Arthritis New Zealand Summer Scholarships Report

Name of Student: Dayang Nurul Izzati binti Awang Shuani
Primary Supervisor's Name: Dr. Paul Hessian
Primary Supervisor's Institution (including address): University of Otago, Department of Medicine
Project Title: Fibroblast subsets, Interleukin-6 (IL-6) and Rheumatoid Inflammation.

Statement Regarding Arthritis New Zealand Sponsorship
This project was supported by a 2021/2022 Arthritis New Zealand Research Summer Scholarship, completed by Izzati Awang Shuani.

Student's Personal Comment About Study/Experience

This summer studentship was a very valuable experience for me. Having no prior experience in research and laboratory work, I found this project to be quite challenging, yet very rewarding in the end. Through this project, I have gained a multitude of new skills such as accurate documentation, reporting, problem-solving, laboratory skills and scientific thinking. In Dr Hessian's lab, I learned how to perform basic laboratory skills such as laboratory safety, measuring and pipetting. I got to learn to utilize different protocols from lab research staff working on similar experiments and conduct the experiments independently. Most of the laboratory experiments that I conducted were Immunofluorescent staining, which I now feel confident in planning and doing; something that I never thought I could do 10 weeks earlier. Thank you to Arthritis NZ for supporting my summer scholarship and the Otago Summer Studentship Programme for giving me this opportunity. A special thanks to Dr. Paul Hessian and Melanie Miller for taking the time to teach and support me during the 10-weeks I spent in the lab.

Summary of the Project (maximum 500 words)

a) Aims of the project

In-house, gene expression analyses suggested fibroblasts are a potential source of proinflammatory IL-6 in subcutaneous rheumatoid nodules (RN). Separately, preliminary immunohistology suggested different fibroblast subsets are present within RN. In this study of RN, the *aims* were to identify fibroblast subsets associated with inherent RN inflammation and establish the possibilities for fibroblasts producing IL-6, as distinct from fibroblasts responding to IL-6.

b) Materials and Methods

Patients and tissues. Rheumatoid nodules (n=6) were obtained following elective surgery from 6 people with Rheumatoid Arthritis (RA), as defined by American College of Rheumatology (ACR) classification criteria[1]. The study was approved by the NZ Health and Disability Ethics Committee (MEC/06/02/003). All participants provided written informed consent. All nodule tissues for histology analysis are preserved at -80^{o}C in TissueTek O.C.T compound (Sakura).

Tissue processing. Tissues were then processed and embedded in paraffin wax, as previously described[2].
Four μm sections of were cut using a rotary microtome and placed in a 40^{o}C water bath before being mounted onto histological glass slides. Sections were adhered by incubation in a 60^{o}C oven for 60 minutes.

RNAScope analysis. Detection of *IL6* mRNA was performed essentially as recommended by the manufacturer, utilising reagents provided in RNAScope 2.5HD brown detection kit (Advanced Cell diagnostics [ACD], USA), and an Hs-IL6 (310371) predesigned probe, with additional in-house variations as previously described[2].

Standard Multiplex Immunofluorescence Staining. Four μm sections were dewaxed and rehydrated before antigen retrieval by heating overnight at 60^{o}C in Tris-EDTA containing 0.05% Tween20, at pH 9.0 (antigen blocking buffer).

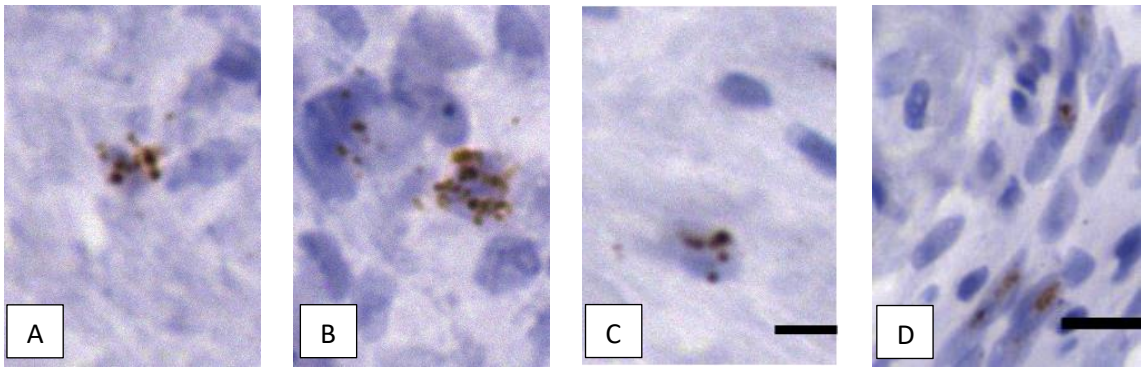
Non-specific binding was blocked using Tris-buffered saline (TBS) containing 0.1% Tween20 and additional 1% BSA, 5% normal donkey serum and 50mM glycine (blocking buffer) at room temperature for 60 minutes. Sections were then incubated with a mixture of either recombinant rabbit monoclonal anti-human fibroblast activation protein-α (FAPα, 1:200, [ERP20021], ab207178) and mouse monoclonal anti-human CD90/Thy1 (1:200, [7E1B11], ab181469), or rabbit monoclonal anti-human CD55 (1:750, [EPR6689], ab133684) and mouse monoclonal anti-human CD68 (1:100, [c68/684), ab201340), diluted in blocking buffer overnight at 4°C. Slides were then washed 4x in TBST, for 5 minutes each before incubation in a mixture of AFplus488-conjugated donkey anti-rabbit (Invitrogen, A32790) and AFplus647-conjugated donkey anti-mouse (Invitrogen, A32787), both diluted 1:1000 in TBS for 60 minutes at room temperature. Slides were washed in the dark 2x with TBST for 5 minutes each, before incubating in autofluorescence quenching solution (ReadyProbes, R37630) for 3 minutes. Slides were then washed in TBS solution 2x for 5 minutes each in the dark before mounting with ProLong Gold Anti-fade Mountant containing DAPI and applying a coverslip.

Immunofluorescence using Tyramide Detection. This protocol was utilised for immunofluorescence staining when primary antibodies were derived from the same species. Dewaxed, rehydrated sections, treated for antigen retrieval (as above) were used. A Tyramide SuperBoost Kit (Alexa Fluor 488, Thermofisher, USA) was used to visualise IL-6 proteins in RN sections according to the manufacturer's protocol. Endogenous peroxidase activity was blocked by incubation with kit 3% hydrogen peroxide solution for 60 minutes at room temperature. Non-specific binding was blocked by incubation with 10% goat serum in a kit buffer (kit blocking buffer) for 60 minutes at room temperature. Slides were incubated with rabbit monoclonal anti-human IL-6 (1:100, [EPR21711], ab233706), diluted in kit blocking buffer for 60 minutes followed by polymerised-horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody. Tyramide-AF488 working solution was applied for 5 minutes followed by reaction stop reagent, according to the manufacturer's instructions. Sections were then washed in TBS before multiplex using mouse monoclonal anti-human CD90/Thy1 and AFplus647 donkey anti-mouse reagent, following the standard IHC protocol as described above.

c) Key results

The RNAScope analysis revealed positive RNA labelling as chromogenic brown dots (Figure 1). In RN, *IL6* mRNA-expressing cells were mainly present in the established palisade layer (Figure 1A, 1B, 1C) and in some vascular areas (Figure 1D).

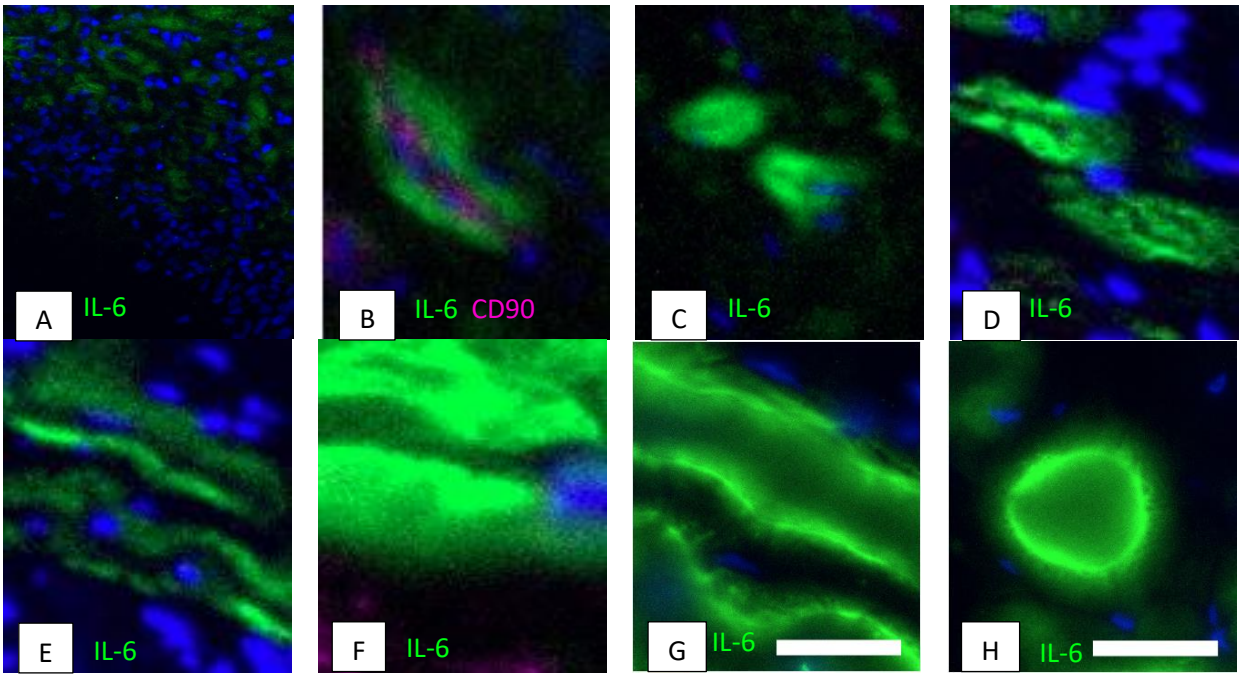
Figure 1. Expression of IL-6 mRNA transcripts in subcutaneous rheumatoid nodules



IL-6 protein, detected by immunofluorescence staining, was mainly detected within established palisade layer and a region immediately outside the palisade layer (Figure 2A), where FAPα^+ and CD55$^+$ fibroblasts were predominant. IL-6 protein was also seen associated with CD90$^+$ fibroblasts surrounding blood vessels, suggesting that CD90$^+$ fibroblasts are a potential source of IL-6 in the vascular area (Figure 2B & 2C).

Several patterns of IL-6 immunofluorescent staining were observed. In the region immediately outside the palisade layer, intense thinner staining was seen, consistent with IL-6 localisation to the cell surface (Figure 2D & 2E). Intense thicker staining is more likely from cells producing IL-6 (Figure 2D & 2F). Surface staining associated with larger, potentially vascular structures was also seen (Figure 2G &2H). The black "shadow" in the centre of these structures (Figure 2G & in cross section, 2H), compared with immunohistochemical staining from published studies, could potentially be a thrombus.

Figure 2. Localisation of IL-6 in subcutaneous rheumatoid nodules



d) Major point of discussion

In this study, we analysed the role of fibroblasts and IL-6 in rheumatoid subcutaneous nodules. *IL6* transcript was not widespread within nodules. Immunofluorescent staining patterns of IL-6 in nodules indicates cells can produce and/or respond to IL-6. We identified $CD90^+$ fibroblasts in vascular regions as one source of IL-6. $CD55^+$ and $FAP\alpha^+$ fibroblasts were also identified, associated with palisading macrophages, but without conclusive evidence these fibroblast subsets could be linked with IL-6. Combined, the results suggest IL-6 production from some fibroblast subsets within nodules but that circulating IL-6 may also influence nodule inflammation.

Perspective: Previous studies have identified functionally distinct subsets of fibroblasts that are associated with RA joint synovial inflammation[3, 4]. RA synovial $CD90^+$ fibroblasts that produce and secrete IL-6 amongst proinflammatory features, occur in the synovial sub-lining layer, localised within a discrete perivascular zone surrounding capillary structures[3, 4]. Similarly, in nodules, $CD90^+$ fibroblasts were found in perivascular locations, producing IL-6.

In RA synovium, $CD55^+$ fibroblasts are found in the synovial lining and produce metalloproteinases that promote bone and cartilage damage[5]. In RN, we found $CD55^+$ fibroblast and a seemingly distinct $FAP\alpha^+$ fibroblast subset, in both the outer regions of palisade layer and surrounding blood vessels. Whether these fibroblast subsets promote the tissue necrosis associated with nodule inflammation remains unknown.

e) Conclusion/Key recommendation

Distinct fibroblast subsets are involved in subcutaneous rheumatoid nodule inflammation. Fibroblasts are one source of IL-6, which together with IL-6 from systemic sources, likely contributes toward nodule inflammation.

References (maximum 500 words)

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