mice
2. mock-infected [redacted]-deficient mice
3. respiratory syncytial virus-infected wild-type mice
4. respiratory syncytial virus-infected [redacted]-deficient mice

The basic set-up for an experiment to study the effect of agonists of inhibitory receptors on disease severity will include the following groups:

5. mock-exposed [redacted]-deficient mice treated with control
6. mock-exposed [redacted]-deficient mice treated with agonist
7. virus/allergen-exposed [redacted]-deficient mice treated with control
8. virus/allergen-exposed [redacted]deficient mice treated with agonist

NB. In case of an agonistic antibody the treatment control will be an isotype-matched control antibody.
This basic set-up could be expanded to include for instance different agonist/control doses, as well as varying frequencies and time points of agonist/control administration depending on the results from pilot experiments. Optimal conditions may vary for different inhibitory receptors. In such cases, treatment controls and mock-exposed controls will be taken along for the additional conditions.

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

Respiratory virus-induced asthma

RSV infection at 8 weeks; & neonatal RSV infection. To characterize the susceptibility of [redacted]-deficient mice to clinically relevant parameters of airway inflammation, remodelling and hyperresponsiveness induced by RSV infections, we will infect [redacted] knockout mice and wild-type mice with RSV as neonates (7 days old pups) or at age 6-8 weeks with RSV type A2 or UV-inactivated RSV. The UV-inactivated RSV will serve as an control for the immunogenic effect of viral proteins and RNA in the absence of productive infection. This will inform us whether the elicited immune response by inactivated, but still immunogenic virus particles is sufficient, or if productive replication-induced epithelial damage is also required for the induction of hyperresponsiveness.

RSV re-infection. In addition, a group of neonatally infected mice will be re-infected with RSV type A2 at an age of 8 weeks, and the clinical parameters will be monitored daily in a blinded fashion after re-infection. At day 5 after the primary or secondary infection with RSV, a subset of mice will be sacrificed for in-depth histopathological analysis of inflammatory and structural parameters by standardised methods.

An illness grading scale will 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; mild to moderate dyspnea
4: ruffled fur, inactive, hunched, and gaunt; moderate to severe dyspnea
5: dead
Illness scores and body weight will be evaluated by a blinded observer. Scores of 4 are not expected to be reached and are considered a humane endpoint. If mice suffer from severe dyspnea (shortness of breath, laboured breathing) leading to excessive discomfort, this is also set as a humane end point.

Also, a sum score for interstitial inflammation, endothelialitis, bronchitis, edema, thrombus, and pleuritis (each scored 0-4) will be calculated. In a separate group of mice, we will measure lung function parameters (compliance and airway resistance) by direct invasive measurements (see below) and evaluate airway inflammation by presence of inflammatory immune cells in lung tissue and broncho-alveolar lavage (BAL) fluid. Moreover, we will assess cytokines in lung tissue, systemic levels of RSV-specific IgG, viral titers in lung, and lung histopathology.

Respiratory virus and aeroallergen-induced asthma
HDM dose study. To determine the minimal HDM dose required for sensitisation, [redacted]-deficient mice and wild-type (WT) controls will be exposed to HDM intranasally three times in one week early in life (e.g. the first week of life) at 25, 5, 1 or 0 µg HDM per treatment. Two weeks after the last HDM treatment, mice will be sacrificed and analysed for sensitization.

HDM rechallenge. Next, we will use the minimal dose inducing HDM sensitization in [redacted]KO mice as neonates for a second experiment. In this second series of experiments, we will treat [redacted]-deficient and wild-type mice for 3 weeks with HDM (3 times per week) followed by 5 HDM or PBS challenges in 1 week at 10 weeks of age. These mice will be used for detailed analysis of lung function, epithelial integrity, pulmonary inflammatory cells, and systemic levels of HDM-specific IgE and IgG.

RSV/HDM challenge. We will perform RSV infections at the first week of age, followed by HDM exposures at weaning (week 3) and test whether the airway epithelial, innate and adaptive immune response to HDM exposure after RSV infection are qualitatively different between [redacted]-deficient and control mice in young mice.

HDM/RSV challenge. We will treat newborn mice with HDM for 3 times during the first week of life, followed by RSV infection at weaning (week 3) and ask whether primary RSV-induced responses are qualitatively or quantitatively different in young [redacted]-deficient mice that had prior sensitization to HDM.

RSV reinfection. Moreover, we will perform re-infection of the mice at 8 weeks of age and test whether long-term effects of RSV infection are different between [redacted]-deficient and wild-type mice that were HDM sensitized at the time of the first RSV infection.

We will select the model that renders the most significant differences between [redacted]-deficient and wild-type mice in clinically relevant parameters of allergic asthma (such as AHR, lung compliance, eosinophilic airway inflammation and airway remodeling) as the experimental model for the inception of asthma for use in the remainder of the project. The role of inhibitory receptors will be investigated in this optimised model using genetic modifications and agonists/antagonists of inhibitory receptors.

Epithelial gene expression signature. In addition, we will examine airway epithelial gene expression signatures in neonatal and adult mice. The airway

epithelium is thought to play a crucial role in asthma inception. We will sort airway epithelial cells from mice with induced asthma and controls, both neonatal and adult mice, using flow cytometry. In this manner, we can examine the effect of asthma-inducing stimuli and inhibitory immune receptor stimulation (by agonist treatment) on airway epithelium.

Airway responsiveness in anaesthetized mice
Mice are intraperitoneally anaesthetized with ketamine 125 mg/kg and medetomidine 0.2 mg/kg. The animals are ventilated (O2/air (1:2)) at a frequency of 150 beats/min. The mice are prepared for the measurement of the following lung parameters: pulmonary resistance (RL) and tidal volume (TV). Increasing doses of methacholine (acetyl-β-methyl-choline chloride) are administered by aerosol generated in a nebulizer. After the first dose of methacholine, pulmonary resistance (RL) dynamic compliance (Cdyn) and tidal volume (TV) are measured for 3 min, and this procedure is repeated for all doses.

Bioluminescence
Mice will be anaesthetized with isoflurane and subsequently injected with 100 µl of luciferin dissolved in phosphate-buffered saline (25 mg/kg). Bioluminescent signals of anaesthetized mice are collected over a ca. 10 min interval. This only applies to mice specifically infected with luciferase-expressing virus.

Inhibitory immune receptor agonists
Intraperitoneal, intravenous, or nebulised administration of inhibitory immune receptor agonist or antagonist will be administered at different time points to assess the effect on the relevant parameters mentioned above. The exact dose and administration method hereof are to be determined in future pilot experiments.

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

Based on estimated variation and the magnitude of biologically significant differences, we expect that an group size of 10 mice is sufficient to detect statistically significant differences. This has proven to be an appropriate group size for other asthma models in literature (Johnson *et al.*, Am J Physiol Lung Cell Mol Physiol, 2015). Additionally, a number of pilot experiments will be performed to determine optimal dosage, frequency, and timing of agonist delivery.

For epithelial gene signature studies, experience has shown that more animals per group are needed to obtain enough epithelial cells to perform reliable measurements. For each experimental group 40 mice are needed for 5 pools of 8 mice (i.e. 5 data points per group).

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Female and male mice (*Mus musculus*) will be used as experimental animals in equal measure. Mice are a well-established research model to study allergic sensitisation. Mouse models of asthma provide insight into the pathogenesis of airway hyperresponsiveness. The induced disease of mice mimics that of humans and clinically relevant parameters (discussed above), such as lung compliance, airway responsiveness, airway inflammation, and airway remodelling, can be assessed in mice (Kumar and Foster, Front Physiol, 2012). Moreover, many essential research tools, including genetic modifications and antibodies, are only available in the mouse system. The mice used in the proposed experiments, both wild-type and genetically modified strains, will be housed and bred at the common breeding facility (Gemeenschappelijk Dierenlaboratorium) at Utrecht University. Age of the mice depends on the exact experiment, as stipulated above.

[redacted]-deficient mice have been generated and will be provided by collaborators. There is no inherent discomfort that results from the genetic ablation of [redacted] Thus, breeding of [redacted] mice is not counted towards the total number of mice used.

In the five-year period of the project, we intend to optimize the house dust mite- and respiratory syncytial virus-induced asthma inception model. Subsequently, we will study the effect of multiple inhibitory immune receptors, [redacted], in the asthma inception model. Depending on the efficiency by which potent agonists to inhibitory receptors are generated, various agonists may also be tested. Experiments will be repeated twice (n=3) to ensure reproducibility of results. Additionally, a number of pilot experiments will be required to determine optimal dosage, frequency, and timing of agonist delivery.

We also reserve a limited number of mice for training purposes and validation of new respiratory syncytial virus batches. Only (new) group members who will be involved in our research for more than one year will be eligible for training in intranasal inoculation, neonatal infections, bronchoalveolar lavage, and other techniques related to the asthma inception model. In our experience, there is variation between respiratory syncytial virus batches and new batches should first be validated, preferably in a direct comparison with a previous batch. Estimated numbers needed: 30 mice/year. For this estimate, we take into account that at least 4 mice need to be used per tested batch (old and new) and the average size of newly made batches of virus (issues of practicality in virus culture prohibit the generation of significantly larger batches). On occasion, a virus batch does not evoke a proper response *in vivo*, in which case a new batch needs to be made and tested again with new (immune naïve) mice.

Estimated total number of mice needed in the 5-year period: 1500 mice. This different types of experiment are discussed above (2A, second box). For the optimisation of the HDM- and RSV-induced asthma model, the different experiments will performed once. Agonists treatment of HDM- and RSV-induced asthma and epithelial cell gene signature studies will be performed in triplicate.

<u>HDM- and RSV-induced asthma model optimisation</u>
RSV infection at 8 weeks
Mice per group: 10
Number of groups: 4 (mock WT, mock KO, RSV WT, RSV KO)
Total number of mice: 40

Neonatal RSV infection
Mice per group: 10
Number of groups: 4 (mock WT, mock KO, RSV WT, RSV KO)
Total number of mice: 40

RSV re-infection
Mice per group: 10
Number of groups: 4 (RSV/mock WT, RSV/mock KO, RSV/RSV WT, RSV/RSV KO; all neonatal RSV infection)
Total number of mice: 40

HDM dose study:
Mice per group: 10
Number groups: 8 (WT or KO (=2x); 0, 1, 5 or 25 µg HDM per treatment (=4x); 2x4=8 groups)
Total number of mice: 80

HDM re-challenge study
Mice per group: 10
Number of groups: 4 (HDM/mock WT, HDM/mock KO, HDM/HDM WT, HDM/HDM KO; all neonatal HDM exposure)
Total number of mice: 40

RSV/HDM challenge
Mice per group: 10
Number of groups: 2 (RSV/HDM WT, RSV/HDM KO; NB. RSV/mock WT vs. RSV/mock KO are already compared in "*RSV re-infection*")
Total number of mice: 20

HDM/RSV challenge
Mice per group: 10
Number of groups: 2 (HDM/RSV WT, HDM/RSV KO; NB. HDM/mock WT vs. HDM/mock KO are already compared in "*HDM re-challenge*")
Total number of mice: 20

Epithelial gene expression signature
Mice per group: 40
Number of groups: 4 (neonatal vs. adult [=2x]; with/without HDM- & RSV-induced asthma [=2x]; 2x2=4)
Reproducibility of results: n = 3
Total number of mice: 480

Agonist treatment
Mice per group: 10
Number of groups: 4 (mock unchallenged, mock asthma, agonist unchallenged, agonist asthma; all PCDH-1 KO mice)
Number of inhibitory receptors: 4
Reproducibility of results: n = 3
Total number of mice: 480

Pilot experiments (e.g. agonist administration during disease model at different intervals or with different doses):
Mice per experiment: 10
Estimated number of inhibitory receptor agonists tested: 4
Different conditions tested: 3 (e.g. timing, dosing, administration route)
Total needed: 120 mice (approximate)

Testing of virus batches and technique training (training is only for new members that join the research group from more than one year):
Mice per experiment: 10 (e.g. for virus batch testing: 2x mock infected, 4x old batch, 4x new batch)
Estimated frequency: 3x per year
Total needed: 10 x 3 x 5 = 150 mice (approximate)

The estimated grand total number of mice needed is therefore (40 + 40 + 40 + 80 + 40 + 20 +20 + 480 + 480 + 120 +150 = 1510) approximately 1500

mice.

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'?

X 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 to respiratory viral infections and allergic sensitisation are highly complex and multifaceted. Interactions between multiple compartments such as bone marrow, circulation, lymphatic system, secondary lymphoid organs, and lungs are involved. 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 immune response it elicits *in vitro*. This requires animal models. Similarly, the use of experimental agonist of inhibitory receptors to test their potential beneficial impact on the course of disease during viral infection 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 infections in mice, we will first study human cells *in vitro* to ensure we only perform the most relevant experiments in mice. Specifically, the expression and capacity of a particular inhibitory receptor to inhibit immune cells that are important to immune-mediated pathology can be tested using human healthy control and patient material. Only receptors that, based on these results, could potentially impact disease severity will be tested *in vivo* for their role in asthma inception. Whether the expression pattern and functional capabilities of the inhibitory receptor are similar in steady-state mice can be assessed using unchallenged mice (as described in appendix #5).

Reduction:
To minimise the number of surplus mice and ensure the highest degree of comparability between wild-type and genetically modified mice, the modified mouse strains will be maintained as heterozygotes. This way, separately maintaining genetically modified and matched wild-type strains is not necessary. This is intended for maintaining the mouse strains, not for breeding mice for experiments. On the eve of experiments, when large numbers of homozygous mice (both genetically modified and wild type) are required, we will temporarily breed mice from homozygous parents to prevent that many heterozygous mice are lost as surplus. Differences due to genetic drift between wild-type and genetically modified mice will be kept to a minimum in this way. This ensures that experimental differences observed between the wild-type and genetically modified mice are due to the intended genetic modification.

Statistical power calculation based on our own previous experience, pilot experiments, and literature will be used to determine the optimal number of mice for each experimental group.

Refinement:
Mice will be housed in groups in cages with nesting opportunities. Virus-inoculated mice will be assessed at least once per day, more if necessary, for clinical signs of infection, including weight loss. 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.

Mice suffering from unexpectedly severe discomfort will be euthanized.

During inoculation with virus, mice will be anaesthetised with isoflurane. No pain medication will be administered during the course of infection, since this could interfere with immune regulation, as we have shown for opioids [redacted]), but also analgesic nonsteriodal anti-inflammatory drugs interfere with immune signaling, e.g. via inhibition of cyclooxygenase enzymes that produce inflammatory lipid mediators. Moreover, discomfort experienced by experimental animals is likely to depend more on other factors such as shortness of breath or fever rather than pain. During invasive lung and heart function measurements, mice will be fully anaesthetised with ketamine and medetomidine.

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?

X No

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

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.

During inoculation with virus, mice will be anaesthetised with isoflurane. No pain medication will be administered during the course of infection, since this could interfere with immune regulation, as we have shown for opioids [redacted] but also analgesic nonsteriodal anti-inflammatory drugs interfere with immune signaling, e.g. via inhibition of cyclooxygenase enzymes that produce inflammatory lipid mediators. Moreover, discomfort experienced by experimental animals is likely to depend more on other factors such as dyspnea or fever rather than pain. During invasive lung and heart function measurements, mice will be fully anaesthetised with ketamine and medetomidine.

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

I. Other aspects compromising the welfare of the animals

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

As a direct result of viral infection, mice may experience discomfort in the form of shortness of breath and fever.
Where applicable, the administration of house dust mite, agonist/antagonist, or luciferin (for bioluminescence) can result in transient mild discomfort, resulting e.g. from intranasal instillation or intraperitoneal injection.
Once successfully induced, asthma symptoms such as shortness of breath can result in discomfort of mice.
While invasive lung function tests would result in severe discomfort, mice will be fully anaesthetised with ketamine and medetomidine during measurements and euthanized at completion.

Explain why these effects may emerge.

These are direct effects of viral respiratory infection and asthma.

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

This discomfort is inherent to the model of house dust mite- and respiratory syncytial virus-induced asthma and cannot be treated without compromising the model.

J. Humane endpoints

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

X No > Continue with question K.

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

Reaching a score of 4 on the illness scale and/or severe dyspnea; for adult mice, loosing more than 20% of original body weight.

Indicate the likely incidence.

Mice with experimentally induced asthma do not, as a rule, die from disease nor reach a humane endpoint. Only in the case of comorbidity, such as sickle cell disease, do mice show reduced survival (Nandedkar *et al.*, Blood, 2008).

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').

Cumulative levels of discomfort are expected to be moderate.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No > Continue with Section 3: 'signatures'.

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

Accurate assessment of lung function requires invasive measurements that are not compatible with continued survival of the test subject.

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

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
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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. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 4 Type of animal procedure: Characterisation and testing of inhibitory immune receptor agonists

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.

In order to study the function and the potential as therapeutic target of inhibitory immune receptors *in vivo*, compounds that can specifically engage these receptors, such as monoclonal antibodies, must be developed. For our project, such monoclonal antibodies will be generated in collaboration with the antibody facility of the UMC Utrecht. Prior to their use in disease models, newly developed compounds should be fully characterized in wild-type mice. Antibodies, for instance, can mediate the (undesired) depletion of cells that express the targeted receptor. Thus, the depleting potential, dissemination, and

pharmacokinetics of novel inhibitory receptor agonists and antagonists administered by multiple routes will be assessed. Following the final administration of agonists, mice may be studied for up to an additional 2 weeks.

Experimental groups
To characterize the *in vivo* effects of the agonists the following groups will be used:

1. Mock-treated wild-type mice
2. Agonist-treated wild-type mice

NB. In case of an agonistic antibody the treatment control will be an isotype-matched control antibody.
This basic set-up can be expanded to include, for instance, comparison of multiple doses, frequencies, and modes of agonist administration.

Relation to other experiments
Prior to performing animal experiments, we will study the expression and function of inhibitory immune receptors on immune cells derived from healthy controls and patients. In this setting, we can determine which inhibitory receptors can modulate immune cells thought to be important to the pathogenesis of several immune-mediated diseases. Only inhibitory receptors thus shown to potentially limit disease will be tested *in vivo*. For inhibitory receptors that were shown to limit disease severity/progression, (i.e. exacerbated disease in genetic knockout or antagonist treated mice), we will develop agonists and their potency will be assessed *in vitro*. Only the most promising/potent *in* vitro agonists will be tested *in vivo*. We will test respiratory viral infection (appendix #1), house dust mite- and respiratory syncytial virus-induced asthma (appendix 3), and systemic lupus erythematosus. The most potent agonists *in vitro* will be characterized in wild-type mice and screened for unwanted side effects, as described here, before testing therapeutic potential in the disease models, as described here.

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

At multiple time points, mice will receive varying doses of agonist/antagonist by injection, either intraperitoneal or intravenous, or as nebulised compound. Over time, the concentration of the administered agonist/antagonist in blood and other compartments will be analysed to assess pharmacokinetics and dissemination. The differentiated immune cell numbers of multiple compartments will be determined to examine possible depletion effects. Following the final administration of agonists, mice may be studied for up to an additional 2 weeks.

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

Groups will comprise up to 6 mice. These numbers are comparable to those used in experiments in published literature that look at (unintended) antibody-mediated cell depletion (Nishikado *et al.*, J Immunol, 2011). Thus, these numbers of mice are expected to reveal conclusively whether the agonists have undesired side effects.
Only *in vitro* verified agonists/antagonists will be used to minimise the number of mice used.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

The disease models in which the agonist/antagonist will ultimately be tested, i.e. respiratory viral infection (appendix #1), house dust mite- and respiratory syncytial virus- induced asthma (appendix #3), and pristane-induced lupus (appendix #6), will employ mice as experimental animal. Hence, the agonists/antagonists *in vivo* characterisation will be performed in mice. Female and male mice (*Mus musculus*) will be used as experimental animals in equal measure. The mice will be housed and bred at the common breeding facility (Gemeenschappelijk Dierenlaboratorium) at Utrecht University. Mice used in the experiments will be approximately 8-12 weeks of age, which confirms to the majority of mice used in the disease models.

Estimated total number of mice: 100 mice/year for total of 500 mice in 5-year period. Experimental groups will consist of 6 mice, with at least 2 groups (i.e.

12 mice per tested agonist). It is difficult to predict the efficiency by which new, potent inhibitory receptor agonists will be generated, which determines how many mice will be required. Nonetheless, the novel cellular immunisation technique developed by our collaborators at UMab (hybridoma facility UMC Utrecht) is highly efficient at generating high affinity antibodies, even against lowly immunogenic membrane proteins. Recently, we generated multiple new agonistic antibodies for the mouse inhibitory receptor [redacted] using this method, which show great promise in initial *in vitro* experiments. We therefore estimate to require ca. 100 mice per year (on average).

Agonist characterisation
- Mice per group: 5
- Administration routes: 3
- Doses: 3
- Number of inhibitory receptors: 4
- Number agonists (per receptor): 2
- Total number of mice: (5 x 3 x 3 x 4 x 2 =) 480

The estimated grand total number of mice needed is therefore approximately 500 mice

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:
Following full *in vitro* characterisation of the potency of agonists/antagonists, their effects on e.g. depletion have to be assessed *in vivo*. There could be unexpected effects that one would not detect *in vitro*, therefore agonists/antagonists must be characterized *in vivo*. Only those agonists/antagonists that show efficacy *in vitro* will be tested *in vivo*.

Reduction:
Full characterisation of the agonists/antagonists will ensure that only optimal agonists/antagonists will be used in the disease models. Fewer mice will therefore be subjected to the discomfort inherent in the disease models. Agonists/antagonists will be extensively tested *in vitro* for efficacy in targeting an inhibitory immune receptor prior to *in vivo* application. For instance, reporter cell lines that measurably (e.g. by production of fluorescent protein) respond to

signaling by inhibitory receptors will be employed to assess the stimulatory/inhibitory function of agonists/antagonists. Only the most promising agonists/antagonists will be subsequently tested *in vivo*. By not progressing to *in vivo* testing with suboptimal agonists/antagonists, this ensures that the number of mice used is reduced to a minimum.

Refinement:
Mice will be housed in groups in cages with nesting opportunities. The wellbeing of mice will be regularly inspected. If unexpected 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.

The wellbeing of mice will be regularly inspected. If unexpected suffering is observed, mice will be euthanized.

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?

X No

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

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?

X No > Continue with question I.

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

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

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

I. Other aspects compromising the welfare of the animals

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

Administration of the inhibitory immune receptor agonists/antagonists may cause mild transient discomfort e.g. from intraperitoneal injection.
Repeated collection of blood causes discomfort.
No adverse effects on wellbeing are expected to result from the agonist/antagonists themselves nor from possible side effect, such as transient immune cell depletion.

Explain why these effects may emerge.

Handling intrinsically causes discomfort for the mice.

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

Well-trained personnel will handle the mice.

J. Humane endpoints

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

X No > Continue with question K.

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

Indicate the likely incidence.

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').

Cumulative levels of discomfort are expected to be mild.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No > Continue with Section 3: ‘signatures’.

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

To assess e.g. immune cell numbers in compartments other than blood, such as spleen and bone marrow, mice need to be terminated. Removal of these organs is not compatible with continued survival of the mice.

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

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

X Yes

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.zbo-ccd.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.	ÙMC Utrecht
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 5 Type of animal procedure: Functional and phenotypical characterisation of genetically modified mice

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.

In our project, we will employ mice that have genetic modifications relating to inhibitory immune receptors to study the function and therapeutic potential of these receptors. This includes mice with genetic ablation of endogenous inhibitory receptors and mice with transgenic modifications. Differences between unchallenged modified and wild-type mice can inform us on effects observed in the disease models (described in other appendices). Moreover, steady-state differences between mice and *in vitro* behaviour of cells obtained from unchallenged mice can help elucidate the regulatory roles of specific inhibitory

receptors. We will study the differential (immune) cell numbers in multiple compartments, including blood, bone marrow, spleen, and lymph nodes; analyse histological changes, for instance lung and skin fibrosis; and assess immune cell function *ex vivo*, e.g. migration and production/release of antimicrobial molecules by neutrophils. Since ablation of inhibitory immune receptors could subtly disturb the regulation of the immune system, the effect of which may only be apparent at advanced age, we will also assess these parameters in aged (1-year old) mice. This will provide further insight into the biological role of the inhibitory immune receptors.

[redacted]

Experimental groups
Unchallenged wild-type and genetically modified mice will be compared against each other. For instance, in an *ex vivo* migration assay the groups may look as follows:

1. Young (8-12 weeks old), unchallenged wild-type mice
2. Young (8-12 weeks old), unchallenged genetically modified mice

In an experiment that examines, for instance, the lung function/histology of aged mice the included experimental groups will be:

1. Young (8-12 weeks old), unchallenged wild-type mice
2. Young (8-12 weeks old), unchallenged genetically modified mice
3. Old (up to ca. 1 year old), unchallenged wild-type mice
4. Old (up to ca. 1 year old), unchallenged genetically modified mice

Relation to other experiments
Prior to performing animal experiments, we will study the expression and function of inhibitory immune receptors on immune cells derived from healthy controls and patients. In this setting, we can determine which inhibitory receptors can modulate immune cells thought to be important to the pathogenesis of several immune-mediated diseases. To study the role of these inhibitory receptors, genetically modified mice will be used in animal models of these diseases. Specific effects (phenotypical and functional) of the genetic ablation will be assessed *ex vivo* with cells obtained from unchallenged wild-type and genetically modified mice.

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

The mice will receive no experimental treatment/challenge to treat/induce disease. Some mice will be aged up to ca. 1 year of age under regular housing conditions.

Airway responsiveness in anaesthetized mice
Mice are intraperitoneally anaesthetized with ketamine 125 mg/kg and medetomidine 0.2 mg/kg. The animals are ventilated (O2/air (1:2)) at a frequency of 150 beats/min. The mice are prepared for the measurement of the following lung parameters: pulmonary resistance (RL) and tidal volume (TV). Increasing doses of methacholine (acetyl-β-methyl-choline chloride) (0.75 -25 mg/ml, 10% puff for 10 seconds) are administered by aerosol generated in a nebulizer.

Using the nebulizer it is possible to adjust the rate of aerosol delivery by adjusting the delivery cycle from 0% to 100%. After the first dose of methacholine, pulmonary resistance (RL) dynamic compliance (Cdyn) and tidal volume (TV) are measured for 3 min, and this procedure is repeated for all doses.

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

None of the genetically modified mice experience discomfort inherent to the genetic modification itself and breeding of the mice is therefore not counted towards the total number of mice used. Possibly, aged mice may experience mild discomfort, but mice kept for breeding will not grow old enough to experience age-related discomfort.

Group sizes will vary based on the type of experiment. For an *ex vivo* experiment such as neutrophil migration, a group size of 3 mice would be sufficient. But when mice are aged to, for instance, assess lung function/histology, groups may reach an size of 10 mice. Power analyses will be based on previous experiments, literature, and pilot experiments.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Mice (*Mus musculus*) of both sexes will be used. In the house dust mite- and respiratory syncytial virus-induced asthma model (appendix #3) as well as systemic lupus erythematosus model (appendix #6) both sexes are also used and the use of both female and male mice as described here will provide further insight into the role of inhibitory immune receptors in those diseases. The mice, both wild type and genetically modified strains, will be housed and bred at the common breeding facility (Gemeenschappelijk Dierenlaboratorium) at Utrecht University. Several genetically modified strains are already present in the breeding facility, [redacted]. Additional genetically modified mouse strains may be obtained from collaborators, including, for instance, [redacted] In general, mice will be around 8-12 weeks old. Aged mice up to ca. 1-year old will also be used.

We reserve a limited number of mice for training purposes. Only (new) group members who will be involved in our research for more than one year will be eligible for training techniques such as blood collection, organ excision, and lung function measurements. Estimated numbers needed: 30 mice/year.

Estimated total number of mice required: 200 mice/year for a total of 1000 mice in the 5-year period. This estimate is based on factors discussed in more detail above. For aging/phenotyping experiments, groups will consist of 10 mice, with at least 4 groups (i.e. 40 mice per experiment) and will be repeated once or twice (i.e. minimum of 80-120 mice per fully confirmed experiment). This will be performed for multiple immune inhibitory receptors, [redacted]. *Ex vivo* functional experiments with cells from unchallenged mice will be guided by results obtained with human cells, both healthy controls and patients, and results obtained from the mouse disease models (appendix #1, #3, #6). Hence, it is difficult to predict exactly what experiments will be performed and the number of mice that are required. For example, respiratory syncytial virus-infected [redacted]-deficient mice show a significantly greater pulmonary neutrophil influx than wild-type mice, which warranted further *ex vivo* neutrophil migration experiments comparing [redacted]-deficient and wild-type neutrophils. This type of experiment requires 2 experimental (unchallenged) groups (i.e. wild-type and genetic knockout) with 3 mice in each group (i.e. 6 mice per experiment), and will be performed at least 3 times (i.e. minimum of 18 mice per fully confirmed [n=3] experiment). Based on the intended aging/phenotyping experiments and experience with *ex vivo* functional tests with cells from unchallenged mice, we estimate that we require ca. 200 mice per year (on average).

Aging experiments

Mice per experiment: 40 (e.g. 10x young WT, 10x young KO, 10x aged WT, 10x aged KO)

Estimated number of inhibitory receptors tested: 4

Number of inhibitory receptors tested: 4
Reproducibility of results: n = 3
Total needed: 40 x 4 x 3 = 480 mice

In vitro experiments:
Mice per experiment: 10 (5x WT, 5x KO)
- Number of mice depends highly on the required cells, e.g. bone marrow is rich in neutrophils, but obtaining sufficient lymph node cells from unchallenged mice is more of a challenge. This is only an educated guess. The cells of interest (and thereby the required number of mice) for *in vitro* experiments will be determined based on data obtained from patient vs. healthy controls and data from the mouse disease models.

Number of inhibitory receptors: 4
Types of experiments: 4 (e.g. migration, immune cell effector functions)
Reproducibility of results: n = 3
Total needed: 10 x 4 x 4 x 3 = 480 mice

Training
A limited number of mice is reserved for training purposes (only for new members that join the research group from more than one year), such as the excision of organs including bone marrow, lymph nodes, spleen, etc.
Expected number of mice needed: 10 / year, for 50 mice in 5-year period.

The estimated grand total number of mice needed is therefore (480 + 480 + 50 = 1010) approximately 1000 mice

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:
Here, we look at the systemic effects of genetic ablation of inhibitory immune receptors in the short- and long-term. Unfortunately, it is not yet possible to simulate the entire immune system, and its interaction with other organs, *in vitro*. Human patients with deleterious mutations are not known or accessible for study, nor would body-wide biopsies be feasible.

Reduction:
Steady-state differences in unchallenged wild-type versus genetically modified mice could provide information useful for the disease models, and strengthen these results. Consequently, fewer mice may need to be used in the disease model experiments. It will also allow us to compare *in vitro* human and mice data to confirm translatability and relevance.

To minimise the number of surplus mice and ensure the highest degree of comparability between wild-type and genetically modified mice, the modified mouse strains will be maintained as heterozygotes. This way, separately maintaining genetically modified and matched wild-type strains is not necessary. This is intended for maintaining the mouse strains, not for breeding mice for experiments. On the eve of experiments, when large numbers of homozygous mice (both genetically modified and wild type) are required, we will temporarily breed mice from homozygous parents to prevent that many heterozygous mice are lost as surplus. Differences due to genetic drift between wild-type and genetically modified mice will be kept to a minimum in this way. This ensures that experimental differences observed between the wild-type and genetically modified mice are due to the intended genetic modification.

Refinement:
Mice will be housed in groups in cages with nesting opportunities. The wellbeing of mice will be regularly inspected. If unexpected 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.

The wellbeing of mice will be regularly inspected. If unexpected suffering is observed, mice will be euthanized. During invasive lung and heart function measurements, mice will be fully anaesthetised with ketamine and medetomidine.

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?

X No

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

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?

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

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

During invasive lung function measurements of a subset of mice, the mice will be fully anaesthetised with ketamine and medetomidine.

I. Other aspects compromising the welfare of the animals

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

There may be mild age-related discomfort in the subset of mice selected for aging experiments.

Explain why these effects may emerge.

Aging may result in mild discomfort as physical fitness declines. This could be amplified in inhibitory receptor-deficient mice. Although effects in previously untested strains of inhibitory receptor-deficient mice are difficult to predict, previous experiments show that 1-year old LAIR-1-deficient mice did not appear to suffer from notable discomfort compared to wild type mice.

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

If unexpectedly severe discomfort 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?

X No > Continue with question K.

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

Indicate the likely incidence.

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’).

Cumulative levels of discomfort are expected to be mild.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No > Continue with Section 3: ‘signatures’.

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

To assess for example immune cell numbers in compartments other than blood, such as spleen and bone marrow, mice need to be terminated. Removal of these organs is not compatible with continued survival of the mice, and neither are invasive lung function measurements.

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

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.zbo-ccd.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. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 6 — Type of animal procedure: Induced auto-immune disease

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.

Autoimmune diseases can be chemically induced in mice to investigate pathogenesis and treatment. Systemic lupus erythematosus (SLE) will be induced by a single intraperitoneal injection of tetramethylpentadecane (TMPD, or commonly known as pristane). To investigate the biological role of inhibitory immune receptors in lupus pathogenesis, lupus will be induced in genetically modified and wild-type mice. Symptoms will develop over the course of 4-6 months. Disease progression will be scored by the assessment of hypergammaglobulinemia, presence of lupus autoantibodies (e.g. anti-nRNP/Sm and anti-dsDNA)

and the development of renal disease. Endpoint outcome parameters include deposition of glomerular immune complexes and glomerular hypercellularity. These are clinically relevant outcomes.

Experimental groups
Comparisons between wild-type and genetically modified mice will include at least 4 groups:

1. mock-injected wild-type mice
2. mock-injected genetically modified mice
3. pristane-injected wild-type mice
4. pristane-injected genetically modified mice

The basic set-up for an experiment to study the effect of agonists of inhibitory receptors on disease severity will include the following groups:

1. mock-injected wild-type mice treated with control
2. mock-injected wild-type mice treated with agonist
3. pristane-injected wild-type mice treated with control
4. pristane-injected wild-type mice treated with agonist

NB. In case of an agonistic antibody the treatment control will be an isotype-matched control antibody.
This basic set-up could be expanded to include for instance different agonist/control doses, as well as varying frequencies and time points of agonist/control administration depending on the results from pilot experiments. Optimal conditions may vary for different inhibitory receptors

Relation to other experiments
For inhibitory receptors that were shown to limit lupus disease severity/progression, (i.e. exacerbated disease in genetic knockout or antagonist treated mice), we will develop agonists and their potency will be assessed *in vitro*. The most promising agonists will be characterized in wild-type mice and screened for unwanted side effects, such as immune cell depletion (appendix #4). Fully vetted agonists will then be tested for amelioration of lupus disease severity as stipulated here. Specific effects (phenotypical and functional) of the genetic ablation will be assessed *ex vivo* with cells obtained from unchallenged wild-type and genetically modified mice (appendix #5). Investigation of inhibitory immune receptors in other mouse disease models, i.e. respiratory viral infection (appendix #1), and house dust mite- and respiratory syncytial virus-induced asthma (appendix #3) will be studied in parallel. Prior to performing animal experiments, we will study the expression and function of inhibitory immune receptors on immune cells derived from healthy controls and patients. In this setting, we can determine which inhibitory receptors can modulate immune cells thought to be important to the pathogenesis of several immune-mediated diseases. Only inhibitory receptors thus shown to potentially limit disease will be tested *in vivo*.

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

Pristane injection
Nature: intraperitoneal injection of approximately 0.5 mL pristane
Frequency: single application
Duration: disease progression will be assessed during ca. 6 months

Administration of agonist or antagonist
Intraperitoneal or intravenous administration of inhibitory immune receptor agonists or antagonists. The exact dose, timing, and frequency of administration are to be determined in future pilot experiments.

In the pristane model of lupus, a single intraperitoneal dose of TMPD results in the development of disease symptoms over the course of 4-6 months. Disease progression will be scored by the assessment of body weight, hypergammaglobulinemia, presence of lupus autoantibodies (e.g. anti-nRNP/Sm and anti-dsDNA) and renal disease. Body weight will be assessed every week. Serum levels of total IgM and IgG as well as the production of autoantibodies will be evaluated by ELISA. To this end, blood (ca. 100 µl) may be drawn once every two weeks by means of superficial tail cuts or mandibular puncture. Determination of proteinuria (dipstick test) will be used to score the development of nephritis. Upon sacrifice of the mice, deposition of glomerular immune complexes and glomerular hypercellularity will be quantified by immunohistochemistry; other (immunological) organs may also be studied by flow cytometry and histology analysis. Depending on the mouse strain (e.g. BALB/c or C57BL/6), animals can also develop signs of arthritis. The severity of the arthritis will be assessed using an established semiquantitative scoring system of 0–4 where 0=normal, 1=mild swelling, 2=moderate swelling, 3=swelling of all joints, and 4=joint distortion and/or rigidity and dysfunction. The cumulative score for all four paws of each mouse (maximum possible score 16, but scores above 10 are not expected) will be used as the arthritis score to represent overall arthritis severity and progression in an animal.

We will study clinically relevant outcome parameters in these mice. We will assess the effect of inhibitory receptors on these outcome parameters by employing genetically modified mouse strains and by the administration of inhibitory receptor agonists or antagonists.

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

Based on estimated variation and the magnitude of biologically significant differences, we expect that an group size of 16 mice is sufficient to detect statistically significant differences, using both male and female mice (Summers *et al*., J Autoimmun, 2010). For mock-treated control mice, a group size of 8 mice is deemed sufficient due to lower variation within groups. Experiments will be repeated once (n=2) to ensure reproducibility of results. Due to the long-term nature of the SLE disease model (ca. 6 months), it is common practice in literature to use sizable experimental groups and confirm the results in two independent experiments, rather than three (Chowdhary *et al*., Rheumatology [Oxford], 2007; Summers *et al*., J Autoimmun, 2010). Additionally, a number of pilot experiments will be performed to determine optimal dosage, frequency, and timing of inhibitory receptor agonist delivery.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Female and male mice (*Mus musculus*) will be used as experimental animals in equal measure. Pristane-induced lupus in mice is a well-established animal model that closely mimics human disease, including key features such as anti-double stranded DNA autoantibody production, an interferon signature, and immune complex deposition-induced nephritis (Reeves *et al.*, Trends Immunol, 2009). Clinically relevant parameters can thus be measured in mice. Many essential research tools, including genetic modifications and antibodies, are only available in the mouse system. The mice used in the proposed experiments, both wild-type and genetically modified strains, will be housed and bred at the common breeding facility (Gemeenschappelijk Dierenlaboratorium) at Utrecht University. Mice used in the experiments will be approximately 8-12 weeks of age, as described in published literature.

In the five-year period of the project, we intend to study the effect of multiple inhibitory immune receptors, [redacted] in the lupus model. Depending on the efficiency by which potent agonists to inhibitory receptors are generated, multiple agonists may also be tested. Additionally, a number of pilot experiments will be performed to determine optimal dosage, frequency, and timing of inhibitory receptor agonist delivery.

Estimated total number of mice needed in the 5-year period: 1500 mice. This estimate is based on factors discussed in more detail above.
Experiments with KO mice (proposal figure Phase 2a):
- Mice per experiment: 48 (e.g. 8x mock WT, 8x mock KO, 16x pristane WT, 16x pristane KO)
- Estimated number of inhibitory receptors tested: 4
- Reproducibility of results: n = 2

Total needed: 48 x 4 x 2 = 384 mice

Experiments with agonist treated mice (proposal figure Phase 4):
Mice per experiment: 24 (e.g. 8x mock & control WT, 8x mock & agonist WT, 16x pristane & control WT, 16x pristane & agonist WT)
Estimated number of inhibitory receptors tested: 4
Estimated number of viruses tested: 2
Reproducibility of results: n = 2
Total needed: 48 x 4 x 2 = 384 mice

Pilot experiments (e.g. agonist administration during disease model at different intervals or with different doses):
Mice per experiment: 20
Estimated number of inhibitory receptor agonists tested: 4
Different conditions tested: 3 (e.g. timing, dosing, administration route)
Total needed: 240 mice

The estimated grand total number of mice needed is therefore (348 + 384 + 240 = 1008) approximately 1000 mice.

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 that induce autoimmune diseases are complex and multifaceted. Interactions between multiple compartments such as bone marrow, circulation, lymphatic system, secondary lymphoid organs, and, particularly in the case of SLE, the kidneys are involved. Certain aspects of the immune response can be separately simulated *in vitro*, but it is not yet possible to study the full course and development of aberrant immune responses and the autoimmune diseases these elicits *in vitro*. This requires animal models. Similarly, the use of experimental agonist of inhibitory receptors 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 the disease, such as the deposition of immune complexes in glomeruli. In order to reduce the number of animals used for experimentation, we will first conduct *in vitro* assays using human cells to ensure we only

perform the most relevant experiments *in vivo*. Specifically, the expression and capacity of a particular inhibitory receptor to inhibit immune cells that are important to immune-mediated pathology can be tested using human healthy control and patient material. Only receptors that, based on these results, could potentially impact disease will be tested *in vivo* for their role in the development/severity of SLE. Whether the expression pattern and functional capabilities of the inhibitory receptor are similar in steady-state mice can be assessed using unchallenged mice (as described in appendix #5).

Reduction:
To minimise the number of surplus mice and ensure the highest degree of comparability between wild-type and genetically modified mice, the modified mouse strains will be maintained as heterozygotes. This way, separately maintaining genetically modified and matched wild-type strains is not necessary. This is intended for maintaining the mouse strains, not for breeding mice for experiments. On the eve of experiments, when large numbers of homozygous mice are required (both genetically modified and wild type), we will temporarily breed mice from homozygous parents to prevent that many heterozygous mice are lost as surplus. Differences due to genetic drift between wild-type and genetically modified mice will be kept to a minimum in this way. This ensures that experimental differences observed between the wild-type and genetically modified mice are due to the intended genetic modification.

Refinement:
Mice will be housed in groups in cages with nesting opportunities. Mice will be regularly inspected (twice per week) for the development of clinical symptoms and body weight will be measured. Humane endpoints have been defined. A maximum arthritic clinical score of 12 is allowed in the experiment. If an animal has a score higher than 12, it will be sacrificed immediately. In addition, animals with arthritis scores between 10 and 12 are expected to have difficulty reaching the lid of the cage. Therefore, food and water (gelpacks) will be provided on the floor of the cage. 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.

Animals with arthritis scores between 10 and 12 are expected to have difficulty reaching the lid of the cage. Therefore, food and water (gelpacks) will be provided on the floor of the cage. Mice suffering from unexpectedly severe discomfort will be euthanized.
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?

X No

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

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.

No pain medication will be administered. As pain killers might influence the outcome of the experiments, as they are anti-inflammatory, it is not possible to use these compounds.

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

I. Other aspects compromising the welfare of the animals

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

Mice may present with mild-erosive arthritis.
Repeated blood collection will cause mild transient discomfort.

Explain why these effects may emerge.

Joint involvement is an feature of lupus. In mice, the frequency of arthritis among pristine-injected mice depends on the strain of the mice (e.g. significantly more common in BALB/c compared to C57BL/6).

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

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.

A maximum arthritic clinical score of 12 is allowed in the experiment. If an animal has a score higher than 12, it will be sacrificed immediately. If mice lose more than 20% of original weight, mice will be euthanised.

Indicate the likely incidence.

The frequency of arthritis among pristine-injected mice depends on the strain of the mice. If any signs of arthritis appear, the severity is expected to be mild. Even mice of a genetic background sensitive to arthritis development (e.g. BALB/c), will only present with mild-erosive arthritis. Scores greater than 10 are not expected to surface.

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').

Cumulative levels of discomfort resulting from induced lupus model are expected to be moderate.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No > Continue with Section 3: 'signatures'.

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

Deposition of glomerular immune complexes and glomerular hypercellularity are critical outcome parameters to our study. To examine these, histopathological analyses must be performed on the kidneys. Removal of kidneys is incompatible with continued survival of the mice.

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

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

X Yes

A. Algemene gegevens over de procedure

1. Aanvraagnummer : 2015.II.572.030
2. Titel van het project : Inhibitory immune receptors as therapeutic targets to dampen injurious immune responses
3. Titel van de NTS : Nieuwe behandelmethoden voor schadelijke afweerreacties

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: 14-09-2015
   - ☐ aanvraag compleet:
   - ☒ in vergadering besproken: 23-09-2015 en 21-10-2015
   - ☒ anderszins behandeld: per email: 26-10-2015
   - ☒ termijnonderbreking(en) van / tot : 30-09-2015 tot 09-10-2015
     23-10-2015 tot 26-10-2015
   - ☐ besluit van CCD tot verlenging van de totale adviestermijn met max. 15 werkdagen:
   - ☐ aanpassing aanvraag:
   - ☒ advies aan CCD: 16-11-2015

7. Eventueel horen van aanvrager
   - Datum: 21-10-2015
   - Plaats: Utrecht
   - Aantal aanwezige DEC-leden: 5 DEC-leden
   - Aanwezige (namens) aanvrager: postdoc en promovendus

   - Strekking van de vragen:
   - De DEC vraagt de onderzoekers nog eens uit te leggen dat de verschillende diermodellen die in het project worden gebruikt aantoonbaar passen in de hoofdvraagstelling.
   - Klopt het dat de te gebruiken agonisten niet in het kader van dit project worden geproduceerd maar verkregen worden van de in huis aanwezige faciliteit die een eigen vergunning heeft voor dierproeven?
   - Hoe worden naast de in de aanvraag genoemde en gepubliceerde inhibitory receptors nieuwe kandidaat receptoren gevonden en hoe wordt dat ingepast in het project?

- Strekking van de antwoorden:
- De uitleg is helder en de onderzoekers wijzen erop dat hier extra aandacht aan is besteed in het projectvoorstel om de overkoepelende hoofdvraagstelling beter tot zijn recht te laten komen (pag. 4 en 8). Voorts is op advies van de DEC een figuur toegevoegd die de relatie van de te gebruiken diermodellen met de hoofddoelstelling van het project weergeeft.
- In de tekst staat nu duidelijk vermeld dat de bereiding van de potentieel agonistisch werkende antistoffen gebeurt in een in house faciliteit met een eigen vergunning voor het maken van antistoffen. Nu duidelijk vermeld in het projectvoorstel (pag. 2, 4 en 6)
- Potentieel agonistische antistoffen (en de counterparts daarvan) verkregen van de in house faciliteit worden in in vitro experimenten getest (veelal celkweken) op hun functie en werkzaamheid. Een aanvullende beschrijving is toegevoegd aan het projectvoorstel en in bijlage 4 (pag. 7).
- Het horen van de aanvrager heeft geleid tot aanpassing van de aanvraag: Ja

8. Correspondentie met de aanvrager
- Datum: 30-09-2015
- Strekking van de vragen:

Projectvoorstel
- 3.1 Achtergrond: U noemt drie remmers, maar de DEC heeft het idee dat u ook nog op zoek bent naar andere remmers. Hoe gaat u dat doen en kunt u enig zicht geven op het succes daarvan?
- 3.4 Onderzoeksstrategie: De DEC zou ter illustratie graag een schema/figuur in het projectvoorstel opgenomen zien, om de samenhang tussen de verschillende bijlagen weer te geven.
- 3.4.1: In de 4e regel van onder staat: “Both house dust mice …”, maar dit moet volgens de DEC mite zijn. Graag wijzigen.
- 3.4.2: Hetgeen geformuleerd is in eerste alinea is sterk gecondenseerd opgeschreven en daardoor niet makkelijk te lezen (bv. betekenis tweede zin is onduidelijk). De DEC raadt u aan dit anders te formuleren.

Alle bijlagen
- Experimentele aanpak en primaire uitkomstparameters: Gezien het feit dat het gaat om fundamenteel exploratief onderzoek, raadt de DEC u aan het aantal dieren globaal te onderbouwen en in grote lijnen de benodigde aantallen dieren weer te geven. U dient in ieder geval in de verschillende bijlagen dezelfde berekeningsstrategie te gebruiken. De DEC raadt u aan dit te overleggen met de IvD.

Bijlage 1

- A. Experimentele aanpak en primaire uitkomstparameters: De DEC adviseert u benauwdheid op te nemen in het scoresysteem. Graag hierover contact opnemen met de IvD.
- A. Experimentele aanpak en primaire uitkomstparameters: De DEC gaat ervan uit dat het RSV luciferase model gevalideerd is. Kunt u hier een referentie voor geven?
- A. Experimentele aanpak en primaire uitkomstparameters: De bron van antagonisten en agonisten graag opnemen in de aanvraag.

Bijlage 2

- A. Experimentele aanpak en primaire uitkomstparameters: U heeft het hier over agonisten en antagonisten, maar antagonisten worden verder niet meer genoemd in deze bijlage.
- B. De dieren: Omdat u niet alleen [redacted] onderzoekt maar ook andere inhibatoire remmers gaat onderzoeken vraagt de DEC zich af of u dan niet ook mannetjes moet gebruiken, net zoals in de overige bijlagen.

Bijlage 3

- A. Experimentele aanpak en primaire uitkomstparameters: De DEC verzoekt u het gebruik van protocadherin-1 deficiënte muizen te onderbouwen. Waarom heeft u gekozen voor dit gen?
- A. Experimentele aanpak en primaire uitkomstparameters: Het is de DEC niet helder waarom u gebruik maakt van een UV- geïnactiveerd RSV. Wat is de ratio voor de UV-bestraling van het virus?

Bijlage 4

- D. Vervanging, vermindering en verfijning: U dient hier aan te geven wat u heeft gedaan aan vermindering in de betreffende bijlage, nu is het te algemeen geformuleerd. Graag scherper herformuleren.

Bijlage 6

- A. Experimentele aanpak en primaire uitkomstparameters: De DEC vraagt zich af waarom u in deze bijlage rekent met N=2 i.p.v. N=3 zoals in de andere bijlagen. Graag verhelderen.

- Datum antwoord: 09-10-2015
- Strekking van de antwoorden:

Projectvoorstel

- 3.1 Achtergrond: Momenteel richten we ons onderzoek voornamelijk op een viertal remmende receptoren, [redacted]. Er zijn huidig geen concrete plannen om dit op korte termijn uit te breiden. Geregeld bestuderen wij echter expressiepatronen van meerdere remmende receptoren op immuuncellen. Als bijv. bij

onderzoek met patiëntmateriaal een bepaalde remmende receptor in het oog springt door de wijze van expressie, houden wij graag de mogelijkheid open om verder onderzoek hieraan te wijden.

- 3.4 Onderzoeksstrategie: Een schema dat een globaal beeld van het project schetst is toegevoegd aan de aanvraag, te weten onder kopje 3.4.3 (projectvoorstel, pag. 8).
- 3.4.1: De typefout is verbeterd.
- 3.4.2: Formulering is aangepast ten behoeve van leesgemak.

Alle bijlagen

- Experimentele aanpak en primaire uitkomstparameters: Een verder uitgewerkte (uniforme) aantallenberekening is toegevoegd aan de verschillende bijlages.

Bijlage 1

- A. Experimentele aanpak en primaire uitkomstparameters: In overleg met de IvD is benauwdheid opgenomen in het scoresysteem en als apart criterium voor het humaan eindpunt (bijlage 1, pag. 2 pag. 7, pag. 9).

- A. Experimentele aanpak en primaire uitkomstparameters: Het gebruik van gemodificeerd RSV dat luciferase tot uitdrukking brengt om in vivo virusreplicatie te visualiseren is inderdaad een gevalideerd model, zie bijv. Rameix-Welti et al., Nat Commun, 2014. Dit geldt ook voor andere virussen die luciferase tot uitdrukking brengen, zoals gemodificeerd mouse hepatitis corona virus, welke onze groep eerder heeft gebruik ([redacted]. De referenties zijn toegevoegd aan de aanvraag (bijlage 1, pag. 2).
- A. Experimentele aanpak en primaire uitkomstparameters: Een verwijzing naar de hybridomafaciliteit van het UMC Utrecht (UMab), zoals ook vermeld in de projectbeschrijving, is toegevoegd (bijlage 1, pag. 1).

Bijlage 2

- A. Experimentele aanpak en primaire uitkomstparameters: Onze focus ligt op het gebruik van agonisten van remmende receptoren om proof-of-concept te verkrijgen voor hun therapeutische toepassingen. Antagonisten zouden kunnen dienen als verdere bevestiging van knock-out modellen, en om een genetische knock-out te simuleren als deze niet beschikbaar is. Deze toelichting is aan de aanvraag toegevoegd (bijlage 2, pag. 1-2).
- B. De dieren: Het doel van dit model is het verkrijgen van mechanistisch inzicht in de immuunrespons die zich voordoet bij virale luchtweginfecties (d.w.z. model/bijlage 1). In het virale luchtweginfectie model gebruiken wij enkel vrouwelijke dieren omdat deze een sterkere (en daarmee schadelijkere) antivirale afweerreactie ten toon stellen dan mannelijke dieren en wij geïnteresseerd zijn in het tegengaan van immuun-gemedieerde schade. Vanwege de verergerde immuunpathologie bij vrouwelijke dieren zijn deze hier een gevoeliger model voor dan mannelijke dieren. Vandaar dat wij bij de verdere bestudering van

mechanistische aspecten ook enkel vrouwelijke dieren gebruiken. Deze uitleg is toegevoegd aan de aanvraag (bijlage 2, pag. 3)

Bijlage 3

- A. Experimentele aanpak en primaire uitkomstparameters: Recent is een duidelijke associatie tussen bepaalde [redacted] genvarianten met astma en eczeem aangetoond (Koppelman et al., Am J Respir Crit Care Med, 2009; Koning et al., Pediatr Allergy Immunol, 2012). Daarnaast interacteert protocadherin-1 SMAD3, waarvan bepaalde genetische varianten ook met astma geassocieerd zijn (Faura Tellez et al., Am J Physiol Lung Cell Mol Physiol, 2015;Moffatt et al., N Engl J Med, 2010). Preliminaire data suggereren dat [redacted] deficiëntie lijkt daarom een passende benadering van een genetische aanleg voor astma. Verder blijkt uit pilotexperimenten dat [redacted] deficiënte muizen gevoeliger zijn voor RSV-infectie, aangezien deze o.a. een verergerde immuuncelinflux in de longen vertonen. Deze toelichting is toegevoegd aan de aanvraag (bijlage 3, pag. 1-2).
- A. Experimentele aanpak en primaire uitkomstparameters: UV-geïnactiveerd virus zal fungeren als controle voor de inoculatie met 'levend' virus. Het UV-geïnactiveerde virus bevat nog steeds immuno-stimulerende componenten zoals virale eiwitten en viraal RNA, maar zal geen productieve infectie op gang brengen. Dit is een betere controle dan enkel fosfaat-gebufferde zoutoplossing (PBS). Deze toelichting is toegevoegd aan de aanvraag (bijlage 3, pag. 2).

Bijlage 4

- D. Vervanging, vermindering en verfijning: Er is nu duidelijker geformuleerd dat vooraf aan het in vivo testen van agonisten/antagonisten eerst in vitro experimenten uitgevoerd zullen worden die duidelijk zullen maken welke agonisten/antagonisten het meest veelbelovend zijn. In vergelijking suboptimale agonisten/antagonisten zullen niet in vivo toegepast worden, hetgeen het aantal benodigde muizen tot een minimum beperkt. (Zie bijlage 4, pag. 3).

Bijlage 6

- A. Experimentele aanpak en primaire uitkomstparameters: Het lupus model is een langdurig, chronisch model. Eén experiment neemt (meer dan) zes maanden in beslag. In de wetenschappelijke literatuur is het gebruikelijk om aanzienlijke groepsgroottes te gebruiken (ook om voortijdige uitval op te kunnen vangen, bijv. vanwege ongerief door gevechten tussen muizen die in groepen zijn gehuisvest) en experimenten éénmalig te herhalen om reproduceerbaarheid te garanderen. Deze toelichting is toegevoegd aan de aanvraag (bijlage 6, pag. 3).

- De antwoorden hebben geleid tot aanpassing van de aanvraag: Ja

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

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. Het project is:
   - ☒ uit wetenschappelijk oogpunt verantwoord.
   - ☐ uit onderwijskundig oogpunt verantwoord.
   - ☐ uit het oogpunt van productiedoeleinden verantwoord.
   - ☐ wettelijk vereist.

2. De in de aanvraag aangekruiste doelcategorie is in overeenstemming met de hoofddoelstelling.

3. De DEC onderschrijft het belang van de doelstelling, die beoogt de kennis over natuurlijke remmers van de immunologische reactie (zijnde receptoren op cellen die een rol spelen in de afweer) uit te breiden en bewijs te verzamelen dat deze remmende receptoren aangrijpingspunten bieden om ongewenste of uit de hand lopende excessieve en daardoor beschadigende afweerreacties in te tomen. Het wordt ingeschat als meer dan een substantieel belang, omdat er een veelvoud van ziekten bestaat waar een verkeerd gerichte of excessief heftig verlopende afweerreactie zorgt voor ernstige immunopathologie. Te denken valt aan autoimmuunziekten (verkeerd gerichte afweer), infecties met bepaalde virussen en allergische reacties (hyperactiviteit van het afweersysteem). Het kunnen activeren van de remsystemen is het finale doel van dit type onderzoek. De onderzoekers zien hun project als een preklinisch exploratief fundamenteel onderzoek dat gezien het feit dat het nog in de beginfase verkeert breed moet worden ingezet. Het projectonderwerp - remmers van het immuunsyteem - kan gezien worden als complementair aan de ontwikkeling van de zogenaamde checkpoint inhibitors die ervoor zorgen dat een immunologische reactie op gang komt. Checkpoint inhibitors worden inmiddels met succes toegepast in de behandeling (immunotherapie) van een aantal vormen van kanker.

4. De gekozen strategie en experimentele aanpak kunnen leiden tot het behalen van de doelstelling binnen het kader van het project. De DEC is ervan overtuigd dat de aanvrager over voldoende expertise en voorzieningen beschikt om de projectdoelstelling met de gekozen strategie/aanpak binnen de gevraagde termijn te realiseren. De strategie omvat *in vitro* vooronderzoek met menselijk materiaal dat al geleid heeft tot het karakteriseren van een aantal inhibitoire receptoren. Ook zijn in het vooronderzoek al een beperkt aantal dierproeven uitgevoerd die een *proof of concept* hebben laten zien van de mogelijkheid om de demping van de immunologische reactie te verkrijgen door activatie van de inhibitoire receptor, met als gevolg minder immuunpathologie c.q. beschadiging. Het project zet in op het gebruik van een aantal muis diermodellen, te weten een model voor virus-geïnduceerde luchtweginfectie (bijlage 1), ontsteking van de luchtwegen, niet-infectieus geïnduceerd (bijlage 2), huismijt en RSV geïnduceerd astma in een k.o. muizenmodel (bijlage 3) en auto-immuniteit in een model voor SLE (bijlage 6). In bijlage 4 worden de nieuwe te testen agonisten (en antagonisten) van de inhibitoire receptoren gekarakteriseerd alvorens te worden gebruikt in de dierproeven. In bijlage 5 worden de genetisch gemodificeerde dieren, gemodificeerd voor de receptoren, nader onderzocht.

5. 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)
   - ☐ Gefokt voor dierproeven (11)
   - ☐ Zwerfdieren (10h)
   - ☐ Hergebruik (1e lid 2)
   - ☐ Huisvesting en verzorging
   - ☐ Locatie: instelling vergunninghouder (10g)

6. Het ongerief als gevolg van de dierproeven is realistisch ingeschat en geclassificeerd. In de bijlagen 1, 2 en 3 is het cumulatieve ongerief ingeschat als matig voor de dieren die geïnfecteerd zijn met een virus (bijlage 1), voor de dieren waarin een steriele ontsteking is geïnduceerd (bijlage 2) en voor de dieren waarin astma optreedt ten gevolge van blootstelling aan huismijt en een virusinfectie (bijlage 3). De score 'matig ongerief' betreft maximaal 75 % van de dieren in deze experimenten; 25 % van de dieren ondergaat mild ongerief. In bijlage 4 en 5, waar respectievelijk agonisten/antagonisten en genetisch gemodificeerde dieren worden gekarakteriseerd, ondergaan de dieren naar schatting licht ongerief. In bijlage 6 is de ongeriefscore geschat op 'matig' in maximaal 75 % van de dieren en op 'licht' in 25 % van de dieren. Gezien het feit dat het hier gaat om exploratief onderzoek zijn de ongerief scores inschattingen op basis van eerdere ervaringen. De verwachting is dat indien de agonisten werken, dat wil zeggen de rem op de immunologische reactie versterken, het percentage dieren dat matig ongerief ondervindt zal dalen.

7. Er zijn geen methoden die de voorgestelde dierproeven geheel of gedeeltelijk zouden kunnen vervangen. *In vitro* vooronderzoek met menselijke cellen heeft geleid tot identificatie van een aantal inhibitoire receptoren. Op deze receptoren wordt in eerste instantie het onderzoek geconcentreerd. Om het hoofddoel te verwezenlijken zijn diermodellen nodig waarin aantoonbare immuunpathologie is opgetreden. Het remmen daarvan is hoofddoel van het project.

8. In het project wordt optimaal tegemoet gekomen aan de vereiste van de vermindering van dierproeven. Het maximale aantal te gebruiken dieren is realistisch ingeschat en voor zover mogelijk in een exploratief onderzoek ook onderbouwd. De DEC heeft het advies gegeven aan de onderzoeker om de getallen af te ronden, omdat het niet realistisch is om een exact aantal te benoemen. In bijlage 5, waarin de genetisch gemodificeerde dieren worden beschreven, is aangegeven dat er extra aandacht is voor het voorkomen van fokoverschot door de fok op uitgekiende tijden te doen plaatsvinden, zodat zoveel mogelijk onderzoekers van de opbrengst gebruik kunnen maken.

9. Het project is in overeenstemming met de vereiste van de verfijning van dierproeven en het project is zo opgezet dat de dierproeven zo humaan mogelijk kunnen worden uitgevoerd. Er is geen sprake van ongewenste milieueffecten. De onderzoekers gebruiken in bijlage 1 en 2 vrouwelijke muizen, omdat het vooronderzoek (inmiddels gepubliceerd) heeft aangetoond dat het beloop van de virusinfectie/ longontsteking in vrouwelijke dieren meer immunopatologie toont dan in mannelijke muizen. Effecten van agonisten ijn daardoor met meer kans op succes te bestuderen in vrouwelijke muizen dan in mannelijke muizen.

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

D. Ethische afweging

De doeleinden van dit vernieuwende fundamentele exploratieve project, zoals geformuleerd onder C3, rechtvaardigen het voorgestelde gebruik van de muizen. De schade die de dieren oplopen is verantwoord, omdat vanuit wetenschappelijk oogpunt verwacht mag worden dat het onderzoek inzicht zal geven in de werking van constitutieve remsystemen in het immuunapparaat zelf, die op termijn mogelijk kunnen worden aangewend om uit de hand lopende immunologische reacties te beteugelen. Gezien het vooronderzoek waarin *proof of concept* is geleverd mag de kans op succes als veelbelovend worden ingeschat. Schade door excessieve immunologische reacties komt voor bij een veelvoud van ziekten. De onderzoekers hebben er daarom voor gekozen drie modellen van ziekte waar immunopathologische schade evident is in het onderzoek te betrekken. De DEC steunt deze brede aanpak mede, omdat dit type onderzoek nog in de beginfase verkeert. Dit alles overwegende oordeelt de DEC unaniem dat het belang van het doel van het project opweegt

tegen het ongerief dat de muizen zullen ondervinden. De DEC acht het gebruik van de dieren ethisch aanvaardbaar.

E. Advies

1. Advies aan de CCD

☐ De DEC adviseert de vergunning niet te verlenen vanwege:

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

☒ De DEC adviseert de vergunning te verlenen.

2. Het uitgebracht advies is gebaseerd op consensus.

[redacted]

[redacted]

11

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

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[redacted]
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centralecommissiedierproeven.nl
0900 28 000 28 (10 ct/min)
info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD115002015322
Bijlagen
2

Datum 24 november 2015
Betreft Ontvangstbevestiging Aanvraag projectvergunning Dierproeven

Geachte [redacted]
Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 24 november 2015.
Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD115002015322. 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).

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlagen:
- Gegevens aanvraagformulier
- Factuur

Gegevens aanvrager

Uw gegevens	
Deelnemersnummer NVWA:	11500
Naam instelling of organisatie:	Instantie voor Dierenwelzijn 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:	[redacted]
Afdeling:	[redacted]
Telefoonnummer:	[redacted]
E-mailadres:	[redacted]

Over uw aanvraag

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

Over uw project

Geplande startdatum:	1 januari 2016
Geplande einddatum:	31 december 2020
Titel project:	Inhibitory immune receptors as therapeutic targets to dampen injurious immune respons
Titel niet-technische samenvatting:	Nieuwe behandelmethoden voor schadelijkeafweerreacties
Naam DEC:	DEC Utrecht
Postadres DEC:	Postbus 85500 3508 GA Utrecht
E-mailadres DEC:	dec-utrecht@umcutrecht.nl

Betaalgegevens

De leges bedragen:	€ 741,-
De leges voldoet u:	na ontvangst van de factuur

Checklist bijlagen

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

Ondertekening

Naam:	[redacted]
Functie:	[redacted]
Plaats:	Utrecht
Datum:	23 november 2015

Centrale Commissie Dierproeven

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Instantie voor Dierenwelzijn Utrecht
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3508 TA UTRECHT

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Onze referentie
Aanvraagnummer
AVD115002015322

Bijlagen
2

Datum 24 november 2015
Betreft Factuur aanvraag projectvergunning Dierproeven

Factuur
Factuurdatum: 24 november 2015
Vervaldatum: 24 december 2015
Factuurnummer: 15700322
Ordernummer: CB. 841910.3.01.011

Omschrijving	**Bedrag**
Betaling leges projectvergunning dierproeven Betreft aanvraag AVD115002015322	€ 741,00

Wij verzoeken u het totaalbedrag vóór de gestelde vervaldatum over te maken op rekening NL28RBOS 056.99.96.066 onder vermelding van het factuurnummer en aanvraagnummer, ten name van Centrale Commissie Dierproeven, Postbus 20401, 2500 EK te 's Gravenhage.

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Centrale Commissie Dierproeven

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Onze referentie
Aanvraagnummer
AVD115002015322

Uw referentie
-

Bijlagen
1

Datum 28 december 2015
Betreft Aanvulling Aanvraag projectvergunning dierproeven

Geachte [redacted],

Op 24 november 2015 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Inhibitory immune receptors as therapeutic targets to dampen injurious immune responses" met aanvraagnummer AVD115002015322. 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:

Projectvoorstel

Onduidelijkheden
- Wij begrijpen dat het aantal te gebruiken dieren een schatting is. Echter, de inschatting van het aantal dieren dient beter onderbouwd te worden. U geeft bijvoorbeeld in appendix 1 en 2 aan dat u meerdere virussen/inflammatory mediators gaat testen. Daarnaast geeft u in appendix 1, 2, 3, 5 en 6 aan dat de experimenten meerdere keren worden uitgevoerd om de reproduceerbaarheid te testen. Kunt u de beslismomenten beter beschrijven, wanneer u bijvoorbeeld beslist om een tweede virus te testen en waarop u de beslissing baseert om een experiment wel/niet te herhalen?

- Appendix 2, vraag B. Hier wordt op pagina 4 bij de 4e paragraaf gesproken over Estimated number of viruses tested. Wij nemen aan dat u hiermee bedoelt estimated number of inflammatory mediators tested. Kunt u dit bevestigen?

- In appendix 5 beschrijft u dieren nodig voor trainingsdoeleinden. Het aantal dieren dat u hiervoor nodig denkt te hebben is 30 per jaar (2e alinea vraag B) of 10 per jaar (10 dieren per jaar). Het is ons niet duidelijk welk aantal correct is. Kunt u dit consistent maken?

- In appendix 5 beschrijft u een fok met dieren waarbij de dieren op latere leeftijd ongerief kunnen ondervinden van de genetische modificatie. U geeft aan dat deze dieren dit ongerief echter niet zullen ondervinden omdat zij deze leeftijd niet zullen bereiken. Omdat deze dieren wel risico lopen dit ongerief te ondervinden, moet deze fok wel als dierproef worden beschouwd en heeft u voor deze fok een vergunning nodig in het kader van de Wod. U dient deze fok dus op te nemen in onderliggende aanvraag, of hiervoor een aparte aanvraag in te dienen.

Wij vragen u deze informatie te verduidelijken.

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.

Bijlage:
- formulier Melding Bijlagen via de post

Centrale Commissie Dierproeven

Melding

Bijlagen via de post

- U wilt één of meerdere bijlagen naar ons versturen? Voeg *altijd* deze Melding Bijlagen toe. Wij weten dan welke documenten van u zijn en hoeveel documenten u opstuurt.
- Meer informatie vindt u op www.centralecommissiedierproeven.nl
- Of bel met ons: 0900 28 000 28 (10 ct/min).

1 Uw gegevens

1.1 Vul de gegevens in.

Naam aanvrager			
Postcode		Huisnummer	

1.2 Bij welke aanvraag hoort de bijlage?
Het aanvraagnummer staat in de brief of de ontvangstbevestiging.

Aanvraagnummer	

2 Bijlagen

2.1 Welke bijlagen stuurt u mee?
Vul de naam of omschrijving van de bijlage in.

☐	
☐	
☐	

3 Ondertekening

3.1 Onderteken het formulier en stuur het met alle bijlagen op naar:

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

Naam	
Datum	- - 20
Handtekening	

13

Instantie voor Dierenwelzijn Utrecht

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Postbus 20401
2500 EK s-GRAVENHAGE

uw kenmerk
ons kenmerk

datum 13 januari 2016
onderwerp Antwoorden AVD115002015322

Mijne Dames en Heren,

Bijgaand zend ik u de antwoorden van de onderzoeker op uw e-mail d.d. 28 december 2015.

Met vriendelijke groet

[redacted]

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

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AVD115002015322

Uw referentie
-

Bijlagen
1

Datum 28 december 2015
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Geachte [redacted]

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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.

Bijlage:
- formulier Melding Bijlagen via de post

- **Wij begrijpen dat het aantal te gebruiken dieren een schatting is. Echter, de inschatting van het aantal dieren dient beter onderbouwd te worden. U geeft bijvoorbeeld in appendix 1 en 2 aan dat u meerdere virussen/inflammatory mediators gaat testen. Daarnaast geeft u in appendix 1, 2, 3, 5 en 6 aan dat de experimenten meerdere keren worden uitgevoerd om de reproduceerbaarheid te testen. Kunt u de beslismomenten beter beschrijven, wanneer u bijvoorbeeld beslist om een tweede virus te testen en waarop u de beslissing baseert om een experiment wel/niet te herhalen?**

Het gebruik van een virus is afhankelijk van resultaten die voortkomen uit experimenten met materiaal van patiënten en gezonde controles. Als uit *in vitro* experimenten blijkt dat een specifieke remmende receptor cellen reguleert waarvan wordt gedacht dat deze een belangrijke rol spelen bij de (schadelijke) afweerreactie tegen een bepaald virus, dan zal infectie met dit virus getest worden in muizen waarbij deze receptor ontbreekt. Dit zal per receptor en virus combinatie bekeken worden. Het gebruik van ieder virus zal eerst *in vitro* ondersteund worden (ook in het geval van meerdere virussen in muizen met dezelfde genetische modificatie). *A priori* is niet met zekerheid vast te stellen of een effect van een remmende receptor virus-specifiek is, waaruit volgt dat mogelijk meerdere virussen getest worden in een muis met dezelfde genetische modificatie.

[redacted]

De huidige *modus operandi* binnen de immunologie dicteert dat experimenten tweemaal herhaald worden (d.w.z. drie onafhankelijke experimenten), waarbij veelal de representatieve resultaten van één experiment getoond worden in publicaties met mogelijk de overige experimenten als bijlage. Zonder herhaling zijn de resultaten van het experiment niet te publiceren, aangezien de *peer review* niet zal worden doorstaan. In uitzonderlijke gevallen, zoals langlopende (maanden durende) dierenexperime met

[redacted]

Bij de herhaling van experimenten ligt onze voorkeur bij reproduceren boven repliceren. Bij een langlopend muisexperiment kan bijvoorbeeld in een eerste experiment naar de acute fase gekeken worden. Vervolgens kan dit herhaald worden met een langer lopend

experiment dat de chronische fase bekijkt, waarin ook de eerdere resultaten van de acute fase bevestigd kunnen worden (Ramos *et al.*, Ann Rheum Dis, 2015). Bij een respiratoire infectie kan een initieel experiment herhaald worden met een titratie van de virusdosis om additionele data te winnen en oude resultaten te bevestigen [redacted]

Desalniettemin blijft enige vorm van replicatie noodzakelijk. Biologische systemen vertonen stochastisch processen en het immuunsysteem wordt beïnvloed door een veelvoud aan lastig beheersbare variabelen. Hieronder vallen bijvoorbeeld: variatie tussen batches van toegediende virussen en/of antilichamen, samenstelling van het microbioom van de muizen, stress bij de muizen door verzorging gerelateerde en experimentele handelingen (bijv. mannelijke dierverzorgers induceren door lichaamsgeur meer stress bij knaagdieren dan vrouwelijke verzorgsters [Sorge *et al.*, Nat Methods, 2014]), hormonale status van vrouwelijke muizen, en circadiaans ritme van de muizen. De kans dat een onbeheersbare, en mogelijk onbekende, verstorende factor de uitkomst van het experiment beïnvloed is altijd aanwezig. Dit kan worden ondervangen door het experiment minstens éénmalig te herhalen.

Ten slotte kan er in de voorbereiding en uitvoering van een experiment helaas altijd sprake zijn van menselijke fouten die resultaten kunnen vertroebelen. Muizen en monsters kunnen verwisseld worden. Bij virustitraties en dosisberekeningen kunnen fouten gemaakt worden. Uiteraard is dit zeer onwenselijk en worden er waar mogelijk maatregelen genomen om dit te voorkomen. Zo wordt na afloop van een experiment de genetische modificatie op eiwitniveau bevestigd (m.b.v. bijv. *flow cytometry*) om verwisseling van materiaal tegen te gaan. Echter, menselijke fouten zijn bij experimentele interventies nooit volledig uit te sluiten. Door herhaling kunnen deze fouten op het spoor gekomen worden en voorkomen worden dat ze in de wetenschappelijke literatuur terecht komen.

Ervaring binnen ons instituut toont aan dat twee vergelijkbare experimenten die met grote zorgvuldigheid en toereikende power zijn uitgevoerd desalniettemin verschillende resultaten kunnen opleveren (Meulenbroek, *et al.*, Chapter 6, ISBN: 978-90-6464-723-9; http://dspace.library.uu.nl/handle/1874/287127). Om met zekerheid onze resultaten te kunnen publiceren is het bevestigen van onze eigen bevindingen essentieel. Reproduceerbaarheid is een leidend beginsel in de wetenschap en om hieraan gehoor te geven achten wij het noodzakelijk om eigen experimenten te herhalen.

- **Appendix 2, vraag B. Hier wordt op pagina 4 bij de 4e paragraaf gesproken over Estimated number of viruses tested. Wij nemen aan dat u hiermee bedoelt estimated number of inflammatory mediators tested. Kunt u dit bevestigen?**

De Commissie heeft gelijk. Het betreft in bijlage 2 inderdaad ‘inflammatory mediators’ en niet ‘viruses.’

- **In appendix 5 beschrijft u dieren nodig voor trainingsdoeleinden. Het aantal dieren dat u hiervoor nodig denkt te hebben is 30 per jaar (2e alinea vraag B) of 10 per jaar (10 dieren per jaar). Het is ons niet duidelijk welk aantal correct is. Kunt u dit consistent maken?**

Het betreft hier een per abuis overgebleven alinea uit een eerdere versie van de bijlage #5. Het correcte aantal is 10 dieren per jaar (totaal: 50 dieren in 5 jaar).

- **In appendix 5 beschrijft u een fok met dieren waarbij de dieren op latere leeftijd ongerief kunnen ondervinden van de genetische modificatie. U geeft aan dat deze dieren dit ongerief echter niet zullen ondervinden omdat zij deze leeftijd niet zullen bereiken. Omdat deze dieren wel risico lopen dit ongerief te ondervinden, moet deze fok wel als dierproef worden beschouwd en heeft u voor deze fok een vergunning nodig in het kader van de Wod. U dient deze fok dus op te nemen in onderliggende aanvraag, of hiervoor een aparte aanvraag in te dienen.**

De Commissie lijkt te verwijzen naar de volgende passage uit de "Working document on genetically altered animals," waarin inderdaad het afvoeren van dieren vóór het optreden van ongerief als gevolg van de genetische modificatie als onvoldoende beoordeeld wordt.

*"Genetically altered lines which retain a risk of the development of a **harmful phenotype** (e.g. age onset of disease or tumours; risk of infection due to compromised immune system) regardless of the applied refinement (e.g. barrier conditions, culling at early age), in line with Article 1(2), their breeding requires project authorisation as the application of refinement does not eliminate the risk."*

Verder worden heterozygote muizen gebruikt voor de instandhouding van lijnen. Heterozygote muizen vertonen geen fenotype. Homozygote muizen worden gebruikt voor de in de aanvraag voorgestelde experimenten.

14

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uw kenmerk
ons kenmerk

datum 15 januari 2016
onderwerp Antwoorden AVD115002015322

Mijne Dames en Heren,

Bijgaand zend ik u de antwoorden van de onderzoeker op uw e-mail d.d. 14 januari 2016.

Met vriendelijke groet

[redacted]

Van: Info-zbo <info@zbo-ccd.nl>
Datum: 14 Jan 2016 16:27:02 GMT+1
Aan: "[redacted]
Kopie: [redacted]

Beste Heer de Leeuw,
Dank voor het toezenden van de antwoorden op de gestelde vragen betreffende aanvraag AVD115002015322.
Nog niet alles is ons echter volledig duidelijk nu.

1) Betreffende de herhaling van experimenten. Het is ons helder dat experimenten herhaald moeten worden om reproduceerbaarheid aan te tonen. We kunnen ons echter voorstellen dat niet alle experimenten 3x hoeven worden uitgevoerd. Als bijvoorbeeld uit de eerste 2 experimenten geen effect van een bepaalde receptor wordt aangetoond, wordt dit experiment dan nog een derde keer uitgevoerd? Kunt u de criteria beschrijven op basis waarvan u besluit een experiment voor een tweede of derde keer uit te voeren?

2) Wat betreft de fok van de genetisch gemodificeerde dieren met mogelijk ongerief op latere leeftijd. Hiermee refereerde de CCD inderdaad aan de passage uit het "working document on genetically altered animals". In uw antwoord dd 13 januari 2015 schrijft u over jarenlange ervaring met de lijnen in kwestie, terwijl u in bijlage 5 van de aanvraag schrijft over "previously untested strains" en "This could be aplified in inhibitory receptor-deficient mice". Dit lijkt niet met elkaar overeen te komen. Kunt u dit nogmaals verhelderen?

Stuur de ontbrekende informatie binnen veertien dagen na de datum van deze brief op. U kunt dit aanleveren via NetFTP. De behandeling van uw aanvraag wordt opgeschort tot het moment dat wij de aanvullende informatie hebben ontvangen.

Met vriendelijke groet,

[redacted]

Centrale Commissie Dierproeven

Instantie voor Dierenwelzijn Utrecht

1) Betreffende de herhaling van experimenten. Het is ons helder dat experimenten herhaald moeten worden om reproduceerbaarheid aan te tonen. We kunnen ons echter voorstellen dat niet alle experimenten 3x hoeven worden uitgevoerd. Als bijvoorbeeld uit de eerste 2 experimenten geen effect van een bepaalde receptor wordt aangetoond, wordt dit experiment dan nog een derde keer uitgevoerd? Kunt u de criteria beschrijven op basis waarvan u besluit een experiment voor een tweede of derde keer uit te voeren?

Als de eerste herhaling het voorafgaande experiment volledig ondersteunt, dan zal er inderdaad niet voor gekozen worden om dit een derde maal te testen. Ieder experiment zal minstens tweemaal uitgevoerd worden, vanwege de eerder beschreven redenen. Bij volledige overeenstemming tussen de twee experimenten zal niet over worden gegaan tot een derde experiment. Als echter reden tot twijfel blijft bestaan (bijv. de grootte van het effect varieert sterk tussen de twee experimenten), wordt uitsluitsel gezocht aan de hand van een tweede herhaling.

2) Wat betreft de fok van de genetisch gemodificeerde dieren met mogelijk ongerief op latere leeftijd. Hiermee refereerde de CCD inderdaad aan de passage uit het "working document on genetically altered animals". In uw antwoord dd 13 januari 2015 schrijft u over jarenlange ervaring met de lijnen in kwestie, terwijl u in bijlage 5 van de aanvraag schrijft over "previously untested strains" en "This could be amplified in inhibitory receptor-deficient mice". Dit lijkt niet met elkaar overeen te komen. Kunt u dit nogmaals verhelderen?

Het blijkt dat een te algemene formulering door ons de verwarring veroorzaakt. Wij kunnen namelijk de mogelijkheid niet volledig uitsluiten dat bij genetische deletie van een zekere remmende receptor muizen van gevorderde leeftijd last ondervinden die toe te schrijven is aan die deletie. Echter, van alle muislijnen die wij momenteel in fok hebben, waaronder Lair1-/- muizen, kunnen wij met zekerheid zeggen dat op gevorderde leeftijd deze muizen geen aantoonbaar ongerief ondervinden die inherent is aan de genetische modificatie. Bovendien, uit de literatuur is verder bekend dat muizen met genetische modificaties in inhibitoire receptoren over het algemeen hieraan geen ongerief ondervinden als zij enkel gefokt worden, ook niet op gevorderde leeftijd. Enkel als de muizen worden blootgesteld aan een experimentele setting, zoals infectie met een virus, kunnen de genetisch gemodificeerde dieren mogelijk hoger ongerief ondervinden dan wildtype muizen. Hoewel de genetisch gemodificeerde muizen op gevorderde leeftijd mogelijk veranderingen in het immuunsysteem vertonen, wat voor ons van interesse is, gaat dit niet direct gepaard met ongerief.

Momenteel hebben wij geen plannen om een muislijn in fok te nemen die ongerief ondervindt die inherent is aan zijn genetische modificatie buiten experimenten om. Zou hier later wel sprake van zijn, dan wordt een nieuwe aanvraag ingediend om de fok van deze dieren te ondervangen.

Vriendelijke groeten,

[redacted]

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Instantie voor Dierenwelzijn 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
AVD115002015322

22 JAN 2016

Datum
Betreft Aanvraag projectvergunning Dierproeven

Geachte [redacted]
Op 24 november 2015 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Inhibitory immune receptors as therapeutic targets to dampen injurious immune respons" met aanvraagnummer AVD115002015322. Wij hebben uw aanvraag beoordeeld.

Op 13 januari 2016 en 15 januari heeft u uw aanvraag aangevuld. Deze aanvulling betrof een nadere onderbouwing van het aantal te gebruiken dieren, tekstuele verduidelijking en nadere uitleg van het fenotype van de te gebruiken GMO's. Deze aanvullingen zijn op ons verzoek verstrekt.

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. U kunt met uw project "Inhibitory immune receptors as therapeutic targets to dampen injurious immune respons" starten. De vergunning wordt afgegeven van 22 januari 2016 tot en met 31 december 2020.
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 16 november 2015. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a, lid 3 van de wet.
Wij kunnen ons vinden in de inhoud van het advies van de Dierexperimentencommissie. Wij nemen dit advies van de commissie over, inclusief de daaraan ten grondslag liggende motivering. De CCD stelt echter wel enkele algemene voorwaarden.

Dit 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).

Met vriendelijke groet,

Centrale Commissie Dierproeven
namens deze:

[redacted]

ir. G. de Peuter
Algemeen Secretaris

Bijlagen:
- Vergunning
  Hiervan deel uitmakend:
  - DEC-advies
  - Weergave wet- en regelgeving

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 22 januari 2016 tot en met 31 december 2020, voor het project "Inhibitory immune receptors as therapeutic targets to dampen injurious immune respons" met aanvraagnummer AVD115002015322, volgens advies van Dierexperimentencommissie DEC Utrecht.
De functie van de verantwoordelijk onderzoeker is [redacted]
De aanvraag omvat de volgende bescheiden:

1 een aanvraagformulier projectvergunning dierproeven, ontvangen op 24 november 2015
2 de bij het aanvraagformulier behorende bijlagen:
a Projectvoorstel, zoals ontvangen per digitale indiening op 24 november 2015;
b Niet-technische Samenvatting van het project, zoals ontvangen per digitale indiening op 24 november 2015;
c Advies van dierexperimentencommissie d.d. 16 november 2015, ontvangen op 24 november 2015;
d De aanvullingen op uw aanvraag, ontvangen op 13 januari 2016 en 15 januari 2016.

Naam proef	Diersoort/ Stam	Aantal dieren	Ernst	Opmerkingen
Respiratory viral infections	Muizen (Mus musculus) / WT en GMO	1500	Matig / moderate	
Non-infectious pulmonary inflammation	Muizen (Mus musculus) / WT en GMO	500	Matig / moderate	
House dust mite- and respiratory syncytial virus-induced asthma	Muizen (Mus musculus) / WT en PCDH1-deficient	1500	Matig / moderate	
Characterisation and testing of inhibitory immune receptor agonists	Muizen (Mus musculus) / WT	500	Licht / mild	
Functional and phenotypical characterisation of genetically modified mice	Muizen (Mus musculus) / WT en GMO	1000	Licht / mild	
Induced auto-immune diseases	Muizen (Mus musculus) / WT en GMO	1000	Matig / moderate	

Voorwaarden

Op grond van artikel 10a1 lid 2 Wod zijn aan een projectvergunning voorwaarden te stellen

De vergunning wordt verleend onder de voorwaarde dat eventuele go/no go momenten goedkeuring van de IvD verkrijgen.

In artikel 10, lid 1a van de wet, wordt bepaald dat het verboden is een dierproef te verrichten voor een doel dat, naar de algemeen kenbare, onder deskundigen heersende opvatting, ook kan worden bereikt anders dan door middel van een dierproef, of door middel van een dierproef waarbij minder dieren kunnen worden gebruikt of minder ongerief wordt berokkend dan bij de in het geding zijnde proef het geval is. Nieuwe onderzoeken naar alternatieven kunnen tot gevolg hebben dat inzichten en/of omstandigheden van het aangevraagde project in de vergunningsperiode wijzigen, gedurende de looptijd van deze vergunning. Indien bovenstaande zich voordoet dient aanvrager dit in overleg met de IvD te melden bij de CCD. De

CCD kan in een dergelijke situatie aan de vergunning nieuwe voorwaarden verbinden en gestelde voorwaarden wijzigen of intrekken.

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.