A Novel Human Synovial Explant Model of Infectious and Inflammatory Arthritis

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Introduction: The synovium is the membrane of the joint space that has a specific tissue architecture relevant to its immune regulating function. The innermost or intimal lining is composed of M2-skewed resident synovial macrophages (RSMs) bound together by tight junctions. Behind these intimal lining cells are interstitial macrophages that renew the intimal lining population and are more M1-skewed. These interstitial macrophages also interact with fibroblast-like synoviocytes, which have been well-evaluated and are known to perpetuate inflammatory responses especially in cases of autoimmune arthritis. *In vitro* evaluation of the synovium often relies on tissue digestion and plating, which destroys this immune-relevant architecture. Here we describe a novel *ex vivo* methodology using synovial explants obtained from routine total joint replacement surgery to study the initial changes of the synovium in response to differing stimuli. While we are using this to study the cells that migrate from the synovium, this can and should also be used to evaluate the changes in the synovium itself, which is a future step of this work.
Methods: This study was approved by the Institutional Review Board at the University of Iowa Hospitals and Clinics. Six patients >18 years of age receiving a scheduled, non-emergent, total joint replacement or resection arthroplasty secondary to infection were recruited. Synovium (knee) or pulvinar (hip) was sterilely obtained during normal operating protocol. Tissue acquired in the operating room was transferred to the laboratory in PBS on ice. Whole synovium was washed twice in PBS, sterilely dissected into 4x4 mm segments, and washed again. Segments were placed into 24-well transwell inserts with 5 µm pores (Corning), a size that should allow monocyte and macrophage migration, but prevent fibroblast migration (**Figure A**). Bottom wells were treated with LPS (1, 10, or 100 µg/mL, Sigma-Aldrich) or MCP-1 (25 ng or 250 ng/mL, Fischer Scientific) in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. The inserts were then placed in the well, and enough media to cover the tissue was placed in the insert (approximately 200-400 µl). The transwell plates were incubated for up to 48 hours at 37°C and 5% CO2. A crystal violet assay was performed on cells adhered to the bottom of the well and the underside of the transwell per standard protocol. Synovial tissue was stored in neutral buffered formalin for histology, immunohistochemistry, and fluorescence microscopy. Migratory cells in solution were counted and analyzed using flow cytometry immediately after the end of the assay. Samples were plated in a 96-well round-bottom plate for single stain, unstained, patient test samples, and/or Fluorescence Minus One (FMO) controls. Controls were plated at $2x10^5$ cells per well, and patient test samples at $1\text{-}2x10^6$ cells per well. Cell markers are shown in **Table 1**. For intracellular staining (OPG, CD68, F11R/JAM-A, TREM2, ZO-1/AQP1), cells were fixed and permeabilized with Fixation Buffer and Intracellular Staining Perm Wash Buffer (BioLegend). Flow cytometry was performed on a Cytek Aurora cytometer (Bethesda, MD). Analysis was performed using FlowJo (Ashland, OR) software.
Results: Viable cells migrated out from the synovium into the lower transwell out to 48 hours and represented >85% of migratory cells. We did not test a longer time frame for these experiments. The migratory synovial myeloid population (Live/CD56-/CD3-/CD11c-/CD20-/CD14+/CD11b+) in a representative patient sample contained OPG+, TREM-2+, ZO-1+, CX3CR1+, and F11R+ populations in the lower transwell media (Figure 1B). Sixty-five percent of CD11b+CD14^{dim} cells migrating out of the synovium were OPG+CX3CR1+, and of those cells, 93.8% were double positive for tight junction markers F11R and ZO-1. However, we noted the pro-inflammatory CD14^{hi} macrophage population displayed the greatest increase in the proposed RSM markers (**Figure B**). We used t-distributed Stochastic Neighbor Embedding (tSNE) plots to evaluate co-expression of RSM-specific markers, and found that ZO-1 may be most specific to identifying migratory RSM in SF. Cells with markers of circulating immune subsets, including neutrophils (CD66b), NK cells (CD56), and T cells (CD3) were also present in the lower transwell chambers. Though there were no significant differences found between treatments in these experiments, in all cases the stimuli resulted in decreased RSM-like cell migration compared to control, indicating these RSMs may remain active in the tissue, and migratory cells present in the control may be in response to tissue trauma, an effect countered by the stimuli. Cells found adhered to the underside of the transwell or in the bottom of the lower well were below the limit of detection by Crystal Violet assay (data not shown).
Discussion: Using live, human, *ex-vivo* tissue we were able to demonstrate viability of immune cells out to 48 hours. This assay has yet to be fully optimized for duration or survival conditions (for example, hypoxia or hyperoxia chambers) which represents significant future work by our group. Regardless, in normal incubation conditions, there was high cell viability, which may be due to the normally-hypoxic nature of synovium *in vivo*. For identification of migratory RSM, we conclude that ZO-1 is likely the most specific RSM marker expressed by myeloid cells, but tight junction markers in conjunction with M2 markers and OPG are necessary for identification.
Significance: This assay represents a feasible *ex-vivo* assay for evaluation of acute synovial changes in settings in infection, inflammation, or even trauma. Depending on the pathology, or lack thereof, of the patient sample, treatments could also be evaluated – for example, immunomodulatory medications. By maintaining the tissue architecture that is essential to the function of the synovial organ, this could also be a bridge between animal and human or *in vitro* and *in vivo* experiments. Alternative transwell pore or well sizes can be used depending on the need for cell/stimulus migration or tissue size, respectively.

Table 1:

Molecular Target	Fluorochrome	Clone	Vendor	Cell of Interest
CD11b	BV605	ICRF44	BioLegend	Myeloid
CD11c	BV785	3.9	BD Biosciences	Dendritic Cell
CD14	Spark Blue 550	63D3	BioLegend	Mac/Mono
CD20	BV785	2H7	BD Biosciences	B cell
CD3	BV510	SK7	BioLegend	T Cell
CD45	AF700	HI30	BioLegend	Leukocytes
CD56	PE-Cy5	5.1H11	BioLegend	NK cell
CD66b	PE	MIH24	BioLegend	Neutrophil
CD68	R718	Y1/82A	BD Biosciences	Macrophage
CX3CR1	BV711	2A9-1	BioLegend	RSM/Mac/Mono
F11R/JAM-1/JAM-A	BV421	M.Ab.F11	BD Biosciences	RSM
OPG/TNFRSF11B	AF488	155321	R&D Systems	RSM
TREM2	APC	237920	R&D Systems	Macrophage
ZO-1/TJP1	Coralite 594	polyclonal	ThermoFisher	RSM
Viability	Live/Dead Blue		ThermoFisher	

Figure 1: