Home Office

NON-TECHNICAL SUMMARY

Tuberculosis pathogenesis & treatment in zebrafish and medaka

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

No answer provided

Animal types	Life stages
Zebra fish	embryo, neonate, juvenile, adult
Medaka	embryo, neonate, juvenile, adult

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?

Our aim is to advance our understanding of how mycobacteria produce tuberculous disease and how the host responds to infection.

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?

Our research is centered on understanding tuberculosis, a contagious infectious disease, caused by the bacterium Mycobacterium tuberculosis. This tuberculosis bacterium has very ancient origins and has been infecting humans at least for 30,000 years. Over the millennia, this highly specialized pathogen has evolved mechanisms to counteract, evade, and even benefit from host processes, particularly immune responses to cause disease. The tuberculosis (TB) bacterium has a complex life cycle in the host that involves distinct processes at each stage. As a result, from new infection to active disease, tuberculosis is delineated by complex steps, which remain largely unknown.

According to the most recent World Health Organization (WHO) report, TB caused disease in ~ 10 million individuals and killed 1.5 million individuals worldwide in 2018 (https://www.who.int/news-room/fact-sheets/detail/tuberculosis). Additionally, tuberculosis has had important social implications for sufferers of this disease over the course of human history. TB sufferers are often not able to work or take care of themselves, and to make matters worse are stigmatized leading to social rejection and isolation even from family members.

The reason that TB continues to be an enormous problem reflects the failure of public health measures that have been implemented more affluent parts of the world - these include ventilation, improved air quality and nutrition. In the face of the inability of most of the world to implement such public measure, it is important to note that the currently available vaccine BCG and the currently available drug regimen to control the global burden have not been adequate to control the disease burden. This scenario is worsened with the emergence of multi drug resistant tuberculosis that is difficult to treat. The work described in this application aims to find completely new ways to treat and prevent TB through a better understanding of how it interacts with the host to produce disease.

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

This project is expected to yield new understanding of the bacterial and host determinants that promote tuberculosis infection and antibiotic resistance. In the next five years, we intend to continue to dissect and identify the genetic determinants responsible for the 47 susceptibility mutants identified during the previous license period. We also expect to find 50 additional mutants based on the number we identified in the last period. We will try to find drugs that can treat (counter) these susceptibilities, and expect to find hundreds of drugs based on our previous experience where the investigation of the mechanism of susceptibility of a single mutant has identified 15 drugs. We are currently also exploring

the virulence mechanism of 5 bacterial determinants, and this will continue over the next five years. These studies will also lead to the identification of new drugs. Based on past experience, we anticipate identifying 5 new drugs from the study of these bacterial virulence mutants.

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

We expect that several of our findings will lead to human clinical studies and trials. During the last year, we have identified a new pathological pathway which led to the identification of two groups of cheap, readily-available oral drugs that can be re-purposed to treat tuberculosis. We anticipate additional findings going to human clinical studies in this next phase of the project license.

Therefore, TB patients will ultimately benefit from these outputs. Since human studies and clinical trials take several years, this may not happen during the period of the project license. However, we expect that some human studies and clinical trials based on our findings will have started during the tenure of this license.

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

We will disseminate our findings through publications and presentations at conferences and in various institutions. This will include the publication of unsuccessful strategies. We will continue to collaborate with human geneticists, with clinical tuberculosis researchers who can take the findings to human clinical studies, and with other zebrafish and mouse researchers, as warranted by the work.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 2,019,000 from egg to neonate; 1,011,000 from egg to adults
- Other fish: No answer provided

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 have developed the zebrafish and medaka as models to study tuberculosis by infecting it with the pathogen *Mycobacterium marinum*, a close relative of the human tuberculosis bacterium. *M. marinum* naturally infects zebrafish and medaka, causing a tuberculosis-like disease. This allows us to study tuberculosis pathogenesis in these organisms as the mechanisms of disease are conserved between these species of fish and humans. The zebrafish has proven to be an ideal model for the study of tuberculosis and has enabled us to address questions that have been elusive in the more traditional models of tuberculosis - mice, guinea pigs, rabbit and more recently nonhuman primates. The medaka has provided information into human resistance to tuberculosis. The model has several advantages over existing tuberculosis animal models which use the human tuberculosis bacterium in mice, guinea pigs, rabbits and nonhuman primates. First, because these fish naturally get tuberculosis with *M.*

marinum, the disease is more similar to human tuberculosis than when the human tuberculosis bacterium is used in non-natural hosts. Second, the transparency of the fish larva allows for the direct visualization of the steps that lead to tuberculosis in live animals; third, the genetic tractability of the fish allows for the effective dissection of the host immunity to tuberculosis - genetic fish mutants that show variation in disease susceptibility can be identified and studied in detail. Fourth, the effective use of pharmacological interventions enables both identification of pathways in conjunction with the genetic studies above; it also helps identify new host- and bacterial-targeting drugs against tuberculosis. These unique benefits have together proved tremendously powerful and have allowed us to make surprising discoveries about tuberculosis pathogenesis that have immediate clinical implications.

We take advantage of the transparency of the the larval stage of the fish. The zebrafish and medaka are transparent during the first stages of their life, and this allows us to perform experiments that last only for a few days when possible. In the larva, we can visualize different stages of infection as they happen in real time in live animals.

For some experiments we use adult animals with a fully developed immune system to ensure that our results obtained in the larvae are reproduced in adults.

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

For genetically altered animal generation, maintenance, gamete recovery and breeding protocols:

Eggs might be injected with proteins, DNA or RNA to generate new genetically altered animal lines. In many cases, we attach fluorescent reporters that are light sensing substances to the DNA introduced into the animals. This allows us to monitor the introduced DNA under a microscope and allows the determination of the exact steps by which disease occurs. Because these experiments are completed before the fish are five days old, these are not regulated procedures. In case of new mutant lines, juveniles or adults will be genotyped by fin tissue removal under anesthesia at 2-3 months of age. Fish regenerate their fins, so these small amounts of tissue removed grow back within two weeks. Founder fish may have additional mutations, so they will be outcrossed with wild type animals to remove these mutations and establish the line that bears only the mutation of interest. Founders will be killed when the next generation has been genotyped. Animals from these new lines will be used to generate eggs by natural spawning. These eggs will be used in other protocols in the Project License . Some of the animals from these new lines may be used to recover gametes to cryopreserve the line (males) or to recover a line that has been cryopreserved (females). Gamete recovery will be performed in a given animal up to 5 times maximum and these animals will be used for natural breeding after this procedure. Animals used for breeding will be kept up to 2.5 years of age. Animals showing clinical signs of disease or damaged during maintenance and breeding procedures will be immediately killed.

For phenotyping of genetically altered animals with mutations causing harmful phenotypes:

Animals undergoing this protocol will be obtained from our genetically altered animal stocks by natural spawning. These animals might be screened by microscopy before five days of age and genotyped by fin tissue removal under anesthesia at 2-5 months of age when phenotyping experiments are performed blinded and the genotype is known only at the end of the experiment. All mutant animals will be killed immediately after showing clinical signs of disease.

For infection protocols:

For any given adult fish, bacteria will be administered by up to two of the described routes and if a subsequent drug/substance is administered, it may be by the same route or a different one with use of a maximum of 3 routes. Generally, for injected substances, we would expect to inject substances only once but there will be instances where a drug such as an antibiotic (e.g. streptomycin) has to be administered daily by intramuscular injection, as is done for human treatment. We will minimize the number of injections per animal. Bacteria will be administered to most adults only once with no further treatments. Survival experiments in adults will normally last for up to 2 months. All animals will be killed at the end of the protocol.

For any given larval fish, we will perform a maximum of three administrations of the infectious agent. We anticipate administering the compound at most once a day whether by soaking, injection or gavage. For compound administration, we will typically use only the immersion method unless the compound is not absorbed through immersion or we require its localization to a specific area. In rare cases, we may need to use up to 2 additional routes of administration. Bacteria will be administered to most zebrafish larvae only once. In many cases, these infected animals will be treated with substances by soaking which is not invasive. In some cases, other substances will be administered before or after infection by injection. Infection experiments in larval fish will be terminated when or before animals reach 14 days of age. All animals will be killed at the end of the protocol.

For fish younger than 30 days used for pilot experiments:

Animals undergoing this protocol will be obtained from our genetically altered animal stocks by natural spawning. These animals might be screened by microscopy up to five days of age. Substances will be administered to find the dosage used in further infection experiments. Pilot experiments in larval fish will be terminated when or before animals reach 14 days of age. All animals will be killed at the end of the protocol.

Use of non-Schedule 1 killing methods:

These methods will be used to kill the animals in experiments where biological samples or the whole animal must be preserved for future analysis. At any stage of the experiment and independent of the age of the animal, fish will be killed by anesthetic overdose (medaka and zebrafish) followed by either 1) Chemical dissociation of tissues (e.g. Phenol, Hot Shot buffer, detergent-based lysis buffers) to preserve genetic or protein material, 2) Chemical Fixation (e.g. Paraformaldehyde, Glutaraldehyde, (dithiobis [succinimidylpropionate]) or Dithio-bismaleimidoethane) to preserve structure of tissues and organs.

Only in exceptional cases where the anesthetic can interfere with instruments used for analysis, only zebrafish will be killed by use of ice slush (where the animal is physically separated from the ice) followed by snap freezing of fish in liquid nitrogen to preserve all components of the animals.

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

The infected adult fish will eventually suffer from the consequences of a tuberculosis-like disease manifesting lethargy and very small surface hemorrhages that are less than 1 mm. These clinical signs would increase in adult fish until they eventually become moribund and die from the infection, within

weeks to months depending on the inoculum. Infected adult fish will be monitored twice daily and will be killed immediately when they show clinical signs of disease and before they become moribund, to decrease suffering.

For larvae, we cannot evaluate the severity of disease as they do not show any clinical signs of disease. However, to decrease suffering, infected larvae will be killed as soon as they become unresponsive to tactile stimuli (i.e. do not swim away when touched with a plastic pipette).

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)?

Protocol 1: 100% Mild

Protocol 2: 100% Mild

Protocol 3: 100% Mild

Protocol 4: 100% Mild

Protocol 5: 80% Mild and 20% Moderate

Protocol 6: 100% Mild

Protocol 7: 100% Mild

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

- Kept alive
- Used in other projects

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?

Tuberculosis is a complex disease involving interactions between bacteria and many different types of host cells. Disease pathogenesis involves multiple cellular processes, including the movement of various types of host immune cells, engulfment of bacteria by these cells and the death of infected host cells and nearby cells. Moreover, multiple interactions between different host cells influence disease. This is exemplified in the tubercle, the hallmark structure of tuberculosis in which multiple host cells, both infected and uninfected, come together and form an organized structure in which the bacteria reside. The tubercle houses the tuberculosis bacteria and is the product of the interaction of multiple

cell types. Adding to the complexity, tuberculosis can occur in multiple host organs and tissues. This multicellular structure is virtually impossible to replicate outside the host.

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

In vitro infections using the various cells that become infected by the tuberculosis bacterium. We use these non-animal alternatives to ask very specific questions that are dependent only on direct interactions between the bacterium and its infected cell on its own, rather than in the context of the complex multicellular structure, the tubercle.

Why were they not suitable?

Human cells in culture do not always behave as they do within the tissues in the host.

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?

Adults included in Protocol 6 are included in these numbers as they are the same animals (200,000 adult zebrafish and 10,000 adult medaka). We have estimated these numbers by the number of animals used under the previous Project License.

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

We use the NC3Rs experimental design assistant in combination with strategies to reduce the number of animals required for each experiment as described below.

We use pilot studies to look for differences caused by genetic mutations in the bacterium or the host, or by drugs that change bacterial or host determinants. This allows us to determine sample size for subsequent studies. For instance, a strong effect will allow us to reduce the number of animals used in subsequent studies.

Control animals are always required including for the pilot studies. When possible, we use siblings to minimize variability and thereby reduce sample size. Similarly, we use randomizations in all experiments: sibling animals from a single clutch are randomized into the various experimental groups. In the case of studies with mutant fish, we design our crosses so as to generate wildtype and mutants that are siblings. We perform genotyping only at the end of the experiment, so that we look for differences in infection without knowing which group the animal belongs to. This avoids any bias based on how our preconceived notion on how a mutation should affect infection.

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 use PREPARE and ARRIVE guidelines at experiment planning stages and when preparing manuscripts for publication, respectively.

For all experiments, we use the minimum number of animals required based on prior experience. For new experiments, we run small pilot studies to assess the impact of a condition and how much it varies among the animals. This then allows to use statistical calculations to determine the minimum number of animals that we can use.

For experiments where the whole animal or tissues from the animal are preserved for further studies such us gene expression of tissue microscopical analysis, these are shared between researchers so the same experiment is not repeated while enough tissue sample remains.

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 larvae, juveniles and adults of zebrafish and medaka. We use larvae rather than adult fish to the extent possible and limit the studies to a few days where possible. We are always seeking to refine our techniques to identify differences in the responses to infection to keep the infected animals for shorter time periods. This means that we can evaluate differences in the response to infection before the animals show any clinical signs of disease. We use adults for key confirmatory experiments only. All procedures in adults and larvae are performed on anaesthetized fish and animals are euthanized immediately once the experiment is completed.

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

The bulk of our experiments involve fish larvae rather than adults. We do not use species such as fruit flies (Drosophila) and worms (*C. elegans*) that are used for certain types of research because these species do not have the same types of immune cells that humans do and that are important to fight against tuberculosis. Fish do have these cells and we have shown them to be similarly important in fish tuberculosis as they are in human tuberculosis.

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

To minimize impacts in animal welfare during pairing to produce eggs, the spawning tanks are placed on shelves covered with a black background. This mimics the natural habitat where the light is entering the water from above and the bottom is dark. We have found this improves the number and quality of eggs that are produced. The tanks used to house the fish are transparent to allow the light enter the tank and the bottom is darker due to the shelving.

Genotyping trays are used to ensure fresh water and food is provided when the animals need to be individually housed. Fish are fed live diets using newly hatched brine shrimp (*Artemia salina*), which stimulates the animals as they chase the prey. Fish are housed in groups where possible, and are feed a suitable diet and follow a regime which has increased survival, development and breeding of the fish. When housing of three or two animals is required, artificial plant material is supplied to provide hiding places in order to decrease aggression. We use black shelving under each tank so as avoid stress from light surfaces and reflection.

Use of small molecule inhibitors or other chemicals (i.e. drugs) as a treatment for experimental groups: if the chemical has been used in the zebrafish or medaka before, we use that dose as a reference for the experiments under this Project License. In the case of new chemicals, pilot experiments will be used to test chemical toxicity. This will be done with small number of animals (5-10). The minimum nontoxic dose of chemical that has an effect in the outcome of the experiment will be used for further experiments.

All experiments will be ended early if it is determined that significant differences between treatments can be observed before the expected endpoint of the study. During all infection studies, any moribund animals (larvae or adults) are killed using a Schedule 1 method. For studies utilizing chemical or other synthetic compounds (e.g. drugs), we use the lowest effective dose that minimizes adverse effects. Furthermore, in the context of severe severity work, all infected animals will be inspected twice daily as this will be enough to spot changes in behavior or clinical signs of infection before animals become moribund .

With respect to severity protocols, we do not feed the larvae for the 14 days post fertilization during which we conduct our experiments. We determined that this was advantageous to the health of the larvae under the conditions of the experiment where the fish water cannot be treated or replaced. The larvae derive all necessary nutrients from the ample high-density lipoproteins stored in their yolk. Restricting food from larvae from hatching to 14 days post-fertilization has been shown by our lab to reduce super infection of the larvae by food-borne commensal infection susceptibility.

Survival studies are a critical component of our goal to understand human tuberculosis. For example, host mortality can result from inappropriate immune responses rather than bacterial growth. In such cases, an analysis of bacterial burden may not provide understanding of a disease process. In these cases, survival studies can provide important insight into central disease processes. When survival studies are necessary, experimental animal numbers are minimized via statistical Power Analysis and up to 20% of animals undergoing this protocol may develop moderate clinical signs of disease. To minimize suffering during these experiments, we kill any animal with clinical signs of disease or moribund immediately using a Schedule 1 method. All infected animals are meticulously inspected twice daily to ensure that moribund animals or animals showing clinical signs of disease are quickly detected in order to further minimize suffering. These checks are conducted by experienced technicians and researchers who have had rigorous training and assessment before conducting checks alone. During these inspections, any action taken is recorded to be available for the researcher in charge of

the experiment, the rest of the technicians doing the checks and the NACWO. Any fish found dead (<10%) or culled because of signs are recorded within the room and also a database. This allows all relevant parties to know the health status of the stocks under experiment and monitor numbers closely to ensure we work within the license allowances. In instances when unexpected number of animals show signs of disease, different from predicted by fish line and bacterial strain and inoculum used, the researcher in charge of the experiment, as well as the NACWO, is notified immediately.

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

Humane methods of euthanasia have been evaluated outside of the current and future acts of UK law relating to the use of animals in scientific procedures and have been cited as having a potential to improve the welfare of animals such as zebrafish RSPCA's 'Guidance on the Housing and Care of Zebrafish', May 2011. The method entails the immersion or exposure of zebrafish to temperatures of $4^{o}C$ or less for periods greater than 2 hours as a means to halt metabolism. Only 39% of chilled fish showed signs of distress, compared to 100% of fish exposed to the anesthetic agent MS-222 (tricaine). After this step, the zebrafish will be subjected to Snap Freezing. The advantage of Rapid Cooling/Snap Freezing is to humanely euthanize zebrafish without causing them distress (Rapid Cooling/Snap Freezing as a means of euthanasia of ectothermic zebrafish for larvae up to 14 days of age. For euthanasia of adult zebrafish this method will require the zebrafish to be held within 2-$4^{o}C$ ice slush until loss of operculum (the support structure on the side of a fish's head which forms a protective cover for the gills) movement followed by introduction to liquid nitrogen to confirm death and preserve tissues (snap freezing). We have received training to perform this procedure from the Zebrafish International Resource Centre and have since presented the method at meetings within the UK. Following this, other facilities have also adapted their protocols to incorporate this method due to its welfare improvement.

We will also use PREPARE and ARRIVE guidelines at experiment planning stages.

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

We will be closely in contact with the NACWO, NIO and NVS to be informed of any new publications or reports regarding 3Rs. The NACWO will attend relevant continued professional development training as well as zebrafish husbandry meetings as with the previous license.