Instantie voor
Dierenwelzijn
Utrecht

	Dossier: AVD1150020174288	
		Aanwezig
1	NTS	X
2	Aanvraagformulier	X
3	Projectvoorstel	X
4	Bijlage beschrijving dierproeven	4X
5	DEC-advies	X
6	Ontvangstbevestiging	X
	Evt. Vragen CCD aan aanvrager	X
	Evt. antwoorden aanvrager	X
7	Beschikking en vergunning	X
	Beoordeling achteraf	X

Centrale Commissie Dierproeven

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	Immuun receptor therapie tegen kanker: effectief en veilig
1.2	Looptijd van het project	5 jaar
1.3	Trefwoorden (maximaal 5)	Immuuntherapie, kanker, immuun receptor

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)

Kanker is een van de belangrijkste doodsoorzaken in de westerse wereld. Kanker is een verzamelnaam voor een zeer gevarieerde ziekte, met als gemeenschappelijk deler een ontspoorde groei van cellen waardoor de normale lichaamsfuncties worden verstoord en de patiënt kan komen te overlijden. In dit project willen we onderzoek doen naar nieuwe en verbeterde therapieën, die als uitgangspunt hebben om het immuunsysteem te richten tegen de tumor en eventuele uitzaaiingen. Het immuunsysteem is vaak verzwakt in de patiënt en ons uitgangspunt is dat door slim gebruik te maken van de anti-tumor eigenschappen van het immuunsysteem we een nieuwe generatie immuuntherapie kunnen ontwikkelen voor een brede patiënten groep.
Door de infrastructuur in onze onderzoeksafdeling, van fundamenteel via preklinisch tot klinisch onderzoek, hebben we een unieke onderzoekslijn

		opgezet om deze nieuwe therapieën naar de patiënt te brengen.
3.2	Welke opbrengsten worden van dit project verwacht en hoe dragen deze bij aan het wetenschappelijke en/of maatschappelijke belang?	Wij verwachten met het onderzoek in dieren de noodzakelijke inzichten te verkrijgen om daadwerkelijk een meer effectieve en veilige immuuntherapie te kunnen introduceren in de klinische praktijk.
3.3	Welke diersoorten en geschatte aantallen zullen worden gebruikt?	Dit onderzoek gebruikt immuundeficiënte muizen (muizen zonder een werkend immuunsysteem) die ook genetisch aangepast kunnen zijn om de humane therapie en de werkingsmechanismen nog beter te kunnen evalueren. We verwachten 10.050 muizen nodig te hebben voor de komende 5 jaar.
3.4	Wat zijn bij dit project de verwachte negatieve gevolgen voor het welzijn van de proefdieren?	De muizen zullen tumoren krijgen in de meeste experimenten. De plaats en grootte van de tumor zullen het ongerief bepalen. Een oppervlakkig groeiende tumor (onder de huid) zal leiden tot gering of matig ongerief en een inwendige tumor in het beenmerg of in vitale organen zal kunnen leiden tot ernstig ongerief gelijk als in de patiënt. De handelingen met de dieren (meten van de tumor, injecties) zullen leiden tot matig ongerief, de behandeling kan bijwerkingen hebben en leiden tot ongerief.
3.5	Hoe worden de dierproeven in het project ingedeeld naar de verwachte ernst?	55% licht; 33% matig en 12% ernstig ongerief
3.6	Wat is de bestemming van de dieren na afloop?	De dieren zullen aan het einde van de experimenten worden gedood en organen worden verzameld om verdere laboratorium testen mee te kunnen uitvoeren die resulteren in inzichten in de interactie van de therapie en de tumor.

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.	In het laboratorium ontwikkelen we complexe *in vitro* modellen om de receptor immuuntherapie te testen zonder het gebruik van muizen. Dit zijn bijvoorbeeld 3D structuren van mini organen en tumoren. Maar door de complexiteit van de ziekte, kunnen we tot op heden de complexe interactie tussen immuuntherapie en tumoren het beste nabootsen in een levend organisme met een werkende bloedvoorziening waarbij de mate waarin de therapie de tumor kan bereiken wordt meegenomen in het geheel. Ook de tijdsduur van de dierexperimenten kan niet worden geëvenaard in *in vitro* systemen. En immuuntherapie wordt juist ontwikkeld om, in tegenstelling tot klassieke therapieën tegen kanker, een langdurige effect te bewerkstelligen.

4.2	**Vermindering** Leg uit hoe kan worden verzekerd dat een zo gering mogelijk aantal dieren wordt gebruikt.	Alleen indien de kandidaat therapie geselecteerd wordt na een uitvoerige *in vitro* test periode wordt deze verder getest en ontwikkeld in de muismodellen. De minimale groepsgrootte nodig om een wetenschappelijke vraag te beantwoorden, wordt bepaald door het gebruik van statistische berekeningen. Om het aantal gebruikte dieren te beperken worden alleen die tumormodellen toegepast met een robuuste en consistente tumorgroei.
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.	Naast het feit dat muizen hanteerbaar en relatief eenvoudig te huisvesten zijn, zijn er vele genetische stammen beschikbaar geschikt voor ons type onderzoek die men niet beschikbaar heeft voor andere diersoorten. Doordat wij zogenaamde gehumaniseerde muismodellen gebruiken (met humane tumoren en humane immuun cellen), kunnen we redelijk eenvoudig de vertaalslag naar de mens maken.
	Vermeld welke algemene maatregelen genomen worden om de negatieve (schadelijke) gevolgen voor het welzijn van de proefdieren zo beperkt mogelijk te houden.	Dagelijkse observatie van de dieren vindt plaats in combinatie met pijnbestrijding en verdoving waar dat nodig is. De dieren zijn gehuisvest in kooien met kooiverrijking en in groepjes muizen bij elkaar. Een welzijnsmonitoring systeem specifiek voor onze experimenten is aanwezig en indien er onverwachte veranderingen in het welzijn van de dieren optreden, worden extra controles ingevoerd om te controleren of het humane eindpunt al is bereikt.

5 In te vullen door de CCD

Publicatie datum	
Beoordeling achteraf	
Andere opmerkingen	

Centrale Con

Aanvraag
Projectvergunning Dierproeven
Administratieve gegevens

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

1 Gegevens aanvrager

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

☒ Ja > *Vul uw deelnemernummer in* 11500

☐ Nee > *U kunt geen aanvraag doen*

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

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

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

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

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	[redacted]	☐ Dhr. ☒ Mw.
Functie	Wetenschappelijk projectleider	
Afdeling	Laboratory of Translational Immunology	
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	[redacted] ☒ Dhr. ☐ Mw.
		Functie	Principal investigato
		Afdeling	Hematology and Laboratory of Translational Immunology
		Telefoonnummer	[redacted]
		E-mailadres	[redacted]
1.7	Is er voor deze projectaanvraag een gemachtigde?	☐ Ja > *Stuur dan het ingevulde formulier Melding Machtiging mee met deze aanvraag*	
		☒ Nee	

2 Over uw aanvraag

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

3 Over uw project

3.1	Wat is de geplande start- en einddatum van het project?	Startdatum	1 - 2 - 2018
		Einddatum	31 - 1 - 2023
3.2	Wat is de titel van het project?	Immune receptor mediated control of tumors	
3.3	Wat is de titel van de niet-technische samenvatting?	Immuun receptor therapie tegen kanker	
3.4	Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?	Naam DEC	DEC Utrecht
		Postadres	Postbus 85500 3508 GA Utrecht
		E-mailadres	dec-utrecht@umcutrecht.nl

4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

☒ Nieuwe aanvraag Projectvergunning € 1684 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

Plaats Utrecht

Datum 04-12-2017

Handtekening [redacted]

Form
Project proposal

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	11500
1.2	Provide the name of the licenced establishment.	UMC Utrecht
1.3	Provide the title of the project.	Immune receptor mediated control of tumours

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.

Background / context

The focus of our laboratory is the development of immune receptor-based immunotherapy against cancer. Cancer immunotherapy has been considered as scientific Breakthrough of the Year for 2013 (Couzin-Frankel, Science 2013;342:1432). This form of therapy received enormous scientific but also

public attention as a result of the clinical successes of checkpoint inhibitors (a class of antibodies) in many types of cancer (Wolchok et al. NEJM 2013;369:122) and Chimeric Antigen Receptor T cells (CAR T cells, a class of engineered T cells) in B cell malignancies (Maude et al. Blood 2017;125:4017). Classical immunotherapies are most successful in tumour types with high mutational load, i.e. those tumours that harbor many genetic alterations as compared to healthy cells. But these strategies are less successful in tumours with low mutational load such as acute myeloid leukemia (AML) and neuroblastoma or intermediate mutational load such as prostate, breast and ovarian cancer (Schreiber and Schumacher, Science 2015;348:69). For those tumour types an interesting alternative therapeutic approach is to use metabolic cancer targeting instead of targeting tumour-specific mutations. One layer of daily cancer immune surveillance consists of gamma/delta T cells (γδT cells), which appear to be more potent than many other subpopulations (Gentles et al. Nat Med 2015;21:938). The major power of γδT cells arises from the fact that γδT cells see cancer not as a genetic, but as a metabolic disease (Gober et al. J Exp Med 2003; 197:163). In addition, γδT cells have unique features of both the innate and adaptive immune system. Innate features include Major Histocompatibility Complex (MHC) independent cellular activation and antigen presenting capacities. Whereas adaptive features of γδT cells include clonal expansion and the formation of immunological memory. γδT cells can recognize and are able to target a broad range of tumour types including haematological and solid tumours (Marcu-Malina et al. Blood 2011;118:50). Importantly, our group showed that the γδT cell receptor (γδTCR) itself is able to distinguish between healthy and malignant stem cells, simply by detecting subtle changes in lipid metabolism (Sebestyen et al. Cell reports 2016;15:1973). These features allow not only a broad clinical application of γδT cells but add a treatment option for those tumour types with low mutational load that do not respond to current available strategies, such as checkpoint inhibitors or classical CAR T cells. Our laboratory has developed the concept of metabolic cancer targeting with TEGs: T cells engineered to Express a defined γδTCR (Gruender et al. Blood 2012;120:5153 and Straetemans et al. Clin Can Res 2015;21:3957). In short, patient derived (autologous) T cells are *ex vivo* engineered to express the tumour-specific γδTCR, cultured until sufficient cell numbers, purified and reinfused into the patient. This led to the initiation of a phase I clinical trial with TEG001 to treat patients with acute myeloid leukaemia and multiple myeloma that have no remaining treatment options.

In our laboratory we have established a research pipeline that aims to increase our knowledge on the mechanism of action of immune receptors for metabolic cancer targeting and to bring the next generation of immune receptor-based immunotherapy against cancer to the patient (see for a schematic overview figure 1).
The first compound, TEG001, was selected through this pipeline and will enter a phase I clinical trial next year. To illustrate the research pipeline and the criteria for compound selection, the development and selection of this first compound is shortly described below.
A) First, using (semi)high-throughput immunological assays a panel of γδT cell clones was screened for their anti-tumour potential. This was measured by cytotoxic activity and cytokine production. When γδT cell clones were targeting a broad panel of tumours, molecular techniques were used to identify their unique gamma/delta T cell receptor (γδTCR) DNA sequence. Immune receptor gene transfer techniques were used to further select potential receptors. To this end the γδTCR DNA was introduced in conventional αβT cells derived from a healthy donor by the use of a retroviral transduction procedure (Marcu-Malina et al. Blood 2011;118:50 and Gruender et al. Blood 2012;120:5153). We have observed that differential tumour recognition can be attributed to the γδTCR gene and can be maintained after transfer of these genes into αβT cells.
B) The platform of immune receptor usage was named TEG (T cells engineered to express a defined γδTCR). Retroviral gene expression and transduction, culture conditions and purification of engineered immune cells were optimized resulting in a GMP-grade *ex vivo* production protocol for TEGs (Straetemans et al. Clin Can Res 2015;21:3957). Sufficient surface expression of the γδTCR, cell yield and purity of TEGs defined the selection towards the functional testing in phase C.
C) A limited panel of candidate TEGs were further used *in vitro* to study the mechanism of action (Gruender et al. Blood 2012;120:5153 and Sebestyen et al. Cell Reports 2016;15:1973) as well as the efficacy and the safety in tumour or healthy target co-culture assays and in more complicated 3D-bone marrow tumour model systems (main read-outs: cytotoxicity and cytokine production). Importantly, we have designed experiments that allow in similar model systems the evaluation of TEGs against tumour cells and healthy cells side by side, as requested by the regulatory authorities. In this phase the number

of candidate receptors was dependent on the assays used, but generally limited to 4 selected receptors including controls.
D) Go/no go moment: If the immune response against a broad panel of tumour targets was confirmed and healthy cells were not targeted and if the platform of immune receptor usage (in this case TEG platform) was stable and suitable for *in vivo* studies, TEGs were selected for evaluation in pre-clinical mouse models.
E) This limited panel of TEGs was evaluated for their anti-tumour activity in pre-clinical mouse models of leukaemia. The leukaemia models of choice were selected based on the following criteria: *in vitro* anti-tumour effector function of TEGs (cytotoxicity and cytokine production), tumour growth data in the specific mouse strain (scientific literature or data from collaborations) and were optimized for the specific application of TEGs (Marcu-Malina et al. Blood 2011;118:50). A series of efficacy-toxicity balance studies have been performed demonstrating that TEG001 cells are able to differentiate between malignant cells versus healthy cells within one model. It was observed that the particular γδT cell receptor sequence and *ex vivo* production procedure influenced the anti-tumour efficacy of TEGs in these models (Gruender et al. Blood 2012;120:5153 and Straetemans et al. Clin Can Res 2015;21:3957). In addition, the same models were applied to engraft the healthy human counterpart cells for targeting by TEG001 cells to test off-target toxicity. No off-target toxicity was observed.
5) Finally, TEG001 lead compound was selected based on its haematological tumour recognition profile and safety profile in the different *in vitro* and *in vivo* efficacy-safety balance assays performed. The TEG001 application including the pre-clinical mouse tumour model data was approved by the Dutch regulatory authorities (Centrale Commissie Mensgebonden Onderzoek (CCMO)) for testing TEG001 in a phase I clinical trial in man. Results from the clinical trial will be used to evaluate the preclinical development strategy, including the translational value of all *in vitro* and *in vivo* models used.

Next generation immunotherapy
In the meantime that we are taking our first receptor-based compound to the clinic, our research is focussed on constant optimizing and implementing new knowledge into the next generation of immunotherapy against cancer using immune receptors that target cancer as a metabolic disease. The working mechanisms of this class of immune receptors are not yet fully understood and we have found that defined receptors work best against certain tumour types. Moreover, the platform and TEG format in which the receptors are used are developed implementing the most resent knowledge in the field. And finally, we believe that combining treatment options would ultimately be significantly improving treatment outcome.

Figure 1 and the legend to figure 1 describe in detail the research pipeline applied in our laboratory for next generation immunotherapy compounds.

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 is to develop the next generation effective and safe anti-cancer immunotherapy based on defined immune receptors. This project focuses on the part of the research pipeline that uses preclinical mouse models for answering the following sub questions:

1. **Testing clinical candidate immune receptors in TEG format, in [redacted] or [redacted] on [redacted] against a defined panel of tumour types for anti-tumour efficacy.**
2. **Elucidate the biological mechanism of action and the mechanisms responsible for bio-distribution.**

3. **Enhancing efficacy of candidate immune receptors by design of combination therapy.**
4. **Evaluate the safety profile of candidate immune receptor-based therapy.**
5. **Defining the optimal criteria for (*ex vivo*) GMP-grade immune receptor production based on their *in vivo* anti-tumour activity, safety profile and/or long-term persistence.**

To bring the next generation immune receptor-based therapy into the clinic, efficacy and safety testing including anti-tumour responses, long-term persistence (up to 6-12 months) and homing of TEGs as monotherapy or in combination with other compounds that are readily available or developed in our laboratory will be assessed in pre-clinical mouse models. Furthermore we explore other immune receptors to use in TEG format and design additional platforms for immune-receptor-based immunotherapy *in vivo*.
These aims can be achieved in this project because of the following reasons:
-Our laboratory has a proven track record in the development of receptor based immune therapy towards a phase I clinical trial including the acquirement of necessary *in vitro* and *in vivo* data in order to receive permission of the authorities to start a phase I clinical trial.
-There are long-standing international collaborations in the field of transplantation (Kaneko et al. Blood 2009;113:1006), cellular immunotherapy (Schmitt et al Clin Can Res 2015;21:5191 and Provasi et al. Nat Med 2012;18:807) and the γδT cell field (Silvas-Santos et al., Nat Rev Imm 2015;15:683). These collaborating scientists are experts in the field of mouse models for immune therapy against cancer.
-Within the University, we have close collaborations with key scientists that developed organoid (tumour) models and are currently exploring the *in vitro* as well as *in vivo* potential of these models for immune receptor based TEG therapy (Fumagalli et al. PNAS 2017;114:E2357). In addition, we have established a collaboration with a research group that has key-expertise in animal pathology and they assist in the performance of histopathology research. Within our own department 2-photon *in vivo* imaging techniques are established and we will apply this technique in the *in vivo* models (Ritsma et al. Science Transl Med 2012;4:158)
-Ultimately, the preclinical *in vivo* studies will contribute to the design of clinical treatment protocols in phase I/II studies to treat cancer. We are therefore in close communication with clinicians, in fact the PI is an MD and a direct need from clinical perspective feeds into our research line (bedside-to-bench-to-bedside). Scientific meetings between scientists and clinicians are organized on weekly basis. In addition, the Cell Therapy Facility, where the GMP-grade cellular products are produced is greatly integrated in our meeting structures resulting in a close network of key expertise's to bring the next generation receptor-based immunotherapy to the clinic.
-Since we have experienced the complete TEG001 compound development process towards a phase I trial, including regulatory approval, we are aware of data that are required in this relative young field of cellular medicine. In the meantime we are in constant communication with the appropriate authorities and regulatory experts in the field to remain updated on the required data for the next generation compounds that are being developed in the research pipeline.

3.3 Relevance

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

Standard chemotherapy, small molecules and radiation therapy are for many tumour types not successful in eradication of metastized tumours due to the occurrence of therapy-resistant tumour variants. In contrast, immunotherapy has shown dramatic effects in metastic melanoma patients or end stage leukemic patients (Wolchock et al. NEJM 2013; 369:122). A major advantage of immunotherapy is the specific targeting of tumours without harming healthy cells and the possible induction of immunological memory. But, even in the era of advanced immunotherapy of cancer, there are solid and haematological tumour types that remain largely resistant to classical immunotherapy. However, for those tumour types γδT cells and their immune receptors provide a novel and promising treatment alternative by targeting the metabolic alterations in malignant cells and leaving healthy cells unharmed. Treatment of patients with TEGs may not only reduce treatment costs (single treatment), but also reduces the need for invasive surgical treatments. An alternative treatment scenario may be treatment of advanced-stage cancer patients leading to reduction of tumour burden and making patients eligible for

surgery in case of solid tumours or stem cell transplantations in case of haematological malignancies.

In addition to an improved immune receptor-based therapy, this project will lead to a better understanding of the biology of targeting mechanisms utilized by defined immune receptors. Although we are primarily focussing on the targeting of tumours in this project, our laboratory has shown that these targeting mechanisms are involved in viral infection clearance as well (Scheper et al. Leukemia 2013; 27:1328) and are of importance in stem cell transplantation immunology, when patients' immune system is temporarily impaired. Therefore, acquired knowledge is of value not only in the field of tumour immunology but also in basic immunology, infection and stem cell transplantation medicine. This will result in improved treatment strategies not only for cancer patients but also in case of infections and will contribute to improve stem cell transplantation treatment regiments.

3.4 Research strategy

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

In our laboratory we have established a pipeline of research aiming to develop next generation immune receptor-based immune therapy against cancer to the patient (see Figure 1). The pre-clinical mouse models are imbedded in this research pipeline and here described in more detail.

First, the mouse experiments are placed in their context.

1. Testing different clinical candidate immune receptors for anti-tumour efficacy

In our laboratory we are selecting candidate immune receptors (mainly γδT cell receptors) for their anti-tumour activity *in vitro* in order to improve efficacy or broaden the possible patient population that we can treat with TEG cells. We have found that some tumours are better targeted by the one but not by the other receptor. An example is a receptor published previously (Scheper et al. Leukemia 2013;27:1328) and part of ongoing fundamental as well as pre-clinical research. Also high-throughput screening techniques have been established to search for alternative or additional immune receptors that can be used for clinical application. The platform that is best for clinical application of defined immune receptors such as the described TEG format, [redacted] or [redacted] with [redacted] will be defined *in vitro*.

Based on their cytotoxic potential *in vitro* (read-out cytotoxicity and cytokine production, see research pipeline figure 1) the most promising receptor format(s) will be developed towards pre-clinical testing in humanized mouse models. Tumour growth reduction and/or increased survival compared to control treatment and immune cell persistence in peripheral blood are the main read-outs for efficacy testing. In order to test long-term efficacy of immune therapy, immunological memory will be evaluated. In case tumour clearance is observed after therapy, mice will be rechallenged with tumour cells to evaluate if immunological memory has been formed (Straetemans et al. Clin Can Res 2015;21:3957). In case tumour cells were responding initially to the therapy but escape at a later stage, the tumours are collected and used for further *in vitro* studies or to transplant the tumour into naïve mice (comparable model as initial model) for treatment to test if intrinsic tumour cell properties (epigenetic mechanisms) or tumour microenvironment is responsible for tumour immune escape (Straetemans et al. Mol Ther 2015;23:396). Control immune receptor engineered cells (TEGs, classical CAR T cells or αβTCR-engineered T cells) (Kuball et al. Blood 2007;109:2331 and Straetemans et al. Mol Ther 2015;23:396) are taken along in experiments throughout the research pipeline in order to evaluate the additional value of our strategy or as positive or negative controls depending on the context and research question.

2. Biological mechanisms: mechanism of action, persistence and biodistribution *in vivo*

In addition to anti-tumour efficacy in each mouse experiment, biological mechanisms are studies as well. Although we are taking our first TEG product to the clinic, there are many open questions remain which could jeopardize a rapid clinical implementation. First, though the target molecule (CD277) has been described recently for the receptor used in TEG001, the exact recognition pathway and its regulation is poorly understood. Although the target molecule is expressed on every cell, the delicate balance between recognizing tumour cells and healthy tissues remains a key question. For other receptors even more questions remain regarding the mechanism of action (Scheper et al. Leukemia 2013;27:1328). These

fundamental questions are studied *in vitro*, however the humanized mouse models add valuable information. For example, in the humanized mouse models the presence of immune receptors (in TEG cells) and their characteristics are studied and can answer questions such as which receptor allows TEG subsets to remain in the circulation (long-term persistence) or which migrate to the tumour site and are effective against the tumour? This can be measured in peripheral blood by flow cytometry or by molecular techniques such as q-PCR, or at the end of the experiment in the tumour or different organs (biodistribution) by histopathological methods or molecular DNA or RNA-based sequencing techniques. Migration to the tumour site and cell-cell interactions will be visualized with intravital 2-photon imaging techniques in a small proportion of animals.

3. Combination therapies (*in vitro* and *in vivo*)

As immunotherapy has reached clinical practice, it becomes obvious that non-responding or relapsed patients may benefit from combining classes of immunotherapy (Vanneman and Dranehoff Nat Rev Canc 2012;12:237). Not only to increase the first hit of anti-tumour response but also to overcome local mechanisms of tolerance and prevent tumour escape from immunotherapy. In case of haematological malignancies the bone marrow microenvironment has been previously suggested to be responsible for immune escape (Mussai et al. Blood 2013;122:749) and several cellular components in the milieu of mostly solid tumours have been studied for their role in suppressing immune therapies (Gajewski et al Nat Imm 2013;14:1014). The expression of [redacted] molecules on primary tumour cells is one of the key resistance mechanism including [redacted] and [redacted] pathways [redacted]. These [redacted] pathways can be [redacted] by [redacted] these molecules, and are interesting examples to implement in the TEG treatment protocol to improve anti-tumour efficacy as demonstrated successful in pre-clinical CAR T cell mouse models [redacted]

4. Safety profile of candidate immune receptor-based therapy

Regulatory authorities for medicinal products recognize that classical (according to OECD guidelines) toxicity studies do not apply for cellular immune receptor based therapy (ATMPs = advanced therapeutic medicinal products), a non-clinical development program has been designed which compares safety and toxicity directly to efficacy by the use of efficacy-toxicity balance studies. A series of these efficacy-toxicity balance studies *in vitro* and *in vivo* have been performed demonstrating that the first TEG compound is able to differentiate between malignant cells versus healthy cells within one model. We will evaluate potential off-target effects *in vivo* as described here. First, if available, immune deficient mice transgenic for the human target molecule are used to evaluate the balance between recognizing healthy cells or tumour cells. Second, if transgenic mice are not available, human healthy cells can be engrafted in immune deficient mice, like their tumour counterpart cells. Once these human cells are engrafted, immune receptor therapy can be applied and the effect on human healthy cells can be evaluated. Examples of human cells that can be engrafted are hematopoietic stem cells (Ratliff *et al.* J Immunol, 2015, 194: 940–949 and our own unpublished results) or Mesenchymal Stromal Cells or Epithelial Progenitor Cells. Monitoring of the presence/targeting of healthy human cells in peripheral blood or organs is the main read-out (flow cytometry, histopathology). Comparable read-outs apply in peripheral blood, tumour site and/or organs as described above. In all safety experiments a clinical scoring system is applied.

A possible safety concern, in case allogenic donor cells are used as vehicle for immune receptors, (a realistic treatment option for heamatological cancer patients) is alloreactivity of the cellular medicine. Potential alloreactivity can be measured in immune deficient mouse graft versus host disease (GVHD) models. The potent alloreactivity of the endogenous immune receptor (αβTCR) on the donor cells can lead to xenoreactivity in mice and these models are used to study these adverse effects of cellular therapy. A clinical scoring system focussed on GVHD symptoms is applied in these experiments.

5. Define criteria for product characteristics to increase long-term anti-tumour effectivity (*in vitro* and *in vivo*). Although, we have established a GMP-grade *ex vivo* production protocol for TEGs in the planned clinical trial, improvement of the protocol is a constant line of research. The *ex vivo* production procedure of receptor engineered cells influences the *in vivo* performance of the cells once adoptively transferred into the patient. The *in vivo* performance is usually defined by long-term anti-tumour control. In order to

have long-term tumour control, engineered immune cells (such as TEGs) need to persist (remain viable and functional) in the host. This is defined as persistence. A way of improving long-term persistence and long-term anti-tumour activity in patients is by optimizing the *ex vivo* production procedure. For instance, the compounds and conditions involved in culturing, like the choice of start material, cytokines, media and many more may influence the *in vivo* performance of adoptively transferred engineered immune cells. Also the ratio of cellular subsets (i.e. CD4+ / CD8+ T cells) or differentiation status of immune cells may have an effect on performance *in vivo*. Since immunological *in vitro* assays are classically designed to evaluate rapid (within 24h) anti-tumour effector responses, *in vitro* assays are not (yet) available to evaluate long-term (months) anti-tumour control including immunological memory (Gattinoni et al. J. Clin. Invest. 2005;115:1616). So far, pre-clinical mouse models are therefore the golden standard in immune receptor-based therapy against cancer for studying long-term anti-tumour effects and immune cell persistence (Zitvogel et al. Nat Rev Cancer 2016;16:759; Gattinoni et al. J. Clin. Invest. 2005;115:1616 and Gattinoni et al. Nat Med 2011;17:1290).

Immune deficient mouse strains as the basis of our pre-clinical *in vivo* models. The main reason for this is the fact that the γδT cell – target cell interactions are not present in mice, because mice lack specific γδT cell subsets and their ligands. Therefore we cannot use mouse T cells and mouse tumour targets in our models. We have designed 'humanized mouse models' to evaluate γδTCR (TEG) – based immunotherapy as described in (Marcu-Malina et al. Blood 2011;118:50, Gruender et al. Blood 2012;120:5153 and Straetemans et al. Clin Can Res 2015;21:3957).

In our research strategy, humanized mouse models represent a valuable additional tool to answer biological questions and provide data on efficacy and safety regarding candidate immune-receptor mediated anti-tumour therapy such as TEG therapy. First, on-target anti-tumour efficacy is an important read-out of these models. An advantage of these models compared to *in vitro* assays is that these models allow long-term growth (months) of (primary) human tumour cells and therefore long-term interaction of tumour cells with immune therapy. A second read-out, unique for humanized tumour models in mice, is the homing (trafficking) of immune cells (such as TEGs) to the tumour and/or tumour microenvironment and evaluation of long-term persistence of engineered immune cells. Third, the tumours that engraft in humanized mice form a complex tumour microenvironment including blood vessel networks that are not (yet) possible to model *in vitro* and receptor-based immune therapy needs to overcome possible barriers to target the complexity of these tumours. Also, changes in the human tumour and its microenvironment under the influence of therapy can be assessed. Another advantage of these humanized models is their ability to engraft human immune receptor engineered cells. We can directly evaluate the potency of our *ex vivo* production protocols (research grade and GMP-grade) that are optimized for human cells and as such these results have important translational value for the initiation of clinical studies using these products. In addition to *in vitro* data, the *in vivo* read-outs provide biological data as well as on-target efficacy and off-target toxicity information that is requested by the authorities before initiation of a clinical trial.
In spite of the advantages, humanized mouse models have disadvantages, as they remain a 'model' for human disease and therapy. Disadvantages include possible preferential outgrowth of subsets of tumour cells, the mouse environment with limitations for the complete support of human immune cells and the absence of a complete functional immune system to interact with tumour growth. Time and costs are another drawback of humanized mouse models compared to relative simple and fast *in vitro* models. In this light we use these models **in addition** to *in vitro* models and are continuously searching for the latest state of the art techniques (such as organoid/tumouroid models) to find the optimal models to answer our scientific and pre-clinical questions.

Overview of the *in vivo* strategy

The current project is divided in 4 different types of mouse experiments (explained in detail under 3.4.2), 3 different tumour models and 1 type of experiment for safety assessment.

We have designed a go/no go moment in the research pipeline before candidate immune receptor based therapy can be tested in humanized mouse models, see also figure 1:

Immune receptors that have not previously been tested in mice will be considered for testing only when:
- Receptors (alone or in combination therapy) are active against a broad panel of tumour cell lines and primary tumour cells, but not against the healthy counterpart cells *in vitro*.
- The stability and formulation of the receptor platform is suitable for *in vivo* studies, in other words only in case stable and suitable dosage of compounds can be produced an *in vivo* study is initiated.

In order to be relevant, the tumour model selected for the therapy should carry the appropriate tumour target. The tumour origin to target *in vivo* is defined based on the following (described in Figure 1):
1) The receptor(s) characteristics with respect to anti-tumour potency;
2) Unmet medical need of cancer types;
3) Other potential therapies in development for the particular tumour origin;
4) Experts opinions (medical doctors in our research group, regulatory experts, medical advisors, scientific data available)
5) The possibility to knock-out or knock-in target molecules to answer questions related to the biological mechanism of action.

The first *in vivo* tests will be done using the same cell lines that have been used during the prior *in vitro* studies, experiment type 1 or 2. These initial tests will include efficacy studies and answer biological questions regarding long-term persistence and biodistribution of the immune receptor based compound. If these first *in vivo* tests are unsuccessful, the immune receptor-based strategy will be discontinued. When successful, it will in general be necessary to perform confirmatory studies in other models, such as patient derived xenograft models (PDX), more stringent and or physiological relevant models with respect to tumour location (orthotopic) or with respect to dosing and treatment regimen (established treatment model) (experiment type 1, 2, 3). Confirmatory studies may also include models lacking the appropriate target (as a negative control) to understand/confirm the mechanism of action.
In addition to confirmatory studies the safety of immune receptor therapy will be assessed in the off-target toxicity and/or GVHD models, experiment type 4.

Evaluation
The research pipeline in figure 1 is a two-directional flowchart as indicated with the arrows. Therefore constant evaluation, feedback and improvement will flow through at any level. For the *in vivo* studies this can result in re-design of *in vivo* experiments and the go/no-go criteria for retesting immune receptors is defined:

Immune receptors that have been tested previously *in vivo* will be retested when:

-Anti-tumour efficacy in *in vitro* experiments confirms that a new tumour type can be targeted and needs pre-clinical *in vivo* evaluation.
-A relevant metastatic model is available to evaluate if immune receptors are effective against metastatic tumour growth.
-There is a clear rationale for improved dosing and treatment schedule (such as more or less stringent testing. i.e. established tumour treatment model).
-The immune receptor treatment was successful *in vivo*, human cell types that are key players in the human tumour microenvironment and their impact on the efficacy of the receptor based immune therapy will be evaluated (immune cells from haematological stem cells, or mesenchymal stromal cells).
-The production protocol (formulation) for immune receptor based therapy is improved and evaluation of improved anti-tumour efficacy cannot be performed in *in vitro* assays and needs pre-clinical *in vivo* evaluation.
-There is a clear indication from *in vitro* assays that combination of immune receptor therapy with agents or treatment modalities may be beneficial for treatment outcome
-Although the rational for combination therapy is clear but there is no suitable *in vitro* model and validation *in vitro* is not possible.

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

The current project contains the following type of mouse experiments:

1. Humanized tumour models: subcutaneous
2. Humanized tumour models: metastatic
3. Humanized tumour models: orthotopic
4. Humanized models: Safety

Humanized tumour models
The humanized tumour models are listed in Table 1.

Table 1. Type of humanized tumour models and procedures

1. Subcutaneous human tumour models				
Solid tumour source	**Metastatic site**	**Site of injection**	**Tumour take rate**	**Discomfort[1]**
Cell line	no	s.c. in flank	High >80%	Mild to moderate[2]
Tumouroid cell line	no	s.c. in flank	High >80%	Mild to moderate
Patient derived primary cells	no	s.c. in flank	Variable >50%	Mild to moderate
2. Metastatic tumour models				
Tumour source	**Metastatic site**	**Site of injection**	**Tumour take rate**	**Discomfort**
A. solid tumour origin	lung	i.v.	High >80%	severe
	liver	Spleen or mesenteric vein	High >80%	severe
B. Heamatological tumour origin				
Cell lines	Bone marrow and peripheral blood	i.v.	Very high >90%	Moderate/ severe
Patient derived primary cells	Bone marrow and peripheral blood	i.v.	Variable[3] >70-90%	Moderate/ severe
3. Orthotopic tumour models				
Tumour source	**Metastatic site**	**Site of injection**	**Tumour take rate**	**Discomfort**
Cell line	No	Depending on origin of tumour[4]	High>80%	Moderate/ severe
Tumouroid cell line	No		High >80%	Moderate/ severe
Patient derived primary cells	no		Variable >50%	Moderate/ severe

[1] Discomfort when humane endpoint is reached
[2] In general low discomfort unless tumour ulceration occurs (this is humane endpoint)
[3] Patient derived primary haematological tumours are screened and selected for high predicted engraftment using genetic biomarkers before application in mouse experiments
[4] Detailed description in attachment 3

Usually we select a tumour model from which previous *in vivo* data is present (from our own work, collaborations or literature). If no previous *in vivo* data is present, we will start with a relevant model that has the least possible level of discomfort. In order to minimize the amount of ineligible study animals, we will select the tumour model with a high take rate and consistent growth characteristics. If needed, growth curve experiments are performed to select the tumour.
For solid tumour types a subcutaneous early treatment model (less stringent) is applied for the first *in vivo* evaluation of the receptor specific anti-tumour efficacy. If successful a more stringent model

(established treatment model) will be used in a confirmation experiment also to increase the clinical translational value. The haematological tumour models are classified into the metastatic tumour models based on site of injection, engraftment site and resulting discomfort level (table 1). Also here, we will start with an early treatment model if applied for the first time and proceed to an established treatment model in case successful anti-tumour efficacy is observed. Immune receptor-based therapy is suited to treat metastasized cancer and therefore relevant metastatic models are applied if available for the defined tumour origin. If the location of the tumour is relevant (e.g. engraftment of primary Acute Myeloid Leukaemia cells is higher when implanted in bone marrow) we will select an orthotopic model (Table 1). Tumour models using patient derived tumour material are usually with less favourable growth/take rate. To prevent low growth rate, genetic markers that predict tumour engraftment in mice are used to select haematological tumours for *in vivo* usage. If those markers are not known, small-scale tumour growth experiments are performed before initiation of the use in the mouse models.

A typical humanized mouse tumour model for immune receptor based therapy evaluation is described below:
The main focus of these experiments is to study therapeutic efficacy against tumours and the biological mechanisms involved. Mice will receive tumour cells and immune therapy treatment shortly thereafter (early treatment model) or treatment will be applied when tumour cells are engrafted and an established tumour is measured (established tumour treatment model). In case of defined receptors treatment will be supported with aminobiphosphonates, like in the *in vitro* assays as well as in our clinical study design, to support the mechanism of action of the immune receptor. Aminobiphosphonates, such as pamidronate or zoledronate, act on the deregulated cancer cell metabolism resulting in increased sensitivity of cancer cells to defined immune receptor treatment. Pamidronate has been applied in our previous humanized mouse tumour models treated with TEGs (Marcu-Malina et al. Blood 2011;118:50, Gruender et al. Blood 2012;120:5153 and Straetemans et al. Clin Can Res 2015;21:3957). In addition, immune cell [redacted] may be injected during the experiments to support survival of human immune cells in the mouse environment. To mimic the human tumour microenvironment and its influence on therapy, human healthy cells can be engrafted in the mice before treatment. These will either be injected together with the tumour cells in the same injection or if needed for proper engraftment these cells will be injected orthotopically.
Tumour growth will be measured regularly by bioluminescence imaging or PET/CT, using caliper measurements to determine the tumour volumes and/or by measuring tumour burden in peripheral blood in case of hematological malignancies. Immune cell (TEG) persistence will be monitored in peripheral blood by flow cytometry. In addition, 2-photon imaging technique will be applied to perform real-life imaging to visualize tumour – immune cell interaction, follow homing of immune cells and migration to or into the tumour and possible tumour clearance. Control groups will be treated with non-functional receptor engineered cells or classical αβTCR or CAR engineered T cells.

Safety models
If new immune receptors pass the go/no go moment for proceeding towards *in vivo* testing, the non-clinical development program contains safety experiments that are designed in parallel with the efficacy experiments to obtain balanced efficacy and safety data. The safety models are summarized in table 2.

Table 2. Safety model and procedures

4. Safety models for immune receptor based therapy				
A. off-target toxicity models	**Site of injection**	**Take**	**Expected clinical parameters**	**Discomfort[1]**
Target molecule transgenic mice	n.a.	n.a.	GVHD-like[2]	Mild [4]
Human healthy cell engraftment	I.v. / s.c./ orthotopic[3]	Cell subset dependent	Depletion of cellular subsets	Mild [4]

B. Graft Versus Host Disease models				
Immune receptor therapy	I.v.	n.a.	High >80%	Mild to severe[5]

[1]In case humane endpoint is reached
[2]GVHD-like symptom scoring system described in appendix 4
[3]Dependent on origin of healthy cells
[4]Dependent on treatment group and model, in previous comparable experiments no clinical symptoms were observed.
[5]Dependent on allo-reactive potential of immune receptor therapy platform

Description animal experiments for safety testing
The main focus of these experiments is to test the safety of the candidate immune receptor based therapy in case anti-tumour efficacy was successful for a defined immune receptor. Different strategies of safety testing in humanized mouse models will be applied:
A. Off-target toxicity
2 strategies are applied for off-target toxicity measurements. First, NSG mice transgenic for human molecules involved in immune receptor recognition mechanisms are used to test safety of defined immune receptor formats. In case the human target molecule is expressed on healthy mouse cells, it is possible to test if healthy cells are being targeted by the receptor. If healthy cells are targeted, GVHD-like symptoms are expected and a scoring system is defined (see attachment 4).
Second, in case no mice are available transgenic for the human target molecule, immune deficient mice can be engrafted with healthy human cells (derived from healthy cord blood stem cells or other stem cells sources such as MSCs or EPCs or fibroblasts) (Li et al., PLoS ONE8(1): e55319. doi:10.1371/journal.pone.0055319). Engrafted stem cells differentiate into cellular subsets and that allows us to study the effect of receptors towards healthy human cells in long-term cell-cell interactions.
B. Xeno Graft Versus Host Disease Model (XenoGVHD)
In addition, the allogeneic potential of immune receptor-based therapy will be tested for immune-receptor platforms that are designed for therapy in an allogeneic setting. One of our aims using the TEG format is to reduce potential GVHD when applying engineered immune cells against cancer in an allogeneic setting, a realistic treatment option in patients that have already received allogeneic stem cell transplantation. Immune deficient mouse strains are used to test immune therapy for xenograft reactions as a model for GVHD (XenoGVHD) (Bondanza et al. Blood 2006; 107:1828, Casucci et al. Blood 2013). A GVHD scoring system is defined see appendix 4.
In A and B similar treatment protocols are applied as in the tumour treatment models described above.

In all animal experiments vital organs, bone marrow, tumours and/or blood will be collected of mice at the end or during the course of the experiment to perform histopathological studies answering efficacy / mechanism of action / biodistribution related questions. Only when during the time-course of the experiments organs need to be collected, additional animals are needed. Otherwise, we collect the organs at the end of the experiment.

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

As described in 3.1 and Figure 1 we have established a research pipeline to develop immune receptor-based immunotherapy against cancer in which all research components, i.e. from fundamental research until clinical practise, are represented in our research group (see figure 1). Moving a receptor (format) from top to bottom in the pipeline, (from concept to clinic) implies that the receptor meets the predefined criteria (go/no go) to move onwards as described in text figure 1 and in the text under 3.4.1 and 3.4.2.

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	Humanized mouse tumour models: subcutaneous
2	Humanized mouse tumour models: metastatic
3	Humanized mouse tumour models: orthotopic

4	Humanized mouse models: safety
5	
6	
7	
8	
9	
10	

Appendix
Description animal procedures

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

1

General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	11500
1.2	Provide the name of the licenced establishment.	UMC Utrecht
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3.4.4.1 Type of animal procedure: Humanized tumour models: subcutaneous

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

In these experiments humanized mice with subcutaneous injected tumours are used as a surrogate for cancer patients to evaluate immune receptors as anti-tumour therapy and their mechanism of action. The primary outcome parameters are inhibition of tumour growth or reduction of tumour size and increased (tumour-free) survival. Secondary parameters are long-term persistence of viable immune cells, biodistribution of immune receptors and immune cells, characteristics of tumours upon treatment, tumour microenvironment including immune cells and tumour therapy escape or resistance.

Pre-conditioning
In general, mice receive a pre-conditioning radiation treatment before tumour and immune receptor treatment as described (Marcu-Malina et al. Blood 2011;118:50, Gruender et al. Blood 2012;120:5153 and Straetemans et al. Clin Can Res 2015;21:3957). First, this results in optimal engraftment of human tumour cells in the mice. Secondly, a pre-conditioning treatment (either radiation or chemotherapy) before immune receptor-based therapy is standard to improve the efficacy of the treatment in clinical practice of CAR T cell therapy and in our current clinical protocol of TEG001 treatment. Mice receive irradiation or chemotherapy before tumour injection (early treatment model) or right before treatment as a combination treatment strategy (established tumour treatment model).

Subcutaneous tumour cell injection and tumour measurement
Solid tumour cells of human origin are being used in the subcutaneous models. The cells may derive from different sources as described in the proposal. The growth and behaviour of the tumours will be monitored by appropriate techniques during the course of the experiment. The readout can be based on

tumour progression (measuring tumour volume and/or imaging), (tumour-free) survival and/or biological effect (based on analysis following the resection/collection of relevant tissue material).
In case subcutaneous tumours, caliper measurements will be used to measure tumours larger than 3 mm.
A valuable alternative applied in our current models is bioluminescence imaging (BLI). Tumour cells producing the enzyme Luciferase that converts Luciferin (injected ip) in a chemical reaction resulting in bioluminescence. This allows us to monitor tumour growth at relevant locations during the course of the experiment without sacrificing the animals. We have experienced in subcutaneous models that BLI data allows a more quantitative and robust evaluation of immune receptor therapy because of the following reasons (recent observations, not published yet). A likely reason for this is the fact that the BLI signal is only from viable cells within a tumour. In case immune receptor therapy leads to tumour necrosis this is not always reflected in tumour size directly. Also these data are completely objective and not sensitive for measuring errors unlike caliper measurements. Therefore, BLI measurements are of added value also in subcutaneous tumour models in case tumour cell lines are modified to express the reporter gene.

Treatment
Selected candidate receptors, such as TEGs, are applied either as monotherapy or as combination therapy. Mice will receive treatment 1-3 days after tumour cell injection (early treatment model) or treatment will be applied when tumour cells are engrafted and an established (palpable) tumour is measured (established tumour treatment model). The treatment generally will be applied in the form of one or two i.v. injections in combination with [redacted] such as [redacted] to support survival of human immune cells in the mouse environment. Also repetitive injections of immune receptors can be applied. A non-functional immune receptor serves as our negative control condition and when relevant classical CAR or αβTCR engineered T cells will be used as positive or negative control conditions. In case of combination therapy, additional injections with defined antibodies or compounds to increase effectivity of immune cells will be applied. Generally these will be applied systemically, but can be added locally at the tumour site as well, depending amongst others on the mechanism of action, half-life and *in vivo* trafficking characteristics. Before evaluation of any combination therapy in the relevant tumour models, extensive literature studies and *in vitro* studies have been performed to predict potential clinical benefit of the immune-receptor-based combination therapy. *In vivo* depletion of therapeutic receptors may be tested in the efficacy models by the delivery of a compound (generally i.v. in the form of an antibody, substrate or enzyme) that can lead to the eradication of these receptors and consequently their anti-tumour effect or potential side effects. Such an 'off-switch' is of great value in clinical practice.

Immune receptor and immune cell persistence tumour in peripheral blood
Immune cell persistence (long-term survival *in vivo*) will be monitored in peripheral blood by flow cytometry. We have established a protocol to quantify cells in peripheral blood of the mice using quantification beads in combination with labeling antibodies. We can measure the immune cells, such as TEG cells, labeled with specific antibodies against the immune receptor and at the same time we can measure phenotypic characteristics of these cells (inhibition markers or differentiation markers). Also, we can measure tumour cells, defined by specific markers, present in peripheral blood. Regular blood sampling allows us to determine if immune cells are viable and effective and in addition if tumour cells are present in the blood. Also, we can isolate DNA and RNA from peripheral blood to measure presence of immune receptors.

Harvesting of organs and blood at the end of the experiment.
During the course of the experiments, but generally at the end of each experiment we will harvest organs, tumours, bone marrow and peripheral blood to analyze the presence of immune cells, isolate RNA and/or DNA or perform histopathological analysis in order to establish a treatment effect and to acquire mechanistic insight in immune receptor-based therapy model.

It should be noted that our strategy is not aiming to develop humanized (tumour) models in mice, but we intend to use suitable, preferably already published (or unpublished via collaborations) tumour models, fine-tune these models and apply them to test candidate immune receptor-based therapies to

metabolically target cancer

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

-Preconditioning will be applied one day before tumour cell injection or before treatment. This will either be whole body irradiation or consists of a chemotherapeutic regiment. A chemotherapeutic regiment will be based on standard clinically used compounds in immunotherapy, such as (a combination of) cyclophosphamide, busulfan or fludarabin injected i.p. on 2-3 consecutive days. Dosing in mice will be based on protocols available for the specific compound and mouse strain and if needed a dose titration will be performed before initiation of the experiment.
-Mice will receive human tumour cells subcutaneously (s.c.) In some experiments when tumours are cleared, mice will be rechallenged with tumour cells to test long-term effect of candidate immunotherapy. The second tumour challenge will be on a different location, i.e. the opposite flank. To be able to evaluate long-term effect of the treatment the experiment duration is maximal 200 days.
-Mice will be treated with candidate receptor-based immunotherapy. The cells (or other format) will be injected usually i.v. or in some occasions locally at the tumour site (s.c). Generally the treatment regimen consists of 2 doses, but can vary from a single dose up to daily injections during a defined period of weeks (max 3 weeks) depending on the receptor platform and research question.
-Candidate immune therapy will be supported by [redacted] Human immune cells need crucial [redacted] for [redacted] which are lacking in the immune deficient mice. Therefore immune cell treatment is supported with cellular [redacted] such as [redacted] or others or a combination. Generally these [redacted] are applied s.c. in a carrier called Incomplete Freunds'Adjuvant (IFA) every 21 days from the moment of treatment, max 10 injections/animal during the course of the experiment.
-For defined receptors, mice are every 21 days i.v. injected with aminobiphosphates (such as pamidronate) from the moment of treatment resembling the treatment of patients with these receptor-based therapies (Straetemans et al. Clin Can Res 2015;21:3957), max 10 injections per animal during the course of the experiment.
-Combination therapy can imply additional injections, preferably combined with the immune cell injections or separately injected i.v. or locally at the tumour site (s.c.). Dosing schedule is dependent on compound used, an example is 250 microgram/injection at the day of receptor treatment followed by 2 additional injections 3 and 6 days later [redacted]). -Caliper measurement: In case superficial growing tumours, tumour size will be measured with calliper until the chosen end point or the humane end point is reached. Calliper measurements are generally 2 times a week and the mice need to be fixed by hand and it takes a few minutes in order to measure the tumour size.
-Blood sampling: in general every 2 weeks or weekly blood sampling will be performed, and 50-70 microliter of blood will be collected per mouse per time point. We will adhere to the recommended blood sampling frequencies and volumes as published by Diehl and colleagues (Diehl et al. J. Appl. Toxicol. 21, 15–23 (2001).
-Imaging techniques: In case biological information of tumours or immune receptors is required, imaging techniques such as Bioluminescence imagaging (BLI) will be applied. During these measurements mice need to be absolutely immobile and will therefore be anesthetized by isoflurane before they receive an i.p. injection of Luciferin. In case both sides will be measured it generally lasts 20 minutes and will take place maximal 1 time a week.
-At the end of the experiments organs, bone marrow and blood will be collected for further research at the pathology department or in our laboratory.

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

Within our group we have established humanized tumour models for immune receptor therapy testing and results from these experiments are used to define size and number of experimental groups for any new experiment. Primary outcome parameters that are used to calculate number of animals are tumour growth and survival.
We make use of statistical methods to calculate the power to optimize group size. To this end an online program will be used such as the following: http://homepage.stat.uiowa.edu/~rlenth/Power/. In this way we will design realistic and statically sound experiments that allow scientific interpretation of the results

obtained.
To illustrate such calculation, a description of a typical experimental design is outlined here. An experiment will generally comprise of several study arms (control group(s) and treatment groups). To demonstrate a 50% improvement in terms of tumour growth rate or survival between two groups (test vs controls) with the overall variability (relative standard deviation) being around 30, we will need a group size of 8 animals (power > 0.9 with $\alpha = 0.05$, two sided). However, if multiple treatment groups are included we need to increase the group size because we need to adjust for multiple comparisons (the α of 0.05 will be divided by the number of test groups minus 1 (e.g. 5-arm study will take α as 0.05/3 = 0.013). Overall, the group size will need to be 10 evaluable animals when performing a 5-arm study.

In order to obtain sufficient evaluable animals we will need more animals from start as will be outlined below.

B. The animals

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

Immune deficient mice
As indicated in the proposal immune deficient mouse strains are the basis of our pre-clinical *in vivo* models. The main reason for this is the fact that the γδT cell – target cell interactions are not present in mice, because mice lack specific γδT cell subsets and their ligands. Therefore we cannot use mouse T cells and mouse tumour targets in our models. Another reason is that in this way we do not need to redesign pre-clinical protocols and *in vitro* assays with mouse immune cells and mouse tumours that result in additional animal usage and possible hurdles when translating again back to the human situation. We have designed 'humanized mouse models' to evaluate γδTCR (TEG) – based immunotherapy that can be used for broader immune receptor-based therapy. Immune deficient mouse strains based on the NOD scid gamma (NSG) mice (official strain name NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) are being bred. These mice carry two mutations on the NOD/ShiLtJ genetic background and lack mature T , B, natural killer (NK) cells, are deficient in multiple cytokine signaling pathways and in many innate immune components such as complement factors

Variants of immune deficient mice
A variant of the NSG strain, the NSG-SGM3, transgenic for 3 human cytokine genes (SC, GMCSF and IL-3) is used in experiments depending on the tumour of interest. Compared to the NSG mice, these mice allow increased engraftment of human primary leukemic samples and healthy human hematopoietic stem cells due to the constant production of non- or poorly cross-reacting human cytokines essential for hematopoietic (tumour) cell engraftment.
NSG mice transgenic for human molecules that are involved in the recognition mechanism can also be used in the tumour models. The usage of the strain is dependent on the immune receptor to test, the tumour and the scientific question in each experiment.

The mice are (or will be) acquired from a commercial or research colony and further bred in house. We will combine both male and female in our experiments. We will preferably use mice between 8-21 weeks of age at the start of the experiment. We would like to end the experiments preferentially before the mice are 1 year old and therefore don't want to start with mice above 21 weeks. In some experiments we would like to rechallenge the mice again with tumour cells to study the long-term persistence of active immune cells (memory response). This requires that mice are not too old at the start of the experiment.

We use a power calculation to define the minimal group size we need, taking into account the expected treatment effect and the number of comparisons we want to make. Usually we don't need more than 10 mice per group (as calculated above under **A**). In case we need to harvest tissues during the experiment to answer research questions we will add additional animals (generally 3 mice) in order to keep the calculated group size for evaluation of primary outcome parameters during the whole experiment. Per experiment we will generally use not more than 5 treatment groups including negative and positive control treatment. If we apply the maximum group size we need 5 x 13 = 65 mice per experiment. Due to biological limitations such as primary tumour samples or human stem cell material but also *in vitro* production limitations of therapeutic cells and compounds, we don't expect to have larger experiments.

We expect to perform 8 experiments per year. This results in 8 x 65 = 520 mice per year and for 5 years a total amount of 5 x 520 =2600 mice
Randomization will take place based on gender in case of the early treatment model. We are using both male and female mice in our experiments. In case of established tumour treatment models randomization also includes tumour size.

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.

Extensive *in vitro* assays have been performed and results are evaluated critically. Only when the candidate immune receptor is meets the go/no go criteria outlined in the general proposal it will be selected for testing in the *in vivo* models . To model tumour cell – immune cell interactions *in vitro* we apply state-of-the-art models and techniques such as the organoid / tumouroid models and the 3D bioprinted bone marrow model in order to gather valuable information without the usage of animals. However, studying the complexity of long-term interactions between tumour cells, environmental factor such as tumour stroma, oxygen supply and immune cells and immunotherapy is not possible (yet) in *in vitro* assay systems. And before a new therapy can enter clinical evaluation pre-clinical *in vivo* testing is required.
We apply a power calculation to minimize the number of animals and scientific literature is constantly used to prevent repetition of already performed experiments. In case new tumour types are being used, growth curves and/or pilot experiments may have to be performed to develop a new model suited for therapeutic testing before complete *in vivo* experiments can be performed. To reduce unnecessary usage of mice only in case robust and consistent tumour cell engraftment will be obtained the model is suited to perform complete experiments. State-of-the-art methods and equipment to follow-up tumour growth (imaging) will be used to minimize discomfort to the animals.

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

Mice will be housed in groups and have standard enrichment in their cage Mice are daily monitored according to local guidelines and an extensive welfare check is performed minimal 2 times a week. In order to minimize suffering, we will adhere to the national and internationally accepted rules (Code of Practice) of handling lab animals in oncology (see P. Workman et al. Guidelines for welfare and use of animals in cancer research. Br J Cancer 2010; 102: 1555-1577). Specifically, animals will be monitored closely for tumour growth or any treatment related (unexpected) discomfort by visual inspection and tumour measurements to identify animals at risk for developing symptoms. When the humane endpoint is reached mice will be humanely killed to prevent severe discomfort.

Repetition and duplication

E. Repetition

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

Not applicable. The proposed research does not relate to legally required research.

Accommodation and care

F. Accommodation and care

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

☒ No

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

G. Location where the animals procedures are performed

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

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

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

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

Injections (i.v., s.c., i.p.) will possibly cause pain, but due to the very short moment of pain, applying pain relieving anaesthesia or analgesia will cause equal discomfort to the mice.

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

During BLI measurements mice will be under anaesthesia using isoflurane. We are in constant communication with the local animal welfare officer in case relevant improved concepts regarding pain relieve are developed and can be implemented in our protocols.

I. Other aspects compromising the welfare of the animals

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

Subcutaneous growing tumours can form ulcerations. Treatments may cause toxic side effects.

Explain why these effects may emerge.

These effects are the consequence of tumour growth and treatment.

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

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

J. Humane endpoints

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

☐ No > Continue with question K.

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

We will adhere to the Code of Practise of lab animals used in oncology, in line with internationally agreed rules (Workman et al. Guidelines for welfare and use of animals in cancer research. Br J Cancer 2010; 102: 1555-1577). In general the most important humane endpoints that apply in the subcutaneous models are:

- A weight loss of more than 15% in 48 hours or total 20% of the initial body weight, measured from the start of the treatment.
- A tumour mass greater than 10% of the body weight, usually 2000 cubic mm in case of superficial measurable lesions (by caliper)
- Skin ulceration/necrosis.

Indicate the likely incidence.

We estimate that around 41% of mice will reach one of the predefined humane endpoints, in the remaining 59% of mice tumour burden is low at the moment the animal is killed for the collection of tissues or is low due to the treatment effect and/or the experiment is ended before the humane endpoint is reached.
In case in the subcutaneous models the humane endpoint is reached it is because of a maximum tumour size (40% of total mice) or due to skin ulceration (1% of total mice).

K. Classification of severity of procedures

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

The tumour model will determine the severity of the discomfort. Induction of the tumour is expected to give the following level of discomfort:
-Injection of tumour cells s.c. through the skin, mild discomfort Tumour growth
-Generally, subcutaneous growing tumours do not cause metastasis within the lifetime of the study and weight loss or any other discomfort will not occur. Generally only the humane endpoint of maximum tumour size is reached and mice experience mild discomfort.
- In 10% of mice skin ulceration of tumours is expected:
The severity of the ulceration and as a consequence the discomfort that the mice experience varies.
It is expected that mice that have mild starting ulceration can experience moderate discomfort (9%).
Severe ulceration is classified as severe discomfort and the humane endpoint is reached (1%).

Interventions
-Simple well-tolerated interventions (injection of cells, aminobiphosphonates, [redacted] or other compounds, chemotherapy or irradiation) will cause discomfort classified as mild.
-Simple but frequent handlings like weighing, caliper measurements and cheek vein bleeding will cause discomfort classified as mild.
-Bioluminescence imaging is classified under mild discomfort due to recovery from anaesthesia.

Table 1 summarizes the expected number of mice per discomfort classification:

	Mild discomfort	**Moderate discomfort**	**Severe discomfort**
Tumour model: Subcutaneous growing			
In %	90%	9%	1%
Total mice in 5 year	2340	234	26

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

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

The condition of the animals reaching the humane endpoint or the use of tissues for further research both require that mice are killed at the end of the experiment.

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'.	11500
1.2	Provide the name of the licenced establishment.	UMC Utrecht
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3.4.4.2 Type of animal procedure: Humanized tumour models: metastatic

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

In these experiments humanized mice with tumours that form metastases are used as a surrogate for cancer patients to evaluate immune receptors as anti-tumour therapy and their mechanism of action. The primary outcome parameters are inhibition or reduction of tumour growth, number of metastases and increased (tumour-free) survival. Secondary parameters are long-term persistence of viable immune cells, biodistribution of immune receptors and immune cells, characteristics of tumours upon treatment, tumour microenvironment including immune cells and tumour therapy escape or resistance.

Pre-conditioning
In general, mice receive a pre-conditioning radiation treatment before tumour and immune receptor treatment as described (Marcu-Malina et al. Blood 2011;118:50, Gruender et al. Blood 2012;120:5153 and Straetemans et al. Clin Can Res 2015;21:3957). First, this results in optimal engraftment of human tumour cells in the mice. Secondly, a pre-conditioning treatment (either radiation or chemotherapy) before immune receptor-based therapy is standard to improve the efficacy of the treatment in clinical practice of CAR T cell therapy and in our current clinical protocol of TEG001 treatment. Mice receive irradiation or chemotherapy before tumour injection (early treatment model) or right before treatment as a combination treatment strategy (established tumour treatment model).

Tumour cell injection and tumour measurement
We can make a distinction between the solid tumour metastatic and the leukemic metastatic models based not only on the origin of the tumour cells (described in general proposal), but also on the injection site, because both determine the site of metastases. The site of metastatic tumour growth will determine

the typical discomfort and as such the level of discomfort for each model (see K for more details). The growth and behaviour of the tumours will be monitored by appropriate techniques during the course of the experiment.

Treatment
Selected candidate receptors, such as TEGs, are applied either as monotherapy or as combination therapy. Mice will receive treatment 1-3 days after tumour cell injection (early treatment model) or treatment will be applied when tumour cells are engrafted and an established tumour is measured (established tumour treatment model). The treatment generally will be applied in the form of one or two i.v. injections in combination with [redacted] of [redacted] in the mouse environment. Also repetitive injections of immune receptors can be applied. A non-functional immune receptor serves as our negative control condition and when relevant classical CAR or αβTCR engineered T cells will be used as positive or negative control conditions. In case of combination therapy, additional injections with defined antibodies or compounds to increase effectivity of immune cells will be applied. Generally these will be applied systemically, but can be added locally at the tumour site as well, depending amongst others on the mechanism of action, half-life and *in vivo* trafficking characteristics. Before evaluation of any combination therapy in the relevant tumour models, extensive literature studies and *in vitro* studies have been performed to predict potential clinical benefit of the immune-receptor-based combination therapy. *In vivo* depletion of therapeutic receptors may be tested in the efficacy models by the delivery of a compound (generally i.v. in the form of an antibody, substrate or enzyme) that can lead to the eradication of these receptors and consequently their anti-tumour effect or potential side effects. Such an 'off-switch' is of great value in clinical practice.

Immune receptor and immune cell persistence and leukemic tumour burden in peripheral blood
Immune cell persistence (long-term survival *in vivo*) will be monitored in peripheral blood by flow cytometry. We have established a protocol to quantify cells in peripheral blood of the mice using quantification beads in combination with labeling antibodies. We can measure the immune cells, such as TEG cells, labeled with specific antibodies against the immune receptor and at the same time we can measure phenotypic characteristics of these cells (inhibition markers or differentiation markers). Also, we can measure tumour cells, defined by specific markers, present in peripheral blood. Regular blood sampling allows us to determine if immune cells are viable and effective and in addition if tumour cells are present in the blood. Also, we can isolate DNA and RNA from peripheral blood to measure presence of immune receptors or tumour cells.

It should be noted that our strategy is not aiming to develop humanized tumour models in mice, but we intend to use suitable, preferably already published (or unpublished via collaborations) tumour models, fine-tune these models and apply them to test candidate immune receptor-based therapies to metabolically target cancer. In case there is no tumour model available for the specific immune receptor therapy small scale tumour growth curve and treatment experiments will be performed before a large study is initiated.

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

-Preconditioning will be applied one day before tumour cell injection or before treatment. This will either be whole body irradiation or consists of a chemotherapeutic regiment. A chemotherapeutic regiment will be based on standard clinically used compounds in immunotherapy, such as (a combination of) cyclophosphamide, busulfan or fludarabin injected i.p. on 2-3 consecutive days. Dosing in mice will be based on protocols available for the specific compound and mouse strain and if needed a dose titration will be performed before initiation of the experiment.

-We have the following experimental tumour metastases routes/models:
A. Solid tumours:
-Intravenous injection into the tail vein resulting in lung metastases. Requires only a single i.v. injection. Occasionally local tumours in the tail may form due to extravasation of tumour cells during the injection. Usually these local tumours remain very small, but if not may become a reason to euthanize the animal.

-Intra-splenic injection resulting in liver metastases. Involves surgery under general anaesthesia and adequate analgesia. Two minutes after the injection, the spleen will be removed (95% of cells will have reached the liver) to prevent outgrowth of a primary tumour in the spleen. Cauterization is used to avoid bleeding. Bleeding may occur (5-10% of cases) and requires immediate killing of the animal.
-Mesenteric vein injection results also in liver metastases (applied in case of colon cancer model). This procedure involves surgery under general anaesthesia and adequate analgesia. A laparotomy will be performed (incision along the abdominal midline), the caecum and the distal small intestine will be exteriorised. Mesenteric vein will be exposed and tumour cells will be injected into the bloodstream. After injection, pressure will be applied on the injection site to limit the bleeding (±5 minutes). The animal will be monitored after the surgery until they are awake and mobile. The animal will be checked regularly and if unexpected discomfort arises, indicative of a humane endpoint, the animal will be euthanized.
B. Haematological tumours
- Intravenous injection into the tail vein of haematological cells (cell lines or primary cells) will result in leukemic outgrowth in the bone marrow and in peripheral blood.
In some experiments when tumours are cleared, mice will be rechallenged with tumour cells to test long-term effect of candidate immunotherapy. To be able to evaluate long-term effect of the treatment the experiment duration is maximal 200 days.
-Mice will be treated with candidate receptor-based immunotherapy. The cells (or other format) will be injected usually i.v. or in some occasions locally at the tumour site (s.c) Generally the treatment regimen consists of 2 doses, but can vary from a single dose up to daily injections during a defined period of weeks (max 3 weeks) depending on the receptor platform and research question.
-Candidate immune therapy will be supported by [redacted]. Human immune cells need crucial [redacted] for [redacted], which are lacking in the immune deficient mice. Therefore immune cell treatment is supported with [redacted] such as [redacted] or others or a combination. Generally these [redacted] are applied s.c. in a carrier called Incomplete Freunds'Adjuvant (IFA) every 21 days from the moment of treatment, max 10 injections/animal during the course of the experiment.
-For defined receptors, mice are every 21 days i.v. injected with aminobiphosphates (such as pamidronate) from the moment of treatment resembling the treatment of patients with these receptor-based therapies (Straetemans et al. Clin Can Res 2015;21:3957), max 10 injections per animal during the course of the experiment.
-Combination therapy can imply additional injections, preferably combined with the immune cell injections or separately injected i.v. Dosing schedule is dependent on compound used, an example is 250 microgram/injection at the day of receptor treatment followed by 2 additional injections 3 and 6 days later (John et al. Clin Can Res 2013;19:5636)
-Blood sampling: in general every 2 weeks or weekly blood sampling will be performed, and 50-70 microliter of blood will be collected per mouse per time point. We will adhere to the recommended blood sampling frequencies and volumes as published by Diehl and colleagues (Diehl et al. J. Appl. Toxicol. 21, 15–23 2001)).
- Tumour development follow-up: In order to evaluate metastatic tumour development, we will need to use appropriate imaging techniques (BLI, SPECT, PET, CT). For this purpose we will mainly use tumours that express luciferase in order to allow bioluminescence imaging (BLI) for easy follow up. Tumour cells producing the enzyme Luciferase that converts Luciferin (injected ip) in a chemical reaction resulting in bioluminescence. This allows us to monitor tumour growth at relevant locations during the course of the experiment without sacrificing the animals. Alternatively, we can use survival as primary study outcome or we can take out tissues at predefined time points to quantify or characterize the metastatic lesions by ex vivo analysis (e.g. flow cytometry). For BLI the duration varies between 5-10 minutes for one side and up to 20 minutes for both sides and will take place maximal once a week. PET/SPECT/CT generally last for more than an hour and will generally only take place once a week. For long term anaesthesia we will use a dedicated life monitoring system that will record respiration rate and control body temperature to minimize any negative impact on the condition of the animal by the duration of the anaesthesia.
-At the end of the experiments organs, bone marrow and blood will be collected for further research at the pathology department (histopathology) or in our laboratory (DNA/RNA isolation, *in vitro* culture and experiments with cultured tumour cells).

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

Within our group we have established humanized tumour models for immune receptor therapy testing and results from these experiments are used to define size and number of experimental groups for any new experiment. Primary outcome parameters that are used to calculate number of animals are tumour growth and survival.
We make use of statistical methods to calculate the power to optimize group size. To this end an online program will be used such as the following: http://homepage.stat.uiowa.edu/~rlenth/Power/. In this way we will design realistic and statically sound experiments that allow scientific interpretation of the results obtained.
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NSG mice transgenic for human molecules that are involved in the recognition mechanism can also be used in the tumour models. The usage of the strain is dependent on the immune receptor to test, the tumour and the scientific question in each experiment.

The mice are acquired from a commercial or research colony and further bred in house. We will combine both male and female in our experiments. We will preferably use mice between 8-21 weeks of age at the start of the experiment. We would like to end the experiments preferentially before the mice are 1 year old and therefore don't want to start with mice above 21 weeks. In some experiments we would like to rechallenge the mice again with tumour cells to study the long-term persistence of active immune cells (memory response). This requires that mice are not too old at the start of the experiment.

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calculated group size for evaluation of primary outcome parameters during the whole experiment. Per experiment we will generally use not more than 5 treatment groups including negative and positive control treatment. If we apply the maximum group size we need 5 x 13 = 65 mice per experiment. Due to biological limitations such as primary tumour samples or human stem cell material but also *in vitro* production limitations of therapeutic cells and compounds, we don't expect to have larger experiments.

Estimated numbers:
We expect to perform

- 4 solid tumour metastases experiments per year
- 8 heamatological tumour metastases experiments per year

Total = 12 experiments per year. This results in 12 x 65 = 780 mice per year and for 5 years a total amount of 5 x 780 =3900 mice
Randomization will take place based on gender in case of the early treatment model. We are using both male and female mice in our experiments. In case of established tumour treatment models randomization also includes tumour size.

The intravital 2-photon imaging experiments are specified separately here because of the nature of the experiments, but may be performed in parallel depending on the research question. We will image presence, trafficking and behavior of immune cells, such as TEGs, labeled with a fluorescent dye, in or around tumour cells and vasculature. Cell-cell interactions are visualized and in each animal we are able to image numerous cells or cell-cell interactions. Therefore, the above described power analysis does not apply for these type of experiments. In fact, within one mouse we are able to measure more than enough images to perform statistically sound experiments. We expect to use maximal 6 mice per experiment due to the time consuming nature of the imaging and preparation per animal (around 2 hours per mouse) and we expect to need one or more pilot experiments to establish the technique for our purpose. Therefore, we estimate for 5 years to be able to perform 5 experiment: 5 x 5 x 6 = 150 mice including the pilot experiments.

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.

Extensive *in vitro* assays have been performed and results are evaluated critically. Only when the candidate immune receptor meets the go/no go criteria as stated in the project proposal it will be selected for testing in the *in vivo* models. To model tumour cell – immune cell interactions *in vitro* we apply state-of-the-art models and techniques such as the organoid / tumouroid models and the 3D bioprinted bone marrow model in order to gather valuable information without the usage of animals. However, studying the complexity of long-term interactions between tumour cells, environmental factor such as tumour stroma, oxygen supply and immune cells and immunotherapy is not possible (yet) in *in vitro* assay systems. And before a new therapy can enter clinical evaluation pre-clinical *in vivo* testing is required.
We apply a power calculation to minimize the number of animals and scientific literature is constantly used to prevent repetition of already performed experiments. In case new tumour types are being used, growth curves and/or pilot experiments may have to be performed to develop a new model suited for therapeutic testing before complete *in vivo* experiments can be performed. To reduce unnecessary usage

of mice only in case robust and consistent tumour cell engraftment will be obtained the model is suited to perform complete experiments. State-of-the-art methods and equipment to follow-up tumour growth (imaging) will be used to minimize discomfort to the animals.

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

Mice will be housed in groups and have standard enrichment in their cage. Mice are daily monitored according to local guidelines and an extensive welfare check is performed minimal 2 times a week. In order to minimize suffering, we will adhere to the national and internationally accepted rules (Code of Practice) of handling lab animals in oncology (see P. Workman et al. Guidelines for welfare and use of animals in cancer research. Br J Cancer 2010; 102: 1555-1577). Specifically, animals will be monitored closely for tumour growth or any treatment related (unexpected) discomfort by visual inspection and tumour measurements to identify animals at risk for developing symptoms. When the humane endpoint is reached mice will be humanely killed to prevent severe discomfort.

Repetition and duplication

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

Not applicable. The proposed research does not relate to legally required research.

Accommodation and care

F. Accommodation and care

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

☒ No

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

G. Location where the animals procedures are performed

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

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

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

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

Injections (i.v., s.c., i.p.) will possibly cause pain, but due to the very short moment of pain, applying pain relieving anaesthesia or analgesia will cause equal discomfort to the mice.

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

Tumour cell injections in spleen and mesenteric vein need surgery and as described above this will be under general anaesthesia and adequate analgesia.
During imaging mice will be under anaesthesia using isoflurane.

I. Other aspects compromising the welfare of the animals

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

Animals that carry tumours in internal organs may experience disfunction of these organs like cancer patients. For example tumour formation in the lungs may result in respiratory problems and tumours in the bone marrow can result in paralysis of the hind legs. Treatments may cause toxic side effects.

Explain why these effects may emerge.

These effects are the consequence of tumour growth and treatment.

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

In general the negative effects on the well-being of the animals by the tumour cannot be prevented. In order to minimize the burden of the tumour, the animals will be monitored at a frequency that is dictated by the model and timely killed when the humane endpoint is met.
For example for the haematological tumour models, a cage lid test is developed in close communication with the local IVD. In short, twice a week mice are placed on the lid of the cage and the cage is being held upside down. In case the mouse is not able to hold on to the cage with one of its paws, the mouse does not pass the cage lid test and the experimental endpoint is reached for this mouse in order to minimise discomfort.
Nevertheless, unforeseen complications may occur. In such cases, we will try to find solutions that will minimize the impact of the complications, for example by providing easy access to food (mush-feeding).

J. Humane endpoints

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

☐ No > Continue with question K.

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

We will adhere to the Code of Practise of lab animals used in oncology, in line with internationally agreed rules (Workman et al. Guidelines for welfare and use of animals in cancer research. Br J Cancer 2010; 102: 1555-1577).
The humane endpoints that apply in the metastases models are:

- A weight loss of more than 15% in 48 hours or total 20% of the initial body weight, measured from the start of the treatment.
- Severe abnormal breathing.
- Severe abnormal behavior
- Not passing the above described cage lid test.

Indicate the likely incidence.

We estimate that around 20% of mice will reach one of the predefined humane endpoints of weight loss, abnormal breathing or abnormal behaviour. And 20% of mice will not pass the cage lid test. In the remaining 60% of mice the experiment is ended is based on imaging or peripheral blood tumour load, or the tumour burden is low at the moment the animal is killed for the collection of tissues or is low due to the treatment effect and/or the experiment is ended before the humane endpoint is reached.

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').

Induction of the tumour is expected to give the following level of discomfort:
i.v. injection through tail vein: mild discomfort
surgical implantation of tumour cells: moderate discomfort
Intravital 2-photon imaging will only be possible in case of surgical implantation of tumour cells: moderate discomfort

Interventions:
-Simple well-tolerated interventions (injection of cells, aminobiphosphonates, [redacted] or other compounds, chemotherapy or irradiation) will cause discomfort classified as mild.
-Simple but frequent handlings like weighing, caliper measurements and cheek vein bleeding will cause discomfort classified as mild.
-Bioluminescence imaging is classified under mild discomfort due to recovery from anaesthesia.
-PET/CT/SPECT will be classified under moderate discomfort due to the duration of anaesthesia.

The tumour dictates the overall discomfort in these models. In case metastatic tumours grow to the size that the humane endpoint of weight loss, breathing or abnormal behaviour is reached these will cause severe discomfort. We expect that animals under this appendix may suffer from severe discomfort in 20% of cases. In those cases where the animal will be sacrificed based on imaging results, cage lid test, collection of tissues, or due to effectivity of therapy will not reach the endpoint, the discomfort level will be less (mild or moderate).

Table 1 summarizes the expected number of mice per discomfort classification:

	Mild discomfort	**Moderate discomfort**	**Severe discomfort**
Tumour model: metastases			
In %	40%	40%	20%
Total mice in 5 year	1620	1620	810

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

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

The condition of the animals reaching the humane endpoint or the use of tissues for further research both require that mice are killed at the end of the experiment.

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]

Appendix
Description animal procedures

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	11500
1.2	Provide the name of the licenced establishment.	UMC Utrecht
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3.4.4.3 Type of animal procedure: Humanized tumour models: orthotopic

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

In these experiments humanized mice with tumours that are orthotopically injected are used as a surrogate for cancer patients to evaluate immune receptors as anti-tumour therapy and their mechanism of action. The primary outcome parameters are inhibition or reduction of tumour growth, and increased (tumour-free) survival. Secondary parameters are long-term persistence of viable immune cells, biodistribution of immune receptors and immune cells, characteristics of tumours upon treatment, tumour microenvironment including immune cells and tumour therapy escape or resistance.

Pre-conditioning
In general, mice receive a pre-conditioning radiation treatment before tumour and immune receptor treatment as described (Marcu-Malina et al. Blood 2011;118:50, Gruender et al. Blood 2012;120:5153 and Straetemans et al. Clin Can Res 2015;21:3957). First, this results in optimal engraftment of human tumour cells in the mice. Secondly, a pre-conditioning treatment (either radiation or chemotherapy) before immune receptor-based therapy is standard to improve the efficacy of the treatment in clinical practice of CAR T cell therapy and in our current clinical protocol of TEG001 treatment. Mice receive irradiation or chemotherapy before tumour injection (early treatment model) or right before treatment as a combination treatment strategy (established tumour treatment model).

Tumour cell injection and tumour measurement
In the orthotopic setting, the site of the tumor cell injection depends on the tumour origin. Usually, a surgical procedure takes place under general anaesthesia and adequate analgesia.
The site of tumor growth will determine the typical discomfort and as such the level of discomfort for

each model. For example, injection of leukemic tumour cells in the femur, injection in the mammary fatpad for breast cancer or injection in the cecum wall for colon cancer modeling. The growth and behaviour of the tumours will be monitored by appropriate techniques during the course of the experiment.

Treatment

Selected candidate receptors, such as TEGs, are applied either as monotherapy or as combination therapy. Mice will receive treatment 1-3 days after tumour cell injection (early treatment model) or treatment will be applied when tumour cells are engrafted and an established tumour is measured (established tumour treatment model). The treatment generally will be applied in the form of one or two i.v. injections in combination with human [redacted] such as [redacted] to [redacted] of [redacted] in the mouse environment. Also repetitive injections of immune receptors can be applied. A non-functional immune receptor serves as our negative control condition and when relevant classical CAR or αβTCR engineered T cells will be used as positive or negative control conditions. In case of combination therapy, additional injections with defined antibodies or compounds to increase effectivity of immune cells will be applied. Generally these will be applied systemically, but can be added locally at the tumour site as well, depending amongst others on the mechanism of action, half-life and *in vivo* trafficking characteristics. Before evaluation of any combination therapy in the relevant tumour models, extensive literature studies and *in vitro* studies have been performed to predict potential clinical benefit of the immune-receptor-based combination therapy. *In vivo* depletion of therapeutic receptors may be tested in the efficacy models by the delivery of a compound (generally i.v. in the form of an antibody, substrate or enzyme) that can lead to the eradication of these receptors and consequently their anti-tumour effect or potential side effects. Such an 'off-switch' is of great value in clinical practice.

Immune receptor and immune cell persistence or leukemic tumour burden in peripheral blood

Immune cell persistence (long-term survival *in vivo*) will be monitored in peripheral blood by flow cytometry. We have established a protocol to quantify cells in peripheral blood of the mice using quantification beads in combination with labeling antibodies. We can measure the immune cells, such as TEG cells, labeled with specific antibodies against the immune receptor and at the same time we can measure phenotypic characteristics of these cells (inhibition markers or differentiation markers). Also, we can measure tumour cells, defined by specific markers, present in peripheral blood. Regular blood sampling allows us to determine if immune cells are viable and effective and in addition if tumour cells are present in the blood. Also, we can isolate DNA and RNA from peripheral blood to measure presence of immune receptors or tumour cells.

It should be noted that our strategy is not aiming to develop humanized tumour models in mice, but we intend to use suitable, preferably already published (or unpublished via collaborations) tumour models, fine-tune these models and apply them to test candidate immune receptor-based therapies to metabolically target cancer. In case there is no tumour model available for the specific immune receptor therapy small scale tumour growth curve and treatment experiments will be performed before a large study is initiated.

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

-Preconditioning will be applied one day before tumour cell injection or before treatment. This will either be whole body irradiation or consists of a chemotherapeutic regiment. A chemotherapeutic regiment will be based on standard clinically used compounds in immunotherapy, such as (a combination of) cyclophosphamide, busulfan or fludarabin injected i.p. on 2-3 consecutive days. Dosing in mice will be based on protocols available for the specific compound and mouse strain and if needed a dose titration will be performed before initiation of the experiment.
-We have the following experimental orthotopic models:
A. Solid tumours, examples are:
- breast cancer cells in the mammary fat pad (Kocaturk and Versteeg. J Vis Exp 2015;96:51967)
-colon cancer cells in the caecal wall (Fumagalli et al. PNAS 2016;114:E2357). Depending on the tumour cell line metastasis are formed in liver and lung and these models are a combination of appendix 2 and 3.
B. Heamatological tumours

- Intra femur injection of haematological cells (cell lines or primary cells) will result in increased leukemic outgrowth in the bone marrow. In this type of model human bone marrow supportive cells can be injected simultaneously at the same site with the tumour cells to study the effect of the presence of these human cells on the therapy.
In some experiments when tumours are cleared, mice will be rechallenged with tumour cells to test long-term effect of candidate immunotherapy. To be able to evaluate long-term effect of the treatment the experiment duration is maximal 200 days.
-Mice will be treated with candidate receptor-based immunotherapy. The cells (or other format) will be injected usually i.v. Generally the treatment regimen consists of 2 doses, but can vary from a single dose up to daily injections during a defined period of weeks (max 3 weeks) depending on the receptor platform and research question.
-Candidate immune therapy will be supported by [redacted]. Human immune cells need crucial [redacted] for [redacted], which are lacking in the immune deficient mice. Therefore immune cell treatment is supported with [redacted] such as [redacted] or others or a combination. Generally these [redacted] are applied s.c. in a carrier called Incomplete Freunds'Adjuvant (IFA) every 21 days from the moment of treatment, max 10 injections/animal during the course of the experiment.
-For defined receptors, mice are every 21 days i.v. injected with aminobiphosphates (such as pamidronate) from the moment of treatment resembling the treatment of patients with these receptor-based therapies (Straetemans et al. Clin Can Res 2015;21:3957), max 10 injections per animal during the course of the experiment.
-Combination therapy can imply additional injections, preferably combined with the immune cell injections or separately injected i.v. Dosing schedule is dependent on compound used, an example is 250 microgram/injection at the day of receptor treatment followed by 2 additional injections 3 and 6 days later (John et al. Clin Can Res 2013;19:5636).
-Blood sampling: in general every 2 weeks or weekly blood sampling will be performed, and 50-70 microliter of blood will be collected per mouse per time point. We will adhere to the recommended blood sampling frequencies and volumes as-published by Diehl and colleagues (Diehl et al. J. Appl. Toxicol. 21, 15–23 2001)).
- Tumour development follow up: In order to evaluate tumour development, we will need to use appropriate imaging techniques (BLI, SPECT, PET, CT). For this purpose we will mainly use tumours that express luciferase in order to allow bioluminescence imaging (BLI) for easy non-invasive follow up. Tumour cells producing the enzyme Luciferase that converts Luciferin (injected ip) in a chemical reaction resulting in bioluminescence. This allows us to monitor tumour growth at relevant locations during the course of the experiment without sacrificing the animals. Alternatively, we can use survival as primary study outcome or we can take out tissues at predefined time points to quantify or characterize the tumour lesions by ex vivo analysis (e.g. flow cytometry). For BLI the duration varies between 5-10 minutes for one side and up to 20 minutes for both sides and will take place maximal once a week. PET/SPECT/CT generally last for more than an hour and will generally only take place once a week. For long-term anaesthesia we will use a dedicated life monitoring system that will record respiration rate and control body temperature to minimize any negative impact on the condition of the animal by the duration of the anaesthesia.
-In a small number of animals we will perform intravital 2-photon imaging to answer specific biological questions regarding migration, cell-cell interactions.
-At the end of the experiments organs, bone marrow and blood will be collected for further research at the pathology department (histopathology) or in our laboratory (DNA/RNA isolation, *in vitro* culture and experiments with cultured tumour cells).

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

Within our group we have established humanized tumour models for immune receptor therapy testing and results from these experiments are used to define size and number of experimental groups for any new experiment. Primary outcome parameters that are used to calculate number of animals are tumour growth and survival.
We make use of statistical methods to calculate the power to optimize group size. To this end an online program will be used such as the following: http://homepage.stat.uiowa.edu/~rlenth/Power/. In this way we will design realistic and statically sound experiments that allow scientific interpretation of the results obtained.

To illustrate such calculation, a description of a typical experimental design is outlined here. An experiment will generally comprise of several study arms (control group(s) and treatment groups). To demonstrate a 50% improvement in terms of tumour growth rate or survival between two groups (test vs controls) with the overall variability (relative standard deviation) being around 30, we will need a group size of 8 animals (power > 0.9 with $\alpha = 0.05$, two sided). However, if multiple treatment groups are included we need to increase the group size because we need to adjust for multiple comparisons (the α of 0.05 will be divided by the number of test groups minus 1 (e.g. 5-arm study will take α as 0.05/3 = 0.013). Overall, the group size will need to be 10 evaluable animals when performing a 5-arm study.

B. The animals

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

Immune deficient mice
We use immune deficient mouse strains as the basis of our pre-clinical *in vivo* models. The main reason for this is the fact that the γδT cell – target cell interactions are not present in mice, because mice lack specific γδT cell subsets and their ligands. Therefore we cannot use mouse T cells and mouse tumour targets in our models. Another reason is that in this way we do not need to redesign pre-clinical protocols and *in vitro* assays with mouse immune cells and mouse tumours that result in additional animal usage and possible hurdles when translating again back to the human situation. We have designed 'humanized mouse models' to evaluate γδTCR (TEG) – based immunotherapy that can be used for broader immune receptor-based therapy. Immune deficient mouse strains based on the NOD scid gamma (NSG) mice (official strain name NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) are being bred. These mice carry two mutations on the NOD/ShiLtJ genetic background and lack mature T , B, natural killer (NK) cells, are deficient in multiple cytokine signaling pathways and in many innate immune components such as complement factors.

Variants of immune deficient mice
A variant of the NSG strain, the NSG-SGM3, transgenic for 3 human cytokine genes (SC, GMCSF and IL-3) is used in experiments depending on the tumour of interest. Compared to the NSG mice, these mice allow increased engraftment of human primary leukemic samples and healthy human hematopoietic stem cells due to the constant production of non- or poorly cross-reacting human cytokines essential for hematopoietic (tumour) cell engraftment.
NSG mice transgenic for human molecules that are involved in the recognition mechanism can also be used in the tumour models. The usage of the strain is dependent on the immune receptor to test, the tumour and the scientific question in each experiment.

The mice are acquired from a commercial or research colony and further bred in house. We will combine both male and female in our experiments. We will preferably use mice between 8-21 weeks of age at the start of the experiment. We would like to end the experiments preferentially before the mice are 1 year old and therefore don't want to start with mice above 21 weeks. In some experiments we would like to rechallenge the mice again with tumour cells to study the long-term persistence of active immune cells (memory response). This requires that mice are not too old at the start of the experiment.

We use a power calculation to define the minimal group size we need, taking into account the expected treatment effect and the number of comparisons we want to make. Usually we don't need more than 10 mice per group (as calculated above under **A**). In case we need to harvest tissues during the experiment to answer research questions we will add additional animals (generally 3 mice) in order to keep the calculated group size for evaluation of primary outcome parameters during the whole experiment. Per experiment we will generally use not more than 5 treatment groups including negative and positive control treatment. If we apply the maximum group size we need 5 x 13 = 65 mice per experiment. Due to biological limitations such as primary tumour samples or human stem cell material but also *in vitro* production limitations of therapeutic cells and compounds, we don't expect to have larger experiments.

Estimated numbers:
We expect to perform 5 orthotopic experiments per year

This results in 5 x 65 = 325 mice per year and for 5 years a total amount of 5 x 325 =1625 mice Randomization will take place based on gender in case of the early treatment model. We are using both male and female mice in our experiments. In case of established tumour treatment models randomization also includes tumour size.

The intravital 2-photon imaging experiments are specified separately here because of the nature of the experiments, but may be performed in parallel depending on the research question. We will image presence, trafficking and behavior of immune cells, such as TEGs, labeled with a fluorescent dye, in or around tumour cells and vasculature. Cell-cell interactions are visualized and in each animal we are able to image numerous cells or cell-cell interactions. Therefore, the above described power analysis does not apply for these type of experiments. In fact, within one mouse we are able to measure more than enough images to perform statistically sound experiments. We expect to use maximal 6 mice per experiment due to the time consuming nature of the imaging and preparation per animal (around 2 hours per mouse) and we expect to need one or more pilot experiments to establish the technique for our purpose. Therefore, we estimate for 5 years to be able to perform 5 experiment: 5 x 5 x 6 = 150 mice including the pilot experiments.

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.

Extensive *in vitro* assays have been performed and results are evaluated critically. Only when the candidate immune receptor meets the go/no go criteria as stated in the project proposal it will be selected for testing in the *in vivo* models. To model tumour cell – immune cell interactions *in vitro* we apply state-of-the-art models and techniques such as the organoid / tumouroid models and the 3D bioprinted bone marrow model in order to gather valuable information without the usage of animals. However, studying the complexity of long-term interactions between tumour cells, environmental factor such as tumour stroma, oxygen supply and immune cells and immunotherapy is not possible (yet) in *in vitro* assay systems. And before a new therapy can enter clinical evaluation pre-clinical *in vivo* testing is required.
We apply a power calculation to minimize the number of animals and scientific literature is constantly used to prevent repetition of already performed experiments. In case new tumour types are being used, growth curves and/or pilot experiments may have to be performed to develop a new model suited for therapeutic testing before complete *in vivo* experiments can be performed. To reduce unnecessary usage of mice only in case robust and consistent tumour cell engraftment will be obtained the model is suited to perform complete experiments. State-of-the-art methods and equipment to follow-up tumour growth (imaging) will be used to minimize discomfort to the animals.

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

Mice will be housed in groups and have standard enrichment in their cage. Mice are daily monitored according to local guidelines and an extensive welfare check is performed minimal 2 times a week. In order to minimize suffering, we will adhere to the national and internationally accepted rules (Code of Practice) of handling lab animals in oncology (see P. Workman et al. Guidelines for welfare and use of animals in cancer research. Br J Cancer 2010; 102: 1555-1577). Specifically, animals will be monitored closely for tumour growth or any treatment related (unexpected) discomfort by visual inspection and

tumour measurements to identify animals at risk for developing symptoms. When the humane endpoint is reached mice will be humanely killed to prevent severe discomfort.

Repetition and duplication

E. Repetition

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

Not applicable. The proposed research does not relate to legally required research.

Accommodation and care

F. Accommodation and care

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

☒ No

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

G. Location where the animals procedures are performed

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

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

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

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

Injections (i.v., s.c., i.p.) will possibly cause pain, but due to the very short moment of pain, applying pain relieving anaesthesia or analgesia will cause equal discomfort to the mice.

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

Tumour cell injections and placing the window for intravital 2-photon imaging need surgery and as described above this will be under general anaesthesia and adequate analgesia.
During BLI or intravital 2-photon imaging mice will be under anaesthesia using isoflurane.

I. Other aspects compromising the welfare of the animals

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

Animals that carry tumours in internal organs may experience disfunction of these organs like cancer patients. For example tumour formation in the lungs may result in respiratory problems and tumours in the bone marrow can result in paralysis of the hind legs. Treatments may cause toxic side effects.

Explain why these effects may emerge.

These effects are the consequence of tumour growth and treatment.

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

In general the negative effects on the well-being of the animals by the tumour cannot be prevented. In order to minimize the burden of the tumour, the animals will be monitored at a frequency that is dictated by the model and timely killed when the humane endpoint is met.
For example for the haematological tumour models, a cage lid test is developed in close communication with the local IVD. In short, twice a week mice are placed on the lid of the cage and the cage is being held upside down. In case the mouse is not able to hold on to the cage with one of its paws, the mouse does not pass the cage lid test and the experimental endpoint is reached for this mouse in order to minimise discomfort.
Nevertheless, unforeseen complications may occur. In such cases, we will try to find solutions that will minimize the impact of the complications, for example by providing easy access to food (mush-feeding).

J. Humane endpoints

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

☐ No > Continue with question K.

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

We will adhere to the Code of Practise of lab animals used in oncology, in line with internationally agreed rules (Workman et al. Guidelines for welfare and use of animals in cancer research. Br J Cancer 2010; 102: 1555-1577).
The humane endpoints that apply in the orthotopic models are:

- A weight loss of more than 15% in 48 hours or total 20% of the initial body weight, measured from the start of the treatment.
- Severe abnormal breathing.
- Not passing the cage lid test

Indicate the likely incidence.

We estimate that around 20% of mice will reach one of the predefined humane endpoints of weight loss or severe abnormal breathing. In the remaining 80% of mice the experimental endpoint is based on imaging or cage lid test results (20% moderate) or peripheral blood tumour load, or the tumour burden is low at the moment the animal is killed for the collection of tissues or is low due to the treatment effect and/or the experiment is ended before the humane endpoint is reached.

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').

Induction of the tumour is expected to give the following level of discomfort:
i.v. injection through tail vein: mild discomfort
surgical implantation of tumour cells: moderate discomfort
Intravital 2-photon imaging will only be perform in limited number of animals: moderate discomfort
Interventions:
-Simple well-tolerated interventions (injection of cells, [redacted] or other compounds, chemotherapy or irradiation) will cause discomfort classified as mild.
-Simple but frequent handlings like weighing, caliper measurements and cheek vein bleeding will cause discomfort classified as mild.
-Bioluminescence imaging is classified under mild discomfort due to recovery from anaesthesia.
-PET/CT/SPECT will be classified under moderate discomfort due to the duration of anaesthesia.

In case tumours grow to the size that the humane endpoint weight loss or severe abnormal breathing is reached these will cause severe discomfort. We expect that animals under this appendix may suffer from severe discomfort in 18% of cases. In those cases where the animal will be sacrificed based on cage lid test (20% moderate discomfort), imaging results, collection of tissues, or due to effectivity of therapy

will not reach the endpoint, the discomfort level will be less (37% mild or 20% moderate).

Table 1 summarizes the expected number of mice per discomfort classification:

	Mild discomfort	**Moderate discomfort**	**Severe discomfort**
Tumour model: orthotopic			
In %	37%	45%	18%
Total mice in 5 year	650	800	325

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

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

The condition of the animals reaching the humane endpoint or the use of tissues for further research both require that mice are killed at the end of the experiment.

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]

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'.	1150
1.2	Provide the name of the licenced establishment.	UMC Utrecht
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3.4.1.4 Type of animal procedure: Humanized mouse models: safety testing

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 main focus of these experiments is to test the safety of the candidate immune receptor-based therapy. Depending on the receptor of choice the model for safety testing is designed. Combination of receptor based-immune therapy with other compounds will be tested for safety too. The compounds/treatments that are used to combine are generally already tested before but not in combination with our receptor therapy. The safety will be tested in non-tumour bearing animals.

Human safety and off-target toxicity of immune therapies and cell-based therapies cannot be predicted with conventional *in vitro* and animal models and this is recognized by regulatory authorities for medicinal products. For this reason, no conventional safety or toxicology studies will be performed for immune receptor based therapy as they are considered not to provide any relevant information in order to assess the safety of this class of medicine in man.
However, a non-clinical development program has been designed which compares safety and toxicity directly to efficacy by the use of efficacy-toxicity balance studies. A series of these efficacy-toxicity balance studies *in vitro* and *in vivo* have been performed demonstrating that TEG001 is able to differentiate between malignant cells versus healthy cells within one model. And this strategy will be applied for next generation compounds and combination therapies as well. This means that efficacy against malignant cells (appendix 1, 2, 3) are directly linked to safety (appendix 4).
In all safety experiments immune receptor therapy protocols as described in the efficacy (tumour models) apply, but the procedures are listed again below. Strategies of safety testing in humanized mouse models are the following:

A. Off-target toxicity models

Target molecule transgenic mice

Mice transgenic for human molecules involved in tumour recognition can be used to test safety of selected receptor formats. An example is an NSG mouse strain transgenic for a human molecule indispensable for the receptor recognition mechanism of malignant cells, but on healthy cells this molecule does not induce targeting of the cell *in vitro*. Long-term interactions of immune receptors with healthy cells expressing the target molecule can be studied *in vivo* in these mice. Although this is not a classical XenoGVHD model as described below, if any off-target toxicity effects can be expected it will be a T cell driven effect, most likely causing GVHD-like symptoms as described in an immunocompetent mouse model (Bendle et al. Nature Medicine 2010;16:565). Since, we have performed extensive *in vitro* safety assays before initiation of the *in vivo* experiment, severe off-target effects are not expected, but to be prepared we will use a GVHD-like clinical symptom scoring system (see below under welfare monitoring for details). For all safety experiments this scoring systems is applied.

Main outcome parameters: GVHD symptom scoring and survival

Secondary parameters: biodistribution and off-target effects in vital organs (immunohistopathology), immune cell persistence and cytokine levels in peripheral blood (Bendle et al. Nature Med 2010;16:565).

In case no off-target toxicities are observed for a selected receptor in this type of experiment, we proceed to combining efficacy in the presence of a tumour target along with safety in the same experiment. The experiment will be performed according to appendix 1, 2 or 3 including the GVHD clinical scoring for off-target toxicity and biodistribution analysis in blood

Human healthy cell engraftment

In case there are no transgenic mice available that express the target molecule for a selected immune receptor, mice are engrafted with human healthy cells as potential target for toxic effects of immune receptor-based therapy. For example, we engraft mice with human hematological cells derived from healthy cord blood stem cells. Engrafted stem cells differentiate into hematological subsets and that allows us to study the effect of therapy against healthy human cells in long-term cell-cell interactions. Other (stem) cell sources may be used too such as Epithelial Progenitor Cells (EPCs) and/or Mesenchymal Stromal Cells (MSCs).

Main outcome parameters: GVHD symptom scoring, survival and depletion of human cellular subsets.

Secondary parameters: biodistribution and off-target effects in vital organs (immunohistopathology), immune cell persistence and cytokine levels in peripheral blood (Bendle et al Nature Med 2010).

B. Modeling Graft Versus Host Disease (GVHD).

Immune deficient mouse models are being established to test for xenograft reactions as a model for GVHD in order to test if cellular therapy is safe in an allogeneic setting (Ito et al. Transplant 2009;87:565, Schroeder and DIPersio Disease Models and Mechanisms 2011;4:318 and Bendle et al Nature Med 2010;16:565). Humanized mouse models can be used to test the allogeneic potential of immune receptor-based therapy as described previously (Bondanza et al. Blood 2006; 107:1828)

Main outcome parameters : GVHD symptom scoring and survival

Secondary parameters: off-target effects and biodistribution of immune cells (TEGs) in vital organs (immunohistopathology), immune cell persistence and cytokine levels in peripheral blood (Bendle et al. Nature Med 2010;16:565). A detailed scoring protocol, combining these parameters into a quantitative scoring is developed before initiation of the study based on previous studies.

Secondary parameters: biodistribution of therapeutic receptors (TEGs) in vital organs, long-term persistence in blood, off-target effects on mouse tissues.

To be able to evaluate long-term effect of the treatment the experiment duration is maximal 200 days.

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

Engraftment with healthy human cells.

Immune deficient mice are engrafted with healthy human (stem) cells usually i.v. or s.c. or orthotopically in the relevant organ or location. Orthotopic implantation of healthy cells will be performed under anesthesia and analgesia according to local guidelines.

The following animal procedures for treatment are identical to those in appendix 1-3.
-Preconditioning will be applied before treatment. This will either be whole body irradiation or consists of a chemotherapeutic regiment. A chemotherapeutic regiment will be based on standard clinically used compounds in immunotherapy, such as (a combination of) cyclophosphamide, busulfan or fludarabin injected i.p. on 2-3 consecutive days. Dosing in mice will be based on protocols available for the specific compound and mouse strain and if needed a dose titration will be performed before initiation of the experiment.
-Mice will be treated with candidate receptor-based immunotherapy. The cells (or other format) will be injected usually i.v. or in some occasions s.c. to mimic representative location of a tumour (for local treatment at tumour site). Generally the treatment regimen consists of 2 doses, but can vary from a single dose up to daily injections during a defined period of weeks (max 3 weeks) depending on the receptor platform and research question.
-Candidate immune therapy will be supported by [redacted]. Human immune cells need crucial [redacted] for [redacted], which are lacking in the immune deficient mice. Therefore immune cell treatment is supported with [redacted] such as [redacted] or others or a combination thereof. Generally these growth factors are applied s.c. in a carrier called Incomplete Freunds' Adjuvant (IFA) every 21 days from the moment of treatment, max 10 injections/animal during the course of the experiment.
-For defined receptors, mice are every 21 days i.v. injected with aminobiphosphates (such as pamidronate) from the moment of treatment resembling the treatment of patients with these receptor-based therapies (Straetemans et al. Clin Can Res 2015;21:3957), max 10 injections per animal during the course of the experiment.
-Combination therapy can imply additional injections, preferably combined with the immune cell injections or separately injected i.v. or locally in some occasions s.c. to mimic representative location of a tumour (for local treatment at tumour site). Dosing schedule is dependent on compound used, an example is 250 microgram/injection at the day of receptor treatment followed by 2 additional injections 3 and 6 days later [redacted]
-Blood sampling: in general every 2 weeks or weekly blood sampling will be performed, and 50-70 microliter of blood will be collected per mouse per time point. Blood is collected in order to monitor safety parameters such as immune cell persistence and cytokine levels in peripheral blood, but also to monitor if healthy cell compartments engrafted in blood, are affected in peripheral (flow cytometry). We will adhere to the recommended blood sampling frequencies and volumes as published by Diehl and colleagues (Diehl et al. J. Appl. Toxicol. 2001;21:15)
-At the end of the experiments organs, bone marrow and blood will be collected for further research at the pathology department or in our laboratory. In case pathological research will be performed CO2 euthanasia is applied. In any other case cervical dislocation will be applied to euthanize the mice.

Welfare monitoring
Clinical symptoms:
The experiments will be observational using the GVHD scoring system based on the following parameters weight loss, hunching, activity, fur texture, skin integrity and diarrhea. The scoring combines the parameters in order to prevent severe discomfort, but allows quantification of the clinical symptoms. In classical GVHD model systems severe body weight loss of >20% is observed, due to severe colitis coinciding with the occurrence of severe diarrhea. Often the skin, liver, and kidneys are other organ systems involved. To prevent severe discomfort, the scoring prevents the occurrence of advanced stages of GVHD. Animals will be humanely killed when a combination of clinical symptoms reach the maximum score. The clinical scoring is combined with sampling of blood to assess physiological parameters such as biodistribution of immune receptors and immune cells (see below), as well as cytokine levels.

Biodistribution: Immune receptor and immune cell persistence in peripheral blood
Immune cell persistence (long-term survival *in vivo*) will be monitored in peripheral blood by flow cytometry. We have established a protocol to quantify cells in peripheral blood of the mice using quantification beads in combination with labeling antibodies. We can measure the immune cells, such as TEG cells, labeled with specific antibodies against the immune receptor and at the same time we can measure phenotypic characteristics of these cells (inhibition markers or differentiation markers). Regular bleeding of mice allows us to determine if immune cells are viable and effective and

Biodistribution and imaging: Immune receptor and immune cell detection with live imaging techniques (BLI or PET/CT)If immune cells are labeled with an appropriate tracer, live imaging techniques can be applied to measure biodistribution in the context of safety. - During these measurements mice need to be absolutely immobile and will therefore be anesthetized by isoflurane before they receive an i.p. injection of Luciferin. In case both sides will be measured it generally lasts 20 minutes and will take place maximal 1 time a week.

Biodistribution and pathology: Immune receptor and immune cell detection and histopathological studies in organs.
During the course of the experiments and/or at the end of each experiment we will harvest organs, bone marrow and peripheral blood to analyze the presence of receptors and immune cells, isolate RNA and/or DNA or perform histopathological analysis in order to evaluate biodistribution and possible on and off-target toxicities. In case of organ collection mice will be euthanized using CO2.

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

We make use of statistical methods to calculate the power to optimize group size. To this end an online program will be used such as the following: http://homepage.stat.uiowa.edu/~rlenth/Power/. In this way we will design realistic and statically sound experiments that allow scientific interpretation of the results obtained.
The primary outcome of the safety study is the GVHD clinical scoring and survival based on these clinical symptoms.
To illustrate such calculation, a description of a typical experimental design is outlined here. An experiment will generally comprise of several study arms (control group(s) and treatment groups). To demonstrate a 50% improvement in terms of survival between two groups (test vs controls) with the overall variability (relative standard deviation) being around 30, we will need a group size of 8 animals (power > 0.9 with $\alpha = 0.05$, two sided). However, if multiple treatment groups are included we need to increase the group size because we need to adjust for multiple comparisons (the α of 0.05 will be divided by the number of test groups minus 1 (e.g. 5-arm study will take α as $0.05/3 = 0.013$). Overall, we need a group size of 10 animals in case 5 treatment groups are used. In case we will harvest organs during the time course of the experiments (in case we are interested in early safety effects or distribution of immune receptors), we will include additional animals (max 3) to keep the appropriate group size for evaluation of treatment effect. This results in maximal 13 mice per group.
In case no clinical symptoms occur biodistribution of immune effector cells will help to make a good assessment whether a treatment is safe or not. Organs will be harvested at the end of the experiment .

B. The animals

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

Because we design balanced efficacy and safety studies the same immune deficient mouse strains, male and female, as in the tumour models for the specific immune receptor-based therapy are used. These were the NSG, NSG SGCM3 or NSG transgenic for human molecules. The experiments under A and B have a comparable design: a maximum of 5 groups per experiment including control groups with 13 animals per group results in 50 animals per experiment. We estimate to perform 5 safety experiments per year. This results in maximum of 65 animals per experiments and a total of 325 animals per year. In 5 years we will use 1625 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.

The experiments described are necessary to determine the safety of immune-receptor based therapy in case this cannot be tested in *in vitro* systems. It is crucial to have safety data acquired before proceeding toward a clinical application in man. We combine the possible and available *in silico*, *in vitro* and *in vivo* models to allow the most efficient safety testing to proceed to the clinic and to prevent unnecessary animal usage. For instance 2D and 3D culture models (bioprinted bone marrow models or organoids) are refined to test safety for immune receptor-based therapy in our laboratory, however complex and long-term cellular interactions cannot (yet) be modeled *in vitro*.

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

Mice will be housed in groups and have standard enrichment in their cage. Mice are daily monitored according to local guidelines and an extensive welfare check is performed minimal 2 times a week and intensified in case needed and we implement timely sacrifice of mice when the condition of the animal deteriorates. Weight loss will be a guiding parameter, but all other signs of discomfort will be taken into account.

Repetition and duplication

E. Repetition

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

Not applicable. The proposed research does not relate to legally required research

Accommodation and care

F. Accommodation and care

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

☒ No

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

G. Location where the animals procedures are performed

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

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

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

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

Injections (i.v., s.c., i.p.) will possibly cause pain, but due to the very short moment of pain, applying pain relieving anaesthesia or analgesia will cause equal discomfort to the mice. The outgrowth of human healthy cells is not likely to result in any discomfort.

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

Anaesthesia and analgesia will be used in case orthotopic implantation of human healthy cells is applied. Anaesthesia will be used in case of live imaging for biodistribution questions.

I. Other aspects compromising the welfare of the animals

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

GVHD-like symptoms may occur as a result of immune receptor therapy as explained above.

Explain why these effects may emerge.

The weight loss may be a result of immune receptor of choice or the allogeneic potential of the immune cells. In any case it is a T cell drive toxicity that result in typical GVHD-like symptoms.

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

We will minimize the suffering of the animals by careful and frequently monitoring of the mice and by timely sacrificing the animal if the humane endpoint defined in the GVHD scoring system is reached. We will apply a very detailed scoring system including all possible parameters in the safety experiments and have this prepared in collaboration with the pathobiology department of the Veterinary Sciences and our scientific collaborators that established a similar GVHD model as we propose.

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.

Using the clinical scoring system to combine scoring (from 0-2) on the different clinical parameters, we prevent mice reaching severe discomfort in the safety experiments. Frequent monitoring will take place to enable this.
The following parameters are included weight loss, hunching, activity, fur texture, skin integrity and diarrhea.

Indicate the likely incidence.

We expect for experiments under A no toxicities, in previous similar experiments no clinical symptoms were scored at all. Under B, we expect in 2 out of 5 treatment groups (40%) GVHD-like symptoms scoring 2.

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').

Interventions
-Simple well-tolerated interventions (injection of cells, aminobiphosphonates, [redacted] or other compounds, chemotherapy or irradiation) will cause discomfort classified as mild.
-Simple but frequent handlings like weighing, and cheek vein bleeding will cause discomfort classified as mild.
-Orthotopic implantation of human healthy cells performed with surgery under anaesthesia and using analgesia is classified as moderate discomfort (50% of experiments under A, 25% of total in this appendix).

Off-target toxicity with GVHD-like symptoms:
A clinical scoring system to combine scoring on the different clinical parameters, will be optimized and discussed with the IvD, to ensure that HEP will be applied when animals reach moderate discomfort. We expect in A no off-target effects, based on results of previous experiments. For the experiments under B

we expect 2 out of 5 experimental groups to have GVHD score 2 which is moderate discomfort, incidentally mice will pass the GVHD humane endpoint and suffer from severe discomfort.

We expect that 60% of the experiments fall under A and 40% under B:
A. 50% moderate (due to orthotopic implantation) and 50% mild discomfort.
B. 2% severe (passed humane endpoint), 35% moderate (due to GVHD-like symptoms) and 60% mild discomfort

Table 1 summarizes the expected number of mice per discomfort classification of this appendix:

	Mild discomfort	**Moderate discomfort**	**Severe discomfort**
Total safety models			
In % and numbers			
A	50% (487 mice)	50% (488 mice)	0%
B	60% (390 mice)	38% (247 mice)	2% (13 mice)
Total	54% (877 mice)	45% (735 mice)	1% (13 mice)*

*due to round off values

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.

Due to the experiment, the condition of a part of the animals will require that the animal is humanely killed. Other animals cannot be re-used in other studies
and will also be killed. And in most of the cases we will use organs for further analysis and animals cannot be reused.

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 UTRECHT

A. Algemene gegevens over de procedure

1. Aanvraagnummer : 2017.II.523.037
2. Titel van het project : Immune receptor mediated control of tumours
3. Titel van de NTS : Immuun receptor therapie tegen kanker: effectief en veilig

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

5. Contactgegevens DEC

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

6. Adviestraject (data dd-mm-jjjj):
   - ☒ ontvangen door DEC: 13-10-2017
   - ☐ aanvraag compleet:
   - ☒ in vergadering besproken: 30-10-2017
   - ☐ anderszins behandeld:
   - ☒ termijnonderbreking(en) van / tot : 06-11-2017/16-11-2017
   - ☐ besluit van CCD tot verlenging van de totale adviestermijn met max. 15 werkdagen:
   - ☐ aanpassing aanvraag:
   - ☒ advies aan CCD: 28-11-2017

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

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

9. Correspondentie met de aanvrager
   - Datum vragen: 06-11-2017
   - Datum antwoord: 16-11-2017
   - Gestelde vragen en antwoorden:

Projectvoorstel

- 3.4 Onderzoeksstrategie, 3.4.2: U schrijft: *"In case of defined receptors treatment will be supported with aminobiphosphates,..."*. Wat is de ratio voor het gebruik van aminobiphosphates? En heeft dat invloed op het welzijn van de dieren? Graag toelichten.
  De rationale van het gebruik van bifosfonaten (bijvoorbeeld, APD pamidronaat dinatrium) is beschreven in het projectvoorstel onder 3.4.2 en hieronder:
  'Aminobiphosphonates, such as pamidronate or zoledronate, act on the deregulated cancer cell metabolism resulting in increased sensitivity of cancer cells to defined immune receptor treatment. Pamidronate has been applied in previous humanized mouse tumour models treated with TEGs (Marcu-Malina et al. Blood 2011;118:50, Gruender et al. Blood 2012;120:5153 and Straetemans et al. Clin Can Res 2015;21:3957).'
  Daarnaast is het toedienen van bifosfonaten nu gespecificeerd in bijlage 1 t/m 4 onder 'K. Classification of severity of procedures' onder eenvoudige handelingen met ongerief classificatie mild. Uit onze eigen ervaring is geen ongerief van de toediening van bifosfonaten gebleken.

Bijlage 1

- A. Experimentele aanpak en primaire uitkomstparameters: De DEC gaat er vanuit dat de tumorcellen op één plaats worden toegediend en indien een tweede challenge nodig is, dat u dat op een andere plaats doet.
  Een tweede tumor challenge zal plaatsvinden op een andere plaats, bijvoorbeeld in de andere flank. Deze tekst is toegevoegd in Bijlage 1 onder A.
- J. Humane eindpunten vs. K. Classificatie van ongerief: Bij het humane eindpunt spreekt u over skin ulceration als eindpunt en bij de classificatie van het ongerief over ulceration. Graag verhelderen en consistent weergeven.
  Er wordt hier bedoeld ulceratie van de huid als gevolg van de subcutane tumorgroei. Dit is aangepast in de tekst in bijlage 1 onder J en K.
- J. Humane eindpunten vs. K. Classificatie van ongerief: Bij de humane eindpunten zegt u: *"In case in the subcutaneous models the humane endpoint is reached it is because of a maximum tumour size (40% of total mice) or due to ulceration (10% of total mice)"*. Vervolgens zegt u bij de classificatie van het ongerief dat: *"Severe ulceration is classified as severe discomfort and the humane endpoint is reached (1%)"*. Het is nu niet helder hoeveel muizen het humane eindpunt zullen halen als gevolg van ulceratie: 1% of 10%. Graag verhelderen en consistent weergeven.
  Het was inderdaad niet juist weergegeven. Onder J moet het zijn: 1% van de muizen bereikt het humane eindpunt door ulceratie van de huid als gevolg van subcutane tumorgroei. Dit is aangepast in de tekst in bijlage 1 onder J en K.

- De antwoorden hebben geleid tot aanpassing van de aanvraag.

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

B. Beoordeling (adviesvraag en behandeling)

1. Het project is vergunningplichtig (dierproeven in de zin der wet).
2. De aanvraag betreft een nieuwe aanvraag.
3. De DEC is competent om hierover te adviseren.
4. Er zijn geen DEC-leden betrokken bij het betreffende project.

C. Beoordeling (inhoud):

1. De aanvraag is toetsbaar en heeft voldoende samenhang. Het uitgangspunt van de onderzoekers is een nieuw soort immunotherapie, gericht tegen bepaalde receptoren op kanker cellen. De tot nu toe toegepaste klassieke immunotherapieën met antistoffen tegen zogenaamde checkpoint inhibitors (membraan antigenen voorkomend op immuuncellen welke betrokken zijn bij het aan- of uitzetten van de immunologische reactie) en met zg. Chimere Antigeen Receptor (CAR) T-cellen (een klasse T-cellen die in vitro zijn getransfecteerd met receptoren tegen tumorantigenen) zijn succesvol. Deze vorm van immunotherapie werkt goed en vooral tegen tumoren met een hoge zogenaamde 'mutational load', dus met veel mutaties. Maar tumoren met een lage 'mutational load' zoals bijv. acute myeloide leukemie zijn veel minder gevoelig voor deze vormen van immunotherapie.
   De onderzoekers hebben in voorgaande projecten een nieuw soort immunotherapie ontwikkeld welke gebruik maakt van een populatie T-cellen (γδT cellen) die natuurlijkerwijze al betrokken is bij de afweer tegen tumoren. Deze klasse van T-cellen kan tumorcellen van gezonde cellen onderscheiden, niet door hun mutaties als kankercel, maar op basis van kleine veranderingen in het lipide metabolisme van de kankercel. Deze klasse T-cellen zou dus ook effectief kunnen zijn bij tumoren met een lage mutational load.
   De onderzoekers hebben dit concept al in het laboratorium ontwikkeld door deze T-cellen te transfecteren met de gewenste receptor en deze tot expressie te brengen. Deze specifieke γδTCellen zijn in vitro gekloneerd en gekweekt (TEG's). Deze eerste TEG001 wordt momenteel in patiënten verder onderzocht. Intussen hebben de onderzoekers diverse γδT-cel klonen ontwikkeld welke onderzocht zijn op tumor specifieke cytotoxiciteit.
   De Commissie heeft uitvoerig stilgestaan bij deze aanvraag vanwege de uitgebreide immunologische kennis welke nodig is om deze aanvraag te begrijpen, maar is unaniem van menig dat de aanvraag zeer goed is opgeschreven. Het gaat hier om zeer hoogwaardig onderzoek waar al veel zorgvuldig vooronderzoek aan vooraf is gegaan in de selectie en werkzaamheid van de γδT-cel klonen (TEG's). Het is logisch dat de verdere werking nu in vivo

moet worden onderzocht op tumoren met een lage mutational load. Het ligt dan ook voor de hand om dit in muismodellen met tumoren te doen, waarbij in de muizen dan humane tumoren worden geïnduceerd/geïmplanteerd en het muizen immuunsysteem genetisch is uitgeschakeld (gehumaniseerde muizen). De aanvraag is daardoor een toetsbaar en navolgbaar geheel, welke het meeste lijkt op voorbeeld 1 uit de "Handreiking Invulling Definitie Project".

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

3. De in de aanvraag aangekruiste doelcategorieën sluiten aan bij de hoofddoelstellingen. Hoewel de termen werkzaamheid en veiligheid onde
rdelen zijn van deze aanvraag is de Commissie van mening dat het hier geen wettelijk voorgeschreven onderzoek betreft voor registratie doeleinden van een nieuwe immunotherapie, maar vooronderzoek om uiteindelijk op basis van werkzaamheid en veiligheid intern de juiste kandidaat te kiezen voor verdere ontwikkeling en trials in de mens. Volgens de onderzoekers is voor dit type onderzoekers geen verder wettelijk verplicht onderzoeknoodzakelijk.

Belangen en waarden

4. Het directe doel van het project is het onderzoeken van nieuwe immuuntherapieën met bepaalde T-cel klonen tegen bepaalde type tumoren in muismodellen. Het uiteindelijke doel van het project is patiënten met een tumor welke niet gevoelig is voor een behandeling met antistoffen of CAR T-cellen te behandelen met in het laboratorium gekweekte gamma/delta T-cellen (klonen) welke verschillen kunnen "zien " in lipide metabolisme van de tumorcel en daarnaast een specifiek antigeen op de tumor herkennen hetgeen leidt tot cytotoxie van de tumor cel. Daarvoor is het eerst nodig het biologische werkingsmechanisme op te helderen en het mechanisme achter de specifieke biodistributie (hoe komen de gekloonde T-cellen in voldoende mate bij de tumor)? De T-cellen worden [redacted] om de distributie te optimaliseren en de werking aan te kunnen tonen. Verder is het belangrijk dat de therapie veilig is en de T-cellen alleen de tumor aanvallen en geen andere cellen. Als laatste stap voor de beslissing of de T-cel kloon geschikt is voor verdere ontwikkeling is het belangrijk of deze in voldoende mate kan worden geweekt met methodieken welke vallen onder Good Manufacturing Procedures omdat alleen deze productie methode volgens de autoriteiten toegelaten zal worden voor humane therapieën. De DEC is daarom van mening dat er in voldoende mate een samenhangende relatie is tussen het directe doel en het uiteindelijke doel.

5. De belangrijkste belanghebbenden in dit onderzoeksproject zijn: proefdieren, onderzoekers, en kankerpatiënten. De morele waarden die voor het proefdier in het geding zijn: de implantatie van een humane tumor en het daarmee gepaard gaande ongerief van de operatie en de groei van de tumor. De morele waarden die voor de onderzoekers worden bevorderd zijn: het verkrijgen van meer fundamentele kennis over het gebruik van specifieke T-cel populaties voor de behandeling van bepaalde tumoren. De morele waarden die voor de patiënten worden

bevorderd is dat er in potentie nieuwe behandel methoden beschikbaar komen met tumor specifieke cytotoxische T-cellen als immunotherapie voor tumoren welke niet of slecht reageren op bijv. een behandeling met antistoffen tegen checkpoint inhibitors.

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

Proefopzet en haalbaarheid

7. De kennis en kunde van de onderzoeksgroep en andere betrokkenen bij de dierproeven zijn ruim voldoende gewaarborgd en dragen eraan bij dat de doelstellingen behaald kunnen worden, dat aan de 3V-beginselen voldaan kan worden en dat voorkomen kan worden dat mens, dier en milieu negatieve effecten ondervinden als gevolg van de dierproeven. Er is heel veel vooronderzoek verricht in het laboratorium met o.a. celkweken, en organoid modellen waardoor veel dieren bespaard zijn. De betrokken onderzoekers zijn zeer ervaren op dit gebied.

8. Het project is goed opgezet, de voorgestelde experimentele opzet en uitkomstparameters sluiten logisch en helder aan bij de aangegeven doelstellingen en de gekozen strategie en experimentele aanpak kan leiden tot het behalen van de doelstelling binnen het kader van het project. Zoals bij C1 al uitgelegd, hebben de onderzoekers al veel in vitro vooronderzoek gedaan. Op basis van die resultaten worden kandidaten gekozen voor verdere werkzaamheid in de verschillende tumormodellen (onderhuids, uitzaaiingen en orthotopische tumoren). De gekozen strategie is voor de Commissie helder en heeft als zodanig geen vragen opgeroepen. Wel heeft de Commissie gediscussieerd over de te gebruiken tumormodellen. Er wordt niet echt toegelicht welke tumormodellen gebruikt zullen worden, maar onderzoekers leggen uit dat ze zullen kiezen voor modellen die bepaalde humane tumoren het beste benaderen. Voor het ongerief van de muis kan het type tumor echter wel uitmaken en ook wat de plaats is (orthotoop, subcutaan, etc.) waar de tumor geïnjecteerd wordt. Maar de DEC is van mening dat het ongerief goed is afgebakend en dat de keuze over welke tumormodellen gebruikt gaan worden zorgvuldig genomen wordt. Verder geven onderzoekers nog aan dat ze zelf geen modellen willen gaan opzetten, maar gebruik zullen maken van reeds bestaande en goed beschreven en gevalideerde tumormodellen. De DEC is derhalve van mening dat het voor de ethische afweging niet uitmaakt dat de tumormodellen niet genoemd worden.

Welzijn dieren

9. Er is geen sprake van de volgende bijzonderheden op het gebied van categorieën van dieren, omstandigheden of behandeling van de dieren:
   - ☐ Bedreigde diersoort(en) (10e lid 4)
   - ☐ Niet-menselijke primaten (10e)
   - ☐ Dieren in/uit het wild (10f)
   - ☐ Niet gefokt voor dierproeven (11, bijlage I EU richtlijn)
   - ☐ Zwerfdieren (10h)

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

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

11. Het cumulatieve ongerief als gevolg van de dierproeven is realistisch ingeschat en geclassificeerd. De onderzoekers hebben de diverse aspecten van pijn en ander ongerief per ingreep goed beschreven in de bijlagen. Ze beschrijven ook welke observaties bijdragen aan het ongerief en hoe dat door hen geclassificeerd wordt. Ook geven ze aan welke complicaties ze verwachten bij de tumorgroei en wanneer ze om die reden een humaan eindpunt gaan toepassen. Deze door de onderzoekers ingeschatte mate van ongerief is geen aanleiding geweest voor een discussie hierover door de Commissie.

12. De integriteit van de dieren wordt fysiek aangetast door de implantatie van de tumor en de groei daarna en de behandeling van de tumor. Ook zal de meting van de tumorgroei onder beeldvormende techniek de dieren in hun fysieke welzijn aantasten omdat zij (kort) worden verdoofd. Ondanks deze fysieke aantasting kunnen de dieren wel hun normale gedrag vertonen. Deze aantastingen in fysiek welzijn zijn onvermijdelijk als de proeven in deze opzet worden uitgevoerd.

13. De humane eindpunten zijn in de bijlage dierproeven goed gedefinieerd en het percentage dieren dat naar verwachting een humaan eindpunt bereikt is goed ingeschat. De beschreven humane eindpunten volgen de 'code practice' voor kankeronderzoek en beschrijven met name de grootte van de tumor of het gaan bloeden of zweren. De genoemde aantallen zijn aangepast na vragen van de commissie en is een schatting gebaseerd op de praktijk. Er worden in het algemeen geen onverwachte verschijnselen verwacht welke aanleiding zouden kunnen zijn voor een humaan eindpunt. Daarnaast zou de behandeling met de T-cellen een zogenaamde graft versus host reactie (GvH) kunnen oproepen. Deze wordt beoordeeld op basis van een gevalideerd score systeem. Ook in dat geval is een humaan eindpunt onvermijdelijk. De onderzoekers geven aan dat dit bij 2 van de 5 behandelgroepen zou kunnen optreden. Het getal berust op eerdere observaties in voorafgaande experimenten en dit lijkt de commissie redelijk ingeschat.

3V's

14. De aanvrager heeft voldoende aannemelijk gemaakt dat er geen geschikte vervangingsalternatieven zijn om uiteindelijk een betrouwbare uitspraak te kunnen doen over de werkzaamheid van de behandeling van tumoren met cytotoxische T-cellen. Daarnaast is het belangrijk uit te zoeken of deze therapie ongewenste bijwerkingen heeft.

15. Het aantal te gebruiken dieren is realistisch ingeschat en er is een heldere strategie om ervoor te zorgen dat tijdens het project met het kleinst mogelijke aantal dieren wordt gewerkt waarmee nog een betrouwbaar resultaat kan worden verkregen. Er is een duidelijk uitgewerkte strategie met beslismomenten voor verder onderzoek. De aantallen zijn daarnaast statistisch onderbouwd. En hebben geen aanleiding gegeven tot discussie bij de Commissieleden tijdens de behandeling van deze aanvraag.

16. Het project is in overeenstemming met de vereiste van verfijning van dierproeven en het project is zodanig opgezet dat de dierproeven zo humaan mogelijk kunnen worden uitgevoerd. De betrokken onderzoekers hebben zeer veel ervaring met muis-tumor modellen. Doordat de groei van de tumoren ook met beeldvormende technieken wordt gevolgd, is niet te verwachten dat dieren onnodig leiden doordat tumoren inwendig te ver doorgroeien.

17. Er is geen sprake van wettelijk vereist onderzoek.

Dieren in voorraad gedood en bestemming dieren na afloop proef

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

19. De dieren worden in het kader van het project gedood om dat het humane eindunt is bereikt, of omdat de tumor en organen moeten worden uitgenomen voor verdere analyse. De dieren worden volgens een, bijlage IV van de EU richtlijn, passende methode gedood.

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

NTS

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

D. Ethische afweging

1. De morele vraag die de DEC dient te beantwoorden is of het belang van dit onderzoek, namelijk kunnen bepaalde specifieke T-cel klonen worden gebruikt als nieuwe immunotherapie voor tumoren met een lage mutatie, de onvermijdelijke aantasting van het welzijn en de integriteit van de gebruikte proefdieren kan rechtvaardigen.

2. Er vindt een beperkte tot aanzienlijke aantasting van welzijn en integriteit van de proefdieren plaats met mild, matig of ernstig ongerief.
   Indien de hierboven genoemde doelstellingen behaald worden, dan zal dit project er toe bijdragen dat de onderzoekers meer wetenschappelijk inzicht hebben verkregen in deze nieuwe

generatie immunotherapie en dat patiënten welke tumoren hebben welke nu niet of slecht gevoelig zijn voor de behandeling met de klassieke immunotherapie dan wel effectief behandeld zou kunnen worden. Het is aannemelijk dat de fundamentele & translationele doelstelling behaald zal worden. Daarvoor is de inzet van proefdieren noodzakelijk, maar de onderzoekers doen al het mogelijke om het ongerief voor de dieren en het aantal dieren tot een minimum te beperken. De Commissie is zeer onder de indruk van alle resultaten welke tot u toe al zijn behaald met in vitro methoden. De directe link van het onderzoeksteam met de kliniek is van grote waarde voor gebruik van de resultaten in de klinische praktijk op langere termijn.

3. Op grond van het bovenstaande is de DEC van oordeel dat de behandeling van bepaalde tumoren met tumor specifieke cytotoxische T-cellen een essentieel belang vertegenwoordigt en dat dit essentiële belang opweegt tegen de deels beperkte deels aanzienlijke aantasting van het welzijn en de integriteit van de proefdieren. Het gebruik van de proefdieren zoals beschreven in de aanvraag is daarmee gerechtvaardigd.

E. Advies

1. Advies aan de CCD

☒ De DEC adviseert de vergunning te verlenen.

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

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

☐ De DEC adviseert de vergunning niet te verlenen vanwege:

- ☐ De vaststelling dat het project niet vergunningplichtig is om de volgende redenen:...
- ☐ De volgende doorslaggevende ethische bezwaren:...
- ☐ De volgende tekortkomingen in de aanvraag:...

2. Het uitgebrachte advies is gebaseerd op consensus.

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

Centrale Commissie Dierproeven

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Onze referentie
Aanvraagnummer
AVD1150020174288
Bijlagen
2

Datum 5 december 2017
Betreft Ontvangstbevestiging aanvraag projectvergunning Dierproeven

Geachte [redacted]

Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 5 december 2017. Het gaat om uw project "Immune receptor mediated control of tumors". Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD1150020174288. Gebruik dit nummer wanneer u contact met de CCD opneemt.

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

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

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

Datum:
5 december 2017
Aanvraagnummer:
AVD1150020174288

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlagen:
- Gegevens aanvraagformulier
- Factuur

Datum:
5 december 2017
Aanvraagnummer:
AVD1150020174288

Gegevens aanvrager

Uw gegevens

Deelnemersnummer NVWA:	11500
Naam instelling of organisatie:	UMC Utrecht
Naam portefeuillehouder of diens gemachtigde:	[redacted]
Postbus:	12007
Postcode en plaats:	3501 AA UTRECHT

Gegevens verantwoordelijke onderzoeker

Naam:	[redacted]
Functie:	Wetenschappelijk projectleider
Afdeling:	Laboratory of Translational Immunology
Telefoonnummer:	[redacted]
E-mailadres:	[redacted]

Gegevens verantwoordelijke uitvoering proces

Naam:	[redacted]
Functie:	[redacted]
Afdeling:	Hematology and Laboratory of Translational Immunology
Telefoonnummer:	[redacted]
E-mailadres:	[redacted]

Datum:
5 december 2017
Aanvraagnummer:
AVD150020174288

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 februari 2018
Geplande einddatum:	31 januari 2023
Titel project:	Immune receptor mediated control of tumors
Titel niet-technische samenvatting:	Immuun receptor therapie tegen kanker
Naam DEC:	DEC Utrecht
Postadres DEC:	Postbus 85500 3508 GA Utrecht
E-mailadres DEC:	dec-utrecht@umcutrecht.nl

Betaalgegevens

De leges bedragen:	€ 1.684,-
De leges voldoet u:	na ontvangst van de factuur

Checklist bijlagen

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

Ondertekening

Naam:	[redacted]
Functie:	[redacted]
Plaats:	Utrecht
Datum:	4 december 2017

Centrale Commissie Dierproeven

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Onze referentie
Aanvraagnummer
AVD1150020174288
Bijlagen
2

Datum 5 december 2017
Betreft Factuur aanvraag projectvergunning Dierproeven

Factuur
Factuurdatum: 5 december 2017
Vervaldatum: 4 januari 2018
Factuurnummer: 174288
Ordernummer: CB.841910.3.01.011

Omschrijving	**Bedrag**
Betaling leges projectvergunning dierproeven Betreft aanvraag AVD1150020174288	€ 1.684,00

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

From: info@zbo-ccd.nl
To: Instantie voor Dierenwelzijn Utrecht
Cc: [redacted]
Subject: Aanhouden AVD1150020174288
Date: 11 January 2018 15:24:38

Geachte [redacted]

Op 05-12-2017 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Immune receptor mediated control of tumors" met aanvraagnummer AVD1150020174288. In uw aanvraag zitten voor ons nog enkele onduidelijkheden. In dit bericht leest u wat wij nog nodig hebben en wanneer u een beslissing kunt verwachten.

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

Onduidelijkheden
- U geeft aan dat de groepsgroottes berekend zijn op basis van o.a. verwachte behandeleffect en het aantal vergelijkingen die u wenst te maken. Daarnaast geeft u in het projectvoorstel de tumor take rate voor verschillende tumormodellen aan. Heeft u bij de berekening van de groepsgroottes rekening gehouden met deze tumor take rates? - In de legenda van figuur 1 verwijst u naar figuur 2 voor uitleg over keuze en beschrijving van de verschillende modellen. Deze figuur 2 is niet meegezonden met de stukken. Kunt u deze alsnog toesturen?

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.

Wanneer een beslissing
De behandeling van uw aanvraag wordt opgeschort tot het moment dat wij de aanvullende informatie hebben ontvangen. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Mocht u vragen hebben, dan kunt u uiteraard contact met ons opnemen.

Met vriendelijke groet,
Namens de Centrale Commissie Dierproeven

[redacted]
www.centralecommissiedierproeven.nl

.....
Postbus 20401 | 2500 EK | Den Haag
.....
T: 0900 2800028
E: info@zbo-ccd.nl

From: [redacted]
To: Instantie voor Dierenwelzijn Utrecht
Cc: [redacted]
Subject: Fwd: Aanhouden AVD1150020174288
Date: 22 January 2018 20:38:50
Attachments: 20180122 figure 1 research scheme [redacted].pdf

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Beste [redacted]

Hierbij de antwoorden voor de CCD vragen.

Voor punt 2: Ik heb de bijlage verbeterd en toegevoegd maar wellicht is het voldoende om aan te geven dat de zin die refereert naar een ander figuur abuis is. Maar dat kun jij misschien beter inschatten dan ik.

Antwoord op vragen CCD:
1) In de voorbeeld berekeningen in de bijlages is geen rekening gehouden met de tumor take.
In de meeste modellen zal dit naar verwachting 80% of meer zijn en is er geen correctie nodig. Indien dit wel nodig is naar aanleiding van de take van de tumor zal dit in de berekeningen van de betreffende experimenten worden meegenomen.
Deze berekeningen zullen altijd in overleg en na review van de IVD worden toegepast voor het bepalen van de uiteindelijke groepsgrootte in de betreffende werkprotocollen en experimenten. Aangezien de tumormodellen met een lage tumor take een zeer klein onderdeel zullen vormen van dit projectvoorstel, zullen de eventuele aanpassing van de groepsgrootte geen invloed hebben op het ingeschatte totaal aantal dieren dat gebruikt zal worden in dit project.
2) In de legenda van figuur 1 was onjuist gerefereerd naar figuur 2. Figuur 2 bestaat niet en de tekst in de legenda is aangepast in toegevoegde pdf.

Indien er nog opmerkingen zijn of ik nog iets moet doen, hoor ik het graag.
Met vriendelijke groet,
[redacted]

Begin forwarded message:

From: <info@zbo-ccd.nl>
Subject: Aanhouden AVD1150020174288
Date: 11 January 2018 at 15:24:34 CET
To: <info@ivd-utrecht.nl>
Cc: [redacted]
Reply-To: <info@zbo-ccd.nl>

Geachte [redacted]

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Met vriendelijke groet,
Namens de Centrale Commissie Dierproeven

[redacted]
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.....
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.....
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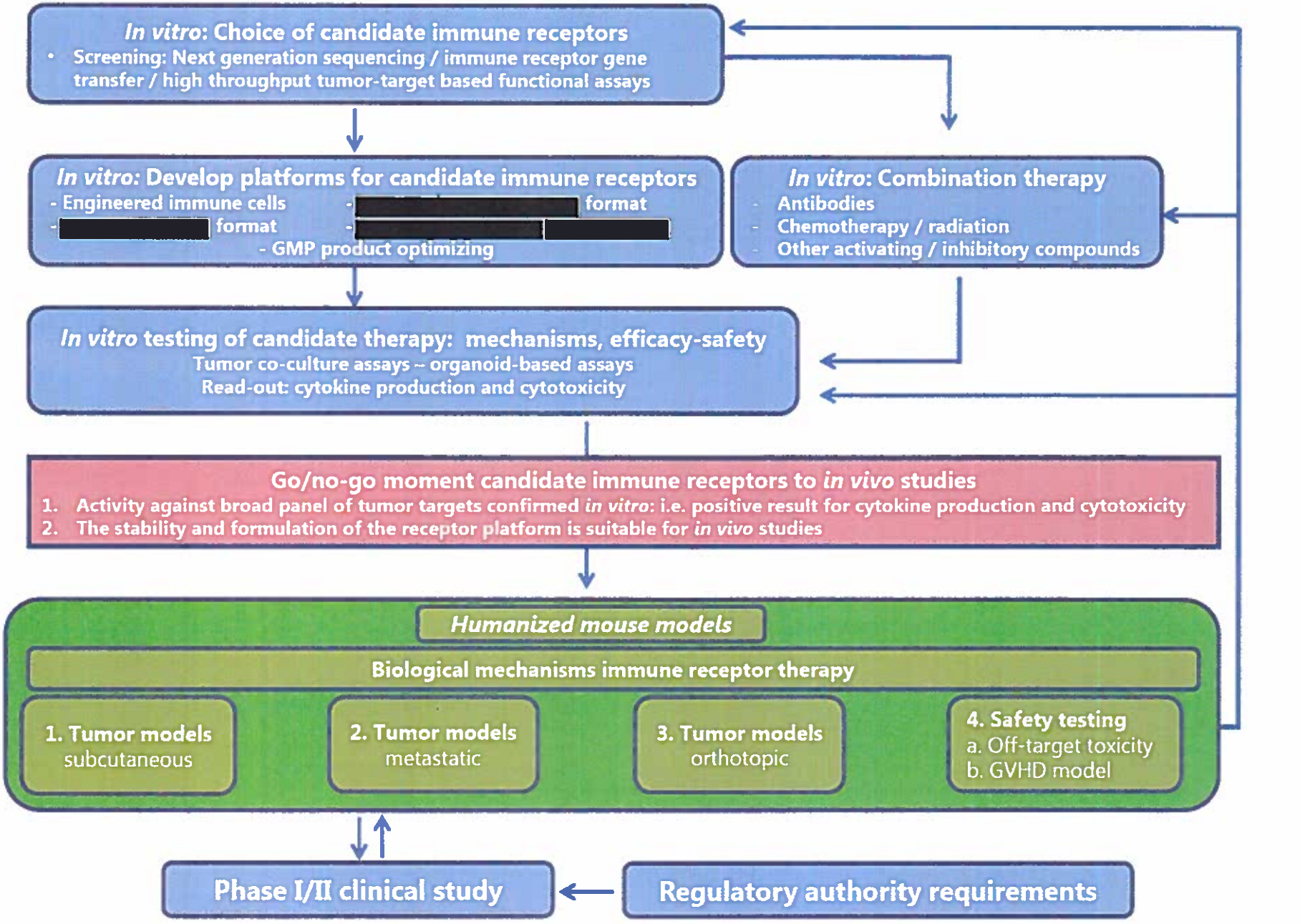
E: info@zbo-ccd.nl

Figure 1. Immune receptor mediated control of tumors: research pipeline

6.3



AVD1150020174288

Legend to figure 1. Immune receptor mediated control of tumors: research pipeline

A. Choice of immune receptors *(in vitro)*.

In order to define possible candidate immune receptors for usage in immunotherapy strategies, high-throughput screening using immune receptor gene transfer, sequencing techniques and functional tumour target-based assays are used to screen for anti-tumor cytotoxic potential (read-out cytotoxicity or cytokine production). Our lab has a strong focus on γδT cell receptors, but also other immune receptors can be potential candidates for further exploration. Broad anti-tumour function (cytotoxic and cytokine production) is requested for a receptor to proceed to the next level. In this phase a very broad and unbiased screening of receptors and tumours is applied and from here we can start with 100 to 10 possible receptor candidates.

B. *In vitro* development of platforms of immune receptors in therapy and combination therapy.

In this phase the potential tumour origin to target are defined based on: 1)the receptor(s) characteristics with respect to anti-tumour potency; 2)unmet medical need of cancer types; 3)other potential therapies in development for the particular tumour origin; 4)experts opinions (medical doctors in our research group, regulatory experts, medical advisors, scientific data available). Once potential candidate receptors are selected the platform for the therapeutic application will be chosen and tested *in vitro*. Possible platforms include the described TEG format but also [redacted] and [redacted] either [redacted] or [redacted] on a [redacted] are also being explored in our laboratory. New formats but also conceptual changes in the production protocol of engineered TEGs may influence their clinical potential and flow into the research pipeline. Based on clear rationale we select compounds to enhance efficacy of immunotherapy and test these in our research pipeline.

C. *In vitro* testing of immunotherapy strategy.

Pre-clinical *in vitro* assays are available in our laboratory that allow effectivity-safety testing of the anti-tumour treatment(s) and answer questions regarding the mechanism of action. Standard 2D co-culture assays with tumour cells and immune effector cells are used to assess the anti-tumour effect such as cytotoxic potential, cytokine production, and proliferation. More advanced 3D-bioprinted models are established to culture cell lines or primary tumour cells in the presence of accessory cells (epithelial cells, mesenchymal stem cells, fibroblasts or others) to mimic the tumour microenvironment. Advantages of 3D-models are a more physiological relevant environment, prolonged cell-cell interactions, successful growth of primary tumour cells and relative long-term assessment of immunotherapy strategies (up to 2-3 weeks). In addition, tumouroids (organoid structures from tumour cells) and healthy organoid culture systems are explored and used to test effectivity and safety of therapy *in vitro*. Microscopy techniques are applied to evaluate anti-tumour potential and answer biological questions. These *in vitro* models allow also to test interesting combinations of treatment options, i.e. TEG cells in combination with [redacted] (i.e. [redacted] antibodies) or other compounds/treatment modalities that can increase anti-tumour efficacy.

D. Go/no go of candidate immunotherapy into humanized mouse models.

Based on the *in vitro* assays that use cytotoxicity and cytokine production as read-out, candidate immune receptors (alone or in combination therapy format) will be selected for further evaluation in pre-clinical humanized mouse models. Receptors (alone or in combination therapy) that are active against a broad panel of tumour cell lines and primary tumour cells, but not against the healthy counterpart cells from a defined tumour origin are selected. Control immune receptors (positive, negative controls) are included in the mouse models. At this stage a limited panel of around 4-6 receptors remained.

E. Humanized mouse models

In the next phase towards clinical application humanized mouse models are used to evaluate selected candidate therapy for biological mechanisms, efficacy and safety questions . 4 different models are used in our research strategy. See project proposal 3.4.2 for a detailed description of these models.

1. Subcutaneous humanized tumour models
2. Metastatic humanized tumour models
3. Orthotopic humanized tumour models.
4. Safety models: A. Off-target toxicity models; B. Graft Versus Host Disease models

F. Phase I/II clinical study

If the candidate therapy proofs effective and safe from the pre-clinical studies, clinical studies will be designed to test the next generation immunotherapy.

The complete pre-clinical dataset including rational, choice of patient population, biological mechanism of action, efficacy-safety balance *in vitro* and *in vivo* will be submitted to the regulatory authorities for approval. Before initiation of a clinical study regulatory authorities may require additional pre-clinical testing in animal models and therefore regulatory feedback flows into our research pipeline.

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info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD1150020174288
Bijlagen
1

Datum 30 januari 2018
Betreft Beslissing aanvraag projectvergunning Dierproeven

Geachte [redacted]

Op 5 december 2017 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Immune receptor mediated control of tumors" met aanvraagnummer AVD1150020174288. Wij hebben uw aanvraag beoordeeld.

Beslissing
Wij keuren uw aanvraag goed op grond van artikel 10a lid 1 van de Wet op de dierproeven (hierna: de wet).

U kunt met uw project starten. De vergunning wordt afgegeven van 1 februari 2018 tot en met 31 januari 2023.
De onderbouwing van deze beslissing vindt u onder 'Overwegingen'.

Procedure

Advies dierexperimentencommissie
Bij uw aanvraag heeft u een advies van de Dierexperimentencommissie (DEC) DEC Utrecht gevoegd. Dit advies is ontvangen op 5 december 2017. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a lid 3 van de wet. Het advies van de DEC is betrokken bij de behandeling van uw aanvraag.

Nadere vragen aanvrager
Op 11 januari 2018 hebben wij u om aanvullingen gevraagd. U heeft tijdig antwoord gegeven. De aanvullingen hadden betrekking op de dieraantallen rekening houdend met tumor take en een missende figuur. Figuur 1 met aangepaste legenda is bijgevoegd bij de antwoorden. Uw antwoord is betrokken bij de behandeling van uw aanvraag.

Overwegingen
Alle hierboven genoemde stukken liggen ten grondslag aan ons besluit.

Wij kunnen ons vinden in de inhoud van het advies van de DEC, inclusief de daaraan ten grondslag liggende motivering.

Beoordeling achteraf
Na afloop van het project zal er een beoordeling plaatsvinden, zoals bedoeld in artikel 10a1 lid 1 sub d en artikel 10a1 lid 3 van de wet. De reden van deze beoordeling achteraf is dat in dit project dieren ernstig ongerief ondergaan. Beoordeling achteraf is nodig vanwege ernstig ongerief bij een deel van de dieren. Deze beoordeling zal uiterlijk januari 2024 plaatsvinden. Meer informatie over de eisen die gesteld worden bij de beoordeling achteraf vindt u in de bijlage 'Weergave wet- en regelgeving'.

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

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

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

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

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

Datum:
30 januari 2018
Aanvraagnummer:
AVD1150020174288

Centrale Commissie Dierproeven
namens deze:

[redacted]

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

Centrale Commissie Dierproeven

Projectvergunning

gelet op artikel 10a van de Wet op de Dierproeven

Verleent de Centrale Commissie Dierproeven aan

Naam:	UMC Utrecht
Adres:	Postbus 12007
Postcode en plaats:	3501 AA UTRECHT
Deelnemersnummer:	11500

deze projectvergunning voor het tijdvak 1 februari 2018 tot en met 31 januari 2023, voor het project "Immune receptor mediated control of tumors" met aanvraagnummer AVD1150020174288, volgens advies van Dierexperimentencommissie DEC Utrecht.
De functie van de verantwoordelijk onderzoeker is Wetenschappelijk projectleider. Voor de uitvoering van het project en voor de overeenstemming ervan met de verleende projectvergunning is Principal investigator verantwoordelijk.
Het besluit is gebaseerd op de volgende (aangepaste) stukken:

1 een aanvraagformulier projectvergunning dierproeven, zoals ontvangen op 5 december 2017
2 de bij het aanvraagformulier behorende bijlagen:
   a Projectvoorstel, zoals ontvangen op 5 december 2017;
   b Bijlagen dierproeven
      - 3.4.4.1 Humanized tumour models: subcutaneous, zoals ontvangen op 5 december 2017;
      - 3.4.4.2 Humanized tumour models: metastatic, zoals ontvangen op 5 december 2017;
      - 3.4.4.3 Humanized tumour models: orthotopic, zoals ontvangen op 5 december 2017;
      - 3.4.4.4 Humanized mouse models: safety testing, zoals ontvangen op 5 december 2017;
   c Niet-technische Samenvatting van het project, zoals ontvangen op 5 december 2017;
   d Advies van Dierexperimentencommissie zoals ontvangen op 5 december 2017
   e De aanvullingen op uw aanvraag, ontvangen op 24 januari 2018.

Aanvraagnummer:
AVD1150020174288

Naam proef	Diersoort/ Stam	Aantal dieren	Ernst
3.4.4.1 Humanized tumour models: subcutaneous			
	Muizen (Mus musculus)	2.600	1,0% Ernstig 9,0% Matig 90,0% Licht
3.4.4.2 Humanized tumour models: metastatic			
	Muizen (Mus musculus)	4.050	20,0% Ernstig 40,0% Matig 40,0% Licht
3.4.4.3 Humanized tumour models: orthotopic			
	Muizen (Mus musculus)	1.775	18,0% Ernstig 45,0% Matig 37,0% Licht
3.4.4.4 Humanized mouse models: safety testing			
	Muizen (Mus musculus)	1.625	1,0% Ernstig 45,0% Matig 54,0% Licht

Voorwaarden

Beoordeling achteraf
In dit project worden dierproeven toegepast die vallen in de categorie ernstig volgens artikel 10b van de wet en wordt daarom voorzien van beoordeling achteraf. Deze beoordeling zal uiterlijk januari 2024 plaatsvinden. Er zal dan beoordeeld worden of de doelstellingen van het project werden bereikt. Daarnaast wordt bekeken of de schade die de dieren hebben ondervonden, het aantal en soorten proefdieren en de ernst de dierproeven conform de vergunning waren.

Ter informatie
Onderstaande informatie is opgenomen op grond van artikel 1d lid 4, artikel 10a1 lid 2, artikel 10 lid 2 en/of artikel 10a3 van de wet.

- Go/ no go momenten worden voor aanvang van elk experiment afgestemd met de IvD.
- Het is verboden een dierproef te verrichten voor een doel dat, naar de algemeen kenbare, onder deskundigen heersende opvatting, ook kan worden bereikt anders dan door middel van een dierproef, of

door middel van een dierproef waarbij minder dieren kunnen worden gebruikt of minder ongerief wordt berokkend dan bij de in het geding zijnde proef het geval is.
- Het is verboden dierproeven te verrichten voor een doel waarvan het belang niet opweegt tegen het ongerief dat aan het proefdier wordt berokkend.
- Overige wettelijke bepalingen blijven van kracht.

Centrale Commissie Dierproeven

Aanvraagnummer:
AVD1150020174288

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.

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.

Centrale Commissie Dierproeven i.o.

Form
Retrospective assessment
(Version July 11, 2018)

- This form should be used to provide information required for the retrospective assessment
- For more information on the retrospective assessment, see the Guidelines to the retrospective assessment on our website (www.centralecommissiedierproeven.nl)
- or contact us by phone (0900-2800028).

1 General information

1.1	Provide the AVD number	AVD1150020174288
1.2	Licenced establishment	Name of the licenced establishment: UMC Utrecht
		Email address contact person: info@ivd-utrecht.nl
		Email address Animal Welfare Body (Optional): info@ivd-utrecht.nl
1.3	Responsible researcher	Title, first name, surname: [redacted]
		Phone number: [redacted]
		Email address: [redacted]
1.1	Title of the project	Immune receptor mediated control of tumors

2 Used animals

2.1	Provide, for each appendix and species, information on the number of animals that have been used. -If not all species stated on the licence have been used, provide an explanation. -If the actual numbers differ from the licenced numbers, provide an explanation.	**Appendix 1 Humanized tumour models: subcutaneous** Number of mice used: 576 Licenced number: 2600 The actual number of animals we used is lower than the licenced number. We calculated the licenced number based on the expectation that we would perform 8 experiments per year. The actual number of experiments was 3-4 per year. Experiments were mainly performed sequentially. This allowed us to use lessons learned from one experiment for the design of the next experiment, optimizing the usage of mice resources. One experiment (including analysis) takes a few months, so performing 8 experiments per year was not realistic.

Appendix 2 Humanized tumour models: metastatic
Number of mice used: 735
Licenced number: 3900

The actual number of animals we used is lower than the licenced number. We calculated the licenced number based on the expectation that we would perform 12 experiments per year. The actual number of experiments was 4 per year. Experiments were mainly performed sequentially. This allowed us to use lessons learned from one experiment for the design of the next experiment, optimizing the usage of mice resources. One experiment (including analysis) takes a few months, so performing 12 experiments per year was not realistic.

Appendix 3 Humanized tumour models: Orthotopic
Number of mice used: 28
Licenced number: 1775

We have overestimated the number of animals needed for orthotopic experiments. The main reason for this was that setting up the orthotopic experiments was more difficult than previously expected.

Appendix 4 Humanized tumour models: Safety testing
Number of mice used: 10
Licenced number: 1625

We have overestimated the number of animals needed for safety experiments. The main reason for this was that for most compounds we were in a starting phase and that mice lack the expression of the human genes targeted by these compounds. Generating mouse strains expressing these specific genes, without having established *in vivo* efficacy in xenograft models is not preferrable.

2.2 Provide, for each appendix and species, information on the severity experienced. -If this differs from the severity estimated in the application, provide an explanation.

Appendix 1 Humanized tumour models: subcutaneous
Discomfort level
Mild: 36% (20%)
Moderate:56% (75%)
Severe: 7% (5%)

Appendix 2 Humanized tumour models: metastatic
Discomfort level
Mild: 22% (5%)
Moderate:64% (80%)
Severe: 14% (15%)

Appendix 3 Humanized tumour models: Orthotopic
Discomfort level
Mild: 0 % (10%)
Moderate:100% (80%)
Severe: 0 % (10%)

Appendix 4 Humanized tumour models: Safety testing
Discomfort level
Mild: 100% (0%)
Moderate: 0% (99%)

Severe: 0% (1%)

Between brackets: estimated discomfort percentages.

For appendix 1 and 2 the estimated moderate discomfort is higher than the real moderate discomfort, so we overestimated the moderate discomfort. The estimated mild discomfort was lower than the real mild discomfort. A reason for this is that for some experiments the experimental aim was not efficacy / survival but other parameters as T cell persistence / proliferation at early time points. Therefore, mice were sacrificed before HEP was reached and low number of procedures were performed (less than 10), consequently mice were classified as mild discomfort.

For appendix 3 and 4 the estimated discomfort differs from the real discomfort. For both, we only did one experiment, with a low number of mice, which could explain why these values differ from the estimated ones. The real discomfort, based on one experiment only, with a low number of mice, is probably not representative and provides insufficient insight.

3 Replacement, reduction and refinement

3.1 Replacement

Have there been any developments in your scientific field which would replace some or all of the use of animals?
– If so, describe these developments.
- If so, to what extent have these developments been implemented in this project?
- If so, to what extent can these developments be used in future projects?

New immune receptor candidates are tested extensively *in vitro* using 2D models (read-out cytotoxicity, cytokine production, proliferation…). Only when the candidate meets the go/no go criteria it will be selected for *in vivo* testing (see 4.2).
Since 2018 we have developed 3D models for different tumour types. New candidates that were selected for testing *in vivo*, were also tested in our 3D model. The outcome of the mouse experiments will also be used to refine our advanced *in vitro* models to gain better predictive power of compound efficacy without the use of animals. However, for this we need a larger data set. This concept will be further developed in an OncodePACT (Nationaal Groeifonds) work package.

3.2 Reduction

Have there been any developments in your scientific field which would lead to a reduction in the number of animals?
- If so, describe these developments
- If so, to what extent have these developments been implemented in this project?

No, there have not been any developments which would lead to a reduction in the number of animals. However, we always try to reduce the number of mice by looking critically at the design of an experiment.
For example:

- We did a pilot experiment to investigate the growth/engraftment of 4 solid tumour cell lines. Per cell line we tested two doses of tumour cells. To reduce number of mice we injected mice on both flanks (each mouse received the same tumor cell line in left and right flank, only different in cell numbers). This did not increase discomfort of the mice,

	- If so, to what extent can these developments be used in future projects? - Were the estimated numbers for each test group appropriate for statistical analysis??	since the sum of both tumour sizes was used as total tumour volume for HEP - We combined experiments that use the same controls to reduce the number of animals we use.
3.3	<u>Refinement</u> Have there been any developments in your scientific field which would reduce the harm to the animals? - If so, describe these developments - If so, to what extent have these developments been implemented in this project? - If so, to what extent can these developments be used in future projects? -Were the used animal monitoring regimes adequate? - Can the humane endpoints be refined?	Over the past 5 years we have adjusted the following things to reduce/ prevent the discomfort of the mice as much as possible: - We prolonged the acclimatization period from 1 week to 2 weeks. In this period we gave the animals breeding chow as a boost - We gave them wet food prior and after irradiation and IL2 to try to reduce the body weight loss - We increased health check frequency when we observed weight loss after IL2/pamidronate injection and irradiation (we monitor these mice daily) - We reduced the IL2 dose to try to minimize side effects - We agreed with the IVD to lower the tumor size that we use to determine HEP for solid tumors (before it was 2000mm3 and now we agreed to use 1500mm3) - We took into account the cumulative discomfort (if some mild procedures are repeated over time, it will be counted as moderate), and decrease the number of different procedures by combining them We will continue to do all of these measures in future projects.

4 Strategy

4.1	Are the animal models used still the most appropriate for this type of study? Please, provide an explanation. -If not, describe when this has been observed, to what extent the animal models have been adapted, and/or the project has (temporarily) been stopped?	Humanized mouse models represent a valuable additional tool to answer biological questions and provide data on efficacy and safety regarding candidate immune receptor mediated anti-tumour therapy. First, on-target anti-tumour efficacy is an important read-out of these models. An advantage of these models compared to in vitro assays is that these models allow long-term growth (months) of (primary) human tumour cells and therefore long-term interaction of tumour cells with immune therapy. A second read-out, unique for humanized tumour models in mice, is the homing (trafficking) of receptor engineered cells to the tumour and/or tumour microenvironment and evaluation of long-term persistence of engineered immune cells. Third, the tumours that engraft in humanized mice form a complex tumour microenvironment including blood vessel networks that are not (yet) possible to model in vitro and receptor-based immune therapy needs to overcome possible barriers to target the complexity of these tumours. Also, changes in the human tumour and its microenvironment under the influence of therapy can be assessed. Another advantage of these humanized models is their ability to engraft human receptor engineered cells. We can directly evaluate the potency of our ex vivo production protocols (research grade and GMP-grade) that are optimized for human cells and as such these results have important translational value for the initiation of clinical studies using these products. In addition to in vitro data, the in vivo read-outs provide biological data as well as on-target efficacy and

	off-target toxicity information that is requested by the authorities before initiation of a clinical trial.
4.2 Were the go/ no go moments described in the application and the criteria for deciding whether the procedure/project will be continued or cancelled adequate to prevent the undue use and/or suffering of animals?	Yes, they were adequate. As described in the CCD, we had designed a go/no go moment in the research pipeline before candidate immune receptor based therapy can be tested in humanized mouse models. Immune receptors that have not previously been tested in mice were considered for testing only when: - Receptors (alone or in combination therapy) are active against a broad panel of tumour cell lines and primary tumour cells, but not against the healthy counterpart cells in vitro - The stability and formulation of the receptor platform is suitable for in vivo studies, in other words only in case stable and suitable dosage of compounds can be produced an in vivo study is initiated.

5 Achievements

5.1 Explain whether, and to what extent, the objectives set out in your application have been achieved.	The main objective of the project was to develop the next generation effective and safe anti-cancer immunotherapy based on defined immune receptors. TEG001 lead compound was selected based on its hematological tumour recognition profile and safety profile in the different *in vitro* and *in vivo* efficacy-safety balance assays performed. The TEG001 application including the pre-clinical mouse tumour model data was approved by the Dutch regulatory authorities (Centrale Commissie Mensgebonden Onderzoek (CCMO)) for testing TEG001 in a phase I clinical trial in man. In the meantime that we are taking our first receptor-based compound to the clinic, our research is focussed on constantly optimizing and implementing new knowledge into the next generation of immunotherapy against cancer using immune receptors that target cancer as a metabolic disease. The working mechanisms of this class of immune receptors are not yet fully understood and we have found that defined receptors work best against certain tumour types. In line with our CCD objectives, new TEG formats (such as TEG003 and TEG004) were created by manipulation of different immune receptors during these years. These new developed TEGs showed improvement in terms of efficacy when compared to TEG001, leading to the next generation of immunotherapy. Moreover, not only new TEG formats have been developed. In line with the CCD, we have created new immunotherapy formats such as bispecifics (soluble format). We have successfully set up an in vivo model to test these bispecifics.

Although we are satisfied with these results we expected to generate and test more new leads. When the CCD was written we expected to have more manpower and funding. This would have led to more experiments.
For the same reason we were not able to set up orthotopic models. Since these models are very complex, we did not have the manpower to set it up. We decided to focus on our strength: solid and metastatic tumour models.

5.2 What other benefits have been accrued from the work to date, and are further benefits expected?

Individual mouse experiments that we have performed during the last 5 years have resulted in a better understanding of the efficacy and shortcomings of our candidate therapeutic concepts. This led to successful improvements of some of our concepts, bringing them closer to the clinic. The outcome of the mouse experiments will also be used to refine our advanced *in vitro* models to gain better predictive power of compound efficacy without the use of animals. However, for this we need a larger data set. This concept will be further developed in an OncodePACT (Nationaal Groeifonds) work package.

We intent to communicate all relevant (positive and negative) findings with the scientific community, which resulted in a high number of mouse experiments included in scientific publication.

- [redacted] 2022 Nat Biotechnol;
- [redacted] 2021, Journal for ImmunoTherapy of Cancer;
- [redacted] 2019, Blood Advances;
- [redacted] 2020, Journal of Leukocyte Biology;
- [redacted] 2021, Front in Immunol)

From 10 mouse experiments we have still manuscripts in preparation. In conclusion, once a model is established, 80% of the experiments are used in publications.

6 Other aspects

6.1 Are there other aspects that may be important for the performance of the retrospective assessment?

In general, no big concerns were encountered during the performance of the experiments. The main issues during the last five years were:

-Delays in starting mice experiments due to T cell production. It is already solved as we further optimized the protocols, so experiments can be started on time.

-We had a breast cancer model in which estrogen pellet was injected. We observed that mice got quite sick after it. Therefore, we stopped these experiments. Further optimization needs to be done if this model is used in the future so discomfort of the mice is reduced.

7 Lessons

7.1	What lessons can be learnt with respect to the design and execution of future projects?	Changes we applied, in consultation with IVD, to increase quality of our research: - To prevent cage effect we house animals from different experimental groups in the same cage - The people performing the animal experiment are blinded for the different experimental groups We will keep doing this in all our future experiments.

8 Signature

8.1

This form must be signed by the establishment licence holder or the portfolio holder. The undersigned declares:

- that the answers to the questions above have been discussed with the Animal Welfare body.
- that the form has been completed truthfully

Name

Date

Signature

Centrale Commissie Dierproeven i.o.

Aanvulling Niet-technische samenvatting

Beoordeling achteraf 20174288-BA

1 Algemene gegevens

1.1	Titel van het project	Immuun receptor therapie tegen kanker: effectief en veilig

2 Gebruik dieren

2.1	Welke diersoorten zijn gebruikt?	muis
2.2	Hoeveel dieren zijn gebruikt?	Appendix 1: 576 Appendix 2: 735 Appendix 3: 28 Appendix 4: 10 Totaal aantal muizen: 1349
2.3	Wat is het werkelijke ongerief dat de dieren hebben ondergaan?	Appendix 1: Licht: 36% Matig:56% Ernstig: 7% Appendix 2: Licht: 22% Matig:64% Ernstig: 14% Appendix 3: Licht: 0% Matig:100% Ernstig: 0% Appendix 4: Licht: 100% Matig: 0% Ernstig: 0%

3 Opbrengsten

3.1	Wat zijn de belangrijkste opbrengsten van het project?	De belangrijkste opbrengst van dit project is dat we nieuwe inzichten hebben verkregen die kunnen bijdrage aan een effectievere en veiligere immuuntherapie voor patiënten met kanker. Het immuunsysteem is

cruciaal in de bescherming tegen bacteriën en virussen, maar speelt ook een belangrijke rol in het opruimen van kankercellen. Immuuntherapie is een nieuwe vorm van kankerbehandeling, die gebaseerd is op dit natuurlijke vermogen van immuuncellen om kankercellen te herkennen en op te ruimen. Ons onderzoek is gericht op het voortdurend verbeteren en ontwikkelen van nieuwe ideeën voor immuuntherapie tegen kanker.
De afgelopen jaren hebben we in ons lab verschillende nieuwe concepten voor immuuntherapie ontwikkeld. Vervolgens hebben we de werkzaamheid van deze nieuwe therapieën getest in verschillende muismodellen.

4 Nieuwe inzichten

4.1 Zijn er nieuwe inzichten die kunnen leiden tot vervanging, vermindering en/of verfijning?

Vervanging:
In het laboratorium zijn wij ook bezig met experimenten zonder het gebruik van muizen. We testen de nieuwe immuuntherapieën bijvoorbeeld ook in 3D modellen. Deze modellen bestaan uit verschillende celtypes en structuren (zoals mini-organen en tumoren), die de omgeving van de tumor in enige mate kunnen nabootsen. We willen de resultaten van 3D modellen vergelijken met de resultaten van de muis modellen, om te kijken hoe voorspellend de 3D modellen zijn. Hier hebben we eerst meer data voor nodig.

Vermindering:
Om het aantal dieren zo laag mogelijk te houden hebben we voor elk experiment heel precies het aantal benodigde muizen berekend wat nodig is om goede wetenschappelijke resultaten te verkrijgen. We gebruiken voortdurend wetenschappelijke literatuur om herhaling van reeds uitgevoerde experimenten te voorkomen.
In geval dat we nieuwe tumor types hebben gebruikt, hebben wij eerst een pilot experiment uitgevoerd om een nieuw model te ontwikkelen. Voor volledige muis experimenten gebruiken we dus altijd een proefopzet die al eerder getest is en werkt.
Om het aantal dieren dat we gebruiken te verminderen hebben we ook experimenten gecombineerd die dezelfde controle groepen nodig hebben.

Verfijning:
De afgelopen 5 jaar hebben we in overleg met de Instantie voor Dierenwelzijn een aantal dingen aangepast om het ongerief bij de muizen te verminderen/voorkomen:

- We hebben de periode waarin de muizen kunnen wennen aan hun nieuwe omgeving, verlengd van 1 week naar 2 weken. In deze twee weken voor de start van het experiment krijgen de muizen fokvoer, wat rijker is aan voedingstoffen, waardoor de muizen goed op gewicht zijn voordat we beginnen met het experiment.
- We geven de muizen week gemaakt voer voor en na behandeling om het gewichtsverlies te verminderen/voorkomen.
- We controleren de muizen dagelijks in het geval dat er gewichtsverlies optreedt tijdens het experiment.

- We hebben de dosis van bepaalde vaste medicatie verlaagd om het ontstaan van bijwerkingen te beperken
- We laten de tumor minder groot groeien.

5 In te vullen door CCD

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Andere opmerkingen	Het betreft een beoordeling achteraf.