

	Dossier: AVD11500202216349	
		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	
7	Beschikking en vergunning	X

NIET-TECHNISCHE PROJECTSAMENVATTING

Naam van het project	Immuuntherapie tegen kanker: een stap naar effectievere en veiligere behandelingen
NTS-identificatiecode	NTS-NL-509643 v.1, 29-12-2022
Land	Nederland
Taal	nl
Duur van het project, uitgedrukt in maanden.	60
Trefwoorden	Immuuntherapie Kanker Immuunsysteem
Doel(en) van het project	Fundamenteel onderzoek: Oncologie Fundamenteel onderzoek: Immuunstelsel Omzettinggericht en toegepast onderzoek: Kanker bij de mens

DOELSTELLINGEN EN VERWACHTE VOORDELEN VAN HET PROJECT

Beschrijf de doelstellingen van het project (bijvoorbeeld het aanpakken van bepaalde wetenschappelijke onduidelijkheden, of wetenschappelijke of klinische behoeften).	<p>Kanker is een van de belangrijkste doodsoorzaken in de westerse wereld.</p> <p>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 tegen de tumor en eventuele uitzaaiingen te richten. Het immuunsysteem is vaak verzwakt en ons uitgangspunt is dat we een nieuwe generatie immuuntherapie kunnen ontwikkelen voor een brede patiëntengroep, door slim gebruik te maken van de anti-tumor-eigenschappen van het immuunsysteem.</p> <p>Door de infrastructuur in onze onderzoeksafdeling, die gaat van fundamenteel via preklinisch tot klinisch onderzoek, hebben we een unieke onderzoekslijn opgezet om deze nieuwe therapieën naar de patiënt te brengen.</p>
Welke potentiële voordelen kan dit project opleveren? Leg uit hoe de wetenschap vooruit kan worden geholpen of mensen, dieren of het milieu uiteindelijk voordeel kunnen hebben bij het project. Maak, waar van toepassing, een onderscheid tussen voordelen op korte termijn (binnen de looptijd van het project) en voordelen op lange termijn (die mogelijk pas worden bereikt nadat het project is afgerond).	<p>Wij verwachten met het onderzoek in dieren de noodzakelijke inzichten te verkrijgen die daadwerkelijk zouden kunnen bijdragen aan effectievere en veiligere immuuntherapie voor patiënten met kanker.</p>

VOORSPELDE SCHADE

<p>In welke procedures worden de dieren gewoonlijk gebruikt (bijvoorbeeld injecties, chirurgische procedures)? Vermeld het aantal en de duur van deze procedures.</p>	<p>In de meeste experimenten zullen de muizen tumoren geïnjecteerd krijgen (1 injectie,). De soort tumor bepaalt waar in het lichaam de tumor gaat groeien en ook hoe de muis geïnjecteerd wordt (bijvoorbeeld in een ader of onder de huid). Daarnaast zullen de muizen injecties krijgen met de te onderzoeken immuuntherapie. Het aantal injecties dat de muizen dan krijgen hangt af van de soort immuuntherapie. De tumorgroei wordt regelmatig (twee keer per week) bijgehouden, om de tumorontwikkeling te volgen en het effect van onze therapie te bepalen.</p>					
<p>Wat zijn de verwachte gevolgen/nadelige effecten voor de dieren, bijvoorbeeld pijn, gewichtsverlies, inactiviteit/verminderde mobiliteit, stress, abnormaal gedrag, en wat is de duur van die effecten?</p>	<p>In de meeste experimenten zullen de muizen tumoren krijgen. 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, zoals 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 ernstig ongerief.</p>					
<p>Welke soorten en aantallen dieren zullen naar verwachting worden gebruikt? Wat zijn de verwachte ernstgraden en de aantallen dieren in elke ernstcategorie (per soort)?</p>	<p>Soort:</p>	<p><i>Totaal aantal</i></p>	<p><i>Geraamde aantallen naar ernstgraad</i></p>			
			<p><i>Terminaal</i></p>	<p><i>Licht</i></p>	<p><i>Matig</i></p>	<p><i>Ernstig</i></p>
	<p>Muizen (<i>Mus musculus</i>)</p>	<p>6010</p>	<p>0</p>	<p>1927</p>	<p>3610</p>	<p>473</p>
<p>Wat gebeurt er met de dieren die aan het einde van de procedure in leven worden gehouden?</p>	<p>Soort:</p>	<p><i>Geraamd aantal te hergebruiken, in het habitat-/houderijsysteem terug te plaatsen of voor adoptie vrij te geven dieren</i></p>				
		<p><i>Hergebruikt</i></p>	<p><i>Teruggeplaatst</i></p>	<p><i>Geadopteerd</i></p>		
<p>Geef de redenen voor het geplande lot van de dieren na de procedure.</p>	<p>De dieren zullen aan het einde van de experimenten worden gedood en organen worden verzameld. Met deze organen kunnen wij de werking van de therapie op de tumor bestuderen.</p>					

TOEPASSING VAN DE DRIE V'S

1. Vervanging

Beschrijf welke diervrije alternatieven op dit gebied voorhanden zijn en waarom zij niet voor het project kunnen worden gebruikt.

In het laboratorium doen we voorwerk zonder proefdieren om de nieuwe 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 bestudeerd. Ook kunnen we met dierproeven beter de lange termijn effecten van onze therapie bestuderen, dit is niet mogelijk in het laboratorium. En immuuntherapie wordt juist ontwikkeld om, in tegenstelling tot klassieke therapieën tegen kanker, een langdurig effect te bewerkstelligen.

2. Vermindering

Leg uit hoe de aantallen dieren voor dit project zijn bepaald. Beschrijf de stappen die zijn genomen om het aantal te gebruiken dieren te verminderen en de beginselen die zijn gebruikt bij het opzetten van de studies. Beschrijf, waar van toepassing, de praktijken die gedurende het hele project zullen worden toegepast om het aantal dieren die in overeenstemming met de wetenschappelijke doelstellingen werden gebruikt, tot een minimum te beperken. Deze praktijken kunnen bijvoorbeeld bestaan uit proefprojecten, computermodellen, het delen van weefsel en hergebruik.

We willen het aantal muizen verminderen door alleen onderzoek te doen naar therapieën die in het laboratorium veel zijn getest en veelbelovend zijn gebleken. Om het aantal muizen zo laag mogelijk te houden, zullen wij heel precies het aantal benodigde muizen berekenen wat nodig is om goede wetenschappelijke resultaten te verkrijgen. Ten slotte, gebruiken wij een proefopzet die al van tevoren is getest en werkt. Dit houdt in dat de muizen die wij gebruiken speciaal voor dit soort onderzoek zijn gefokt en ook dat de tumoren die wij inspuiten getest zijn.

3. Verfijning

Geef voorbeelden van de specifieke maatregelen (bv. verscherpte monitoring, postoperatieve behandeling, pijnbestrijding, training van dieren) die in verband met de procedures moeten worden genomen om de welzijnskosten (schade) voor de dieren tot een minimum te beperken. Beschrijf de mechanismen om gedurende de looptijd van het project nieuwe verfijningstechnieken in gebruik te nemen.

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.

Licht de keuze van de soorten en de bijbehorende levensstadia toe

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. 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.

VOOR EEN BEOORDELING ACHTERAF GESELECTEERD PROJECT

Project geselecteerd voor BA?	ja
Termijn voor BA	31-01-2029
Reden voor de beoordeling achteraf	
Bevat ernstige procedures	ja
Maakt gebruik van niet-menselijke primaten	
Andere reden	
Toelichting van de andere reden voor de beoordeling achteraf	

AANVULLENDE VELDEN

Link naar de eerdere versie van de NTS buiten het EC-systeem	
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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 neem telefonisch contact op. (0900-2800028).

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 Wat voor aanvraag doet u?

Nieuwe aanvraag > Ga verder met vraag 1.3
 Wijziging > Vul hiernaast het AVD nummer van uw vergunde project in en ga verder met vraag 2.1
 Melding > Vul hiernaast het AVD nummer van uw vergunde project in en ga verder met vraag 2.2

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

Naam instelling of organisatie	UMC Utrecht			
Titel, voorletters en achternaam van de portefeuillehouder	Titel	Voorletters	Achternaam	<input checked="" type="checkbox"/> Dhr. <input type="checkbox"/> Mw
	██████	████	██████	

E-mailadres contactpersoon	info@ivd-utrecht.nl			
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Titel, voorletters en achternaam van de diens gemachtigde (indien van toepassing)	Titel	Voorletters	Achternaam	<input type="checkbox"/> Dhr. <input type="checkbox"/> Mw
	n.v.t.			

E-mailadres gemachtigde				
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Vul de gegevens van het postadres in.

Straat en huisnummer	Instantie voor Dierenwelzijn Utrecht		50
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Postcode en plaats	3584CJ	UTRECHT	
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Postbus, postcode en plaats	80125	3508TC	UTRECHT
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1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	████████████████████	<input type="checkbox"/> Dhr. <input checked="" type="checkbox"/> Mw.
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Functie	████████████████████
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Afdeling	Centre for Translational Immunology
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Telefoonnummer	██████████
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1.5	<i>(Indien van toepassing)</i> Vul hier de gegevens in van de plaatsvervangende verantwoordelijke onderzoeker.	E-mailadres	[REDACTED]
		(Titel) Naam en voorletters	<input type="checkbox"/> Dhr. <input type="checkbox"/> Mw.
		Functie	
		Afdeling	
1.6	<i>(Indien van toepassing)</i> Vul hier de gegevens in van de persoon aan wie de portefeuillehouder de verantwoordelijkheid inzake de algemene uitvoering van het project en de overeenstemming daarvan met de projectvergunning heeft gedelegeerd.	E-mailadres	
		(Titel) Naam en voorletters	<input type="checkbox"/> Dhr. <input type="checkbox"/> Mw.
		Functie	
		Afdeling	
1.7	<i>(Optioneel)</i> Vul hier de gegevens in van de Instantie voor Dierenwelzijn	Telefoonnummer	030-2531569
		E-mailadres	info@ivd-utrecht.nl
1.8	Is er voor deze projectaanvraag een gemachtigde?	<input type="checkbox"/> Ja > <i>Stuur dan het ingevulde formulier Melding Machtiging mee met deze aanvraag</i> <input checked="" type="checkbox"/> Nee	

2 Over uw aanvraag

2.1	Gaat uw aanvraag over een <i>wijziging</i> op een vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn?	<input checked="" type="checkbox"/> Nee > Ga verder met vraag 3 <input type="checkbox"/> Ja > Geef hier onder kort de wijziging en de onderbouwing daarvan weer. Geef in de originele formulieren (niet-technische samenvatting, projectvoorstel en bijlage dierproeven) duidelijk aan (bij voorbeeld in een andere kleur) waar de projectaanvraag wijzigt. Ga daarna verder met vraag 6.
2.2	Gaat uw aanvraag over een <i>melding</i> op een vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn?	<input checked="" type="checkbox"/> Nee > Ga verder met vraag 3 <input type="checkbox"/> Ja > Geef hier onder weer wat deze melding inhoudt 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 - 2023 Einddatum (t/m) 31 - 1 - 2028
3.2	Wat is de titel van het project?	Immune receptor mediated control of tumours
3.3	Wat is de titel van de niet-technische samenvatting?	Immuuntherapie tegen kanker: een stap naar effectievere en veiligere behandelingen
3.4	Wat is de naam van de Dierexperimentencommissie (DEC) van voorkeur?	Naam DEC DEC-Utrecht
		Postadres Postbus 85500 3508 GA Utrecht
		E-mailadres dec-utrecht@umcutrecht.nl

4 Factuurgegevens

4.1 (indien factuuradres afwijkt van de gegevens uit vraag 1.3) Vul de gegevens van het factuuradres in.	Naam: UU-ASC		Afdeling:	
	Straat:		Huisnummer:	
	Postcode:	Plaats:		
4.2 (optioneel) Vul hier het ordernummer van de instelling in.	Postbus: 80.011		Postcode: 3508TA	
	E-mail: asc.factuur@uu.nl		Plaats: UTRECHT	
	Ordernummer: CB.841910.3.01.011			

5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?	Verplicht	
	<input checked="" type="checkbox"/> Projectvoorstel	Aantal bijlage(n) dierproeven 4
	<input checked="" type="checkbox"/> Niet-technische samenvatting	
Overige bijlagen, indien van toepassing		
	<input type="checkbox"/> Melding Machtiging	
	<input type="checkbox"/>	

6 Ondertekening

6.1 Print het formulier uit, onderteken het en stuur het inclusief bijlagen via de beveiligde e-mailverbinding naar de CCD en per post naar de Centrale Commissie Dierproeven (voor adresgegevens zie website)	<p>Ondertekening door de portefeuillehouder namens de instellingsvergunninghouder of gemachtigde (zie 1.8). De ondergetekende verklaart:</p> <ul style="list-style-type: none"> 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 C 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.
	<p>Naam</p> <p>Functie</p> <p>Plaats</p> <p>Datum</p> <p>Handtekening</p>





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 the Guidelines to the project licence application form for animal procedures on 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.	<input checked="" type="checkbox"/> Basic research
	<input checked="" type="checkbox"/> Translational or applied research
	<input type="checkbox"/> Regulatory use or routine production
	<input type="checkbox"/> Research into environmental protection in the interest of human or animal
	<input type="checkbox"/> Research aimed at preserving the species subjected to procedures
	<input type="checkbox"/> Higher education or training
	<input type="checkbox"/> Forensic enquiries
	<input type="checkbox"/> 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 2.1.

Background / context

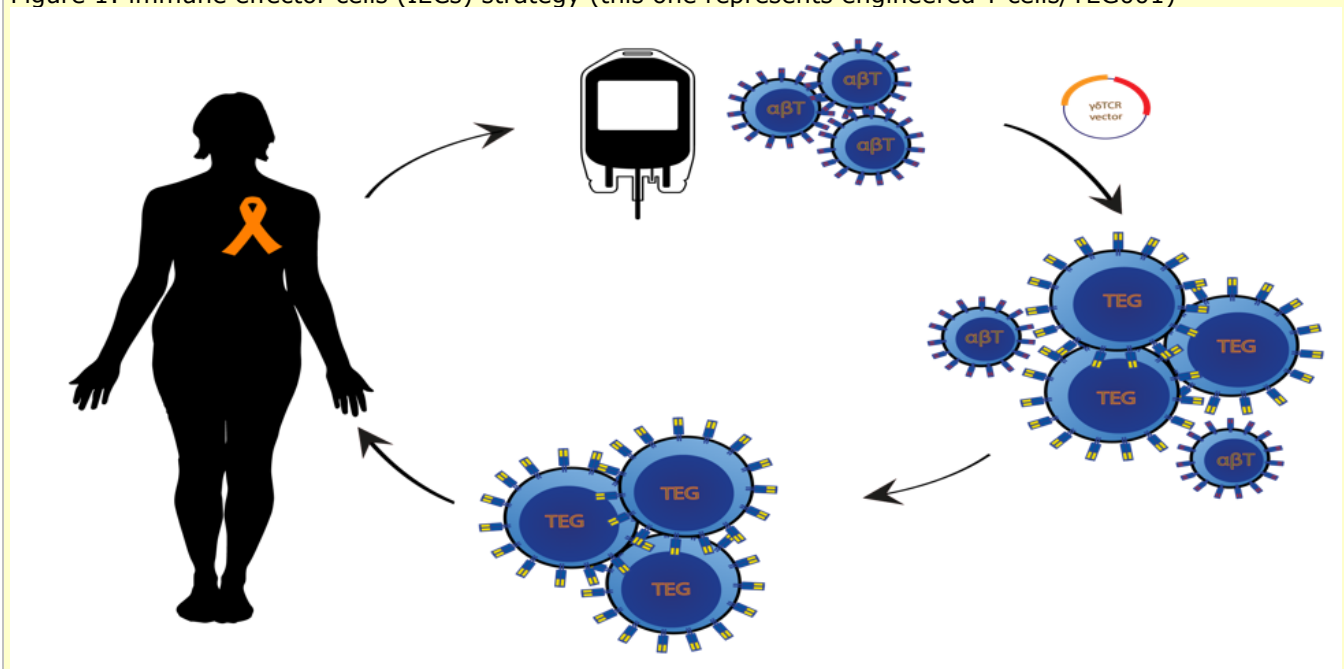
The focus of our laboratory is the development of immune receptor-based immunotherapy against cancer. Cancer immunotherapy has been considered as scientific Breakthrough of the Year for 2013 (Couzin-Frankel, Science 2013;342:1432). This form of therapy received enormous scientific but also public attention 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 ($\gamma\delta$ T cells), which appear to be more potent than many other subpopulations (Gentles et al. Nat Med 2015;21:938). The major power of $\gamma\delta$ T cells arises from the fact that $\gamma\delta$ T cells see cancer not as a genetic, but as a metabolic disease (Gober et al. J Exp Med 2003; 197:163). In addition, $\gamma\delta$ T 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 $\gamma\delta$ T cells include clonal expansion and the formation of immunological memory. $\gamma\delta$ T cells can recognize and are able to target a broad range of tumour types including hematological and solid tumours (Marcu-Malina et al. Blood 2011;118:50). Importantly, our group showed that the $\gamma\delta$ T cell receptor ($\gamma\delta$ TCR) 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 $\gamma\delta$ T 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.

Strategies in our lab

Our laboratory has developed two strategies to use the concept of metabolic cancer targeting by $\gamma\delta$ T cells. The first strategy involves immune effector cells (IECs), either genetically modified or not modified. One example of such IECs that we are optimizing in our current *in vitro* and *in vivo* studies are TEGs: T cells engineered to Express a defined $\gamma\delta$ TCR (Gruender et al. Blood 2012;120:5153 and Straetemans et al. Clin Can Res 2015;21:3957). In short, patient derived (autologous) $\alpha\beta$ T cells are *ex vivo* engineered to express the tumour-specific $\gamma\delta$ TCR, cultured until sufficient cell numbers, purified and reinfused into the patient (figure 1).

Figure 1: immune effector cells (IECs) strategy (this one represents engineered T cells/TEG001)



This led to the initiation of a phase I clinical trial with TEG001 to treat patients with acute myeloid leukaemia (AML) and multiple myeloma (MM) that have no remaining treatment options.

This extends current therapies with CAR T which will be also investigated in this proposal side by side or alone, as well non engineered immune effector cells.

Sebestyen et al. Nat Rev Drug Discov. 2020 19(3):169-184).

The second strategy makes use of soluble formats or molecules (Sols), including bispecific molecules, nanoparticles and checkpoint molecules (figure 2). We are currently exploring the use of Gamma delta Anti-CD3 Bispecific molecules (GABs), where we combine the tumour targeting capacity of the $\gamma\delta$ TCR with a T cell recruitment domain. These soluble compounds can efficiently redirect cytotoxic T cells towards tumour cells of both solid and hematological origin (Van Diest et al. JITC 2021;9(11)). Also this therapy will be benchmarked with approved and novel pre-clinical bispecific concepts.

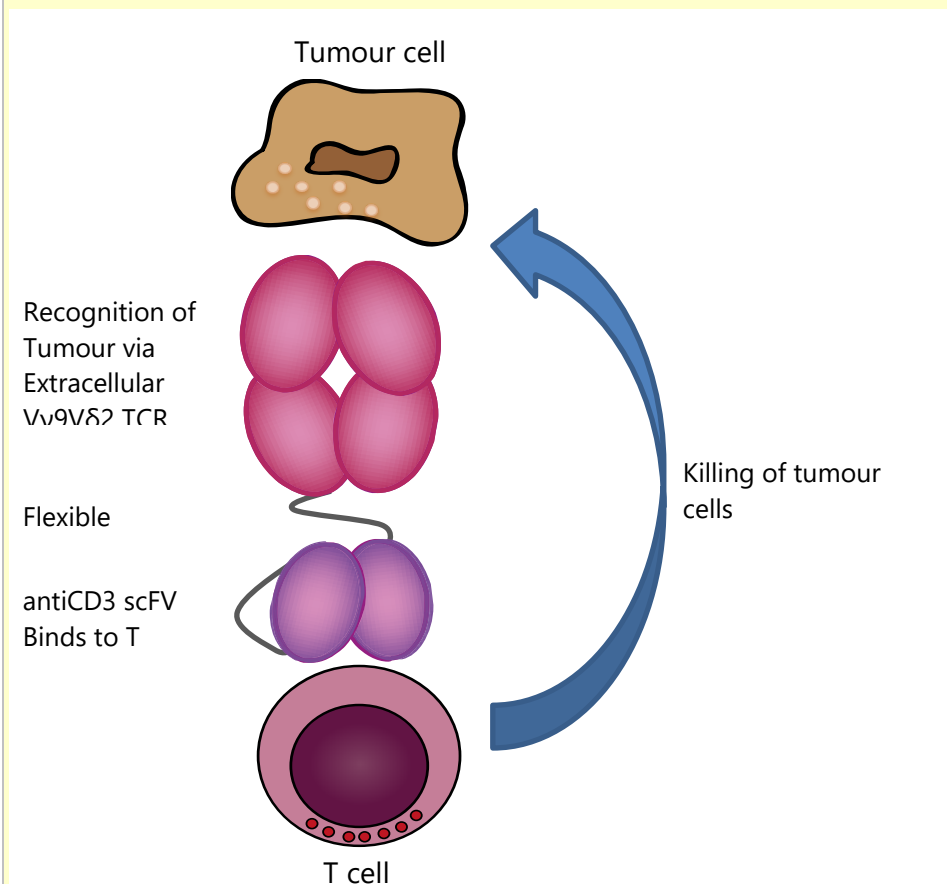


Figure 2: A soluble format strategy (this one represents bispecific molecules)

3.2 Purpose

3.2.1 Describe the project's immediate and ultimate goals. Describe to which extent achieving the project's immediate goal will contribute to achieving the ultimate goal.

- If applicable, describe all subobjectives

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 approaches:

1. **Testing clinical candidate immune receptors in IECs and Sols against a defined panel of tumour types for anti-tumour efficacy.**
2. **Elucidating the biological mechanism of action and the mechanisms responsible for bio-distribution.**
3. **Enhancing efficacy of candidate IECs and [REDACTED]**
4. **Evaluate the safety profile of candidate immune receptor-based ([REDACTED] [REDACTED] [REDACTED] 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.**

3.2.2 Provide a justification for the project's feasibility.

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 our candidate receptors as monotherapy (IECs and Sols) 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 (IECs) 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 $\gamma\delta$ T 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, [REDACTED] 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, we have established the Innovation Center for Advanced Therapies (ICAT), an integrated facility consisting of a Biofabrication pilot facility. GMP simulation and production facility (Cell Therapy Facility). The realization of these facilities is aimed at facilitating the processes of discovery, predication and intervention for the development of new GMP-grade products. We thus have a great environment for researching new immunotherapeutic targets and subsequently bringing the next generation receptor-based immunotherapies 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.

3.2.3 Are, for conducting this project, other laws and regulations applicable that may affect the welfare of the animals and/or the feasibility of the project?

No

Yes > Describe which laws and regulations apply en describe the effects on the welfare of the animals and the feasibility of the project.

[Click or tap here to enter text.](#)

3.3 Relevance

3.3.1 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 metastasised tumours due to the occurrence of therapy-resistant tumour variants. In contrast, immunotherapies have emerged as novel, promising treatment option. For both solid and hematological cancers, various efforts have been made to establish immunotherapeutic opportunities, such as adoptive transfer of engineered or non-engineered T cells and checkpoint inhibitors. Especially checkpoint inhibitors have shown noticeably beneficial results, especially in melanoma and non-small cell lung cancer, even leading to the approval of some immune checkpoint inhibitors for various types of solid and hematological cancers (Darvin et al. Exp Mol Med 2018;1-11). But also adoptive transfer with T cells is improving rapidly. The use of, for instance, chimeric antigen receptor (CAR) T cell therapy is approved by the FDA for the treatment of several types of lymphoma and multiple myeloma (Sengsayadeth et al. E J Haem 2022;6-10). 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 hematological tumour types that remain largely resistant to classical immunotherapy. However, for those tumour types $\gamma\delta$ T 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 IECs or Sols 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 hematological 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 regimens.

3.3.2 Who are the project's stakeholders? Describe their specific interests.

Researchers: involved in the design and execution of the experiments (in vitro but also in vivo), are ambitious to find new therapies to treat patients.

Patients: would benefit from our projects, as we are trying to find a novel treatment option for them.

Clinicians: would benefit from new state-of-art therapies for their patients.

Animals: are used in the experiments, their main concern is that the projects are well-designed with as little discomfort as possible.

Industry: involved in making products out of the candidate immunotherapies that we are exploring.

KWF: financially supports our research with various grants; want to be kept updated with our progress and hope to contribute to finding a novel therapeutic option for cancer patients.

3.4 Strategy

3.4.1 Provide an overview of the overall design of the project (strategy). If applicable, describe the different phases in the project, the coherence, the milestones, selection points and decision criteria.

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 3 below). 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 (IECs and Sols) for their anti-tumour activity *in vitro* in order to improve efficacy or broaden the possible patient population that we can treat. We have found that some tumours are better targeted by the one but not by the other receptor. Also *in vitro* and *in silico* 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 (IECs and Sols) (Oates et al. *Oncoimmunology* 2013;2:e22891; Van Diest et al. *JITC* 2021;9(11);Lo et al. *J Immunol* 2013, 191: 5107–5114) will be defined *in vitro*.

Based on their cytotoxic, anti-tumour potential *in vitro* (read-out cytotoxicity and cytokine production, proliferation and migration capacity, see research pipeline figure 3) the most promising IECs and Sols 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, PBMCs or $\alpha\beta$ TCR-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 studied as well. Although we are taking our first TEG product to the clinic, there are many open questions remaining which could jeopardize a rapid clinical implementation. First, though the target molecule (CD277) has been described recently for the receptor used in TEG001 (Sebestyen et al. *Cell Reports* 2016; 1973-1985 and Payne et al. *Science* 2022; 942-949), 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 (Sebestyen et al. *Nat Rev Drug Discov.* 2020 19(3):169-184). 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 IECs) 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 (Schmidt, C Nature 2017; 552(7683)). 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 hematological 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).

4. Safety profile of candidate immune receptor-based (IECs and Sols) 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; Johanna et al. 2019 Journal for immunotherapy of cancer, 1-13) 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 hematological 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 ($\alpha\beta$ TCR) 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*) of IECs and Sols.

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 (IECs) 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 IECs (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 (Mengus et al. ACS Biomaterials Science & Engineering 2017; 314-323).). So far, pre-clinical mouse models are 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;and Gattinoni et al. Nat Med 2011;17:1290). It is the best most accepted and valued model currently in our field.

Immune deficient mouse strains are the basis of our pre-clinical *in vivo* models. The main reason for this is the fact that the $\gamma\delta$ T cell – target cell interactions are not present in mice, because mice lack specific $\gamma\delta$ T 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 IEC or Sols – 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; van Diest et al. 2021, *Journal for ImmunoTherapy of Cancer*).

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 IEC and Sol 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 IECs 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 (IECs and Sols) 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 IECs . 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.

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 injection of	Tumour take rate	Discomfort
Cell line	no	s.c. in flank	High >80%	Mild to moderate ¹
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 injection of	Tumour take rate	Discomfort
A. solid tumour origin	lung	i.v.	High >80%	severe
	liver	Spleen or mesenteric vein	High >80%	severe
B. Hematological 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 ² >70-90%	Moderate/severe
3. Orthotopic tumour models				
Tumour source	Metastatic site	Site injection of	Tumour take rate	Discomfort
Cell line	No	Depending on origin of tumour ³	High >80%	Moderate/severe
Tumouroid cell line	No		High >80%	Moderate/severe
Patient derived primary cells	no		Variable >50%	Moderate/severe

¹In general low discomfort unless tumour ulceration occurs (this is humane endpoint)

² Patient derived primary hematological tumours are screened and selected for high predicted engraftment using genetic biomarkers before application in mouse experiments

³ 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 experimental 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 hematological 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 hematological 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 growth factors (cytokines) 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. IEC and Sols persistence will be monitored in peripheral blood by e.g. flow cytometry and mass spectrometry. 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 $\alpha\beta$ TCR, PBMCs 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 next to our 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	of	Take	Expected clinical parameters	Discomfort
Target molecule transgenic mice	n.a.		n.a.	GVHD-like ¹	Mild ³
Human healthy cell engraftment	i.v. / orthotopic ²	s.c./	Cell subset dependent	Depletion of cellular subsets	Mild ³
B. Graft Versus Host Disease models					
Immune receptor therapy	i.v.		n.a.	High >80%	Mild to severe ⁴

¹GVHD-like symptom scoring system described in appendix 4

²Dependent on origin of healthy cells

³Dependent on treatment group and model, in previous comparable experiments no clinical symptoms were observed.

⁴Dependent 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 (IECs and Sols) 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 ONE 8(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 (IEC and Sols) therapy will be tested for immune-receptor platforms that are designed for therapy in an allogeneic setting. One of our aims using for instance 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.

3.4.2 Provide a justification for the strategy described above.

The current project is divided in 4 different types of mouse experiments (explained in detail under 3.4.1), 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 3 (above).

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:

- 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.

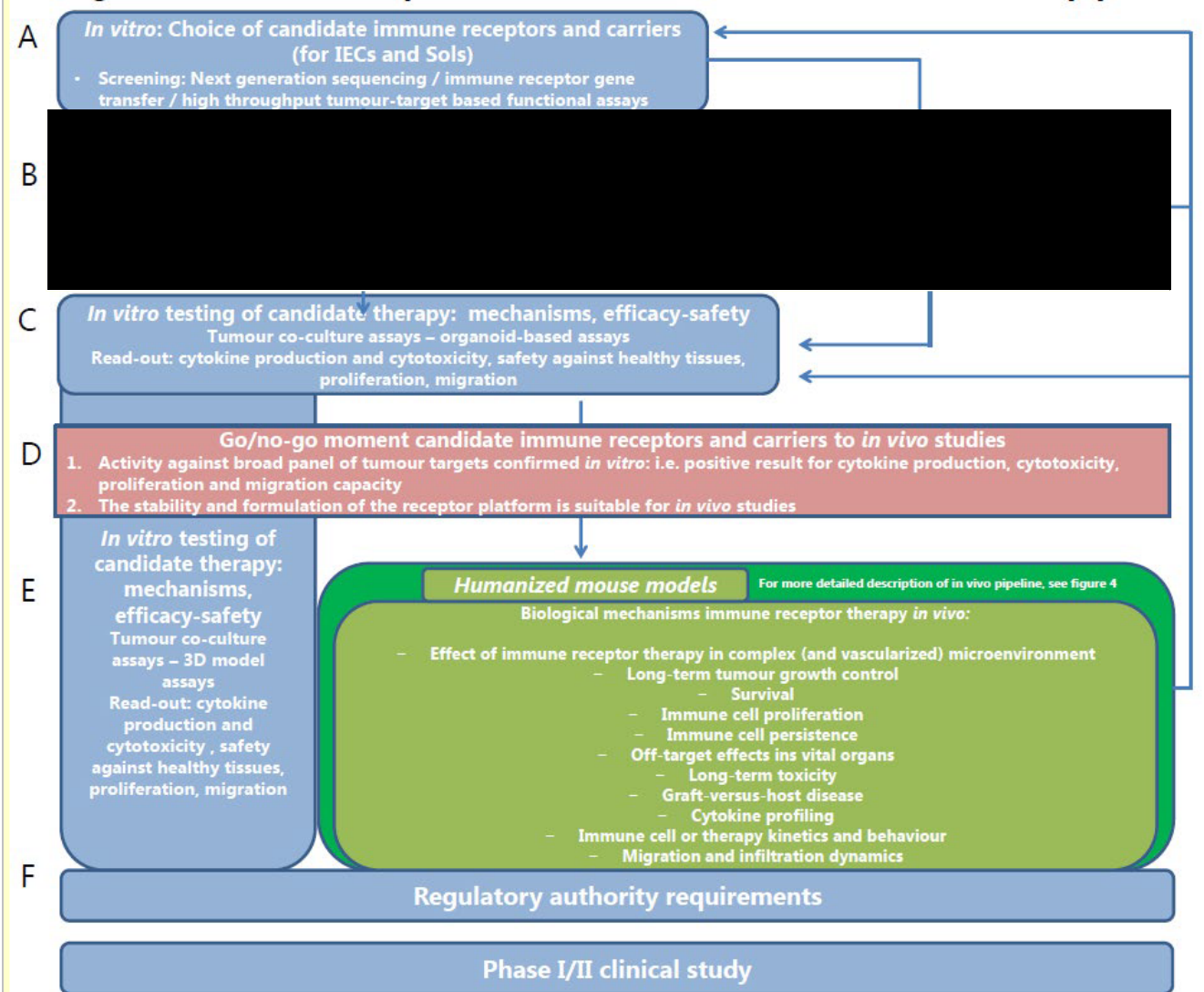
When successful, it will in general be necessary to perform confirmatory studies in other models, such as patient derived xenograft models (PDX), more stringent and or physiological relevant models with respect to tumour location (orthotopic) or with respect to dosing and treatment regimen (established 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

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 3).

Figure 3. Immune receptor mediated control of tumours: research pipeline



A. Choice of immune receptors and carriers (*in vitro*) for IECs and Sols.

In order to define possible candidate immune receptors and the optimal carrier for usage in immunotherapy strategies involving immune effector cells (IECs) and soluble molecules (Sols), high-throughput screening using immune receptor gene transfer, sequencing techniques and functional tumour target-based assays are used

to screen for anti-tumour cytotoxic potential (read-out cytotoxicity or cytokine production). Our lab has a strong focus on $\gamma\delta$ T cell receptors, but also other immune receptors can be potential candidates for further exploration. Broad anti-tumour function (cytotoxic and cytokine production) is required 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 and carriers for IECs and Sols and in 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 soluble immune receptors, [REDACTED]

[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. [REDACTED]

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

Based on the **results of *in vitro*** assays that use cytotoxicity, cytokine production, proliferation and migration capacity 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 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. **Specific criteria are challenging to select upfront, as this differs substantially per research question. Guidelines for criteria are for instance significant reactivity against tumour cell lines or a two-fold increase in cytokine production or cytotoxicity, proliferation or migration compared to baseline/negative control, in combination with negative results against healthy counterparts. We would like to refer to 2 key publications (Johanna et al. 2020, Journal of Leukocyte Biology; Johanna et al. 2021, Front in Immunol), where we have assessed some specific criteria, such as significant differences in cytokine release (IFN γ) *in vitro*, but also graft-versus-host symptoms and T cell persistence as criteria to continue *in vivo* experiments.**

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 figure 4 for a detailed description when a specific mouse model is chosen.

The listed parameters are the read-outs that are specifically measured in the *in vivo* models. *In vivo* models are key for answering research questions concerning these read-outs, as our *in vitro* models cannot evaluate these parameters accurately or our models are technically insufficient to address them.

If we observe shortcomings of our candidate therapy in this step, for example insufficient tumour control due to poor persistence or poor kinetics of the candidate therapy, this will feed back into our design and *in vitro* pipelines.

F. Phase I/II clinical study

If the candidate therapy proves 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 rationale, 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.

To illustrate the research pipeline and the criteria for compound selection, the development and selection of the lead of TEG001, one of our first compounds, is shortly described below.

A) First, using (semi)high-throughput immunological assays a panel of $\gamma\delta$ T cell clones was screened for their anti-tumour potential. This was measured by cytotoxic activity and cytokine production. When $\gamma\delta$ T cell clones were targeting a broad panel of tumours, molecular techniques were used to identify their unique gamma/delta T cell receptor ($\gamma\delta$ TCR) DNA sequence. Immune receptor gene transfer techniques were used to further select potential receptors. To this end the $\gamma\delta$ TCR DNA was introduced in conventional $\alpha\beta$ T 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 $\gamma\delta$ TCR gene and can be maintained after transfer of these genes into $\alpha\beta$ T cells.

B) The platform of immune receptor usage was named TEG (T cells engineered to express a defined $\gamma\delta$ TCR). 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 $\gamma\delta$ TCR, 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 (Sebestyén et al. Cell Reports 2016;15:1973, Sebestyén et al. Nature reviews Drug discovery, 2020; 169-184, Vyborova et al. J Clin Inv 2020; 4637-4651 and Dekkers et al. Nature Biotechnology 2022: 1-10) 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 $\gamma\delta$ T 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 (Johanna et al. J Immunother Cancer 2019;1-13).

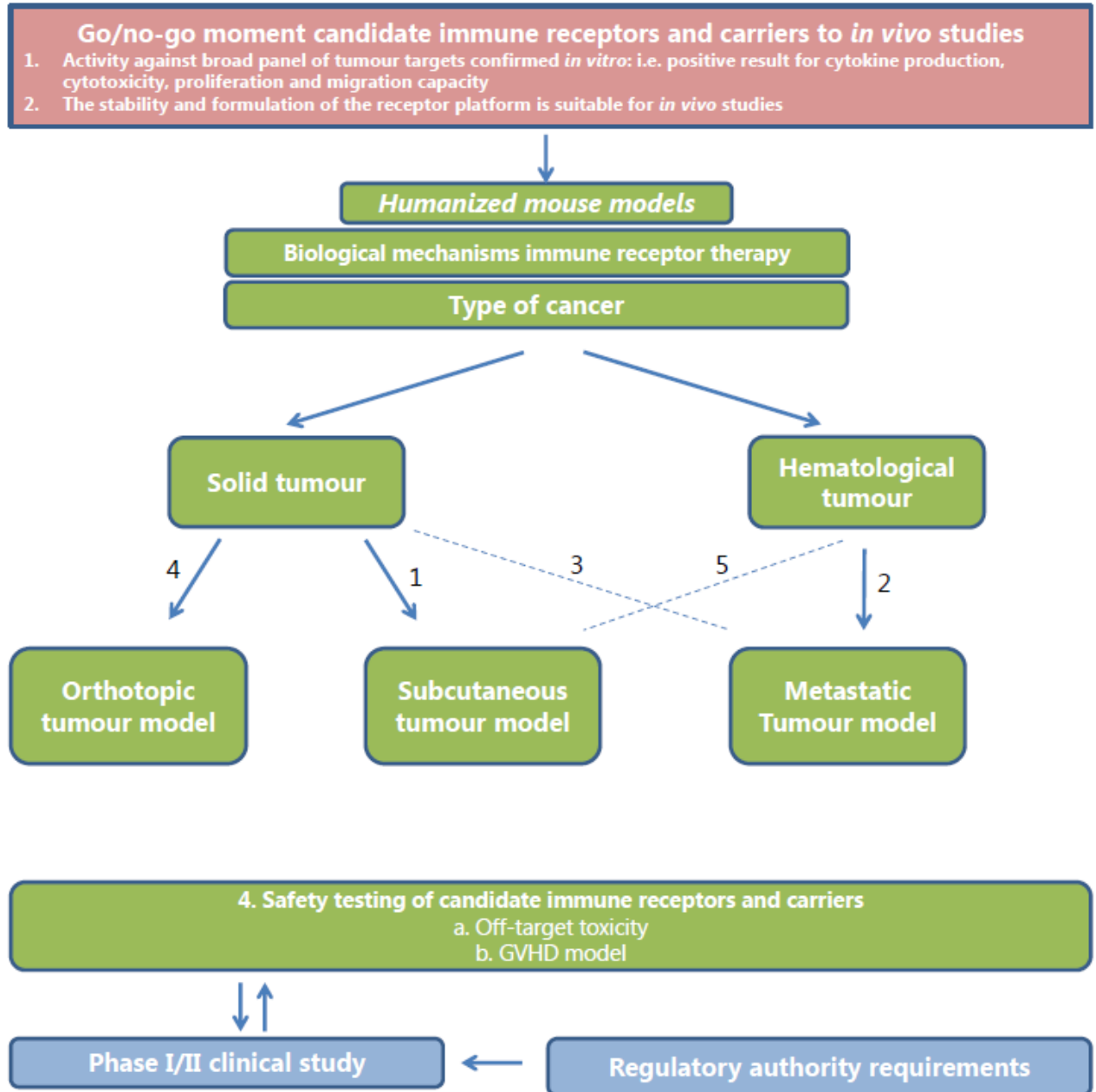
F) Finally, TEG001 lead compound was selected based on its hematological tumour recognition profile and safety profile in the different *in vitro* and *in vivo* efficacy-safety balance assays performed. The TEG001 application including the pre-clinical mouse tumour model data was approved by the Dutch regulatory authorities (Centrale Commissie Mensgebonden Onderzoek (CCMO)) for testing TEG001 in a phase I clinical trial in man. 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 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. Moreover, the platform, that include IECs and Sols, are developed implementing the most recent knowledge in the field. And finally, we believe that combining treatment options would ultimately be significantly improving treatment outcome.

The research pipeline in figure 3 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. The choice of animal model after *in vitro* experiments is further explained in figure 4 below.

Figure 4. Pipeline for *in vivo* experiments with selected therapies



After the *in vitro* experiments and passing the GO/NO GO moment, a candidate immune therapy will be tested *in vivo*, to assess parameters that could not be evaluated *in vitro* (see figure 3, E). The critical question from the perspective of resources and animal welfare remains once a candidate reached as outlined in figure 3 the stage of animal testing, what is the best of the described models, or do we even need to modified models in line with many different possibilities as described in the appendices. The choice of animal model depends first and foremost on the tumour target (solid versus hematological). E.g. for solid tumours we aim to increase access to the tumour side, this will be done in the subcutaneous mouse model (1) while for hematological tumours the primary route is the metastatic tumour model, where the tumour is injected intravenously (2). A second option for solid tumours is the metastatic tumour model (3; dashed line indicating 'non-standard'). This model will for instance be chosen if we want to test our candidate receptor's effectivity against lung or liver metastases (see appendix 3). Solid tumours could also be studied orthotopically (4). An example of this would be to study the effect of our candidate therapies in the brain. Such a complex model will enable us to e.g.

address and possibly enhance activity once environment (such as the blood-brain barrier) is involved. Finally, hematological tumours might also be injected subcutaneously to study efficacy of candidate therapies (5; dashed line indicating 'non-standard'). In previous experiments with for instance bispecific molecules, we noticed that intravenous injection of hematological tumour cells combined with a negative control already showed background targeting of the tumour cells, while this was not the case with a subcutaneous injection. The window to study the efficacy of the bispecific was therefore much better in a subcutaneous model. In this case, we performed a 'non-standard' model (hematological tumour in a subcutaneous tumour model) next to the 'standard' choice. It can happen that for some tumour types we perform the 'standard' or 'non-standard' model for the different purposes of our experiments (i.e. different candidate therapies). Finally, toxicity assays will only be performed if efficacy has been observed in the first design with acceptable toxicity. By following this pipeline, we limit the amount of mouse experiments, as we will only select the most suitable model for the experiment and create the best information for future clinical trial designs. The choice for a specific tumour model, where it fits in our strategy and the argumentation will also be described in detail in the work protocols of the corresponding experiment, which will be monitored by the IVD. **Experiments using a specific new lead/carrier combination will be preferentially performed sequentially. This allows us to use lessons learned from one experiment for the design of the next experiment, optimizing the usage of mice resources. In general, we will test a lead/carrier combination under the same conditions maximum twice, where 2 negative results (e.g. no improvement in tumour control; or see figure 3 for a list of read-outs) will be seen as a no-go. When a lead/carrier combination is showing a positive result for a parameter described above (figure 3), it can be selected for further testing in a different model or with different conditions. However, this depends on the specific results in the model and the (new) research question and will be evaluated on in the work protocols, which will be monitored by the IVD.**

Past experience improvements to increase output of the pipeline as well as animal welfare.

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. 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.

This is an overall success rate of around 60%. This also included experiments that have been used to establish a model, such for e.g. testing efficacy of bispecific compounds. If we look at models that were already established, 80% of our experiments are published as indicator for efficiency of data generation, once a pipeline is established. During these experiments, a maximum of 9% of the animals experienced severe discomfort (see tables below for the amount and percentages of discomfort per model). Despite of an already rather low rate of mouse discomfort we further aimed to reduce discomfort during the last years by:

- Increasing health checking frequency when we observe weight loss after intense procedures (such as IL2 injections and irradiation)
- Offer mice wet food prior and after irradiation and IL2 to reduce the body weight loss
- Lower the tumour size that we use to determine humane endpoint (HEP) for solid tumours (before it was 2000mm³ and now we agreed to use 1500mm³)
- Combine experiments that use same controls to reduce the number of animals that we use
- Use cumulative discomfort in account (if some MILD procedures are repeated over time, it will be counted as MODERATE), and decrease the number of different procedures by combining them

In summary we believe that our high success rate in terms of generating meaningful data as well as constant efforts to reduce discomfort of mice justifies our strategy (Figure 3 and 4). In addition our models have been shown to be predictive for clinical efficacy, even more pronouncing the importance of the models, but also the needed flexibility to allow addressing impact of novel compounds.

3.4.3 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: systemic/metastatic
3	Humanized mouse tumour models: orthotopic
4	Humanized mouse models: safety
5	Click or tap here to enter text.
6	Click or tap here to enter text.
7	Click or tap here to enter text.
8	Click or tap here to enter text.
9	Click or tap here to enter text.
10	Click or tap here to enter text.



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 on the project proposal, see the Guidelines to the project licence application form for animal procedures on 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	Serial number	Type of animal procedure
<i>Use the numbers provided at 3.4.3 of the project proposal.</i>	3.4.4.1	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 (solid or hematological) are used as a surrogate for cancer patients to evaluate immune receptors - IECs and Sols (as mentioned in the project proposal) - 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 and hematological 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 of subcutaneous tumours, calliper 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 i.p.) 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 calliper 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 (IECs and Sols), 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 human growth factors (s.c.) such as IL-2 or IL-15 to support survival of human immune cells in the mouse environment. Peripheral blood mononuclear cells (PBMCs) can be injected, i.e. for the Sols experiments (see figure 2), as these contain the cells necessary for the functioning of the candidate therapy. 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, $\alpha\beta$ TCR engineered T cells or PBMCs will be used as positive or negative control conditions.

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 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

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

Schematic overview of experimental approach

To illustrate the main procedures the animals might undergo during the experiments with either IECs or Sols, a timeline of two examples of experiments (both for IECs and Sols, figure 1 and 2, respectively) are added below.

The duration of the experiment, as well as the timing of bleeding and BLI might differ per specific research question and per tested immunotherapy. When combining therapies, these timelines might vary. This depends on which therapies are combined and which extra procedures need to be done. So far, we have not performed any combination experiments, however we are developing combinational therapies *in vitro*. The timeline and procedures for combination experiments in *in vivo* will be specified in the work protocols.

Timeline experiments with IECs

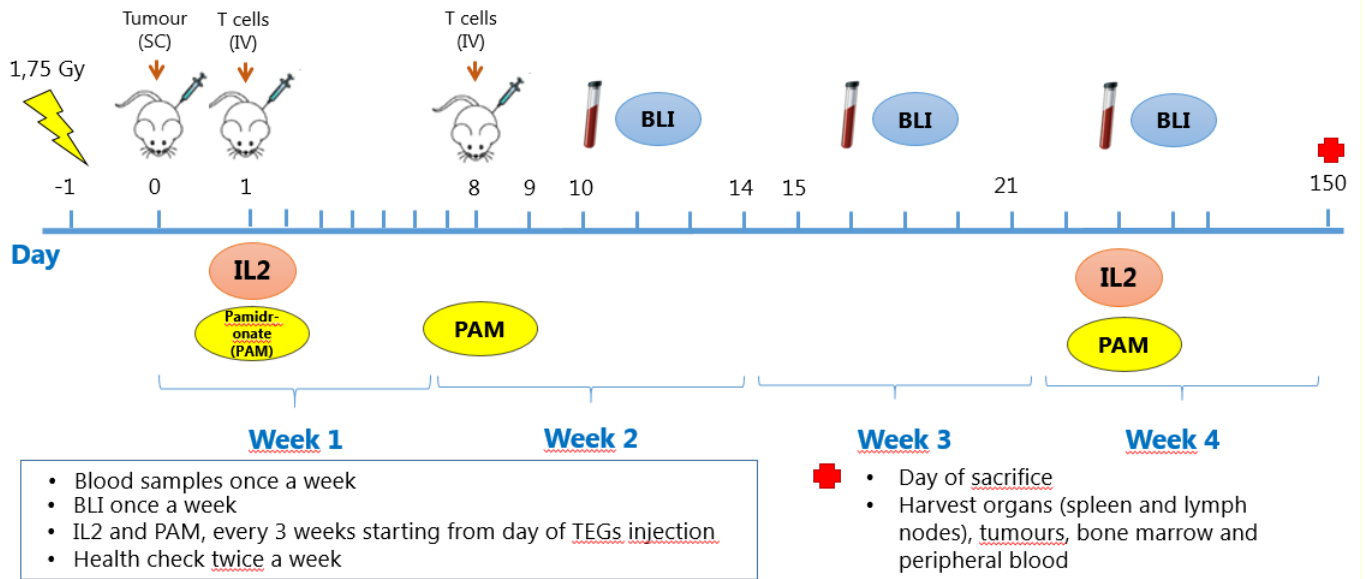


Figure 1: Example of a timeline of an experiment involving IECs

Timeline experiments with Sols

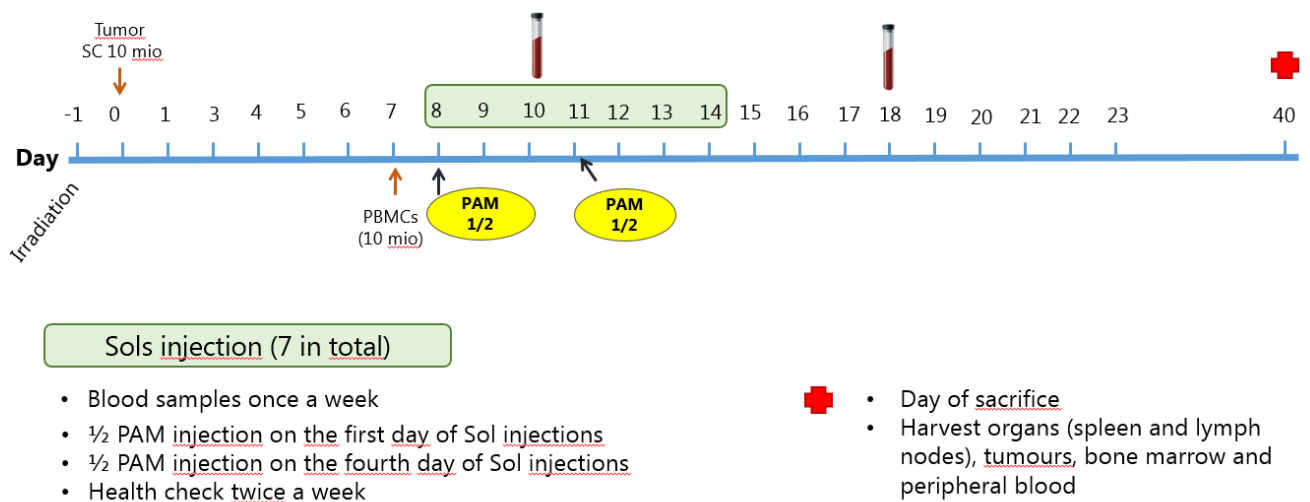


Figure 2: Example of a timeline of an experiment involving Sols

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 fludarabine 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 (total time max 3 weeks, but maximum amount of daily injections is 7 consecutive injections) depending on the receptor platform and research question.

-Candidate immune therapy will be supported by human growth factors. Human immune cells need crucial growth factors for their survival, which are lacking in the immune deficient mice. Therefore immune cell treatment is supported with cellular growth factors such as human IL-2 IL-7, IL-15, IL-21 or others or a combination. Generally these growth factors are applied s.c. in a carrier called Incomplete Freund's Adjuvant (IFA), that will make sure the growth factors are released slowly, every 21 days from the moment of treatment, max 10 injections/animal during the course of the experiment.

-For defined receptors, mice are repetitively (e.g. every 21 days) i.v. injected [redacted] aminobiphosphates (such as pamidronate (PAM)) 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]

-Calliper 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 weekly blood sampling will be performed, or more frequently depending on the experiment, 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 imaging (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 once or twice a week until the end of the experiment, depending on the experimental setup (end time differs between experiments, but up to 150 days).

-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.

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.

B. The animals

Specify the species, origin, life stages, estimated numbers, gender, genetic alterations and, if important for achieving the immediate goal, the strain.

Serial number	Species	Origin	Life stages	Number	Gender	Genetically altered	Strain
3.4.4.1	Mice (mus musculus)	Registered breeder	8-21 weeks of age	2420	Male and female	NOD.Cg-Prkdcscid I12rgtm1Wjl/SzJ NSG-SGM3	NOD scid gamma (NSG)

Provide justifications for these choices

Species	As mentioned in our project proposal, humanized mouse models represent a valuable additional tool to answer biological questions and provide data on efficacy and safety regarding candidate IEC and Sol mediated anti-tumour therapy (Olson et al. Cancer Discov 2018; 1358-1365).
Origin	All mice will be bought from a registered breeder to ensure that the e.g. breeding conditions are all the same, limiting the variability between our experiments.
Life stages	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.
Number	<p>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. Based on experience in previous experiments, we don't need more than 10 mice per group. In case we need to harvest tissues during the experiment (and not only at the end), to specifically answer research questions at different timepoints for instance), we will add additional animals (generally 3 mice) in order to keep the calculated group size for evaluation of the primary outcome parameters during the whole experiment. Per experiment we will generally use not more than 6 treatment groups including negative and positive control treatment. If we apply the maximum group size we need $6 \times 13 = 78$ mice per experiment. Due to biological limitations such as primary tumour samples or human stem cell material but also <i>in vitro</i> production limitations of therapeutic cells and compounds, we don't expect to have larger experiments.</p> <p>We expect to perform 6 experiments per year. This results in $6 \times 78 = 468$ mice per year and for 5 years a total amount of $5 \times 468 = 2340$ mice.</p> <p>For pilot studies or training purposes we might need additional mice. We estimate that we will not need more than 80 mice (~5% of the total mice requested) based on the amount of pilot studies/training we have performed in the previous years.</p> <p>The total amount of mice for subcutaneous experiments will be: $2340 + 80 = 2420$.</p> <p>Randomization will take place based on gender in case of the early treatment model. We are using both male and female mice (or a combination) in our experiments. In case of established tumour treatment models randomization also includes tumour size.</p>
Gender	We will combine both male and female in our experiments to decrease the amounts of animals used and to not limit our findings to a specific gender. For some studies, however, it is important that there is only one gender (for instance for breast cancer research), then we will specifically use one or the other.
Genetic alterations	Immune deficient mouse strains based on the NOD scid gamma (NSG) mice (official strain name NOD.Cg-Prkdc ^{scid} I12rg ^{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

	<p>deficient in multiple cytokine signaling pathways and in many innate immune components such as complement factors.</p> <p>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.</p>
Strain	<p>Immune deficient mice As indicated in the proposal, immune deficient mouse strains are the basis of our pre-clinical <i>in vivo</i> models. The main reason for this is the fact that the $\gamma\delta$ T cell – target cell interactions are not present in mice, because mice lack specific $\gamma\delta$ T 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 <i>in vitro</i> 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 IEC and Sols – based immunotherapy that can be used for broader immune receptor-based therapy.</p> <p>Humanized mouse models represent a valuable additional tool to answer biological questions and provide data on efficacy and safety regarding candidate 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 <i>in vitro</i> 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 IECs 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 <i>in vitro</i> and receptor-based immune therapy (IECs and Sols) 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 IECs. We can directly evaluate the potency of our <i>ex vivo</i> 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 <i>in vitro</i> data, the <i>in vivo</i> 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.</p>

C. Accommodation and care

Is the housing and care of the animals used in experimental procedures in accordance with Annex III of the Directive 2010/63/EU?

Yes

No > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices

Click or tap here to enter text.

D. Pain and compromised animal welfare

Will the animals experience pain during or after the procedures?

No

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 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.

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

Subcutaneous growing tumours can form ulcerations. Treatments may cause toxic side effects, weightloss can be observed shortly after irradiation or after injecting specific growth factors (such as IL2).

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).

E. 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 F

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:

1. 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.

2. A tumour mass greater than 10% of the body weight, usually 2000 cubic mm in case of superficial measurable lesions (by calliper)

3. Skin ulceration/necrosis

NOTE: As observed in previous studies (WP 4288-2-05 and 4288-2-11), after irradiation and IL2 injection, mice might experience weight loss between 20-35%. This weight loss was restored after 1-2 weeks while mice were monitored daily. Therefore, we have discussed with the IVD that the limit of weight loss is now set at 35% and mice will be monitored every day once the weight loss is larger than 20%.

If the mice still:

1. show active behaviour and eat/drink per normal,

2. do not reach 17.0 gram in weight

3. do not experience more than 35% weight loss,

then we will not euthanise the mice but will monitored them in daily basis until they show recovery and reach stable weight (around 1 -2 weeks).

Furthermore, mice that experience weight loss has to show increased in weight within 3 days.

When such case occurs, IvD and veterinarian will be contacted for further assessment if necessary.

4. Failure to eat or drink for a period longer than 24 hours

5. Tumours that result in abnormal locomotion or cause abnormal vocalization, behavior or function

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).

F. Classification of severity of procedures

Provide information on the experimental factors contributing to the discomfort of the animals and indicate to which category these factors are assigned ('non-recovery', 'mild', 'moderate', 'severe'). In addition, provide for each species and treatment group information on the expected levels of cumulative discomfort (in percentages).

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, growth factors or other compounds, chemotherapy or irradiation) will cause discomfort classified as mild.

-Simple but frequent handlings like weighing, calliper measurements and cheek vein bleeding will cause discomfort classified as mild.

-Bioluminescence imaging is classified under mild discomfort due to recovery from anaesthesia.

Note: A maximum of 10 mild procedures (injections, blood draws, BLI measures) in total which may include a maximum of 3 BLI measurements would count as MILD discomfort. Anything above this will be counted as MODERATE. This only counts for the type of experiments with tumours where most procedures have at least a week resting time in between and only if the discomfort due to e.g. tumour growth or IL-2 injections does not increase. The number of procedures can be decreased by combining if possible (E.g.: injecting i.p. animals or taking a blood draw while the animal is under anaesthesia for a BLI measure, could be as one procedure). Extreme weight loss experienced due to irradiation of the mice and IL-2 injections will be counted as SEVERE.

In figure 3 below, the discomfort of the animals per experiment performed in the last 5 years is stated. The numbers in this overview serve as an estimation for the discomfort for the next 5 years.

Type experiment 1: Humanized tumour models: subcutaneous						
Mild discomfort		Moderate discomfort		Severe discomfort		
WP no	Total mice	WP no	Total mice	WP no	Total mice	
4288-1-01	28	4288-1-01	12	4288-1-01	0	
4288-1-02	5	4288-1-02	0	4288-1-02	0	
4288-1-03	35	4288-1-03	2	4288-1-03	3	
4288-1-05	7	4288-1-05	3	4288-1-05	2	
4288-1-06	0	4288-1-06	40	4288-1-06	0	
4288-1-07	1	4288-1-07	39	4288-1-07	0	
4288-1-08	11	4288-1-08	0	4288-1-08	19	
4288-1-09	10	4288-1-09	0	4288-1-09	10	
4288-1-10	0	4288-1-10	59	4288-1-10	1	
4288-1-11	0	4288-1-11	24	4288-1-11	0	
4288-1-12		4288-1-12		4288-1-12		
4288-1-13	0	4288-1-13	24	4288-1-13	1	
4288-1-14		4288-1-14		4288-1-14		
4288-1-15	34	4288-1-15	7	4288-1-15	5	
4288-1-16	5	4288-1-16	39	4288-1-16	1	
4288-1-17	20	4288-1-17	0	4288-1-17	0	
4288-1-18		4288-1-18		4288-1-18		
4288-1-19	0	4288-1-19	50	4288-1-19	0	
4288-1-20	8	4288-1-20	12	4288-1-20	0	
Total	164	Total	311	Total	42	
Total number			517			
Discomfort level		%				
Mild		32				
Moderate		60				
Severe		8				

Figure 3: The discomfort levels of animals used in the pasts 5 years, based on each experiment performed

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

	Mild discomfort	Moderate discomfort	Severe discomfort
Tumour model: Subcutaneous growing			
In %	90% 32%	9% 60%	1% 8%
Total mice in 5 year	2340 774	234 1452	26 194

G. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement	To model tumour cell – immune cell interactions <i>in vitro</i> 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
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	factor such as tumour stroma, oxygen supply and immune cells and immunotherapy is not possible (yet) in <i>in vitro</i> assay systems. And before a new therapy can enter clinical evaluation pre-clinical <i>in vivo</i> testing is required.
Reduction	Extensive <i>in vitro</i> assays have been performed and results are evaluated critically. Only when the candidate immune receptor (IECs or Sols) meets the go/no go criteria outlined in the general proposal it will be selected for testing in the <i>in vivo</i> models. 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 and/or training purposes may have to be performed to develop a new model suited for therapeutic testing before complete <i>in vivo</i> 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. Also, we combine experiments that use same controls to reduce the number of animals that we use.
Refinement	State-of-the-art methods and equipment to follow-up tumour growth (imaging) will be used to minimize discomfort to the animals. Additionally, we give animals breeding chow as a boost, use tube handling and increase health checking frequency when we observed weight loss after e.g. IL2 injections and irradiation.

Are adverse environmental effects expected? Explain what measures will be taken to minimise these effects.

No

Yes > Describe the environmental effects and explain what measures will be taken to minimise these effects.

Click or tap here to enter text.

H. Re-use

Will animals be used that have already been used in other animal procedures ?

No > Continue with question I.

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.

Click or tap here to enter text.

I. Repetition

Explain for legally required animal procedures what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, describe why duplication is required.

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

J. 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 K.

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.

Click or tap here to enter text.

3. End of experiment

K. Destination of the animals

Will the animals be killed during or after the procedures?

No > Provide information on the destination of the animals.

Click or tap here to enter text.

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.

Click or tap here to enter text.

Yes > Will a method of killing be used for which specific requirements apply?

No > Describe the method of killing.

Mice will be killed by cervical dislocation or CO2.

Yes > Describe the method of killing that will be used and provide justifications for this choice.

Click or tap here to enter text.

If animals are killed for non-scientific reasons, justify why it is not feasible to rehome the animals.

Click or tap here to enter text.



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 on the project proposal, see the Guidelines to the project licence application form for animal procedures on 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	Serial number	Type of animal procedure
<i>Use the numbers provided at 3.4.3 of the project proposal.</i>	3.4.4.2	Humanized tumour models: systemic/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 systemic tumours (in the case of hematological tumours) or tumours that form metastases are used as a surrogate for cancer patients to evaluate immune receptors - IECs and Sols (as mentioned in the project proposal) - 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 systemic 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, IECs and Sols, are applied either as monotherapy or as combination therapy. Mice will receive treatment 1-3 days after tumour cell injection (early treatment model; in order to explore whether a candidate therapy could eradicate the tumour from the beginning on and avoid tumour outgrowth) 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 growth factors (s.c.) such as IL-2 or IL-15 to support survival of human immune cells in the mouse environment. Peripheral blood mononuclear cells (PBMCs) can be injected, i.e. for the Sols experiments (see figure 2), as these contain the cells necessary for the functioning of the candidate therapy. 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, $\alpha\beta$ TCR engineered T cells or PBMCs will be used as positive or negative control conditions. In case of combination therapy, additional injections with defined [REDACTED] 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 labeled with specific antibodies against the immune receptor and at the same time we can measure phenotypic characteristics of these cells (such as 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.

Harvesting of organs and blood

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

Schematic overview of experimental approach

To illustrate the main procedures the animals might undergo during the experiments with either IECs or Sols, a timeline of two examples of experiments (both for IECs and Sols, figure 1 and 2, respectively) are added below. The duration of the experiment, as well as the timing of bleeding and BLI might differ per specific research question and per tested immunotherapy. When combining therapies, these timelines might vary. This depends on which therapies are combined and which extra procedures need to be done. So far, we have not performed any combination experiments, however we are developing combinational therapies *in vitro*. The timeline and procedures for combination experiments in *in vivo* will be specified in the work protocols.

Timeline experiments with IECs

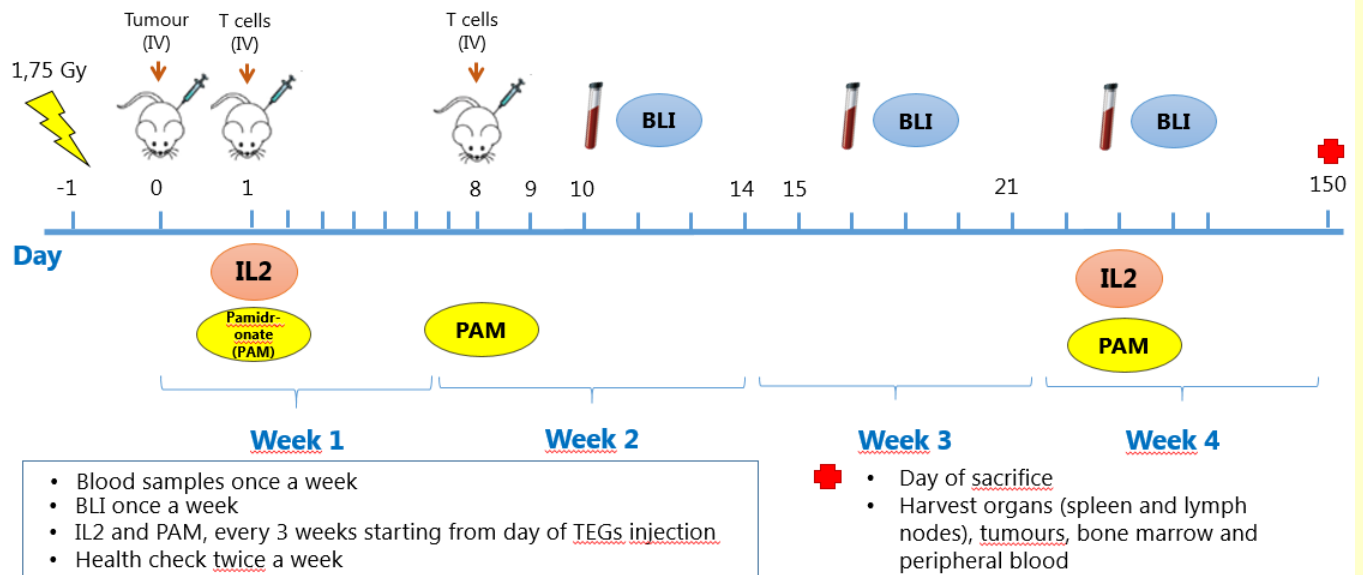


Figure 1: Example of a timeline of an experiment involving IECs

Timeline experiments with Sols

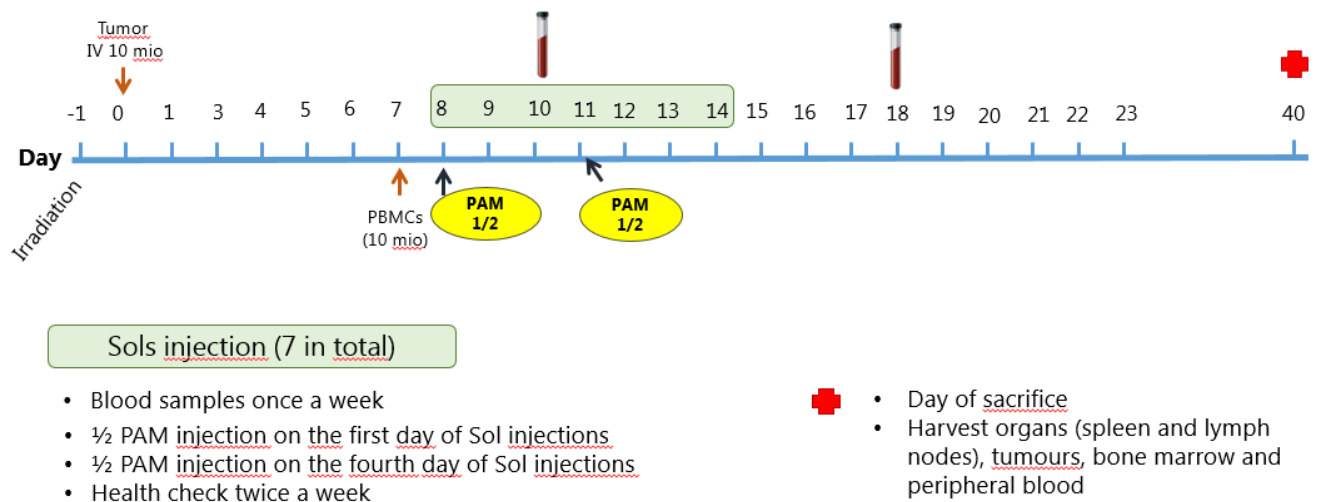


Figure 2: Example of a timeline of an experiment involving Sols

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 fludarabine 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.

-Mesenteric vein injection results 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. Hematological tumours

- Intravenous injection into the tail vein of hematological 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 (total time max 3 weeks, but maximum amount of daily injections is 7 consecutive injections) depending on the receptor platform and research question.

-Candidate immune therapy will be supported by human growth factors. Human immune cells need crucial growth factors for their survival, which are lacking in the immune deficient mice. Therefore immune cell treatment is supported with cellular growth factors such as human IL-2 IL-7, IL-15, IL-21 or others or a combination. Generally these growth factors are applied s.c. in a carrier called Incomplete Freund's 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 repetitively (e.g. every 21 days) i.v. injected with [redacted] or 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 [redacted]

- Blood sampling: in general weekly blood sampling will be performed, or more frequently depending on the experiment,, 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 systemic or 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, the amount of animals that reach HEP can say something about the effectivity of the

treatment 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 once or twice a week, depending on the experimental setup. 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)
Some experiments require

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, life stages, estimated numbers, gender, genetic alterations and, if important for achieving the immediate goal, the strain.

Serial number	Species	Origin	Life stages	Number	Gender	Genetically altered	Strain
3.4.4.2	Mice (mus musculus)	Registered breeder	8-21 weeks of age	2070	Male and female	NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ NSG-SGM3	NOD scid gamma (NSG)

Provide justifications for these choices

Species	As mentioned in our project proposal, humanized mouse models represent a valuable additional tool to answer biological questions and provide data on efficacy and safety regarding candidate IEC and Sol mediated anti-tumour therapy (Olson et al. Cancer Discov 2018; 1358-1365).
Origin	All mice will be bought from a registered breeder to ensure that the e.g. breeding conditions are all the same, limiting the variability between our experiments.
Life stages	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.
Number	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

	<p>need more than 10 mice per group. 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 6 treatment groups including negative and positive control treatment. If we apply the maximum group size we need $6 \times 13 = 78$ mice per experiment. Due to biological limitations such as primary tumour samples or human stem cell material but also <i>in vitro</i> production limitations of therapeutic cells and compounds, we don't expect to have larger experiments.</p> <p>Estimated numbers: We expect to perform</p> <ul style="list-style-type: none"> - 5systemic/metastatic experiments per year <p>This results in $5 \times 78 = 390$ mice per year and for 5 years a total amount of $5 \times 390 = 1950$ mice.</p> <p>For pilot studies, replacement of mice or training purposes we might need additional mice. We estimate that we will not need more than 120 mice (~5% of the total mice requested) based on the amount of pilot studies/training we have performed in the previous years. 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.</p> <p>The total amount of mice needed for 5 years = $1950 + 120 = 2070$.</p>
Gender	<p>We can have both male and female in our experiments to decrease the amounts of animals used and to not limit our findings to a specific gender. For some studies, however, it is important that there is only one gender (for instance for breast cancer research), then we will specifically use one or the other.</p>
Genetic alterations	<p>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.</p> <p>Variants of immune deficient mice</p> <p>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.</p> <p>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.</p>
Strain	<p>Immune deficient mice</p> <p>As indicated in the proposal, immune deficient mouse strains are the basis of our pre-clinical <i>in vivo</i> models. The main reason for this is the fact that the $\gamma\delta$ T cell – target cell interactions are not present in mice, because mice lack specific $\gamma\delta$ T 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 <i>in vitro</i> 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 IEC and Sols – based immunotherapy that can be used for broader immune receptor-based therapy.</p> <p>Humanized mouse models represent a valuable additional tool to answer biological questions and provide data on efficacy and safety regarding candidate receptor mediated anti-tumour therapy. First, on-target anti-tumour efficacy is an important read-out of these models. An</p>

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 IECs 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 (IECs and Sols) 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 IECs. 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.

C. Accommodation and care

Is the housing and care of the animals used in experimental procedures in accordance with Annex III of the Directive 2010/63/EU?

Yes

No > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices

Click or tap here to enter text.

D. Pain and compromised animal welfare

Will the animals experience pain during or after the procedures?

No

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 tumour itself might also induce pain, we will carefully check the well-being of the mice and if human endpoints are reached, the animal will be euthanized.

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 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.

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

Animals that carry tumours in internal organs may experience dysfunction 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, weightloss can be observed shortly after irradiation or after injecting specific growth factors (such as IL2).

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 hematological tumour models, a cage lid test is developed in close communication with the local IVD and designated veterinarian. 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).

E. 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 F

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

We will adhere to the Code of Practice 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:

1. 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.

-Failure to eat or drink for more than 24 hrs

-Persistent hypothermia

-Bloodstained or mucopurulent discharge from any orifice

-Hind-limb paralysis or weakness

-Anaemia as indicated by symptoms such as pale feet, or hematological measures

-Significant abdominal distension or where ascites burden exceeds 10% of the bodyweight of age-matched controls.

-Incontinence or diarrhoea over a 48-h period

-Tumours that interfere with locomotion or cause abnormal vocalisation, animal behaviour or function

NOTE: As observed in previous studies (WP 4288-2-05 and 4288-2-11), after irradiation and IL2 injection, mice might experience weight loss between 20-35%. This weight loss was restored after 1-2 weeks while mice were monitored daily. Therefore, we have discussed with the IVD that the limit of weight loss is now set at 35% and mice will be monitored every day once the weight loss is larger than 20%.

If the mice still :

1. show active behaviour and eat/drink per normal,
2. do not reach 17.0 gram in weight
3. do not experience more than 35% weight loss,

then we will not euthanise the mice but will monitor them in daily basis until they show recovery and reach stable weight (around 1 -2 weeks).

Furthermore, mice that experience weight loss have to show increased in weight within 3 days.

When such case occurs, IVD and veterinarian will be contacted for further assessment if necessary.

•

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 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.

F. Classification of severity of procedures

Provide information on the experimental factors contributing to the discomfort of the animals and indicate to which category these factors are assigned ('non-recovery', 'mild', 'moderate', 'severe'). In addition, provide for each species and treatment group information on the expected levels of cumulative discomfort (in percentages).

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, growth factors 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.

Note: A maximum of 10 mild procedures (injections, blood draws, BLI measures) in total which may include a maximum of 3 BLI measurements would count as MILD discomfort. Anything above this will be counted as MODERATE. This only counts for the type of experiments with tumours where most procedures have at least a week resting time in between and only if the discomfort due to e.g. tumour growth or IL-2 injections does not increase. The number of procedures can be decreased by combining if possible (E.g.: injecting i.p. animals or taking a blood draw while the animal is under anaesthesia for a BLI measure, could be as one procedure). Extreme weight loss experienced due to irradiation of the mice and IL-2 injections will be counted as SEVERE.

In general, 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 or if the metastasis affects the functioning of the specific organ(s), these will cause severe discomfort. We expect that animals under this appendix may suffer from severe discomfort in 9-20% of cases (based on previous experiments). 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).

In figure 3 below, the discomfort of the animals per experiment performed in the last 5 years is stated. The numbers in this overview serve as an estimation for the discomfort for the next 5 years.

Mild discomfort		Moderate discomfort		Severe discomfort			
WP no	Total mice	WP no	Total mice	WP no	Total mice		
4288-2-01	12	4288-2-01	0	4288-2-01	0	Total number of mice in all experiments un appendix 2	579
4288-2-03	37	4288-2-03	3	4288-2-03	0		
4288-2-04	4	4288-2-04	1	4288-2-04	0		
4288-2-05	34	4288-2-05	10	4288-2-05	0		
4288-2-06	0	4288-2-06	40	4288-2-06	0	Discomfort level	%
4288-2-08	3	4288-2-08	16	4288-2-08	1	Mild	28
4288-2-09	10	4288-2-09	18	4288-2-09	0	Moderate	63
4288-2-10	8	4288-2-10	30	4288-2-10	2	Severe	9
4288-2-11	6	4288-2-11	37	4288-2-11	13		
4288-2-12	0	4288-2-12	46	4288-2-12	14		
4288-2-13	0	4288-2-13	30	4288-2-13	0		
4288-2-14	13	4288-2-14	29	4288-2-14	6		
4288-2-15	0	4288-2-15	11	4288-2-15	5		
4288-2-16	14	4288-2-16	27	4288-2-16	7		
4288-2-17	0	4288-2-17	12	4288-2-17	0		
4288-2-18	0	4288-2-18	53	4288-2-18	7		
4288-2-19	20	4288-2-19	0	4288-2-19	0		
4288-2-20		4288-2-20		4288-2-20			
4288-2-21		4288-2-21		4288-2-21			
Total	161	Total	363	Total	55		

Figure 3: The discomfort levels of animals used in the pasts 5 years, based on each experiment performed

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

	Mild discomfort	Moderate discomfort	Severe discomfort
Tumour model: systemic/metastatic			
In %	40% 28%	40% 63%	20% 9%
Total mice in 5 year	1620 580	1620 1304	810 186

G. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement	To model tumour cell – immune cell interactions <i>in vitro</i> 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 (see figure 1 in the research proposal). 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 <i>in vitro</i> assay systems. And before a new therapy can enter clinical evaluation pre-clinical <i>in vivo</i> testing is required.
Reduction	Extensive <i>in vitro</i> assays have been performed and results are evaluated critically. Only when the candidate immune receptor (IECs or Sols) meets the go/no go criteria outlined in the general proposal it will be selected for testing in the <i>in vivo</i> models.

	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 <i>in vivo</i> 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. Also, we combine experiments that use same controls to reduce the number of animals that we use.
Refinement	State-of-the-art methods and equipment to follow-up tumour growth (imaging) will be used to minimize discomfort to the animals. Additionally, we give animals breeding chow as a boost, use tube handling and increase health checking frequency when we observed weight loss after e.g. IL2 injections and irradiation.

Are adverse environmental effects expected? Explain what measures will be taken to minimise these effects.

No

Yes > Describe the environmental effects and explain what measures will be taken to minimise these effects.

[Click or tap here to enter text.](#)

H. Re-use

Will animals be used that have already been used in other animal procedures ?

No > Continue with question I.

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.

[Click or tap here to enter text.](#)

I. Repetition

Explain for legally required animal procedures what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, describe why duplication is required.

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

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

No > Continue with question K.

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.

[Click or tap here to enter text.](#)

3. End of experiment

K. Destination of the animals

Will the animals be killed during or after the procedures?

No > Provide information on the destination of the animals.

[Click or tap here to enter text.](#)

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.

[Click or tap here to enter text.](#)

Yes > Will a method of killing be used for which specific requirements apply?

No > Describe the method of killing.

Mice will be killed by cervical dislocation or CO2.

Yes > Describe the method of killing that will be used and provide justifications for this choice.

[Click or tap here to enter text.](#)

If animals are killed for non-scientific reasons, justify why it is not feasible to rehome the animals.

[Click or tap here to enter text.](#)



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 on the project proposal, see the Guidelines to the project licence application form for animal procedures on 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	Serial number	Type of animal procedure
<i>Use the numbers provided at 3.4.3 of the project proposal.</i>	3.4.4.3	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 - IECs and Sols (as mentioned in the project proposal) - 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 tumour cell injection depends on the tumour origin. Usually, a surgical procedure takes place under general anaesthesia and adequate analgesia.

The site of tumour 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, intracranial injection to simulate brain tumours 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, IECs and Sols, 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 growth factors (s.c.) such as IL-2 or IL-15 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 $\alpha\beta$ TCR engineered T cells will be used as positive or negative control conditions. In case of combination therapy, additional injections with defined [REDACTED] 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 [REDACTED] 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 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 fludarabine 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).

- Intracranial injection of brain tumour cells (Qazi et al. Methods Mol Biol 2014)

Depending on the tumour cell line metastasis can be formed in liver and lung and these models are a combination of appendix 2 and 3.

B. Hematological tumours

- Intra femur injection of hematological 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 human growth factors. Human immune cells need crucial growth factors for their survival, which are lacking in the immune deficient mice. Therefore immune cell treatment is supported with cellular growth factors such as human IL-2 IL-7, IL-15, IL-21 or others or a combination. Generally these growth factors are applied s.c. in a carrier called Incomplete Freund's 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 repetitively (e.g. every 21 days) i.v. injected with [REDACTED] with aminobiphosphates (such as pamidronate (PAM)) 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 [REDACTED]

-Blood sampling: in general weekly blood sampling will be performed, or more frequently depending on the experiment, 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 i.p.) 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 once or twice a week, depending on the experimental setup. 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.

For some experiments, we want to do specific imaging techniques to illustrate more closely the effect of our selected immune therapy candidates, such as intravital 2-photon imaging experiments. This imaging technique is specified separately here because of the nature of the experiments, but may be performed in parallel depending on the research question. With such a live imaging technique, we are able to study T cell dynamics and behaviour in the tumour microenvironment. This relates to the migration and infiltration dynamics and immune cell behaviour read-outs (see figure 3 in project proposal). We will image presence, trafficking and behaviour of immune cells labeled with a fluorescent dye, in or around tumour cells and vasculature. In each animal we are able to image numerous cells or cell-cell interactions. If for a specific experimental setup the choice for intravital 2-photon imaging is desired, the argumentation and strategy will be further specified in the work protocol of that experiment.

-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.

As mentioned in the project proposal, experiments with orthotopic tumour models will be performed sequentially in order to use lessons learned from one experiment for the design of the next experiment, optimizing the usage of mice resources.

B. The animals

Specify the species, origin, life stages, estimated numbers, gender, genetic alterations and, if important for achieving the immediate goal, the strain.

Serial number	Species	Origin	Life stages	Number	Gender	Genetically altered	Strain
3.4.4.3	Mice (mus musculus)	Registered breeder	8-21 weeks of age	1130	Male and female	NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ NSG-SGM3	NOD scid gamma (NSG)

Provide justifications for these choices

Species	As mentioned in our project proposal, humanized mouse models represent a valuable additional tool to answer biological questions and provide data on efficacy and safety regarding candidate IEC and Sol mediated anti-tumour therapy (Olson et al. Cancer Discov 2018; 1358-1365).
Origin	All mice will be bought from a registered breeder to ensure that the e.g. breeding conditions are all the same, limiting the variability between our experiments.
Life stages	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.
Number	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. In case we need to harvest tissues during the experiment (and not only at the end), to specifically answer research questions at different timepoints for instance, we will add additional animals (generally 3 mice) in order to keep the calculated group size for evaluation of the primary outcome parameters during the whole experiment. Per experiment we will generally use not more than 6 treatment groups including negative and positive control treatment. If we apply the maximum group size we need $6 \times 13 = 78$ mice per experiment. Due to biological limitations such as primary tumour samples or human stem cell material but also <i>in vitro</i> production limitations of therapeutic cells and compounds, we don't expect to have larger experiments. Estimated numbers:

	<p>We expect to perform 2 orthotopic experiments per year. In the past 5 years, 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. We are now establishing more models <i>in vitro</i> and therefore we expect to perform more experiments than in the last 5 years.</p> <p>This results in $2 \times 78 = 156$ mice per year and for 5 years a total amount of $5 \times 156 = 780$ mice.</p> <p>Since we have not established a lot of these orthotopic models, we have calculated a larger amount of animals needed for training purposes. We will use 200 mice (25% of total mice needed for orthotopic experiments) in these 5 years for training purposes and pilot studies. 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 (or a combination). In case of established tumour treatment models randomization also includes tumour size.</p> <p>For the 2 photon imaging experiments, the above described power analysis does not apply. 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 (including pilot experiments) due to the time consuming nature of the imaging and preparation per animal (around 2 hours per mouse) and we expect to need one to maximum three pilot experiments to establish the technique for our purpose (in case we run into technical issues). Therefore, we estimate per year to be able to perform 5 experiments maximum (including pilot experiments): $6 \times 5 = 30$ mice per year including the pilot experiments. In 5 years, we expect to use $30 \times 5 = 150$ mice in total (including pilot experiments).</p> <p>$780 + 200 + 150 = 1130$</p>
Gender	We will combine both male and female in our experiments to decrease the amounts of animals used and to not limit our findings to a specific gender.
Genetic alterations	<p>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.</p> <p>Variants of immune deficient mice</p> <p>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.</p> <p>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.</p>
Strain	<p>Immune deficient mice</p> <p>As indicated in the proposal, immune deficient mouse strains are the basis of our pre-clinical <i>in vivo</i> models. The main reason for this is the fact that the $\gamma\delta$ T cell – target cell interactions are not present in mice, because mice lack specific $\gamma\delta$ T 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 <i>in vitro</i> 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 IEC and Sols – based immunotherapy that can be used for broader immune receptor-based therapy.</p> <p>Humanized mouse models represent a valuable additional tool to answer biological questions and provide data on efficacy and safety regarding candidate receptor mediated anti-tumour</p>

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 IECs 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 (IECs and Sols) 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 IECs. 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.

C. Accommodation and care

Is the housing and care of the animals used in experimental procedures in accordance with Annex III of the Directive 2010/63/EU?

Yes

No > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices

Click or tap here to enter text.

D. Pain and compromised animal welfare

Will the animals experience pain during or after the procedures?

No

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.

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

Animals that carry tumours in internal organs may experience dysfunction 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, weightloss can be observed shortly after irradiation or after injecting specific growth factors (such as IL2).

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 hematological 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).

E. 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 F

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

We will adhere to the Code of Practice 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:

1. 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 (due to paralysis of the hindlegs).
- For solid orthotopic tumour models (including breast cancer, colon cancer, brain tumours), a tumor volume of more than 1500 mm³ (Humane intervention points for rodent cancer models – McGill)
- For solid orthotopic tumour models, a tumor burden more than 10% baseline body weight (Humane intervention points for rodent cancer models – McGill)

NOTE: As observed in previous studies (WP 4288-2-05 and 4288-2-11), after irradiation and IL2 injection, mice might experience weight loss between 20-35%. This weight loss was restored after 1-2 weeks while mice were monitored daily. Therefore, we have discussed with the IVD that the limit of weight loss is now set at 35% and mice will be monitored every day once the weight loss is larger than 20%.

If the mice still :

1. show active behaviour and eat/drink per normal,
 2. do not reach 17.0 gram in weight
 3. do not experience more than 35% weight loss,
- then we will not euthanise the mice but will monitored them in daily basis until they show recovery and reach stable weight (around 1 -2 weeks).

Furthermore, mice that experience weight loss has to show increased in weight within 3 days.

When such case occurs, IVD and veterinarian will be contacted for further assessment if necessary.

- Failure to eat or drink for more than 24 hrs
- Persistent hypothermia
- Bloodstained or mucopurulent discharge from any orifice
- Hind-limb paralysis or weakness
- Anaemia as indicated by symptoms such as pale feet, or hematological measures
- Significant abdominal distension or where ascites burden exceeds 10% of the bodyweight of age-matched controls.
- Incontinence or diarrhoea over a 48-h period
- Tumours that interfere with locomotion or breathing or cause abnormal vocalisation, animal behaviour or function

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.

F. Classification of severity of procedures

Provide information on the experimental factors contributing to the discomfort of the animals and indicate to which category these factors are assigned ('non-recovery', 'mild', 'moderate', 'severe'). In addition, provide for each species and treatment group information on the expected levels of cumulative discomfort (in percentages).

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 performed in limited number of animals: moderate discomfort due to recovery from surgery

Interventions:

-Simple well-tolerated interventions (injection of cells, aminobiphosphonates, growth factors 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.

Note: A maximum of 10 mild procedures (injections, blood draws, BLI measures) in total which may include a maximum of 3 BLI measurements would count as MILD discomfort. Anything above this will be counted as MODERATE. This only counts for the type of experiments with tumours where most procedures have at least a week resting time in between and only if the discomfort due to e.g. tumour growth or IL-2 injections does not increase. The number of procedures can be decreased by combining if possible (E.g.: injecting i.p. animals or taking a blood draw while the animal is under anaesthesia for a BLI measure, could be as one procedure). Extreme weight loss experienced due to irradiation of the mice and IL-2 injections will be counted as SEVERE.

In case tumours grow to the size that the humane endpoint weight loss or severe abnormal breathing is reached these will cause severe discomfort. As we have not performed many experiments with orthotopic models, it is hard to estimate the expected percentages per cumulative discomfort. As an estimation, we will use the cumulative discomfort percentages of the subcutaneous model (see appendix 1; as this more closely resembles the orthotopic injection than our systemic/metastatic model). Our experiments with the orthotopic models will show how accurate these pre-defined percentages are. In case the numbers are not correct, we will adjust the discomfort percentages in our work protocols and make an amendment to the existing appendix and project.

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

	Mild discomfort	Moderate discomfort	Severe discomfort
Tumour model: orthotopic			
In %	37% 32%	45% 60%	18% 8%
Total mice in 5 year	650 362	800 678	325 90

G. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement	To model tumour cell – immune cell interactions <i>in vitro</i> 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 <i>in vitro</i> assay systems. And before a new therapy can enter clinical evaluation pre-clinical <i>in vivo</i> testing is required.
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Reduction	Extensive <i>in vitro</i> assays have been performed and results are evaluated critically. Only when the candidate immune receptor (IECs or Sols) meets the go/no go criteria outlined in the general proposal it will be selected for testing in the <i>in vivo</i> models. 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 <i>in vivo</i> 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. Also, we combine experiments that use same controls to reduce the number of animals that we use.
Refinement	State-of-the-art methods and equipment to follow-up tumour growth (imaging) will be used to minimize discomfort to the animals. Additionally, we give animals breeding chow as a boost, use tube handling and increase health checking frequency when we observed weight loss after e.g. IL2 injections and irradiation.

Are adverse environmental effects expected? Explain what measures will be taken to minimise these effects.

No

Yes > Describe the environmental effects and explain what measures will be taken to minimise these effects.

Click or tap here to enter text.

H. Re-use

Will animals be used that have already been used in other animal procedures ?

No > Continue with question I.

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.

Click or tap here to enter text.

I. Repetition

Explain for legally required animal procedures what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, describe why duplication is required.

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

J. 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 K.

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.

Click or tap here to enter text.

3. End of experiment

K. Destination of the animals

Will the animals be killed during or after the procedures?

No > Provide information on the destination of the animals.

Click or tap here to enter text.

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.

Click or tap here to enter text.

Yes > Will a method of killing be used for which specific requirements apply?

No > Describe the method of killing.

Mice will be killed by cervical dislocation or CO2.

Yes > Describe the method of killing that will be used and provide justifications for this choice.

Click or tap here to enter text.

If animals are killed for non-scientific reasons, justify why it is not feasible to rehome the animals.

Click or tap here to enter text.



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 on the project proposal, see the Guidelines to the project licence application form for animal procedures on 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	Serial number	Type of animal procedure
<i>Use the numbers provided at 3.4.3 of the project proposal.</i>	3.4.1.4	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 - IECs and Sols (as mentioned in the project proposal). 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 (IECs and Sols). 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 DIPerslo 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 (IECs and Sols) 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 (IECs and Sols) 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 regimen. A chemotherapeutic regimen will be based on standard clinically used compounds in immunotherapy, such as (a combination of) cyclophosphamide, busulfan or fludarabine 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 (IECs or Sols). The cells (or other format) will be injected usually i.v. or in some occasions s.c. or orthotopic 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 human growth factors. Human immune cells need crucial growth factors for their survival, which are lacking in the immune deficient mice. Therefore immune cell treatment is supported with cellular growth factors such as human IL-2 IL-7, IL-15, IL-21 or others or a combination thereof. Generally these growth factors are applied s.c. in a carrier called Incomplete Freund's 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 repetitively (e.g. every 21 days) i.v. injected with [redacted] aminobiphosphates (such as pamidronate) from the moment of treatment resembling the treatment of patients with these receptor-based therapies (Straetmans 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 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, life stages, estimated numbers, gender, genetic alterations and, if important for achieving the immediate goal, the strain.

Serial number	Species	Origin	Life stages	Number	Gender	Genetically altered	Strain
3.4.1.4	Mice (mus musculus)	Registered breeder	8-21 weeks of age	390	Male and female	NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ NSG-SGM3	NOD scid gamma (NSG)

Provide justifications for these choices

Species	As mentioned in our project proposal, humanized mouse models represent a valuable additional tool to answer biological questions and provide data on efficacy and safety regarding candidate IEC and Sol mediated anti-tumour therapy (Olson et al. Cancer Discov 2018; 1358-1365).
Origin	All mice will be bought from a registered breeder to ensure that the e.g. breeding conditions are all the same, limiting the variability between our experiments.
Life stages	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.

Number	<p>In the past 5 years, 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 not a lot could be tested for safety already. We are now developing more compounds that need to be tested for safety <i>in vivo</i> and therefore we expect to perform more experiments than in the last 5 years.</p> <p>The experiments have a comparable design as in previous appendices: a maximum of 6 groups per experiment including control groups with 13 animals per group results in 78 animals per experiment. We estimate to perform 1 safety experiment per year. This results in a maximum total of 78 animals per year. In 5 years we will use 390 mice.</p>
Gender	<p>We will combine both male and female in our experiments to decrease the amounts of animals used and to not limit our findings to a specific gender. For some studies, however, it is important that there is only one gender (for instance specific therapies for breast cancer research), then we will specifically use one or the other.</p>
Genetic alterations	<p>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.</p> <p>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.</p>
Strain	<p>Immune deficient mice As indicated in the proposal, immune deficient mouse strains are the basis of our pre-clinical <i>in vivo</i> models. The main reason for this is the fact that the $\gamma\delta$ T cell – target cell interactions are not present in mice, because mice lack specific $\gamma\delta$ T 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 <i>in vitro</i> 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 IEC and Sols – based immunotherapy that can be used for broader immune receptor-based therapy.</p>

C. Accommodation and care

Is the housing and care of the animals used in experimental procedures in accordance with Annex III of the Directive 2010/63/EU?

Yes

No > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices

[Click or tap here to enter text.](#)

D. Pain and compromised animal welfare

Will the animals experience pain during or after the procedures?

No

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.

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.

E. 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 F

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 as much as possible. Frequent monitoring will take place to enable this.

The following parameters are included weight loss, hunching, activity, fur texture, skin integrity and diarrhoea.

Humane endpoints more specified:

1. 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.

NOTE: As observed in previous studies (WP 4288-2-05 and 4288-2-11), after irradiation and IL2 injection, mice might experience weight loss between 20-35%. This weight loss was restored after 1-2 weeks while mice were monitored daily. Therefore, we have discussed with the IVD that the limit of weight loss is now set at 35% and mice will be monitored every day once the weight loss is larger than 20%.

If the mice still:

1. show active behaviour and eat/drink per normal,
2. do not reach 17.0 gram in weight
3. do not experience more than 35% weight loss,

then we will not euthanise the mice but will monitored them in daily basis until they show recovery and reach stable weight (around 1 -2 weeks).

Furthermore, mice that experience weight loss has to show increased in weight within 3 days.

When such case occurs, IvD and veterinarian will be contacted for further assessment if necessary.

2. The following GvHD symptom scoring system will be used to estimate the amount of discomfort for each animal during the whole experiment.

Humane endpoint is reached in case score 2 is reached for an individual parameter or a total score of 4 is reached.

a. Hunching:

- score 0 normal

- score 1 only at rest
- score 2 severe and impaired movements
- b. Activity
 - score 0 normal
 - score 1 mild to moderately decreased
 - score 2 stationary unless stimulated
- c. Fur texture
 - score 0 normal
 - score 1 mild to moderately ruffling
 - score 2 severe ruffling/poor grooming
- d. Skin integrity
 - score 0 normal
 - score 1 scaling of paws and tail
 - score 2 obvious areas of denuded skin
- e. Diarrhoea
 - score 0 normal
 - score 1 green/yellow droppings
 - score 2 excrement sticking around anus

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.

F. Classification of severity of procedures

Provide information on the experimental factors contributing to the discomfort of the animals and indicate to which category these factors are assigned ('non-recovery', 'mild', 'moderate', 'severe'). In addition, provide for each species and treatment group information on the expected levels of cumulative discomfort (in percentages).

Interventions

- Simple well-tolerated interventions (injection of cells, aminobiphosphonates, growth factors 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).

Note: A maximum of 10 mild procedures (injections, blood draws, anaesthesia) in total which may include a maximum of 3 anaesthesia procedures would count as MILD discomfort. Anything above this will be counted as MODERATE. This only counts for the type of experiments where most procedures have at least a week resting time in between and only if the discomfort due to e.g. tumour growth or IL-2 injections does not increase. The number of procedures can be decreased by combining if possible (E.g.: injecting i.p. animals or taking a blood draw while the animal is under anaesthesia for a BLI measure, could be as one procedure).

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), 38% moderate (due to GVHD-like symptoms) and 60% mild discomfort

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

	Mild discomfort	Moderate discomfort	Severe discomfort
Total safety models			
In % and numbers			

A	50% - 117	50% -117	0%
B	60% -	38%- 59	2% - 3
Total	54% -211	45% -176	1% - 3

*due to round off values

G. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement	To model cell – immune cell interactions <i>in vitro</i> we apply state-of-the-art models and techniques such as the organoid models and the 3D bioprinted bone marrow model in order to simulate the effect of our therapies on healthy cells and gather valuable information without the usage of animals. However, studying the complexity of long-term interactions between healthy tissue cells (stroma) and immune cells and immunotherapy is not possible to mimick completely in <i>in vitro</i> assay systems And before a new therapy can enter clinical evaluation pre-clinical <i>in vivo</i> testing is required.
Reduction	Extensive <i>in vitro</i> assays have been performed and results are evaluated critically. Only when the candidate immune receptor (IECs or Sols) meets the go/no go criteria outlined in the general proposal it will be selected for testing in the <i>in vivo</i> models. We apply a power calculation to minimize the number of animals and scientific literature is constantly used to prevent repetition of already performed experiments. For safety testing, as much as possible will be assessed <i>in vitro</i> , thereby reducing unnecessary use of animals, for instance by doing co-culture assays with healthy cells/healthy tissue/healthy organoids with our therapy (also combining different cell types). Also, we combine experiments that use same controls to reduce the number of animals that we use.
Refinement	State-of-the-art methods and equipment to follow-up tumour growth (imaging) will be used to minimize discomfort to the animals. Additionally, tube handling will be used to minimize discomfort for the animals.

Are adverse environmental effects expected? Explain what measures will be taken to minimise these effects.

No

Yes > Describe the environmental effects and explain what measures will be taken to minimise these effects.

[Click or tap here to enter text.](#)

H. Re-use

Will animals be used that have already been used in other animal procedures ?

No > Continue with question I.

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.

[Click or tap here to enter text.](#)

I. Repetition

Explain for legally required animal procedures what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, describe why duplication is required.

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

J. 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 K.

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.

[Click or tap here to enter text.](#)

3. End of experiment

K. Destination of the animals

Will the animals be killed during or after the procedures?

No > Provide information on the destination of the animals.

[Click or tap here to enter text.](#)

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.

[Click or tap here to enter text.](#)

Yes > Will a method of killing be used for which specific requirements apply?

No > Describe the method of killing.

Mice will be killed by cervical dislocation or CO2.

Yes > Describe the method of killing that will be used and provide justifications for this choice.

[Click or tap here to enter text.](#)

If animals are killed for non-scientific reasons, justify why it is not feasible to rehome the animals.

[Click or tap here to enter text.](#)

A. Algemene gegevens over de procedure

1. Aanvraagnummer : AVD11500202216349
2. Titel van het project : Immune receptor mediated control of tumours
3. Titel van de NTS : Immuuntherapie tegen kanker: een stap naar effectievere en veiligere behandelingen

4. Type aanvraag:

- nieuwe aanvraag projectvergunning
 wijziging van vergunning met nummer :

5. Contactgegevens DEC

Naam DEC : DEC Utrecht
Telefoonnummer contactpersoon : 06 - 31118069
Emailadres contactpersoon : dec-utrecht@umcutrecht.nl

6. Adviestraject (data dd-mm-jjjj):

- ontvangen door DEC: 22-08-2022
 aanvraag compleet:
 in vergadering besproken: 24-08-2022 en 16-11-2022
 anderszins behandeld: 24-08-2022 en 16-11-2022
 termijnonderbreking(en) van / tot : 26-08-2022 / 02-11-2022 en 21-11-2022 / 22-11-2022
 besluit van CCD tot verlenging van de totale adviestermijn met max. 15 werkdagen:
 aanpassing aanvraag:
 advies aan CCD: 28-11-2022

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

8. Eventueel horen van aanvrager

- Datum: 24-08-2022
- Plaats: online via Teams
- Aantal aanwezige DEC-leden: 7
- Aanwezige (namens) aanvrager: 4 onderzoekers
- Gestelde vragen en verstrekte antwoorden: De DEC heeft de onderzoeker o.a. gehoord over het missende overzicht van eerdere uitkomsten met daarbij de geleerde lessen en de mate van ongerief (was deze zoals vooraf ingeschat?). Het is de DEC niet duidelijk of een geselecteerde kandidaat in alle modellen gebruikt zal worden of in een deel. De DEC mist een beslisboom met criteria waaruit duidelijk wordt welke modellen ingezet worden, op welke manier en met name waarom deze ingezet worden. De aanvraag zal herschreven worden en er zal hiervoor overlegd worden met de IvD.

Uit het gesprek zijn onderstaande vragen, zoals vermeld bij punt A9, voortgekomen, die schriftelijk aan de onderzoekers werden voorgelegd.

- Het horen van de aanvrager heeft geleid tot aanpassing van de aanvraag.

Eventueel horen van aanvrager

- Datum: 16-11-2022
- Plaats: Utrecht
- Aantal aanwezige DEC-leden: 5
- Aanwezige (namens) aanvrager: 4 onderzoekers
- Gestelde vragen en verstrekte antwoorden: De DEC heeft de onderzoeker o.a. gehoord over het ontbreken van een go/no-go moment voor de stap naar in vivo onderzoek. Er mist een definitie van een positief resultaat op basis waarvan men naar de volgende stap gaat. Onderzoekers geven aan dat een definitie moeilijk aan te geven is vanwege de breedte van de aanvraag. Er zullen minstens 3 replicaties moeten optreden bij het in vitro onderzoek voordat men verder gaat. Als er geen activiteit optreedt, gaat men niet verder. Per stap wordt gekeken welk model het meest geschikt is. Onderzoekers beginnen met het model dat ze het best kennen (subcutaan). Er zijn te weinig resources om alles tegelijk te kunnen uitvoeren, dus onderzoekers zijn zelf kritisch in hun keuzes. Ze willen graag nog keuzevrijheid hebben naar aanleiding van de uitkomsten en hebben daarom in de aanvraag niet alle stappen al vastgelegd.

Daarnaast zijn vragen gesteld over het orthotope model: dit zal als laatst worden uitgevoerd, omdat het ingewikkeld is en niet alle onderzoekers dit experiment kunnen uitvoeren. Er wordt maar één tumor gebruikt. De DEC verzoekt de onderzoekers deze stapsgewijze aanpak meer te beschrijven en aan te geven dat er een maximum is aan het aantal uit te voeren experimenten.

Tot slot is gesproken over off-target effecten met betrekking tot de veiligheid van potentiële therapieën waarbij het muismodel niet goed voorspellend kan zijn voor langdurige effecten. Er worden stamcellen ingezet om te kijken of deze aangevallen worden, met een follow-up na 200 dagen. Uit het gesprek zijn onderstaande vragen, zoals vermeld bij punt A9, voortgekomen, die schriftelijk aan de onderzoekers werden voorgelegd.

- Het horen van de aanvrager heeft geleid tot aanpassing van de aanvraag.

9. Correspondentie met de aanvrager

- Datum vragen: 26-08-2022
- Datum antwoord: 02-11-2022
- Gestelde vragen en antwoorden:

Algemeen

- De DEC mist een overzicht van uitkomsten uit het voorgaande onderzoek met daarbij o.a. de geleerde lessen, de aantallen gebruikte dieren en een overzicht van het daadwerkelijke ongerief (was dit zoals vooraf ingeschat?).

In het research proposal is aan het einde van 3.4.2. een alinea toegevoegd met 'Past experience improvements to increase output of the pipeline as well as animal welfare'.

Hier beschrijven wij, als we kijken naar de afgelopen 5 jaar, wat wij hebben geleerd en veranderd. In de appendices is verder het aantal dieren per experiment en hoeveel dieren een bepaald ongerief heeft ondergaan in de afgelopen jaren beschreven.

- Daarnaast is het de DEC niet duidelijk of een geselecteerde kandidaat in alle modellen gebruikt zal worden of in een deel. De DEC mist een beslisboom met criteria waaruit duidelijk wordt welke modellen ingezet worden, op welke manier en met name waarom deze ingezet worden.

In het research proposal is aan het einde van 3.4.2. (justification for the strategy) een figuur met daaronder uitleg toegevoegd van de route die een kandidaat receptor therapie doormaakt en welke modellen daarvoor gebruikt worden. Daarnaast zullen de keuze voor het model in detail worden uitgelegd in de werk protocollen (dit staat ook vermeld in de figuur en tekst bijbehorend bij de figuur).

- De antwoorden hebben geleid tot aanpassing van de aanvraag.

Correspondentie met de aanvrager

- Datum vragen: 21-11-2022
- Datum antwoord: 22-11-2022
- Gestelde vragen en antwoorden:

Algemeen

- De DEC vraagt u de track changes te verwijderen en wijzigingen rood te maken om de tekst meer leesbaar te maken.

Wij hebben de track changes verwijderd en wijzigingen op de vragen rood gemaakt in de tekst van het project proposal en bijlage 3.

- Zoals besproken tijdens de vergadering vraagt de DEC u een nog meer stapsgewijze benadering te beschrijven, die u ook mondeling toegelicht heeft: go/no-go momenten met bijbehorende criteria op basis waarvan u verder gaat naar een volgende stap. Als het mogelijk is, dan deze ook graag toevoegen in figuur 3. *Het is van tevoren lastig om definitieve criteria vast te stellen, aangezien deze verschillend kunnen zijn afhankelijk van de onderzoeksvraag. Richtlijnen voor criteria zijn bijvoorbeeld in vitro een two-fold increase in vergelijking met baseline/negatieve controle of significante verschillen in in vitro assays die uitgevoerd worden voor het ontwikkelen van een muisexperiment (zie figuur 3). Wij willen graag verwijzen naar 2 van onze publicaties (Johanna et al. 2020, Journal of Leukocyte Biology; Johanna et al. 2021, Front in Immunol), waar een aantal in vitro en in vivo criteria zijn aangegeven, zoals significante verschillen in cytokine release in in vitro assays, maar ook off-target toxicity, graft-versus-host symptomen en T cell persistentie als in vivo criteria. Extra informatie over go/no go criteria is in het onderschrift bij figuur 3 toegevoegd.*
- Kunt u aangeven of er een maximum aantal uit te voeren experimenten is? *De richtlijn is per lead/carrier combinatie maximaal twee keer testen in hetzelfde model onder dezelfde condities, waarbij 2 negatieve experimenten worden gezien als no go (bijvoorbeeld geen verbetering in tumor controle, zie voor criteria figuur 3 van het*

project proposal). Wanneer een lead/carrier combinatie positief test kan ervoor gekozen worden om dezelfde combinatie te testen in een ander model of met andere condities, dit hangt echter af van de resultaten in het betreffende model en de (nieuwe) onderzoeksvraag. Dit is ook aangegeven in figuur 4.

- Kunt u aangeven of er een risico gaat optreden van resistentie tegen bepaalde therapie?

Helaas kan voor alle therapieën een bepaalde resistentie optreden. Wij verwachten dat resistenties kunnen optreden tegen de therapieën die wij ontwikkelen en zullen deze bestuderen en kennis gebruiken om resistenties te voorkomen. Zo kunnen wij met deze kennis bijvoorbeeld combinatietherapieën in de toekomst ontwikkelen, die de kans op resistentie verminderen.

Bijlage 3

- A. Experimentele aanpak en primaire uitkomstparameters: kunt u aangeven of u de orthotopie tumormodellen in parallel wilt uitvoeren of serieel?
Wij hebben bij figuur 4 van de project proposal een zin bijgevoegd: 'Experiments using a specific new lead/carrier combination will be preferentially performed sequentially. This allows us to use lessons learned from one experiment for the design of the next experiment, optimizing the usage of mice resources.' Er is een extra zin toegevoegd in bijlage 3 (onder A) Experimental approach and primary outcome parameters) dat wij willen leren van de uitkomsten van orthotopie tumormodellen en daardoor experimenten sequentieel willen uitvoeren.

- De antwoorden hebben geleid tot aanpassing van de aanvraag.

10. Eventuele adviezen door experts (niet lid van de DEC): n.v.t.

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. De aanvragers beschrijven een onderzoek naar alternatieve therapeutische behandeling van tumoren die gebaseerd is op metabole eigenschappen van kankercellen in plaats van gericht op tumorspecifieke mutaties. Het onderzoek is gebaseerd op de observatie dat bepaalde immuuncellen (gamma/delta T-cellen ($\gamma\delta$ T-cellen)), krachtiger lijken te zijn dan veel andere subpopulaties. De belangrijkste kracht van deze T-cellen is dat deze kanker niet als een genetische, maar als een stofwisselingsziekte zien. Dat is het aangrijpingspunt van een breed gedragen onderzoek waar meerdere instellingen aan deelnemen. De onderzoekers hebben twee strategieën ontwikkeld

om het concept van metabole kanker gericht op $\gamma\delta$ T-cellen te gebruiken. De eerste strategie omvat immuueffectorcellen (IEC's), genetisch gemodificeerd of niet gemodificeerd. Een voorbeeld van dergelijke IEC's die de onderzoekers momenteel optimaliseren in hun huidige *in vitro* en *in vivo* studies zijn TEG's: T-cellen die een gedefinieerde $\gamma\delta$ TCR tot expressie brengen. Men wil uiteindelijk patiënt afgeleide (autologe) $\alpha\beta$ T-cellen met de tumorspecifieke $\gamma\delta$ TCR kweken tot voldoende cel aantallen, zuiveren en aan de patiënt gaan toedienen. Deze aanvraag beschrijft het *in vivo* deel waarbij een selectie aan kandidaten (elders getest op potentie) wordt onderzocht in verschillende stappen met criteria voor selectie voor de volgende fase, en volgt voorbeeld 1 uit de nieuwe handreiking 'definitie project'. De DEC heeft aanvullende vragen gesteld, met name over de stappen en beslismomenten die nu helderder zijn beschreven.

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 doelcategorie(ën), te weten fundamenteel onderzoek en translationeel onderzoek, sluit(en) aan bij de hoofddoelstelling(en).

Belangen en waarden

4. Het directe doel van het project is het onderzoek naar immuun-receptor gemedieerde controle van tumoren. Het uiteindelijke doel van het project is met deze specifieke therapie bepaalde vormen van kanker te remmen in ontwikkeling of uitzaaien. De DEC is van mening dat er een duidelijke relatie is tussen het directe en het uiteindelijke doel, en dat het doel gerechtvaardigd is in de context van het kankeronderzoek en de behoeften vanuit de patiënten met bepaalde tumoren.
5. De belangrijkste belanghebbenden in dit onderzoeksproject zijn weergegeven in onderstaande tabel:

Belanghebbende	Morele waarden die worden bevorderd
Onderzoekers	Kennis vergaren, publiceren, bijdragen aan effectieve kankertherapie
Patiënten en hun behandelaars	Zicht op een behandeling die de ontwikkeling van de kanker remt, uitzaaiing tegengaat
Familie van patiënten	Hopen dat hun familielid de ziekte kan overwinnen
Industrie	Maken de kandidaat immuuntherapie onder GMP condities
Maatschappij	Kanker is een veelvoorkomende ziekte die hoge ziektelast en sterfte en hoge kosten met zich mee brengt
Subsidieverstrekker (KWF)	Maken het financieel mogelijk dat het onderzoek kan worden uitgevoerd

	Morele waarden die in het geding zijn:
Proefdieren	Het ervaren van pijn en ongerief omdat de dieren ziek worden gemaakt, behandelingen worden getest en uiteindelijk gedood moeten worden

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 voldoende gewaarborgd en dragen eraan bij dat de doelstellingen behaald kunnen worden, dat aan de 3V-beginselen voldaan kan worden en dat voorkomen kan worden dat mens, dier en milieu negatieve effecten ondervinden als gevolg van de dierproeven. De onderzoekers hebben ruime ervaring in de ontwikkeling van receptor gebaseerde immuuntherapie naar een fase I klinische studie, met inbegrip van het verkrijgen van noodzakelijke *in vitro* en *in vivo* gegevens om toestemming van de autoriteiten te verkrijgen om een fase I klinische studie te starten. Er zijn langdurige internationale samenwerkingen op het gebied van transplantatie, cellulaire immunotherapie en het $\gamma\delta$ T-cel veld. Deze samenwerkende wetenschappers zijn experts op het gebied van muismodellen voor immuuntherapie tegen kanker. Binnen de universiteit zijn er ook nauwe samenwerkingen met belangrijke wetenschappers die (tumor) organoïden hebben ontwikkeld.
8. Het project is goed opgezet, de voorgestelde experimentele opzet en uitkomstparameters sluiten logisch en helder aan bij de aangegeven doelstellingen. De gekozen strategie en experimentele aanpak kunnen leiden tot het behalen van de doelstelling binnen het kader van het project. Er is een stapsgewijze aanpak met selectiecriteria in achtereenvolgens 1) selectie van kandidaat therapie (*in vitro* en *in silico*), 2) onderzoek naar werkingsmechanisme en biodistributie, 3) onderzoek naar combinaties van therapie (*in vitro* en *in vivo*), 4) veiligheid van de therapie omdat er mogelijk een graft versus host reactie zou kunnen optreden en 5) effectiviteit van de potentiële therapie in verschillende tumormodellen. De DEC kan zich vinden in deze stappen en ook in het feit dat veiligheid (kans op GvHD) in gehumaniseerde modellen eerst wordt onderzocht en dan pas het meest belastende tumoronderzoek.

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. Per bijlage zijn de handelingen die de dieren ondergaan uitgeschreven met het daarbij behorend ongerief. Ook het cumulatieve ongerief dat een dier zal ondergaan is goed herleidbaar en naar het oordeel van de IvD en de DEC goed en realistisch ingeschat. De onderzoekers hebben ook het aantal dieren met werkelijk ongerief over de afgelopen jaren weergegeven waardoor er een goed inzicht is gegeven over het ongerief dat verwacht kan worden, omdat het immers grotendeels over hetzelfde type experimenten gaat.
12. De integriteit van de dieren wordt fysiek en gedragsmatig aangetast doordat dieren stoffen krijgen toegediend, er biologisch materiaal wordt afgenomen en tumoren worden veroorzaakt in de dieren, waardoor zij zich ziek kunnen gaan voelen. De tumoren moeten gemeten worden of via beeldvormende technieken onder anesthesie worden beoordeeld.
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. Per bijlage worden specifieke humane eindpunten vermeld door de opzet van de proef (tumorgroei, necrose, ernstige GvHD, etc.) en daarnaast nog algemene klinische verschijnselen zoals gewichtsverlies waarvoor een scoresysteem wordt gebruikt.

3V's

14. De aanvrager heeft voldoende aannemelijk gemaakt dat er geen geschikte vervangingsalternatieven zijn. De eerste onderzoeken worden met bijvoorbeeld 3D-structuren van mini-organen en tumoren uitgevoerd, maar door de complexiteit van de ziekte kan tot op heden de complexe interactie tussen immuuntherapie en tumoren het beste worden nagebootst in een levend organisme met een werkende bloedvoorziening, waarbij de mate waarin de therapie de tumor kan bereiken wordt bestudeerd. Ook kunnen met behulp van dierproeven beter de lange termijn effecten van de therapie worden bestudeerd. Dit is niet mogelijk in het laboratorium. En immuuntherapie wordt juist ontwikkeld om een langdurig effect te bewerkstelligen.
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. De onderzoekers willen het aantal muizen verminderen door alleen onderzoek te doen naar therapieën die in het laboratorium veel zijn getest en veelbelovend zijn gebleken. Om het aantal muizen zo laag mogelijk te houden, wordt het aantal benodigde muizen exact berekend om goede wetenschappelijke resultaten te verkrijgen. Er worden gevalideerde proefopzetten gebruikt.

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. 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.
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 (tenzij er sprake is van een geslachtsgebonden tumor) in gelijke mate worden ingezet.
19. De dieren worden in het kader van het project gedood om zo veel mogelijk biologisch materiaal te verzamelen voor verder onderzoek. De dieren worden op een passende wijze, in overeenstemming met bijlage IV van de EU richtlijn, 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 het onderzoek naar immuun-receptor gemedieerde behandeling van kanker, de onvermijdelijke aantasting van het welzijn en de integriteit van de gebruikte proefdieren kan rechtvaardigen.
2. Er vindt een beperkte aantasting van welzijn en integriteit van 1927 proefdieren met licht ongerief, een aanzienlijke aantasting van 3610 muizen met matig ongerief en 473 muizen met ernstig ongerief plaats.

Indien de hierboven genoemde doelstellingen behaald worden, dan zal dit project er toe bijdragen dat patiënten met bepaalde vormen van kanker met deze specifieke therapie behandeld kunnen worden, zodat de ziekte geremd kan worden en/of uitzaaiingen worden voorkomen. De DEC ziet de unieke aanpak van het onderzoek. Het is aannemelijk dat de fundamentele en 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.

3. Op grond van het bovenstaande is de DEC van oordeel dat het onderzoek naar immuun-receptor gemedieerde behandeling van tumoren een substantieel belang vertegenwoordigt en dat dit substantiële belang opweegt tegen de deels beperkte en deels aanzienlijke aantasting van het welzijn en de integriteit van de proefdieren. De relatie tussen het directe en het uiteindelijk doel is voldoende helder. Het is aannemelijk dat de directe doelstelling behaald zal worden. De commissie is overtuigd van de kwaliteit van het werk van de aanvrager. De aanvrager heeft voldoende aannemelijk gemaakt dat er geen geschikte vervangingsalternatieven zijn, dat het doel niet met minder dieren behaald kan worden, dat de gebruikte aanpak de meest verfijnde is en dat er geen sprake zal zijn van onbedoelde negatieve effecten voor mens, dier en milieu als gevolg van de dierproeven. 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. De volgende knelpunten/dilemma's zijn naar voren gekomen tijdens het beoordelen van de aanvraag en het opstellen van het advies. Binnen de context heeft de DEC gesproken met de onderzoeker en daarna tijdens haar afweging over de flowchart met de beslismomenten en het inzetten van de tumormodellen. De antwoorden van de onderzoeker zijn in lijn met wat ze zeiden tijdens de meeting. Het is niet exact te formuleren waar het onderzoek ophoudt en waar het nog interessant zou zijn voor verder onderzoek. De DEC heeft er echter wel vertrouwen in

dat de onderzoekers niet toch extra onderzoek zouden doen. Dat is mede gezien het feit dat het een academische onderzoeksgroep is met gelimiteerde financiën en personele bezetting. Daarnaast kan dit nog ondervangen worden als de IvD bij de tumorexperimenten een rechtvaardiging vraagt en toeziet dat niet onnodig alle modellen worden ingezet omdat daar in de tekst nog wel ruimte voor is (in het algemeen, meestal, etc.).



> Retouradres Postbus 93118 2509 AC Den Haag

UMC Utrecht

[Redacted]

Postbus 12007

3508 GA UTRECHT



**Centrale Commissie
Dierproeven**

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centralecommissiedierproeven.nl
0800 789 0789
info@zbo-ccd.nl

Onze referentie

Aanvraagnummer
AVD11500202216349

Bijlagen

2

Datum 22 augustus 2022

Betreft Ontvangstbevestiging aanvraag projectvergunning Dierproeven

Geachte [Redacted]

Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 19 augustus 2022. Het gaat om uw project "Immune receptor mediated control of tumours". Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD11500202216349. 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, stuur een e-mail naar info@zbo-ccd.nl of neem telefonisch contact met ons op: 0800 789 0789.

Datum:

22 augustus 2022

Aanvraagnummer:

AVD11500202216349

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlagen:

- Gegevens aanvraagformulier
- Factuur



Gegevens aanvrager

Uw gegevens

Deelnemersnummer NVWA: 11500
Naam instelling of organisatie: UMC Utrecht
Naam portefeuillehouder of diens gemachtigde: [REDACTED]
Postbus: 12007
Postcode en plaats: 3508 GA UTRECHT

Gegevens verantwoordelijke onderzoeker

Naam: [REDACTED]
Functie: [REDACTED]
Afdeling: Centre for Translational Immunology
Telefoonnummer: [REDACTED]
E-mailadres: [REDACTED]

Over uw aanvraag

Wat voor aanvraag doet u? 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 2023
Geplande einddatum: 31 januari 2028
Titel project: Immune receptor mediated control of tumours
Titel niet-technische samenvatting: Immuuntherapie tegen kanker: een stap naar effectievere en veiligere behandelingen
Naam DEC: DEC-Utrecht
Postadres DEC: Postbus 85500 3508 GA Utrecht
E-mailadres DEC: dec-utrecht@umcutrecht.nl

Betaalgegevens

De leges bedragen: € 2.212,-
De leges voldoet u: na ontvangst van de factuur

Checklist bijlagen

Verplichte bijlagen: Projectvoorstel
 Beschrijving Dierproeven
 Niet-technische samenvatting

Ondertekening

Naam:

[REDACTED]

Functie:

[REDACTED]

Plaats:

Utrecht

Datum:

16 augustus 2022



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Onze referentie
Aanvraagnummer
AVD11500202216349
Bijlagen
2

Datum 22 augustus 2022
Betreft Factuur aanvraag projectvergunning Dierproeven

Factuur

Factuurdatum: 22 augustus 2022
Vervaldatum: 21 september 2022
Factuurnummer: 2216349
Ordernummer: CB.841910.3.01.011

Omschrijving	Bedrag
Betaling leges projectvergunning dierproeven Betreft aanvraag AVD11500202216349	€ 2.212,00

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 te 's Gravenhage.

From: info@zbo-ccd.nl
To: [Instantie voor Dierenwelzijn Utrecht](#)
Cc: [redacted]; dec-utrecht@umcutrecht.nl
Subject: Aanhouden AVD11500202216349
Date: vrijdag 9 december 2022 09:58:12

Geachte [redacted]

Op 19-08-2022 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Immune receptor mediated control of tumours" met aanvraagnummer AVD11500202216349. 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

In bijlage 2 van de dierproeven geeft u bij B aan 2070 dieren voor uw onderzoek in te willen zetten. De aantallen in de tabel bij F zijn samen 2097 dieren. Kunt u de tabel consistent maken met de aantallen die u aangeeft bij B?

In bijlage 4 van de dierproeven geeft u bij E algemene criteria als humane eindpunten aan. Kunt u specifieker formuleren welke criteria u als humaan eindpunt hanteert?

In bijlage 4 van de dierproeven geeft u bij F het cumulatieve ongerief van dieren aan. Wanneer dit bij elkaar wordt opgeteld komt er een percentage van 110% uit. Kunt u de tabel aanpassen zodat het percentage cumulatief ongerief 100% wordt?

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 dit bericht 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 93118 | 2509 AC | Den Haag

.....
T: 0800 789 0789

E: info@zbo-ccd.nl

Dit bericht kan informatie bevatten die niet voor u is bestemd. Indien u niet de geadresseerde bent of dit bericht abusievelijk aan u is gezonden, wordt u verzocht dat aan de afzender te melden en het bericht te verwijderen.

De Staat aanvaardt geen aansprakelijkheid voor schade, van welke aard ook, die verband houdt met risico's verbonden aan het elektronisch verzenden van berichten.

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Onze referentie

Aanvraagnummer
AVD11500202216349

Bijlagen

3

Datum 19 december 2022

Betreft Beslissing aanvraag projectvergunning Dierproeven

Geachte [Redacted]

Op 19 augustus 2022 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Immune receptor mediated control of tumours" met aanvraagnummer AVD11500202216349. Wij hebben uw aanvraag beoordeeld.

Beslissing

Wij keuren uw aanvraag goed. Uit artikel 10a, eerste lid van de Wet op de dierproeven (hierna: de wet) volgt daarom dat het is toegestaan om uw project uit te voeren binnen de gestelde vergunningsperiode. Deze vergunning wordt afgegeven voor de periode van 1 februari 2023 tot en met 31 januari 2028.

Aan de vergunning hebben wij de volgende voorwaarde verbonden op grond van artikel 10a1, tweede lid van de wet.

Beoordeling achteraf

In dit project worden dierproeven toegepast die vallen in de categorie ernstig volgens artikel 10b van de wet. Daarom bent u verplicht om na afloop van de vergunning in een Beoordeling achteraf over uw project te rapporteren. Deze beoordeling zal uiterlijk januari 2029 plaatsvinden. Er zal dan conform artikel 10a2, derde lid van de wet, beoordeeld worden of de doelstellingen van het project werden bereikt.

De onderbouwing van deze beslissing vindt u onder 'Overwegingen'.

Procedure

Datum:

19 december 2022

Aanvraagnummer:

AVD11500202216349

Advies dierexperimentencommissie

Wij hebben advies gevraagd bij de dierexperimentencommissie DEC-Utrecht (hierna: DEC). Dit advies is ontvangen op 28 november 2022. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a, derde lid van de wet.

Nadere vragen aanvrager

Op 9 december 2022 hebben wij u om aanvullingen gevraagd. U heeft tijdig antwoord gegeven. Het verzoek om aanvullingen had betrekking op de aantallen dieren in bijlage 2 van de dierproeven, en de humane eindpunten en het percentage cumulatief ongerief in bijlage 4 van de dierproeven. Uw reactie is betrokken bij de behandeling van uw aanvraag.

Overwegingen

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 moet er een beoordeling plaatsvinden zoals bedoeld in artikel 10a1, eerste lid, onder d en artikel 10a1, derde lid van de wet. De reden van deze beoordeling achteraf is dat in dit project dieren ernstig ongerief ondergaan. Deze beoordeling zal uiterlijk januari 2029 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 93118, 2509 AC 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. Nadat u een bezwaarschrift heeft ingediend kunt u een voorlopige voorziening vragen bij de voorzieningenrechter van de rechtbank in de vestigingsplaats van de vergunninghouder. U moet dan wel kunnen aantonen dat er sprake is van een spoedeisende situatie.

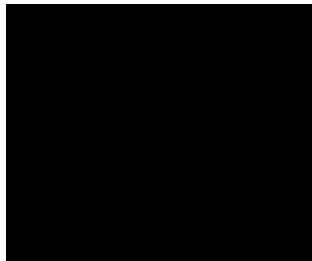
Voor de behandeling van een voorlopige voorziening is griffierecht verschuldigd. Op

<http://www.rechtspraak.nl/Organisatie/Rechtbanken/Pages/default.aspx> kunt u zien onder welke rechtbank de vestigingsplaats van de vergunninghouder valt.

Meer informatie

Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl, stuur een e-mail naar info@zbo-ccd.nl of neem telefonisch contact met ons op: 0800 789 0789.

Centrale Commissie Dierproeven
namens deze:



Bijlagen:

- Projectvergunning
- DEC-advies
- Weergave wet- en regelgeving

Datum:

19 december 2022

Aanvraagnummer:

AVD11500202216349



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: 3508 GA UTRECHT
Deelnemersnummer: 11500

deze projectvergunning voor het tijdvak 1 februari 2023 tot en met 31 januari 2028, voor het project "Immune receptor mediated control of tumours" met aanvraagnummer AVD11500202216349, na advies van dierexperimentencommissie DEC-Utrecht. De functie van de verantwoordelijk onderzoeker is [REDACTED]

[REDACTED] Het besluit is gebaseerd op de volgende (aangepaste) stukken:

- 1 een aanvraagformulier projectvergunning dierproeven, zoals ontvangen op 19 augustus 2022
- 2 de bij het aanvraagformulier behorende bijlagen:
 - a Projectvoorstel, zoals ontvangen op 28 november 2022;
 - b Bijlagen dierproeven
 - 3.4.3.1. Humanized tumour models: subcutaneous, zoals ontvangen op 28 november 2022;
 - 3.4.3.2. Humanized tumour models: systemic/metastatic, zoals ontvangen op 13 december 2022;
 - 3.4.3.3. Humanized tumour models: orthotopic, zoals ontvangen op 28 november 2022;
 - 3.4.3.4. Humanized mouse models: safety testing, zoals ontvangen op 13 december 2022;
 - c Niet-technische Samenvatting van het project, zoals ontvangen op 28 november 2022;
 - d Advies van dierexperimentencommissie, zoals ontvangen op 28 november 2022
 - e De aanvullingen op uw aanvraag, zoals ontvangen op 13 december 2022.

Aanvraagnummer: AVD11500202216349

Naam proef	Diersoort/ Stam	Aantal dieren	Ongerief
3.4.3.1. Humanized tumour models: subcutaneous			
	Muizen (Mus musculus)	2.420	8,0% Ernstig 60,0% Matig 32,0% Licht
3.4.3.2. Humanized tumour models: systemic/metastatic			
	Muizen (Mus musculus)	2.070	9,0% Ernstig 63,0% Matig 28,0% Licht
3.4.3.3. Humanized tumour models: orthotopic			
	Muizen (Mus musculus)	1.130	8,0% Ernstig 60,0% Matig 32,0% Licht
3.4.3.4. Humanized mouse models: safety testing			
	Muizen (Mus musculus)	390	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. Daarom bent u verplicht om na afloop van de vergunning in een Beoordeling achteraf over uw project te rapporteren. Deze beoordeling zal uiterlijk januari 2029 plaatsvinden. Er zal dan conform artikel 10a2, derde lid van de wet, beoordeeld worden of de doelstellingen van het project werden bereikt.

Geldende voorschriften

Wij wijzen u op onderstaande geldende voorschriften, die volgen uit artikel 1d, vierde lid, artikel 10, eerste lid 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.

Aanvraagnummer: AVD11500202216349

- 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.



Aanvraagnummer:

AVD11500202216349

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, derde lid van de wet. Uit artikel 10b, eerste lid van de wet 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, eerste lid van de wet de wijziging eerst voorgelegd worden en mag deze pas doorgevoerd worden na goedkeuren door de Centrale Commissie Dierproeven. Artikel 10b, tweede en derde lid van de wet schrijven 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 van de wet 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

Aanvraagnummer:
AVD11500202216349

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 van de wet 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 13c van de wet 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 13d van de wet 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, eerste lid onder d en derde lid 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.