	Inventaris Wob-verzoek W16-08S								
		wordt verstrekt				**weigeringsgronden**			
nr.	**document**	**reeds openbaar**	**niet**	**geheel**	**deels**	**10.1.c**	**10.2.e**	**10.2.g**	**11.1**
	NTS2015253								
1	Aanvraagformulier				x		x	x	
2	Projectvoorstel			x					
3	Niet-technische samenvatting oud			x					
4	Bijlage beschrijving dierproeven 1 oud				x		x	x	
5	Bijlage beschrijving dierproeven 2 oud			x					
6	Bijlage beschrijving dierproeven 3 oud			x					
7	Bijlage beschrijving dierproeven 4 oud			x					
8	Bijlage beschrijving dierproeven 5 oud				x		x	x	
9	Bijlage beschrijving dierproeven 6 oud			x					
10	Bijlage beschrijving dierproeven 7 oud			x					
11	Bijlage beschrijving dierproeven 8 oud			x					
12	Bijlage beschrijving dierproeven 9 oud			x					
13	DEC-advies			x					
14	Ontvangstbevestiging				x		x	x	
15	Mail vragen en antwoorden DEC 16-10-2015				x		x	x	
16	Brief verzoek aanvulling				x		x	x	
17	Reactie aanvulling				x		x	x	
18	Projectvoorstel herzien			x					
19	Bijlage beschrijving dierproeven 1 herzien				x		x	x	
20	Bijlage beschrijving dierproeven 2 herzien			x					
21	Bijlage beschrijving dierproeven 3 herzien			x					
22	Bijlage beschrijving dierproeven 4 herzien			x					
23	Bijlage beschrijving dierproeven 5 herzien				x		x	x	
24	Bijlage beschrijving dierproeven 6 herzien			x					
25	Bijlage beschrijving dierproeven 7 herzien			x					
26	Bijlage beschrijving dierproeven 8 herzien			x					
27	Bijlage beschrijving dierproeven 9 herzien			x					
28	Niet-technische samenvatting herzien	x							
29	Mail vragen en antwoorden onderzoeker 1-12-2015			x			x	x	
30	Aanvullende informatie			x			x	x	
31	Advies CCD		x						x
32	Beschikking			x			x	x	

33	Vergunning			x			x	x	
34	Mail beschikking 17-12-2015			x			x	x	

Centrale Commissie Dierproeven

0 6 OKT. 2015

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* 11600 1253
☐ Nee > *U kunt geen aanvraag doen*

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

Naam instelling of organisatie	Academisch Ziekenhuis Leiden	
Naam van de portefeuillehouder of diens gemachtigde	Leiden Universitair Medisch Centrum	
KvK-nummer	27366422	
Straat en huisnummer	Albinusdreef	2
Postbus	9600	
Postcode en plaats	2300 RC	Leiden
IBAN	NL11DEUT0451001400	
Tenaamstelling van het rekeningnummer	LUMC	

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.

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	[redacted]	☒ Dhr. ☐ Mw.
Functie	Projectleider/Senior Researcher	
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	Zie 1.4	☐ Dhr. ☐ Mw.
		Functie		
		Afdeling		
		Telefoonnummer		
		E-mailadres		
1.7	Is er voor deze projectaanvraag een gemachtigde?	☐ Ja > *Stuur dan het ingevulde formulier Melding Machtiging mee met deze aanvraag*		
		☒ Nee		

2 Over uw aanvraag

2.1	Wat voor aanvraag doet u?	☒ Nieuwe aanvraag > Ga verder met vraag 3
		☐ Wijziging op (verleende) vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn Vul uw vergunde projectnummer in en ga verder met vraag 2.2
		☐ Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn Vul uw vergunde projectnummer in en ga verder met vraag 2.3
2.2	Is dit een *wijziging* voor een project of dierproef waar al een vergunning voor verleend is?	☐ Ja > Beantwoord dan in het projectplan en de niet-technische samenvatting alleen de vragen waarop de wijziging betrekking heeft en onderteken het aanvraagformulier
		☐ Nee > Ga verder met vraag 3
2.3	Is dit een *melding* voor een project of dierproef waar al een vergunning voor is verleend?	☐ Nee > Ga verder met vraag 3
		☐ Ja > Geef hier onder een toelichting en ga verder met vraag 6

3 Over uw project

3.1	Wat is de geplande start- en einddatum van het project?	Startdatum	01 - 11 - 2015
		Einddatum	01 - 11 - 2020
3.2	Wat is de titel van het project?	Metabolic control of dendritic cell-driven t cell polarization: dissecting underlying signals	
3.3	Wat is de titel van de niet-technische samenvatting?	Het manipuleren van de stofwisseling van verkennercellen om het afweersysteem te sturen	
3.4	Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?	Naam DEC	DEC Leiden
		Postadres	Secretariaat DEC Leiden Postzone: T7-P, LUMC Postbus 9600 2300 RC Leiden
		E-mailadres	pdc-lumc-dierexperimentencommissie@lumc.nl

4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

☒ Nieuwe aanvraag Projectvergunning € 741 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	Gemandateerd vergunninghouder
Plaats	Leiden
Datum	29/9/2015
Handtekening	[redacted]

Leids Universitair
Medisch Centrum

Begeleidend schrijven

afdeling Divisie 4
postzone T7-P
afzender [redacted]
bezoekadres Einthovenweg 20, Leiden
telefoon
fax
e-mail PDC-LUMC-projectvergunning@lumc.nl

aan Centrale Commissie Dierproeven

onze referentie
uw referentie
datum 1 oktober 2015
onderwerp Administratieve gegevens
AVD116002015253
aantal pagina's 1

Geachte heer/mevrouw ,

Bijgaand doen wij u toekomen: de administratieve gegevens van AVD116002015253: Metabolic control of dendritic cell-driven t cell polarization: dissecting underlying signals.

- ☐ naar aanleiding van uw telefonisch/schriftelijk verzoek d.d.
- ☐ op verzoek van
- ☐ volgens afspraak
- ☐ retour met dank voor inzage
- ☐ met verzoek om advies / bericht / nadere informatie
- ☑ met verzoek de (verdere) behandeling over te nemen
- ☐ ter kennisname / inzage
- ☐ ter doorzending aan
- ☐ gaarne retour
- ☐

Met vriendelijke groet,

[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'.	11600
1.2	Provide the name of the licenced establishment.	Leids Universitair Medisch Centrum
1.3	Provide the title of the project.	Metabolic control of dendritic cell-driven t cell polarization: dissecting underlying signals

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

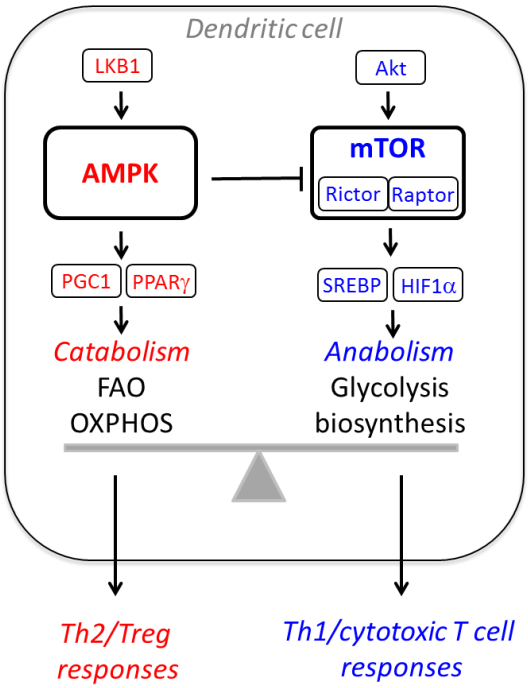
Describe the project (motivation, background and context) with respect to the categories selected in 2.

- 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.

Dendritic cells (DCs) are key regulators of both immunity and tolerance by controlling activation and polarization of effector T helper cells (Th) and regulatory T cell (Treg) responses. Therefore, there is a major focus on developing approaches to manipulate DC function for immunotherapy. It is becoming

increasingly clear that cellular metabolism dictates the fate and function of immune cells. However, still little is known about the metabolic pathways that regulate DC function and shape their immune-polarizing properties.
Recently published and preliminary data indicate that strongly immunogenic DCs that prime Th1 and cytotoxic CD8 T cell responses are metabolically characterized by a switch to anabolic metabolism fuelled by glycolysis, whereas Th2- or Treg-polarizing tolerogenic DCs display catabolic metabolism dependent on mitochondrial fatty acid oxidation (FAO) and oxidative phosphorylation (OXPHOS), and that these metabolic states are required for their immune-polarizing capacities (see figure 1). However which signalling pathways are important for the regulation of these metabolic properties of DCs and thereby their immunepolarizing function in vitro and in vivo is still unclear. In other cell types mTOR and AMPK, as major sensors of nutrient availability and bioenergetics of a cell, have been shown be crucial regulators of anabolic and catabolic metabolism, respectively. We therefore hypothesize that the balance between mTOR and AMPK signalling determines the T cell-polarizing properties of DCs through control of the balance between anabolic and catabolic metabolism in these cells (see figure 1). Thus, we predict that strong AMPK and/or low mTOR activation favours DCs to promote Th2/Treg responses, whereas tipping the balance in the opposite direction licences DCs to effectively prime Th1 and cytotoxic CD8 T cell responses.

Figure 1: Role of AMPK/mTOR in DC metabolism?



3.2 Purpose

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

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

The main objective of the current project is to define the role of the mTOR and AMPK signalling axis in controlling DC metabolism and thereby the immune polarizing properties of these cells in vitro and in vivo. The main researcher has ample experience with and expertise in the biology and metabolism of DCs and has access to various transgenic mouse strains to study AMPK and mTOR signalling in DCs in vivo. Moreover, the department has the required technology available to study cellular metabolism and has expertise with the different in vivo models that will be used to address this main aim. This makes this

project highly feasible and as such we anticipate that the proposed timeframe to address the main objective is realistic.

3.3 Relevance

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

The field of immunometabolism is a novel and very rapidly developing research area. Most of our understanding in this area has been gained from studies with T cells and macrophages. However, still little is known about the metabolic pathways that support DC activation or their immune-polarizing functions. The current proposal aims to fill this gap. Manipulation of metabolism in DCs to promote or inhibit immunity is a novel concept and it is to be expected that there is great practical significance in understanding how metabolism is linked to function in DCs. There is a major interest in the use of DCs as targets for immune-intervention and for vaccine strategies, because of their powerful immune stimulatory as well as regulatory functions. The few studies that have been performed on DC metabolism so far have mostly used in vitro models. However, in vitro models to study cellular metabolism lack the metabolic complexity of situations in vivo, where in contrast to in vitro systems, nutrient availability and oxygen tension may be rapidly fluctuating or limiting. Therefore, since cellular metabolism is highly dependent on the nutrient availability in the microenvironment DCs reside in, it is of crucial importance to also understand how metabolism of DCs shapes their function in tissues in vivo. Hence, the results from the current proposal in which both in vitro and in vivo models are used, will not only provide novel insights in the basic understanding of the molecular mechanisms through which DCs in vivo become conditioned to prime different classes of immune responses that will be of interest to immunologists, but will also lead to the identification of metabolic regulators in DCs that could potentially be targeted to manipulate the biology of these cells in situ to our advantage for therapeutic purposes in humans. For instance promoting the development of tolerogenic DCs has great potential as a therapeutic approach in settings where unwanted effector T cell responses need to be controlled, such as following transplantation to prevent rejection, in auto-immune disorders or allergies (of which the latter will be studied in this project). Conversely, rendering DCs more immunogenic to promote more robust cellular and humoral immunity is central for improving DC-based vaccination efficacy to potentially treat certain infectious diseases and cancers, such as melanoma. In this respect it is important to note that several drugs targeting metabolic regulators (for example: rapamycin targeting mTOR and metformin targeting AMPK), are currently, albeit for different purposes, already used in the clinic. We therefore anticipate that findings from this project can readily be translated into clinically relevant settings such as DC-based immunotherapy. While others are testing the effects of different cytokines, adjuvants and antigens in improving DC-based immunotherapies, this project aims to contribute to this field from a unique angle by identifying metabolic signaling pathways in DCs that can be manipulated to regulate their function.

3.4 Research strategy

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

To assess the role of the AMPK and mTOR signalling axes in the regulation of DC metabolism and thereby their immunepolarizing function, the central strategy will be to test well-defined in vitro and in vivo models of DC-dependent Th2/Treg and Th1/Cytotoxic T cell immune polarization in different mouse strains with DC-specific deletions of key components within those signaling pathways.

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.

Part 1) As a first step elucidate the role of the AMPK and mTOR signalling axes in the regulation of DC metabolism and thereby their immunepolarizing function, AMPK and mTOR signalling and their downstream targets/transcription factors will be pharmacologically or genetically promoted or inhibited in DC cultured from bone marrow or isolated from naive WT mice in which the DC populations have been expanded in vivo using following inoculation with a Flt3-L expressing tumour. These cells will be characterized metabolically and will be analysed for their ability to prime and polarize t cell responses in vitro in a T cell coculture assay. Not only mTOR and AMPK itself but also its downstream targets such as transcription factors PGC1 (master regulators of mitochondrial biogenesis and metabolism that is activated by AMPK and inhibited by mTOR), HIF1a (a key regulator of glycolysis downstream of mTOR) and SREBP (regulator of Fatty acid synthesis downstream of mTOR) will be targeted to assess what

factors downstream of AMPK/mTOR signalling control DC metabolism and function (see figure 1).

In parallel we will generate mice with DC-specific deletions of key components within the AMPK and mTOR signalling pathways. To this end, mice expressing cre recombinase under the control of the DC-specific marker CD11c, will be crossed to mice with floxed alleles of components within the AMPK and mTOR signalling pathways. In this respect mouse strain are available with floxed genes encoding Raptor and rictor (key proteins of the mTORC1 and 2 complexes, respectively) and LKB1 (a central activator of AMPK) and AMPK (see figure 1). In addition, if data from the WT mice as described above reveal that particular targets downstream of AMPK/mTOR signalling are important in controlling DC driven T cell polarization, CD11c-cre mice will be crossed to mice harbouring floxed genes of these targets. Since most of these cre-lox combinations has never been crossed before, DC frequency and numbers and surface markers expression will be assessed in naïve transgenic mice to determine whether these genes are important in DC homeostasis in naïve mice. Next, the DCs of these transgenic mice will generated as described above and characterized in vitro for 1) absence of expression of targeted genes 2) change in metabolism and 3) T cell-polarizing capacity in vitro.

animal experiments involved (see figure 2):
#1 Generation of DCs for in vitro T cell-coculture assays

Next, to test whether AMPK and mTOR signalling axes are also important in the regulation of DC metabolism and thereby their immunepolarizing function in vivo, the mutant mice strains will be used in well-defined in vivo models of DC-driven T cell polarization

Part 2) First the mutant mice will be analysed for their capacity to generate a local polarized immune response by different immune-polarizing antigens/adjuvants in a model of subcutaneous immunization. For this purpose antigens/adjuvants will be used that are known to promote Th2/Treg, Th1 or cytotoxic CD8 T cell responses in these setting. If the transgenic mice with DC-specific deletions of AMPK/mTOR signalling molecules show an altered immunepriming/polarizing capacity, the migratory ability of the Skin DCs will be determined using a FITC-painting model, to be able to distinguish whether the immune-phenotype is due to an aberrant capacity of the DCs to migrate or a consequence of altered DC-T cell communication.

animal experiments involved (see figure 2):
#2 subcutaneous immunization model
#3 FITC-painting DC migration model

Part 3) the roles of AMPK and mTOR signalling axes in DC-mediated immunepolarization during systemic infection will be determined by using well-studied infection models of the parasitic helminth schistosoma , malaria and Listeria, which are each characterized by a unique DC-mediated immunepolarization profile: i.e. Th2/Treg responses by schistosoma infection, Cytotoxic CD8 T cell responses by Malaria infection and Th1 responses by Listeria infection. Since these immune responses are critical in controlling pathogen burden and infection-induced pathology, these parameters will be assessed as well to address whether potential effects on immunepolarization translate into changes in immunopathology and/or resistance to infection.

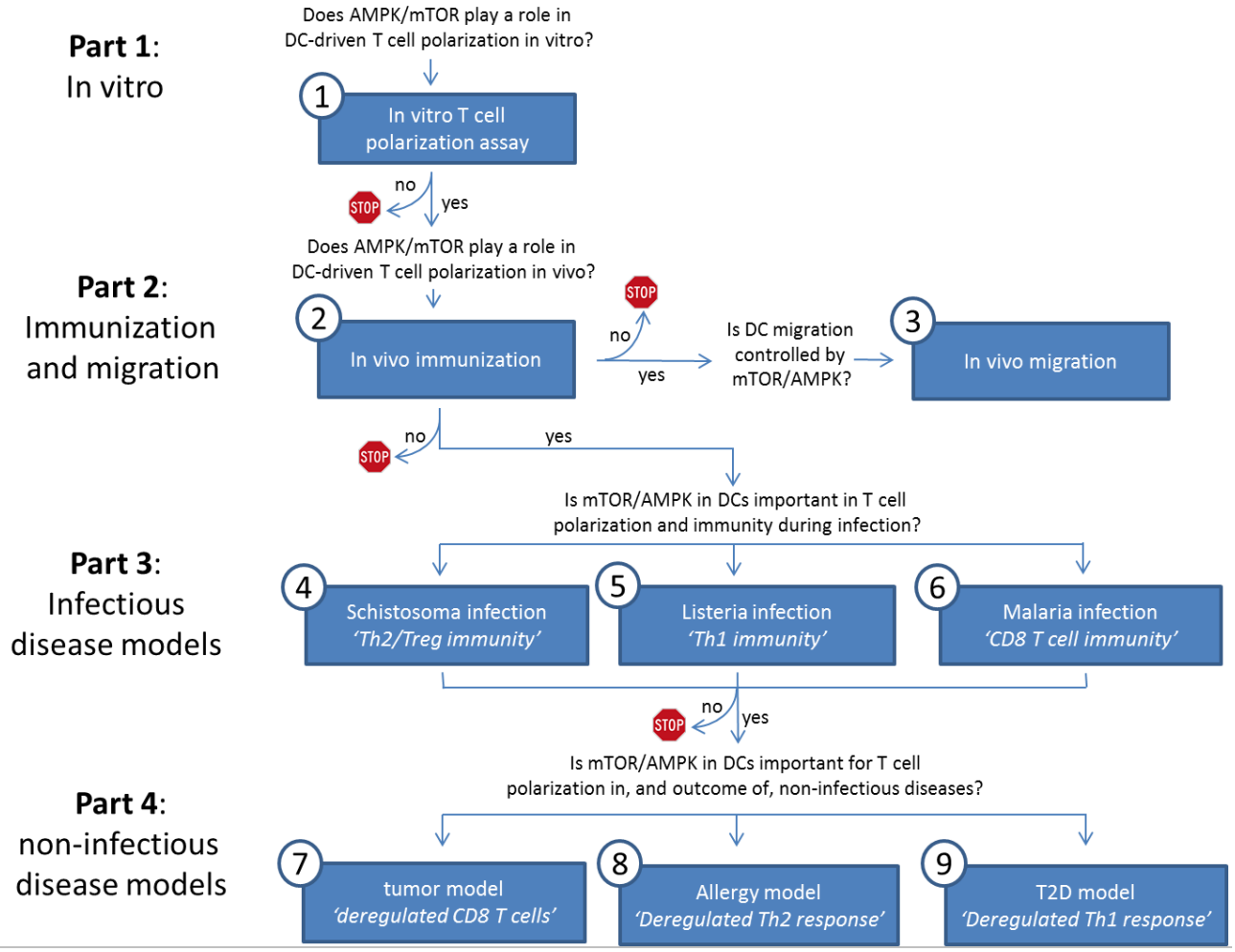
animal experiments involved (see figure 2):
#4 schistosoma mansoni infection model
#5 Malaria infection model
#6 Listeria monocytogenes infection model

Part 4) Finally, the effects of DC-specific perturbations in mTOR/AMPK signalling in immune polarization and disease outcome will be assessed in models of allergic asthma, tumour growth and high fat diet induced Type 2 diabetes. These type of diseases that have seen a rapid increase in prevalence in the western world in recent decades and are each characterized by a distinct form of immune-deregulation (ie deregulated Th2/Treg in the case of Allergic asthma, suppressed Th1/cytotoxic T cell responses in the case of tumour development and a switch from Th2/Treg to Th1/cytotoxic T cell responses in metabolic

tissues in High fat diet (HFD)-induced Type 2 diabetes). These models will provide key mechanistic insights into how DC metabolism regulates immunepolarization and thereby disease outcome. Moreover, findings from these studies can lead to the identification of druggable targets in metabolic signalling pathways in DCs that can be used to develop novel therapeutic approaches to treat these highly prevalent diseases.

animal experiments involved (see figure 2):
#7 Melanoma tumor model
#8 Allergic Asthma model
#9 High fat diet-induced Type II Diabetes model

Figure 2: outline research strategy



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.

1) The project will start with the metabolic characterization of, and analysis of the T cell polarizing capacity of DCs cultured or isolated from WT mice in which mTOR and AMPK signalling axes will be pharmacologically or genetically inhibited or promoted. These data will be compared with data obtained from DCs isolated from transgenic mice with DC-specific deletions of key components within the AMPK and mTOR signalling pathways.

- Milestone (1): established whether the AMPK and mTOR signalling axes control DC metabolism and thereby T cell polarizing capacity in vitro

- Go / no-Go moments (1): If this part shows no efficient deletion of the targeted genes and/or no effect

of manipulation of AMPK/mTOR signalling axis on T cell polarization in vitro, part 2-4 will not be performed.

2) Next, using the mutant mice, subcutaneous immunization will be performed, which is a simple, yet powerful, in vivo model for a first assessment of whether local immune priming and polarization by DCs is regulated by AMPK/mTOR signalling in these cells. In addition, to be able to determine whether any immune-priming/polarizing phenotype is due to an aberrant capacity of the DCs to migrate a FITC painting model will be used.

- Milestone (2): established whether DC-AMPK/mTOR regulates immune polarization in subcutaneous immunization model and migration of skin DCs.

- Go / no-Go moments (2): If the subcutaneous immunization model (part 2) shows that there is no immunological phenotype in mice that harbour DC- specific deletions in AMPK or mTOR signalling pathway, part 3 and 4 will not be performed

3) When these experiments reveal there is a defect in immune priming and/or polarization when DCs have defects in AMPK/mTOR signalling, the next phase of this project will focus on addressing whether these immunological observations can be extrapolated to models of highly prevalent systemic infectious diseases: schistosomiasis, malaria and listeria. Not only are these infection models chosen for their clinical relevance but also because each of these infections are well-known to elicit unique highly polarized immune responses; Th2/Treg responses in the case of schistosomiasis, primarily cytotoxic T cell responses in the case of malaria and strong Th1 responses in response to listeria infection. Each of these specific responses are essential for protection against these infections. This makes them ideal immunological models to test our hypothesis in the context of systemic infections as well as what it means for disease outcome.

- Milestone (3): Defined the role of DC-AMPK/mTOR in immune polarization, pathology and protection against, schistosomiasis, malaria and Listeria.

- Go / no-Go moments (3):If the infection models show that there is no immunological phenotype in mice that harbour DC-specific deletions in AMPK or mTOR signalling pathway part 4 will not be performed

4) The final part of this project aims to define whether the balance in mTOR/AMPK signalling in DCs is also an important determinant of immune polarization and thereby disease outcome in models of highly prevalent non-communicable western diseases: allergic asthma, melanoma tumour growth and high fat diet (HFD)-induced Type II Diabetes. The immunological basis for each of these diseases is different as they have have been shown to be caused by an aberrant Th2 response, a defective Th1/cytotoxic T cell response, and a switch from Th2/Treg to Th2/cytotoxic T cell responses, respectively, which we hypothesize will all be controlled by the balance in AMPK/mTOR signalling in DCs. The use of these non-infectious disease models is also highly relevant for a different reason, since these models in conjunction with the aforementioned infection and immunization models enable one to assess whether our hypothesis is generalizable to both non-infectious and infectious diseases or only hold true for certain settings.

- Milestone (4): Defined the role of DC-AMPK/mTOR in immune polarization and disease progression during allergic asthma, tumour development and HFD-induced type II diabetes

Taken together, these distinct infectious and non-infectious models form the basis for a coherent set of targeted experiments to address the main objective of this project, of which the results may contribute to the design of metabolism based approaches to manipulate DCs function in clinical settings to potentially help to boost immunity against infectious diseases or tumours as well as to treat non-communicable inflammatory diseases such as allergies and HFD-driven Type II diabetes.

General Go/no-Go moments: If, for example, in part 1 or 2 only mTOR signalling, but not AMPK, in DCs appears to play a role in regulating immunepolarization, mice with defects in DC-specific defects in AMPK signalling will not be studied in part 3 and 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	DC generation for T cell priming/polarizarition assay in vitro
2	subcutaneous immunization
3	FITC-painting
4	Model of Schistosoma mansoni infection
5	Model of Malaria infection
6	Model of Listeria infection
7	Melanoma Tumor model
8	Model of allergic asthma
9	Model of High fat diet-induced type II diabetes
10	

Format
Niet-technische samenvatting

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

1 Algemene gegevens

1.1	Titel van het project	Het manipuleren van de stofwisseling van verkennercellen om het afweersysteem te sturen
1.2	Looptijd van het project	5 jaar
1.3	Trefwoorden (maximaal 5)	stofwisseling, dendritische cellen, afweerresponse, T cel polarisatie

2 Categorie van het project

2.1 In welke categorie valt het project.

U kunt meerdere mogelijkheden kiezen.

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

3 Projectbeschrijving

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

Om gezond te blijven hebben we een goed werkend afweersysteem nodig, om ons te beschermen tegen bijvoorbeeld infectieziekten. Wanneer ons afweersysteem niet meer goed werkt, kan dat niet alleen leiden tot een verhoogde kans op een infectieziekte, maar ook tot een verhoogde kans om verschillende aandoeningen zoals auto-immuunziektes, allergieën en zelfs kanker te ontwikkelen.

De verkennercellen, ofwel dendritische cellen, spelen een cruciale rol spelen in de activering en regulering van ons afweersysteem. Er is daarom grote interesse in het ontwikkelen van therapieën die gericht zijn op het manipuleren van deze cellen, zodat we ons afweersysteem dusdanig kunnen

		beïnvloeden dat we eerder genoemde ziekten kunnen voorkomen of genezen. In dendritische cellen vinden verschillende stofwisselingsprocessen plaats. Eerdere proeven met dendritische cellen in kweekschalen hebben aangetoond dat het specifieke type stofwisseling dat actief is in de cel bepaalt welke afweerrespons dendritische cellen op dat moment activeren. Het is echter nog niet bekend of de afweerrespons in een levend dier ook op deze manier wordt gereguleerd. Het huidige project heeft daarom als doel te bepalen of ook in levende dieren de stofwisselingseigenschappen van dendritische cellen bepalen welke afweerrespons er door deze cellen wordt geactiveerd. Om dit te onderzoeken zal gebruik worden gemaakt van dieren die, door middel van genetische modificatie, dendritische cellen hebben met een aangepaste stofwisseling. Van deze dieren zal vervolgens worden onderzocht of ze beter of juist minder goed beschermd zijn tegen bepaalde infecties, maar ook tegen de ontwikkeling van tumoren, allergieën of Type II diabetes; alle drie aandoeningen waarin het afweersysteem een belangrijke rol speelt. Na uitvoering van dit project zullen we weten welke stofwisselingsprocessen in dendritische cellen belangrijk zijn voor de activering van specifieke afweerresponsen, en daarmee al dan niet bescherming bieden tegen het oplopen van verschillende ziektes. Deze kennis kan vervolgens als basis dienen voor de ontwikkeling van nieuwe immuuntherapieën waarbij, door het manipuleren van de stofwisseling in dendritische cellen, bepaalde ziektes voorkomen of mogelijk zelfs genezen kunnen worden.
3.2	Welke opbrengsten worden van dit project verwacht en hoe dragen deze bij aan het wetenschappelijke en/of maatschappelijke belang?	Vanuit wetenschappelijk oogpunt, zullen we na uitvoering van dit project weten welk type stofwisseling van belang is voor het aansturen van bepaalde typen afweerresponsen door dendritische cellen in proefdieren. Vervolgens, kan deze kennis de basis vormen voor de ontwikkeling van nieuwe therapieen waarbij deze stofwisselingsroutes in dendritische cellen gemanipuleerd worden, met als doel het afweersysteem te sturen in een richting waarmee hierboven genoemde ziekten voorkomen of genezen kunnen worden.
3.3	Welke diersoorten en geschatte aantallen zullen worden gebruikt?	muizen maximaal 4000
3.4	Wat zijn bij dit project de verwachte negatieve gevolgen voor het welzijn van de proefdieren?	De negatieve gevolgen van dit project zullen voor het welzijn van de muizen beperkt blijven. Enkel bij infectie met de bilharzia (Schistosoma) worm en malaria en het tumor model bestaat er een kans dat de proefdieren daar vanwege koorts, gewichtsverlies en/of fysiek ongemak, hinder van ondervinden. Dit zal echter nauwkeurig gemonitord worden en ingegrepen worden wanneer zulke tekenen zich voordoen.
3.5	Hoe worden de dierproeven in het project ingedeeld naar de verwachte ernst?	In fase 1 van het project zullen dieren worden gedood 1) na lokale immunisatie of 2) om afweercellen te kunnen isoleren en deze in kweek schalen in het lab te bestuderen. Bij deze procedures schatten we dat het ongerief laag is. In de 2 fase zullen de dieren geïnfecteerd worden met parasieten (Malaria of Bilharzia) of een bacterie (Listeria). Tijdens deze infecties bestaat de kans dat bij de parasitaire infecties matig, en in een uiterst geval, ernstig ongerief ontstaat bij de dieren. Dit zal nauwkeurig gemonitord worden en wanneer ernstig ongerief gedetecteerd wordt zullen de betreffende dieren worden gedood. In de 3^{e} en laatste fase, waarin Astma, Tumor groei en Type II diabetes als modellen worden gebruikt, is alleen bij de tumor groei kans om matig ongerief als gevolg van fysiek ongemak door de groei van de tumor. Voor Astma en Type II diabetes zal het

		ongerief minimaal blijven.
3.6	Wat is de bestemming van de dieren na afloop?	Alle dieren worden gedood tijdens of aan het einde van de proef, teneinde de organen en de afweerrespons van de dieren volledig te kunnen bestuderen

4 Drie V's

4.1	**Vervanging** Geef aan waarom het gebruik van dieren nodig is voor de beschreven doelstelling en waarom proefdiervrije alternatieven niet gebruikt kunnen worden.	Voor dit project kan geen gebruik gemaakt worden van proefdiervrije alternatieven omdat de complexiteit van de regulatie van afweerresponsen door dendritische cellen in levende dieren onvolledig kan worden nagebootst in kweek schaaltjes in het lab. Daarnaast is het zo dat ondanks dat simpele modellen met gekweekte cellen hebben aangetoond dat stofwisseling in dendritische cellen een grote invloed heeft op hun functie, is het nog niet duidelijk hoe dat zich vertaalt naar de situatie in een levend organisme. Bijvoorbeeld de veranderingen in beschikbaarheid van voedingstoffen in een levend dier zijn moeilijk te modelleren in kweekschalen, maar zullen wel een groot effect hebben op de stofwisseling en daarmee de functie van dendritische cellen in een dier.
4.2	**Vermindering** Leg uit hoe kan worden verzekerd dat een zo gering mogelijk aantal dieren wordt gebruikt.	Om het aantal muizen te verminderen zullen we eerst met behulp van celkweken in het lab testen of dendritische cellen met bepaalde genetische defecten in stofwisseling een veranderde capaciteit hebben om het afweersysteem te aan te sturen. Alleen als er op basis van deze celkweken een immunologisch effect is van de metabole defecten in dendritische cellen, zullen proefdier modellen gebruikt worden. Als laatste zullen we conform de wet, het minimum aantal muizen gebruiken wat nodig is om biologisch en statistisch significante data te genereren.
4.3	**Verfijning** Verklaar de keuze voor de diersoort(en). Verklaar waarom de gekozen diermodel(len) de meest verfijnde zijn, gelet op de doelstellingen van het project.	Voor dit project zullen muizen worden gebruikt, omdat de stofwisseling en het afweer systeem van deze diersoort sterk overeenkomen met die van de mens. Daarnaast zijn er voor deze diersoort genetische gemodificeerde dieren beschikbaar waarin selectief dendritische cellen bepaalde afwijkingen in hun stofwisseling hebben. Dat biedt de unieke mogelijkheid om specifiek de rol van stofwisseling van dendritische cellen in de regulatie van het afweersysteem in levende dieren te bestuderen. Om dit te onderzoeken, zullen deze muizen getest worden in ziektemodellen waarvan bekend is dat 1) dendritische cellen een centrale rol spelen in het reguleren van de afweerrespons tijdens deze ziektes 2) ze model staan voor ziektes die veelvuldig bij de mens voorkomen en een grote impact hebben op de volksgezondheid. De ziektemodellen die gebruikt zullen worden in deze studie zijn bepaalde infecties (Malaria, Bilharzia en Listeria) en niet infectieuze ziektemodellen waarbij het immuunsysteem een belangrijke rol speelt (allergische astma, tumorgroei en obesitas gedreven Type 2 diabetes).
	Vermeld welke algemene maatregelen genomen worden om de negatieve (schadelijke) gevolgen voor het welzijn van de proefdieren zo beperkt mogelijk te houden.	Alle injecties die bij deze ziektemodellen toegepast zullen worden, zullen onder narcose worden uitgevoerd, om zo min mogelijk stress en pijn te berokkenen. Daarnaast zal bij het Malaria en listeria infectie model, een gemuteerde parasiet en bacterie worden gebruikt waar de dieren niet of minder ziek van worden, zodat het meeste ongerief wordt voorkomen. Mochten de dieren tijdens de proeven toch meer ongerief ondervinden dan gewenst, dan zullen we de dieren doden aan de hand van 'humane

eindpunten' die specifiek voor deze proeven van tevoren zijn vastgesteld

5 In te vullen door de CCD

Publicatie datum	
Beoordeling achteraf	
Andere opmerkingen	

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'.	11600
1.2	Provide the name of the licenced establishment.	Leids Universitair Medisch Centrum
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 1 Type of animal procedure: DC generation for in vitro T cell polarization assay

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.

To study whether AMPK and mTOR control DC metabolism and thereby their immune polarizing properties, we will first use an in vitro DC-T cell coculture model. To this end murine DCs will be differentiated from bone marrow from WT naïve mice and the AMPK/mTOR signalling pathways will be manipulated pharmacologically or genetically and after which the DCs will be exposed to various pathogen-derived immune-polarizing compounds. Next, their metabolic properties will be studies as well as their capacity to prime and polarize T cell responses in a coculture system with CD8 and CD4 T cells. In addition, we will take advantage of transgenic mice that have CD11c+ DC-specific deletions in genes involved in AMPK and mTOR signalling. CD11c+ DCs will be isolated from spleens from these animals. However, since DCs are a scarce cell population, we aim to significantly increase the yield of DCs by pre-inoculating these mice with a FLT3L-expressing tumour cell line, that results in a strong expansion of DCs populations (from 1% in spleen to up to 40% in spleen). These DCs will then be stimulated and signalling, metabolic pathway activation and T cell priming and polarizing capacity will be analysed.

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

Murine DCs will be generated from bone marrow in 8 day in vitro cultures in the presence of Flt3-L. To this end, bone-marrow will be extracted from femurs and tibia of naïve wild-type mice that are euthanized by an overdose Nembutal. To generate different T cell-polarizing DCs, bone marrow-derived DCs will be conditioned by well-known Th1-/cytotoxic T cell- l (Heat killed Lysteria), Th2- (SEA, Soluble Egg Antigens from the parasitic worm S. mansoni) or Treg-promoting (S. mansoni derived LysoPS) for 24 h in the presence of endotoxin free ovalbumin (OVA). During this period activity of the mTOR and AMPK signalling axes will be assessed (Flow cytometry, Western blot) as well as changes in their rates of

mitochondrial (catabolic) or glycolytic (anabolic) metabolism (metabolite tracing experiments, metabolic flux analysis).

The use of specific inhibitors or lentiviral transduction of small-hairpin (sh)RNA containing constructs allows for inhibition of these metabolic signalling pathways. Conversely, to promote activation of these metabolic signalling pathways key components of these pathways will be overexpressed using a similar transduction approach. Specifically, mTOR pathway can be manipulated using inhibitors or hairpins/overexpression constructs against mTOR (rapamycin/Torin), HIF1a - a transcription factor downstream of mTOR that controls glycolysis- (YC-1), or SREBP - key regulator of anabolic lipid metabolism. The AMPK signalling axis can te targeted using activators (merformin, AICAR) or inhibitors (dorsomorphin) of AMPK, or activators of PGC1 (resveratrol). LKB1, AMPK and PGC1 activity can also be manipulated genetically through a lentiviral transduction of hairpin or overexpression constructs. For this purpose, a genome-wide shRNA library is available at the LUMC.

To address the immunological consequences of manipulation of these pathways, DC phenotype will be assessed following the treatments by determining surface activation marker expression and cytokine production. To define the effects of manipulation of MAPK/mTOR signalling axes in DCs on their T cell priming and polarizing capacity, the DCs conditioned for 24 h will be cultured at various ratios with naive OVA-specific OT-II CD4+ T cells or OT-I CD8+ T cells labelled with CFSE (carboxyfluorescein diacetate succinimidyl ester). On day 3, T cell proliferation will be assessed by flow cytometry as dilution of CFSE. Cytokine production by T cells will be determined on day 6

In addition, we will take advantage of transgenic mice that have DC-specific deletions in genes involved in AMPK and mTOR signalling. The following C57BL/6 CD11c-cre 'x'-flox strains will be created, where 'x' will be a gene involved in:
1) AMPK signalling directly (for instance LKB1 and AMPK1)
2) mTOR signalling directly (for instance Raptor and Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 and PPARg)
4) metabolic regulation downstream of mTOR signalling: (for instance HIF1a and SREBP)

Importantly, only genes present in the signalling pathways and downstream target genes that were identified using pharmacological and hairpin knockdown approaches in WT mice to play a role in metabolism and the T cell priming and polarizing capacity of DC (part 1), will be evaluated using CD11c-cre /flox mice.

These CD11c-cre+ mice as well as CD11cre- control mice will be injected intradermally (i.d.) in their back with FLT3L expressing B16 tumour cells (1x10e5 in 50ul) under isoflurane anaesthesia. Mice will be monitored for tumour growth every other day. After 10 days or when tumour size reaches 1 cm3 in diameter (before it may cause distress to the animals), the mice will be euthanized by i.p. injection of an overdose Nembutal and the expanded DC populations will be isolated from spleen and lymph nodes. These DCs will then be stimulated and signalling, metabolic pathway activation and T cell priming and polarizing capacity will be analysed in vitro 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.

To establish right inhibitor and stimulation concentrations and optimization of the use of shRNA to knockdown genes we expect to need 25 mice. Once these experimental conditions are optimized we need 5 WT, 5 OT-I and 5 OT-II mice to determine the metabolic requirements for T cell polarization. This is based on experience with these DC / T cell coculture assays [redacted] et al, JEM, 2012; [redacted] et al, Nat imm, 2014) where have a standard deviation of around 20%. We hope to observe a 30% difference between the experimental groups, and we set the power at 80% and determine p-value of 0.05. With these parameters, the experiments need to be performed 5 times to be able to draw reliable conclusions. The same number of mice will be needed for the experiments with DCs isolated from conditional knockout mice and their respective controls following FLT3L -tumour challenge: 5 cre+ and 5 cre- mice with 5 OT-I and 5 OT-II

B. The animals

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

C57BL/6 WT mice n=30 (20 mice for setting up the experiments, 10 for metabolic and T cell polarization assays)
C57BL/6 OT-I mice n=20 (10 for coculture with WT DCs and 10 for coculture with CD11c-Cre+ and Cre- DCs)
C57BL/6 OT-II mice n=20 (10 for coculture with WT DCs and 10 for coculture with CD11c-Cre+ and Cre- DCs)
These mice will be purchased from Harlan and Jackson laboratories and used between 6-12 weeks of age.
Both male and females will be used.

C57BL/6 CD11c-cre+ / cre- 'x'-flox/flox, rederived in house, n=10 per strain (5 cre+ and 5 cre-) where 'x' will be a gene involved in:
1) AMPK signalling directly (for instance LKB1 or AMPK1)
2) mTOR signalling directly (for instance Raptor or Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 or PPARg)
4) metabolic regulation downstream of mTOR signalling: (for instance HIF1a or SREBP)

Of note, only genes present in the signalling pathways and downstream target genes that were identified using pharmacological and hairpin knockdown approaches in WT mice to play a role in metabolism and the T cell priming and polarizing capacity of DC (part 1), will be evaluated using CD11c-cre/flox mice, with a maximum of a total of 6 different target genes.

Breeding will be setup with homozygous flox breeding pairs of which one of the breeding pair is heterozygous for CD11c-cre, whereas the other will be CD11c-cre negative. Offspring should then be 50% CD11c-cre+ and 50% CD11c-cre negative. The CD11c-Cre negative littermates will be used as controls for CD11c-Cre+ mice in the same experiment

Total maximum number of mice: 30 WT + 20 OTI + 20 OTII + 6x10 cre/flox = 130 mice

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:
Research involving mice is essential for the proposed in vitro research, since this is the only model organism in which the genetic tools are available to study DC-specific deletions in AMPK/mTOR signalling cascades. Moreover, mice are needed for acquisition of sufficient numbers of tissue resident DCs, which are hard to come by from other sources. Finally, the use of murine DCs, as opposed to human DCs, enables one to more reliably predict whether in vitro findings can be extrapolated to murine in vivo models that are used in this project as well.
Reduction:
To reduce the number of mice needed, we will implant mice with a FLT3-L expressing tumour. This enables one to isolate up to 100x more DCs from lymph nodes and spleen than from untreated mice.

This way we can significantly reduce the number of mice needed to acquire the same number of DCs for in vitro studies. In addition, DC generated or isolated from a single mouse allow to test various experimental conditions in in vitro cultures. This would requires multiple mice if tested directly in vivo. Furthermore, littermate controls (CD11c-Cre+ and CD11c-Cre-) will be used to reduce experimental variation and thereby reduce the number of mice needed per experimental group. In addition, only genes present in the signalling pathways and downstream target genes that were identified using pharmacological and hairpin knockdown approaches in WT mice to play a role in metabolism and the T cell priming and polarizing capacity of DC (part 1), will be evaluated using CD11c-cre flox mice. Finally, experiments will be performed by highly skilled and experienced staff, which will reduce intra-experimental variation and as such will result in fewer animals that will be needed per experiment to obtain reliable and statistically significant data.
Refinement:
Subcutaneous injection of the FLT3-L expressing tumour cells will be performed under anaesthesia, which takes only a few minutes. If any of the tumour bearing animals develop any state described as humane endpoint in the next section, the animals will be humanely sacrificed. Finally, experiments will be performed by staff that are highly skilled and experienced with animal handling. As a result all procedures with the animals will be performed with the greatest care and minimal animal distress will be ensured.

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

For the mice bearing Flt3L-expressing tumours, a human endpoint is chosen when in the unlikely case tumour size reaches 2 cm3, when it physically interferes with normal behaviour, when the intradermally located tumour starts ulcerating or when rapid weight loss occurs (> 15% in 1-2 days). In addition, the animals will be observed on a daily basis and be humanely sacrificed in case the following defined end points are reached: visible pain (abnormal posture and/or movement), significant weight loss (>20%), abnormal behaviour (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanized.

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.

The research question that is being addressed with the current set of experiments is novel and has not be studied before. An extensive literature search has revealed that these trangenic mice have not been used before in this kind of experimental model.

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

☒ 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.

Before i.d. injection of Flt3L-expressing tumour cells, mice will be put under anaesthesia by using isoflurane (5%) mixed with oxygen. During the injection, mice will be maintained under anaesthesia using a mouth piece. To ensure anaesthesia has set in well, rear foot reflex will be tested. During the maintenance phase isoflurane dosage is reduced (to 2-3%) to prevent overdosing.

I. Other aspects compromising the welfare of the animals

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

development of a solid subcutaneous tumour beyond 2 cm3 may negatively affect the wellbeing of the mice

Explain why these effects may emerge.

the tumour may induce pain in the surrounding tissue or may physically interfere with free movement of the animal

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

the tumor will be established on the back of the mouse, to minimize interference with normal behaviour. In addition, the tumor only needs to grow a couple of millimeters to express sufficient FLt3L to significantly expand DC populations. Mice will be sacrificed for DC isolation well before humane endpoints are reached. Nonetheless, humane endpoints are in place as described below

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.

For mice carrying the FLT3L-expressing tumors, a human endpints is chosen when tumor size reaches 2 cm3, when it physically interferes with normal behaviour, when the intradermally located tumor starts ulcerating or when rapid weight loss (> 15% in 1-2 days). In addition, the animals will be observed on a daily basis and be humanely sacrificed in case the following defined end points are reached: visible pain (abnormal posture and/or movement), significant weight loss (>20%), abnormal behavior (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanised. .

Indicate the likely incidence.

Very unlikely

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').

anesthesia/recovery from anathesia: mild

development of Flt3-ligand expressing tumor: mild

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.

To extract Bone marrow or spleen and lymph nodes to isolate and culture DCs mice need to sacrificed.

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

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

☒ Yes

Appendix
Description animal procedures

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1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	11600
1.2	Provide the name of the licenced establishment.	Leids Universitair Medisch Centrum
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 2 Type of animal procedure: model of subcutaneous 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.

The well-established model of subcutaneous immunization with various immune polarizing agents will be used to assess whether mice that harbour DCs with defects in AMPK or mTOR signalling have an altered metabolism and immune response following immunization. In this model immune polarizing compounds will be injected into the hind footpad of mice, where locally DCs will be activated and subsequently be migrating to the draining lymph node to prime and polarize T cell responses. This makes this model a highly suitable to interrogate the role of AMPK/mTOR signalling axes in controlling DCs metabolism and in the DC-driven polarization of local immune responses in vivo.

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

For footpad injections, antigens that drive Th1 and cytotoxic T cell responses (LPS and Heat Killed Listeria monocytogenes) or Th2/Treg responses (schistsosome derived soluble egg antigens (SEA) and omega-1) will be administered subcutaneously (s.c.) in 30µL of volume into one hind footpad under isoflurane anaesthesia. In all these injections, ovalbumin (OVA) will be co-administered to be able to track te response of Ova-specific T cells. The other hind footpad will be injected with the same volume of PBS as control.

Group 1: SEA+OVA, s.c. footpad injection
Group 2: HKLM+OVA s.c. footpad injection
Group 3: omega-1+OVA, s.c. footpad injection
Group 4: LPS+OVA. s.c. Footpad injection
Group 5: OVA, s.c. footpad injection (protein control)

The following readouts will be performed on each group:

1) to assess the metabolic status of the cells in vivo, immunized mice will be injected i.p with a 250ul bolus of a fluorescently labelled glucose analog (2NBDG) or free fatty acid (BODIPY-C12) 2 days after immunization. 15 minutes later the mice will be euthanized by cervical dislocation, popliteal lymph nodes will be harvested and DC will be analysed for 2NBDG and BODPIY-C12 uptake by flowcytometry as a measure for glycolytic and oxidative metabolism, respectively.

2) For immunological readouts, The mice will be euthanized by an overdose Nembutal 7 days later, the popliteal lymph nodes will be collected and the immune response to the immunization will then be assessed by flow cytometry and ex vivo restimulation. In this experiment, we will monitor the induction of OVA-antigen specific regulatory and IL-10 producing cells as well as Th1, Th2 and Th17 immunity.

Per group the following number of mice will be needed:
a) metabolic analysis: day 2 (PBS control) n=3
b) metabolic analysis: day 2 (2NBDG) n=4
c) metabolic analysis: day 2 (Bodipy-C12) n=4
d) immunological analysis: day 7 n=6

The following C57BL/6 CD11c-cre 'x'-flox strains will be used, where 'x' will be a gene involved in:
1) AMPK signalling directly (for instance LKB1 and AMPK1)
2) mTOR signalling directly (for instance Raptor and Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 and PPARg)
4) metabolic regulation downstream of mTOR signalling: (for instance HIF1a and SREBP)

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role in metabolism and the T cell priming and polarizing capacity of DC in the vitro model (part 1), will be evaluated using CD11c-cre flox mice.

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

Based on our experience with the fluorescent metabolite tracing in vivo, we expect a standard deviation of 10% for this type of experiment. We hope to observe a 25% difference between the experimental groups, and we set the power at 80% and determine p-value of 0.05. According to power analysis, we need 4 mice per group in order to obtain significant differences.

Based on our experience with the immune response induced following footpad injection, we expect a standard deviation of 12% for this type of experiments. We hope to observe a 20% difference between the experimental groups, and we set the power at 80% and determine p-value of 0.05. According to power analysis, we need 6 mice per group in order to obtain significant differences

B. The animals

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

C57BL/6 CD11c-cre+ / cre- 'x'-flox/flox, rederived in house, n=170 per strain (85 cre+ and 85 cre-) where 'x' will be a gene involved in:
1) AMPK signaling directly (for instance LKB1 or AMPK1)
2) mTOR signaling directly (for instance Raptor or Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 or PPARg)
4) metabolic regulation downstream of mTOR signaling: (for instance HIF1a or SREBP)
These mice will be used between 6-12 weeks of age and both males and females will be used.

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role in metabolism and the T cell priming and polarizing capacity of DC in the vitro model (part 1), will be evaluated using CD11c-cre flox mice, with a maximum of a total of 6 different

target genes.

Breeding will be setup with homozygous flox breeding pairs of which one of the breeding pair is heterozygous for CD11c-cre, whereas the other will be CD11c-cre negative. Offspring should then be 50% CD11c-cre+ and 50% CD11c-cre negative. The CD11c-Cre negative littermates will be used as controls for CD11c-Cre+ mice in the same experiment

Total maximum number of mice: 6 x 170 = 1020

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:
Research involving mice is essential for the proposed research. The metabolic properties of DCs and how they shape their immune polarizing function (which is the focus of this project), are expected to be largely dictated by the constantly changing micro-environment they reside in in situ. This cannot be mimicked in vitro. Finally, these studies need to be performed in mice, since this is the only model organism in which the tools are available to study DC-specific deletions in AMPK/mTOR signalling cascades. Therefore, this study can only be properly performed in vivo in mice.
Reduction:
For all animal experimentation a minimum number of mice will be used to still achieve statistically significant data. To reduce the number of mice needed the contralateral footpad will serve as the negative control for the immunization in the other footpad. This saves the use of a separate group of control mice. Finally, littermate controls (CD11c-Cre+ and CD11c-Cre-) will be used to reduce experimental variation and thereby reduce the number of mice needed per experimental group. Finally, experiments will be performed by staff that are highly skilled and experienced with animal handling. As a result all procedures with the animals will be performed with the greatest care and minimal animal distress will be ensured.
Refinement
Subcutaneous injection of antigens will be performed under anaesthesia, which takes only a few minutes. If any of the animals develop any state described as humane endpoint in the next section, the animals will be humanely sacrificed. Finally, experiments will be performed by staff that are highly skilled and experienced with animal handling. As a result all procedures with the animals will be performed with the greatest care and minimal animal distress will be ensured.

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

subcutaneous immunization will be performed under anaesthesia, to minimize animal pain or fear. While we do not expect severe complications as a result of this procedure, the animals will be observed on a daily basis and be humanely sacrificed in case the following defined end points are reached: visible pain (abnormal posture and/or movement, significant swelling of the foot and/or limping), significant weight loss (>20%), abnormal behaviour (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and

euthanized.

Repetition and duplication

E. Repetition

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

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III

☒ 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.

Before immunization mice will be put under anaesthesia by using isoflurane (5%) mixed with oxygen. During immunization, mice will be maintained under anaesthesia using a mouth piece. To ensure anaesthesia has set in well, rear foot reflex will be tested. During the maintenance phase isoflurane dosage is reduced (to 2-3%) to prevent overdosing.

I. Other aspects compromising the welfare of the animals

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

infection of the injection site

Explain why these effects may emerge.

needle ruptures the skin which may cause an opportunistic infection

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

Sterile needles and siringes will be used to minimize chances of infection

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.

Although human endpints are unlikely to be the animals will be observed on a daily basis and be humanely sacrificed in case the following defined end points are reached: visible pain (abnormal swelling of the immunized footpad, posture and/or movement), significant weight loss (>20%), abnormal behavior (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanised.

Indicate the likely incidence.

Very unlikely

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').

anaesthesia/recovery from anaesthesia: mild
Immunisation: moderate

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.

To analyse the immune response occurring in the draining lymph node (popliteal) mice need to sacrificed.

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

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

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.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'.	11600
1.2	Provide the name of the licenced establishment.	Leids Universitair Medisch Centrum
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3 Type of animal procedure: Fitc painting

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.

o study whether AMPK and mTOR control DC metabolism and thereby their capacity to migrate from the peripheral sites to draining lymph nodes, a FITC painting model will be used in the transgenic mice with DC-specific deletions of key components of the AMPK/mtor signalling axes. In this model a solution of FITC (a harmless fluorophore) will be applied on bare skin on the flanks of a mouse. skin resident DCs will take up the FITC and migrate to draining lymph nodes, where their FITC signal will be quantified 24 h later. This will provide a robust measurement for the migratory capacity DCs in these transgenic mice.

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

Mice with DC-specific deletion of key components of mTOR and AMPK signalling axes or Cre- controls will be shaved on the flanks and a 200ul 1% FITC acetone solution will be applied on the skin. 24 h later the mice will be euthanized with an overdose Nembutal and the drain ng brachial lymph nodes will be collected and analysed for the presence of FITC-positive migratory DCs.

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

Based on previous experiments with the FITC painting model we expect a standard deviation of around 15% for this type of experiments. We hope to observe a 25% difference between the experimental groups, and we set the power at 80% and determine p-value of 0.05. According to power analysis, we need 6 mice per group in order to obtain significant differences.

B. The animals

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

C57BL/6 CD11c-cre+ / cre- 'x'-flox/flox, rederived in house, n=12 per strain (6 cre+ and 6 cre-) where 'x' will be a gene involved in:
1) AMPK signalling directly (for instance LKB1 or AMPK1)
2) mTOR signalling directly (for instance Raptor or Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 or PPARg)
4) metabolic regulation downstream of mTOR signalling: (for instance HIF1a or SREBP)
These mice will be used between 6-12 weeks of age and both male and females will be used.

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role in metabolism and the T cell priming and polarizing capacity of DC in the immunization model (part 2), will be evaluated using CD11c-cre flox mice, with a maximum of a total of 6 different target genes.

Breeding will be setup with homozygous flox breeding pairs of which one of the breeding pair is heterozygous for CD11c-cre, whereas the other will be CD11c-cre negative. Offspring should then be 50% CD11c-cre+ and 50% CD11c-cre negative. The CD11c-Cre negative littermates will be used as controls for CD11c-Cre+ mice in the same experiment

Total maximum number of mice: 6x12 cre/flox = 72 mice

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:
Migration of DCs is a bio energetically demanding process that is likely to be dictated by nutrient availability in their constantly changing microenvironments during migration in vivo. This complexity cannot be fully mimicked in in vitro studies. Finally, these studies need to be performed in mice, since this is the only model organism in which the tools are available to study DC-specific deletions in AMPK/mTOR signalling cascades. Therefore, this study can only be properly performed in vivo in mice.
Reduction:
To reduce the number of mice needed, both flanks will be FITC painted of every mouse to maximize the data generated from every mouse. Littermate controls (CD11c-Cre+ and CD11c-Cre-) will be used to reduce experimental variation and thereby reduce the number of mice needed per experimental group. Finally, only genes present in the signalling pathways and downstream target genes that were identified to play a role in metabolism and the T cell priming and polarizing capacity of DC in the immunization model (part 2), will be evaluated using CD11c-cre flox mice in this FITC painting model. Finally, experiments will be performed by highly skilled and experienced staff, which will reduce intra-experimental variation and as such will result in fewer animals that will be needed per experiment to obtain reliable and statistically significant data.
Refinement
Only the area of the flank that will be FITC painted will be shaved, minimizing distress to the animals. Shaving and FITC painting will be performed under anaesthesia. If any of the animals develop any state described as humane endpoint in the next section, the animals will be humanely sacrificed. Finally,

experiments will be performed by staff that are highly skilled and experienced with animal handling. As a result all procedures with the animals will be performed with the greatest care and minimal animal distress will be ensured.

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

Topical application of FITC will be performed under anaesthesia, to minimize animal fear. While we do not expect complications as a result of FITC painting, the animals will be observed after they wake up from anaesthesia to check whether they experience any discomfort/itching from the FITC painting. The mice will be humanely sacrificed in case the following defined end points are reached: visible pain (abnormal posture and/or movement, severe itching), abnormal behaviour (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanized.

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.

The research question that is being addressed with the current set of experiments is novel and has not be studied before. An extensive literature search has revealed that these trangenic mice have not been used before in this kind of experimental model.

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

☒ 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.

Before topical application of FITC, mice will be put under anaesthesia by using isoflurane (5%) mixed with oxygen. During the injection, mice will be maintained under anaesthesia using a mouth piece. To ensure anaesthesia has set in well, rear foot reflex will be tested. During the maintenance phase isoflurane dosage is reduced (to 2-3%) to prevent overdosing.

I. Other aspects compromising the welfare of the animals

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

not very likely, but Itching from the FITC painting

Explain why these effects may emerge.

pplication of the aceton may cause some temporary itching of the skin

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

he mice will be observed after they woke up from anaesthesia for the FITC painting. If they experience severe itching (determined by trying to scratch the treated flanks, the mice will be euthanized

J. Humane endpoints

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

☐ No > Continue with question K.

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

Although human endpints are unlikely to be reached in this experimentation, the animals will be observed and humanely sacrificed in case the following defined end points are reached: visible pain (severe scratching), abnormal behavior (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanised.

Indicate the likely incidence.

Very unlikely

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').

anaesthesia/recovery from anaesthesia: mild

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.

To analyze migrated FITC-carrying DCs in Draining lymph nodes mice need to be sacrificed.

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

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

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
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1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	11600
1.2	Provide the name of the licenced establishment.	Leids Universitair Medisch Centrum
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 4 Type of animal procedure: Schistosoma mansoni infection

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.

Helminths are the strongest natural inducers of type 2 immune responses. Therefore, to study whether mice that harbour DCs with defects in AMPK or mTOR signalling have an altered capacity to prime Type 2 immune a helminth infection model with Schistosoma mansoni will be used. The larval stages of this parasite infect the host via penetration through the skin after which they develop in to adult worms in the mesenteric plexus over a period of 5 weeks. Around weeks 6 post infection the female worms start to lay eggs, which get trapped in the liver and gut wall. The eggs trigger a string Th2 immune response by the host which peaks around week 8 post infection. However, despite this acute response the host is unable to clear the infection, and as a results the infection enters the chronic phase (>12 weeks) which is characterized by a dampened type 2 immune response and the development of a regulatory immune response comprising Tregs. Dendritic cells are play a crucial role priming and regulation of Type 2 immune responses during S mansoni infection. This makes this model a highly suitable to interrogate the role of metabolic pathways in DCs that regulate their capacity to prime Th2 responses during the acute phase of the infection and Treg development during the chronic stages of the infection .

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

The animals will be infected with the larval stages of the helmith S. mansoni for 8 weeks. In short, the belly of every mouse will be quickly shaved and then be anesthetized with an i.p. injection of a mixture of Esketamine (50 mg/kg) and Dexdomitor (0.5 mg/kg). Next, the mice will be placed on their backs on a heated matrass and a metal ring will be placed on their shaved bellies. Subsequently, a solution of 1 ml of water (containing the cercariae) will be pipetted into the ring. After 30 min the ring and the remaining fluid will be removed and the animals will be turned on their bellies. To help the mice to recover quickly

from their anaesthesia, they will receive an i.p. injection with anti-sedan (0,20 ml). The mice are not anesthetized during the shaving procedure, because this is only a short period where the mice are 'hand-fixed' (30 sec). The concentration of the anaesthesia is optimized to ensure the mice are only anesthetized for 45 min. The infection with the cercariae is maximized at 12 mice at the same time to make the procedure and safety as optimized possible for the researchers involved.

8 weeks post infection the mice will be sacrificed by an overdose Nembutal and the following parameters will be analysed:
hepatic and mesenteric lymph nodes and a piece of liver will be collected and cell composition will be determined by flow cytometry. Specifically, hallmarks of type 2 immune responses will be analysed: presence of IgG1-switched B cells, eosinophils and Th2/Treg cells. The T cells will be characterized by ex vivo antigen specific and polyclonal restimulation and staining for foxp3 as a marker for Tregs.
For the chronic infection time point (16 week post infection), the same parameters will be assessed. in addition Liver sections will be fixed for histological analysis to establish whether the transgenic mice are more or less prone to develop egg-induced liver fibrosis as a result of the infection, which is a prototypical immunopathological consequence of Type 2 immune response during chronic S. mansoni infection.

Group 1: naïve control mice n=12
Group 2: acute S. mansoni infection (8 weeks) n=12
group 3: chronic S. mansoni infection (16 weeks) n=12

The following C57BL/6 CD11c-cre 'x'-flox strains will be used, where 'x' will be a gene involved in:
1) AMPK signalling directly (for instance LKB1 and AMPK1)
2) mTOR signalling directly (for instance Raptor and Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 and PPARg)
4) metabolic regulation downstream of mTOR signalling: (for instance HIF1a and SREBP)

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role T cell polarization in the immunization model (part 2), will be evaluated using CD11c-cre flox mice in this model.

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

In previous experiments (Hussaarts et al, FASEB J, 2015; Smits et al, JACI, 2007) we observed an average of 20% stdev in parameters regarding the Th2 response parameters. Based on these data and an anticipated difference between WT and knockout mice of at least 25% we need 9 mice per group, to be able have sufficient power (80%) to obtain significant differences (with a $p = 0.05$). From experience we know that about 15% of the infected animals develops hypersplenomegaly. According to human endpoints they will need to be taken out of the experiment. In addition, in around 5% of the animals worms will not fully develop. To compensate for these animals, we would like to include 20% extra animals (= ~3) in the infected groups (total 9+3=12 per group).

B. The animals

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

C57BL/6 CD11c-cre+ / cre- 'x'-flox/flox, rederived in house, n=72 per strain (36 cre+ and 36 cre-) where 'x' will be a gene involved in:
1) AMPK signalling directly (for instance LKB1 or AMPK1)
2) mTOR signalling directly (for instance Raptor or Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 or PPARg)
4) metabolic regulation downstream of mTOR signalling: (for instance HIF1a or SREBP)
These mice will be used between 6-12 weeks of age and both male and females will be used.

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role in metabolism and the T cell priming and polarizing capacity of DC in the immunization model (part 2), will be evaluated using CD11c-cre flox mice, with a maximum of a total of

6 different target genes.

Breeding will be setup with homozygous flox breeding pairs of which one of the breeding pair is heterozygous for CD11c-cre, whereas the other will be CD11c-cre negative. Offspring should then be 50% CD11c-cre+ and 50% CD11c-cre negative. The CD11c-Cre negative littermates will be used as controls for CD11c-Cre+ mice in the same experiment

Total maximum number of mice: 6x72 cre/flox = 432 mice

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:
Research involving mice is essential for the proposed research. The metabolic properties of DCs and how they shape their immune polarizing function (which is the focus of this project), are expected to be largely dictated by the constantly changing micro-environment they reside in in situ. This cannot be mimicked in vitro. Finally, these studies need to be performed in mice, since this is the only model organism in which the tools are available to study DC-specific deletions in AMPK/mTOR signalling cascades. Therefore, this study can only be properly performed in vivo in mice.
Reduction:
To reduce the number of mice needed, only the transgenic mice that gave an altered Th2 immune response in the immunization model will be tested in this infection model. In addition, littermate controls (CD11c-Cre+ and CD11c-Cre-) will be used to reduce experimental variation and thereby reduce the number of mice needed per experimental group. Finally, experiments will be performed by highly skilled and experienced staff, which will reduce intra-experimental variation and as such will result in fewer animals that will be needed per experiment to obtain reliable and statistically significant data.
Refinement
Infection will be performed under anaesthesia, which takes 45 minutes. If any of the animals develop any state described as humane endpoint in the next section during the infection, the animals will be humanely sacrificed. Finally, experiments will be performed by staff that are highly skilled and experienced with animal handling. As a result all procedures with the animals will be performed with the greatest care and minimal animal distress will be ensured.

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

infection will be performed under anaesthesia, to minimize animal pain or fear. While we do not expect severe complications as a result of the initial infection the laying of eggs by the adult worms (starting around week 6), may lead to illness in case any the transgenic mice have a defect in their ability to mount a Th2 response. Therefore, the animals will be observed 3 times a week from week 5 onwards and will be humanely sacrificed in case the following defined end points are reached: visible pain (abnormal posture and/or movement), significant weight loss (>20%), abnormal behaviour (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria,

the animals will be taken out of the experiment and euthanized.

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.

The research question that is being addressed with the current set of experiments is novel and has not be studied before. An extensive literature search has revealed that these trangenic mice have not been used before in this kind of experimental model.

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

☒ 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.

The animals will be infected with the larval stages of the helmith S. mansoni. To this end mice will be anesthetized with an i.p. injection of a mixture of Esketamine (50 mg/kg) and Dexdomitor (0.5 mg/kg). To ensure anaesthesia has set in well, the rear foot reflex will be tested. To prevent hypothermia, the mice will be placed on their backs on a heated matrass during anaesthesia.

I. Other aspects compromising the welfare of the animals

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

infection with S mansoni may cause liver dysfunction

Explain why these effects may emerge.

S. mansoni derived eggs get trapped in the liver

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

as described in J (humane endpoints), mice will be sacrificed if mice display pain, abnormal behaviour, weight loss as a consequence of hepatic dysfunction.

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.

While we do not expect severe complications as a result of the initial infection the laying of eggs by the adult worms (starting around week 6), may lead to illness in case any the transgenic mice have a defect in their ability to mount a protective Th2 response. Therefore, the animals will be observed 3 times a week from week 5 onwards and will be humanely sacrificed in case the following defined end points are reached: visible pain (abnormal posture and/or movement), significant weight loss (>20%), abnormal behavior (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanised.

Indicate the likely incidence.

possible

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').

anaesthesia/recovery from anaesthesia: mild
Helminth S. mansoni infection: moderate and in some specific cases severe

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.

To analyse the immune response occurring in the draining lymph node (mesenteric and hepatic) and liver, mice need to sacrificed.

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

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

☒ Yes

Appendix
Description animal procedures

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1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	11600
1.2	Provide the name of the licenced establishment.	Leids Universitair Medisch Centrum
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 5 Type of animal procedure: Malaria infection model

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.

Malaria infection drives strong type 1 immunity. During a blood meal by a plasmodium-infected mosquito, sporozoites are injected into the skin and reach the bloodstream, after which the parasites develop into a liver stage around day 2. Protection against this stage of the infection is predominantly dependent on CD8 cytotoxic T cell responses. After day 2, a merozoite blood stage develops that infects erythrocytes and causes blood stage malaria that leads to clinical symptoms. In contrast to the liver stage, blood stage parasitemia is thought to be primarily controlled by Th1 immune responses. In both stages of the infection, it has been shown that priming of T cells is dependent on dendritic cells. This makes a liver and blood stage malaria infection model highly suitable study whether mice that harbour DCs with defects in AMPK or mTOR signalling have an altered capacity to prime Type 1 immune responses (ie cytotoxic T cell responses and Th1 responses, respectively).
(A) First, to investigate whether metabolism in DCs controls their capacity to prime Th1 responses during malaria, a murine blood-stage malaria infection model with Plasmodium chabaudi AS (Pc) will be used, since C57/B6 mice are resistant to this infection and clear it after about 2 weeks. The peak of the primary T cell response in spleen lies around day 5-7 post infection and the peak of parasitemia lies around day 7-9. In this model T cell responses in spleen as well as parasitemia and anaemia will be studied.
(B) Second, to investigate whether metabolism in DCs controls their capacity to prime CD8 cytotoxic Tcell responses during malaria, we will be using a genetically attenuated parasite (GAP) of P. yoelii that can only develop into the liver stage. This attenuated parasite line also express the reporter protein GFP-Luciferase which will allow to determine parasite liver loads in live mice by real time in vivo imaging after infection/administration of defined doses of attenuated sporozoites This will allow for the characterisation of CD8 T cell immune responses specifically generated against the liver stages, which is thought be

crucial for protection against subsequent infections. Moreover, following injection of luciferin, parasite burden can be monitored real-time in vivo without the need to sacrifice mice.

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

(A)

Before experimental mice can be infected with infected red blood cells (iRBCs), 2 WT mice will be infected i.p. (200ul, 10000 Pc AS iRBCs), with parasite stored in liquid nitrogen or blood of an infected mice. Parasitemia will be monitored in these mice by taking a drop of blood (1/3 of a capillary, i.e.~25uL) from the tail vein and a blood smear will be made to count the percentage of parasitized erythrocytes per 100 erythrocytes. When up to 40% infection is reached, these mice will be euthanized with an overdose Nembutal and blood will be collected to obtain fresh Pc–infected RBCs that can be used blood-stage infection of experimental mice. The protocol for maintenance and storage of blood stage parasites is defined under a different DEC protocol ([redacted]) that is operational within our department. iRBC that are not used for infection of experimental mice will be stored in liquid nitrogen until needed for infection of new mice.

To study the primary immune response against Pc blood-stage infection, mice will be infected i.p. (200ul) with 1x10e5-1x10e6 infected RBC diluted in PBS. At day 5, the peak of the immune response, a group of mice will be euthanized with an overdose Nembutal and analysed for the following parameters:
1) DCs and T cell numbers in spleens and blood will be quantified and analysed for their phenotype.
2) Splenocytes will be re-stimulated with iRBC or polyclonally to assess cytokine production by the T cells.
3) blood will be collected as described below to assess parasitemia and anaemia

To assess whether any change in T cell response affect the ability of these mice to control the infection a second group of infected mice will be followed for up to 2 weeks to monitor weight, parasitemia and anaemia on day 3,5,7,10,and 13, after which the mice will be euthanized. To quantify parasitemia, mice will be bled by taking a drop of blood (1/3 of a capillary, i.e.~25uL) from the tail vein and a blood smear will be made to count the percentage of parasitized erythrocytes per 100 erythrocytes. Experiments will be terminated when the parasitemia reaches a level greater than 40% in the mice, before signs of severe anaemia and organ failure occur.

Experimental groups:

Group 1: naïve control mice (immunology)	n=6
Group 2: Pc infected mice (immunology)	n=6
Group 3: naïve control mice (parasitemia	n=11
Group 4: Pc infected mice (parasitemia)	n=11

The following C57BL/6 CD11c-cre 'x'-flox strains will be used, where 'x' will be a gene involved in:
1) AMPK signalling directly (for instance LKB1 and AMPK1)
2) mTOR signalling directly (for instance Raptor and Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 and PPARg)
4) metabolic regulation downstream of mTOR signalling: (for instance HIF1a and SREBP)

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role T cell polarization in the immunization model (part 2), will be evaluated using CD11c-cre flox mice in this model.

(B)

The protocol for obtaining/isolation GAP sporozoites from salivary glands of infected mosquitoes for

immunization and challenge is defined under a different DEC protocol ([redacted]) that is operational within our department

To study the primary immune response against Plasmodium yoelii, mice will be infected i.v. (200ul, 10000 parasites), under anaesthesia, through tail vein injection, with sporozoites of a genetically attenuated parasite (GAP) of Py that can only develop to the liver stage and expresses luciferase.

To quantify parasite liver loads, real time in vivo imaging will be performed at one time point 2 days after administration of sporozoites, by measuring luciferase activity/luminescence emanating from parasites present in the liver using the IVIS-Lumina system; the reduction in the liver load can be very accurately quantified and will give a measure how effective the host immune response is at limiting liver stage development. Mice are anesthetized with isoflurane, after which they are subcutaneously injected with luciferin (100mg/kg body weight). Eight minutes after injection mice are placed under the CCD camera, whereupon luciferase activity/luminescence is measured during a period of 2 minutes (the total period of anaesthesia per mouse is max 10 min).

At day 7 mice will be euthanized with an overdose Nembutal and analysed for the following immunological parameters:
1) DCs and Py specific T cells in spleens, hepatic lymph nodes and liver will be quantified and analysed for their phenotype.
2) Splenocytes and liver will be re-stimulated with Py specific peptide (CS) or polyclonally to assess cytokine production by the T cells.

Experimental groups:

Group 1: naïve control mice n=10
Group 2: Py infected mice n=10

The following C57BL/6 CD11c-cre 'x'-flox strains will be used, where 'x' will be a gene involved in:
1) AMPK signalling directly (for instance LKB1 and AMPK1)
2) mTOR signalling directly (for instance Raptor and Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 and PPARg)
4) metabolic regulation downstream of mTOR signalling: (for instance HIF1a and SREBP)

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role T cell polarization in the immunization model (part 2), will be evaluated using CD11c-cre flox mice in this model.

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

or (A): Based on earlier studies with the Pc blood stage infection model in C57BL/6 mice (Borges da Silva et al, PloS Path, 2015; Stevenson et al, J Immunol, 1995) we expect a standard deviation of around 10% for the immunological parameters we are interested and 15% for the parasitological reads. We hope to observe a 20% difference between the experimental groups, and we set the power at 80% and determine p-value of 0.05. According to power analysis, we need 5 mice per group for the immunology and 9 mice per groups for the parasitology, in order to observe significant differences. From experience we know that about 10-20% do not get properly infected. Therefore, we included 1 and 2 additional mice per group respectively.

For (B): Based on earlier studies with the P.y. liver stage infection model in our lab we experienced the larger variability in the immunological parameters such as CD8 T cell IFNg production than parasitic loads. For the CD8 t cell responses we observed a standard deviation of around 20%. We hope to observe a 25% difference between the experimental groups, we set the power at 80% and determine p-value of 0.05. According to a 2-tailed power analysis (G*power 3.1), we need 9 mice per group in order to obtain significant differences. From experience we know that about 10-20% do not get properly infected. Therefore, we included an additional mouse per group

B. The animals

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

C57BL/6 CD11c-cre+ / cre- 'x'-flox/flox, rederived in house, n=108 per strain (54 cre+ and 54 cre-) where 'x' will be a gene involved in:
1) AMPK signalling directly (for instance LKB1 or AMPK1)
2) mTOR signalling directly (for instance Raptor or Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 or PPARg)
4) metabolic regulation downstream of mTOR signalling: (for instance HIF1a or SREBP)
These mice will be used between 6-12 weeks of age and both male and females will be used.

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role in metabolism and the T cell priming and polarizing capacity of DC in the immunization model (part 2), will be evaluated using CD11c-cre flox mice, with a maximum of a total of 6 different target genes.

Breeding will be setup with homozygous flox breeding pairs of which one of the breeding pair is heterozygous for CD11c-cre, whereas the other will be CD11c-cre negative. Offspring should then be 50% CD11c-cre+ and 50% CD11c-cre negative. The CD11c-Cre negative littermates will be used as controls for CD11c-Cre+ mice in the same experiment .

Total maximum number of mice: 6x108 cre/flox = 648 mice

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:
Research involving mice is essential for the proposed research.. The metabolic properties of DCs and how they shape their immune polarizing function (which is the focus of this project), are expected to be largely dictated by the constantly changing micro-environment they reside in in situ. This cannot be mimicked in vitro. Finally, these studies need to be performed in mice, since this is the only model organism in which the tools are available to study DC-specific deletions in AMPK/mTOR signalling cascades. Therefore, this study can only be properly performed in vivo in mice.
Reduction:
To reduce the number of mice needed, only the transgenic mice that gave an altered Type 1 immune response in the immunization model will be tested in this infection model. If the transgenic mice do not display an altered immunological profile compared to littermate controls as determined in (A), (B) will not be performed. In the current project we will be using a genetically attenuated strain of Py that expresses luciferase. following injection of luciferin, this will allow for monitoring parasite burden real time in vivo at multiple time points in the same mice without the need to sacrifice mice. This reduces the number of mice needed for these experiments. In addition, Measurement of luciferase activity with an I-CCD Video camera is a very sensitive and accurate method to analyse parasite loads in live animals. As only small variations in luminescence signals between mice belonging to the same group are observed, a

smaller number of animals per group is required in comparison with other more traditional methods of analysing parasite loads (e.g. Histology, RT-PCR). In addition, littermate controls (CD11c-Cre+ and CD11c-Cre-) will be used to reduce experimental variation and thereby reduce the number of mice needed per experimental group. Finally, experiments will be performed by highly skilled and experienced staff, which will reduce intra-experimental variation and as such will result in fewer animals that will be needed per experiment to obtain reliable and statistically significant data.
Refinement
Infection will be performed under isoflurane anaesthesia. In the current project we will be using a genetically attenuated strain of Py that can only develop to the liver stage. This will ensure that blood stage malaria cannot develop and as such no overt disease is to be expected. Moreover, following injection of luciferin, parasite burden can be monitored real-time in vivo without the need to sacrifice mice. If any of the animals develop any state described as humane endpoint in the next section during the infection, the animals will be humanely sacrificed. Finally, experiments will be performed by staff that are highly skilled and experienced with animal handling. As a result all procedures with the animals will be performed with the greatest care and minimal animal distress will be ensured.

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

infection will be performed under isoflurane anaesthesia, to minimize animal pain or fear. We do not expect severe complications as a result of infection with GAP Py since these parasites cannot develop into blood stage malaria that can cause overt disease. However, Pc blood stage infection may lead to illness due to weight loss hypothermia or anaemia, in case any the transgenic mice have a defect in their ability to mount protective Type 1 immune responses. Therefore, these animals will be observed daily following the Pc infection and will be humanely sacrificed in case the following defined end points are reached: >40% positive blood smear, visible pain (abnormal posture and/or movement), significant weight loss (>20%), abnormal behaviour (isolation, abnormal reaction to stimuli, no food and water intake) or hypothermia (<34C). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanized.

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.

The research question that is being addressed with the current set of experiments is novel and has not be studied before. An extensive literature search has revealed that these trangenic mice have not been used before in this kind of experimental model.

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

☒ 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.

Before Luciferine administration s.c. or sporozoite infection i.v., mice will be put under anaesthesia by using isoflurane (5%) mixed with oxygen. During the injection, mice will be maintained under anaesthesia using a mouth piece. To ensure anaesthesia has set in well, rear foot reflex will be tested. During the maintenance phase isoflurane dosage is reduced (to 2-3%) to prevent overdosing.

I. Other aspects compromising the welfare of the animals

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

infection of the injection site

Explain why these effects may emerge.

needle ruptures the skin which may cause an opportunistic infection

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

Sterile needles and syringes will be used to minimize chances of infection

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.

Animals will be humanely sacrificed in case the following defined end points are reached: >40% positive blood smear, visible pain (abnormal posture and/or movement), significant weight loss (>20%), abnormal behavior (isolation, abnormal reaction to stimuli, no food and water intake) or hypothermia (<34C). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanised.

Indicate the likely incidence.

possible

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').

iv injection: mild
WT infection: moderate/severe

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.

To analyse the immune response occurring in spleen and liver the draining lymph node, mice need to sacrificed.

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

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

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.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'.	11600
1.2	Provide the name of the licenced establishment.	Leids Universitair Medisch Centrum
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 6 Type of animal procedure: Listeria monocytogenes infection model

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.

Intracellular bacterial infections drive strong type 1 immunity characterized by Th1 and cytotoxic T cell responses. Therefore, to study whether mice that harbour DCs with defects in AMPK or mTOR signalling have an altered capacity to prime Type 1 immune responses a intracellular bacterial infection model with Listeria monocytogenes (Lm) will be used. Following i.v. infection, the peak of the T cell response lies at day 7 post infection, and while the peak of the bacterial load is around day 3 and is generally cleared by day 7. Dendritic cells play a crucial role in priming and regulation of Type 1 immune responses during this infection. This makes this model a highly suitable to interrogate the role of metabolic pathways in DCs that regulate their capacity to prime Type 1 immune responses. (A) The primary immune response to the infection as well as (B) lasting immunity will be studied.

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

(A)
To study the primary immune response against Listeria, mice will be infected i.v. (200ul), through tail vain injection, with a subclinical dose of Lm expressing ovalbumin (OVA) (2500CFU). At day 3 and 5 after the infection mice will be bled by cheek bleeding under isoflurane anaesthesia and analysed for OVA specific circulating CD4 and CD8 T cells. At day 3 and 7 mice will be euthanized with an overdose Nembutal and analysed for the following parameters:
1) DCs and OVA specific T cells in spleens and liver (the two main sites of infection) will be quantified and analysed for their phenotype.
2) Splenocytes and liver will be restimulated with OVA or polyclonally to assess cytokine production by

the T cells.
3) Bacterial loads will be determined in spleens and livers to assess whether the mutant mice have an altered ability to control infection.

To track proliferative responses of T cells in vivo in response to the infection, 5000 CFSE and CTV-labelled OVA-specific CD4 (OTII) and CD8 (OTI) T cells will be adoptively transferred i.v. in 200ul PBS, 1 day prior to infection as described above, in a separate group of mice. At day 3 and 7 mice will be euthanized with an overdose Nembutal and analysed for proliferation and phenotype of transferred T cells.

Experimental groups:

Group 1: naïve control mice	n=8
Group 2: Lm infected (Day 3)	n=8
Group 3: Lm infected (Day 7)	n=8
Group 1: naïve control mice + transferred T cells	n=8
Group 2: Lm infected (Day 3) + transferred T cells	n=8
Group 3: Lm infected (Day 7) + transferred T cells	n=8

The following C57BL/6 CD11c-cre 'x'-flox strains will be used, where 'x' will be a gene involved in:
1) AMPK signalling directly (for instance LKB1 and AMPK1)
2) mTOR signalling directly (for instance Raptor and Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 and PPARg)
4) metabolic regulation downstream of mTOR signalling: (for instance HIF1a and SREBP)

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role in the T cell priming and polarizing capacity of DC in the immunization model (part 2), will be evaluated using CD11c-cre flox mice in this model.

(B)
To study the ability of these mice to mount protective/lasting Type 1 immunity, mice will be infected i.v. (200ul), through tail vain injection, with a replication deficient strain of Lm-(delta ActA) expressing OVA (10e7 CFU). 21 days after the primary infection and when the memory response has been established, mice will be challenged i.v. (200ul), through tail vain injection, with a LD50 dose of WT Lm expressing ovalbumin (OVA) (10e5 CFU). At day 3 mice will be euthanized with an overdose Nembutal and analysed for the following parameters:
1) OVA specific CD4 and CD8 T cells in spleens and liver (the two main sites of infection) will be quantified and analysed for their phenotype.
2) Splenocytes and liver will be re-stimulated with OVA or polyclonally to assess cytokine production by the T cells.
3) Bacterial loads will be determined in spleens and livers to assess whether the mutant mice have an altered ability to mount protective immunity

Experimental groups:

Group 1: naïve control mice (control)	n=8
Group 2: Primary infection only (control)	n=8
Group 3: Secondary infection only (control)	n=8
Group 4: Primary + secondary infection	n=8

The following C57BL/6 CD11c-cre 'x'-flox strains will be used, where 'x' will be a gene involved in:
1) AMPK signalling directly (for instance LKB1 and AMPK1)
2) mTOR signalling directly (for instance Raptor and Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 and PPARg)
4) metabolic regulation downstream of mTOR signalling: (for instance HIF1a and SREBP)
Importantly, only genes present in the signalling pathways and downstream target genes that were

identified to play a role in the T cell priming and polarizing capacity of DC in the immunization model (part 2), will be evaluated using CD11c-cre flox mice in this model.

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

Based on earlier findings (Wensveen et al. Immunity 2010, van der Windt et al. Immunity 2012), we observed an in-group variation of around 40% with this infection in terms of immunological outcome and bacterial loads. Based on preliminary experiments we anticipate a difference between WT and knout mice of around 50%. We would need 8 mice per group, to be able have sufficient power (80%) to obtain significant differences (with a p = 0.05).

B. The animals

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

C57BL/6 OTI n=5
C57BL/6 OTII n=5
Mice are obtained from Harlan or Jackson laboratories and used at 6-8 weeks of age

C57BL/6 CD11c-cre+ / cre- 'x'-flox/flox, rederived in house, n=160 per strain (80 cre+ and 80 cre-) where 'x' will be a gene involved in:
1) AMPK signaling directly (for instance LKB1 or AMPK1)
2) mTOR signaling directly (for instance Raptor or Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 or PPARg)
4) metabolic regulation downstream of mTOR signaling: (for instance HIF1a or SREBP)
These mice will be used between 6-12 weeks of age and both male and females will be used.

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role in metabolism and the T cell priming and polarizing capacity of DC in the immunization model (part 2), will be evaluated using CD11c-cre flox mice, with a maximum of a total of 6 different target genes.

Breeding will be setup with homozygous flox breeding pairs of which one of the breeding pair is heterozygous for CD11c-cre, whereas the other will be CD11c-cre negative. Offspring should then be 50% CD11c-cre+ and 50% CD11c-cre negative. The CD11c-Cre negative littermates will be used as controls for CD11c-Cre+ mice in the same experiment

Total maximum number of mice: 5 OTI + 5 OTII + 6x160 cre/flox = 970 mice

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:
Research involving mice is essential for the proposed research. The metabolic properties of DCs and how they shape their immune polarizing function (which is the focus of this project), are expected to be

largely dictated by the constantly changing micro-environment they reside in in situ. This cannot be mimicked in vitro. Finally, these studies need to be performed in mice, since this is the only model organism in which the tools are available to study DC-specific deletions in AMPK/mTOR signalling cascades. Therefore, this study can only be properly performed in vivo in mice.
Reduction:
To reduce the number of mice needed, only the transgenic mice that gave an altered Type 1 immune response in the immunization model. If the transgenic mice do not display an altered immunological profile compared to littermate controls as determined in (A), (B) will not be performed. Furthermore, littermate controls (CD11c-Cre+ and CD11c-Cre-) will be used to reduce experimental variation and thereby reduce the number of mice needed per experimental group. In addition, only genes present in the signalling pathways and downstream target genes that were identified to play a role in the T cell priming and polarizing capacity of DC in the immunization model (part 2), will be evaluated using CD11c-cre flox mice in this model. Finally, experiments will be performed by highly skilled and experienced staff, which will reduce intra-experimental variation and as such will result in fewer animals that will be needed per experiment to obtain reliable and statistically significant data.
Refinement
Infection will be performed under anaesthesia. A replication deficient Lm strain will be used, which eliminates the risk of developing clinical signs of disease. If any of the animals develop any state described as humane endpoint in the next section during the infection, the animals will be humanely sacrificed. Finally, experiments will be performed by highly skilled and experienced staff, which will reduce intra-experimental variation and as such will result in fewer animals that will be needed per experiment to obtain reliable and statistically significant data.

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

infection and cheek bleeding will be performed with under isoflurane anaesthesia, to minimize animal pain or fear. While we do not expect severe complications as a result of the initial infection the challenge infection, may lead to illness in case any the transgenic mice have a defect in their ability to mount a Type 1 immune responses. Therefore, the animals will be observed daily following the secondary infection and will be humanely sacrificed in case the following defined end points are reached: visible pain (abnormal posture and/or movement), significant weight loss (>20%), abnormal behaviour (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanized.

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.

The research question that is being addressed with the current set of experiments is novel and has not be studied before. An extensive literature search has revealed that these trangenic mice have not been used before in this kind of experimental model.

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

☒ 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.

Before i.v. infection or cheek bleeding, mice will be put under anaesthesia by using isoflurane (5%) mixed with oxygen. During the procedure, mice will be maintained under anaesthesia using a mouth piece. To ensure anaesthesia has set in well, rear foot reflex will be tested. During the maintenance phase isoflurane dosage is reduced (to 2-3%) to prevent overdosing.

I. Other aspects compromising the welfare of the animals

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

infection of the injection site

Explain why these effects may emerge.

needle ruptures the skin which may cause an opportunistic infection

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

Sterile needles and syringes will be used to minimize chances of infection

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.

While we do not expect severe complications as a result of the initial infection the challenge infection, may lead to illness in case any the transgenic mice have a defect in their ability to mount a Type 1 immune responses. Therefore, the animals will be observed daily following the secondary infection and will be humanely sacrificed in case the following defined end points are reached: visible pain (abnormal posture and/or movement), significant weight loss (>20%), abnormal behavior (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanised.

Indicate the likely incidence.

possible

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').

iv injection: mild

WT Lm OVA infection: moderate

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.

To analyse the immune response occurring in spleen and liver the draining lymph node, mice need to sacrificed.

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

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

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.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'.	11600
1.2	Provide the name of the licenced establishment.	Leids Universitair Medisch Centrum
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: 7 Type of animal procedure: Melanoma tumor model

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.

While carcinogenesis itself is considered to be triggered by cell-intrinsic genetic mutations, tumor growth and the clinical outcome is thought to be primarily a function of how well the immune system is able to mount an anti-tumor response. For this anti-tumor immune response cell mediated immunity mediated by Th1 and cytotoxic T cells is particularly important. Therefore there is great significance in understanding how DCs become conditioned to prime Th1 and Cytotoxic T cell responses during cancer. To establish whether the balance in AMPK vs mTOR signaling in DCs controls their T cell polarizing and priming function and thereby regulates their capacity to drive an anti-tumor responses, we will make use of a B16-OVA melanoma tumor model which is a model for highly prevalent type of skin cancer in humans and of which its clinical outcome is highly dependent on effective anti-melanoma Th1 and cytotoxic T cell responses. The different transgenic mice will be subcutaneously injected with the OVA-expressing tumor cells and tumor growth will be followed as a function of several immunological parameters, including OVA-specific CD4+ and CD8+ T cell numbers systemically and around the tumor as well as production of effector molecules by these cells..

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

The mice will be injected intradermally (i.d.) in their back with B16 melanoma cells expressing OVA (1x105 in 50ul) under isoflurane anesthesia. Mice will be monitored for tumor growth periodically. When tumor size of one or multiple animals reaches 2 cm in diameter (before it may cause distress to the animals), all mice will be euthanized by i.p. injection of an overdose Nembutal and tumors will be analyzed for immune cell infiltrate.

The following time scheme will be applied for the melanoma tumor model

day 0: i.d. injection of B16 melanoma cells expressing OVA (1x105 in 50ul)
day 7-14: When reaching tumor size of 2cm max, euthanasia by i.p. injection of an overdose Nembutal

The following groups will be setup:
1) x- flox mice naïve n=5
2) Cd11c-cre+ x-flox mice naive n=5
3) x-flox mice tumor challenged n=12
4) Cd11c-cre+ x-flox mice tumor challenged n=12

The following C57BL/6 CD11c-cre 'x'-flox strains will be used, where 'x' will be a gene involved in:
1) AMPK signaling directly (for instance LKB1 and AMPK1)
2) mTOR signaling directly (for instance Raptor and Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 and PPARg)
4) metabolic regulation downstream of mTOR signaling: (for instance HIF1a and SREBP)

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role T cell polarization in the immunization model (part 2), will be evaluated using CD11c-cre flox mice in this model.

The following readouts will be performed to determine whether mTOR/AMPK signaling in DCs play a role in protection and

- During tumor growth (day 8 until day 21) tumor sizes will be measured with digital calipers every 3 days and every day after day 14

- On day 14, mice will be bled by cheek bleeds and frequencies and effector phenotype of circulating OVA-specific CD8 T cells will be determined.

- At time of harvest (when tumors of one or multiple mice reach 2 cm in diameter, or after 21 days), mice will be euthanized by i.p. injection of an overdose Nembutal and tumors will be excised to determine with final tumor volumes and weights.

- In addition, tumor infiltrating cells and cells from draining inguinal lymph nodes will be isolated by mechanical dissociation and passing through 70 µm cell strainers to obtain single cell suspensions. Cellular composition will be assessed by flow cytometry and specifically screened for the presence of different T helper subsets and Cytotoxic T cells. LN cells will be restimulated ex vivo to determine to Tcell cytokine profile in these mice using intracelluar cytokine staining and ELISA

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

In previous experiments (Amiel et al, JI, 2012) we experienced a considerable variability in tumor growth between different individual mice (41% stdev) and based on these data and an anticipated difference between WT and knout mice of at least 40% we need 12 mice per group with tumors, to be able have sufficient power (80%) to obtain significant differences (with a p = 0.05).

B. The animals

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

C57BL/6 CD11c-cre+ / cre- 'x'-flox/flox, rederived in house, n=34 per strain (17 cre+ and 17 cre-) where 'x' will be a gene involved in:
1) AMPK signaling directly (for instance LKB1 or AMPK1)
2) mTOR signaling directly (for instance Raptor or Rictor)

3) metabolic regulation downstream of AMPK signalling (for instance PGC1 or PPARg)
4) metabolic regulation downstream of mTOR signaling: (for instance HIF1a or SREBP)
These mice will be used between 6-12 weeks of age and both male and females will be used.

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role in metabolism and the T cell priming and polarizing capacity of DC in the immunization model (part 2), will be evaluated using CD11c-cre flox mice, with a maximum of a total of 6 different target genes.

Breeding will be setup with homozygous flox breeding pairs of which one of the breeding pair is heterozygous for CD11c-cre, whereas the other will be CD11c-cre negative. Offspring should then be 50% CD11c-cre+ and 50% CD11c-cre negative. The CD11c-Cre negative littermates will be used as controls for CD11c-Cre+ mice in the same experiment

Total maximum number of mice: 6x34 cre/flox = 204mice

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:
Research involving mice is essential for the proposed research. The immune responses involved in tumor development are extremely complex involving multiple different players at different times. Furthermore, the metabolic properties of DCs and how they shape their immune polarizing function (which is the focus of this project), are expected to be largely dictated by the constantly changing micro-environment they reside in in situ, especially in the context of tumors which are likely to compete with DCs for nutrients. This cannot be mimmicked in vitro. Finally, these studies need to be performed in mice, since this is the only model organism in which the tools are available to study DC-specific deletions in AMPK/mTOR signaling cascades. Therefore, this study can only be properly performed in vivo in mice.
Reduction:
To reduce the number of mice needed, only the transgenic mice that gave an altered Th1/cytotoxic T cell immune response in the immunization model will be tested in this tumor model. Moreover, littermate controls (CD11c-Cre+ and CD11c-Cre-) will be used to reduce experimental variation and thereby reduce the number of mice needed per experimental group. Finally, experiments will be performed by highly skilled and experienced staff, which will reduce intraexperimental variation and as such will result in fewer animals that will be needed per experiment to obtain reliable and statistically significant data.
Refinement
Injection of the tumor cells and bleeding of the mice will be performed under anesthesia, which takes only a few minutes. the tumor cells will be injected on the back of the mice to minimize physical discomfort. If any of the animals develop any state described as humane endpoint in the next section, the animals will be humanely sacrifized. Finally, experiments will be performed by staff that are highlyskilled and experienced with animal handling. As a result all procedures with the animals will be performed with the greatest care and minimal animal distress will be ensured.

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

Injection of the tumor cells and bleeding of the mice will be performed under anesthesia, which takes only a few minutes. the tumor cells will be injected on the back of the mice to minimize physical discomfort. If any of the animals develop any state described as humane endpoint in the next section, the animals will be humanely sacrifized.

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.

The research question that is being addressed with the current set of experiments is novel and has not be studied before. An extensive literature search has revealed that these trangenic mice have not been used before in this kind of experimental model.

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

☒ 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.

Before tumour injection mice will be put under anaesthesia by using isoflurane (5%) mixed with oxygen. During injection, mice will be maintained under anaesthesia using a mouth piece. To ensure anaesthesia has set in well, rear foot reflex will be tested. During the maintenance phase isoflurane dosage is reduced (to 2-3%) to prevent overdosing.

I. Other aspects compromising the welfare of the animals

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

development of a solid subcutaneous tumor beyond 2 cm3 may negatively affect the welbeing of the mice

Explain why these effects may emerge.

the tumor may induce pain in the surrounding tissue or may physically interfere with free movement of the animal

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

the tumor will be establisshed on the back of the mouse, to minimize interference with normal behaviour. In addition humane endpoints are in place as described below.

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.

In accordance with the Code of Practice for 'Kankeronderzoek in proefdieren', A human endpoint is chosen when tumor size reaches 2 cm3, when it physically interferes with normal behaviour, when the intradermally located tumor starts ulcerating or when rapid weight loss (> 15% in 1-2 days). In addition, the animals will be observed on a daily basis and be humanely sacrificed in case the following defined end points are reached: visible pain (abnormal posture and/or movement), significant weight loss (>20%), abnormal behavior (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanised.

Indicate the likely incidence.

unlikely

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').

anaesthesia/recovery from anaesthesia: mild
discomfort from tumor growth: moderate

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.

To analyse the immune response occurring in and around the tumor and draining Lymph nodes, mice need to be sacrificed.

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

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

☒ Yes

Appendix
Description animal procedures

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- 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'.	11600
1.2	Provide the name of the licenced establishment.	Leids Universitair Medisch Centrum
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: 8 Type of animal procedure: House dust mite Allergic Asthma model

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.

Allergic Asthma is the result of strong induction of Th2 immune responses by DCs in response to allergens in the lung that culminates in allergic airway inflammation. Therefore there is great significance in understanding how DCs become conditioned to prime Th2 responses during allergic asthma and how we can manipulate that process. To establish whether the balance in AMPK vs mTOR signalling in DCs controls their T cell polarizing function in the context of allergic asthma a model of allergic asthma will be used based on human allergen house dust mite (HDM). In this model mice are sensitized and challenged by allergens only via the intranasal route, which is similar to the natural route of allergen entry in humans and therefore is more relevant to allergic processes found in humans. Immunological outcomes that will be analysed following allergen sensitization and challenge are: DC phenotype and nature of T cell responses in the lung and draining lymph nodes. In addition, disease outcome in terms of airway inflammation will be assessed by analysing airway resistance, cellular composition of the Bal fluid and lung histology. This will determine whether mice with DC-specific deletions in the AMPK or mTOR signalling axis are more or less prone to develop Th2 immune responses and as a result allergic asthma.

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

C57BL/6 mice will be sensitized by i.t. application of 10µg HDM allergen in 30µL PBS on day 1. Next, mice will be challenged for 5 consecutive days (day 8-12) by i.t. application of 10µg HDM allergen. I.t. application of allergens in these models will be done under narcosis with isofluraan. The mice will be euthanized 3 days after the last challange (day 15) by an overdose Nembutal and the following readout

will be performed:
The following readouts will be performed:

• A set of N=5 mice per group will be used to measure the airway resistance after challenge with a metacholine (concentration curve 3.125, 6.25, 12.5, 25, 50, 100mg/mL metacholine in PBS) by FlexiVent. Metacholine challenge results in a faster increase in airway resistance in mice with allergic airway inflammation compared to control mice. One day after the last allergen challenge, mice will be anesthetized by i.p injection of ketamine (100mg/kg) and xylazine (10mg/kg), and afterwards the own breathing of the mouse is blocked by i.p. injection of pancuronium (0,8mg/kg in 100ul). A small incision is made in the trachea and a small tube is inserted that allows the connection of the mouse respiratory tract to the FlexiVent machine, which takes over the breathing during the measurement. After the measurement, the mice will be euthanized by an overdose of Nembutal. Then, the vena cava will be cut and venous blood will be collected for serum IgE measurements.

• A set of N=5 mice will be used for the assessment of cellular composition of the BAL fluid, the lungs and the medLNs (eosinophilia, neutrophilia, macrophages, T cells). The mice will be euthanized one day after the last challenge by an overdose of Nembutal. A tube will be inserted into the trachea and the lungs flushed with 3x1mL of PBS to collect the BAL fluid. Afterwards, the lungs will be flushed with ca. 50mL of Hanks buffer and collected together with the medLNs. One lobe of the lung will be used to measure cytokine and chemokine mRNA expression levels by PCR. In addition medLN mcells and the remainder of the lung and will be used for cell isolation for ex vivo restimulation assays.

• The lungs of a set of N=4 mice will be used for histology. The mice will be euthanized one day after the last challenge by an overdose Nembutal. A tube will be inserted into the trachea, the lungs will be inflated with 1mL of OCT and snap-frozen in liquid nitrogen. These lungs will be used for histological hematoxylin/eosin (H&E) and periodic acid-schiff (PAS) staining for assessment of lung inflammation.

The following time scheme will be applied for the HDM model:

day 1: sensitisation by i.t. application of 10 HDM allergen in 50µL PBS; control mice receive PBS only
day 8-12 : challenge by i.t. application of 10µg HDM allergen alone, control mice receive PBS
day 15: euthanasia by i.p. injection of an overdose Nembutal

Group 1: x-flox mice: Sensitization by PBS i.t., challenge by i.t. PBS N=14
(negative control)

Group 2: x-flox mice: Sensitisation by i.t. HDM , challenge by i.t. HDM N=14
(control asthmatic group)

Group 3: CD11c-cre+ x-flox mice: Sensitization by PBS i.t., challenge by i.t. PBS N=14
(negative control group)

Group 4: CD11c-cre+ x-flox mice: Sensitisation by i.t. HDM , challenge by i.t. HDM N=14
(Test asthmatic group)

The following C57BL/6 CD11c-cre 'x'-flox strains will be used, where 'x' will be a gene involved in:
1) AMPK signalling directly (for instance LKB1 and AMPK1)
2) mTOR signalling directly (for instance Raptor and Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 and PPARg)
4) metabolic regulation downstream of mTOR signalling: (for instance HIF1a and SREBP)

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role T cell polarization in the immunization model (part 2), will be evaluated using CD11c-cre flox mice in this model.

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

Based on our experience with the OVA/alum model, we expect a standard deviation of 13.5% for this type of experiments. We hope to observe a 25% difference between the experimental groups, and we set the power at 80% and determine p-value of 0.05. According to power analysis, we need 5 mice per group in order to obtain significant differences. From experience we know that about 80% about respond to the treatment and induce asthma. Therefore, we included one additional mice per group. 5 mice will be used for the measurement of airway hyper responsiveness by Flexivent, 5 mice for cell composition of different tissues by flow cytometry, for assessment of mRNA expression and ex vivo restimulations and 4 mice for histology.

B. The animals

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

C57BL/6 CD11c-cre+ / cre- 'x'-flox/flox, rederived in house, n=56 per strain (28 cre+ and 28 cre-) where 'x' will be a gene involved in:
1) AMPK signalling directly (for instance LKB1 or AMPK1)
2) mTOR signalling directly (for instance Raptor or Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 or PPARg)
4) metabolic regulation downstream of mTOR signalling: (for instance HIF1a or SREBP)
These mice will be used between 6-12 weeks of age and both male and females will be used.

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role in metabolism and the T cell priming and polarizing capacity of DC in the immunization model (part 2), will be evaluated using CD11c-cre flox mice, with a maximum of a total of 6 different target genes.

Breeding will be setup with homozygous flox breeding pairs of which one of the breeding pair is heterozygous for CD11c-cre, whereas the other will be CD11c-cre negative. Offspring should then be 50% CD11c-cre+ and 50% CD11c-cre negative. The CD11c-Cre negative littermates will be used as controls for CD11c-Cre+ mice in the same experiment

Total maximum number of mice: 6x56 cre/flox = 336 mice

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:
Research involving mice is essential for the proposed research. The immune responses involved in Allergic asthma are extremely complex involving multiple different players at different times. Furthermore, the metabolic properties of DCs and how they shape their immune polarizing function (which is the focus of this project), are expected to be largely dictated by the constantly changing micro-environment they reside in in situ. This cannot be mimmicked in vitro. Finally, these studies need to be

performed in mice, since this is the only model organism in which the tools are available to study DC-specific deletions in AMPK/mTOR signalling cascades. Therefore, this study can only be properly performed in vivo in mice.
Reduction:
To reduce the number of mice needed, only the transgenic mice that gave an altered Th2/Treg cell immune response in the immunization model will be tested in this Allergy model. Littermate controls (CD11c-Cre+ and CD11c-Cre-) will be used to reduce experimental variation and thereby reduce the number of mice needed per experimental group. Finally, experiments will be performed by staff that are highly skilled and experienced with animal handling. As a result all procedures with the animals will be performed with the greatest care and minimal animal distress will be ensured.
Refinement
Intranasal application of allergens and assessment of airway resistance will be performed under anaesthesia, which takes only a few minutes. If any of the animals develop any state described as humane endpoint in the next section, the animals will be humanely sacrificed.
Finally, experiments will be performed by highly skilled and experienced staff, which will reduce intra-experimental variation and as such will result in fewer animals that will be needed per experiment to obtain reliable and statistically significant data.

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

Intranasal application of allergens and assessment of airway resistance will be performed under anaesthesia, to minimize animal pain or fear. While we do not expect severe complications as a result of inducing allergic airway inflammation. In addition, the animals will be observed on a daily basis and be humanely sacrificed in case the following defined end points are reached: visible pain (abnormal posture and/or movement), significant weight loss (>20%), abnormal behaviour (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanized.

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.

The research question that is being addressed with the current set of experiments is novel and has not be studied before. An extensive literature search has revealed that these trangenic mice have not been used before in this kind of experimental model.

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

☒ 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.

Before intratrachial application of allergens, mice will be put under anaesthesia by using isoflurane (5%) mixed with oxygen. During the procedure, mice will be maintained under anaesthesia using a mouth piece. To ensure anaesthesia has set in well, rear foot reflex will be tested. During the maintenance phase isoflurane dosage is reduced (to 2-3%) to prevent overdosing.

I. Other aspects compromising the welfare of the animals

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

development of wheezing/shallow breathing

Explain why these effects may emerge.

Induction of Allergic asthma may reduce longfunction and cause bronchocontriction causing signs of wheezing

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

see humane endpoint

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.

Although human endpints are unlikely to be the animals will be observed on a daily basis and be humanely sacrificed in case the following defined end points are reached: visible pain (abnormal breathing, posture and/or movement), significant weight loss (>20%), abnormal behavior (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanised.

Indicate the likely incidence.

Very unlikely

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').

anaesthesia/recovery from anaesthesia: mild
development of allergic asthma: mild and in some specific cases moderate

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.

To analyse the immune response occurring in the lung and associated Lymph nodes, mice need to sacrificed. Lung inflammation based on histology can only be assessed after sacrificing the mice.

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

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

☒ Yes

Appendix
Description animal procedures

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- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
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1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	11600
1.2	Provide the name of the licenced establishment.	Leids Universitair Medisch Centrum
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: 9 Type of animal procedure: model of obesity induced type 2 diabetes

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.

Obesity is well-known to be a risk factor for the development of peripheral insulin resistance and type 2 diabetes. It is becoming increasingly clear that insulin resistance in metabolic tissues is for a large part determined by immune cells residing in these tissues. In obesity it has been shown that low grade inflammation in metabolic tissues (fat, liver and muscle) caused by a type 1 polarized immune cell infiltrate (Th1 and cytotoxic CD8 T cells, M1 macrophages, NK cells), promotes insulin resistance. Conversely, healthy metabolic homeostasis and good insulin sensitivity is underpinned by the presence ofTh2, Treg and M2 macrophages in these tissues. DCs appear to play an important role in regulating the polarization profile of these immune cells in metabolic tissues. Therefore a model of high fat diet induced obesity and induction of insulin resistance is an excellent model to establish whether mice that harbour DCs with defects in AMPK or mTOR signalling have an altered immune response in the metabolic tissues and whether they are more or less susceptible to obesity induced insulin resistance and development of type 2 diabetes .

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

Mice will be put either on normal chow or on high-fat diet (HFD, 40% of energy derived from fat) for 12 weeks. During these 12 weeks the following parameters will be determined:
Body weight: once every week
Food intake (per cage): continuous monitoring (by weighting food pellets)
Blood sampling (1/2 of a capillary, i.e 35 uL) on 4h fasted mice: wk 0, 6 and 12 for determination of fasting plasma glucose/insulin/lipids.

At week 10:
Insulin Tolerance Test. An i.p injection of insulin (0.75U/kg of 100mU/ml insulin dissolved in PBS with 0.2% BSA) will be performed in 4h-fasted mice, followed by determination of plasma glucose levels (with handmeter) at t=0, t=15, t=30, t=60, t=120 minutes by taking a drop of blood and collecting blood sample (1/3 of a capillary, i.e.~25uL) from the tail vein in order to determine plasma insulin levels.

At week 12:
Glucose tolerance test (GTT): An i.p injection of glucose (1.25 g/kg body weight of 20% D-glucose) will be performed in 4h-fasted mice, followed by determination of plasma glucose levels (with handmeter) at t=0, t=15, t=30, t=60, t=120 minutes by taking a drop of blood and collecting blood sample (1/3 of a capillary, i.e.~25uL) from the tail vein in order to determine plasma insulin levels.

At week 12: After 2-3 days of recovery post GTT, the animals will be killed by cervical dislocation and various peripheral organs (liver, white adipose tissues [gonadal, perirenal, subcutaneous], brown adipose tissue, skeletal muscle [gastrocnemius], heart, kidney and spleen), will be collected, weighted and divided in various parts for subsequent analysis (western blot, qPCR, Immunohistochemistry, and FACS for immune cell composition.)

In summary the following experimental groups will be used:
1) mice on normal chow diet n=12
2) mice on High fat diet n=12

The following C57BL/6 CD11c-cre 'x'-flox strains will be used, where 'x' will be a gene involved in:
1) AMPK signalling directly (for instance LKB1 and AMPK1)
2) mTOR signalling directly (for instance Raptor and Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 and PPARg)
4) metabolic regulation downstream of mTOR signalling: (for instance HIF1a and SREBP)

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role T cell polarization in the immunization model (part 2), will be evaluated using CD11c-cre flox mice in this model.

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

Various kind of parameters will be measured in this experiment, so we use the most variable one (GTT) in order to calculate the sample size (2 sided T-test).
Based on earlier findings (Hussaarts et al, Faseb J, 2015), we observed an in-group variation of 15% with this Test. We anticipate a difference between WT and knout mice of around 15%. We would need 10 mice per group, to be able have sufficient power (80%) to obtain significant differences (with a p = 0.05). Taken into consideration the 10-20% of non-responders to HFD and the 10-20%, we ask for using 12 animals per group.

B. The animals

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

C57BL/6 CD11c-cre+ / cre- 'x'-flox/flox, rederived in house, n=48 per strain (24 cre+ and 24 cre-) where 'x' will be a gene involved in:
1) AMPK signalling directly (for instance LKB1 or AMPK1)
2) mTOR signalling directly (for instance Raptor or Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 or PPARg)
4) metabolic regulation downstream of mTOR signalling: (for instance HIF1a or SREBP)
These mice will be used between 6-8 weeks of age and both male and females will be used.

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role in metabolism and the T cell priming and polarizing capacity of DC in the

immunization model (part 2), will be evaluated using CD11c-cre flox mice, with a maximum of a total of 6 different target genes.

Breeding will be setup with homozygous flox breeding pairs of which one of the breeding pair is heterozygous for CD11c-cre, whereas the other will be CD11c-cre negative. Offspring should then be 50% CD11c-cre+ and 50% CD11c-cre negative. The CD11c-Cre negative littermates will be used as controls for CD11c-Cre+ mice in the same experiment

Total maximum number of mice: 6x48 cre/flox = 288 mice

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:
Research involving mice is essential for the proposed research. The metabolic properties of DCs and how they shape their immune polarizing function (which is the focus of this project), are expected to be largely dictated by the constantly changing micro-environment they reside in in situ. This cannot be mimicked in vitro. Furthermore the effects on high fat diet on whole body metabolism and the metabolic regulation of an interplay between various tissues can only be studied in vivo. Finally, these studies need to be performed in mice, since this is the only model organism in which the tools are available to study DC-specific deletions in AMPK/mTOR signalling cascades. Therefore, this study can only be properly performed in vivo in mice.
Reduction:
To reduce the number of mice needed the Finally, littermate controls (CD11c-Cre+ and CD11c-Cre-) will be used to reduce experimental variation and thereby reduce the number of mice needed per experimental group. Finally, experiments will be performed by highly skilled and experienced staff, which will reduce intra-experimental variation and as such will result in fewer animals that will be needed per experiment to obtain reliable and statistically significant data.
Refinement
High-fat diet treatment will not induce any discomfort. If, however, any of the animals develop any state described as humane endpoint in the next section, the animals will be humanely sacrificed. Finally, experiments will be performed by staff that are highly skilled and experienced with animal handling. As a result all procedures with the animals will be performed with the greatest care and minimal animal distress will be ensured.

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

While we do not expect severe complications as a result HFD the animals will be observed on a daily basis and be humanely sacrificed in case the following defined end points are reached: visible pain (abnormal posture and/or movement, significant swelling of the foot and/or limping), significant weight loss (>20%), abnormal behaviour (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanized.

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.

The research question that is being addressed with the current set of experiments is novel and has not be studied before. An extensive literature search has revealed that these trangenic mice have not been used before in this kind of experimental model.

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

☒ 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?

none

Explain why these effects may emerge.

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

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of

humane endpoints to prevent further distress?

☐ No > Continue with question K.

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

Although human endpoints are very unlikely needed to be implemented, the animals will be observed on a daily basis by the animal caretakers and be humanely sacrificed in case the following defined end points are reached: visible pain (abnormal posture and/or movement), significant weight loss (>20%), abnormal behavior (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanised.

Indicate the likely incidence.

Very unlikely

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').

High fat diet = mild
Determination of body weight = mild
Blood sampling = moderate
4h fasting for GTT and ITT: mild
i.p. injection of either glucose or insulin = mild
Cervical dislocation = mild

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.

To analyse the immune response occurring and metabolic profile of various tissues mice need to be sacrificed.

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

DEC-advies

A. Algemene gegevens over de procedure

1. Aanvraagnummer: AVD116002015253
2. Titel van het project: Metabolic control of dendritic cell-driven t cell polarization: dissecting underlying signals.
3. Titel van de NTS: Het manipuleren van de stofwisseling van verkennercellen om het afweersysteem te sturen.
4. Type aanvraag:
   - ✓ nieuwe aanvraag projectvergunning
   - ☐ wijziging van vergunning met nummer
5. Contactgegevens DEC:
   - naam DEC: DEC Leiden
   - telefoonnummer contactpersoon: [redacted]
   - mailadres contactpersoon: pdc-lumc-dierexperimentencommissie@lumc.nl
6. Adviestraject (data dd-mm-jjjj):
   - ✓ ontvangen door DEC: 06-08-2015
   - ✓ aanvraag compleet:24-08-2015
   - ✓ in vergadering besproken: 13-08-2015 & 03-09-2015
   - ☐ anderszins behandeld: n.v.t.
   - ✓ termijnonderbreking(en) van 18-08-2015 tot 24-08-2015
   - ☐ besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen
   - ✓ aanpassing aanvraag: 24-08-2015
   - ✓ advies aan CCD: 18-09-2015, na overleg met vergunninghouder
7. Eventueel horen van aanvrager
   - N.v.t.
8. Correspondentie met de aanvrager
   - Datum vragen: 18-08-2015
   - Datum antwoord:24-08-2015
   - Strekking van de vragen: De DEC heeft bij de aanvrager aanvullende

informatie ingewonnen met betrekking tot de niet technische samenvatting. De informatie in de NTS was in sommige opzichten te technische geschreven. Door de DEC is gevraagd dit aan te passen en de NTS op een aantal punten uit te breiden.

Bij de projectaanvraag spitste de discussie in de DEC zich primair op het ongerief, de groepsgrootte en de humane eindpunten in de diverse appendices. Naar aanleiding van deze vragen en suggesties is het projectvoorstel inclusief de NTS naar tevredenheid door de aanvrager aangepast.

9. Eventuele adviezen door experts (niet lid van de DEC)
   - Aard expertise: proefdiergeneeskundige praktijk
   - Deskundigheid expert: proefdierkundig adviseur met uitgebreide veterinaire ervaring.
   - Datum verzoek: 04-08-2015
   - Datum expert advies: 13-08-2015
   - Strekking van het verzoek: De DEC heeft gevraagd of het gekozen footpad model de beste keuze is voor het verkrijgen van resultaten uit de popliteus lymfeklier gezien het bijkomende ongerief. De expert heeft bevestigd dat dit een bekend model is en voor het betreffende experiment een logische keuze.

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 over deze projectaanvraag te adviseren. De benodigde expertise op dit wetenschappelijke terrein is aanwezig binnen de DEC.
4. Vanwege betrokkenheid bij het betreffende project is een aantal DEC-leden, met het oog op onafhankelijkheid en onpartijdigheid, niet betrokken bij de advisering: Geen van de DEC leden is betrokken bij het betreffende project.

C. Beoordeling (inhoud):

1. Het project is:
   - ✓ uit wetenschappelijk oogpunt verantwoord
   - □ uit onderwijskundig oogpunt verantwoord
   - □ uit het oogpunt van productiedoeleinden verantwoord
   - □ wettelijk vereist
2. De in de aanvraag aangekruiste doelcategorie is in overeenstemming met de hoofddoelstelling.
3. Het primaire doel van de projectaanvraag is om de invloed van het metabolisme van dendritische cellen en de daaruit volgende polarisatie van de immuunrespons i.e. de activatie op een Th2/Treg respons of een Th1/cytotoxic respons, te onderzoeken. De DEC onderschrijft het wetenschappelijk belang van deze projectaanvraag en erkent dat *in vivo* studies noodzakelijk zijn om de onderzoeksvraag te beantwoorden. Naast een wetenschappelijk belang, ziet de DEC ook een mogelijk toekomstige klinische relevantie van het onderzoek in deze projectaanvraag. De DEC erkent het essentieel belang van dit onderzoek om het metabolisme in DC's te detecteren.
4. Naar de overtuiging van de DEC beschikt de aanvrager over voldoende expertise en voorzieningen om de projectdoelstelling met de gekozen strategie binnen de gevraagde termijn te realiseren. De benadering en uitwerking van de proefopzet met de verschillende diermodellen is goed onderbouwd met diverse go/no-go momenten die onnodig gebruik van dieren voorkomen.
5. Alle dieren worden gefokt bij een geregistreerd fokbedrijf voor het gebruik in dierproeven, er is geen sprake van afwijkende huisvesting en/of hergebruik. Er is geen sprake van bedreigde diersoorten, niet-menselijke primaten, zwerfdieren en/of dieren uit het wild. De toegepaste methoden voor anesthesie en euthanasie zijn conform de Richtlijn.
6. De Commissie heeft geconstateerd dat de onderzoeker zich houdt aan de "Code of Practice Dierproeven in het Kankeronderzoek" als basis voor de humane eindpunten. Het ongerief als gevolg van de dierproeven is realistisch ingeschat en geclassificeerd

7. In het project wordt de keuze voor het diermodel duidelijk onderbouwd. De betrokken dieren en diermodellen zijn het meest geschikt voor deze studieopzet. De desbetreffende dierproef berokkent de dieren het minste pijn, lijden, angst of blijvende schade. De DEC is ervan overtuigd dat voor zover mogelijk *in vitro* studies worden uitgevoerd maar dat er voor de vervolgvraagstellingen geen alternatieven beschikbaar zijn dan het voorgestelde gebruik van intacte dieren om de doelstelling van dit project te realiseren.
8. In het project wordt optimaal tegemoet gekomen aan de vereisten van vermindering van dierproeven. De onderzoeksgroep heeft jarenlange ervaring met dit soort experimenten en door het gebruik van duidelijke go/no-go momenten wordt per experiment het minimum benodigde aantal dieren ingezet. Technieken en procedures worden zorgvuldig toegepast. Dit alles is gebaseerd op jarenlange ervaring. Het maximale aantal te gebruiken dieren is realistisch geschat.
9. De uitvoering van het project is in overeenstemming met de vereisten van verfijning van dierproeven en is zo opgezet dat de dierproeven met zo min mogelijk ongerief worden uitgevoerd. Bij de opzet van dit onderzoek wordt rekening gehouden met dierenwelzijn door het gebruik van adequate anesthesie waar nodig, het gebruik van genetische gemodificeerde dieren waarin selectief dendritische cellen bepaalde afwijkingen in hun stofwisseling hebben en het gebruik van een gemuteerde parasiet en bacterie bij het Malaria en listeria infectie model waar de dieren niet of minder ziek van worden, zodat het meeste ongerief wordt voorkomen.

   De DEC is ervan overtuigd dat de beschreven dierproeven zo humaan mogelijk zullen worden uitgevoerd.
10. De niet-technische samenvatting is een evenwichtige weergave van het project en begrijpelijk geformuleerd. De NTS voldoet daarmee aan de eisen zoals gesteld in artikel 10.a.1.7 van de Wod.

D. Ethische afweging

Het fundamenteel wetenschappelijke onderzoek in dit project is van essentieel belang. Bij het uitvoeren van de dierproeven wordt een adequate invulling gegeven aan de vereisten op het gebied van de vervanging, vermindering en

verfijning van de dierproeven. De DEC is van mening dat de resultaten zullen bijdragen aan het verkrijgen van meer kennis over of in levende dieren de stofwisselingseigenschappen van dendritische cellen bepalen welke afweerrespons er door deze cellen wordt geactiveerd. De DEC acht het belang van de fundamenteel wetenschappelijke doelstelling essentieel en schat de kans op het realiseren van de doelstellingen in als hoog. De verkregen fundamenteel wetenschappelijke kennis kan vervolgens als basis dienen voor de ontwikkeling van nieuwe immuuntherapieën waarbij, door het manipuleren van de stofwisseling in dendritische cellen, bepaalde ziektes voorkomen of mogelijk zelfs genezen kunnen worden. De onderzoeksgroep beschikt over een ruime ervaring met de gekozen onderzoeksstrategie en met de voorgestelde typen dierproeven. De DEC onderschrijft dat de doelstellingen niet zonder het gebruik van proefdieren kunnen worden behaald en acht het gebruik van het aantal dieren en het daarmee samenhangende gering tot ernstig ongerief bij de dieren gerechtvaardigd.

E. Advies

1. Advies aan de CCD
   - De DEC adviseert de vergunning niet te verlenen vanwege:
     - De vaststelling dat het project niet vergunningplichtig is
     - De volgende doorslaggevende ethische bezwaren
     - De volgende tekortkomingen in de aanvraag
   - De DEC adviseert de vergunning te verlenen onder de volgende voorwaarden
     - Op grond van het wettelijk vereiste dient de projectleider bij beëindiging van het project een beoordeling achteraf aan te leveren die is afgestemd met de IvD.
     - Voor de uitvoering van dit project is tevens een ministeriële ontheffing vereist
     - Overige door de DEC aan de uitvoering verbonden voorwaarden
   - ✓ **De DEC adviseert de vergunning te verlenen**
2. Het uitgebrachte advies is gebaseerd op consensus.

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Academisch Ziekenhuis Leiden
Dhr. Leiden Universitair Medisch Centrum
Postbus 9600
2300 RC LEIDEN

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
AVD116002015253

Bijlagen
2

Datum 2 oktober 2015
Betreft Ontvangstbevestiging Aanvraag projectvergunning Dierproeven

Geachte heer Leiden Universitair Medisch Centrum,
Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 1 oktober 2015.
Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD116002015253. Gebruik dit nummer wanneer u contact met de CCD opneemt.

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

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

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

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlagen:
- Gegevens aanvraagformulier
- Factuur

Gegevens aanvrager

Uw gegevens	
Deelnemersnummer NVWA:	11600
Naam instelling of organisatie:	Academisch Ziekenhuis Leiden
Naam portefeuillehouder of diens gemachtigde:	Dhr. Leiden Universitair Medisch Centrum
KvK-nummer:	27366422
Straat en huisnummer:	Albinusdree 2
Postbus:	9600
Postcode en plaats:	2300 RC LEIDEN
IBAN:	NLIIDEUT0451001400
Tenaamstelling van het rekeningnummer:	LUMC

Gegevens verantwoordelijke onderzoeker	
Naam:	[redacted]
Functie:	Projectleider/Senior Researcher
Afdeling:	[redacted]
Telefoonnummer:	[redacted]
E-mailadres:	[redacted]

Over uw aanvraag

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

Over uw project

Geplande startdatum:	1 november 2015
Geplande einddatum:	1 november 2020
Titel project:	Metabolic control of dendritic cell-driven t cell polarization: dissecting underlying signals
Titel niet-technische samenvatting:	Het manipuleren van de stofwisseling van verkennercellen om het technische samenvatting? ' afweersysteem to sturen
Naam DEC:	DEC Leiden
Postadres DEC:	Secretariaat DEC Leiden Postzone: T7-P, LUMC Postbus 9600 2300 RC Leiden
E-mailadres DEC:	pdc-lumc-dierexperimentencommissie@lumc.nl

Betaalgegevens

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

Checklist bijlagen

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

Ondertekening

Naam:	[redacted]
Functie:	Gemandateerd vergunninghouder
Plaats:	Leiden
Datum:	29 september 2015

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

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info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD116002015253

Bijlagen
2

Datum 2 oktober 2015
Betreft Factuur aanvraag projectvergunning Dierproeven

Factuur
Factuurdatum: 2 oktober 2015
Vervaldatum: 1 november 2015
Factuurnummer: 15700253

Omschrijving	**Bedrag**
Betaling leges projectvergunning dierproeven Betreft aanvraag AVD116002015253	€ 741,00

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

[redacted]

Van: pdc-lumc-dierexperimentencommissie@lumc.nl
Verzonden: vrijdag 16 oktober 2015 15:10
Aan: info@zbo-ccd.nl
Onderwerp: RE: AVD116002015253

Categorieën: Dossier: [redacted]

Beste [redacted]

Bedankt voor de vragen. De DEC zou hier als volgt op willen reageren:

1) - Topical application: in verband met de nauwkeurigheid (hoeveelheid + locatie) van de applicatie is het van belang dat deze niet wordt gecompromitteerd door beweging van de muis. Ook het feit dat de oplossing enige tijd nodig heeft om in te trekken betekent dat de muis voor langere tijd gefixeerd moet worden. De DEC acht het ongerief als gevolg van een kort durende sedatie vergelijkbaar met het ongerief als gevolg van de langere fixatie en is van mening dat de hogere reproduceerbaarheid van het experiment door het toepassen van isofluraan, om zo verzekerd te zijn van een goede, consistente en nauwkeurige applicatie opweegt tegen het extra ongerief dat dit oplevert.
   - i.v. injectie: de betreffende onderzoeker doet de iv injecties altijd retro-orbital onder korte isofluraan sedatie. Dit gaat zeer snel en
   heeft een zeer hoge successrate. De DEC is van mening dat deze techniek niet meer ongerief met zich meebrengt dan wanneer bij
   i.v. injectie via de staartvene de muis verwarmd moet worden en in een restrainer gedwongen wordt.
   - wangprik: dit is een verschrijving van de onderzoeker geweest die helaas niet is opgemerkt tijdens de vergadering, maar waarvan
   de DEC meent dat deze zonder narcose dient te geschieden.

2) De DEC is het met u eens dat er voor de controlegroepen geen extra dieren nodig zijn in verband met uitval. De onderzoeker heeft aangegeven dat er voor de controle groepen geen extra dieren gebruikt zullen worden.

Met vriendelijke groet,

[redacted]

[redacted]
Secretaris DEC/IvD
[redacted]
Postzone: T7-P
Postbus 9600
2300 RC Leiden
Tel.: [redacted]
Aanwezig: di, do & vr (halve dag)

From: Info-zbo [mailto:info@zbo-ccd.nl]
Sent: dinsdag 13 oktober 2015 16:56
To: [redacted]
Cc: Info-zbo
Subject: AVD116002015253

Beste DEC,
Onlangs ontvingen wij uw advies betreffende aanvraag AVD116002015253, Metabolic control of dendritic cell-driven t cell polarization: dissecting underlying signals.
Onze dank hiervoor. Na bekijken van deze aanvraag hebben wij nog enkele vragen.

1) Enkele handelingen met de dieren worden uitgevoerd onder anesthesie, terwijl dit bij deze handelingen niet altijd gebruikelijk is om dit onder anesthesie uit te voeren, en anesthesie mogelijk meer i.p.v. minder ongerief zal opleveren. Het gaat hierbij om handelingen als: topical application of FITC (bijlage 3), i.v.

injectie (bijlage 6) en wangprik (bijlage 6 en 7). Is deze anesthesie besproken in uw DEC? Kunt u uw mening hierover aan ons melden?

2) In bijlage 4, 5, 8 en 9, worden extra dieren aangevraagd vanwege “worms will not fully develop”, “10-20% is not properly infected”, “80% of animals induce asthma” en “non-responders to HFD”. We begrijpen deze extra dieren in de behandelde groepen. In de beschrijving worden echter deze extra dieren ook aangevraagd in de controlegroepen (die dus geen behandeling ondergaan). Dit lijkt ons overbodig, aangezien de controlegroepen dus geen uitvallers zullen hebben vanwege de beschreven redenen. Kunt u hier de mening van de DEC over geven?

Kunt u z.s.m. en wel uiterlijk maandag 19 oktober antwoorden op deze vragen aan ons sturen?

Vanwege problemen met onze mailbox: kunt u a.u.b. een bevestiging sturen dat u deze e-mail hebt ontvangen?

Met vriendelijke groet,

[redacted]

Centrale Commissie Dierproeven **www.centralecommissiedierproeven.nl**
Postbus 20401 | 2500 EK | Den Haag
Let op: vanaf nu heeft de CCD een nieuw e-mailadres info@zbo-ccd.nl. Heeft u ons oude e-mail adres in uw adressenboek, dan vragen we u om dat aan te passen.

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Centrale Commissie Dierproeven

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Centrale Commissie Dierproeven

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Onze referentie
Aanvraagnummer
AVD116002015253

Uw referentie
uw ref

Bijlagen
1

Datum 26 oktober 2015
Betreft Aanvulling Aanvraag projectvergunning dierproeven

Geachte [redacted]

Op 01 oktober 2015 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project “Metabolic control of dendritic cell-driven t cell polarization: dissecting underlying signals” met aanvraagnummer AVD116002015253. In uw aanvraag zitten voor ons nog enkele onduidelijkheden. In deze brief leest u wat wij nog nodig hebben en wanneer u een beslissing kunt verwachten.

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

Projectvoorstel
Wij hebben contact opgenomen met de DEC over enkele onduidelijkheden in uw aanvraag. Op basis van dit contact met de DEC willen wij u vragen uw projectvoorstel op enkele punten aan te passen:
- U beschrijft dat u de wangprik uitvoert onder anesthesie. Deze handeling dient echter, naar onze mening en de mening van de DEC zonder narcose te geschieden. Graag dit aanpassen in de aanvraag.
- In bijlagen 4, 5, 8 en 9 worden extra dieren aangevraagd vanwege “worms will not fully develop”, “10-20% is not properly infected”, “80% of animals induce asthma” en “non-responders to HFD”. We begrijpen deze extra dieren in de behandelde groepen. In de beschrijving worden echter deze extra dieren ook aangevraagd in de controlegroepen (die dus geen behandeling ondergaan). Dit lijkt ons overbodig, aangezien de controlegroepen dus geen uitvallers zullen hebben vanwege de beschreven redenen. De DEC geeft aan dat de onderzoeker heeft aangegeven dat er voor de controle groepen geen extra dieren gebruikt zullen worden. Graag dit aanpassen in de aanvraag.

Verder maken wij u erop attent dat in de Code of Practice voor het immuniseren van proefdieren is opgenomen dat dieren die geïmmuniseerd zijn in de voetzool moeten worden gehouden in kooien met extra dikke bedding.

Niet technische samenvatting
Graag ontvangen wij een Niet technische samenvatting waarin bovengenoemde wijzigingen in het projectvoorstel zijn doorgevoerd.

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. Na ontvangst van uw reactie op de in deze brief gestelde vragen, zullen wij uw aanvraag verder inhoudelijk beoordelen.

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

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlage:
- formulier Melding Bijlagen via de post

Centrale Commissie Dierproeven

Melding

Bijlagen via de post

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

1 Uw gegevens

1.1 Vul de gegevens in.

Naam aanvrager			
Postcode		Huisnummer	

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

Aanvraagnummer	

2 Bijlagen

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

☐	
☐	
☐	

3 Ondertekening

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

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

Naam	
Datum	- - 20
Handtekening	

Leids Universitair Medisch Centrum

department Instantie voor Dierenwelzijn
postal zone PDC, T7-9

sender [redacted], DVM
visiting address Einthovenweg 20, 2333 ZC Leiden
phone [redacted]
e-mail PDC-LUMC-IvD@lumc.nl

to **Centrale Commissie Dierproeven**

Postbus 20401
2500 EK Den Haag

our reference 15013
your reference Aanvraagnummer AVD116002015253
date November 7, 2015
subject Ontbrekende informatie aanvraag projectvergunning dierproeven AVD116002015253 .

Geachte Centrale Commissie Dierproeven,

In antwoord op uw vragen in de brief aangaande de aanvulling projectvergunning dierproeven met aanvraagnummer AVD116002015253 is het projectvoorstel en de bijbehorende niet-technische samenvatting op door u genoemde punten aangepast.

Echter, bij het corrigeren van de bijlagen aangaande de aantallen dieren benodigd voor de controlegroepen is gebleken dat er een fout in bijlage 8 van het projectvoorstel staat. In deze bijlage is de correctie voor het niet aanslaan voor de behandeling wél beschreven in de vraag over de statistische methoden maar niet in de uiteindelijke berekening opgenomen. Dit punt is tevens in het projectvoorstel aangepast en daarmee is het maximaal benodigde aantal dieren 360 voor bijlage 8.

Het maximaal aantal benodigde dieren voor het volledige project omvat nu 4016 in plaats van de eerder vermelde 4100.

Namens de instellingsvergunninghouder LUMC,

[redacted]

Voorzitter Instantie voor Dierenwelzijn LUMC

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'.	11600
1.2	Provide the name of the licenced establishment.	Leids Universitair Medisch Centrum
1.3	Provide the title of the project.	Metabolic control of dendritic cell-driven t cell polarization: dissecting underlying signals

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

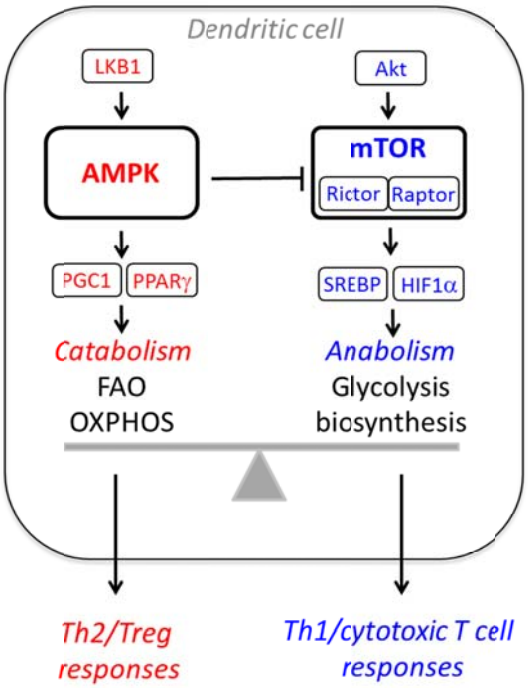
Describe the project (motivation, background and context) with respect to the categories selected in 2.

- 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.

Dendritic cells (DCs) are key regulators of both immunity and tolerance by controlling activation and polarization of effector T helper cells (Th) and regulatory T cell (Treg) responses. Therefore, there is a major focus on developing approaches to manipulate DC function for immunotherapy. It is becoming

increasingly clear that cellular metabolism dictates the fate and function of immune cells. However, still little is known about the metabolic pathways that regulate DC function and shape their immune-polarizing properties.
Recently published and preliminary data indicate that strongly immunogenic DCs that prime Th1 and cytotoxic CD8 T cell responses are metabolically characterized by a switch to anabolic metabolism fuelled by glycolysis, whereas Th2- or Treg-polarizing tolerogenic DCs display catabolic metabolism dependent on mitochondrial fatty acid oxidation (FAO) and oxidative phosphorylation (OXPHOS), and that these metabolic states are required for their immune-polarizing capacities (see figure 1). However which signalling pathways are important for the regulation of these metabolic properties of DCs and thereby their immunepolarizing function in vitro and in vivo is still unclear. In other cell types mTOR and AMPK, as major sensors of nutrient availability and bioenergetics of a cell, have been shown be crucial regulators of anabolic and catabolic metabolism, respectively. We therefore hypothesize that the balance between mTOR and AMPK signalling determines the T cell-polarizing properties of DCs through control of the balance between anabolic and catabolic metabolism in these cells (see figure 1). Thus, we predict that strong AMPK and/or low mTOR activation favours DCs to promote Th2/Treg responses, whereas tipping the balance in the opposite direction licences DCs to effectively prime Th1 and cytotoxic CD8 T cell responses.

Figure 1: Role of AMPK/mTOR in DC metabolism?



3.2 Purpose

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

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

The main objective of the current project is to define the role of the mTOR and AMPK signalling axis in controlling DC metabolism and thereby the immune polarizing properties of these cells in vitro and in vivo. The main researcher has ample experience with and expertise in the biology and metabolism of DCs and has access to various transgenic mouse strains to study AMPK and mTOR signalling in DCs in vivo. Moreover, the department has the required technology available to study cellular metabolism and has expertise with the different in vivo models that will be used to address this main aim. This makes this

project highly feasible and as such we anticipate that the proposed timeframe to address the main objective is realistic.

3.3 Relevance

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

The field of immunometabolism is a novel and very rapidly developing research area. Most of our understanding in this area has been gained from studies with T cells and macrophages. However, still little is known about the metabolic pathways that support DC activation or their immune-polarizing functions. The current proposal aims to fill this gap. Manipulation of metabolism in DCs to promote or inhibit immunity is a novel concept and it is to be expected that there is great practical significance in understanding how metabolism is linked to function in DCs. There is a major interest in the use of DCs as targets for immune-intervention and for vaccine strategies, because of their powerful immune stimulatory as well as regulatory functions. The few studies that have been performed on DC metabolism so far have mostly used in vitro models. However, in vitro models to study cellular metabolism lack the metabolic complexity of situations in vivo, where in contrast to in vitro systems, nutrient availability and oxygen tension may be rapidly fluctuating or limiting. Therefore, since cellular metabolism is highly dependent on the nutrient availability in the microenvironment DCs reside in, it is of crucial importance to also understand how metabolism of DCs shapes their function in tissues in vivo. Hence, the results from the current proposal in which both in vitro and in vivo models are used, will not only provide novel insights in the basic understanding of the molecular mechanisms through which DCs in vivo become conditioned to prime different classes of immune responses that will be of interest to immunologists, but will also lead to the identification of metabolic regulators in DCs that could potentially be targeted to manipulate the biology of these cells in situ to our advantage for therapeutic purposes in humans. For instance promoting the development of tolerogenic DCs has great potential as a therapeutic approach in settings where unwanted effector T cell responses need to be controlled, such as following transplantation to prevent rejection, in auto-immune disorders or allergies (of which the latter will be studied in this project). Conversely, rendering DCs more immunogenic to promote more robust cellular and humoral immunity is central for improving DC-based vaccination efficacy to potentially treat certain infectious diseases and cancers, such as melanoma. In this respect it is important to note that several drugs targeting metabolic regulators (for example: rapamycin targeting mTOR and metformin targeting AMPK), are currently, albeit for different purposes, already used in the clinic. We therefore anticipate that findings from this project can readily be translated into clinically relevant settings such as DC-based immunotherapy. While others are testing the effects of different cytokines, adjuvants and antigens in improving DC-based immunotherapies, this project aims to contribute to this field from a unique angle by identifying metabolic signaling pathways in DCs that can be manipulated to regulate their function.

3.4 Research strategy

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

To assess the role of the AMPK and mTOR signalling axes in the regulation of DC metabolism and thereby their immunepolarizing function, the central strategy will be to test well-defined in vitro and in vivo models of DC-dependent Th2/Treg and Th1/Cytotoxic T cell immune polarization in different mouse strains with DC-specific deletions of key components within those signaling pathways.

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.

Part 1) As a first step elucidate the role of the AMPK and mTOR signalling axes in the regulation of DC metabolism and thereby their immunepolarizing function, AMPK and mTOR signalling and their downstream targets/transcription factors will be pharmacologically or genetically promoted or inhibited in DC cultured from bone marrow or isolated from naive WT mice in which the DC populations have been expanded in vivo using following inoculation with a Flt3-L expressing tumour. These cells will be characterized metabolically and will be analysed for their ability to prime and polarize t cell responses in vitro in a T cell coculture assay. Not only mTOR and AMPK itself but also its downstream targets such as transcription factors PGC1 (master regulators of mitochondrial biogenesis and metabolism that is activated by AMPK and inhibited by mTOR), HIF1a (a key regulator of glycolysis downstream of mTOR) and SREBP (regulator of Fatty acid synthesis downstream of mTOR) will be targeted to assess what

factors downstream of AMPK/mTOR signalling control DC metabolism and function (see figure 1).

In parallel we will generate mice with DC-specific deletions of key components within the AMPK and mTOR signalling pathways. To this end, mice expressing cre recombinase under the control of the DC-specific marker CD11c, will be crossed to mice with floxed alleles of components within the AMPK and mTOR signalling pathways. In this respect mouse strain are available with floxed genes encoding Raptor and rictor (key proteins of the mTORC1 and 2 complexes, respectively) and LKB1 (a central activator of AMPK) and AMPK (see figure 1). In addition, if data from the WT mice as described above reveal that particular targets downstream of AMPK/mTOR signalling are important in controlling DC driven T cell polarization, CD11c-cre mice will be crossed to mice harbouring floxed genes of these targets. Since most of these cre-lox combinations has never been crossed before, DC frequency and numbers and surface markers expression will be assessed in naïve transgenic mice to determine whether these genes are important in DC homeostasis in naïve mice. Next, the DCs of these transgenic mice will generated as described above and characterized in vitro for 1) absence of expression of targeted genes 2) change in metabolism and 3) T cell-polarizing capacity in vitro.

animal experiments involved (see figure 2):
#1 Generation of DCs for in vitro T cell-coculture assays

Next, to test whether AMPK and mTOR signalling axes are also important in the regulation of DC metabolism and thereby their immunepolarizing function in vivo, the mutant mice strains will be used in well-defined in vivo models of DC-driven T cell polarization

Part 2) First the mutant mice will be analysed for their capacity to generate a local polarized immune response by different immune-polarizing antigens/adjuvants in a model of subcutaneous immunization. For this purpose antigens/adjuvants will be used that are known to promote Th2/Treg, Th1 or cytotoxic CD8 T cell responses in these setting. If the transgenic mice with DC-specific deletions of AMPK/mTOR signalling molecules show an altered immunepriming/polarizing capacity, the migratory ability of the Skin DCs will be determined using a FITC-painting model, to be able to distinguish whether the immune-phenotype is due to an aberrant capacity of the DCs to migrate or a consequence of altered DC-T cell communication.

animal experiments involved (see figure 2):
#2 subcutaneous immunization model
#3 FITC-painting DC migration model

Part 3) the roles of AMPK and mTOR signalling axes in DC-mediated immunepolarization during systemic infection will be determined by using well-studied infection models of the parasitic helminth schistosoma , malaria and Listeria, which are each characterized by a unique DC-mediated immunepolarization profile: i.e. Th2/Treg responses by schistosoma infection, Cytotoxic CD8 T cell responses by Malaria infection and Th1 responses by Listeria infection. Since these immune responses are critical in controlling pathogen burden and infection-induced pathology, these parameters will be assessed as well to address whether potential effects on immunepolarization translate into changes in immunopathology and/or resistance to infection.

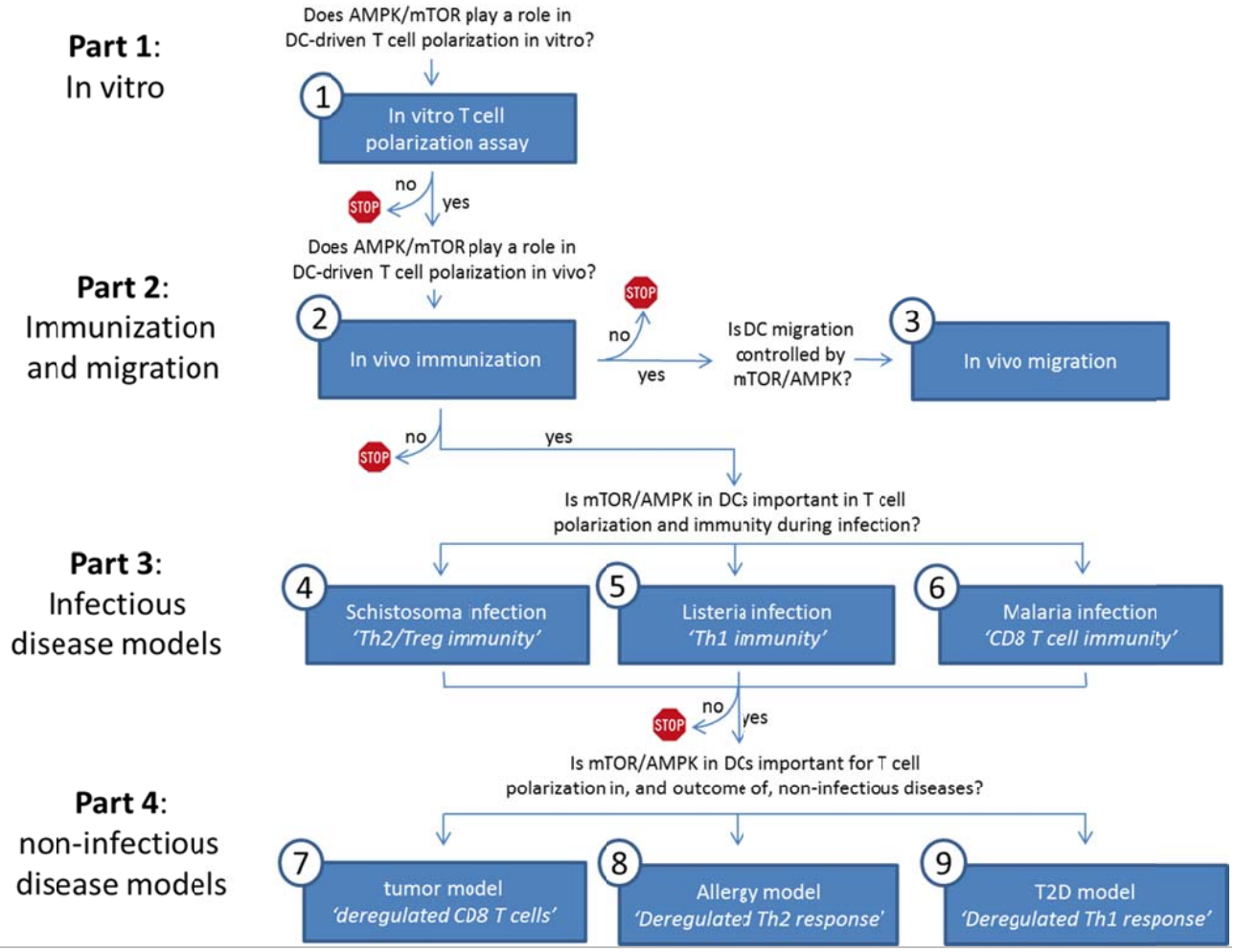
animal experiments involved (see figure 2):
#4 schistosoma mansoni infection model
#5 Malaria infection model
#6 Listeria monocytogenes infection model

Part 4) Finally, the effects of DC-specific perturbations in mTOR/AMPK signalling in immune polarization and disease outcome will be assessed in models of allergic asthma, tumour growth and high fat diet induced Type 2 diabetes. These type of diseases that have seen a rapid increase in prevalence in the western world in recent decades and are each characterized by a distinct form of immune-deregulation (ie deregulated Th2/Treg in the case of Allergic asthma, suppressed Th1/cytotoxic T cell responses in the case of tumour development and a switch from Th2/Treg to Th1/cytotoxic T cell responses in metabolic

tissues in High fat diet (HFD)-induced Type 2 diabetes). These models will provide key mechanistic insights into how DC metabolism regulates immunepolarization and thereby disease outcome. Moreover, findings from these studies can lead to the identification of druggable targets in metabolic signalling pathways in DCs that can be used to develop novel therapeutic approaches to treat these highly prevalent diseases.

animal experiments involved (see figure 2):
#7 Melanoma tumor model
#8 Allergic Asthma model
#9 High fat diet-induced Type II Diabetes model

Figure 2: outline research strategy



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.

1) The project will start with the metabolic characterization of, and analysis of the T cell polarizing capacity of DCs cultured or isolated from WT mice in which mTOR and AMPK signalling axes will be pharmacologically or genetically inhibited or promoted. These data will be compared with data obtained from DCs isolated from transgenic mice with DC-specific deletions of key components within the AMPK and mTOR signalling pathways.

- Milestone (1): established whether the AMPK and mTOR signalling axes control DC metabolism and thereby T cell polarizing capacity in vitro

- Go / no-Go moments (1): If this part shows no efficient deletion of the targeted genes and/or no effect

of manipulation of AMPK/mTOR signalling axis on T cell polarization in vitro, part 2-4 will not be performed.

2) Next, using the mutant mice, subcutaneous immunization will be performed, which is a simple, yet powerful, in vivo model for a first assessment of whether local immune priming and polarization by DCs is regulated by AMPK/mTOR signalling in these cells. In addition, to be able to determine whether any immune-priming/polarizing phenotype is due to an aberrant capacity of the DCs to migrate a FITC painting model will be used.

- Milestone (2): established whether DC-AMPK/mTOR regulates immune polarization in subcutaneous immunization model and migration of skin DCs.

- Go / no-Go moments (2): If the subcutaneous immunization model (part 2) shows that there is no immunological phenotype in mice that harbour DC- specific deletions in AMPK or mTOR signalling pathway, part 3 and 4 will not be performed

3) When these experiments reveal there is a defect in immune priming and/or polarization when DCs have defects in AMPK/mTOR signalling, the next phase of this project will focus on addressing whether these immunological observations can be extrapolated to models of highly prevalent systemic infectious diseases: schistosomiasis, malaria and listeria. Not only are these infection models chosen for their clinical relevance but also because each of these infections are well-known to elicit unique highly polarized immune responses; Th2/Treg responses in the case of schistosomiasis, primarily cytotoxic T cell responses in the case of malaria and strong Th1 responses in response to listeria infection. Each of these specific responses are essential for protection against these infections. This makes them ideal immunological models to test our hypothesis in the context of systemic infections as well as what it means for disease outcome.

- Milestone (3): Defined the role of DC-AMPK/mTOR in immune polarization, pathology and protection against, schistosomiasis, malaria and Listeria.

- Go / no-Go moments (3):If the infection models show that there is no immunological phenotype in mice that harbour DC-specific deletions in AMPK or mTOR signalling pathway part 4 will not be performed

4) The final part of this project aims to define whether the balance in mTOR/AMPK signalling in DCs is also an important determinant of immune polarization and thereby disease outcome in models of highly prevalent non-communicable western diseases: allergic asthma, melanoma tumour growth and high fat diet (HFD)-induced Type II Diabetes. The immunological basis for each of these diseases is different as they have have been shown to be caused by an aberrant Th2 response, a defective Th1/cytotoxic T cell response, and a switch from Th2/Treg to Th2/cytotoxic T cell responses, respectively, which we hypothesize will all be controlled by the balance in AMPK/mTOR signalling in DCs. The use of these non-infectious disease models is also highly relevant for a different reason, since these models in conjunction with the aforementioned infection and immunization models enable one to assess whether our hypothesis is generalizable to both non-infectious and infectious diseases or only hold true for certain settings.

- Milestone (4): Defined the role of DC-AMPK/mTOR in immune polarization and disease progression during allergic asthma, tumour development and HFD-induced type II diabetes

Taken together, these distinct infectious and non-infectious models form the basis for a coherent set of targeted experiments to address the main objective of this project, of which the results may contribute to the design of metabolism based approaches to manipulate DCs function in clinical settings to potentially help to boost immunity against infectious diseases or tumours as well as to treat non-communicable inflammatory diseases such as allergies and HFD-driven Type II diabetes.

General Go/no-Go moments: If, for example, in part 1 or 2 only mTOR signalling, but not AMPK, in DCs appears to play a role in regulating immunepolarization, mice with defects in DC-specific defects in AMPK signalling will not be studied in part 3 and 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	DC generation for T cell priming/polarizarition assay in vitro
2	subcutaneous immunization
3	FITC-painting
4	Model of Schistosoma mansoni infection
5	Model of Malaria infection
6	Model of Listeria infection
7	Melanoma Tumor model
8	Model of allergic asthma
9	Model of High fat diet-induced type II diabetes
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'.	11600
1.2	Provide the name of the licenced establishment.	Leids Universitair Medisch Centrum
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 1 — Type of animal procedure: DC generation for in vitro T cell polarization assay

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.

To study whether AMPK and mTOR control DC metabolism and thereby their immune polarizing properties, we will first use an in vitro DC-T cell coculture model. To this end murine DCs will be differentiated from bone marrow from WT naïve mice and the AMPK/mTOR signalling pathways will be manipulated pharmacologically or genetically and after which the DCs will be exposed to various pathogen-derived immune-polarizing compounds. Next, their metabolic properties will be studies as well as their capacity to prime and polarize T cell responses in a coculture system with CD8 and CD4 T cells. In addition, we will take advantage of transgenic mice that have CD11c+ DC-specific deletions in genes involved in AMPK and mTOR signalling. CD11c+ DCs will be isolated from spleens from these animals. However, since DCs are a scarce cell population, we aim to significantly increase the yield of DCs by pre-inoculating these mice with a FLT3L-expressing tumour cell line, that results in a strong expansion of DCs populations (from 1% in spleen to up to 40% in spleen). These DCs will then be stimulated and signalling, metabolic pathway activation and T cell priming and polarizing capacity will be analysed.

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

Murine DCs will be generated from bone marrow in 8 day in vitro cultures in the presence of Flt3-L. To this end, bone-marrow will be extracted from femurs and tibia of naïve wild-type mice that are euthanized by an overdose Nembutal. To generate different T cell-polarizing DCs, bone marrow-derived DCs will be conditioned by well-known Th1-/cytotoxic T cell- l (Heat killed Lysteria), Th2- (SEA, Soluble Egg Antigens from the parasitic worm S. mansoni) or Treg-promoting (S. mansoni derived LysoPS) for 24 h in the presence of endotoxin free ovalbumin (OVA). During this period activity of the mTOR and AMPK signalling axes will be assessed (Flow cytometry, Western blot) as well as changes in their rates of

mitochondrial (catabolic) or glycolytic (anabolic) metabolism (metabolite tracing experiments, metabolic flux analysis).

The use of specific inhibitors or lentiviral transduction of small-hairpin (sh)RNA containing constructs allows for inhibition of these metabolic signalling pathways. Conversely, to promote activation of these metabolic signalling pathways key components of these pathways will be overexpressed using a similar transduction approach. Specifically, mTOR pathway can be manipulated using inhibitors or hairpins/overexpression constructs against mTOR (rapamycin/Torin), HIF1a - a transcription factor downstream of mTOR that controls glycolysis- (YC-1), or SREBP - key regulator of anabolic lipid metabolism. The AMPK signalling axis can te targeted using activators (merformin, AICAR) or inhibitors (dorsomorphin) of AMPK, or activators of PGC1 (resveratrol). LKB1, AMPK and PGC1 activity can also be manipulated genetically through a lentiviral transduction of hairpin or overexpression constructs. For this purpose, a genome-wide shRNA library is available at the LUMC.

To address the immunological consequences of manipulation of these pathways, DC phenotype will be assessed following the treatments by determining surface activation marker expression and cytokine production. To define the effects of manipulation of MAPK/mTOR signalling axes in DCs on their T cell priming and polarizing capacity, the DCs conditioned for 24 h will be cultured at various ratios with naive OVA-specific OT-II CD4+ T cells or OT-I CD8+ T cells labelled with CFSE (carboxyfluorescein diacetate succinimidyl ester). On day 3, T cell proliferation will be assessed by flow cytometry as dilution of CFSE. Cytokine production by T cells will be determined on day 6

In addition, we will take advantage of transgenic mice that have DC-specific deletions in genes involved in AMPK and mTOR signalling. The following C57BL/6 CD11c-cre ‘x’-flox strains will be created, where ‘x’ will be a gene involved in:
1) AMPK signalling directly (for instance LKB1 and AMPK1)
2) mTOR signalling directly (for instance Raptor and Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 and PPARg)
4) metabolic regulation downstream of mTOR signalling: (for instance HIF1a and SREBP)

Importantly, only genes present in the signalling pathways and downstream target genes that were identified using pharmacological and hairpin knockdown approaches in WT mice to play a role in metabolism and the T cell priming and polarizing capacity of DC (part 1), will be evaluated using CD11c-cre /flox mice.

These CD11c-cre+ mice as well as CD11cre- control mice will be injected intradermally (i.d.) in their back with FLT3L expressing B16 tumour cells (1x10e5 in 50ul) under isoflurane anaesthesia. Mice will be monitored for tumour growth every other day. After 10 days or when tumour size reaches 1 cm3 in diameter (before it may cause distress to the animals), the mice will be euthanized by i.p. injection of an overdose Nembutal and the expanded DC populations will be isolated from spleen and lymph nodes. These DCs will then be stimulated and signalling, metabolic pathway activation and T cell priming and polarizing capacity will be analysed in vitro 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.

To establish right inhibitor and stimulation concentrations and optimization of the use of shRNA to knockdown genes we expect to need 25 mice. Once these experimental conditions are optimized we need 5 WT, 5 OT-I and 5 OT-II mice to determine the metabolic requirements for T cell polarization. This is based on experience with these DC / T cell coculture assays ([redacted] et al, JEM, 2012; [redacted] et al, Nat imm, 2014) where have a standard deviation of around 20%. We hope to observe a 30% difference between the experimental groups, and we set the power at 80% and determine p-value of 0.05. With these parameters, the experiments need to be performed 5 times to be able to draw reliable conclusions. The same number of mice will be needed for the experiments with DCs isolated from conditional knockout mice and their respective controls following FLT3L -tumour challenge: 5 cre+ and 5 cre- mice with 5 OT-I and 5 OT-II

B. The animals

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

C57BL/6 WT mice n=30 (20 mice for setting up the experiments, 10 for metabolic and T cell polarization assays)
C57BL/6 OT-I mice n=20 (10 for coculture with WT DCs and 10 for coculture with CD11c-Cre+ and Cre- DCs)
C57BL/6 OT-II mice n=20 (10 for coculture with WT DCs and 10 for coculture with CD11c-Cre+ and Cre- DCs)
These mice will be purchased from Harlan and Jackson laboratories and used between 6-12 weeks of age.
Both male and females will be used.

C57BL/6 CD11c-cre+ / cre- 'x'-flox/flox, rederived in house, n=10 per strain (5 cre+ and 5 cre-) where 'x' will be a gene involved in:
1) AMPK signalling directly (for instance LKB1 or AMPK1)
2) mTOR signalling directly (for instance Raptor or Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 or PPARg)
4) metabolic regulation downstream of mTOR signalling: (for instance HIF1a or SREBP)

Of note, only genes present in the signalling pathways and downstream target genes that were identified using pharmacological and hairpin knockdown approaches in WT mice to play a role in metabolism and the T cell priming and polarizing capacity of DC (part 1), will be evaluated using CD11c-cre/flox mice, with a maximum of a total of 6 different target genes.

Breeding will be setup with homozygous flox breeding pairs of which one of the breeding pair is heterozygous for CD11c-cre, whereas the other will be CD11c-cre negative. Offspring should then be 50% CD11c-cre+ and 50% CD11c-cre negative. The CD11c-Cre negative littermates will be used as controls for CD11c-Cre+ mice in the same experiment

Total maximum number of mice: 30 WT + 20 OTI + 20 OTII + 6x10 cre/flox = 130 mice

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:
Research involving mice is essential for the proposed in vitro research, since this is the only model organism in which the genetic tools are available to study DC-specific deletions in AMPK/mTOR signalling cascades. Moreover, mice are needed for acquisition of sufficient numbers of tissue resident DCs, which are hard to come by from other sources. Finally, the use of murine DCs, as opposed to human DCs, enables one to more reliably predict whether in vitro findings can be extrapolated to murine in vivo models that are used in this project as well.
Reduction:
To reduce the number of mice needed, we will implant mice with a FLT3-L expressing tumour. This enables one to isolate up to 100x more DCs from lymph nodes and spleen than from untreated mice.

This way we can significantly reduce the number of mice needed to acquire the same number of DCs for in vitro studies. In addition, DC generated or isolated from a single mouse allow to test various experimental conditions in in vitro cultures. This would requires multiple mice if tested directly in vivo. Furthermore, littermate controls (CD11c-Cre+ and CD11c-Cre-) will be used to reduce experimental variation and thereby reduce the number of mice needed per experimental group. In addition, only genes present in the signalling pathways and downstream target genes that were identified using pharmacological and hairpin knockdown approaches in WT mice to play a role in metabolism and the T cell priming and polarizing capacity of DC (part 1), will be evaluated using CD11c-cre flox mice. Finally, experiments will be performed by highly skilled and experienced staff, which will reduce intra-experimental variation and as such will result in fewer animals that will be needed per experiment to obtain reliable and statistically significant data.
Refinement:
Subcutaneous injection of the FLT3-L expressing tumour cells will be performed under anaesthesia, which takes only a few minutes. If any of the tumour bearing animals develop any state described as humane endpoint in the next section, the animals will be humanely sacrificed. Finally, experiments will be performed by staff that are highly skilled and experienced with animal handling. As a result all procedures with the animals will be performed with the greatest care and minimal animal distress will be ensured.

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

For the mice bearing Flt3L-expressing tumours, a human endpoint is chosen when in the unlikely case tumour size reaches 2 cm3, when it physically interferes with normal behaviour, when the intradermally located tumour starts ulcerating or when rapid weight loss occurs (> 15% in 1-2 days). In addition, the animals will be observed on a daily basis and be humanely sacrificed in case the following defined end points are reached: visible pain (abnormal posture and/or movement), significant weight loss (>20%), abnormal behaviour (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanized.

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.

The research question that is being addressed with the current set of experiments is novel and has not be studied before. An extensive literature search has revealed that these trangenic mice have not been used before in this kind of experimental model.

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

☒ 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.

Before i.d. injection of Flt3L-expressing tumour cells, mice will be put under anaesthesia by using isoflurane (5%) mixed with oxygen. During the injection, mice will be maintained under anaesthesia using a mouth piece. To ensure anaesthesia has set in well, rear foot reflex will be tested. During the maintenance phase isoflurane dosage is reduced (to 2-3%) to prevent overdosing.

I. Other aspects compromising the welfare of the animals

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

development of a solid subcutaneous tumour beyond 2 cm3 may negatively affect the wellbeing of the mice

Explain why these effects may emerge.

the tumour may induce pain in the surrounding tissue or may physically interfere with free movement of the animal

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

the tumor will be established on the back of the mouse, to minimize interference with normal behaviour. In addition, the tumor only needs to grow a couple of millimeters to express sufficient FLt3L to significantly expand DC populations. Mice will be sacrificed for DC isolation well before humane endpoints are reached. Nonetheless, humane endpoints are in place as described below

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.

For mice carrying the FLT3L-expressing tumors, a human endpints is chosen when tumor size reaches 2 cm3, when it physically interferes with normal behaviour, when the intradermally located tumor starts ulcerating or when rapid weight loss (> 15% in 1-2 days). In addition, the animals will be observed on a daily basis and be humanely sacrificed in case the following defined end points are reached: visible pain (abnormal posture and/or movement), significant weight loss (>20%), abnormal behavior (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanised. .

Indicate the likely incidence.

Very unlikely

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').

anesthesia/recovery from anathesia: mild

development of Flt3-ligand expressing tumor: mild

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.

To extract Bone marrow or spleen and lymph nodes to isolate and culture DCs mice need to sacrificed.

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

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

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
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- 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'.	11600
1.2	Provide the name of the licenced establishment.	Leids Universitair Medisch Centrum
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 2 — Type of animal procedure: model of subcutaneous 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.

The well-established model of subcutaneous immunization with various immune polarizing agents will be used to assess whether mice that harbour DCs with defects in AMPK or mTOR signalling have an altered metabolism and immune response following immunization. In this model immune polarizing compounds will be injected into the hind footpad of mice, where locally DCs will be activated and subsequently be migrating to the draining lymph node to prime and polarize T cell responses. This makes this model a highly suitable to interrogate the role of AMPK/mTOR signalling axes in controlling DCs metabolism and in the DC-driven polarization of local immune responses in vivo.

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

For footpad injections, antigens that drive Th1 and cytotoxic T cell responses (LPS and Heat Killed Listeria monocytogenes) or Th2/Treg responses (schistsosome derived soluble egg antigens (SEA) and omega-1) will be administered subcutaneously (s.c.) in 30µL of volume into one hind footpad under isoflurane anaesthesia. In all these injections, ovalbumin (OVA) will be co-administered to be able to track te response of Ova-specific T cells. The other hind footpad will be injected with the same volume of PBS as control.

Group 1: SEA+OVA, s.c. footpad injection
Group 2: HKLM+OVA s.c. footpad injection
Group 3: omega-1+OVA, s.c. footpad injection
Group 4: LPS+OVA. s.c. Footpad injection
Group 5: OVA, s.c. footpad injection (protein control)

The following readouts will be performed on each group:

1) to assess the metabolic status of the cells in vivo, immunized mice will be injected i.p with a 250ul bolus of a fluorescently labelled glucose analog (2NBDG) or free fatty acid (BODIPY-C12) 2 days after immunization. 15 minutes later the mice will be euthanized by cervical dislocation, popliteal lymph nodes will be harvested and DC will be analysed for 2NBDG and BODPIY-C12 uptake by flowcytometry as a measure for glycolytic and oxidative metabolism, respectively.

2) For immunological readouts, The mice will be euthanized by an overdose Nembutal 7 days later, the popliteal lymph nodes will be collected and the immune response to the immunization will then be assessed by flow cytometry and ex vivo restimulation. In this experiment, we will monitor the induction of OVA-antigen specific regulatory and IL-10 producing cells as well as Th1, Th2 and Th17 immunity.

Per group the following number of mice will be needed:
a) metabolic analysis: day 2 (PBS control) n=3
b) metabolic analysis: day 2 (2NBDG) n=4
c) metabolic analysis: day 2 (Bodipy-C12) n=4
d) immunological analysis: day 7 n=6

The following C57BL/6 CD11c-cre 'x'-flox strains will be used, where 'x' will be a gene involved in:
1) AMPK signalling directly (for instance LKB1 and AMPK1)
2) mTOR signalling directly (for instance Raptor and Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 and PPARg)
4) metabolic regulation downstream of mTOR signalling: (for instance HIF1a and SREBP)

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role in metabolism and the T cell priming and polarizing capacity of DC in the vitro model (part 1), will be evaluated using CD11c-cre flox mice.

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

Based on our experience with the fluorescent metabolite tracing in vivo, we expect a standard deviation of 10% for this type of experiment. We hope to observe a 25% difference between the experimental groups, and we set the power at 80% and determine p-value of 0.05. According to power analysis, we need 4 mice per group in order to obtain significant differences.

Based on our experience with the immune response induced following footpad injection, we expect a standard deviation of 12% for this type of experiments. We hope to observe a 20% difference between the experimental groups, and we set the power at 80% and determine p-value of 0.05. According to power analysis, we need 6 mice per group in order to obtain significant differences

B. The animals

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

C57BL/6 CD11c-cre+ / cre- 'x'-flox/flox, rederived in house, n=170 per strain (85 cre+ and 85 cre-) where 'x' will be a gene involved in:
1) AMPK signaling directly (for instance LKB1 or AMPK1)
2) mTOR signaling directly (for instance Raptor or Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 or PPARg)
4) metabolic regulation downstream of mTOR signaling: (for instance HIF1a or SREBP)
These mice will be used between 6-12 weeks of age and both males and females will be used.

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role in metabolism and the T cell priming and polarizing capacity of DC in the vitro model (part 1), will be evaluated using CD11c-cre flox mice, with a maximum of a total of 6 different

target genes.

Breeding will be setup with homozygous flox breeding pairs of which one of the breeding pair is heterozygous for CD11c-cre, whereas the other will be CD11c-cre negative. Offspring should then be 50% CD11c-cre+ and 50% CD11c-cre negative. The CD11c-Cre negative littermates will be used as controls for CD11c-Cre+ mice in the same experiment

Total maximum number of mice: 6 x 170 = 1020

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:
Research involving mice is essential for the proposed research. The metabolic properties of DCs and how they shape their immune polarizing function (which is the focus of this project), are expected to be largely dictated by the constantly changing micro-environment they reside in in situ. This cannot be mimicked in vitro. Finally, these studies need to be performed in mice, since this is the only model organism in which the tools are available to study DC-specific deletions in AMPK/mTOR signalling cascades. Therefore, this study can only be properly performed in vivo in mice.
Reduction:
For all animal experimentation a minimum number of mice will be used to still achieve statistically significant data. To reduce the number of mice needed the contralateral footpad will serve as the negative control for the immunization in the other footpad. This saves the use of a separate group of control mice. Finally, littermate controls (CD11c-Cre+ and CD11c-Cre-) will be used to reduce experimental variation and thereby reduce the number of mice needed per experimental group. Finally, experiments will be performed by staff that are highly skilled and experienced with animal handling. As a result all procedures with the animals will be performed with the greatest care and minimal animal distress will be ensured.
Refinement
Subcutaneous injection of antigens will be performed under anaesthesia, which takes only a few minutes. If any of the animals develop any state described as humane endpoint in the next section, the animals will be humanely sacrificed. Moreover, experiments will be performed by staff that are highly skilled and experienced with animal handling. As a result all procedures with the animals will be performed with the greatest care and minimal animal distress will be ensured. Finally, in line with the code of practice, mice that received footpad injection will be housed in cages with extra thick bedding.

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

subcutaneous immunization will be performed under anaesthesia, to minimize animal pain or fear. While we do not expect severe complications as a result of this procedure, the animals will be observed on a daily basis and be humanely sacrificed in case the following defined end points are reached: visible pain (abnormal posture and/or movement, significant swelling of the foot and/or limping), significant weight loss (>20%), abnormal behaviour (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators

and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanized.

Repetition and duplication

E. Repetition

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

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III

☒ 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.

Before immunization mice will be put under anaesthesia by using isoflurane (5%) mixed with oxygen. During immunization, mice will be maintained under anaesthesia using a mouth piece. To ensure anaesthesia has set in well, rear foot reflex will be tested. During the maintenance phase isoflurane dosage is reduced (to 2-3%) to prevent overdosing.

I. Other aspects compromising the welfare of the animals

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

infection of the injection site

Explain why these effects may emerge.

needle ruptures the skin which may cause an opportunistic infection

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

Sterile needles and syringes will be used to minimize chances of infection

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.

Although human endpints are unlikely to be the animals will be observed on a daily basis and be humanely sacrificed in case the following defined end points are reached: visible pain (abnormal swelling of the immunized footpad, posture and/or movement), significant weight loss (>20%), abnormal behavior (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanised.

Indicate the likely incidence.

Very unlikely

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').

anaesthesia/recovery from anaesthesia: mild
Immunisation: moderate

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.

To analyse the immune response occurring in the draining lymph node (popliteal) mice need to sacrificed.

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

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

☒ Yes

Appendix
Description animal procedures

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- 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'.	11600
1.2	Provide the name of the licenced establishment.	Leids Universitair Medisch Centrum
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3 Type of animal procedure: Fitc painting

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.

o study whether AMPK and mTOR control DC metabolism and thereby their capacity to migrate from the peripheral sites to draining lymph nodes, a FITC painting model will be used in the transgenic mice with DC-specific deletions of key components of the AMPK/mtor signalling axes. In this model a solution of FITC (a harmless fluorophore) will be applied on bare skin on the flanks of a mouse. skin resident DCs will take up the FITC and migrate to draining lymph nodes, where their FITC signal will be quantified 24 h later. This will provide a robust measurement for the migratory capacity DCs in these transgenic mice.

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

Mice with DC-specific deletion of key components of mTOR and AMPK signalling axes or Cre- controls will be shaved on the flanks and a 200ul 1% FITC acetone solution will be applied on the skin. 24 h later the mice will be euthanized with an overdose Nembutal and the drain ng brachial lymph nodes will be collected and analysed for the presence of FITC-positive migratory DCs.

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

Based on previous experiments with the FITC painting model we expect a standard deviation of around 15% for this type of experiments. We hope to observe a 25% difference between the experimental groups, and we set the power at 80% and determine p-value of 0.05. According to power analysis, we need 6 mice per group in order to obtain significant differences.

B. The animals

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

C57BL/6 CD11c-cre+ / cre- 'x'-flox/flox, rederived in house, n=12 per strain (6 cre+ and 6 cre-) where 'x' will be a gene involved in:
1) AMPK signalling directly (for instance LKB1 or AMPK1)
2) mTOR signalling directly (for instance Raptor or Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 or PPARg)
4) metabolic regulation downstream of mTOR signalling: (for instance HIF1a or SREBP)
These mice will be used between 6-12 weeks of age and both male and females will be used.

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role in metabolism and the T cell priming and polarizing capacity of DC in the immunization model (part 2), will be evaluated using CD11c-cre flox mice, with a maximum of a total of 6 different target genes.

Breeding will be setup with homozygous flox breeding pairs of which one of the breeding pair is heterozygous for CD11c-cre, whereas the other will be CD11c-cre negative. Offspring should then be 50% CD11c-cre+ and 50% CD11c-cre negative. The CD11c-Cre negative littermates will be used as controls for CD11c-Cre+ mice in the same experiment

Total maximum number of mice: 6x12 cre/flox = 72 mice

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:
Migration of DCs is a bio energetically demanding process that is likely to be dictated by nutrient availability in their constantly changing microenvironments during migration in vivo. This complexity cannot be fully mimicked in in vitro studies. Finally, these studies need to be performed in mice, since this is the only model organism in which the tools are available to study DC-specific deletions in AMPK/mTOR signalling cascades. Therefore, this study can only be properly performed in vivo in mice.
Reduction:
To reduce the number of mice needed, both flanks will be FITC painted of every mouse to maximize the data generated from every mouse. Littermate controls (CD11c-Cre+ and CD11c-Cre-) will be used to reduce experimental variation and thereby reduce the number of mice needed per experimental group. Finally, only genes present in the signalling pathways and downstream target genes that were identified to play a role in metabolism and the T cell priming and polarizing capacity of DC in the immunization model (part 2), will be evaluated using CD11c-cre flox mice in this FITC painting model. Finally, experiments will be performed by highly skilled and experienced staff, which will reduce intra-experimental variation and as such will result in fewer animals that will be needed per experiment to obtain reliable and statistically significant data.
Refinement
Only the area of the flank that will be FITC painted will be shaved, minimizing distress to the animals. Shaving and FITC painting will be performed under anaesthesia. If any of the animals develop any state described as humane endpoint in the next section, the animals will be humanely sacrificed. Finally,

experiments will be performed by staff that are highly skilled and experienced with animal handling. As a result all procedures with the animals will be performed with the greatest care and minimal animal distress will be ensured.

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

Topical application of FITC will be performed under anaesthesia, to minimize animal fear. While we do not expect complications as a result of FITC painting, the animals will be observed after they wake up from anaesthesia to check whether they experience any discomfort/itching from the FITC painting. The mice will be humanely sacrificed in case the following defined end points are reached: visible pain (abnormal posture and/or movement, severe itching), abnormal behaviour (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanized.

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.

The research question that is being addressed with the current set of experiments is novel and has not be studied before. An extensive literature search has revealed that these trangenic mice have not been used before in this kind of experimental model.

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

☒ 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.

Before topical application of FITC, mice will be put under anaesthesia by using isoflurane (5%) mixed with oxygen. During the injection, mice will be maintained under anaesthesia using a mouth piece. To ensure anaesthesia has set in well, rear foot reflex will be tested. During the maintenance phase isoflurane dosage is reduced (to 2-3%) to prevent overdosing.

I. Other aspects compromising the welfare of the animals

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

not very likely, but Itching from the FITC painting

Explain why these effects may emerge.

pplication of the aceton may cause some temporary itching of the skin

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

he mice will be observed after they woke up from anaesthesia for the FITC painting. If they experience severe itching (determined by trying to scratch the treated flanks, the mice will be euthanized

J. Humane endpoints

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

☐ No > Continue with question K.

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

Although human endpints are unlikely to be reached in this experimentation, the animals will be observed and humanely sacrificed in case the following defined end points are reached: visible pain (severe scratching), abnormal behavior (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanised.

Indicate the likely incidence.

Very unlikely

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').

anaesthesia/recovery from anaesthesia: mild

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.

To analyze migrated FITC-carrying DCs in Draining lymph nodes mice need to be sacrificed.

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

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- 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'.	11600
1.2	Provide the name of the licenced establishment.	Leids Universitair Medisch Centrum
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 4 Type of animal procedure: Schistosoma mansoni infection

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.

Helminths are the strongest natural inducers of type 2 immune responses. Therefore, to study whether mice that harbour DCs with defects in AMPK or mTOR signalling have an altered capacity to prime Type 2 immune a helminth infection model with Schistosoma mansoni will be used. The larval stages of this parasite infect the host via penetration through the skin after which they develop in to adult worms in the mesenteric plexus over a period of 5 weeks. Around weeks 6 post infection the female worms start to lay eggs, which get trapped in the liver and gut wall. The eggs trigger a string Th2 immune response by the host which peaks around week 8 post infection. However, despite this acute response the host is unable to clear the infection, and as a results the infection enters the chronic phase (>12 weeks) which is characterized by a dampened type 2 immune response and the development of a regulatory immune response comprising Tregs. Dendritic cells are play a crucial role priming and regulation of Type 2 immune responses during S mansoni infection. This makes this model a highly suitable to interrogate the role of metabolic pathways in DCs that regulate their capacity to prime Th2 responses during the acute phase of the infection and Treg development during the chronic stages of the infection .

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

The animals will be infected with the larval stages of the helmith S. mansoni for 8 weeks. In short, the belly of every mouse will be quickly shaved and then be anesthetized with an i.p. injection of a mixture of Esketamine (50 mg/kg) and Dexdomitor (0.5 mg/kg). Next, the mice will be placed on their backs on a heated matrass and a metal ring will be placed on their shaved bellies. Subsequently, a solution of 1 ml of water (containing the cercariae) will be pipetted into the ring. After 30 min the ring and the remaining fluid will be removed and the animals will be turned on their bellies. To help the mice to recover quickly

from their anaesthesia, they will receive an i.p. injection with anti-sedan (0,20 ml). The mice are not anesthetized during the shaving procedure, because this is only a short period where the mice are 'hand-fixed' (30 sec). The concentration of the anaesthesia is optimized to ensure the mice are only anesthetized for 45 min. The infection with the cercariae is maximized at 12 mice at the same time to make the procedure and safety as optimized possible for the researchers involved.

8 weeks post infection the mice will be sacrificed by an overdose Nembutal and the following parameters will be analysed:
hepatic and mesenteric lymph nodes and a piece of liver will be collected and cell composition will be determined by flow cytometry. Specifically, hallmarks of type 2 immune responses will be analysed: presence of IgG1-switched B cells, eosinophils and Th2/Treg cells. The T cells will be characterized by ex vivo antigen specific and polyclonal restimulation and staining for foxp3 as a marker for Tregs.
For the chronic infection time point (16 week post infection), the same parameters will be assessed. in addition Liver sections will be fixed for histological analysis to establish whether the transgenic mice are more or less prone to develop egg-induced liver fibrosis as a result of the infection, which is a prototypical immunopathological consequence of Type 2 immune response during chronic S. mansoni infection.

Group 1: naïve control mice n=9
Group 2: acute S. mansoni infection (8 weeks) n=12
group 3: chronic S. mansoni infection (16 weeks) n=12

The following C57BL/6 CD11c-cre 'x'-flox strains will be used, where 'x' will be a gene involved in:
1) AMPK signalling directly (for instance LKB1 and AMPK1)
2) mTOR signalling directly (for instance Raptor and Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 and PPARg)
4) metabolic regulation downstream of mTOR signalling: (for instance HIF1a and SREBP)

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role T cell polarization in the immunization model (part 2), will be evaluated using CD11c-cre flox mice in this model.

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

In previous experiments (Hussaarts et al, FASEB J, 2015; Smits et al, JACI, 2007) we observed an average of 20% stdev in parameters regarding the Th2 response parameters. Based on these data and an anticipated difference between WT and knockout mice of at least 25% we need 9 mice per group, to be able have sufficient power (80%) to obtain significant differences (with a $p = 0.05$). From experience we know that about 15% of the infected animals develops hypersplenomegaly. According to human endpoints they will need to be taken out of the experiment. In addition, in around 5% of the animals worms will not fully develop. To compensate for these animals, we would like to include 20% extra animals (= ~3) in the infected groups (total 9+3=12 per group).

B. The animals

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

C57BL/6 CD11c-cre+ / cre- 'x'-flox/flox, rederived in house, n=66 per strain (33 cre+ and 33 cre-) where 'x' will be a gene involved in:
1) AMPK signalling directly (for instance LKB1 or AMPK1)
2) mTOR signalling directly (for instance Raptor or Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 or PPARg)
4) metabolic regulation downstream of mTOR signalling: (for instance HIF1a or SREBP)
These mice will be used between 6-12 weeks of age and both male and females will be used.

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role in metabolism and the T cell priming and polarizing capacity of DC in the immunization model (part 2), will be evaluated using CD11c-cre flox mice, with a maximum of a total of

6 different target genes.

Breeding will be setup with homozygous flox breeding pairs of which one of the breeding pair is heterozygous for CD11c-cre, whereas the other will be CD11c-cre negative. Offspring should then be 50% CD11c-cre+ and 50% CD11c-cre negative. The CD11c-Cre negative littermates will be used as controls for CD11c-Cre+ mice in the same experiment

Total maximum number of mice: 6x66 cre/flox = 396 mice

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:
Research involving mice is essential for the proposed research. The metabolic properties of DCs and how they shape their immune polarizing function (which is the focus of this project), are expected to be largely dictated by the constantly changing micro-environment they reside in in situ. This cannot be mimicked in vitro. Finally, these studies need to be performed in mice, since this is the only model organism in which the tools are available to study DC-specific deletions in AMPK/mTOR signalling cascades. Therefore, this study can only be properly performed in vivo in mice.
Reduction:
To reduce the number of mice needed, only the transgenic mice that gave an altered Th2 immune response in the immunization model will be tested in this infection model. In addition, littermate controls (CD11c-Cre+ and CD11c-Cre-) will be used to reduce experimental variation and thereby reduce the number of mice needed per experimental group. Finally, experiments will be performed by highly skilled and experienced staff, which will reduce intra-experimental variation and as such will result in fewer animals that will be needed per experiment to obtain reliable and statistically significant data.
Refinement
Infection will be performed under anaesthesia, which takes 45 minutes. If any of the animals develop any state described as humane endpoint in the next section during the infection, the animals will be humanely sacrificed. Finally, experiments will be performed by staff that are highly skilled and experienced with animal handling. As a result all procedures with the animals will be performed with the greatest care and minimal animal distress will be ensured.

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

infection will be performed under anaesthesia, to minimize animal pain or fear. While we do not expect severe complications as a result of the initial infection the laying of eggs by the adult worms (starting around week 6), may lead to illness in case any the transgenic mice have a defect in their ability to mount a Th2 response. Therefore, the animals will be observed 3 times a week from week 5 onwards and will be humanely sacrificed in case the following defined end points are reached: visible pain (abnormal posture and/or movement), significant weight loss (>20%), abnormal behaviour (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria,

the animals will be taken out of the experiment and euthanized.

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.

The research question that is being addressed with the current set of experiments is novel and has not be studied before. An extensive literature search has revealed that these trangenic mice have not been used before in this kind of experimental model.

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

☒ 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.

The animals will be infected with the larval stages of the helmith S. mansoni. To this end mice will be anesthetized with an i.p. injection of a mixture of Esketamine (50 mg/kg) and Dexdomitor (0.5 mg/kg). To ensure anaesthesia has set in well, the rear foot reflex will be tested. To prevent hypothermia, the mice will be placed on their backs on a heated matrass during anaesthesia.

I. Other aspects compromising the welfare of the animals

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

infection with S mansoni may cause liver dysfunction

Explain why these effects may emerge.

S. mansoni derived eggs get trapped in the liver

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

as described in J (humane endpoints), mice will be sacrificed if mice display pain, abnormal behaviour, weight loss as a consequence of hepatic dysfunction.

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.

While we do not expect severe complications as a result of the initial infection the laying of eggs by the adult worms (starting around week 6), may lead to illness in case any the transgenic mice have a defect in their ability to mount a protective Th2 response. Therefore, the animals will be observed 3 times a week from week 5 onwards and will be humanely sacrificed in case the following defined end points are reached: visible pain (abnormal posture and/or movement), significant weight loss (>20%), abnormal behavior (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanised.

Indicate the likely incidence.

possible

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').

anaesthesia/recovery from anaesthesia: mild
Helminth S. mansoni infection: moderate and in some specific cases severe

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.

To analyse the immune response occurring in the draining lymph node (mesenteric and hepatic) and liver, mice need to sacrificed.

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

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

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
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1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	11600
1.2	Provide the name of the licenced establishment.	Leids Universitair Medisch Centrum
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 5 — Type of animal procedure: Malaria infection model

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.

Malaria infection drives strong type 1 immunity. During a blood meal by a plasmodium-infected mosquito, sporozoites are injected into the skin and reach the bloodstream, after which the parasites develop into a liver stage around day 2. Protection against this stage of the infection is predominantly dependent on CD8 cytotoxic T cell responses. After day 2, a merozoite blood stage develops that infects erythrocytes and causes blood stage malaria that leads to clinical symptoms. In contrast to the liver stage, blood stage parasitemia is thought to be primarily controlled by Th1 immune responses. In both stages of the infection, it has been shown that priming of T cells is dependent on dendritic cells. This makes a liver and blood stage malaria infection model highly suitable study whether mice that harbour DCs with defects in AMPK or mTOR signalling have an altered capacity to prime Type 1 immune responses (ie cytotoxic T cell responses and Th1 responses, respectively).
(A) First, to investigate whether metabolism in DCs controls their capacity to prime Th1 responses during malaria, a murine blood-stage malaria infection model with Plasmodium chabaudi AS (Pc) will be used, since C57/B6 mice are resistant to this infection and clear it after about 2 weeks. The peak of the primary T cell response in spleen lies around day 5-7 post infection and the peak of parasitemia lies around day 7-9. In this model T cell responses in spleen as well as parasitemia and anaemia will be studied.
(B) Second, to investigate whether metabolism in DCs controls their capacity to prime CD8 cytotoxic Tcell responses during malaria, we will be using a genetically attenuated parasite (GAP) of P. yoelii that can only develop into the liver stage. This attenuated parasite line also express the reporter protein GFP-Luciferase which will allow to determine parasite liver loads in live mice by real time in vivo imaging after infection/administration of defined doses of attenuated sporozoites This will allow for the characterisation of CD8 T cell immune responses specifically generated against the liver stages, which is thought be

crucial for protection against subsequent infections. Moreover, following injection of luciferin, parasite burden can be monitored real-time in vivo without the need to sacrifice mice.

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

(A)

Before experimental mice can be infected with infected red blood cells (iRBCs), 2 WT mice will be infected i.p. (200ul, 10000 Pc AS iRBCs), with parasite stored in liquid nitrogen or blood of an infected mice. Parasitemia will be monitored in these mice by taking a drop of blood (1/3 of a capillary, i.e.~25uL) from the tail vein and a blood smear will be made to count the percentage of parasitized erythrocytes per 100 erythrocytes. When up to 40% infection is reached, these mice will be euthanized with an overdose Nembutal and blood will be collected to obtain fresh Pc–infected RBCs that can be used blood-stage infection of experimental mice. The protocol for maintenance and storage of blood stage parasites is defined under a different DEC protocol ([redacted]) that is operational within our department. iRBC that are not used for infection of experimental mice will be stored in liquid nitrogen until needed for infection of new mice.

To study the primary immune response against Pc blood-stage infection, mice will be infected i.p. (200ul) with 1x10e5-1x10e6 infected RBC diluted in PBS. At day 5, the peak of the immune response, a group of mice will be euthanized with an overdose Nembutal and analysed for the following parameters:
1) DCs and T cell numbers in spleens and blood will be quantified and analysed for their phenotype.
2) Splenocytes will be re-stimulated with iRBC or polyclonally to assess cytokine production by the T cells.
3) blood will be collected as described below to assess parasitemia and anaemia

To assess whether any change in T cell response affect the ability of these mice to control the infection a second group of infected mice will be followed for up to 2 weeks to monitor weight, parasitemia and anaemia on day 3,5,7,10,and 13, after which the mice will be euthanized. To quantify parasitemia, mice will be bled by taking a drop of blood (1/3 of a capillary, i.e.~25uL) from the tail vein and a blood smear will be made to count the percentage of parasitized erythrocytes per 100 erythrocytes. Experiments will be terminated when the parasitemia reaches a level greater than 40% in the mice, before signs of severe anaemia and organ failure occur.

Experimental groups:

Group 1: naïve control mice (immunology)	n=5
Group 2: Pc infected mice (immunology)	n=6
Group 3: naïve control mice (parasitemia	n=9
Group 4: Pc infected mice (parasitemia)	n=11

The following C57BL/6 CD11c-cre ‘x’-flox strains will be used, where ‘x’ will be a gene involved in:
1) AMPK signalling directly (for instance LKB1 and AMPK1)
2) mTOR signalling directly (for instance Raptor and Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 and PPARg)
4) metabolic regulation downstream of mTOR signalling: (for instance HIF1a and SREBP)

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role T cell polarization in the immunization model (part 2), will be evaluated using CD11c-cre flox mice in this model.

(B)

The protocol for obtaining/isolation GAP sporozoites from salivary glands of infected mosquitoes for

immunization and challenge is defined under a different DEC protocol ([redacted]) that is operational within our department

To study the primary immune response against Plasmodium yoelii, mice will be infected i.v. (200ul, 10000 parasites), under anaesthesia, through tail vein injection, with sporozoites of a genetically attenuated parasite (GAP) of Py that can only develop to the liver stage and expresses luciferase.

To quantify parasite liver loads, real time in vivo imaging will be performed at one time point 2 days after administration of sporozoites, by measuring luciferase activity/luminescence emanating from parasites present in the liver using the IVIS-Lumina system; the reduction in the liver load can be very accurately quantified and will give a measure how effective the host immune response is at limiting liver stage development. Mice are anesthetized with isoflurane, after which they are subcutaneously injected with luciferin (100mg/kg body weight). Eight minutes after injection mice are placed under the CCD camera, whereupon luciferase activity/luminescence is measured during a period of 2 minutes (the total period of anaesthesia per mouse is max 10 min).

At day 7 mice will be euthanized with an overdose Nembutal and analysed for the following immunological parameters:
1) DCs and Py specific T cells in spleens, hepatic lymph nodes and liver will be quantified and analysed for their phenotype.
2) Splenocytes and liver will be re-stimulated with Py specific peptide (CS) or polyclonally to assess cytokine production by the T cells.

Experimental groups:

Group 1: naïve control mice n=9
Group 2: Py infected mice n=10

The following C57BL/6 CD11c-cre 'x'-flox strains will be used, where 'x' will be a gene involved in:
1) AMPK signalling directly (for instance LKB1 and AMPK1)
2) mTOR signalling directly (for instance Raptor and Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 and PPARg)
4) metabolic regulation downstream of mTOR signalling: (for instance HIF1a and SREBP)

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role T cell polarization in the immunization model (part 2), will be evaluated using CD11c-cre flox mice in this model.

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

or (A): Based on earlier studies with the Pc blood stage infection model in C57BL/6 mice (Borges da Silva et al, PloS Path, 2015; Stevenson et al, J Immunol, 1995) we expect a standard deviation of around 10% for the immunological parameters we are interested and 15% for the parasitological reads. We hope to observe a 20% difference between the experimental groups, and we set the power at 80% and determine p-value of 0.05. According to power analysis, we need 5 mice per group for the immunology and 9 mice per groups for the parasitology, in order to observe significant differences. From experience we know that about 10-20% do not get properly infected. Therefore, we included 1 and 2 additional mice per group respectively.

For (B): Based on earlier studies with the P.y. liver stage infection model in our lab we experienced the larger variability in the immunological parameters such as CD8 T cell IFNg production than parasitic loads. For the CD8 t cell responses we observed a standard deviation of around 20%. We hope to observe a 25% difference between the experimental groups, we set the power at 80% and determine p-value of 0.05. According to a 2-tailed power analysis (G*power 3.1), we need 9 mice per group in order to obtain significant differences. From experience we know that about 10-20% do not get properly infected. Therefore, we included an additional mouse per group

B. The animals

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

C57BL/6 CD11c-cre+ / cre- 'x'-flox/flox, rederived in house, n=100 per strain (50 cre+ and 50 cre-) where 'x' will be a gene involved in:
1) AMPK signalling directly (for instance LKB1 or AMPK1)
2) mTOR signalling directly (for instance Raptor or Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 or PPARg)
4) metabolic regulation downstream of mTOR signalling: (for instance HIF1a or SREBP)
These mice will be used between 6-12 weeks of age and both male and females will be used.

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role in metabolism and the T cell priming and polarizing capacity of DC in the immunization model (part 2), will be evaluated using CD11c-cre flox mice, with a maximum of a total of 6 different target genes.

Breeding will be setup with homozygous flox breeding pairs of which one of the breeding pair is heterozygous for CD11c-cre, whereas the other will be CD11c-cre negative. Offspring should then be 50% CD11c-cre+ and 50% CD11c-cre negative. The CD11c-Cre negative littermates will be used as controls for CD11c-Cre+ mice in the same experiment .

Total maximum number of mice: 6x100 cre/flox = 600 mice

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:
Research involving mice is essential for the proposed research.. The metabolic properties of DCs and how they shape their immune polarizing function (which is the focus of this project), are expected to be largely dictated by the constantly changing micro-environment they reside in in situ. This cannot be mimicked in vitro. Finally, these studies need to be performed in mice, since this is the only model organism in which the tools are available to study DC-specific deletions in AMPK/mTOR signalling cascades. Therefore, this study can only be properly performed in vivo in mice.
Reduction:
To reduce the number of mice needed, only the transgenic mice that gave an altered Type 1 immune response in the immunization model will be tested in this infection model. If the transgenic mice do not display an altered immunological profile compared to littermate controls as determined in (A), (B) will not be performed. In the current project we will be using a genetically attenuated strain of Py that expresses luciferase. following injection of luciferin, this will allow for monitoring parasite burden real time in vivo at multiple time points in the same mice without the need to sacrifice mice. This reduces the number of mice needed for these experiments. In addition, Measurement of luciferase activity with an I-CCD Video camera is a very sensitive and accurate method to analyse parasite loads in live animals. As only small variations in luminescence signals between mice belonging to the same group are observed, a

smaller number of animals per group is required in comparison with other more traditional methods of analysing parasite loads (e.g. Histology, RT-PCR). In addition, littermate controls (CD11c-Cre+ and CD11c-Cre-) will be used to reduce experimental variation and thereby reduce the number of mice needed per experimental group. Finally, experiments will be performed by highly skilled and experienced staff, which will reduce intra-experimental variation and as such will result in fewer animals that will be needed per experiment to obtain reliable and statistically significant data.
Refinement
Infection will be performed under isoflurane anaesthesia. In the current project we will be using a genetically attenuated strain of Py that can only develop to the liver stage. This will ensure that blood stage malaria cannot develop and as such no overt disease is to be expected. Moreover, following injection of luciferin, parasite burden can be monitored real-time in vivo without the need to sacrifice mice. If any of the animals develop any state described as humane endpoint in the next section during the infection, the animals will be humanely sacrificed. Finally, experiments will be performed by staff that are highly skilled and experienced with animal handling. As a result all procedures with the animals will be performed with the greatest care and minimal animal distress will be ensured.

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

infection will be performed under isoflurane anaesthesia, to minimize animal pain or fear. We do not expect severe complications as a result of infection with GAP Py since these parasites cannot develop into blood stage malaria that can cause overt disease. However, Pc blood stage infection may lead to illness due to weight loss hypothermia or anaemia, in case any the transgenic mice have a defect in their ability to mount protective Type 1 immune responses. Therefore, these animals will be observed daily following the Pc infection and will be humanely sacrificed in case the following defined end points are reached: >40% positive blood smear, visible pain (abnormal posture and/or movement), significant weight loss (>20%), abnormal behaviour (isolation, abnormal reaction to stimuli, no food and water intake) or hypothermia (<34C). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanized.

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.

The research question that is being addressed with the current set of experiments is novel and has not be studied before. An extensive literature search has revealed that these trangenic mice have not been used before in this kind of experimental model.

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

☒ 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.

Before Luciferine administration s.c. or sporozoite infection i.v., mice will be put under anaesthesia by using isoflurane (5%) mixed with oxygen. During the injection, mice will be maintained under anaesthesia using a mouth piece. To ensure anaesthesia has set in well, rear foot reflex will be tested. During the maintenance phase isoflurane dosage is reduced (to 2-3%) to prevent overdosing.

I. Other aspects compromising the welfare of the animals

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

infection of the injection site

Explain why these effects may emerge.

needle ruptures the skin which may cause an opportunistic infection

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

Sterile needles and syringes will be used to minimize chances of infection

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.

Animals will be humanely sacrificed in case the following defined end points are reached: >40% positive blood smear, visible pain (abnormal posture and/or movement), significant weight loss (>20%), abnormal behavior (isolation, abnormal reaction to stimuli, no food and water intake) or hypothermia (<34C). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanised.

Indicate the likely incidence.

possible

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').

iv injection: mild
WT infection: moderate/severe

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.

To analyse the immune response occurring in spleen and liver the draining lymph node, mice need to sacrificed.

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

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

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
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1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	11600
1.2	Provide the name of the licenced establishment.	Leids Universitair Medisch Centrum
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 6 Type of animal procedure: Listeria monocytogenes infection model

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.

Intracellular bacterial infections drive strong type 1 immunity characterized by Th1 and cytotoxic T cell responses. Therefore, to study whether mice that harbour DCs with defects in AMPK or mTOR signalling have an altered capacity to prime Type 1 immune responses a intracellular bacterial infection model with Listeria monocytogenes (Lm) will be used. Following i.v. infection, the peak of the T cell response lies at day 7 post infection, and while the peak of the bacterial load is around day 3 and is generally cleared by day 7. Dendritic cells play a crucial role in priming and regulation of Type 1 immune responses during this infection. This makes this model a highly suitable to interrogate the role of metabolic pathways in DCs that regulate their capacity to prime Type 1 immune responses. (A) The primary immune response to the infection as well as (B) lasting immunity will be studied.

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

(A)
To study the primary immune response against Listeria, mice will be infected i.v. (200ul), through tail vain injection, with a subclinical dose of Lm expressing ovalbumin (OVA) (2500CFU). At day 3 and 5 after the infection mice will be bled by cheek bleeding and analysed for OVA specific circulating CD4 and CD8 T cells. At day 3 and 7 mice will be euthanized with an overdose Nembutal and analysed for the following parameters:
1) DCs and OVA specific T cells in spleens and liver (the two main sites of infection) will be quantified and analysed for their phenotype.
2) Splenocytes and liver will be restimulated with OVA or polyclonally to assess cytokine production by

the T cells.
3) Bacterial loads will be determined in spleens and livers to assess whether the mutant mice have an altered ability to control infection.

To track proliferative responses of T cells in vivo in response to the infection, 5000 CFSE and CTV-labelled OVA-specific CD4 (OTII) and CD8 (OTI) T cells will be adoptively transferred i.v. in 200ul PBS, 1 day prior to infection as described above, in a separate group of mice. At day 3 and 7 mice will be euthanized with an overdose Nembutal and analysed for proliferation and phenotype of transferred T cells.

Experimental groups:

Group 1: naïve control mice n=8
Group 2: Lm infected (Day 3) n=8
Group 3: Lm infected (Day 7) n=8
Group 1: naïve control mice + transferred T cells n=8
Group 2: Lm infected (Day 3) + transferred T cells n=8
Group 3: Lm infected (Day 7) + transferred T cells n=8

The following C57BL/6 CD11c-cre 'x'-flox strains will be used, where 'x' will be a gene involved in:
1) AMPK signalling directly (for instance LKB1 and AMPK1)
2) mTOR signalling directly (for instance Raptor and Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 and PPARg)
4) metabolic regulation downstream of mTOR signalling: (for instance HIF1a and SREBP)

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role in the T cell priming and polarizing capacity of DC in the immunization model (part 2), will be evaluated using CD11c-cre flox mice in this model.

(B)
To study the ability of these mice to mount protective/lasting Type 1 immunity, mice will be infected i.v. (200ul), through tail vain injection, with a replication deficient strain of Lm-(delta ActA) expressing OVA (10e7 CFU). 21 days after the primary infection and when the memory response has been established, mice will be challenged i.v. (200ul), through tail vain injection, with a LD50 dose of WT Lm expressing ovalbumin (OVA) (10e5 CFU). At day 3 mice will be euthanized with an overdose Nembutal and analysed for the following parameters:
1) OVA specific CD4 and CD8 T cells in spleens and liver (the two main sites of infection) will be quantified and analysed for their phenotype.
2) Splenocytes and liver will be re-stimulated with OVA or polyclonally to assess cytokine production by the T cells.
3) Bacterial loads will be determined in spleens and livers to assess whether the mutant mice have an altered ability to mount protective immunity

Experimental groups:

Group 1: naïve control mice (control) n=8
Group 2: Primary infection only (control) n=8
Group 3: Secondary infection only (control) n=8
Group 4: Primary + secondary infection n=8

The following C57BL/6 CD11c-cre 'x'-flox strains will be used, where 'x' will be a gene involved in:
1) AMPK signalling directly (for instance LKB1 and AMPK1)
2) mTOR signalling directly (for instance Raptor and Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 and PPARg)
4) metabolic regulation downstream of mTOR signalling: (for instance HIF1a and SREBP)
Importantly, only genes present in the signalling pathways and downstream target genes that were

identified to play a role in the T cell priming and polarizing capacity of DC in the immunization model (part 2), will be evaluated using CD11c-cre flox mice in this model.

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

Based on earlier findings (Wensveen et al. Immunity 2010, van der Windt et al. Immunity 2012), we observed an in-group variation of around 40% with this infection in terms of immunological outcome and bacterial loads. Based on preliminary experiments we anticipate a difference between WT and knout mice of around 50%. We would need 8 mice per group, to be able have sufficient power (80%) to obtain significant differences (with a p = 0.05).

B. The animals

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

C57BL/6 OTI n=5
C57BL/6 OTII n=5
Mice are obtained from Harlan or Jackson laboratories and used at 6-8 weeks of age

C57BL/6 CD11c-cre+ / cre- 'x'-flox/flox, rederived in house, n=160 per strain (80 cre+ and 80 cre-) where 'x' will be a gene involved in:
1) AMPK signaling directly (for instance LKB1 or AMPK1)
2) mTOR signaling directly (for instance Raptor or Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 or PPARg)
4) metabolic regulation downstream of mTOR signaling: (for instance HIF1a or SREBP)
These mice will be used between 6-12 weeks of age and both male and females will be used.

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role in metabolism and the T cell priming and polarizing capacity of DC in the immunization model (part 2), will be evaluated using CD11c-cre flox mice, with a maximum of a total of 6 different target genes.

Breeding will be setup with homozygous flox breeding pairs of which one of the breeding pair is heterozygous for CD11c-cre, whereas the other will be CD11c-cre negative. Offspring should then be 50% CD11c-cre+ and 50% CD11c-cre negative. The CD11c-Cre negative littermates will be used as controls for CD11c-Cre+ mice in the same experiment

Total maximum number of mice: 5 OTI + 5 OTII + 6x160 cre/flox = 970 mice

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:
Research involving mice is essential for the proposed research. The metabolic properties of DCs and how they shape their immune polarizing function (which is the focus of this project), are expected to be

largely dictated by the constantly changing micro-environment they reside in in situ. This cannot be mimicked in vitro. Finally, these studies need to be performed in mice, since this is the only model organism in which the tools are available to study DC-specific deletions in AMPK/mTOR signalling cascades. Therefore, this study can only be properly performed in vivo in mice.
Reduction:
To reduce the number of mice needed, only the transgenic mice that gave an altered Type 1 immune response in the immunization model. If the transgenic mice do not display an altered immunological profile compared to littermate controls as determined in (A), (B) will not be performed. Furthermore, littermate controls (CD11c-Cre+ and CD11c-Cre-) will be used to reduce experimental variation and thereby reduce the number of mice needed per experimental group. In addition, only genes present in the signalling pathways and downstream target genes that were identified to play a role in the T cell priming and polarizing capacity of DC in the immunization model (part 2), will be evaluated using CD11c-cre flox mice in this model. Finally, experiments will be performed by highly skilled and experienced staff, which will reduce intra-experimental variation and as such will result in fewer animals that will be needed per experiment to obtain reliable and statistically significant data.
Refinement
Infection will be performed under anaesthesia. A replication deficient Lm strain will be used, which eliminates the risk of developing clinical signs of disease. If any of the animals develop any state described as humane endpoint in the next section during the infection, the animals will be humanely sacrificed. Finally, experiments will be performed by highly skilled and experienced staff, which will reduce intra-experimental variation and as such will result in fewer animals that will be needed per experiment to obtain reliable and statistically significant data.

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

infection will be performed with under isoflurane anaesthesia, to minimize animal pain or fear. While we do not expect severe complications as a result of the initial infection the challenge infection, may lead to illness in case any the transgenic mice have a defect in their ability to mount a Type 1 immune responses. Therefore, the animals will be observed daily following the secondary infection and will be humanely sacrificed in case the following defined end points are reached: visible pain (abnormal posture and/or movement), significant weight loss (>20%), abnormal behaviour (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanized.

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.

The research question that is being addressed with the current set of experiments is novel and has not be studied before. An extensive literature search has revealed that these trangenic mice have not been used before in this kind of experimental model.

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

☒ 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.

Before i.v. infection, mice will be put under anaesthesia by using isoflurane (5%) mixed with oxygen. During the procedure, mice will be maintained under anaesthesia using a mouth piece. To ensure anaesthesia has set in well, rear foot reflex will be tested. During the maintenance phase isoflurane dosage is reduced (to 2-3%) to prevent overdosing.

I. Other aspects compromising the welfare of the animals

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

infection of the injection site

Explain why these effects may emerge.

needle ruptures the skin which may cause an opportunistic infection

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

Sterile needles and syringes will be used to minimize chances of infection

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.

While we do not expect severe complications as a result of the initial infection the challenge infection, may lead to illness in case any the transgenic mice have a defect in their ability to mount a Type 1 immune responses. Therefore, the animals will be observed daily following the secondary infection and will be humanely sacrificed in case the following defined end points are reached: visible pain (abnormal posture and/or movement), significant weight loss (>20%), abnormal behavior (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanised.

Indicate the likely incidence.

possible

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').

iv injection: mild

WT Lm OVA infection: moderate

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.

To analyse the immune response occurring in spleen and liver the draining lymph node, mice need to sacrificed.

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

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

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.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'.	11600
1.2	Provide the name of the licenced establishment.	Leids Universitair Medisch Centrum
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: 7 — Type of animal procedure: Melanoma tumor model

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.

While carcinogenesis itself is considered to be triggered by cell-intrinsic genetic mutations, tumor growth and the clinical outcome is thought to be primarily a function of how well the immune system is able to mount an anti-tumor response. For this anti-tumor immune response cell mediated immunity mediated by Th1 and cytotoxic T cells is particularly important. Therefore there is great significance in understanding how DCs become conditioned to prime Th1 and Cytotoxic T cell responses during cancer. To establish whether the balance in AMPK vs mTOR signaling in DCs controls their T cell polarizing and priming function and thereby regulates their capacity to drive an anti-tumor responses, we will make use of a B16-OVA melanoma tumor model which is a model for highly prevalent type of skin cancer in humans and of which its clinical outcome is highly dependent on effective anti-melanoma Th1 and cytotoxic T cell responses. The different transgenic mice will be subcutaneously injected with the OVA-expressing tumor cells and tumor growth will be followed as a function of several immunological parameters, including OVA-specific CD4+ and CD8+ T cell numbers systemically and around the tumor as well as production of effector molecules by these cells..

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

The mice will be injected intradermally (i.d.) in their back with B16 melanoma cells expressing OVA (1x105 in 50ul) under isoflurane anesthesia. Mice will be monitored for tumor growth periodically. When tumor size of one or multiple animals reaches 2 cm in diameter (before it may cause distress to the animals), all mice will be euthanized by i.p. injection of an overdose Nembutal and tumors will be analyzed for immune cell infiltrate.

The following time scheme will be applied for the melanoma tumor model

day 0: i.d. injection of B16 melanoma cells expressing OVA (1x105 in 50ul)
day 7-14: When reaching tumor size of 2cm max, euthanasia by i.p. injection of an overdose Nembutal

The following groups will be setup:
1) x- flox mice naïve n=5
2) Cd11c-cre+ x-flox mice naive n=5
3) x-flox mice tumor challenged n=12
4) Cd11c-cre+ x-flox mice tumor challenged n=12

The following C57BL/6 CD11c-cre 'x'-flox strains will be used, where 'x' will be a gene involved in:
1) AMPK signaling directly (for instance LKB1 and AMPK1)
2) mTOR signaling directly (for instance Raptor and Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 and PPARg)
4) metabolic regulation downstream of mTOR signaling: (for instance HIF1a and SREBP)

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role T cell polarization in the immunization model (part 2), will be evaluated using CD11c-cre flox mice in this model.

The following readouts will be performed to determine whether mTOR/AMPK signaling in DCs play a role in protection and

• During tumor growth (day 8 until day 21) tumor sizes will be measured with digital calipers every 3 days and every day after day 14

• On day 14, mice will be bled by cheek bleeds and frequencies and effector phenotype of circulating OVA-specific CD8 T cells will be determined.

• At time of harvest (when tumors of one or multiple mice reach 2 cm in diameter, or after 21 days), mice will be euthanized by i.p. injection of an overdose Nembutal and tumors will be excised to determine with final tumor volumes and weights.

• In addition, tumor infiltrating cells and cells from draining inguinal lymph nodes will be isolated by mechanical dissociation and passing through 70 µm cell strainers to obtain single cell suspensions. Cellular composition will be assessed by flow cytometry and specifically screened for the presence of different T helper subsets and Cytotoxic T cells. LN cells will be restimulated ex vivo to determine to Tcell cytokine profile in these mice using intracelluar cytokine staining and ELISA

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

In previous experiments (Amiel et al, JI, 2012) we experienced a considerable variability in tumor growth between different individual mice (41% stdev) and based on these data and an anticipated difference between WT and knout mice of at least 40% we need 12 mice per group with tumors, to be able have sufficient power (80%) to obtain significant differences (with a p = 0.05).

B. The animals

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

C57BL/6 CD11c-cre+ / cre- 'x'-flox/flox, rederived in house, n=34 per strain (17 cre+ and 17 cre-) where 'x' will be a gene involved in:
1) AMPK signaling directly (for instance LKB1 or AMPK1)
2) mTOR signaling directly (for instance Raptor or Rictor)

3) metabolic regulation downstream of AMPK signalling (for instance PGC1 or PPARg)
4) metabolic regulation downstream of mTOR signaling: (for instance HIF1a or SREBP)
These mice will be used between 6-12 weeks of age and both male and females will be used.

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role in metabolism and the T cell priming and polarizing capacity of DC in the immunization model (part 2), will be evaluated using CD11c-cre flox mice, with a maximum of a total of 6 different target genes.

Breeding will be setup with homozygous flox breeding pairs of which one of the breeding pair is heterozygous for CD11c-cre, whereas the other will be CD11c-cre negative. Offspring should then be 50% CD11c-cre+ and 50% CD11c-cre negative. The CD11c-Cre negative littermates will be used as controls for CD11c-Cre+ mice in the same experiment

Total maximum number of mice: 6x34 cre/flox = 204mice

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:
Research involving mice is essential for the proposed research. The immune responses involved in tumor development are extremely complex involving multiple different players at different times. Furthermore, the metabolic properties of DCs and how they shape their immune polarizing function (which is the focus of this project), are expected to be largely dictated by the constantly changing micro-environment they reside in in situ, especially in the context of tumors which are likely to compete with DCs for nutrients. This cannot be mimmicked in vitro. Finally, these studies need to be performed in mice, since this is the only model organism in which the tools are available to study DC-specific deletions in AMPK/mTOR signaling cascades. Therefore, this study can only be properly performed in vivo in mice.
Reduction:
To reduce the number of mice needed, only the transgenic mice that gave an altered Th1/cytotoxic T cell immune response in the immunization model will be tested in this tumor model. Moreover, littermate controls (CD11c-Cre+ and CD11c-Cre-) will be used to reduce experimental variation and thereby reduce the number of mice needed per experimental group. Finally, experiments will be performed by highly skilled and experienced staff, which will reduce intraexperimental variation and as such will result in fewer animals that will be needed per experiment to obtain reliable and statistically significant data.
Refinement
Injection of the tumor cells will be performed under anesthesia, which takes only a few minutes. the tumor cells will be injected on the back of the mice to minimize physical discomfort. If any of the animals develop any state described as humane endpoint in the next section, the animals will be humanely sacrifized. Finally, experiments will be performed by staff that are highlyskilled and experienced with animal handling. As a result all procedures with the animals will be performed with the greatest care and minimal animal distress will be ensured.

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

Injection of the tumor cells will be performed under anesthesia, which takes only a few minutes. the tumor cells will be injected on the back of the mice to minimize physical discomfort. If any of the animals develop any state described as humane endpoint in the next section, the animals will be humanely sacrified.

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.

The research question that is being addressed with the current set of experiments is novel and has not be studied before. An extensive literature search has revealed that these trangenic mice have not been used before in this kind of experimental model.

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

☒ 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.

Before tumour injection mice will be put under anaesthesia by using isoflurane (5%) mixed with oxygen. During injection, mice will be maintained under anaesthesia using a mouth piece. To ensure anaesthesia has set in well, rear foot reflex will be tested. During the maintenance phase isoflurane dosage is reduced (to 2-3%) to prevent overdosing.

I. Other aspects compromising the welfare of the animals

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

development of a solid subcutaneous tumor beyond 2 cm3 may negatively affect the welbeing of the mice

Explain why these effects may emerge.

the tumor may induce pain in the surrounding tissue or may physically interfere with free movement of the animal

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

the tumor will be establisshed on the back of the mouse, to minimize interference with normal behaviour. In addition humane endpoints are in place as described below.

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.

In accordance with the Code of Practice for 'Kankeronderzoek in proefdieren', A human endpoint is chosen when tumor size reaches 2 cm3, when it physically interferes with normal behaviour, when the intradermally located tumor starts ulcerating or when rapid weight loss (> 15% in 1-2 days). In addition, the animals will be observed on a daily basis and be humanely sacrificed in case the following defined end points are reached: visible pain (abnormal posture and/or movement), significant weight loss (>20%), abnormal behavior (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanised.

Indicate the likely incidence.

unlikely

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').

anaesthesia/recovery from anaesthesia: mild
discomfort from tumor growth: moderate

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.

To analyse the immune response occurring in and around the tumor and draining Lymph nodes, mice need to be sacrificed.

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

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

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.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'.	11600
1.2	Provide the name of the licenced establishment.	Leids Universitair Medisch Centrum
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: 8 Type of animal procedure: House dust mite Allergic Asthma model

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.

Allergic Asthma is the result of strong induction of Th2 immune responses by DCs in response to allergens in the lung that culminates in allergic airway inflammation. Therefore there is great significance in understanding how DCs become conditioned to prime Th2 responses during allergic asthma and how we can manipulate that process. To establish whether the balance in AMPK vs mTOR signalling in DCs controls their T cell polarizing function in the context of allergic asthma a model of allergic asthma will be used based on human allergen house dust mite (HDM). In this model mice are sensitized and challenged by allergens only via the intranasal route, which is similar to the natural route of allergen entry in humans and therefore is more relevant to allergic processes found in humans. Immunological outcomes that will be analysed following allergen sensitization and challenge are: DC phenotype and nature of T cell responses in the lung and draining lymph nodes. In addition, disease outcome in terms of airway inflammation will be assessed by analysing airway resistance, cellular composition of the Bal fluid and lung histology. This will determine whether mice with DC-specific deletions in the AMPK or mTOR signalling axis are more or less prone to develop Th2 immune responses and as a result allergic asthma.

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

C57BL/6 mice will be sensitized by i.t. application of 10µg HDM allergen in 30µL PBS on day 1. Next, mice will be challenged for 5 consecutive days (day 8-12) by i.t. application of 10µg HDM allergen. I.t. application of allergens in these models will be done under narcosis with isofluraan. The mice will be euthanized 3 days after the last challange (day 15) by an overdose Nembutal and the following readout

will be performed:
The following readouts will be performed:

• A set of N=6 mice per group will be used to measure the airway resistance after challenge with a metacholine (concentration curve 3.125, 6.25, 12.5, 25, 50, 100mg/mL metacholine in PBS) by FlexiVent. Metacholine challenge results in a faster increase in airway resistance in mice with allergic airway inflammation compared to control mice. One day after the last allergen challenge, mice will be anesthetized by i.p injection of ketamine (100mg/kg) and xylazine (10mg/kg), and afterwards the own breathing of the mouse is blocked by i.p. injection of pancuronium (0,8mg/kg in 100ul). A small incision is made in the trachea and a small tube is inserted that allows the connection of the mouse respiratory tract to the FlexiVent machine, which takes over the breathing during the measurement. After the measurement, the mice will be euthanized by an overdose of Nembutal. Then, the vena cava will be cut and venous blood will be collected for serum IgE measurements.

• A set of N=6 mice will be used for the assessment of cellular composition of the BAL fluid, the lungs and the medLNs (eosinophilia, neutrophilia, macrophages, T cells). The mice will be euthanized one day after the last challenge by an overdose of Nembutal. A tube will be inserted into the trachea and the lungs flushed with 3x1mL of PBS to collect the BAL fluid. Afterwards, the lungs will be flushed with ca. 50mL of Hanks buffer and collected together with the medLNs. One lobe of the lung will be used to measure cytokine and chemokine mRNA expression levels by PCR. In addition medLN mcells and the remainder of the lung and will be used for cell isolation for ex vivo restimulation assays.

• The lungs of a set of N=4 mice will be used for histology. The mice will be euthanized one day after the last challenge by an overdose Nembutal. A tube will be inserted into the trachea, the lungs will be inflated with 1mL of OCT and snap-frozen in liquid nitrogen. These lungs will be used for histological hematoxylin/eosin (H&E) and periodic acid-schiff (PAS) staining for assessment of lung inflammation.

The following time scheme will be applied for the HDM model:

day 1: sensitisation by i.t. application of 10 HDM allergen in 50µL PBS; control mice receive PBS only
day 8-12 : challenge by i.t. application of 10µg HDM allergen alone, control mice receive PBS
day 15: euthanasia by i.p. injection of an overdose Nembutal

Group 1: x-flox mice: Sensitization by PBS i.t., challenge by i.t. PBS N=14
(negative control)

Group 2: x-flox mice: Sensitisation by i.t. HDM , challenge by i.t. HDM N=16
(control asthmatic group)

Group 3: CD11c-cre+ x-flox mice: Sensitization by PBS i.t., challenge by i.t. PBS N=14
(negative control group)

Group 4: CD11c-cre+ x-flox mice: Sensitisation by i.t. HDM , challenge by i.t. HDM N=16
(Test asthmatic group)

The following C57BL/6 CD11c-cre 'x'-flox strains will be used, where 'x' will be a gene involved in:
1) AMPK signalling directly (for instance LKB1 and AMPK1)
2) mTOR signalling directly (for instance Raptor and Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 and PPARg)
4) metabolic regulation downstream of mTOR signalling: (for instance HIF1a and SREBP)

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role T cell polarization in the immunization model (part 2), will be evaluated using CD11c-cre flox mice in this model.

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

Based on our experience with the OVA/alum model, we expect a standard deviation of 13.5% for this type of experiments. We hope to observe a 25% difference between the experimental groups, and we set the power at 80% and determine p-value of 0.05. According to power analysis, we need 5 mice per group in order to obtain significant differences. From experience we know that about 80% about respond to the treatment and induce asthma. Therefore, we included one additional mice per group. 6 mice will be used for the measurement of airway hyper responsiveness by Flexivent, 6 mice for cell composition of different tissues by flow cytometry, for assessment of mRNA expression and ex vivo restimulations and 4 mice for histology.

B. The animals

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

C57BL/6 CD11c-cre+ / cre- 'x'-flox/flox, rederived in house, n=30 per strain (30 cre+ and 30 cre-) where 'x' will be a gene involved in:
1) AMPK signalling directly (for instance LKB1 or AMPK1)
2) mTOR signalling directly (for instance Raptor or Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 or PPARg)
4) metabolic regulation downstream of mTOR signalling: (for instance HIF1a or SREBP)
These mice will be used between 6-12 weeks of age and both male and females will be used.

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role in metabolism and the T cell priming and polarizing capacity of DC in the immunization model (part 2), will be evaluated using CD11c-cre flox mice, with a maximum of a total of 6 different target genes.

Breeding will be setup with homozygous flox breeding pairs of which one of the breeding pair is heterozygous for CD11c-cre, whereas the other will be CD11c-cre negative. Offspring should then be 50% CD11c-cre+ and 50% CD11c-cre negative. The CD11c-Cre negative littermates will be used as controls for CD11c-Cre+ mice in the same experiment

Total maximum number of mice: 6x60 cre/flox = 360 mice

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:
Research involving mice is essential for the proposed research. The immune responses involved in Allergic asthma are extremely complex involving multiple different players at different times. Furthermore, the metabolic properties of DCs and how they shape their immune polarizing function (which is the focus of this project), are expected to be largely dictated by the constantly changing micro-environment they reside in in situ. This cannot be mimmicked in vitro. Finally, these studies need to be

performed in mice, since this is the only model organism in which the tools are available to study DC-specific deletions in AMPK/mTOR signalling cascades. Therefore, this study can only be properly performed in vivo in mice.
Reduction:
To reduce the number of mice needed, only the transgenic mice that gave an altered Th2/Treg cell immune response in the immunization model will be tested in this Allergy model. Littermate controls (CD11c-Cre+ and CD11c-Cre-) will be used to reduce experimental variation and thereby reduce the number of mice needed per experimental group. Finally, experiments will be performed by staff that are highly skilled and experienced with animal handling. As a result all procedures with the animals will be performed with the greatest care and minimal animal distress will be ensured.
Refinement
Intranasal application of allergens and assessment of airway resistance will be performed under anaesthesia, which takes only a few minutes. If any of the animals develop any state described as humane endpoint in the next section, the animals will be humanely sacrificed.
Finally, experiments will be performed by highly skilled and experienced staff, which will reduce intra-experimental variation and as such will result in fewer animals that will be needed per experiment to obtain reliable and statistically significant data.

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

Intranasal application of allergens and assessment of airway resistance will be performed under anaesthesia, to minimize animal pain or fear. While we do not expect severe complications as a result of inducing allergic airway inflammation. In addition, the animals will be observed on a daily basis and be humanely sacrificed in case the following defined end points are reached: visible pain (abnormal posture and/or movement), significant weight loss (>20%), abnormal behaviour (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanized.

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.

The research question that is being addressed with the current set of experiments is novel and has not be studied before. An extensive literature search has revealed that these trangenic mice have not been used before in this kind of experimental model.

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

☒ 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.

Before intratrachial application of allergens, mice will be put under anaesthesia by using isoflurane (5%) mixed with oxygen. During the procedure, mice will be maintained under anaesthesia using a mouth piece. To ensure anaesthesia has set in well, rear foot reflex will be tested. During the maintenance phase isoflurane dosage is reduced (to 2-3%) to prevent overdosing.

I. Other aspects compromising the welfare of the animals

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

development of wheezing/shallow breathing

Explain why these effects may emerge.

Induction of Allergic asthma may reduce longfunction and cause bronchocontriction causing signs of wheezing

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

see humane endpoint

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.

Although human endpints are unlikely to be the animals will be observed on a daily basis and be humanely sacrificed in case the following defined end points are reached: visible pain (abnormal breathing, posture and/or movement), significant weight loss (>20%), abnormal behavior (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanised.

Indicate the likely incidence.

Very unlikely

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').

anaesthesia/recovery from anaesthesia: mild
development of allergic asthma: mild and in some specific cases moderate

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.

To analyse the immune response occurring in the lung and associated Lymph nodes, mice need to sacrificed. Lung inflammation based on histology can only be assessed after sacrificing the mice.

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

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

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.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'.	11600
1.2	Provide the name of the licenced establishment.	Leids Universitair Medisch Centrum
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: 9 Type of animal procedure: model of obesity induced type 2 diabetes

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.

Obesity is well-known to be a risk factor for the development of peripheral insulin resistance and type 2 diabetes. It is becoming increasingly clear that insulin resistance in metabolic tissues is for a large part determined by immune cells residing in these tissues. In obesity it has been shown that low grade inflammation in metabolic tissues (fat, liver and muscle) caused by a type 1 polarized immune cell infiltrate (Th1 and cytotoxic CD8 T cells, M1 macrophages, NK cells), promotes insulin resistance. Conversely, healthy metabolic homeostasis and good insulin sensitivity is underpinned by the presence ofTh2, Treg and M2 macrophages in these tissues. DCs appear to play an important role in regulating the polarization profile of these immune cells in metabolic tissues. Therefore a model of high fat diet induced obesity and induction of insulin resistance is an excellent model to establish whether mice that harbour DCs with defects in AMPK or mTOR signalling have an altered immune response in the metabolic tissues and whether they are more or less susceptible to obesity induced insulin resistance and development of type 2 diabetes .

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

Mice will be put either on normal chow or on high-fat diet (HFD, 40% of energy derived from fat) for 12 weeks. During these 12 weeks the following parameters will be determined:
Body weight: once every week
Food intake (per cage): continuous monitoring (by weighting food pellets)
Blood sampling (1/2 of a capillary, i.e 35 uL) on 4h fasted mice: wk 0, 6 and 12 for determination of fasting plasma glucose/insulin/lipids.

At week 10:
Insulin Tolerance Test. An i.p injection of insulin (0.75U/kg of 100mU/ml insulin dissolved in PBS with 0.2% BSA) will be performed in 4h-fasted mice, followed by determination of plasma glucose levels (with handmeter) at t=0, t=15, t=30, t=60, t=120 minutes by taking a drop of blood and collecting blood sample (1/3 of a capillary, i.e.~25uL) from the tail vein in order to determine plasma insulin levels.

At week 12:
Glucose tolerance test (GTT): An i.p injection of glucose (1.25 g/kg body weight of 20% D-glucose) will be performed in 4h-fasted mice, followed by determination of plasma glucose levels (with handmeter) at t=0, t=15, t=30, t=60, t=120 minutes by taking a drop of blood and collecting blood sample (1/3 of a capillary, i.e.~25uL) from the tail vein in order to determine plasma insulin levels.

At week 12: After 2-3 days of recovery post GTT, the animals will be killed by cervical dislocation and various peripheral organs (liver, white adipose tissues [gonadal, perirenal, subcutaneous], brown adipose tissue, skeletal muscle [gastrocnemius], heart, kidney and spleen), will be collected, weighted and divided in various parts for subsequent analysis (western blot, qPCR, Immunohistochemistry, and FACS for immune cell composition.)

In summary the following experimental groups will be used:
1) mice on normal chow diet n=10
2) mice on High fat diet n=12

The following C57BL/6 CD11c-cre 'x'-flox strains will be used, where 'x' will be a gene involved in:
1) AMPK signalling directly (for instance LKB1 and AMPK1)
2) mTOR signalling directly (for instance Raptor and Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 and PPARg)
4) metabolic regulation downstream of mTOR signalling: (for instance HIF1a and SREBP)

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role T cell polarization in the immunization model (part 2), will be evaluated using CD11c-cre flox mice in this model.

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

Various kind of parameters will be measured in this experiment, so we use the most variable one (GTT) in order to calculate the sample size (2 sided T-test).
Based on earlier findings (Hussaarts et al, Faseb J, 2015), we observed an in-group variation of 15% with this Test. We anticipate a difference between WT and knout mice of around 15%. We would need 10 mice per group, to be able have sufficient power (80%) to obtain significant differences (with a p = 0.05). Taken into consideration the 10-20% of non-responders to HFD and the 10-20%, we ask for using 12 animals per group.

B. The animals

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

C57BL/6 CD11c-cre+ / cre- 'x'-flox/flox, rederived in house, n=44 per strain (22 cre+ and 22 cre-) where 'x' will be a gene involved in:
1) AMPK signalling directly (for instance LKB1 or AMPK1)
2) mTOR signalling directly (for instance Raptor or Rictor)
3) metabolic regulation downstream of AMPK signalling (for instance PGC1 or PPARg)
4) metabolic regulation downstream of mTOR signalling: (for instance HIF1a or SREBP)
These mice will be used between 6-8 weeks of age and both male and females will be used.

Importantly, only genes present in the signalling pathways and downstream target genes that were identified to play a role in metabolism and the T cell priming and polarizing capacity of DC in the

immunization model (part 2), will be evaluated using CD11c-cre flox mice, with a maximum of a total of 6 different target genes.

Breeding will be setup with homozygous flox breeding pairs of which one of the breeding pair is heterozygous for CD11c-cre, whereas the other will be CD11c-cre negative. Offspring should then be 50% CD11c-cre+ and 50% CD11c-cre negative. The CD11c-Cre negative littermates will be used as controls for CD11c-Cre+ mice in the same experiment

Total maximum number of mice: 6x44 cre/flox = 264 mice

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:
Research involving mice is essential for the proposed research. The metabolic properties of DCs and how they shape their immune polarizing function (which is the focus of this project), are expected to be largely dictated by the constantly changing micro-environment they reside in in situ. This cannot be mimicked in vitro. Furthermore the effects on high fat diet on whole body metabolism and the metabolic regulation of an interplay between various tissues can only be studied in vivo. Finally, these studies need to be performed in mice, since this is the only model organism in which the tools are available to study DC-specific deletions in AMPK/mTOR signalling cascades. Therefore, this study can only be properly performed in vivo in mice.
Reduction:
To reduce the number of mice needed the Finally, littermate controls (CD11c-Cre+ and CD11c-Cre-) will be used to reduce experimental variation and thereby reduce the number of mice needed per experimental group. Finally, experiments will be performed by highly skilled and experienced staff, which will reduce intra-experimental variation and as such will result in fewer animals that will be needed per experiment to obtain reliable and statistically significant data.
Refinement
High-fat diet treatment will not induce any discomfort. If, however, any of the animals develop any state described as humane endpoint in the next section, the animals will be humanely sacrificed. Finally, experiments will be performed by staff that are highly skilled and experienced with animal handling. As a result all procedures with the animals will be performed with the greatest care and minimal animal distress will be ensured.

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

While we do not expect severe complications as a result HFD the animals will be observed on a daily basis and be humanely sacrificed in case the following defined end points are reached: visible pain (abnormal posture and/or movement, significant swelling of the foot and/or limping), significant weight loss (>20%), abnormal behaviour (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanized.

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.

The research question that is being addressed with the current set of experiments is novel and has not be studied before. An extensive literature search has revealed that these trangenic mice have not been used before in this kind of experimental model.

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

☒ 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?

none

Explain why these effects may emerge.

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

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of

humane endpoints to prevent further distress?

☐ No > Continue with question K.

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

Although human endpoints are very unlikely needed to be implemented, the animals will be observed on a daily basis by the animal caretakers and be humanely sacrificed in case the following defined end points are reached: visible pain (abnormal posture and/or movement), significant weight loss (>20%), abnormal behavior (isolation, abnormal reaction to stimuli, no food and water intake). If distress of the animals is observed by the animal caretakers, this will be reported to the investigators and according to the aforementioned criteria, the animals will be taken out of the experiment and euthanised.

Indicate the likely incidence.

Very unlikely

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').

High fat diet = mild
Determination of body weight = mild
Blood sampling = moderate
4h fasting for GTT and ITT: mild
i.p. injection of either glucose or insulin = mild
Cervical dislocation = mild

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.

To analyse the immune response occurring and metabolic profile of various tissues mice need to be sacrificed.

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

[redacted]

Van: PDC-LUMC-projectvergunning@Lumc.nl
Verzonden: dinsdag 1 december 2015 10:31
Aan: info@zbo-ccd.nl
CC: [redacted]
Onderwerp: RE: Aanvraag projectverguning AVD116002015253
Bijlagen: AVD116002915253 aanvullende informatie deel 3 en 4.docx

Opvolgingsmarkering: Opvolgen
Markeringsstatus: Voltooid

Categorieën: Dossier: [redacted]

Geachte mevrouw [redacted]

Graag maken wij gebruik om aanvullende informatie te geven die het oordeel van de CCD kunnen beïnvloeden en wellicht kunnen overtuigen de vergunningaanvraag in zijn geheel te honoreren. Wij zijn ons er van bewust dat hiermee de termijn ook verschoven wordt om de Commissie de mogelijkheid te geven dit te beoordelen.

Wij zijn van mening dat er in de beoordeling van de projectvergunningaanvraag een interpretatiefout gemaakt is en hopen u met de aanvullende informatie de juiste beoordeling te kunnen laten maken. Bijgevoegd treft u de aanvullende informatie van de onderzoeker aangaande de (on)afhankelijkheid van de delen 3 en 4 van de projectvergunningaanvraag.

Hopende op een positief besluit.
Met vriendelijke groet,
[redacted]
==========

[redacted]
Chair Animal Welfare Body

Leiden University Medical Center / Leiden University
PO Box 9600, Post-zone T7-P
2300 RC Leiden
Tel. [redacted]
e-mail: [redacted]

mail about IvD/AWB, Animal Welfare, Laboratory Animal Experiments: PDC-LUMC-IvD@lumc.nl
mail about Project Licences: PDC-LUMC-projectvergunning@lumc.nl
mail about Laboratory Animal Science Course: pdc-lumc/cursuspdk@lumc.nl

Intranet-site PDC

From: Info-zbo [mailto:info@zbo-ccd.nl]
Sent: woensdag 25 november 2015 13:01
To: Projectvergunningen Wet op de dierproeven (BEHDIV5
Subject: RE: Aanvraag projectverguning AVD116002015253

Beste Heer [redacted]
Bedankt voor uw vraag. In principe kunt u pas een bezwaar indienen op een beschikking nadat de beschikking verstuurd is. In uw geval zou dit u extra werk opleveren aangezien de beschikking pas verstuurd kan worden nadat u de NTS heeft aangepast. Daarom stel ik u in dit geval, naast de mogelijkheid om na ontvangst van het besluit bezwaar aan te tekenen, het volgende voor: Als u denkt dat wij een fout gemaakt hebben, of als er nieuwe feiten of omstandigheden zijn kunt u deze aangeven. Wij zullen dan inhoudelijk beoordelen of wij deze reactie voorleggen aan de CCD, met het verzoek uw aanvraag in heroverweging te nemen.

Wij moeten dan wel de termijn opschorten tot het hernieuwde oordeel van de CCD geveld is. Tot die tijd mag u dan dus niet beginnen met het project.

Indien u gebruik wilt maken van deze mogelijkheid en u dient uiterlijk 1 december 12.00 uur uw verzoek in, dan kan dit worden besproken in de eerstvolgende CCD-vergadering.

Met vriendelijke groet,

[redacted]

Centrale Commissie Dierproeven

Van: PDC-LUMC-projectvergunning@Lumc.nl [mailto:PDC-LUMC-projectvergunning@Lumc.nl]
Verzonden: maandag 23 november 2015 11:01
Aan: info@zbo-ccd.nl
Onderwerp: RE: Aanvraag projectverguning AVD116002015253

Geachte mevrouw [redacted]

Wij hebben uw bericht ontvangen en na overleg met de onderzoeker concluderen wij dat mogelijk de verwoording voor het 4e deel niet helder genoeg is. Echter voordat wij onze reactie geven, kunt u mij aangeven welke termijnen gebruikt zullen worden wanneer wij met uitleg toch de hele aanvraag vergund willen hebben?

Namens de vergunninghouder en onderzoeker,
Met vriendelijke groet,
[redacted]
Voorzitter IvD LUMC
==========

[redacted]
Chair Animal Welfare Body

Leiden University Medical Center / Leiden University
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mail about Project Licences: PDC-LUMC-projectvergunning@lumc.nl
mail about Laboratory Animal Science Course: pdc-lumc/cursuspdk@lumc.nl

Intranet-site PDC

From: Info-zbo [mailto:info@zbo-ccd.nl]
Sent: vrijdag 13 november 2015 14:15
To: [redacted]Projectvergunningen Wet op de dierproeven (BEHDIV5
Cc: [redacted]DIV4)
Subject: RE: Aanvraag projectverguning AVD116002015253

Beste Meneer [redacted]
Mogelijk heeft u onderstaande e-mail niet ontvangen wegens technische problemen in ons e-mail verkeer. Vandaar nogmaals dit bericht.

Met vriendelijke groet,

[redacted]

Van: Info-zbo [mailto:info@zbo-ccd.nl]
Verzonden: donderdag 12 november 2015 16:14
Aan: [redacted]; 'pdc-lumc-projectvergunning@lumc.nl'

CC: [redacted]'; info@zbo-ccd.nl
Onderwerp: Aanvraag projectverguning AVD116002015253

Beste meneer [redacted]

Dank voor het toezenden van de aangepaste aanvraag AVD116002015253 getiteld: “Metabolic control of dendritic cell-driven t cell polarization: dissecting underlying signals”. De CCD heeft uw aanvraag besproken.
Omdat het voor de CCD niet voldoende helder is wat exact in deel 4 zal worden uitgevoerd, zal de CCD alleen de eerste 3 delen van de projectaanvraag vergunnen. De experimenten uit te voeren in deel 4 zijn helder genoeg beschreven, maar omdat de uitvoer van deze experimenten afhankelijk lijken van de resultaten van de eerste 3 delen, is de CCD van mening dat nog onvoldoende bekend is over deze vierde stap om deze te kunnen beoordelen.

Vanwege deze gedeeltelijke vergunning wil ik u vragen de NTS hierop aan te passen, zowel in de beschrijvingen als in de dieraantallen. U kunt deze NTS aanleveren via NetFTP.

Ik realiseer me dat ik u eerder gevraagd heb het project en daarmee ook de NTS aan te passen, wat achteraf gezien misschien niet handig is gebleken. Echter, wij hebben u toch eerder deze vraag gesteld om het proces voor u zo snel mogelijk te laten verlopen, zodat de definitieve beslissing eerder uw kant op zou kunnen komen. In dit geval leidt dat helaas tot een tweede aanpassing van de NTS.

De behandeling van uw aanvraag wordt opgeschort tot het moment dat wij de nieuwe NTS hebben ontvangen. Zodra wij de aangepaste NTS van u hebben ontvangen (het is niet nodig het hele project opnieuw in te zenden) zullen wij u de definitieve beschikking toezenden.

Met vriendelijke groet,

[redacted]

Centrale Commissie Dierproeven **www.centralecommissiedierproeven.nl**

.....
Postbus 20401 | 2500 EK | Den Haag
.....
T: 0900 2800028
E: info@zbo-ccd.nl (Let op: nieuw e-mail adres)

Onlangs heeft de CCD de aanvraag AVD116002015253 getiteld “Metabolic control of dendritic cell-driven t cell polarization: dissecting underlying signals” besproken. U heeft ons laten weten dat de CCD alleen de eerste 3 delen van de projectaanvraag gaat vergunnen. Als we het goed begrijpen is dit omdat, ondanks dat de experimenten in deel 4 helder genoeg zijn beschreven, de uitvoer van deze experimenten afhankelijk lijkt van de resultaten van de eerste 3 delen, waardoor de CCD van mening dat nog onvoldoende bekend is over deze vierde stap om deze te kunnen beoordelen.

Wij zouden graag op dit voorlopige besluit willen reageren om hopelijk de CCD ervan te overtuigen de volledige projectaanvraag te vergunnen. De projectaanvraag bestaat uit 4 delen waarvan in deel 1 en 2 in vitro en lokale immunisatie experimenten staan beschreven. Op basis van deze eerste 2 delen zullen vervolgens deel 3 en 4 uitgevoerd worden waarin muizenmodellen staan beschreven van respectievelijk infectieuze en non-infectieuze ziektes. In de aanvraag hebben we deel 4 afhankelijk gemaakt van deel 3 door de introductie van een go/no-go moment. Wat we hier echter niet mee hebben willen impliceren is dat deel 4 van secundair belang is. In tegendeel, vanuit wetenschappelijk oogpunt zijn deel 3 en deel 4 twee gelijkwaardige onderdelen die elkaar juist complementeren: het ene beschrijft muizen modellen van infectieziektes terwijl het andere richt zich juist op modellen van non-infectieuze/niet overdraagbare ziektes. We willen hier graag benadrukken dat de ziektemodellen die in beide delen staan beschreven immunologisch zeer goed gekarakteriseerd zijn en daarom zorgvuldig zijn gekozen om de centrale vraagstelling van deze aanvraag te kunnen beantwoorden (= wat is de rol van AMPK/mTOR signalering in metabolisme van dendritische cellen (DCs) en daarmee in hun T cel activerende en polariserende eigenschappen). Namelijk, voor elk van de 6 modellen beschreven in deel 3 en 4, spelen DCs geactiveerde specifieke T cel responsen een centrale rol in de bescherming tegen of de beschreven infectieziektes (deel 3) of juist in de manifestatie van de beschreven veel voorkomende niet infectieuze ziektes (deel 4). Zo bieden in de infectiemodellen van onderdeel 3 (Listeria, Schistosoma en malaria infectie) respectievelijk een Th1, Th2/Treg en een cytotoxische T cel respons bescherming tegen deze ziektes, terwijl in de modellen beschreven in deel 4 (Type 2 diabetes, astma en kanker) een dysfunctie in respectievelijk diezelfde Th1, Th2/Treg en cytotoxische T cel responsen tot deze niet-overdraagbare ziektes kunnen leiden. Beide onderdelen zullen dus verschillende maar complementaire informatie opleveren over de rol van metabolisme in DCs in T cel polarisatie en daarmee pathogenese van zowel infectie- als niet-infectieuze ziektes met elk verschillende etiologiën. Al met al is deel 4 dus een cruciaal onderdeel van de aanvraag en essentieel om een volledig en afgerond beeld te krijgen van hoe belangrijk het metabolisme van DCs is hun T cel polariserende eigenschappen en daarmee de bescherming tegen, of ontwikkeling van verschillende ziektes, zowel infectieus als niet-infectieus.

Gezien de hierboven beschreven gelijkwaardigheid tussen deel 3 en 4, rijst wellicht de vraag waarom we dan deel 4 afhankelijk gemaakt hebben van deel 3 (= alleen deel 4 uit te voeren als er een fenotype in deel 3 te zien is) en bijvoorbeeld niet beide delen samen afhankelijk hebben gemaakt van deel 2. We hebben weloverwogen voor een afhankelijkheid gekozen tussen deel 3 en 4 om het aantal experimentele dieren tot het minimum te beperken wat nodig is om de vraagstelling te kunnen beantwoorden. In de praktijk zal dit betekenen dat wanneer blijkt dat een transgene muizenlijn in een der modellen in deel 3 geen verwachte resultaten oplevert (= geen fenotype), zal het complementaire model in deel 4, betreffende hetzelfde type T cel respons, niet uitgevoerd worden. Dus concreet, als bijvoorbeeld in deel 3 mTOR signalering belangrijk blijkt te zijn voor Th2 sturing door DCs (op basis *S. mansoni* infectie) maar niet Th1 of CD8 T cel responsen (op basis van Listeria en malaria infectie), dan zal alleen het model in deel 4 getest worden waarin Th2 polarisatie belangrijk is voor pathogenese (in dit geval astma). Deze strategie zorgt ervoor dat geen experimenten ingezet zullen worden waarvoor er een aanwijzing bestaat dat de verwachte resultaten niet behaald kunnen worden en garandeert daarmee een minimalisering van het benodigde aantal proefdieren, met maximaal resultaat. Wat we graag willen benadrukken is dat, terwijl deze afhankelijkheid op zich als duidelijk doel heeft het aantal proefdieren te beperken, de volgorde van deze afhankelijkheid tussen deel 4 en deel 3 in zekere zin willekeurig gekozen is, aangezien beide onderdelen op gelijke voet staan en niet vanuit wetenschappelijk oogpunt van elkaar afhankelijk zijn. Met andere woorden; we hadden dit ook kunnen omkeren en dus deel 3 afhankelijk kunnen maken van deel 4. We zien in dat dit misschien niet helder is verwoord in de aanvraag, maar hopen dat met dit nieuwe

inzicht de CCD begrijpt dat het ook hierom niet logisch zou zijn als deel 3 wel, maar deel 4 niet vergund zou worden.

Wij hopen dat de CCD als gevolg van de bovengenoemde uitleg in de projectaanvraag, zijn huidige beslissing in deze wil heroverwegen en de volledige projectaanvraag alsnog wil honoreren.

Wij hopen u hiermee voldoende te hebben geïnformeerd.

Met vriendelijke groeten,

[redacted]

Centrale Commissie Dierproeven

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Onze referentie
Aanvraagnummer
AVD116002015253

Bijlagen
1

17 DEC. 2015

Datum
Betreft Aanvraag projectvergunning dierproeven

Geachte [redacted]
Op 1 oktober 2015 hebben wij digitaal uw projectvoorstel voor een projectvergunning dierproeven ontvangen. Op 6 oktober hebben wij per post het aanvraagformulier ontvangen. Het gaat om uw project "Metabolic control of dendritic cell-driven t cell polarization: dissecting underlying signals" met aanvraagnummer AVD116002015253. Wij hebben uw aanvraag beoordeeld.

Op 6 november 2015 heeft u uw aanvraag aangevuld en herzien. Op verzoek van de CCD heeft u de NTS en de projectaanvraag inclusief bijlagen aangepast. Op 2 december heeft u aanvullende informatie ingediend betreffende de toetsbare eenheid van de aanvraag. Deze aanvullende informatie is betrokken bij de beoordeling van de aanvraag.

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. Deze voorwaarden zijn algemene voorwaarden. De algemene voorwaarde betreffende artikel 10, lid 1a van de wet wordt gesteld bij vergunningen met een langere looptijd. Dit om te voldoen aan datgene wat volgt uit dit artikel. De algemene voorwaarde betreffende instemming van go / no-go momenten door de IvD wordt gesteld om onnodige inzet van proefdieren te voorkomen.
U kunt met uw project "Metabolic control of dendritic cell-driven t cell polarization: dissecting underlying signals" starten. De vergunning wordt afgegeven van 17 december 2015 tot en met 1 november 2020. De begindatum van deze vergunning wijkt af van de aangevraagde datum omdat deze in het verleden ligt. 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.

Procedure
Bij uw aanvraag heeft u een advies van de Dierexperimentencommissie DEC Leiden gevoegd. Dit advies is opgesteld op 18 september 2015. 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 16 oktober 2015 heeft de DEC gereageerd op onze vragen. Deze vragen betroffen de noodzaak van anesthesie bij bepaalde handelingen en het aantal reservedieren in controlegroepen.
Wij kunnen ons vinden in de inhoud van het advies van de Dierexperimentencommissie. Wij nemen dit advies van de commissie over, inclusief de daaraan ten grondslag liggende motivering, met uitzondering van de afwijkingen zoals hierboven gemotiveerd.

Dit advies en de in de bijlage opgenomen beschrijving van de artikelen van de wet- en regelgeving zijn de grondslag van dit besluit.

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

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

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

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

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

De Centrale Commissie Dierproeven
namens deze:

[redacted]

ir. G. de Peuter
Algemeen Secretaris

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

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. In dit project worden dierproeven toegepast waarbij die vallen in de categorie ernstig volgens artikel 10b van de wet en wordt daarom voorzien van beoordeling achteraf.
Deze beoordeling zal uiterlijk 01 november 2021 plaatsvinden. Er zal dan beoordeeld worden of de

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[redacted]

Van: Info-zbo
Verzonden: donderdag 17 december 2015 14:38
Aan: [redacted] 'pdc-lumc-projectvergunning@lumc.nl'
CC: [redacted]
Onderwerp: Beschikking AVD116002015253
Bijlagen: Projectvergunning en Decadvies.pdf; Beschikking116002015253.pdf

Geachte heer, mevrouw,

Deze beschikking is ook per post verstuurd.

Met vriendelijke groet,

Centrale Commissie Dierproeven www.centralecommissiedierproeven.nl
Nationaal Comité advies dierproevenbeleid www.ncadierproevenbeleid.nl
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