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NON-TECHNICAL SUMMARY

Effects of human gene mutations and follicular T cell products on immune responses and disease

Project duration

5 years 0 months

Project purpose

- (a) Basic research
- (b) Translational or applied research with one of the following aims:
  - (i) Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Autoimmunity, Autoantibodies, Follicular T cells, B cells, Tumours

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

> The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand how a healthy immune system is normally regulated to avoid reacting against innocuous environmental antigens (to prevent allergies) or self-antigens (to prevent autoimmunity) while still being reactive to self-tumour antigens, microbes, and infected cells.

An important parallel aim is to trial new therapeutic agents emanating from our work to prevent and/or treat autoimmune disease (like lupus, pemphigus, multiple sclerosis, autoimmune arthritis and colitis) and allergies.

A retrospective assessment of these aims will be due by 04 May 2028

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve it's aims and if not, why not?

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

There are over 100 different human autoimmune diseases and the incidence is increasing at rates of 3-9% per year. Together, these autoimmune diseases affect ~ 5% of the population. These diseases like lupus, pemphigus, rheumatoid arthritis, inflammatory bowel disease (colitis) and multiple sclerosis, can cause severe symptoms that can be debilitating and sometimes life-threatening. To date there are no cures for any of these diseases. They are generally treated with high dose steroids and treatments that tend to dampen the entire immune system and can lead to multiple side-effects and organ damage over time. Furthermore, sometimes, some of the most serious disease manifestations including kidney damage and leukopenias (e.g. thrombocytopenia) do not respond to these treatments. Although new treatments are being developed, it is impossible to know which patient will respond to which therapy, so a lot of time and money is wasted prescribing these treatments one after another until one works. For treatments to be more effective, it is very important to improve our understanding of the root causes of each autoimmune disease in each patient or group of patients.

Allergies are also on the rise and can cause lethal anaphylactic shock in susceptible individuals. The limited treatment options are not effective in a large proportion of cases.

Cancer is a leading cause of death worldwide; the prognosis and life expectancy of many cancers is improving thanks to developments in immunotherapy, where boosting our own immune systems provides the ability to better fight tumours.

At the core of all these pathologies is the establishment of immune cell tolerance processes that in healthy individuals prevent reactions against self or innocuous environmental antigens, while allowing immune cells to fight tumours. How these processes work is incompletely understood.

What outputs do you think you will see at the end of this project?

New knowledge and information on the causes of autoimmune disease and allergies, or on how to better fight cancers, that will peer reviewed and published in respected international publications. The new insights will also be communicated at national and international conferences. Some of the knowledge may be translated into new treatments. We will also be testing novel therapeutics we are developing, mostly based around a small soluble protein shown by our laboratory to be effective at suppressing autoimmune B cells and IgE production (allergies associated antibodies) in mice. We will test the therapeutic potential of neuritin and neuritin derivatives in several autoantibody mediated disease in mice (e.g. collagen-induced arthritis, K/BxN arthritis, pemphigus, lupus, inflammatory bowel disease and a multiple sclerosis models). We currently hold a 'method of use' patent and are aiming to obtain a 'composition of matter' patent and develop the product for commercial application. This will be done with the help of a translation team, venture capitalists and pharmaceutical partners. Other treatments will also aim to silence TLR7 signaling in mice with lupus-like disease. We are interacting with pharma companies that have developed specific RNA-based TLR7-blocking compounds, which we will be trialling alongside other commercially-available inhibitors.

Our work investigating the effects of follicular regulatory T cells and their products on B cells and antibodies in cancer may reveal novel immunotherapy targets.

Who or what will benefit from these outputs, and how?

This knowledge will allow clinicians to refine the diagnosis of patients and will provide information as to which groups of patients will respond better to which treatments. This may be undertaken in different ways. For example, we have determined that TLR7 overactivity drives the expansion of a rare B cell subset known as DN2. A simple flow cytometric assay enumerating DN2 B cells in patients with autoimmunity may therefore identify those that will more greatly benefit from TLR7 inhibition. Whilst the few individual patients whose gene variants have been introduced into mouse models may benefit from refined diagnosis and/or more targeted treatment within the life of this project license, larger groups of patients will see the benefit after we have completed the project. Some of our findings (i.e. inhibition of autoantibody-producing plasma cells by Tfr products) may be applicable to a large number of B cell-driven diseases (e.g. pemphigus, rheumatoid arthritis, lupus, thyroiditis, anti-phospholipid Sd, Sjögren's Sd and vasculitis). Other findings, such as the therapeutic value of TLR7 inhibition, may be selectively beneficial for patients with systemic autoimmunity to nucleic acids (i.e. lupus). While the latter may be translatable to the clinic within the time-frame of this PPL (there are already biologicals in pre-clinical trials inhibiting TLR7), taking our novel compounds to the clinic may take longer.

How will you look to maximise the outputs of this work?

We will maximise the outputs of this work through already established collaborations with clinicians and scientists in the UK, US, Australia, China and several European countries. We will also collaborate with pharmaceutical and commercial companies. The principal investigator has done extensive outreach work to patient groups or societies in her previous position, and will aim to continue to do this in the UK. The knowledge will be disseminated in publications and conferences.

Species and numbers of animals expected to be used

- Mice: 31500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using mice because their immune system is 99% identical to that of humans, and we have been able to recapitulate some human diseases in mice, by introducing the genetic mutations found in the human patients. As a result, the models are valid for the study of the immune pathways that are faulty because of the mutations.

For most studies we will use young and adult mice, since the diseases we are interested in develop in late childhood, adolescence and adulthood.

Typically, what will be done to an animal used in your project?

For this project, we have developed new mouse models of human disease by introducing the genetic mutations found in the patients. We first need to validate these models and see if they develop the clinical manifestations of autoimmune disease found in humans (for example the swollen joints and tiredness that accompany several forms of autoimmunity). We will take blood samples and cells from the mice to understand the abnormalities that are linked with disease development. Development of disease manifestations can take from weeks of life to 6 months of age, depending on the symptoms: for example, in lupus mouse models, autoantibodies usually appear in the first 2-3 months, whereas kidney disease usually takes 6 months to develop.

The first few experiments with each new line will validate the model by allowing the mice to develop end-organ damage, and once the model is validated, further experiments will not go as far and we will use the earlier manifestations as readout of disease.

Some mouse lines do not or may not develop disease overtly. For those lines, we will inject them with substances that mimic environmental or other known disease triggers (like viral infections). In some

case we will expose mice to a transient increase in temperature that might change the presentation of self-proteins and make them immunogenic and accelerate the onset of autoimmune disease.

A particular strain of mouse will develop tumours spontaneously and these mice will be used to investigate how B cells contribute to the control of the tumour and how T cell-derived factors may or may not limit B cell-dependent tumour control.

In order to understand whether the immune system is generally overactive or there are selective defects, we will immunise the mice with proteins or other agents that are typically used to induce antibody responses, allergies, and/or inflammation, and investigate whether such responses are protective or cause unwanted reactions. These experiments typically last several weeks.

In order to understand which cells are responsible for causing disease, we will irradiate mice to remove their immune cells, and then inject them with a mixture of immune cells that have or do not have the disease-causing mutation, with a marker that can tell apart their origin. This will help us pinpoint very precisely the cells that trigger disease, so they can be targeted for treatment. Normally, we need to wait at least 8 weeks after the mice have been irradiated and injected with the new immune cells, for the cells to reach stable numbers and the mice to be analysed.

In some cases, we will need to remove components of the immune system, to check whether they are necessary for disease development. This will be achieved by injecting antibodies that neutralise such components, or by genetic manipulation of the mice. In some cases, the mice will need to be treated with a substance like a hormone or an inactivated toxin, in order to activate the genetic deletion in a particular cell type. From the moment the substances are injected, the experiments typically last a few weeks.

Finally, some mouse models of autoimmune disease will be treated with therapies based on natural products of our immune system that we have published, for which we understand at least in part the mechanism of action, aimed at improving the condition. These treatments are expected to last for a few weeks to a couple of months.

What are the expected impacts and/or adverse effects for the animals during your project?

Immunization experiments are expected to cause no or minimal side effects.

Injection of antibodies or substances to delete immune components will also cause minimal adverse effects in mice bred under SPF conditions, although elimination of immune subsets like T cells may make mice more susceptible to infections by commensal organisms and may need antibiotic treatment.

Irradiation and reconstitution of mice is expected to cause mild transient adverse effects and rarely death due to poor immune reconstitution. Mice poorly reconstituted or undergoing an alloreaction will show signs of illness that will prompt increased monitoring and if needed, humane killing.

Exposing mice, especially female to a transient increase in body temperature should not cause any harm except for mild transient discomfort.

In systemic autoimmune disease models exemplified by lupus, mice will develop manifestations of autoimmune disease that typically include tiredness, general malaise, abdominal discomfort and

decreased survival. These manifestations tend to be chronic and will prompt closer monitoring and humane killing when needed. More specific ones will depend on the autoimmune disease:

In mouse models of arthritis, the majority if not all mice will develop swollen and painful joints, which may affect mobility and food intake (causing weight loss). In the spontaneous model of autoimmune arthtitis (K/BxN) these manifestations tend to be chronic and will prompt closer monitoring and humane killing at the indicated stage. In the induced model of autoimmune arthritis (collagen-induced arthritis), joint inflammation lasts for 10 days to 3 weeks after the second injection, after which mice typically recover. Mice will typically be culled as soon as they reach the peak. Only when the effectiveness of treatments or removing immune system cells or components is being investigated will mice with swollen and painful joints be kept alive (being closely monitored), but mice will be treated with regular analgesics.

In mouse carrying mutations or receiving treatments like DSS that cause inflammatory bowel disease (IBD), a proportion of mice may develop diarrhoea causing weight loss. DSS-induced colitis peaks in severity at day 6 and mice gradually recover. In most of our experiments mice will be taken down as soon as they peak or even before then.

In a mouse model of pemphigus, the majority of mice develop irritation in the mouth and skin from 1-2 weeks after cell transfer. Minor mucosal/skin lesions can be detected by 4 weeks and disease peaks at 6 weeks post transfer. The skin and mucosal lesions may be irritating and painful, and therefore need to treated regularly with analgesics, and given soft food. Mice will be killed humanely as soon as skin erosions (i.e. ulcers) are found.

In mouse models of multiple sclerosis (experimental allergic encephalomyelitis (EAE)), mice will develop progressive paralysis starting from the tip of the tail from 8-10 days after immunization, with the course of disease rarely exceeding 14 days. Most mice will be humanely culled as soon as they reach the severity limit.

In mouse models of allergies, mice could develop anaphylaxis within 10 minutes from the second allergen challenge that will prompt humane killing.

Mice developing mammary tumours may experience mild discomfort at the site of the tumour and compromised overall well-being.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Immunisation experiments: MILD

Injection of substances that deplete components of the immune system or induce genetic deletions: MILD

Sublethal irradiation and immune reconstitution experiments: MODERATE in up to 5-10% in which immune reconstitution fails or there may be some unwanted alloreactivity. Otherwise, this is a mild procedure. Very rarely, and only in a particular type of experiment in which the mutant donor cells

trigger autoimmune disease, the proportion of mice developing moderate disease may increase to 50%.

Heat exposure: MILD

Mouse models of autoimmune disease:

a) Lupus: most genetic mutations will induce MILD (80%) to MODERATE (20%) disease, with exceptions like TLR7 gain-of-function mice (kika/ramos) in which disease may be SEVERE (20%) in mice over 12 weeks of age.

b) Autoimmune arthritis models: Collagen-induced arthritis: Expected severity = MILD (70%); Highest severity that can be experienced = MODERATE (30%). In K/BxN mice, most mice develop arthritis spontaneously and may peak within 2-3 weeks after weaning, which means that nearly 100% will reach MODERATE disease severity. These mice will be monitored very closely and culled humanely to avoid severe disease.

c) Colitis models: MILD (50%); highest severity that can be experienced = MODERATE (50%)

d) Pemphigus vulgaris model: MODERATE in the majority of the mice due to the development skin and mucosal lesions that can eventually ulcerate, at which point mice will be immediately culled.

e) Experimental autoimmune encephalitis: SEVERE in the majority of the mice (80-100%). Even though mice will be culled immediately if they develop total hind limb paralysis, mice may experience reduced mobility, eventually developing partial hind limb paralysis.

f) Allergies: SEVERE in less than 10% in WT and up to 100% in GA animals because anaphylactic shock develops from 5-10 minutes from the second injection of allergen, and even though each individual mouse is closely monitored during those 10 minutes and culled as soon as the first symptoms are observed, the very rapid fatal progression makes it very difficult to assess the degree of suffering.

g) Tumour models: Expected severity = MILD (50%); Highest severity that can be experienced = MODERATE (50%)

h) Infection models: Expected severity = MILD (20%); MODERATE (70%); Highest severity that can be experienced = SEVERE (10%),

What will happen to animals at the end of this project?

- Killed

A retrospective assessment of these predicted harms will be due by 04 May 2028

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The focus of the experiments in this application is the discovery of genetic and cellular mechanisms that regulate antibody production relevant to vaccination and immune diseases including autoimmunity and allergies. Over the last few years, we have managed to undertake close to 50% of all our experimental work using human primary cells, or cell lines. There are however a few areas of our work that unfortunately, to date, still require experiments using mouse models:

1. Obtain definitive proof that a particular genetic mutation found in human patients causes disease, particularly in the context of rare diseases in which there are few patients in the world that carry mutations in a given gene. Before generating the mouse model, extensive functional screens are performed to obtain biochemical and molecular evidence that a gene mutation alters protein function. Only when this evidence is compelling, we need to prove that alteration is the cause of the disease. It is then necessary to isolate that mutation from the other hundreds of rare gene variants found in each individual and introduce that single mutation in mice in which their genetic make-up is otherwise identical and are also exposed to the same and highly controlled environment. Pathogenesis of immunological disease is generally very similar in mice and humans, and the function of individual genes and mechanisms can be resolved in the uniform genetic and environmental background of laboratory mice.

2. Establish the mechanism by which a gene mutation causes disease or breaches immune "tolerance checkpoints". These checkpoints can act during B cell development in the bone marrow, or during their selection and transition through a number of maturation stages as lymphocytes recirculate through different organs, or during interactions with specialised T cells at defined niches in secondary lymphoid organs (i.e. "germinal centres") that spontaneously occur during infection or vaccination. None of these developmental stages nor germinal centres have yet been recapitulated in vitro and therefore require animal models.

3. To elucidate in which cell a particular mutated gene is acting to cause disease. Mixed bone marrow chimeras (ie reconstituting the immune system of sub-lethally irradiated mice by injection of a mixture of labelled mutant and wild-type stem cells) allow us to determine in which cells the mutation is acting to cause disease.

4. To identify which diseases can be treated with our newly discovered regulatory proteins produced by follicular T cells.

Our program is generating the most accurate models of disease to date, because mice are generated to harbour the mutations found to cause disease in patients. In turn, these more animal models will be more relevant and therefore immediately translatable to human disease and therefore accelerate the path to discovery of more effective treatments.

Which non-animal alternatives did you consider for use in this project?

We have considered non-animal alternatives, some of which we will still use.

1. Before undertaking any research in mice to understand mechanisms of action of the human mutations, we try and use a broad range of in vitro reporter assays to try to narrow down the pathway. We transfect a spectrum of human B, T, myeloid and embryonic kidney cell lines with plasmids containing the human gene variants, to inform as to which may be the affected pathway, and whether a particular mutation is pathogenic, gain-of-function or loss-of-function.

2. We use short term cultures of fresh human tonsil cells in which most cells including germinal centre B cells can survive for some amount of time.

3. We routinely model the effects of mutations on protein structure and function using a number of bioinformatic tools. These insights help focus the experimental work and formulate more specific hypotheses.

4. We routinely investigate the consequences of genetic mutations on protein production, stability, localization and cell migration using in vitro assays. For example, we test whether a plasmid carrying a human mutation within the coding sequence can make the normal amount of RNA and protein, or whether the mutation results in nonsense-mediated decay, less protein expression, or the function of the protein in well-validated assays.

These efforts have proven very successful at reducing the use of mice. Indeed, my laboratory was 100% mouse-focused 5 years ago, and today, mouse work represents less than 50% of our research activity, as we have gradually designed better assays that can be performed in-vitro using human peripheral blood mononuclear cells (PBMCs) from both patients and healthy controls, as well as tonsils extracted during routine pediatric tonsillectomies. We are also trialling thick-tonsil section based organoid cultures, although survival of B cell subsets of interest is still severely compromised.

Why were they not suitable?

Further reduction using some of the alternatives described above is impossible at the moment, given that protective immune responses in the context of infection or immunisation involve interactions between T and B cells, innate cells and stromal cells in various organs both as the cells mature and as they encounter microbes, commensal bacteria, or physiological stressors. Such interactions and effective activation to mount a productive long-lived memory antibody response to date have not been successfully recapitulated in vitro.

In a similar line, despite ongoing efforts by many groups around the world including ours, negative selection of self-reactive B cells during their different developmental stages cannot be induced in cell cultures. These cellular processes and the tissue in which they physiologically occur, are inaccessible in live humans and, with rare exceptions, the complexity of human genetic and environmental variation makes it impossible to tease out the critical genes and pathways without experimental validation in laboratory mice.

We continuously read and screen the literature, conference presentations and abstracts, and wherever possible develop tissue culture assays to investigate genes and mechanisms for vaccine development

and autoimmune disease prevention and treatment without the use of animals once these mechanisms have been established by in vivo experiments.

A retrospective assessment of replacement will be due by 04 May 2028

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your experience?

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Estimating the numbers required for breeding and propagating gene variant and transgenic strains while generating sufficient mice for experiments: We estimate 40 non-genetically-altered mice will be needed as breeding partners per year per strain. Our breeding strategies are often complex and entail generating a large number of mice per year. We often need to intercross strains carrying several alleles of interest that: a) reduces the frequency of mice of interest per litter and b) often results in an inability to use mice until the 2nd or 3rd generation. We also regularly back-cross our strains to avoid genetic drift (these mice are of no use experimentally but are necessary to ensure animal welfare in adoptive transfer and bone marrow chimera experiments). Therefore, we anticipate we will need 3 breeder pairs per strain. Breeder mice produce on average a litter of 6 mice every 5 weeks. That means 10 litters x 6 mice/litter x 3 breeders, per year and per strain = 180, plus 6 breeders = 186/strain. For 30 strains = 5580/year.

Typically, each experiment will include 5-10 mouse per genotype, depending on how large the expected change is between experimental and control groups in order to detect statistically-significant differences. Most experiments will require 3 different genotypes (i.e wild type mice, mice with one copy of the mutated gene and mice with two copies of the mutated gene). Thus, for protocols that do not require intervention (e.g. injection), we will typically use 15-30 mice. By contrast, protocols that require challenges and analysis of tissues/organs before and after challenge, will require additional groups of treated mice (the total number per experiment will depend on the number of time-points that need to be evaluated by tissue dissection).

To ensure reproducibility, we will repeat experiments at least 2 times. For experiments where we expect, based on previous experience or pilot projects, a small effect size or larger variability between animals we will increase the experimental groups to achieve a similar statistical power.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In order to minimise the numbers used we will:

Have a very clear idea of the question we want to answer and the required readout.

Plan carefully every experiment, to include every necessary control in all experiments, as well as include randomization and blinding at all required steps.

Perform the required small pilot projects to estimate the size of effect we are expecting and narrow the number of doses to be used etc.

Take advantage of a number of online tools including the NC3Rs Experimental Design assistant.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will perform pilot experiments to test the doses of the products we are going to inject as well as to estimate the effect sizes mentioned above.

We plan to collaborate with other groups interested in exploring similar pathways so we distribute the work and do not duplicate efforts

We will maximise the use of genetically altered animals so we take all the required readouts from the smallest number of animals (serial serum samples, lymphoid organs for flow cytometric analysis and preparation of cell suspensions for in vitro cultures, tissues at necropsy for histological examination)

For mouse models in which there are few mice of the desired homozygous genotype born (i.e. Prkcd-/- mice), we will generate bone marrow chimeric mice from a single mutant donor, to save having to breed large numbers of mice to obtain the required genotypes.

A retrospective assessment of reduction will be due by 04 May 2028

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

There are two types of models that we use depending on the project:

1. In order to understand the genes and pathways that cause systemic autoimmunity (diseases like lupus), we are introducing in mice the same gene variants that we find in humans. This ensures that the models of disease are the most relevant to human disease. In these models, mice develop lupus-like disease and other related systemic autoimmune conditions that closely recapitulate the human condition. When possible – i.e. for studies of cell types and general mechanisms, we will prioritise the use of our newly generated lupus models that cause less pain and distress (i.e. Tlr7 gain-of-function). Those that cause more severe manifestations (such as for example Trex1-deficiency) will only be used when we need to understand the differences between pathways and find the key biomarkers that can identify each pathway.

2. In order to test the efficacy of our recently discovered therapies in autoimmune and allergic diseases (i.e. autoimmune arthritis, inflammatory bowel disease, multiple sclerosis) we need to use models of disease that are antibody-driven, and in which production of antibodies to a specific antigen can be monitored.

Given the mechanism of action of neuritin (preventing the generation of autoantibodies), we have no choice but to use the only validated models of arthritis and pemphigus, that have been proven to be antibody-dependent in serum-transfer assays. These are the K/BxN model of arthritis driven by GPI autoantibodies, and the model of pemphigus induced upon transfer of desmoglein3-deficient cells into C57BL/6 mice, driven by DSG3 autoantibodies. Experimental models of allergies such as OVA or peanut-induced allergies are typically mediated by antigen specific anti-IgE antibodies, that can be monitored .

For experiments where we want to understand other roles of Tfr cells, we will choose collagen-induced arthritis as the best accepted and mildest model of arthritis.

In the case of multiple sclerosis, mouse models are somewhat limited: some do not fully recapitulate the human relapsing remitting disease, and the ones that do, are not B cell dependent. Thus we are constrained to use the only mouse model that is B cell-dependent so we can test our B cell-modulating therapies. This is the modified EAE model selected in our protocol.

Monitoring the development, cell of origin and kinetics/persistence of these antibodies in the presence or absence of neuritin-based or similar treatments will be crucial readouts, besides monitoring the development of clinical signs and symptoms.

Why can't you use animals that are less sentient?

Development of autoantibodies requires a mature immune system, and autoimmunity is not observed during immature life stages or in species that are less sentient. The pathogenesis of these diseases is studied over time and cannot be studies at a single time point.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will pay a lot of attention to define very carefully the question we want to answer so that we can identify and put in place the earliest possible end points. For example, in order to find out if our treatment with neuritin is effective at preventing pemphigus disease, valuable readouts can be measured before mice develop the full clinical manifestations (i.e. appearance of autoantibodies), therefore preventing the suffering associated with these condition. So whenever possible, early readouts will be prioritized and experiments terminated before mice become unwell.

We also plan to undertake very close monitoring of the animals. We have developed a separate clinical scoring sheet for each model of autoimmunity so that we can very quickly and continuously assess the more likely sources of pain and distress in each model so as to manage them efficiently.

We will also have in our protocols very clear pain management plans to prevent suffering. While the arthritis and pemphigus autoimmune mouse models have been widely used in the literature without the use of analgesia, we are aware that they can involve significant discomfort or pain to the animals. We are planning a trial on the K/BxN arthritis and pemphigus mouse models to test if the use of Buprenorphine has any significant effect on the production of total and antigen-specific antibodies, inflammatory cytokines, and clinical scores.

In the case of mice developing tumours, besides mice being very closely monitored, the tumours themselves will be examined regularly and experiments will be terminated if tumours ulcerate, metastasise or reach a certain diameter or growth.

Environmental enrichment will be provided, guided by the most up-to-date practices in our facility. These will include more and softer bedding/nesting material for mice with arthritis and sore skin due to pemphigus, food on the floor for animals with arthritis and EAE, and mashed food for animals with pemphigus.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the local guidelines, as well as those from NC3Rs (https://nc3rs.org.uk/) with particular attention to the themes of Analgesia" and "Humane end-points"; ARRIVE (https://www.nc3rs.org.uk/arrive-guidelines) and PREPARE guidelines.

Important guidance for pain management will be sought from the online book: Recognition and Alleviation of Pain in Laboratory Animals (National Research Council (US) Committee on Recognition and Alleviation of Pain in Laboratory Animals; Washington (DC): National Academies Press (US); 2009.

We will also follow best practice and refinement as published in the following papers:

For administration of substances:

D. B. Morton , M. Jennings , A. Buckwell. Refining procedures for the administration of substances. Laboratory Animal Limited (LAL) 2001. https://doi.org/10.1258/0023677011911345

For arthritis research:

Hawkins P, Armstrong R, Boden T, et al. Applying refinement to the use of mice and rats in rheumatoid arthritis research. Inflammopharmacology. 2015;23(4):131-150. doi:10.1007/s10787-015-0241-4

For EAE research:

Wolfensohn S, Hawkins P, Lilley E, Anthony D, Chambers C, Lane S, Lawton M, Voipio HM, Woodhall G. Reducing suffering in experimental autoimmune encephalomyelitis (EAE). J Pharmacol Toxicol Methods. 2013 May-Jun;67(3):169-76. doi: 10.1016/j.vascn.2013.01.009. Epub 2013 Jan 26. PMID: 23357188.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will seek to read the communications and advice produced by our NC3R liaison officer on advances in the 3Rs. I will continuously read the literature to find progresses that may enable substituting some of our models for better ones and further reducing animal work. My team will continue to optimise the use of organoids using human tonsil to understand immune cell interactions so that we reduce the need to investigate these in vivo.

A retrospective assessment of refinement will be due by 04 May 2028

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?