A standardized protocol for the isolation and culture of normal and arthritogenic murine synovial fibroblasts

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Method Article

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Abstract

Introduction

Rheumatoid arthritis \(RA) is a chronic inflammatory disease affecting mainly the synovial joints. The chronic phase of disease is characterized by the hyperproliferation of synovial tissue and formation of pannus, resulting in the progressive erosion of cartilage and bone tissue and leading to disability. Despite the uncertainties on the autoimmune versus autoinflammatory nature of RA, the requirement of non-immune cell function such as synovial fibroblasts \(SFs) in the loss of tissue integrity has been widely accepted [1]. SFs are CD45-negative cells of mesenchymal origin involved in supporting and lubricating the joint by providing nutrients and proteoglycans [2]. Interestingly, it was shown that human RA-SFs are activated and contribute to hyperplasia and destruction [3] and mixed populations of human synoviocytes when implanted into Severe Combined Immuno-Deficiency \(SCID) mice retain their ability to destroy cartilage in the absence of a functioning immune system [4]. Indeed, cells from arthritic joints in culture show increased invasiveness, matrix degradation and increased cell adhesion [5]. A murine experimental paradigm that strongly supports this notion came from the generation of human-TNF transgenic mice \(henceforth noted as Tg197), a representative model of chronic inflammatory polyarthritis [6]. In the Tg197 model, TNF signalling through TNFRI in SFs appears to be sufficient for the orchestration of full-blown pathology [7]. Additionally, SFs from the same mouse can transfer arthritic pathology to healthy recipients upon intra-articular transfer [8]. Further understanding of the biology of the SF and the mechanisms leading to its arthritogenic properties should be key to unravelling the contribution of this specific cell type in the pathogenesis of chronic arthritis in human. A well-standardized protocol for _ex vivo_ culturing of murine SFs is currently missing from the literature and should prove useful both for unravelling the importance of SFs in disease as well as for the development of novel therapeutic approaches for several chronic joint diseases. We present here a detailed protocol for efficient isolation of primary murine SFs, which after 20 days of cell culturing, retain their original characteristics of constitutive CD90.2, VCAM-1 and ICAM-1 expression. The method presented is time-effective, and the materials needed are minimum compared to other published methods for mouse tissue [9-11]. More importantly, it results in an SF population of high yield and purity.

Reagents

• Three 8 week old WT mice **\!CAUTION** Experiments involving animals must conform to national and institutional regulations. • Ethanol 100% and Ethanol 70% • Methanol • Water For Injection \(WFI) \(Fresenius Kabi) • Hanks balanced salt solution\(HBSS) without $CaCl_2$ and $MgCl_2$ \(Invitrogen Cat.#14170) • Penicillin/streptomycin \(Invitrogen Cat.#15140) • Nystatin \(Sigma Cat.#N4014) • Collagenase from Clostridium histolyticum type IV \(Sigma Cat.#C5138) **\!CAUTION** Handle with caution as it is harmful to the skin. • 10x Dulbecco's modified Eagle's media with 4.5g/lt D-Glucose, 8mg/lt Phenol red, without $NaHCO_3$, without Sodium pyruvate, and without L-glutamine \(10xDMEM;

Biochrom AG, Cat.#F0455) • Fetal bovine serum \(FBS; Biochrom AG, Cat.#S0115) heat inactivated • L-Glutamine \(Invitrogen Cat.#25030) • 10x Dulbecco's phosphate-buffered saline \(DPBS) without $CaCl_2$ and $MgCl_2$ \(Invitrogen Cat.#14200) • Trypsin-EDTA1x \(Invitrogen #25-3000-62) • VCAM-1 \(or CD106) purified rat antibody \(BD biosciences Cat.#553330) \(5µg/ml) • Goat Fab2 anti-rat PE-antibody \(SouthernBiotech Cat.#3052-09) \(0.25µg/ml) • ICAM-1 \(CD54) antibody PE labelled \(BD biosciences Cat.#553253) \(1µg/ml) • Mac-1 \(CD11b) antibody FITC-labelled \(BD biosciences Cat.#553310) \(0.4µg/ml) • CD90.2 antibody \(Thy1.2) biotinylated \(BD biosciences Cat.#553011) \(1µg/ml) • PE-Streptavidin secondary antibody \(BD biosciences Cat.#554061) \(0.33µg/ml) • Vimentin \(Chemicon Cat.#AB1620) **REAGENT SETUP** • HBSS solution: HBSS solution without $CaCl_2$ and $MgCl_2$ supplemented with antibiotics Penicillin/streptomycin \(1%) and Nystatin \(2%). • DMEM 1x preparation in water for injection: 10% 10x DMEM stock solution, 10% FBS, 1% L-Glutamine, 1% Penicillin/streptomycin, 3.2% Sodium bicarbonate, 200-250µl NaOH 1N. • Collagenase IV solution: for isolation of SFs from 3 mice 20mg of collagenase IV are dissolved in 2ml HBSS supplemented with Pen/Strep and Nystatin, and filtered with a 0.22µm filter **\!CRITICAL** Prepare a fresh solution just before use.

Equipment

• Humidified tissue culture incubator \(37 °C, 5% CO_2) • Laminar flow hood for cell culture equipped with UV light for decontamination • Water bath with temperature control \(set at 37 °C) and shaker option • Flow cytometer \(i.e., BD FACS Canto II Flow cytometry system, BD Biosciences) • Microdissecting instruments: dissecting scissors; forceps—straight and angled, scalpel • Centrifuge \(no temperature control is needed) • Optical microscope • 15 ml plastic conical tubes \(Greiner) • 50 ml plastic conical tubes \(Greiner) • 10ml plastic pipettes \(SARSTEDT Cat.#86.1254.001) • 25ml plastic pipettes \(SARSTEDT Cat.#86.1685.001) • 100mm tissue culture dishes \(Corning inc. Cat#430167) • 150mm tissue culture dishes \(Corning inc. Cat.#430599) • T-75 flasks \(Corning inc. Cat.#430720) • T-175 flasks \(Corning inc. Cat.#431079) • 96-well clear flat bottom tissue culture plates \(Corning Cat. #3596) • Sterile medium filters \(0.22 µm)

Procedure

A. ISOLATION OF PRIMARY SYNOVIAL FIBROBLASTS FROM MOUSE JOINTS 1. Euthanize the mice using carbon dioxide and according to guidelines from the Institutional Animal Care and Use Committee of BSRC "Alexander Fleming". 2. Rinse microdissecting instruments with 70% Ethanol and then immerse them in an antimicrobial for a few seconds. **\!CRITICAL STEP** Repeat the decontamination step of the instruments after cutting off the joints of each mouse. 3. Place the mouse in the face-up position and rinse it with 100% Ethanol. 4. Peel back the skin and remove soft tissues from the hind legs using scissors and forceps. **\!CRITICAL STEP** Be careful not to leave any remaining skin covering the toes. 5. Cut ankle joint off before the tibial muscle including the toes, and spray again with 100% ethanol. 6. Place ankle joints in a tube filled with HBSS and antibiotics. Repeat steps 4-6 for all joints. **\!CRITICAL

STEP** From this step on work should be done in a laminar flow hood. 7. Place the cut joints into a 100mm tissue culture plate containing 70% ETOH, for some seconds, and then transfer them to an HBSS-containing dish. 8. Place one joint in a fresh empty 100mm dish and cut through the joint space with the scalpel. Hold each toe digit with the scalpel and pull with the forceps. **\!CRITICAL STEP** Avoid smashing the bones so as to prevent contamination by bone marrow cells. 9. Repeat steps 8-9 for all joints. 10. Prepare fresh and filtered collagenase IV in HBSS at a high concentration \(10mg/ml). 11. Add 18ml DMEM 10% FCS to the plate containing the processed tissue from 3 mice. 12. Place the dissected joints and tissue in 50ml tubes with freshly-made and filtered collagenase IV \(2 ml of 10mg/ml solution) \[final concentration \(1mg/ml) in DMEM 10% FBS 1% L-Glutamine, and 1% Pen/Strep]. 13. Incubate tissues with collagenase solution in the shaking water bath at 37 °C for 50min to 1h, at maximum shaking speed. **\!CRITICAL STEP** Adjust the time of collagenase digestion according to the enzymatic activity of the lot used. The range of activity of batches used in standardization of this protocol was between 450-600 units/mg. 14. Following completion of incubation time, vortex vigorously to release cells. 15. Transfer supernatant to a new 50ml tube. Place 20ml of fresh DMEM in the tube containing the digested joints. Repeat the procedure and add the supernatant to the new tube. 16. Centrifuge tubes at 1100rpm for 10min at room temperature. 17. Re-suspend cell pellet in fresh DMEM supplemented with 10% FBS, 1% L-Glutamine, and 1% Pen/Strep. 18. Seed tissues/cells into 100mm dishes \(dissected tissue from 1 mouse into one 100mm plate or 75 cm^2 flask) and place in a humidified tissue culture incubator \(37 °C, 5% CO_2). **TIMING: 2.0-2.5h \(although it might be shorter if cells from less than 6 mice are isolated).** **Culture of isolated synovial fibroblasts** 19. Monitor isolated cells daily, and change medium every 3 days **\!CRITICAL STEP** Be careful not to remove the small pieces of tissue during the first 1 week after isolation. 20. Once groups of cells are observed, remove the floating pieces of tissue. 21. Trypsinize when confluence reaches 90-100% of the plate and transfer to a 150mm plate \(or 175cm^2 flask) \(split 1:2). Usually cells reach confluency 7-10 days after isolation. After first passage, confluency is reached 3-4 days later. **\!CRITICAL STEP** Avoid long incubations with Trypsin \(2-4 min incubation with pre-warmed trypsin is effective for fibroblast detachment whereas macrophages that are highly adhesive will remain attached. Thus, it is highly recommended to change flasks/plates in every passage).

Timing

==~== 18-20 days

Troubleshooting

See **Table 1**.

Anticipated Results

After 3 passages, cells are trypsinized, stained at 4 °C for 20 min with the corresponding antibodies in PBA \(PBS, 3% FBS, 0.2% NaN_3) and analyzed by Fluorescence-activated cell sorting \(FACS) on a BD FACS Canto II flow cytometer using the WinMDI 2.9 FACS analysis software. The resulting cultures are >80-90% CD90.2 positive and <1% Mac1 positive \(**Figure 1a**), >85% VCAM-1 positive \(**Figure 1b**), and >80% ICAM-1-positive \(**Figure 1c**). Under the optical microscope the cells exhibit the typical spindle-like shape of fibroblasts \(**Figure 1d**). They are also tested for Vimentin expression, a mesenchymal marker, and they are found positive \(**Figure 1e**). At this stage they are ready to use. From one mouse ~10^7 cells are obtained after three passages.

References

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Figures

Step number	Problem	Possible reason	Solution
A20-21	Low yield of synovial fibroblasts.	1) Poor Collagenase IV digestion 2) Split ratio too high (more than 1:2) 3)Fungi/Mycoplasma or other microbial contamination	1) Check the amount/units of collagenase added 2) Avoid splitting the cells more than 1:2 3) Always sterilize surgical tools and decontaminate them with antimicrobial detergents and ethanol
A20-21	Low percentage of CD90+ cells (when the total number of cells obtained is within anticipated range)	Contamination of synovial fibroblasts with other cell types	Avoid cutting through the bone while isolating the cells (step A9). Always change plasticware in every passage.
A20-21	Fungi contamination of cultured cells	Contamination during the part of the procedure that is done out of the hood (steps A1-7) or inadequate handling within the hood	1) Take care to sterilize all surgical tools and decontaminate them with antimicrobial detergents and ethanol. 2) Add Amphotericin B antifungal solution to HBSS and culture medium in recommended by supplier concentration. 3) Make sure you remove all the skin from the toes as this could be a source of contamination.
A20	No adherent cells 72h after seeding	1) Incubator problem (CO_2 level, no water) 2) Possible fungi contamination 3) Collagenase IV overdigestion 4) Too much debris found on the surface of the plate	1) Check incubator status 2) Observe the cells under the microscope for possible contamination 3) Avoid adding collagenase IV for more than 1h or in higher amounts than recommended 4)Place the medium to a newplate, avoiding transfer of the debris

Figure 1

Table 1 *TROUBLESHOOTING*

Figure 1

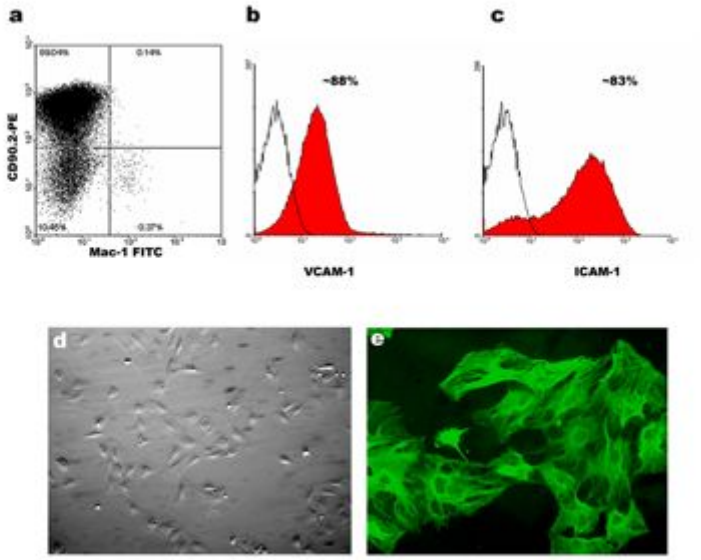

Figure 2

Figure 1 Isolated synovial fibroblasts from WT mice were subjected to FACS or immunocytochemistry analysis on passage 3 after seeding to verify purity. a) Density plot showing expression of CD90.2 on the majority of the isolated cells (89%) whereas very few Mac-1 positive myeloid cells can be detected (0.37%). Single stained samples were used for compensation set up, b) Overlaid histogram showing VCAM-1 expression (red shaded area) on SFs compared to unstained control (empty area), c) Overlaid histogram showing ICAM-1 expression (red shaded area) on SFs or unstained control (empty area). d) Phase contrast image of isolated SFs in culture under a Nicon E300 inverted fluorescence microscope equipped with a Nikon digital camera (10x), e) Immunocytochemical staining of isolated SFs with vimentin antibody under a Nicon eclipse inverted fluorescence microscope (20x).