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NON-TECHNICAL SUMMARY

Investigating how factors outside the cell ('the extracellular matrix') control immune responses.

Project duration

5 years 0 months

Project purpose

- (a) Basic research

Key words

Leukocyte, Migration, Inflammation, Brain, Extracellular Matrix

Animal types	Life stages
Mice	Embryo and egg, Neonate, Juvenile, Adult, Pregnant 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?

To understand how proteins and sugars outside of the cell (extracellular matrix) control movement of immune system cells and the wider immune response.

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?

Movement of immune cells from the blood to tissues is critical in allowing us to fight infections by bacteria and viruses. However, movement of immune cells is also central to problems associated with a wide range of diseases including inflammatory arthritis, stroke, atherosclerosis and cancer.

The extracellular matrix is a network of factors (proteins and sugars) released by cells to provide scaffolds which provide structure to tissues. We are now beginning to understand that the extracellular matrix plays a more specific and dynamic role in biological processes beyond simply being a structural support.

Specifically the extracellular matrix lines blood vessels and forms a barrier called the glycocalyx. We now know that the glycocalyx is also found on the majority of cells of the immune system and controls the movement of immune cells from the blood vessels into tissues, a process that is vital in fighting infection. A more complete understanding of the glycocalyx will allow us to understand how immune cells are recruited during disease and help to inform develop of drugs to treat and limit immune cell recruitment and help treat people with a wide range of diseases.

This project will define how these processes work during the body's normal response to inflammatory stimuli, e.g. infection and sterile injury. In the longer term these findings can be used to understand how these processes go wrong in disease, e.g. rheumatoid arthritis.

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

The main outputs of this project will be new information explaining how immune cells are recruited and migrate to fight infection and also during inflammatory based diseases.

In the longer term these insights will drive development of new ways to control immune cell recruitment and produce new drugs.

This knowledge will be critical to understanding and finding new ways to treat patients with a wide range of diseases, such as auto-immune disease (rheumatoid arthritis) and vascular diseases, such as stroke, atherosclerosis and cancer.

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

This project will investigate how the extracellular scaffold (glycocalyx) helps to control the immune system response to infection. Importantly, this includes the positive elements of the immune system allowing us to fight infections, but also diseases where the immune system goes wrong. The chemokine system controls the movement of immune cells from the blood to tissue, vital for fighting infections but also important in inflammatory based disease. We have previously failed to make drugs that target the chemokine system during inflammatory diseases. Inflammatory diseases remain a key hindrance to quality of life and health in the UK and globally. Information from this project will benefit researchers to better understand how the chemokine works to develop new drugs in the future.

This project will also produce findings that will help to improve the use of a new approach where the thickness of the extracellular matrix barrier (glycocalyx) is being used to diagnose disease and decide on the best treatment approach.

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

The data generated, both positive and neutral, will mostly be suitable for sharing via publication or by being uploaded to publicly accessible data repositories.

Any outputs will be assigned a digital object identifier (DOI) to facilitate sharing and accessibility of them. Outputs beyond traditional publications may include video demonstrations of our validated protocols that would be of use to the wider community. I will also continue to undertake a variety of engagement events (e.g. public science days) to communicate our findings to the wider public.

Species and numbers of animals expected to be used

- Mice: 4500

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.

Mice are chosen as they have been used in related approaches, paving the way for my research and providing vital details that will inform our design and understanding of this work.

Using mice for this proposal will enable comparison with existing knowledge and allow future development of drugs based on our work.

The life stages chosen will be to undertake experiments on mice at the adult stage of development. This will allow comparison to previous work which was done on adult mice.

The use of genetically altered mice will allow us to determine the mechanisms underlying the function of the glycocalyx during immune cell recruitment. Knowledge that is crucial for the benefits of this proposal.

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

In the first experimental approach animals with have an air pouch produced, on the animals back, by injection of sterile air under the skin (taking approximately 2 minutes), while the animal is anaesthetised, on 3 separate occasions, each 48 hours apart to allow recovery. Air pouch formation will also induce localised blood vessel formation containing an endothelial cell (cells that line the interior surfaces of blood vessels) glycocalyx structure. Air pouch formation will be carried out using aseptic technique to minimise risk of infection and pain relief will be given peri- and post-operatively on the advice of the NVS (Named Veterinary Surgeon). Special substances, called reagents, will either be injected into the air pouch to induce bacterial infection on a maximum of 3 occasions and/or used to inhibit immune cell recruitment, the process by which immune cells are attracted to sites of inflammation, on a maximum of 4 occasions. The animal will be humanely killed up to 2 weeks later, but usually within 48 hours. Any animal showing mild/moderate signs (e.g., mild/moderate piloerection, intermittent shivering, mild paleness, weight loss of 10%) will receive supportive care (e.g., extra heat, food treats, fluid supplements) and will be more closely monitored or will be humanely killed if there is no improvement.

In the second experimental approach animals will undergo surgery to perform a 3 mm craniotomy (surgical procedure where a section of the skull is removed) and cover this by implanting a glass window (4 x 4 mm) secured to the skull by dental cement. This is then surrounded by a metal head plate also secured to the skull by dental cement. Surgery typically takes 1 hour per mouse and the majority of mice will have 48 hours to recover from surgery prior to imaging. Analgesics will be administered pre-surgery to alleviate any pain. Inflammation will be minimised by use of good surgical technique. Weight loss will be monitored and fluid and diet supplemented, where necessary, including "ground feeding". Post-operative pain will be carefully managed, regularly assessed and alleviated as far as possible. Analgesics will be administered as recommended by the NVS . We can then inject reagents to promote and/or reduce immune cell recruitment and attach detectable fluorescent markers (known as labelling) to the endothelial glycocalyx and immune cells in the circulation and the brain. This will be done by intravenous injection on a maximum of 3 occasions, by intra-peritoneal injection on a maximum 2 occasions, or subcutaneous on a maximum 2 occasions. The majority (90%) of imaging experiments will be performed under terminal anaesthesia, and within 48 hours, from the step of induction of immune cell migration. Some cases will require longitudinal imaging up to two weeks after induction of immune cell recruitment. Any animal showing mild/moderate signs (e.g., mild/moderate piloerection, intermittent shivering, weight loss of 10%) will receive supportive care (e.g., extra heat, food treats, fluid supplements) and will be more closely monitored or will be humanely killed if there is no improvement.

In the third experimental approach THE ANIMAL will undergo surgery to expose the muscle blood vessels, before or after injection of reagents. This will be to promote and/or reduce immune cell recruitment and to label the blood vessel endothelial glycocalyx and immune cells within the blood vessels and surrounding tissue. If done by intravenous injection, it will be on a maximum of 3 occasions, intra-peritoneal injection on a maximum 2 occasions, or subcutaneous on a maximum of 2 occasions. All of the imaging following muscle blood vessel exposure will be performed under terminal

anaesthesia. The majority (80%) will be culled within 24 hours of induction of immune cell recruitment whilst the rest will be culled within 2 weeks of induction of immune cell recruitment.

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

Air pouches are made while the animals are anaesthetised and they display minimal adverse effects. Injection of reagents to induce immune cell recruitment may cause low levels of inflammation over the next few hours, however, the mice do not display signs of discomfort.

Cranial window implantation, imaging and induction of immune cell recruitment are performed under anaesthesia, as a result animals have few associated adverse effects. Post-operative pain will be assessed and alleviated where possible. Analgesics will be administered as recommended by the NVS. Local bleeding lasting no more than minutes is possible during surgery to implant the cranial windows and weight loss (1-4 days) after surgery is also possible, but animals generally recover quickly.

Muscle exposure and imaging of immune cell recruitment are performed under terminal anaesthesia, as a result animals have few associated adverse effects. Injection of reagents to induce immune cell recruitment may cause low levels of inflammation over the next few hours, however, the mice do not display signs of discomfort.

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)?

The expected severity of breeding is mild.

The expected severities of the experiments are moderate and will consist of 100% of the animals involved.

What will happen to animals used in this project?

- Killed
- 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?

Currently there are no good in vitro cellular models of the glycocalyx, partly because we do not have a good understanding of the glycocalyx structure in live animals. Thus, it is essential to do these

measurements in live animals

It is not possible to replicate the complexities involved in immune cell recruitment using cell-based assays, therefore, these experiments must be undertaken in live animals.

Using genetically manipulated animals is critical to understand the molecular mechanisms that drive immune cell recruitment and pathology in brain inflammation and precludes other approaches.

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

The first alternative to be considered are cell-based models of immune cell migration, including transwell and adhesion under flow systems. Transwell systems monitor the movement of purified immune cells in a plastic dish. Adhesion under flow systems analyse movement of immune cells in the presence of mechanical flow produced by a machine, this models the effect of blood flow.

Glycocalyx function in immune cell recruitment: Using biophysical approaches we have shown that certain factors (proteins) can remodel the components that make up the glycocalyx, specifically glycosaminoglycan (GAG) sugar chains. We have used *in vitro* cellular approaches to show that this ability of chemokines to re-model the endothelial glycocalyx increases endothelial layer permeability. Targeting this effect has reduced immune cell migration over the endothelium *in vitro*, demonstrating a possible future therapeutic approach. We have also shown that changes in GAG biochemistry (sulphation) controls the ability of these sugars to bind to chemokines and present them on endothelial cells. Given the limitation of in vitro cellular systems detailed elsewhere it is now vital to assess these findings using animal models.

Another consideration is the developing field of organoid cultures to recreate live animal conditions. These are simplified and miniaturised models of organs produced in vitro.

Why were they not suitable?

Following on from biophysics and *in vitro* cell models it is now vital to use *in vivo* approaches.

Neither of these systems (or organoids) can replicate the glycocalyx. We do not have a complete understanding of the glycocalyx structure at different parts of the blood vessel system, this must first be established to allow comparison for cell-based studies.

The complexity of immune cell recruitment cannot be recreated in cell-based experiments. The air pouch model, live cranial window imaging and muscle vasculature exposure approaches allow analysis of the role of the glycocalyx in immune cell recruitment in the brain and in peripheral tissues.

Surgical implantation of a cranial window is the only way to directly image the glycocalyx and immune cell recruitment. Information we acquire on the glycocalyx and leukocyte recruitment will be used to inform development of in vitro/organoid models

Our findings will help to try and inform development of better non-animal model systems for these processes.

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 worked with a statistician and used the National Centre for the Replacement, Refinement and Reduction of Animals in Research experimental design assistant ('NC3r's' EDA) to calculate the number of animals needed. This involved using data from our own preliminary, and past, experiments to calculate the minimum number of animals that we need to use to enable the experiments to produce statistically significant data. We have also used previous annual return figures to calculate the numbers of animals we will need to breed for this project.

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

Where possible multiple read-outs will be measured in the same experiment, e.g. glycocalyx structure before and after treatment with a reagent that produces immune cell recruitment.

The 'NC3r's' experimental design assistant has been used, and will continue to be used, to determine the minimum number of animals to provide sufficient power to analyse relevant effects sizes of treatments.

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

Where necessary, breeding will be designed to produce the number of animals needed and experiments will be undertaken on animals bred to optimise the number of animals used in this project.

Pilot studies will be undertaken when starting new experiments to inform experimental design, i.e. power calculations, again optimising animal use. Where possible these will be informed by cell-based analysis.

A continued effort will be made to share tissue from experimental animals. In particular it may be possible to undertake analysis of the glycocalyx from tissue from animals which have been humanely killed and also from tissues following systemic treatment with mediators of immune cell recruitment. Tissue from experiments analysing the glycocalyx in the brain blood vessel system may allow analysis of the glycocalyx in other tissues as well and inform future studies by ourselves and others.

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.

The air pouch, cranial window and muscle exposure approaches will be run separately but the data from all, in combination, will allow analysis of immune cell recruitment and the role of the glycocalyx using the minimum possible pain, suffering, distress or lasting harm to the animals involved.

The air pouch model will firstly be used to screen the numbers and types of immune cells recruited in response to different reagents and for compounds that may inhibit this process. This reduces the need for the more invasive cranial window approach, that involves surgery, thus acting as a refinement by using a less invasive experimental approach.

Implanting cranial windows involves surgery but is the only way to simultaneously directly image the glycocalyx and immune cell recruitment within a tissue. Thus the associated pain and suffering is the least possible to achieve this outcome.

Exposure of muscle vasculature will allow direct imaging of the glycocalyx and immune cell recruitment in peripheral vasculature and will be performed under terminal anaesthesia, minimising pain and suffering.

All three involve moderate severity and direct glycocalyx analysis (via cranial windows and muscle vasculature exposure) will be undertaken whilst the animal is anaesthetised. In the majority of cases mediators of immune cell recruitment, that may produce some inflammation in the animal, will be administered whilst the animal is under terminal anaesthesia.

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

Our knowledge of the production and relevance of the glycocalyx is uncertain beyond its existence and importance to inflammation. For this reason analysis must begin in adult mice where we know the glycocalyx is formed, regulates immune cell recruitment and replicates effects seen in humans in health and disease.

Where possible glycocalyx imaging experiments will be undertaken during terminal anaesthesia. The only exceptions will be unavoidable as they will involve analysis of the glycocalyx over time and therefore the same animal must be analysed then allowed to recover before further anaesthesia and glycocalyx analysis. Specifically we will initially perform experiments under terminal anaesthesia and successfully collect and analyse data before progressing to longer time points needed to analyse the glycocalyx over time.

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

Monitoring of all animals will be continually undertaken as described alongside all possible post-operative care and pain management.

The air pouch model will firstly be used to screen the numbers and types of immune cells recruited in response to different reagents and for compounds that may inhibit this process. This reduces the need for the more invasive cranial window approach, that involves surgery, thus acting as a refinement by using a less invasive experimental approach.

Most up to date handling techniques will be utilised throughout, all operators will be highly skilled and/or continually trained and updated with the latest refined approaches, e.g. handling.

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

All relevant NC3R's guidance and updates will be engaged with, including sign posting to published studies, e.g. FRY, D. 2014. Chapter 8 - Experimental Design: Reduction and Refinement in Studies Using Animals. In: TURNER, K. B. V. (ed.) Laboratory Animal Welfare. Boston: Academic Press. Smith AJ, Clutton RE, Lilley E, Hansen KEA, Brattelid T. PREPARE: guidelines for planning animal research and testing. Lab Anim. 2018 Apr;52(2):135-141. doi: 10.1177/0023677217724823. Epub 2017 Aug 3. PMID: 28771074; PMCID: PMC5862319. Morton DB, Jennings M, Buckwell A, Ewbank R, Godfrey C, Holgate B, Inglis I, James R, Page C, Sharman I, Verschoyle R, Westall L, Wilson AB; Joint Working Group on Refinement. Refining procedures for the administration of substances. Report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement. British Veterinary Association Animal Welfare Foundation/Fund for the Replacement of Animals in Medical Experiments/Royal Society for the Prevention of Cruelty to Animals/Universities Federation for Animal Welfare. Lab Anim. 2001 Jan;35(1):1-41. doi: 10.1258/0023677011911345. PMID: 11201285.

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

Continued engagement with the NC3R's resources will be undertaken. In particular continued use of the experimental design assistant (NC3R's EDA) will facilitate regular assessment of the 3R's and recent developments whilst also enhancing experimental design.

Attendance of workshops organised by the animal facility will be undertaken as well as continued consultation with staff at the facility.