Investigating platelet function in microglial activation and Alzheimer's disease.

Student: Bismah Mateen, Manchester Metropolitan University

Supervisors:
Dr Sarah Jones
Dr Amanda Unsworth
Dr Xenia Sawkulycz

Lay Summary:
Platelets are small cells in the blood responsible for blood clotting. In recent years however, it has been suggested that they might also play a role in the development of Alzheimer's disease (AD). In this study we used a rat model of AD to investigate whether platelet number and function are altered in AD. To investigate whether platelet activation and specifically the release of fibrinogen contributes to microglial damage, a human microglial cell line was used, and cells stimulated with fibrinogen or platelet releasate. Alterations in cell morphology and the expression of iNOS, as a marker of inflammation was then assessed using fluorescence microscopy.

Aims:

1) To investigate platelet function in a rat model of Alzheimer's disease
2) To investigate the effect of platelet activation on microglial cells

How did you address the aim?
To investigate whether platelet number and function is altered in AD, we collaborated with Dr Herve Boutin (University of Manchester). Dr Boutin is currently carrying out a study to investigate alterations in the brain of wild type (WT) and transgenic AD rats (TgF33-AD) with and without infection (LPS injection). Rats were culled at 18 months old, and we obtained a citrated blood sample and the legs to prepare bone marrow.

Full blood counts were performed using a Sysmex XS-300. Platelet rich plasma (PRP) and platelet poor plasma (PPP) were prepared for aggregation using centrifugation, and light transmission aggregometry was performed using a Chronolog-700. To assess platelet aggregation, PRP was stimulated with ADP (10μM) and collagen (10μg/ml) for 5min at 37°C under constant stirring conditions (Fig. 1).

The bone marrow from the femur bones was fixed in 4% paraformaldehyde. The bone marrow was then removed from the bone and embedded with paraffin wax to enable histology and immunohistochemistry at a later date to analyse megakaryocytes to investigate whether there are any differences between the WT and TG rats.

A) B)

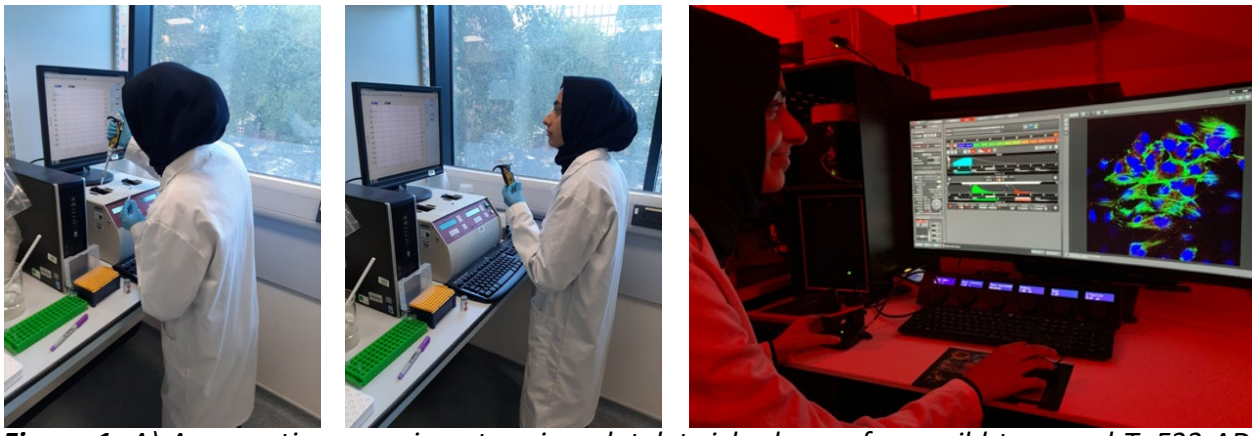

Figure 1: *A) Aggregation experiments using platelet rich plasma from wild type and TgF33-AD rats. B) Fluorescent microscopy to analyse cell morphology and inflammation in microglial cells following stimulation with the releasate from activated platelets.*

To investigate whether fibrinogen activates and alters the morphology of microglial cells, the HMC-3 cell line was seeded into 12 well plates. Cells were serum starved overnight then treated with fibrinogen (1mg/ml or 2.5mg/ml), LPS (100nG/ml) as a positive control, or vehicle control. The plates were fixed, and immunofluorescence performed using DAPI for the nucleus, phalloidin for the actin fibres and an anti-iNOS antibody as an inflammatory marker.

In order to see whether wash platelets activate or protect microglia cells, cells were serum starved overnight then treated for 4h with platelet releasate obtained from washed platelets activated via centrifugation, LPS (10ng/mL), LPS and platelet releasate or Tyrode's alone. The plates were fixed, and immunofluorescence performed in the same was as described above.

What did you find out?

Full blood counts revealed no significant difference in platelet count or size between WT and TG rats (Figure 2). However, there was a decrease in platelet size following LPS stimulation. Figure 2C shows a significant reduction in platelet distribution width (PDW) in LPS treated wild type (WT) rats compared to TG rats. A significant decrease in platelet large cell ration (P-LCR) was also observed in TG AD rats treated with LPS compared to control TG AD rats. Caution must be taken when analysing the data however due to the low animal numbers.

Aggregation assays were extremely variable, and the low animal numbers makes the data difficult to analyse (Figure 3) and no conclusions can really be drawn. Of the 16 rats across all four groups, platelet aggregation was observed in PRP from 12 animals in response to ADP (100μM) and only 7 animals in response to collagen (10μg/ml).

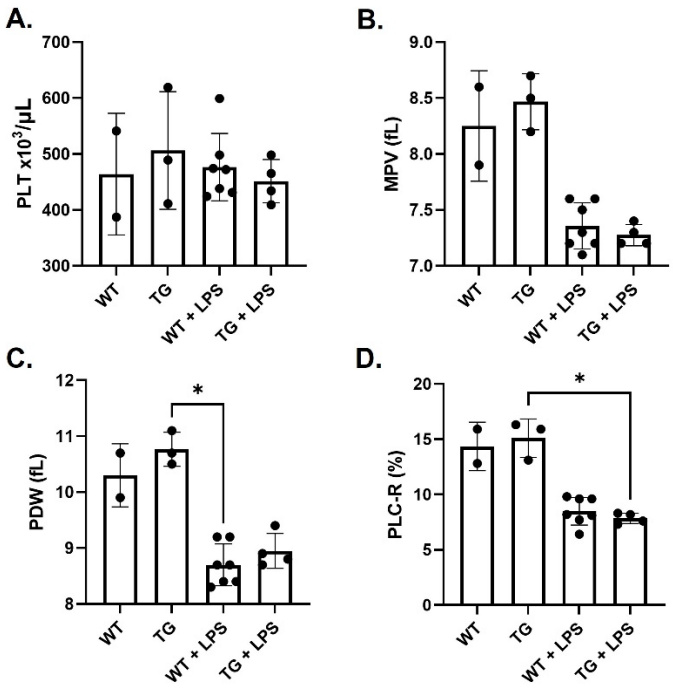

Figure 2: Platelet count and size. *Full blood counts were performed on whole citrated blood from 18-month-old wild type (WT) rats and transgenic AD rat following LPS or control treatment. Data displayed show A. platelet count (PLT), B. mean platelet volume (MPV), C. Platelet distribution width (PDW), and D. platelet large cell ration (P-LCR). Data represents mean±SD. *P<0.05.*

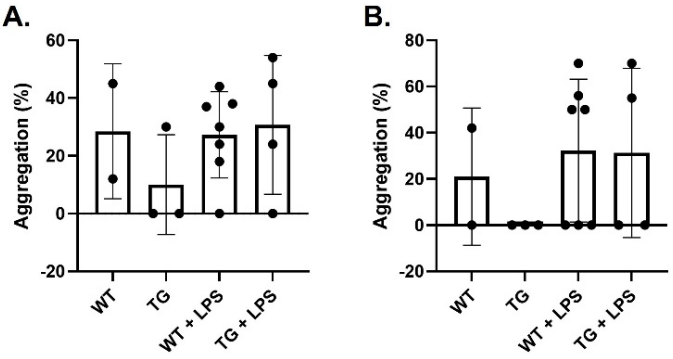

Figure 3: Platelet aggregation responses in WT and AD rats. *Aggregation assays were performed in platelet rich plasma from 18-month-old wild type (WT) rats and transgenic AD rat following LPS or control treatment. Data displayed show A. maximum aggregation in response to ADP (100μM) and B. Collagen (10μg/ml).*

Investigations into microglial activation following stimulation with fibrinogen demonstrated significant staining in the microglial cells treated with fibrinogen, both at 1mg/mL and 2.5mg/mL, suggesting that fibrinogen does activate microglial cells as well as clear morphological changes to the microglial cells. There were significantly less cells in the fibrinogen treated cells, as during the treating process the media clotted as the fibrinogen turned into fibrin in the media, therefore

whilst staining the cells were removed from within the clotted media (Fig. 4). Control cells showed no positive staining for iNOS, while the LPS treated microglial cells showed abundant iNOS level, indicating activation (Fig. 4).

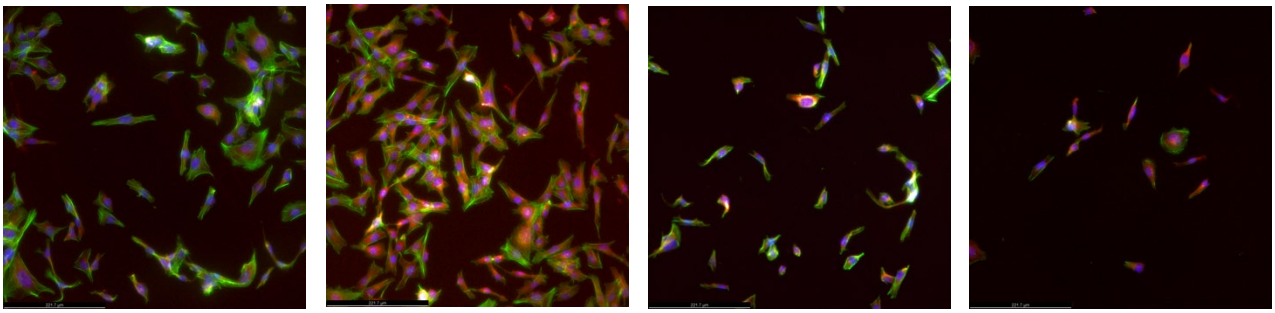

Figure 4: Microglial cell activation in response to fibrinogen. *Control microglial cells, LPS treated microglial cells, 1mg/ml fibrinogen treated microglial cells, 2.5mg/ml fibrinogen treated microglial cells (left to right) stained with DAPI=blue=nucleus, phalloidin=green=actin fibres, iNOS=red=inflammatory marker.*

Significant iNOS staining can be observed in the microglial cells treated with LPS as well as when treated with wash platelets, indicating activation. Interestingly, when microglial cells were treated with LPS and wash platelets there was no iNOS or phalloidin staining suggesting the wash platelets may protect microglial cells from the effect of LPS, but the cells morphology does show proof of changes.

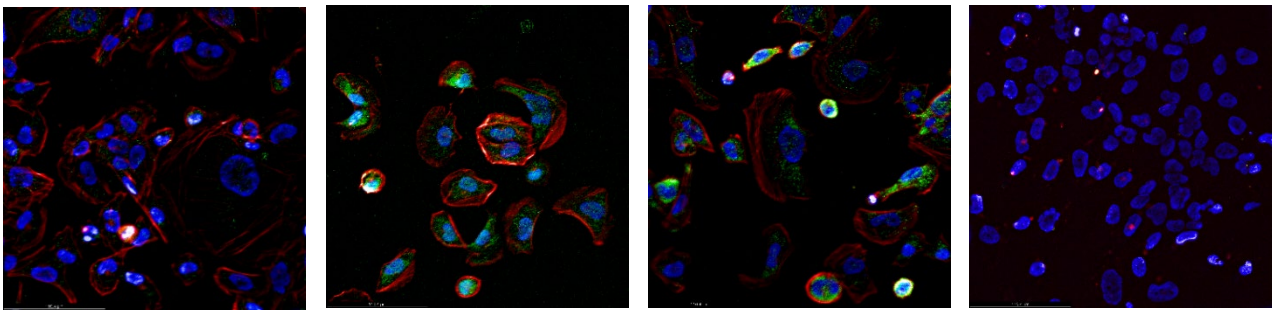

Figure 5: Microglial cell activation in response to platelet lysates. *Control microglial cells, LPS (10ng/mL) treated microglial cells, platelet releasate treated microglial cells, and LPS and releasate treated microglial cells (left to right) stained with DAPI=blue=nucleus, Rhodamine phalloidin=red=actin fibres, 488 iNOS=green=inflammatory marker.*

What did you learn from participating in this project?

I enjoyed many aspects of this project, from working independently in the tissue culture lab growing my cells for my experiments to working with the Thrombosis Group. This opportunity gave me a chance to learn new techniques that I will need for the rest of my career, namely aseptic techniques in a class II cabinet for cell culture. The weekly meetings really made me feel like a part of the team and it developed my confidence and ability to summarise scientific points and get my point across in a very short amount of time. Microscopy is so important as without it you would simply not be able to see the effects of your treatments so visually and clearly. I

received specific training on a number of microscopes including STELLARIS Confocal, Leica DM6000i Live Cell Imager, and the Thunder Imaging System. I learnt about the different sample preparations required for each and the importance of design planning experiments. Even before imaging you must stain according to which microscope you would like to use and analysis and make adjustments as needed after imaging, therefore forward planning is essential**.**

How has this project affected your long-term goals?

This studentship has really reinforced my passion and interest in biological research. The laboratory experience and advice that I have gained during the studentship from the successful scientists that I have worked alongside, has helped me to clarify my future career ambitions. I now have a clear overall goal, and feel better equipped to achieve this, through the experience that this studentship has offered me. I also know that I have the continued support of my supervisors and the PhD students and postdocs that I worked with. My short-term goal is to get accepted onto the Scientist Training Programme (STP) and specialise in haematology and transfusion. This would allow me to combine a patient facing role, with laboratory science and research that can have direct patient benefit. I would then like to develop my career further by completing the Higher Specialist Scientist Training Programme (HSST), leading to a consultant role.