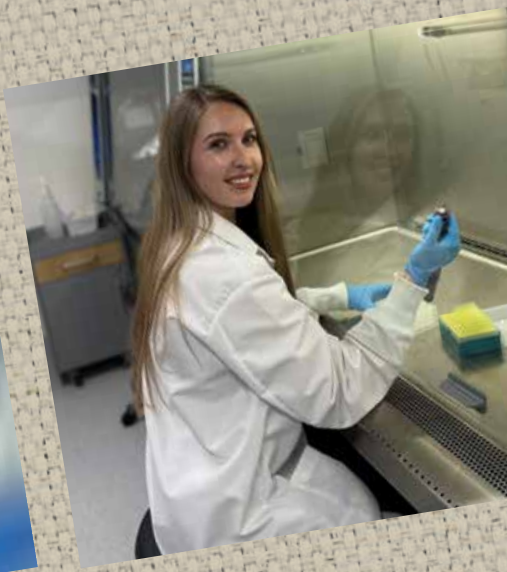
2024

RESEARCH REVIEW



Research For Life

WELLINGTON MEDICAL
RESEARCH FOUNDATION

RESEARCH REVIEW 2024

COVER PHOTO:
Grant and Fellowship recipients:
Cerys Blackshaw, Sarah Sczelecki and Dr Abby Sharrock

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Research For Life
The Wellington Medical
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Contents

TRAVEL GRANT REPORTS

Editorial

BY PROFESSOR REBECCA GRAINGER

Good things take time. Slow and steady wins the race. Rome wasn't built in a day. These idioms remind us that consistent effort, with focus and purpose are required to achieve great things.

When reflecting on this years' research reports I recalled my own doctoral studies and at the time those years felt like the longest three years of my life. That was just the warmup. A friend and colleague often reminds me that academic medicine is a marathon not a sprint. The laboratory research of my doctorate was supported by a Wellington Medical Research Foundation grant (now RFL) and enabled me to learn the foundations of medical research. I've been learning ever since. Behind the short summaries in this research report is persistent focused effort from the talented emerging medical researchers in Wellington, supported by their supervisors and mentors and institutions. The work reported usually takes 1-2 years, sometimes longer, and summarizes many hours of laboratory work, data collection, analysis, reading, thinking and writing. Please take some time to read about their work.

These reports describe novel science providing enhanced understanding of disease or opportunities to optimize management of diverse conditions including cancer, gynaecological and cardiovascular disease, inflammation, catastrophic health events, and infections. Many describe incredible biological detail with work undertaken right here in Wellington. Much of the work is for doctoral degrees. Just as these insights are foundational building blocks to future improvements in medical science and healthcare, doctoral degrees are foundational to future careers in medical research. Good things will take time.

The reports have several authors. As is usual in scientific reporting, the first author generally did most of the work. They are generally the doctoral students who applied for the RFL grant to support their research. The middle authors have supported the work in some way. The last author is the senior research leader in the team. These people provide essential guidance and training to our young scientists at all stages of the project, from the idea to design and conduct and reporting. Our young talent could not be developed here in Wellington but for these senior scientists and researchers who have developed the team and infrastructure to undertake medical research - no small feat in New Zealand, where research funding is relatively limited and highly competitive. Slow and steady wins the race.

The reports this year come from the three powerhouses of medical research in Wellington: the Malaghan Institute of Medical Research, Te Herenga Waka Victoria University of Wellington, and University of Otago Wellington. The RFL grants support direct research costs, but the host institution provides essential infrastructure and often covers other necessary laboratory costs for the work reported. Just as Rome wasn't built in a day, these institutions have developed over decades to be able to support the exciting work that happens in health and medical research in Wellington. The impact of the work is evidenced in the travel grant reports. These reports capture the excitement of presenting your work to a relevant audience, and the feedback after presenting often guides future direction. Since our institutions often have very limited support for conference travel, RFL is very pleased to enable the dissemination of our researchers work.

As well as acknowledging the work of our grant recipients, I would like to acknowledge the work of two longstanding contributors to RFL. After almost three decades of service to RFL Professor Brett Delahunt is leaving the Board. Professor Delahunt was chair of the Research Advisory Committee (RAC) for many years and invited me onto the RAC about a decade ago. I learnt a great deal from him as a member of RAC and remain grateful to have observed his analytic ability and intellect firsthand. I hope I have maintained the rigor of the RAC assessment process so that all RFL grant funding goes to high quality science. I remain grateful to Professor Delahunt and wish him all the best with his ongoing commitments to Anatomic Pathology and research.

Associate Professor Peter Larsen was already a member of the RAC when I joined and has been a highly valued committee member who has always contributed in an active, insightful and balanced manner. I am very grateful to Peter for his years of service and wish him all the best. I would also like to thank and acknowledge the rest of my team on the RAC, Dr Lisa Connor, Dr Oliver Gasser, Dr Rachel Perret, Dr Jeremy Owen and Professor Anne La Flamme for their time and expertise.

If any grant recipients are reading this, and perhaps facing challenges in the day to day of their research, please remember, good things take time and slow and steady wins the race. And the race is a marathon, not a sprint. We all wish you every success and look forward to reading about it.

RESEARCH ADVISORY COMMITTEE

Professor Rebecca Grainger (Chair)
Dr Lisa Connor
Professor Anne La Flamme
Associate Professor Peter Larsen
Dr Jeremy Owen
Dr Olivier Gasser
Dr Rachel Perret

Guest members

Dr Kathryn Hally

RESEARCH REPORTS

Confirmation of a new FISH assay for CAR T cell transgene visualisation and enumeration

A WILSON[1,2,3], N DASYAM[1,2], R PERRET[1], R WEINKOVE[1,3,4]

[1] Malaghan Institute of Medical Research, Wellington, New Zealand
[2] Hugh Green Technology Centre, Wellington, New Zealand
[3] Department of Pathology and Molecular Medicine, University of Otago, Wellington, New Zealand
[4] Wellington Blood & Cancer Centre, Te Whatu Ora Health New Zealand, Capital, Coast, and Hutt Valley, Wellington, New Zealand

KEY FINDINGS

This research project aimed to confirm results generated by a new fluorescence in situ hybridization (FISH) assay for transgene visualisation and enumeration. Results generated using our new FISH assay for transgene insertion site visualisation and enumeration in CAR T cells were able to be confirmed by droplet digital PCR (ddPCR), and estimated transgene copies per cell were comparable between methods. Overall, our new FISH method holds potential for use as an additional safety measure before releasing clinical gene-edited cell therapy products for infusion into a patient.

OBJECTIVES

For CAR T cells used in our FISH experiments, we wished to confirm the observed number of transgene copies by an orthogonal method. ddPCR provides a higher genomic resolution than qPCR- based methods, which are the current gold standard. Although not the gold standard for the enumeration of transgene copy numbers in clinical gene-edited cell therapy products, ddPCR was the most suitable method of confirmation for our novel data as the technology can estimate transgene copies at a much higher resolution than qPCR.

To address our aim, we performed the following:

1. Extracted gDNA from a population of CAR T cells, and performed confirmatory ddPCR to estimate average transgene copy numbers.
2. Compared transgene copy number estimates generated by both ddPCR and FISH-based techniques.

METHODS

gDNA from CAR T cells was extracted using the NucleoSpin Tissue Mini kit according to the manufacturer's protocol. A ddPCR reaction mastermix was prepared using a 2x ddPCR Supermix for Probes (no dUTP) (Bio-Rad), and primer-probe sets targeting RPP30 (a housekeeping gene) and the Woodchuck Hepatitis Virus post-transcriptional regulatory element (WPRE) region contained within the transgene. ddPCR primer probe sets were hybridized to transgenic and genomic sequences in CAR T cells, followed by amplification and detection of transgene copy numbers using standard ddPCR protocols. Results generated by ddPCR were directly compared to results generated using our FISH assay. Average transgene copy numbers for FISH were estimated after scoring 200 nuclei within a 66% CAR T cell population processed for cytogenomic analysis.

RESULTS

Using gDNA extracted from a 66% CAR T cell population utilized in our previous FISH work, we performed ddPCR in triplicate using primer-probe sets targeting WPRE (transgenic target) and RPP30 (housekeeping gene, two copies per genome), resulting in an absolute concentration of WPRE copies per microlitre (Figure 1). From these data, we then estimated transgene copy numbers per cell by taking the ratio of transgene copies per microlitre to half the number of housekeeping gene copies (under the assumption there are two copies of RPP30 per genome), which produced an average of 1.33 transgene copies per cell.

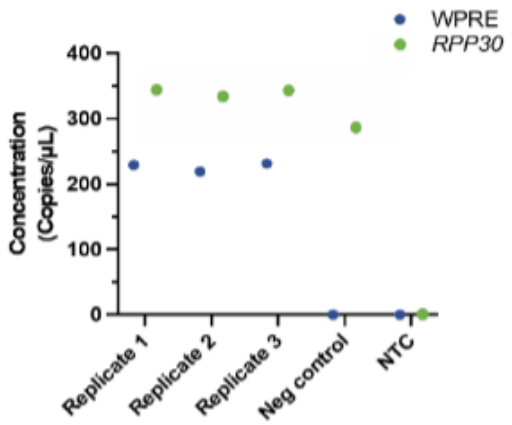


FIGURE 1 Transgene copies per microlitre detected in CAR T cell gDNA by ddPCR. Replicates 1-3 are representative of three individual ddPCR reactions using a 66% CAR T cell population. A non-transduced T cell gDNA sample was used as a negative control. Abbreviations: WPRE, Woodchuck Hepatitis Virus post-transcriptional regulatory element; NTC, no template control.

After obtaining an average transgene copy number by ddPCR, a direct comparison was made to the average transgene copy number for a 66% CAR T cell population generated by our new FISH-based technique. Enumeration data generated as part of the applicant's PhD indicated that the 66% CAR T cell population features an average of 1.28 transgene copies per cell, after accounting for transduction efficiency (66%, estimated through flow cytometric methods) and reaction efficiency (63%, determined by the percentage of nuclei with two clear RPP30 FISH signals). These data are presented in Table 1.

Whilst estimated transgene copies per cell produced by our FISH assay are slightly lower than transgene copies per cell estimated by ddPCR (1.28 copies/cell vs. 1.33 copies/cell), this may be attributable to a lower overall reaction efficiency of our FISH assay when compared to the high efficiency of ddPCR assays. We believe both FISH and ddPCR assays may work synergistically to accurately estimate transgene copies per cell in addition to visualising transgene insertion sites at a single-cell resolution within a CAR T cell population.

NUMBER OF TRANSGENE FISH SIGNALS	FREQUENCY OF SIGNAL PATTERNS IN CAR-POSITIVE NUCLEI	TOTAL NUMBER OF OBSERVED SIGNALS (NUMBER*FREQUENCY)
1	51	51
2	33	66
3	9	27
4	3	12
5+	1	5

TABLE 1 Enumeration data generated by FISH for 200 CAR T cell nuclei. Data generated as part of the applicant's PhD studies using a 66% CAR T cell population. Abbreviations: FISH, fluorescence in situ hybridization; CAR, chimeric antigen receptor.

CONCLUSION

Our data confirms that our new FISH-based method for transgene enumeration and visualisation in CAR T cells can produce similar estimations of transgene copies per cell to ddPCR methods. With further development and refinement, our FISH technique holds promise for the routine detection of transgene copy numbers in other gene-edited cell therapy products as an additional quality control approach prior to patient infusion.

A calming influence from your MAITs: Unveiling MAIT cell mechanisms safeguarding lung tissue during influenza

INTERIM REPORT

H BOSWELL, I HERMANS, L CONNOR

KEY FINDINGS

Our work aims to harness the activity of Mucosal Associated Invariant T (MAIT) cells to shift the lung immune landscape towards an anti-inflammatory state, aiming to protect against influenza infection. We performed single-cell RNA sequencing of lung immune cells and developed a new protocol to prepare single-cell suspension of lung tissue enriched with MAIT cells, a rare population. Sequence libraries have been generated and bioinformatic analysis is about to start. Insights from our RNA sequencing will help us understand how activating MAIT cells can mitigate the negative effects of influenza infection.

OBJECTIVES

This project aimed to develop a method to isolate MAIT and other immune cells that protect the lung from damage during influenza. We aim to achieve this by performing single-cell RNA sequencing of isolated cells.

METHODS

MAIT cells represent a very small percentage of a lung immune compartment. These numbers can be increased by performing intranasal administration of the MAIT cell activating ligand, 5-OP-RU (Fig 1A). To assess how enhancement of MAIT cells can alter the lung immune landscape, two 'vaccine groups' were produced. Mice were given intranasal treatment of 5-OP-RU or a vehicle control three times, two weeks apart. These mice were culled at day 49 and the lungs harvested. To assess how MAIT cell enhancement can alter the course of an influenza infection, two 'influenza groups' were made. In the influenza groups mice were treated with the vaccination schedule and given influenza two weeks after the final 5-OP-RU or vehicle treatment. These mice were culled at day three post influenza infection to look at the changes in MAIT cells and other antigen presenting cells which have the potential to aid viral clearance and lung pathology. Lungs from three mice per experimental condition were collected for single-cell RNA sequencing.

Isolated lung immune cells were obtained for single-cell RNA sequencing by cell sorting (Fig 1B). The lung tissue was processed to obtain single cell suspensions. Cells were tagged with fluorescent antibodies to detect live cells, immune cells and MAIT cells. Cells were run through an influx cell sorter to obtain live MAIT and immune cell populations. The cells were mixed at a ratio of 1:10 MAIT to immune cells. Once mixed, the cells were tagged with specific oligonucleotide antibodies to identify each treatment group, mixed, and run on the BD Rhapsody. The BD Rhapsody works in a well and magnetic bead-based selection of RNA. Cells were gently flowed over a cartridge containing microwells, a magnetic bead capable of catching RNA was then added to each well. The cells were then lysed, RNA bound to the bead, which were magnetically removed from the wells. The RNA was converted to stable cDNA and sequenced at the Ramaciotti Centre for Genomics, UNSW Sydney. The data is currently being analysed, using the Seurat package in R to identify the transcripts for each cell and each cells phenotype and function.

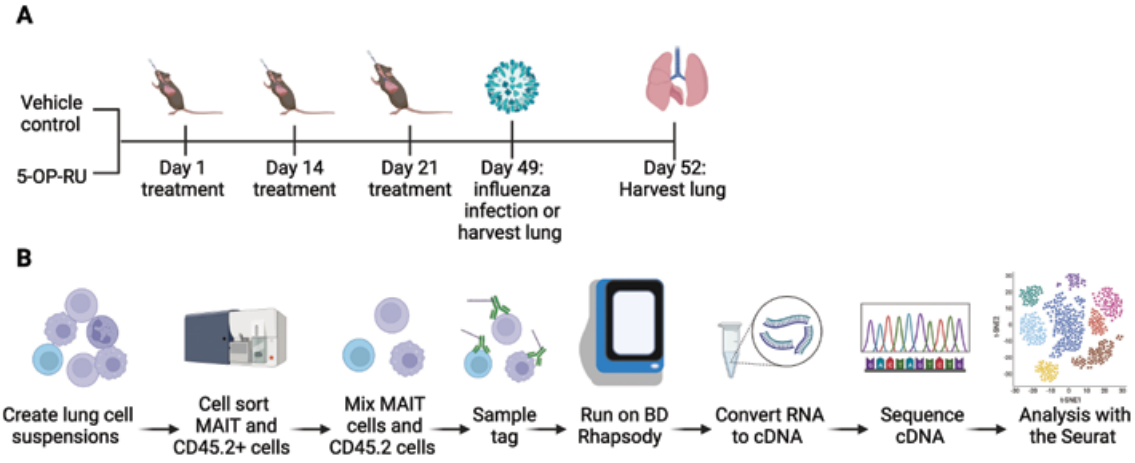


FIGURE 1: Schematic detailing the A) timeline of 5-OP-RU dosage schedule and B) experimental design of single-cell RNA sequencing experiment.

RESULTS OR FINDINGS

We developed a cell sorting method to isolate and enrich MAIT cells from the lung, addressing their rarity in the murine lung which makes them difficult to detect with single-cell RNA sequencing. Using an influx cell sorter (Fig 2), we stained cells with fluorescent antibody markers for MAIT cells (TCRß and MR1 tetramer), total immune cells (CD45.2) as well as markers for high abundant cell types such as monocytes and B cells (Ly6G and CD19). Populations that were doublets, dead, or low quality were separated out from MAIT cells or non-MAIT immune cells based on their fluorescence and size (Fig 2). MAIT cells and non-MAIT immune cells were isolated in the 'MAIT cell sort gate' and 'Live immune cell sort gate' and mixed at a 1:10 ratio to enrich for MAIT cells while retaining the lung immune cell environment.

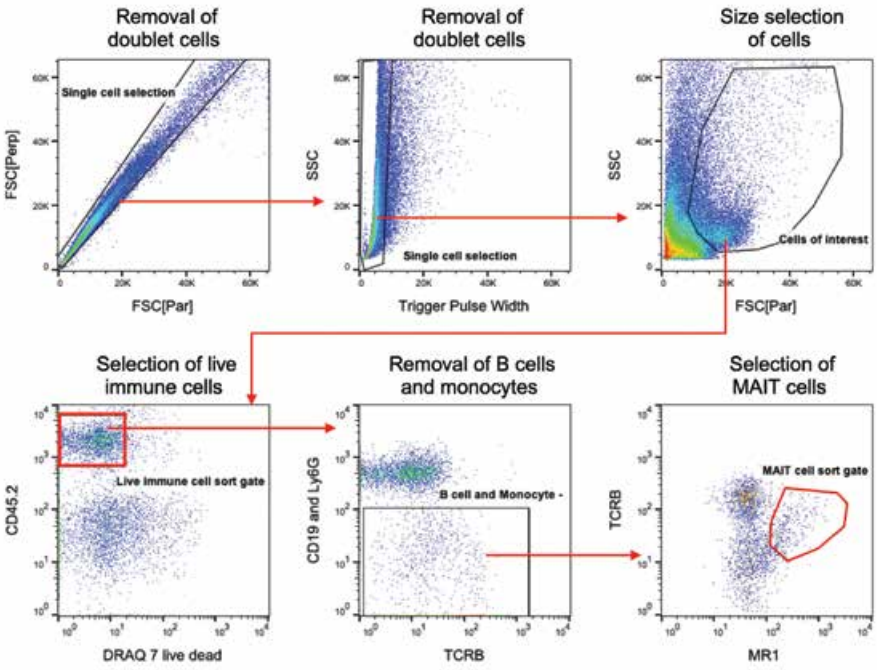


FIGURE 2: Cell sort example of 5-OP-RU and immune cell purification by the influx cell sorter.
The enriched populations were processed for single-cell RNA sequencing, which typically results in significant cell loss, necessitating the collection of large numbers of cells from the cell sorter. We isolated 200,000 immune cells and between 8000 to 20,000 MAIT cells, depending on treatment group (Table 1). After pooling, RNA extraction, and collection, we obtained 36,097 cells from the vaccine groups and 41,945 from the influenza groups for analysis. Clustering algorithms require at least 100 cells per cell type, so we collected a surplus to ensure that sufficient quality for analysis. We are currently in the quality control and cell identification stages of the analysis.

SAMPLE	NUMBER OF CD45.2+ IMMUNE CELLS SORTED	NUMBER OF MAIT CELLS SORTED	RATIO OF MAIT:CD45.2+ IMMUNE CELLS RAN ON BD RHAPSODY	FINAL NUMBER SUCCESSFULLY CAPTURED WITH BEAD FOR SEQUENCING
Vehicle	200,000	8,001	1:10	36,097
5-OP-RU	200,000	20,003		
Vehicle + influenza	200,000	10,000	1:10	41,945
5-OP-RU + influenza	200,000	20,000		

TABLE 1: Cell number and viability of cells isolated from murine lung by the influx cell sorter and ran on the BD Rhapsody.

The analysis of this dataset is in progress using Seurat package in R. Seurat allows for selection of high-quality cells and clustering analysis to group immune cells with similar gene expression together. The cell subset each cluster represents can be identified based on transcriptomic signatures. Differential gene analysis will be performed to identify key pathways that have altered between the MAIT enriched groups and vehicle controls, and how this alters during an influenza infection.

CONCLUSION

We have successfully developed a novel method to prepare single-cell suspensions of lung cells enriched with MAIT cells. High quality sequence data from mice treated with MAIT ligand 5-OP-RU and infected with Influenza has been generated. This data will help us understand how enriching MAIT cells can aid in protection during influenza infection.

Te Herenga Waka
Victoria University of Wellington
Malaghan Institute of Medical Research

Engineering self-protection enzymes to enable CAR T-cells to combat solid tumours with precision chemotherapy

A SHARROCK, R WEINKOVE, M TERCEL, D ACKERLEY

KEY FINDINGS

My research has employed both database mining and function-based screening to uncover two classes of enzymes capable of protecting human cells against a promising next-generation DNA alkylating drug. I have demonstrated that the cellular location of these protective enzymes strongly influences their activity, with one functioning best in the nucleus whilst the other functions best in the cytoplasm. This suggests that one plays a specific DNA repair role, while the other may be detoxifying the drug directly. Additionally, I have determined that substantially more protection can be conferred when one enzyme from each class is co-expressed in human cells.

AIMS

Chemotherapy and immune cell therapies are two essential forms of cancer treatment, however they are largely incompatible with one another, as immune cells are inherently sensitive to chemotherapeutic agents. The overarching goal of this research is to identify and characterise novel protective enzymes that can effectively shield engineered immune cells from specific forms of chemotherapy-induced DNA damage. Previously, I identified top candidate protective enzymes through bioinformatic analyses and function-based metagenomic screening, characterised their protective activities in a model human cell line, and attempted to enhance their activities through directed evolution. I now aim to i) investigate whether the protective function of these enzymes can be enhanced by manipulating their cellular localisation, and ii) determine whether protective enzymes of different classes can be co-expressed in human cells to provide complementary forms of protection against a next-generation DNA alkylating drug.

METHODS

To explore the impact of cellular localisation on enzymatic activity, nuclear localisation signal (NLS) sequences were fused to the N-terminus of two lead protective enzymes from different classes; i) one identified through metagenomic screening, classed as a putative transcriptional regulator ('MG'), and ii) one identified through bioinformatic analyses ('Alkrep'), classed as a DNA repair protein. Gene constructs encoding each protein were synthesised, cloned into a mammalian expression vector and expressed stably in model human cell lines (HEK-293). These cell lines were then exposed to increasing concentrations of a DNA alkylating drug, and their sensitivity was compared using cell viability assays. To detect intracellular localisation of the native enzymes and confirm nuclear trafficking of the NLS fusions, each enzyme was also fused to a green fluorescent protein partner, and fluorescence confocal microscopy was used to detect the intracellular localisation of each protein.

To investigate whether co-expression of 'MG' and 'Alkrep' can enhance cell protection against DNA alkylating drugs, a single gene construct encoding both enzymes, separated by a self-cleaving peptide sequence, was designed and expressed stably in HEK-293 cells. Cell viability assays were conducted to assess the drug sensitivity of this cell line, compared to HEK-293 cell lines stably expressing either MG or Alkrep alone.

RESULTS

A comparison of the drug sensitivity of HEK-293 cell lines expressing MG and Alkrep enzymes, with or without NLS sequences, revealed that nuclear localisation strongly influences their activity. Fusion of a NLS to Alkrep slightly increased its protective effect, whilst fusion of a NLS to

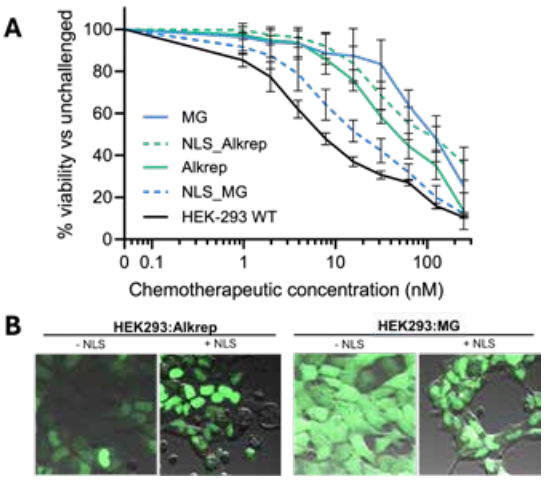


FIGURE 1. A. Drug sensitivity of HEK-293 cell lines expressing protective enzymes with or without a fused NLS. **B.** Confocal fluorescence microscope images of HEK-293 cells expressing GFP-tagged proteins with or without a fused NLS.

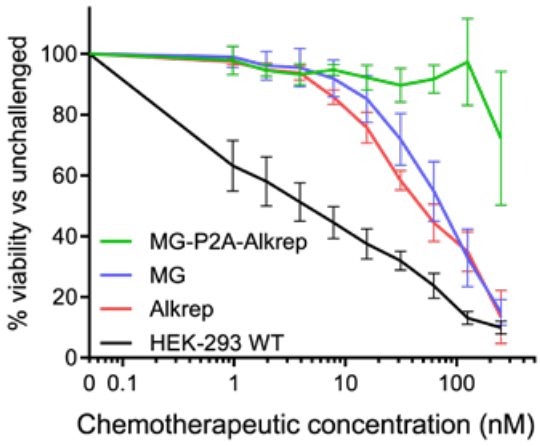


FIGURE 2. Drug sensitivity of HEK-293 cell lines expressing one, or both, protective enzymes.

MG decreased its ability to protect HEK-293 cells against DNA alkylation (Figure 1A.). The former result is consistent with the putative function of Alkrep, annotated as a DNA repair protein presumed to act upon DNA in the nucleus. Nuclear localisation of the NLS-tagged fluorescent fusion constructs was confirmed through fluorescence microscopy (Figure 1B.). We also observed the native localisation of MG and Alkrep, which appear to be expressed primarily in the cytoplasm and nucleus, respectively.

To investigate whether co-expression of MG and Alkrep could protect HEK-293 cells against DNA alkylating drug toxicity more effectively than each protein on its own, a HEK-293 cell line expressing both proteins was generated and tested. Cell viability assays indicated that HEK-293 cells expressing both proteins are substantially more protected against DNA alkylating toxicity than cells expressing either protein alone (Figure 2.).

CONCLUSION

Through bioinformatic analyses and metagenomic screening, I have identified two protective enzymes of different classes, MG and Alkrep, capable of shielding HEK-293 cells from the toxicity of a DNA alkylating drug. By comparing the sensitivity of HEK-293 cell lines expressing either enzyme localised to different regions of the cell, I determined that the MG enzyme functions best in the cytoplasm, whilst the Alkrep enzyme function can be improved by maximising localisation in the nucleus. Confirming the native cytoplasmic localisation of the metagenomic protein brings us one step closer to understanding its protective mechanism. I also determined that co-expression of MG and Alkrep substantially enhances drug resistance in a model human cell line. This project is ongoing, and our future plans involve testing and characterising these enzymes in human chemo/immunotherapy models.

Te Herenga Waka
Victoria University of Wellington

In vitro characterisation of the specific pattern of immunomodulation in macrophages treated with a collection of novel compounds

E-R GRANT[1], J MCCONE[2], R KEYZERS[2], J HARVEY[2], A LA FLAMME[1,3]

[1] Centre for Biodiscovery, School of Biological Sciences, Victoria University of Wellington, New Zealand.

[2] Centre for Biodiscovery, School of Chemical and Physical Sciences, Victoria University of Wellington, New Zealand.

[3] Malaghan Institute of Medical Research, Wellington, New Zealand.

KEY FINDINGS

Macrophages were exposed to pro-inflammatory stimuli and treated with the novel Btk inhibitor (-)-TAN-2483B. Exposure to (-)-TAN-2483B led to the formation of macrophages with a wound-healing phenotype, shown by increased production of anti-inflammatory mediators and reduced pro-inflammatory cytokines and harmful reactive oxidants. These results indicate that (-)-TAN-2483B may be able to resolve inflammatory processes in other the macrophage-like cells found in the brain called microglia.

INTRODUCTION

Macrophages adapt to their microenvironment, which makes them an ideal model for screening novel compounds for immunomodulatory activity. *In vitro* studies show two distinct states of macrophage activation at opposing ends of the polarisation spectrum. Pro-inflammatory M1 macrophages have important roles in immunosurveillance and pathogen clearance, whereas wound-healing M2 macrophages are involved in resolution of inflammation and maintenance of tissue homeostasis. Imbalance in the functional activation state of macrophages can lead to chronic inflammation and autoimmune disease formation. Using a macrophage-focused screening platform, we have identified compounds with immunomodulatory activity that modulate the activation states of macrophages.

(-)-TAN-2483B is a metabolite that was first discovered in fungal cultures and was completely synthesised from D-mannose in 14 steps (McCone et al., 2020). (-)-TAN-2483B is a selective inhibitor of Bruton's tyrosine kinase (Btk) (IC50 of 5.1μM). Btk is an important signalling molecule in a variety of immune cells, including macrophages, and is implicated in the formation of chronic inflammatory diseases such as multiple sclerosis.

OBJECTIVES

The aim of this project was to investigate the immunomodulatory effect of novel compounds on macrophages, which was addressed in 2 specific objectives:

Objective 1: Assess the immunomodulatory potential of (-)-TAN-2483B to re-polarise pro-inflammatory macrophages.

Objective 2: Investigate changes in macrophage protein expression upon treatment with (-)-TAN-2483B.

RESULTS

Objective 1: Assess the immunomodulatory potential of (-)-TAN-2483B to re-polarise pro-inflammatory macrophages.

Through our macrophage-focused screening platform, we identified the novel Btk inhibitor (-)-TAN-2483 as a compound which modulated macrophages away from an induced pro-inflammatory state ***(Figure 1a)***. In BMDM generated by GM-CSF and IL-3, which are inflammatory, (-)-TAN-2483B reduced the production of pro-inflammatory mediators IL-12, IL-6, and NO, whilst it increased production of anti-inflammatory cytokine IL-10 in a dose-dependent

manner. In M-CSF macrophages, which are neutral, we compared the activity of (-)-TAN-2483B to ibrutinib, a potent Btk inhibitor with well characterised off-target effects ***(Figure 1b, 1c)***. At the 2.5μM concentration, (-)-TAN-2483B did not increase IL-10 production as seen in GM-CSF+IL-3 BMDM; however potent NO inhibition was still observed, along with a modest reduction in IL-12. This immunomodulatory profile is distinct from Ibrutinib.

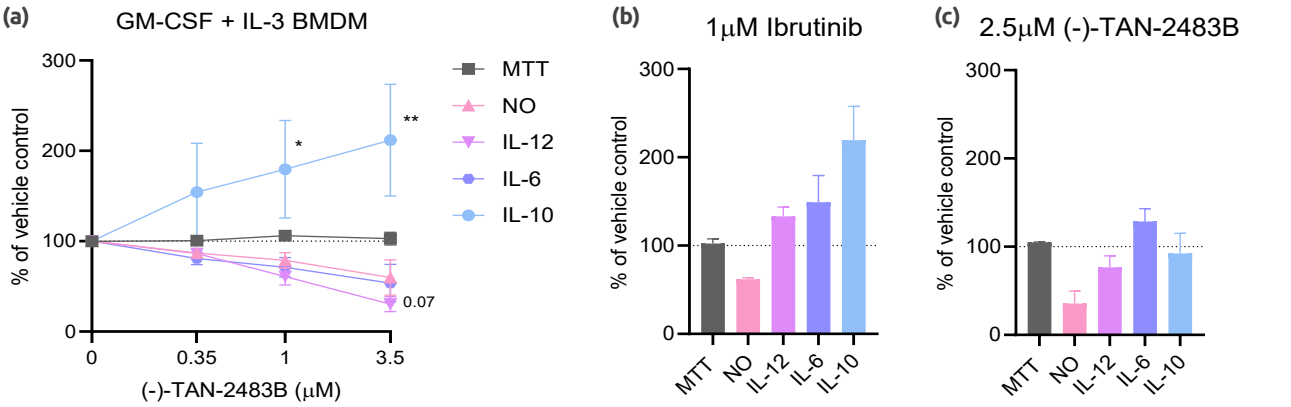


FIGURE 1: BMDM were generated over a period of 9 days by either **(a)** GM-CSF and IL-3, or **(b, c)** M-CSF methods. BMDM were IFN-γ primed for 18 hours, then stimulated with LPS and co-treated with (-)-TAN-2483B, Ibrutinib, equivalent vehicle control, or media for 48 hours. Cells were subject to MTT assay and supernatants were used for Griess (for NO detection) and ELISA (for IL-12, IL-6, and IL-10). Results are from three independent replicate experiments, and data is represented as mean ± SEM. *p≤0.05, **p≤0.01 by two-way ANOVA with Dunnett's multiple comparisons.

Based upon the results of immunomodulatory screening, we sought to analyse in greater depth the potential of the novel Btk inhibitor (-)-TAN-2483B to repolarise pro-inflammatory macrophages towards a phenotype associated with wound-healing and immune resolution. We also wanted to determine the mechanism by which (-)-TAN-2483B reduced the ability of these macrophages to produce NO. The enzyme inducible nitric oxide synthase (iNOS) facilitates the conversion of L-arginine into NO, therefore we hypothesised that (-)- TAN-2483B may be targeting iNOS to inhibit NO production. We determined that (-)-TAN-2483B reduced the expression of M1-associated markers CD40 and CD80, and modestly upregulated CD206 expression, a marker associated with wound-healing macrophages ***(Figure 2)***. In addition, iNOS expression was downregulated, providing confirmation of the mechanism by which (-)-TAN-2483B reduces NO production. CD163, a marker indicative of a regulatory macrophage phenotype, was also significantly reduced upon (-)-TAN-2483B treatment. Overall, the changes elicited by (-)-TAN-2483B treatment do not match a traditional M2 wound-healing phenotype, nor do they match ibrutinib's effects, displaying the unique effect of (-)-TAN-2483B.

Objective 2: Investigate changes in macrophage protein expression upon treatment with (-)-TAN-2483B.

We investigated whether the immunomodulatory impact of (-)-TAN-2483B treatment observed in macrophages was due to inhibited phosphorylation of: 1) Btk-Tyr223; 2) Syk-Tyr352 (the kinase directly upstream of Btk); and/ or 3) downstream signalling proteins (STAT3, p65 NFκB, ERK1/2, or p38-MAPKα). (-)-TAN-2483B modestly reduced Btk phosphorylation and left Syk phosphorylation unaltered. In contrast, ibrutinib significantly inhibited both Syk and Btk phosphorylation ***(Figure 3a, 3b)***. Thus, the immunomodulatory impacts of (-)-TAN-2483B treatment in macrophages were likely due to downstream effects of this subtle Btk inhibition. In support of this idea, the phosphorylation of signalling proteins p38-MAPKα, STAT3, and p65 NFκB but not ERK1/2 were significantly inhibited by (-)-TAN-2483B treatment ***(Figure 3c)***.

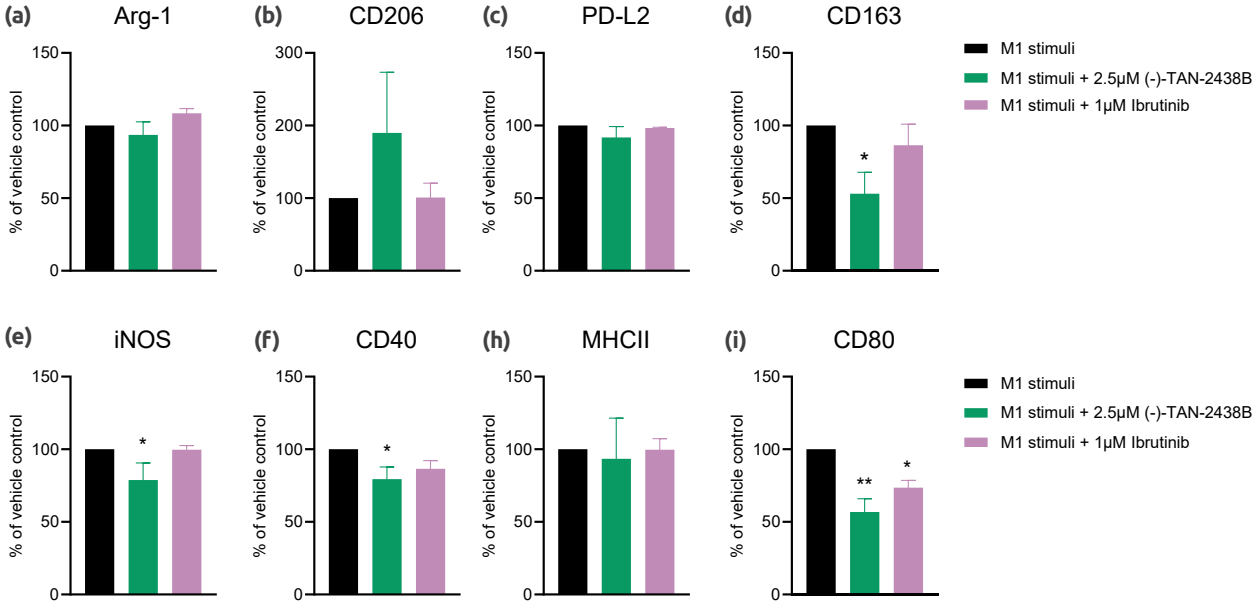


FIGURE 2: BMDM were stimulated with M1 stimuli IFN-γ and LPS, and co-incubated with (-)-TAN-2483B (2.5μM), Ibrutinib (1μM) or equivalent vehicle controls for 24 hours. Macrophages were stained for M2-associated markers **(a)** Arg-1, **(b)** CD206, **(c)** PD-L2 and **(d)** CD163, and M1-associated markers **(e)** iNOS, **(f)** CD40, **(g)** MHCII, and **(h)** CD80. Results are from three independent replicate experiments, and data is represented as mean ± SEM. *p≤0.05, **p≤0.01 by one-way ANOVA with Dunnett's multiple comparisons.

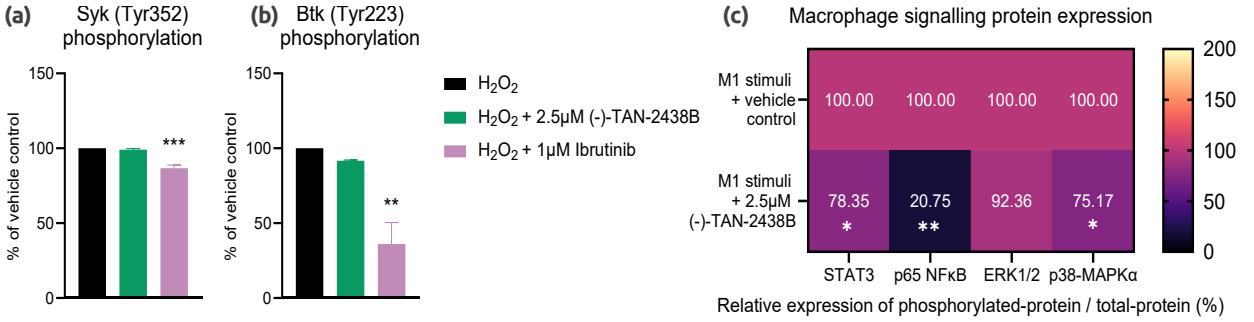


FIGURE 3: BMDM were pre-treated with (-)-TAN-2483B (2.5μM), Ibrutinib (1μM), or equivalent vehicle control, then stimulated with H2O2 for 15 minutes. Macrophages were stained for **(a)** Syk-p (Tyr352) and **(b)** Btk-p (Tyr223). Results are from three independent replicate experiments, and data is represented as mean ± SEM. *p≤0.05, **p≤0.01 by one-way ANOVA with Dunnett's multiple comparisons. **(c)** BMDM were pre-treated with (-)-TAN-2483B (2.5μM) or vehicle control, then stimulated with LPS for 0.5, 1, and 2 hours. After cell lysis, lysates were used to detect phosphorylated- and total-protein levels of STAT3, p65 NFκB, ERK1/2, and p38-MAPKα. Results are from three independent replicate experiments, and data is represented as the mean. *p≤0.05, **p≤0.01 by unpaired t-test..

CONCLUSION

In this project, we have confirmed the novel Btk inhibitor (-)-TAN-2483B is an immunomodulatory compound with the ability to re-polarise macrophages away from a pro-inflammatory state, and these activities are distinct from ibrutinib. We discovered that the reduction in NO production by these macrophages is a result of reduced iNOS expression by (-)-TAN-2483B treatment. We also uncovered that the immunomodulatory effects of (-)-TAN-2483B treatment in macrophages is likely due to downstream effects of Btk inhibition, such as reduced activation of proteins STAT3, p65 NFκB, and p38-MAPα. These findings show us that (-)-TAN-2483B holds promise as an immunomodulator to reduce the harmful pro-inflammatory effects of dysregulated or overactive Btk signalling.

Te Herenga Waka
Victoria University of Wellington
University of Canterbury
Ferrier Research Institute, Australian Synchrotron

Structural and functional characterisation the FAM171 family of neuronal proteins

T BIRD, D WOOD, Z TILLET, F DE POL, G MITTELSTÄDT, R DOBSON, D COMOLETTI

KEY FINDINGS

Protein structure determines protein function, thus, characterising a proteins three-dimensional structure allows us to understand at a molecular level how a protein interacts with other proteins and ligands within the human body. The FAM171 neuronal receptors are an understudied family of three proteins with no published functional or structural data despite their genetic implications in multiple types of cancer, and neurodegenerative diseases. In this ongoing project we have identified an interaction network within the FAM171 family. We have also found that despite high sequence and predicted structural similarity, each FAM171 protein has distinct structural features that can be inferred by different biochemical behaviours.

OBJECTIVES

1. Determination of the binding characteristics of the FAM171 family of proteins. Specifically,
    a. Identification of new FAM171 binding partners.
    b. Biophysical characterisation of these interactions using complementary methods.
2. Determination of the three-dimensional structures of each protein and protein complex. Specifically,
    a. The oligomeric state of individual proteins and stoichiometry of interacting proteins.
    b. Low-resolution, in-solution structures.
    c. High-resolution crystal structures.

METHODS

a. Identification of new FAM171 binding partners utilised an ELISA-based proteomic screen. Proteins were expressed in mammalian cells and interactions between the three FAM171 proteins and a library of ~1000 neuronal proteins were then assessed in a modified sandwich ELISA format.

b. Large scale expression of FAM171 proteins required generation of stable cell lines using HEK293 GnTI- cells. Protein was secreted into the cell culture media and purified using Protein A affinity resin, followed by size exclusion chromatography (SEC).

c. Binding affinities were determined using purified protein in an affinity ELISA.

d. The oligomeric state of proteins was assessed using sedimentation velocity analytical ultracentrifugation (AUC) experiments. These were carried out at the University of Canterbury using an Optima AUC at 10°C with an An-50 Ti rotor at 50,000 rpm. All purified proteins were used at concentrations of 2 and 20μM.

e. Small angle x-ray scattering (SAXS) was also used for investigating protein behaviour in-solution. These experiments were carried out on the SAXS/WAXS beamline at the Australian Synchrotron. SEC-SAXS with protein at a concentration of 5mg/mL with a co-flow setup was used for data collection.

f. Determination of high-resolution protein structure utilised protein crystallography techniques. 576 crystallisation conditions were screened for each protein. FAM171A1 crystallised readily in more than ten unique conditions and approximately 200 FAM171A1 crystals were tested for diffraction on the MX1/2 beamlines at the Australian Synchrotron.

RESULTS

Our ELISA-based proteomic screen identified a novel interaction network within the FAM171 family, where FAM171 A1 and A2 both exhibit self-interaction as well as both binding to FAM171B **(Figure 1A)**. To investigate these interactions in further detail, we purified the extracellular domain of each FAM171 protein from mammalian cells to ensure proper folding and glycosylation. Each protein expressed exceptionally well (>50mg/L conditioned media) which has allowed for a larger range of characterisation experiments to be performed. Purification by SEC also revealed that each protein behaves differently in solution, despite their predicted structural similarities **(Figure 1B)**. AUC experiments provided further evidence for protein oligomerisation. A1 was found to be a stable trimer whilst A2 and B shift between monomeric and trimeric states in a concentration-dependent manner. A2 and B appear to bind with a 1:1 ratio.

Initial SAXS experiments show each FAM171 protein to form trimers at high concentrations, which is consistent with SEC and AUC experiments. Future experiments will utilise the new BioSAXS beamline at the Australian Synchrotron, examining protein behaviour at a greater range of concentrations and hopefully confirming the monomer-trimer equilibrium.

Relative binding affinities for the interactions found in our screen were in the nanomolar range **(Figure 1C)**, suggesting strong interactions. In particular, the A2 and B interaction was found to be stronger than A2 binding to itself. Future in vitro experiments will explore if these interactions are inter- or intra-cell and their potential role in facilitating cell communication or adhesion.

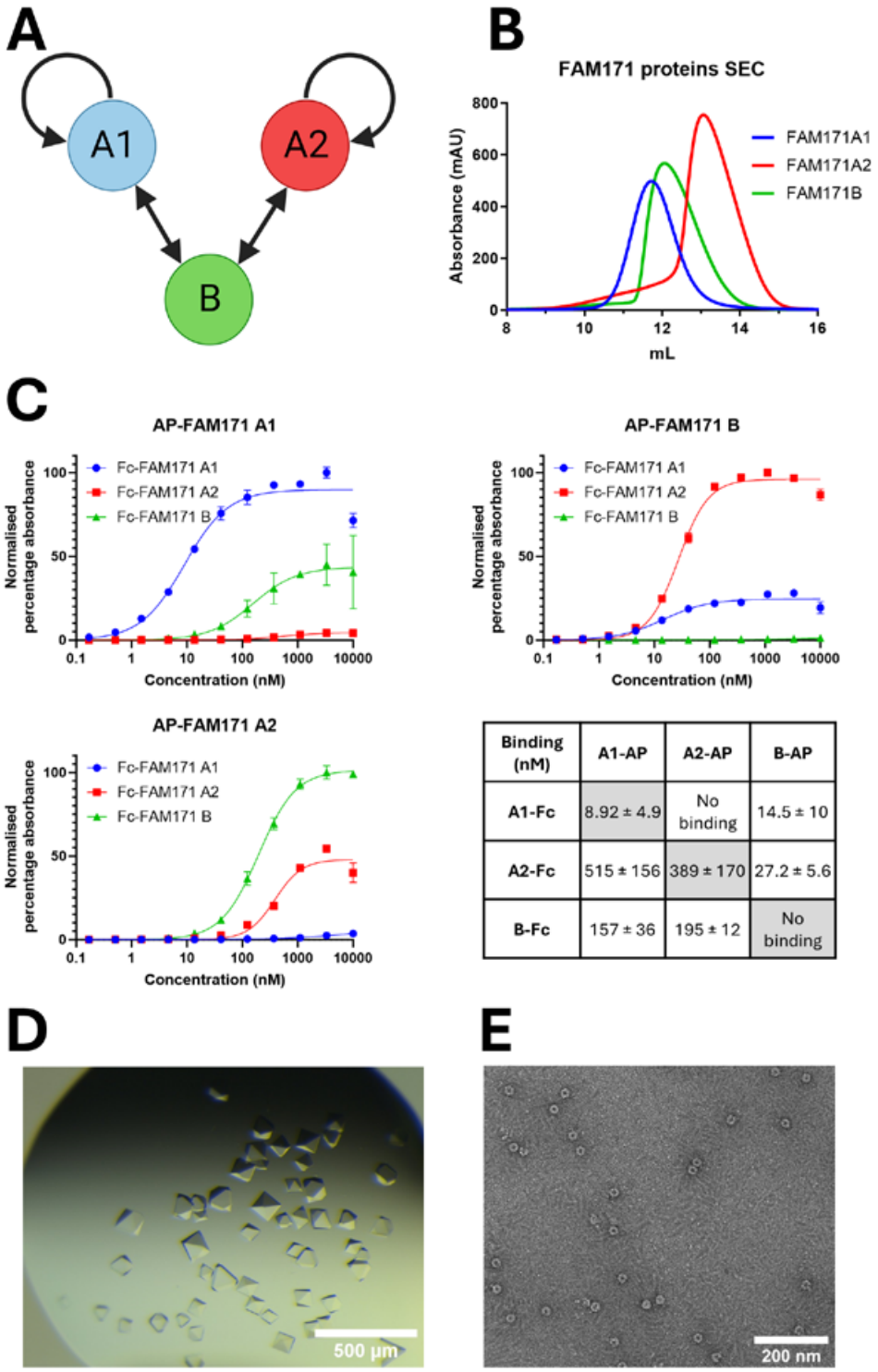
X-ray crystallography was used extensively to attempt to elucidate a high-resolution molecular structure for each protein and definitively confirm the oligomeric state of each protein.

Unfortunately, the best diffracting crystals for A1 **(Figure 1D)** and B only diffracted to a resolution of 4.52 Å and 5.65 Å, respectively. Ideally a resolution of 3.5 Å or lower is required for a detailed protein structure with precise amino acid positions. Fortunately, we have received another Research for Life grant to continue this work at the University of Melbourne using an alternative technique, cryo electron microscopy. Early negative stain results for FAM171A1 look promising **(Figure 1E)**.

CONCLUSION

We have made good progress in resolving the structure and function of the FAM171 family of proteins. Large scale expression and purification of the FAM171 proteins has allowed us to show how they interact with each other and themselves. Complementary techniques show each protein forms trimers of varying stability.

Using the resources from a new Research for Life grant, we have started expression and purification of truncated protein constructs which we expect to be easier to work with and streamline protein structure elucidation. We will utilise cryo-EM and BioSAXS facilities in Melbourne in order to gain high-resolution and in-solution protein structures. A cell-based assay will also be used to assess whether the FAM171 interactions are transcellular. We hope an increased understanding of the structure and function of FAM171 proteins will provide insights into their roles in human diseases.



Binding (nM)	A1-AP	A2-AP	B-AP
A1-Fc	8.92 ± 4.9	No binding	14.5 ± 10
A2-Fc	515 ± 156	389 ± 170	27.2 ± 5.6
B-Fc	157 ± 36	195 ± 12	No binding

FIGURE 1. (A) The FAM171 interaction network. **(B)** Size exclusion chromatography traces of purified FAM171 proteins demonstrating different sizes in-solution. Larger protein species elute first (smaller elution volume). **(C)** Affinity ELISA graphs using purified protein confirming the FAM171 interaction network identified in the proteomic screen. Data was normalised and fit using the specific binding with Hill slope function in GraphPad Prism 9. Binding affinities with 95% confidence intervals are shown in the table bottom right. Self-interactions are highlighted in grey. **(D)** Example of FAM171A1 crystals taken to the Australian Synchrotron for analysis. Crystals grown in hanging drops using in 0.6M trimethylamine N-oxide dihydrate (TMAO), 0.1M Tris pH 8.5, 16% PEG monomethyl ether 2000. **(E)** Negative stain electron microscopy image of purified FAM171A1 at 10 µg/mL using 1% uranyl acetate.

Te Herenga Waka
Victoria University of Wellington

SARS-CoV-2 specific cross-reactive clones arise within lineages of mono-reactive clones

I MONTGOMERIE[1], R MCKENZIE[2], S VON DRAKE[2], S SMALL[2], O PALMER[2], N MASON[2], S. DRAPER[2], L CONNOR[1]

[1] School of Biological Sciences, Victoria University of Wellington, Wellington, New Zealand

[2] Malaghan Institute of Medical Research

KEY FINDINGS

In this work we find that cross-reactive antibodies are specifically amplified by our novel SARS-CoV-2 vaccine. These antibodies are generated by mutations that arise in the antibody genes during the immune response.

OBJECTIVES

COVID-19, the disease caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has had a profound impact on human health at a global scale and is responsible for the deaths of more than 7 million people. The goal of this project is to assess the capacity of a novel vaccine to preferentially amplify antibodies against immunological targets on SARS-CoV-2 that are conserved between variants of concern, and thus, predicted to provide cross-protection against new and emerging variants.

SARS-CoV-2 mediates cell entry through the viral spike protein binding to its receptor protein, angiotensin converting enzyme 2 (ACE2) on host epithelial cells (Carnell et al.; Ou et al., 2020; Starr et al., 2020). As such, the spike protein has been the target for most vaccine strategies (Martinez-Flores et al., 2021; Walls et al., 2020). The SARS-CoV-2 spike is a homotrimer, where each monomer is composed of an S1 and S2 domain (Ou et al., 2020). The S1 domain consists of an N terminal domain (NTD) followed by the receptor binding domain (RBD) which directly interacts with ACE2. Immunisation with a tandem dimer of the RBD generates high levels of neutralising antibodies compared to the RBD monomer (Pan et al., 2021), and protects mice from infection following live SARS-CoV-2 challenge (Dai et al., 2020). We have designed a heterodimer subunit vaccine, which consists of a tandem RBD dimer, where each RBD is derived from different SARS-CoV-2 variants of concern. One of the RBDs in the dimer is from the Delta variant, and one is from the Omicron variant. We hypothesise that our heterodimer design will improve the generation of cross-protective antibodies.

Antibodies are produced by immune cells called B cells. Antibodies bind to their targets through a specific region of the protein called the “variable region”. During B cell development, random segments of DNA are combined to generate unique variable regions for each antibody. Therefore, each B cell encodes a unique antibody. B cells express the antibody they encode on their surface. When the antibody is expressed on the surface of the cell it is called the B cell receptor (BCR). When the antibody on the surface of the cell (the BCR) binds its target, this activates the cell. This will cause the B cell to divide, leading to the expansion of a specific B cell clone. In addition, B cells undergo a process called affinity maturation. During affinity maturation B cells introduce random mutations in the variable region of their antibody genes. The B cells then “test” their newly mutated BCR against the pathogen target. B cells carrying mutations in the variable regions that improve antigen binding are preferentially expanded. This improves the quality of the antibody response overall.

METHODS

We utilised flow cytometry to identify and isolate B cells expressing cross-reactive antibodies from immunised mice. Because B cells express the antibody they encode on their surface, we can identify B cells that are specific for a protein of interest by labelling cells with a fluorescently tagged version of this protein. We fluorescently labelled SARS-CoV-2 proteins from different variants of concern, using

different florescent wavelengths for the different variants of concern, so that we could identify B cells that bound the SARS-CoV-2 proteins from more than one variant of concern. We could compare B cells that bound to multiple variants to B cells that only bound to one or two. We could then use next generation immunoglobulin sequencing to investigate how these antibodies are generated by our novel pan-coronavirus vaccine approach.

RESULTS

Objective one: identifying cross protective SARS-CoV-2 RBD binding antibodies.

We have implemented a flow cytometry-based method to identify B cells which bind a broad panel of spike proteins. We compared the frequency of cross-reactive B cells following immunisation with our Delta-Omicron heterodimer vaccine, a Delta-Delta homodimer vaccine, and a full-length spike vaccine as a benchmark. We saw that our heterodimer vaccine generated higher frequencies of B cells that bound the Delta variant RBD, as well as the ancestral strain of SARS-CoV-2 (Wuhan stain) RBD and 2002 pandemic SARS-CoV-1 RBD.

Objective two: sequencing SARS-CoV-2 cross-reactive B cells..

We analysed cross binding among B cells that were specific for the RBD from the Delta variant of SARs-CoV-2. Of the Delta binding cells, we identified cells that bound the ancestral strain of SARS-CoV-2 (Wuhan stain) or bound the 2002 pandemic strain SARS-CoV-1. Cells that bound neither of these additional variants were considered single binding, and cells that bound both additional variants were considered highly cross reactive. We also sorted cells that bound only one of the additional RBDs, two RBDs in total. Having sorted the binding cells into these four populations, the cells were sent for next generation sequencing, and the antibody sequences were analysed. Lineage analysis will allow an assessment of how cross-reactive SARS-CoV-2 B cells are generated.

We hypothesise cross-reactive antibodies could be induced by two separate non-mutually exclusive mechanisms. During affinity maturation, antibody genes are mutated to generate higher affinity antibodies. High affinity antibodies are more likely to bind an RBD derived from a variant different from the one against which they were raised. The second hypothesis is that the heterodimer may specifically amplify

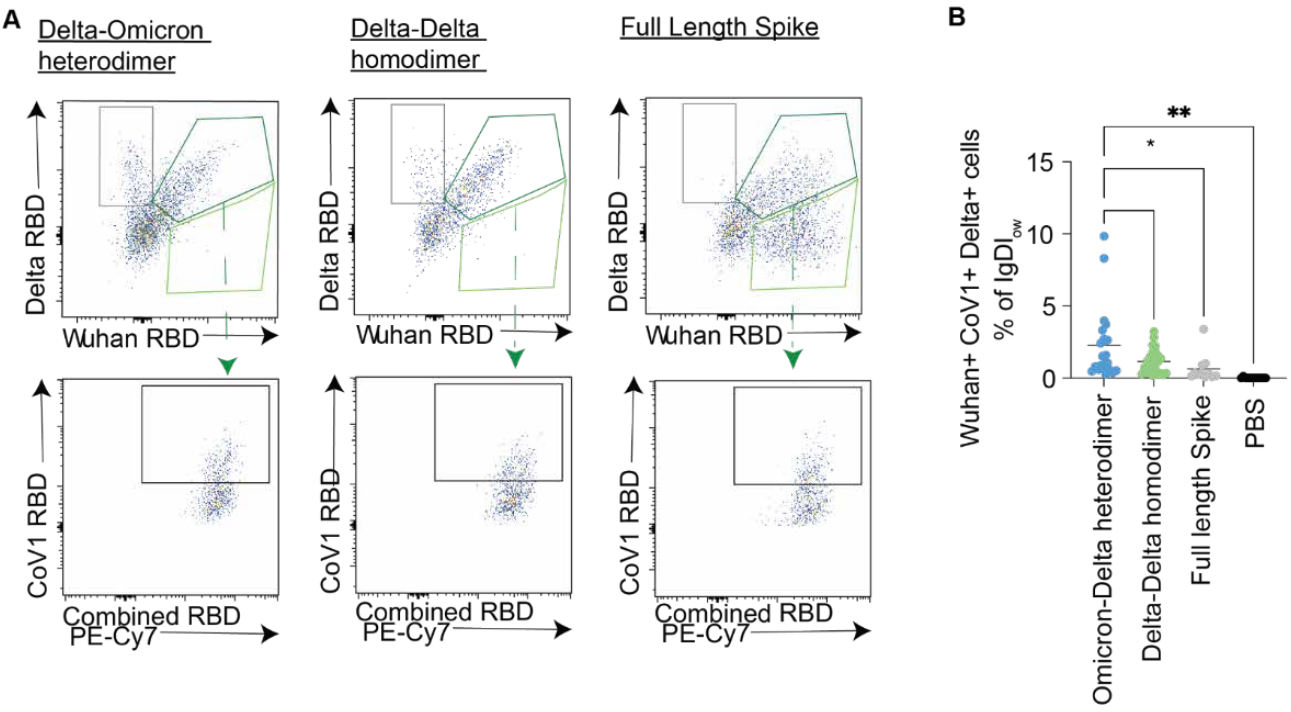


FIGURE 1. Heterodimer-based vaccine increases cross-reactivity of B cells. Mice were immunised twice, 21 days apart, and culled on day 28. The cross-binding capacity of B cells was addressed using fluorescent probes against the SARS-CoV-2 RBD from the Delta and ancestral (Wuhan) variant, and against the SARS-CoV-1 RBD. Representative plots are shown in (A) and (b) show triple binding cells as a frequency of IgDl B cells.

B cell clones encoding antibodies that recognise conserved regions shared by both variant strains contained within the heterodimer. If hypothesis one is correct, and cross-reactive SARS-CoV-2 binding B cells are generated as the affinity of the antibody increases, then cross-reactive cells may be found among many lineages, interspersed with cells that bind only one SARS-CoV-2 variant. However, if hypothesis two is correct, cross-reactive cells B cell may cluster within discrete lineages. This would indicate that early during the response to the vaccine, B cell clones that are specific to conserved epitopes are selected into the immune response at the expense of less conserved epitopes. We found that triple binding clones were frequently shared with the double or single binding populations (Figure 2). This is more consistent with hypothesis one, in which cross-reactive antibodies are generated through affinity maturation during the immune response.

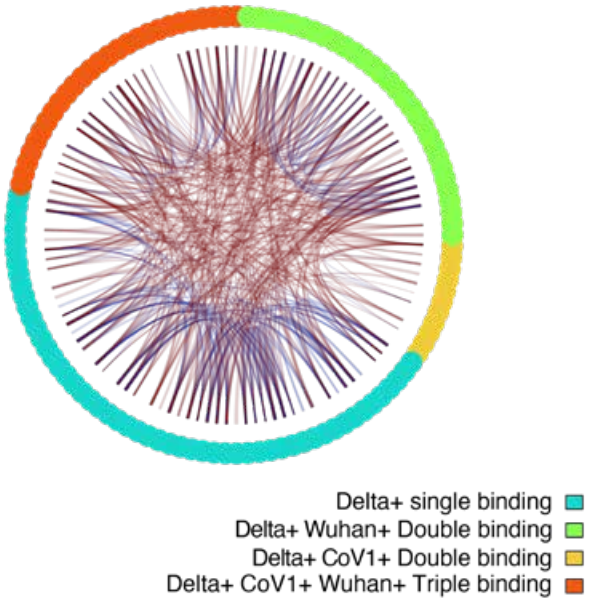


FIGURE 2. CIRCOS plots summarise the similarity matrix between triple binding B cells (red), Delta and Wuhan double positive cells (green), Delta and CoV-1 positive cells (yellow) and delta single binding cells (teal). A link is created between two cells with similar sequences, more similar cells have wider links. Links within categories are coloured blue, and the links between categories are dark red.

CONCLUSION

We found that the heterodimer design of the vaccine encourages the amplification of cross-reactive B cell clones. These clones are more likely generated through somatic hypermutation.

Te Herenga Waka
Victoria University of Wellington

Characterising the progenitor to theca cell transition in ovarian follicles

Z CLARK, S SCZELECKI

KEY FINDINGS

We successfully optimised the conditions for ovarian tissue collection and spatial gene expression experiments. We also completed objectives outside the scope of the initial proposal by performing spatial gene expression experiments on pre- and post-pubertal/aged female mouse ovaries. Preliminary results include data that will be used to characterise key stages of the progenitor to theca cell transition.

OBJECTIVES

The theca cell layer of the ovarian follicle is instrumental in the development and ensuing release of a mature egg (ovulation). Stroma-resident theca cell progenitors are recruited to the developing follicle and differentiate in response to stage-specific paracrine signals (1,2) . Emerging evidence in models of infertility indicates that modifying the stroma microenvironment (for example by reducing fibrosis) increased ovulation (3). Thus, we hypothesise that exposure of stroma-based theca progenitors to paracrine stressors affects differentiated theca cell functions, including ovulation. To begin to test this hypothesis, we will characterise the changes that occur during the progenitor to theca cell transition in situ, using spatial gene expression technologies. This research has two specific objectives:

1. To optimise the conditions for tissue (ovary) collection and sample preparation for spatial gene expression experiments.
2. To identify and describe key milestones in the progenitor to theca cell transition using spatial gene expression.

For this project we requested funding for the consumables and reagents required for Objective 1.

METHOD DEVELOPMENT AND RESULTS

Mammalian ovarian follicle development is hierarchical and sequential (5,6) and so each follicle in the mammalian ovary is at a unique stage of development. Thus, theca cell progenitors and differentiated theca cells are present in the ovary, but will be differentially localised (e.g., in the stroma and associated with growing follicles, respectively). We aimed to exploit this naturally- occurring feature of mammalian follicle development and spatial gene expression technology (Stereo-seq, STOmics) to identify theca cell progenitors in situ and track theca cell differentiation.

Ovaries from naturally cycling, pre- (14-15 day old) and post-pubertal/aged (1 year old) female mice were recovered in accordance with THW VUW Animal Ethics Committee-approved protocols. Tissue blocks were prepared in duplicate whereby one ovary from 2-5 animals was snap-frozen in embedding compound (OCT). A requirement of spatial gene expression experiments is that sample collection and processing must result in high-quality RNA and conserved tissue morphology. First, to assess RNA quality, the first ten cryosections were collected and measured using a BioAnalyzer. Subsequent cryosections were sectioned onto positively charged slides and systematically evaluated following either haematoxylin and eosin staining or gross observation of ovarian morphology. Our samples resulted in high-quality RNA (RIN >7) and highly conserved tissue morphology enabling the identification of follicles at several different stages of development. These results confirmed that our sample preparation methods had been optimised.

Using these optimised sample preparation methods, we proceeded to the optimisation of the spatial gene expression protocol. Prior to mounting onto specialised Stereo-seq slides, tissue was landmarked by light microscopy. When the cryosection contained the follicles at several different stages of development, the next section was mounted onto either a Stereo-seq Chip P or T Slide (STOmics) for optimisation, or experimental sample preparation, respectively.

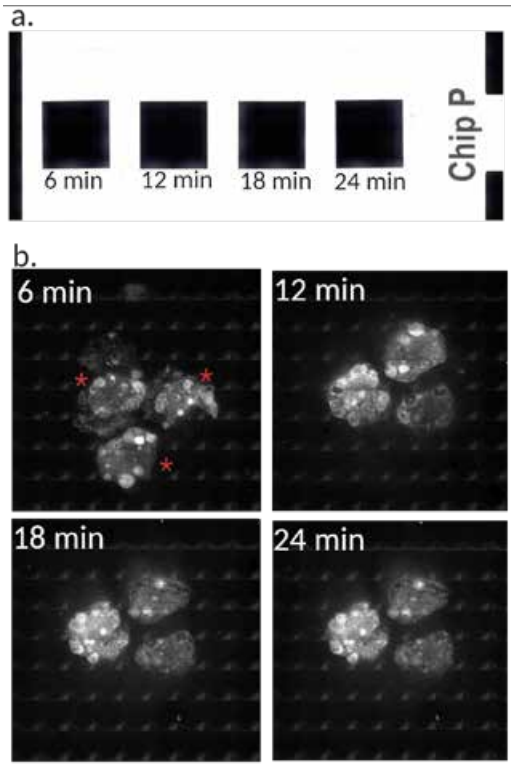


FIGURE 1: Tissue permeabilisation optimisation
a. Stereo-seq Chip P slide, each square on the slide is a 10 mm x 10 mm chip (capture area) where tissue sections were mounted and then permeabilised for either 6, 12, 18 or 24 minutes. **b.** Representative results from the permeabilisation optimisation experiment for ovarian tissue from 1-year old mice. Sections from a tissue block containing an ovary (each indicated by*) from three, 1-year old female mice were permeabilised for 6, 12, 18 or 24 minutes. Images were evaluated using Image J to identify the image with highest fluorescence signal but the lowest signal diffusion, representing the optimal permeabilisation time.

The optimal tissue permeabilisation times for the pre- and post-pubertal tissue samples were determined using the Stereo-seq Permeabilization Set for Chip-on-a-slide kit. Serial sections from blocks containing either ovaries from pre- or post-pubertal animals (Figure 1) were mounted onto the four capture areas on a Stereo-seq Chip P slide and imaged. The optimal permeabilisation time for pre-pubertal samples was 18 minutes whilst for the post-pubertal/aged samples, it was 12 minutes.

Having achieved Objective 1, we used the optimised protocol to perform spatial transcriptomics experiments on tissue blocks containing ovaries from 14-15 day old (pre-pubertal) and 1-year old (post-pubertal/aged) female mice. Slides (pre-pubertal, n=2; post-pubertal, n=2) were imaged and libraries were prepared for sequencing. Preliminary data from one of the 14-15 day old samples are presented in Figure 2. Although, outside of the scope of the current research, sequencing and associated bioinformatic analyses of remaining samples are in progress.

CONCLUSIONS

We have successfully completed the tissue-specific optimisation required to perform spatial gene expression experiments on mouse ovarian tissue. We also prepared experimental samples from pre- (n=2) and post-pubertal (n=2) female mice and generated preliminary data (Figure 2) from one of the pre-pubertal samples. This indicated that we can identify ovarian structures (follicles, oocytes, etc.) based on transcriptomic profiles. The remaining samples are being sequenced and subsequent bioinformatic analyses will identify theca progenitors and differentiated theca cells in pre- and post-pubertal/aged tissues. Additionally, this project provides a robust dataset for interrogating the functions and relationships of different ovarian cell types in situ.

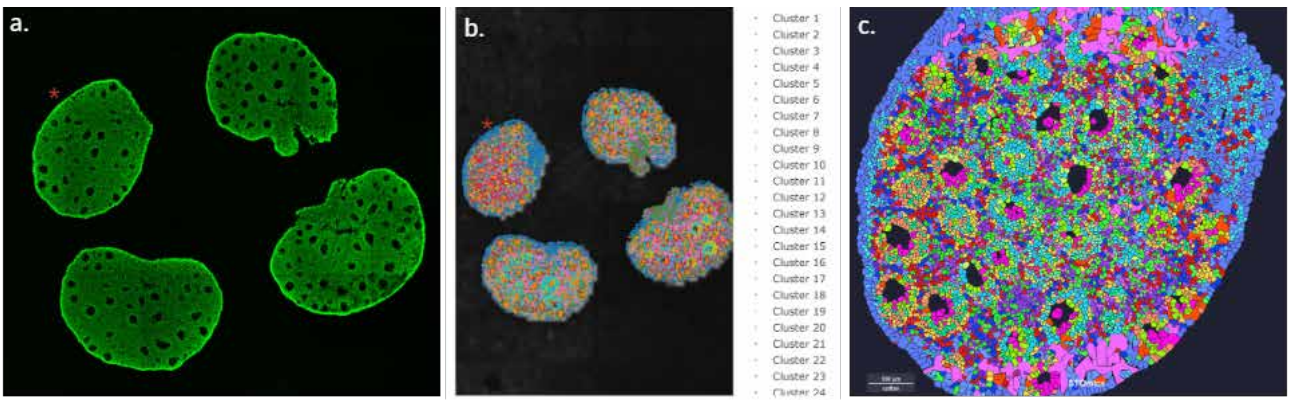


FIGURE 2: Preliminary spatial transcriptomics data from ovarian tissue of four 14-15 day old female mice. a.) ssDNA-stained image generated for quality control (QC) prior to permeabilization. QC ensures that subsequent transciptomic data can be mapped to co-ordinates in a.) b.) initial overlay of spatial and transciptomic data c.) Cell bin by transciptomic profile of the ovary indicated by * in a.) and b.).

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CENTRE FOR EPIGENETIC AND GENETIC RESEARCH
University of Otago, Wellington

Oxidative stress causes epigenetic changes that may promote tumour development

A STEVENS, F RICH, M HAMPTON

KEY FINDINGS

This project aided our understanding of the biological consequences of inflammation associated oxidants on our cells. We also expanded this research to investigate the intricate dynamics of DNA methylation in the hours following CD8+ T cell activation, during a critical yet understudied temporal window. This research unveils a nuanced tapestry of global and site-specific methylation changes. The temporal perspective of early activation adds depth to the evolving field of epigenetic immunology, offering insights with implications for therapeutic innovation and expanding our understanding of epigenetic modulation in immune function. We anticipate that this interaction may contribute towards the subsequent development or progression of cancer, however it may also be of wider relevance to inflammation associated diseases, such as Alzheimer's.

AIMS

The primary goal of the study was to determine whether sub lethal concentrations of the oxidant hypothiocyanous acid (HOSCN) can modify the pattern of DNA methylation. The Secondary goal of the study was to perform a bioinformatic prediction of how the impacted gene regions may contribute towards disease development or progression. We also used our experimental design to investigate the epigenetic changes that occur in primary immune cells following activation.

INTRODUCTION

At the most fundamental level, cancer is a genetic condition that results from a cumulative series of unfavourable cell changes. According to the eight hallmarks of cancer recently comprised by Douglas Hanahan, cancer cells can sustain proliferative signalling, evade growth suppressors, resist cell death, enable replicative immortality, induce/access vasculature, activate invasion and metastasis, reprogramme cellular metabolism, and avoid immune destruction (Hanahan, 2022).

Carcinogenic pathways are regulated by tumour suppressor genes and proto-oncogenes, and disruption to their expression or integrity is causative in many cancers (Kontomanolis et al., 2020). When DNA damage occurs, it is usually remedied efficiently by repair machinery during cell division. Therefore, a combination of gene alterations is often required to initiate and drive the cancer.

While mutational events have a known role in cancer pathology, the implication of epigenetic mechanisms in disease progression is less understood. Hypermethylation of promoters in tumour suppressor genes and global hypomethylation of oncogenes are commonly observed molecular features in cancer, particularly those that are inflammation-associated (Ehrlich, 2002). Oxidative stress is a common feature of inflammation-driven cancers and promotes aggressive tumour developments. This can occur by promoting genomic disruptions, or by modification of key regulatory proteins that direct how genes are turned on and off. Neutrophils are our most abundant white blood cells and accumulate at sites of infection and inflammation where they can produce strong oxidants. At sites of infection and inflammation the heme enzyme myeloperoxidase generates the chemical HOCl, and its primary targets are most likely to be extracellular methionine and amines. Resultant chloramines, such as the glycine chloramine or the oxidant hypothiocyanous acid are prominent under physiological conditions. Their lower reactivity provides them the opportunity to pass into neighbouring cells and alter cell function.

We hypothesize that exposure to immune derived oxidants can impact the pattern of chemical signatures on genomic DNA. These chemical signatures, such as DNA methylation are important epigenetic regulators of gene activity

and act at the interface between the environment and gene regulation. The methylation of cytosine alters DNA structure and gene expression and impacts all aspects of cell function. Understanding how DNA methylation patterns are regulated by cellular and environmental stimuli is a central question for managing health and disease and is a major contemporary challenge in human genetics.

FINDINGS

This grant aided in the development of *in vitro* assays that were designed to interrogate the cellular consequences of oxidant exposure on genomic DNA methylation. We developed two new assays to investigate sub lethal HOSCN exposure. This was investigated in the context of DNA replication in synchronised cells and also in the context of prolonged and repeated exposure. HOSCN exposure did not coincide with substantial decreases in DNA methylation, which was contrary to our hypothesis and the implications from prior experiments. However, we used our experimental design to map widespread *de novo* Genomic DNA methylation change following CD8+ T-cell activation and proliferation.

This novel line of research mapped the intricate dynamics of DNA methylation in the immediate hours following CD8+ T-cell activation, during a critical yet understudied temporal window. DNA methylation is an epigenetic modification central to regulation of gene expression and directing immune responses. Our investigation spanned 96 hours post-activation and unveiled a nuanced tapestry of global and site-specific methylation changes. In total 15,626 significant differentially methylated CpGs were identified across the genome (Figure 1 and 2). Approximately 120 of these CpGs exhibited a log2FC above 1.5. The corresponding change in raw methylation values was approximately equivalent to 2.5%, which suggests that relatively small and widespread changes may contribute substantially towards biological outcomes. These changes in DNA methylation appeared to be directing biological processes corresponding to cell activation and proliferation, with a smaller subset directing cell signalling and immune responses. Neither of these observations have been previously reported in the scientific literature. This research reinforces the central role that DNA methylation contributes in directing and regulating cellular processes (Figure 3). The temporal perspective of early activation adds depth to the evolving field of epigenetic immunology, offering insights with implications for therapeutic innovation and expanding our understanding of epigenetic modulation in immune function.

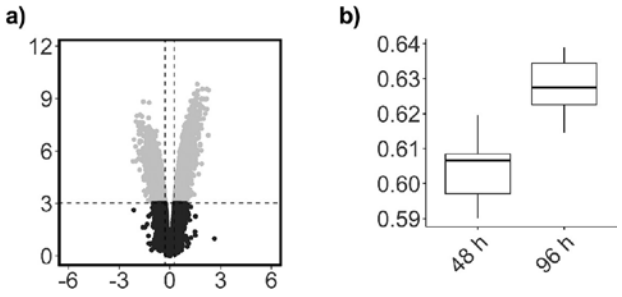

FIGURE 1. Global DNA methylation (β-values) for all probes. (A) Scatterplot with log2 fold changes on the x-axis versus statistical significance on the y-axis -log10P-value. Grey dots represent probes with an adj. P < 0.05. (B) Relative average methylation levels for each timepoint. Mean methylation levels for β-values are presented as percentages.

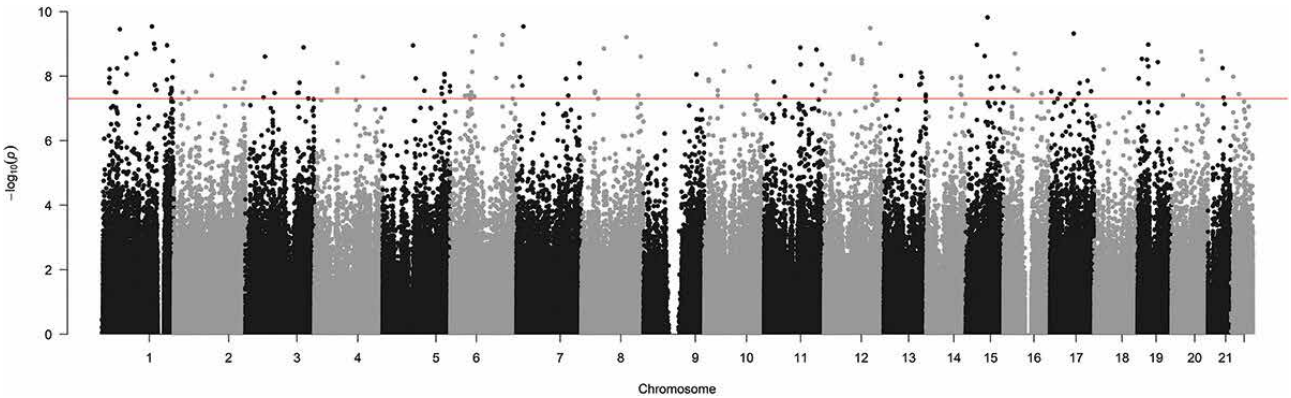

FIGURE 2. Manhattan plot of all probes (M-Values) across the array. To summarize the results, each probe was mapped to the corresponding genomic region and unadjusted significance was plotted across the whole genome. All probes are ordered per chromosome position along the x-axis, and p-values as the –log10(p-values) are presented on the y-axis. Genome-wide significance was determined using the "Benjamini, Hochberg" method within Limma and is approximately represented by the horizontal red line. This analysis demonstrated that the significant changes were distributed across the genome and appeared to be concentrated at specific genomic locations.

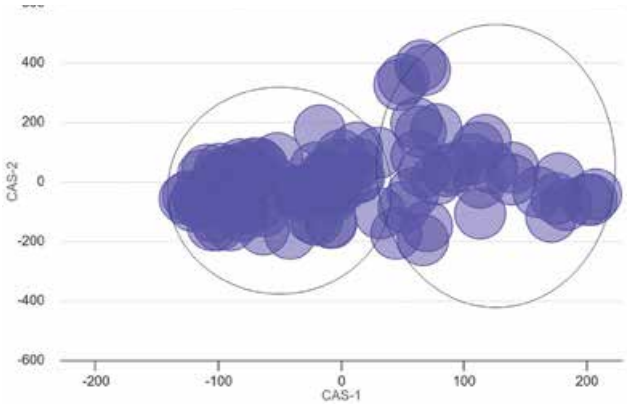

FIGURE 3. MDS plot of top 100 significant pathways. Co-occurrence Association Score (CAS) is presented on the x and y axis. CAS is a measure used to quantify the frequency of co-occurrences of two genetic ontology terms in a single gene annotation relative to random chance. Pathways contained within the left circle generally correspond with activation and proliferation, whereas pathways within the right circle generally correspond with cell signalling and immune response. The genes corresponding to the topmost significant CpGs were assessed for significant biological relevance by pathway analysis within the genetic ontology databases.

CONCLUSION

Our investigation into CD8+ T cell activation and DNA methylation dynamics presents new insights into the epigenetic events steering stimulatory responses. The activation of CD8+ T cells through CD28 and CD3 antibodies instigates a cascade of significant alterations in DNA methylation profiles. The observed changes, though subtle, may orchestrate important downstream effects in the immune response. Understanding the epigenetic regulation of T cells soon after activation contributes valuable insights to the broader field of immunology and opens avenues for future investigations into the development of targeted therapies that can recalibrate immune responses in the context of various diseases. These novel findings have demonstrated an interesting avenue for immune directed epigenetic regulation of gene activity. We are currently building on these findings and plan to expand the findings in disease contexts.

PUBLICATIONS AND OUTPUTS

Successful award of funding from the WMRF was crucial for completion of this research and contributed towards the following publication:

Seddon AR, Damiano OM, Hampton MB, Stevens AJ. Widespread genomic de novo DNA methylation occurs following CD8+ T cell activation and proliferation. Epigenetics. 2024 Dec 31;19(1):2367385.

Two additional publications are in progress:

"Inflammation and DNA methylation in Alzheimer's disease: mechanisms of epigenetic remodelling by immune cell oxidants in the ageing brain." Seddon, A.R, MacArthur, C.P, Hampton M.B, Stevens A.J. Accepted pending minor amendments: Redox Report.

"From Chronic Inflammation to Cancer: The Impact of Oxidative Stress on DNA Methylation" Olivia Damiano, Aaron J. Stevens, Diane Kenwright, Annika R. Seddon. Manuscript in preparation.

Student Support:

This funding supported BBioMedSci(Hons) students, Olivia Damiano in 2024 and Caitlin MacArthur in 2023. Olivia has now progressed into a PhD with our department, on a similar research theme.

Presentation at international Conferences:

Stevens AJ, Seddon A, Das A, Hampton M,. Site-specific decreases in DNA methylation in replicating cells following exposure to oxidative stress. European Society of human Genetics, Glasgow. June 10-15th 2023. Hybrid Poster Presentation

Stevens AJ, Seddon A, Das A, Hampton M,. Site-specific decreases in DNA methylation in replicating cells following exposure to oxidative stress. Genemappers 2024, Christchurch. Aug 18-23rd 2024. Hybrid Poster Presentation

METHODS

Cell viability and proliferation

Cell viability was assessed using flow cytometry prior to initial stimulation and then at 24 and 72 h post treatment. The percentage of viable cells was assessed by the exclusion of PI. Growth rate after treatment was determined by live cell counts 24 and 72 h post treatment and expressed as a percent growth (current day count – previous day count)/ previous day count x100).

DNA isolation and bisulfite conversion

DNA was extracted from cell samples using a GeneJet DNA extraction kit (ThermoFisher Scientific, USA) according to the manufacturer's instructions. Sodium bisulphite treatment of genomic DNA was performed on 1000 ng of DNA using the Zymo Research EZ DNA methylation kit (Zymo Research, Irvine, CA), according to the manufacturer's recommended protocol for use on the Illumina Infinium MethylationEPIC 850K array (Illumina, Inc., San Diego, CA, USA). Genome-wide DNA methylation profiles were assayed with the Illumina Infinium MethylationEPIC 850k array, at AgResearch Ltd (Invermay, New Zealand). Analysis was performed on all samples in a single batch.

DNA methylation detection and bioinformatic analysis of DNA methylation

DNA methylation profiles were assessed using the Illumina methylationEPIC v1.0 bead chip array. Methylated and non-methylated values were determined using the minfi pipeline, within R. Probes were annotated against the Infinium MethylationEPIC v1.0 B5 Manifest File, which includes genomic coordinates for hg38 and gene regulatory features from Gencode. Data analyses were performed using the Minfi and Limma Bioconductor software packages within the R statistical program (www.R-project.org). All packages, programs and pipelines used in these analyses are freely available, and the workflow was based upon published scripts. Any additional scripts are available from the authors on request. All samples passed quality control together using Subset-quantile Within Array Normalization (SWAN) and variance-stabilizing transformation. Probes containing detection p-values >0.05 for 1% or more samples were excluded from further analysis. One sample failed to pass the quality control criteria, and both replicates were removed from the analysis. Because the cell samples were of the same sex (female), all chromosomes were retained; however, probes identified as having polymorphic hybridizing potential and homology to common SNPs were removed. The final data frame contained 808,953 probes available for analysis. Multidimensional scaling of the top 1000 methylation values was performed using pairwise distance method for gene selection, and normalized, filtered methylation values. Hierarchical clustering was performed on a 'minkowski' distance matrix calculated using the β-values for all probes, regardless of significance.

The methylation status of each probe was calculated using normalized probe signals represented as methylation values (M-values) and β-values. M-values were generated within Minfi as the log2 ratio of the signal intensities of methylated probe divided by the unmethylated probe. Unless stated otherwise, statistical analyses were performed using the M-values. β-values (average DNA methylation level for each probe) were used for data visualization and range from 0 (unmethylated) to 1 (methylated). β-values were generated by dividing the methylated probe signal with the sum of the methylated and unmethylated probe signals. Normalized β-values and M-values were manually assessed for fit.

Differentially methylated positions which are correlated with treatment were identified using a linear regression model within the Limma package, with adjustment for multiple testing. To control for interindividual DNA methylation, paired samples were used from the same participants at both time points and replicate was incorporated as a factor into the statistical design. DNA methylation profiles from the 48 h cell aliquots were subtracted from the 96 h samples. This analysis did not include the cells exposed to GlyCl. The topmost significant, differentially methylated CpG positions were identified by significance and were also assessed using a log2FC <1.5 in Limma's topTable algorithm. Adjustment for multiple correction was performed using the 'Benjamini, Hochberg' method within Limma and Quantile – Quantile plots for the linear regression model were assessed at each time point using the observed against expected – log10(p-value. The observed data show a minor inflation of p-values smaller than expected by chance.

Pathway analysis was performed by comparison gene ontology database (GO) using the Gometh algorithm within the missMethyl R package, with correction for probe bias. Pathway analysis was performed on significant CpG positions that mapped to the regions located from –1500 to –200 base pairs upstream of the transcription start site, and within the first gene exon. The output from the gene ontology analysis was then assessed using the Navigo online algorithm, with Resnik semantic similarity-based scoring.

Differentially methylated regions

Differentially methylated regions were interrogated within the Minfi package using the statistical package DMRcate. A methylation differential cut-off of 10 across 5 or more adjacent CpGs was used, and significance was determined using a false discovery rate (FDR) of 0.05.

University of Otago,
Wellington

Novel flow cytometry techniques to detect extracellular vesicles for diagnosing endometriosis

INTERIM REPORT

E PATERSON, J GIRLING, C HENRY

KEY FINDINGS

This project developed techniques to use flow cytometry to examine single, fluorescently labelled extracellular vesicles. There were no differences in plasma EVs with endometrial stromal cell markers between people with endometriosis and without endometriosis. Methods developed in this study will be applied to cervico-vaginal fluid EVs, a biofluid with increased biological relevance for endometriosis biomarkers.

OBJECTIVES

Endometriosis is a common, painful condition defined by the presence of tissue resembling the endometrium (the lining of the uterus) growing outside the uterus. Currently, an endometriosis diagnosis can only be confirmed through surgery, resulting in an approximate eight-year diagnostic delay in Aotearoa. Furthermore, with a non-specific symptom profile and poor sensitivity of pre-surgical imaging, around one third of surgeries for suspected endometriosis find no evidence of disease (Paterson et al., 2023). Thus, non-invasive diagnostic biomarkers are critically required to shorten the time to diagnosis and predict which patients will benefit from surgical treatment.

This project focuses on extracellular vesicles (EV) as biomarkers of endometriosis. EVs are membrane delimited particles released by cells that are involved in cell-to-cell communication and cellular homeostasis. EVs have been implicated in the pathogenesis of endometriosis in a multitude of studies, yet only a few studies have investigated EVs in the context of diagnostic biomarkers.

The overall aim of this project was to develop methodologies to measure EVs using flow cytometry to investigate potential biomarkers of endometriosis. Specifically:

Objective 1: Demonstrate ability to detect single, fluorescently labelled vesicles using flow cytometry.

Objective 2: Measure the proportion of plasma EVs carrying endometrial stromal cell markers (CD10, CD90 and CD140b) and evaluate their potential as diagnostic biomarkers.

METHODS

Blood samples were collected from consenting people having surgery for suspected endometriosis at Wellington and Kenepuru Hospitals. Whole blood was processed into plasma and stored at -80°C until use. Based on surgical and histological reports, patients were grouped into either the endometriosis group or no endometriosis group.

EVs were isolated from 500µL of plasma using size exclusion chromatography, enriching for EVs sized 70-1000nm. EVs were stained overnight at 4°C with antibodies targeting markers of endometrial stromal cells, anti-CD10-BB515, anti-CD90-BV711 and anti-CD140b-PE. The next day, EVs were fluorescently labelled with Aco-430, a membrane dye used to identify the EV population. Samples were then analysed on a three-laser Cytek Aurora flow cytometer equipped with the enhanced small particle detection module. Each experimental day, 8-Peak rainbow beads and polystyrene beads sized 70-400nm were acquired and resulting data was used to calibrate FCS files from arbitrary units to standardised units using FCMpass software.

Aco-430$^+$ events were deemed EVs. To determine particles detected were vesicular in nature, samples were analysed pre and post detergent lysis. Two-tailed ratio paired t-test were performed to assess reduction in EVs (Aco-430$^+$ events). To demonstrate that vesicles detected were single vesicles (rather than clusters of vesicles), samples were analysed at five different dilutions. The Aco-430 MFI of EVs was plotted, and simple linear regression was performed on the EV count for each dilution recorded.

CD10, CD90 and CD140b gates were then applied to the EV population. The CD10$^+$, CD90$^+$, CD10$^+$CD90$^+$ and CD140b$^+$ EVs were presented as a proportion of the total EV population. Median fluorescence intensity (MFI)

statistics were acquired for each population, as a measure of antigen density. To compare the endometriosis and no endometriosis groups, Mann-Whitney tests with Holm-Šídák correction for multiple comparisons were performed.

RESULTS

Data calibration into standardised units determined that the lower limit of detection of the cytometer was 100nm. An upper limit of 1000nm was set based on calibrated SSC-H.

Detergent lysis of EVs showed a significant reduction in EV events by 88.6±4.1% after lysis (p=0.011, n=3), demonstrating particles detected were true EV events rather than other non-membranous particles such as protein complexes (Figure 1A). Samples had a linear relationship between sample dilution factor and the number of EV events detected, with a consistent Aco-430 MFI (Figure 1B).

The final cohort consisted of 15 participants per group. There were no differences in the proportions of CD10+, CD90+, CD10+CD90+ or CD140b+ EVs between groups (Figure 2A) or MFIs (Figure 2B) were identified between groups. This held true when analysing small EVs (EVs < 200nm) and large EVs (EVs > 200nm) separately (data not shown).

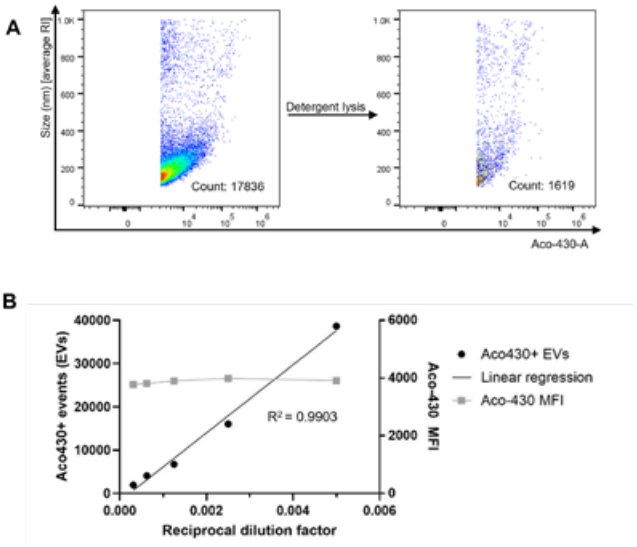

FIGURE 1: Single vesicle detection. A) Representative plot showing significant reduction in EVs (Aco-430+ events) after detergent lysis. B) Representative graph showing a linear reduction in EV count with sample dilution and a constant Aco-430 median fluorescence intensity (MFI).

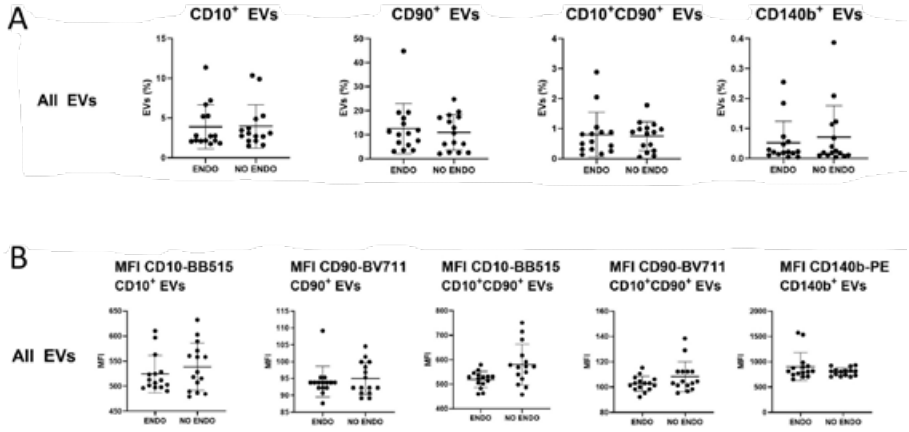

FIGURE 2: Plasma EVs in people with endometriosis and surgically confirmed controls. A) CD10+, CD90+ CD10+CD90+ and CD140b+ EVs, expressed as a proportion of the total plasma EV population (Aco-430+ events). B) Median fluorescence intensity (MFI) of CD10+, CD90+ CD10+CD90+ and CD140b+ EVs. Data is presented as mean ± standard deviation. N=15 per group. No differences between groups were identified (adjusted p value > 0.05, Mann-Whitney tests with Holm-Šídák correction for multiple comparisons).

CONCLUSION

We have developed methods for measuring fluorescently labelled EVs using spectral flow cytometry. There was no difference in the antigen density or proportions of plasma EVs with endometrial stromal cell markers between people with and without endometriosis.

The final steps for this research will be applying the protocols developed in this study to alternative EV samples. We will be isolating EVs from cervico-vaginal fluid sampled using vaginal swabs and analysing markers identified by proteomic analysis. We hypothesise that the biological relevance of cervicovaginal fluid may yield more promising results, and we are excited to apply our novel techniques developed in the first phases of this research project.

University of Otago,
Wellington

NETendo: Neutrophil Extracellular Traps in endometriosis

INTERIM REPORT

K BORCHOWSKY, E PATERSON,
C JONES, K HALLY, C HENRY

KEY FINDINGS

The proportion of neutrophils with appendant Neutrophil Extracellular Traps (NET burden) was significantly greater in those with non-endometriosis pelvic symptoms compared to those with endometriosis and to healthy controls. There was no significant difference in NET burden between endometriosis patients and healthy controls. NET burden did not significantly vary throughout the menstrual cycle.

AIMS

This study aimed to compare NET burden in neutrophils from the venous blood of endometriosis patients to those having undergone surgery with no endometriosis found, and to healthy control using a novel flow cytometry panel. This study also aimed to characterise NET burden throughout the menstrual cycle.

METHODS

Patient recruitment: Consenting participants undergoing surgery for suspected endometriosis were recruited from Wellington and Kenepuru hospitals on the day of surgery. Two ethylenediaminetetraacetic acid (EDTA) tubes and one serum separation tube (SST) were collected prior to commencing anaesthesia. Depending on surgical and histopathological findings, participants were grouped into the 'endometriosis' group (if endometriosis was found) or the 'symptomatic control' group (if no endometriosis was found). Furthermore, blood was collected from healthy volunteers with no endometriosis-type symptoms forming the asymptomatic control group.

Menstrual phenotyping: Menstrual phenotyping was done through last menstrual period (LMP) dating and hormonal analysis (Figure 1). During consenting, participants provided the date of the first day of their LMP, period regularity and hormonal contraceptive usage. Participant using hormonal contraceptives were placed into the 'hormonal' group. Participant who did not report the first day of their LMP, had not menstruated within the last 3 months or reported 'irregular' periods were excluded from menstrual phenotyping. Participants expecting their period within the next 14 days were placed into the 'secretory' group and, if not, into the 'proliferative' group. Participants 14 days from the first day of their LMP were assigned to the 'ovulation' group. Participants not using hormonal contraceptives who were not excluded from menstrual phenotyping then underwent hormonal analysis. Blood from the SST was rested for a minimum of 30 minutes before centrifugation at 10,000rpm for 10 minutes. The SST was then sent to Awanui Labs, Wellington Hospital for quantification of menstrual hormones. Using reference ranges provided by the lab, participants were grouped into the proliferative, secretory or ovulation group. If hormonal analysis and LMP grouping matched, participants were kept in the assigned group. If the grouping did not match, participants were excluded from menstrual phenotyping.

Staining: Processing began as soon as possible after sample collection, and always within four hours. 2mL of blood from the EDTA tubes were used to isolate neutrophils through negative magnetic bead separation. Neutrophil concentration was acquired by flow cytometry and, if required, cells were resuspended in phosphate buffered saline to a concentration of 4×10^6 cells/mL. Neutrophils were then stained with optimised antibodies titres in accordance with a seven-colour flow cytometry panel designed to identify NET appendant neutrophils (Jones et al., 2024). Markers used included neutrophil markers (CD15 and CD66b), putative markers of NETosis (extracellular DNA, myeloperoxidase (MPO), neutrophil elastase (NE) and citrullinated histones) and a viability marker (Zombie NIR). Immediately after staining, samples were acquired on a full-spectrum flow cytometer.

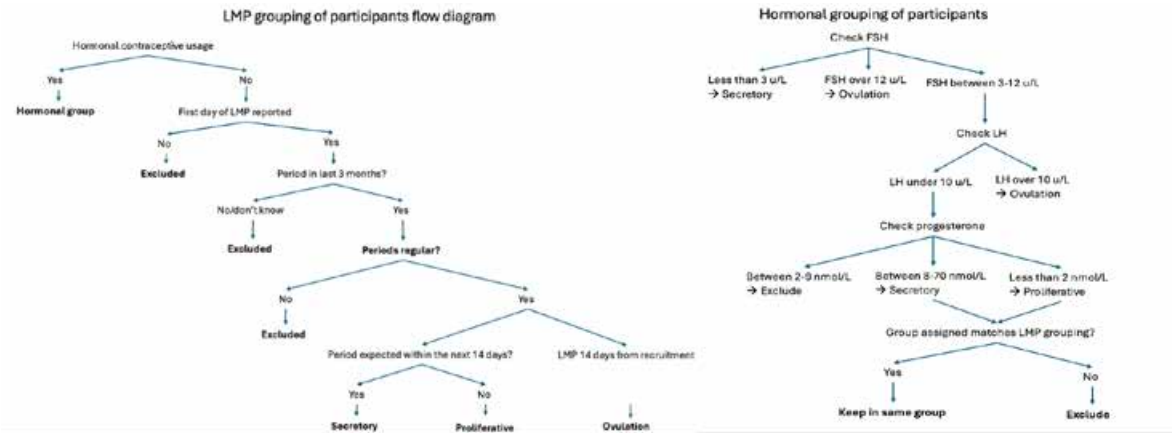

FIGURE 1. Diagram demonstrating menstrual phenotyping. Participants using hormonal contraceptives were assigned to the 'hormonal' group. Participants were subsequently excluded if they did not report the first day of their LMP, had not menstruated within the last 3 months or had irregular periods. Participants expecting their period within 14 days were assigned to the secretory group, or if not, to the proliferative group. Participants who had their LMP 14 days prior to recruitment were assigned to the ovulation group. LMP grouping was then validated with hormonal analysis. If the groups matched, the participant was kept in the LMP group. When hormonal grouping did not match LMP grouping, participants were excluded from menstrual phenotyping.

Gating strategy: An example gating strategy can be found in (Figure 2). Granulocytes were identified by their forward scatter (FSC) and side scatter (SSC) profiles. After doublet exclusion, neutrophils were identified through positive surface expression of the two neutrophil markers. Cells which were Zombie NIR^{bright} were deemed necrotic and excluded. Live, NET appendant neutrophils were identified through their positive surface expression of putative markers of NETosis, namely extracellular DNA, MPO, NE and citrullinated histones. NET burden was defined as the proportion of neutrophils which were NET positive.

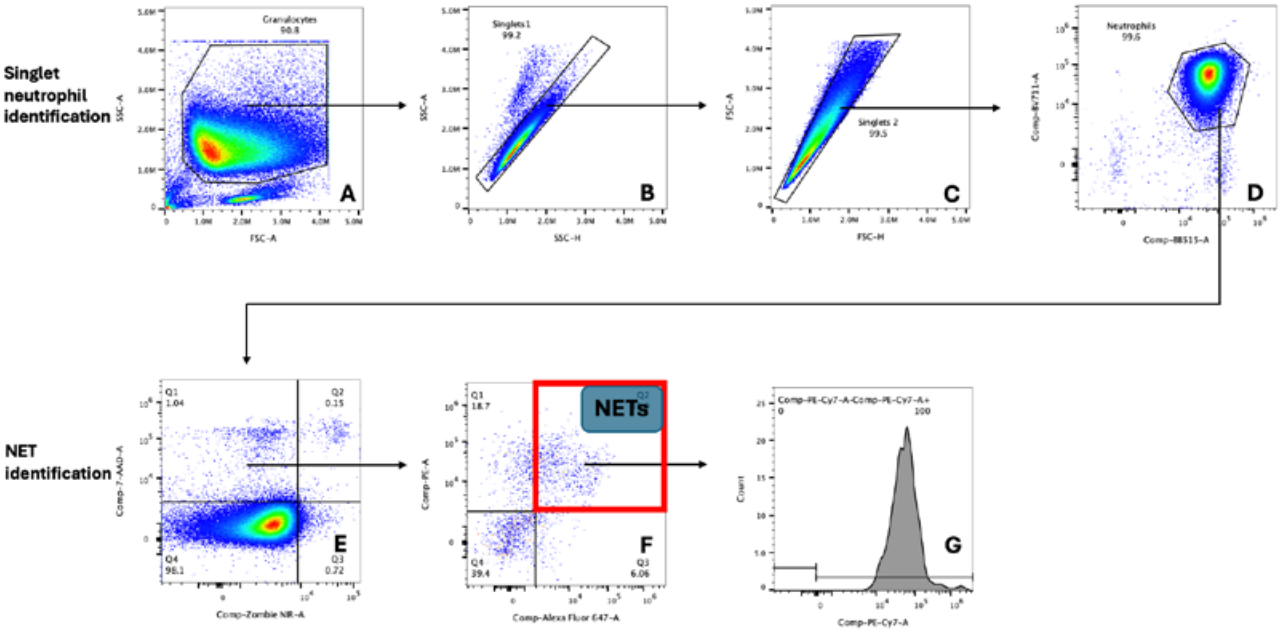

FIGURE 2. An example gating strategy. Singlet neutrophils were first identified as being FSC^{high}SSC^{high}CD15^{+}CD66b^{+} (A-D). Necrotic neutrophils were excluded through excluding Zombie NIR^{bright} cells (E). NET appendant neutrophils were identified through their expression of extracellular DNA, MPO and NE (E-F). Expression of citrullinated histones was also examined, however, in all cases, close to all NETs expressed this marker (G).

Statistics: A one-way analysis of variance (ANOVA) was performed. Post-hoc Tukey tests were subsequently performed to confirm which groups had significantly different mean NET burdens.

RESULTS

To date, 11 endometriosis patients, 6 symptomatic controls and 6 asymptomatic controls have been recruited. NET burden did not significantly differ in endometriosis patients compared to asymptomatic controls (0.17±0.12% vs 0.11±0.07%, p=0.60) and was significantly lower compared to symptomatic controls (0.17±0.12% vs 0.34±0.14%, p=0.02) (Figure 3A). 8 participants were excluded from menstrual phenotyping. 10 participants used hormonal contraceptives, 2 participants were in the proliferative phase, 4 in the secretory phase and 0 were ovulating. NET burden did not differ significantly between menstrual groups (Figure 3B).

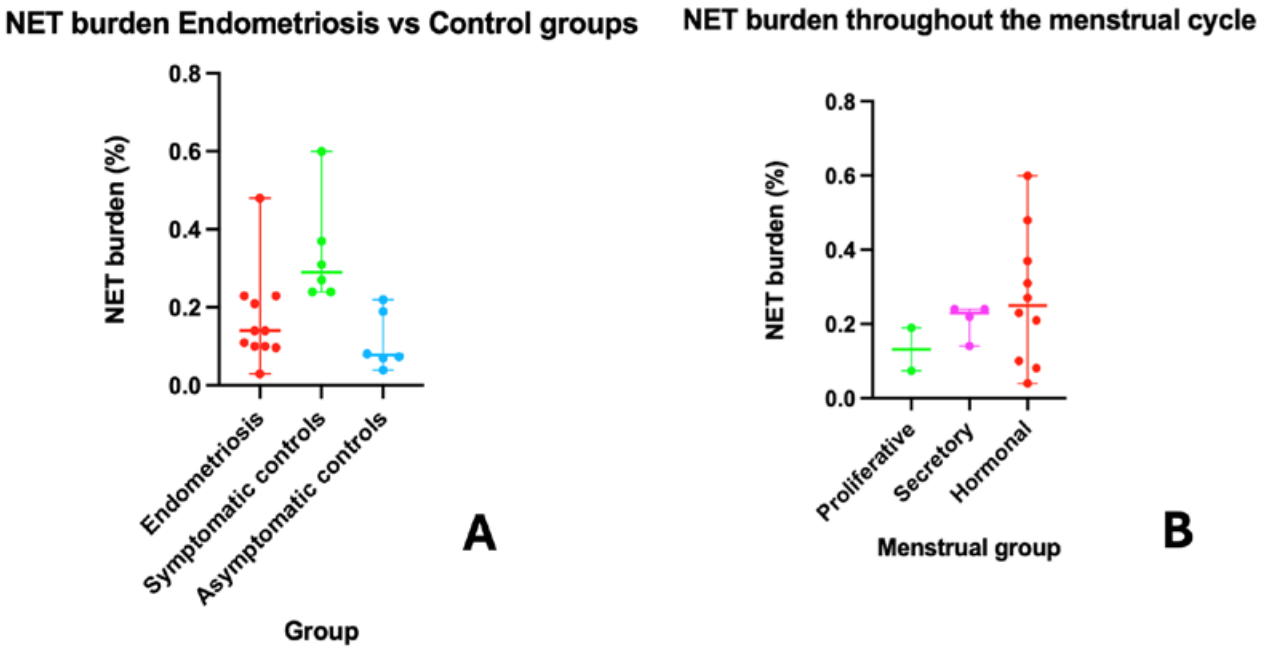

FIGURE 3. Comparison of NET burdens between: A. Endometriosis patients, symptomatic and asymptomatic controls. B. Those in different phases of the menstrual cycle and those taking hormonal contraceptives

CONCLUSION

Venous NET burden is not elevated in endometriosis cases compared asymptomatic controls. NET burden was significantly higher in symptomatic controls, indicating increased systemic inflammation in those with non-endometriosis pelvic symptoms. NET burden did not significantly vary throughout the menstrual cycle. Recruitment is currently ongoing to validate these findings in a larger cohort.

University of Otago, Wellington

Investigating the role of T cells in endometrial cancer treatment response

H VAN DER WOUDE, K HALLY, M CURRIE, C HENRY

KEY FINDINGS

A subset of immune cells (T cells) was isolated from the blood of healthy volunteers and treated with current endometrial cancer therapeutic, levonorgestrel (LNG). LNG had a mild immunosuppressive effect on T cells but did not affect T cell survival or subset distribution.

OBJECTIVE

The levonorgestrel intra-uterine device (LNG-IUS) is the current primary form of surgery-sparing treatment for early-stage endometrial cancer. However, response rates for this therapy are as low as 43% based on results from an Australian/New Zealand clinical trial [1]. Progesterone has reported immunosuppressive properties in the literature [2, 3], and this may influence response to LNG treatment. The overall aim of this work was to determine the effect of direct LNG treatment on T cells.

METHOD

Whole blood was donated from three female healthy volunteers. Inclusion criteria was no hormonal medication for minimum 3 months prior to blood draw, and participants were in the follicular phase of their menstrual cycle. The follicular phase has low endogenous progesterone circulation so as not to interfere with LNG treatment, which is a synthetic progesterone. T cells were isolated from the immune cell fraction of the blood using negative magnetic bead isolation.

To ensure that the population of immune cells isolated was indeed T cells, a purity assessment was conducted post-isolation using flow cytometry. CD3 is a classical T cell receptor and was used to determine the proportion of immune cells (using CD45) that were T cells. All isolations resulted in >95% T cells.

The hypothesis of this work was that LNG would be immunosuppressive. To ensure that any immunosuppressive signal was able to be captured, T cells were stimulated using commonly used commercially available stimulation antibodies (CD3/CD28). After 48 hours, T cells were harvested and stained with a panel of antibodies to identify T cell subsets and activation status using spectral flow cytometry.

RESULTS

LNG treatment did not affect T cell survival **(Figure 1A)** or the distribution of T cell subsets **(Figure 1B,C)**. LNG did not affect activation marker expression in CD4+ or NKT-like cells **(Figure 2)**. However, within the CD8+ T cell subset, expression of activation markers CD25 **(Figure 2Bii)** and PD-1 **(Figure 2Cii)** were reduced compared to control.

CONCLUSION

Overall, the data from T cells obtained from healthy donors and cultured in 2D monolayer demonstrates a mild suppressive effect of LNG.

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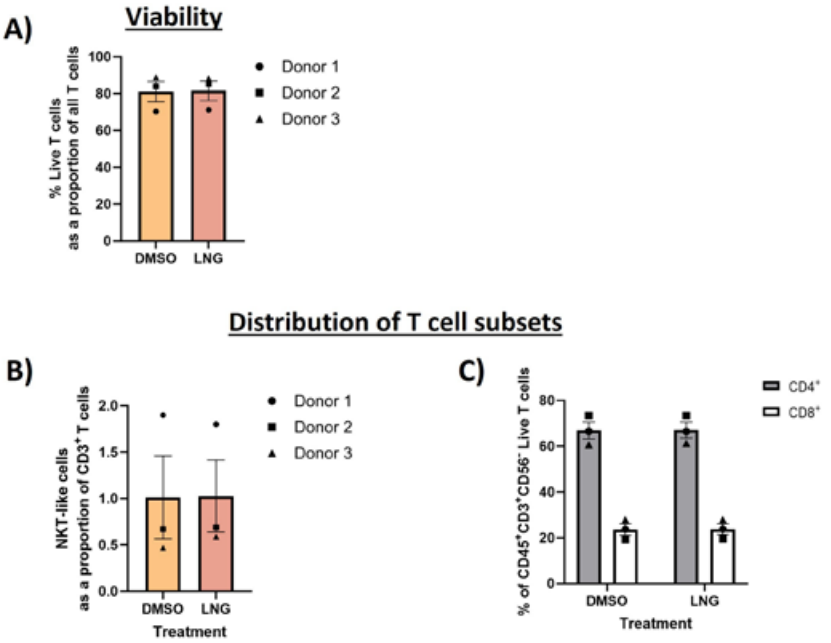


FIGURE 1. Viability **(A)** and distribution of T cell subsets, including natural killer T-like (NKT-like) cells **(B)** and CD4+:CD8+ T cell ratio. T cells were obtained from the blood of three healthy volunteers and cultured with CD3/CD28 stimulation for 48 h with dimethyl sulfoxide (DMSO; vehicle control) or levonorgestrel (LNG; 4 µg/mL) treatment. Following treatment, cells were harvested and stained for analysis using spectral flow cytometry.

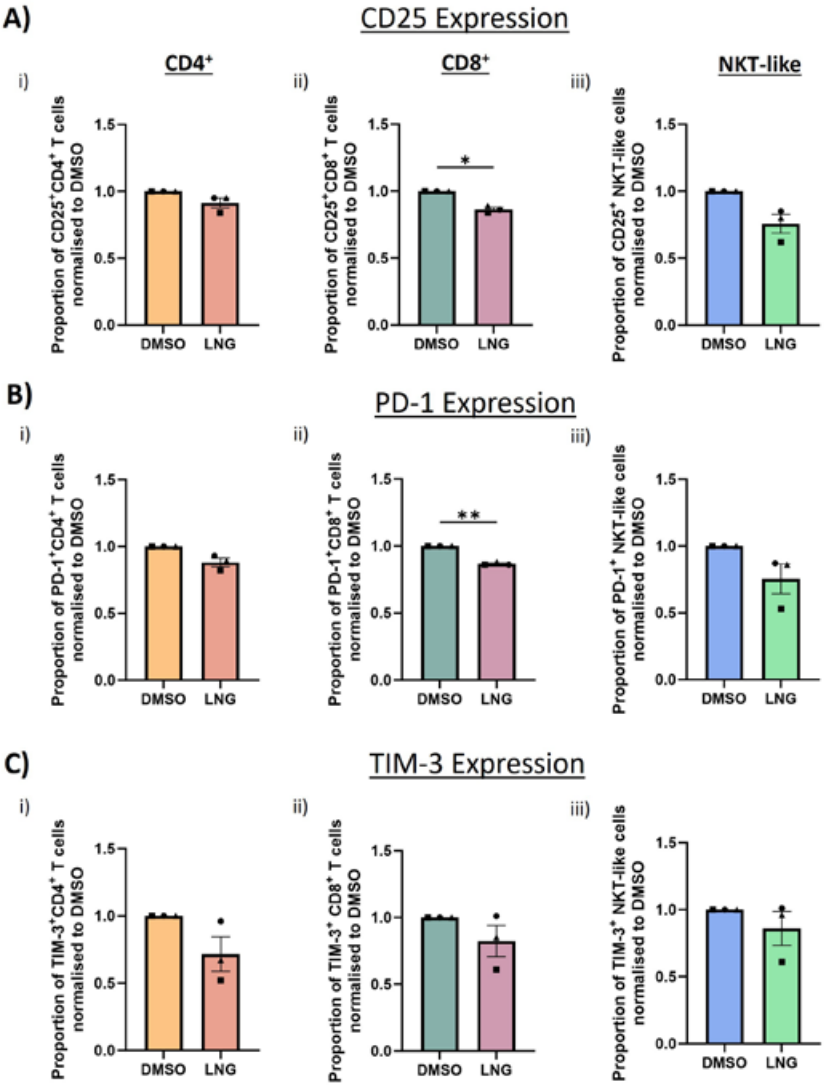


FIGURE 2. Expression of activation markers CD25 **(A)**, PD-1 **(B)** and TIM-3 **(C)** within three T cell subsets – CD4 **(i)**, CD8 **(ii)** and NKT-like **(iii)**. T cells were obtained from the blood of three healthy volunteers and cultured with CD3/CD28 stimulation for 48 h with dimethyl sulfoxide (DMSO; vehicle control) or levonorgestrel (LNG; 4 µg/mL) treatment. Following treatment, cells were harvested and stained for analysis using spectral flow cytometry.

DEPARTMENT OF SURGERY AND ANAESTHESIA

University of Otago, Wellington

Investigating the transcriptomic profile of neutrophils after acute myocardial infarction

K HALLY, Z MOLTSCHANIWSKYJ, A HOLLEY, P LARSEN

KEY FINDINGS

The function of neutrophils, which are important immune cells, is highly dynamic after a heart attack. We highlight that one pathway – the formation of neutrophil extracellular traps (NETs) – is a function that is activated very early on after onset of symptoms of a heart attack and quickly 'turned off'. Because of this early upregulation, we are investigating NETs as a potential treatment target for heart attacks.

OBJECTIVE

Acute myocardial infarction (AMI), commonly called a heart attack, is associated with significant morbidity and mortality and is the leading cause of death in Aotearoa New Zealand. Understanding how the body responds to AMI – for example, by monitoring the immune response – may reveal targets for treatment to improve heart health in this context.

Neutrophils are cells of the innate immune system, which is the 'arm' of this system that is activated first in response to inflammation caused by a pathogen (e.g., during infection) or damage to cells in the body (e.g., during an AMI). Neutrophils specialise in early detection of inflammation, and function to contain inflammation at the site of damage. Because these cells are activated early, we hypothesize that tracking their immune activity is the most accurate way of understanding how and when the immune response to an AMI starts, rises to a peak, and resolves.

In summary, we aimed to understand the neutrophil-mediated immune response to AMI in a clinical setting.

METHODS

Recruiting individuals with AMI. For this study, we recruited 10 individuals with AMI who presented to Wellington Hospital within 48 hours of onset of ischaemia (often, chest pain, shortness of breath, nausea, etc.). Individuals consented to serial in-hospital peripheral blood draws and a follow-up blood draw at 7-10 days after onset of symptoms. Time to drawing the first blood sample was within 40-hours of onset of chest pain (21±13 hours post symptom onset) and a blood sample was also drawn on discharge (2.6±0.6 days post symptom onset) and at follow-up (8.9±1.1 days post symptom onset).

Isolating neutrophils from peripheral blood samples. Neutrophils were isolated from each blood sample, and total RNA was extracted. RNA (ribonucleic acid) contains genetic information about how a neutrophil is functioning at the time of the blood draw. Analysing the RNA – by using a technique called RNA sequencing – allows us to determine how neutrophils were responding to AMI. Identifying genes that are differentially expressed in neutrophils over the acute period (within 40 hours of symptom onset), sub-acute period (2-3 days) and remote period (about 9 days) after AMI can provide important insights into the evolving immune response. We hypothesized that changes in gene expression during the acute period would reflect an upregulation of inflammatory neutrophil-centric functions. We also hypothesized that the transition to the sub-acute and remote time points would induce a shift in gene expression that indicates wound healing, tissue remodelling and resolution of inflammation.

Analysis of neutrophil RNA sequencing. We performed analysis to understand the function of genes that were differentially expressed at timepoints 2 (sub-acute) and 3 (remote), compared to timepoint 1 (acute). This was done in two ways: (1) Gene ontology (GO) enrichment analysis and (2) KEGG pathway analysis both identify the biological processes/pathways that these differentially expressed genes are known to be involved in. Because these analyses use different databases, they generate unique results.

RESULTS

Description of the AMI cohort. Our cohort is representative of AMI patients: this cohort has a mean age of 60 years, a mean BMI of 29 (classified as overweight), and a high proportion of participants presented with hypertension (40%), dyslipidaemia (70%), Type 2 diabetes (20%) and a family history of coronary artery disease (30%). However, all individuals were male: while males are at a higher risk of experiencing AMI, we recognize that an all-male cohort is a limitation of this dataset. Most of the participants were diagnosed with ST elevation myocardial infarction (STEMI), which is caused by full blockage of blood flow within the coronary artery, and which often results in a large area of heart muscle at risk of infracting (70%). The remaining 30% were diagnosed with non-ST elevation myocardial infarction (NSTEMI), where there is partial blockage of the coronary artery and, therefore, a smaller area of muscle at risk compared to a STEMI. Most participants (70%) were of New Zealand European ethnicity, with representation of other ethnicities including Indian and Māori.

A comparison of neutrophil function at timepoint 1 (acute) and timepoint 2 (sub-acute). During the acute phase of an AMI, we identified 79 differentially expressed genes between timepoints 1 and 2 (the two in-hospital timepoints). These were significantly enriched across 1066 GO terms. These terms support the idea that neutrophil function is dynamic even during the acute phase of an AMI, with dynamic changes in functions such as cytokine production pathways, integrin binding and serine peptidase activity. Of note from the KEGG pathway analysis, neutrophil extracellular trap (NET) formation is significantly downregulated at timepoint 2. NET formation is a highly inflammatory process where neutrophils expel intracellular content – including proteins involved in coagulation and inflammation – into the extracellular space. This indicates that NETosis is activated early on after AMI but is a function that is already being 'turned off' at 2-3 days after symptom onset. NET formation is of interest to our research group, as we have previously demonstrated that an increase in the concentration of circulating NET components (alongside a marker of hypercoagulation) is linked to a higher risk of adverse cardiovascular events within 1 year after AMI.

A comparison of neutrophil function at timepoint 1 (acute) and timepoint 3 (remote). 488 significantly differentially expressed genes were identified between these two timepoints. KEGG pathway analysis revealed upregulation of 29 pathways and downregulation of 32 pathways. The upregulation of cGMP-PKG signalling pathway at timepoint 3 suggests enhanced cellular signalling and vascular regulation, potentially contributing to vascular repair and adaptation post-AMI. Upregulation of the glycosaminoglycan biosynthesis pathway indicates active production of heparan sulfate and heparin, which play key roles in modulating inflammation and coagulation. Downregulation of the ribosome pathway indicates a potential reduction in protein synthesis typically associated with cell proliferation, differentiation and apoptosis, suggesting a shift in neutrophil function to a 'quieter' state. NET formation was also significantly down regulated at timepoint 3 compared to both timepoint 1 and 2. This suggests that NETosis peaks very shortly after symptom onset, is reduced at a sub-acute point and reduced even further at a remote timepoint.

CONCLUSION

We demonstrate that neutrophil function changes dynamically within a very short window (3 days) after symptom onset. Of note, NET formation is activated very early (within 40 hours) and is significantly downregulated at the remaining two time points (2-10 days). We are currently investigating the dynamics of NET formation after AMI by using a different technique – flow cytometry to identify NET structures on the surface of neutrophils – to confirm this neutrophil RNA sequencing data. Given the early upregulation of NETosis, this could be a potential treatment target for individuals with AMI.

University of Otago, Wellington

Investigating CD133^{+ve} endometrial cancer stem cells as drivers of Mirena® treatment resistance

M DORE, S FILOCHE, C JONES, C HENRY

KEY FINDINGS

This study successfully isolated cancer stem cells from an endometrial cancer (EC) cell line using flow cytometry. The isolated EC cancer stem cells showed increased stem cell marker expression, enhanced sphere formation, and heightened resistance to levonorgestrel (LNG), a hormone-based EC treatment, compared to non-cancer stem cells. Furthermore, protein expression of CD133 in endometrial biopsy samples of people treated with LNG showed promise for monitoring treatment response clinically.

OBJECTIVES

EC is becoming increasingly common among younger people. While total removal of the uterus is the standard treatment option, it is not suitable for many people with EC. The levonorgestrel intra-uterine system (LNG-IUS or Mirena®) offers a non-surgical alternative but is ineffective in about one-third of patients, and the cellular mechanism of LNG resistance remains unknown. Cancer stem cells are frequently implicated in cancer treatment resistance, therefore, we aimed to explore the role of cancer stem cells in resistance to Mirena® treatment of EC. This study was conducted with four main objectives: 1) Determine the best method for isolating cancer stem cells from EC cell lines; 2) Analyse the behavioural and molecular characteristics of isolated cancer stem cells, including LNG resistance; 3) Investigate if cancer stem cells transfer stem-like properties to non-cancer stem cells via extracellular vesicles (EVs); and 4) Assess CD133 protein expression in endometrial biopsy samples to evaluate its potential role in predicting or monitoring response to the Mirena®.

METHODS

CD133, an established cancer stem cell marker, was utilised to identify EC stem cells using flow cytometry. Cell surface CD133 expression was investigated in six EC cell lines to identify the best cell line for downstream analysis. CD133^{+ve} cancer stem-like cells were then isolated from the Ishikawa EC cell line using fluorescent-activated cell sorting.

The stem cell phenotype of isolated CD133^{+ve} cells was confirmed by analysing protein and mRNA expression of established cancer stem cell markers using Western Blot and RT-qPCR, and by investigating sphere-forming ability, a recognised characteristic of cancer stem cells.

Proliferation and invasion of CD133^{+ve} cells was compared to CD133^{-ve} (non-stem-like) cells using a viability assay and a 3D spheroid invasion assay, respectively. The in-vitro viability of CD133^{+ve} and CD133^{-ve} cells was compared after exposure to escalating concentrations of LNG for 72 hours.

To investigate if cancer stem cells can transfer stem-like properties to non-cancer stem cells via EVs, EVs were isolated from the cell culture media of CD133^{+ve} cells using size exclusion chromatography. CD133^{+ve} EVs were then co-cultured with CD133^{-ve} cells for 48 hours. Subsequently, CD133 mRNA and protein expression, alongside LNG resistance was investigated in the co-cultured cells.

Finally, CD133 protein expression was investigated in endometrial biopsy samples from people with EC treated with the Mirena® at Te Whatu Ora – Capital, Coast and Hutt Valley, using immunohistochemistry. CD133 protein expression was graded on a scale of 0-3, based on staining intensity, and correlated with histological response to the Mirena®.

RESULTS

Flow cytometric analyses revealed variable CD133 populations among six EC cell lines. The Ishikawa EC cell line had the highest proportion of CD133^{+ve} cells (83.2%) and

was chosen for downstream analysis. Isolated CD133^{+ve} cells were confirmed to have a stem-like phenotype.

CD133^{+ve} cells appeared to be more invasive than CD133^{-ve} cells, however, there was no difference in proliferation. Additionally, CD133^{+ve} cells were significantly more resistant to LNG treatments **(Figure 1)**. Co-culturing CD133^{-ve} cells with CD133^{+ve} EVs led to an upregulation of CD133 protein and mRNA expression in CD133^{-ve} cells but did not impact LNG resistance.

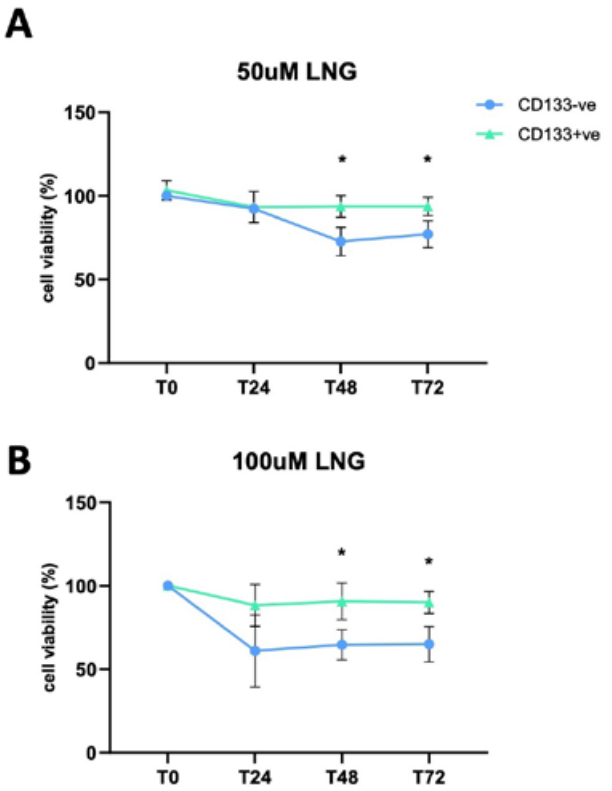


FIGURE 1. Effect of LNG treatment on CD133^{+ve} and CD133^{-ve} Ishikawa cells.
CD133^{+ve} Ishikawa cells are more viable than CD133^{-ve} Ishikawa cells when exposed to increasing doses of LNG. A) Cell viability of CD133+ve (green) and CD133^{-ve} (blue) Ishikawa cell lines in 50μM LNG over 72 hours. B) Cell viability of CD133^{+ve} (green) and CD133^{-ve} (blue) Ishikawa cell lines in 100μM LNG over 72 hours. Results are expressed as mean ± SD, experiments performed in triplicate (n=3). Differences between CD133^{+ve} and CD133^{-ve} cells were analysed using an unpaired T-test. Significance is shown via (*). * P <0.05.

Finally, the clinical relevance of stem cells in LNG resistance was assessed. CD133 protein expression did not show value in predicting response to Mirena® treatment. However, in people showing complete histological response to Mirena® treatment (n=11), CD133 expression decreased during treatment (p=0.04). There were no observable trends in people showing histological non-response to treatment (n=6). Representative images of CD133 staining are shown in **Figure 2**.

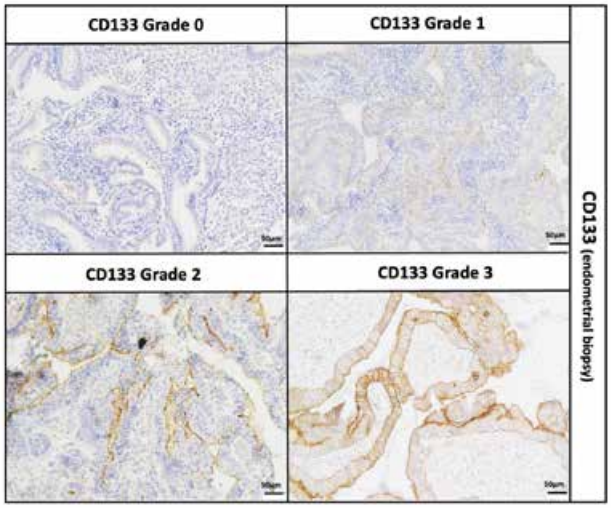


FIGURE 2. Representative images of CD133 protein expression in endometrial biopsy samples.
Grading scores ranged from 0 (no staining in epithelial cells) to 3 (strong staining in epithelial cells of samples). Images taken at 20x magnification.

CONCLUSION

We have demonstrated that EC stem-like cells may contribute to Mirena® treatment resistance. CD133^{+ve} stem-like cells displayed increased stem cell marker expression, enhanced sphere formation, and greater LNG resistance compared to CD133^{-ve} cells, indicating a distinct stem-like phenotype. Additionally, CD133 protein expression in endometrial biopsy samples warrants further investigation as a potential marker for monitoring response to Mirena®.

University of Otago, Wellington

SPLIT ENZ: Survivorship of Patients post Long Intensive care stay, exploration in a New Zealand cohort

INTERIM REPORT

PRIMARY INVESTIGATOR:

LYNSEY SUTTON *MNclin; PGdip, RNdip* [1,2]

[1] Clinical Nurse Specialist (CNS), Wellington Intensive Care Unit, Wellington Regional Hospital, Health New Zealand, Capital, Coast and Hutt Valley, Riddiford Street, Newtown, Wellington, New Zealand, 6021

[2.] PHD student, Department of Psychological Medicine, University of Otago, Wellington, New Zealand.

SUPERVISORY TEAM AND SECONDARY CO-AUTHORS:

DR. PAUL SKIRROW *DClinPsychol, PGDipClinNeuropsych, MPhil., BSc (hons)*

Associate Professor **DR. ELLIOT BELL** *PhD; PGDipClinPsych, MA, BA (Hons), BCA, FNZCCP*

Associate Professor **SUSANNA EVERY-PALMER** *MBChB, MSc, FRANZCP, PhD*

DR MARK WEATHERALL, Department of Medicine, University of Otago, Wellington

REPORT SUMMARY

This is a 5–6-year part time PhD research exploring the survivorship journey, experience, outcomes and post Intensive Care Syndrome (PICS) for patients who have had a critical illness in the Intensive Care Unit (ICU). The timeframe for this study is aiming for completion at the end of 2025 and is progressing within well. This is a mixed methods study with two arms: quantitative and qualitative. The quantitative component is a prospective cohort study using validated tools (questionnaires) to assess and quantify the level of disability in the 12 months following critical illness. Functional disability, Health-related quality of life, mental health, and cognition are explored at three time periods in the year following critical illness. Currently the study has completed recruitment, meeting its aim of over 100 patients with 118 patients recruited by Dec 2023. The results for this part of the study will hopefully be available by the end of 2025, currently still undergoing data collection.

The Qualitative study has finished and is currently being reported (with first manuscript at peer review stage with journal). This important study explores the journey and survivorship experience for participants once home. This design uses a nested convenience sample conducting semi-structured Interviews at around 6-8 months post discharge home. In total 11 participants were recruited, and a model of recovery was created.

An equally important part of the qualitative research is an exploration of unmet needs during the recovery journey. The major aim is to identify the ongoing needs (met and unmet) in the year following critical illness. Understanding what is missing will help highlight strategies to better meet the needs for patients recovering from critical illness in the future. This study is still being reported and aiming for publication by October 2024.

The following summaries of both those qualitative explorations is given below:

1. **Earthquakes, and aftershocks: What is the survivorship journey through critical illness in Aotearoa, New Zealand? A grounded theory study.**

Aim

To date, very little research has been published exploring the survivorship journey and recovery after critical illness in New Zealand.

Methods

This Constructivist Grounded Theory study aims to explore this journey, highlighting the impact of critical illness disability, memories and experiences, and transition through hospital to recovery at home in the first seven months.

Results

The "Seismic Survivorship Model" in critical illness links the patient's journey to the phases of an earthquake. This central phenomenon describes the survivorship journey with progression through the following core phases: foreshocks, mainshock of the critical illness, seismic waves of the illness leading to emergency management, aftershocks and disaster management in the Intensive Care Unit, and the aftermath experience on the ward. Participants move on to the rebuild during rehabilitation, and reconstruction and regeneration begins once home. The survivorship journey is tinged with physical disability, psychological distress, delirium, and cognitive dysfunction, of being trapped and dependent on others, and complications and symptoms persist for many months.

Conclusion

For most participants this journey is a time of deep reflection on what life was like before, of processing illness and disability, and living with new vulnerabilities. In time, this culminates in an acceptance of a changed life once the main shock and aftermath of critical illness has been managed.

2. **Aftershocks and critical illness: What are the needs of survivors during recovery once home?**

Background

There is little to no research exploring what the needs are of critical illness survivors during recovery at home in Aotearoa, New Zealand.

Objective

To explore and report common themes around needs unmet during recovery at home.

Methods

This is a thematic analysis of findings from a Constructivist Grounded Theory study exploring the survivorship journey for survivors of critical illness in Aotearoa, New Zealand. Eleven participants were recruited and interviewed.

Results

The survivorship journey after critical illness is arduous, burdensome and complicated. As well as physical disability, there is psychological fragility, vulnerability, and persistent complications and symptoms. Access to rehabilitation, home help, hygiene assistance and gardening support were the main functional needs identified. Not everyone who required help received it and there were regional disparities in services offered. Psychological support for participants to process the aftermath of critical illness, navigate new burdens and vulnerabilities and manage the emotional aftermath of delirium is greatly needed. Information and reassurance around cognitive dysfunction and recovery trajectories after critical illness are crucial. Little to no follow up exists for participants once home, a missed opportunity to address all these issues.

Conclusion

There were multiple unmet needs for participants around information, psychological support, reassurance, functional supports, discharge processes, and financial implications. A follow up service may provide an opportunity to improve fractured care and services, referrals, provide information, understanding, and reassurance together in a tidy package.

University of Otago, Wellington

The effects of dual anti-platelet therapy (DAPT) on the platelet-neutrophil axis and the formation of neutrophil extracellular traps (NETs)

S HUMMEL, K HALLY, P LARSEN

KEY FINDINGS

Dual anti-platelet therapy (DAPT) is the current gold standard for treating heart attacks as DAPT prevents cellular components in the blood, called platelets, from aggregating and thus reduces recurrent adverse cardiovascular events. However, heart attacks have a complex pathophysiology, involving a variety of immune cells that can interact with platelets. Neutrophils have gained a new appreciation in the pathophysiology of heart attacks due to their ability to form Neutrophil extracellular traps (NETs), a pro-inflammatory mechanism that has been associated with recurrent adverse cardiovascular events. We have previously demonstrated that platelets can dampen NET formation and as such, seems to immunomodulate the production of this pro-inflammatory mechanism. Here, we found that DAPT did not affect the ability of platelets to dampen NET production, but that platelets also seem to aggregate more strongly following DAPT.

OBJECTIVES

We aimed to investigate the effects of DAPT on the interplay of platelets and neutrophils.

METHODS

Patient recruitment: We recruited 13 healthy volunteers (five males and eight females), aged between 45 to 70, into a prospective interventional study with DAPT (aspirin and ticagrelor) as the intervention. Exclusion criteria for healthy subjects were known cardiovascular or inflammatory disease, disorders associated with platelet dysfunction, acute illness within six weeks prior to recruitment, pregnancy, diabetes mellitus, a history of adverse drug reaction, treatment with cardiovascular and immune-modulating drugs or antiplatelet agents within seven days prior to recruitment, having had a vaccine within the last six weeks or awaiting a COVID-19 test result at any point throughout the study. Ten of these healthy volunteers were asked to provide a blood sample before and after treatment with DAPT for isolation of treatment-naïve and DAPT-treated platelets. The other three healthy volunteers were not receiving any DAPT and were asked to provide a blood sample for isolation of treatment-naïve neutrophils.

Administration of DAPT. Ten healthy subjects in the intervention arm received DAPT for two days. DAPT consisted of a combination of aspirin (300mg loading dose on day 1, 100mg maintenance dose on day 2) and ticagrelor (180mg loading dose on day 1, 90mg maintenance dose on day 1 and day 2). This mimics the routine of DAPT administration given to individuals with AMI in Aotearoa New Zealand.

Platelet and neutrophil isolation and *in vitro* co-culture. To test the effects of DAPT on the ability of platelets to dampen NET formation, platelets were isolated from ACD-anticoagulated whole blood from ten volunteers in the interventional part of the study and resuspended in PBS to 5 x 10^7 platelets/mL. Neutrophils were isolated directly from EDTA-anticoagulated whole blood from three treatment-naïve volunteers and resuspended in cell culture media to 2 x 10^6 neutrophils/mL. Platelets and neutrophils were co-cultured and simultaneously stimulated with various concentrations of PMA (4 nM to 500 nM), or Ionomycin (5 µM to 75 µM), both classical NET agonists, or FSL-1 (0.016 µg/mL to 1 µg/mL), a neutrophil activator, but does not induce NETosis, or left unstimulated, for two hours at 37˚C/ 5% CO_2.

Measuring NETosis. We used Myeloperoxidase activity and SytoxGreen fluorescence as surrogate markers of NETosis. Co-cultures were cultured in the presence of the

cell-impermeable nucleic acid stain SytoxGreen. Briefly, upon NET formation, DNA is expelled from the cells and the cell-impermeable nucleic acid stain is able to bind to the extracellular DNA and the amount of NET formation can be inferred from the fluorescent intensity. Additionally, we utilized the peroxidase activity of MPO, a peroxidase found within NETs. Following co-culture, free-floating MPO was removed by discarding the supernatant prior to vigorous resuspension of the neutrophil monolayer, which contains NET-appendant MPO. Using a colorimetric assay, NET release was inferred proportionally from MPO activity.

Measuring platelet aggregation. To assess the extent to which platelets aggregated, we utilized Light Transmission Aggregometry (LTA). Briefly, we measured how much light passed through the co-cultures at time 0 and after 2 hours of stimulation. As platelets aggregate, more light passes through the culture, and the extent of aggregation can be inferred proportionally from the amount of light detected.

STATISTICS

We calculated fold changes (signal:noise) ratios for all assays using the unstimulated samples as their own control. Samples were then normalized to their pre-DAPT values. We performed an unpaired t-test with Welsh's correction and assumed normal Gaussian distribution (α = 5% significance level) to compare NET formation pre- and post-DAPT and used Šídák's multiple comparisons tests to test whether platelets aggregation was changed pre- and psot-DAPT. Values are displayed as mean ± SEM.

RESULTS

To assess whether DAPT treatment affects the ability of platelets to dampen NET formation, we measured MPO activity and SytoxGreen fluorescence as surrogate markers of NETosis pre- and post-DAPT treatment in platelet-neutrophil co-cultures. Neutrophil SytoxGreen expression and MPO activity was not statistically significant between pre- and post-DAPT treatment across all concentrations of PMA (Figure 1A, D), Ionomycin (Figure 1B, E) and FSL-1 (Figure 1C, F).

To assess whether DAPT treatment affected the ability of platelets to aggregate in response to NET-inducing agonists, we measured LTA pre- and post-DAPT treatment in platelet-neutrophil co-cultures. Platelets aggregated significantly more strongly than pre-DAPT platelets in co-culture, both in response to PMA (Figure 2A) and Ionomycin (Figure 2B) across all concentrations.

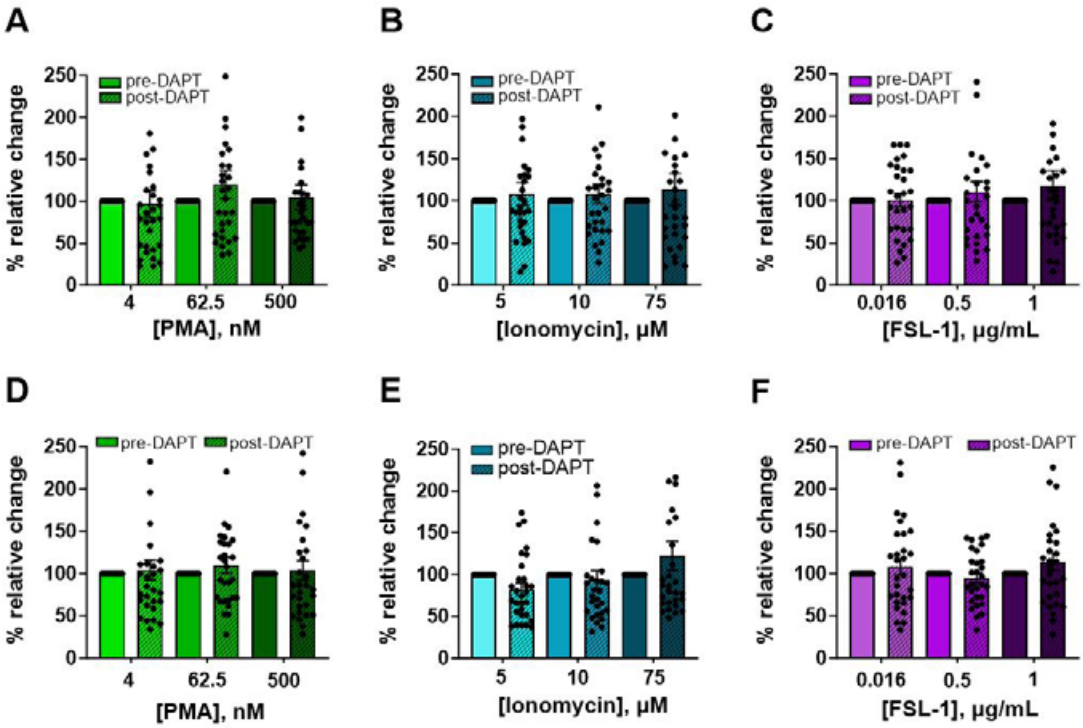


FIGURE 1. DAPT treatment does not alter the effect of platelets on NET formation. Platelets from ten healthy participants, both pre- and post-DAPT treatment, were co-cultured with treatment-naïve neutrophils from three healthy participants. SYTOX Green Fluorescence (A, B) and NET-associated MPO activity (C, D) in platelet-neutrophil co-cultures did not differ pre- and post-DAPT treatment. Mean ± SEM of n=30 replicates.

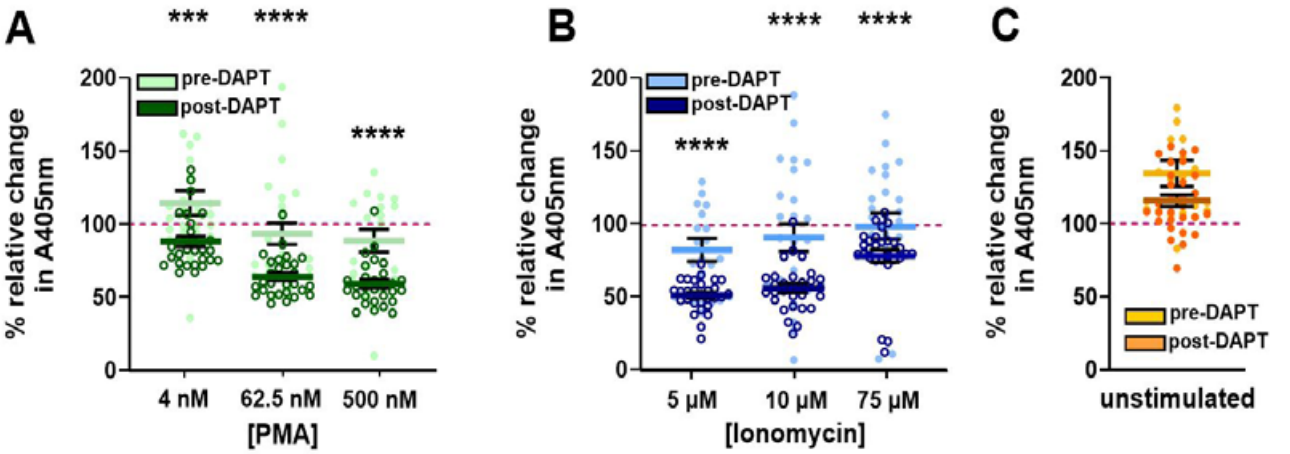


Figure 2. DAPT alters platelet aggregation in response to NET agonists in neutrophil-platelet co-cultures. Platelets from ten healthy participants, both pre- and post-DAPT, were co-cultured with treatment-naïve neutrophils from three healthy participants. Post-DAPT platelets aggregate significantly more strongly than pre-DAPT platelets in co-culture, both in response to PMA **(A)** and Ionomycin **(B)** across all concentrations. Platelet aggregation remained unchanged in co-cultures with neutrophils in CCM **(C)**. Mean ± SEM. Washed platelets were obtained from ACD vacutainers. ***p<0.001, ****p<0.0001 with Šídák's multiple comparisons tests.

CONCUSION

We assessed the effects of DAPT on platelet-mediated NET dampening and observed that NET production was unchanged in co-cultures pre- and post-DAPT in response to NET-inducing stimulants. This suggests that the pathways that cause platelets to aggregate are not inhibited by DAPT. This is further supported by our observation that platelets aggregate more strongly in response to NET-inducing agonists post-DAPT treatment. While DAPT has shown to reduce recurrent adverse events for patients diagnosed with heart attacks due to reduced platelet aggregation, further research is needed to understand why DAPT may prime platelets for a stronger aggregatory response when exposed to NET-inducing agonists. It may be that DAPT primes platelets to respond strongly if the body is exposed to dangerous pathogens that produce NET-inducing chemicals but prevents platelets from aggregating in less severe situations.

RESEARCH PROJECT & TRAVEL GRANTS

Research Project Grants 2024

THE FOLLOWING PROJECTS HAVE BEEN APPROVED FOR FUNDING IN THE FINANCIAL YEAR ENDED 30 JUNE 2024 OR ARE LONGER TERM PROJECTS FROM PREVIOUS FUNDING ROUNDS. THESE WILL BE REPORTED ON IN SUBSEQUENT RESEARCH REVIEWS.

Evaluating Kappa Opioid Receptor Localisation and Expression in Multiple Sclerosis

MARTIN DALEFIELD

Martin Dalefield, M.Phil., received a Research for Life grant of up to $18,000 to conduct research into multiple sclerosis, an autoimmune disorder that is the most common cause of non-traumatic neurologic disability in young people. Several studies have found that drugs that target the kappa opioid receptor are beneficial in promoting recovery in preclinical models of multiple sclerosis, but it remains unclear whether these preclinical results will translate into benefits for human patients. By working with the post-mortem brains of patients with multiple sclerosis, Mr. Dalefield will work to clarify the specific location of the kappa opioid receptor and how these changes in MS. This will allow us to better understand how drugs targeting this receptor can used to benefit patients with multiple sclerosis. Mr. Dalefield is a PhD student in the School of Biological Sciences at Victoria University of Wellington supervised by Associate Professor Bronwyn Kivell.

Defining the role of immunometabolism in multiple sclerosis

GEORGIA LENIHAN-GEELS

Dr Georgia Lenihan-Geels received a grant of up $21,921 to undertake research to improve our understanding of immune cell metabolism in progressive multiple sclerosis patients. Aotearoa has one of the highest rates of multiple sclerosis worldwide and there is a lack of effective treatment options for those with progressive forms of multiple sclerosis. Recent findings have shown that patients with secondary progressive multiple sclerosis have higher levels of activated immune cells in the blood. Dr Lenihan-Geels' research aims to better understand the influence of these cells on MS disease outcomes, such as fatigue and cognition. Dr Lenihan-Geels is a postdoctoral researcher at Victoria University of Wellington - Te Herenga Waka.

Investigating differences in the peripheral immune signature of females and males with Acute Myocardial Infarction (AMI).

CERYS BLACKSHAW

Cerys Blackshaw received a grant of up to $15,295 to undertake research to help women with cardiovascular disease (CVD). CVD is the leading cause of mortality for women worldwide, but research shows that there are inequalities of care between women and men. International reviews have shown that females have worse health outcomes due to a lack of comparable care between males and females. In a New Zealand context, we have found that females are experiencing less guideline-based interventions but have seen no effect on health outcomes. Cerys' research is investigating if these differences are due to systemic bias in the hospital system, or due to a physical difference in the way the disease impacts women compared to men. Cerys is a PhD student at Otago University, working in the Surgery and Anaesthesia Department at Wellington Regional Hospital.

A narrative inquiry exploring the experiences of survivors of long-term intensive care and their support people in Aotearoa New Zealand

AMY BEST

Ms Amy Best received a grant of up to $2,000 grant to undertake research to help former critically ill patients and families who have experienced a prolonged stay in the intensive care unit (ICU). Most people admitted to an ICU have a relatively brief length of stay, however, up to ten percent do not recover rapidly, experiencing a prolonged stay (≥8 days). Internationally there is very limited knowledge on prolonged ICU stays as well as the complexities, vulnerabilities, and traumas that a protracted stay creates for survivors and their family. Ms Best's research is creating new knowledge by exploring survivors' and support peoples' lived experience territory across the first year following discharge from hospital in New Zealand. Through the narrative process, health, and healthcare issues as well as the support needs of these people be illuminated, thus, this research has the potential to improve the care and support ICU survivors receive. Ms Best is a PhD candidate at Massey University School of Nursing and a senior staff nurse in the ICU at Wellington Regional Hospital.

Investigate The Effect of Kappa Opioid Agonism in Food Allergies Model

HASANAH HAMIZAN

Hasanah Hamizan received a grant of up to $18,832 to unpack the potential benefits of the kappa opioid receptor agonists as alternative food allergies treatment. Hasanah's research will explore the little-known mechanisms and immune response (particularly mast cell activity) involved during kappa opioid receptor agonists treatment in animal models. Hasanah is a second year PhD student working under the supervision of Prof Anne La Flamme in the School of Biological Sciences and Centre for Biodiscovery at Victoria University of Wellington.

Modelling the transition between goal-directed to habitual drug-seeking behaviour in the rat

JUAN CANALES

Professor Juan Canales received a grant of up to $16,971 to develop a new model to study the transition from voluntary drug use to habitual, compulsive drug taking. Addiction can be seen as a habit in the sense that it involves a repetitive pattern of behaviour that is extremely hard to break. Drug habits become strongly associated with drug-related cues and contexts, such as drug paraphernalia, that are able to induce uncontrollable urges, contributing to the maintenance of a substance use disorder. Such drug habits often persist in spite of negative consequences. Professor Canales will develop a new animal model of drug habit formation that will provide new insights into the role of learning mechanisms in addiction and pave the way for the design of new medical treatments and interventions for substance use disorders. Professor Canales is Head of School for the School of Psychology at Te Herenga Waka – Victoria University of Wellington.

Generating a single-cell atlas of the murine lung in health and disease

KERRY HILLIGAN AND DAVID SESTER

Dr Kerry Hilligan and Dr David Sester received a grant of up to $10,000 to undertake research into respiratory infectious diseases, such as the flu virus, using a new cutting-edge technology. Spectral cytometry enables the simultaneous analysis of 50+ parameters from each individual sample at a single-cell level, enabling data collection on millions of cells. Dr Hilligan and Dr Sester will use this information to learn about the early immune response following a flu infection to identify potential targets for future vaccines. Dr Hilligan is a Senior Research Fellow and Dr Sester is Head of Cytometry at the Malaghan Institute.

Developing 3D models of breast cancer for therapeutic study

HELENA ABOLINS-THOMPSON

Helena Abolins-Thompson received a grant of up to $24,374 to establish 3D models of different subtypes of breast cancer. She will be comparing integrated spheroid models derived from immortalised cell lines to patient-derived organoid models generated from primary patient samples. This will be done in an exclusively Māori patient cohort. Māori patients are at higher risk of breast, presenting at later and more advanced stages. Confocal microscopy and viability assays will be used to assess the effect of clinically relevant chemotherapies on these models with the wider goal of ensuring Māori are represented in therapeutic testing platforms, which will allow further exploration into more appropriate and equitable treatments for Māori patient cohorts. Helena is a PhD candidate in the Surgical Cancer Research Group at the University of Otago, Wellington.

A comparison of post meal glycaemic excursion after ingestion of konjac glucomannan rice, cauliflower rice and brown rice (control) in type 1 diabetes.

DR AMBER PARRY STRONG

Dr Amber Parry Strong received a Research for Life grant of up to $6,859 to undertake research to help people with Type 1 Diabetes. Type 1 Diabetes affects around 11000 adults in New Zealand and her research team are always trying to find solutions that work clinically for people on a practical level. This project is looking at a high fibre foodstuff called konjac which can be eaten as rice or noodles, and the effect this has on blood glucose levels in type 1 diabetes. Dr Parry Strong is a Research Fellow at the Centre for Endocrine, Diabetes and Obesity Research at Wellington Hospital.

A Feasibility Study for Ovarian Cancer Biomarker Detection in Vaginal Swabs

DR SARAH SCZELECKI

Dr Sarah Sczelecki, A/Prof Janet Pitman and Dr Claire Henry received a Research for Life grant of up to $16,434 to undertake research to assess a potential ovarian cancer screening methodology. Ovarian cancer is the most lethal gynaecological cancer, partly due to the lack of a diagnostic test for general population screening. This research will investigate the feasibility of ovarian cancer diagnostic testing using vaginal swabs collected similarly to the new national cervical screening self-swabbing programme.

Forewarned is forearmed: Investigating and mitigating resistance to the clinical antibiotic candidate niclosamide

HANNAH LEE-HARWOOD

Dr Hannah Lee-Harwood received a Research for Life grant of up to $13,950 to undertake research that will support the clinical progression of niclosamide, a repurposed anti-parasite drug that is under current evaluation as an antibacterial drug candidate. Owing to the widespread emergence of life-threatening antibiotic resistant bacterial pathogens, new antibiotics are urgently needed. Niclosamide is a promising candidate that has strong anti-bacterial activity. However, to support its clinical development, it is important to perform a comprehensive assessment of the future mechanisms of antibiotic resistance that are likely to emerge. Using an innovative method to search for potential resistance genes present in environmental bacteria, Hannah has shown that there are several classes of gene that encode niclosamide-detoxifying protective elements. She is now testing these to assess the risk they pose, and identifying alternative antibiotic sensitivities that arise as bacteria gain resistance to niclosamide. These insights will inform potential countermeasures to combat future strains of niclosamide-resistant bacteria.

Characterising platelet activation in response to vascular surgery

KATHRYN HALLY

Dr Kathryn Hally has received a $15,770 Research For Life grant to explore how platelet activation changes after vascular surgery. Although a necessary process, surgery is associated with significant activation of Grant-in-aid of research application, March 2024 Hally, Thompson, La Flamme6platelets, the clotting cells in blood. As these cells are hyperactivated after surgery, they may contribute to the risk of experiencing a cardiovascular event after surgery such as a heart attack. Patients that are undergoing surgery to treat peripheral vascular disease are at a heightened risk of these types of events, because they often have several cardiovascular risk factors. Kathryn's research aims to investigate the change in platelet activation that occurs over time after vascular surgery. The research team will use this information to study platelet activation biomarkers for their ability to predict risk following vascular surgery in future research. This current research will be conducted by Emma Thompson, a BBioMedSc(Hons) student at UOW, who will be supervised by Kathryn and Professor Anne La Flamme (Te Herenga Waka).

The effects of in vivo anti-platelet therapy on neutrophils

CERIDWYN JONES

PhD student Ceridwyn Jones received a Research for Life grant of up to $16,472 to undertake research to investigate the neutrophil response to anti-platelet therapy. Anti-platelet therapy is the gold standard treatment to manage heart attacks and prevent recurrent cardiovascular events in Aotearoa New Zealand, however a third of New Zealander's will still experience a recurrent event within a year after their heart attack. Ceridwyn's research is exploring the off-target effects of anti-platelet therapy on neutrophils, as a key mediator of inflammation after a heart attack. Ceridwyn is in the third year of her PhD at Victoria University of Wellington.

Investigating the temporality of monocyte function after acute myocardial infarcation

KATHRYN HALLY

Dr Kathryn Hally, Lecturer at the University of Otago Wellington (UOW), has received a Research For Life grant of up to $11,274 to characterise the function of monocytes in response to a heart attack. Monocytes are an important circulating immune cell that migrate into the affected heart tissue and are involved in healing after a heart attack. It was previously thought that monocytes are helpful in this context by supporting tissue repair. However, Kathryn and her research team have recently demonstrated that monocytes remain activated up to 10 days after a heart attack. Constant activation of these cells may maintain an inflammatory environment within the heart tissue, and lead to harm. To strengthen these findings, this current research aims to understand how monocytes function during this time after a heart attack: are monocytes involved in maintaining inflammation or facilitating tissue repair? This research will involve analysing monocytes from a biobank of cryopreserved peripheral blood mononuclear cells (PBMCs) that have previously been collected from heart attack patients, and will be conducted by Michael Roberts, a BBioMedSc(Hons) student at UOW. Michael will be supervised by Kathryn, alongside Dr Ana Holley, Lecturer at UOW, and AProf Peter Larsen, Head of Department of Surgery and Anaesthesia at UOW.

Bartonella quintana interactions with the host innate immune system

BRIANNA OTTO

Brianna Mouariki Otto has received a Research for Life grant of up to $12,500 to carry out research on the interactions between the human pathogen Bartonella quintana and innate immune cells. B. quintana causes a disease known as trench fever in humans hosts but can also persist chronically in the bloodstream. It also causes serious complications, such as endocarditis, in some patients. Very little is understood about how B. quintana avoids the immune response to cause disease. Preliminary data suggests that B. quintana resides in an uncharacterised vacuole in host macrophages and that it avoids or delays the bactericidal activity of macrophages. The aim of this research is to determine how long B. quintana survive inside macrophages and to use microscopy and staining of cellular markers to characterise the vacuoles that B. quintana resides in. The function of macrophages following phagocytosis will also be assessed.

Enhancing mucosal immunity by determining the immunological basis of pulmonary tertiary lymphoid structure formation using micro-dissection through photoconversion

DR KIT MOLONEY-GEANY

Dr Kit Moloney-Geany and Alfonso J. Schmidt received up to $24,139 as part of a Research for Life grant to explore how the lungs develop immunity towards the flu virus. By using cutting-edge technologies, the research team will use a specialised microscope to isolate desired regions in flu-infected mouse lung tissue. By isolating specific structures known as tertiary lymphoid structures, the researchers will be able to analyse the interactions and development of individual cells that are vital to fighting the flu virus. By better understanding how these structures are formed, the research team hopes to inform on what components of a vaccine would be needed to induce these structures and boost the potency of flu vaccines.

Investigating the effect of kappa opioid receptor agonists on the differentiation and function CD4+ T cell subsets

BROOKE WALDRAM

Brooke Waldram received a Research for Life grant of up $13,351 to investigate the effect of kappa opioid receptor (KOR) agonists on CD4+ T helper cell responses. These cells can be either pro- or anti-inflammatory and play a key role in the initiation and progression of multiple sclerosis (MS), an autoimmune disease that affects the central nervous system. The KOR agonist nalfurafine has been shown to reduce disease severity and promote remyelination in an animal model of MS. This research aims to understand the immunomodulatory effects of nalfurafine treatment and how it might alter CD4+ T cell responses in models of MS. Brooke is a master's student supervised by Prof Anne La Flamme at Victoria University of Wellington.

Investigating Fibrosis in Liver Cells during Bartonella Infection

ZOE KING

Zoë King has received a Research for Life grant of up to $13,800 to undertake research into the mechanisms underlying development of liver fibrosis during Bartonella henselae infection. B. henselae is an underdiagnosed but prevalent bacterial pathogen in New Zealand. Transmitted to individuals from infected cats, B. henselae typically causes a self-limiting illness, cat scratch disease. However, some patients develop serious complications from the infection, including liver infection and fibrosis. Fibrosis, characterised by the proliferation of extracellular matrix proteins, such as collagen, contributes to numerous diseases. The aim of this research is to characterise the interactions between B. henselae and liver cells, including those that promote fibrosis, and to identify B. henselae virulence factors that are involved. Findings could lead to better prevention, treatment, and diagnosis of B. henselae liver infections.

Assessment of Peripheral Human Lymphocytes in Response to Nalfurafine Treatment

MACKENZIE KIERNAN

Mackenzie Kiernan from Victoria University of Wellington has received a Research for Life grant of up to $19,410 to assess the clinical relevance of a new treatment for multiple sclerosis. This chronic inflammatory condition results in the breakdown of nerves, potentially leading to significant pain and eventual paralysis in individuals affected. This novel treatment has demonstrated excellent pre-clinical efficacy, and the aim of this research is to determine whether it could be an effective treatment for multiple sclerosis.

Characterising the immunomodulatory properties and mechanism of action of a novel Btk inhibitor in B cells and microglia

ELYSHA-ROSE GRANT

Elysha-Rose Grant received a Research for Life grant of up to $13,450 to investigate potential treatments for multiple sclerosis. Multiple sclerosis is a chronic inflammatory disease that affects approximately 1 in 1000 people in Aotearoa New Zealand, and results in symptoms that include vision loss, fatigue, and paralysis. In Elysha's research, she will investigate how a potential therapeutic impacts the function of B cells and microglia, immune cells that play an important role in multiple sclerosis. Elysha-Rose Grant is a PhD student under the supervision of Professor Anne La Flamme at the School of Biological Sciences, Victoria University of Wellington.

## The role of colorectal tumour-derived extracellular vesicles in modulating primary human monocytes **KIRSTY DANIELSON**	Dr Kirsty Danielson received a Research for Life grant of up to $16,100 to undertake research on how tumour cells talk to immune cells in bowel cancer. Bowel cancer affects more than 1200 New Zealanders every year and studying the way that tumour cells interact with the immune system could help with the development of new treatments. Dr Danielson is Group Leader of the Surgical Cancer Research Group at the University of Otago, Wellington.

## Unlocking therapeutic potential: Repurposing drugs to treat invasive meningioma **MATTHEW MONRO**	Matthew Munro received a RFL grant of $ 14,000 to research meningioma, the most common brain tumour, with incidence rates three times greater in women than men. Patients with symptoms can undergo surgical removal, but high-grade cases are likely to recur due to tumour cell invasion into the brain. However, if invasion were slowed or prevented, recurrence would be less likely, and more cases could be monitored without requiring removal and associated post-surgical morbidity. We have preliminary evidence that the Pharmac-subsidised drugs sunitinib, mebendazole and everolimus can reduce meningioma invasion. In this context, I will identify differences between pre- and post-drug treated meningiomas. We will perform a thorough proteomic and metabolomic analysis to determine the mechanisms through which repurposed drugs affect cellular invasion. Understanding the unique molecular mechanisms involved in meningioma invasion and progression will help identify potential therapeutic targets. Our goal of identifying repurposed drugs that are off-patent and relatively inexpensive aims to contribute to health care equity for all New Zealanders by enabling wider access to treatment throughout the community.

Travel Grant Reports

CERIDWYN JONES

INTERNATIONAL SOCIETY FOR THE ADVANCEMENT OF CYTOMETRY (ISAC) ANNUAL SCIENTIFIC CONFERENCE CYTO, EDINBURGH IN MAY 2024

Ceridwyn Jones

With the support of the Wellington Medical Research Foundation, I was able to attend and present her PhD research at the International Society for Advancement of Cytometry (ISAC) annual scientific meeting - CYTO2024, in Edinburgh, Scotland in May this year. CYTO is the premier international cytometry conference, and consisted of workshops, plenary sessions, and presentations from leading academics in the field.

I presented my research on the development of a flow cytometry-based methodology to characterise neutrophils – a type of white blood cell – which are understudied in this context due to their inability to be stored and high degree of protein co-expression on their surface. Presenting this novel work at CYTO allowed me to receive invaluable feedback from esteemed experts in cytometry, which was particularly useful given the challenging development of this technique. During my presentation, I received encouragement on this work from highly regarded academics and was interviewed and filmed about this research. I was also able to establish a collaboration with the other attending scientist who researches neutrophils – Associate Professor Jiří Hrdý from Charles University in Prague.

Attending the workshops at CYTO allowed me to develop knowledge and skills in advanced cytometry techniques, including imaging flow cytometry and high-dimensional clustering algorithms; techniques highly relevant to my current and future research. The in-depth workshops at CYTO on these techniques included panels consisting of experts and/or founders of these techniques, and have equipped me with the technical expertise to incorporate their use into my research, which will elevate the overall quality of my work.

Overall, my attendance at CYTO2024 was inspiring, rewarding, and invaluable for my current PhD and future career.

CERYS BLACKSHAW

71ST ANNUAL MEETING OF THE CARDIAC SOCIETY OF AUSTRALIA AND NEW ZEALAND (CSANZ), ADELAIDE, AUSTRALIA

Cerys Blackshaw

CSANZ is an excellent conference to attend as a burgeoning cardiac researcher, as it has plenty of lectures to attend in a wide range of topics. Esteemed speakers were present from all over the world, including several local and impressive names. The program consists of four days, with both lecture and networking functions, as well as poster and mini-oral presentations.

Having the opportunity to attend CSANZ was an amazing experience for me, as it gave me the first chance to present my research to fellow academics from other research groups. Presenting my poster allowed me to engage with several researchers, both academic and clinical, to show them my research, and to gain new insight and ideas.

The program may be jam-packed full of interesting lectures, but I also made time to attend a 'women in cardiology' afternoon tea, which was an incredible experience where the voices of the women in STEM were highlighted and heard. It was incredible to have a space to connect one-on-one with the lecturers and scientists I had been listening to, and to have empowering conversations with the strong women leading my field.

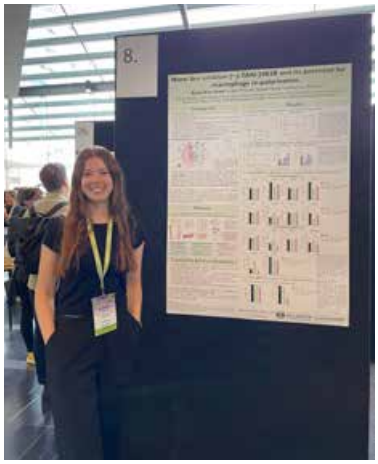
In addition, the program encouraged me to develop a new research interest in the cardiology field. I was also able to develop this interest through a lab visit with Professor John Beltrame's lab group, who were gracious and informative hosts.

Through attendance at this conference as a second-year PhD student, I have developed international connections I can build on throughout the rest of my PhD, as well as developing new project ideas to bring new research into the Wellington area. I have also developed personal and professional skills relating to presenting my work to peers and have gained experience and knowledge into cardiology research going on outside of New Zealand.

Overall, the 71st Annual CSANZ meeting was an incredible and inspirational experience for me, and something I look forward to building on throughout the rest of my PhD. I am very grateful to Research for Life for the support and funding to attend this conference.

ELYSHA-ROSE GRANT

51ST ANNUAL SCIENTIFIC MEETING OF AUSTRALIAN AND NEW ZEALAND SOCIETY FOR IMMUNOLOGY



Elysha-Rose Grant

Thanks to the generous support from the Wellington Medical Research Foundation, I was able to travel to Auckland and present at the 51st Annual Scientific Meeting of Australian and New Zealand Society for Immunology (ASI), held in December 2023.

I am a PhD student at Te Herenga Waka, Victoria University of Wellington (VUW). During my PhD, in collaboration with chemists at VUW, I am investigating a drug that specifically inhibits Btk, a pro-inflammatory molecule found in immune cells. The ASI conference held in Auckland last year provided me with the first opportunity to present the findings of my research so far. I presented a poster titled "Novel Btk inhibitor (–)-TAN-2483B and its potential for macrophage re-polarisation." In this poster, I presented results on the anti-inflammatory impacts of this novel fungal natural product on macrophages, and delved into the mechanism by which this inhibitor elicits its effects. These findings highlighted a potential for this novel Btk inhibitor to be used therapeutically to treat inflammatory disorders with aberrant Btk signalling, such as in multiple sclerosis. During the 2-hour poster session, I had the opportunity to discuss my project and gain meaningful insights and feedback from experts in my field, as well as from researchers specialising in other areas. The ASI conference ran over 5 days, and consisted of a variety of presentations, specialised workshops, and poster sessions. This provided me with many opportunities to interact with scientists across different fields and learn about a huge range of research being carried out in immunology, all of which have inspired future directions in my research.

Overall, attending this conference was a helpful and rewarding experience that helped me immensely in my PhD journey, and I am very grateful for the funding I received from Research for Life that allowed me attend.

HANNAH LEE-HARWOOD

ENZYME ENGINEERING XXVII IN OCTOBER 2023

Hannah Lee-Harwood

Receiving the Research for Life travel grant enabled me to attend the conference Enzyme Engineering XXVII in October 2023. Enzyme Engineering is the premier conference in my field and was the first international meeting I attended.

The poster session was a great opportunity to share my PhD work and connect with others who share similar research interests. I was approached by a researcher from Denmark who is investigating enzymatic methods to formulate analogues of niclosamide with improved bioavailability. They were interested in the mechanisms of niclosamide resistance I presented, and we have since discussed a possible collaboration.

Two of my favourite talks were from scientists at Merck. I am interested in the development of biosynthetic pathways that can provide greener, novel routes to the production of medically relevant compounds. The two speakers presented on enzyme cascades that Merck has extensively engineered to produce important medical compounds such as islatravir, an HIV drug, and belzutifan, an anti-cancer drug. This work, along with other talks on AI in protein engineering, has renewed my interest in how these techniques can be combined with the functional metagenomic screening methods I used during my PhD to discover and engineer new enzymes for industrial applications.

Thank you again to Research for Life for assisting in this vital part of my career development.

HANNAH VAN DER WOUDE

EUROPEAN ASSOCIATION FOR CANCER RESEARCH (EACR) GOODBYE FLAT BIOLOGY: NEXT GENERATION CANCER MODELS CONFERENCE IN BERLIN

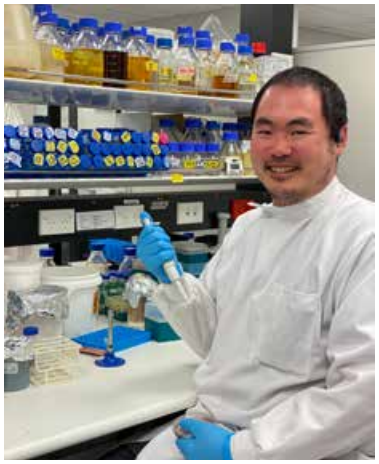
Hannah van der Woude

I am a third year PhD student in the Department of Surgery and Anaesthesia at the University of Otago Wellington. My PhD is focussed on endometrial cancer research. Specifically, I have developed a new model of studying endometrial cancer using whole patient tissue which is more biologically relevant compared to current research methods as it retains the multicellular environment and cellular interactions of the tumour. I have used this model to investigate the effect of treatment on the immune cells that reside within the tumour in an attempt to better understand treatment response. I received funding from Research For Life to attend the European Association for Cancer Research (EACR) Goodbye Flat Biology: Next Generation Cancer Models conference in Berlin in October 2023. This was my first international scientific conference, and it was an exciting opportunity to meet researchers and learn about some of the incredible work that is being conducted around the world. This was invaluable as, in my early career stage, I am interested in institutes that produce good quality research where I might want to work in the future. Additionally, the conference gave me the opportunity to present my research to fellow cancer scientists, building my confidence in science communication and networking.

The EACR, is a global community for those working and studying in cancer research. Their mission is "The advancement of cancer research for the public benefit: from basic research to prevention, treatment and care." The Goodbye Flat Biology conference was centred on new and innovative ways to model cancer in the lab that better reflect the way that tumours grow inside the body. With more biologically relevant models, we hope to increase the chances of success for novel therapeutics. The grant money received went towards paying for my return flights from Wellington, New Zealand to Berlin, Germany. As it was a relatively small conference with 200 attendees, I attended every presentation that was scheduled, which spanned three days. I also attended the "Speed Networking Session" where I was able to chat to academics about their career path and possible future collaborations. As a cancer biologist, I was fascinated by the various methods and techniques used to research cancer that go beyond the traditional "cells in a dish" technique. My poster was well received and gave me the opportunity to discuss my research with others who are interested in implementing my model into their own work. It also allowed me to brainstorm future projects and work through problems with people I normally wouldn't interact with. This all gave me insight into my data that I wouldn't have otherwise realised and has helped with the writing of my thesis upon my return. Overall, attendance at EACR:Goodbye Flat Biology was hugely beneficial to my current research and future career.

HUNG-EN LAI

2ND AUSNZ CHEMICAL BIOLOGY OF NATURAL PRODUCTS (CBNP) SYMPOSIUM 2023, AND THE 2023 SYNTHETIC BIOLOGY AUSTRALASIA (SBA) CONFERENCE



Hung-En Lai

After spending around 10 hours on the plane, I was excited to be greeted by the warm weather and sunshine in Perth, Australia, where two conferences are taking place back-to-back on 13-17 Nov 2023. The first was the 2nd AUSNZ Chemical Biology of Natural Products (CBNP) Symposium 2023, and the second was the 2023 Synthetic Biology Australasia (SBA) Conference, both hosted in the beautiful University of Western Australia (UWA) campus in Perth. The CBNP symposium was organized by Yit-Heng Chooi (UWA), and featured four keynote speakers (Nancy Keller – University of Wisconsin-Madison, Irina Vetter – University of Queensland, Marnix Medema – University of Wagenigen, Jeremy Owen – Victoria University of Wellington), and about 30 speakers from the Australasia region, with around 70 researchers attended over 13-14 Nov. I was honored to present our group's recent work titled "Partition-seq: cost-effective and retrievable archive of biosynthetic gene clusters in New Zealand soil microbiome", which aims to survey the biosynthetic potential of the soil microbiome that is largely uncultivable in laboratory conditions. There were fascinating talks presented by other speakers on fungal terpenoid biosynthesis, bioinformatic tools for predicting structure and bioactivity of putative natural products based on DNA sequences, bioprospecting of marine and plant natural products, and new methodologies in accessing chemical derivatives of natural products.

The SBA conference then took place on 15-17 Nov, attracting about 250 people in the synthetic biology communities mostly from Australasia, but also further afield from Europe and the United States. The SBA conference was organized by Georg Fritz (UWA) and featured several plenary speakers including Birger Moller (Copenhagen), Sabine Flitsch (Manchester), Rachel Ankenny (Adelaide) and Sally Gras (Melbourne). There are also seven break-out sessions where attendees can choose to attend one out of two sessions running in parallel, thus providing more speaking opportunities for researchers. Some of my favourite talks include Tristan de Rond (Auckland) on marine sponge terpenoid natural products, Max Cryle (Melbourne) on non-ribosomal peptide (NRP) biosynthesis and engineering, Matthew Gilliham (Adelaide) on the newly established Plant for Space (P4S) consortium, and Coco Xie (UWA) on fungal NRP biosynthesis. The next SBA is scheduled to be held in New Zealand in 2025, which will be a great opportunity for more NZ-based synthetic biologists to attend and connect with the global synthetic biology communities.

With the support from Research for Life which I am grateful for, it was a great pleasure and honor to present our work at the CBNP and SBA conferences, with constructive feedback from our peers in the natural product and synthetic biology communities. I made new connections and the interactions have inspired me to come up with new directions for my future research. I look forward to future conferences further afield to connect with the global natural product and synthetic biology communities, building more fruitful relationships and potential collaboration network.

DR KATHRYN HALLY

CYTO2024, EDINBURGH, MAY 2024

Kathryn co-hosts a workshop on reproducibility in longitudinal flow cytometry studies at CYTO2024. From left to right: Dr Ana Longhini (Memorial Sloan Kettering Cancer Centre), Dr Kathryn Hally (University of Otago), Sam Small (Malaghan Institute of Medical Research), Megan McCausland (Q2 Solutions) and Dr Laura Ferrer-Font (BD Biosciences, shown on screen).

Dr Kathryn Hally was awarded a travel grant to attend CYTO2024, which was hosted by the International Society for the Advancement of Cytometry (ISAC) in Edinburgh, Scotland, in May 2024. CYTO2024 is the premier international conference on cytometry and brings together experts involved in research, development and education in analytical cytometry. Dr Hally, who is a Lecturer at the University of Otago, Wellington, is an early career cytometrist and researcher. She particularly enjoyed networking with emerging and well-established cytometrists to advance her research and education goals. To highlight this point, Kathryn co-hosted a workshop with the goal of understanding the cytometry community's position on reproducibility in longitudinal cytometry studies. The workshop team, featured in the photo, have formed a strong collaboration with the aim to publish best practices on this topic. Kathryn wishes to thank Research For Life for their support, as this was an immensely beneficial experience.

NIAMH WALSH

NEW ZEALAND SOCIETY FOR ONCOLOGY (NZSO) CONFERENCE IN NAPIER

Niamh Walsh

In September, I attended the New Zealand Society for Oncology (NZSO) conference in Napier. I was a speaker and presented my research that I conducted over summer under the supervision of Dr Annie Wong. This was focused on melanoma patients with brain metastases and their clinical outcomes when treated with immune checkpoint inhibitors (ICI).

Melanoma is a very pertinent issue, as Aotearoa has the highest incidence of melanoma worldwide. ICI have revolutionised the treatment of metastatic melanoma and increased the median survival from months to three years. Despite this, brain metastases affect 30-70% of patients and remain an unmet need as ICI are less effective intracranially. Dual ICI is the standard therapy however, only single agent therapy is funded in Aotearoa, leading to Kiwis having poorer outcomes compared to the rest of the world.

The start of this trip was not smooth, as due to high winds my flight out of Dunedin was cancelled and there were no other flights leaving that day. Considering this, I had to present my research remotely and then drive straight to the airport after where I was able to successfully make it to Napier on my second attempt.

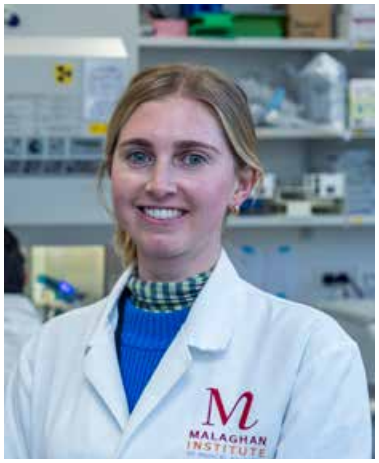
Presenting my research remotely posed its own challenges, as I was not able to see the audience and found it harder to connect with them as I was not able to gauge the audiences' responses to my presentation. Despite these challenges, the presentation resulted in a large discussion amongst health professionals about how this research affects their clinical practice, and how these results show that patients with late-stage disease are still able to respond to treatment and live for much longer than expected without disability.

I attended the Medical Oncology Group of Australia (MOGA) conference earlier that year in Perth to present this work. This was a great opportunity, but it was incredibly valuable to present this research on home soil due to its insights on Kiwis living with melanoma. This project was a national audit, involving collaboration across multiple cancer centres in New Zealand. It was incredibly valuable to reunite with oncologists I had worked with, and finally meet those I collaborated with remotely over summer in person, finally putting a face to the names I had constantly badgered with emails.

This was the first research project I had undertaken, and under Annie's supervision I was able to be provided opportunities I never would have thought of. Cancer touches the lives of us all and to try make a difference to the lives of patients living with late-stage melanoma has been an incredibly rewarding experience. I am extremely grateful to Research for Life for their support in enabling me to attend this conference and further my professional identity and interests.

OLIVIA BURN

2024 CD1-MR1 CONFERENCE IN HOBART, AUSTRALIA 26-29 FEBRUARY 2024



Olivia Burn

I am very grateful to have received a Research for Life travel grant to attend the 2024 CD1-MR1 conference in Hobart, Australia. This biennial conference is a crucial platform for sharing cutting-edge research about unconventional T cells, particularly natural killer T (NKT) cells, which are a significant focus of my research. My project aims to design better treatments for liver cancer, with Hepatocellular carcinoma – one of the most diagnosed and least survivable cancers in Aotearoa-New Zealand – as a primary focus. Through a highly successful collaboration between the Malaghan Institute and Ferrier Research Institute, we have developed a unique mRNA vaccine platform that harnesses the activity of NKT cells to activate the immune system. My research is assessing whether this mRNA vaccine can be used to overcome immunosuppressive networks present in liver tumours and improve the efficacy of other immunotherapies, such as immune checkpoint inhibitors (ICI), which currently have a minimal impact. To begin this assessment, I have been on secondment at Mount Sinai Hospital in New York to learn animal models of liver cancer and gather early efficacy data. This conference took place halfway through my placement and provided a valuable space to discuss my early findings with attendees, receiving feedback on the promising data I had already collected and advice on experimental design and protocols that could help to further understand the anti-tumour response generated by the vaccine. Their feedback has been very helpful as I now look to return to the Malaghan Institute, establish the liver cancer models there, and continue the project with an aim of gathering enough preclinical data to progress this vaccine to clinical stages. Furthermore, the networking sessions offered a great opportunity to initiate discussion with other laboratories about potential collaborations, which could further amplify the impact and scope of my research. An additional highlight was my colleague presenting our research on a malaria vaccine using this same mRNA platform, and hearing how well this data was received by the scientific community.

REBECCA PALMER

51ST ANNUAL SCIENTIFIC MEETING OF THE AUSTRALIAN AND NEW ZEALAND SOCIETY FOR IMMUNOLOGY (ASI) 2023 IN AUCKLAND, NEW ZEALAND

Rebecca Palmer

I am working towards my PhD in immunology at the Malaghan Institute of Medical Research in Te Whanganui a Tara, Wellington. In the early months of my first year, I had the opportunity to attend the 51st annual scientific meeting of the Australian and New Zealand Society for Immunology (ASI) 2023 in Auckland, New Zealand. This was the first conference I attended, and it was all made possible thanks to the generosity and support from Research for Life and their travel scholarship.

This conference provided a wonderful experience to connect with researchers at similar stages of their careers, paving the way for potential future collaborations. I explored a range of innovative and creative research, which inspired and motivated me in my own work.

For this conference, I presented a poster based on my research as a research officer before beginning my PhD. My work investigated the role of metabolism in specialized cells known as dendritic cells, focusing on how metabolism changes when encountering different infectious agents. Understanding these changes is crucial for grasping how these cells protect the body from dangerous microbes. Through my poster, I demonstrated a range immunological techniques and highlighted previous research within the group. I received valuable feedback and suggestions for future research, enhancing my understanding and direction.

Presenting this poster was an excellent opportunity to sharpen my communication and presentation skills which I believe are integral to productive and meaningful science. Additionally, I attended other poster sessions and oral presentations from researchers from Aotearoa, Australia, America, and Switzerland. Engaging with these researchers at the poster sessions and the student mixer allowed me to learn about their perspectives and creativity in overcoming scientific challenges. These conversations were particularly memorable and inspiring, motivating me as a young scientist.

Overall, attending the ASI 2023 conference was a transformative experience. It broadened my research horizons, helped me establish valuable connections, and significantly contributed to my professional development. I look forward to applying the insights and feedback I gained to my future research endeavours.

SARAH SCZELECKI

THE SOCIETY FOR THE STUDY OF REPRODUCTION ANNUAL MEETING IN OTTAWA, CANADA IN 2023

Sarah Sczelecki

With the generous support of the travel award from Research for Life, I was able to attend The Society for the Study of Reproduction Annual meeting in Ottawa, Canada in 2023. The topics at this international conference are a perfect intersection of my research interests, i.e., ovarian biology and cancer development. Attendance also provided many opportunites to interact with experts in these fields.

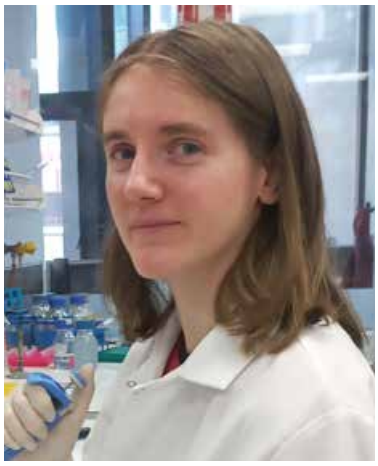
My abstract was selected for an oral presentation in the Reproductive Cancers session, where I discussed the main findings of my PhD research. This included some preliminary work funded by Research For Life, where I validated a new mouse model of early ovarian cancer development, which was then leveraged to aid in the discovery of novel candidate biomarkers of early ovarian cancer detection in human patients. This was the first international conference where I was selected for an oral presentation, so this opportunity was extremely beneficial to my career progression. The research presented was well received and sparked conversations within the session. I hope to attend on a consistent basis to continue to build the international connections I made.

The theme of the 55th annual meeting was '*Reproduction: Odyssey of Discovery*', thus focussed on cutting-edge technologies or advances in knowledge of reproductive biology. One keynote presentation was given by Rebecca Robker from the University of Adelaide. Her group is pioneering work on the role of the ovarian stroma in infertility and ovarian aging. This work is relevant to my research because there is a link between ovarian fibrosis (occurs with aging) and ovarian cancer development. This and other sessions provided ideas for my future research pursuits and was a fantastic opportunity to observe how ovarian research is carried out internationally and what technologies are being utilised to study disease.

Overall, the conference was an excellent opporunity to network with experts in the reproductive biology field and gave my research exposure on an international stage. I hope to maintain some of the connections I made, which may transform into international research collaborations in the future. I am extremely grateful to Research for Life for this opportunity to attend such a fantastic conference.

SARAH MESSENGER

THE ENZYME ENGINEERING XXVII CONFERENCE, SINGAPORE



Sarah Messenger

With the generous support of Research for Life, I was able to travel to Singapore to present my research at the Enzyme Engineering XXVII conference. This conference series is the leading international forum for discussion of enzyme engineering technology and applications. The 5-day conference program was packed with exciting presentations on Enzyme Engineering for Medical Applications, Synthetic Biology, and New Enzyme Technologies among other topics. These talks highlighted new technologies and methods which I am now applying in my own research. Major themes were the engineering of enzymes for the degradation of plastics, and the shift to "rational design" for the improvement of enzyme catalysis. Importantly, I was able to network with the other attendees and made meaningful connections to leading researchers in the field. This included potential collaborations for my lab.

For the first time, I presented research which has recently been published in Nature Chemical Biology. My poster presentation received significant interest, and I was awarded a Merit Poster Award. My presentation described the high-throughput engineering of non-ribosomal peptide drugs, produced by non-ribosomal peptide synthetase (NRPS) enzymes. This class of compounds are of particular interest for drug discovery and development, with many derivatives already approved for treatment of cancer and microbial infections, examples including the antibiotics penicillin and daptomycin and anti-cancer compounds oprozomib and dactinomycin. I developed a 'metagenomic domain substitution' approach which takes advantage of the richness of NRPS genes present in soil. Over 1000 unique NRPS gene fragments were substituted into a model system, yielding 467 compounds, of which 16 were unique versions. This strategy is transferable to any bacterial NRPS system which offers scope for the development of novel therapeutic compounds in the future, such as antibiotic and anti-cancer compounds.

This trip was complemented by a visit to the world-leading Singapore Consortium for Synthetic Biology (SINERGY) facilities led by Professor Matthew Chang at the National University of Singapore. This was a unique experience to see a world-class synthetic biology laboratory which contained a lot of robotic equipment.

SONJA HUMMEL

VASCULAR DISCOVERY 2024 CONFERENCE IN CHICAGO, ILLINOIS

Sonja Hummel

Thanks to the support from Research for Life, I was able to attend the Vascular Discovery 2024 conference in Chicago, Illinois. With the topic "From Genes to Medicine", this conference brought together cardiologists and researchers alike and provided the scientists, doctors, and researchers in the cardiovascular space a meeting place to present and discuss world-class research. I immensely valued the opportunity to attend this meeting and discuss some of my PhD research with experts in the field.

I especially valued the opportunity to attend a talk by Kimberley Martinod. Her research on Neutrophil Extracellular Traps has been inspiring and her work has been frequently cited in my own research. A personal highlight of the conference was having the opportunity to discuss my own research with Professor Martinod during the poster presentations. Her questions about techniques and the experimental design of my research were a great chance to prepare for my thesis defence and her praise for the results I presented were encouraging. Additionally, as a female in science, having an established and accomplished female scientist approve and congratulate me on my research increased my confidence about my post-PhD career path. This was an instrumental experience that further encouraged me to be a confident woman in science.

Presenting my poster also benefitted me in improving my presentation and communication skills, both soft skills that are instrumental for a successful career as well as wonderful opportunity to practice for a PhD defence. I found it highly valuable to engage with researchers, scientists, and industry professionals alike and learn about their individual career pathways. These conversations have given me a great insight into the varying roles available to someone post-PhD, as well as connections that I would not have been able to form without attending the conference. Moreover, I was awarded the Paul Dudley White International Scholar Award by the American Heart Association for the highest-ranked scientific abstract submitted from New Zealand. This award is a great honour and I feel humbled that my research has been recognized as some of the best science presented from this part of the world.

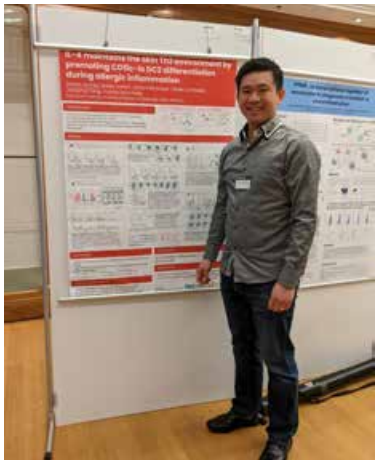
Lastly, I am always amazed at the range of research that is presented at these conferences. At Vascular Discovery 2024, presentations about the loss of Y chromosome and its relationship to an increased risk in cardiovascular death in males was fascinating. It provided me with an insight about the varied research aspects that exist within the cardiology space.

Overall, I am grateful to have been given the opportunity to attend this conference. I believe, attending this conference has been hugely beneficial for my professional development.

Paul Dudley White International Scholarship recipients. (I am third from the left, front row)

SOTARA OCHIAI

THE EUROPEAN MACROPHAGES AND DENDRITIC CELL SOCIETY (EMDS) MEETING HELD IN GHENT, BELGIUM FROM 18TH TO THE 20TH OF OCTOBER



Sotara Ochiai

I received a $3000 travel grant to attend the European Macrophages and Dendritic Cell Society (EMDS) Meeting held in Ghent, Belgium from 18th to the 20th of October. This esteemed event brought together over 600 researchers including world-leaders in myeloid cell biology. I presented my recent results through a poster titled, 'IL-4 maintains the skin Th2 environment by promoting CD11b-lo DC2 differentiation during allergic inflammation'. The study delves into how IL-4 cytokine sustains the allergic environment in the skin by fostering the generation of 'pro-allergic' dendritic cells.

My presentation sparked lively discussions among conference attendees and was well received. I also forged meaningful connections with esteemed speakers and fellow researchers for potential collaborations. I engaged in discussions with several researchers about CRISPR/Cas9 technology for gene editing, as well as cell-specific depletion using neutralising antibodies. These exchanges provided me with valuable insights into specific experimental procedures. I am immensely grateful to Research For Life for providing me with the opportunity to present my work to the myeloid cell biology community and participate in in-person discussions. This has significantly expanded the impact and reach of my research.

Three Minute Thesis (3MT)

Research For Life supports Masters and PhD level research by sponsoring a $500 prize for the University of Otago, Wellington 3MT competition.

This year the prize was won by Elle Johnson. She is being presented with her prize by Darryl Carpenter, CE of Cancer Society Wellington

NIWA Science Fair

Research For Life also provides $300 annually for prizes in the Wellington NIWA Science Fair for schools. It rewards projects which look at some aspect of health.

The following exhibits won Research For Life WMRF Special Prizes:

ANIKA FYFE YEAR 7 STUDENT FROM ST FRANCIS DE SALES
Won $100 with an exhibit called “My fight to eat cake!”.

TOBIAS BURGESS YEAR 8 STUDENT FROM EVANS BAY INTERMEDIATE
Won $50 with an exhibit called “Eat Like an won $100 with an exhibit called “Sugar Rush”.

BHAVIA PARMAR YEAR 8 STUDENT FROM ST MARK’S SCHOOL
Won $100 with an exhibit called “Hunger Games”.

Research For Life

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RESEARCH FOUNDATION

www.researchforlife.org.nz

PO Box 14186, Kilbirnie, Wellington 6241