2024 Arthritis Australia Grant-In-Aid Project Report

Synovial neutrophils in early rheumatoid arthritis: relationship with clinical outcome and the development of improved handling procedures.

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Background

Neutrophils are the most abundant white blood cell in the human circulation and in the synovial fluid of patients with inflammatory arthritis, including rheumatoid arthritis (RA). In RA, neutrophils become activated and release inflammatory factors which contribute to joint damage and exacerbate the inflammatory response. However, despite their undisputed significant role in driving inflammation in RA, neutrophils are notoriously difficult to study due to their short life span once removed from the host, and the inability to store (cryopreserve) or expand them in vitro. In the clinical setting, the frequent age range for functional analyses of neutrophil samples is <8 hours following specimen collection. This brief window frequently poses a challenge for clinicians and scientists in the rheumatology space, particularly due to factors such as transport from the clinic/theatre where the collection takes place to the laboratory where the specimens are examined.

This project was therefore designed to address this challenge by establishing novel methodology to allow the efficient preservation of synovial fluid neutrophils to enable future studies in the role of these cells in driving the pathogenesis of the inflammatory arthritides, and to enable studies into neutrophil-targeting therapeutic strategies for these diseases.

Project Aims:

The project was built around two main aims:

1. To characterise neutrophil infiltration into the synovial tissue in an early RA cohort in relation to clinical parameters at
    a) baseline, prior to treatment, and
    b) 6-months post-treatment.
2. To optimise the cryopreservation of synovial fluid neutrophils, with respect to (a) cell viability and expression of activation markers, and (b) cell function.

Summary of completed work

Aim 1.

For this aim, a method for scoring neutrophil infiltration into synovial tissue biopsies, sectioned and stained with Haemotoxylin and Eosin was established. Neutrophils were judged manually by two independent observers by their characteristic multi-lobed nuclei and pale, neutral cytoplasm. Four scores were taken for each sample: (1) Number of neutrophils present in the vessels ('marginating' neutrophils), (2) Number of neutrophils present in the tissue ('tissue-infiltrating' neutrophils), (3) Combined total raw score (combined marginating and tissue-infiltrating count), and (4) Total neutrophil semiquantitative score. Neutrophils were counted in six fields of view as per Kraan, et al. [1], and semiquantitative scoring was adapted from Tak, et al. [2].

A total of n=50 synovial tissue samples from patients with early, ACPA+ RA patients from the ARBITRATE cohort were included in the study. Baseline clinical parameters including DAS28CRP, disease duration, ESR, CRP, tender and swollen joint counts, RF and CRP titres were correlated with neutrophil infiltration scores. To assess the relationship between

neutrophil infiltration and treatment response, neutrophil infiltration was compared in patients who achieved remission (DASCRP<2.4) at 3, 6, and 12 months with patients who did not.

For validation of our developed score, we stained sections from the same patients by immunohistochemistry with anti-CD15 (a commonly used marker for tissue-infiltrating neutrophils Spengler, et al. [3] and found 100% concordance between CD15 SQA and total neutrophil SQA, suggesting our scoring method was appropriate. For further validation, we compared neutrophil scores from n=21 patients, whose samples had previously undergone bulk RNA sequencing analysis. CIBERSORT deconvolution was used to determine neutrophil fractions from the sequencing data sets, and then these fractions were correlated with manual neutrophil scores. We observed a strong positive correlation between neutrophil infiltration as judged by CIBERSORT and neutrophil infiltration judged by manual scoring, further supporting the accuracy of our scoring method.

When assessing clinical parameters, we found strong moderate correlations between degree of neutrophil infiltration (both manual scores and CIBERSORT fractions) and baseline disease activity. Neutrophil infiltration scores correlated with CRP and RF titre, while no association between neutrophil scores were observed with disease duration, tender or swollen joint counts or CCP status or titre. Finally, following stratification by remission status, we found a significant difference between neutrophil infiltration at baseline in patients who did not achieve remission at 3 months compared to patients who did; no significance was found between patient groups at 6 and 12 months.

Aim 2

For aim 2a, we examined the effect of cryopreservation on neutrophil viability and expression of activation markers frozen under varying conditions in order to optimise their storage. We found that following preservation of synovial neutrophils directly in synovial fluid with the addition of 10% DMSO (cryoprotectant), proportions of viable neutrophils were significantly higher than those preserved in conventional freezing media (mean viability: 83.41% viable compared with 52.82%, respectively). However, we found no significant difference in expression of activation markers (CD66b, MPO, and CD11b) between thawed cells from either preservation condition.

For aim 2b, we examined the functional capacity of neutrophils post-preservation using two methods: (1) flow cytometric oxidative burst assay (dihydrorhodamine-123; DHR-123 assay), and (2) the flow cytometric phagocytosis assay (pHrodo *S. aureus* bioparticles).

As part of (1), the DHR-123 assay was optimised in the Rheumatology laboratory at Flinders Medical Centre. Following the initial optimisation period, the results from n=3 experiments revealed that neutrophils preserved directly in synovial fluid had a significantly lower oxidative index at rest (i.e., without stimulation by PMA) than neutrophils preserved in conventional freezing media, with a 3.18-fold increase in basal DHR-123 median fluorescent intensity (raw basal values were 7821 versus 24848, respectively). Interestingly however, following PMA stimulation, conventionally preserved neutrophils had a greater oxidative response than neutrophils preserved in synovial fluid (oxidative index of 5.73 versus 1.85, respectively). Together, these results suggest that neutrophils preserved in synovial fluid are indeed less activated at rest than neutrophils preserved conventionally, and are more resistant to further PMA-induced activation. Meanwhile, for (2), n=3 experiments showed no significant difference in phagocytic capability of neutrophils preserved in synovial fluid compared with conventionally preserved neutrophils.

Additionally, as is common with research, our approach to answering our research questions was altered throughout the course of the project. In 2024, we had the opportunity to interrogate extended bulk RNA sequencing datasets from the ARBITRATE cohort, enabling us to assess expression of neutrophil and chemokine genes in neutrophil-rich synovial tissue samples. We found a strong positive correlation between both neutrophil and chemokine gene expression and our manually determined scores.

Changes to the project/Unforeseen circumstances

Throughout the project duration, there were notable circumstances that delayed progress:

- The Rheumatology Synovial Tissue Research Laboratory was relocated from level 2 in Flinders Medical Centre up to level 5 in October 2024. Though the move was not far, the disruption of relocating the office and laboratory equipment led to delays in being able to complete the project.
- The study was further delayed by CIA's parental leave career interruption (July 2024-January 2025), leading to an extension of the project's end date from 31 December 2024 to 31 March 2025.

Ongoing work and future directions

Work to increase the sample size of our functional studies is currently ongoing within the laboratory. Additionally, we have now in collaboration with Harvard University, Boston, USA, performed Xenium spatial transcriptomic analysis (10X Genomics) on selected samples from the ARBITRATE cohort. As a result, we are currently working with the RESET RA transcriptomic team to annotate neutrophils in these datasets, which will allow deeper interrogation into the function and crosstalk of RA synovial tissue neutrophils.

Significance

The findings from this project advance our understanding of neutrophils in early RA and address the long-standing technical challenge of cryopreservation of these short-lived cells. By demonstrating that neutrophil infiltration in synovial tissue is linked to baseline disease activity and early treatment response, our findings strengthen the evidence for neutrophils as key drivers of inflammation in RA. The development of a novel cryopreservation method—using synovial fluid as a medium—has enabled improved viability and functionality of neutrophils after freezing, which may provide a valuable tool for future functional and therapeutic studies. Finally, our results pave way for more sophisticated analyses, including spatial transcriptomic approaches, which are currently underway.

Outputs from this work

While still an ongoing project, there have been several outputs generated from this work:

- On May 4th 2025, project findings were presented at the 2025 Australian Rheumatology Association meeting in Adelaide, South Australia in an oral presentation titled, "Neutrophil infiltration into the early rheumatoid arthritis synovial tissue is associated with disease activity and early remission".
- On May 13th, findings were presented at the RESET RA Annual Team Meeting in Woolloongabba, Queensland in an invited oral presentation titled, "Flinders Rheumatology: the ARBITRATE Study, Synovial Tissue Neutrophils, and Somatic Mutations".
- An abstract of the project findings has been submitted for presentation at the 2025 American Congress of Rheumatology (ACR) Annual Meeting to be held in Chicago,

USA in October 2025, titled, “Synovial Tissue Neutrophils are Associated with Disease Activity and Early Remission in Rheumatoid Arthritis”.

- A manuscript of the project findings is currently in preparation.

References

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3. Spengler J, Lugonja B, Ytterberg AJ, et al. Release of Active Peptidyl Arginine Deiminases by Neutrophils Can Explain Production of Extracellular Citrullinated Autoantigens in Rheumatoid Arthritis Synovial Fluid. *Arthritis & rheumatology* 2015;67(12):3135-45.

Acknowledgements

This work was funded by the ARA Research Fund. We thank Arthritis Australia for their support, as well as all study participants and consumer advisors.