Home Office

NON-TECHNICAL SUMMARY

Novel immunotherapeutic strategies to treat Cancer

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
- (c) Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Cancer,, antibody,, immunotherapy,, immune-modulation, immune system

Animal types	Life stages
Rats	adult
Mice	adult, neonate

Retrospective assessment

> The Secretary of State has determined that a retrospective assessment of this licence is not required.

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 overall aim of this project is to explore the utility of new anti-cancer reagents for use in the clinic. The specific objectives are:

1. To produce and characterize new anti-cancer reagents.

2. To determine how these reagents work and how they can be improved.

3. To develop strategies to promote/modulate the immune response to cancer and to understand how this happens

4. To understand how tumours form with the aim of developing reagents that can stop them.

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?

In 2020 there were more than 19 million new cases of cancer diagnosed alongside 10 million cancer-related deaths worldwide. With such a high worldwide healthcare burden, the need for effective anti-cancer treatments is paramount. Although the conventional treatments of surgery, chemotherapy and radiotherapy have been effective in the treatment of certain cancers and in improving outcomes for many patients, frequently these treatments fail and tumours come back. Understanding why tumours form and how they overcome treatment is critical for developing more effective treatments. In the last 2 decades it has become increasingly clear that tumour cells interact with the host immune system. This knowledge has led to a huge surge in the field of immunotherapy – which studies how the immune system can be leveraged to treat diseases including cancer. The idea is to redirect or reinvigorate the immune response against cancer, in therapies that are highly specific and effective with the potential to prevent tumour recurrence, in the same way we become immunised against measles or mumps.

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

Immunotherapy offers the potential for treating a wide range of cancers in a highly focussed and specific way. The immune system has the amazing ability to distinguish pathogens from the host and can do the same in recognising, detecting and removing cancer cells.

The most successful immunotherapy drugs explored to date are monoclonal antibodies (abbreviated as mAb). Using these cancer-specific reagents, we can direct the patient's own immune system to destroy unwanted cells while leaving normal tissue mostly untouched. Consequently, unlike conventional treatment, mAb treatment will not be associated with long-lasting toxicity. In addition, it has the potential through what is termed "immunological memory", which forms the basis of vaccines, to mean that the patient will continue to recognise and kill their tumour cells on an ongoing basis, reducing the potential for relapse. In this project we will deliver these benefits through the development and characterization of new immunotherapeutic reagents and treatment strategies.

Potential benefits from our work also arise from our ability to develop new combinations of treatments in which mAb are given alongside conventional and other emerging methods, including other mAb. The ultimate aim is to develop and /or improve treatment options for patients with a range of cancers. However, this can only be achieved through a far better understanding of the interaction between the cancer cells and the immune system.

Therefore, during our programme of work we anticipate discovering and patenting new reagents and approaches for cancer treatment alongside our partners such as Cancer Research UK and Biotech/pharmaceutical companies. Several reagents developed under our current and previous licences have been or are already in clinical trials.

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

There will be multiple beneficiaries from our studies. In addition to those cancer patients who may benefit from our discoveries in the longer-term, we will publish our results in peer-reviewed journals and present at scientific conferences in the short-term. This manuscripts will be available on eprints 3 months after publication. Our data will be of interest to scientists, pharmaceutical companies and clinicians - helping them to make further progress towards deeper understanding and ultimately hopefully new and better cancer drugs in the future. Moreover, often our aim in cancer is to stimulate the immune system to attack the cancer cells and the same is often true in infection. Therefore, the principles established during these studies should also be applicable to immunotherapy against infectious diseases for both clinical and veterinary applications. Furthermore, the opposite effect is required in the treatment of autoimmune disorders (a condition in which the body's immune system mistakenly attacks healthy tissues) and so drugs that we develop, should they prove to be inhibitors rather than stimulators (reduces the effect rather than boosts it), or understanding that we gain that is not of use in the treatment of cancer, may well be of use for the treatment of these diseases and for the benefit of human health.

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

We will maximise our outputs by publishing our findings widely and freely. Where possible we publish in open access journals, with full disclosure of the associated raw data, enabling our studies and results to be accessible to all. We have an excellent track record in publishing our findings (>180 to date with myself as an author), including those where approaches were not ultimately successful. In addition, almost all of our work is performed in collaboration with other academic groups and/or Biotech/Industry partners and so shared knowledge and expertise further maximises our outputs.

Species and numbers of animals expected to be used

- Mice: 14304
- Rats: 16

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.

Whilst all attempts are made to reduce the use of animals by using in vitro methods (e.g. isolating and performing experiments on various types of blood cells that are an essential part of the immune system outside of the body) and explant studies (using tissue grown in a culture medium out of the body), it is inevitable in work of this nature, assessing the immune system, that in vivo investigation must be undertaken. Animal models are critical to facilitate in vivo (in a living animal) proof of concept and efficacy in an amenable and manipulable system, not possible in humans. We are investigating the in vivo effects and interactions between various arms of the immune system, with a view to therapeutic application in humans. These interactions occur between different tissues and organs, and as such they cannot be fully reproduced in vitro (in an artificial environment) and so systemic in vivo studies remain fundamental to the study of new complex tumour microenvironments and therapeutics.

Rodents (principally mice) will be used for this study since they are the most appropriate species of mammal that have the following characteristics:

1. The cellular and molecular interactions of the mouse immune system are broadly similar to those of humans, allowing us to investigate clinically relevant immunotherapeutic strategies and mechanisms in these animals;

2. Individual mice within a given inbred strain are considered genetically ‘identical’, thereby reducing variability and allowing valid conclusions to be drawn from experimental data.

3. Numerous tumour models have been established in mice that are strain specific; although none fully capture every aspect of the human disease, models can be selected that allow specific questions to be addressed to facilitate translation.

4. Numerous strains of genetically altered mice have been developed. These genetically altered mice allow more meaningful modelling of human disease. These experiments are simply not possible in humans and will enable us to validate and extend our findings in a ‘human’ setting and to help inform the generation of novel agents for use in patients.

Mice will be used as they possess a mammalian immune system, reflecting that of humans.

For the raising of certain monoclonal antibodies we will use rats, as they allow the generation of antibodies to mouse targets (not possible to raise in mice) to serve as tool reagents and for dissection

of mechanism of action prior to development of human antibodies.

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

A series of different types of experiments are proposed which enable us to introduce and measure immune-modulatory agents (including antibodies) and assess their effects on the immune system as well as tumour cells.

In experiments designed to develop new antibody drugs, mice will be injected with material to enable them to mount an immune response over a period of weeks, before the mice are euthanised and their B cells isolated to make hybridomas.

In experiments designed to measure effects on the immune system, mice will be injected with material (such as an antibody) and then the immune response measured by taking peripheral blood (much as we do for patients) and measuring changes in the immune cells (typically over a period of days to weeks). A proportion of mice will be euthanised and their tissues examined to understand how the immune response is developing in the different organs. These experiments are often done in the presence of a growing tumour, introduced earlier through injection, allowing us to assess infiltration of immune cells into the tumour after different treatments. A proportion of mice are then followed to measure tumour growth over time in experiments that may last 1-2 months, allowing us to determine if the treatments have cured the mice or delayed the tumour growth.

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

We typically identify mice by ear notching - this is expected to result in only mild and transient pain with no healing problems.

Mice will be injected with various immunomodulatory substances using a combination of volumes, routes and frequencies. Injections will cause momentary needle stick pain minimised by using the needle of the smallest suitable gauge. The minimum number of administrations and routes will always be used to achieve the scientific objectives. Typically, the animals will not receive more than 3-4 intraperitoneal (i.p.) injections a week or 2-3 intravenous (i.v.) injections a week. On some occasions they may receive 2 injections on a given day through a combination of different routes.

For some more invasive procedures, general anaesthesia will be used, to ensure the animals feel no pain.

For some experiments, mice will receive tumour cells through various routes; e.g. injected subcutaneously (under the skin) using a suitable vehicle. The tumours grow at this site and can be measured over time using digital calipers. Mice are euthanised at a size that is deemed to impact their normal behaviour, which may vary for different tumour models, based upon previous research. Throughout, the health status of the animal is the primary consideration used to define humane endpoints. Experiments will be terminated, or individual mice euthanised at the earliest signs of tumour-associated symptoms such as piloerection (goose bumps making the fur stand up), restricted movement, abnormal posture, abnormal gait (movement when walking), hunching, and/or weight loss up to, but not reaching, 20% body weight.

In many of our experiments, designed to measure immune changes, peripheral blood is taken from a superficial vessel. Pain from bleeding is controlled by suitable anaesthesia/analgesic with rapid healing and no long-lasting harm.

When the immune system is stimulated, for example with immunomodulatory antibodies, this can result in symptoms similar to those experienced during an infection (lethargy, fever etc.). This can result in mice becoming less mobile, exhibiting pilo-erection etc. Typically these symptoms are transient (first few hours) but can recur as the immune response develops (e.g. after several days) potentially resulting in further effects such as weight loss. These effects are therefore carefully monitored with mice euthanised if their symptoms become more severe or pass defined humane endpoints.

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)?

During our current project licence the proportion of mice experiencing sub-threshold, mild, moderate or severe severities was as follows and we expect similar proportions in our new project licence:

sub-threshold 0.2%

mild 80.2%

moderate 19.6%

severe <0.1%

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

- Killed

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?

Whilst all attempts are made to reduce the use of animals by using in vitro methods (in vitro experiments on various immune cells and explant studies using tissues grown outside of the body - see below), it is inevitable in work of this nature, involving the immune system that investigation in vivo (in an entire living organism) must be undertaken. The animal models proposed are critical to facilitate in vivo proof of concept and efficacy in an amenable and manipulable system, not possible in humans. We are investigating the in vivo effects and interactions between various arms of the immune system, with a view to therapeutic application in humans. These interactions occur between different tissues

and organs, and as such they cannot be fully reproduced in vitro and so systemic in vivo studies remain fundamental to the study of new complex tumour microenvironments and therapeutics. Further, advances, such as the availability of genetically altered animals and the development of more specific and sensitive techniques and reagents, continually permit a refinement and reduction in the types of experiments and the numbers of animals that are required (e.g. as highlighted by the organisation 'Fund for the Replacement of Animals in Medical Experiments', http://www.frame.org.uk) and this is something to which we remain committed. The increasing availability of genetically altered mice has allowed genetic dissection and more meaningful modelling of human disease.

Specifically, mice will be used for this study since they are the most appropriate species of mammal that have the following characteristics:

1. The cellular and molecular interactions of the mouse immune system are broadly similar to those of humans, allowing us to investigate clinically relevant immunotherapeutic strategies and mechanisms in these animals;

2. Individual mice within a given inbred strain are considered genetically 'identical', thereby reducing variability and allowing valid conclusions to be drawn from experimental data.

3. Numerous tumour models have been established in mice that are strain specific; although none fully capture every aspect of the human disease, models can be selected that allow specific questions to be addressed to facilitate translation.

4. Numerous strains of genetically altered mice have been developed, often replacing a mouse gene with its human counterpart, allowing us to study these interactions in vivo for the first time. For example, the human target of an antibody may replace the mouse counterpart, allowing antibodies directed against the human target to be tested. These experiments are simply not possible in humans and will enable us to validate and extend our findings in a 'human' setting and to help inform the generation of novel agents for use in patients.

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

As a group, we have sought to increase our access to primary human material to reduce the requirement for animals wherever possible. We are now able to purchase lymphocyte "cones" from the local blood transfusion service and this enables us to perform experiments using human peripheral blood lymphocytes. These cones are a natural by-product produced when people donate platelets, and contain many other immune cells of interest. We also have links with clinicians resulting in access to primary human tumours enabling us to investigate the effects of mAbs on human T cells infiltrating a tumour site in vitro. While we ultimately still need to use mice to study the influence of mAbs on a growing tumour and in a whole body system, better access to human material has enabled us to answer some clinically-relevant questions without the use of mice. It is difficult to accurately quantify the reductions in mouse numbers that this change has facilitated since the two experimental systems are not interchangeable but address distinct scientific questions. However, undoubtedly access to human material and the ethical clearance to address basic scientific questions enables us to replace animals in some circumstances.

In an attempt to help predict efficacy and toxicity of certain mAb we made use of the lymphocyte cones and have developed and adopted an in vitro assay using human cells as a replacement to mouse in vivo studies.

We are also evaluating in vitro protocols (phage display) to produce mAbs to our selected targets. This will reduce the number of animals used in the coming project licence, although these make up a very small proportion of the total animal usage.

Finally, we have also explored the use of organoid/ three-dimensional (3D) in vitro systems to study more tissue-relevant impacts of our immune interventions. Organoids and other 3D systems are typically small, self-organized 3D tissue cultures attempting to replicate the complexity of an organ/tissue, or to express selected aspects of it. Over a period of 6 years we were able to develop a 3D system to mimic certain aspects of a particular type of lymphoma microenvironment. Importantly, this model did not incorporate T cells, nor tumour-derived immune cells and so was unable to fully mimic the situation in lymphoma patients. It also represents a model of just a single type of lymphoma, with a similar amount of development required to produce equivalent models for other lymphoma sub-types and tumours. Therefore, as above we see these models as complementary to, rather than fully replacing the mouse models - with each addressing distinct scientific questions.

Why were they not suitable?

We are investigating the in vivo effects and interactions between various arms of the immune system, with a view to therapeutic application in humans. These interactions occur between different tissues and organs, and as such they cannot be fully reproduced in vitro. Each of the systems we have developed above, model different aspects of the immune response but none fully recapitulate the complexity and inter-relationships of a whole organism and so systemic in vivo models remain fundamental to our studies.

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?

I have a current project licence and have been monitoring annual usage. This new project licence (PPL) represents a reduction of almost 50% in comparison to our estimate from our current PPL and takes into account our shift to use primary human tissues where possible as well as likely refinements over the coming years, alongside principal investigators within our research group holding their own project licences.

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

Our experimental design is always considered in light of how the animal numbers can be reduced whilst ensuring meaningful and reproducible results. We are committed to reducing animal waste, implementing the three Rs, and maximising the reproducibility of research and so follow the PREPARE guidelines (https://norecopa.no/prepare) when considering our experiments.

As a first means to reduce the numbers of mice in our project, inbred mouse strains will be used to reduce variability of response and so allow reduced numbers sufficient to deliver reproducible data. Related to this, we routinely use age- and sex-matched mice that have been bred in the same facility (ideally litter matched where control and genetically altered mice are being compared) to minimise variability.

We have been using a range of tumour models for some time and so are well aware of the reproducibility of controls and appropriate mouse numbers required in tumour growth and therapy experiments. Where new models or treatments are introduced, we will first perform pilot studies to inform the design of larger studies, including dosing regimen, expected humane endpoint etc, as well as to monitor for any signs of adverse events. For example, 2 mice per group would be inoculated with a new tumour at different cell numbers to establish tumour growth kinetics etc. even where data exists in the literature as we are cognisant of the impacts of the local environment, microbiome etc. Similarly, a single mouse would first be treated with a new experimental treatment to judge safety, immune response etc, before proceeding to larger studies.

We are also cognisant of ensuring we minimise experimental bias. Accordingly, in experiments where we implement more subjective humane end points as part of a tumour therapy experiment (e.g. abdominal palpation as opposed to caliper measurements), an experienced and treatment-group blinded animal technician is consulted regarding outcome.

Where we have useful data (from historical or pilot experiments), power analyses will be used to help guide the optimal numbers of mice needed for each experiment, taking into account expected magnitudes of impact. Power analysis is performed using the PS: Power and Sample Size Calculation programme: www.biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize):

For immunotherapy experiments, where we are using inbred strains of mice, intra-group variability is reduced. From experience, we have found that we can use considerably fewer animals per group, usually 5, to detect such a difference at the 5% significance level in many of our models (e.g. EG7, MC38, B16-OVA and TC-1).

For monitoring immunological responses we typically use 5 mice per group for each experiment. From our experience, this number of mice/group allows us to identify with a statistical power of 95% a 3-fold difference in the number of CD8 T cells between 2 groups at the 5% significance level.

Therefore, the proposed mechanistic studies will be performed with experiments utilising groups of 5 mice, in a standard vehicle versus drug manner. We aim to test the candidate immune stimulators (identified by prior human and murine in vitro experiments) for efficacy in a selection of tumours. Throughout, replicate experiments will be performed to ensure reproducibility and when appropriate studies may be combined to increase sample size. To assess differences in animal survival between

groups appropriate statistical tests will be used (e.g. Kaplan Meier curves will be analysed by Log rank test).

Should substances or tumour models be used for the first time, pilot studies will be performed on individual mice and humane endpoints established as indicated above. Substances will be administered and monitored after 1, 4 and then 24h for adverse effects before proceeding; a dose escalation schedule conforming to accepted practice will be used should adverse effects be predicted. When agents are used for the first time in the laboratory, their dose will be based on previous published data whenever possible.

When new tumour lines are introduced they have to go through quarantine to ensure safe introduction into the animal facility. As an additional measure of reduction, we use this period to also monitor growth and humane endpoints reducing the need for additional animal use.

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

Mice used across experiments are inbred thereby minimising intra-group variability and allowing reduced mouse numbers for experiments. We also wherever possible when breeding Genetically altered strains will use their non Genetically altered (e,g, transgene negative) littermate controls when available. Experiments are always designed with the fewest animals consistent with obtaining statistically valid results. We have performed Power analysis to determine the numbers of mice required to deliver statistically significant results, although through experience we find we can often use smaller numbers of animals without sacrificing statistical significance as they are inbred strains. Where appropriate, small pilot experiments are carried out to determine factors such as dose or route of administration.

Where multiple inter-relating parameters are to be evaluated, to prevent use of excess mice, we will employ higher dimensional analysis tools. Significant technological advances have enabled more information to be obtained from one individual mouse than was previously possible (e.g. using multi-parameter flow cytometry and RNA-seq technology), enabling multiple parameters to be assessed simultaneously from small samples. These technologies thereby facilitate longitudinal studies (repeated observations of the same variables over time) and reduce the need to cull multiple mice at different time points to sample from the spleen for instance. For example, we are moving from 3 colour flow cytometry to 8 and more colour flow cytometry, reducing the sample input requirements accordingly.

Since the start of our current PPL, we have instigated a policy that new in vivo experiments must be detailed on a ‘study-plan’ detailing: the aims, number and strain of mice, substances/cells administered, treatment dates, confirmation of required competencies and/or delegation of procedures, any known or anticipated adverse effects, and appropriate risk assessments. This has to be submitted to myself and the Named Animal Care & Welfare Officer (NACWO; person who is responsible for checking the welfare of the animals) before an experiment is allowed to begin.

This has facilitated a more detailed dialogue between PPL holder (PPLh) and personal licence holder (PILh) prior to work and if necessary a discussion about the number of mice being used. More recently this process has included approval by representatives of the Animal Welfare Ethical Review Body (AWERB) committee. This serves to ensure animal numbers are optimised.

Other optimisations to reduce animal use include:

Tumour cells being stored frozen when possible to prevent mice being used to passage tumour in vivo.

Consideration of freezing additional animal tissues (whole and as single cell suspensions) when an animal is sacrificed to provide controls for staining in different immune situations, relieving the need to cull specific mice for this comparative purpose.

Similarly, harvesting blood (for serum production), spleens for lymphocytes and bone marrow (to generate macrophages) when appropriate mice are culled for other purposes.

The in vitro use of immune cells isolated from mouse tissues offers several benefits and aids in optimising number of animals used for in vivo experiments. Importantly, this strategy is in line with the 3Rs. Due to the relatively low numbers of cells required to perform in vitro assays, such experimental approaches allow the screening of a high number of drugs or combination thereof, with potential for in vivo activity, while minimising the number of animals required. Furthermore, they allow refining doses, and identifying potential mechanisms of action and adverse reactions, prior to in vivo testing. In addition, in vitro assays using mouse immune cells represent a powerful reductionist approach to study their activation. They allow the isolation and analysis of distinct cell subsets, and the direct assessment of drugs on each individual cell population, otherwise difficult to accomplish.

Furthermore, having identified that the hIgG2 isotype is especially powerful for driving immune-stimulation in vivo, we are now following up on the molecular basis behind these findings with colleagues in Biology and Chemistry, using molecular simulations and other in silico approaches (reducing the need for animal experiments). We plan to develop prediction tools to guide our studies, further reducing the numbers of animals required.

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.

We will use rodents for these studies as they are the most appropriate species of mammal that have the following characteristics:

1. The cellular and molecular interactions of the mouse immune system are broadly similar to those of humans, allowing us to investigate clinically relevant immunotherapeutic strategies and mechanisms in these animals;

2. Individual mice within a given inbred strain are considered genetically ‘identical’, thereby reducing variability and allowing valid conclusions to be drawn from experimental data with as few animals as possible.

3. Numerous tumour models have been established in mice that are strain specific;

4. Numerous strains of genetically altered mice have been developed such as those expressing tumour antigens or human receptors (part of the cell which binds to a substance) of interest (human CD20, human OX40 etc.) and mice with genes of interest removed or added in order to elucidate immune mechanisms e.g. mice in which a mouse Fc gamma Receptor (FcgR) has been replaced with its human counterpart. If required we will source new human knock-in (KI) mice to enable us to validate our findings in a ‘human’ setting and to help generate novel agents for use in patients as we have done.

When evaluating immunomodulatory and immunotherapeutic approaches, we begin with in vitro evaluation and progress to pre-clinical animal models when sufficient promise is obtained. Our in vitro studies may include assays to determine the ability of reagents to kill or affect the growth or survival of target cells, and to recruit immune effector mechanisms. Where appropriate, we will first establish effects on in vitro cell lines and ex vivo primary material. However, in vitro lines and primary cells become adapted to cell culture conditions and are not present in a complex multicellular tissue/organ and therefore do not best recapitulate in vivo responses, hence the need for animal experiments.

In order to understand the mechanisms operating, cells may be obtained from unmodified, wild type (WT) or genetically altered animals with deficient/altered immune effector systems e.g. FcgR or complement knock-out, mice deficient in key effector molecules. For those reagents that show promise in vitro, we will determine if this translates into an in vivo effect using the most appropriate models. Typically, we would first examine reagents in the absence of tumour, to assess immunomodulation, without unnecessarily increasing the harm to animals by adding to the cumulative harm from further interventions and/or activities of the tumour. We ensure humane end-points are established that minimise the harm to the animal without compromising the veracity of the experimental data.

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

Our work largely involves study of the immune system, which is a multi-faceted, interacting system that is spread throughout the body and organs, linked by the vasculature. The cellular and molecular interactions of the rodent immune system are broadly similar to those of humans, allowing us to investigate clinically relevant immunotherapeutic strategies and mechanisms in these animals - less sentient species have very different immune systems (e.g. zebrafish lack adaptive immunity). More immature animals also do not display mature immune systems (the immune system becomes educated with age and development) that we seek to investigate.

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

We continually review our procedures and if unnecessary harms are identified or can be reduced, modified protocols are implemented through discussion with the NACWOs, PPL holder, academic lead for the BRF and named vet surgeon (NVS) as required. We also seek to ensure the best housing and conditions for the animals are provided.

As examples, of our commitment to these aspirations over the last 4 years we have:

1) Moved to a new purpose-built animal facility. This unit is more conducive to high quality in vivo research utilising a 'barrier' system meaning that all materials coming into the unit are clean and enter via a dedicated positive-pressure pass-through hatch. All personnel entering must now change into scrubs, and pass through an air-shower on entering (and leaving). The new facility also houses a separate quarantine facility that is no longer inside the main unit, but attached and accessible separately, with dedicated technicians only permitted.

2) All new strains of mice entering quarantine undergo rederivation before offspring are allowed in the main unit. All new cell lines have to be screened in mice in the quarantine room, and serum from sentinels assessed for the presence of pathogens before cells are allowed to enter the main unit. All mice are now housed in individually ventilated cages (IVCs). These changes minimise the risk of infection with obvious potential implications for the welfare of the animals and the quality of the research. In addition, the IVCs permit less disturbance to the animals as an additional refinement.

3) We have also implemented a number of new policies to improve welfare and reduce adverse experiences. All mice undergoing an injection now undergo a 'second check' by the person performing that procedure, within 30 mins-4 hours. This was instigated to prevent animal welfare impact should an unanticipated adverse event occur. If a procedure is delegated to a technician, this second check also incorporates a 'positive handover' between the technician and the PILh to transfer the responsibility for the mice back to the PILh to ensure it is always clear where responsibility for welfare ultimately lies.

4) In response to changing guidelines a number of further and specific refinements have been put in place. These include:

(a) that CO2 entering a chamber during schedule 1 culling, enters from the top rather than from the bottom in line with current best practice. Flow rate is 20% of chamber volume/min. 3 min dwell time, where the CO2 is stopped but the chamber remains untouched so they remain in maximum CO2. Nb: the machine is serviced annually to check that it is still within parameters and we check that there is adequate CO2 pressure before starting and stay in the room during the CO2 delivery.

(b) that mice are handled by 'cupping' or by gently moving them using a dedicated tunnel, prior to any restraint to ensure mice are calm and habituated to the person performing injections, serving to reduce anxiety when handling mice.

(c) a policy that needles are only to be used once. This is to prevent mice receiving an injection with a blunted needle. Initially during the early part of my current project licence (PPL), the refinement was to be used less than 5 times but last year we implemented a new single use policy unless specific justification is provided that is approved by the BRF management committee.

(d) we have now transitioned towards venesection (tail pricking) to take blood samples instead of tail tipping. While this is not always possible (for instance when mice are receiving concurrent i.v. injections) new PILh are now trained in venesection and established PILh who regularly bleed mice have undergone re-training.

(e) During the course of this PPL, we instigated a policy that new in vivo experiments must be detailed on a 'study-plan' that encompass the experimental aims and how they address the PPL objectives, number and strain of mice, substances/cells administered, treatment dates, confirmation of required competencies and/or delegation of procedures, any known or anticipated adverse effects, welfare measures and monitoring employed and appropriate risk assessments (see study-plan template in Reductions above). This study-plan has to be submitted to the PPL and then responsible NACWO for approvals prior to experiment commencement. This has ensured that there is evidence of an appropriately detailed dialogue between PPLh and PILh prior to work and as necessary enabled a discussion about the number of mice used and procedures administered.

5) We also have a new system that records training competencies for individual PILh to ensure that training on all procedures is renewed every 3 years. Training is conducted by dedicated trainers using Directly Observed Practical (DOP) skills forms written specifically for this purpose. As PPLh I have access to these records, and reference is made to them on the 'study plan' (see above) ensuring that PILh also review their records regularly.

6) An additional refinement implemented has been to modify the humane endpoint for our CT26 tumour experiments; reducing the tumour size from 400mm2 to 300mm2 for most experiments; this allows us to obtain sufficient scientific information whilst reducing the risk of tumours impairing the welfare of the animal such as through impaired movement.

7) When possible, genotyping protocols for new transgenic (Tg) mice have been refined to allow PCR only protocols for screening, reducing the need to bleed mice prior to experimentation. Moreover, some existing transgenic strains (e.g. human CD20 transgenic) that were previously screened via blood are now done so by polymerase chain reaction (PCR; a technique for amplifying genetic material useful in transgenes) from ear notches reducing the number of procedures the mice undergo.

8) We have also adopted the practice of transferring male mouse nesting material, not substrate, to minimise male mouse aggression (the nest has calming pheromones in it, but the sawdust aggravates aggression as it holds the testosterone).

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

There are a number of detailed publications and guidelines for the welfare and use of animals in cancer research on cancer models that provide excellent guidance in the methodologies, study design and best practice that we will follow and adapt as appropriate to our research (Guidelines for the welfare and use of animals in cancer research Workman, P., Aboagye, E., Balkwill, F. et al. Guidelines for the welfare and use of animals in cancer research. Br J Cancer 102, 1555–1577 (2010). https://doi.org/10.1038/sj.bjc.6605642).

We will follow the ARRIVE guidelines https://arriveguidelines.org/ which provide a checklist of the minimum information required to be reported by groups using animals in research. ARRIVE guidelines are essential to help overcome issues in science such as reproducibility, reducing bias and the correct use of statistical methods of analysis.

In addition, we will follow and consult NORECOPA https://norecopa.no/3r-guide: Norway's National Consensus Platform for the advancement of the 3Rs (Replacement, Reduction and Refinement associated with animal experiments) database platform and PREPARE https://norecopa.no/prepare (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines for better science experiments using animals to ensure that we are using the best models for our research.

Finally, regular communication within our research group and communication with our peers at seminars and conferences ensures we're aware of any new or updated best practice. Best practice information is also disseminated by our Named Information Officer.

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

We have a very active Biomedical research facility (BRF) users group, meeting approx 3 times per year to discuss issues that arise within the animal facility. These are attended by the PILh, PPLh, NACWO, Home Office Liaison Contact (HOLC) and establishment licence holder. Any issues and incidents that PILh need to be aware of are discussed at this meeting. Further to this, this platform is used to disseminate information from Animals in Science Regulation Unit (ASRU), National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) and other organisations and to provide details of training opportunities to enhance welfare and research practice. We have also embarked upon a strategy of ensuring that PPLh and PILh undergo regular refresher training at least every 3 years to ensure that they remain abreast of changes to best practices and aware of their responsibilities under the Animals (Scientific Procedures) Act 1986 (ASPA). These advances are further communicated through our active team of NACWOs and regular email updates. We will also stay up to date with specific cancer groups, databases and alternatives (NC3Rs) for cancer models such as https://resources.researchanimaltraining.com/faqs/breast-cancer-research-alternatives-database; https://data.jrc.ec.europa.eu/dataset/352f7dfd-05cf-434b-a96a-7e270dc76573.