	Inventaris Wob-verzoek W17-12								
			wordt verstrekt			weigeringsgronden			
nr.	documenten NTS20172205	reeds openbaar	niet	geheel	deels	10.1.c	10.2.e	10.2.g	11.1
1	Aanvraagformulier				x		x	x	
2	Projectvoorstel			x					
3	Niet-technische samenvatting	x							
4	Bijlage beschrijving dierproeven 1			x					
5	Bijlage beschrijving dierproeven 2			x					
6	Bijlage beschrijving dierproeven 3			x					
7	Bijlage beschrijving dierproeven 4			x					
8	Bijlage beschrijving dierproeven 5			x					
9	Bijlage beschrijving dierproeven 6			x					
10	Bijlage beschrijving dierproeven 7			x					
11	DEC-advies				x		x	x	
12	Ontvangstbevestiging				x		x	x	
13	Verzoek aanvulling aanvraag				x		x	x	
14	Reactie verzoek aanvulling			x					
15	Advies CCD		x						x
16	Beschikking en vergunning				x		x	x	

1 9 JUN 2017

1.

Centrale Commissie Dierproeven

Aanvraag
Projectvergunning Dierproeven
Administratieve gegevens

- U bent van plan om één of meerdere dierproeven uit te voeren.
- Met dit formulier vraagt u een vergunning aan voor het project dat u wilt uitvoeren. Of u geeft aan wat u in het vergunde project wilt wijzigen.
- Meer informatie over de voorwaarden vindt u op de website www.centralecommissiedierproeven.nl. of in de toelichting op de website.
- Of bel met 0900-2800028 (10 ct/min).

1 Gegevens aanvrager

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

☒ Ja > *Vul uw deelnemernummer in*	30100
☐ Nee > *U kunt geen aanvraag doen*	

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

Naam instelling of organisatie	Stichting Het Nederlands Kanker Instituut - Antoni van Leeuwenhoek Ziekenhuis	
Naam van de portefeuillehouder of diens gemachtigde	[redacted]	
KvK-nummer	40530817	

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	Plesmanlaan	121
Postbus	90203	
Postcode en plaats	1066 CX	Amsterdam
IBAN	NL71DEUT0626343534	
Tenaamstelling van het rekeningnummer	Stichting Het Nederlands Kanker Instituut – Antoni van Leeuwenhoek Ziekenhuis	

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

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

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	Instantie voor Dierenwelzijn
		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	1 - 8 - 2017
		Einddatum	1 - 8 - 2022
3.2	Wat is de titel van het project?	Study of immune cells after infection and tumor challenge to uncover fundamental principles to improve immunotherapy	
3.3	Wat is de titel van de niet-technische samenvatting?	Studie aan immuun cellen na infectie en tumor challenge om fundamentele principes te ontrafelen voor immunotherapie	
3.4	Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?	Naam DEC	NKI
		Postadres	t.a.v. ; Postbus 90203; 1006 BE Amsterdam
		E-mailadres	

4 Betaalgegevens

4.1	Om welk type aanvraag gaat het?	☒ Nieuwe aanvraag Projectvergunning € 2.113,- Lege ☐ Wijziging € Lege
4.2	Op welke wijze wilt u dit bedrag aan de CCD voldoen. *Bij een eenmalige incasso geeft u toestemming aan de CCD om eenmalig het bij 4.1 genoemde bedrag af te schrijven van het bij 1.2 opgegeven rekeningnummer.*	☐ Via een eenmalige incasso ☒ Na ontvangst van de factuur

5 Checklist bijlagen

5.1	Welke bijlagen stuurt u mee?	Verplicht ☒ Projectvoorstel ☒ Niet-technische samenvatting Overige bijlagen, indien van toepassing ☐ Melding Machtiging ☒

6 Ondertekening

6.1 Print het formulier uit, onderteken het en stuur het inclusief bijlagen via de beveiligde e-mailverbinding naar de CCD of per post naar:

Centrale Commissie Dierproeven
Postbus 20401
2500 EK Den Haag

Ondertekening door de instellingsvergunninghouder of gemachtigde (zie 1.7). De ondergetekende verklaart:

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

Naam	[redacted]
Functie	Instantie voor Dierenwelzijn
Plaats	Amsterdam
Datum	12 - 6 - 2017
Handtekening	[redacted]

Centrale Commissie Dierproeven

Form
Project proposal

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

1 General information

1.1 Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	30100
1.2 Provide the name of the licenced establishment.	Stichting Het Nederlands Kanker Instituut (NKI) – Antoni van Leeuwenhoek Ziekenhuis
1.3 Provide the title of the project.	Study of immune cells after infection and tumor challenge to uncover fundamental principles to improve immunotherapy

2 Categories

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

- ☒ Basic research
- ☐ Translational or applied research
- ☐ Regulatory use or routine production
- ☐ Research into environmental protection in the interest of human or
- ☐ 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.

T cells constitute an essential line of immune defense that mediates protection during viral infection in an antigen-specific manner. Upon infection, antigen-specific naive T cells expand and differentiate into

effector cells that contribute to viral clearance through granzyme B-driven cytotoxicity and production of the pro-inflammatory cytokine IFN-γ. T cells are also important after resolution of infection, because of their capacity to develop into memory cells that provide enhanced protection upon secondary encounter with the pathogen. Therefore, it is highly relevant to study how T cells are available to achieve up to life-long protection against re-infection.

T cells have also been found to infiltrate tumor tissue and to develop responses against tumors, but in such a setting they are often ineffective to combat disease in the absence of treatment. The underlying reason resides at least in part in the expression of inhibitory molecules such as PD1 and CTLA4 on these tumor-infiltrating T cells. The upregulated expression of inhibitory molecules on tumor-infiltrating T cells allows tumor cells to block the ability of these lymphocytes to eliminate tumor cells. In recent years, immunotherapy has emerged as a promising strategy in the treatment of cancer (Couzin-Frankel, Science, 2013, 'Breakthrough of the year 2013: Cancer immunotherapy'). In particular, immunotherapies that activate T cells have been found effective in the elimination of tumor cells. Checkpoint blockade therapy overcomes immune suppression through blocking antibodies that relieve inhibitory pathways through PD1 (pembrolizumab) and CTLA4 (ipilimumab). The efficacy of these therapies is thought to rely on the re-activation of suppressed T cell responses directed against the tumor cells. A second successful approach works through the re-infusion of in vitro expanded T cells derived from excised tumors. Tumor-infiltrating T cells can be expanded to large numbers in vitro and these cultured cells have been found to substantially accelerate the clearance of tumor cells. Both of these therapies highlight the importance of T cells for the treatment of cancer. Although these therapies have clear benefits and can completely clear disease in a substantial fraction of the patients, they do not work efficiently in other patients or in different types of cancer. Thus, further knowledge on T cell differentiation is required to improve T cell immunotherapy for cancer treatment.

As also noted above, an important pathway that suppresses T cell responses against tumor cells is mediated by the inhibitory receptor PD1 that is expressed on tumor-infiltrating lymphocytes and blockade of this pathway forms an effective immunotherapy in many cancer patients (Hamid et al., N Engl J Med, 2013). The identification of PD1 as a target for intervention in immunotherapy originates from infection experiments in mice with the LCMV virus (Barber et al., Nature, 2006). In the chronic model of LCMV infection PD1 is upregulated on virus-specific T cells and suppresses T cell responses against the virus. Treatment of LCMV infected mice with blocking anti-PD1 antibodies reinstates T cell responses that clear infection. The applicability of PD1 as a target for intervention in immunotherapy shows by example that the chronic infection model of LCMV can be instrumental to identify further targets for immunotherapy. Therefore, we aim to study T cell differentiation in chronic infection models such as with chronic LCMV variants. Obviously, differences exist between T cell responses in chronic infection and in tumor settings (e.g. due to the systemic presence of completely foreign T cell epitopes from the virus versus locally expressed self but potentially modified T cell epitopes from the tumor cells). Despite these differences, T cell responses against chronic infection and during tumor development are remarkably similar, suggesting that targets from chronic infection models can provide useful starting points for further study in tumor models.

As the development of efficient T cell responses is impaired in cancer patients and in in vivo mouse models of cancer, a different approach is required to study how T cells with high potential to eliminate tumor cells differentiate. Therefore, our second approach is to study how T cells develop in acute infection models, as these models have been instrumental in the understanding of the differentiation of effective T cell responses. Acute infection induces the formation of pathogen-specific effector T cells that contribute to pathogen clearance. After sterile immunity is achieved the T cell response contracts, but memory cells that can mount highly effective secondary responses upon re-encounter with the same pathogen remain. The ability of memory cells to persist in combination with their potential to develop highly specific responses makes them highly useful for therapeutic purposes in patients. Therefore, we are studying effector and memory T cell differentiation to unravel how T cell development is instructed by environmental and intrinsic cues, how naïve T cells differentiate into distinct subsets with unique properties, how effector functions are established, and how T cells engage other immune cells upon activation.

We aim to apply the fundamental knowledge gained in these chronic and acute infection models to find immunization strategies and in vitro culture protocols to generate effector or memory T cells with high potential to combat infection and / or tumor growth. Our research is designed to ultimately understand how the qualities of effector and memory T cells can be optimally used in therapy for treatment of infection and / or cancer.

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 ultimate objective of this project is to assess novel and improved strategies for T cell immunotherapy and to translate these findings to a human cancer or infection setting. Obviously, development of therapies to extend the survival of cancer or infection patients form a long-term goal that is unachievable within the 5 years of this project. Nevertheless, we expect that our work on this project will contribute to substantial improvements in our understanding of T cell differentiation that is essential for the further development of T cell immunotherapy.

We aim to expand our knowledge on T cell differentiation using the study of memory T cells that develop after infection with influenza, lymphocytic choriomeningitis virus (LCMV) or the bacterium Listeria monocytogenes (Lm). These infection models are chosen as they have been used in the past in our lab and many other labs to study T cell differentiation. This has resulted in a wealth of knowledge, availability of important tools and expertise that strongly supports further work on these infection models. We will focus on how T cell differentiation is influenced by internal cues (transcription factors, histone modifying enzymes, RNA binding factors) and external cues (signals from antigen-presenting cells, other immune cells, or stromal cells). We will study the different aspects of the memory T cells that include longevity and turnover, metabolism, migration, the ability to form secondary responses, the acquisition of effector functions, and their potential to activate or inhibit the actions of other immune cells. We will make use of these findings to recapitulate formation of memory T cells with desired properties in cell cultures. These in vitro expanded memory T cells will be tested for their potential in vivo to protect against infection and tumor challenge.

In this project, we expect to achieve our goals in a stepwise manner. First, we will identify target genes of memory T cells in primary infection models (milestone 1). To identify target genes of interest we will consider intrinsic signals in T cells and extrinsic signals from the environment. Of utmost importance for the selection of relevant target genes is that they contribute to the differentiation, expansion and / or acquisition of effector functions of T cells as mentioned in further detail in our research strategy under point 3.4. Next, we will analyze the role of these target genes in the efficacy of memory cells to respond in re-challenge settings upon re-encounter with the same pathogen (milestone 2) or upon tumor challenge (milestone 3). Finally, immunization strategies to induce memory T cells or adoptive transfer strategies with cultures of memory T cells will be developed based on the acquired information to establish protocols that can achieve clearance of infection or tumor cells (milestone 4).

These milestones appear achievable in light of past accomplishments. We have extensively contributed to the understanding of T cell differentiation through our in vivo work on infection models. For example, we have previously described the role of costimulation through TNF related molecules such as the CD70-CD27 and GITRL-GITR ligand-receptor pairs in the establishment of T cell responses (Van Gisbergen et al., 2011, Immunity; van Olffen et al, 2009, JI; Pascutti et al, 2015, PLOS Pathogen). We have also addressed the transcriptional regulation of anti-viral T cell responses and found how transcription factors such as Notch and Hobit instruct the formation of effector and memory T cell responses (van Gisbergen et al, 2012, Nat. Immunol.; Backer et al, 2014, Nat. Immunol.; Hombrink et al, 2016, Nat. Immunol.; Mackay et al., 2016, Science). In addition, we have a team of skilled researchers in the field of infection and tumor immunology and labs that have state of the art equipment enabling adequate analysis of immune responses using flow cytometry, microscopy, mass spectrometry and molecular techniques.

We anticipate that establishing these milestones allows us to introduce improvements to the longevity, proliferative capacity and / or the effector functions of cultured T cells to further unlock these cells for use as therapeutic products. Currently, we participate in ongoing immunotherapy trials on the use of tumor-infiltrating lymphocytes in cancer patients and hold a competitive position in cellular

immunotherapy, as the lab contains one of the production centers of tumor-infiltrating T cells for adoptive transfer into melanoma patients in Europe. This means that the expertise and facilities for follow-up research are in place within our institute. Therefore, translation of our in vivo mouse studies into a clinical setting can be implemented on site for the improvement of existing products of tumor-infiltrating lymphocytes and the development of novel T cell products.

3.3 Relevance

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

Scientific:
In the past years large steps have been made in the understanding of how T cell responses against the tumor are suppressed during cancer formation. This has resulted in the identification of many targets including PD-1 and CTLA4 that can now be exploited for the treatment of cancer patients. In addition, substantial progress has been made in the use of in vitro expanded T cells as cellular products for the treatment of cancer patients. This is in particular the case for melanoma patients, as complete responses have been achieved in a large proportion of these patients using adoptive T cell therapy. Collectively, these studies suggest that T cells are not only essential in protection against viruses and other pathogens, but can also be applied in therapies that are effective in protection against cancer. Therefore, it is of pivotal importance to improve our fundamental understanding of T cell differentiation to identify and validate further targets in order to design good clinical studies and to ultimately establish better strategies to apply T cells for the treatment of cancer or infection patients.

Social:
Health and well-being are generally considered to be of primary value by the population. Cancer and infectious disease are factors that have a very negative impact on the health and well-being of many people, not only for patients, but also for their relatives and friends. Moreover, the loss of lives and morbidity also has considerable negative economic consequences (high cost of care, loss of productivity). This project can provide fundamental understanding of T cell differentiation to establish better T cell therapy that reduces adverse effects, morbidity, and mortality and may therefore be of importance to patients that will benefit from these therapies.

3.4 Research strategy

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

The aim of the project is to identify targetable properties of T cells that are potentially useful to optimize T cell therapy in cancer or infection patients using a large set of experiments. We will focus on different aspects of T cell differentiation outlined below to search for such targets.

Research Topics:

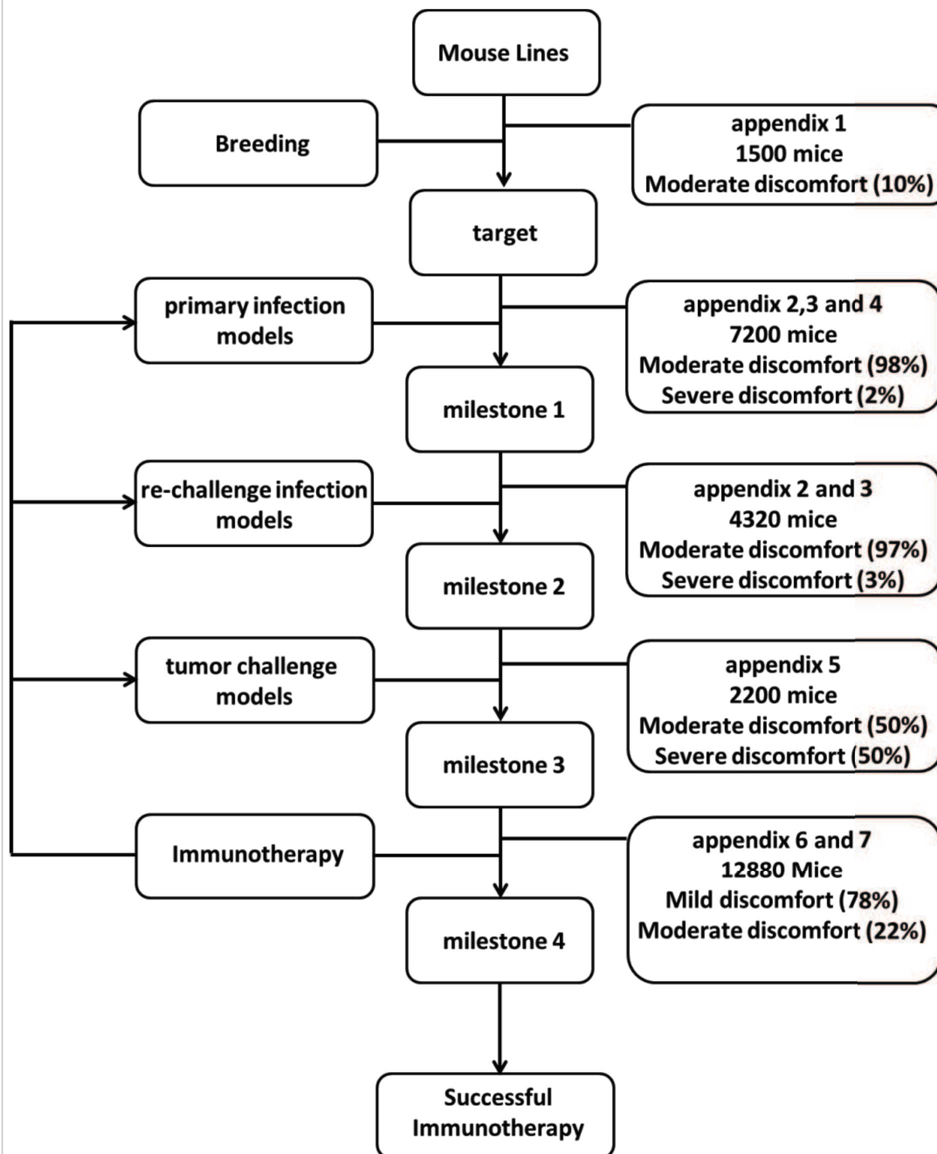
1. Identification of intrinsic signals in T cells that drive T cell differentiation. This line of research concerns transcription factors and target genes of these transcription factors that regulate T cell development and function. Others and we have found that transcription factors play an essential role in the development of the different subsets of effector and memory T cells that arise during an immune response. The instrumental role of these transcription factors can be applied to induce the differentiation of memory T cells with desired properties. In addition, this line of research concerns RNA-binding proteins that regulate effector function of memory T cells through RNA stability. Others and we have found that RNA stability and translation play a very important role in the regulation of effector functions of memory T cells. We envision that RNA-binding proteins can be utilized to enhance the effector functions of memory T cells.

2. Identification of extrinsic signals that T cells acquire for example from antigen-presenting cells, other immune or stromal cells to differentiate into effector or memory cells. Others and we have shown that antigen (signal 1) in combination with co-stimulation (signal 2) and cytokines (signal 3) are important signals that T cells acquire from surrounding cells during their differentiation process in immune responses. These signals can be used to instruct the formation of effector and memory T cells with optimal properties for therapy.

These research questions will result in the identification of key molecules involved in T cell differentiation that may provide useful novel targets of intervention in immunotherapy against cancer. For this purpose, we will uniformly address the research questions using a standard set of experiments as outlined below under points 3.4.2 and 3.4.3. The analysis will make use of genetically modified mice that over-express, lack or label specific molecules or genes of interest. These genetically modified mice will be compared against the relevant wild-type mice of the same background. Alternatively, blocking or stimulating antibodies or small molecule inhibitors will be used to modulate any of the pathways of interest. The effect of these reagents will be tested against the appropriate control conditions.

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.

The different components of the project are aimed at understanding T cell differentiation by making use of a set of infection and tumor models, in which the formation, properties and potential of pathogen and tumor-specific T cells can be analyzed. Ultimately, these findings will be used to design novel immunotherapy strategies. These immunotherapies will then be tested for their efficacy against challenge in infection and tumor models. The general setup of the experiments is displayed in the flow chart depicted below.



We largely use existing mouse models for our infection experiments, but, if existing models are not

available, it is possible that the project requires the development of novel mouse models. In general these new models will not display discomfort under standard conditions, but in rare cases it can be necessary that mice are bred with discomfort to study targets of interest (**appendix 1**). The type of infection impacts on T cell differentiation. Important parameters that affect pathways of T cell differentiation are the duration and the location of the infection. Therefore, we aim to study T cell differentiation using pathogens that induce chronic versus acute infection and using pathogens that induce local versus systemic infection. We aim to establish T cell differentiation in local infection models using the influenza virus, which exclusively targets the lungs (**appendix 2**). T cell differentiation in systemic infection will be studied using either LCMV or Lm that both induce infection in multiple tissues (**appendix 3**). Chronic infection will be studied using variant viruses of LCMV that persist and that therefore induce the differentiation of exhausted T cells (**appendix 4**). The potential of memory CD8 T cells will be studied in re-challenge experiments. Re-challenge can be achieved in the influenza model (**appendix 2**) or through serial infection with LCMV and Lm containing the same T cell epitopes (**appendix 3**). In addition, the efficacy of memory T cells in a tumor challenge setting to clear the tumor cells will be studied (**appendix 5**). In vitro studies will be established to mimic the in vivo formation of memory T cells under culture conditions (**appendix 6**). Alternatively, immunization strategies will be applied to induce the formation of memory T cells in vivo (**appendix 7**). The in vitro or in vivo induced memory T cells will be probed for their protective potential after transfer into mice and upon challenge with pathogens or tumor cells (**appendices 2-5**).

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.

As outlined above, we aim to use different types of infection models to study T cell differentiation. These infection models will be run in parallel, as they are aimed at the understanding of the properties of pathogen-specific T cells under different conditions. Influenza infection (**appendix 2**) induces local infection in the lungs and can reveal different aspects of T cell differentiation than acute LCMV or Lm infection (**appendix 3**) that induce systemic infection. Similarly, the induction of chronic LCMV infection (**appendix 4**) creates a different setting, in which antigen and inflammation are constitutively present and will therefore be studied in parallel to the acute infection models. The choice between the type of infection model depends on which model provides the best possible chance of success in respect to what is known in the literature.

As depicted in the flow chart, in **step 1**, we will first analyze different time-points after primary infection within any of these models. This setup will allow analysis of effector T cell differentiation in early stages (first 2 weeks after infection) and memory T cell differentiation in late stages (more than 1 month after infection). If we find clear evidence that molecules of interest are involved in any of the aspects of effector or memory T cell differentiation (**milestone 1**), we will progress to the next step. In **step 2**, the efficacy of the memory T cells will be studied in pathogen re-challenge experiments. These experiments can be done in either of 2 ways. Mice will be re-challenged with a pathogen containing the same epitopes to enable analysis of the potential of the memory cells in a secondary response. Alternatively, pathogen-specific memory T cells will be adoptively transferred into recipients that will be challenged with the same pathogen. If in these experiments, we find clear evidence that the memory cells provide protection against secondary infection (**milestone 2**), we will progress to the next step. In **step 3**, the efficacy of the memory T cells will be studied in tumor challenge experiments (**appendix 5**). Similar to the previous step, if in these experiments we find clear evidence that the memory cells also provide protection against tumor challenge (**milestone 3**), we will progress to the next step. In **step 4**, we will analyze whether we can recapitulate the formation of protective effector or memory T cells through in vitro or in vivo manipulation. Such T cells will be induced during culture protocols (**appendix 6**). Alternatively, immunization strategies (**appendix 7**) will be developed to induce memory T cells in vivo. These immunotherapy strategies will then be tested for their efficacy to provide protection in infection and tumor models (**appendices 2-5**). If we are able to induce memory cells with protective capacities in vitro or in vivo, we have achieved the final goal of the project (**milestone 4**). We consider the ultimate goal of this protocol the establishment of successful immunotherapy that has protective potential in the in vivo infection and / or tumor models. Further studies will be required to validate the procedures to generate protective memory cells in different tumor models to ultimately translate these findings to the human setting. These protocols are not part of the current application, but of previously requested and granted protocols (e.g. the protocol entitled pre-clinical intervention studies in mice for prevention and treatment of cancer).

The project reflects ongoing work of our group that until this point has run within the old format of the animal ethics protocols. Therefore, we have parts of the project that have already reached 1, 2 or 3 of the milestones described above, and thus, it is possible that experiments under the current project start off immediately at step 1, 2, 3 or 4.

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	Breeding with discomfort
2	Local infection with influenza
3	Systemic infection with Listeria monocytogenes or LCMV
4	Chronic and acute LCMV infection
5	Tumor challenge
6	Homeostasis studies
7	Immunization
8	
9	
10	

Centrale Commissie Dierproeven

Appendix
Description animal procedures

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	30100
1.2	Provide the name of the licenced establishment.	Stichting Het Nederlands Kanker Instituut – Antoni van Leeuwenhoek ziekenhuis

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	Type of animal procedure
01	Breeding with discomfort

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.

We perform experiments on wildtype and transgenic mouse lines that are maintained through breeding in the facility. Most of our current lines are without discomfort. To study immune cell differentiation we are generating novel mouse lines that may have unanticipated discomfort. The breeding of mice with discomfort occurs for 2 reasons.

1. Breeding of lines with known discomfort for maintenance
2. Breeding of lines with known discomfort for experiments

In some cases, such as for the generation of new lines through genetic manipulation, through crossing of existing lines, or through crossing onto a different background (e.g. 129 mice onto C57Bl/6 mice) the discomfort during breeding is unknown. Therefore, the breeding of new lines will be monitored for discomfort for at least 2 generations to determine whether the mice display any signs of discomfort under standard conditions. If no signs of discomfort are apparent in the first 2 generations, the new lines will be considered without discomfort. If signs of discomfort are apparent, measures will be taken to overcome the discomfort. For example, if discomfort develops after a certain age, we will euthanize mice before development of discomfort. Furthermore, when discomfort requires a specific configuration of alleles, such alleles will be separated (or kept heterozygous) for line maintenance. If the discomfort cannot be overcome, the new lines will be considered breeding with discomfort and further breeding for maintenance and experiments will occur on this protocol.

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

Nature:
Breeding pairs will be set up with one male and one or two females to meet the minimal requirements for mice in maintenance of the lines or in production for experiments. Maintenance breeding requires low numbers of breeding pairs (typically 2 pairs) and production breeding requires high numbers of breeding pairs (typically 5 pairs). The number of production breeding pairs depends on usage of the mice in the experiments. It should be noted that the frequency of obtaining the desired genotypes in the offspring can be as low as 25%, if for example heterozygote parents are used for breeding, or well below 25%, if multiple genotypes have to be taken into consideration. These circumstances can necessitate a higher number of breeding pairs (above 5 pairs), reflecting the efficiency of obtaining the desired offspring.

In general, the lines with spontaneous discomfort will be maintained through breeding of heterozygotes with wildtypes, which eliminates the generation of homozygotes, which can be expected to have a more severe phenotype. For experimental purposes, the lines will then be maintained using heterozygote x heterozygote breeding pairs, enabling the formation of homozygote offspring (25%) and wild type littermates (25%) that can be used as controls.

Material will be obtained from the mice to identify the genotypes of the offspring. Toe clips will be taken at 1 week of age or earclips at 3 or more weeks of age to enable screening of the genotypes by PCR on genomic DNA. For certain genotypes, typing can only be done through analysis of blood samples. Therefore, we will toeclip or earclip these mice for identification purposes and subsequently draw blood samples at 3 or more weeks of age.

The generation of surplus mice will be kept at a minimum. It is impossible to completely avoid the generation of mice that are not required for maintenance and / or that cannot be used in experiments. During the crossing of lines the number of generated surplus mice can be substantial (e.g. 75%, if heterozygotes are mated to obtain homozygote offspring). These mice will be euthanized as soon as possible after the genotypes have been identified.

Breeding pairs will be terminated before the breeder mice reach 1 year of age and the breeder mice will then be sacrificed. New breeding pairs will be set up as replacements.

Frequency:
Breeding pairs will be mated until the female has had 6 litters.

Time:
Breeding pairs will be set up with mice of at least 8 weeks old. Breeding pairs will be retired after 6 litters or when the breeder mice are 1 year old.

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

The number of mice depends on the requirements for maintenance, experiments and the efficiency of obtaining the desired genotypes. We will make use of optimal breeding strategies to meet these requirements as closely as possible and to minimize the number of surplus mice.

B. The animals

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

The animals are obtained from registered commercial or in-house suppliers or through in-house generation of new lines. Breeder mice can be wild-type or genetically modified animals of at least 8 weeks of age and at the most 1 year of age.

We expect to use up to 100 mice per line per year and 1 new line per year. This results in 5x 100 mice after five years for the first line if the line is not discontinued, 4x 100 mice for the second line, 3x 100 mice for the third line, 2x 100 mice for the fourth line and 1x 100 mice for the fifth line. Therefore, we expect to use 1500 mice in total on this protocol. Based on past experience of using genetically modified models in immune cell differentiation, we expect that most of the new lines are without discomfort and therefore do not need to be bred on the protocol.

C. Re-use

Will the animals be re-used?

☒ 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.

The breeding protocol supports the experiments described in the other appendices. Optimization of the breeding strategy to the experimental requirements will reduce the usage of mice. We can use males and females interchangeably in the majority of our experiments, which will optimize the usage of the mice obtained through breeding. In heterozygote breedings, the wild-type offspring will be used as littermate controls for the knockout offspring, which will also reduce the number of surplus mice.

When discomfort requires homozygosity for specific alleles, lines will be maintained heterozygously, which will avoid discomfort. For those lines were this is not the case, as many alleles will be maintained homozygously as possible without compromising experimental set up. For instance, mice will be bred homozygously for floxed alleles for maintenance purposes and crossed with hemizygous Cre mice (either on a homo- or heterzygous background for these same floxed alleles, depending on whether this causes discomfort) to generate experimental mice. This is the most parsimonious strategy to obtain sufficient experimental mice and limit discomfort.

Finally, when discomfort develops with age, mice will be sacrificed before reaching the critical age, unless they are needed for experiments in which the cause of discomfort is part of the experiment.

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

The animals will be housed in groups with cage enrichment to prevent stress. The animals will be closely monitored for discomfort (examining posture, tendency to isolate from the group, fur). Monitoring will occur on a weekly basis or more frequently, if discomfort has been identified. If a genetically modified mouse line appears to display discomfort associated with the genotype under normal breeding conditions, an adequate solution to diminish discomfort will be sought with the help of animal welfare experts. If discomfort develops after a certain age, mice will be euthanized before this age, unless this interferes with experimental demands (in which case they will fall under experimental protocols).

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.

Not applicable

Accommodation and care

F. Accommodation and care

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

☒ No

☐ 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?

☒ 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.

☒ 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.

Animals in this appendix will experience at most moderate discomfort. If spontaneous discomfort is higher, mice will be culled. If mice experience pain as part of the spontaneous discomfort, they will be culled. Analgesia is not feasible, as that would require constitutive administration of pain relief. Anesthesia with isoflurane will be applied during ear clipping.

I. Other aspects compromising the welfare of the animals

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

We do not anticipate discomfort in most newly developed mouse lines, as we are mostly interested in genetic modifications that affect T cell differentiation. As functional effector or memory T cells are not essential in mice housed under SPF conditions, it is unlikely that our new mouse models will display discomfort associated with the genotype. However, it is possible that T cell defects result in the development of autoimmunity in some of the strains.

Explain why these effects may emerge.

Autoimmunity will develop in strains that are deficient for regulatory T cells. These cells prevent spurious activation of the immune system. Disease manifestations develop after the mice develop an adaptive immune system (T cells and B cells), and typically become overt after 12 days of age.

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

The mice will be once weekly monitored for discomfort. If signs of discomfort are apparent the mice will be more closely (up to once daily) monitored. If the signs of discomfort are recurrent in a substantial fraction of the mice, the line will be considered breeding with discomfort. In these cases, in close collaboration with the veterinarian measures to alleviate the discomfort of the mice will be considered. The mice will be terminated if the signs of discomfort exceed the humane endpoints defined below. When possible, mice will be euthanized before development of discomfort. If such measures are not effective to lower the discomfort below the thresholds of the humane endpoints, the line will be discontinued, unless the cause of discomfort is part of the study.

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.

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

We have defined the following humane endpoints:
- Loss of more than 20% body weight
- Severe breathing problems
- Severe ulceration of the skin
- Bloody stool

These humane endpoints are related to autoimmune disease that develops in mice lacking regulatory T cells.

Indicate the likely incidence.

The percentage depends on the genotype. For example in mice lacking regulatory T cells, discomfort may develop in 50% of mice due to the X-chromosomal defect in FoxP3 that only affects hemizygous males.

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’).

We expect that most of the mice (90%) on this protocol will experience at most mild discomfort and some of the mice (10%) will experience moderate discomfort. Mice will be euthanized before development of severe discomfort.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☒ No

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

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

Centrale Commissie Dierproeven

5.

Appendix
Description animal procedures

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	30100
1.2	Provide the name of the licenced establishment.	Stichting Het Nederlands Kanker Instituut – Antoni van Leeuwenhoek ziekenhuis

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	Type of animal procedure
02	Local infection with influenza

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.

General design of animal procedures:
In order to study the immune response to local infection, the widely used acute infection model of influenza infection will be employed. Mice will typically be inoculated with influenza via intranasal routes under isoflurane anesthesia to establish infection. In some experiments other routes of infection will be employed (e.g. through intravenous or intraperitoneal injection).

We will infect naïve mice to study primary immune responses. We are interested in early timepoints (up to day 9 p.i.) to study innate responses, in the peak of the primary adaptive response at day 10 p.i. to study the formation of adaptive responses, in the contraction of the adaptive response (day 11 to 14 p.i.), and in the memory phase of the adaptive response after clearance of the infection (after day 14 p.i.).

We will infect immune mice to study recall responses. For this purpose, mice will be sequentially infected with the same or different strains of influenza. These distinct influenza strains contain the same internal T cell epitope, but different viral coat epitopes (e.g. HKx31 and A/PR8/34). In some cases, the mice will be sequentially infected with influenza and other pathogens that contain the same epitope (e.g. Flu-OVA and Listeria-OVA). These heterologous settings will ensure that memory T cells formed in the first response can respond to the re-challenge in the absence of neutralizing antibodies that immediately clear secondary infection. After the sequential infections, the recall immune response will be followed at similar time-points as displayed above for the primary immune response.

We will also employ an alternative approach to study primary and secondary responses using adoptive transfer of T cells. To study primary responses, naïve T cells of wildtype or TCR transgenic mice (e.g. OT-I or F5 mice), in some cases carrying additional genetically modified alleles, will be isolated and transferred using intravenous injection into recipient mice that will subsequently be infected with influenza. The mice providing donor cells for primary responses come from appendix 6. In this manner, the primary response of the donor antigen-specific T cells can be analyzed at

similar time-points as described above for endogenous T cells. To study secondary responses, memory or in vitro activated T cells of wildtype or TCR transgenic mice will be isolated and transferred using intravenous injection into recipient mice that will subsequently be infected with influenza. The mice providing donor cells for secondary responses come from this appendix (influenza primed mice) or appendix 3, 4, 5 or 7. In this manner, the secondary response of the donor antigen-specific T cells can be analyzed at similar time-points as described above for endogenous T cells.

In some experiments, we will employ immunotherapy through immunization of the mice. To enable these experiments, mice will be immunized with immunogens (antigens, TLR ligands, adjuvans etc) as described in detail in appendix 7 to study the protective effect against infection. It is also relevant to study the impact of therapeutic immunization (immunization after establishment of infection), as in many cases help can only be offered to diseased individuals. Therefore, mice will be challenged with the pathogen and subsequently be immunized with immunogens as described in detail in appendix 7.

We will use inbred mice on a common background, typically wildtype versus transgenic or knock out mice on a C57BL/6 background in the infection experiments. Alternatively, mixed bone marrow chimeric mice will be used that have a wildtype and / or a transgenic / knockout compartment. We will employ this strategy to ensure that the infection is similarly controlled in the wildtype and in the transgenic setting. Mixed bone marrow chimeric mice will be generated by 2x irradiation (typically 4 to 5 Gray) of recipient mice and adoptive transfer of donor bone marrow by intravenous injection. Mice will be used 2-3 months after bone marrow reconstitution. Injections with antibodies can be used to label immune populations (e.g circulating CD8 T cells with non-depleting anti-CD8 antibodies), to deplete immune or hematopoietic populations (e.g. CD8 T cells with depleting anti-CD8 antibodies), to rescue the survival of specific immune populations (e.g. ARTC2 nanobodies to rescue CD8 T cell subsets) or to block specific immune processes (e.g. anti-LFA1 antibodies to block adhesion/migration of immune cells). In addition, in some experiments, small molecule inhibitors will be used to block specific immune processes (e.g. FTY720 to block migration of immune cells). Some genetic models require the addition of doxycycline (eg tet on / tet off system), tamoxifen (eg the ERT2-CRE system), diphtheria toxin (eg DTR transgenic mice), poly IC (eg Mx1-CRE system) or other to be specified reagents to induce or suppress expression of the molecules of interest.

Small volumes of blood within the effective guidelines (no more than 300 ul blood per mouse per 14 days) will be drawn from the tail vein of the mice at various time points to study the kinetics of the immune response after infection. At the end of the experiment, the mice will be humanely euthanized to enable harvest of the organs (e.g. lymph nodes, spleen, bone marrow, liver, gut, kidneys and lungs) for study of the viral load and the immune response within these organs.

Primary outcome parameters:
The immune response will be monitored using flow cytometric analysis, which allows for the identification and the phenotyping of immune cell populations within different organs of experimental and control mice. The analysis of these immune cell populations will allow assessment of the degree of inflammation and determination of the size and type of innate and adaptive immune responses (e.g. the responses of monocytes, neutrophils, CD4 and CD8 T cells, and B cells). In addition to flow cytometry, histology will be used to assess inflammation and pathology within the indicated organs that are targeted by the influenza infection. Measurements of the viral loads within the organs and blood are performed to study the kinetics of viral clearance in transgenic or treated animals compared to controls. *In vitro* experiments will be performed with immune cells from the isolated tissues to assess the potential of the immune cells in restimulation experiments. In some experiments, antibody titers will be determined by ELISA.

For some experiments, we will employ intravital imaging to study in vivo T cell responses. For this purpose, infected animals will be anesthetized using isoflurane. The tissue of interest will be prepared and positioned to enable in vivo imaging. The animals will be kept on a heating pad during the imaging procedure for maintenance of body temperature. In vivo imaging will take no more than 4 hours per animal and after the procedure the animals are euthanized using CO2 asphyxiation.

We expect that 50% of the mice will experience primary infection and that 50% will experience recall infections of which the majority (90%) will receive no more than one recall infection. We expect that 33.3% of the mice will not receive further treatment besides the infections, 33.3% will receive adoptive transfers of immune or hematopoietic cells and 33.3% will receive immunizations or other treatments as described above. From the majority of the mice (90%) blood will be taken prior to sacrifice. A minority of the mice will be analyzed by intravital imaging (less than 10%). All mice will be sacrificed at the end of the experiment.

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

Nature:
Mice with an inbred background (e.g. C57BL/6) will be inoculated with a sublethal dose of influenza (typically 2500 tissue-culture infectious dose (TCID50) in naïve mice and 25000 TCID50 in influenza immune mice). Influenza immune mice have been previously primed or contain adoptive transfer of memory T cells that are known to provide enhanced protection against infection. Upon depletion of current stocks of influenza, new stocks will be validated *in vivo* using

titration around the current dose. The virus will typically be inoculated in PBS in a volume of 50 microliters. The virus is applied through intranasal inhalation under isoflurane anesthesia. In some cases influenza is administered through a different route (via intraperitoneal or intravenous injection) for comparison to intranasal infection.

Frequency
Infection with influenza will occur once per mouse for the study of primary responses and twice or more for the study of recall responses. Mice can acquire neutralizing antibodies that achieve rapid sterile immunity after infection and therefore, re-challenge responses will typically occur with different influenza strains (e.g. HKx31 and A/PR8/34).

Mice will typically be bled once or twice only to establish the efficacy of infection (assessed by the size of the virus-specific T cell response). In some experiments, mice will be bled more frequently to follow kinetics of the response (no more than 300 ul per mouse per 14 days).

In some cases, mice will be weighed up to daily after infection to assess disease severity (when immunity is compromised due to genetic deficiency).

Duration
Inoculation of the mice with the influenza virus takes no more than 5 minutes. It is expected to cause only mild discomfort for less than one day due to the inoculation. After the onset of infection, the mice may experience moderate discomfort for a maximum period of 7 to 14 days after infection. During this period, mice may lose up to 20% of their starting body weight (primary HKx31 infection, secondary infection HKx31 and A/PR8/34). In some cases, it can be necessary to use A/PR8/34 in primary infection to study the infection-associated immunopathology that is much more apparent after infection with this strain than with other influenza strains. After primary infection with A/PR8/34, mice may lose up to 30% of their starting body weight and transiently show visible signs of discomfort (hunched posture, reduced activity and ruffled fur). Importantly, infections are sublethal and have been carefully titrated to be at the minimal doses that achieve a robust innate and adaptive immune response. It is also important to note that these signs of discomfort are transient and mice will completely recover within 14 days after infection. Mice will be in experiment for up to 90 days after infection unless in unusual cases the longevity of the memory T cells is studied. In case of re-challenge experiments, mice can be in experiment for 90 days after the last infection (e.g. 180 days for secondary infection).

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

In order to minimize the number of animals required to achieve scientifically reliable and reproducible data, the number of animals required for the experiments is calculated through power analysis. Normally distributed data will ultimately be tested by student's t test or ANOVA depending on the number of groups or variables that will be measured. Non-parametric tests will be applied in the absence of a normal distribution and power testing will take that into account.

To exemplify, an infection experiment will typically comprise two study arms (eg control group versus knockout group). To demonstrate a 50% improvement in terms of immune response size between two groups (test vs controls) with the overall variability (relative standard deviation) being around 30, we will need a group size of 5 evaluable animals per group (power > 0.8 with α = 0.05, two sided). The infection model is very consistent, and therefore, we expect only minimal loss of mice due to the inability to achieve infection or due to other causes (less than 10%). Thus, to account for losses during experimentation we will use 6 mice per group in this experiment. Consequently, we will need a group size of 6 animals per study arm, thus 2 x 6 = 12 animals per study.

The above example may not be representative for all of the experiments under this appendix. The exact group sizes will for instance depend on the number of groups and the parameters that will be analyzed in any given experiment. Therefore, each experiment will require separate power analyses and approval of the institutional animal welfare body (IvD) to determine the relevant group sizes.

B. The animals

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

Species
We will use inbred mice, because the immune response in these mice is well characterized and the reagents to measure the immune cell populations are available. Usage of inbred strains also reduces genetic variability and thus minimizes the number of required animals. The proposed influenza infection models are well-established mouse models for acute infection with intracellular pathogens, with which we have extensive prior experience. The availability of inbred strains (to enhance reproducibility) and genetically modified strains makes this the best model system to study the immune response to local primary and secondary infection in vivo.

For the experimentation, the gender of the mice is in most cases not relevant, which allows for optimal use of female and male mice from available stock.

Origin
Animals will be obtained through in-house breeding for wildtype or transgenic or knockout mouse lines. When available, littermates will be used in experiments as controls. If required, wild-type inbred control mice will be purchased from a certified commercial supplier to serve as controls.

Estimated Numbers
We will require approximately 2880 mice for these infection models.

These numbers are calculated based upon the number of molecules assessed, the number of time points, the number of immune therapies including cell transfers and immunizations and the number of groups and the size of the group. In the five years of the project, we expect to study 10 molecules after infection. These targets are assessed at four time points after primary infection and at four time points after secondary infection. We expect to study the role of these molecules in 3 different settings (control, adoptive T cell transfer and after immunization). For each time point two groups are examined (group 1: 6 control mice, group 2: 6 experimental mice). This requires 10 molecules x 3 settings x 8 time points x 6 animals x 2 groups = 2880 animals.

Life Stages
Young adult mice of approximately 8-16 weeks old will be used, as these mice have fully developed immune populations for protection against infection in the experiments. In case bone marrow chimeric mice are used, mice will be 4-6 months old due to the time it takes to reconstitute the immune compartments.

C. Re-use

Will the animals be re-used?

☒ 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
Mice will be used in experiments to assess the immune response to influenza virus. The immune response to these pathogens is a temporal cascade of complex interactions between different cell types, receptors, signaling molecules, transcription factors etc. *In vitro* studies using human samples or cell lines cannot mimic these complex interactions and no *in vitro* system exists for the generation of immune responses. There is also no computer modeling *(in silica)* that can accurately reproduce and predict the complex immune response of a living animal. Extensive searches in DB-ALM and ZEBET databases were also not able to suggest possible alternatives to the proposed animal experiments. Therefore, these studies can only be carried out in live animals.

The availability of inbred strains (to enhance reproducibility) and genetically modified strains makes the proposed influenza model the best system to study local immune responses in vivo.

Reduction
Power analyses will be done for each animal procedure to determine the minimum sample size of each group. The power analysis will be reviewed by the institutional animal welfare body (IvD) in order to obtain statistically significant and biologically relevant readouts from the proposed experiments. We manage to minimize the number of mice for these experiments by using inbred and therefore genetically identical mouse strains. This allows for smaller sample sizes and thus usage of lower total amounts of animals. We have reduced these experiments to include only crucial time points during the immune response. In addition, we perform kinetic analyses of immune responses in blood without directly sacrificing the animals. As blood samples do not reflect immune cell populations within the organs, it is required that at selected time points subgroups of animals will be humanely killed to obtain the organs.

Refinement
Animals are housed together in stable social groups with nesting and cage accessories (e.g. cage furniture) for their comfort.

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

1) To minimize animal discomfort, all experiments will be performed with the minimal dose of influenza virus that reproducibly infects the animals and generates a robust immune response. Animals will be housed in stable social groups at all times to minimize distress, as mice are social animals. Animals exhibiting any of the humane endpoints mentioned in Section J will be euthanized to reduce suffering. Specifically, mice will be weighed once every 3 days in the first 2 weeks after infection and if weight loss exceeds 20% of starting weight at the day of infection, mice will be euthanized to prevent further suffering. In case of primary A/PR8/34 infection 30% weight loss of starting weight at the day of infection will be used as cutoff.

2) There are no risks for adverse effects on the environment, as the infected mice are contained in DM-II facilities and the material is analyzed in ML-II facilities.

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.

Not applicable

Accommodation and care

F. Accommodation and care

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

☒ No

☐ 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?

☒ 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.

☒ 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.

Mice will be inoculated with influenza virus under isoflurane anesthesia. During the course of infection no measures can be taken to relieve pain, as such measures will interfere with the immune response that

we intend to study.

I. Other aspects compromising the welfare of the animals

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

The majority of the mice (90%) will receive primary infection with strains other than A/PR8/34. These mice will demonstrate moderate discomfort during infection, including weight loss, reduced activity, ruffled fur and hunched posture. For some experiments (10%) primary A/PR8/34 infection is required, which will induce severe discomfort, which is reflected in more severe weight loss of up to 30% of starting weight. The loss of bodyweight is due to the infection of target tissues (lungs) and / or the resultant immune response directed at the pathogen that may cause collateral tissue damage. These effects transiently induce loss of appetite and consequently loss of bodyweight in the infected mice. As A/PR8/34 induces higher levels of immunopathology, the observed weight loss in these mice is more severe than for other influenza strains (up to 30% instead of up to 20% of starting body weight). It is important to note that the discomfort is transient (similar for all influenza strains including A/PR8/34). The infected mice do not demonstrate any symptoms of disease after day 14 post infection, since mice typically clear the infection within this timeframe. The observed weight loss is also transient and mice regain weight after the peak of infection at around day 10 after infection and are back at starting weight after about 14 days post-infection. In the proposed study, we include experimental time points of up to 90 days post infection, which means that mice can be without discomfort during a large part of the experiment. It is known that occasionally some mice will demonstrate disease symptoms more severely than expected or that disease symptoms persist due to inability to resolve infection. If symptoms are more severe or if they persist (that is mice do not recover within 14 days), these mice will be sacrificed as also described below under humane endpoints.

In case bone marrow chimeric mice are used in the experiments discomfort due to irradiation may arise (irradiation sickness). The irradiation is required to eliminate the host compartment of the immune system to make space for the donor bone marrow. The effects are transient (less than 2 weeks) as reconstitution with donor bone marrow results in full recovery of the irradiated mice. In rare cases when reconstitution is insufficient, irradiated mice will be eliminated on similar criteria as after infection as described under humane endpoints.

Explain why these effects may emerge.

These effects are a result of the infection and the accompanying inflammation that may cause moderate discomfort in the animals

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

The administration of analgesia is impossible, because of its potential inhibition of the immune response, which we intend to study in this project. To minimize severity of infection, we will use the viral inoculum in the lowest possible dose that induces a robust and reproducible immune response in the mice without causing excessive discomfort.

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.

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

Removal from this study will be based on critical clinical criteria including: 1) abnormal weight loss of greater than 20% of initial weight (or 30% of initial weight in case of primary A/PR8/34 infection), 2) severe breathing problems, 3) absence of recovery of observed weight loss at day 14 after infection and 4) any other serious symptoms of disease. In the case of sporadic or unpredicted problems, mice will be separated into different subgroups when possible (e .g. fighting), or killed humanely, if the health of the animal is impaired beyond the expected infectious symptoms.

Indicate the likely incidence.

We expect the occurrence of such complications and subsequent removal based on the critical clinical parameters as rare (approximately 5% of experimental mice).

K. Classification of severity of procedures

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

The expected level of discomfort associated with influenza infection except for primary A/PR8/34 infection is moderate. Primary A/PR8/34 infection can induce severe discomfort related to enhanced immune pathology. In this appendix, 90% of the infected mice will experience moderate discomfort related to influenza infection other than primary A/PR8/34 infection and 10% will experience severe discomfort related to A/PR8/34 infection.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

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

The animals need to be euthanized at the end of the experiment in order to harvest vital organs at key time points post infection. This is necessary in order to measure and assess immune responses in target organs of the infection and in the lymphoid organs.

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

Centrale Commissie Dierproeven

6.

Appendix
Description animal procedures

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1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	30100
1.2	Provide the name of the licenced establishment.	Stichting Het Nederlands Kanker Instituut – Antoni van Leeuwenhoek ziekenhuis

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	Type of animal procedure
03	Systemic infection with Listeria monocytogenes or LCMV

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.

General design of animal procedures:
In order to study the immune response to systemic infection, the widely used acute infection models of *Listeria monocytogenes* (*L.m.*) and the lymphocytic choriomeningitis virus (LCMV) will be employed. Mice will be inoculated with *L.m.* via intravenous, intraperitoneal, oral or intragastric routes to establish *L.m.* infection, and / or with LCMV via intravenous or intraperitoneal routes to establish LCMV infection. In some experiments other routes of infection may be employed (e.g. intranasal infection).

We will infect naïve mice to study primary immune responses. We are interested in early timepoints (up to day 7 p.i.) to study innate responses, in the peak of the primary adaptive response at day 8-10 p.i. to study the formation of adaptive responses, in the contraction of the adaptive response (day 11 to 14 p.i.), and in the memory phase of the adaptive response after clearance of the infection (after day 14 p.i.).

We will infect immune mice to study recall responses. Mice will be re-challenged with a second homologous infection (2x L.m. or 2x LCMV) or a second heterologous infection. For heterologous recall responses, first mice will be infected with LCMV or L.m. and then after at least 30 days the same mice will be infected with a different pathogen containing the same epitope as LCMV or *L.m* (e.g. LCMV → *L.m.* containing the gp33 epitope of LCMV). The heterologous settings will ensure that memory T cells formed in the first response can respond to the re-challenge in the absence of neutralizing antibodies that can immediately clear secondary infection. After the sequential infections, the recall immune response will be followed at similar time-points as displayed above for the primary immune response.

We will also employ an alternative approach to study primary and secondary responses using adoptive transfer of T cells. To study primary responses, naïve T cells of wildtype or TCR transgenic mice (e.g. OT-I or P14 mice), in some cases carrying additional genetically modified alleles, will be isolated and transferred using intravenous injection into

recipient mice that will subsequently be infected with LCMV or *L.m*. The mice providing donor cells for primary responses come from appendix 6. In this manner, the primary response of the donor antigen-specific T cells can be analyzed at similar time-points as described above for endogenous T cells. To study secondary responses, memory or in vitro activated T cells of wildtype or TCR transgenic mice will be isolated and transferred using intravenous injection into recipient mice that will subsequently be infected with LCMV or *L.m*. The mice providing donor cells for secondary responses come from this appendix (LCMV or *L.m.* primed mice) or appendix 2, 4, 5 or 7. In this manner, the secondary response of the donor antigen-specific T cells can be analyzed at similar time-points as described above for endogenous T cells.

In some experiments, we will employ immunotherapy through immunization of the mice. To enable these experiments, mice will be immunized with immunogens (antigens, TLR ligands, adjuvans etc) as described in detail in appendix 7 to study the protective effect against infection. It is also relevant to study the impact of therapeutic immunization (immunization after establishment of infection), as in many cases help can only be offered to diseased individuals. Therefore, mice will be challenged with the pathogen and subsequently be immunized with immunogens as described in detail in appendix 7.

We will use inbred mice on a common background, typically wildtype versus transgenic or knock out mice on a C57BL/6 background in the infection experiments. Alternatively, mixed bone marrow chimeric mice will be used that have a wildtype and / or a transgenic / knockout compartment. We will employ this strategy to ensure that the infection is similarly controlled in the wildtype and in the transgenic setting. Mixed bone marrow chimeric mice will be generated by 2x irradiation (typically 4 to 5 Gray) of recipient mice and adoptive transfer of donor bone marrow by intravenous injection. Mice will be used 2-3 months after bone marrow reconstitution. Injections with antibodies can be used to label immune populations (e.g circulating CD8 T cells with non-depleting anti-CD8 antibodies), to deplete immune or hematopoietic populations (e.g. CD8 T cells with depleting anti-CD8 antibodies), to rescue the survival of specific immune populations (e.g. ARTC2 nanobodies to rescue CD8 T cell subsets) or to block specific immune processes (e.g. anti-LFA1 antibodies to block adhesion/migration of immune cells). In addition, in some experiments, small molecule inhibitors will be used to block specific immune processes (e.g. FTY720 to block migration of immune cells). Some genetic models require the addition of doxycycline (eg tet on / tet off system), tamoxifen (eg the ERT2-CRE system), diphtheria toxin (eg DTR transgenic mice), poly IC (eg Mx1-CRE system) or other to be specified reagents to induce or suppress expression of the molecules of interest.

Small volumes of blood within the effective guidelines (no more than 300 ul blood per mouse per 14 days) will be drawn from the tail vein of the mice at various time points to study the kinetics of the immune response after infection. At the end of the experiment, the mice will be humanely euthanized to enable harvest of the organs (e.g. lymph nodes, spleen, bone marrow, liver, gut, kidneys and lungs) for study of the viral or bacterial load and the immune response within these organs.

Primary outcome parameters:
The immune response will be monitored using flow cytometric analysis, which allows for the identification and the phenotyping of immune cell populations within different organs of experimental and control mice. The analysis of these immune cell populations will allow assessment of the degree of inflammation and determination of the size and type of innate and adaptive immune responses (e.g. the responses of monocytes, neutrophils, CD4 and CD8 T cells, and B cells). In addition to flow cytometry, histology will be used to assess inflammation and pathology within the indicated organs that are targeted by the *L.m.* or LCMV infection. Measurements of the bacterial or viral loads within the organs and blood are performed to study the kinetics of bacterial or viral clearance in transgenic / knockout or treated animals compared to controls. *In vitro* experiments will be performed with immune cells from the isolated tissues to assess the potential of the immune cells in restimulation experiments. ELISAs will be performed to determine antibody titers.

For some experiments, we will employ intravital imaging to study in vivo T cell responses. For this purpose, infected animals will be anesthetized using isoflurane. The tissue of interest will be prepared and positioned to enable in vivo imaging. The animals will be kept on a heating pad during the imaging procedure for maintenance of body temperature. In vivo imaging will take no more than 4 hours per animal and after the procedure the animals are euthanized using CO2 asphyxiation.

We expect that 50% of the mice will experience primary infection and that 50% will experience recall infections of which the majority (90%) will receive no more than one recall infection. We expect that 33.3% of the mice will not receive further treatment besides the infections, 33.3% will receive adoptive transfers of immune or hematopoietic cells and 33.3% will receive immunizations or other treatments as described above. From the majority of the mice (90%) blood will be taken prior to sacrifice. A minority of the mice will be analyzed by intravital imaging (less than 10%). All mice will be sacrificed at the end of the experiment.

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

Nature:

***L.m.* infection:**
Mice with an inbred background (e.g. C57BL/6) will be inoculated with a sublethal dose of *L.m.* (typically 25.000 colony forming units (CFU) in naïve mice and approximately 250.000 CFU in *L.m.* immune mice). *L.m.* immune mice have been primed or contain adoptive transfer of memory T cells that are known to provide enhanced protection against infection. Upon depletion of current stocks of *L.m.* bacteria, new stocks will be generated and new stocks will be validated *in vivo* using titration around the current dose. The bacteria will typically be inoculated in PBS in a volume of 200 microliters. *L.m.* bacteria are applied intravenously through injection in the tail vein, through intraperitoneal injection, or through intragastric injection or by oral administration. Intravenous and intraperitoneal injection procedures result in systemic infection, whereas intragastric injection and oral administration target infection preferentially to the small intestine. In some cases different routes of inoculation are applied.

LCMV infection:
Mice with an inbred background (e.g. C57BL/6) will be inoculated with a sublethal dose of LCMV Armstrong (typically $1x10^5$ plaque forming units (PFU) in naïve mice and approximately $1x10^6$ PFU in LCMV immune mice). LCMV immune mice have been primed or contain adoptive transfer of memory T cells that are known to provide enhanced protection against infection. Upon depletion of current stocks of LCMV virus, new stocks will be validated *in vivo* using titration around the current dose. The virus will be inoculated in PBS in a volume of 200 microliters. LCMV virus is applied intravenously through injection in the tail vein or through intraperitoneal injection. Intravenous and intraperitoneal injection procedures both result in systemic infection. In some cases different routes of inoculation are applied.

Frequency
Infection with *L.m.* or LCMV will occur once per mouse for the study of primary responses and twice or more for the study of recall responses. Mice can acquire neutralizing antibodies that achieve rapid sterile immunity after infection and therefore re-challenge will typically occur by sequential infection with different pathogens (e.g. first *L.m.* and then LCMV, or alternatively, first LCMV and then *L.m*).

Mice will typically be bled once or twice only to establish the efficacy of infection (assessed by the size of the pathogen-specific T cell response). In some experiments, mice will be bled more frequently to follow kinetics of the response (no more than 300 ul per mouse per 14 days).

In some cases, mice will be weighed up to daily after infection to assess disease severity (when immunity is compromised due to genetic deficiency).

Duration
Inoculation of the mice with the LCMV virus or the *L.m.* bacteria takes no more than 5 minutes. It is expected to cause only mild discomfort for less than one day due to the injection. After the onset of infection, the mice may experience moderate discomfort for a maximum period of 7 to 14 days after infection. During this period, mice are typically asymptomatic, but can show visible signs of discomfort such as weight loss of up to 20% of starting weight, ruffled fur, reduced activity and hunched posture. Importantly, infections are sublethal and have been carefully titrated to be at the minimal doses that achieve a robust innate and adaptive immune response. It is also important to note that these signs of discomfort are transient and mice will completely recover within 14 days after infection. Mice will be in experiment for up to 90 days after infection unless in unusual cases the longevity of the memory T cells is studied. In case of re-challenge experiments, mice can be in experiment for 90 days after the last infection (e.g. 180 days for secondary infection).

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

In order to minimize the number of animals required to achieve scientifically reliable and reproducible data, the number of animals required for the experiments is calculated through power analysis. Normally distributed data will ultimately be tested by student's t test or ANOVA depending on the number of groups or variables that will be measured. Non-parametric tests will be applied in the absence of a normal distribution and power testing will take that into account.

To exemplify, an infection experiment will typically comprise two study arms (eg control group versus knockout group). To demonstrate a 50% improvement in terms of immune response size between two groups (test vs controls) with the overall variability (relative standard deviation) being around 30, we will need a group size of 5 evaluable animals per group (power > 0.8 with $\alpha = 0.05$, two sided). The infection model is very consistent, and therefore, we expect only minimal loss of mice due to the inability to achieve infection or due to other causes (less than 10%). Thus, to account for losses during experimentation we will use 6 mice per group in this experiment. Consequently, we will need a group size of 6 animals per study arm, thus 2 x 6 = 12 animals per study.

The above example may not be representative for all of the experiments under this appendix. The exact group sizes will for instance depend on the number of groups and the parameters that will be analyzed in any given experiment. Therefore, each experiment will require separate power analyses and approval of the institutional animal welfare body (IvD) to determine the relevant group sizes.

B. The animals

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

Species
We will use inbred mice, because the immune response in these mice is well characterized and the reagents to measure the immune cell populations are available. Usage of inbred strains also reduces genetic variability and thus minimizes the number of required animals. The proposed *L.m.* and LCMV infection models are well-established mouse models for acute infection with intracellular pathogens, with which we have extensive prior experience. The availability of inbred strains (to enhance reproducibility) and genetically modified strains makes these models the best systems to study the immune response to systemic primary and secondary infections in vivo.

For the experimentation, the gender of the mice is in most cases not relevant, which allows for optimal use of female and male mice from available stock.

Origin
Animals will be obtained through in-house breeding for wild-type or transgenic or knockout mouse lines. When available, littermates will be used in experiments as controls. If required, wild-type inbred control mice will be purchased from a certified commercial supplier to serve as controls.

Estimated Numbers
We will require approximately 5760 mice for these infection models.

These numbers are calculated based upon the number of molecules assessed, the number of time points, the number of immune therapies including cell transfers and immunizations and the number of groups and the size of the group. In the five years of the project, we expect to study 10 molecules after infection. These targets are assessed at four time points after primary infection and at four time points after secondary infection. We expect to study the role of these molecules in 3 different settings (control, adoptive T cell transfer and after immunization). For each time point two groups are examined (group 1: 6 control mice, group 2: 6 experimental mice). This requires 10 molecules x 3 settings x 8 time points x 6 animals x 2 groups x 2 types of infection models (Listeria and LCMV) = 5760 animals.

Life Stages
Young adult mice of approximately 8-16 weeks old will be used, as these mice have fully developed immune populations for protection against infection in the experiments. In case bone marrow chimeric mice are used, mice will be 4-6 months old due to the time it takes to reconstitute the immune compartments.

C. Re-use

Will the animals be re-used?

☒ 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
Mice will be used in experiments to assess the immune response to LCMV virus or *L.m.* bacteria. The immune response to these pathogens is a temporal cascade of complex interactions between different cell types, receptors, signaling molecules, transcription factors etc. *In vitro* studies using human samples or cell lines cannot mimic these complex interactions and no *in vitro* system exists for the generation of immune responses. There is also no computer modeling *(in silica)* that can accurately reproduce and predict the complex immune response of a living animal. Extensive searches in DB-ALM and ZEBET databases were also not able to suggest possible alternatives to the proposed animal

experiments. Therefore, these studies can only be carried out in live animals. The availability of inbred strains (to enhance reproducibility) and genetically modified strains makes this these infection models the best systems to study systemic immune responses in vivo.

Reduction
Power analyses will be done for each animal procedure to determine the minimum sample size of each group. The power analysis will be reviewed by the institutional animal welfare body (IvD) in order to obtain statistically significant and biologically relevant readouts from the proposed experiments. We manage to minimize the number of mice for these experiments by using inbred and therefore genetically identical mouse strains. This allows for smaller sample sizes and thus usage of lower total amounts of animals. We have reduced these experiments to include only crucial time points during the immune response. In addition, we perform kinetic analyses of immune responses in blood without directly sacrificing the animals. As blood samples do not reflect immune cell populations within the organs, it is required that at selected time points subgroups of animals will be humanely killed to obtain the organs.

Refinement
Animals are housed together in stable social groups with nesting and cage accessories (e.g. cage furniture) for their comfort.

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

1) To minimize animal discomfort, all experiments will be performed with the minimal dose of LCMV virus or *L.m.* bacteria that reproducibly infects the animals and generates a robust immune response. Animals will be housed in stable social groups at all times to minimize distress, as mice are social animals. Animals exhibiting any of the humane endpoints mentioned in Section J will be euthanized to reduce suffering.

2) There are no risks for adverse effects on the environment, as the infected mice are contained in DM-II facilities and the material is analyzed in ML-II facilities.

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.

Not applicable

Accommodation and care

F. Accommodation and care

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

☒ No

☐ 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?

☒ 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.

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

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

During the course of infection no measures can be taken to relieve pain, as such measures will interfere with the immune response that we intend to study.

☐ 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 inoculated with a sublethal dose of LCMV virus or *L.m.* bacteria can demonstrate moderate discomfort during infection, including weight loss, reduced activity, ruffled fur and hunched posture. The loss of bodyweight is due to the infection of target tissues (multiple tissues including liver, gut and kidneys) and / or the resultant immune response directed at the pathogen that may cause collateral tissue damage. These effects may transiently induce loss of appetite and consequently loss of bodyweight in the infected mice. It is important to note that the discomfort is transient. The infected mice do not demonstrate any symptoms of disease after day 14 post infection, since mice typically clear the infection within this timeframe. The observed weight loss is also transient and mice regain weight after the peak of infection at around day 8 after infection and are back at starting weight after about 14 days post-infection. In the proposed study, we include experimental time points of up to 90 days post infection, which means that mice can be without discomfort during a large part of the experiment. It is known that occasionally some mice will demonstrate disease symptoms more severely than expected or that disease symptoms persist due to inability to resolve infection. If symptoms are more severe or if they persist (that is mice do not recover within 14 days), these mice will be sacrificed as also described below under humane endpoints.

In case bone marrow chimeric mice are used in the experiments discomfort due to irradiation may arise (irradiation sickness). The irradiation is required to eliminate the host compartment of the immune system to make space for the donor bone marrow. The effects are transient (less than 2 weeks) as reconstitution with donor bone marrow results in full recovery of the irradiated mice. In rare cases when reconstitution is insufficient, irradiated mice will be eliminated on similar criteria as after infection as described under humane endpoints.

Explain why these effects may emerge.

These effects are a result of the infection and the accompanying inflammation that may cause moderate discomfort in the animals

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

The administration of analgesia is impossible, because of its potential inhibition of the immune response, which we intend to study in this project. To minimize severity of infection, we will use the bacterial inoculum in the lowest possible dose that induces a robust, reproducible immune response in the mice without causing excessive discomfort.

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.

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

Removal from this study will be based on critical clinical criteria including: 1) weight loss indicative of more than 20% of the initial weight, 2) severe breathing problems, 3) absence of recovery of observed weight loss at day 14 after infection and 4) any other serious symptoms of disease. In the case of sporadic or unpredicted problems, mice will be separated into different subgroups when possible (e .g. fighting), or killed humanely, if the health of the animal is impaired beyond the expected infectious symptoms.

Indicate the likely incidence.

We expect the occurrence of such complications and subsequent removal based on the critical clinical parameters as rare (approximately 5% of experimental mice).

K. Classification of severity of procedures

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

The expected level of discomfort associated with infection is moderate. In this appendix, it is expected that 100% of the infected mice will experience moderate discomfort.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

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

The animals need to be euthanized at the end of the experiment in order to harvest vital organs at key time points post infection. This is necessary in order to measure and assess immune responses in target organs of the infection and in the lymphoid organs.

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

Centrale Commissie Dierproeven

Appendix
Description animal procedures

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	30100
1.2	Provide the name of the licenced establishment.	Stichting Het Nederlands Kanker Instituut – Antoni van Leeuwenhoek ziekenhuis

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	Type of animal procedure
04	Infection with acute and chronic LCMV

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.

General design of animal procedures:
In order to study the immune response to acute versus chronic infection, the widely used infection models of the lymphocytic choriomeningitis virus (LCMV) will be employed. Different strains of LCMV induce acute infection (LCMV WE and LCMV Armstrong) or chronic infection (LCMV Clone 13 and LCMV Docile). Therefore, it is possible to directly compare T cell responses of the same specificity in acute and chronic immune responses. Mice will be inoculated with LCMV via intravenous or intraperitoneal routes to establish infection. In some experiments other routes of infection may be employed (e.g. intranasal infection).

We will infect naïve mice to study acute and chronic immune responses against LCMV. We are interested in early timepoints (up to day 7 p.i.) to study innate responses, in the peak of the primary adaptive response at day 8 p.i. to study the formation of adaptive responses, in the contraction of the adaptive response (day 9 to 14 p.i.), and in the memory phase of the adaptive response after clearance of acute infection (after day 14 p.i.) or in the chronic phase of the adaptive response at late timepoints (after day 14 p.i.).

We will also employ an alternative approach to study acute and chronic immune responses using adoptive transfer of T cells. For this purpose, T cells of wildtype or TCR transgenic mice (e.g. P14 mice), in some cases carrying additional genetically modified alleles, will be isolated and transferred using intravenous injection into recipient mice that will subsequently be infected with acute or chronic strains of LCMV. The mice providing donor cells for these responses come from this appendix (LCMV primed mice) or appendix 2, 3, 4, 5, 6 or 7. In this manner, the acute or chronic immune response of the donor antigen-specific T cells can be analyzed at similar time-points as described above for endogenous T cells.

In some experiments, we will employ immunotherapy through immunization of the mice. To enable these experiments, mice will be immunized with immunogens (antigens, TLR ligands, adjuvans etc) as described in detail in appendix 7 to

study the protective effect against infection. It is also relevant to study the impact of therapeutic immunization (immunization after establishment of infection), as in many cases help can only be offered to diseased individuals. Therefore, mice will be challenged with the pathogen and subsequently be immunized with immunogens as described in detail in appendix 7.

We will use inbred mice on a common background, typically wildtype versus transgenic or knock out mice on a C57BL/6 background in the infection experiments. Alternatively, mixed bone marrow chimeric mice will be used that have a wildtype and / or a transgenic / knockout compartment. We will employ this strategy to ensure that the infection is similarly controlled in the wildtype and in the transgenic setting. Mixed bone marrow chimeric mice will be generated by 2x irradiation (typically 4 to 5 Gray) of recipient mice and adoptive transfer of donor bone marrow by intravenous injection. Mice will be used 2-3 months after bone marrow reconstitution. Injections with antibodies can be used to label immune populations (e.g circulating CD8 T cells with non-depleting anti-CD8 antibodies), to deplete immune or hematopoietic populations (e.g. CD8 T cells with depleting anti-CD8 antibodies), to rescue the survival of specific immune populations (e.g. ARTC2 nanobodies to rescue CD8 T cell subsets) or to block specific immune processes (e.g. anti-LFA1 antibodies to block adhesion/migration of immune cells). In addition, in some experiments, small molecule inhibitors will be used to block specific immune processes (e.g. FTY720 to block migration of immune cells). Some genetic models require the addition of doxycycline (eg tet on / tet off system), tamoxifen (eg the ERT2-CRE system), diphtheria toxin (eg DTR transgenic mice), poly IC (eg Mx1-CRE system) or other to be specified reagents to induce or suppress expression of the molecules of interest.

Small volumes of blood within the effective guidelines (no more than 300 ul blood per mouse per 14 days) will be drawn from the tail vein of the mice at various time points to study the kinetics of the immune response after infection. At the end of the experiment, the mice will be humanely euthanized to enable harvest of the organs (e.g. lymph nodes, spleen, bone marrow, liver, gut, kidneys and lungs) for study of the viral load and the immune response within these organs.

Primary outcome parameters:
The immune response will be monitored using flow cytometric analysis, which allows for the identification and the phenotyping of immune cell populations within different organs of experimental and control mice. The analysis of these immune cell populations will allow assessment of the degree of inflammation and determination of the size and type of innate and adaptive immune responses (e.g. the responses of monocytes, neutrophils, CD4 and CD8 T cells, and B cells). In addition to flow cytometry, histology will be used to assess inflammation and pathology within the indicated organs that are targeted by LCMV infection. Measurements of the viral loads within the organs and blood are performed to study the kinetics of viral clearance in transgenic / knockout or treated animals compared to controls. *In vitro* experiments will be performed with immune cells from the isolated tissues to assess the potential of the immune cells in restimulation experiments. In some experiments, antibody titers will be determined by ELISA.

For some experiments, we will employ intravital imaging to study in vivo T cell responses. For this purpose, infected animals will be anesthetized using isoflurane. The tissue of interest will be prepared and positioned to enable in vivo imaging. The animals will be kept on a heating pad during the imaging procedure for maintenance of body temperature. In vivo imaging will take no more than 4 hours per animal and after the procedure the animals are euthanized using CO2 asphyxiation.

We expect that 50% of the mice will experience acute primary infection and that 50% will experience chronic primary infection. We expect that 33.3% of the mice will not receive further treatment besides the infections, 33.3% will receive adoptive transfers of immune or hematopoietic cells and 33.3% will receive immunizations or other treatments as described above. From the majority of the mice (90%) blood will be taken prior to sacrifice. A minority of the mice will be analyzed by intravital imaging (less than 10%). All mice will be sacrificed at the end of the experiment.

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

Nature:
To induce acute immune responses, mice with an inbred background (e.g. C57BL/6) will be inoculated with a sublethal dose of LCMV Armstrong (typically $1x10^5$ plaque forming units (PFU)). To induce chronic immune responses, mice will be inoculated with a sublethal dose of LCMV Clone 13 (typically $1x10^6$ PFU). Upon depletion of current stocks of LCMV virus, new stocks will be validated *in vivo* using titration around the current dose. The virus will be inoculated in PBS in a volume of 200 microliters. LCMV virus is applied intravenously through injection in the tail vein or through intraperitoneal injection. Both injection procedures result in systemic infection.

Frequency
Infection with LCMV will only occur once per mouse.

Mice will typically be bled once or twice only to establish the efficacy of infection (assessed by the size of the pathogen-specific T cell response). In some experiments, mice will be bled more frequently to follow kinetics of the response (no more than 300 ul per mouse per 14 days).

In some cases, mice will be weighed up to daily after infection to assess disease severity (when immunity is compromised due to genetic deficiency).

Duration
Inoculation of the mice with the LCMV virus takes no more than 5 minutes. It is expected to cause only mild discomfort for less than one day due to the injection. After the onset of acute or chronic infection, the mice may experience moderate discomfort for a maximum period of 7 to 14 days after infection. During this period, mice are typically asymptomatic, but can show visible signs of discomfort such as weight loss of up to 20% of starting weight, ruffled fur, reduced activity and hunched posture. Mice experiencing acute infection completely clear infection within 14 days and are asymptomatic thereafter. Mice experiencing chronic infection do not clear the virus within the first 2 months after inoculation, but the viral loads are strongly reduced after 14 days, which results in asymptomatic disease during this stage. Importantly, infections are sublethal and have been carefully titrated to be at the minimal doses that achieve a robust innate and adaptive immune response. Mice will be in experiment for up to 90 days after infection unless in unusual cases the longevity of the memory T cells is studied.

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

In order to minimize the number of animals required to achieve scientifically reliable and reproducible data, the number of animals required for the experiments is calculated through power analysis. Normally distributed data will ultimately be tested by student's t test or ANOVA depending on the number of groups or variables that will be measured. Non-parametric tests will be applied in the absence of a normal distribution and power testing will take that into account.

To exemplify, an infection experiment will typically comprise two study arms (eg control group versus knockout group). To demonstrate a 50% improvement in terms of immune response size between two groups (test vs controls) with the overall variability (relative standard deviation) being around 30, we will need a group size of 5 evaluable animals per group (power > 0.8 with $\alpha = 0.05$, two sided). The infection model is very consistent, and therefore, we expect only minimal loss of mice due to the inability to achieve infection or due to other causes (less than 10%). Thus, to account for losses during experimentation we will use 6 mice per group in this experiment. Consequently, we will need a group size of 6 animals per study arm, thus 2 x 6 = 12 animals per study.

The above example may not be representative for all of the experiments under this appendix. The exact group sizes will for instance depend on the number of groups and the parameters that will be analyzed in any given experiment. Therefore, each experiment will require separate power analyses and approval of the institutional animal welfare body (IvD) to determine the relevant group sizes.

B. The animals

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

Species
We will use inbred mice, because the immune response in these mice is well characterized and the reagents to measure the immune cell populations are available. Usage of inbred strains also reduces genetic variability and thus minimizes the number of required animals. The proposed LCMV infection models are well-established mouse models for acute and chronic infection with intracellular pathogens, with which we have extensive prior experience. The availability of inbred strains (to enhance reproducibility) and genetically modified strains makes this the best model system to study the immune response to acute versus chronic infection in vivo.

For the experimentation, the gender of the mice is in most cases not relevant, which allows for optimal use of female and male mice from available stock.

Origin
Animals will be obtained through in-house breeding for wild-type or transgenic or knockout mouse lines. When available, littermates will be used in experiments as controls. If required, wild-type inbred control mice will be purchased from a certified commercial supplier to serve as controls.

Estimated Numbers
We will require approximately 2880 mice for these infection models.

These numbers are calculated based upon the number of molecules assessed, the number of time points, the number of immune therapies including cell transfers and immunizations and the number of groups and the size of the group. In the five years of the project, we expect to study 10 molecules after infection. These targets are assessed at four time points after acute infection and at four time points after chronic infection. We expect to study the role of these molecules in 3

different settings (control, adoptive T cell transfer and after immunization). For each time point two groups are examined (group 1: 6 control mice, group 2: 6 experimental mice). This requires 10 molecules x 3 settings x 8 time points x 6 animals x 2 groups = 2880 animals.

Life Stages
For acute LCMV infection (LCMV Armstrong), young adult mice of approximately 8-16 weeks old will be used (50% of the mice), as these mice have fully developed immune populations for protection against infection in the experiments. For chronic LCMV infection (LCMV Armstrong Clone 13), mice will be used of 4-6 weeks of age (50% of the mice), as incomplete development of the immune system is required to establish chronic infection. In case bone marrow chimeric mice are used, mice will be 4-6 months old due to the time it takes to reconstitute the immune compartments.

C. Re-use

Will the animals be re-used?

☒ 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
Mice will be used in experiments to assess the immune response to LCMV virus. The immune response to these pathogens is a temporal cascade of complex interactions between different cell types, receptors, signaling molecules, transcription factors etc. *In vitro* studies using human samples or cell lines cannot mimic these complex interactions and no *in vitro* system exists for the generation of immune responses. There is also no computer modeling *(in silica)* that can accurately reproduce and predict the complex immune response of a living animal. Extensive searches in DB-ALM and ZEBET databases were also not able to suggest possible alternatives to the proposed animal experiments. Therefore, these studies can only be carried out in live animals. The availability of inbred strains (to enhance reproducibility) and genetically modified strains makes this the best model system to study the immune response to systemic chronic infection in vivo.

Reduction
Power analyses will be done for each animal procedure to determine the minimum sample size of each group. The power analysis will be reviewed by the institutional animal welfare body (IvD) in order to obtain statistically significant and biologically relevant readouts from the proposed experiments. We manage to minimize the number of mice for these experiments by using inbred and therefore genetically identical mouse strains. This allows for smaller sample sizes and thus usage of lower total amounts of animals. We have reduced these experiments to include only crucial time points during the immune response. In addition, we perform kinetic analyses of immune responses in blood without directly sacrificing the animals. As blood samples do not reflect immune cell populations within the organs, it is required that at selected time points subgroups of animals will be humanely killed to obtain the organs.

Refinement
Animals are housed together in stable social groups with nesting and cage accessories (e.g. cage furniture) for their comfort.

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

1) To minimize animal discomfort, all experiments will be performed with the minimal dose of LCMV virus that reproducibly infects the animals and generates a robust immune response. Animals will be housed in stable social groups at all times to minimize distress, as mice are social animals. Animals exhibiting any of the humane endpoints mentioned in Section J will be euthanized to reduce suffering. Specifically, mice will be weighed once every 3 days in the first 2 weeks after infection and if weight loss exceeds 20% of starting weight at the day of infection, mice will be euthanized to prevent further suffering.

2) There are no risks for adverse effects on the environment, as the infected mice are contained in DM-II facilities and

the material is analyzed in ML-II facilities.

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.

Not applicable

Accommodation and care

F. Accommodation and care

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

☒ No

☐ 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?

☒ 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.

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

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

During the course of infection no measures can be taken to relieve pain, as such measures will interfere with the immune response that we intend to study.

☐ 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 inoculated with a sublethal dose of LCMV virus can demonstrate moderate discomfort during infection, including weight loss, reduced activity, ruffled fur and hunched posture. The loss of bodyweight is due to the infection of target tissues (multiple tissues including liver, gut and kidneys) and / or the resultant immune response directed at the pathogen that may cause collateral tissue damage. These effects may transiently induce loss of appetite and consequently loss of bodyweight in the infected mice. It is important to note that the discomfort is transient. The infected mice do not demonstrate any symptoms of disease after day 14 post infection, since mice typically clear the infection within this timeframe (acute infection) or are able to reduce the viral titers substantially, although not completely (chronic infection). The observed weight loss is also transient and mice regain weight after the peak of infection at around day 8

after infection and are back at starting weight after about 14 days post-infection. In the proposed study, we include experimental time points of up to 90 days post infection, which means that mice can be without discomfort during a large part of the experiment. It is known that occasionally some mice will demonstrate disease symptoms more severely than expected or that disease symptoms persist due to inability to resolve infection. If symptoms are more severe or if they persist (that is mice do not recover within 14 days), these mice will be sacrificed as also described below under humane endpoints.

In case bone marrow chimeric mice are used in the experiments discomfort due to irradiation may arise (irradiation sickness). The irradiation is required to eliminate the host compartment of the immune system to make space for the donor bone marrow. The effects are transient (less than 2 weeks) as reconstitution with donor bone marrow results in full recovery of the irradiated mice. In rare cases when reconstitution is insufficient, irradiated mice will be eliminated on similar criteria as after infection as described under humane endpoints.

Explain why these effects may emerge.

These effects are a result of the infection and the accompanying inflammation that may cause moderate discomfort in the animals

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

The administration of analgesia is impossible, because of its potential inhibition of the immune response, which we intend to study in this project. To minimize severity of infection, we will use the virus in the lowest dose that induces a robust, reproducible immune response in the mice without causing excessive discomfort.

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.

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

Removal from this study will be based on critical clinical criteria including: 1) abnormal weight loss of greater than 20% of initial weight, 2) severe breathing problems, 3) absence of recovery of observed weight loss at day 14 after infection and 4) any other serious symptoms of disease. In the case of sporadic or unpredicted problems, mice will be separated into different subgroups when possible (e .g. fighting), or killed humanely, if the health of the animal is impaired beyond the expected infectious symptoms.

Indicate the likely incidence.

We expect the occurrence of such complications and subsequent removal based on the critical clinical parameters as rare (approximately 5% of experimental mice).

K. Classification of severity of procedures

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

The expected level of discomfort associated with infection is moderate. In this appendix, we expect that 100% of the infected mice will experience moderate discomfort.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

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

The animals need to be euthanized at the end of the experiment in order to harvest vital organs at key time points post infection. This is necessary in order to measure and assess immune responses in target organs of the infection and in the lymphoid organs.

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

Centrale Commissie Dierproeven

Appendix
Description animal procedures

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	30100
1.2	Provide the name of the licenced establishment.	Stichting Het Nederlands Kanker Instituut – Antoni van Leeuwenhoek ziekenhuis

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	Type of animal procedure
05	Tumor challenge

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.

General design of animal procedures:
This appendix describes the use of transplantable tumor models in mice as a surrogate for cancer patients to study the efficacy of adoptive T cell transfer as an anti-cancer therapy. Mice will be injected/implanted with a tumor cell line (e.g. the melanoma cell line B16 or the colon carcinoma cell line MC38) at an ectopic site (usually subcutaneously in the right and / or left flank) or an orthotopic site (e.g breast cancer cell lines in the fat pads) as a surrogate for primary tumor formation or systemically to mimic metastasis formation. The tumor cell lines cover a wide range in immunogenicity (e.g. MC38 is immunogenic, whereas B16 is not immunogenic). Transplantable tumor models do not completely reflect the formation of endogenous tumors. Despite these translation inconsistencies, the original findings on the efficacy of anti-PD1 therapy have been documented using transplantable tumor models. As these findings translated well into more advanced tumor models, the transplantation of tumor cell lines is our model of choice for technical reasons. Injection of the tumor cell lines can be easily implemented in our transgenic and knockout models, as no time-consuming crosses between mouse lines are required to perform the experiments. Moreover, tumor cell lines with model antigens of T cells are readily available, enabling the study of tumor-specific T cell responses. The selection of the specific tumor cell line for transplantation will be based on the type and/or genetic abnormalities of the tumors in the patients that we would like to mimic. After implantation the cancer cells will form solid tumors or metastases, given that in general these transplantable tumor models are reproducible and robust in terms of tumor take rate and growth consistency.

To study the impact of immunotherapy on the growth and behavior of these tumors, the animals will be treated by adoptive T cell transfers. To this end, mice will receive intravenous injections of tumor-specific T cells before or after tumor challenge. The transferred T cells consist of isolated populations of naïve,

effector, memory or in vitro activated T cells of wildtype or TCR transgenic mice (e.g. OT-I or F5 mice). The naïve donor T cells come from mice on appendix 6. The effector or memory donor T cells come from tumor challenged mice on this protocol or from mice infected with influenza, LCMV or Listeria on distinct protocols under appendices 2-4 or after immunization on the distinct protocol described under appendix 7. In some cases, the transferred T cells carry additional genetically modified alleles. In this manner, the response and impact of the donor antigen-specific T cells can be analyzed.

In some experiments, we will employ immunotherapy through immunization of the mice. To enable these experiments, mice will be immunized with immunogens (antigens, TLR ligands, adjuvans etc) as described in detail in appendix 7 to study the protective effect against tumor challenge. It is also relevant to study the impact of therapeutic immunization (immunization after establishment of tumor growth), as in many cases help can only be offered to diseased individuals. Therefore, mice will be challenged with the tumor cell lines and subsequently be immunized with immunogens in a similar manner as described in detail in appendix 7.

We will use inbred mice on a common background, typically wildtype versus transgenic or knock out mice on a C57BL/6 background in the tumor challenge experiments. Alternatively, mixed bone marrow chimeric mice will be used that have a wildtype and / or a transgenic / knockout compartment. We will employ this strategy to ensure that the tumor challenge is similarly controlled in the wildtype and in the transgenic setting. Mixed bone marrow chimeric mice will be generated by 2x irradiation (typically 4 to 5 Gray) of recipient mice and adoptive transfer of donor bone marrow by intravenous injection. Mice will be used 2-3 months after bone marrow reconstitution. Injections with antibodies can be used to label immune populations (e.g circulating CD8 T cells with non-depleting anti-CD8 antibodies), to deplete immune or hematopoietic populations (e.g. CD8 T cells with depleting anti-CD8 antibodies), to rescue the survival of specific immune populations (e.g. ARTC2 nanobodies to rescue CD8 T cell subsets) or to block specific immune processes (e.g. anti-LFA1 antibodies to block adhesion/migration of immune cells). In addition, in some experiments, small molecule inhibitors will be used to block specific immune processes (e.g. FTY720 to block migration of immune cells). Some genetic models require the addition of doxycycline (eg tet on / tet off system), tamoxifen (eg the ERT2-CRE system), diphtheria toxin (eg DTR transgenic mice), poly IC (eg Mx1-CRE system) or other to be specified reagents to induce or suppress expression of the molecules of interest.

Small volumes of blood within the effective guidelines (no more than 300 ul blood per mouse per 14 days) will be drawn from the tail vein of the mice at various time points to study the kinetics of the immune response after infection. At the end of the experiment, the mice will be humanely euthanized to enable harvest of the organs (e.g. lymph nodes, spleen, bone marrow, liver, gut, kidneys and lungs) for study of the viral load and the immune response within these organs.

The aim of these experiments is to develop better adoptive T cell treatments to ultimately obtain results that may be applied / translated to the clinic.

Primary outcome parameters:
The clinical readout of the tumor models will be based on tumor progression (physical measurement of tumor size or imaging), survival (humane endpoints) and / or biological effect based on analysis following the resection / collection of relevant tissue material.

The immune response will be monitored using flow cytometric analysis, which allows for the identification and the phenotyping of immune cell populations within different organs of experimental and control mice. The analysis of these immune cell populations will allow assessment of the degree of inflammation and determination of the size and type of innate and adaptive immune responses (e.g. the responses of monocytes, neutrophils, CD4 and CD8 T cells, and B cells). In addition to flow cytometry, histology will be used to assess inflammation and pathology within the tumor. Measurements of tumor growth are performed to study the impact of the immune system in controlling the tumor tissue. *In vitro* experiments will be performed with immune cells from the isolated tissues to assess the potential of the immune cells in restimulation experiments.

For some experiments, we will employ intravital imaging to study in vivo T cell responses. For this purpose, tumor-bearing animals will be anesthetized using isoflurane. The tissue of interest (tumor) will be prepared and positioned to enable in vivo imaging. The animals will be kept on a heating pad during the imaging procedure for maintenance of body temperature. In vivo imaging will take no more than 4 hours per animal and after the procedure the animals are euthanized using CO_2 asphyxiation.

We expect that 50% of the mice will experience primary tumor challenge and that 50% will experience metastasis challenge. We expect that 33.3% of the mice will not receive further treatment besides the tumor challenge, 33.3% will receive adoptive transfers of immune or hematopoietic cells and 33.3% will receive immunizations or other treatments as described above. From the majority of the mice (90%) blood will be taken prior to sacrifice. A minority of the mice will be analyzed by intravital imaging (less than 10%). All mice will be sacrificed at the end of the experiment.

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

Nature:

1: Ectopic or systemic transplantation
Induction of ectopic tumors typically occurs via subcutaneous injection and induction of metastases occurs typically through intravenous injection of tumor cell suspensions of a cell line into an animal. If necessary, the applications will be performed under appropriate anesthesia and analgesia. As a result of the transplantation procedures, tumors or metastases will develop, but time of onset and speed of growth will vary per model. In general, models with a high tumor take rate and consistent tumor growth will be used. A tumor model that has less optimal take rate and consistency may be used, but only in such cases, in which a tumor model has unique features that are not available in other models. For example, there are just a few human Her2 expressing breast cancer cell lines that are available, but most of them have a relatively poor tumor take. Many tumor models are already available or will be developed and characterized under a separate CCD project license. However, when such models, such as existing cell lines from the ATCC or other collaborators are introduced into our animal facility, it will be necessary to run a pilot test to determine/confirm the tumor take rate and growth consistency.

2: Tumor development and progression follow-up
In case of superficial tumors we can use palpation and/or caliper measurement to measure tumor growth. To this end, the mouse will be fixed by hand and the tumor palpated. Tumors larger than about 3x3x3 mm will be measured by calipers. In cases of follow up of tumor volume when tumors are not superficial and/or to obtain biological information from the tumor (growing superficial and inside internal organ sites), we will need to use appropriate imaging techniques (IVIS, SPECT, MRI, PET, CT and/or ultrasound). Examples are to monitor distribution of a radiolabeled antibody or to measure apoptosis by 99mTC-Annexin. As part of the imaging procedure, the injection of a radioactive tracer (for PET and SPECT) or contrasting agent (MRI, CT, ultrasound) may be used. If not yet available, these imaging procedures will be developed and validated under a separate CCD project license. In some cases, tumor cells have been transduced with soluble luciferase and blood sampling (25 ul from the tail) can then be used to monitor tumor growth. Duration and frequency varies between 5-10 minutes (typical for optical imaging (IVIS) or ultrasound to more than an hour for MRI, PET, SPECT and CT. During the imaging procedures the animals will be unconscious under anesthesia. For long term anesthesia (MRI, PET, SPECT and CT) we will use a dedicated life monitoring system that will record respiration rate and control body temperature to minimize any negative impact on the condition of the animal by the duration of the anesthesia. For ultrasound imaging the hair on the skin will be shaved. Food fasting of animal (with access to water) for a maximum of 12 hours can be part of the imaging procedure, such as for FDG.

3: T cell transfer
To study how T cells can be applied in tumor clearance, naïve, effector, memory or in vitro activated T cells of wildtype or TCR transgenic mice (e.g. OT-I mice) will be isolated and transferred into recipient mice before or after tumor challenge. In this manner, the anti-tumor response of the donor T cells will be followed over time. In parallel, the impact of the transferred T cells on tumor growth will be analyzed.

4: Euthanasia
At the end of the study the animal will be killed by an approved method (e.g. CO2 asphyxiation, cervical dislocation or overdosis anesthesia).

Frequency:
Tumor challenge will occur once per mouse for the study of anti-tumor T cell responses.

Mice will typically be bled once or twice only to establish the size of the systemic anti-tumor T cell response. In some experiments, mice will be bled more frequently to follow kinetics of the response, starting from day 1 after tumor challenge and maximally 5 times and no more frequently than every other day.

Duration:
Inoculation of the mice with tumor cells takes no more than 5 minutes. It is expected to cause only mild discomfort for less than one day due to the inoculation. After inoculation of the tumor cells, the mice experience moderate discomfort (primary tumor model) or severe discomfort (metastasis model) due to tumor growth for a period, which depends on the tumor model. In general, humane endpoints are reached within 3-8 weeks. Importantly, in many cases, mice will be sacrificed at earlier time-points to study the developing anti-tumor innate and adaptive immune responses.

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

A typical tumor challenge study will comprise several study arms (eg control group(s) versus treatment groups). To demonstrate a 50% improvement in terms of tumor growth rate or survival between two groups (test vs controls) with the overall variability (relative standard deviation) being around 30, we will need a group size of 8 evaluable animals per group (power > 0.9 with $\alpha = 0.05$, two sided). In order to obtain sufficient evaluable animals we will need more animals, as not all mice develop a tumor, or develop a tumor outside the predefined time window (too rapid, too slow). These mice will be taken off-study (censored). Whenever possible we will use models with high tumor take and consistent growth characteristics, but some models may have a lower take rate. Overall, we expect that on average about 25% of the tumor challenged mice cannot be used for this reason. Consequently, we will need a group size of 11 animals per study arm, thus 2 x 11 = 22 animals per study. Randomization/stratification of the mice will take place once the tumor has reached a predefined size in a predefined time window.

B. The animals

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

Species
We will use inbred mice, because the immune response in these mice is well characterized and the reagents to measure the immune cell populations are available. Mice are considered the most appropriate and, therefore, most frequently used animal model in oncology because of their short generation time and the ease of genetic modifications. Like humans, they are mammals with similar organ structures, sharing many similarities in genetic composition. There is a wealth of information on -omics data and many advanced bio-molecular tools for genetic modification are available. Moreover, there is a wide range of available tumor models, cell lines, xenografts that are transplantable into (immune compromised) mice. Usage of inbred strains also reduces genetic variability and thus minimizes the number of required animals. The proposed tumor models are well-established mouse models, with which we have extensive prior experience. The availability of inbred strains (to enhance reproducibility) and genetically modified strains makes this the best model system to study the immune response to tumor challenge in vivo.

For the experimentation, the gender of the mice is in most cases not relevant, which allows for optimal use of female and male mice from available stock.

Origin
Animals will be obtained through in-house breeding for wildtype or transgenic or knockout mouse lines. When available, littermates will be used in experiments as controls. If required, wild-type inbred control mice will be purchased from a certified commercial supplier to serve as controls.

Estimated numbers:
We expect to perform 20 studies with tumor models (primary and metastatic tumor models combined) per year, resulting in a total of 20 x 22 = 440 mice per year (**2200 in 5 years**). Of note is that only about 75% of these mice will actually be included in the studies and will undergo the discomfort of the handlings. The remaining 25% will not be eligible for study (e.g. no tumor take or too long tumor latency) and will be humanely killed before discomfort due to tumor growth will occur.

Life Stages
Young adult mice of approximately 8-16 weeks old will be used, as these mice have fully developed

immune populations for protection against tumor challenge in the experiments. In case bone marrow chimeric mice are used, mice will be 4-6 months old due to the time it takes to reconstitute the immune compartments.

C. Re-use

Will the animals be re-used?

☒ 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:
Evaluations of the proposed concepts are being done in cell culture experiments and in infection experiments prior to the tumor challenge experiments. The results thereof are evaluated critically and only if these tests/concepts are considered sufficiently promising, the step towards in vivo testing in tumor experiments will be taken. Since cancer is a complex disease, it is necessary to study the treatment of the disease in vivo. Cell culture, organoids or computer models, are not sophisticated enough for this purpose as the interaction between the tumor and host environmental factors such as the stroma, oxygen supply, the immune system and metabolism is not accounted for. Therefore, replacement of the tumor challenge experiments is not possible.

Reduction:
The proposed number of evaluable animals per study arm (n=11) is based on our experience with this type of experiments and in line with generally accepted protocols in the literature. Further reduction of animal numbers per cohort will deteriorate the statistical power.

Refinement:
State-of-the-art methods and equipment for tumor induction and follow up of tumor growth (imaging) will be used to minimize discomfort to the animals.

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

We realize that the procedures/handlings that will be conducted under this protocol will inevitably cause suffering of the animals in these studies. In order to minimize suffering, we will adhere to the national (Code of Practice) and internationally accepted rules (Code of Practice) of handling lab animals in oncology (see P. Workman et al. Guidelines for welfare and use of animals in cancer research. Br J Cancer 2010; 102: 1555-1577). Under these rules, the animals will be humanely killed when the humane endpoint is reached. Within the Institute, we have standard operation procedures (SOP) for animal handlings. Importantly, this also includes a SOP for analgesia that should reduce suffering from pain to a minimum. Next to that, we will use state-of-the-art imaging techniques that allow for non-invasive follow up of tumor growth, which is important in case of tumors growing inside internal organs, as this will help to identify animals at risk for developing symptoms. While under anesthesia for imaging, the animals are kept in a temperature controlled environment. For long term imaging (MRI, PET/SPECT/CT) the respiration will be checked to balance the depth of the anesthesia using Life-monitoring systems.

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.

Not applicable

Accommodation and care

F. Accommodation and care

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

☒ No

☐ 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?

☒ 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.

☒ 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.

In some case isoflurane anesthesia is necessary for the inoculation of the tumor cells. After tumor challenge, no measures can be taken to relieve pain, as such measures will interfere with the immune response that we intend to study.

I. Other aspects compromising the welfare of the animals

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

Animals will be monitored using appropriate techniques and at a frequency that is appropriate for the model. The discomfort caused by the tumor will depend on the model. In general, moderate for superficial tumors such as skin and mammary tumors that do not cause symptomatic metastases. The discomfort caused by tumors or metastases growing inside internal organs can be severe when grown to a size that the humane endpoints are reached (e.g. loss of body weight >20% of the initial weight, abnormal breathing, abnormal posture, etc.). Animals carrying tumors in internal organs may develop dysfunction of involved organs or other complications (e.g. obstruction of airway or gastro-intestinal tract) just like cancer patients.

Explain why these effects may emerge.

These effects are a consequence of tumor growth and treatments

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

In general the negative effects on the well-being of the animals by the tumor cannot be prevented. In order to minimize the burden of the tumor, the animals will be monitored at a frequency that is dictated by the model and timely killed when the humane endpoint as described below is met. Nevertheless, unforeseen complications may occur. In such cases, we will try to find solutions that will minimize the impact of the unforeseen complications, for example by providing easy access to food (mush-feeding), taking into account the humane endpoints as listed below.

In case bone marrow chimeric mice are used in the experiments discomfort due to irradiation may arise (irradiation sickness). The irradiation is required to eliminate the host compartment of the immune system to make space for the donor bone marrow. The effects are transient (less than 2 weeks) as reconstitution with donor bone marrow results in full recovery of the irradiated mice. In rare cases when reconstitution is insufficient, irradiated mice will be eliminated on similar criteria as after infection as described under humane endpoints.

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.

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

We will adhere to the Code of Practise of lab animals used in oncology, in line with internationally agreed rules (see P. Workman et al. Guidelines for welfare and use of animals in cancer research. Br J Cancer 2010; 102: 1555-1577). In general the most important humane endpoints that apply are:
• A weight loss of more than 20% of the initial body weight, measured from the start of the treatment and in case of adult animals.
• A tumor mass greater than 10% of the body weight, usually 2000 cubic mm in case of more superficial measurable lesions (by caliper) and/or skin ulceration/necrosis.
• Severe abnormal breathing.

Indicate the likely incidence.

Mice are unable to clear tumors unless therapy is successful. Therefore, humane endpoints are required in survival experiments. Often, mice are killed at an earlier stage to study the anti-tumor immune response.

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').

We expect that up to 25% of the mice will be taken out of experiment, because they will not have tumor take. Due to failure of tumor development, these mice will be terminated with mild discomfort only. The remainder of the mice will develop a tumor and / or metastases. The expected level of discomfort associated with tumor challenge is moderate (37.5% of the mice) and with metastasis challenge severe (37.5% of the mice).

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

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

The animals need to be euthanized at the end of the experiment in order to harvest vital organs at key time points post infection. This is necessary in order to measure and assess immune responses in target organs of the infection and in the lymphoid organs. In some experiments, the condition of the animals at the end of the experiment will require that the animal is humanely killed.

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

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

☒ Yes

Centrale Commissie Dierproeven

9.

Appendix
Description animal procedures

1) This appendix should be enclosed with the project proposal for animal procedures.
2) A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
3) For more information, see our website (www.centralecommissiedierproeven.nl).
4) 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'.	30100
1.2	Provide the name of the licenced establishment.	Stichting Het Nederlands Kanker Instituut – Antoni van Leeuwenhoek ziekenhuis

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	Type of animal procedure
06	Homeostasis studies

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.

General design of animal procedures:
The primary purpose of the in vitro studies is to obtain organs, tissues and cells from mice for use in the experiments. In particular, T cells or other immune cells are isolated from the obtained material to set up in vitro cultures. These cultures will be used to analyze optimal strategies for the expansion and maintenance of T cells or other immune cells. In addition, the cultured immune cells, in particular T cells, will be used as donor cells in adoptive transfer studies. In the adoptive transfer experiments, we will require donor cells from mice that do not undergo infection or tumor challenge themselves. These donor mice will be analyzed on this protocol. In case the recipient mice undergo immunization, infection or tumor challenge, these mice will be analyzed on the appropriate protocols in appendices 2-5 and 7. Finally, the in vitro experiments also include a wide array of experimental approaches to study T cell differentiation under steady state conditions including transcriptional profiling, flow cytometry, mass spectrometry, histology and other approaches.

We will also employ an alternative approach to study immune cells under homeostasic conditions after adoptive transfer. For this purpose, immune cells, in particular T cells of wildtype or TCR transgenic mice (e.g. P14 mice), in some cases carrying additional genetically modified alleles, will be isolated and transferred into recipient mice. The mice providing donor cells for these responses come from this appendix (steady state mice) or from mice that have been infected, challenged with tumor cells or immunized on appendix 2, 3, 4, 5 or 7. The recipient mice will subsequently be sacrificed for analysis without prior immunization, infection or tumor challenge. In this manner, the donor immune cells, often antigen-specific T cells, can be analyzed at distinct time-points after adoptive transfer.

We will use inbred mice on a common background, typically wildtype versus transgenic or knock out mice

on a C57BL/6 background in the homeostasis experiments. Alternatively, mixed bone marrow chimeric mice will be used that have a wildtype and / or a transgenic / knockout compartment. We will employ a chimeric strategy to ensure a similar environment of wildtype and the transgenic compartment. Mixed bone marrow chimeric mice will be generated by 2x irradiation (typically 4 to 5 Gray) of recipient mice and adoptive transfer of donor bone marrow by intravenous injection. Mice will be used 2-3 months after bone marrow reconstitution. It should be noted that the making of bone marrow chimeric mice as described under appendix 2, 3, 4, 5 and 7 will require donor bone marrow from mice that did not undergo further experimental procedures before sacrifice. These donor mice are sacrificed on this protocol to obtain the required bone marrow. Injections with antibodies can be used to label immune populations (e.g circulating CD8 T cells with non-depleting anti-CD8 antibodies), to deplete immune or hematopoietic populations (e.g. CD8 T cells with depleting anti-CD8 antibodies), to rescue the survival of specific immune populations (e.g. ARTC2 nanobodies to rescue CD8 T cell subsets) or to block specific immune processes (e.g. anti-LFA1 antibodies to block adhesion/migration of immune cells). In addition, in some experiments, small molecule inhibitors will be used to block specific immune processes (e.g. FTY720 to block migration of immune cells). Some genetic models require the addition of doxycycline (eg tet on / tet off system), tamoxifen (eg the ERT2-CRE system), diphtheria toxin (eg DTR transgenic mice), poly IC (eg Mx1-CRE system) or other to be specified reagents to induce or suppress expression of the molecules of interest.

In some cases, small volumes of blood within the effective guidelines (no more than 300 ul blood per mouse per 14 days) will be drawn from the tail vein of the mice before sacrifice.

At the end of the experiment, the mice will be humanely euthanized to enable harvest of the organs (e.g. lymph nodes, spleen, bone marrow, liver, gut, kidneys and lungs) for analysis and / or culture of the immune cells within these organs.

Primary outcome parameters:
Steady state populations of immune cells will be monitored using flow cytometric analysis, which allows for the identification and the phenotyping of immune cell populations (e.g. monocytes, neutrophils, CD4 and CD8 T cells, and B cells) within different organs of the mice. The analysis of these immune cell populations will allow assessment of innate and adaptive immunity under steady state conditions. In addition to flow cytometry, histology will be used to assess the location of immune populations within the organs (e.g. lymph nodes, spleen, bone marrow, liver, gut, kidneys and lungs). *In vitro* experiments will be performed with immune cells from the isolated tissues to assess the potential of the immune cells in in vitro stimulation experiments.

For some experiments, we will employ intravital imaging to study the in vivo activity of immune cells, in particular T cells. For this purpose, animals kept under steady state conditions will be anesthetized using isoflurane. The tissue of interest will be prepared and positioned to enable in vivo imaging. The animals will be kept on a heating pad during the imaging procedure for maintenance of body temperature. In vivo imaging will take no more than 4 hours per animal and after the procedure the animals are euthanized using CO_2 asphyxiation.

We expect that 60% of the mice will not receive any treatment before sacrifice, 20% will receive adoptive transfers of immune or hematopoietic cells and 20% will receive other treatments as described above. From a proportion of the mice (40%) blood will be taken prior to sacrifice. A minority of the mice will be analyzed by intravital imaging (less than 10%). All mice will be sacrificed at the end of the experiment.

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

Nature
Animals will be euthanized without prior infection or tumor challenge to obtain organs, tissues and cell preparations. However, a substantial proportion of the animals will receive adoptive transfer of immune cells, antibody treatment or other treatments as described above. Euthanization will be performed using CO_2 asphyxiation or cervical dislocation, as these procedures limit discomfort of the mice and allow for optimal isolation of the material.

Frequency
Mice will typically be bled once or twice only to establish the efficacy of infection (assessed by the size of the virus-specific T cell response). In some experiments, mice will be bled more frequently to follow

kinetics of the response (no more than 300 ul per mouse per 14 days).

Duration
Mice will be analyzed directly (sacrifice without prior treatment) or within 90 days after treatment consisting of adoptive transfer of immune cells, antibody treatment or other treatments as described above.

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

In order to minimize the number of animals required to achieve scientifically reliable and reproducible data, the number of animals required for the experiments is calculated through power analysis. Normally distributed data will ultimately be tested by student's t test or ANOVA depending on the number of groups or variables that will be measured. Non-parametric tests will be applied in the absence of a normal distribution and power testing will take that into account. The exact groups sizes will be calculated based on power analysis and approved in conjunction with the institutional animal welfare body (IvD).

The availability of cells from the tissues is often a limitation in *in vitro studies*. Pooling of material from several mice may in many cases be required to obtain sufficient cell numbers for experimentation. Therefore, in addition to statistical methods an important consideration to calculate the required mice for *in vitro* studies is the number of mice that are necessary to obtain sufficient cells for analysis. Therefore, we will choose experimental approaches that minimize the amount of mice needed per sample.

To minimize the number of mice used for in vitro experiments, we will also optimally use the available tissues through sharing of the available material of sacrificed mice.

B. The animals

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

The animals are obtained from registered commercial or in-house suppliers or through in-house generation of new lines. Mice used for the in vitro experiments can be wild-type or genetically modified animals in the young adult range of life (approximately 8 to 16 weeks). For some experiments younger mice are used (1 to 8 weeks) to study the stages of T cell development that occur from pup to adulthood (less than 5% of the experiments). For other experiments, older mice up to 1 year of age are used to study the effect of aging on T cell differentiation (less than 5% of the experiments). In case bone marrow chimeric mice are used, mice will be 4-6 months old due to the time it takes to reconstitute the immune compartments. For the experimentation, the gender of the mice is in most cases not relevant, which allows for optimal use of female and male mice from available stock.

The estimated number of mice for use in *in vitro* experiments depends on the type of assay, the amount of available cells, the number of experimental conditions during culture and the number of analyzed genotypes. It should be noted that the number of available cells per mouse can be very low for certain rare cell types (eg certain types of memory T cells) and therefore require the pooling of cells from several mice for one experiment. For example, mass spectometry analysis requires cell numbers in the order of $1x10^6$ cells, whereas T cell populations are in the order of $1x10^5$ cells, necessitating 10 mice per sample. Based on the large number of variables in the above considerations, a statistical approach is not feasible. We expect to use up to 2000 mice per year resulting in 10,000 mice in total on this protocol.

C. Re-use

Will the animals be re-used?

☒ 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:
Material of mice will be used for in vitro study of immune cell biology. Although human immune cells can be used for some purposes, the possibility to use genetically modified mice still makes this organism essential to understand immune cell biology at the molecular level.

In addition, the in vitro experiments are also essential to support the infection experiments of this application. The experiments are designed to minimize the amount of animals used in infection experiments, as the information obtained in the in vitro studies will be used to optimize settings for the infection experiments. Therefore, we expect that using mice in vitro experiments can limit the usage of mice in infection experiments.

Reduction:
Power analyses will be done for each animal procedure to determine the minimum sample size of each group. The power analysis will be reviewed by the institutional animal welfare body (IvD) in order to obtain statistically significant and biologically relevant readouts from the proposed experiments. We manage to minimize the number of mice for these experiments by using inbred and therefore genetically identical mouse strains. This allows for smaller sample sizes and thus usage of lower total amounts of animals. We will further reduce the number of mice by optimal usage of the tissues of sacrificed animals for different experiments that can be run in parallel.

Refinement:
Animals are housed together in stable social groups with nesting and cage accessories (e.g. cage furniture) for their comfort until sacrifice for analysis.

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

The mice are killed for direct use of tissues in in vitro experiments or after experimental procedures that induce mild discomfort only (injection of antibodies, adoptive transfer of cells, and other treatments as described above). Therefore, measures to minimize suffering other than those related to the killing of the mice are not applicable. Animals are euthanized using CO2 asphyxiation or cervical dislocation as these procedures induce rapid death with minimal associated discomfort.

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.

Not applicable

Accommodation and care

F. Accommodation and care

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

☒ No

☐ 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?

☒ 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.

☐ 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?

Most animals (60%) are directly terminated for use in in vitro experiments and therefore adverse effects other than those related to termination of the mice cannot occur. The remainder of the mice (40%) will experience only mild discomfort due to adoptive transfer of immune cells, injection of antibodies and other procedures as described under A that are unlikely to include further adverse effects.

In case bone marrow chimeric mice are used in the experiments discomfort due to irradiation may arise (irradiation sickness). The irradiation is required to eliminate the host compartment of the immune system to make space for the donor bone marrow. The effects are transient (less than 2 weeks) as reconstitution with donor bone marrow results in full recovery of the irradiated mice. In rare cases when reconstitution is insufficient, irradiated mice will be eliminated on similar criteria as after infection as described under humane endpoints.

Explain why these effects may emerge.

Not applicable

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

The sacrifice of the animals will occur in a humane manner using CO_2 asphyxiation or cervical dislocation. These methods ensure rapid death with minimal levels of associated discomfort in the procedure.

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.

☐ 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').

The expected level of discomfort is mild for all of the mice with the exception of mice that are used as recipients in the making of bone marrow chimeric mice. We expect to use 500 mice for these purposes. Therefore, 95% of the mice will experience mild discomfort and 5% moderate discomfort.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

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

It is necessary to kill the animals to obtain tissues including lymph nodes, spleen, liver, gut and kidneys and white blood cells from these tissues for use in *in vitro* experiments

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

Centrale Commissie Dierproeven

10.

Appendix
Description animal procedures

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	30100
1.2	Provide the name of the licenced establishment.	Stichting Het Nederlands Kanker Instituut – Antoni van Leeuwenhoek ziekenhuis

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	Type of animal procedure
07	Immunization

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.

General design of animal procedures:
We will make use of immunization strategies to study the impact of antigens and / or adjuvants on immune responses. To stimulate antigen-specific T cell populations, T cell model proteins and peptide antigens such as for example ovalbumin protein or peptides derived of ovalbumin will be used. To activate innate populations of T cells, we will make use of their cognate antigens that include for example alpha-GalCer for NKT cells. To stimulate large populations of T cells, we will make use of T cell super-antigens (such as Mls-1, staphylococcus enterotoxin A and B (SEA and SEB), TSST-1 and SPEA) that will enable the activation of T cells independent of their cognate antigen. We will employ T cell-dependent and T cell-independent B cell antigens (including TNP/NP-Ficoll, TNP/NP-keyhole limpet hemocyanin (KLH), TNP/NP-chicken gamma-globulin (CGG), r-phycoerythrin (R-PE), and hen egg lysozyme (HEL)) to study the impact of T cell help on B cell responses. Antigenic stimulation of T cells will be used in the presence of adjuvants to boost and / or modify T cell responses. Adjuvants such as incomplete Freund's adjuvants (IFA) and alum induce antigen precipitation to ensure that an antigen depot remains present, thereby increasing the timeframe, in which antigens can stimulate immune responses. Stimulants of pattern recognition receptors such as the Toll-like receptor (TLR) ligands CpG, poly-IC, LPS, ATP and NAD will also be used as adjuvants, as they stimulate antigen-presenting cells (APCs), which enhances the capacity of these APCs to induce T cell responses. Also stimulants that induce local or systemic sterile inflammation such as DNFB (skin), DNBS (gut) and paracetamol (liver) will be applied to study resulting immune responses.

We will study primary immune responses. We are interested in early timepoints (up to day 5 p.i.) to study innate responses, in the peak of the primary adaptive response at day 6 to 10 p.i. to study the formation of adaptive responses, in the contraction of the adaptive response (day 11 to 14 p.i.), and in the memory phase of the adaptive response (after day 14 p.i.).

We will use prime-boost strategies to study recall responses. For this purpose, mice will be sequentially immunized and

challenged (in some cases more than two times) with the antigens mentioned above. After the sequential immunizations, the recall response will be followed at similar time-points as displayed above for the primary immune response.

We will also employ an alternative approach to study primary and recall responses using adoptive transfer of T cells. To study primary responses, naïve T cells of wildtype or TCR transgenic mice (e.g. OT-I or F5 mice), in some cases carrying additional genetically modified alleles, will be isolated and transferred into recipient mice that will subsequently be immunized. The mice providing donor cells for primary responses come from appendix 6. In this manner, the primary response of the donor antigen-specific T cells can be analyzed at similar time-points as described above for endogenous T cells. To study secondary responses, memory or in vitro activated T cells of wildtype or TCR transgenic mice will be isolated and transferred using intravenous injection into recipient mice that will subsequently be immunized. The mice providing donor cells for secondary responses come from this appendix (immunized mice) or appendix 2, 3, 4 or 5. In this manner, the secondary response of the donor antigen-specific T cells can be analyzed at similar time-points as described above for endogenous T cells.

The ultimate purpose of the immunizations is to study whether the procedures provide protection against infection or against tumor challenge. These experiments will not be performed on this protocol, but as described on the protocols in appendices 2-5, in which the infection and tumor challenge experiments are described.

We will use inbred mice on a common background, typically wildtype versus transgenic or knock out mice on a C57BL/6 background in the immunization experiments. Alternatively, mixed bone marrow chimeric mice will be used that have a wildtype and / or a transgenic / knockout compartment. We will employ this strategy to ensure the wildtype and in the transgenic setting. Mixed bone marrow chimeric mice will be generated by 2x irradiation (typically 4 to 5 Gray) of recipient mice and adoptive transfer of donor bone marrow by intravenous injection. Mice will be used 2-3 months after bone marrow reconstitution. Injections with antibodies can be used to label immune populations (e.g circulating CD8 T cells with non-depleting anti-CD8 antibodies), to deplete immune or hematopoietic populations (e.g. CD8 T cells with depleting anti-CD8 antibodies), to rescue the survival of specific immune populations (e.g. ARTC2 nanobodies to rescue CD8 T cell subsets) or to block specific immune processes (e.g. anti-LFA1 antibodies to block adhesion/migration of immune cells). In addition, in some experiments, small molecule inhibitors will be used to block specific immune processes (e.g. FTY720 to block migration of immune cells). Some genetic models require the addition of doxycycline (eg tet on / tet off system), tamoxifen (eg the ERT2-CRE system), diphtheria toxin (eg DTR transgenic mice), poly IC (eg Mx1-CRE system) or other to be specified reagents to induce or suppress expression of the molecules of interest.

Small volumes of blood within the effective guidelines (no more than 300 ul blood per mouse per 14 days) will be drawn at various time points to study the kinetics of the immune response after immunization. At the end of the experiment, the mice will be humanely euthanized to enable harvest of the organs (e.g. lymph nodes, spleen, bone marrow, liver, gut, kidneys and lungs) for study of the immune response within these organs.

Primary outcome parameters:
The immune response will be monitored using flow cytometric analysis, which allows for the identification and the phenotyping of immune cell populations within different organs of experimental and control mice. The analysis of these immune cell populations will allow assessment of the degree of inflammation and determination of the size and type of innate and adaptive immune responses (e.g. the responses of monocytes, neutrophils, CD4 and CD8 T cells, and B cells). In addition to flow cytometry, histology will be used to assess inflammation and pathology within the organs (e.g. lymph nodes, spleen, bone marrow, liver, gut, kidneys and lungs). *In vitro* experiments will be performed with immune cells from the isolated tissues to assess the potential of the immune cells in restimulation experiments. In some experiments, antibody titers will be determined by ELISA.

For some experiments, we will employ intravital imaging to study in vivo T cell responses. For this purpose, immunized animals will be anesthetized using isoflurane. The tissue of interest will be prepared and positioned to enable in vivo imaging. The animals will be kept on a heating pad during the imaging procedure for maintenance of body temperature. In vivo imaging will take no more than 4 hours per animal and after the procedure the animals are euthanized using CO2 asphyxiation.

We expect that 50% of the mice will experience one immunization and that 50% will experience multiple prime boost immunizations of which the majority (90%) will receive no more than one recall challenge. We expect that 33.3% of the mice will not receive further treatment besides the immunizations, 33.3% will receive adoptive transfers of immune or hematopoietic cells and 33.3% will receive other treatments as described above. From the majority of the mice (90%) blood will be taken prior to sacrifice. A minority of the mice will be analyzed by intravital imaging (less than 10%). All mice will be sacrificed at the end of the experiment.

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

Nature:
Mice with an inbred background (e.g. C57BL/6) will be inoculated with the immunization reagents mentioned above. If

concentrations have not been previously determined, stocks of immunization reagents will be carefully validated *in vivo* using titration experiments. The immunization agents will be inoculated in PBS in a volume of max 200 microliters unless indicated otherwise below. Immunizations are applied through different routes to target different organs: intranasal inhalation under isoflurane anesthesia will be done for local immunization of the lungs (max 50 ul), intraperitoneal or intravenous injection for systemic immunization, subcutaneous or intradermal injection (max 5x 10 ul) for local immunization of the skin, and intramuscular immunization to mimic the commonly used immunization route in humans for vaccination.

Frequency
Immunization will occur once per mouse for the study of primary responses and multiple times (up to 10 times) for the study of recall responses.

Mice will typically be bled once or twice only to establish the efficacy of immunization (assessed by the size of the T cell response). In some experiments, mice will be bled more frequently to follow kinetics of the response, starting from day 1 after immunization and maximally 5 times and no more frequently than every other day.

Duration
Inoculation of the mice with the immunization reagents takes no more than 5 minutes. It is expected to cause only mild discomfort for less than one day due to the inoculation. After the onset of inflammation, the mice may experience mild discomfort for a maximum period of 7 to 14 days. During this period, mice are in general asymptomatic, but in some cases may lose up to 20% of their starting body weight, and transiently show visible signs of discomfort (hunched posture, reduced activity and ruffled fur). Importantly, immunization reagents are carefully titrated to be at the minimal doses that achieve a robust innate and adaptive immune response. It is also important to note that these signs of discomfort are transient and mice will completely recover within 14 days after immunization. Mice will be in experiment for up to 90 days after immunization unless in unusual cases the longevity of the memory T cells is studied. In case of re-challenge experiments, mice can be in experiment for 90 days after the last immunization (e.g. 180 days for a secondary immunization).

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

In order to minimize the number of animals required to achieve scientifically reliable and reproducible data, the number of animals required for the experiments is calculated through power analysis. Normally distributed data will ultimately be tested by student's t test or ANOVA depending on the number of groups or variables that will be measured. Non-parametric tests will be applied in the absence of a normal distribution and power testing will take that into account.

To exemplify, an immunization experiment will typically comprise two study arms (eg control group versus knockout group). To demonstrate a 50% improvement in terms of immune response size between two groups (test vs controls) with the overall variability (relative standard deviation) being around 30, we will need a group size of 5 evaluable animals per group (power > 0.8 with $\alpha = 0.05$, two sided). The immunization models are very consistent, and therefore, we expect only minimal loss of mice due to the inability to achieve immunization or due to other causes (less than 10%). Thus, to account for losses during experimentation we will use 6 mice per group in this experiment. Consequently, we will need a group size of 6 animals per study arm, thus 2 x 6 = 12 animals per study.

The above example may not be representative for all of the experiments under this appendix. The exact group sizes will for instance depend on the number of groups and the parameters that will be analyzed in any given experiment. Therefore, each experiment will require separate power analyses and approval of the institutional animal welfare body (IvD) to determine the relevant group sizes.

B. The animals

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

Species
We will use inbred mice, because the immune response in these mice is well characterized and the reagents to measure the immune cell populations are available. Usage of inbred strains also reduces genetic variability and thus minimizes the number of required animals. The proposed immunization models are well-established mouse models to study immune responses, with which we have extensive prior experience. The availability of inbred strains (to enhance reproducibility) and genetically modified strains makes this the best model system to study the impact of immunization strategies on immune responses.

For the experimentation, the gender of the mice is in most cases not relevant, which allows for optimal use of female and male mice from available stock.

Origin

Animals will be obtained through in-house breeding for wildtype or transgenic or knockout mouse lines. When available, littermates will be used in experiments as controls. If required, wild-type inbred control mice will be purchased from a certified commercial supplier to serve as controls.

Estimated Numbers
We will require approximately 2880 mice for these immunization models.

These numbers are calculated based upon the number of molecules assessed, the number of time points, the number of immune therapies including cell transfers and immunizations and the number of groups and the size of the group. In the five years of the project, we expect to study 10 molecules after infection. These targets are assessed at four time points after single immunization and at four time points after prime boost immunization. We expect to study the role of these molecules in 3 different settings (control, adoptive T cell transfer and after other treatments as described above). For each time point two groups are examined (group 1: 6 control mice, group 2: 6 experimental mice). This requires 10 molecules x 3 settings x 8 time points x 6 animals x 2 groups = 2880 animals.

Life Stages
Young adult mice of approximately 8-16 weeks old will be used, as these mice have fully developed immune populations that are required to study the impact of the immunization experiments. In case bone marrow chimeric mice are used, mice will be 4-6 months old due to the time it takes to reconstitute the immune compartments.

C. Re-use

Will the animals be re-used?

☒ 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
Mice will be used in experiments to assess the impact of immunization strategies on the immune response. The immune response is a temporal cascade of complex interactions between different cell types, receptors, signaling molecules, transcription factors etc. *In vitro* studies using human samples or cell lines cannot mimic these complex interactions and no *in vitro* system exists for the generation of immune responses. There is also no computer modeling *(in silica)* that can accurately reproduce and predict the complex immune response of a living animal. Extensive searches in DB-ALM and ZEBET databases were also not able to suggest possible alternatives to the proposed animal experiments. Therefore, these studies can only be carried out in live animals.

The availability of inbred strains (to enhance reproducibility) and genetically modified strains makes the proposed immunization models the best systems to study potential effects on the immune response in vivo.

Reduction
Power analyses will be done for each animal procedure to determine the minimum sample size of each group. The power analysis will be reviewed by the institutional animal welfare body (IvD) in order to obtain statistically significant and biologically relevant readouts from the proposed experiments. We manage to minimize the number of mice for these experiments by using inbred and therefore genetically identical mouse strains. This allows for smaller sample sizes and thus usage of lower total amounts of animals. We have reduced these experiments to include only crucial time points during the immune response. In addition, we perform kinetic analyses of immune responses in blood without directly sacrificing the animals. As blood samples do not reflect immune cell populations within the organs, it is required that at selected time points subgroups of animals will be humanely killed to obtain the organs.

Refinement
Animals are housed together in stable social groups with nesting and cage accessories (e.g. cage furniture) for their comfort.

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

1) To minimize animal discomfort, all experiments will be performed with the minimal dose of immunization reagents that reproducibly generates a robust immune response. Animals will be housed in stable social groups at all times to minimize distress, as mice are social animals. Animals exhibiting any of the humane endpoints mentioned in Section J will be euthanized to reduce suffering.

2) There are no risks for adverse effects on the environment, as the mice and the used reagents are contained in standard facilities.

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.

Not applicable

Accommodation and care

F. Accommodation and care

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

☒ No

☐ 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?

☒ 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.

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

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

After immunization, no measures can be taken to relieve pain, as such measures will interfere with the immune response that we intend to study.

☐ 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 inoculated with immunization reagents can demonstrate mild discomfort, as they may cause inflammation, The discomfort may include weight loss, reduced activity, ruffled fur and hunched posture. It is important to note that the discomfort is related to inflammation and is transient. The immunized mice do not demonstrate any symptoms of disease after clearance of the inoculated pro-inflammatory reagents. In most cases, reagents and related symptoms will clear within 7 to 14 days. In the proposed study, we include experimental time points of up to 90 days post immunization, which means that mice can be without discomfort during a large part of the experiment. It is known that occasionally some mice will demonstrate disease symptoms more severely than expected. If this occurs, these mice will be sacrificed according to the humane endpoints described below.

In case bone marrow chimeric mice are used in the experiments discomfort due to irradiation may arise (irradiation sickness). The irradiation is required to eliminate the host compartment of the immune system to make space for the donor bone marrow. The effects are transient (less than 2 weeks) as reconstitution with donor bone marrow results in full recovery of the irradiated mice. In rare cases when reconstitution is insufficient, irradiated mice will be eliminated on similar criteria as after infection as described under humane endpoints.

Explain why these effects may emerge.

These effects are a result of the inflammation that may cause moderate discomfort in the animals

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

The administration of analgesia is impossible, because of its potential inhibition of the immune response, which we intend to study in this project. To minimize severity of inflammation, we will use the inoculum used for immunization in the lowest possible dose that induces a robust and reproducible immune response in the mice without causing excessive discomfort.

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.

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

Removal from this study will be based on critical clinical criteria including: 1) weight loss indicative of more than 20% of the initial weight, 2) severe breathing problems, 3) absence of recovery of observed weight loss at day 14 after immunization and 4) any other serious symptoms of disease. In the case of sporadic or unpredicted problems, mice will be separated into different subgroups when possible (e .g. fighting), or killed humanely, if the health of the animal is impaired beyond the expected symptoms.

Indicate the likely incidence.

We expect the occurrence of such complications and subsequent removal based on the critical clinical parameters as rare (approximately 5% of experimental mice).

K. Classification of severity of procedures

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

The expected level of discomfort associated with immunized mice is moderate. In this appendix,.100% of the immunized mice will experience moderate discomfort.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

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

The animals need to be euthanized at the end of the experiment in order to harvest vital organs at key time points after the immunization. This is necessary in order to measure and assess immune responses in target organs of the immunization and in the lymphoid organs.

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

11.

Dierexperimentencommissie NKI
Plesmanlaan 121
1066 CX   AMSTERDAM

Centrale Commissie Dierproeven

8 juni 2017

A. Algemene gegevens over de procedure

1. Aanvraagnummer:
2. Titel van het project: Study of immune cells after infection and tumor challenge to uncover fundamental principles to improve immunotherapy
3. Titel van de NTS: Studie aan immuuncellen na infectie en tumorchallenge om fundamentele principes te ontrafelen voor immunotherapie

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

5. Contactgegevens DEC:
   - naam DEC: DEC NKI
   - telefoonnummer contactpersoon:
   - e-mailadres contactpersoon:

6. Adviestraject (data dd-mm-jjjj):
   ☒ ontvangen door DEC: 24-02-2017
   ☐ aanvraag compleet
   ☒ in vergadering besproken: 08-03-2017 en 12-04-2017
   ☐ anderszins behandeld
   ☒ termijnonderbreking(en) van 14-03-2017 – 23-03-2017 en 24-04-2017 – 02-05-2017
   ☐ besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen
   ☒ aanpassing aanvraag: 23-03-2017 en 02-05-2017
   ☒ advies aan CCD: 08-06-2017

7. De inhoud van dit project is afgestemd met de IvD en deze heeft geen bezwaren tegen de uitvoering van het project binnen deze instelling.

8. Eventueel horen van aanvrager: n.v.t.
   - Datum:
   - Plaats:
   - Aantal aanwezige DEC-leden:
   - Aanwezige (namens) aanvrager:
   - Gestelde vraag / vragen:
   - Verstrekt(e) antwoord(en)

- Vragen (datum: 14-03-2017) en *antwoorden* (datum: 23-03-2017):

  Algemeen:
- In het projectdeel en in de appendices worden erg veel details vermeld die voor een toetsing op projectniveau niet relevant zijn.
- De DEC doet de suggestie de kanker therapie doelstelling te relativeren en de aanvraag meer op fundamenteel onderzoek naar immunotherapie te richten. De DEC denkt dat de modellen (m.n. LCMV) wellicht ver van de reële kankersituaties staan: hoge en ook uniforme expressie van echt vreemde antigenen, daar waar in kanker de expressie misschien niet zo hoog en ook niet uniform zal zijn. Bovendien zullen de antigenen misschien niet 'echt' vreemd zijn.
- De DEC doet de suggestie meer te verduidelijken per appendix welk percentage van de dieren welk ongerief zal ondergaan.

  Project Proposal
- 3.1. (2e alinea): de DEC doet de suggestie om een korte duidelijke uitleg van het principe van de checkpoint blockade therapy toe te voegen.
- 3.2 (laatste alinea).: Dit is lang lopend onderzoek. De DEC doet de suggestie om de doelstelling te verduidelijken (Wat is al bereikt; wat denkt u komende 5 jaar te bereiken etc.). Hierbij kan ook verwezen worden naar de milestones zoals beschreven bij 3.4.3.

  Appendix 1.
- 2B: Het aantal dieren dat wordt gevraagd lijkt de DEC laag. Het verdient aanbeveling dit goed na te gaan en uit te leggen hoe het aantal tot stand komt.
- 2H: de DEC meent dat de dieren mogelijk wel pijn kunnen ondervinden.
- 2J/K de DEC acht een mate van ongerief van meer dan "moderate" niet acceptabel voor het aanhouden van dieren en is van mening dat dieren die dit ervaren uit proef genomen moeten worden als ze niet worden gebruikt in een experimenteel protocol. Graag de tekst en de humane eindpunten hierop aanpassen.
- Appendix 2 en 3
- 2H Graag aangeven dat de dieren wel pijn kunnen ondervinden en toelichten waarom en/of onder welke omstandigheden u afziet van pijnbestrijding.
- 2I/J Graag de reden van het gewichtsverlies toelichten en uitleggen onder welke omstandigheden gewichtsverlies tot een humaan eindpunt leidt.
- Appendix 4 : zie appendix 2 en 3
- 2I De Dec verzoekt de onderzoeker de gevolgen van de bestraling te vermelden.

  Appendix 5
- 2H: Graag aangeven dat de dieren wel pijn kunnen ondervinden en toelichten waarom en/of onder welke omstandigheden u afziet van pijnbestrijding. Onder A (bij 'Nature') wordt pijnbestrijding wel genoemd.

  Appendix 6
- 2A: bij primary parameters staat dat er wel handelingen voorafgaand aan het doden met de dieren wordt gedaan. Dit is niet in overeenstemming met de volgende alinea waarin de procedures die de dieren ondergaan worden beschreven en de tekst beschreven bij I.

- 2A: de DEC verzoekt de onderzoeker bij de onderbouwing van de aantallen te beschrijven dat de afweging door de IvD zal worden gemaakt aan de hand van de experimenten waar de weefsels voor nodig zijn en dat het hier een orgaandonatie protocol betreft.
- 2K: de DEC beschouwt dit als een ex vivo protocol en verzoekt de onderzoeker het antwoord te herformuleren.

- Appendix 7
- 2H: Graag aangeven dat de dieren wel pijn kunnen ondervinden en toelichten waarom en/of onder welke omstandigheden u afziet van pijnbestrijding.
- 2I/K: de DEC meent dat “mild” te laag is ingeschat. Graag onderbouwen waarom u dat vindt en/of zonodig aanpassen.
- NTS: De DEC heeft een aantal redactionele en tekstuele opmerkingen gemaakt die er toe zouden moeten bijdragen dat de titel en de tekst beter de inhoud van het project dekken.
- *De aanvrager heeft een groot deel van de suggesties in de aanvraag en de NTS verwerkt en ook de antwoorden op de vragen grotendeels tot tevredenheid van de DEC in de aanvraag verwerkt. De DEC heeft daarop in tweede instantie nog aanvullende vragen gesteld.*

- 2^{e} ronde vragen (datum: 24-04-2017) en *antwoorden* (datum: 02-05-2017)

  Proposal:
- -Algemeen m.b.t. 3.1: De DEC is van mening dat nog steeds te veel de nadruk wordt gelegd op het feit dat het hier om kankeronderzoek zou gaan. Dit schept een niet geheel juist beeld van het project.
- 3.2.: 2e laatste alinea: de DEC verzoekt de onderzoeker de “accomplishments” te onderbouwen met publicaties.
- 3.2.: laatste alinea: de DEC meent dat de vraag naar de “achievability” nog niet afdoende is beantwoord.

  Appendix 1:
- 2B: de DEC is van mening dat de aantallen dieren in deze bijlage en het ongerief nog steeds niet navolgbaar zijn beschreven.
- H: graag verduidelijken dat geen pijnbestrijding zal worden gegeven; anesthesie is geen vorm van analgesie. Mochten dieren wel onverhoopt pijn ondervinden, dan gaat de DEC er van uit dat deze niet aangehouden worden.

  Appendix 5
- de DEC blijft van mening dat het kankeraspect in deze bijlage meer gerelativeerd zou moeten worden, omdat vooral een immunologisch model beschreven wordt. De tumoren lijken vooral te zijn gekozen om hun (bekende) eigenschappen voor wat betreft hun interactie met het immuunsysteem.

  Appendix 6
- K: volgens de DEC is voor alle dieren in deze appendix het ongerief “mild”.

- *Alle suggesties en antwoorden op de vragen zijn door de aanvrager verwerkt in de aanvraag en de NTS is overeenkomstig aangepast.*

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

B. Beoordeling (adviesvraag en behandeling)

1. Het project is vergunningplichtig (dierproeven in de zin der wet).
2. De aanvraag betreft een nieuwe aanvraag.
3. De DEC is competent om hierover te adviseren.
4. Geen van de DEC-leden is betrokken bij deze projectaanvraag.

C. Beoordeling (inhoud)

1. Deze aanvraag heeft een concrete doelstelling en kan getypeerd worden als een project. De opzet komt het best overeen met voorbeeld 4b uit de handreiking 'Invulling definitie project' van de CCD. Het is helder welke handelingen individuele dieren zullen ondergaan. Hierdoor is ook duidelijk welk ongerief individuele dieren zullen ondergaan.
   In dit project wordt met behulp van infectiemodellen en tumormodellen onderzoek gedaan naar intrinsieke en extrinsieke signalen die de T-cel differentiatie regelen, met de bedoeling om beïnvloedbare ("targetable") eigenschappen van T-cellen te vinden die kunnen worden ingezet voor het optimaliseren van immunotherapie, onder andere bij kanker. De inzichten zullen worden gebruikt om nieuwe immunotherapeutische strategieën te ontwerpen die vervolgens getest en vervolmaakt zullen worden in dezelfde modellen (challenge met infecties en tumoren). Voor het onderzoek worden bestaande (infectie)modellen gebruikt, met bekende infectieuze agentia en tumoren. De aanvrager hanteert een heldere, stapsgewijze strategie.
   De DEC is er van overtuigd dat de aanvrager gedurende het project op zorgvuldige wijze besluiten zal nemen over de voortgang van het project en dat er niet onnodig dieren gebruikt zullen worden.

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

3. De in de aanvraag aangekruiste doelcategorie, fundamenteel onderzoek, is in overeenstemming met de hoofddoelstelling.

Belangen en waarden

4. Het directe doel van het project is het bestuderen van T-celdifferentiatie, om de intrinsieke en extrisieke signalen die die differentiatie regelen te identificeren. Uiteindelijk moet dit leiden tot het vinden van beïnvloedbare ("targetable") eigenschappen van T-cellen die kunnen worden ingezet voor het optimaliseren van immunotherapie, onder andere bij kanker. Het uiteindelijke doel is dus het optimaliseren van immunotherapie door het ontwerpen van beter immunisatiestrategieën en in vitro protocollen voor het kweken van T-cellen die in staat zijn infecties en tumoren te bestrijden. Het verband tussen het directe doel en het uiteindelijke doel is niet direct aanwezig binnen dit project, maar op termijn wel reëel. Het doel van deze

projectaanvraag is gerechtvaardigd binnen de context van het onderzoeksveld.

5. De belangrijkste belanghebbenden in deze projectaanvraag zijn de proefdieren, de onderzoekers, het betreffende onderzoeksveld en op langere termijn patiënten en de samenleving.

Voor de proefdieren geldt dat hun welzijn en integriteit worden aangetast. De dieren zullen beperkt worden in hun natuurlijke gedrag en gedurende de proeven zullen de dieren stress ondervinden en pijn ondergaan. Uiteindelijk zullen ze in het kader van het onderzoek gedood worden. De dieren hebben er belang bij hiervan gevrijwaard te blijven.
Voor de onderzoekers geldt dat ze belangrijke nieuwe wetenschappelijke inzichten kunnen publiceren, hetgeen vaak de sleutel is voor het verkrijgen van nieuwe onderzoeksmiddelen en -mogelijkheden. Naar de mening van de DEC dient dat geen rol te spelen in de ethische afweging over de toelaatbaarheid van het gebruik van proefdieren. Voor de rechtvaardiging van dit onderzoek gaat het uiteindelijk om de vraag of het belangrijke maatschappelijke en wetenschappelijke doelen dient (gezondheid, kennis). Dit onderzoek is in de eerste plaats fundamenteel van aard en levert informatie en kennis op die van belang is voor de voortgang van het onderzoek in dit veld.

6. Er is geen sprake van belangwekkende milieueffecten. Geïnfecteerde muizen worden conform de daarvoor geldende regels zo gehuisvest dat de betreffende micro-organismen niet in het mileu terecht kunnen komen.

Proefopzet en haalbaarheid

7. De kennis en kunde van de onderzoeksgroep en andere betrokkenen bij de dierproeven zijn voldoende gewaarborgd. De commissie is overtuigd van de kwaliteit van het werk van de aanvrager, zoals blijkt uit de in de aanvraag vermelde publicaties van deze onderzoeksgroep. De aanvragers beschikken over voldoende kennis en kunde om te kunnen voldoen aan alle zorgvuldigheidseisen omtrent het verrichten van dierproeven.

8. De doelstellingen van het project zijn realistisch en de voorgestelde experimentele opzet en uitkomstparameters sluiten hier logisch bij aan. De DEC is dan ook van mening dat het project goed is opgezet, en dat deze strategie en experimentele aanpak kunnen leiden tot het behalen van de doelstellingen binnen het kader van het project.

Welzijn dieren

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

10. De huisvesting en verzorging van de dieren vinden plaats conform de eisen in bijlage III van

richtlijn 2010/63/EU.

11. Het cumulatieve ongerief als gevolg van de dierproeven is realistisch ingeschat en geclassificeerd. 41% Van de dieren ondergaat cumulatief licht ongerief, hoofdzakelijk als gevolg van bijvoorbeeld de adoptieve transfer van cellen en infecties die met lichte symptomen verlopen. 55% van de dieren ondergaat cumulatief matig ongerief, hoofdzakelijk als gevolg van een challenge met een tumor of infectie die verloopt met symptomen die tot matig ongerief leiden of door bestraling. Vier procent van de dieren ondergaat naar verwachting ernstig ongerief door challenge met tumoren die metastaseren of een ernstig verlopende influenza infectie. Dit laatste betreft uitsluitend de dieren die met een virulente influenzastrain worden geïnfecteerd. De wetenschappelijke noodzaak daarvoor is goed onderbouwd.
   Het cumulatief ongerief voor de dieren dient te worden ingeschat als licht voor 41% van de dieren, matig voor 55% van de dieren en ernstig voor 4% van de dieren.

12. Elke dierproef brengt instrumenteel gebruik van speciaal voor dat doel in gevangenschap gefokte dieren met zich mee, hetgeen op zich al opgevat kan worden als een aantasting van hun integriteit. Omdat dit voor elk project geldt, vermeldt de DEC hier alleen zaken die kenmerkend zijn voor dit specifieke project. De integriteit van de dieren wordt aangetast door het induceren van tumoren en het infecteren van de dieren met virussen en bacteriën die aandoeningen veroorzaken. Dit leidt in de eerste plaats tot een matige of ernstige aantasting van het welzijn, maar het valt niet uit te sluiten dat dit heeft ook invloed op het gedrag en zelfredzaamheid van de dieren.

13. Voor dieren waarbij een tumor wordt geïnduceerd worden de humane eindpunten van de Code of Practice voor dieren in het kankeronderzoek gehanteerd. In veel gevallen worden de dieren echter gedood voor analyse voordat ze een humaan eindpunt bereiken. Voor dieren met infectieziekten zijn heldere op de betreffende aandoeningen afgestemde humane eindpunten gedefinieerd die in de meeste gevallen ernstig ongerief zullen kunnen voorkomen. De criteria voor humane eindpunten zijn voldoende specifiek gedefinieerd en toegesneden op de experimenten. De commissie is het eens met de inschattingen en met de gehanteerde humane eindpunten.

3V's

14. De aanvrager heeft voldoende aannemelijk gemaakt dat er geen geschikte vervangingsalternatieven zijn. De complexe interactie tussen een tumor en het immuunsysteem en de reactie van het immuunsysteem op een infectie kan op dit moment slechts in vivo bestudeerd worden. Voor een deel van de experimenten wordt voorafgaand in vitro onderzoek gedaan en ook wordt er waar mogelijk (ex vivo) onderzoek gedaan met weefsels van dieren die voor dat doel zijn gedood. Het is niet mogelijk om de vraagstellingen van dit project zonder proefdieren te beantwoorden.

15. Het maximale aantal te gebruiken dieren is realistisch ingeschat en is proportioneel ten opzichte van de gekozen onderzoeksopzet en de looptijd. De onderzoekers hanteren een goede strategie om ervoor te zorgen dat er met het kleinst mogelijk aantal dieren wordt gewerkt waarmee nog een wetenschappelijk betrouwbaar resultaat kan worden verkregen. Door de stapsgewijze aanpak wordt onnodig gebruik van proefdieren voorkomen.

16. Het project is in overeenstemming met de vereiste van de verfijning van dierproeven. De dieren

worden niet langer dan noodzakelijk in het experiment gehouden en er worden adequate humane eindpunten gehanteerd. Daarbij wordt de “Code of Practice” voor het kankeronderzoek gevolgd. De DEC is ervan overtuigd dat de beschreven proefopzet de meest verfijnde is en dat de dierproeven zo humaan mogelijk worden uitgevoerd.

17. Het project betreft geen wettelijk vereist onderzoek.

Dieren in voorraad gedood en bestemming dieren na afloop proef

18. De aanvrager geeft aan dat het geslacht van de dieren in verreweg de meeste gevallen niet relevant is en verwacht in het project gebruik te maken van zowel mannelijke, als vrouwelijke dieren in gelijke hoeveelheden.

19. De dieren zullen in het kader van het project gedood worden. Dit is noodzakelijk om weefsels en organen op verschillende tijdpunten tijdens de infectie of na afloop te kunnen uitnemen voor verder onderzoek en/of om te voorkomen dat de zich verder ontwikkelende tumor ongerief zal gaan veroorzaken. Een deel van d dieren wordt gedood om weefsels te verkrijgen voor ex vivo onderzoek. In het algemeen acht de DEC het niet raadzaam om dieren de een ernstige infectie met een gevaarlijk micro-organisme hebben doorgemaakt in leven te laten. De gebruikte dodingsmethode staat vermeld in bijlage IV van richtlijn 2010/63/EU.

20. Er worden in deze projectaanvraag geen landbouwhuisdieren, honden, katten of niet-humane primaten gedood om niet-wetenschappelijke redenen.

NTS

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

D. Ethische afweging

1. Rechtvaardigt het belang van de doelstelling van het project het ongerief dat de dieren wordt aangedaan, en is aan alle zorgvuldigheidseisen (3V’s) voldaan?

2. Vrijwel alle dieren ondergaan een lichte of matige aantasting van welzijn en integriteit. Circa 4% van het totaal aantal dieren ondervindt ernstig ongerief (beschreven in C9 tot C20). De doelstellingen kunnen niet zonder dieren behaald worden. De onderzoekers doen al het mogelijke om het lijden van de dieren en het aantal dieren te beperken.
   Uiteindelijk doel van het project is de eigenschappen van T-cellen zo beïnvloeden dat ze succesvol kunnen worden ingezet voor immunotherapie bij infecties en kanker. Dit onderzoek is in de eerste plaats fundamenteel van aard en richt zich op het verwerven van de kennis die daarvoor nodig is. Een verbetering van de mogelijkheden om ernstige infecties en kanker te behandelen, waardoor de patiënt uitzicht heeft op genezing of een langere overlevingstijd met een beter kwaliteit van leven, acht de DEC van groot belang.

3. De DEC is overtuigd van het grote belang van de doelstelling van dit project, namelijk het verwerven van inzichten die op termijn kunnen bijdragen aan het optimaliseren van immunotherapie. De commissie is daarnaast overtuigd van de kwaliteit van het onderzoek van de aanvrager. De DEC is van mening dat het project goed is opgezet, en dat de gekozen strategie en experimentele aanpak kunnen leiden tot het behalen van de doelstelling binnen het kader van

het project. De aanvrager heeft voldoende aannemelijk gemaakt dat er geen geschikte vervangingsalternatieven zijn, dat het doel niet met minder dieren behaald kan worden, dat de gebruikte aanpak de meest verfijnde is en dat zij zal kunnen voorkomen dat mens, dier en het milieu onbedoelde negatieve effecten ondervinden als gevolg van de dierproeven.
De DEC is van oordeel dat het hierboven geschetste grote belang van de doelstelling de onvermijdelijke nadelige gevolgen van dit onderzoek voor de dieren, in de vorm van angst, pijn of stress, rechtvaardigt. Aan de eis dat het belang van het onderzoek op dient te wegen tegen het ongerief dat de dieren wordt berokkend, is voldaan.

E. Advies

1. Advies aan de CCD

   ☐ De DEC adviseert de vergunning te verlenen

   ☒ De DEC adviseert de vergunning te verlenen onder de volgende voorwaarden
   - Op grond van het wettelijk vereiste (art. 10a1, lid 3) dient de projectleider bij beëindiging van het project een beoordeling achteraf aan te leveren die is afgestemd met de IvD.

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

2. Het uitgebrachte advies is gebaseerd op consensus.

3. Er zijn geen knelpunten of dilemma's geconstateerd – zowel binnen als buiten de context van het project - die de verantwoordelijkheid en competentie van de DEC overstijgen.

Met vriendelijke groet,

12.

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Stichting Het Nederlands Kanker Instituur - Antoni van Leeuwenhoek Ziekenhuis
[redacted]
Postbus 90203
1066 CX AMSTERDAM

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
AVD3010020172205

Bijlagen
2

Datum 16 juni 2017
Betreft Ontvangstbevestiging aanvraag projectvergunning Dierproeven

Geachte [redacted]

Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 14 juni 2017. Het gaat om uw project "Study of immune cells after infection and tumor challenge to uncover fundamental principles to improve immunotherapy". Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD3010020172205. 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.

Datum:
16 juni 2017
Aanvraagnummer:
AVD3010020172205

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

Datum:
16 juni 2017
Aanvraagnummer:
AVD3010020172205

Gegevens aanvrager

Uw gegevens

Deelnemersnummer NVWA:	30100
Naam instelling of organisatie:	Stichting Het Nederlands Kanker Instituur - Antoni van Leeuwenhoek Ziekenhuis
Naam portefeuillehouder of diens gemachtigde:	
KvK-nummer:	40530817
Straat en huisnummer:	Plesmanlaan 121
Postbus:	90203
Postcode en plaats:	1066 CX AMSTERDAM
IBAN:	NL71DEUT0626343534
Tenaamstelling van het rekeningnummer:	Stichting Het Nederlands Kanker Instituut – Antoni van Leeuwenhoek Ziekenhuis

Gegevens verantwoordelijke onderzoeker

Naam:	
Functie:	
Afdeling:	
Telefoonnummer:	
E-mailadres:	

Datum:
16 juni 2017
Aanvraagnummer:
AVD3010020172205

Gegevens verantwoordelijke uitvoering proces

Naam:	
Functie:	Instantie voor Dierenwelzijn
Afdeling:	
Telefoonnummer:	
E-mailadres:	

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 augustus 2017
Geplande einddatum:	1 augustus 2022
Titel project:	Study of immune cells after infection and tumor challenge to uncover fundamental principles to improve immunotherapy
Titel niet-technische samenvatting:	Studie aan immuun cellen na infectie en tumor challenge om fundamentele principes te ontrafelen voor immunotherapie
Naam DEC:	NKI
Postadres DEC:	Postbus 90203;1006 BE; Amsterdam
E-mailadres DEC:	

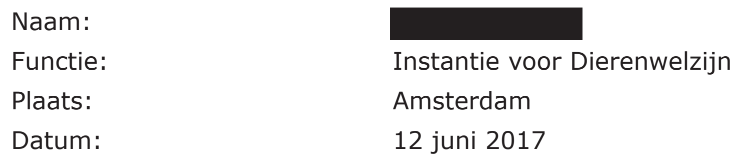
Betaalgegevens

De leges bedragen:	€ 2.113,-
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:	
Functie:	Instantie voor Dierenwelzijn
Plaats:	Amsterdam
Datum:	12 juni 2017

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Stichting Het Nederlands Kanker Instituut - Antoni van Leeuwenhoek Ziekenhuis
Postbus 90203
1066 CX AMSTERDAM

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
AVD3010020172205

Bijlagen
2

Datum 16 juni 2017
Betreft Factuur aanvraag projectvergunning Dierproeven

Factuur
Factuurdatum: 16 juni 2017
Vervaldatum: 16 juli 2017
Factuurnummer: 172205

Omschrijving	Bedrag
Betaling leges projectvergunning dierproeven Betreft aanvraag AVD3010020172205	€ 2.113,00

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

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Stichting Het Nederlands Kanker Instituur - Antoni van Leeuwenhoek Ziekenhuis

Postbus 90203
1066 CX AMSTERDAM

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
AVD3010020172205

Datum 29 juni 2017
Betreft aanvraag projectvergunning Dierproeven

Geachte

Op 14 juni 2017 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Study of immune cells after infection and tumor challenge to uncover fundamental principles to improve immunotherapy" met aanvraagnummer AVD3010020172205. 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:

Niet technische samenvatting
In de NTS wordt gesproken over terminaal ongerief bij een deel van de dieren, wat niet terug te vinden is in het projectvoorstel en de bijlagen. Dieren die zonder voorafgaande handeling worden gedood dienen als "licht" geclassificeerd te worden. Graag dit aanpassen in de NTS.

Onduidelijkheden
De aanvrager zal nog de volgende vragen gesteld worden:
- In de NTS wordt gesproken over terminaal ongerief bij een deel van de dieren, wat niet terug te vinden is in het projectvoorstel en de bijlagen. Dieren die zonder voorafgaande handeling worden gedood dienen als "licht" geclassificeerd te worden. Graag dit aanpassen in de NTS.

- U beschrijft de zoektocht naar targets voor verbetering van T-cel immunotherapie. Kunt u aangeven aan welke criteria potentiële targets moeten voldoen alvorens u deze in vivo gaat testen? M.a.w. waarop baseert u

uw keuze voor de potentiële targets?

- In bijlage 3.4.4.5 beschrijft u 20% (vraag B) tot 25% (vraag A) dieren die niet bruikbaar zijn door bijvoorbeeld geen tumortake. Welk percentage is correct? Indien nodig de dieraantallen hierop aanpassen (in aanvraag en NTS).

- In bijlage 3.4.4.5 beschrijft u niet of u mannelijke of vrouwelijke dieren gebruikt. Graag benoemen en indien niet beide geslachten gebruikt worden, onderbouw waarom. Als onvoldoende (wetenschapopelijk) is onderbouwd waarom het gebruik van één geslacht noodzakelijk is, kan de CCD een voorwaarde opleggen dat beide geslachten in gelijke mate gebruikt moeten worden.

- U beschrijft in meerdere bijlagen het maken van bone marrow chimeric animals. Heeft u bij de inschatting van het aantal dieren dat u nodig heeft in dit project rekening gehouden met de donordieren voor het maken van deze chimeric animals?

- U heeft de ongeriefsinschatting van bijlage 3.4.4.6 op licht gezet. Heeft u bij de inschatting van het ongerief in bijlage 3.4.4.6 rekening gehouden met de gevolgen van de bestraling voor het maken van de BM-chimeren? De DEC heeft ons bevestigd dat het gebruik van beenmerg-chimeren zou leiden tot matig ongerief. Kunt u aangeven om hoeveel dieren het in deze bijlage gaat die dus matig ongerief zullen ondervinden?

Graag ontvangen wij aangepaste documenten.

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

Bijlagen:
- Melding bijlagen
- Niet technische samenvatting

AVD3010020172205

Puntsgewijs antwoord van onderzoeker op vragen van de CCD:

- In de NTS wordt gesproken over terminaal ongerief bij een deel van de dieren, wat niet terug te vinden is in het projectvoorstel en de bijlagen. Dieren die zonder voorafgaande handeling worden gedood dienen als "licht" geclassificeerd te worden. Graag dit aanpassen in de NTS.

De wijziging is uitgevoerd zoals gevraagd. Zie hiervoor NTS onder punt 3.5 en de betreffende bijlage 1 onder punt K waar deze oorspronkelijk als terminaal en nu als mild geclassificeerde muizen op vermeld staan.

- U beschrijft de zoektocht naar targets voor verbetering van T-cel immunotherapie. Kunt u aangeven aan welke criteria potentiële targets moeten voldoen alvorens u deze in vivo gaat testen? M.a.w. waarop baseert u uw keuze voor de potentiële targets?

De potentiele targets worden geselecteerd op basis van de volgende overwegingen. De werking van T cel immunotherapie is afhankelijk van het verkrijgen van zoveel mogelijk van de meest geschikte T cellen met het meest geschikte gereedschap om geïnfecteerde cellen of tumorcellen op te ruimen. Potentiele targets worden dus gezocht in moleculen die een belangrijke rol spelen in de expansie, differentiatie en effector functie van T cellen. Dit is nu vermeld in de aanvraag. Zie hiervoor de project proposal onder punt 3.2.

- In bijlage 3.4.4.5 beschrijft u 20% (vraag B) tot 25% (vraag A) dieren die niet bruikbaar zijn door bijvoorbeeld geen tumortake. Welk percentage is correct? Indien nodig de dieraantallen hierop aanpassen (in aanvraag en NTS).

Het gedeelte van de experimentele muizen zonder tumor take moet zijn 25% en dat percentage is nu consistent aangehouden. Zie hiervoor bijlage 5 onder punt B en K en voor aanpassing in de aantallen van de NTS onder punt 3.5.

- In bijlage 3.4.4.5 beschrijft u niet of u mannelijke of vrouwelijke dieren gebruikt. Graag benoemen en indien niet beide geslachten gebruikt worden, onderbouw waarom. Als onvoldoende (wetenschappelijk) is onderbouwd waarom het gebruik van één geslacht noodzakelijk is, kan de CCD een voorwaarde opleggen dat beide geslachten in gelijke mate gebruikt moeten worden.

Op basis van eerdere experimenten, verwachten we geen verschillen tussen mannen en vrouwen in de vermelde plannen. We zijn dus van plan om mannen en vrouwen door elkaar te gebruiken. In het geval van adoptive transfer proeven (bv T cellen van een muis injecteren in een andere muis) zullen vanwege risico op afstoting strikt proeven worden gedaan met of alleen mannen of alleen vrouwen. We zullen dan ervoor zorgen dat er dan proeven met alleen mannen worden afgewisseld met proeven met alleen vrouwen. Dit is nu vermeld in de aanvraag. Zie hiervoor bijlage 5 onder punt B.

- U beschrijft in meerdere bijlagen het maken van bone marrow chimeric animals. Heeft u bij de inschatting van het aantal dieren dat u nodig heeft in dit

project rekening gehouden met de donordieren voor het maken van deze chimeric animals?

Deze dieren zijn opgenomen onder bijlage 6 waarin dieren zonder voorafgaande handelingen kunnen worden geofferd zoals het geval is voor de donor dieren voor het maken van chimere muizen. Dit is nu duidelijk vermeld. Zie hiervoor bijlage 6 onder punt A.

- U heeft de ongeriefsinschatting van bijlage 3.4.4.6 op licht gezet. Heeft u bij de inschatting van het ongerief in bijlage 3.4.4.6 rekening gehouden met de gevolgen van de bestraling voor het maken van de BM-chimeren? De DEC heeft ons bevestigd dat het gebruik van beenmerg-chimeren zou leiden tot matig ongerief. Kunt u aangeven om hoeveel dieren het in deze bijlage gaat die dus matig ongerief zullen ondervinden?

We verwachten 500 dieren te gebruiken als ontvangers voor BM-chimere proeven onder bijlage 6. Dit houdt dus in dat er 500 dieren zijn met matig ongerief vanwege de classificatie van het maken van chimere muizen dmv bestraling als matig ongerief. De aanpassing in de bijlage is gemaakt zoals gevraagd. Zie hiervoor bijlage 6 onder punt K en aanpassing van de aantallen in de NTS onder punt 3.5.

16.

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Stichting Het Nederlands Kanker Instituut - Antoni van Leeuwenhoek Ziekenhuis

Postbus 90203
1066 CX AMSTERDAM

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
AVD3010020172205

Bijlagen
1

Datum 18 juli 2017
Betreft Beslissing aanvraag projectvergunning Dierproeven

Geachte

Op 14 juni 2017 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Study of immune cells after infection and tumor challenge to uncover fundamental principles to improve immunotherapy" met aanvraagnummer AVD3010020172205. Wij hebben uw aanvraag beoordeeld.

Op 4 juli 2017 heeft u uw aanvraag aangevuld. In antwoord op onze vragen zijn de dieraantallen en de ongeriefsclassificatie in de NTS consistent gemaakt met die in het projectvoorstel, zijn de criteria waarop potentiële targets worden gekozen verhelderd, is het percentage tumortake verduidelijkt, is aangegeven van welk geslacht de dieren zullen worden gebruikt en is het ongerief voor de bone marrow chimeric animals meegewogen in bijlage 3.4.4.6.

Beslissing
Wij keuren uw aanvraag goed op grond van artikel 10a van de Wet op de Dierproeven (hierna: de wet). Hierbij gelden de voorwaarden zoals genoemd in de vergunning.
De algemene voorwaarde(n) zijn opgenomen op grond van artikel 1d lid 4, artikel 10a1 lid 2, artikel 10 lid 2 en/of artikel 10a3 van de wet.

U kunt met uw project "Study of immune cells after infection and tumor challenge to uncover fundamental principles to improve immunotherapy" starten. De vergunning wordt afgegeven van 1 augustus 2017 tot en met 31 juli 2022. Deze termijn is anders dan in uw aanvraag, omdat een vergunning een maximale looptijd van 5 jaar kan hebben.

Overige wettelijke bepalingen blijven van kracht.

Beoordeling achteraf
Na afloop van het project zal er een beoordeling plaatsvinden, zoals bedoeld in artikel 10a1, lid 1d en lid 3, in de wet. Meer informatie over de eisen bij een beoordeling achteraf vindt u in de bijlage.
Beoordeling achteraf dient plaats te vinden wegens ernstig ongerief van een deel van de dieren.

Procedure
Bij uw aanvraag heeft u een advies van de Dierexperimentencommissie NKI gevoegd. Dit advies is opgesteld op 8 juni 2017. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a, lid 3 van de wet. Wij hebben de DEC om aanvullende informatie gevraagd. Op 27 juni en 4 juli 2017 heeft de DEC gereageerd op onze vragen. Naar aanleiding van een door ons gestelde vraag heeft de DEC de ongeriefsclassificatie in bijlage 3.4.4.6 bijgesteld van licht naar matig voor 5% van de dieren.
Wij kunnen ons vinden in de inhoud van het advies van de Dierexperimentencommissie. Dit advies van de commissie nemen wij over, inclusief de daaraan ten grondslag liggende motivering. Er worden aanvullende algemene voorwaarde(n) gesteld.
Het DEC-advies en de in de bijlage opgenomen beschrijving van de artikelen van de wet- en regelgeving zijn de grondslag van dit besluit.

Bezwaar
Als u het niet eens bent met deze beslissing, kunt u binnen zes weken na verzending van deze brief schriftelijk een bezwaarschrift indienen.
Een bezwaarschrift kunt u sturen naar Centrale Commissie Dierproeven, afdeling Juridische Zaken, postbus 20401, 2500 EK Den Haag.

Bij het indienen van een bezwaarschrift vragen we u in ieder geval de datum van de beslissing waartegen u bezwaar maakt en het aanvraagnummer te vermelden. U vindt deze nummers in de rechter kantlijn in deze brief.

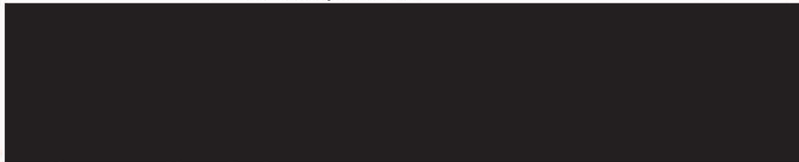
Bezwaar schorst niet de werking van het besluit waar u het niet mee eens bent. Dat betekent dat dat besluit wel in werking treedt en geldig is. U kunt tijdens deze procedure een voorlopige voorziening vragen bij de Voorzieningenrechter van de rechtbank in de woonplaats van de aanvrager. U moet dan wel kunnen aantonen dat er sprake is van een spoedeisend belang.

Voor de behandeling van een voorlopige voorziening is griffierecht verschuldigd. Op
http://www.rechtspraak.nl/Organisatie/Rechtbanken/Pages/default.aspx kunt u zien onder welke rechtbank de vestigingsplaats van de aanvrager valt.

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

Datum:
18 juli 2017
Aanvraagnummer:
AVD3010020172205

Centrale Commissie Dierproeven



Algemeen Secretaris

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

Centrale Commissie Dierproeven

Projectvergunning

gelet op artikel 10a van de Wet op de Dierproeven

Verleent de Centrale Commissie Dierproeven aan

Naam:	Stichting Het Nederlands Kanker Instituut - Antoni van Leeuwenhoek Ziekenhuis
Adres:	Postbus 90203
Postcode en plaats:	1066 CX AMSTERDAM
Deelnemersnummer:	30100

deze projectvergunning voor het tijdvak 1 augustus 2017 tot en met 31 juli 2022, voor het project "Study of immune cells after infection and tumor challenge to uncover fundamental principles to improve immunotherapy" met aanvraagnummer AVD3010020172205, volgens advies van Dierexperimentencommissie NKI. Er worden aanvullende algemene voorwaarde(n) gesteld.
De functie van de verantwoordelijk onderzoeker is [redacted] Voor de uitvoering van het project is Instantie voor Dierenwelzijn verantwoordelijk.
De aanvraag omvat de volgende bescheiden:

1 een aanvraagformulier projectvergunning dierproeven, ontvangen op 14 juni 2017
2 de bij het aanvraagformulier behorende bijlagen:
a Projectvoorstel, zoals ontvangen per digitale indiening op 4 juli 2017;
b Niet-technische Samenvatting van het project, zoals ontvangen per digitale indiening op 4 juli 2017;
c Advies van dierexperimentencommissie d.d. 8 juni 2017, ontvangen op 14 juni 2017.
d De aanvullingen op uw aanvraag, ontvangen op 4 juli 2017

Aanvraagnummer:
AVD3010020172205

Naam proef	Diersoort/ Stam	Aantal dieren	Ernst	Opmerkingen
3.4.4.1 Breeding with discomfort				
	Muizen (Mus musculus) /	1.500	10% Matig 90% Licht	
3.4.4.2 Local infection with influenza				
	Muizen (Mus musculus) /	2.880	10% Ernstig 90% Matig	
3.4.4.3 Systemic infection with Listeria monocytogenes or LCMV				
	Muizen (Mus musculus) /	5.760	100% Matig	
3.4.4.4 Infection with acute and chronic LCMV				
	Muizen (Mus musculus) /	2.880	100% Matig	
3.4.4.5 Tumor challenge				
	Muizen (Mus musculus) /	2.200	38% Ernstig 38% Matig 25% Licht	
3.4.4.6 Homeostasis studies				
	Muizen (Mus musculus) /	10.000	5% Matig 95% Licht	

Aanvraagnummer:
AVD3010020172205

3.4.4.7 Immunization				
	Muizen (Mus musculus) /	2.880	100% Matig	

Voorwaarden

Op grond van artikel 10a1 lid 2 van de Wet op de dierproeven zijn aan een projectvergunning voorwaarden te stellen
In dit project worden dierproeven toegepast die vallen in de categorie ernstig volgens artikel 10b van de wet en wordt daarom voorzien van beoordeling achteraf.Deze beoordeling zal uiterlijk juli 2023 plaatsvinden. Er zal dan beoordeeld worden of de doelstellingen van het project werden bereikt. Daarnaast wordt bekeken of de schade die de dieren hebben ondervonden, het aantal en soorten proefdieren en de ernst de dierproeven conform de vergunning waren.

De vergunning wordt verleend onder de voorwaarde dat go/no go momenten worden afgestemd met de IvD.

In artikel 10, lid 1 sub a 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 afstemming met de IvD te melden bij de CCD. De CCD kan in een dergelijke situatie aan de vergunning nieuwe voorwaarden verbinden en gestelde voorwaarde wijzigen of intrekken.

Centrale Commissie Dierproeven

Aanvraagnummer:
AVD3010020172205

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.

Beoordeling achteraf
Volgens artikel 10a1, lid 1d en lid 3 van de wet worden projecten waarbij niet-menselijke primaten worden gebruikt, projecten die als ernstig ingedeelde dierproeven omvatten of een dierproef die leidt tot ernstige mate van pijn, lijden, angst of blijvende schade die waarschijnlijk langdurig zal zijn en niet kan worden verzacht, achteraf beoordeeld worden.