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NON-TECHNICAL SUMMARY

Gamma delta T cells and Body Surface Immunity

Project duration

5 years 0 months

Project purpose

- (a) Basic research

Key words

cancer, immunosurveillance, inflammation, T cells, epithelium

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

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 is to describe the biology of intraepithelial lymphocytes (IEL) which comprise a large compartment of immune cells sitting at body surfaces, such as the skin, gut or reproductive tract. IEL are particularly enriched in evolutionarily conserved T cells known as gamma delta cells, which can play important roles in key physiologic processes such as dietary adaptation, and in protecting against infections, inflammation and cancer. However they are very poorly understood. To redress this, we aim to establish how specific sets of gamma delta T cells become associated with particular tissues during their development; how they are retained; which forms of local disorder they can respond to; and the biological outcomes of their actions.

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?

Body surfaces are the most susceptible anatomical sites to infection, cancer, and inflammation that collectively make up some of the most common and demanding clinical burdens. They can also be sites for resection and transplantation. By understanding how local gamma delta T cells protect their local environments from various types of disorder or disruption, we can add to our fundamental immunological knowledge while potentially improving clinical diagnostics and therapeutic strategies. Indeed, our previous work provided the foundation for two biotech companies committed to applying gamma delta T cell biology to cancer and inflammatory disease with first-in-human trials commencing in autumn 2021.

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

The following outputs can be anticipated:

- Peer-reviewed primary research publications. To place this in context, our research led to the publication of 25 peer-reviewed primary research papers in the 4.5 years covered by our current license.

- Patents. Our work has led to novel discoveries which have been protected as intellectual property by our host institutions, so that they may be commercialised for practical clinical application(s).

- Abstracts. Our work is primarily undertaken by post-doctoral researchers and PhD students who are encouraged to attend international meetings to which they submit abstracts for presentation and discussion.

- PhD theses. Our laboratory is currently training 4 PhD students whose written, published theses can be anticipated to contain work undertaken as part of this project.

- Public engagement via research updates on open-access websites, etc.

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

We consider the following impacts:

- Short-term: publications describing our results may have immediate impacts on: [i] other research teams, influencing their own experimental plans, studies, and interpretations; [ii] post-doctoral trainees whose career progressions can be largely shaped by the outcomes of the research; [iii] in rare cases, the impact may be to influence current public health policy, as was recently achieved in the context of COVID-19.

- Short-to-medium term: awarding of PhD degrees to those undertaking studies described in this licence application.

- Short-to-medium term: the establishment of UK and international collaborations to expand upon our findings, as manifest in our successful application to the MRC and its Indian counterpart (DBT) for Anglo-Asian COVID-19 studies.

- Medium-to-longer-term: impacts on textbook immunological knowledge.

- Medium-to-longer-term: the results, including patents filed, may accelerate the development of novel diagnostic and therapeutic modalities for infection, cancer and inflammation.

- Longer-term: changes in clinical practise.

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

We shall disseminate our findings via the outputs described above: papers, abstracts, theses.

We shall disseminate our findings via conference presentations.

Where appropriate, we shall disseminate the implications of our findings by public engagement.

We shall upload our data and provide lay narratives on open-access web-portals, etc.

We receive advice from experts in science translation about which of our findings are appropriate for filing patents, thereby maximising opportunities for clinical development of our findings.

Species and numbers of animals expected to be used

- Mice: 77,275 split over 20 protocols over 5 years

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.

The mouse is the ideal organism for our research for five reasons.

First, it is a long-standing choice of immunologists via which they have successfully identified many of the key operating principles of the immune system, such as the connectedness of innate and adaptive immunity, and made advances upon which major clinical interventions are based, e.g. the successful introduction of cytokine blockade for treating rheumatoid arthritis.

Second, there is an immense spectrum of reagents available permitting the thorough, incisive, and comprehensive obtainment of reliable information from the experiments undertaken.

Third, there is an extensive and evolving bibliography of protocols that inform the design of experiments, promoting reproducibility, reducing excessive need for pilot studies, and enhancing the reduction and refinement of animal use.

Fourth, reflecting extensive breeding programmes and the ease with which it may be genetically engineered, the mouse has emerged as an extraordinarily powerful mechanistic system, permitting the unequivocal assignment of function to particular genes and molecules. It is important to recognise here that there will be occasions when our parallel studies in humans implicate a specific gene or pathway in, for example, regulating cancer immunosurveillance. The power of mouse genetics permits this implication to be tested irrefutably by assessing cancer progression in mice rendered mutant for that gene/pathway. This means that the mice may experience higher severity of discomfort when compared to wild-type mice challenged with tumour cells or carcinogens, and hence will be appropriately monitored (see below).

Fifth, many protocols have been developed and are being refined for highly effective, reproducible longitudinal monitoring.

We examine all developmental stages of this organism, because our research seeks to understand the developmental association of T cells with tissues which often occurs in the fetal and neonatal periods; the cells' responses and functions in adults; and the long-term consequences, e.g. the durability of immunological memory in older individuals.

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

The main foci of our research are cancer, infection, inflammation and transplantation.

Many animals used will not be treated in any way, but will at different ages be culled solely as sources of different cells and tissues for study in the laboratory; hence they will experience no ill effects.

For experimental models, mice will be injected with and/or otherwise exposed to cells; and/or to agents such as chemical irritants, toxins, ultraviolet light or other mutagens of the kind that humans are commonly exposed; and/or to microbes or viruses, so as to induce cancer or infection and/or inflammation. They may also be injected with and/or otherwise exposed to cells and/or agents, such a chemical inhibitors or biological substances such as immune cell stimulants known as cytokines, that are known or hypothesised to ameliorate or to enhance susceptibility to cancer or infection or inflammation or transplant rejection. In both cases, cells/agents will be administered by the most suitable route leading to very minor effects on the animals.

In some cases, fragments of organs, specifically skin, will be explanted into a recipient mouse, so that the immune response to that may be assessed.

The number of procedures we use will be kept to the minimum needed to answer our research questions with the highest levels of certainty. At any signs of undue distress in the experimental mice, the experiment will be terminated, and the course-of-events recorded to inform future practice.

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

The majority of the mice will experience no ill effects. Most animals in our breeding and monitoring programme will experience little and/or only transient adverse effects. It is possible that the inter-breeding of novel genetically altered strains will be accompanied by unanticipated adverse effects, such as we observed over a brief period for a very small percentage of mice deficient in butyrophilin-like 1 (Btnl1) which developed hydrocephaly. Our intensive monitoring of our animals means that any adverse events are detected quickly; animals culled; and alternative breeding strategies and/or experimental methods (e.g. bone marrow transplantation of cells from one genotype to another) are adopted.

Different challenge models can be anticipated to provoke different outcomes depending on the age and the immune status of the host. Following some challenges, particularly in young mice and in potentially immunodeficient strains, a standard quantitative measure of the host response is weight loss (more correctly termed weight retardation although this retardation is only temporary, after which the mice develop at the same rate as normal). Some other potential signs of distress may include hunched posture, lack of appetite and ruffled fur. Nevertheless, challenged animals will be monitored closely during periods of potential distress to ensure that appropriate action is promptly taken should any develop severe clinical symptoms.

Mice exposed to cancer cells and/or carcinogenic agents, and those carrying genetic mutations predisposing to cancer may develop cancers for which we have approved protocols for tracking and measurement. The time to develop cancer can vary considerably depending on the nature of the challenge/mutations and the genetic background of the mouse, particularly in relation to its immunocompetence.

When human cells are used for adoptive transfer, the animals may develop human cancers, phenotypically similar to the human disease. The time to develop cancer will again vary depending on the primary tumour cells engrafted and whether or not other cells, e.g. human immune cells, are co-administered. In our experience, the mice are not overtly affected by the cancer growth. It is expected that the mice will have a mild systemic effect.

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)?

Most mice on our breeding and monitoring protocols that experience any discomfort at all will experience mild severity. As mentioned above, inter-breeding of novel mutant strains can be accompanied by unanticipated adverse effects. In such cases, breeding and monitoring will be classified as moderate severity.

All the mice on our experimental protocols will fall under the categories of mild/moderate severity.

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?

The aim of our studies, as considered above, is to understand how body surface gamma delta T cells in vivo offer local protection from infection, cancer, and inflammation, and how they regulate the response to organ transplantation. Clearly, some substantial component of our work needs to be undertaken in vivo, although we quickly move to studies in vitro to investigate the underlying biochemical and cell biological processes that underpin events in vivo (see below). We examine immune responses in vivo in humans, but those studies are limited by three key factors: [i] tissue-resident T cells are our main focus of interest and yet it is challenging to monitor immune phenotypes in tissues as opposed to blood; [ii] it is extremely unusual to find humans whose genotypes allow unequivocal causative association of a gene and/or biochemical pathway with a biomedical outcome / phenotype; [iii] it is appropriately impossible to challenge humans with a spectrum of infectious agents, potential carcinogens, and inflammatory substances. To overcome some of these issues, we are using human (and mouse) organoids much more than before, and trying to overcome a substantial limitation on their current use, namely the appropriate anatomical intercalation of tissue-intrinsic immune cells, particularly gamma delta T cells, with their host tissue organoids. Importantly, our track record shows that as soon as we make key findings in animal model systems, we set up experiments using human material to test their implications for human health and disease.

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

As is evident from our publication record, we have where appropriate undertaken human studies (e.g. immune-profiling) and mathematical models and shall continue to do so. Likewise, we have established experimental protocols that employ human and mouse cell lines and organoids that reduce the need for investigations *in vivo*.

Why were they not suitable?

As considered above, the irrefutable power and value of non-animal alternatives falls far short of recapturing the spatial and temporal dynamics of the interactions between tissue-intrinsic immune cells and their host tissues both at steady-state and after those tissues have been challenged by clinically relevant agents, including microbes, carcinogens, inflammatory substances, and tissue grafts. Nonetheless, knowledge gained from non-animal alternative systems has helped us refine the design of our animal experiments, enhancing reproducibility and thereby reducing the numbers of animals included in our studies, and limiting the scale of severity.

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?

We have estimated the number of animals we will use based on two parameters:

1) The number of mice we have used in these protocols over the last five years; note that this number has evolved as our knowledge has grown and the incisiveness of experimental protocols has improved (see Appendix C).

2) The projected number of mice that we estimate we shall use on different protocols in the next five years based on our current research focus and the progression of the field in the global scientific community.

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

We have undertaken many steps in this regard.

1. We have and shall continue to investigate the ready availability of genetically-altered strains from the community, prior to generating any *de novo*. If new lines need to be generated, we shall employ the most efficient techniques such as Crispr-Cas, which reduce animal numbers by virtue of the fact that targeted animals can be generated within fewer generations, particularly when we wish to examine the impact of a genetic change on different mouse background strains.

2. We have been extremely scrupulous in our choice of experimental systems that have wide dynamic ranges that permit us in single experiments to measure immunological improvements and deficiencies, respectively, and that have low likelihood of batch effect variation, that we monitor with programmes such as Tableau. As part of good laboratory practice, protocols for each experiment defining the objectives, experimental procedures, intended effects, and endpoints will be circulated to all those involved in the care of the mice.

3. We select challenge agents based on: [i] ready availability in defined forms that permit high experimental reproducibility in standard control strains; [ii] our own experience of the challenges and/or ready availability of robust protocols within the community; [iii] our relevant scientific knowledge, e.g. that roles for the agent in gamma delta T cell biology have not already been discounted based on rigorous experimentation; [v] pharmacogenetics, e.g. the probability that an agent may yield important biological data based on clinical observations made in the realms of human genetics and treatment responses, respectively.

4. In our choice of challenge agents and experimental protocols, we consider that all the competences and reagents required for optimal downstream outcomes analysis exist, thereby maximising the amount of information to be obtained from single experiments. As an example of our choice of a tumour challenge model, we ask if so-called antigen-MHC tetramer reagents are available to detect tumour-specific alpha beta T cell responses that may be regulated by gamma delta T cells.

5. In every instance that the experimental design permits, we shall use a non-animal alternative (human immunophenotyping; organoids; immune cell - tumour cell co-cultures in vitro, etc).

6. All models used will be assessed such that we shall employ the minimum severity of disease (e.g. tumour burden; inflammation; infection) required to show an effect.

7. We are not wedded to a single approach. For example, in asking what information skin gamma delta IEL receive at steady state from Skint proteins, one could use mice in which Skint1 can be inducibly deleted. But, we chose instead to plan experiments in which we block the Skint1- gamma delta IEL interaction by intradermally administering anti-Skint1 antibody to ears of WT mice. This reduces animal use because it does not require a complex breeding programme for the mutant strain, and because (for example) a contralateral ear injected with control immunoglobulin can be used as a control for anti-Skint in a single animal, rather than requiring a second, control mouse in which gene deletion was not induced.

8. Where pathophysiologic responses to challenges are to be described, e.g. immune surveillance of cancer; capacity to mount immunity to infection; acceptance of graft, power calculations will be undertaken to determine the minimum numbers of mice required to demonstrate a phenotypic effect. Ideally, we use power of >95% and P=0.01 as thresholds, since our experience (Abeler-Dörner et al., [2020] Nature Immunology 21, 86-100) shows that conventionally-used thresholds are often too lax for many experiments, and may result in the longer run in more mice being used in attempts to resolve the consequent experimental uncertainties. Reaching stringent standards is promoted by well-established experimental systems with high signal-to-noise across broad dynamic ranges (above); nonetheless, to limit animal group sizes it will on many occasions be satisfactory to apply thresholds of >80%. Typically, t-tests or chi-squared tests (or non-parametric equivalents) will be suitable for respectively comparing time to phenotype onset, disease burden, or quantitative immunophenotype between groups, but given the multiplicity of measurements and group comparisons we shall also frequently employ ANOVA. According to the number of groups compared, corrections may be required, e.g. Benjamini Hochberg, to reduce false discovery rates.

An informative power calculation for a typical experiment is as follows: suppose WT mice have 25% less tumours than genetically engineered mice deficient specifically in interferon gamma production by gamma delta T cells, n=40 vs n=30, sd=8, then to reveal a significant difference between the groups requires 23 mice in each group.

9. In many cases, the numbers of animals required will be reduced by longitudinal measurement of responses, by serial blood analysis and/or by optimised and ever-evolving intravital imaging protocols. Hence, the immune response to a challenge may be measured weekly in a set of six mice over a period of six weeks, rather than requiring six mice to be sacrificed weekly across that period. Such longitudinal usage provides essential information on the development or not of immunological memory, but does require approval for successive sequential administration of agents and blood sampling.

10. Unnecessary variation in animal cohorts will be excluded by use of gender and age-matched controls housed under identical conditions, as we have published, and likewise transgenic, knockout, and “knock-in” mice will routinely be generated or obtained on the same genetic background, and, where relevant, not subject to experimentation until the appropriate number of backcrosses has been completed.

11. Guidance in experimental design can be consulted at: https://eda.nc3rs.org.uk/experimental-design

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

Apart from good experimental design, we shall use the following measures to optimise the number of animals used in our project:

- Pilot studies using limited number of mice to determine feasibility of new experiments
- Detailed consultation with others who have used the relevant protocols
- Where possible, tissue will be shared between other members of our laboratory so that we optimise information gained from the single unit of analysis – the individual mouse.
- Mice will be bred efficiently; i.e.: only enough breeding pairs will be set up to meet the requirement of ongoing experiments.

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.

Models and Methods:

We shall mainly use mouse models of:

[i] tumours (subcutaneous, intradermal and metastatic tumour models).

[ii] inflammation (administration of agents by gavage, intranasally, intravaginally, or epicutaneously) to induce inflammation of the relevant body surfaces.

[iii] infection, via gavage, intranasal, intravaginal, or epicutaneous exposure with systemic exposures as means to test, for example, the impact of local protection on systemic immunity.

[iv] vaccines and adjuvants via gavage, intranasal, intravaginal, or epicutaneous exposure with systemic exposures as means to test, for example, the impact of local protection on systemic immunity.

[v] wound healing.

[vi] skin-graft transplantation.

Suffering and discomfort:

Using past experience, the literature, consultation with colleagues locally and globally, all mouse models that we plan to use will be assessed such that we shall employ the minimum discomfort severity burdens required to show effects. For example, in B16 melanoma tumour challenges, we primarily employ the B16 F0 subline for skin tumours because it is demonstrably less aggressive than the F10 subline that is widely used by others. Moreover we undertake pilot dose response studies before adopting final SOPs.

Because the value of our data will be enhanced by comparison with similar studies by the community, mice will be killed at stages of disease-progression consistent with those widely used throughout the UK and international scientific laboratories.

Most animals produced under the breeding protocols are not expected to exhibit any adverse phenotypes and most immunodeficient mice are also unlikely to show adverse symptoms because of the high health status of the animal facilities. However, it is not possible to fully predict the nature or severity of any potential mutation: mutations in gamma delta T cell functions critical for gastrointestinal cancer immuno-surveillance may impact upon steady-state barrier functions, for example, water retention. With these risks anticipated, newly developed or obtained strains will be closely monitored and animals exhibiting any unexpected harmful phenotypes will be killed by schedule 1, or in the case of particular scientific interest, advice will be sought, first from the NVS, and if appropriate, then from the local Home Office Inspector.

Invasive mouse procedures required are minimal. Analgesics will be used as required.

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

Gamma delta T cells emerged in and have been conserved in vertebrates, so there is no capacity to employ nematode or fly genetics in their study. While some immune studies make use of fish, they are inappropriate for our studies because of the paucity of reagents, methods, and supporting literature, and because the contexts that we study would be at an even greater physiologic distance from clinical settings.

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

We shall refine the procedures we are using by increased monitoring according to clearly articulated guidelines, e.g. FELASA “Working Group on Pain and distress”; EC “Endorsed Severity Assessment”; and NCRI. Thus guided, we shall determine the earliest endpoint possible on an experiment-by-

experiment basis to allow a valid scientific outcome to be achieved. Our approaches to this, e.g. consultation with colleagues; reference to the literature; our own experience, are described above.

Assessment will be made of pain and distress, as measured by normal and provoked behaviour; movement; physical signs such as altered respiration rate; animal posture (huddling or hunching) skin and coat changes such as piloerection or overgrooming; inactivity; body weight; inflammation of injection sites; and comments on the animal's general appearance.

Where procedures seem likely to be distressing to animals (e.g. the highly accurate measurement of subcutaneous tumour burdens using calipers), mice may be anaesthetized although this will be avoided if accurate measurements can be obtained without anaesthesia.

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

As mentioned above, we will follow best practice guidance published by:

- FELASA "Working Group on Pain and distress"
- EC "Endorsed Severity Assessment"
- NCRI guidelines
- National Centre for the Replacement, Refinement and Reduction of Animals in Research guidelines

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

I will stay informed about the advances in the 3Rs by regularly staying updated with the guidelines specified in the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs): (https://www.nc3rs.org.uk).

I shall regularly appraise my staff of any changes to the guidelines and ensure we try, at every juncture to implement these advances effectively.