Appendix
Description animal procedures

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	50200
1.2	Provide the name of the licenced establishment.	Biomedical Primate Research Centre
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 1 Type of animal procedure: DRF and PK/PD analysis of Treg inducing therapeutic agents in non-human primates

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 this project proposal novel immune-therapeutics that aim to expand and enhance the function of regulatory T-cells (T_{REG}) and which are specifically designed for use in humans, will be evaluated in rhesus macaques as a prerequisite step before further in animal studies of human disease and clinical studies in human volunteers and patient are performed. The experimental setup is chosen to allow determination of the pharmacokinetic/pharmacodynamic (PK/PD) profile, perform limited dose finding and to monitor for occurrence of adverse events, especially with respect to cytokine release syndrome and pathological changes in organ function. Animals will receive the therapeutic agent by either subcutaneous, intramuscular (at one or multiple sites) or intravenous injection. Blood is taken at multiple time points on the day of the first injection (for PK analysis) and then regularly until the end of the study for; a) PK analysis, b) to study changes in T_{REG} and other leucocyte subset number and activation (for PD analysis), c) release of cytokines in the blood, d) clinical chemistry, e) hematology. The clinical chemistry and hematology analysis will serve to monitor unintended side effects, like changes in liver, kidney function etc. and vascular integrity. Experimental groups are; one control group receiving only vehicle, two to three groups receiving different doses of the therapeutic agent. The control group is needed to be able to correct for the changes induced by sedation alone, for instance on clinical chemistry parameters.

Criteria to select candidates for testing are:

- The therapeutic agent must have been tested in relevant cell cultures for absence of toxicity and *in vitro* efficacy.

- The therapeutic agent, or a surrogate monoclonal antibody (mAb) binding to a homologous target in case of IL-2/mAb complexes, must have been evaluated in rodent models for immunomodulatory effect and absence of toxicity.
- The evaluation in non-human primates is part of the pre-clinical evaluation of the therapeutic agent.

The primary outcome parameters are:

1. Selective increase in the absolute number of T_{REG} (over the other leucocyte subsets) and/or increase in expression of activation and proliferation markers on T_{REG}.

The secondary outcome parameters are:

1. Absent or very limited increase in pro-inflammatory cytokines in the blood.
2. Absent or very limited increase in clinical chemistry values indicative of organ damage or vascular leakage (in access above values induced by the sedation alone).
3. Clinical symptoms
4. Functional characteristics of the *in vivo* expanded T_{REG}

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

The therapeutic agent will be injected either subcutaneously, intramuscularly or intravenously depending on the nature of the compound. Local reactions will be recorded in case of subcutaneous and intramuscular injection. The compound is either given once or multiple times using at least a 1 day interval, with a maximum number of 10 injections. Blood is taken at multiple time points on the day of injection for PK analysis. Subsequently, blood is taken at regular time intervals for PK/PD analysis, cytokine analysis and clinical chemistry and hematology (as described above). In general, blood volumes to be taken will not exceed a maximum of 1% of the body weight per 4 weeks (and 0.7% max per bleeding). Injections and blood draw will be performed under sedation. In case a daily blood draw is required then the animals will receive tube feeding to supplement food intake, which is otherwise reduced because of fasting before sedation. The animals will be monitored for general behavior as well as specific signs for anaphylaxis, including alertness, posture, dehydration, nausea, breathing pattern, temperature increase. A clinical scoring system will be prepared and applied based on these symptoms and used as clinical end-point. At the end of the study, animals will either return to the experimental stock or are humanely euthanized in case full pathology assessment is required.

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

The number of animals per test group will be determined on the basis of experience gained in *in vivo* experiments in rodents and in evaluation of therapeutic mAb in macaques. Our collaborators showed significant increased T_{REG} blood cell counts in a previous study, involving 3 animals per group, where Proleukin treatment was used (P=0.0016 mean +/- SD 16 +/- 5 vs 294 +/- 62, control vs hIL-2, unpaired t-test with equal SD, unpublished data). It is therefore expected that a group size of 3 animals will suffice for the PK/PD analysis and that testing of max two to three doses of the therapeutic agent will provide sufficient information for dose range-finding (DRF) needed for FIH enabling studies. Those latter studies will not be performed at our institute but will be performed by our collaborators of this study.

B. The animals

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

Either *Macaca mulatta* or *Macaca fascicularis* (adult; female or male), 30 animals. Macaques are the animals of choice to evaluate cytokine and mAb based immunomodulatory therapies. The reason is that macaques are phylogenetically closely related to humans to allow sufficient cross recognition of therapeutic agents that are specifically designed to bind human target molecules. Furthermore, the similarity in the cellular composition and functional characteristics of the immune system and physiological responses allows adequate evaluation of PK, desired therapeutic effects as well as potential adverse events.

It is expected that at most four different compounds need to be tested, using two to three doses, with three animals per group. The aim is to test two compounds simultaneously, which makes it possible to share a common vehicle control group. Each study is performed either with all male or all female animals. This is needed because of the known difference in prevalence of autoimmune diseases and the reported difference in percentage of T_{REG} between males and females (1). Typically, a study is comprised of 2 dose groups x 2 test compounds + 1 control group = 5 groups. With 3 animals per group this results in 15 animals per experiment. We expect to perform two experiments over a three-year period (testing 4 new compounds in total); i.e. 30 animals in total.

1. Afshan G, Afzal N, Qureshi S. CD4+CD25(hi) regulatory T cells in healthy males and females mediate gender difference in the prevalence of autoimmune diseases. Clin Lab. 2012;58(5-6):567-71.

C. Re-use

Will the animals be re-used?

☐ No, continue with question D.

☒ Yes > Explain why re-use is considered acceptable for this animal procedure.

Animals to be used in these experiments may have been used in other studies, provided that they did not previously receive immunosuppressive therapy or mAb administration. Given the long lifespan of this species reuse will take place in the legal framework described in art. 1 of the law on animal testing.

Are the previous or proposed animal procedures classified as 'severe'?

☒ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

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

Replacement.

As part of the non-clinical evaluation, cytokine and mAb therapies are first tested in *in vitro* immunopharmacology studies to measure relative cell/antigen specificity of the compound by flow cytometry and cell based assays or competitive immune-assays. In addition, binding to human and animal target tissues will be determined using immunohistochemical techniques. However, further *in vivo* studies are usually needed because the *in vitro* culture procedure, the limited time span of culture of primary immune cells as well as an underrepresentation of the complex multicomponent tissue dependent interactions taking place in the organism make full extrapolation to the human situation rather unprecise. Although rodent models are very valuable for proof of principle studies and to investigate the mechanism of action of new therapeutic agents, they are not suitable to evaluate the efficacy and possible adverse effects of therapeutic agents that are specifically tailored to bind human target molecules or involve human mAb. The only suitable animal model to investigate these aspects are non-human primates that express target molecules that are sufficiently homologous to be recognized and that have the appropriate immunoregulatory molecules and physiology to allow adequate assessment of efficacy and adverse events. Studies to evaluate mAb based immunotherapies are usually performed in macaque species because of their close phylogenetic relationship to humans, their well characterized immune-system and the availability of the required immunological tools.

Reduction.

Only the minimum number of animals needed will be used. Based on preceding experiments in rodents and evaluation of other cytokine and mAb immunomodulatory therapies in macaques, the maximum number of animals per group needed to effectively determine PK/PD characteristics and monitor for inadvertent general immune activation is three animals. Furthermore, we aim to evaluate different doses at the same

time and to include more than one candidate therapeutic, so that a single control group can be used. The control group is needed to correct for effects induced by the vehicle or by the sedation alone.
Refinement.
Supplementary nutritious and calorie-rich diet is administered (via gavage) when animals need to be daily sedated for blood sampling. The gavage is given during the sedation for a maximum period of five days. This reduces the possible negative effects of fasting for the purpose of frequent sedation. The animals are trained to work as much as possible voluntarily on invasive biotechnological actions such as giving sedation. The number of blood samplings, and the collected volumes of blood are reduced to a minimum. All observations will be documented and registered in a clinical scoring form which is part of the digital database at the institute. For the experiments, a standard clinical scoring form (behaviour, appetite and stool) will be extended for clinical signs that may be indicative of vascular leakage syndrome (e.g. alertness, posture, dehydration, nausea, breathing pattern, temperature increase).

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

Animals will be socially housed with a socially compatible animal, whenever possible. There is an extensive program for enrichment in our institute.
All experimental procedures will be performed under sedation using ketamine. Each time an animal is sedated, the animal will be weighed, the body temperature will be taken, and the animal will be closely examined. Our institute uses a customized database that documents all individual animals in the institute. General observations like behaviour, appetite and stool are part of this database. This database thus facilitates early recognition of minor changes in these general parameters. During the study, care will be taken to avoid pain. In case an animal suffers from pain, a veterinarian will be informed, and the animal will receive analgesics to relief the pain, if necessary. Appropriate measures will be taken in case clinical symptoms indicative of vascular leakage syndrome are observed in order to minimize discomfort.
In case a daily blood draw is required then the animals will receive tube feeding. This is necessary, because the daily sedations of the animals necessitate fasting of the animals, and the food intake during this period would otherwise be very limited.
Regular analysis of haematological and clinical chemistry parameters is part of the experiment. If necessary, judged by the veterinarian, measures will then be taken to treat the animal.
The studies will be performed according the guidelines of the “Wet Milieubeheer”, and will cause no adverse effects on the environment.

Repetition and duplication

E. Repetition

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

Not applicable.

Accommodation and care

F. Accommodation and care

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

☒ No

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

G. Location where the animals procedures are performed

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

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

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

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

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

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

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

Pain relief therapy will be applied when substantial induration is seen at the site of injection. Animals will also receive pain relief therapy if there are any other circumstances that indicate that pain can reasonably be expected to occur or animals show signs of illness indicative of pain. Analgesics known not to interfere with the induction of the treatment will be used.

I. Other aspects compromising the welfare of the animals

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

1. Discomfort due to injection
2. Stress because of sedation and recovery
3. Reduced food intake because of frequent blood collection
4. Injection of cytokines or immunomodulatory mAb can cause a cytokine release syndrome leading to vascular leakage.

Explain why these effects may emerge.

1. When the therapeutic agent is given by injection, this can cause local pain and irritation
2. Animals will be repeatedly sedated for delivery of therapeutic agent and blood sampling. Nausea can sometimes be observed during recovery from the sedation.
3. Especially during daily sedation food intake will be reduced.
4. Especially after intravenous injection of therapeutic agent an immediate hyper-immune activation can potentially occur. This risk is increased when the therapeutic agent is given for the second time. Antibodies against the therapeutic agent may also induce anaphylaxis. However, because of the short duration of the experiment this is unlikely.

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

1. Animals will be sedated for delivery of therapeutic agent. Only rarely are strong adverse effects seen. Should granuloma formation be observed, then the animal will be sedated, the wound will be cleaned and analgesics are applied.
2. Recovery of the animals will be monitored and the veterinarian will be consulted in case of problems.
3. Animals will receive tube feeding. This is applied during sedation.
4. Injection of therapeutic agent will be done by a veterinarian. Animals will be monitored closely during recovery and appropriate measures will be taken in case of shock.

J. Humane endpoints

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

☐ No > Continue with question K.

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

Animals are monitored twice daily and a clinical scoring list is used to assess if an animal shows clinical symptoms suggestive of progress to a vascular leakage syndrome. In combination with the clinical chemistry and hematology data this information will be used to assess in consultancy with the veterinarian the likelyhood of immediate progress to vascular leakage syndrome. In that case the animal will be euthanized immediately in order to avoid unnecesary suffering and a full necropsy will be performed.

Indicate the likely incidence.

The risk of development of vascular leakage syndrome is very low (<5%). The estimate is based on other studies as well as the *in vitro* and *in vivo* analysis in rodents that is performed before the therapeutic agents will be tested in macaques.

K. Classification of severity of procedures

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

The total amount of discomfort is estimated as moderate. This is mainly caused by the frequent sedation needed for collection of blood.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

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

Animals will be euthanized in case they show signs of vascular leakage syndrome. In addition, animals need to be euthanized for some studies in case full pathological assessment of the tissues is needed for evaluation of possible adverse events. In other cases this is not needed and animals will return to the experimental stock. The decision depends on the target molecule involved and whether it is possible to obtain the required information from other models or not

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

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

☒ Yes