Aus der Medizinischen Klinik der Universität Heidelberg

Innere Medizin V: Hämatologie, Onkologie und Rheumatologie

(Ärztlicher Direktor: Prof. Dr. med. Carsten Müller-Tidow)

Abteilung für Rheumatologie

(Sektionsleiter: Prof. Dr. med. Hanns-Martin Lorenz)

Bidirectional Interaction of Differently Activated and Polarized Monocytes/Macrophages and Stromal Cells in Context of Tissue Remodeling in Chronic Inflammatory Disease

Inauguraldissertation

zur Erlangung des Doctor scientiarum humanarum

an der

Medizinischen Fakultät Heidelberg

der

Ruprecht-Karls-Universität

vorgelegt von

Jia Li

aus

Shanxi, VR China

2023

Dekan: Herr Prof. Dr. med. Hans-Georg Kräusslich

Doktorvater: Herr Prof. Dr. med. Hanns-Martin Lorenz

Contents

Abbreviations

Abbreviation (Sort alphabetically by the first letter)	Meaning
aIL-6	Anti-IL6 Receptor Antibody
ARG-1	Arginase 1
aSMA	Alpha Smooth Muscle Actin
b-actin	Beta-actin
COL-1	Collagen 1
COX-2	Cyclooxygenase-2
DMSO	Dimethyl Sulfoxide
ELISA	Enzyme-Linked ImmunoSorbent Assay
FACS	Fluorescence-Activated Cell Sorting
Fib	Fibroblast
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
IFNg	Interferon Gamma
IL-10	Interleukin 10
IL-1b	Interleukin 1 Beta
IL-4	Interleukin 4
IL-6	Interleukin 6
JAKi	Janus Kinase Inhibitor
LPS	Lipopolysaccharide
MACS	Magnetic Cell Separation
M-CSF	Macrophage Colony-Stimulating Factor
MED	Medium
MMP-3	Matrix Metalloproteinase-3
Mph	Macrophage
OA	Osteoarthritis
PBMC	Peripheral Blood Mononuclear Cell
RA	Rheumatoid Arthritis
RPL-13	Ribosomal Protein L13
SN	Supernatant
SSc	Systemic Sclerosis
TGFb	Transforming Growth Factor Beta
TNFa	Tumor Necrosis Factor Alpha

1 Introduction

1.1 Background

1.1.1 Immune System

The Immune system is an highly complex network (Nossal 1987) including immune cells, immune molecules and immune organs (Yatim and Lakkis 2015). In order to protect body from virus, bacteria and parasites, the immune system plays an important role in maintaining health and protecting the body from diseases (Childs et al. 2019).

The immune system can be divided into two main parts: the innate immune system and the adaptive immune system (Parkin and Cohen 2001). The innate immune system serves as the first line of defense for the body, including the physical defense such as the skin, and the immune cells such as macrophages, neutrophils, and natural killer cells. These cells possess the ability to directly recognize and destroy invading pathogens without the need for prior exposure. In contrast, the adaptive immune system can recognize and destroy pathogens specifically after prior activation. The adaptive system is consisting of B cells, T cells and antigen-presenting cells such as dendritic cells. These cells work together to produce specific antibodies that recognize and destroy invading pathogens.

The primary function of the immune system is to protect the body from harmful invaders, such as viruses, bacteria, and parasites. The immune system works on them by recognizing and destroying these invaders, as well as by preventing their entry into the body through physical barriers such as the skin and mucous membranes. On the other hand, the immune system also an important role in maintaining homeostasis, or balance, within the body. For example, Irregularities in Regulatory T cells (Treg) function and number can result in loss of tolerance and autoimmune diseases(Eggenhuizen et al. 2020).

However, the dysfunction of immune system will cause many types of diseases, including autoimmune diseases, immunodeficiencies, and allergies. The autoimmune diseases will happen when the

immune system incorrectly recognize and attack the tissues or organs of themselves. On the other hand, immunodeficiencies is due to the disability of immune system. The reason of allergies is overactive immune response.

Altogether, immune system is a complex and crucial system and it plays a key role in protecting the body from harmful invaders and keeping overall healthy. This research will focus on macrophages, an important component of innate immunity.

1.1.2 Innate Immune System

The innate immune system serves as the body's first line of defense, consisting of physical barriers like the skin and mucous membranes, as well as immune cells like macrophages, neutrophils, natural killer cells, and dendritic cells(Marshall et al. 2018). Physical barriers such as the skin and mucous membranes in the respiratory, gastrointestinal, and genitourinary tracts serve to prevent harmful invaders from entering the body. Immune cells are a diverse group of cells that collaborate to defend the body against invading pathogens, such as bacteria, viruses, and parasites(Melo and Dvorak 2012).

The innate immune system plays a crucial role in preventing infection and maintaining overall health. Firstly it can recognize and respond to invading pathogens to prevent severe infection; secondly, macrophages and dendritic cells can be used as antigen presenting cells in order to recruit other immune cells to the area of infection; in addition, innate immune system plays a crucial role in promoting inflammation, for example, classically polarized macrophages as a pro-inflammatory phenotype help to destroy invading pathogens.

On the other hand, dysfunctions of the innate immune system can result in some immune related diseases, including immunodeficiencies, autoimmune diseases, and chronic inflammatory diseases. For example, innate immunity is inextricably linked in the research and treatment of acquired immune deficiency syndrome (AIDS)(Board et al. 2022); As introduced by Scherer et al, the innate immune system is deeply involved in the autoimmune progression of rheumatoid arthritis (Scherer et al. 2020);

the innate immune system is also involved in the progression of osteoarthritis. When endogenous danger/damage-associated molecular patterns (DAMPs) are produced from diseased joint tissues, it sets off a cycle of progressive innate immune activation and additional tissue damage, contributing to the progression of osteoarthritis (Scanzello et al. 2008).

This study will focus on macrophages, an important component of innate immune cells.

1.1.3 Monocytes/Macrophages

1.1.3.1 Origin

Monocytes are generated in the bone marrow from hematopoietic stem cells (HSCs) and undergo a series of successive stages of differentiation. Common myeloid progenitor cells develop into granulocyte/macrophage colony-forming units (GM-CFU), which can further differentiate into either macrophage colony-forming units (M-CFU) or granulocyte colony-forming units (G-CFU). (Ruytinx et al. 2018). Before differentiating into monocytes, promonocytes originated from M-CFU leave bone marrow and enter peripheral blood. Under normal circumstances, monocytes circulate in the blood flow for a short period before undergoing spontaneous apoptosis (Fahy et al. 1950). Monocytes differentiate into macrophages in response to differentiation factors, which allows them to evade apoptosis and become long-lived cells present in almost every organ of the body. (Wiktor-Jedrzejczak and Gordon 1996) . Alternatively, monocytes have the ability to migrate into various tissues and differentiate into tissue-resident macrophages with specialized functions in response to specific environment(Zhao et al. 2018).

1.1.3.2 General Function

Monocytes and macrophages are essential components of innate immune system. They play critical roles in maintaining tissue homeostasis, including phagocytosing and digesting external particle and cellular debris. Macrophages also work for recruiting and activating other immune cells per cytokines and chemokines secretion. Different phenotypes of macrophages promote tissue repair and

regeneration, while dysfunction of macrophages contributes to the pathogenesis and progression of various disease, such as rheumatoid arthritis.

1.1.3.3 Macrophage Polarization

Plasticity is a hallmark of cells within the monocyte-macrophage lineage. (Gordon and Martinez 2010). The plasticity of macrophages can be regulated by different specific tissue microenvironments. Monocytes demonstrate distinct and polarized functional characteristics when exposed to cytokines and microbial products (Mantovani et al. 2004). After polarization, the polarized macrophages can be roughly divided into M1 and M2. M1 macrophages (classically activated macrophages) are typically activated by Th1 cytokines, such as Interferon-gamma (IFNg). Tumor Necrosis Factor alpha (TNFa), and bacterial lipopolysaccharide (LPS) can also induce M1 macrophages. For in vitro experiments, the combination of IFNg and LPS are commonly used to induce M1 polarization (Orecchioni et al. 2019).

On the other side, M2 macrophages (alternatively activated macrophages) including M2a, M2b and M2c are regarded as the anti-inflammatory phenotypes. M2a macrophages can be induced by IL-4 or IL-13 through the activation of STAT6 via the IL-4 receptor alpha (IL-4Rα). M2b macrophages are induced by combined exposure to immune complexes and TLR or IL-1R agonists, while M2c macrophages are induced by IL-10 and glucocorticoid hormones (Porta et al. 2015; Wynn and Vannella 2016).

M1 macrophages are functionally characterized by their production of pro-inflammatory cytokines, including interleukin-12 (IL-12), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α). They also produce several chemokines, such as CXCL8 (interleukin-8 or IL-8), CCL2 (monocyte chemoattractant protein-1 or MCP-1), and CXCL10 (interferon gamma-induced protein 10 or IP-10) (Atri et al. 2018). On the one hand, M1 macrophages have strong abilities of phagocytosis, and play an important role in destroying microbial pathogens; on the other hand, M1 macrophages as a type of antigen presenting cell contribute to antigen presentation and activation of the adaptive immune response. Taken, together, M1 macrophages are involved in the early stages of immune response to

infection or injury with their pro-inflammatory and microbicidal function (Mantovani et al. 2004).
M2 macrophages are known to produce anti-inflammatory cytokines like interleukin-10 (IL-10) and transforming growth factor beta (TGF-β) as well as the immunomodulatory enzyme arginase, and they play an important role in clearing apoptotic cells and performing anti-inflammatory effects(Xu et al. 2006).
Macrophages are trying their best to find a best balance range in response to the changes of human body, while dysfunction of macrophages polarization is involved in pathogenesis of various disease.

1.1.4 Stromal Cells

Stromal cells--a type of cells of mesodermal origin that are present in certain types of connective tissue, providing structural support to surrounding tissues and organs. Unique groups of stromal cells that possess distinct morphologies and functions consist of vascular endothelial cells, pericytes, adipocytes, fibroblasts, and multipotent mesenchymal stromal cells (mesenchymal stem cells, MSC). (Bussard et al. 2016). Stromal cells can also play important roles in regulating tissue homeostasis, inflammation, and immune responses.

1.1.4.1 Fibroblasts

Fibroblasts which can be found in connective tissue is one type of stromal cell. They are responsible for production and maintenance of the extracellular matrix (ECM), which can mechanically support tissues and regulate the structure and function of tissues. In addition, fibroblasts produce the growth factors and cytokines supporting cell proliferation, migration and differentiation.

1.1.4.1.1 Synovial Fibroblasts

Synovial fibroblasts are a kind of fibroblasts resident in synovial membrane. It plays an important role in keeping joint homeostasis and regulating inflammation (Wei et al. 2021). In healthy joints, synovial fibroblasts produce synovial fluid to keep joint structure integrity, and contribute to joints move smoothly.:. On the other hand, synovial fibroblasts can be overactivated by immigrating immune cells

like monocytes/macrophages, T cells and B cells and their cytokine products such as tumor necrosis factor-alpha (TNFa) and Interleukin (IL) -1b, as found in the joints in RA (rheumatoid arthritis) patients. Then they produce pro-inflammatory cytokines and chemokines themselves, promoting further attraction of immune cells and joint inflammation (Croft et al. 2019). Activated fibroblasts also produce Matrix-metalloproteases, such as MMP-3, which enables their invasion into cartilage and finally cartilage destruction (Störch et al. 2016). In terms of some chronic joint inflammatory diseases such as OA (osteoarthritis) and RA, COX-2 as an enzyme that plays a crucial role in the synthesis of prostaglandins can be highly expressed in response to pro-inflammatory cytokines such as TNFa and IL-1b (Kawashima et al. 2013). It contributes to joint inflammation and pain. Excessive amount of ECM such as collagen is produced by fibroblasts in some areas of patients' joints, promoting synovial hyperplasia, fibrosis and joints stiffness (Maglaviceanu et al. 2021). In this process, alpha-smooth-muscle actin, (aSMA) as a marker of myofibroblast differentiation in which fibroblasts possess contractile properties and extracellular matrix (ECM) remodeling abilities can be highly expressed (Kasperkovitz et al. 2005).

1.1.4.1.2 Dermal Fibroblasts

Dermal fibroblasts are a type of cell that reside in the dermis, which is the second layer of skin located below the epidermis (Thulabandu et al. 2018). The dermis is crucial in providing structural support to the skin and is composed of a variety of cell types, including dermal fibroblasts, histiocytes, adipocytes, and immune cells (Brown and Krishnamurthy 2022).

Dermal fibroblasts are the biggest part of cells in dermis. They are responsible for producing extracellular matrix components in order to maintain the strength, elasticity and structural support to the skin. They are crucial in maintaining skin integrity and strength (Thangapazham et al. 2014). Dermal fibroblasts also contribute to the wound healing process and tissue repair (Eming et al. 2014). On the one hand, when the skin is damaged, they migrate to the injured area differentiate into aSMA producing myofibroblasts, characterized by special changes in the cytoskeleton and upregulation of aSMA and produce ECM such as collagen and elastin in order to repair the damaged tissue directly. Dermal fibroblasts also can repair the damaged tissue with the property of the immune response in infections. They can produce the related cytokines and chemokines which help to recruit required immune cells to the site under damage or infection. After the wound healing process is finished the

differentiated myofibroblasts eventually undergo apoptotic cell death.

However, dermal fibroblasts can exhibit dysfunction in various conditions, including aging, skin diseases, and connective tissue disorders (Lago and Puzzi 2019; Tabib et al. 2021). .

For instance, in SSc patients, dermal fibroblasts become overactivated, mostly via cytokines involving IL-6 and transforming growth factor-beta (TGFbeta) and aberrantly differentiate into myofibroblasts, thereby mimicking an overactivated would healing process. The SSc myofibroblasts are not able to finish normal apoptosis, contributing to their accumulation and production of much more extracellular matrix components than the required amount, resulting in ECM overaccumulation in the skin and lung and leading to the process of fibrosis that is still difficult to treat and represents one of the highest mortalities. Research indicates that SSc fibroblasts possess an unusual gene expression profile and abnormal signaling pathways, which are involved in fibroblast activation and collagen production (Zehender et al. 2021), involving the TGF-β signaling pathway.

1.2 Literature Review—Bidirectional Interactions between Monocytes/Macrophages and Stromal Cells

Macrophages and fibroblasts are two closely related types of cells that play crucial roles in various physiological and pathological processes. The interaction between these two cell types has been extensively explored in the context of several diseases, including Systemic sclerosis (SSc) and rheumatoid arthritis (RA).

1.2.1 Interactions between Synovial Fibroblasts and Macrophages

The tissue resident cells like synovial fibroblasts and resident macrophages make up the basis of the joint microenvironment. They play important roles in maintaining joint homeostasis by sharing their common properties of wound healing and tissue repair.

Synovial fibroblasts are a type of connective tissue cell that produces the synovial fluid, which helps

lubricate and nourish the joint. A study (Marsh et al. 2021) demonstrated that lubricin and hyaluronic acid produced by lining layer fibroblasts form the synovial fluid contribute to lubricate joints and keep them from damage. They also help to the development of joint cartilage and bone (Noss and Brenner 2008). Resident macrophages are responsible for the immune response and cleaning up debris and damaged cells within the joint. Both cell types work in concert to maintain joint health and respond to damage. Synovial fibroblasts release growth factors and extracellular matrix components to help repair damaged tissue, while resident macrophages recruit other immune cells to the area to aid in the healing process.

However, the complex interaction between synovial fibroblasts and macrophages is thought to contribute to the development and progression of many joint disorders, including Osteoarthritis and rheumatoid arthritis. The interaction between macrophages and synovial fibroblasts is believed to play a critical role in the progression of RA or OA. Recent research (Bondeson et al. 2006) has shown that depletion of CD14+ cells in PBMCs-synovial cells coculture significantly reduces the secretion of macrophage-derived cytokines such as IL-1b and TNFa, as well as other proteins like IL-6, IL-8, MMP-3, and ADAMTS4 that are primarily secreted by synovial fibroblasts. A recent study suggests that the Heparin-binding EGF-like growth factor positive (HBEGF+) macrophage-dependent epidermal growth factor receptor (EGFR) response could be involved in destructive tissue behaviors in the joints of patients with RA. Specifically, the process promotes abnormal fibroblast activity and the formation of pannus (Kuo et al. 2019). Pyroptosis is a type of cell death that occurs in response to proinflammatory signals and it is tightly related with inflammation(Yu et al. 2021). Macrophages pyroptosis can be also involved in joint damage. Li Zhang et al, showed pyroptosis of synovial macrophage may occur in pathological processes of knee osteoarthritis (KOA) and inhibition pyroptosis of macrophages alleviates synovitis and fibrosis in KOA model rats (Zhang et al. 2019).

Under certain circumstances, synovial fibroblasts are capable of modifying the differentiation and polarization of monocytes and macrophages, indicating a bidirectional interaction between these cell types. Few studies have dealt with this question so far. According to a study conducted by Laura T Donlin and colleagues (Donlin et al. 2014), synovial fibroblasts are capable of effectively inhibiting the TNF-mediated induction of an IFN-β autocrine loop in Mphs, as well as downstream expression of

IFN-stimulated genes (ISGs) such as the chemokines CXCL9 and CXCL10, which are commonly associated with classical macrophage activation. In another study carried out by Lilian Quero et al. (Quero et al. 2020), it was proposed that imbalanced levels of miR-221-3p and miR-155-5p in the synovium of rheumatoid arthritis (RA) may interfere with the anti-inflammatory function of M2-like macrophages and facilitate the development of an inflammatory microenvironment in the synovium.

The bidirectional interaction between synovial fibroblasts and macrophages is a critical mechanism in the development and progression of chronic inflammatory joint diseases. This reciprocal interaction between these two cell types results in the formation of a complex synovial microenvironment that can either promote or suppress chronic inflammation and joint destruction. Dysfunction of this normal interaction may take part in various inflammatory joint diseases.

Therefore, understanding this bidirectional interaction is crucial for developing effective therapeutic strategies for inflammatory joint diseases.

1.2.2 Interactions between Dermal Fibroblasts and Macrophages

Both dermal fibroblasts and macrophages are key cell types that play important roles in maintaining body homeostasis and responding to injury and infection. Previous studies have reported the significance of bidirectional interactions between these two cell types. The current knowledges regarding to the bidirectional interactions between dermal fibroblasts and macrophages were summarized in this part.

Several studies have suggested that monocytes or macrophages are selectively recruited to specific dermal environments, and they may play a role in the pathogenesis of certain dermal diseases, such as SSc. A recent study (Dufour et al. 2018) showed that TGFβ can synergize together with IL17A to promote the IL-6 and CCL2 production in human dermal fibroblasts, which provides an important mechanism for recruiting macrophages to fibroblasts. Another group (Galindo et al. 2001) reported

that the expression of chemokines by fibroblasts in systemic sclerosis is characterized by abnormal regulation of monocyte chemoattractant protein 1 (MCP-1/CCL2) expression. In a study conducted by Rajan Bhandari et al.(Bhandari et al. 2020), macrophages were found to acquire pro-fibrotic properties after being co-cultured with plasma from systemic sclerosis (SSc) patients. The macrophages were then co-cultured with dermal fibroblasts using transwell plates. The expression of alpha-smooth muscle actin (aSMA) in the dermal fibroblasts co-cultured with the macrophages was found to be significantly higher compared to the control group.

Conversely few mostly newer studies suggest that dermal fibroblasts might be able to alter the differentiation and polarization of monocytes and macrophages. Soldano et al. (Soldano et al. 2018) reported that the percentage of macrophages positive for both M1-related surface markers such as CD80, CD86, and TLR4, and M2-related surface markers including CD163, CD204, and CD206, were higher in patients with systemic sclerosis (SSc) compared to healthy donors. A recent study indicated that dermal fibroblasts can influence the differentiation of macrophages through their exosomes. Rajan Bhandari et al. (Bhandari et al. 2023) reported that the expression levels of surface markers CD163, CD206, MHC class II, and CD16 were higher after activation by exosomes derived from dermal fibroblasts of systemic sclerosis (SSc) patients compared to those of healthy donors. Furthermore, the expression of cytokines, including IL6, IL-10, IL12p40, and TNFa, was also found to be upregulated in SSc patient-derived exosomes.

In summary, the interaction between monocytes/macrophages and stromal cells is extremely complex, as they try to maintain a healthy balance in the body. The interaction between fibroblasts and macrophages is certainly bidirectional, with each cell type having the ability to influence the behavior of the other. While fibroblasts can modify the differentiation and polarization of macrophages, macrophages can acquire specific properties when co-cultured with fibroblasts. This reciprocal relationship emphasizes the significance of the microenvironment in the progression of chronic inflammatory diseases.

Identifying the reasons for the dysfunction of this balance will shed light on the pathology of various chronic inflammatory diseases. Finding effective targets to restore this balance will have significant implications for treating these conditions.

1.3 Research Hypothesis

Based on the biological characteristics of monocytes/macrophages and tissue stromal cells and recent research, macrophages are involved in the pathogenesis of chronic inflammatory diseases in some way. Relevant targeted drugs have been put into clinical use or are currently undergoing clinical trials. However, the pathogenesis of such diseases is complex, and the side effects of targeted drugs are still an important consideration in clinical use. Further exploration of the pathogenesis of chronic inflammatory diseases and the continuous search for appropriate therapeutic targets are necessary. On the other hand, macrophages are powerful with different functional characteristics due to their strong plasticity to regulate their differentiation or polarization status in response to changes in the microenvironment. Exploring the role of stromal cells in regulating macrophages in tissues can further clarify the regulatory mechanism of macrophages under physiological and pathological conditions. By regulating the differentiation or polarization process of macrophages, the treatment of relevant diseases may be achieved.

In order to investigate the interactions between stromal cells and monocytes/macrophages, several research hypotheses are stated in this thesis project.

i. Can differently activated and polarized macrophages activate fibroblasts (Dermal Fibroblasts, Synovial Fibroblasts) in different ways?
ii. Can polarized macrophages regulate differently activated fibroblasts as found in a chronic inflammatory environment (RA, SSc)?
iii. Can differently activated fibroblasts affect the polarization of macrophages?
iv. Which signaling pathways are involved in the interaction?
v. Can biological response modifiers that are already used in the treatment of patients (such as monoclonal antibodies or small molecule inhibitors) counteract or improve the observed effects?

A more comprehensive understanding of these events could increase the chances of successfully

treating various inflammatory diseases.

2 Materials and Methodology

2.1 Materials

2.1.1 Plastics

Table 2.1

Product	Specification	Cat No	Supplier
Nunc™ EasYFlask™ Cell Culture Flasks	75cm2	156475	Thermofisher
Nunc™ Cell-Culture Treated Multidishes	6-well	140675	Thermofisher
Nunc™ Cell-Culture Treated Multidishes	24-well	142475	Thermofisher
Falcon® Conical Centrifuge Tubes	50ml	352070	Corning
Falcon® Conical Centrifuge Tubes	15ml	352096	Corning
EP tube	1,5ml		
Nunc™ CryoTubes cryogenic vial		368632	Thermofisher
Millicell Cell Culture Insert	12mm, polycarbonate, 0.4 µm pore	PIHP01250	Millipore
Stripette™ Serological Pipets	25 mL	4489	Corning
Stripette™ Serological Pipets	10ml	4488	Corning
Stripette™ Serological Pipets	5ml	4487	Corning
TipOne Filter Pipette Tip, sterile	1ml	S11221730	Starlab
TipOne Filter Pipette Tip, sterile	200ul	S11208710	Starlab
TipOne Filter Pipette Tip, sterile	10ul	S11203710	Starlab

2.1.2 Cell Culture Media/Supplements

Table 2.2

Product	Cat No	Applier
Dulbeccos Modified Eagles Medium/Nutrient Mixture F-12 Ham	D6421	Sigma
Sodium bicarbonate solution	S8761	Sigma
RPMI 1640 Medium	31870-025	Gibco
Fetal Calf Serum (FCS)	26140079	Gibco
Penicillin-Streptomycin (5000 U/mL)	15070-063	Gibco
L-Glutamine solution	G7513	Sigma
HEPES solution	H0887	Sigma

Sodium Pyruvate Solution	S8636	Sigma
MEM Non-essential Amino Acid Solution (100x)	M7145	Sigma
ITS+ Premix Universal Culture Supplement	354352	Corning
Dulbecco's Phosphate Buffered Saline	D8537	Sigma
β-Mercapto-EtOH	31350-010	Gibco
Accutase	A6964	Sigma
Dimethyl Sulfoxide for Molecular Biology (DMSO)	D8418	Sigma

2.1.3 Recipe of Cell Culture Medium, MACS/FACS Buffer and Cell Freezing Reagent

Tab 2.3

Solution	Constituents	Volume/Concentration
Serum-containing culture medium for Monocytes/Macrophages/Fibroblasts (in Plates)	RPMI 1640 Medium	42.45ml
	Penicillin/Streptomycin	500μL (1/100)
	L-Glutamine solution	500μL (1/100)
	FCS	5ml (1/10)
	Sodium Pyruvate	500μL (1/100)
	NEAA	500μL (1/100)
	HEPES solution	500μL (1/100)
	β-Mercapto-EtOH	50μL (1/1000)
Serum-free culture medium for Monocytes/Macrophages/Fibroblasts (in Plates)	RPMI 1640 Medium	46.95ml
	Penicillin/Streptomycin	500μL (1/100)
	L-Glutamine solution	500μL (1/100)
	ITS+ Premix	500μL (1/100)
	Sodium Pyruvate	500μL (1/100)
	NEAA	500μL (1/100)
	HEPES solution	500μL (1/100)
	β-Mercapto-EtOH	50μL (1/1000)

Synovial Fibroblast Culture Medium (in Flasks)	DMEM F-12	43.75ml
	FCS	5ml (10%)
	Penicillin/Streptomycin	500µL (1/100)
	L-Glutamine solution	500µL (1/100)
	Sodium bicarbonate	250µL (1/50)
Dermal Fibroblast Culture Medium (in Flasks)	DMEM F-12	46.25ml
	FCS	2.5ml (5%)
	Penicillin/Streptomycin	500µL (1/100)
	L-Glutamine solution	500µL (1/100)
	Sodium bicarbonate	250µL (1/50)
MACS/FACS Buffer	PBS	47.3ml
	10%BSA	2.5ml (1/20)
	0.5M EDTA	200µL (1/250)
Cell Freezing Reagent	FCS	45ml (90%)
	DMSO	5ml (10%)

2.1.4 Recombinant Cytokines and Chemicals for Cell Treatment

Tab 2.4

Product	Catalogue No	Supplier
TGF-β1 (CHO derived)	100-21C-10UG	PEPROTECH
IL-1β	200-01B-2UG	PEPROTECH
TNF-α	300-01A-10UG	PEPROTECH
GM-CSF (Leukine®)		Sanofi
IFN-γ	300-02-20UG	PEPROTECH
M-CSF	300-25-10UG	PEPROTECH
IL-4	200-04-20UG	PEPROTECH
Lipopolysaccharide (LPS)	L8274-10MG	Sigma

2.1.5 Inhibitors

Tab 2.5

Product	Catalogue No	Supplier
Tocilizumab (anti-IL6 receptor antibody)		TargetMol
Upadacitinib (JAK inhibitor)	T7503	Roche-Chugai

2.1.6 PBMC/Monocytes Isolation Related Reagent, Consumables and Systems

Table 2.6

Product	Catalogue No	Supplier
Pancol	P0460500	PAN^{TM}BIOTECH
CD14 MicroBeads, human	130-050-201	Miltenyi Biotec
LS Columns	130-042-401	Miltenyi Biotec
MACS MultiStand	130-042-303	Miltenyi Biotec

2.1.7 FACS Related Reagent, Consumables, Antibodies and System

Tab 2.7

Product	Catalogue No	Supplier
FcR Blocking Reagent, human	130-059-901	Miltenyi Biotec
CD14 Antibody, anti-human, FITC	130-113-146	Miltenyi Biotec
CD80 Antibody, anti-human, PE	305208	BioLegend
CD80 Antibody, anti-human, FITC	305206	BioLegend
CD163 Antibody, anti-human, PE	333606	BioLegend
CD206(MMR) Antibody, anti-human, PE	321106	BioLegend
CD209(DC-SIGN) Antibody, anti-human, PE	130-099-707	Miltenyi Biotec
CD2 Antibody, anti-human, PE	130-091-115	Miltenyi Biotec
CD14 Antibody, anti-human, PE	301806	BioLegend
CD16 Antibody, anti-human, FITC	347523	BD Biosciences
CD19 Antibody, anti-human, PE	130-113-169	Miltenyi Biotec
Round Bottom Polystyrene Test Tube, without Cap, Sterile, 5ml	352052	Corning
FACS Canto™ Flow Cytometer	337173	BD Biosciences

2.1.8 ELISA Related Reagent, Consumables, Commercial Kits and Read-out System

Table 2.8

Product	Catalogue No	Supplier
Greiner Bio-One 96-well Half Area ELISA Microplates	07-000-720	Fisherscientific
TMBS TMB Super Sensitive One	TMBS-1000-01	BioFX

Component HRP Microwell Substrate		
DPBS (10X), no calcium, no magnesium	14200-067	Gibco
Human IL-6 DuoSet	DY206	R&D Systems
Human Total MMP-3 DuoSet	DY513	R&D Systems
Human IL-10 DuoSet	DY217B	R&D Systems
Human TNF-alpha DuoSet	DY210	R&D Systems
Microplate reader-Vmax	V04171	MILENIA KINETIC ANALYZER

2.1.9 Primers for qRT-PCR

Tab 2.9

Gene	Oligo Name	Sequence (5'-3')
Beta-actin	**Beta-actin -Fwd**	5' CGTGGACATCCGTAA AGACC 3'
	Beta-actin -Rev	5' ACATCTGCTGGAAGGTGGAC 3'
RPL-13	**RPL-13 -Fwd**	5' CGAGGTTGGCTGGAAGTACC 3'
	RPL-13 -Rev	5' CTTCTCGGCCTGTTTCCGTAG 3'
aSMA	**aSMA -Fwd**	5' CGGTGCTGTCTCTCTATGCC 3'
	aSMA -Rev	5' CGCTCAGTCAGGATCTTCA 3'
COX-2	**COX-2-Fwd**	5' CATCAGGCACAGGAGGAAG 3'
	COX-2-Rev	5' ACAATGCTGACTATGGCTACAA 3'
Col-1 A1	**Col-1 A1-Fwd**	5' CACTGGTGATGCTGGTCCTG 3'
	Col-1 A1-Rev	5' CGAGGTCACGGTCACGAAC 3'
IL-10	**IL-10-Fwd**	5' TTCCAGTGTCTCGGAGGGAT
	IL-10-Rev	5' GCTGGCCACAGCTTTCAAGA

Arg-1	**Arg-1-Fwd**	5' GTCTGTGGGAAAAGCAAGCG
	Arg-1-Rev	5' CACCAGGCTGATTCTTCCGT

2.1.10 Reagents and Consumables for qPCR

Tab 2.10

Product	Catalogue No	Supplier
RNaseZAP	R2020	Merck
High Pure RNA Isolation Kit	11828665001	Roche
QuantiTect Reverse Transcription Kit	205313	Qiagen
0,2ml 8-Strip PCR Tube, Individually Attached Flat Caps Natural	A1402-3700	Starlab
PowerUp™ SYBR™ Green Master Mix	A25742	Applied Biosystems
MicroAmp™ Fast Optical 96-Well-Reaktionsplatte, 0,1 ml	4346907	Applied Biosystems
Step One Plus™ Real-Time PCR System	272001974	Applied Biosystems

2.2 Methods

2.2.1 Human Peripheral Blood Mononuclear Cells (PBMCs) Isolation

To isolate peripheral blood mononuclear cells (PBMCs), the method of density gradient centrifugation was used to separate them from fresh peripheral blood of healthy donors. Briefly, in total 16 pieces of 50ml Falcon tubes were prepared and labeled. PBS was added to four tubes with 20ml/tube, and Pancoll was transferred to eight tubes with 20ml/tube as well. Then 20ml of heparinized peripheral blood was transferred to each tube with PBS. The mixture of PBS and blood was carefully transferred

to the eight separation tubes and centrifuged at 1900rpm for 22 minutes at 18°C without brake. The layer between Pancoll and plasma contained the PBMCs, which were carefully sucked and transferred into four Falcon tubes. All tubes were filled to 50ml with PBS, and they were centrifuged at 1700rpm for 7 minutes at 4°C, after which the supernatant of each tube was discarded. Then, 10ml of PBS was added to the first tube, and the cells were resuspended and transferred into the second tube to enrich PBMCs. This process was repeated until all resuspension was transferred into the fourth tube. The tubes were washed twice with 10ml of PBS, and finally, the final tube was filled to 40ml and the cells were counted. Meanwhile, the last Falcon was put into the centrifuge at 1600rpm for 6 minutes at 4°C.

2.2.2 Monocytes Isolation

The isolation of monocytes was conducted using the magnetic-activated cell sorting (MACS) method, with the use of Magnetic Microbeads and related MACS systems. Briefly, PBMCs were first centrifuged, and the supernatant was then removed. Next, MACS buffer and CD14+ Microbeads were added to the PBMCs, which were then incubated at 4°C for 15 minutes. The recipe for the microbeads incubation mixture, as shown in Table 2.11, was utilized in accordance with the manufacturer's instructions. After centrifugation, the PBMCs were resuspended with MACS buffer and passed through a magnet and column. The cells were subsequently counted and resuspended with serum-free monocytes medium or 10%FACS containing medium. Finally, purity tests were conducted under flow cytometry to measure the expression of CD2, **CD14**, CD16, and CD19.

Table 2.11

MACS buffer (mix with beads)	Beads	MACS buffer for selection
80μL per 1×10^7 PBMCs	20μL per 1×10^7 PBMCs	500μL per 1×10^8 PBMCs
Dose:	Dose:	Dose:

2.2.3 Macrophage Differentiation and Polarization in Vitro

Monocytes isolated from PBMCs with a purity of at least 90% were used in subsequent functional assays. Purified and resuspended monocytes were seeded into 24-well plates at a density of 2 x

10^6/ml in M1 and M2 complete medium respectively. The cells were then treated according to the **Table 2.12 and Table 2.13**. On day 10, cell culture supernatants were harvested, immediately stored at -20°C after centrifugation, and used for further cytokine measurements. Accutase (Gibco) with a volume of 200 µl/well was used to detach the cells. The polarization of M1/M2 macrophages was verified by FACS (Fluorescence-Activated Cell Sorting) with the expression of CD14, CD80, CD163, and CD206. The concentration of IL-10 (Interleukin-10), IL-12 (Interleukin-12), and TNFa (Tumor Necrosis Factor Alpha) in cell culture supernatants was measured via ELISA (Enzyme-Linked ImmunoSorbent Assay).

-M1 macrophages polarization

Table 2.12

	Day 0	Day 4	Day 8	Day 9	Day 10
Operation	Plate Seeding	1/2 Medium Change	1/2 Medium Change		Cells and Supernatant Harvest
Additions	GM-CSF	GM-CSF INFg LPS	GM-CSF INFg LPS	LPS*	

GM-CSF Granulocyte-macrophage colony-stimulating factor (Peprotech); **IFNg** Interferon gamma (Peprotech); **LPS** Lipopolysaccharides

Medium: Serum-free Macrophage complete medium

Plates seeding: 2 x10^6/ml cells/well (24 well plate);

Total volume: 1ml/well

Concentration of cytokines and other additions: GM-CSF 10ng/ml, IFN- ɣ 20ng/ml, LPS 20ng/ml, LPS* 10ng/ml

Table 2.13

-M2 macrophages polarization

	Day 0	Day 4	Day 8	Day 9	Day 10

Operation	Plate Seeding	1/2 Medium Change	1/2 Medium Change		Cells and Supernatant Harvest
Additions	M-CSF	M-CSF IL-4	M-CSF IL-4	LPS	

M-CSF Macrophage colony-stimulating factor (Peprotech); **IL-4** Interleukin-4(Peprotech)

Medium: Serum-contain Macrophage complete medium

Plates seeding: 2 x10^6/ml cells/well (24 well plate);

Total volume: 1ml/well

Concentration of cytokines and other additions: M-CSF 20ng/ml; IL-4 20ng/ml; LPS 10ng/ml

Careful consideration of these factors was necessary to ensure successful differentiation and polarization of macrophages in vitro. Prior to the development of mature methods for M1/M2 macrophage polarization, several crucial elements were taken into consideration in the procedure. These included the types of cell culture plates used, methods for isolating monocytes, the recipe of the cell culture medium, the number of cells seeded per well, the duration of cell culture, the methods used for cell detachment, the types and amounts of activating cytokines used, and the time points for adding cytokines.

2.2.4 M1/M2 Macrophages Supernatant Preparation and Collection for functional assays

To prepare and collect pure M1/M2 macrophage supernatants free of stimulatory cytokines (“pure supernatants”), purified monocytes were seeded into 24-well plates at a density of 2 x 10^6/ml in M1 and M2 complete medium, respectively. The cells were then treated according to the **Table 2.14** and **Table 2.15**. On day 8, the cells were washed twice with PBS and filled with fresh complete medium. On day 9, LPS (Lipopolysaccharides) was added to induce cytokine release. The cell culture supernatant was harvested on day 10, and after centrifugation, stored at -20℃ for later use. M1/M2 macrophages were verified by Flow Cytometer with the expression of CD14, CD80, CD163, and

CD206 after supernatant harvest. The concentration of IL-10 (interleukin-10), IL-12 (interleukin-12), and TNFa (tumor necrosis factor alpha) in cell culture supernatant was measured via ELISA (Enzyme-Linked ImmunoSorbent Assay).

Table 2.14

-M1 macrophages supernatant preparation

	Day 0	Day 4	Day 7	Day 8	Day 9	Day 10
Operation	Plate Seeding	1/2 Medium Change	1/2 Medium Change	Cell Washing with PBS		Cells and Supernatant Harvest
Additions	GM-CSF	GM-CSF INFg LPS	GM-CSF INFg LPS		LPS*	

GM-CSF Granulocyte-macrophage colony-stimulating factor (Peprotech); **IFNg** Interferon gamma (Peprotech); **LPS** Lipopolysaccharides

Medium: Serum-free Mphs complete medium

Plates seeding: 2 x10^6/ml cells/well (24 well plate);

Total volume: 1ml/well

Concentration of cytokines and other additions: GM-CSF 10ng/ml, IFN- ɣ 20ng/ml, LPS 20ng/ml, LPS* 10ng/ml

Table 2.15

-M2 macrophages supernatant preparation

	Day 0	Day 4	Day 7	Day 8	Day 9	Day 10
Operation	Plate Seeding	1/2 Medium Change	1/2 Medium Change	Cell Washing with PBS		Cells and Supernatant Harvest
Additions	M-CSF	M-CSF IL-4	M-CSF IL-4		LPS	

M-CSF Macrophage colony-stimulating factor (Peprotech); **IL-4** Interleukin-4(Peprotech)

Medium: 10%FCS Mphs complete medium

Plates seeding: 2 x10^6/ml cells/well (24 well plate);

Total volume: 1ml/well

Concentration of cytokines and other additions: M-CSF 20ng/ml; IL-4 20ng/ml; LPS 10ng/ml

2.2.5 Origin and Cell Culture of Synovial Fibroblasts and Dermal Fibroblasts

Adult human Synovial fibroblasts were isolated from synovial membrane (SM) tissues of KOA (Knee Osteoarthritis) patients who underwent arthroplasty. Only OA-Fibs from passages 4 to 8 were used for further experimental studies. Dermal fibroblasts(D-Fibs) were derived from skin biopsy. Only D-Fibs from passage 6-9 were utilized for further experiments. The details of the OA-Fibs and D-Fibs were listed in the table below.

Table 2.16

Name of Cell Line	Primary Cell Extraction Date	Passage
OA-G2F3	19.10.2011	4-8
OA-G2F4	10.11.2011	4-8
D-Fib 25.05.11	25.05.2011	6-8
D-Fib 01.06.11	01.06.2011	6-8

The OA-Fibs were all cultured in 75cm^2 Falcon flasks with OA-Fibs complete medium and incubated at 37°C with 5% CO_2. The complete cell culture medium was changed once a week, and Accutase (Gibco) was used for cell detachment. Cell inheritance was performed when the cells grew too densely in the flasks.

2.2.6 The Effects of Differently Polarized Macrophages Supernatant on Different Fibroblasts in Vitro

2.2.6.1 The Effects of M1 Macrophages Supernatant (M1SN) on Synovial Fibroblasts In Vitro

OA Fibroblasts (OA-Fibs) were isolated from the synovial membrane (SM) of OA patients. The cell lines named OA-G2F3 and OA-G2F4, with passages 4 to 8, were used in this experiment. The flow chart in **Figure 2.1** presented a concise overview of the experimental steps. Briefly, OA-Fibs were detached from flasks using Accutase (Gibco) and seeded into 24-well plates at a density of 25,000/well in 500µl. After overnight culturing, the medium was changed to either SF media (Fib serum-free complete medium) after washing twice with PBS. Then, every two duplicated wells were treated with Medium, TNFa+IL1b (tumor necrosis factor alpha +Interleukin 1 beta) and TGFb (Transforming growth factor beta) respectively, and cultured for 72 hours. After twice cells washing with PBS, 350µl medium were added into each well, and the different pre-stimulated cells were treated with 148.5µl medium+1.5µl LPS and M1SN respectively. After 72 hours of cell culturing, the cell culture supernatant of different samples was harvested for ELISA measurement. Lysis Buffer from High Purity RNA Isolation Kits (Roche) was added after sufficient washing with PBS for lysate collection. The concentration of cytokines for OA-Fibs pre-stimulation was listed in the **Table 2.17**.

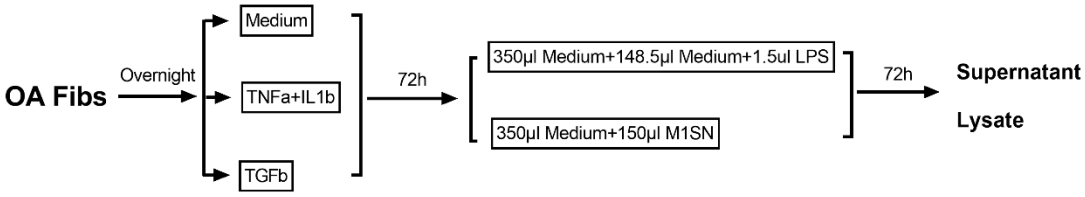


Figure 2.1

Table 2.17

Cytokines	TNFa	IL-1b	TGFb
Concentration	10ng/ml	1ng/ml	20ng/ml

To determine the optimal experimental protocols, a number of other factors were considered, including the addition of Lipopolysaccharides (LPS) and the use of serum-containing or serum-free medium. Experiments were conducted with these different elements.

2.2.6.2 The Effects of M2 Macrophages Supernatant (M2SN) on Synovial Fibroblasts In Vitro

For this experiment, the OA Fibroblasts (OA-Fibs) cell lines OA-G2F3 and OA-G2F4, with passages 4 to 8, were used. The flow chart in **Figure 2.2** presented a concise overview of the experimental steps. Briefly, the OA Fibs were detached from Falcon flasks using Accutase (Gibco) and seeded into 24-well plates at a density of 25,000/well cells per well in 500µl.After overnight culturing, the medium was changed to serum-containing complete medium (SC media) after washing twice with PBS. Then, every two duplicated wells were treated with Medium, TNF+IL1b (tumor necrosis factor alpha +Interleukin 1 beta) and TGFb (Transforming growth factor beta) respectively, and cultured for 72 hours. After washing the cells twice with PBS, 350µl of medium was added to each well, and the different pre-stimulated cells were treated with 148.5 medium+1.5µl LPS and M2SN, respectively. After 72 hours of culturing, the cell culture supernatant of different samples was harvested for ELISA measurement. Lysis Buffer from High Purity RNA Isolation Kits (Roche) was added after sufficient washing with PBS for lysate collection. The concentration of cytokines for OA-Fibs pre-stimulation was listed in the **Table 2.18**.

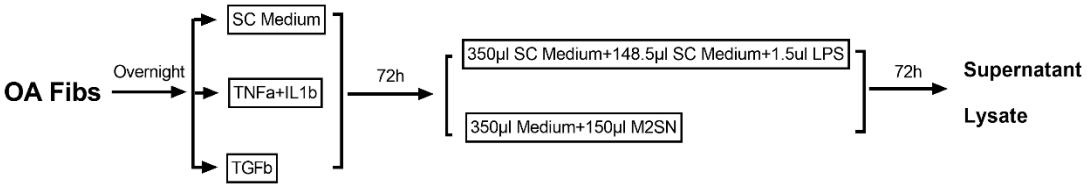


Figure 2.2

Table 2.18

Cytokines	TNFa	IL-1b	TGFb
Concentration	10ng/ml	1ng/ml	20ng/ml

2.2.6.3 The Effects of M1 Macrophages Supernatant (M1SN) on Dermal Fibroblasts In Vitro

In this experiment, Dermal Fibroblasts (D-Fibs) with cell lines named D-Fib 25.01.11 and D-Fib 01.06.11, at passages 6 to 8, were used. The flow chart in **Figure 2.3** provides a concise overview of the experimental steps. Briefly, D-Fibs were detached from flasks using Accutase (Gibco) and seeded into 24-well plates at a density of 25,000 cells per well in 500µl of culture medium. After overnight culturing, the medium was changed to SF media (Fibroblasts serum-free complete medium) following twice washing with PBS. Then, every two duplicated wells were treated with Medium, TNFa+IL1b (tumor necrosis factor alpha +Interleukin 1 beta), or TGFb (Transforming growth factor beta), respectively, and cultured for 72 hours. After twice washing the cells with PBS, 350µl of medium was added to each well, and the different pre-stimulated cells were treated with 148.5µl of medium+1.5µl LPS and M1SN respectively. After 72 hours of cell culturing, the cell culture supernatant of different samples was harvested for ELISA measurement. Lysis Buffer from High Purity RNA Isolation Kits (Roche) was added after sufficient washing with PBS for lysate collection. The concentration of cytokines for D-Fibs pre-stimulation was listed in **Table 2.19**.

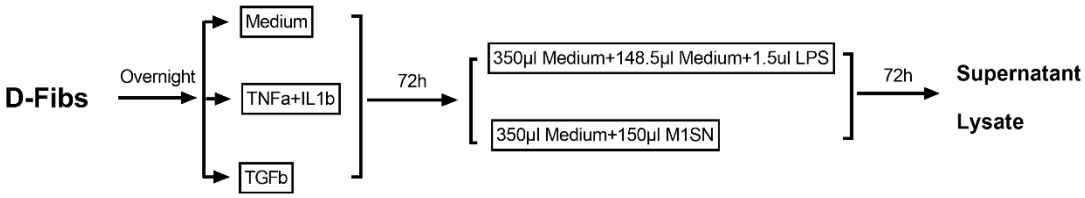


Figure 2.3

Table 2.19

Cytokines	TNFa	IL-1b	TGFb
Concentration	10ng/ml	1ng/ml	20ng/ml

2.2.6.4 The Effects of M2 Macrophages Supernatant (M1SN) on Dermal Fibroblasts In Vitro

In this experiment, we used the Dermal Fibroblasts (D-Fibs) cell lines D-Fib 25.01.11 and D-Fib 01.06.11 with passages 6 to 8. The experimental steps are presented in **Figure 2.4**. Briefly, the D-Fibs were detached from Falcon flasks using Accutase (Gibco) and seeded into 24-well plates at a density of 25,000 cells/well in volume of 500µl. After overnight culturing, medium was changed to fibroblast serum-containing complete medium (SC media) after twice washing with PBS. Then, every two duplicated wells were treated with Medium, TNFa+IL1b (tumor necrosis factor alpha + Interleukin 1 beta), and TGFb (Transforming growth factor beta), respectively, and cultured for 72 hours. After washing the cells twice with PBS, 350µl of medium was added to each well, and the different pre-stimulated cells were treated with 148.5 medium+1.5µl LPS and M2SN, respectively. After another 72 hours of culturing, the cell culture supernatant of different samples was harvested for further ELISA measurement. Lysis Buffer from High Purity RNA Isolation Kits (Roche) were added into each well after sufficient washing with PBS for lysate collection. The concentration of cytokines for D-Fibs pre-stimulation was listed in **Table 2.20**.

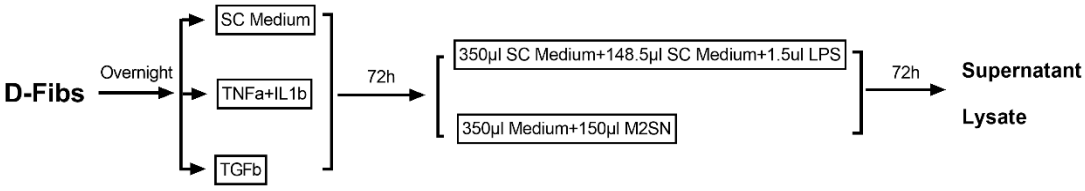


Figure 2.4

Table 2.20

Cytokines	TNFa	IL-1b	TGFb
Concentration	10ng/ml	1ng/ml	20ng/ml

2.2.7 The Effects of Bidirectional Polarized Macrophages (M1/M2) on Synovial Fibroblasts in Vitro—Transwell Co-culture Assay

The flowchart depicted in **Figure 2.5** provides a succinct summary of the experimental procedures. In this experiment, OA Fibroblasts (OA-Fibs) cell lines OA-G2F3 and OA-G2F4, with passages 4 to 8, were seeded into 24-well Transwell inserts (Millicell-PCF) after detachment from Falcon flasks. Accutase (Gibco) was used in this procedure, and the cells were seeded at a density of 25,000/inserts in 500µl of serum-free complete medium. Two duplicated wells were prepared for each sample. As shown in the graph below, inserts with differently pre-stimulated OA Fibs were moved into polarized macrophage (Mphs) plates on 7th culture day of Mphs. After 72 hours of culturing, the cell culture supernatant of different samples was harvested for ELISA measurement. The OA-Fibs were detached from inserts with Accutase and then moved to 15ml Falcon tubes. The cells were resuspended and washed twice with PBS at 4℃, and then Lysis Buffer from High Purity RNA Isolation Kits (Roche) was added after centrifugation for lysate collection. The concentration of cytokines for OA-Fibs pre-stimulation was listed in the **Table 2.21**.

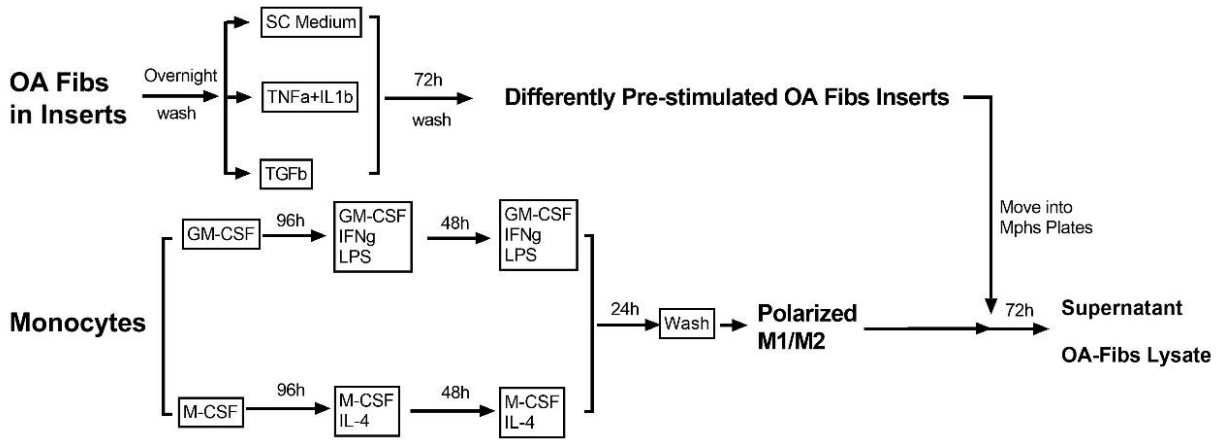


Figure 2.5

Table 2.21

Cytokines	TNFa	IL-1b	TGFb
Concentration	10ng/ml	1ng/ml	20ng/ml

2.2.8 The Effects of M1 Supernatant (M1SN) on Synovial Fibroblasts in Vitro—Time Curves

In this experiment, the OA Fibroblasts (OA-Fibs) cell line OA-G2F4 with passage 7 was used. The flowchart depicted in **Figure 2.6** provides a succinct summary of the experimental procedures. Briefly, the OA Fibs were detached from Falcon flasks using Accutase (Gibco) and seeded into 24-well plates at a density of 25,000/well in 500µl of serum-free complete medium. After overnight culture, the medium was changed to new serum-free complete medium after washing twice with PBS. Two duplicated wells were prepared for each group of samples. After 72 hours of cell culture, the cells were twice washed with PBS, and then 350µl medium was added to each well. Controls were treated with 148.5µl medium+1.5µl LPS, and 150µl M1SN was added to the test groups. All the samples were divided into C1-C4 (Control 1- Control 4) and T1-T4 (Test 1- Test 4) related to 12h, 24h, 48h and 72h time points. After 12 hours of culture, the supernatant and lysates of C1 and T1 were harvested, and then the supernatant of C2-C4 and T2-T4 was removed. The cell treatments were repeated for C2-C4 and T2-T4 before being put into the cell culture incubator. The steps were repeatedly conducted at later time points. The cell culture supernatant of different samples was harvested for ELISA measurement, and Lysis Buffer from High Purity RNA Isolation Kits (Roche) was added after sufficient washing with PBS for lysate collection.

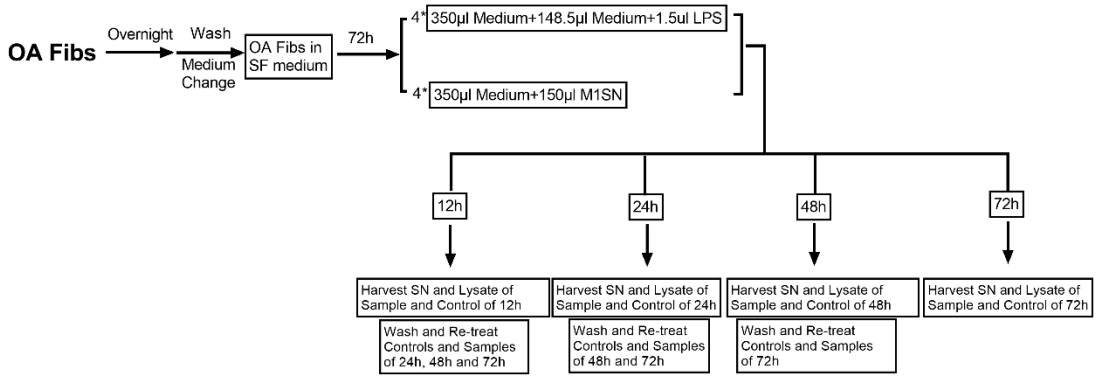


Figure 2.6

2.2.9 The Effects of Differently Polarized Macrophages (M1/M2) on M1 pre-treated Synovial Fibroblasts in Vitro—Transwell Co-culture Assay

The flowchart presented in **Figure 2.7** offers a summary of the experimental procedures. Monocytes were isolated from PBMCs with a purity test result of ≥90%. The purified and resuspended monocytes were seeded into 24-well plates with a density of 2 x10^6/ml in M1 and M2 complete medium, respectively. For the OA Fibroblasts (OA-Fibs) cell line named OA-G2F3 with passage 8, the OA Fibs were seeded into 24-well Transwell inserts (Millicell-PCF) after detachment from Flasks using Accutase (Gibco) with a density of 25,000/inserts and a volume of 500µl per well. After an overnight culture, the medium was changed with SF media (Fibs serum-free complete medium) after twice washing with PBS. Then, 6 inserts in total were treated with 10ng/ml TNFa (tumor necrosis factor alpha) +1ng/ml IL1b (Interleukin 1 beta) and cultured for 72 hours. The date of OA-Fibs seeding should match the date of M1/M2 Macrophages culture. In other words, the date of OA-Fibs finishing 72 hours of pre-treatment with TNFa+IL-1b should match the 7th day of Macrophages culture. The 6 inserts were then moved into M1 after washing with PBS for both of them. After a 72-hour co-culture and twice washing with PBS, the 6 inserts were divided into 3 groups and then moved into MED, new M1 and M2, respectively. The date of insert transfer should match the 10th of M1 and M2 culture. After another 72 hours of co-culture, cell culture supernatant of different samples was harvested for ELISA measurement. OA-Fibs were detached from inserts with Accutase, and then the cells of each sample were moved into 15ml Falcons. Resuspending and washing by PBS with 4℃ were conducted twice, and then Lysis Buffer from High Purity RNA Isolation Kits (Roche) was added after centrifugation for lysate collection.

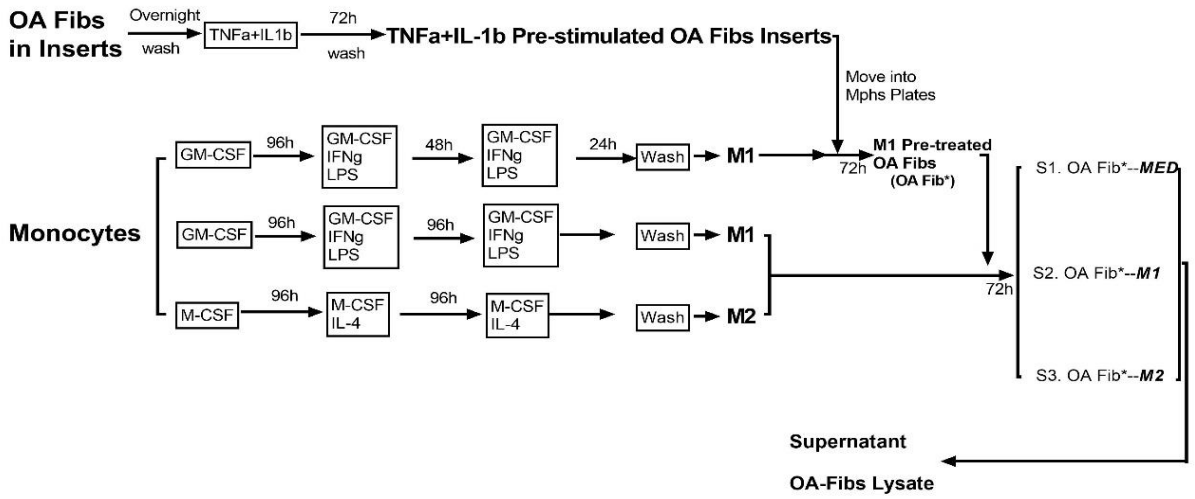


Figure 2.7

2.2.10 The Role of Janus kinase (JAK) inhibitor Played on the Interaction between M1 Macrophages Supernatant (M1SN) and Synovial Fibroblasts

For the JAK inhibition experiments, upadacitinib, an inhibitor of the JAK/STAT signaling pathway was used. The OA Fibroblasts (OA-Fibs) cell lines OA-G2F3 and OA-G2F4 with passages 5-7 were used. Briefly, 8 wells of OA Fibs were detached from Flasks using Accutase (Gibco) and seeded into 24-well plates with a density of 25,000/well and a volume of 500µl. After an overnight culture, the medium was changed with new SF media (Fibs serum-free complete medium) after being washed twice with PBS. After 3 days of culture and twice washing with PBS, 4 wells of cells were pre-treated with DMSO (1:25,000), and the other four wells of cells were pre-treated with 400nM upadacitinib (JAKi) for 30 minutes. Then, the cells were treated according to the following arrangement:

Sample 1. A mixture of DMSO (1:100,000) with 498.5µl Medium + 1.5µl LPS

Sample 2. A mixture of DMSO (1:100,000) with 350µl Medium + 150µl M1SN

Sample 3. A mixture of JAKi (100nM) with 498.5µl Medium + 1.5µl LPS

Sample 4. A mixture of JAKi (100nM) with 350µl Medium + 150µl M1SN

DMSO and JAKi with the same concentration, as shown above, were repeatedly added to the related samples every 24 hours. After three days of cell culture, the cell culture supernatant of different

samples was harvested for ELISA measurement, and Lysis Buffer from High Purity RNA Isolation Kits (Roche) was added after sufficient washing with PBS for lysate collection.

2.2.11 Effects of Differently Activated Synovial Fibroblasts Supernatant on Differently Polarized Macrophages (M1/M2)

2.2.11.1 Differently Activated OA Fibroblasts Supernatant Preparation and Collection

The OA Fibroblasts (OA-Fibs) cell lines OA-G2F3 and OA-G2F4 with passages 6-7 were used in this experiment. Briefly, cells were seeded into 6-well plates at a density of 250,000/well and with a volume of 2.5ml/well. After overnight culture, the medium was changed with new SF media (Fibs serum-free complete medium) after twice washing with PBS, and then the cells were treated with different conditions as indicated in **Table 2.22**. After 3 days of pre-culture and twice washing with PBS, 2.5ml of new medium was added into each well. The supernatants of differently activated OA fibroblasts were collected after another 3 days, and stored at -20°C for later use.

Table 2.22

Name	Fib SN	Fib*SN	Fib$^{\#}$SN	Fib^{g}SN
Pre-Treatment	Medium	10ng/ml TNFa 1ng/ml IL-1b	20ng/ml TGFb	10ng/ml IFNg

2.2.11.2 The Effects of Differently Activated OA Fibroblasts Supernatant on M1 and M2

The concise experimental steps were demonstrated through the flowchart depicted in **Figure 2.8**. Purified monocytes were isolated from PBMCs with a purity test result of ≥90%. The monocytes were resuspended and seeded into 24-well plates at a density of 1 x10^6/ml with a volume of 1ml. The monocytes were then treated with different protocols to differentiate to M1, and M2 macrophages, as

shown in the figure below. On the 7th day of macrophage culture, differently activated OA fibroblast supernatants (Fib SN, Fib*SN, Fib#SN and Fib^{g}SN) were added into M1, and M2, respectively. After an additional 72 hours of culture, the cell culture supernatants of different samples were harvested for ELISA measurement, and the cells were collected for FACS measurement.

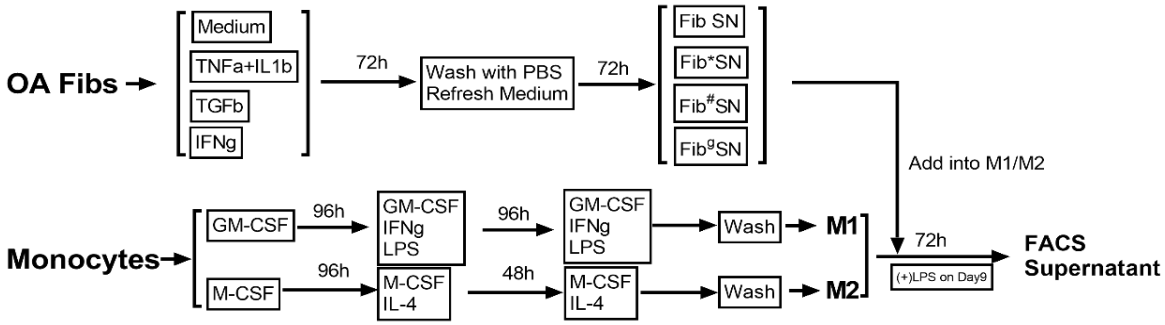


Figure 2.8

2.2.12 The Effects of TNFa+IL1b Activated OA Fibroblasts Supernatant on M0 Macrophages

The flowchart illustrated in **Figure 2.9** demonstrated the succinct experimental steps. Purified monocytes were isolated from PBMCs, and the purity of the monocytes was tested to be at least 90%. The monocytes were then resuspended and seeded into 24-well plates at a density of 1 x10^{6}/ml with a volume of 1ml. To differentiate the monocytes into M0 macrophages, a specific protocol was followed as depicted in the figure below. On the 7th day of macrophage culture, Medium control, Medium cultured fibroblasts supernatant (Fib SN), and TNFa+IL1b activated OA fibroblast supernatants (Fib*SN) were added separately into M0 macrophages. After an additional 72 hours of culture, the cell culture supernatants from each sample were collected for ELISA measurement, and the cells were harvested for FACS measurement. In the additional experiments, LPS was added on the 9th day to test its effects.

The second part of the study utilized a Transwell co-culture assay between M0 macrophages and inserts containing Fibroblasts pre-treated with either Medium control (Fib only insert) or TNFa+IL1b (Fib*insert). The aim was to investigate the cell-cell interactions between M0 and Fibroblasts. Purified

monocytes were treated with the same protocol as the first part of the experiment. The Fib only inserts and Fib*inserts were moved into M0 on the 7th day of M0 culture. The cell culture supernatants were harvested for cytokine measurement, and the cells were collected for analysis using a flow cytometer.

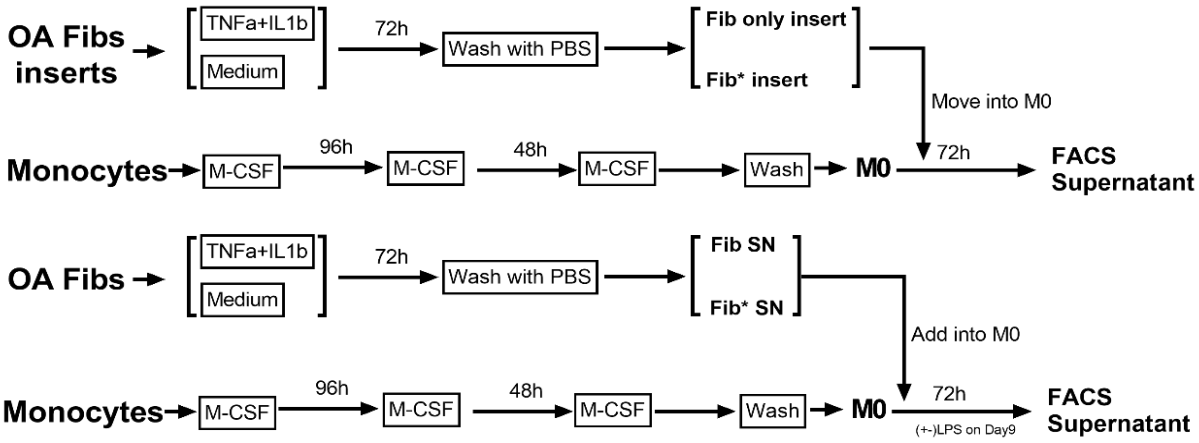


Figure 2.9

2.2.13 Treating Macrophages with anti-IL6 (aIL-6)

Tocilizumab is a humanized monoclonal antibody that targets the IL6-receptor and is used in treatment of rheumatic diseases (Schoels et al. 2013). The experiment involved seeding resuspended monocytes into 24-well plates at a density of 1 x10^6/ml with a volume of 1ml. The monocytes were then treated with the protocol to differentiate them into M0, as mentioned above. On the 7th day of the culture, the cells were pre-incubated with Medium and 200µg/ml aIL-6 separately for 30 minutes. The cells were then treated as the arrangement:

Sample 1. 500µl Medium

Sample 2. A mixture of 350µl Medium + 150µl Fib SN

Sample 3. A mixture of 350µl Medium + 150µl Fib*SN

Sample 4. A mixture of 50µg/ml aIL-6 with 500µl Medium

Sample 5. A mixture of 50µg/ml aIL-6 with 350µl Medium + 150µl Fib SN

Sample 6. A mixture of 50µg/ml aIL-6 with 350µl Medium + 150µl Fib*SN

On 10th day of culture, the cells and cell culture supernatants from different samples were harvested for flow cytometer analysis and ELISA measurements.

2.2.14 Treating Macrophages with Janus kinase (JAK) inhibitor and DMSO

JAKs are intracellular signaling enzymes that mediate intracellular signal transduction via cytokine receptors, such as IL-6.. Upadacitinib is a small molecule inhibitor that has anti-inflammatory and immunosuppressive effects via its selective inhibition of Januskinases (JAK), especially JAK 1 and is used in treatment of rheumatic diseases. In the JAK inhibition experiments resuspended monocytes were seeded into 24-well plates at a density of 1 x10^6/ml with a volume of 1ml. The monocytes were then treated with different methods to differentiate them into M0, as mentioned above. On 7th day of the culture, the cells were pre-incubated with DMSO (1:2,500) and 4μM JAKi separately for 30 minutes. The cells were then treated as the arrangement:

Sample 1. A mixture of DMSO (1:10,000) with 500μl Medium
Sample 2. A mixture of DMSO (1:10,000) with 350μl Medium + 150μl Fib SN
Sample 3. A mixture of DMSO (1:10,000) with 350μl Medium + 150μl Fib*SN
Sample 4. A mixture of 1μM JAKi with 500μl Medium
Sample 5. A mixture of 1μM JAKi with 350μl Medium + 150μl Fib SN
Sample 6. A mixture of 1μM JAKi with 350μl Medium + 150μl Fib*SN

On 10th day of culture, the cells and cell culture supernatants from different samples were harvested for FACS and ELISA measurements.

2.2.15 Coculture of M0 macrophages with supernatant of polarized macrophages

M1 macrophages supernatant (M1SN) were harvested and stored at -20℃ as the protocol mentioned earlier. Purified and resuspended monocytes were seeded into 24-well plates with the density of 1 x10^6/ml and the volume of 1ml. The monocytes were then treated with the M0 differentiation protocol to differentiate them into M0, as shown in **Figure 2.10**. On the 7th day of macrophage culture, M1SN

was added into M0 macrophages. After another 72 hours, the cells and cell culture supernatant were harvested for later measurement.

The second part of the experiment aimed to explore the cell-cell interactions between M0 and M1. As depicted in **Figure 2.10**, monocytes with a density of 250,000/insert were seeded into Transwell inserts for 24-well plates, and both the insert and M0 macrophages were treated with the protocol mentioned earlier. On the 7th day of M1 insert and M0 culture, the M1 insert was moved into M0 after sufficient washing with PBS. After an additional 72 hours of co-culture, the cells from the 24-well plate were harvested for measurement with flow cytometer, and the supernatants from different samples were collected for ELISA measurement.

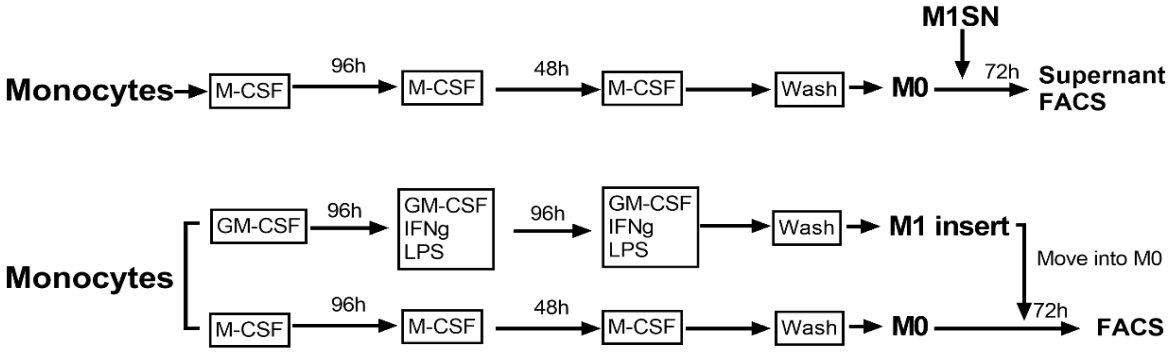


Figure 2.10

2.2.16 Treating Macrophages with Janus kinase (JAK) inhibitor and DMSO

In the JAK inhibition experiments, Upadacitinib, a clinically used JAK inhibitor (JAKi) for rheumatoid arthritis, was applied. Briefly, resuspended monocytes were seeded into 24-well plates with the density of 1 x106/ml and the volume of 1ml. The monocytes were then treated with the M0 differentiation protocol to differentiate them into M0, as mentioned earlier. On 7th day of culture, the cells were pre-incubated with DMSO (1:2,500) and 4μM JAKi separately for 30 minutes. The cells were then treated as the arrangement:

Sample 1. A mixture of DMSO (1:10,000) with 500μl Medium+1.5μl LPS

Sample 2. A mixture of DMSO (1:10,000) with 350μl Medium + 150μl M1SN (using protocol as mentioned in **Part 2.2.4**)

Sample 3. A mixture of 1μM JAKi with 500μl Medium+1.5μl LPS

Sample 4. A mixture of 1μM JAKi with 350μl Medium + 150μl M1SN

After an additional 72 hours, the cells and cell culture supernatants from different samples were harvested for FACS and ELISA measurements.

2.2.17 RNA Isolation, cDNA synthesis and qRT-PCR

Total RNA was isolated using the High Pure RNA Isolation Kit (Roche). Total RNA was extracted from 2 duplicated wells of each group following the manufacturer's instructions. Briefly, lysates of each sample were harvested with Lysis Buffer. Total RNAs were extracted according to the instruction of the commercial kit. The concentration of extracted total RNA was measured using a Nanodrop and stored at -80℃ for later cDNA synthesis.

cDNA was synthesized from the total RNA using the QuantiTect Reverse Transcription Kit (Qiagen), following the manufacturer's instructions. The amount of total RNA used for cDNA synthesis depended on the concentration measured after the total RNA extraction.

The relative transcript expression levels were determined by quantitative real-time PCR (qRT-PCR) using the Applied Biosystems™ PowerUp™ SYBR™ Green Master Mix in the Step One Plus™ Real-Time PCR System. The details of the primers used for qRT-PCR are shown in **Table 2.9**. The gene expression was normalized to the Ct value of the housekeeping genes---beta-actin and RPL-13. The relative gene expression was calculated using the $2^{-\Delta Ct}$ method.

2.2.18 Determination of Cytokine Levels in Culture Supernatant – ELISA (Enzyme-Linked ImmunoSorbent Assay)

The concentration of TNF-α, IL-6, IL-10, and MMP-3 in the supernatants of cell cultures was determined using ELISA (R&D Systems, Duoset ELISA), following the manufacturer's instructions. Briefly, a capture antibody specific to the antigen was coated onto a 96-well microplate and left to incubate overnight. The plate was washed three times with washing buffer (0.05% Tween 20 in PBS)

and then incubated with blocking buffer (2.5% BSA in PBS). The supernatants, in appropriate dilutions, were added to the wells and incubated overnight at 4°C. To establish a standard curve, duplicate wells containing known concentrations of the standard protein or media alone were included. After incubation, the plate was washed three times and then incubated with a specific detection antibody for 2 hours. The wells were washed again, and the enzyme solution (horseradish peroxidase) was added to the wells for 20 minutes in darkness to bind to the secondary antibody. After another washing step, the substrate (TMB) was added, and the chemical reaction between the substrate and the enzyme led to a color change. The optical density was directly proportional to the amount of enzyme-linked antibody present, and thus the amount of antigen. The enzymatic reaction was stopped with the addition of an H2SO4 acid solution. Plates were read on a microplate reader at 450nm, and the absorbance proportional to the amount of specific antigen was calculated according to the standard curve.

2.2.19 Flow Cytometry

The cell surface markers were measured via Flow Cytometry. Briefly, cells were resuspended by the mixture of Fc blocking reagent (Miltenyi Biotec) in MACS buffer (showed in **Table 2.3**) with 1:100 dilution and incubated in 4℃ with darkness for 10 minutes. The cells were separated into FACS tubes (Corning) with different labels, and then fluorescently-labeled antibodies were added to the sample to label the cells. The details of the antibodies were showed in **Table 2.7**. Samples after staining were moved into refrigerator and incubated in 4℃ for 30min. After incubation, 1ml FACS buffer (showed in **Table 2.3**) were added into each sample in order to wash the rest of the antibodies, and then samples were moved into centrifuge at 1600rpm for 6 minutes at 4°C. The sample is then loaded into the flow cytometer, which draws the sample through a flow cell. The data generated by the flow cytometer is analyzed using specialized software. Histograms were analyzed using FlowJo_v10.8.1 and the Mean and Median of fluorescence intensity were analyzed by GraphPad Prism 7.

2.2.20 Statistical Analysis

Statistical analysis was conducted using GraphPad Prism 7. The mean and standard error of the mean (S.E.M.) were calculated for all values, and differences between groups were assessed using paired t-tests. A two-tailed significance level of $p < 0.05$ was considered to be statistically significant. Significant results were indicated using asterisks, where * denotes a p-value less than 0.05, ** denotes a p-value less than 0.01, and *** denotes a p-value less than 0.001.

2.2.21 Ethic Statement

The use of venous blood as well as dermal and synovial tissue for scientific purposes was certified and granted by the ethics committee of the University of Heidelberg (Ethic committee agreement number **S-404/2017** and **S-508/2009**, respectively).

3 Results

3.1 Macrophages Differentiation and Polarization in Vitro

3.1.1 Monocytes Isolation and Purity Test

Table 3.1

Donor No.	Date	Number of PBMCs	Number of Monocytes(mio)	Percentage of Monocytes
1	2020/10/2	171mio	-------	------
2	2020/10/7	169mio	17.45	10.33%
3	2020/10/23	200mio	9.6	4.80%
4	2020/11/20	208mio	21	10.10%
5	2020/12/8	200mio	17	8.50%
6	2021/1/12	96mio	10	10.42%
7	2021/1/20	170mio	25.8	15.18%
8	2021/2/19	164mio	22	13.41%
9	2021/3/12	240mio	10.4	4.33%
10	2021/4/13	194mio	18	9.28%
11	2021/4/16	224mio	28	12.50%
12	2021/4/30	168mio	24	14.29%
13	2021/5/12	198mio	31.7	16.01%
14	2021/6/21	139mio	12.5	8.99%
15	2021/7/16	140mio	18	12.86%
16	2021/8/6	171mio	21	12.28%
17	2021/8/23	188mio	19	10.11%
18	2021/9/21	200mio	29	14.50%
19	2021/9/24	120mio	14	11.67%
20	2021/10/22	200mio	37.3	18.65%
21	2021/12/19	179mio	18	10.06%
22	2021/12/27	160mio	22	13.75%
23	2022/1/13	125mio	14	11.20%
24	2022/1/28	147mio	28	19.05%
25	2022/2/4	265mio	36	13.58%
26	2022/2/18	163mio	19	11.66%
27	2022/3/2	230mio	26	11.30%
28	2022/3/14	141mio	18	12.77%
29	2022/3/18	171mio	28	16.37%
30	2022/4/1	210mio	28	13.33%
31	2022/5/18	231mio	18	7.79%
32	2022/6/10	172mio	26	15.12%

33	2022/6/19	155mio	22	14.19%
34	2022/8/4	251mio	38	15.14%
35	2022/8/29	155mio	18	11.61%
36	2022/9/9	231mio	18	7.79%
37	2022/9/14	140mio	15.8	11.29%
38	2022/9/15	190mio	22.5	11.84%
39	2022/9/26	143mio	26	18.18%
40	2022/10/26	94mio	12	12.77%
41	2022/11/7	250mio	14	5.60%
42	2022/11/15	350mio	52	14.86%
43	2022/11/29	192mio	29	15.10%
Mean±S.E.M (n=40)		189±7 mio	22.8±1.3 mio	12.23±0.55%

In this project, monocytes from n=43 healthy donors were utilized, as shown in **Table 3.1**. The first donor's monocytes were isolated using the natural attachment method, but they were not used for subsequent experiments due to impurities. Monocytes from the other donors were isolated using the MACS method， as described in the methods section. Monocytes from the 6th and 40th donors were isolated from 60ml and 40ml peripheral blood respectively. In addition to the 1st, 6th, and 40th donors, PBMCs and monocytes were isolated from 80ml peripheral blood of other n=40 donors. The average number of PBMCs isolated from all 40 donors was 189±7 million. The average number of monocytes isolated from all 40 donors was 22.8±1.3 million, and the percentage of monocytes isolated was 12.23±0.55%. Purity tests were conducted via flow cytometry after isolation to ensure the quality of the monocytes before proceeding with further experiments.

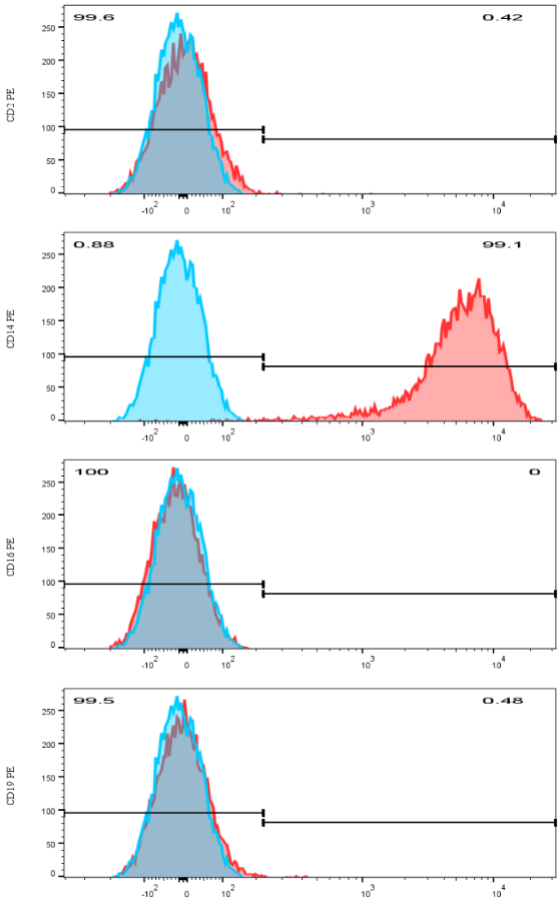


Figure 3.1

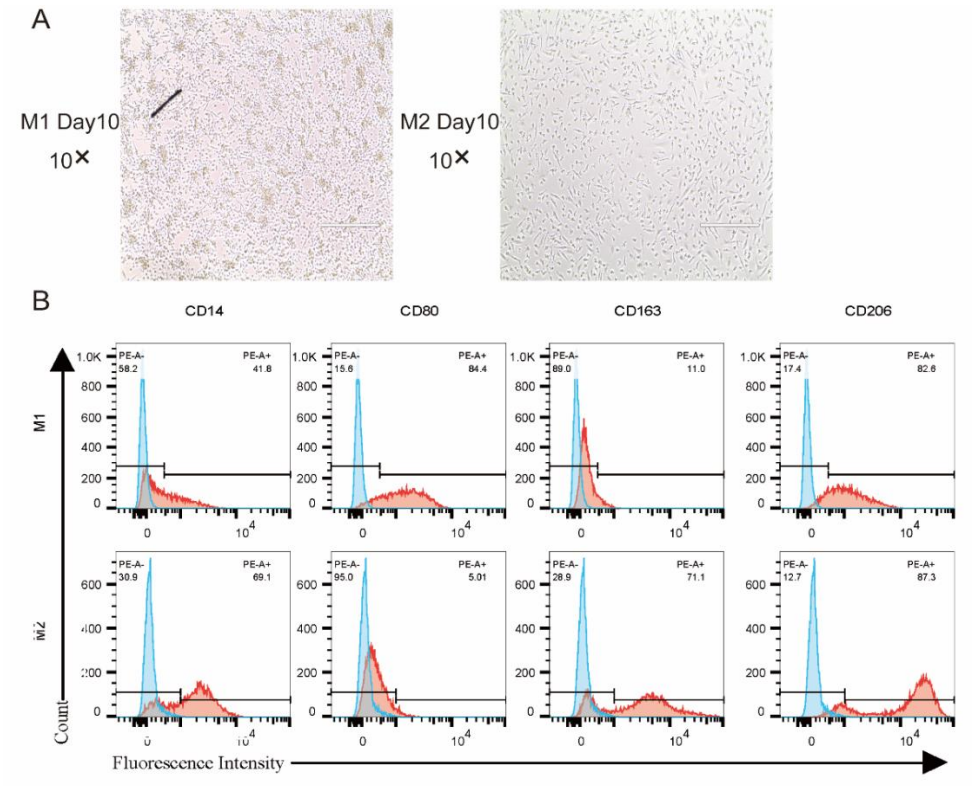
As shown in **Figure 3.1**, the representative histogram demonstrates that CD14, a specific marker for monocytes, was positively expressed at a level higher than 90%. Negative expression of CD2, CD16, and CD19, which are markers for T cells, natural killer cells, and B cells, respectively, was also confirmed.

3.1.2 Macrophages (M1/M2) Polarization

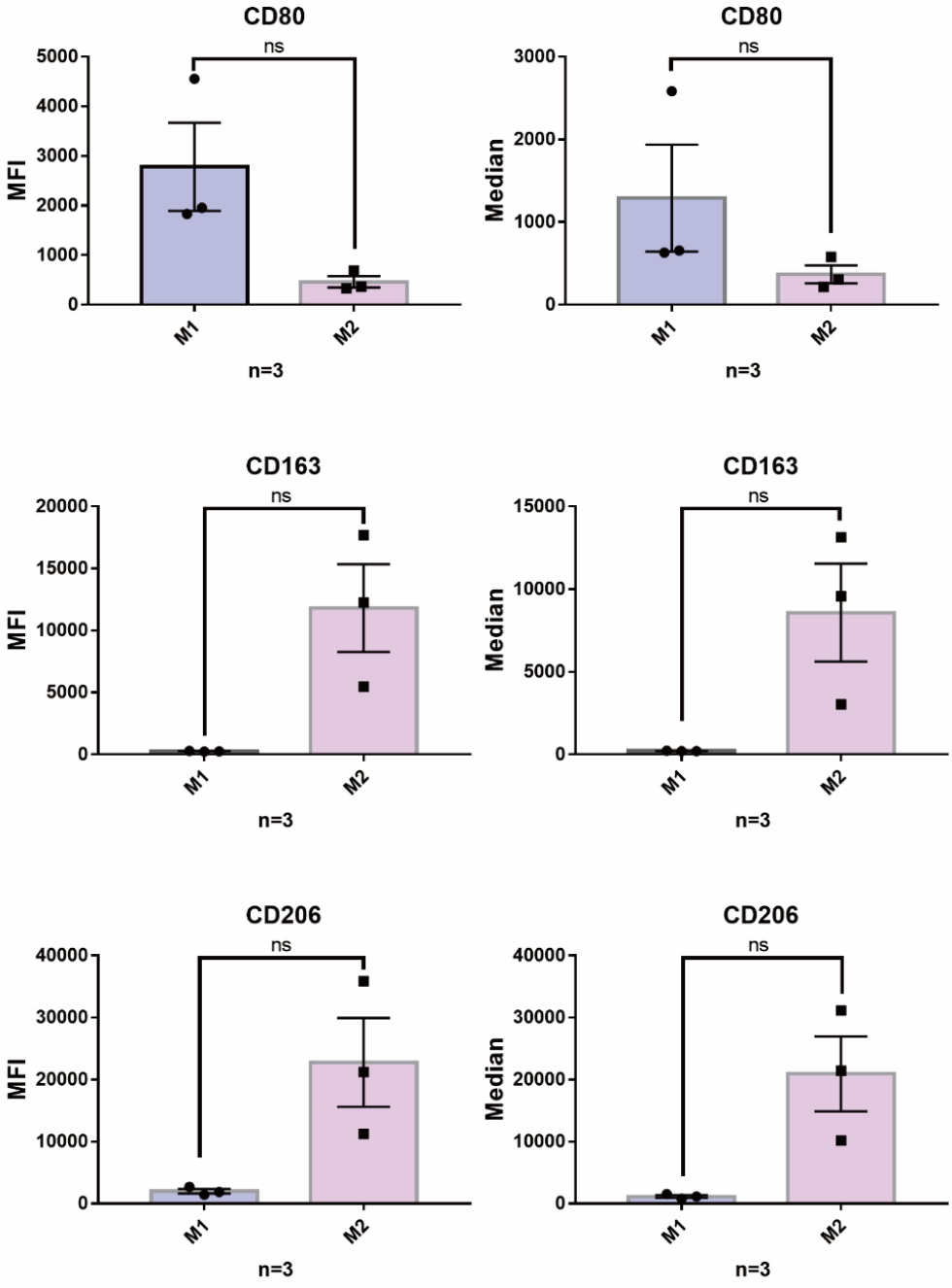
Highly purified Monocytes were seeded into 24-well plates at a density of 2mio cells/well. The suspended cells were then treated with activation and differentiation cytokines according to the protocols outlined in the methods section. Briefly, M1 polarization was induced by IFNg and LPS after an activation period with GM-CSF. For M2 polarization macrophage (Mph) activation was performed with M-CSF, followed by addition of M2- stimulatory cytokine IL-4. After treatments and culture, the cells were harvested on day 10. **Figure 3.2 A** displays a group of example microscopic images of M1 and M2 after polarization on day 10 (10×magnification). As shown in **Figure 3.2 B**, a group of

representative histograms indicates that CD14, as a marker for macrophages, was highly expressed in both M1 and M2 polarization cultures. CD80, one of the specific markers for M1, was highly expressed in IFNg and LPS-activated Mph, while it was weakly expressed in M2 cultures. For M2 polarization the high expression of CD163 and CD206 is considered to be representative. CD163 was poorly expressed in M1 induction cultures but highly expressed in M2 cultures. Both M1 and M2 displayed a CD206 signal to some extent, but only in M2 cultures a significantly positive population could be found, as demonstrated in **Figure 3.2 C**.

In order to determine the cytokine expression variance between M1 and M2, supernatants were harvested on day 10 after addition of a low concentration of LPS at day 9 for the last 24h hours of culture. This step is known to support secretion of all cytokines after a short-term incubation. According to the literature and own previous research M1/M2 specific cytokine secretion was distinguished by ELISA with TNFa and IL-12 being considered representative for M1, while IL-10 being representative for M2- macrophages. As shown in **Figure 3.2 D**, the expression of TNFa was about 6464.1±794.5 pg/ml for M1 versus 1638±521.7 pg/ml in M2, for IL-10: 1932±1293 pg/ml in M2 vs 4.903±4.903pg/ml in M1 (n=3), and the expression of IL-12 was 4127±943.3 pg/ml in M1 (n=3), while IL-12 for M2 were not successfully measured.



C


CD80
ns
MFI
5000
4000
3000
2000
1000
0
M1
M2
n=3
CD80
ns
Median
3000
2000
1000
0
M1
M2
n=3
CD163
ns
MFI
20000
15000
10000
5000
0
M1
M2
n=3
CD163
ns
Median
15000
10000
5000
0
M1
M2
n=3
CD206
ns
MFI
40000
30000
20000
10000
0
M1
M2
n=3
CD206
ns
Median
40000
30000
20000
10000
0
M1
M2
n=3

D

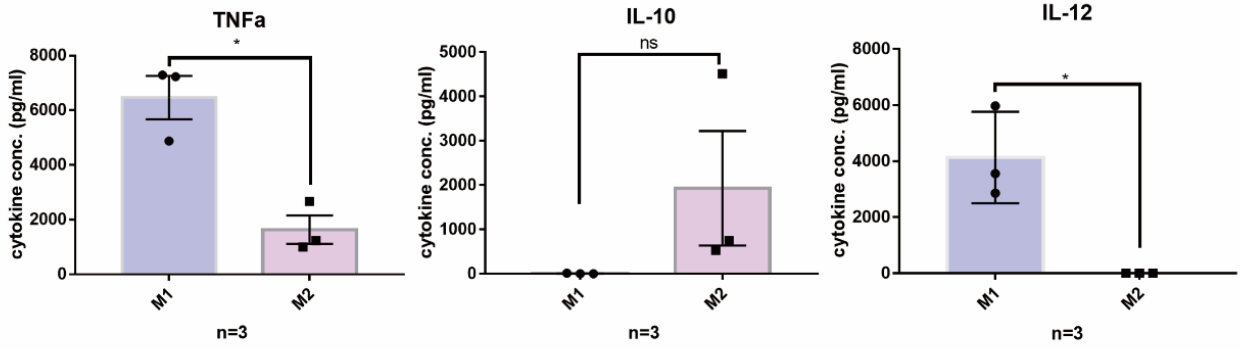

TNFa
*
cytokine conc. (pg/ml)
8000
6000
4000
2000
0
M1
M2
n=3
IL-10
ns
cytokine conc. (pg/ml)
5000
4000
3000
2000
1000
0
M1
M2
n=3
IL-12
*
cytokine conc. (pg/ml)
8000
6000
4000
2000
0
M1
M2
n=3

Figure 3.2: M1/M2 macrophage polarization. (A) Example images of M1 and M2 after polarization on day 10 (10x magnification). **(B)** Expression of surface markers CD14, CD80, CD163, and CD206 without LPS addition on day 9. **(C)** Mean and median fluorescence intensity of CD80, CD163, and CD206 on day 10. **(D)** Cytokine measurements (TNFa, IL-10, and IL-12) from supernatants of M1/M2 with LPS addition on day 9.

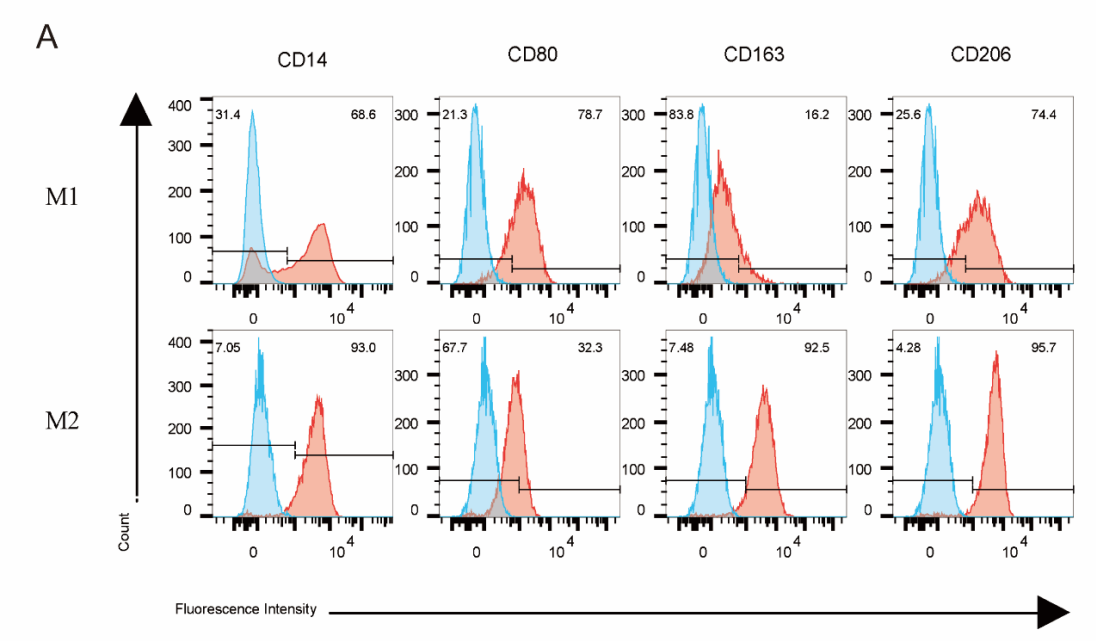
3.1.3 Preparation of pure M1/M2 Supernatants

For functional tests, i.e. later addition of Mph culture supernatant (SN) to fibroblasts (Fib) the Mph SN should not contain any exogenous stimulating or polarizing cytokines anymore ("pure SN") to exclude any potential effects on the Fib. In order to generate pure M1 and M2 supernatants for subsequent experiments, freshly isolated monocytes that passed the purity test were seeded into 24-well plates at a density of 2mio cells/well, and then treated according to the protocol described in the methods section. At day 8 supernatant was completely removed and an additional washing step was performed to remove any remaining traces of cytokines. LPS was added again on day 9 to stimulate cytokine production. On day 10 the now so called "pure" M1/M2 supernatants could be harvested, not containing any exogenous stimulating cytokines any more but the self-produced cytokines that accumulated within the previous 48 hours. To confirm that the M1/M2 phenotype was retained, the surface marker expression profile was re-assessed by flow cytometry. As shown in in a group of representative histograms in **Figure 3.3 A**, CD14 was expressed at rates of 68.6% and 93.0% in M1 and M2, respectively. CD80 was expressed to a slightly higher extent in M2 compared to the first experiment without LPS addition, however still significantly below the M1 polarized macrophages (32% vs 78 %). Likewise CD163, a specific surface marker for M2, was expressed at an extremely high level (92.5%) in M2, while its expression in M1 was only 16.2%. In contrast M1 and M2 both expressed CD206, with positive ratios of 74.4% and 95.7%, respectively, indicating that CD163 and CD206, although described as characteristic M2 marker, might not always be regulated in the same way. **Figure 3.3 B** shows the mean and median fluorescence intensity of 3 experiments. The expression of CD80 for M1 was still higher than that for M2, while CD14, CD163, and CD206 expression was significantly higher in M2 compared to M1. The cytokine production of these pure M1/M2 supernatants is displayed in **Figure 3.3 C**: the expression of IL-12 was not detected in either M1 or M2 supernatants. The concentration of IL-10 in M1 was too low to be measured, while in M2 supernatants it was 1338

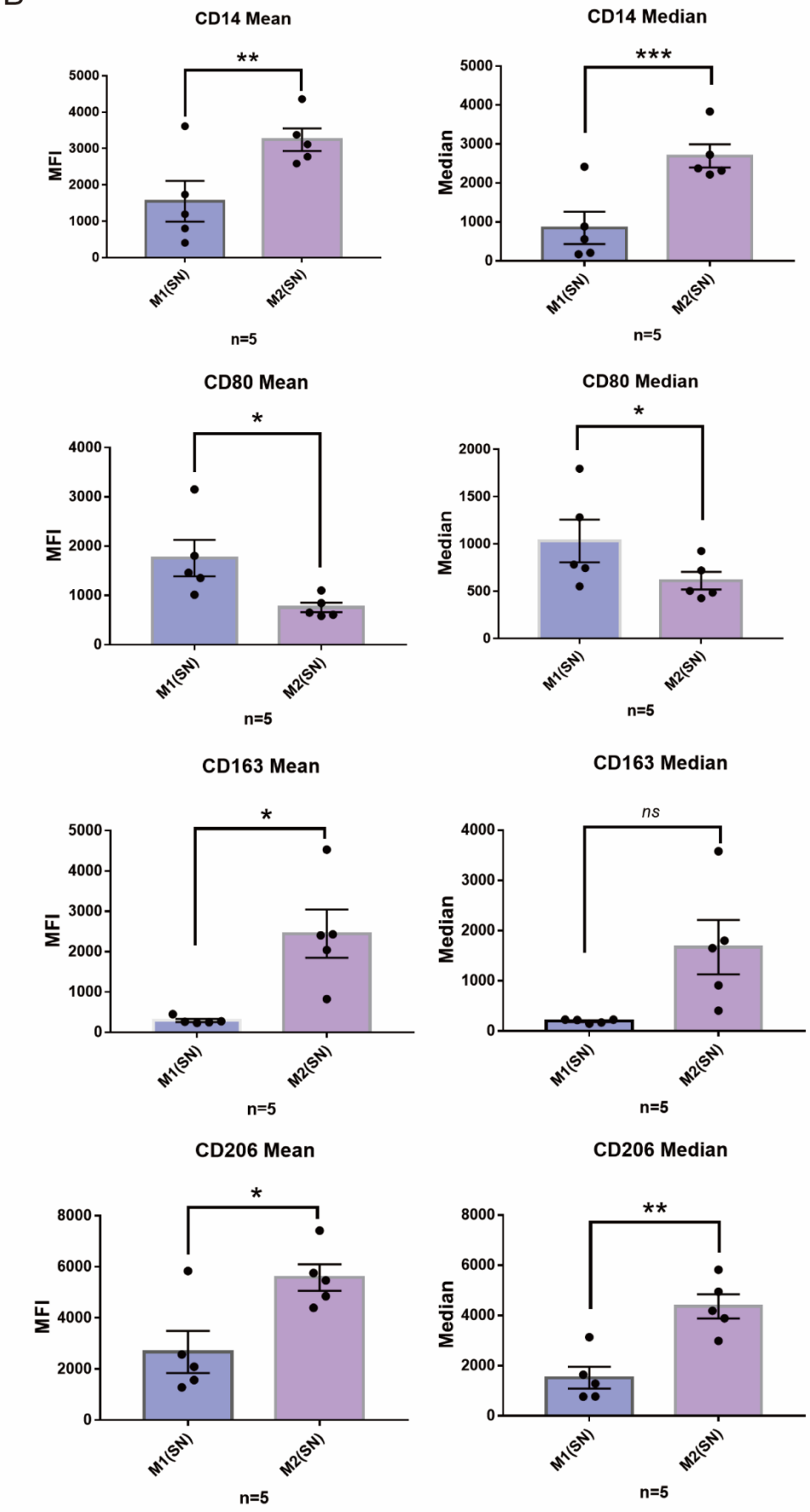
± 198.3 pg/ml. The mean concentration of TNFa in M1 supernatant was 218.2 ± 30.59 pg/ml, while it was 1669 ± 189.7 pg/ml in M2 supernatant. IL-6 was highly expressed in M1 2943 ± 698.1 pg/ml, and the mean concentration for M2 was 1057 ± 146.5 pg/ml.

In summary, adjustment of the culture protocol to generate “pure” SN for functional assays (removal of stimulatory cytokines, washing and readdition of fresh media at day 8, plus addition of LPS at d9, see also M&M) slightly modified the extent of surface marker expression for both M1 and M2 at the time of supernatant harvest but still met the academic consensus, with M1 expressing high levels of CD80 and M2 expressing high levels of CD163 and CD206.

Regarding cytokine secretion IL-12 could not be successfully measured in neither M1 or M2 pure supernatant due to low expression within the last 48 hrs of the experiment. A high amount of IL-10 was expressed by M2 but not in M1 at all. While the amount of TNFa in the M2 SN was not much different from the concentrations before the wash steps the concentration of TNFa in M1 supernatants went below M2. This could be explained by the fact that TNFa is an “early” cytokine, which stands in contrast to the “late” cytokine IL-10, that is known to be upregulated in the later phase of immune responses. The washing step therefore might have affected IL-10 much less than TNFa of which the vast amount was supposed to be already produced during the first days of the polarization phase.



B


CD14 Mean
**
MFI
5000
4000
3000
2000
1000
0
M1(SN)
M2(SN)
n=5
CD14 Median
***
Median
5000
4000
3000
2000
1000
0
M1(SN)
M2(SN)
n=5
CD80 Mean
*
MFI
4000
3000
2000
1000
0
M1(SN)
M2(SN)
n=5
CD80 Median
*
Median
2000
1500
1000
500
0
M1(SN)
M2(SN)
n=5
CD163 Mean
*
MFI
5000
4000
3000
2000
1000
0
M1(SN)
M2(SN)
n=5
CD163 Median
ns
Median
4000
3000
2000
1000
0
M1(SN)
M2(SN)
n=5
CD206 Mean
*
MFI
8000
6000
4000
2000
0
M1(SN)
M2(SN)
n=5
CD206 Median
**
Median
8000
6000
4000
2000
0
M1(SN)
M2(SN)
n=5

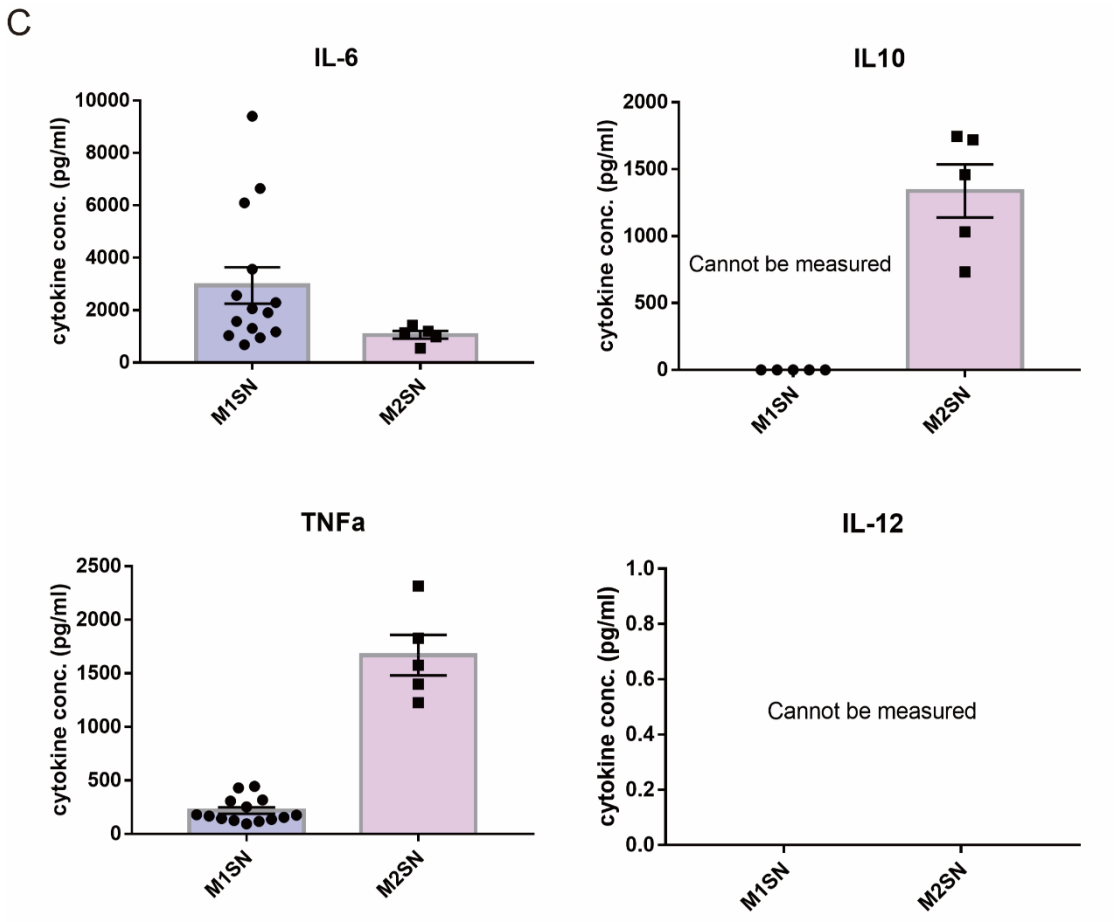


Figure 3.3: Pure M1/M2 supernatant preparation. (A) Expression of surface markers CD14, CD80, CD163, and CD206. **(B)** Mean and median fluorescence intensity of CD14, CD80, CD163, and CD206. **(C)** Cytokine measurements (TNFa, IL6, IL-10, and IL-12) from pure supernatants of M1/M2.

3.2 The Effects of Polarized Macrophages on Synovial Fibroblasts in Vitro

3.2.1 Effects of M1 Macrophage Supernatant on Differently Activated Synovial Fibroblasts in Vitro

Previous results from us and other groups had demonstrated that pre-stimulation of Fibs with TNFa+IL1b for about 3 days induces an inflammatory phenotype similar to the one found in Rheumatoid Arthritis and accompanied by high transcriptional expression of COX-2, induced by proinflammatory cytokines such as TNFa and IL-1ß. Prestimulation with TGFb on the other hand supports conversion into a myofibroblast- like profibrotic phenotype by inducing expression of high

amounts of aSMA and COL-1, as found in fibrotic rheumatic diseases such as Systemic Sclerosis. The aim was to study whether polarized Mph might modify the different activation modes of Fibs, thereby rather supporting or counteracting their pathogenic properties. **Figure 3.4 A** demonstrates the main setup of the experiment. Since M1-SN contains LPS according to the protocol mentioned in the methods section, LPS was proportionally added to the control subgroup together with serum-free fibroblast medium. Fibs with M1-SN was set as experimental subgroup.

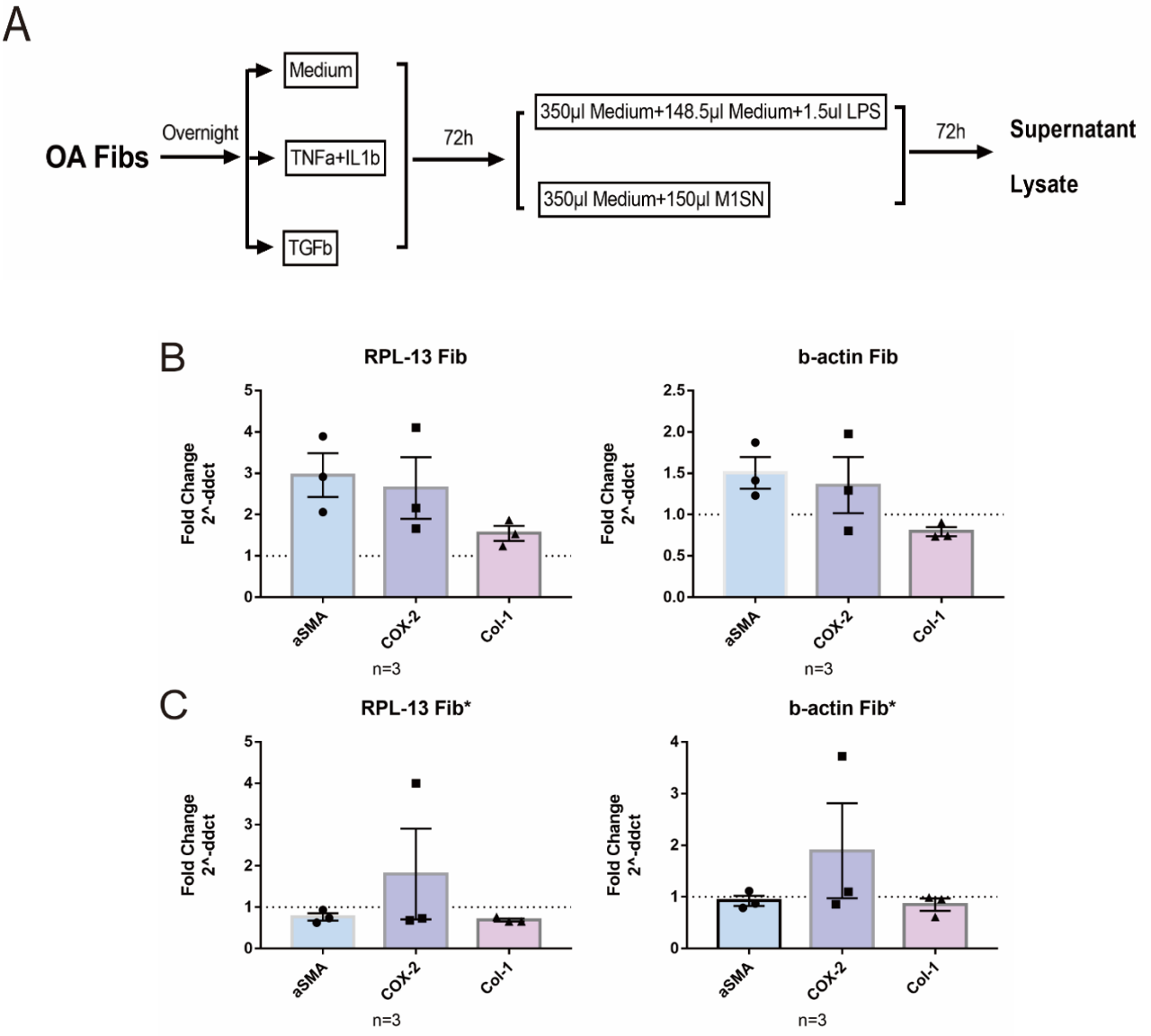
Figure 3.4 B-D shows the transcriptional expression of Fibs inflammatory and fibrotic markers, including COX-2, aSMA and COL-1. Most of the results using both RPL-13 and b-actin as the housekeeping gene (HKG) displayed similar tendencies, aSMA, as a pro-fibrotic marker for Fibs, was slightly increased in the Fib-only group after treatment with M1-SN (mean fold change over 2.5 (RPL-13 as HKG) and 1.5 (b-actin as HKG), respectively).This tendency was more pronounced in Fib^{-}group prestimulated with TGFb ($Fib^{\#}$). where aSMA expression increased significantly in presence of M1-SN to more than 10- (with RPL-13 as HKG) and 5-fold (both with b-actin as HKG), respectively. Likewise COL-1 which is also considered a profibrotic marker was accordingly upregulated by M1-SN in the $Fib^{\#}$ group.

In the Fib group prestimulated with TNFa/IL-1b (Fib*), the change in aSMA or COL-1 expression after M1-SN treatment was not as evident as in the fib only or $Fib^{\#}$ group. COL-1 expression increased in the Fib-only group with mean fold change around 3 (RPL-13 as HKG) and 1.5 (b-actin as HKG) individually after M1-SN treatment, while it was slightly downregulated by M1-SN in the Fib* group.

The expression of the proinflammatory marker COX-2 after addition of M1-SN displayed a variable distribution across the three independent experiments. In the $Fib^{\#}$ group. However, the expression of COX-2 was consistently reduced in all 3 experiments with mean decrease to around 0.8 and 0.4 (RPL-13 and b-actin, respectively) in presence of M1-SN, pointing to a potential opposing trend of the Cox2 and aSMA expression pattern.

Figure 3.4 E demonstrates the cytokine secretion of differently preactivated Fib after co-culture with M1-SN. When cultured alone IL-6 concentration was below 5000 pg/ml in the Fib alone and the $Fib^{\#}$ group and already more than 40.000 pg/ml in the TNFa/IL-1b pre-stimulated group. M1-SN significantly increased both IL-6 and MMP-3 secretion of Fib and $Fib^{\#}$ by almost 10fold approaching the amount of the Fib* cultures.

In summary, synovial Fibs with different stimulations were highly activated by M1-SN resulting in an IL-6 and MMP-3 surge. Regarding gene expression application of M1 SN led to slight upregulation of both aSMA and COL-1 profibrotic gene expression. In Fib$^{\#}$, pre-stimulated with TGFb these pro-fibrotic effects were most emphasized by M1-SN, while COX-2 was hardly influenced.



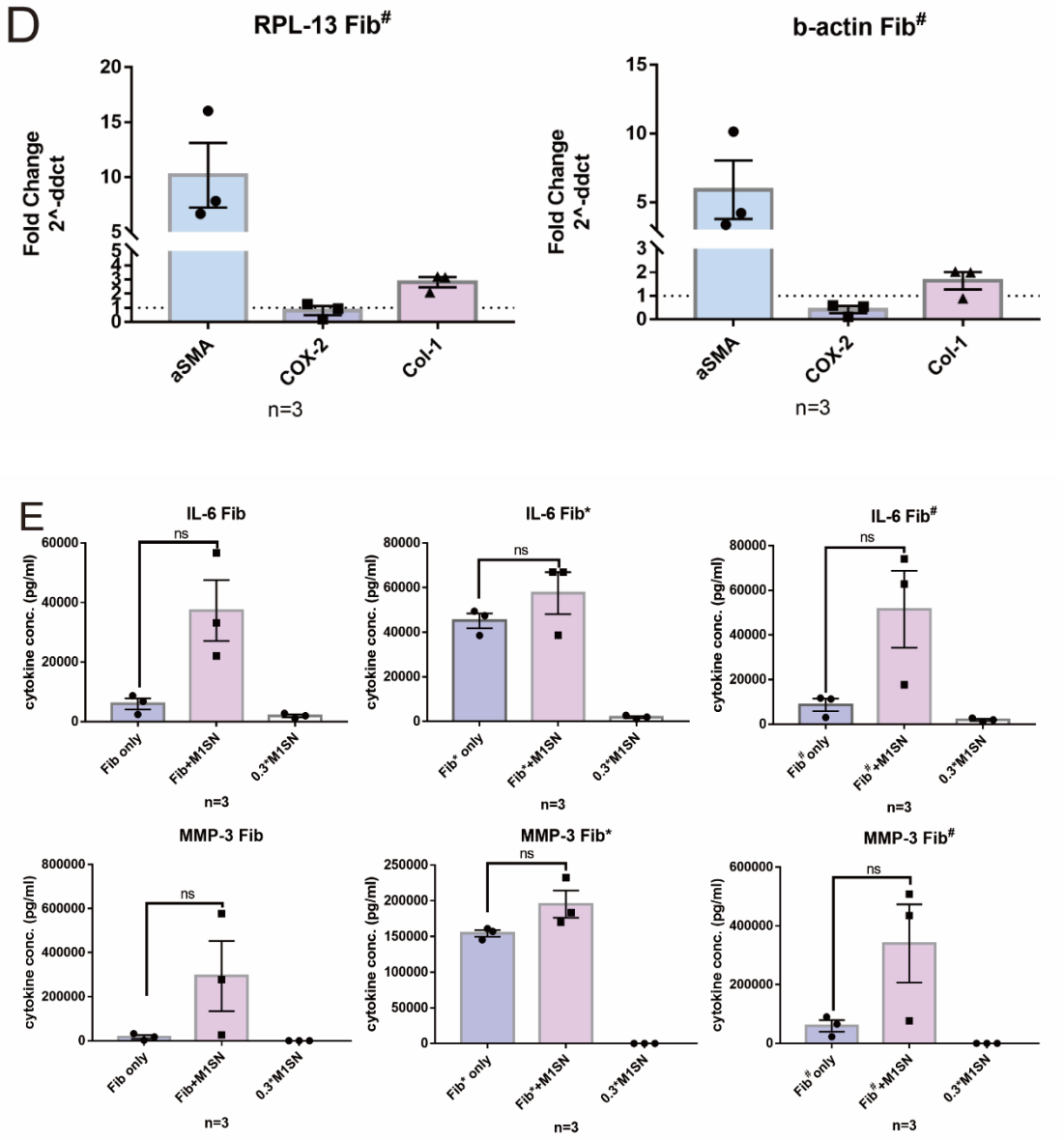
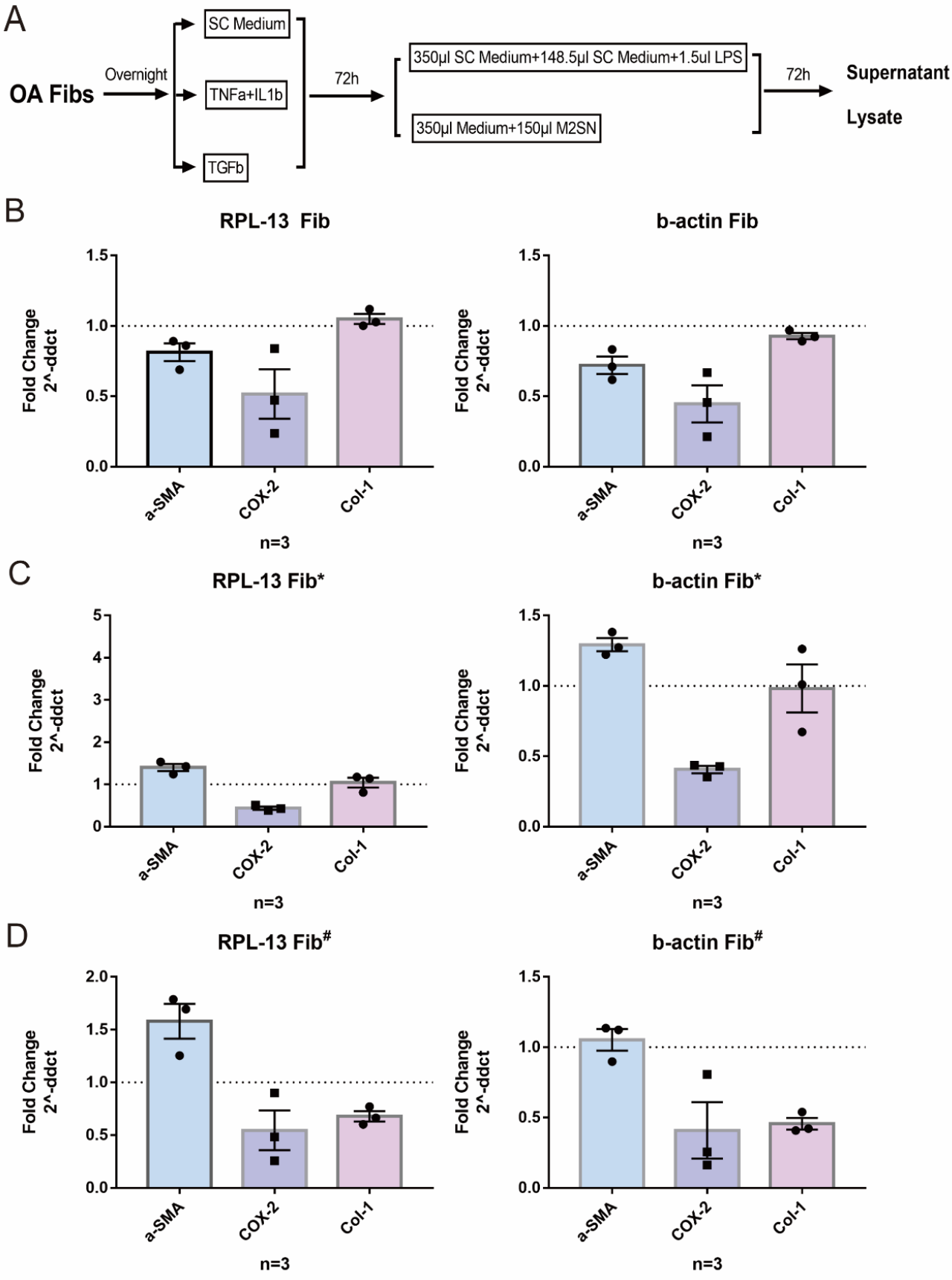


Figure 3.4: Effects of M1 supernatant on differently activated synovial fibroblasts. (A) Group settings and experimental setup. **(B-D)** Transcriptional expression of Fibs proinflammatory and profibrotic markers assessed by qPCR, using RPL-13 and beta-actin as the housekeeping gene. (depicted is the mean ± SEM of n= 3 independent experiments). **(E)** Cytokine expression of IL-6 and MMP-3 measured by ELISA. (depicted is the mean ± SEM of n= 3 independent experiments). P-values of cytokine concentrations were calculated by paired t-test, and results are denoted as follows: ns = no significance, *: P<0.05, **: P<0.01, ***P<0.001.

3.2.2 The Effects of M2 Macrophage Supernatant on Differently Activated Synovial Fibroblasts in Vitro

M2 Mphs are known for their anti-inflammatory properties. In order to investigate the effects of M2 on synovial Fibs in different states, a series of experiments were conducted. **Figure 3.5 A** shows the main experimental setup. As explained in M&M serum-containing fibroblast medium was used throughout the experiments, and pre-stimulation setups were the same as those used in **Part 3.2.1**. The Fibs were divided into three groups - Fib only, Fib*, and Fib$^{\#}$ - to simulate the resting, inflammatory, and fibrotic states of synovial Fibs. Fibs with M2SN were set as the experimental subgroup, and Fibs with medium and LPS were set as the MED control.

Figure 3.5 B-D presents the transcriptional expression of Fibs inflammatory and fibrotic markers - COX-2, aSMA and COL-1. M2SN reduced Cox-2 expression in synovial Fibs in all types of pre-stimulation groups, with the expression of COX-2 significantly reduced after M2SN addition. aSMA and COL-1 were only slightly influenced with aSMA with a mean fold change around 0.81 (RPL-13 HKG) and 0.72 (b-actin as HKG) respectively in the Fib only group, while in the Fib* and Fib$^{\#}$ groups, M2SN slightly increased pro-aSMA expression, but the mean fold change was only less than 1.6 with both RPL-13 and b-actin as HKGs. The expression of COL-1 did not show significant changes with a fold change around 1 with both RPL-13 and b-actin as HKGs in the Fib only group and Fib* group, but it was reduced with a fold change of 0.68 (RPL-13 as HKG) and 0.46 (b-actin as HKG) respectively in the Fib$^{\#}$ group after M2SN addition. **Figure 3.5 E** presents the cytokine secretion after M2SN addition. M2SN had no significant influence on the expression of IL-6 in all groups, with or without pre-stimulation, after M2SN addition, according to the analysis of three experiments. MMP-3 was significantly reduced in the M2SN subgroup of unstimulated Fib (4474 ± 693.3) compared to the MED control (12287 ± 3969 pg/ml), while MMP-3 secretion did not show significant changes after M2SN addition in both Fib* group and Fib$^{\#}$ group. In summary M2-SN has little influence on differently prestimulated synovial Fibs. These results stand in contrast to the experiments with M1-SN, where M1-SN had induced a statistically significant increase of IL-6 in all kind of prestimulated Fib and a significant increase of profibrotic markers.


A
OA Fibs
Overnight
SC Medium
TNFa+IL1b
TGFb
72h
350µl SC Medium+148.5µl SC Medium+1.5ul LPS
350µl Medium+150µl M2SN
72h
Supernatant
Lysate
B
RPL-13 Fib
b-actin Fib
Fold Change 2^-ddct
a-SMA
COX-2
Col-1
n=3
C
RPL-13 Fib*
b-actin Fib*
D
RPL-13 Fib#
b-actin Fib#

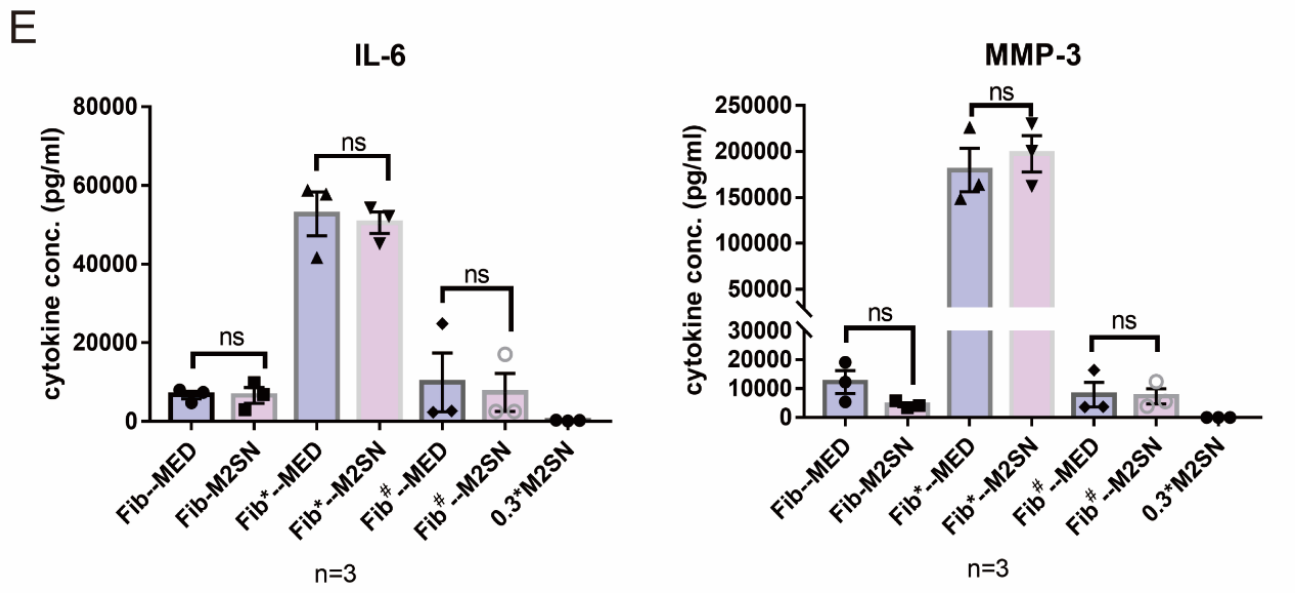


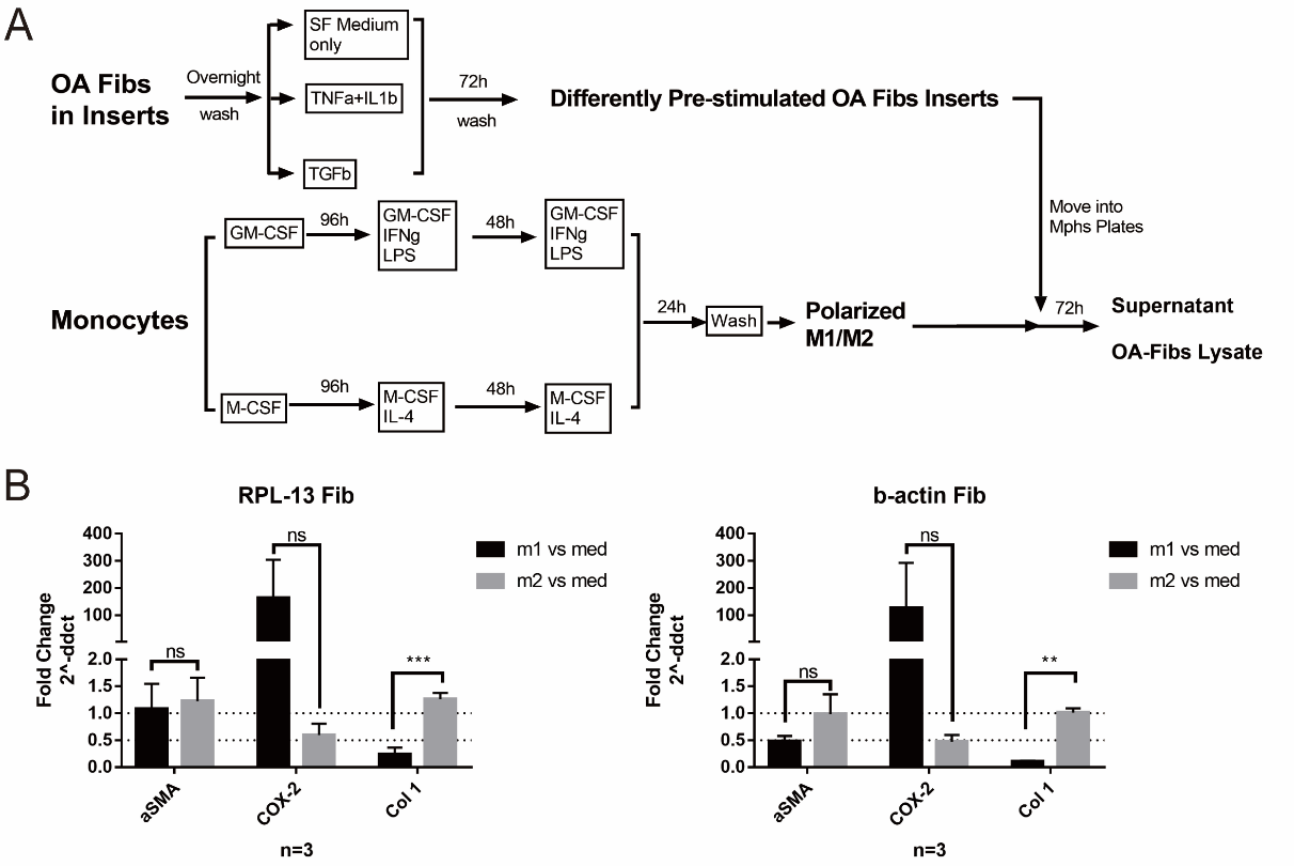
Figure 3.5: Effects of M2 supernatant on differently activated synovial fibroblasts. (A) Group settings and experimental setup. **(B-D)** Transcriptional expression of Fibs inflammatory and fibrotic markers assessed by qPCR, with RPL-13 and beta-actin used as housekeeping genes. The data is presented as the mean ± SEM of n=3 independent experiments. **(E)** Cytokines expression of IL-6 and MMP-3 measured by ELISA, with the data presented as the mean ± SEM of n=3 independent experiments. P-values of cytokine concentrations were calculated by paired t-test, and results are denoted as follows: ns = no significance, *: P<0.05, **: P<0.01, ***P<0.001.

3.2.3 The Effects of M1/M2 Macrophages on Differently Activated Synovial Fibroblasts - in Vitro--Transwell Co-culture Assay

The next experiments aimed to investigate the cell-cell interaction between M1/M2 and synovial Fibs using Transwell co-culture assay, following previous experiments that examined the effects of polarized M1/M2 supernatants on differently activated synovial Fibs. **Figure 3.6 A** shows the experimental setup, where synovial Fibs were seeded into inserts of 24-well plates at a density of 25,000 cells/insert and pre-treated with medium only (Fib), TNFa+IL1b (Fib*) and TGFb (Fib$^{\#}$) respectively for 72 hours after changing to serum-free medium. After washing with PBS, the inserts were transferred to M1 or M2 wells, and the cells lysate and co-culture supernatant were harvested 72 hours later.

Figure 3.6 B-D displays the transcriptional expression of three important markers, aSMA, COX-2 and COL-1, with RPL-13 and b-actin used as housekeeping genes. The expression of COX-2 was highly upregulated in all pre-stimulation groups after M1 co-culture, while COL-1 was significantly reduced with fold change<0.5 after M1 co-culture. aSMA was downregulated in almost all pre-stimulation

groups with a fold change less than 1 after M1 co-culture, except for the results of Fib only group with RPL-13 as the housekeeping gene. The expression of COL-1 was slightly upregulated by M2 co-culture in each group. In Fib only and Fib# groups, M2 co-culture downregulated COX-2 expression, while it increased in Fib* group. The expression of aSMA was slightly upregulated by M2SN in both Fib only and Fib# groups, but it was reduced slightly in the Fib* group. Interestingly the effects of the Mph- SN appear to be very stable since they were independent of the different prestimulatory conditions of the Fibs. Taken together these results stand in sharp contrast to the previous results with M1-SN and M2- SN. One reason might be the mutual influence of both cell populations via soluble factors. The cytokine secretion profile of the cocultures, however resembled closely the results from the experiments with application of M1 and M2 SN: (**Figure 3.6 E**). In Fib only and Fib# groups, both IL-6 and MMP-3 expression were significantly upregulated after M1 co-culture, and IL-6 expression increased slightly after M2 co-culture, while MMP-3 expression was not evidently changed after M2 co-culture. In the Fib* group, IL-6 and MMP-3 expression increased as well after M1 co-culture, and they were slightly downregulated after M2 co-culture.



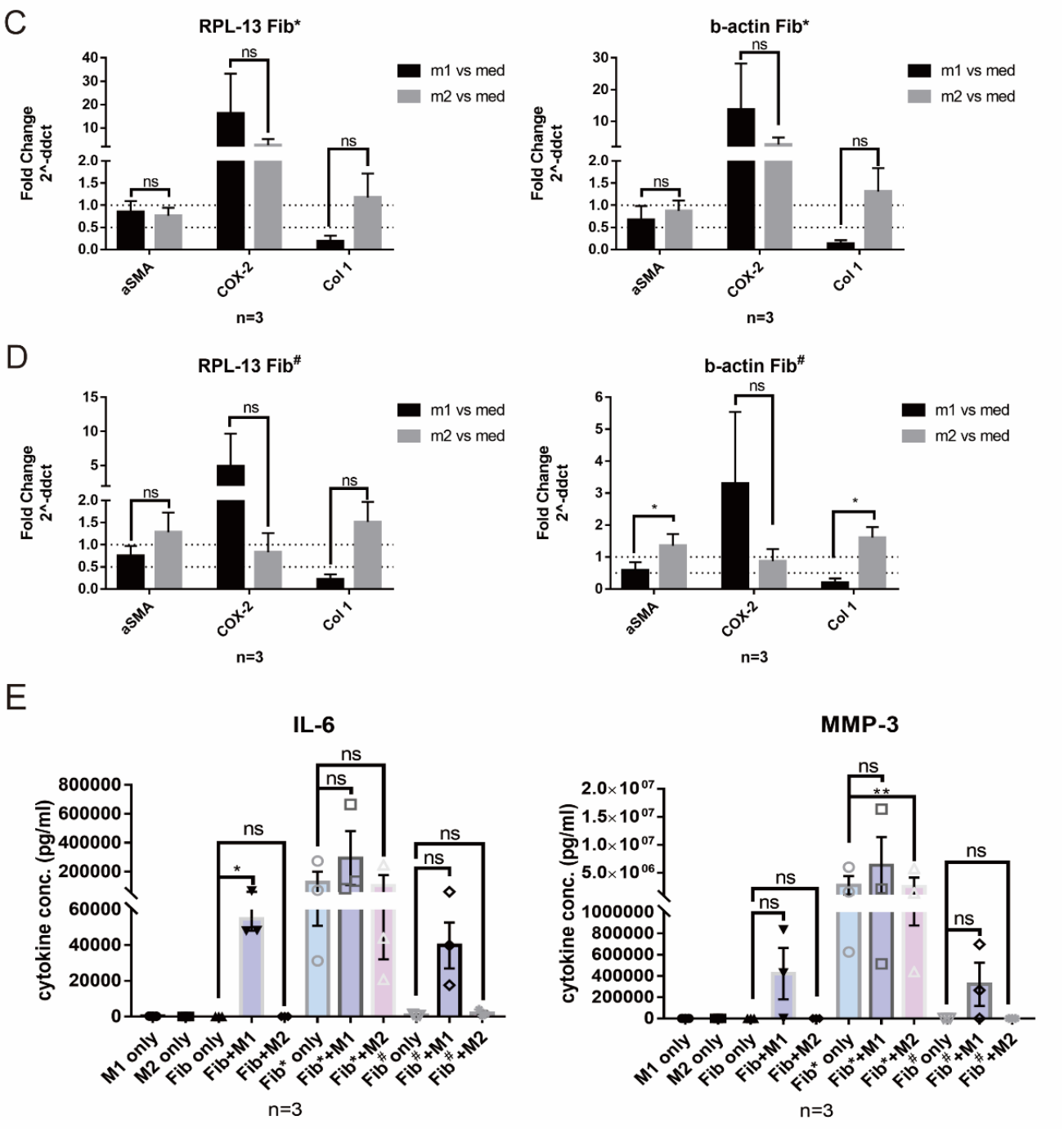


Figure 3.6: Effects of M1/M2 on differently activated synovial fibroblasts—Transwell Co-culture. **(A)** Group settings and experimental setup. **(B-D)** Transcriptional expression of Fibs inflammatory and fibrotic markers assessed by qPCR, with RPL-13 and beta-actin used as housekeeping genes. The data is presented as the mean ± SEM of n=3 independent experiments. **(E)** Cytokines expression of IL-6 and MMP-3 measured by ELISA, with the data presented as the mean ± SEM of n=3 independent experiments. P-values of fold change measured via qPCR and cytokine concentrations were calculated by paired t-test, and results are denoted as follows: ns = no significance, *: P<0.05, **: P<0.01, ***P<0.001.

3.2.4 The Effects of M1 Supernatant (M1-SN) on Resting Synovial Fibroblasts in Vitro—Time Curves

The transcriptional expression of aSMA, COL-1 and Cox-2 in fib cocultured with M1 in the Transwell co-culture assay were found to be rather opposite to those of culture with the M1-SN. The cytokines however were displaying a similar tendency in both SN- and coculture experiments. While the secreted IL-6 and MMP-3 represent their accumulation over 3 days, gene expression is variable over time following stimulation, thereby always representing a “snapshot” of the current time point. Therefore experiments with collection of RNA at different time points of continuous stimulation were conducted, as shown in **Figure 3.7 A**. As described in the methods section, synovial Fibs were seeded into 24-well plates at a density of 25,000/well. After media changing and 3 days culture as usual, samples were harvested at various time points of 12h, 24h, 48h, and 72h. Medium refreshing and cells stimulation were conducted at the same time in all settings.

Figure 3.7 B-D displays the transcriptional expression of aSMA, COX-2 and COL-1, with fold changes compared to the Medium control groups at time points 12h, 24h, 48h, and 72h after stimulation with M1-SN. Based on the analysis of three independent experiments, the trends in the expression of aSMA and COL-1 were similar. Initially, from the start of the experiment until 48h, their expression showed no change or rather a downward trend. However, their expression significantly increased from 48h to 72h. In contrast, COX-2 expression exhibited an opposite trend compared to aSMA and COL-1. It was highly upregulated during the first 12 hours, followed by a rapid decrease from time point 12h to 48h. After this, the trend slowed down, and even showed a slight increase. At the time point of 72h the gene expression pattern finally resembled the results as seen before. **Figure 3.7 E** displays the cytokine concentration at each time point of sample harvest. As expected, since M1-SN was added to the experimental group, IL-6 significantly increased, followed by a gradual slowing down of the trend. As for MMP-3 expression, it maintained a stable upward trend. However, from the time point of 48h, there was a sign of easing up.

Taken together, IL-6 and MMP-3 secretion is inversely correlated with COX-2 expression, and positively correlated with SMA and COL-1 expression. The pro-fibrotic effects of M1-SN on resting synovial Fibs with aSMA and COL-1 expression underwent a process of an initial slight decrease

followed by a rapid increase.

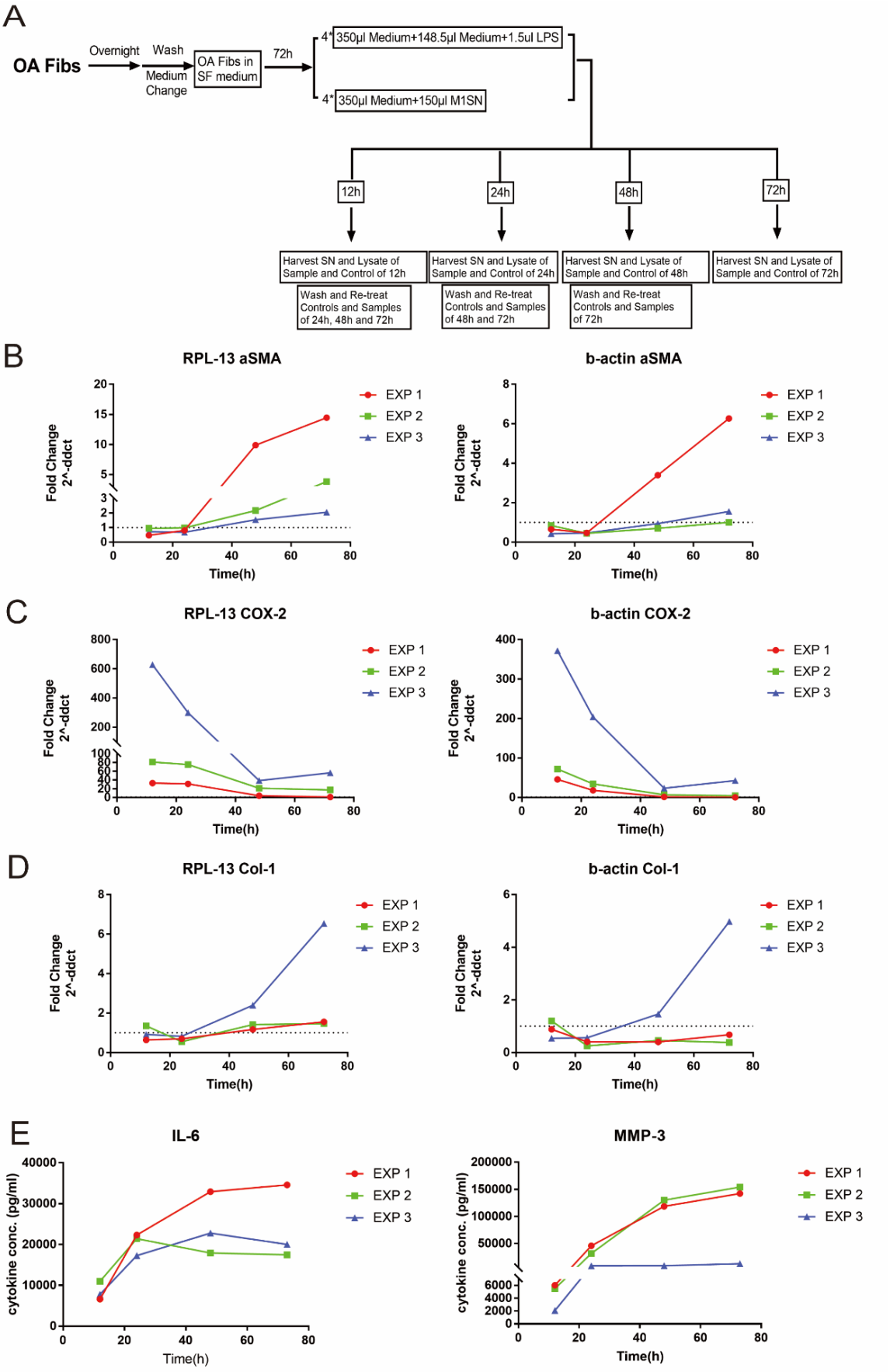


Figure 3.7: Effects of M1 Supernatant on resting synovial fibroblasts—Time Curves. (A) Group settings and experimental setup. **(B-D)** Time curves of the transcriptional expression of Fibs inflammatory and fibrotic markers, which were assessed by qPCR with fold changes compared to the Medium control group. RPL-13 and beta-actin were used as housekeeping genes. The figure depicts the mean ± SEM of n=3 independent experiments. **(E)** Cytokine expression of IL-6 and MMP-3 measured by ELISA. The figure depicts the mean ± SEM of n=3 independent experiments.

3.2.5 The Effects of Differently Polarized Macrophages (M1/M2) on M1 pre-treated OA-Fibs in Vitro—Transwell Co-culture Assay

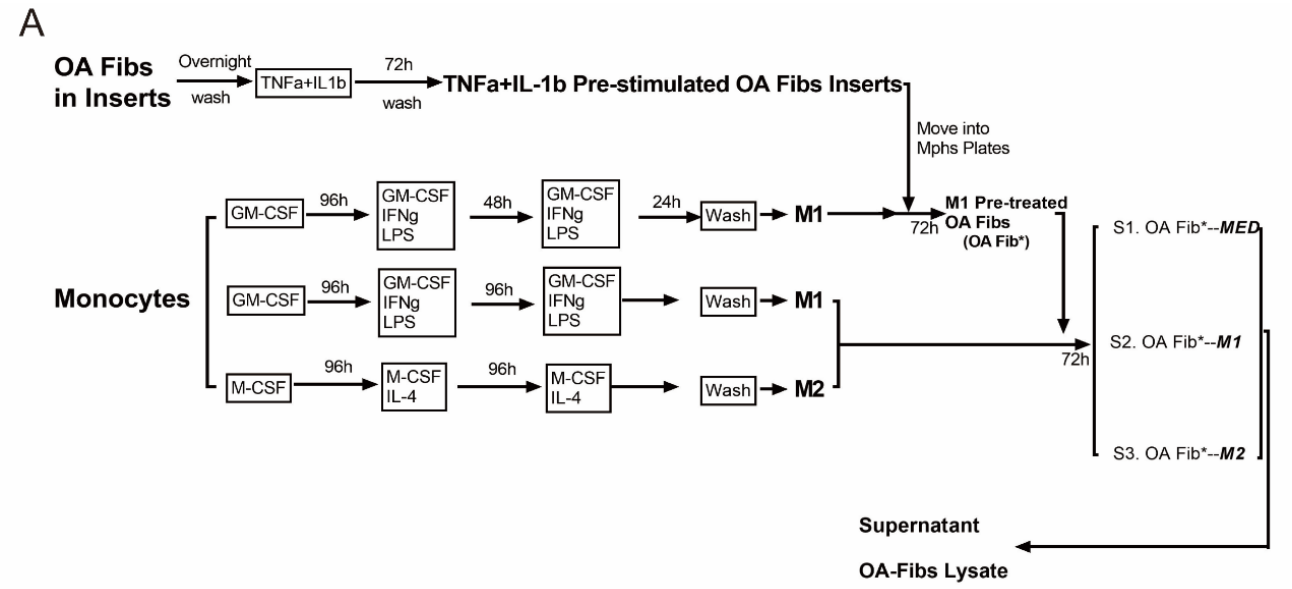
To explore the effects of M1 and M2 on synovial Fibs pre-treated with TNFa+IL1b and M1, a single trial was designed and conducted, as shown in **Figure 3.8 A**. As described in the methods section, synovial Fibs pre-stimulated with TNFa+IL1b in 3 inserts were moved into M1 Mphs for a 3-day co-culture. Then, the Transwell inserts were moved into new medium, new M1, and new M2, respectively, for another 3-day co-culture. The lysate and supernatant samples were harvested on the 13th day of the whole experiment.

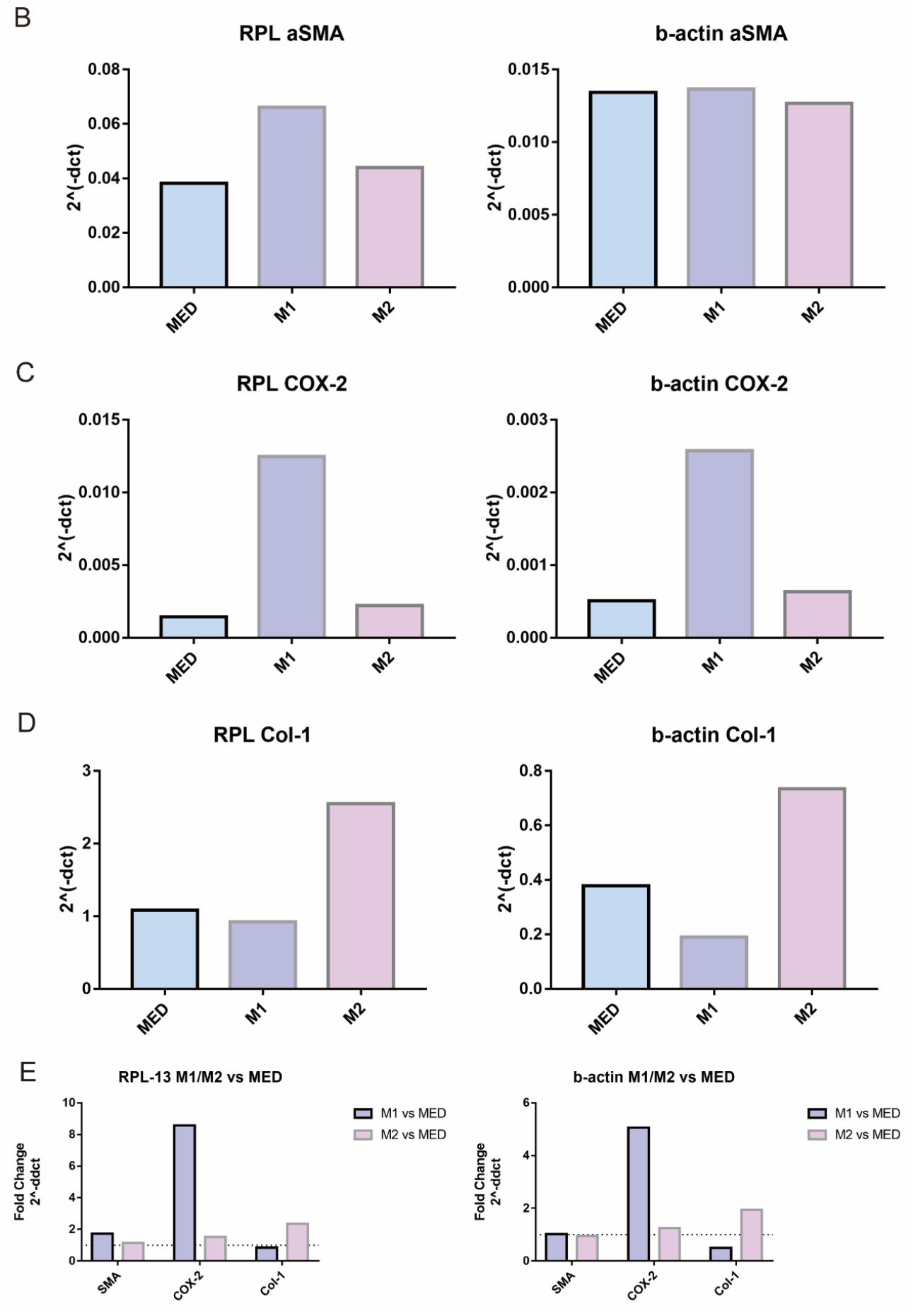
Figures 3.8 B-D display the transcriptional expression calculated by 2-dCt, and **Figures 3.8 E** show the fold change of it. In the M1 co-culture group, COX-2 expression was definitely upregulated with a fold change of 8.5 (RPL-13 as HKG) and 5.1 (beta-actin as HKG) compared to the Medium control group. Interestingly, aSMA expression was also slightly upregulated with a fold change of 1.73 (RPL-13 as HKG) and 1.02 (beta-actin as HKG) after another 3 days of M1 co-culture, which is the opposite result of the Transwell co-culture assay in **Part 3.2.3**. COL-1 expression was reduced with a fold change of 0.9 (RPL-13 as HKG) and 0.5 (beta-actin as HKG) after another 3 days of M1 co-culture. In the M2 group, M2 co-culture upregulated COL-1 expression with a fold change of 2.36 (RPL-13 as HKG) and 1.94 (beta-actin as HKG), and COX-2 expression was also upregulated a little bit with a fold change of 1.52 (RPL-13 as HKG) and 1.25 (beta-actin as HKG), while aSMA did not evidently change after another 3 days of M2 co-culture.

Figure 3.8 F demonstrates cytokine expression of IL-6 and MMP-3. M1 co-culture significantly increased both IL-6 (13814.8 pg/ml vs. 2390.8 pg/ml) and MMP-3 (883313 pg/ml vs. 526330 pg/ml) expression. M2 co-culture also evidently upregulated MMP-3 (811945 pg/ml vs. 526330 pg/ml), while

it only slightly upregulated IL-6 expression (3225.7 pg/ml vs. 2390.8 pg/ml).

In summary, the expression of COX-2 and COL-1 was not evidently changed after another 3 days of M1 or M2 co-culture compared to the results of the 3-day Transwell co-culture assay in **Part 3.2.3**. Surprisingly, the expression of aSMA was upregulated after another 3 days of M1 co-culture. The difference between M1-SN co-culture (**Part 3.2.1**) and M1 Transwell co-culture (**Part 3.2.3**) is that synovial Fibs had reactions with M1-SN immediately after M1-SN addition because IL-6, TNFa, and some other M1-secreted cytokines were already present in M1-SN. However, M1 inserts in the Transwell co-culture assay required more time to gradually secrete these cytokines. The longer period of M1 co-culture improved aSMA expression. However, this was a single trial, so the results of the experiment might not be strictly stable. In terms of this experiment




B
RPL aSMA
2^(-dct)
0.08
0.06
0.04
0.02
0.00
MED
M1
M2
b-actin aSMA
0.015
0.010
0.005
0.000
C
RPL COX-2
b-actin COX-2
0.003
0.002
0.001
D
RPL Col-1
b-actin Col-1
3
2
1
0
0.8
0.6
0.4
0.2
0.0
E
RPL-13 M1/M2 vs MED
b-actin M1/M2 vs MED
Fold Change
2^-ddct
M1 vs MED
M2 vs MED
10
8
6
4
2
SMA
COX-2
Col-1

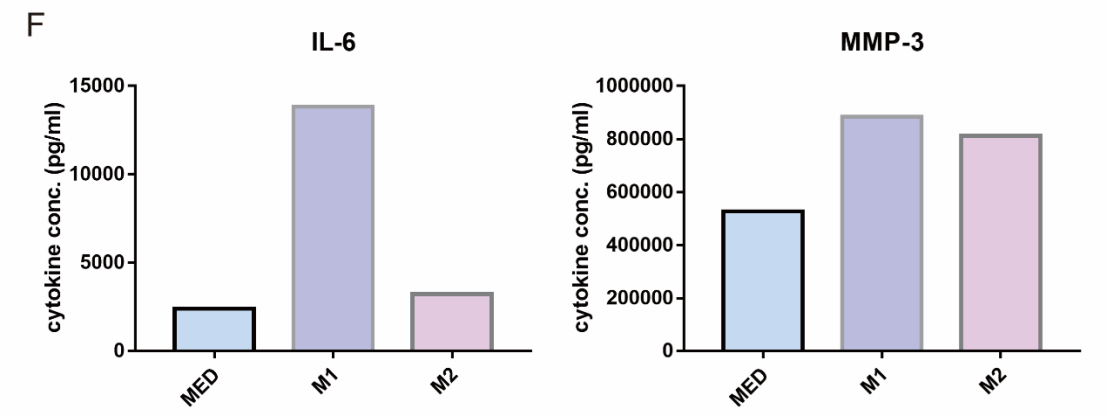


Figure 3.8: Effects of M1 Supernatant on resting synovial fibroblasts—Long period of Transwell co-culture. (A) Group settings and experimental setup. **(B-D)** Transcriptional expression assessed by qPCR with aSMA, COX-2 and COL-1 calculated by 2-dCt. **(E)** Transcriptional expression of aSMA, COX-2 and COL-1 with fold changes compared to the control group. **(F)** Cytokines expression of IL-6 and MMP-3 measured by ELISA.

3.2.6 Janus kinase inhibitors (JAKi) Inhibited Pro-inflammatory and Pro-Fibrotic Effects of M1 Supernatant on Synovial Fibroblasts

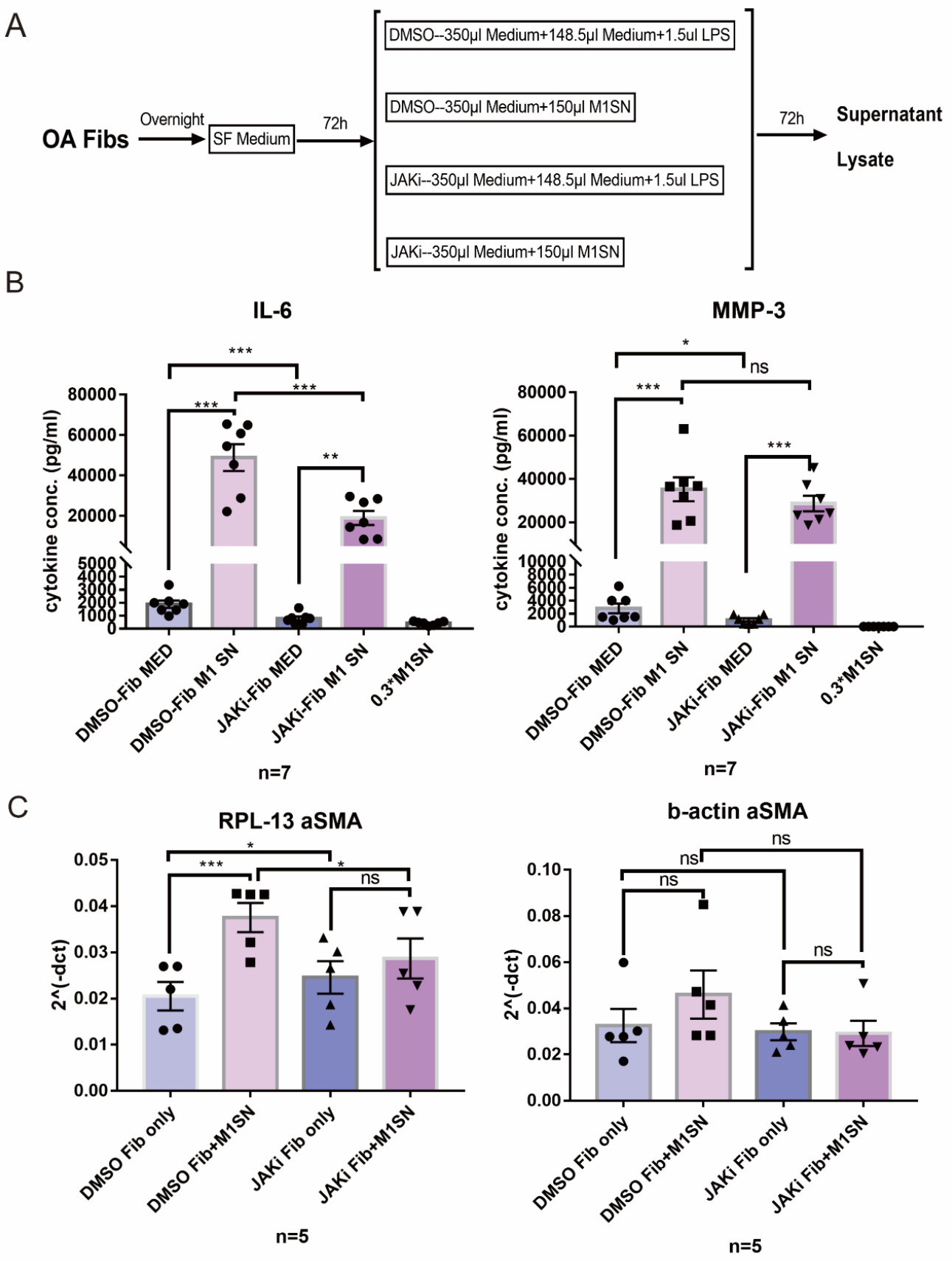
As demonstrated in **Part Fig 3.7,** M1-SN led to early upregulation of pro-inflammatory transcription factor Cox-2 and later upregulation of the pro-fibrotic transcription factors aSMA and COL-1 in synovial Fibs, with a surge of IL-6 being one of the characteristic features of the experiments. The significant roles of IL-6 (Li et al. 2022) in promoting both pro-inflammatory and pro-fibrotic effects have been supported by numerous studies. To further investigate the underlying mechanisms of M1 effects on synovial Fibs and to determine whether these effects are related to the JAK/STAT signaling pathway, experiments were conducted under use of the selective JAK-inhibitor Upadacitinib, that preferentially blocks JAK1 signaling, thereby inhibiting IL-6 induced STAT-3 phosphorylation. **Figure 3.9 A** illustrates the experimental setup and group allocation. As described in the methods section, synovial Fibs were seeded into 24-well plates for overnight culture, and the medium was subsequently changed to serum-free medium. After 72 hours culture, the cells were treated respectively**.** Three days later, the lysate and supernatant were collected for subsequent measurement.

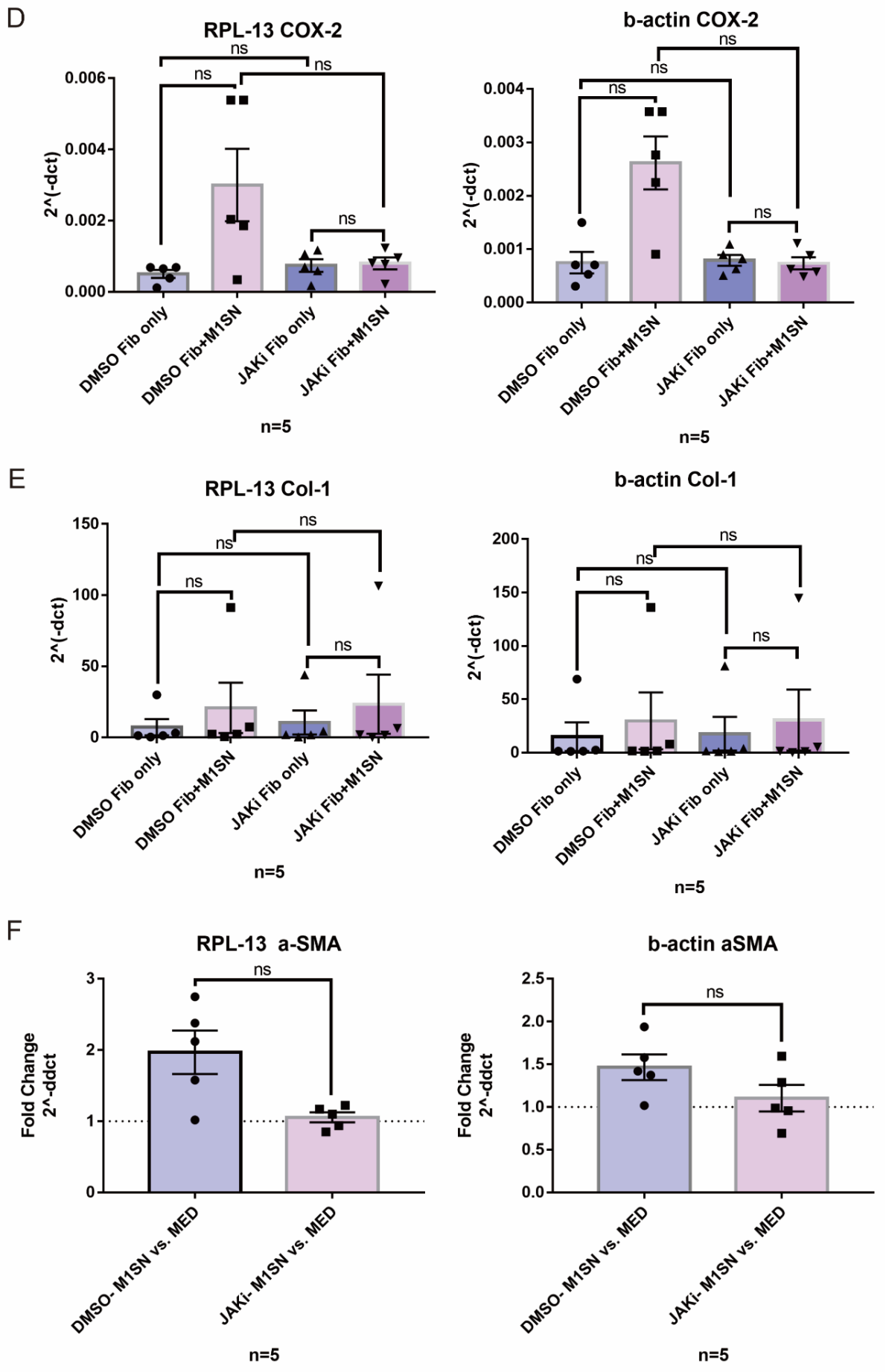
As shown in **Figure 3.9 B**, the expression levels of IL-6 and MMP-3 were assessed for each

experimental group. The levels of IL-6 and MMP-3 in the DMSO-Fib M1-SN group were approximately 26 and 25 times higher, respectively, than those in the DMSO-Fib MED control group. However, the expression levels were significantly reduced in both JAKi-M1-SN group and JAKi-Fib MED control group.

Figures 3.9 C-E illustrate the transcriptional expression levels of aSMA, COX-2 and COL-1 using RPL-13 and beta-actin as the housekeeping genes and calculated by the 2-dCt method. Meanwhile, **Figures 3.9 F-H** display the corresponding fold changes calculated per 2-ddCt. In the DMSO Fib control group, the expression level of aSMA was upregulated with a mean fold change of 1.97 (using RPL-13 as HKG) and 1.47 (using beta-actin as HKG), while the fold change significantly decreased to 1.06 and 1.10, respectively, in the presence of JAKi thereby almost representing the state without M1-SN. The expression level of COX-2 was significantly increased after M1-SN co-culture with a mean fold change of 4.98 (using RPL-13 as HKG) and 4.58 (using beta-actin as HKG) in the DMSO group, but decreased sharply to 1.04 and 1.03, respectively, in the JAKi groups. Similarly, the expression level of COL-1 was upregulated after M1-SN co-culture, with a fold change of 2.12 (using RPL-13 as HKG) and 1.85 (using beta-actin as HKG), but reduced to 1.15 and 1.06, respectively.

Taken together, the results indicate that the M1 Mph supernatant has both pro-inflammatory and pro-fibrotic effects on synovial Fibs, but these effects were significantly suppressed in the presence of JAKi. Moreover, the JAK signaling pathway and IL-6 were identified as key mechanisms involved in this process.


A
OA Fibs
Overnight
SF Medium
72h
DMSO--350µl Medium+148.5µl Medium+1.5ul LPS
DMSO--350µl Medium+150µl M1SN
JAKi--350µl Medium+148.5µl Medium+1.5ul LPS
JAKi--350µl Medium+150µl M1SN
72h
Supernatant
Lysate
B
IL-6
cytokine conc. (pg/ml)
DMSO-Fib MED
DMSO-Fib M1 SN
JAKi-Fib MED
JAKi-Fib M1 SN
0.3*M1SN
n=7
MMP-3
cytokine conc. (pg/ml)
DMSO-Fib MED
DMSO-Fib M1 SN
JAKi-Fib MED
JAKi-Fib M1 SN
0.3*M1SN
n=7
C
RPL-13 aSMA
2^(-dct)
DMSO Fib only
DMSO Fib+M1SN
JAKi Fib only
JAKi Fib+M1SN
n=5
b-actin aSMA
2^(-dct)
DMSO Fib only
DMSO Fib+M1SN
JAKi Fib only
JAKi Fib+M1SN
n=5


D
RPL-13 COX-2
b-actin COX-2
2^(-dct)
ns
DMSO Fib only
DMSO Fib+M1SN
JAKi Fib only
JAKi Fib+M1SN
n=5
E
RPL-13 Col-1
b-actin Col-1
F
RPL-13 a-SMA
b-actin aSMA
Fold Change 2^-ddct
DMSO- M1SN vs. MED
JAKi- M1SN vs. MED

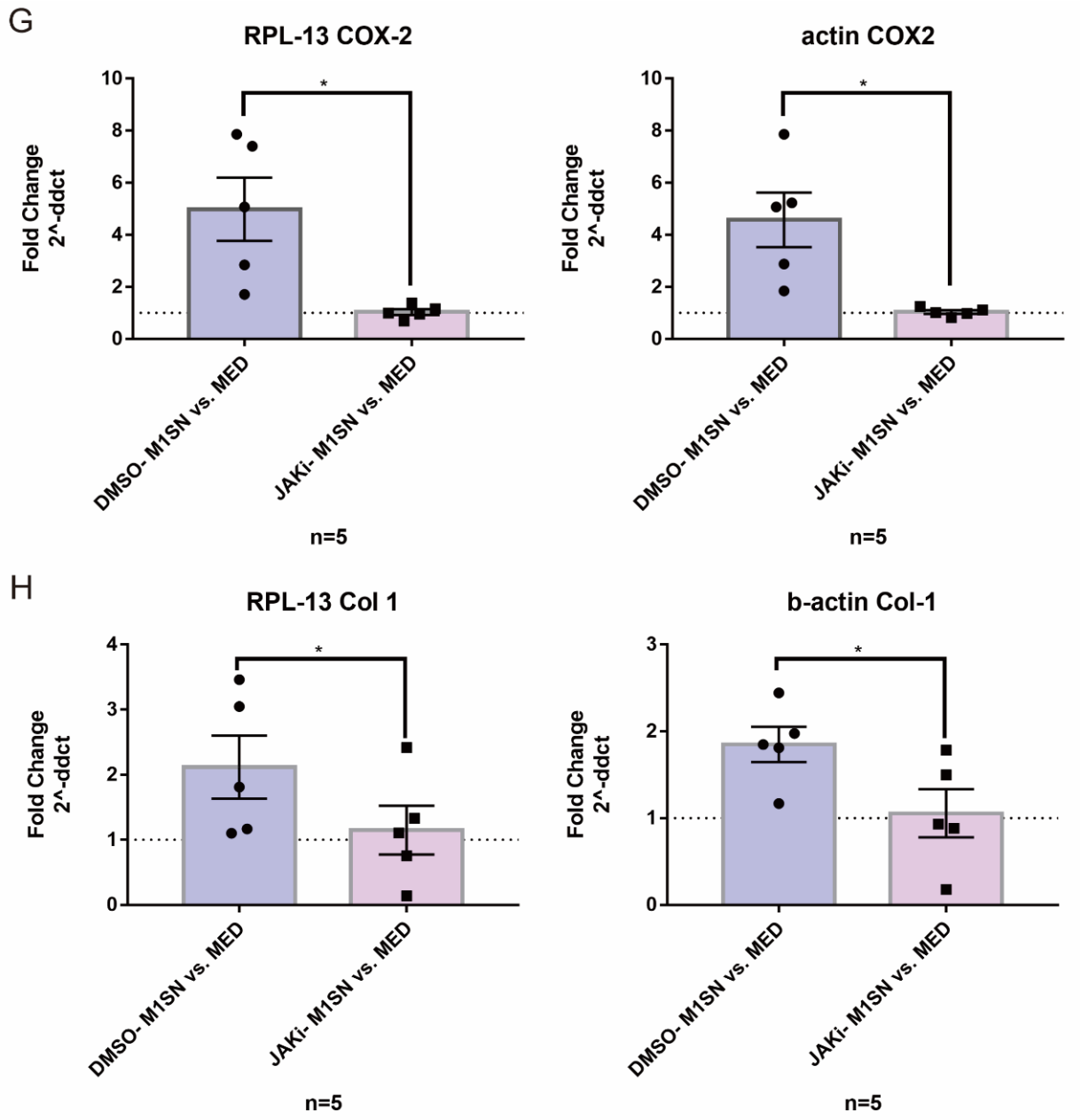
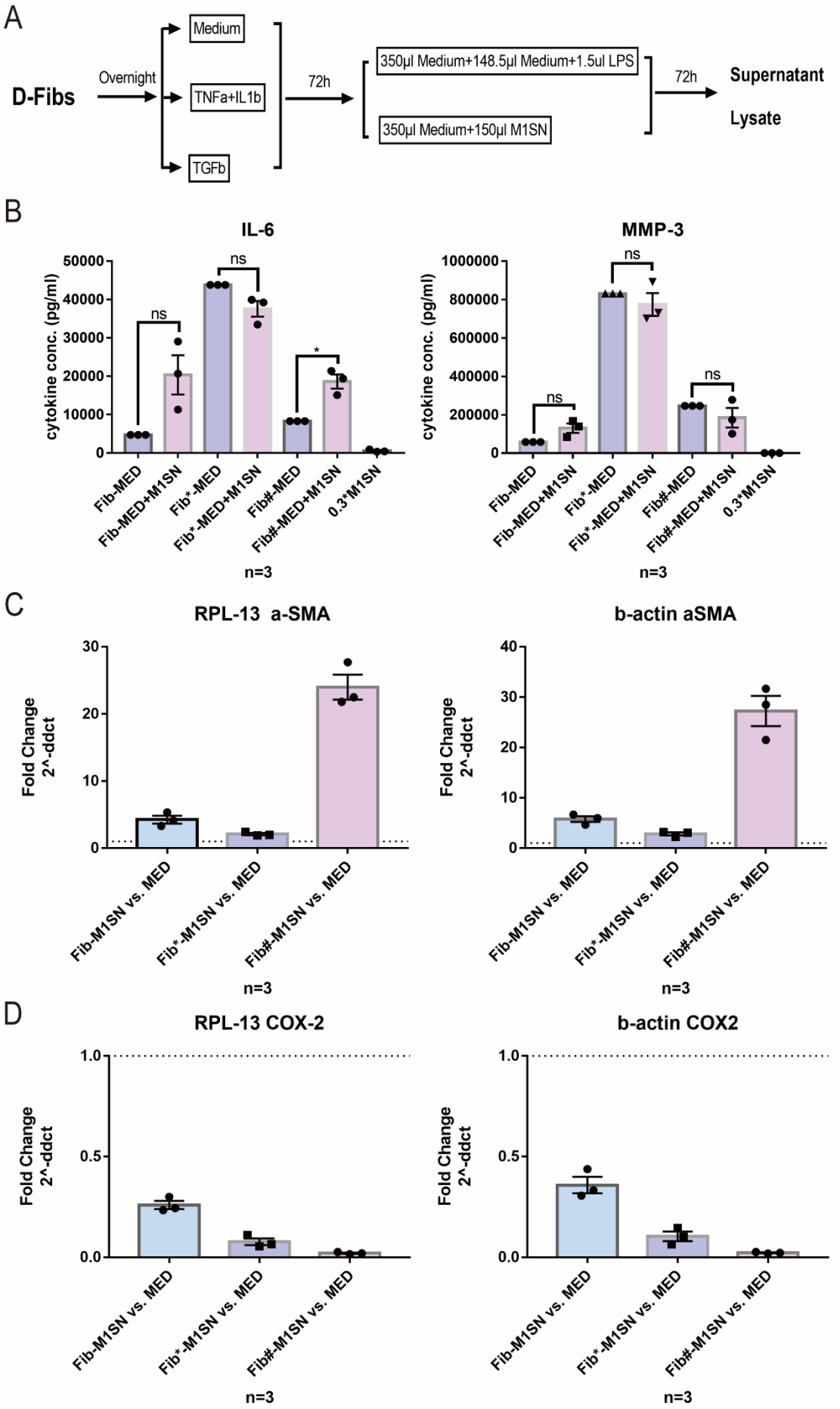


Figure 3.9: JAKi Inhibited Pro-inflammatory and Pro-Fibrotic Effects of M1 Supernatant on Synovial Fibroblasts. (A) Experimental setup and groups setting. **(B)** Cytokines expression of IL-6 and MMP-3 measured by ELISA. (depicted is the mean ± SEM of n=3 independent experiments). **(C-E)** Transcriptional expression calculated with 2-dCt of Fibs inflammatory and fibrotic markers assessed by qPCR. RPL-13 and beta-actin were used as house-keeping gene. (depicted is the mean ± SEM of n=3 independent experiments). **(F-H)** Fold change of transcriptional expression. (depicted is the mean ± SEM of n=3 independent donors per group). P-values were calculated by Paired t test; ns = no significance, *: P<0.05, **: P<0.01, ***P<0.001.

3.2.7 The Effects of M1 Macrophages Supernatant on Differently Activated Dermal Fibroblasts

Previous parts of the project have shown the effects of M1-SN on synovial Fibs, and this part focuses on exploring the interaction between M1-SN and dermal Fibs. **Figures 3.10 A** illustrate the experimental setup and group allocation. The same protocol as in **Part 3.2.1** were utilized. **Figure 3.10 B** shows the expression of IL-6 and MMP-3. The expression of IL-6 was significantly upregulated by M1-SN in the Fib only and Fib$^{\#}$ groups, while it was slightly downregulated compared to the MED control in the Fib* group. Regarding MMP-3 expression, it was significantly upregulated after M1-SN co-culture in the Fib only group, but not evidently changed in the Fib* and Fib$^{\#}$ groups. **Figure 3.10 C-E** displays the fold change in the transcriptional expression of aSMA, COX-2, and COL-1 in each group compared to the MED control. The expressions of fibrotic markers, aSMA and COL-1, were upregulated after M1-SN co-culture in each pre-stimulation group. The most significant upregulation of aSMA was observed in the TGFb-prestimulated Fib$^{\#}$ group with a mean fold change of 24.00 (using RPL-13 as HKG) and 27.23 (using beta-actin as HKG), while the most significant upregulation of COL-1 was observed in the Fib* group with a mean fold change of 14.38 (using RPL-13 as HKG) and 19.73 (using beta-actin as HKG). In contrast, COX-2 expression was significantly downregulated in each group after M1-SN co-culture with less than 0.5-fold change compared to the MED control, and it was extremely reduced in the Fib* and Fib$^{\#}$ groups.

In summary, M1-SN exhibited very strong pro-fibrotic effects, as demonstrated by the evident upregulation of aSMA and COL-1 in all 3 Fibs groups, independent from pre-stimulation. Interestingly, with the upregulation of aSMA and COL-1 in the presence of M1-SN, COX-2 expression showed an inverse correlation and was significantly reduced in each group.


A
D-Fibs
Overnight
Medium
TNFa+IL1b
TGFb
72h
350µl Medium+148.5µl Medium+1.5ul LPS
350µl Medium+150µl M1SN
72h
Supernatant
Lysate
B
IL-6
MMP-3
cytokine conc. (pg/ml)
ns
*
Fib-MED
Fib-MED+M1SN
Fib*-MED
Fib*-MED+M1SN
Fib#-MED
Fib#-MED+M1SN
0.3*M1SN
n=3
C
RPL-13 a-SMA
b-actin aSMA
Fold Change 2^-ddct
Fib-M1SN vs. MED
Fib*-M1SN vs. MED
Fib#-M1SN vs. MED
n=3
D
RPL-13 COX-2
b-actin COX2
Fold Change 2^-ddct
Fib-M1SN vs. MED
Fib*-M1SN vs. MED
Fib#-M1SN vs. MED
n=3

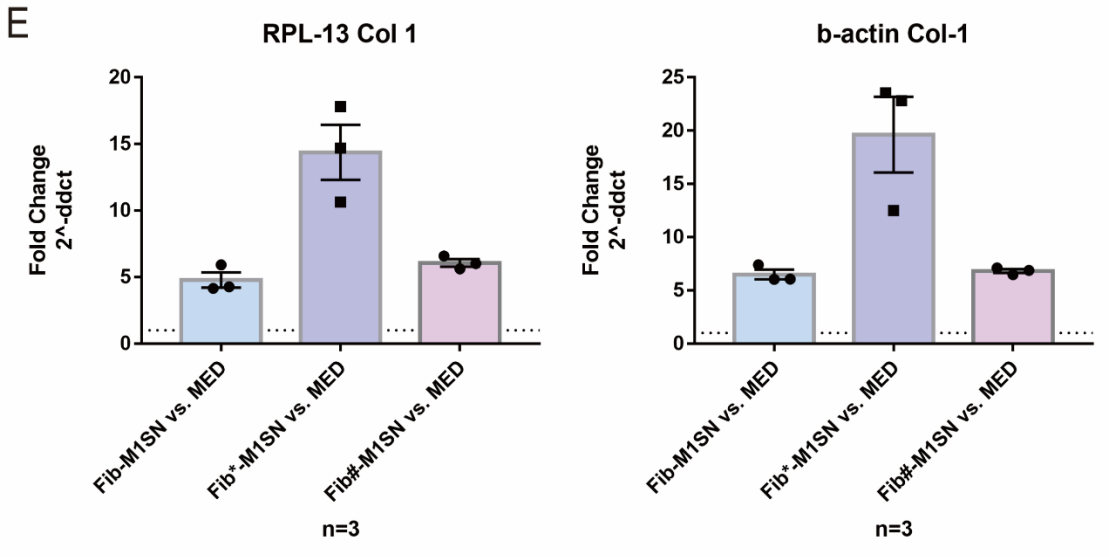


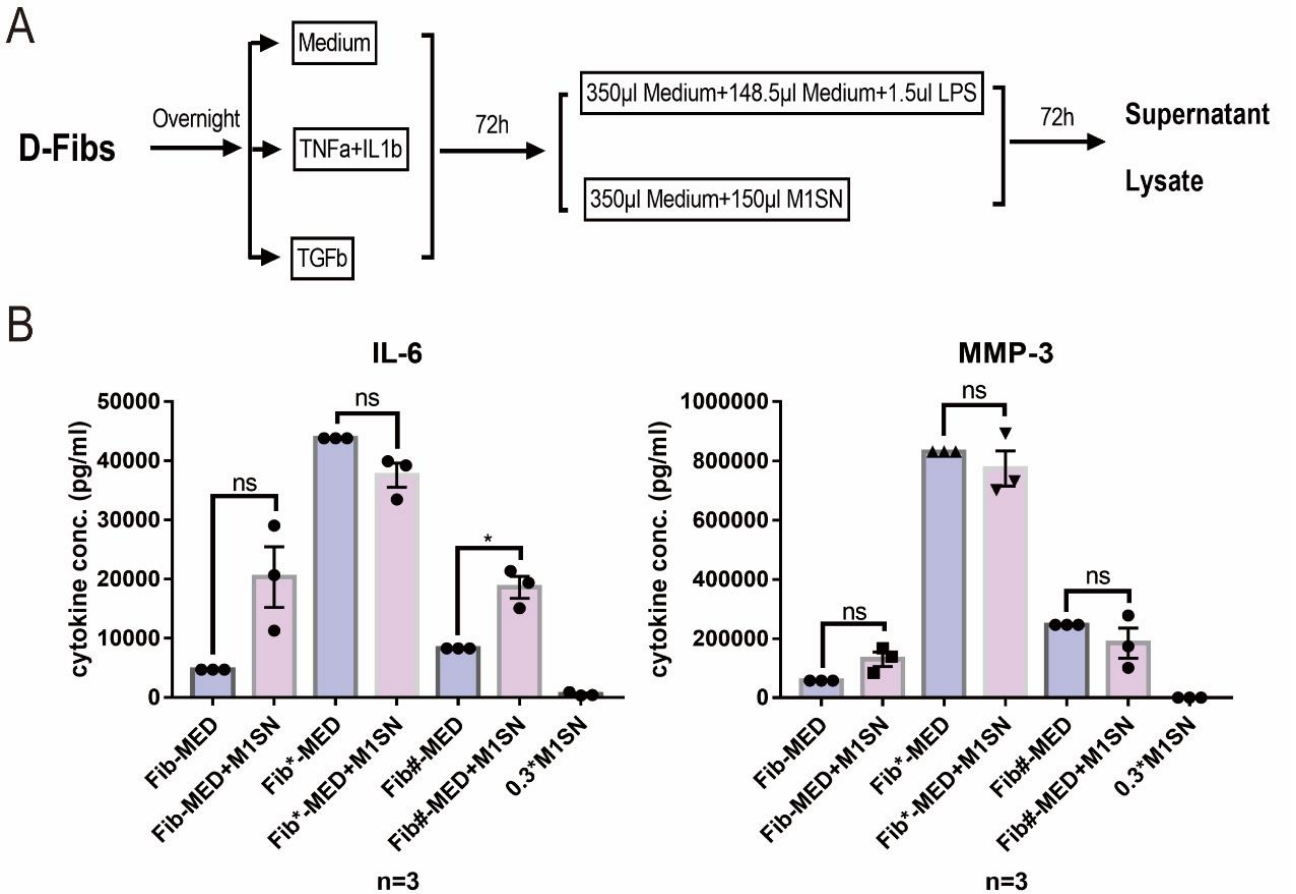
Figure 3.10: Effects of M1 supernatant on differently activated dermal fibroblasts. (A) Group settings and experimental setup. **(B)** Cytokines expression of IL-6 and MMP-3 measured by ELISA. (depicted is the mean ± SEM of n=3 independent donors per group). P-values of cytokines concentration were calculated by Paired t test; ns = no significance, *: P<0.05, **: P<0.01, ***P<0.001. **(C-E)** Fold changes of transcriptional expression of Fibs inflammatory and fibrotic markers assessed by qPCR. RPL-13 and beta-actin were both used as house-keeping gene. (depicted is the mean ± SEM of n=3 independent donors per group).

3.2.8 Role of Janus kinase inhibitors (JAKi) during Interaction between M1 Supernatant and Dermal Fibroblasts

In Part 3.2.7, it was demonstrated that M1-SN had pro-fibrotic effects on dermal Fibs. Similar to the synovial Fib, IL-6 was significantly upregulated in fib after M1-SN co-culture. Therefore again the selective JAK1 inhibitor (Upadacitinib) was used to evaluate the potential involvement of this pathway in M1- mediated effects on the fib in a similar way as before in context with synovial fib (3.2.8). **Figure 3.11 A** shows the experimental setup and group setting. The same protocol as in **Part 3.2.6** was utilized. **Figure 3.11 B** demonstrates the expression of IL-6 and MMP-3 in these experiments with three independent donors. The expression of IL-6 and MMP-3 was highly upregulated after M1-SN co-culture in the DMSO control group; the expression of IL-6 was significantly downregulated with JAKi presence, and the difference between M1-SN co-culture and MED control in the JAKi group were significantly smaller than the difference between M1-SN co-culture and MED control in the

DMSO control group. In other words, the effects of M1-SN on IL-6 expression were successfully suppressed, but JAKi had little effect on the expression of MMP-3.

Figure 3.11 C-E indicates the fold changes of transcriptional expression of aSMA, COX-2, and COL-1. There was no significant change in aSMA expression between the JAKi group and the MED control. The expression of COX-2 was significantly downregulated with a mean fold change of 0.47 (using RPL-13 as HKG) and 0.54 (using b-actin as HKG) with M1-SN exposure, and it was much reduced in the JAKi group with mean fold change decreased to 0.15 (using RPL-13 as HKG) and 0.22 (using b-actin as HKG). In the JAKi group, the fold change of COL-1 expression was slightly increased compared with the DMSO control. In summary, JAKi can definitely lower the IL-6 expression compared with the DMSO control, but the JAK/STAT signaling pathway was not the key pathway for the pro-fibrotic effects of M1-SN on dermal Fib regulation.



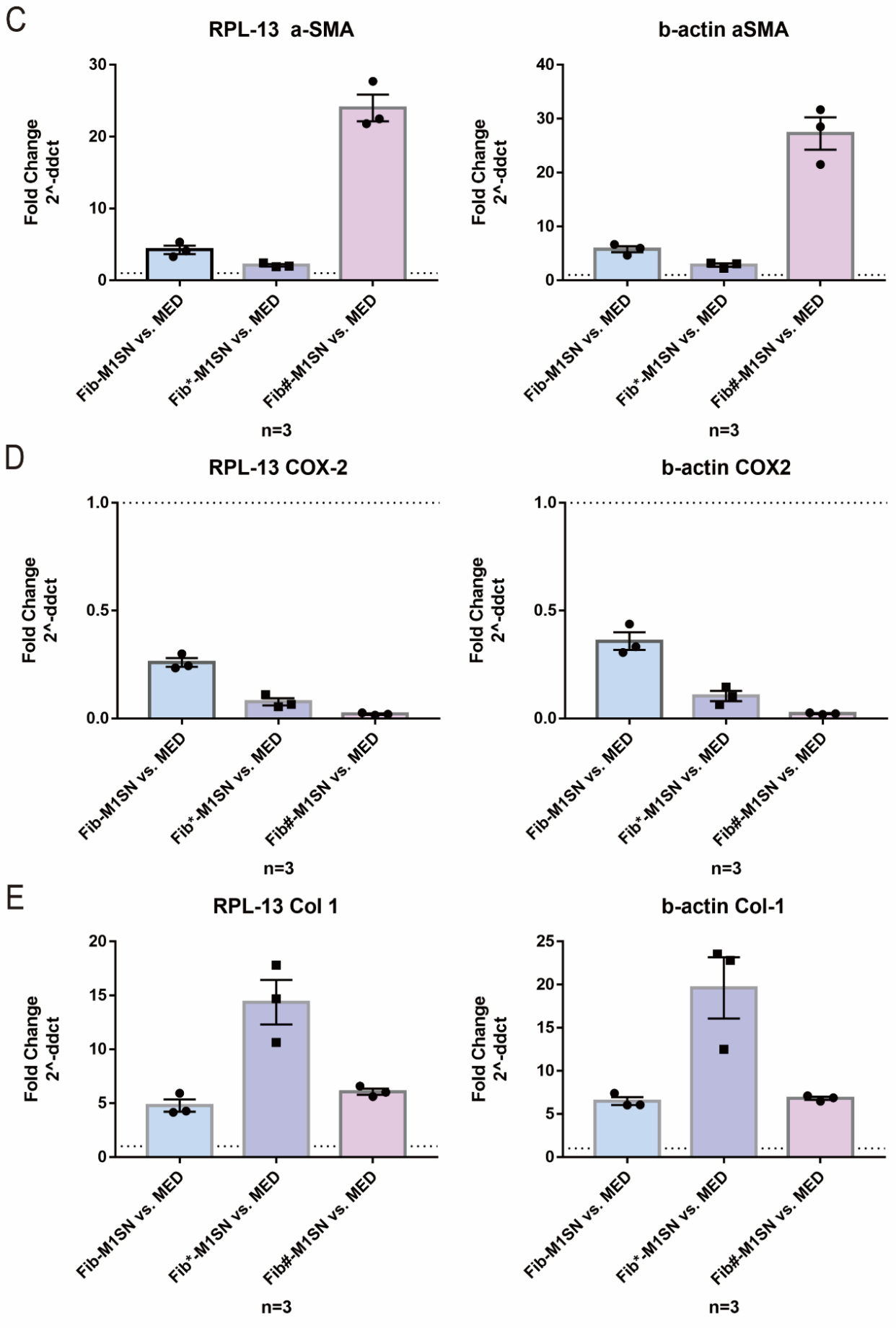

C
RPL-13 a-SMA
Fold Change 2^-ddct
30
20
10
0
Fib-M1SN vs. MED
Fib*-M1SN vs. MED
Fib#-M1SN vs. MED
n=3
b-actin aSMA
Fold Change 2^-ddct
40
30
20
10
0
Fib-M1SN vs. MED
Fib*-M1SN vs. MED
Fib#-M1SN vs. MED
n=3
D
RPL-13 COX-2
Fold Change 2^-ddct
1.0
0.5
0.0
Fib-M1SN vs. MED
Fib*-M1SN vs. MED
Fib#-M1SN vs. MED
n=3
b-actin COX2
Fold Change 2^-ddct
1.0
0.5
0.0
Fib-M1SN vs. MED
Fib*-M1SN vs. MED
Fib#-M1SN vs. MED
n=3
E
RPL-13 Col 1
Fold Change 2^-ddct
20
15
10
5
0
Fib-M1SN vs. MED
Fib*-M1SN vs. MED
Fib#-M1SN vs. MED
n=3
b-actin Col-1
Fold Change 2^-ddct
25
20
15
10
5
0
Fib-M1SN vs. MED
Fib*-M1SN vs. MED
Fib#-M1SN vs. MED
n=3

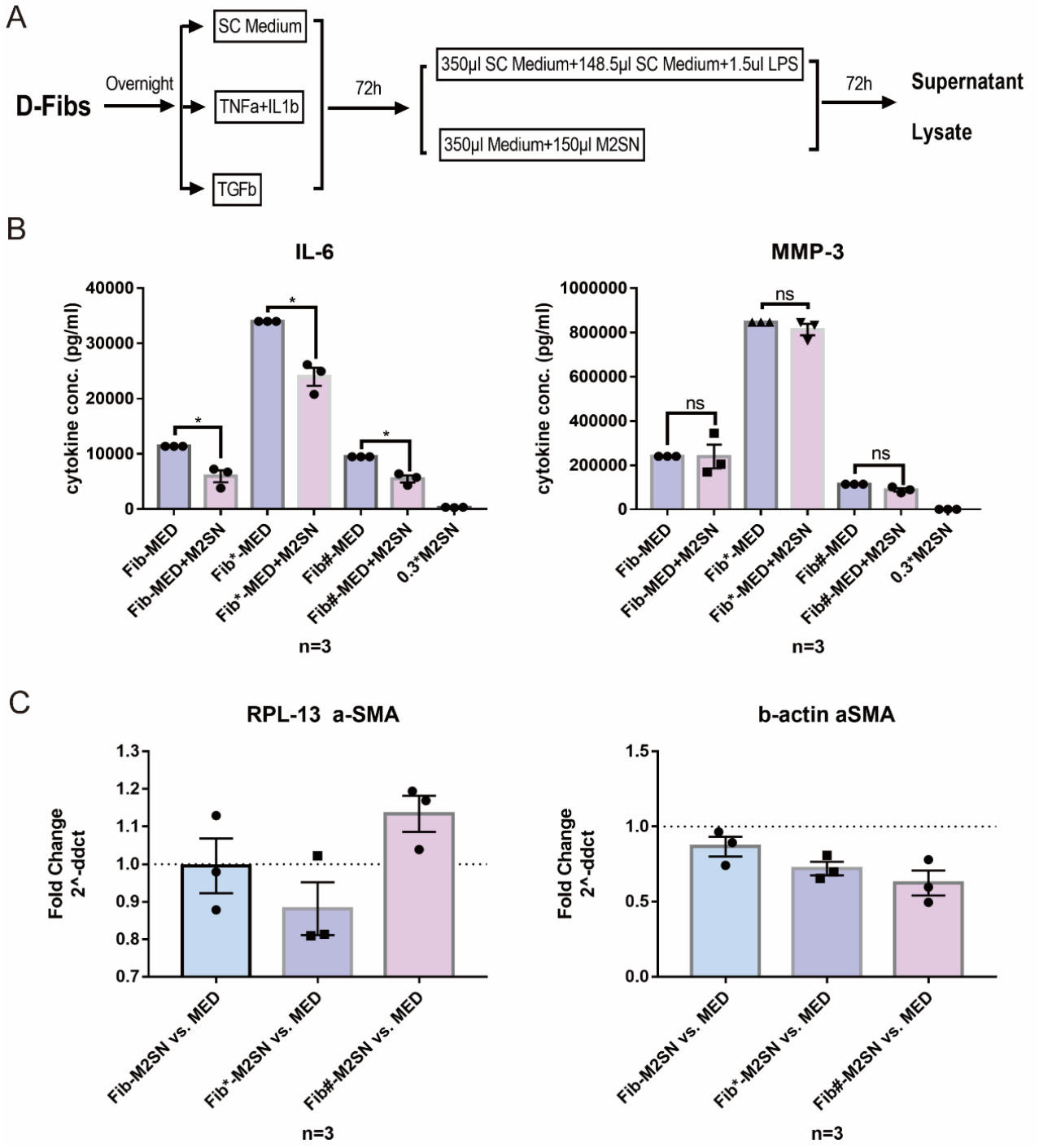
Figure 3.11: The effects of JAKi on the interaction between M1-SN on resting dermal fibroblasts. (A) Group settings and experimental setup. **(B)** Cytokines expression of IL-6 and MMP-3 measured by ELISA. (depicted is the mean ± SEM of n=3 independent donors per group). **(C-E)** Transcriptional expression of Fibs inflammatory and fibrotic markers assessed by qPCR. RPL-13 and beta-actin were used as house-keeping gene. (depicted is the mean ± SEM of n=3 independent donors per group). P-values were calculated by Paired t test; ns = no significance, *: P<0.05, **: P<0.01.

3.2.9 The Effects of M2 Macrophage Supernatant on Differently Activated Dermal Fibroblasts

In Part 3.2.2, the anti-inflammatory effects of M2SN on synovial Fibs were demonstrated. This part of the study was designed to investigate the effects of M2SN on dermal Fibs. The experimental setups and group settings are shown in **Figure 3.12 A**, and the same protocol as **Part 3.2.2** was used with 3 independent donors of M2SN. **Figure 3.12 B** displays the changes in MMP-3 and IL-6 expression after M2SN co-culture. The results showed that the expression of IL-6 was significantly downregulated in all groups after M2SN exposure, while the reaction of MMP-3 expression was less reacted after M2SN co-culture. **Figure 3.12 C-E** shows the gene expression analysis of aSMA, COX-2, and COL-1 expression by qRT-PCR. In the Fib$^{\#}$ group, the gene expression of aSMA slightly increased using RPL-13 as the housekeeping gene, while the results using beta-actin as the housekeeping gene showed an opposite trend. The expression of aSMA slightly reduced in the Fib* group with a fold change of 0.88 (using RPL-13 as HKG) and 0.72 (using beta-actin as HKG). In the Fib only group, the variation of aSMA expression could not be precisely determined when RPL-13 was used as the housekeeping gene, while the mean fold change of 0.87 using beta-actin as the housekeeping gene showed a slight downregulation of aSMA expression. The expressions of COX-2 in the Fib and Fib$^{\#}$ group were significantly reduced with a mean fold change of less than 0.5 using both RPL-13 and beta-actin as HKGs after M2SN co-culture, and it also slightly decreased in the Fib* group. In the Fib only group, the gene expression of COL-1 was reduced with a mean fold change of 0.77 (using RPL-13 as HKG) and 0.67 (using beta-actin as HKG) after M2SN co-culture. In the Fib$^{\#}$ group, the downregulation of COL-1 was much more evident with a mean fold change of 0.49 using beta-actin as the housekeeping gene. However, in the Fib* group, it was not possible to confirm this effect

precisely with the experiments conducted with M2-SN from these 3 independent donors.

In summary, the results suggest that M2SN has evident anti-inflammatory effects with the ability to downregulate COX-2 on resting and TGFb pre-treated (Fib#) dermal Fibs, and it also has slightly anti-fibrotic effects in these two groups. However, the effect of M2SN on the TNFa+IL1b pre-stimulation group (Fib*) was not highly significant.



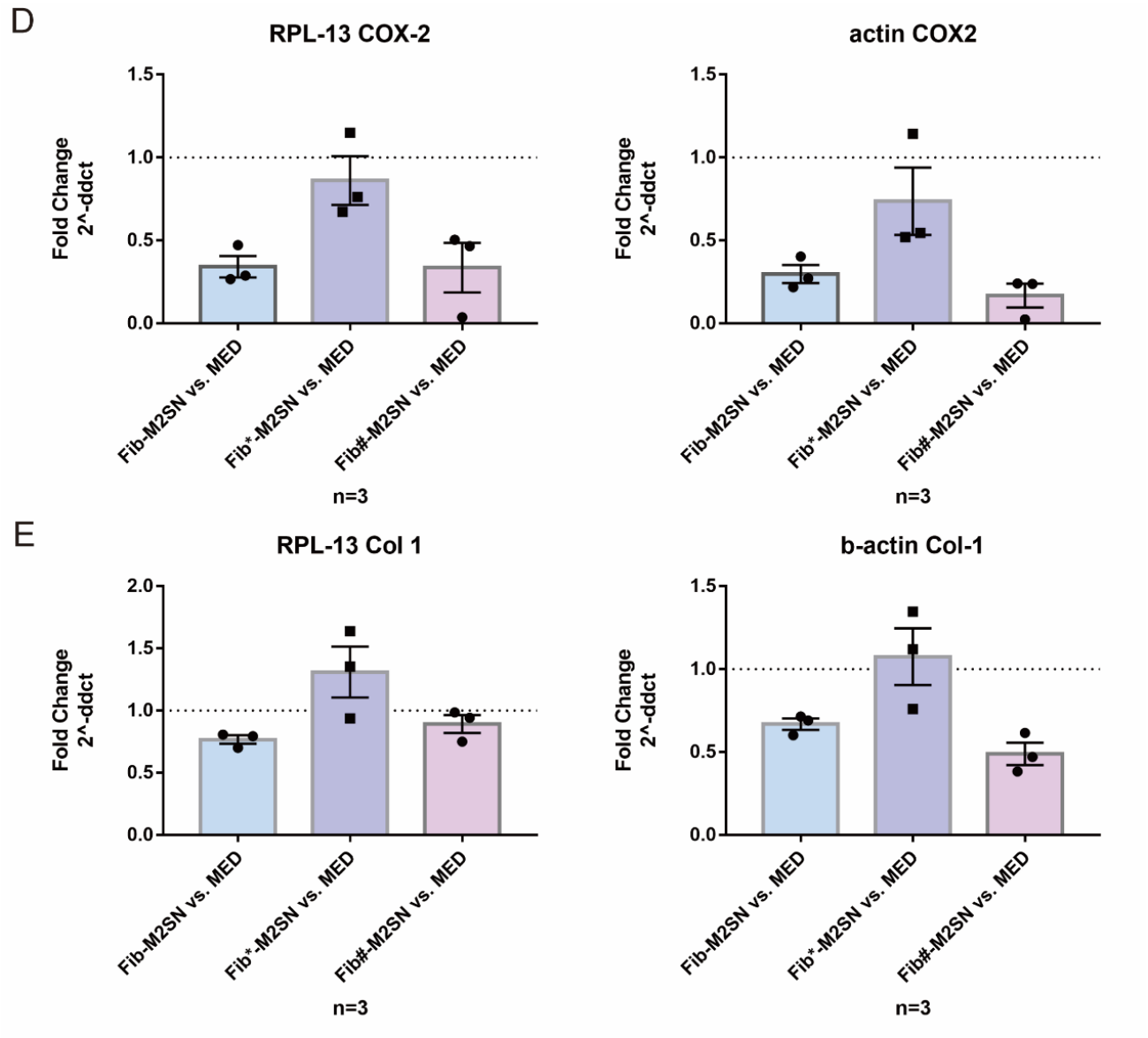


Figure 3.12: Effects of M2 supernatant on differently activated dermal fibroblasts. (A) Group settings and experimental setup. **(B)** Cytokines expression of IL-6 and MMP-3 measured by ELISA. (depicted is the mean ± SEM of n=3 independent donors per group). **(C-E)** Transcriptional expression of aSMA, COX-2 and COL-1 with fold changes assessed by qPCR. RPL-13 and beta-actin were both used as house-keeping gene. (depicted is the mean ± SEM of n=3 independent donors per group). P-values of cytokines concentration were calculated by Paired t test; ns = no significance, *: P<0.05.

3.3 The Effects of Differently Activated Fibroblasts on Macrophages

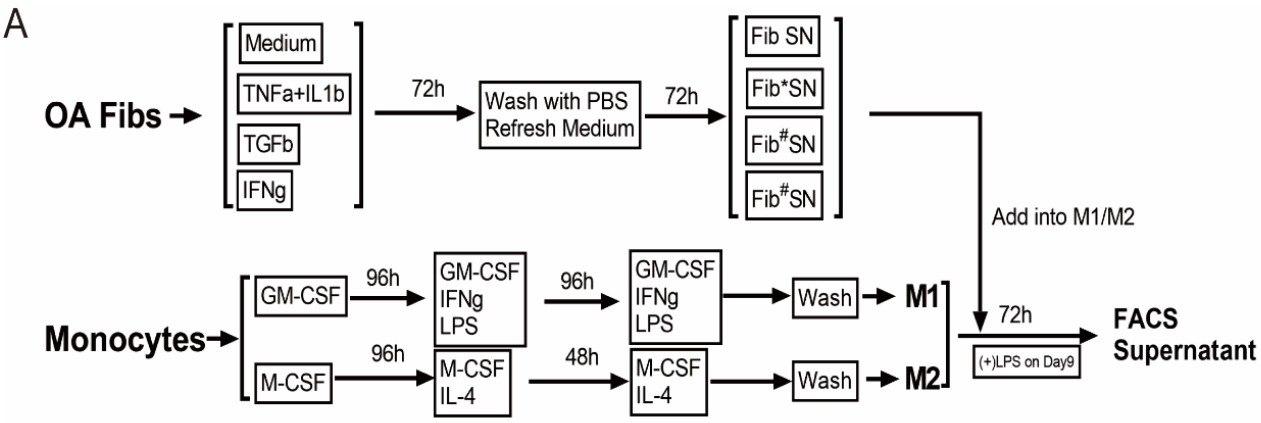
3.3.1 The Effects of Differently Activated Fibroblast Supernatant on polarized M1/M2 Macrophages - Preliminary-Experiment

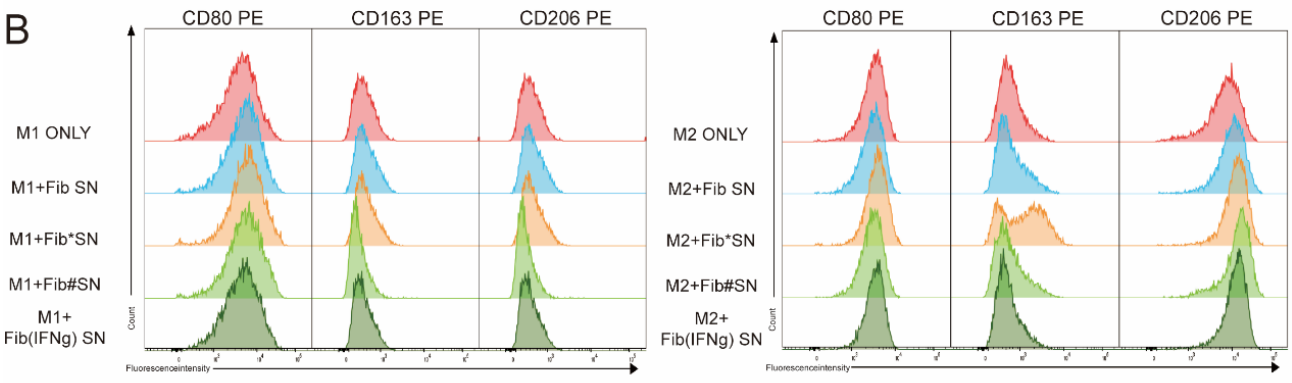
To explore the effects of differently activated synovial Fibs on differentiation of Mphs, a single pre-experiment was conducted. **Figure 3.13 A** shows the experiment setup and group setting. OA Fibs (synovial fibroblasts) were pre-stimulated with Medium control, TNFa+IL1b, TGFb and IFNg respectively. Then the Fib SN, Fib*SN, Fib$^{\#}$SN and this time also IFNg pre-stimulated Fib (Fib^{g}SN) were harvested and added to M1 and M2 respectively. The Mphs and supernatant were harvested 72 hours later for next measurement via ELISA and Flow cytometry. **Figure 3.13 B** show the flow cytometry histograms for the experiment. The fluorescence intensity of the CD163 marker in the M2+Fib*SN sample was noticeably higher than that of the other samples. **Figures 3.13 C-E** display the mean and median fluorescence intensity of three representative markers--CD80 for M1 Mphs, CD163 and CD206 for M2 Mphs. The expression of CD80 was slightly upregulated after co-culture with differently pre-stimulated Fibs supernatants, and M1 after Fib*SN coculture showed highest expression compared with M1 co-cultured by other type of Fibs supernatant. However, the differences between groups were relatively small. The expression of CD163 and CD206 did not change significantly after supernatant co-culture compared to the M1-only control. For the M2 Mphs, the expression of CD163 was significantly upregulated, with a mean fluorescence intensity even twice as high after co-culture with Fib*SN compared to M2 co-cultured with other types of Fibs supernatant. The different Fibs supernatant had little effect on CD80 expression, while CD206 expression was slightly upregulated after the different supernatant – fib co-cultures, but the differences were not significant.

Figures 3.13 F show the IL-10 and TNFa concentrations measured by ELISA. In the M1 cocultures, the IL-10 concentration in the M1-Fib*SN group increased to 1.5 fold compared to the M1-only control, while the other groups did not change significantly compared to the M1-only control. The expression of TNFa in the M1-Fib$^{\#}$SN group was slightly downregulated, while the expression of TNFa in the other groups remained stable after Fibs supernatants co-culture. For the M2 Mphs, both the IL-10 and

TNFa expressions were downregulated after co-culture with the in M2-Fib*SN supernatant, with the IL-10 expression reduced to about 1/3 of the M2-only control and the TNFa expression reduced to around 1/5 of the M2-only control. The expression of IL-10 and TNFa in the M2-Fib$^{\#}$SN group was also downregulated with around 1/2 and 2/5, respectively, of the M2-only control. The IL-10 and TNFa expressions in the other groups were not significantly changed.

Taken together, the differently activated Fibs supernatants did not have an important impact on the M1 Mphs but there was an effect on M2 Mphs: Fib*SN induced significantly higher CD163 expression in M2, which could make M2 Mphs shift more towards a M2 phenotype accompanied by a downregulation of TNFa. However, IL-10 expression was also reduced.





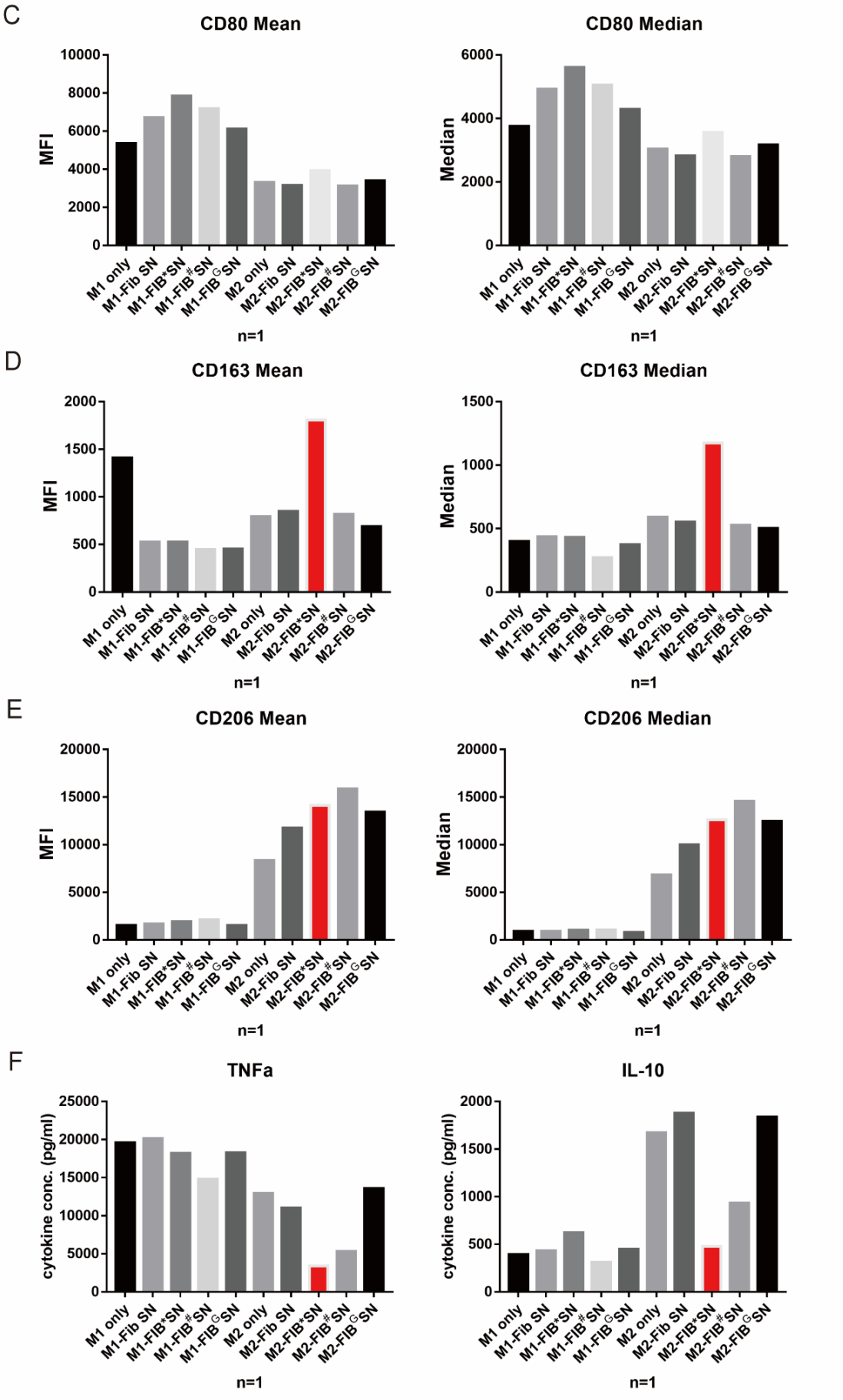

C
CD80 Mean
MFI
10000
8000
6000
4000
2000
0
M1 only
M1-Fib SN
M1-FIB*SN
M1-FIB#SN
M1-FIBGSN
M2 only
M2-Fib SN
M2-FIB*SN
M2-FIB#SN
M2-FIBGSN
n=1
CD80 Median
Median
6000
4000
2000
0
n=1
D
CD163 Mean
MFI
2000
1500
1000
500
0
n=1
CD163 Median
Median
1500
1000
500
0
n=1
E
CD206 Mean
MFI
20000
15000
10000
5000
0
n=1
CD206 Median
Median
20000
15000
10000
5000
0
n=1
F
TNFa
cytokine conc. (pg/ml)
25000
20000
15000
10000
5000
0
n=1
IL-10
cytokine conc. (pg/ml)
2000
1500
1000
500
0
n=1

Figure 3.13: The Effects of Differently Activated Fibroblasts Supernatant on Macrophages---Pre-Experiment. (A) Group settings and experimental setup. **(B)** Representative histogram of M1/M2 expression of CD80, CD163 and CD206 after differently activated Fibs supernatant co-culture. **(C-E)** Mean and median fluorescence intensity of CD80, CD163 and CD206 measured by Flow Cytometer. **(F)** Cytokines expression of IL-10 and TNFa measured by ELISA.

3.3.2 The Effects of TNFa+IL1b Pre-stimulated Synovial Fibroblasts on M0 Macrophages

As described in **Part 3.3.1**, Fib*SN induced CD163 upregulation on M2 Mphs. In order to prove the M2-driving potential of Fib*SN, further experiments were conducted, including yet undifferentiated Mph (M0) that had been activated by M-CSF only. **Figure 3.14 A** shows the experiments setups and groups setting. As described in the methods section, both Fibs supernatants and Fibs in inserts were added to M0 on day 7 of the M0 culture. **Figure 3.14 B-G** demonstrates the mean and median of fluorescence intensity of 3 representative marker, CD80 for M1, CD163 and CD206 for M2 respectively. **Figure 3.14 B-D** shows the data from the experiments without LPS addition at 24 hours before cells harvest with 3 samples--M0 only control, M0+Fib*SN and M0+Fib*insert. The expression of CD80 was slightly upregulated after Fib*SN co-culture and Fib*insert transwell co-culture. The expression of CD163 was highly upregulated in the M0+Fib*insert group, while Fib*SN did not evidently upregulate CD163 expression with current data. The expression of CD206 was significantly increased in M0+Fib*SN group, but as for the M0+Fib*insert group, the data from three out of the four experiments exhibited a gradual increase in expression while the data of one of the experiments showed an extremely high upregulation of CD206 after Transwell co-culture. As shown in **Figure 3.14 M**, the concentration of TNFa was not successfully measured from each sample. IL-10 with a very low concentration was successfully measured in the M0+Fib*SN group, but this was not the case in other groups.

In order to try to explore the changes of cytokines from the experiments within the setups, the experiments with LPS addition at 24 hours before cells harvest were conducted twice. The comparison with in total of 2 samples--M0 only and M0+Fib*SN was made. **Figure 3.14 E-G** demonstrates the mean and median of fluorescence intensity. The results were similar as the results

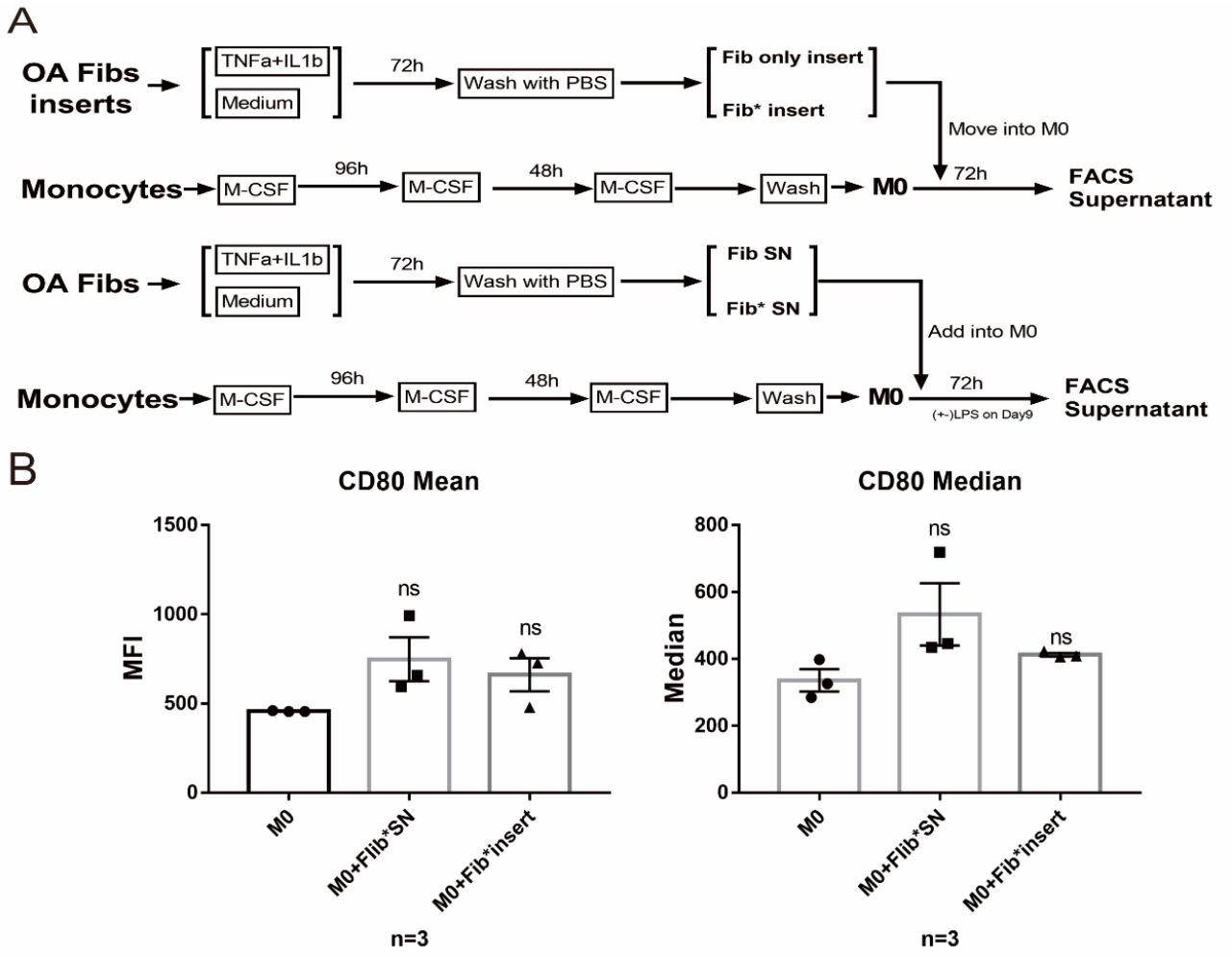
of the experiments without LPS addition. CD80 expression were slightly upregulated in M0+Fib*SN compared with M0+LPS control, and the upregulation of CD163 and CD206 were much evidently compared with control group especially for the expression of CD206. As demonstrated in **Figure 3.14 N**, the concentration of IL-10 was a little bit higher than it in control group, and the TNFa expression was hardly downregulated in M0+Fib*SN group compared with control group.

In order to rule out the possibility of interferences of resting Fibs, one additional single experiment with the additional samples--M0+FibSN and M0+Fib insert was conducted. **Figure 3.14 H-L** demonstrates the mean and median of fluorescence intensity of CD80, CD163 and CD206. **Figure 3.14 H-I** shows the data of the experiment without LPS addition regarding CD163 and CD206. The expression of CD163 was clearly upregulated to around double compared to the M0 control in M0+Fib*SN and M0+Fib*insert groups, while it was reduced clearly in the group of M0+Fib SN. The sample M0+Fib insert as another positive control group did not evidently change compared to M0 control. CD206 expression of group of Fib*SN and Fib*insert were significantly increased compared to the M0 control, and the positive controls--M0+Fib SN and M0+Fib insert were not clearly changed. **Figure 3.14 O** indicate the results regarding the cytokines TNFa and IL-10. In tendency the data showed a similar result as former experiments. Only very low value (87.67pg/ml) of IL-10 concentration of M0+Fib*SN group was successfully measured per ELSIA, and the IL-10 concentration of other samples was too low to be measured. The expression of TNFa also failed to be measurable per ELISA.

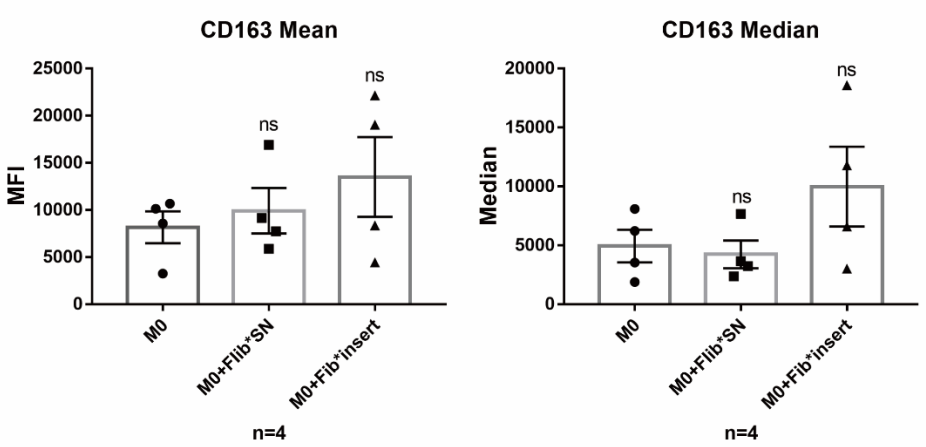
A single experiment with LPS addition at 24 hours before harvest was conducted as well. **Figure 3.14 J-L** indicate the mean and median of fluorescence intensity of CD80, CD163 and CD206. The expression of CD80 was not evidently changed from the comparison of M0+Fib SN, M0+Fib*SN and M0 only control. The expression of CD163 was evidently upregulated in the M0+Fib*SN group compared with the M0 control, but in the M0+Fib SN group, it decreased to around 1/2 as the M0 control. In terms of CD206 expression, it was significantly upregulated in the M0+FIb*SN group, while there were no evident differences between M0+Fib SN and the M0 control. **Figure 3.14 P** shows the IL-10 and TNFa concentration of each sample. The expression of IL-10 was slightly increased in M0+Fib*SN group(1859pg/ml) compared with M0 control (1781pg/ml), while the expression of IL-10 in M0+FibSN group were twice that of M0 only control. In terms of TNFa expression, this was significantly downregulated in both M0+FibSN and M0+Fib*SN groups, and the downregulation in the

M0+Fib*SN group was much evident.

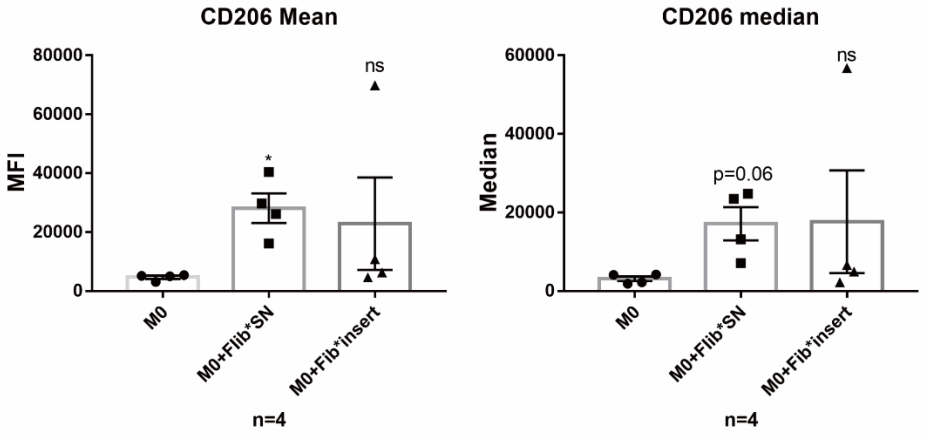
Taken together, synovial Fibs pre-stimulated with proinflammatory TNFa+IL1b can definitely induce both CD163 and CD206 expression in M0 Mphs. In terms of cytokines, Fib*SN can also induce measurable IL-10 secretion of M0 even in absence of LPS addition, and it also can downregulate TNFa expression of M0, thereby supporting M2 polarization.



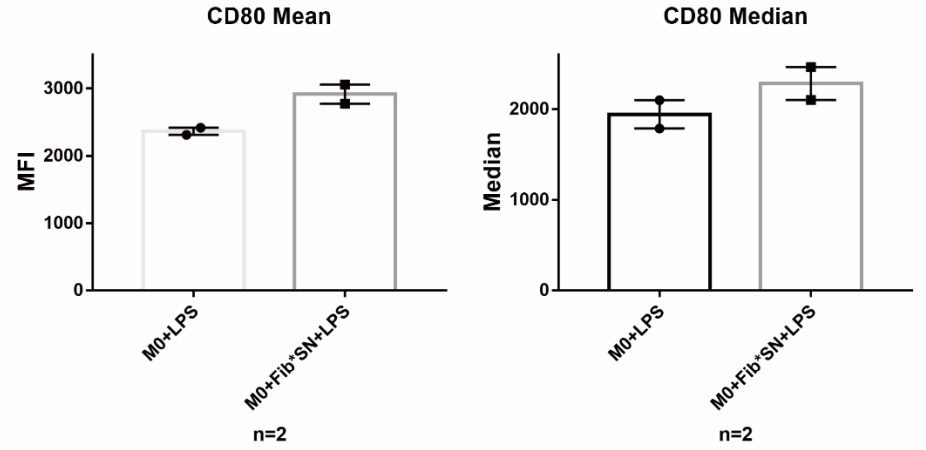
C


CD163 Mean
MFI
25000
20000
15000
10000
5000
0
ns
ns
M0
M0+Flib*SN
M0+Fib*insert
n=4
CD163 Median
Median
20000
15000
10000
5000
0
ns
ns
M0
M0+Flib*SN
M0+Fib*insert
n=4

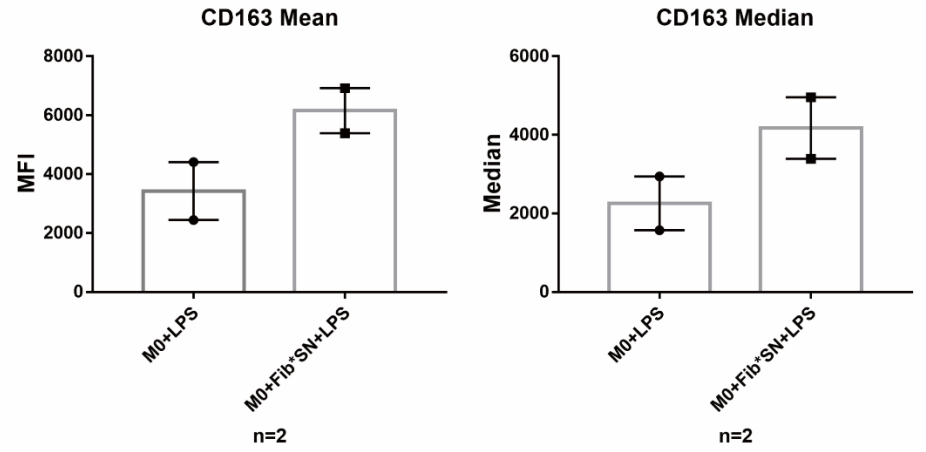
D


CD206 Mean
MFI
80000
60000
40000
20000
0
ns
*
M0
M0+Flib*SN
M0+Fib*insert
n=4
CD206 median
Median
60000
40000
20000
0
ns
p=0.06
M0
M0+Flib*SN
M0+Fib*insert
n=4

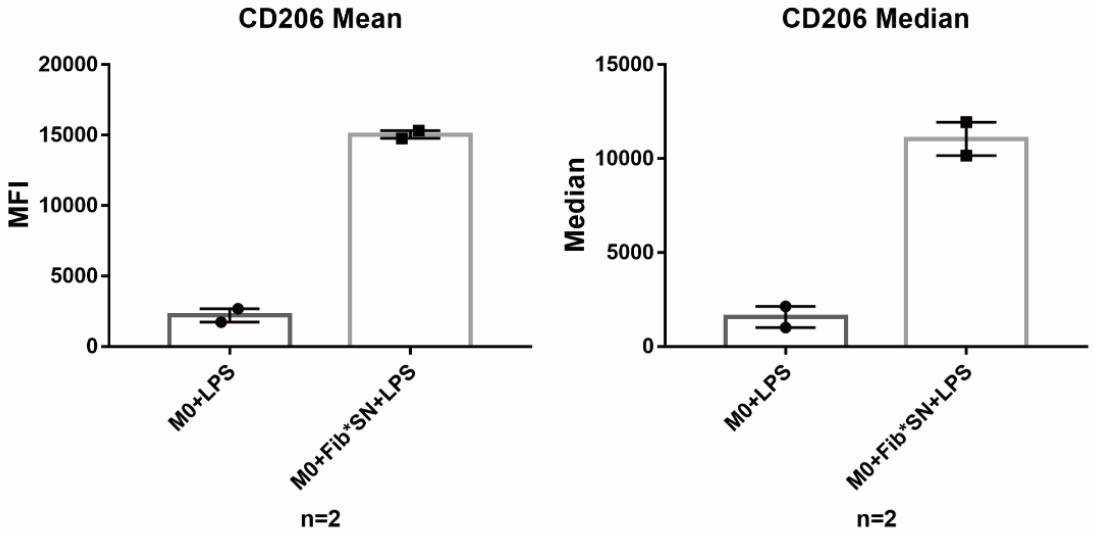
E


CD80 Mean
MFI
3000
2000
1000
0
M0+LPS
M0+Fib*SN+LPS
n=2
CD80 Median
Median
2000
1000
0
M0+LPS
M0+Fib*SN+LPS
n=2

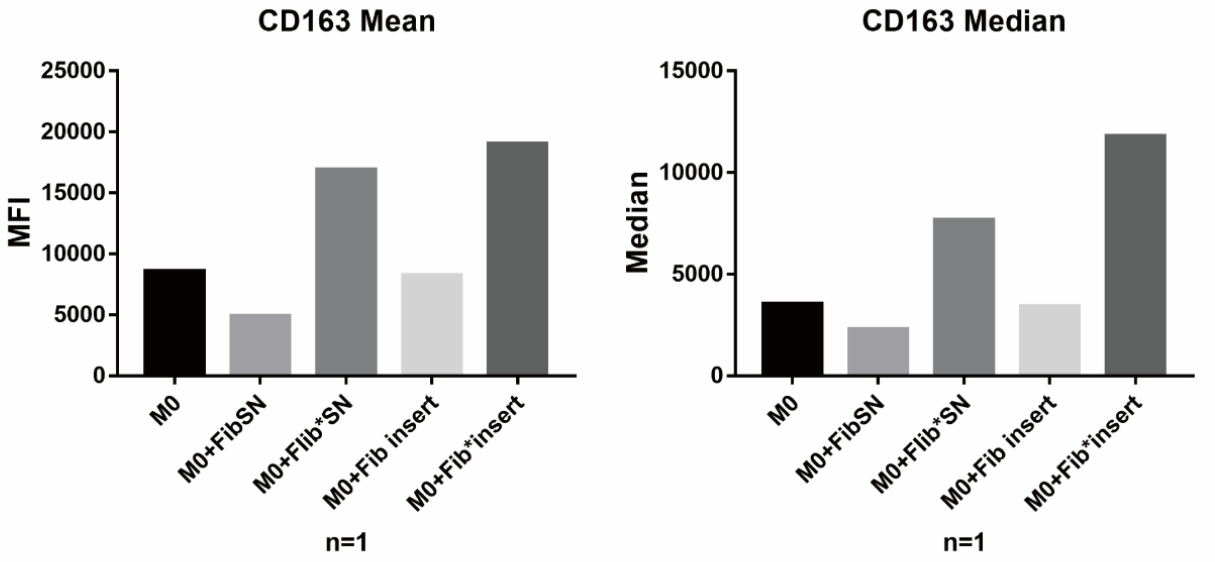
F


CD163 Mean
MFI
8000
6000
4000
2000
0
M0+LPS
M0+Fib*SN+LPS
n=2
CD163 Median
Median
6000
4000
2000
0
M0+LPS
M0+Fib*SN+LPS
n=2

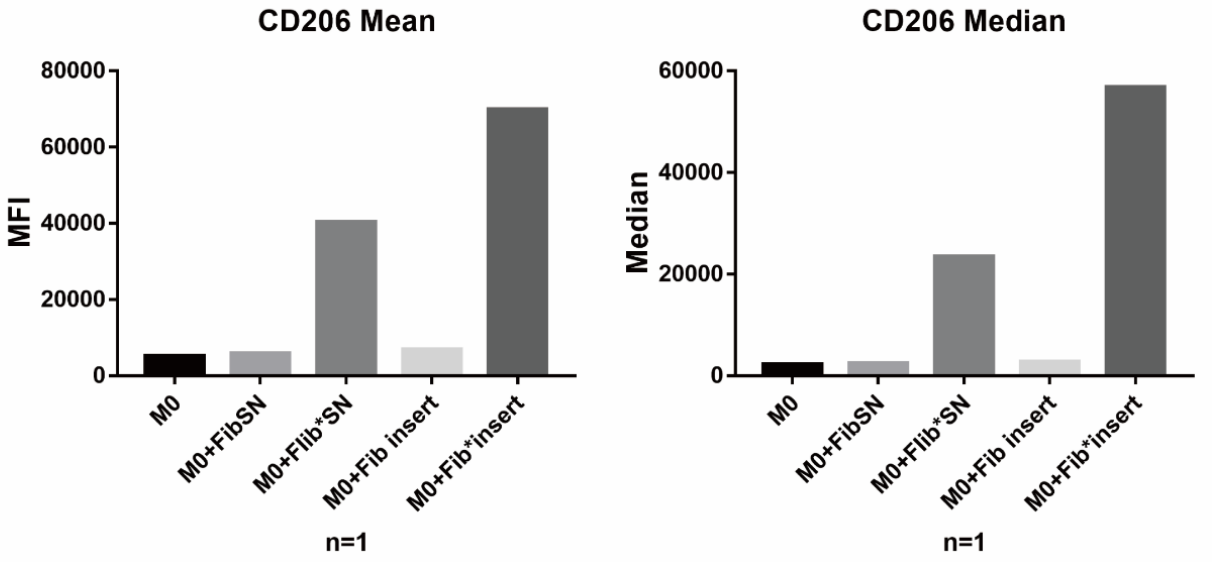
G


CD206 Mean
MFI
20000
15000
10000
5000
0
M0+LPS
M0+Fib*SN+LPS
n=2
CD206 Median
Median
15000
10000
5000
0
M0+LPS
M0+Fib*SN+LPS
n=2

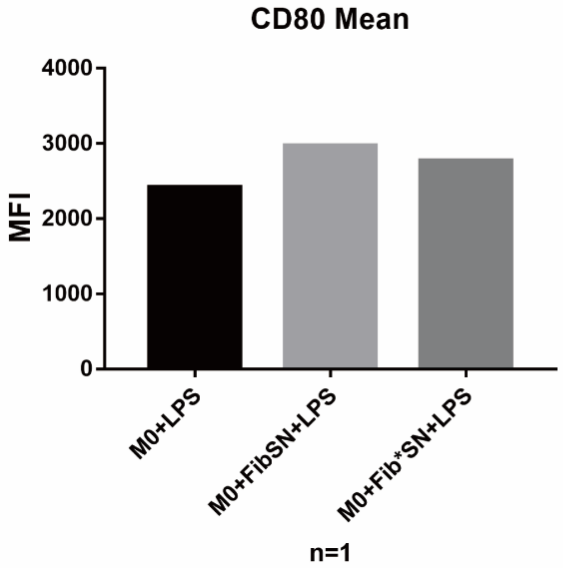
H

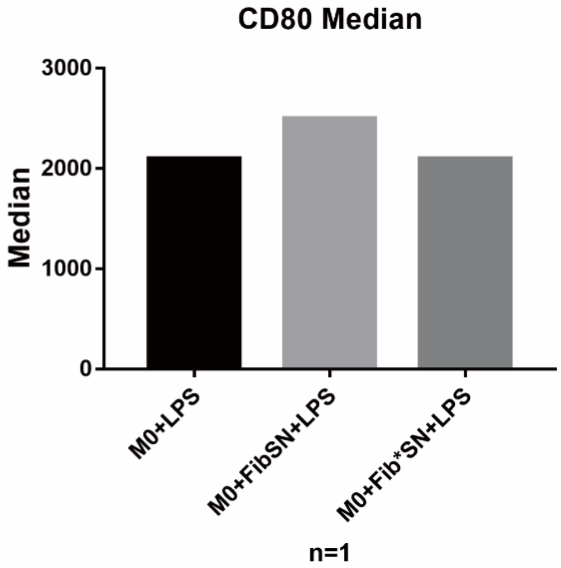

CD163 Mean
MFI
25000
20000
15000
10000
5000
0
M0
M0+FibSN
M0+Flib*SN
M0+Fib insert
M0+Fib*insert
n=1
CD163 Median
Median
15000
10000
5000
0
M0
M0+FibSN
M0+Flib*SN
M0+Fib insert
M0+Fib*insert
n=1

I

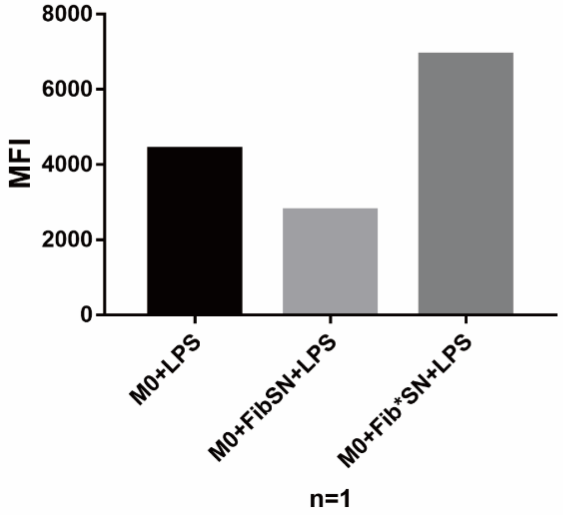

CD206 Mean
MFI
80000
60000
40000
20000
0
M0
M0+FibSN
M0+Flib*SN
M0+Fib insert
M0+Fib*insert
n=1
CD206 Median
Median
60000
40000
20000
0
M0
M0+FibSN
M0+Flib*SN
M0+Fib insert
M0+Fib*insert
n=1

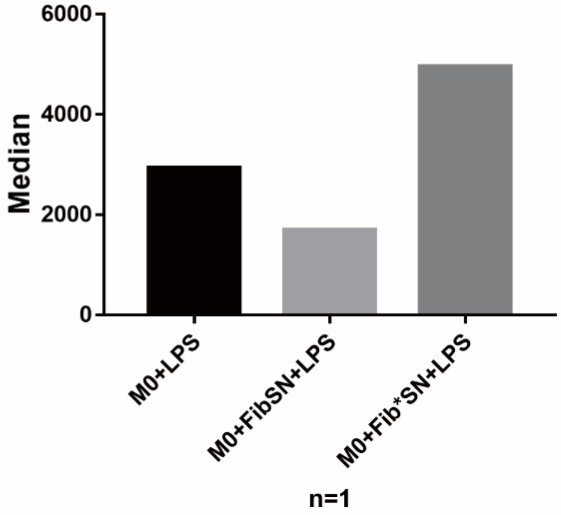
J


CD80 Mean
MFI
4000
3000
2000
1000
0
M0+LPS
M0+FibSN+LPS
M0+Fib*SN+LPS
n=1

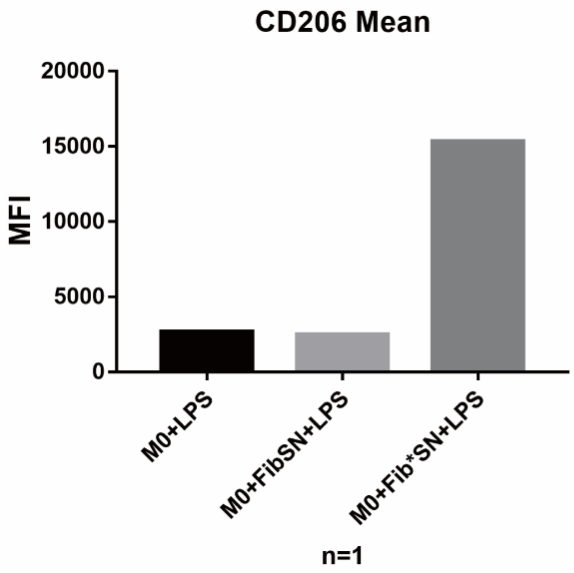

CD80 Median
Median
3000
2000
1000
0
M0+LPS
M0+FibSN+LPS
M0+Fib*SN+LPS
n=1

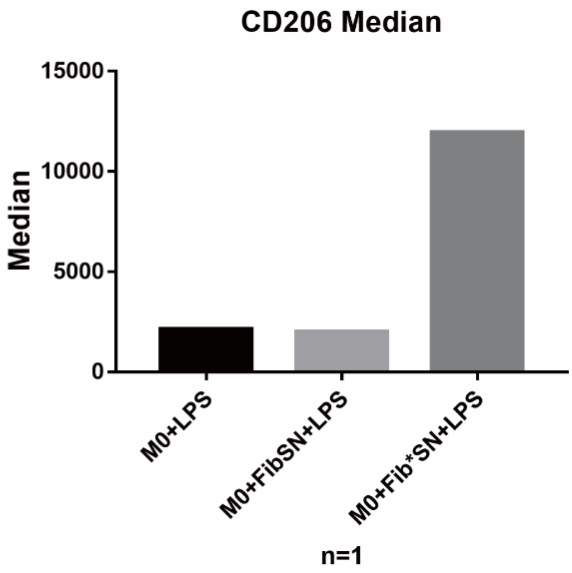
K


CD163 Mean
MFI
8000
6000
4000
2000
0
M0+LPS
M0+FibSN+LPS
M0+Fib*SN+LPS
n=1


CD163 Median
Median
6000
4000
2000
0
M0+LPS
M0+FibSN+LPS
M0+Fib*SN+LPS
n=1

L


CD206 Mean
MFI
20000
15000
10000
5000
0
M0+LPS
M0+FibSN+LPS
M0+Fib*SN+LPS
n=1


CD206 Median
Median
15000
10000
5000
0
M0+LPS
M0+FibSN+LPS
M0+Fib*SN+LPS
n=1

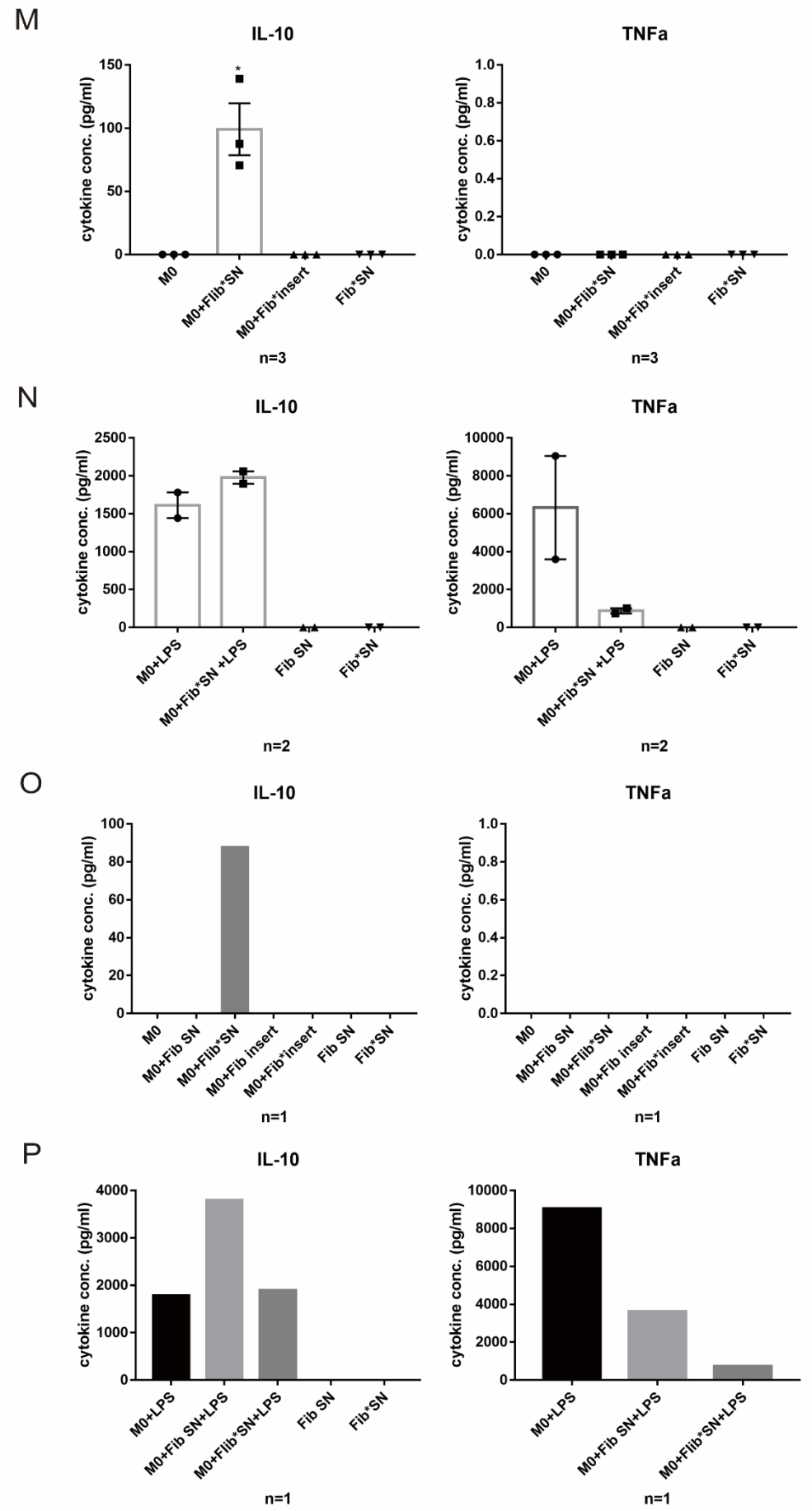

M
IL-10
TNFa
cytokine conc. (pg/ml)
150
100
50
0
1.0
0.8
0.6
0.4
0.2
0.0
*
M0
M0+Flib*SN
M0+Fib*insert
Fib*SN
n=3
N
2500
2000
1500
1000
500
10000
8000
6000
4000
M0+LPS
M0+Fib*SN +LPS
Fib SN
n=2
O
100
80
60
40
20
M0+Fib SN
M0+Fib insert
n=1
P
4000
3000
M0+Fib SN+LPS
M0+Flib*SN+LPS

Figure 3.14: The Effects of TNFa+IL1b Activated Fibroblasts Supernatant on M0 Macrophages. **(A)** Group settings and experimental setup. **(B-D)** Mean and median of fluorescence intensity of CD80, CD163 and CD206 measured by Flow Cytometer. (depicted is the mean ± SEM of n=4 independent experiments). P-values of mean and median of fluorescence intensity were calculated by Paired t test compared with control group; ns = no significance, *: P<0.05. **(E-G)** Mean and median of fluorescence intensity for the experiments with LPS addition. **(H-I)** Mean and median of fluorescence intensity of CD163 and CD206 for the single improved experiment without LPS addition. **(J-L)** Mean and median of fluorescence intensity of CD80, CD163 and CD206 for the single improved experiment with LPS addition. **(M-N)** Cytokines expression of IL-10 and TNFa measured by ELISA for the experiments with or without LPS addition. Statistical analysis was conducted for at least 3 times repeated experiments (depicted is the mean ± SEM of n=3 independent donors per group). P-values of cytokines concentration were calculated by Paired t test compared with control group; ns = no significance, *: P<0.05. **(O-P)** Cytokine expression of IL-10 and TNFa measured by ELISA for the single improved experiments with or without LPS addition.

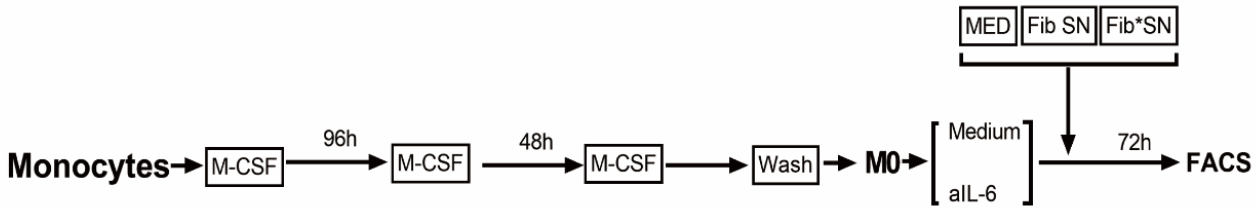
3.3.3 Effects of IL-6 receptor blocking and JAK inhibition during the synovial Fib* - induced Polarization of M0 into M2

The potential of Fib*SN as driving force of M2 Mphs were primarily proven in **Part 3.3.2** with CD163 and CD206 surface markers upregulation, IL-10 upregulation and TNFa downregulation after Fib*SN co-culture. A surge of IL-6 from synovial Fibs after TNFa+IL1b pre-stimulation could be observed. Therefore experiments were designed to explore whether IL-6 or an IL-6 related signaling pathway was involved in the phenomenon. **Figure 3.15 A-B** shows the experiment setups and group setting. Monocytes after 7 days culture with the former protocol of M0 were washed on day 7, then M0 Mphs were pre-treated with aIL-6 (Tocilizumab) or medium alone, and JAKi (Upadacitinib) or DMSO respectively for 30 min. After 30min pre-incubation, medium control, Fib SN and Fib*SN were added to the experiments separately. The cells were harvested on day 10 and surface markers were measured by flow cytometry. **Figure 3.15 C-D** demonstrates the mean and median fluorescence intensity of CD163 and CD206 expression in the experiments in which the comparison between aIL-6 and medium were made. The expression of CD163 were significantly upregulated with over twice fluorescence intensity as medium control and M0+FibSN positive control group; The aIL-6 groups regarding the expression of CD163 were suppressed as a whole, and the gap between M0+Fib*SN

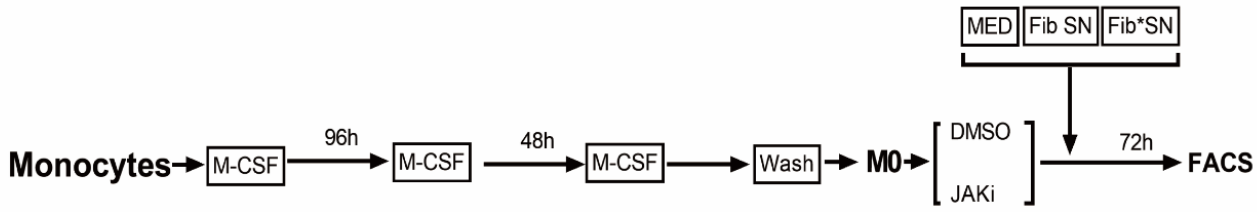
and control groups in the aIL-6 setup was reduced significantly. The expression of CD206 was clearly upregulated in the Fib*SN group compared with control groups in medium control part; but it was not suppressed with aIL-6 presence, but the gap between Fib*SN group and control groups increased evidently. **Figure 3.15 E-F** presented the effects of JAKi on driving force of Fib*SN on M0 Mphs. CD163 expression were highly upregulated in M0+Fib*SN group compared with control groups, whereas CD163 expression of M0+Fib*SN with JAKi presence were precisely downregulated, and CD163 in control groups did not evidently change. The expression of CD206 was clearly upregulated in M0+Fib*SN group compared with control groups, while the upregulation of CD206 was evidently inhibited compared with DMSO controls. In order to validate the results at the gene level, qPCR measurements were conducted for a single experiment. **Figures 3.15 G-H** show the transcriptional expression levels of IL-10 with and without aIL-6 treatment. The results indicated that Fib*SN significantly upregulated the expression of IL-10, while its expression was reduced after the treatment of aIL-6. **Figure 3.15 I-J** represent the transcriptional expression levels of Arg-1 with and without aIL-6 treatment, where Fib*SN slightly upregulated Arg-1 expression with b-actin as the housekeeping gene. However, the effect of aIL-6 was not representative. In **Figure 3.15 K-L**, the state of IL-10 transcriptional expression with and without JAKi treatment is illustrated. Fib*SN evidently upregulated IL-10 expression, while the effect of JAKi on the expression levels was not representative. **Figure 3.15 M-N** demonstrate the state of Arg-1 transcriptional expression with and without JAKi treatment, where Fib*SN upregulated Arg-1 expression, but the effect was evidently suppressed by JAKi.

Taken together, Fib*SN has strong effects of M2 induction and aIL-6 which can block IL-6 receptors was able to effectively reduce CD163 and IL-10 expression, but upregulated CD206. The JAK/STAT pathway inhibitor however can precisely suppress the effects of the M2 driving force of Fib*SN with CD163, CD206 and Arg-1 being downregulated. The weakness of the experiments is that secretion of cytokines cannot be measured without LPS addition.

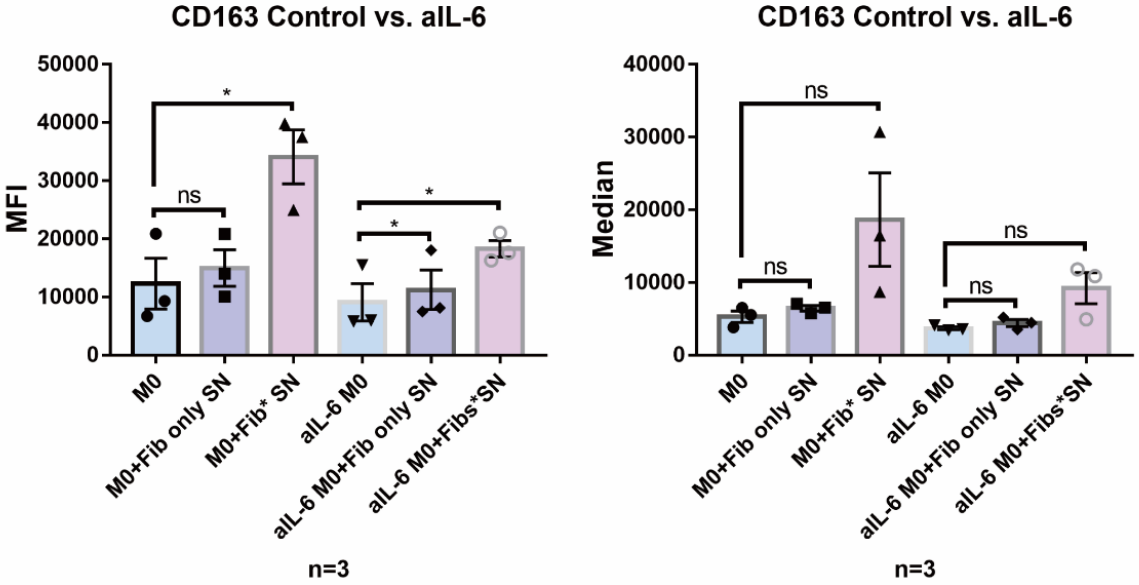
A


MED
Fib SN
Fib*SN
Monocytes
M-CSF
96h
M-CSF
48h
M-CSF
Wash
M0
Medium
aIL-6
72h
FACS

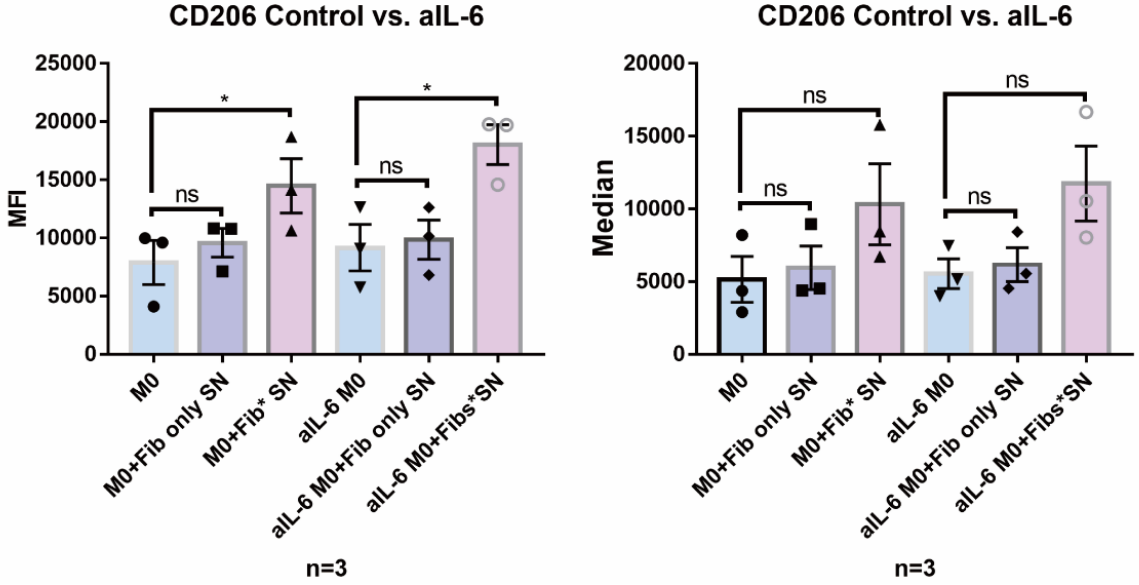
B


MED
Fib SN
Fib*SN
Monocytes
M-CSF
96h
M-CSF
48h
M-CSF
Wash
M0
DMSO
JAKi
72h
FACS

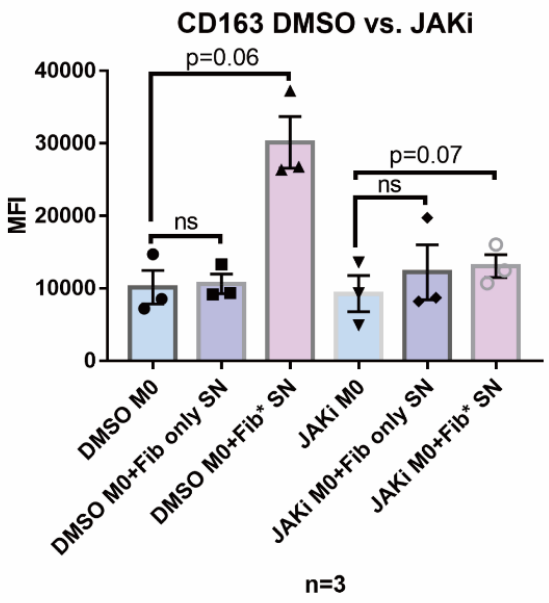
C

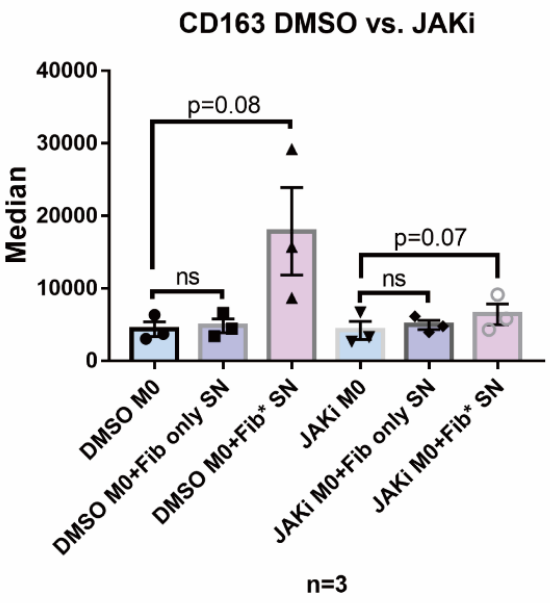

CD163 Control vs. aIL-6
MFI
50000
40000
30000
20000
10000
0
*
ns
*
*
M0
M0+Fib only SN
M0+Fib* SN
aIL-6 M0
aIL-6 M0+Fib only SN
aIL-6 M0+Fibs*SN
n=3
CD163 Control vs. aIL-6
Median
40000
30000
20000
10000
0
ns
ns
ns
ns
M0
M0+Fib only SN
M0+Fib* SN
aIL-6 M0
aIL-6 M0+Fib only SN
aIL-6 M0+Fibs*SN
n=3

D

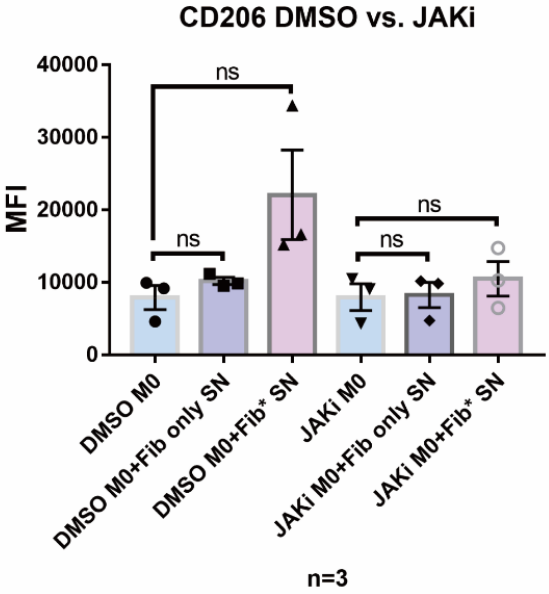

CD206 Control vs. aIL-6
MFI
25000
20000
15000
10000
5000
0
*
ns
ns
*
M0
M0+Fib only SN
M0+Fib* SN
aIL-6 M0
aIL-6 M0+Fib only SN
aIL-6 M0+Fibs*SN
n=3
CD206 Control vs. aIL-6
Median
20000
15000
10000
5000
0
ns
ns
ns
ns
M0
M0+Fib only SN
M0+Fib* SN
aIL-6 M0
aIL-6 M0+Fib only SN
aIL-6 M0+Fibs*SN
n=3

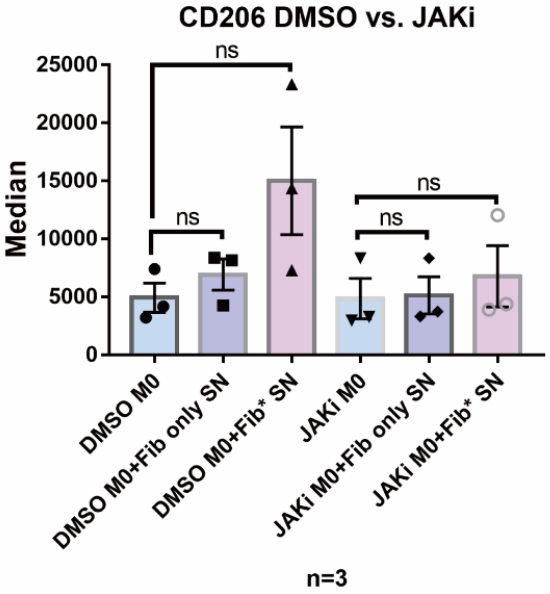
E


CD163 DMSO vs. JAKi
MFI
40000
30000
20000
10000
0
p=0.06
ns
p=0.07
ns
DMSO M0
DMSO M0+Fib only SN
DMSO M0+Fib* SN
JAKi M0
JAKi M0+Fib only SN
JAKi M0+Fib* SN
n=3

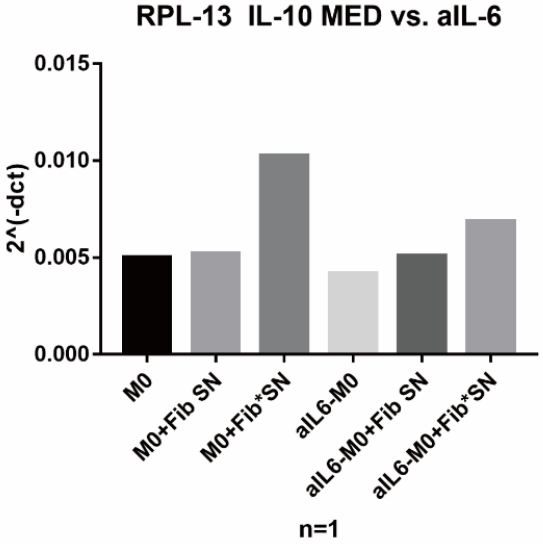

CD163 DMSO vs. JAKi
Median
40000
30000
20000
10000
0
p=0.08
ns
p=0.07
ns
DMSO M0
DMSO M0+Fib only SN
DMSO M0+Fib* SN
JAKi M0
JAKi M0+Fib only SN
JAKi M0+Fib* SN
n=3

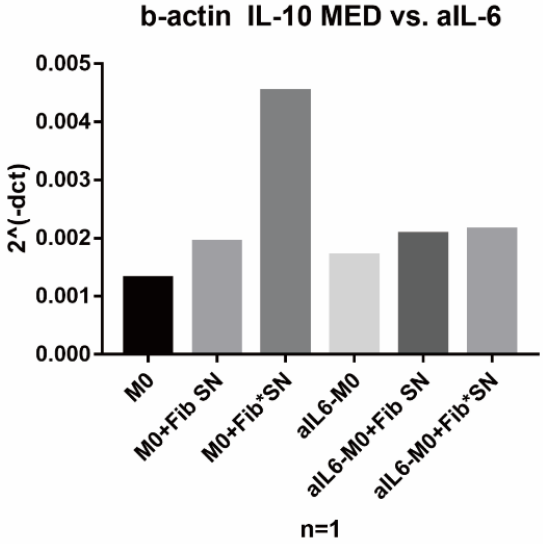
F


CD206 DMSO vs. JAKi
MFI
40000
30000
20000
10000
0
ns
ns
ns
ns
DMSO M0
DMSO M0+Fib only SN
DMSO M0+Fib* SN
JAKi M0
JAKi M0+Fib only SN
JAKi M0+Fib* SN
n=3

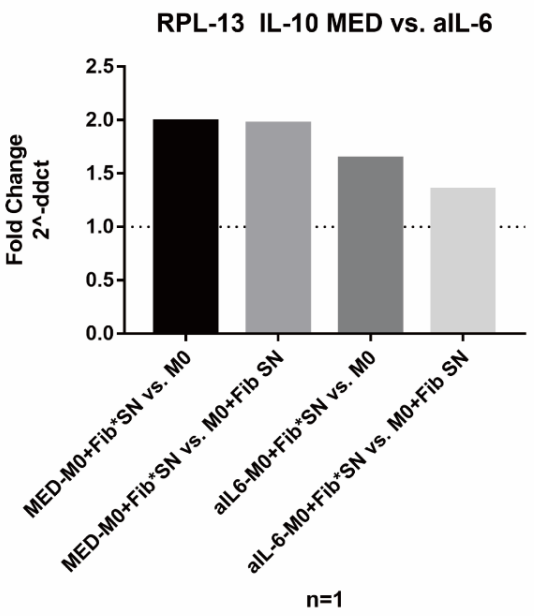

CD206 DMSO vs. JAKi
Median
25000
20000
15000
10000
5000
0
ns
ns
ns
ns
ns
DMSO M0
DMSO M0+Fib only SN
DMSO M0+Fib* SN
JAKi M0
JAKi M0+Fib only SN
JAKi M0+Fib* SN
n=3

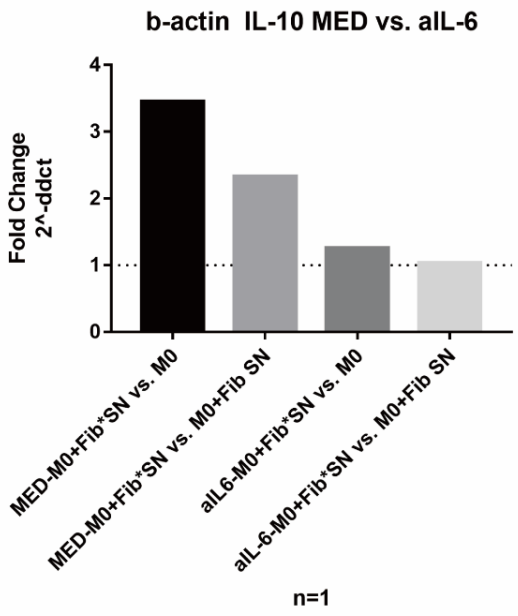
G


RPL-13 IL-10 MED vs. aIL-6
2^(-dct)
0.015
0.010
0.005
0.000
M0
M0+Fib SN
M0+Fib*SN
aIL6-M0
aIL6-M0+Fib SN
aIL6-M0+Fib*SN
n=1

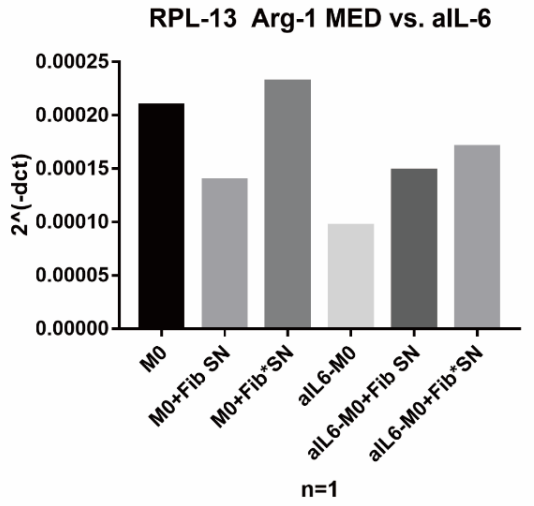

b-actin IL-10 MED vs. aIL-6
2^(-dct)
0.005
0.004
0.003
0.002
0.001
0.000
M0
M0+Fib SN
M0+Fib*SN
aIL6-M0
aIL6-M0+Fib SN
aIL6-M0+Fib*SN
n=1

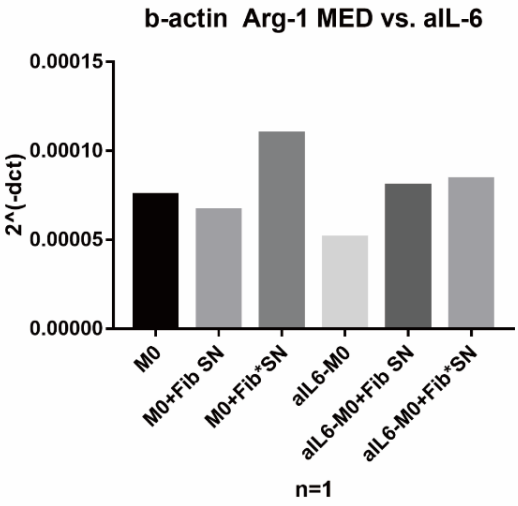
H


RPL-13 IL-10 MED vs. aIL-6
Fold Change 2^-ddct
2.5
2.0
1.5
1.0
0.5
0.0
MED-M0+Fib*SN vs. M0
MED-M0+Fib*SN vs. M0+Fib SN
aIL6-M0+Fib*SN vs. M0
aIL-6-M0+Fib*SN vs. M0+Fib SN
n=1

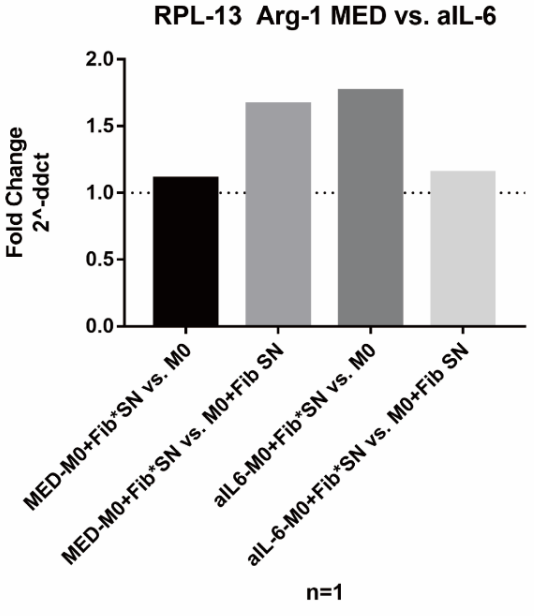

b-actin IL-10 MED vs. aIL-6
Fold Change 2^-ddct
4
3
2
1
0
MED-M0+Fib*SN vs. M0
MED-M0+Fib*SN vs. M0+Fib SN
aIL6-M0+Fib*SN vs. M0
aIL-6-M0+Fib*SN vs. M0+Fib SN
n=1

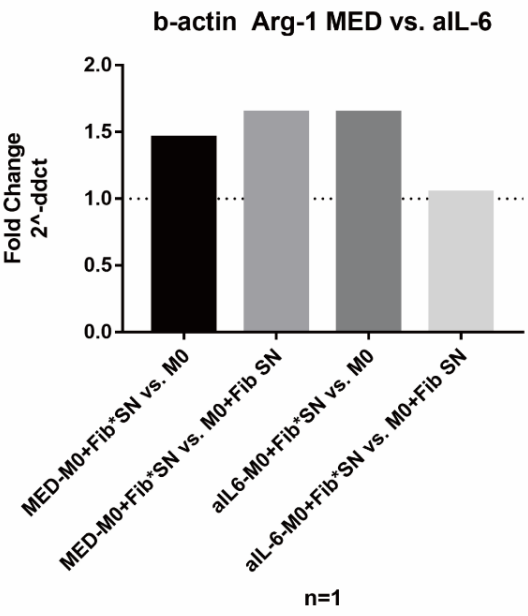
I


RPL-13 Arg-1 MED vs. aIL-6
2^(-dct)
0.00025
0.00020
0.00015
0.00010
0.00005
0.00000
M0
M0+Fib SN
M0+Fib*SN
aIL6-M0
aIL6-M0+Fib SN
aIL6-M0+Fib*SN
n=1

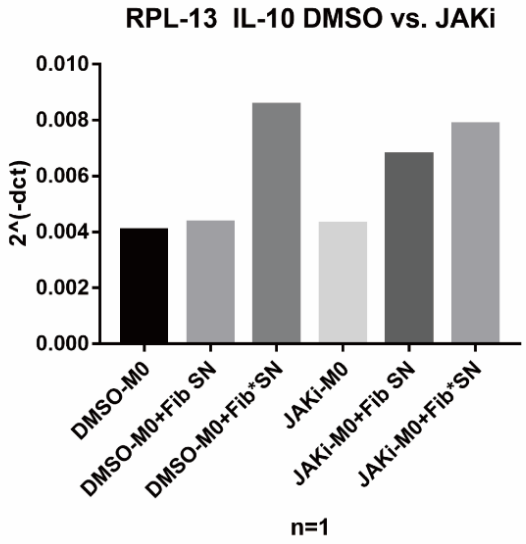

b-actin Arg-1 MED vs. aIL-6
2^(-dct)
0.00015
0.00010
0.00005
0.00000
M0
M0+Fib SN
M0+Fib*SN
aIL6-M0
aIL6-M0+Fib SN
aIL6-M0+Fib*SN
n=1

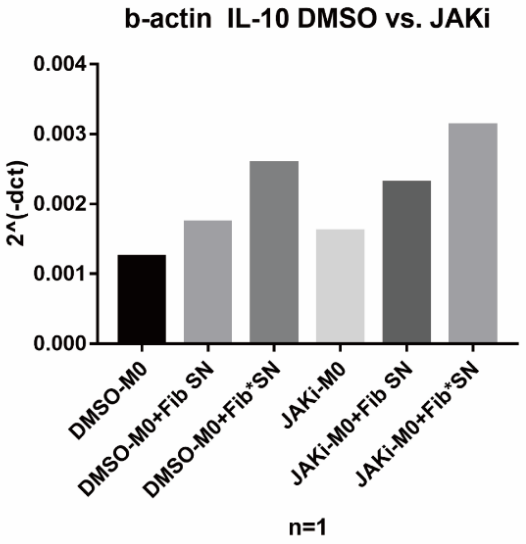
J


RPL-13 Arg-1 MED vs. aIL-6
Fold Change 2^-ddct
2.0
1.5
1.0
0.5
0.0
MED-M0+Fib*SN vs. M0
MED-M0+Fib*SN vs. M0+Fib SN
aIL6-M0+Fib*SN vs. M0
aIL-6-M0+Fib*SN vs. M0+Fib SN
n=1

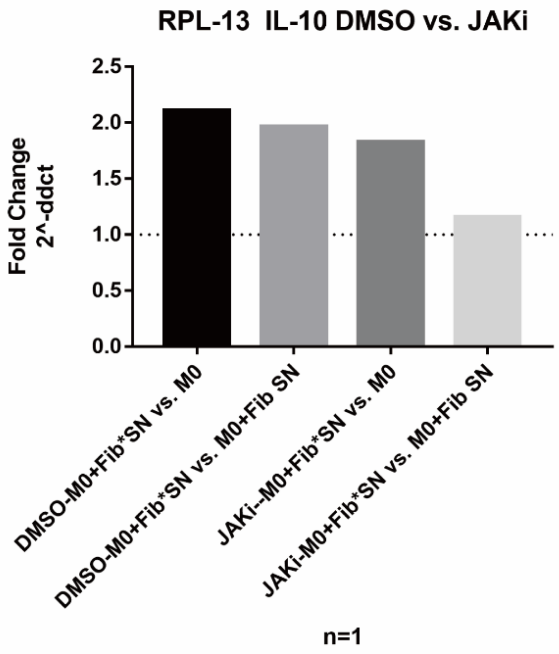

b-actin Arg-1 MED vs. aIL-6
Fold Change 2^-ddct
2.0
1.5
1.0
0.5
0.0
MED-M0+Fib*SN vs. M0
MED-M0+Fib*SN vs. M0+Fib SN
aIL6-M0+Fib*SN vs. M0
aIL-6-M0+Fib*SN vs. M0+Fib SN
n=1

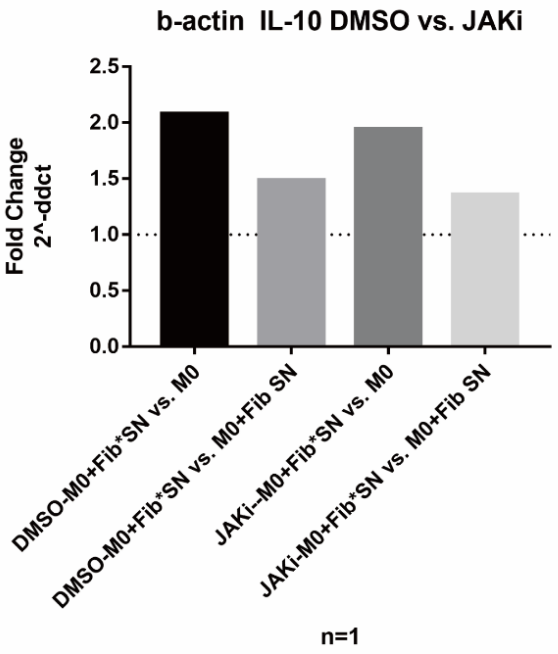
K


RPL-13 IL-10 DMSO vs. JAKi
2^(-dct)
0.010
0.008
0.006
0.004
0.002
0.000
DMSO-M0
DMSO-M0+Fib SN
DMSO-M0+Fib*SN
JAKi-M0
JAKi-M0+Fib SN
JAKi-M0+Fib*SN
n=1

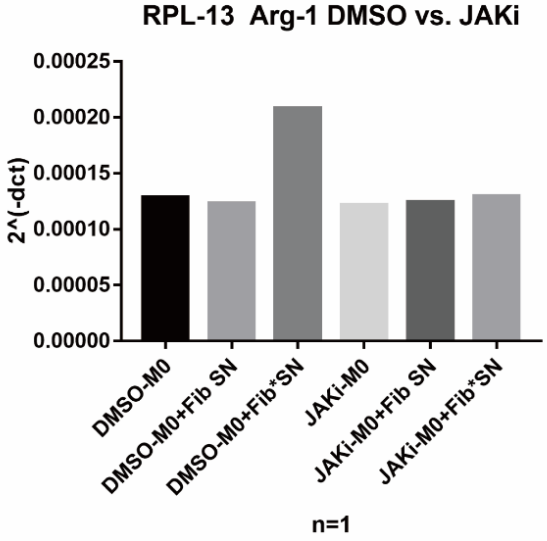

b-actin IL-10 DMSO vs. JAKi
2^(-dct)
0.004
0.003
0.002
0.001
0.000
DMSO-M0
DMSO-M0+Fib SN
DMSO-M0+Fib*SN
JAKi-M0
JAKi-M0+Fib SN
JAKi-M0+Fib*SN
n=1

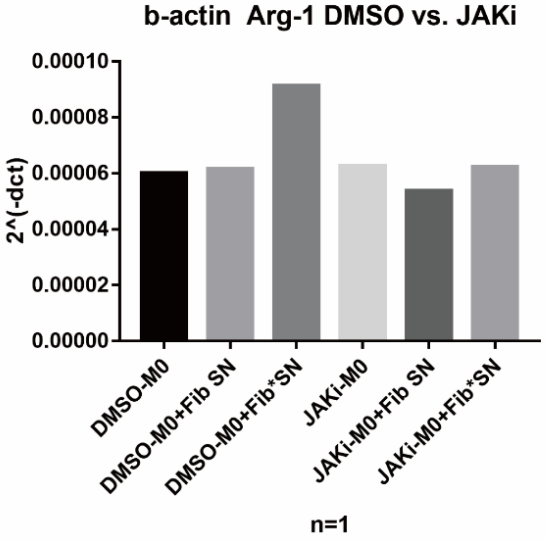
L


RPL-13 IL-10 DMSO vs. JAKi
Fold Change
2^-ddct
2.5
2.0
1.5
1.0
0.5
0.0
DMSO-M0+Fib*SN vs. M0
DMSO-M0+Fib*SN vs. M0+Fib SN
JAKi--M0+Fib*SN vs. M0
JAKi-M0+Fib*SN vs. M0+Fib SN
n=1


b-actin IL-10 DMSO vs. JAKi
Fold Change
2^-ddct
2.5
2.0
1.5
1.0
0.5
0.0
DMSO-M0+Fib*SN vs. M0
DMSO-M0+Fib*SN vs. M0+Fib SN
JAKi--M0+Fib*SN vs. M0
JAKi-M0+Fib*SN vs. M0+Fib SN
n=1

M


RPL-13 Arg-1 DMSO vs. JAKi
2^(-dct)
0.00025
0.00020
0.00015
0.00010
0.00005
0.00000
DMSO-M0
DMSO-M0+Fib SN
DMSO-M0+Fib*SN
JAKi-M0
JAKi-M0+Fib SN
JAKi-M0+Fib*SN
n=1


b-actin Arg-1 DMSO vs. JAKi
2^(-dct)
0.00010
0.00008
0.00006
0.00004
0.00002
0.00000
DMSO-M0
DMSO-M0+Fib SN
DMSO-M0+Fib*SN
JAKi-M0
JAKi-M0+Fib SN
JAKi-M0+Fib*SN
n=1

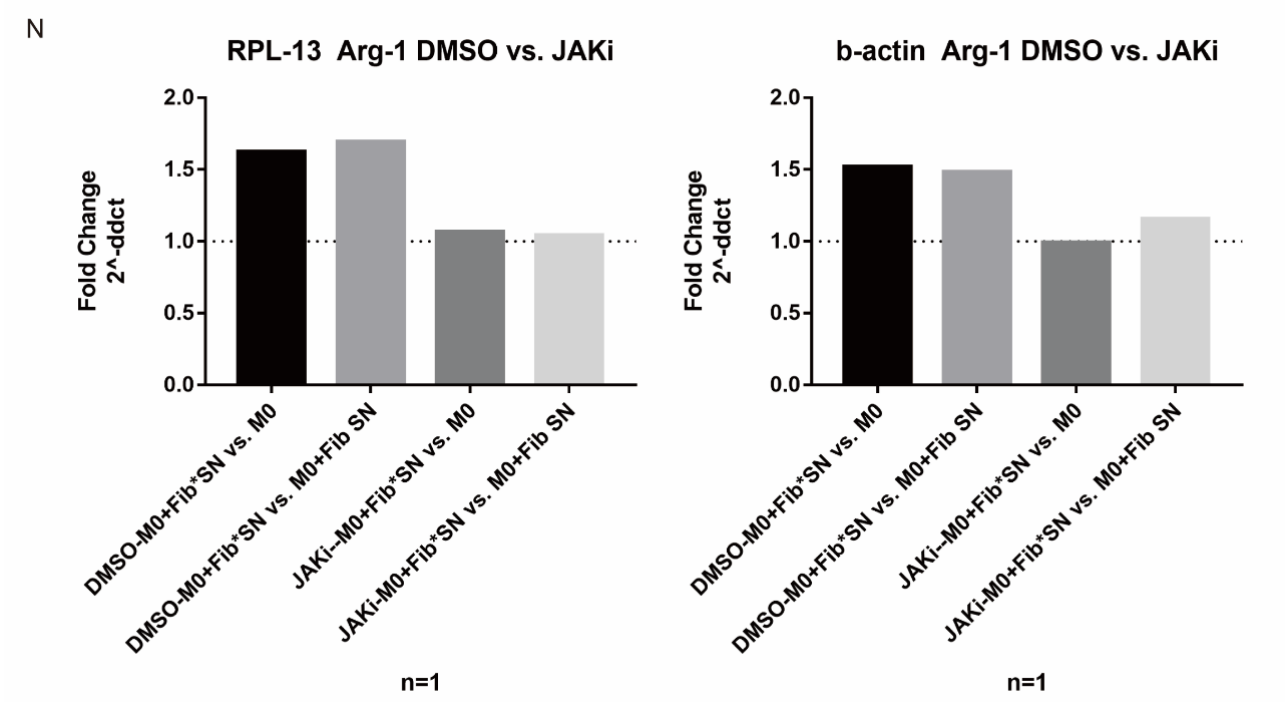


Figure 3.15: anti-IL6 and JAKi suppressed the effects of supernatant from TNFa+IL1b pre-stimulated fibroblasts on M0 macrophages. (A-B) Group settings and experimental setup. **(C-F)** Mean and median of fluorescence intensity of CD80, CD163 and CD206 measured by Flow Cytometer. (depicted is the mean ± SEM of n=3 independent experiments). **(G, I, K, M)** Transcriptional expression calculated with 2-dCt of IL-10 and Arg-1 assessed by qPCR. RPL-13 and beta-actin were used as house-keeping gene. **(H, J, L, N)** Fold change of transcriptional expression. P-values of mean and median of fluorescence intensity were calculated by Paired t test; ns = no significance, *: P<0.05.

3.4 The influence of M1 Macrophages on polarization of M0 Macrophages

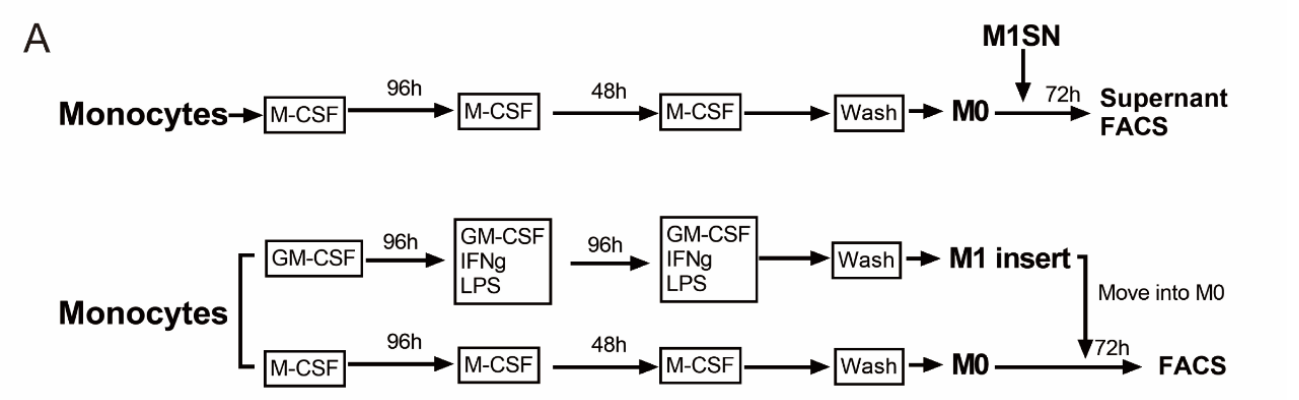
3.4.1 The Effects of M1-SN and M1 Transwell Co-culture on M0 Macrophages

To explore mutual regulation of Mph differentiation, preliminary experiments were conducted. **Figure 3.16 A** show the experiment setups. The experiment was divided into two parts: the first is incubation of M0 with M1-SN, and the second part consist of M0+M1 Transwell co-culture. M1-SN were harvested using the protocol in **Part 3.1.3** and it was added into M0 on day 7 of M0 culture, then cells

and culture supernatant were harvested another 3 days later for further measurement. Additionally, M1 were cultured in inserts using the protocol in **Part 3.2.3** and moved into M0 wells on day 7 of M0 culture. After another 3 days culture, the cells in the 24 well plate were harvested for further measurement.

The mean and median fluorescence intensity of CD80, CD163 and CD206 with M1-SN were presented in **Figures 3.16 B**. It suggests that the expression of CD80 was slightly downregulated compared to the M0 control. The expression of CD163 in the M0+M1-SN group diminished to around half of the M0 only control, while CD206 expression was clearly upregulated to around twice that of the M0 control. M0+M1 insert had little effect on CD80 expression, while it upregulated both CD163 and CD206 expression. **Figure 3.16 C** shows the TNFa concentration in the cell culture supernatant. The expression of TNFa was significantly downregulated in the M0+M1-SN group, even though the M1-SN added to the experiment contained a certain amount of TNFa.

Taken together, M1-SN has a M2 driving force on M0 Mphs. Mphs might have mutual regulatory potential that might contribute to their plasticity.



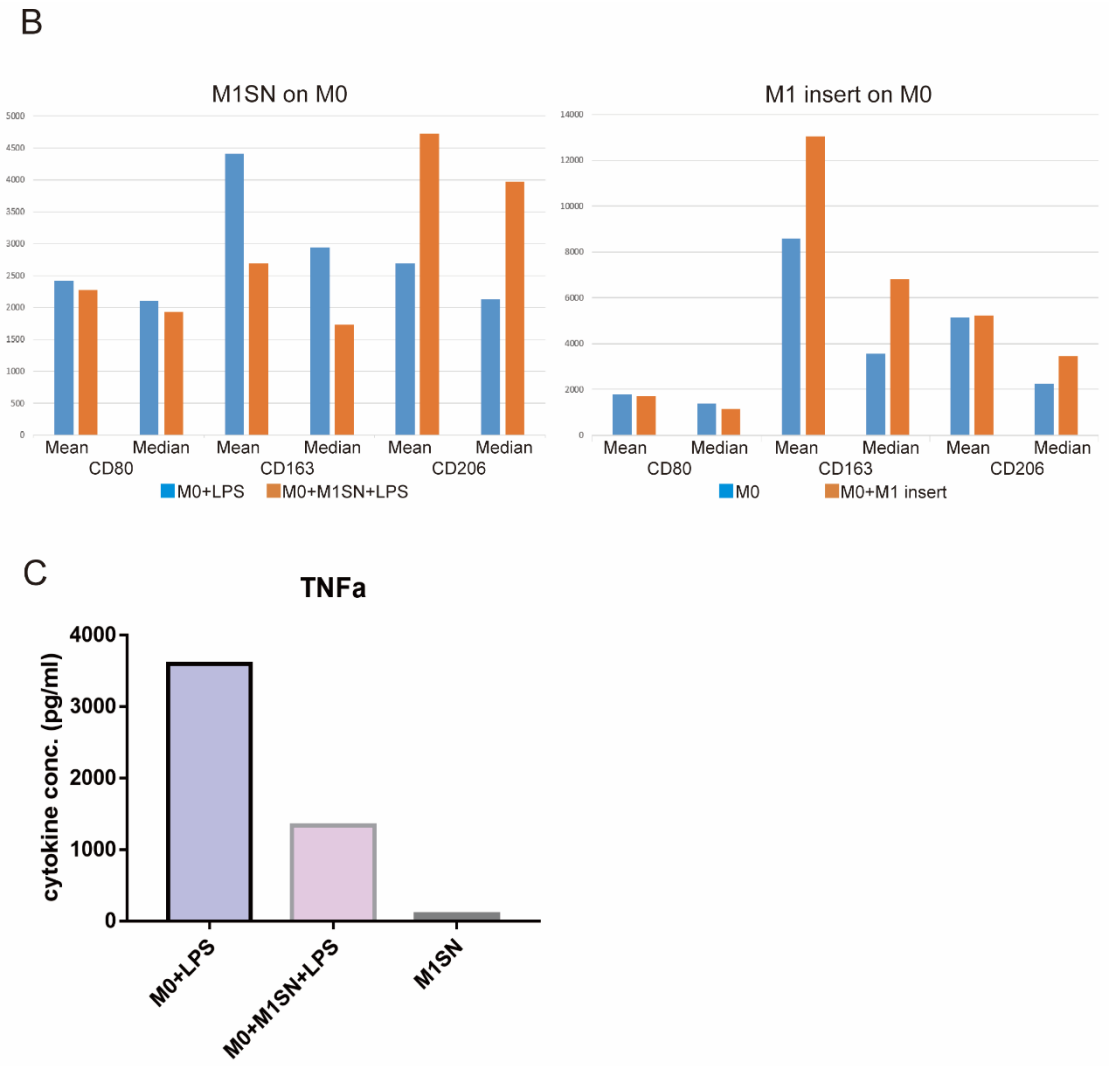


Figure 3.16: The Effects of M1-SN and M1 Transwell Co-culture on M0 Macrophages. (A) Group settings and experimental setup. **(B)** Mean and median of fluorescence intensity of CD80, CD163 and CD206 measured by Flow Cytometer. **(C)** Cytokines expression of TNFa measured by ELISA.

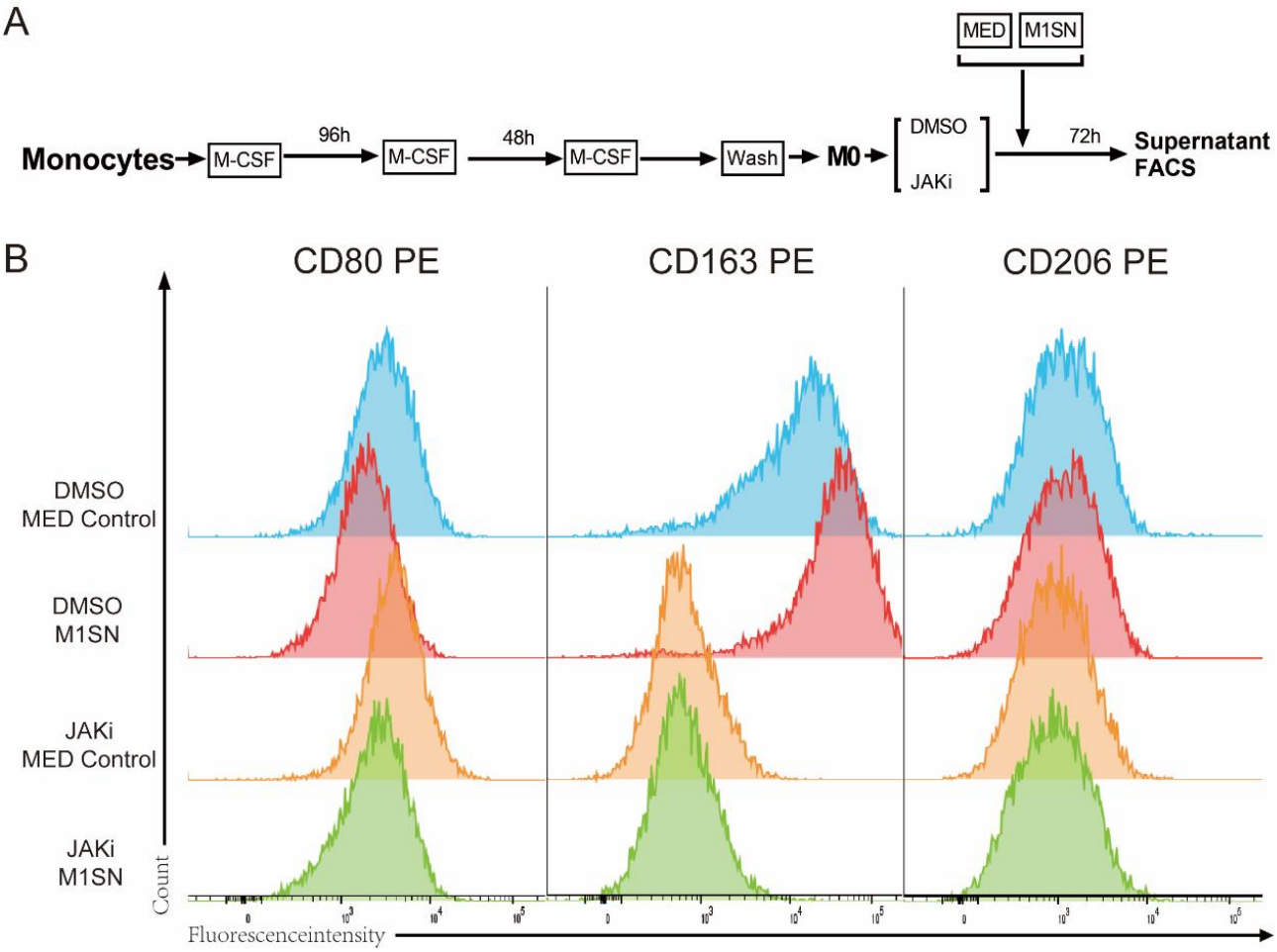
3.4.2 Janus kinase inhibitors (JAKi) interfere with the M1 induced polarization of M0 to M2

In **Part 3.4.1**, a mutual regulation of Mphs was primarily observed with the expression of CD163 and CD206 upregulation when M0 interacted with M1-SN or by M1 coculture in inserts. JAK/STAT signaling pathway was proven before to be involved in CD163 and CD206 induction of Mphs with Fib*SN presence (**Part 3.3.3**). To further prove the effects of M1-SN on regulation of Mphs and reveal the mechanism of this phenomenon, the experiments were repeated with JAKi (Upadacitinib). **Figure**

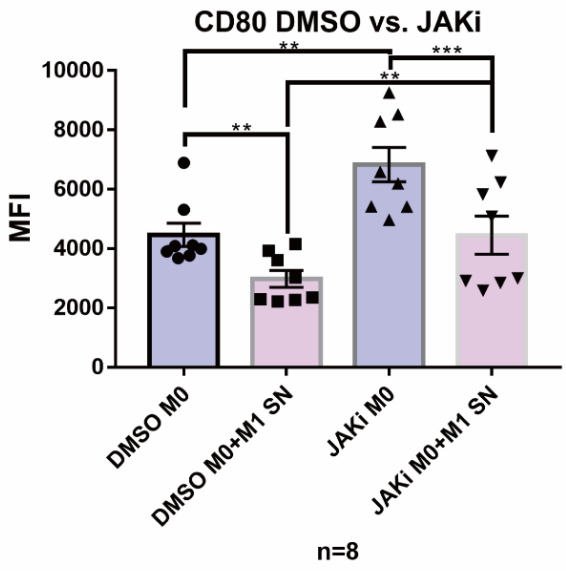
3.17 A shows the experiment setups and group settings. M0 Mphs were cultured until the day 7 with the former protocol, and then pre-treated with JAKi and DMSO control respectively, then M1-SN and MED control were added into DMSO-M0 and JAKi M0. LPS was added into MED control with the same percentage as M1-SN at the same time. JAKi and DMSO were repeatedly added into each group on day 8 and day 9. Cells and supernatants were harvested on day 10 for further measurement. **Figure 3.17 B** demonstrates a representative histogram of CD80, CD163 and CD206 expression measured by flow cytometry. The fluorescence intensity of CD80 in the DMSO-M0+M1-SN group was relatively lower compared with the DMSO-M0 Control, while it clearly increased with presence of JAKi. The expression of CD163 was upregulated in the DMSO-M0+M1-SN group compared to the control group, while its expression was extremely inhibited in the JAKi groups. Expression of CD206 fluorescence intensity was hardly influenced. **Figure 3.17 C-E** presents the mean and median fluorescence intensity of CD80, CD163 and CD206 expression. In total 4 independent experiments were conducted. The expression of CD80 was around 1/3 downregulated in DMSO-M0+M1-SN group compared to DMSO-M0 control group; regarding the comparison between DMSO-M0 control and JAKi-M0 control, CD80 was evidently higher expressed in the JAKi group than in the DMSO-M0 control, and the effect of CD80 downregulation by M1-SN was also suppressed with JAKi presence. CD163 was upregulated in presence of M1-SN, as expected, but its expression was hardly affected by JAKi, and the gap between DMSO-M0+M1-SN and DMSO-M0 control was clearly smaller. The expression of CD206 was not evidently interfered by M1-SN and JAKi. **Figure 3.17 F-I** demonstrates the cytokine expression of IL-6, IL-10 and TNFa. The expression of the pro-inflammatory cytokines IL-6 and TNFa showed a positive correlation with CD80 expression. IL-6 expression in DMSO-M0+M1-SN group was downregulated to around 1/5 of the DMSO M0 control; IL-6 expression in JAKi-M0 group surged to around 3 times above the DMSO-M0 control in presence of JAKi; even though IL-6 expression in the JAKi-M0+M1-SN group was also downregulated compared with JAKi-M0 group, the gap between the JAKi-M0 and JAKi-M0+M1-SN groups increased much higher than between DMSO-M0 control and DMSO-M0 groups. The concentration of TNFa revealed a similar trend as the results of IL-6. The expression of TNFa that was significantly upregulated by JAKi was also downregulated by M1-SN in both the DMSO groups and JAKi groups. IL-10 was evidently upregulated in presence of M1-SN. There was a slight downregulation by JAKi but this was not significant.

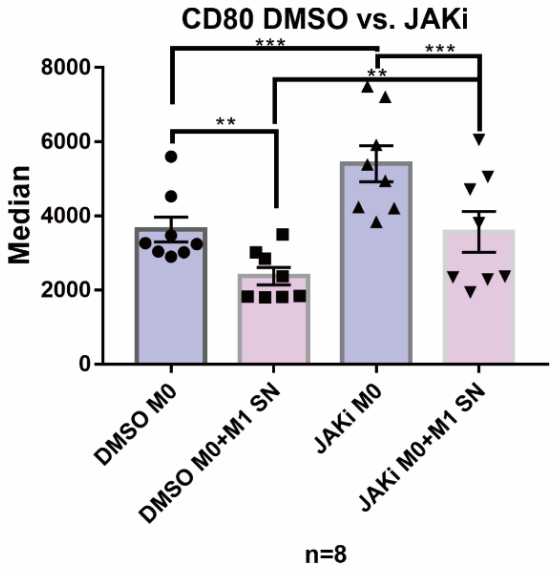
Taken together, CD163 as a specific surface marker of M2 Mphs were significantly upregulated by M1-SN, and the pro-inflammatory cytokines--IL-6 and TNFa were evidently downregulated with M1-SN presence. On the other hand, JAKi forcefully suppressed the effects of M1-SN on M0 Mphs. Addition of JAKi decreased M1-mediated upregulation of CD163 and inhibited M1-mediated downregulation of pro-inflammatory cytokines--IL-6 and TNFa.

In summary, the experiments revealed a regulatory effect of M1 Mphs on polarization of M0 Mphs towards M2 and this effect appears at least partially dependent on the JAK1 signaling pathway.

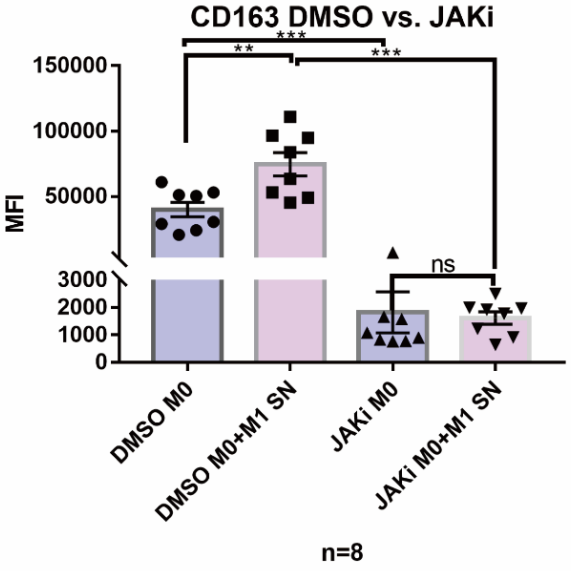


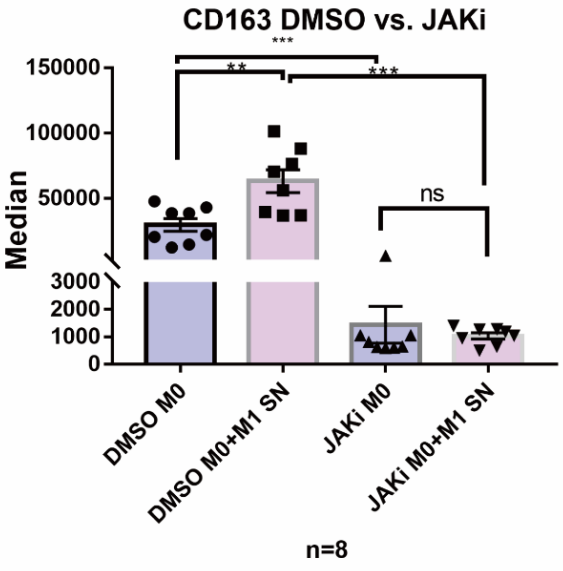
C


CD80 DMSO vs. JAKi
MFI
10000
8000
6000
4000
2000
0
**
**
**
***
DMSO M0
DMSO M0+M1 SN
JAKi M0
JAKi M0+M1 SN
n=8

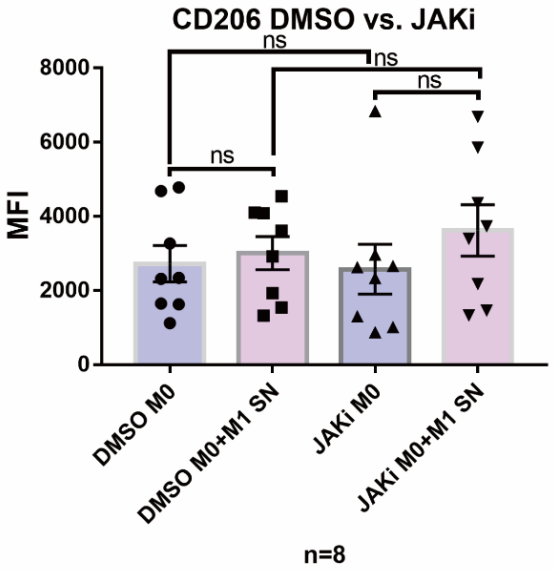

CD80 DMSO vs. JAKi
Median
8000
6000
4000
2000
0
***
**
**
***
DMSO M0
DMSO M0+M1 SN
JAKi M0
JAKi M0+M1 SN
n=8

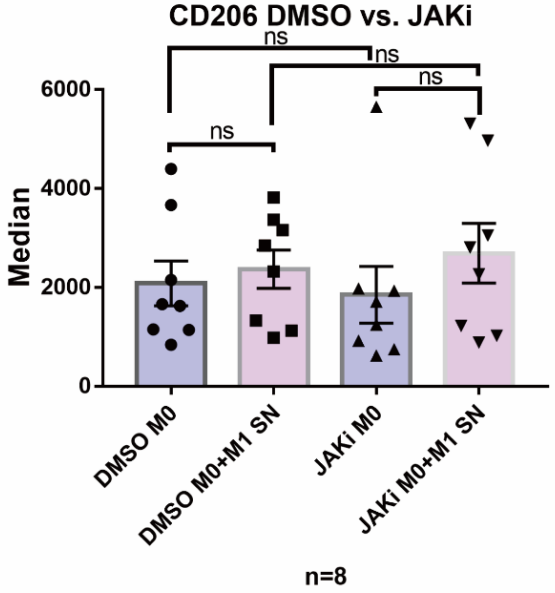
D


CD163 DMSO vs. JAKi
MFI
150000
100000
50000
3000
2000
1000
0
**
***
***
ns
DMSO M0
DMSO M0+M1 SN
JAKi M0
JAKi M0+M1 SN
n=8


CD163 DMSO vs. JAKi
Median
150000
100000
50000
3000
2000
1000
0
***
**
***
ns
DMSO M0
DMSO M0+M1 SN
JAKi M0
JAKi M0+M1 SN
n=8

E


CD206 DMSO vs. JAKi
MFI
8000
6000
4000
2000
0
ns
ns
ns
ns
DMSO M0
DMSO M0+M1 SN
JAKi M0
JAKi M0+M1 SN
n=8


CD206 DMSO vs. JAKi
Median
6000
4000
2000
0
ns
ns
ns
ns
DMSO M0
DMSO M0+M1 SN
JAKi M0
JAKi M0+M1 SN
n=8

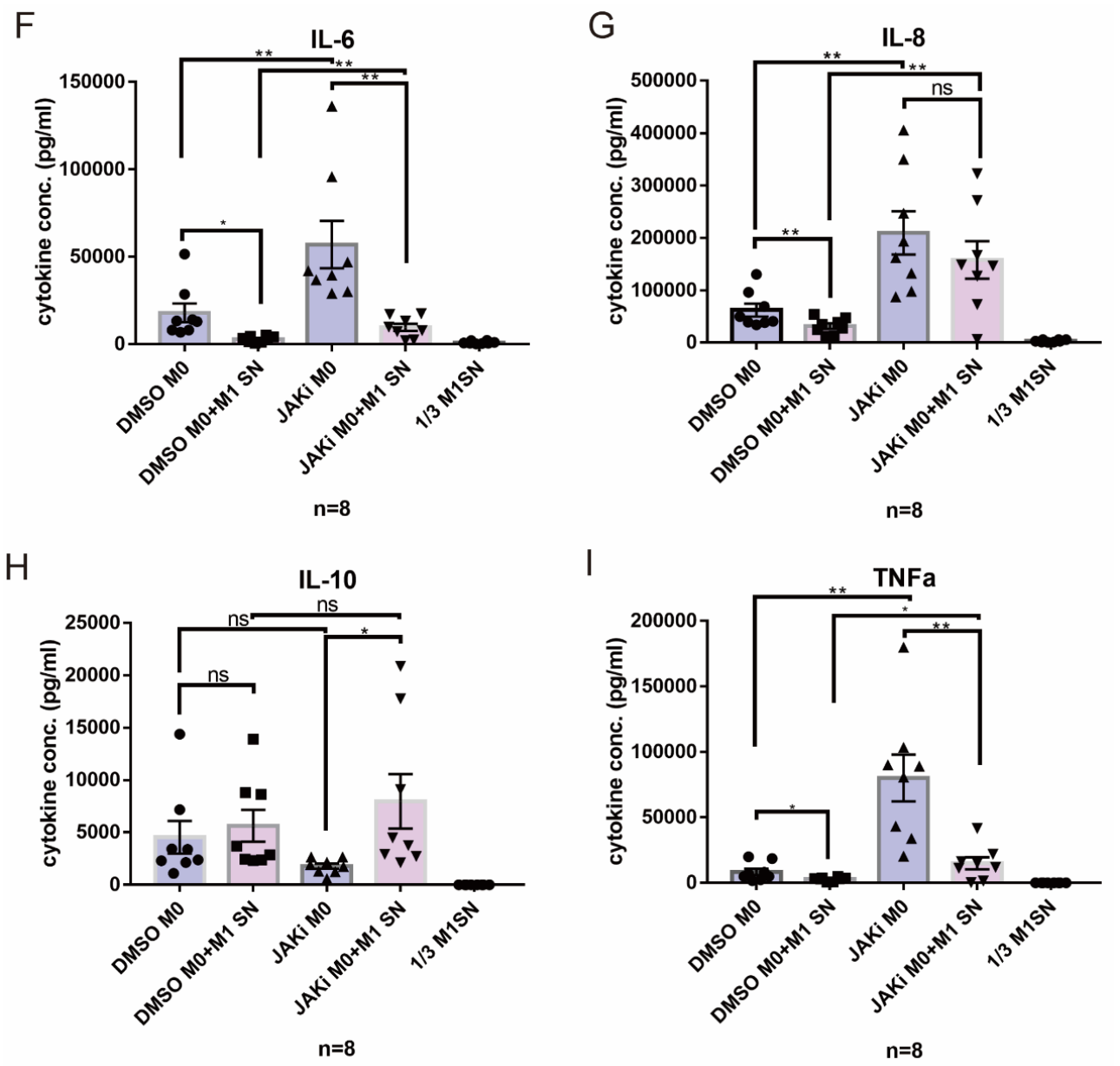
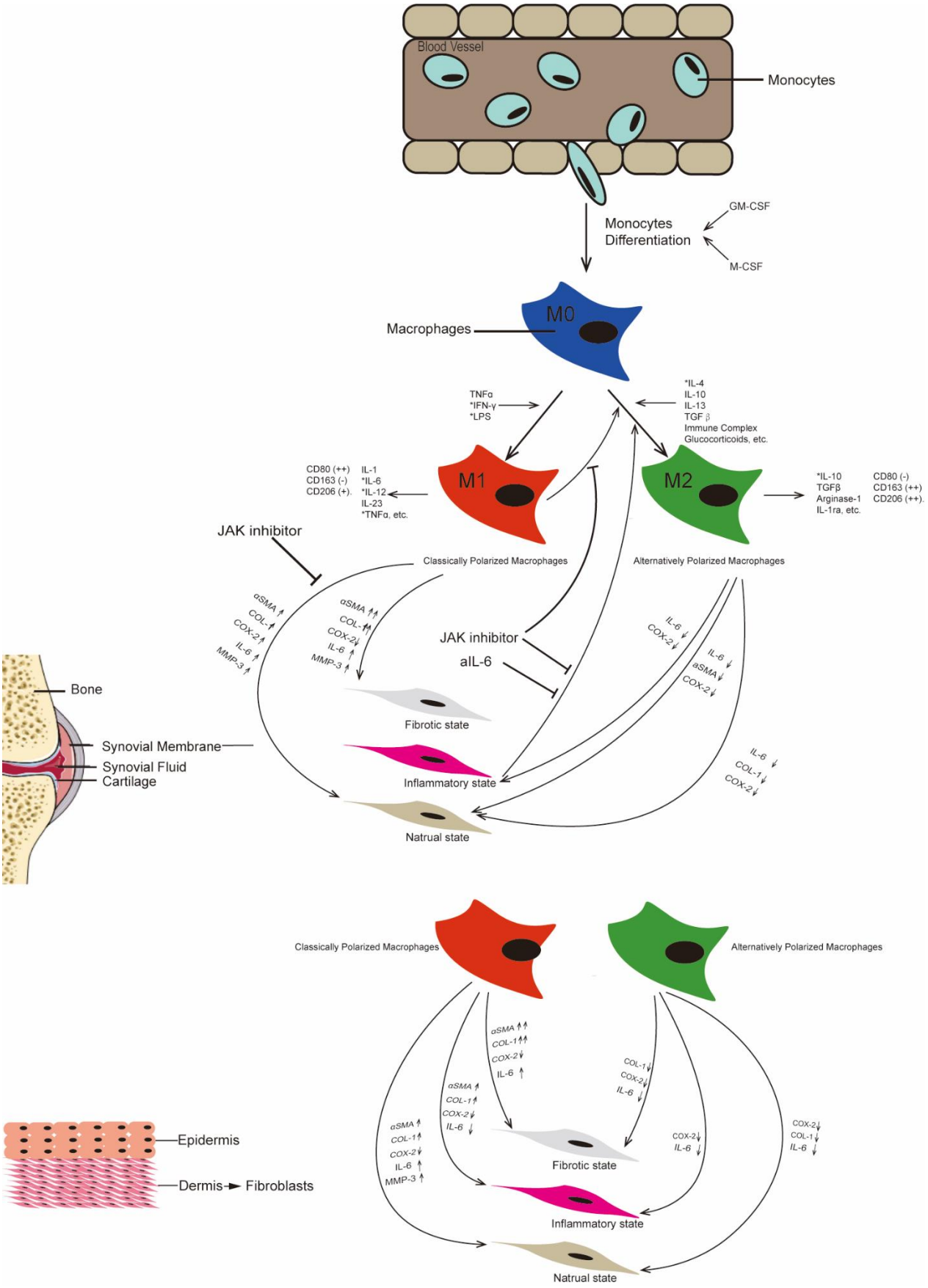


Figure 3.17: JAKi Inhibited the M1 induced polarization of M0 to M2. (A) Group settings and experimental setup. **(B)** Representative histogram of M0 expression of CD80, CD163 and CD206. **(C-E)** Mean and median fluorescence intensity of CD80, CD163 and CD206 measured by Flow Cytometer. (depicted is the mean ± SEM of n=8 independent experiments). **(F-I)** Cytokines expression of IL-6, IL-10 and TNFa measured by ELISA. (depicted is the mean ± SEM of n=8 independent experiments). P-values were calculated by paired t-test, and results are denoted as follows: ns = no significance, *: P<0.05, **: P<0.01, ***P<0.001.

Below is an **illustrated summary** for this project.


Blood Vessel
Monocytes
GM-CSF
Monocytes Differentiation
M-CSF
Macrophages
M0
TNFα
*IFN-γ
*LPS
*IL-4
IL-10
IL-13
TGF β
Immune Complex
Glucocorticoids, etc.
CD80 (++)
CD163 (-)
CD206 (+).
IL-1
*IL-6
*IL-12
IL-23
*TNFα, etc.
M1
M2
*IL-10
TGFβ
Arginase-1
IL-1ra, etc.
CD80 (-)
CD163 (++)
CD206 (++).
JAK inhibitor
Classically Polarized Macrophages
Alternatively Polarized Macrophages
αSMA
COL-1
COX-2
IL-6
MMP-3
JAK inhibitor
aIL-6
Fibrotic state
Inflammatory state
Natrual state
Bone
Synovial Membrane
Synovial Fluid
Cartilage
Classically Polarized Macrophages
Alternatively Polarized Macrophages
Fibrotic state
Inflammatory state
Natrual state
Epidermis
Dermis
Fibroblasts

4 Discussion

4.1 The Influence of Differently Polarized Macrophages on Fibroblasts.

Synovial fibroblasts and macrophages are both important cell types that play a role in the pathogenesis of various joint diseases, including osteoarthritis (OA) and rheumatoid arthritis (RA)(Bernabei et al. 2023; Patel et al. 2023; Tofigh et al. 2023). This study explored how differently polarized macrophages **impact** differently activated synovial fibroblasts, in order to reveal the mechanism which macrophages involved in the progression of OA or RA in different stages.

4.1.1 The Effects of M1 Macrophages on Fibroblasts.

M1 macrophages, which secrete pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFa), IL-1b had significant pro-inflammatory effects on synovial fibroblasts in transwell cocultures. This was proven by the upregulation of cyclooxygenase-2 (COX-2), and matrix metalloproteinase-3 (MMP-3) in synovial fibroblasts after co-culture with M1. and fits to the current understanding of the role of macrophages and TNF/IL-1b, respectively, during inflammation in the rheumatic joint (Bilsborrow et al. 2019). IL-6 as a marker for activated fibroblasts which contributes to both pro-inflammatory (Houssiau et al. 1988) and pro-fibrotic (Yang et al. 2022) effects was also upregulated by M1 co-culture.

Of note, aSMA and Col-1 were downregulated in the M1-SF Transwell co-culture assay, at first suggesting that M1 macrophages may have anti-fibrotic effects on synovial fibroblasts in this setting. However, these results stand in contrast to the results of experiments where where M1-SN was used, While M1-SN still induced upregulateion of MMP-3 and IL-6, similar like the coculture, aSMA and COL-1 were clearly upregulated. This was also confirmed in experiments where M1-SN was added to dermal fibroblasts.Moreover in dermal Fib COX_2 was even downregulated..

Interestingly, experiments on time curves revealed a possible reason why the SF-M1SN co-culture assay and M1-SF Transwell co-culture assay showed opposite outcomes for aSMA and Col-1 markers. Specifically, aSMA and Col-1 initially showed a downtrend from the beginning of the experiments to

the 12-hour time point, but then this trend reversed to an uptrend from the 48-hour test time point to the end of the experiments. The concentration of IL-6 showed a sharp surge from the beginning to the 12-hour time point, but then the trend became gentler. These findings suggest that the pro-fibrotic effects of SF may be dependent on the concentration of IL-6, and that IL-6 plays a key role in the pro-fibrotic process of SF. These results may also explain why the M1-SF Transwell co-culture assay showed an anti-fibrotic phenomenon. Specifically, the Transwell co-culture assay may require a much longer co-culture period in order to allow for the accumulation of IL-6 and the subsequent pro-fibrotic effects on synovial fibroblasts. In terms of COX-2, it showed a sharp decrease from the beginning to the 12-hour test point, after which the downtrend became much gentler. The variation of COX-2 and pro-fibrotic markers such as aSMA and Col-1 appeared to be anti-correlated, which may explain why COX-2 was downregulated after M1SN co-culture in the group of SF pre-stimulated with TGFb (Fib$^{\#}$), where the pro-fibrotic effects of M1SN were much more evident.

The use of SF pre-stimulated with TNFa+IL1b (Fib*) simulated an active phase of OA or RA, while the use of TGFb prestimulated Fib (Fib$^{\#}$) simulated a later phase of OA or RA(Maini et al. 1995; Noack and Miossec 2017; Zhang et al. 2021), where hyperplasia of the synovial membrane and fibrotic effects are much more evident and reflected in joint stiffness (Liao et al. 2021; Remst et al. 2015; Sarkar et al. 2022). In both of these parts, M1 macrophages played an important role in promoting disease progression of OA or RA. These findings suggest that M1 macrophages may be a key player in the pathogenesis of OA or RA, with distinct effects on synovial fibroblasts depending on the stage of the disease. Targeting M1 macrophages or their related signaling pathways may represent a potential therapeutic strategy for the treatment of OA or RA.

JAK inhibitor (Upadacitinib) was used before in previous studies for treating fibroblasts of different origin (Wang et al. 2020) The current study now explored whether the JAKi was also effective on Fib in presence of pro-inflammatory or pro-fibrotic effects of M1. The presence of JAKi led to a suppression of almost all the related markers measured in these experiments, including aSMA, COX-2, Col1, and IL-6. These findings suggest that the pro-inflammatory and pro-fibrotic effects of M1 macrophages on SF are regulated via the JAK/STAT signaling pathway. This study provides further support for the feasibility of using Upadacitinib as a first-line clinical drug in the treatment of RA or a

potential clinical drug in the treatment of OA. Targeting the JAK/STAT signaling pathway may represent an effective strategy for inhibiting the pro-inflammatory and pro-fibrotic effects of M1 macrophages on SF, which may in turn release pain and stiffness in patients with OA or RA.

4.1.2 The Effects of M2 Macrophages on Synovial Fibroblasts.

M2 macrophages are a distinct group of macrophages that are generally associated with anti-inflammatory functions with the properties such as the production of anti-inflammatory cytokines, including IL-10, and are involved in tissue repair and regeneration. M2 macrophages are typically activated in response to anti-inflammatory signals (Wynn and Vannella 2016).

This study investigated the effects of M2 macrophages on synovial fibroblasts, and it revealed rather opposite effects compared to M1 macrophages. Specifically, in the resting fibroblasts group, aSMA and COX-2 were both downregulated in the presence of M2 macrophage supernatant (M2SN). However, the much meaningful effects were observed in the Fib* groups, which consisted of fibroblasts pre-stimulated with TNFa+IL1b to simulate an active phase of OA or RA. In this group, M2 macrophages effectively downregulated COX-2 expression, suggesting that M2 macrophages play an anti-inflammatory role in the inflammatory phase of SF. In the Fib$^{\#}$ group, which was used to simulate a fibrotic phase of OA or RA, M2SN downregulated one of the fibroblast fibrotic markers (Col-1), indicating that M2SN also played an anti-fibrotic role in the fibrotic phase of synovial fibroblasts.

4.1.3 The Effects of Macrophages on Dermal Fibroblasts.

Fibrosis is a typical character of systemic sclerosis (SSc) (Jinnin 2010). In SSc, dermal fibroblasts (DF) become overactivated and produce excessive amounts of ECM components, leading to fibrosis in the skin and other tissues(Kowal-Bielecka et al. 2017). In order to investigate the effects of macrophages on DF, the same experimental methods were conducted on DF as well.

In this part of the study, M1 macrophage supernatant was found to evidently upregulate the

expression of aSMA and Col-1 in each group, with particularly strong effects on aSMA expression in Fib# group and Col-1 expression in Fib* group. The expression of COX-2 were hardly downregulated in each group. In other words, M1 macrophages played a pro-fibrotic role in the progression of dermal fibrosis. The study also used a JAK inhibitor (Upadacitinib) to investigate whether the JAK/STAT signaling pathway regulated the effects of M1 macrophages on DF. However, the results showed that JAKi only downregulated the expression of IL-6 and COX-2, and did not suppress other pro-fibrotic markers. These findings suggest that the pro-fibrotic effects of M1 macrophages on dermal fibroblasts may be regulated by pathways other than the JAK/STAT signaling pathway.

In addition, in Fib# group, which simulated a fibrotic state of dermal fibroblasts, M2 macrophages were found to successfully downregulate the expression of Col-1. This indicates that M2 macrophages may also have anti-fibrotic effects on DF. The ability of M2 macrophages to modulate the fibrotic response of dermal fibroblasts suggests that targeting the M2 macrophage may represent a potential therapeutic strategy for the treatment of dermal fibrosis.

Taken together, M1 and M2 macrophages as two polarized macrophages phenotypes which presented opposite effects on SF and DF. M1 macrophages could take part in the process of OA or RA, and JAK/STAT signaling pathway regulated the process with Upadacitinib effectively suppressed the effects. M1 macrophages were involved in the progression of SSc as well, with the significant pro-fibrotic effects on DF. However, M2 played a role of counterpart of M1, with its properties of anti-inflammatory and anti-fibrotic effects.

Throughout the study, differently polarized macrophages or macrophage supernatants were utilized. Polarization refers to the process by which cells become divided into two or more distinct, opposing subgroups. This study aimed to investigate the effects of macrophages in polarized states on fibroblast. According to the experimental results of this study, in the resting fibroblasts groups, M1 and M2 macrophages showed an unbiased, opposite effect on fibroblasts. In the Fib* groups, M2 macrophages more prominently exhibited anti-inflammatory effects, while in the Fib# groups, M2 macrophages more prominently exhibited anti-fibrotic effects. These findings suggest that in different extreme microenvironments in vitro, macrophages from healthy donors almost intelligently display its

most appropriate role.

Macrophages from healthy donors can display targeted effects, however, it is currently unknown whether monocytes from patients with different rheumatic diseases such as osteoarthritis (OA), rheumatoid arthritis (RA), or systemic sclerosis (SSc) have similar differentiation or polarization conditions. The human immune system is extremely complex. In RA, CD4+ T cells, also known as T helper cells, are believed to be crucial in its development(Cope et al. 2007). Upon activation, CD4+ T cells can become activated and differentiate into various subtypes, including Th1, Th2, and Th17 cells, which produce cytokines such as IFN-γ, IL-4, and IL-17, respectively. These cytokines can activate other immune cells, leading to the production of autoantibodies and the chronic inflammation and tissue damage characteristic of RA. NK cells from SSc patients have been found to produce increased levels of cytokines, including interferon-gamma (IFNg) and TNFa, which promote inflammation and tissue damage(Conigliaro et al. 2011).

The immune system of patients with different diseases may have a markedly different immune microenvironment compared to healthy donors. Within this complex immune microenvironment, it is unclear whether the monocytes from patients with various rheumatic diseases can differentiate or polarize into macrophage phenotypes that are necessary in their respective immune microenvironments.

4.2 The influence of differently activated fibroblasts on polarization of Macrophages

Polarized macrophages display strong targeted effects in response to special in vitro microenvironments, such as Fib*-simulated inflammatory environments and Fib#-simulated fibrosis environments. Research findings indicate that macrophage polarization states include multiple subtypes, such as M1, M2a, M2b, and M2c (Colin, Chinetti-Gbaguidi, & Staels). However, the human body's microenvironment is extremely complex, and macrophage states will not solely exist in the polarized state and play their roles.

M2 macrophages are known as a group of anti-inflammatory phenotypes, with secretion of IL-4, IL-10, Arginase-1 and TGFb(Xu et al. 2023). They play important roles in promoting tissue repair and regeneration.

The results of the current study showed that Fib* led to the upregulation of M2 macrophage-specific markers, including CD163 and CD206, while pro-inflammatory cytokines TNFa and IL-6 were downregulated in the presence of Fib*SN. This suggests that the inflammatory cytokines stimulated SF induced the differentiation of macrophages from M0 to M2, resulting in the performance of anti-inflammatory effects in vitro. The mechanism highlights the plasticity of macrophages to differentiate into an anti-inflammatory phenotype in the joint inflammatory environment, thereby exerting their anti-inflammatory effects.

With the presence of anti-IL6 (Tocilizumab), CD163 expression was significant downregulated, while the expression of CD206 was slightly upregulated. The pro-inflammatory cytokines expression of TNFa and IL-6 overall decreased, while the ability of aIL-6 treated Fib*-M2 still kept the ability of downregulation of TNFa and IL-6 compared to control groups. The expression of CD206 was not suppressed by anti-IL6, instead it was slightly upregulated. Combined with the results of cytokines secretion, CD206 could played a compensatory role with anti-IL6 presence in order to keep the anti-inflammatory ability. In other words, IL-6 appears to be the key factor in the M0-M2 induction mechanism under Fib*SN stimulation, and the use of an anti-IL6 drug like Tocilizumab has few side effects on the process of M0-M2 differentiation under an inflammatory microenvironment.

Upadatinib (JAKi) was also utilized in this study. After treatment with JAKi, the expression of both CD163 and CD206 was significantly suppressed overall. Regarding cytokines, the expression of IL-6 and TNFa surged to several times higher than the DMSO-control groups, and the ability of Fib*SN to reduce IL-6 and TNFa expression was weakened, although Fib*SN still decreased their expression.

Upadacitinib is widely used as a first-line clinical treatment for RA, with its mechanism of JAK inhibition, specifically JAK1, that preferentially activates STAT3.. According to the results of the last part of the project, JAKi significantly suppressed the pro-inflammatory effects of M1SN on synovial fibroblasts.

However, it also inhibited the process of differentiation from M0 to M2 when interacting with Fib*SN. In other words, the use of JAKi to treat diseases such as RA may block the differentiation of the anti-inflammatory phenotype of macrophages, potentially placing a significant burden on the human immune system.

4.3 The mutual regulation of Macrophage polarization

The results from the last part of the study demonstrated the remarkable plasticity of macrophages in response to external inflammatory environments. The results of this part revealed that macrophages possess a self-counterregulatory mechanism in response to the changes of their own state.

M1 macrophages are known as the pro-inflammatory phenotype of macrophages, characterized by high expression of CD80 and the secretion of pro-inflammatory cytokines such as TNFa, IL-1b, and IL-6(Cutolo et al. 2022). On the other hand, TNFa have the ability of inducing M1 macrophages (Wynn and Vannella 2016). Interestingly, when co-cultured with M1 macrophages supernatant, M0 macrophages exhibited M2 properties, as indicated by downregulated expression of CD80 and upregulated expression of CD163. Furthermore, the pro-inflammatory cytokines IL-6 and TNFa were downregulated in the presence of M1SN compared to the M0 control group, and the anti-inflammatory cytokine IL-10 was upregulated with the presence of M1SN compared to the M0 control group. In other words, M1SN mediated the differentiation of M0 macrophages towards the M2 phenotype in vitro.

The mechanism of autoregulation is related to the JAK/STAT signaling pathway. After treatment with JAKi (Upadacitinib), the downregulation of CD80 and upregulation of CD163 were both reversed. In the JAKi groups, the expression of CD80 clearly increased, while the expression of CD163 was significantly reduced. Additionally, the secretion of IL-6 and TNFa increased several times compared to the concentration in the DMSO control groups. The reaction of macrophages to JAKi stands in contrast to the effects seen in presence of fibroblasts and to the reaction of fibroblasts themselves. In other words, Jaki significantly disrupts the ability of macrophages to self-regulate in response to their own inflammatory state.

The expression of the surface marker-CD206 was not significantly affected by the presence of JAKi,

but was slightly upregulated based on the available data from the study. The concentration of IL-10 in the JAKi-M0 group was clearly reduced compared to the DMSO-M0 control group, while the expression of IL-10 in the JAKi-M0+M1SN group was even higher than in the DMSO-M0+M1SN group. These findings suggest the existence of potential self-regulatory mechanisms that are not regulated by the JAK/STAT signaling pathway to cope with changes in the inflammatory state of macrophages.

4.4 Prospects, Weakness and Challenges

This study provides a new perspective on the function of macrophages and fibroblasts , two key players of tissue remodeling in rheumatic diseases, such as RA and SSc . It might help to develop new therapeutic approaches aiming to influence the macrophage plasticity to reconstitute a M1/M2 balance.

Our study has some limitations. For example it has not yet been explored whether macrophages in patients with autoimmune diseases such as RA or SSc would react the same way as macrophages from healthy donors, regarding polarization potential or reaction on signals of differently prestimulated Fib or how they would influence them. In addition, this study only explored the interaction between macrophages and fibroblasts in vitro. However, the human immune system is extremely complex. Not only monocytes or macrophages themselves have the ability to regulate each other in response to a different immune microenvironment. The interactions of B cells, T cells and NK cells with macrophages in the immune microenvironment are still under investigation. Therefore, the results with JAKi might not completely reflect the in vivo effects. The much stimulated IL10 secretion ability in JAKi treated groups after adding M1SN could give a hint that there are more than one self-regulatory mechanisms in the human body to make macrophages differentiate in different directions in response to changes in their own and external states. The experiments in vivo are further required to prove whether or under what condition JAKi could aggravate infections.

This study reveals a putative discrepancy of the JAKi, on one side it acted anti-fibrotic and anti-inflammatory regarding fibroblast function – as described in the literature and evidenced from its clinical efficiency, But on the other side it was inhibiting M2 polarization of M0 macrophages. This has not been described so far and needs to be thoroughly investigated in other in vitro and in vivo settings.

Restoring the macrophage balance from patients with autoimmune diseases such as RA or SSc could be a potential therapeutic approach. Another challenge is to develop targeted therapies that can selectively modulate macrophage polarization and function without compromising the overall immune response. Nevertheless, the study provides a solid foundation for further research and potential therapeutic interventions for chronic inflammatory diseases.

Altogether, the results demonstrated the pro-inflammatory and anti-inflammatory effects of differently polarized macrophages on both synovial fibroblasts and dermal fibroblasts; the macrophage plasticity in response to the self-state or external inflammatory environment was revealed. Most effects were regulated by the JAK/STAT signaling pathway. On the one hand, the efficiency of Upadacitinib in treatment of RA was proven in the context not only of Fib but in presence of macrophages; on the other hand, the results that JAKi inhibited the differentiation of macrophages from M0 to M2 revealed a potential side effect of JAKi that could be disadvantageous in treatment of some diseases.

5 Summary

Background: Activated macrophages (Mph) can be subdivided in at least 2 major subgroups according to their polarization into classical pro (M1-Mph) or anti- inflammatory (M2-Mph) subtypes. Together with fibroblasts they are considered key mediators of tissue remodeling in chronic rheumatic diseases. Mph possess high plasticity and it is suggested that an imbalance of M1/M2 Mph polarization might contribute to aberrant activation of fibroblasts in tissue destructive diseases, as seen in RA or fibrotic diseases, such as systemic sclerosis. Basing on this observation some researchers already discuss a therapeutic modulation of Mph as an interesting option to counteract pathologic tissue remodeling. However, the causes of disrupted M1/M2 homeostasis and the cross-talk with the inflammatory microenvironment, is still not completely understood.

Objective: In order to investigate the interactions between stromal cells and monocytes/macrophages, several research hypotheses are stated in this thesis project:

i. Can differently activated and polarized macrophages activate fibroblasts (Dermal Fibroblasts, Synovial Fibroblasts) in different ways?
ii. Can differently activated fibroblasts affect the polarization of macrophages?
iii. Which signaling pathways are involved in the interaction?
iv. Can biological response modifiers that are already used in the treatment of patients (such as monoclonal antibodies or small molecule inhibitors) counteract or improve the observed effects?

Methods: Macrophages were isolated from highly purified monocytes out of peripheral blood from healthy donors and polarized into M1 and M2 Mph over several days. CD80 and secretion of TNFa and IL12 were considered as representative for M1 and expression of CD163, CD206 and IL-10 representative for M2. Fibroblasts (Fib) were either used unstimulated or they were pre-stimulated for several days by TNFa/IL1b to induce a proinflammatory RA-like phenotype (characterized by strong upregulation of MMP-3, IL-6 and COX-2) or they were pre-stimulated by TGFb to induce a profibrotic myofibroblast-like phenotype as described in SSc (characterized by transcriptional upregulation of aSMA and COL-1). Both Mph and Fib were either cocultured together in cell culture inserts (Transwell) or cells were incubated with cell culture supernatants (SN) generated from respective cell type, generated over a duration of 3 days.

Results: M1-SN and M1-coculture with Fib induced a significant upregulation of IL-6 and MMP-3, especially in TNFa/IL-1b pre-stimulated Fib. M1-coculture induced a high expression of COX-2 and downregulation of fibrotic markers while M1 Mph supernatant had also pro-fibrotic effects on synovial Fibs, as evidenced by upregulation of aSMA and COL-1. These effects were significantly suppressed in the presence of the selective JAK inhibitor (JAKi) Upadacitinib. M2-SN and M2-Fib coculture both downregulated COX-2 in almost all fibroblast phenotypes, thereby exhibiting anti-inflammatory effects and JAKi further downregulated COX-2 expression. Only in TNFa/IL-1 pre-activated Fib this effect was very weak, suggesting that a strong inflammatory environment counteracts immunoregulatory effects by M2-Mph.
In further experiments, the influence of Fib on the polarization of Mph was investigated by incubating Fib supernatant from TNFa/IL-1b pre-stimulated Fib with undifferentiated M0 Mph. As a result, the M0-Mph shifted towards the M2 phenotype and expressed high levels of CD163, CD206 and IL-10. Application of an anti-IL-6 receptor Ab (tocilizumab) and JAKi both inhibited the development of this anti-inflammatory phenotype
At last the effects of M1- on M0 polarization were meassured. CD163 was significantly upregulated by M1-SN, and the pro-inflammatory cytokines-IL-6 and TNFa were evidently downregulated with M1-SN presence, suggesting again a M2- polarizing effect. On the other hand, JAKi forcefully suppressed the effects of M1-SN on M0 Mphs. These experiments revealed a regulatory effect of M1 on polarization of M0 towards M2 and this effect appears at least partially dependent on the JAK1 signaling pathway.

This study demonstrates, that polarized Mph have s strong modulatory effect on the Fib phenotype, regarding transcriptional expression of characteristic markers. On the other hand, pre-activated pro-inflammatory Fib and polarized M1 themselves have also a significant effect on Mph polarization, supporting M2 differentiation. This could potentially reflect a physiological negative feedback mechanism and is at least partially dependent on the JAK/STAT signaling pathway. Interestingly JAKi and anti-IL-6 that successfully abrogate proinflammatory and profibrotic responses in Fib, also inhibited differentiation of Mph into regulatory M2, thereby pointing to a new functional aspect of this Immunomodulator.

6 Zusammenfassung

Hintergrund: Aktivierte Makrophagen (Mph) können entsprechend ihrer Polarisierung in mindestens 2 große Untergruppen, klassische pro- (M1-) und anti-inflammatorische (M2-) Makrophagen unterteilt werden. Zusammen mit Fibroblasten gelten sie als Schlüsselmediatoren des Gewebeumbaus bei chronisch-rheumatischen Erkrankungen. Mph besitzen eine hohe Plastizität, und es wird vermutet, dass ein Ungleichgewicht der M1/M2-Mph-Polarisation zu einer pathogenen Aktivierung von Fibroblasten führen könnte, welche beispielsweise die Knorpeldestruktion bei RA oder Fibrose bei systemischer Sklerose vermitteln. Basierend auf dieser Beobachtung diskutieren einige Forscher bereits eine therapeutische Modulation von Mph als interessante Option, um dem pathologischen Gewebeumbau entgegenzuwirken. Die Ursachen für die gestörte M1/M2-Homöostase und die Wechselwirkung mit der entzündlichen Mikroumgebung sind jedoch noch nicht vollständig verstanden.

Ziel: Um die Wechselwirkungen zwischen Stromazellen und Monozyten/Makrophagen zu untersuchen, werden in diesem Dissertationsprojekt mehrere Hypothesen formuliert:

i. Können unterschiedlich aktivierte und polarisierte Makrophagen Fibroblasten auf unterschiedliche Weise aktivieren?
ii. Können unterschiedlich aktivierte Fibroblasten die Polarisation von Makrophagen beeinflussen?
iii. Welche Signalwege sind an der Interaktion beteiligt?
iv. Können Modulatoren der biologischen Reaktion, die bereits bei der Behandlung von Patienten verwendet werden (wie monoklonale Antikörper oder niedermolekulare Inhibitoren), den beobachteten Wirkungen entgegenwirken oder diese verbessern?

Methoden: Makrophagen wurden aus hochgereinigten Monozyten aus peripherem Blut gesunder Spender isoliert und über mehrere Tage in M1 und M2 Mph polarisiert. CD80 und die Sekretion von TNFa und IL12 wurden als repräsentativ für M1 und die Expression von CD163, CD206 und IL-10 als repräsentativ für M2 gewertet. Fibroblasten (Fib) wurden entweder unstimuliert verwendet oder sie wurden für mehrere Tage durch TNFa/IL1b vorstimuliert, um einen proinflammatorischen RA- ähnlichen Phänotyp zu induzieren (gekennzeichnet durch eine starke Hochregulation von MMP-3, IL-6 und COX-2), oder mit TGFb vorstimuliert, um einen Myofibroblasten-ähnlichen profibrotischen Phänotyp zu generieren, (gekennzeichnet durch die Genexpression von aSMA and COL-1). Sowohl Mph als auch Fib wurden

entweder zusammen in Zellkultureinsätzen (Transwell) kokultiviert oder Zellen wurden mit Zellkulturüberständen (SN) inkubiert.

Resultate: M1-SN und M1-Kokultur mit Fib induzierten eine signifikante Hochregulation von IL-6 und MMP-3, insbesondere in TNFa/IL-1b-vorstimulierten Fib. Die M1-Kokultur induzierte eine hohe Expression von COX-2 und eine Herunterregulierung fibrotischer Marker, während der M1-Mph-Überstand auch profibrotische Wirkungen auf synoviale Fibs hatte, wie durch die Hochregulierung von aSMA und COL-1 belegt wurde. Diese Wirkungen wurden in Gegenwart eines selektiven JAK-Inhibitors (JAKi) Upadacitinib signifikant unterdrückt. Die Kokultur von M2- SN und M2 mit Fib regulierte COX-2 in fast allen Fibroblasten-Phänotypen herunter und zeigte dadurch entzündungshemmende Wirkungen. JAKi regulierte die COX-2-Expression weiter herunter. Nur bei TNFa/IL-1b-voraktivierten Fib war dieser Effekt sehr schwach ausgeprägt, was darauf hindeutet, dass eine starke entzündliche Umgebung den immunregulatorischen Wirkungen von M2-Mph entgegenwirkt.

In weiteren Versuchen wurde der Einfluss von Fib auf die Polarisierung von Mph untersucht indem Fib-Überstand von TNFa/IL-1b vorstimulierten Fib mit undifferenzierten M0 Mph inkubiert wurde. Als Ergebnis verschob sich der M0- in Richtung des M2-Phänotyps und exprimierte hohe Mengen an CD163, CD206 und IL-10. Die Anwendung eines Anti-IL-6-Rezeptor-Ak (Tocilizumab) und JAKi hemmten beide die Entwicklung dieses anti-inflammatorischen Phänotyps.

Zuletzt wurden die Auswirkungen von M1- auf die M0-Polarisation gemessen. CD163 wurde durch M1-SN signifikant hochreguliert, und die entzündungsfördernden Zytokine –IL-6 und TNFa– wurden offensichtlich durch die Anwesenheit von M1-SN herunterreguliert, was wiederum auf einen M2-polarisierenden Effekt hindeutet wie er bereits bei der Wirkung von M1-SN auf Fib beobachtet worden war. JAKi hemmte wiederum die M2-induzierende Wirkung von M1-SN auf M0 Mphs.

Diese Studie zeigt, dass polarisierte Mph eine starke modulierende Wirkung auf den Fib-Phänotyp hinsichtlich der transkriptionellen Expression charakteristischer Marker haben. Andererseits haben voraktivierte pro-inflammatorische Fib und polarisierte M1 selbst auch einen signifikanten Effekt auf die Mph-Polarisation und unterstützen die M2-Differenzierung. Dies könnte potentiell einen physiologischen negativen Rückkopplungs-mechanismus widerspiegeln und ist zumindest teilweise von IL-6 bzw dem JAK/STAT-Signalweg abhängig. Interessanterweise hemmten JAKi und anti-IL-6, welche erfolgreich proinflammatorische und profibrotische Reaktionen in Fib aufheben, auch die Differenzierung von Mph in regulatorische M2, was auf einen neuen funktionellen Aspekt dieses Immunmodulators hinweist.

7 Reference List

Atri, C., Guerfali, F. Z. and Laouini, D. (2018). **Role of Human Macrophage Polarization in Inflammation during Infectiou s Diseases**. International journal of molecular sciences *19 (6)*, 1801, doi: 10.3390/ijms19061801.

Bernabei, I., So, A., Busso, N. and Nasi, S. (2023). **Cartilage calcification in osteoarthritis: mechanisms and clinical rel evance**. Nature reviews. Rheumatology *19 (1)*, 10-27, doi: 10.1038/s41584-022-00875-4.

Bhandari, R., Ball, M. S., Martyanov, V., Popovich, D., Schaafsma, E., Han, S., ElTanbouly, M., Orzechowski, N. M., Carns, M., Arroyo, E., Aren, K., Hinchcliff, M., Whitfield, M. L. and Pioli, P. A. (2020). **Profibrotic Activation of Human Macrophages in Systemic Sclerosis**. Arthritis & rheumatology (Hoboken, N.J.) *72 (7)*, 1160-1169, doi: 10.1002/art.41243.

Bhandari, R., Yang, H., Kosarek, N. N., Smith, A. E., Garlick, J. A., Hinchcliff, M., Whitfield, M. L. and Pioli, P. A. (2023). **Human dermal fibroblast-derived exosomes induce macrophage activation in systemic sclerosis**. Rheumatology (Oxford, England) *62 (SI)*, SI114-SI124, doi: 10.1093/rheumatology/keac453.

Bilsborrow, J. B., Doherty, E., Tilstam, P. V. and Bucala, R. (2019). **Macrophage migration inhibitory factor (MIF) as a therapeutic target f or rheumatoid arthritis and systemic lupus erythematosus**. Expert opinion on therapeutic targets *23 (9)*, 733-744, doi: 10.1080/14728222.2019.1656718.

Board, N. L., Moskovljevic, M., Wu, F., Siliciano, R. F. and Siliciano, J. D. (2022). **Engaging innate immunity in HIV-1 cure strategies**. Nature reviews. Immunology *22 (8)*, 499-512, doi: 10.1038/s41577-021-00649-1.

Bondeson, J., Wainwright, S. D., Lauder, S., Amos, N. and Hughes, C. E. (2006). **The role of synovial macrophages and macrophage-produced cytokines in driving aggrecanases, matrix metalloproteinases, and other destructive and inflammatory responses in osteoarthritis**. Arthritis research & therapy *8 (6)*, R187, doi: 10.1186/ar2099.

Brown, T. M. and Krishnamurthy, K. (2022). **Histology, Dermis**. In: StatPearls, StatPearls Publishing.

Bussard, K. M., Mutkus, L., Stumpf, K., Gomez-Manzano, C. and Marini, F. C. (2016). **Tumor-associated stromal cells as key contributors to the tumor microe nvironment**. Breast cancer research : BCR *18 (1)*, 84, doi: 10.1186/s13058-016-0740-2.

Childs, C. E., Calder, P. C. and Miles, E. A. (2019). **Diet and Immune Function**. Nutrients *11 (8)*, 1933, doi: 10.3390/nu11081933.

Conigliaro, P., Scrivo, R., Valesini, G. and Perricone, R. (2011). **Emerging role for NK cells in the pathogenesis of inflammatory arthrop athies**. Autoimmunity reviews *10 (10)*, 577-581, doi: 10.1016/j.autrev.2011.04.017.

Cope, A. P., Schulze-Koops, H. and Aringer, M. (2007). **The central role of T cells in rheumatoid arthritis**. Clinical and experimental rheumatology *25 (5 Suppl 46)*, S4-11.

Croft, A. P., Campos, J., Jansen, K., Turner, J. D., Marshall, J., Attar, M., Savary, L., Wehmeyer, C., Naylor, A. J., Kemble, S., Begum, J., Dürholz, K., Perlman, H., Barone, F., McGettrick, H. M., Fearon, D. T., Wei, K., Raychaudhuri, S., Korsunsky, I., Brenner, M. B., Coles, M., Sansom, S. N., Filer, A. and Buckley, C. D. (2019). **Distinct fibroblast subsets drive inflammation and damage in arthritis**. Nature *570 (7760)*, 246-251, doi: 10.1038/s41586-019-1263-7.

Cutolo, M., Campitiello, R., Gotelli, E. and Soldano, S. (2022). **The Role of M1/M2 Macrophage Polarization in Rheumatoid Arthritis Syno vitis**. Frontiers in immunology *13*, 867260, doi: 10.3389/fimmu.2022.867260.

Donlin, L. T., Jayatilleke, A., Giannopoulou, E. G., Kalliolias, G. D. and Ivashkiv, L. B. (2014). **Modulation of TNF-induced macrophage polarization by synovial fibrobla sts**. Journal of immunology (Baltimore, Md. : 1950) *193 (5)*, 2373-2383, doi: 10.4049/jimmunol.1400486.

Dufour, A. M., Alvarez, M., Russo, B. and Chizzolini, C. (2018). **Interleukin-6 and Type-I Collagen Production by Systemic Sclerosis Fib roblasts Are Differentially Regulated by Interleukin-17A in the Presen ce of Transforming Growth Factor-Beta 1**. Frontiers in immunology *9*, 1865, doi: 10.3389/fimmu.2018.01865.

Eggenhuizen, P. J., Ng, B. H. and Ooi, J. D. (2020). **Treg Enhancing Therapies to Treat Autoimmune Diseases**. International journal of molecular sciences *21 (19)*, 7015, doi: 10.3390/ijms21197015.

Eming, S. A., Martin, P. and Tomic-Canic, M. (2014). **Wound repair and regeneration: mechanisms, signaling, and translation**. Science translational medicine *6 (265)*, 265sr266, doi: 10.1126/scitranslmed.3009337.

Fahy, R. J., Doseff, A. I. and Wewers, M. D. (1950). **Spontaneous human monocyte apoptosis utilizes a caspase-3-dependent pa thway that is blocked by endotoxin and is independent of caspase-1**. Journal of immunology (Baltimore, Md. : 1950) *163 (4)*, 1755-1762.

Galindo, M., Santiago, B., Rivero, M., Rullas, J., Alcami, J. and Pablos, J. L. (2001). **Chemokine expression by systemic sclerosis fibroblasts: abnormal regul ation of monocyte chemoattractant protein 1 expression**. Arthritis and rheumatism *44 (6)*, 1382-1386, doi: 10.1002/1529-0131(200106)44:6<1382::AID-ART231>3.0.CO;2-T.

Gordon, S. and Martinez, F. O. (2010). **Alternative activation of macrophages: mechanism and functions**. Immunity *32 (5)*, 593-604, doi: 10.1016/j.immuni.2010.05.007.

Houssiau, F. A., Devogelaer, J. P., Van Damme, J., de Deuxchaisnes, C. N. and Van Snick, J. (1988). **Interleukin-6 in synovial fluid and serum of patients with rheumatoid arthritis and other inflammatory arthritides**. Arthritis and rheumatism *31 (6)*, 784-788, doi: 10.1002/art.1780310614.

Jinnin, M. (2010). **Mechanisms of skin fibrosis in systemic sclerosis**. The Journal of dermatology *37 (1)*, 11-25, doi: 10.1111/j.1346-8138.2009.00738.x.

Kasperkovitz, P. V., Timmer, T. C., Smeets, T. J., Verbeet, N. L., Tak, P. P., van Baarsen, L. G., Baltus, B., Huizinga, T.

W., Pieterman, E., Fero, M., Firestein, G. S., van der Pouw Kraan, T. C. and Verweij, C. L. (2005). **Fibroblast-like synoviocytes derived from patients with rheumatoid arthritis show the imprint of synovial tissue heterogeneity: evidence of a link between an increased myofibroblast-like phenotype and high-inflammation synovitis**. Arthritis Rheum *52 (2)*, 430-441, doi: 10.1002/art.20811.

Kawashima, M., Ogura, N., Akutsu, M., Ito, K. and Kondoh, T. (2013). **The anti-inflammatory effect of cyclooxygenase inhibitors in fibroblast-like synoviocytes from the human temporomandibular joint results from the suppression of PGE2 production**. J Oral Pathol Med *42 (6)*, 499-506, doi: 10.1111/jop.12045.

Kowal-Bielecka, O., Fransen, J., Avouac, J., Becker, M., Kulak, A., Allanore, Y., Distler, O., Clements, P., Cutolo, M., Czirjak, L., Damjanov, N., Del Galdo, F., Denton, C. P., Distler, J. H. W., Foeldvari, I., Figelstone, K., Frerix, M., Furst, D. E., Guiducci, S., Hunzelmann, N., Khanna, D., Matucci-Cerinic, M., Herrick, A. L., van den Hoogen, F., van Laar, J. M., Riemekasten, G., Silver, R., Smith, V., Sulli, A., Tarner, I., Tyndall, A., Welling, J., Wigley, F., Valentini, G., Walker, U. A., Zulian, F., Müller-Ladner, U. and Coauthors, E. (2017). **Update of EULAR recommendations for the treatment of systemic sclerosi s**. Annals of the rheumatic diseases *76 (8)*, 1327-1339, doi: 10.1136/annrheumdis-2016-209909.

Kuo, D., Ding, J., Cohn, I. S., Zhang, F., Wei, K., Rao, D. A., Rozo, C., Sokhi, U. K., Shanaj, S., Oliver, D. J., Echeverria, A. P., DiCarlo, E. F., Brenner, M. B., Bykerk, V. P., Goodman, S. M., Raychaudhuri, S., Rätsch, G., Ivashkiv, L. B. and Donlin, L. T. (2019). **HBEGF⁺ macrophages in rheumatoid arthritis induce fibroblas t invasiveness**. Science translational medicine *11 (491)*, eaau8587, doi: 10.1126/scitranslmed.aau8587.

Lago, J. C. and Puzzi, M. B. (2019). **The effect of aging in primary human dermal fibroblasts**. PloS one *14 (7)*, e0219165, doi: 10.1371/journal.pone.0219165.

Li, Y., Zhao, J., Yin, Y., Li, K., Zhang, C. and Zheng, Y. (2022). **The Role of IL-6 in Fibrotic Diseases: Molecular and Cellular Mechanis ms**. International journal of biological sciences *18 (14)*, 5405-5414, doi: 10.7150/ijbs.75876.

Liao, B., Guan, M., Tan, Q., Wang, G., Zhang, R., Huang, J., Liu, M., Chen, H., Li, K., Bai, D. and Zhu, Y. (2021). **Low-intensity pulsed ultrasound inhibits fibroblast-like synoviocyte p roliferation and reduces synovial fibrosis by regulating Wnt/β-catenin signaling**. Journal of orthopaedic translation *30*, 41-50, doi: 10.1016/j.jot.2021.08.002.

Maglaviceanu, A., Wu, B. and Kapoor, M. (2021). **Fibroblast-like synoviocytes: Role in synovial fibrosis associated wit h osteoarthritis**. Wound repair and regeneration : official publication of the Wound Heal ing Society [and] the European Tissue Repair Society *29 (4)*, 642-649, doi: 10.1111/wrr.12939.

Maini, R. N., Elliott, M. J., Brennan, F. M. and Feldmann, M. (1995). **Beneficial effects of tumour necrosis factor-alpha (TNF-alpha) blockad e in rheumatoid arthritis (RA)**. Clinical and experimental immunology *101 (2)*, 207-212, doi: 10.1111/j.1365-2249.1995.tb08340.x.

Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A. and Locati, M. (2004). **The chemokine system in diverse forms of macrophage activation and pol arization**. Trends in immunology *25 (12)*, 677-686, doi: 10.1016/j.it.2004.09.015.

Marsh, L.-J., Kemble, S., Reis Nisa, P., Singh, R. and Croft, A. P. (2021). **Fibroblast pathology in inflammatory joint disease**. Immunological reviews *302 (1)*, 163-183, doi: 10.1111/imr.12986.

Marshall, J. S., Warrington, R., Watson, W. and Kim, H. L. (2018). **An introduction to immunology and immunopathology**. Allergy, asthma, and clinical immunology : official journal of the Can adian Society of Allergy and Clinical Immunology *14 (Suppl 2)*, 49, doi: 10.1186/s13223-018-0278-1.

Melo, R. C. N. and Dvorak, A. M. (2012). **Lipid body-phagosome interaction in macrophages during infectious dise ases: host defense or pathogen survival strategy?** PLoS pathogens *8 (7)*, e1002729, doi: 10.1371/journal.ppat.1002729.

Noack, M. and Miossec, P. (2017). **Selected cytokine pathways in rheumatoid arthritis**. Seminars in immunopathology *39 (4)*, 365-383, doi: 10.1007/s00281-017-0619-z.

Noss, E. H. and Brenner, M. B. (2008). **The role and therapeutic implications of fibroblast-like synoviocytes in inflammation and cartilage erosion in rheumatoid arthritis**. Immunological reviews *223*, 252-270, doi: 10.1111/j.1600-065X.2008.00648.x.

Nossal, G. J. (1987). **Current concepts: immunology. The basic components of the immune syste m**. The New England journal of medicine *316 (21)*, 1320-1325, doi: 10.1056/NEJM198705213162107.

Orecchioni, M., Ghosheh, Y., Pramod, A. B. and Ley, K. (2019). **Macrophage Polarization: Different Gene Signatures in M1(LPS+) vs. Cla ssically and M2(LPS-) vs. Alternatively Activated Macrophages**. Frontiers in immunology *10*, 1084, doi: 10.3389/fimmu.2019.01084.

Parkin, J. and Cohen, B. (2001). **An overview of the immune system**. Lancet (London, England) *357 (9270)*, 1777-1789, doi: 10.1016/S0140-6736(00)04904-7.

Patel, J. P., Konanur Srinivasa, N. K., Gande, A., Anusha, M., Dar, H. and Baji, D. B. (2023). **The Role of Biologics in Rheumatoid Arthritis: A Narrative Review**. Cureus *15 (1)*, e33293, doi: 10.7759/cureus.33293.

Porta, C., Riboldi, E., Ippolito, A. and Sica, A. (2015). **Molecular and epigenetic basis of macrophage polarized activation**. Seminars in immunology *27 (4)*, 237-248, doi: 10.1016/j.smim.2015.10.003.

Quero, L., Tiaden, A. N., Hanser, E., Roux, J., Laski, A., Hall, J. and Kyburz, D. (2020). **miR-221-3p Drives the Shift of M2-Macrophages to a Pro-Inflammatory Fu nction by Suppressing JAK3/STAT3 Activation**. Frontiers in immunology *10*, 3087, doi: 10.3389/fimmu.2019.03087.

Remst, D. F. G., Blaney Davidson, E. N. and van der Kraan, P. M. (2015). **Unravelling osteoarthritis-related synovial fibrosis: a step closer to solving joint stiffness**. Rheumatology (Oxford, England) *54 (11)*, 1954-1963, doi:

10.1093/rheumatology/kev228.

Ruytinx, P., Proost, P., Van Damme, J. and Struyf, S. (2018). **Chemokine-Induced Macrophage Polarization in Inflammatory Conditions**. Frontiers in immunology *9*, 1930, doi: 10.3389/fimmu.2018.01930.

Sarkar, A., Chakraborty, D., Kumar, V., Malhotra, R. and Biswas, S. (2022). **Upregulation of leucine-rich alpha-2 glycoprotein: A key regulator of inflammation and joint fibrosis in patients with severe knee osteoarth ritis**. Frontiers in immunology *13*, 1028994, doi: 10.3389/fimmu.2022.1028994.

Scanzello, C. R., Plaas, A. and Crow, M. K. (2008). **Innate immune system activation in osteoarthritis: is osteoarthritis a chronic wound?** Current opinion in rheumatology *20 (5)*, 565-572, doi: 10.1097/BOR.0b013e32830aba34.

Scherer, H. U., Häupl, T. and Burmester, G. R. (2020). **The etiology of rheumatoid arthritis**. Journal of autoimmunity *110*, 102400, doi: 10.1016/j.jaut.2019.102400.

Schoels, M. M., van der Heijde, D., Breedveld, F. C., Burmester, G. R., Dougados, M., Emery, P., Ferraccioli, G., Gabay, C., Gibofsky, A., Gomez-Reino, J. J., Jones, G., Kvien, T. K., Murakami, M., Nishimoto, N. and Smolen, J. S. (2013). **Blocking the effects of interleukin-6 in rheumatoid arthritis and othe r inflammatory rheumatic diseases: systematic literature review and me ta-analysis informing a consensus statement**. Annals of the rheumatic diseases *72 (4)*, 583-589, doi: 10.1136/annrheumdis-2012-202470.

Soldano, S., Trombetta, A. C., Contini, P., Tomatis, V., Ruaro, B., Brizzolara, R., Montagna, P., Sulli, A., Paolino, S., Pizzorni, C., Smith, V. and Cutolo, M. (2018). **Increase in circulating cells coexpressing M1 and M2 macrophage surfac e markers in patients with systemic sclerosis**. Annals of the rheumatic diseases *77 (12)*, 1842-1845, doi: 10.1136/annrheumdis-2018-213648.

Störch, H., Zimmermann, B., Resch, B., Tykocinski, L.-O., Moradi, B., Horn, P., Kaya, Z., Blank, N., Rehart, S., Thomsen, M., Lorenz, H.-M., Neumann, E. and Tretter, T. (2016). **Activated human B cells induce inflammatory fibroblasts with cartilage -destructive properties and become functionally suppressed in return**. Annals of the rheumatic diseases *75 (5)*, 924-932, doi: 10.1136/annrheumdis-2014-206965.

Tabib, T., Huang, M., Morse, N., Papazoglou, A., Behera, R., Jia, M., Bulik, M., Monier, D. E., Benos, P. V., Chen, W., Domsic, R. and Lafyatis, R. (2021). **Myofibroblast transcriptome indicates SFRP2^{hi} fibroblast pr ogenitors in systemic sclerosis skin**. Nature communications *12 (1)*, 4384, doi: 10.1038/s41467-021-24607-6.

Thangapazham, R. L., Darling, T. N. and Meyerle, J. (2014). **Alteration of skin properties with autologous dermal fibroblasts**. Int J Mol Sci *15 (5)*, 8407-8427, doi: 10.3390/ijms15058407.

Thulabandu, V., Chen, D. and Atit, R. P. (2018). **Dermal fibroblast in cutaneous development and healing**. Wiley interdisciplinary reviews. Developmental biology *7 (2)*, 10.1002/wdev.1307, doi: 10.1002/wdev.307.

Tofigh, R., Hosseinpourfeizi, M., Baradaran, B., Teimourian, S. and Safaralizadeh, R. (2023). **Rheumatoid arthritis**

and non-coding RNAs; how to trigger inflammation. Life sciences *315*, 121367, doi: 10.1016/j.lfs.2023.121367.

Wang, F., Sun, L., Wang, S., Davis, J. M., 3rd, Matteson, E. L., Murad, M. H., Luo, F. and Vassallo, R. (2020). **Efficacy and Safety of Tofacitinib, Baricitinib, and Upadacitinib for Rheumatoid Arthritis: A Systematic Review and Meta-Analysis**. Mayo Clinic proceedings *95 (7)*, 1404-1419, doi: 10.1016/j.mayocp.2020.01.039.

Wei, K., Nguyen, H. N. and Brenner, M. B. (2021). **Fibroblast pathology in inflammatory diseases**. The Journal of clinical investigation *131 (20)*, e149538, doi: 10.1172/JCI149538.

Wiktor-Jedrzejczak, W. and Gordon, S. (1996). **Cytokine regulation of the macrophage (M phi) system studied using the colony stimulating factor-1-deficient op/op mouse**. Physiological reviews *76 (4)*, 927-947, doi: 10.1152/physrev.1996.76.4.927.

Wynn, T. A. and Vannella, K. M. (2016). **Macrophages in Tissue Repair, Regeneration, and Fibrosis**. Immunity *44 (3)*, 450-462, doi: 10.1016/j.immuni.2016.02.015.

Xu, S., Xiong, Y., Fu, B., Guo, D., Sha, Z., Lin, X. and Wu, H. (2023). **Bacteria and macrophages in the tumor microenvironment**. Frontiers in microbiology *14*, 1115556, doi: 10.3389/fmicb.2023.1115556.

Xu, W., Roos, A., Schlagwein, N., Woltman, A. M., Daha, M. R. and van Kooten, C. (2006). **IL-10-producing macrophages preferentially clear early apoptotic cells**. Blood *107 (12)*, 4930-4937, doi: 10.1182/blood-2005-10-4144.

Yang, R., Tang, Y., Hou, J., Yu, M., Long, Y., Yamuhanmode, A., Li, Q., Li, F., Zhang, Y., Warsame, M., Zhang, C. and Shen, H. (2022). **Fibrosis in frozen shoulder: Activation of IL-6 through PI3K-Akt signa ling pathway in synovial fibroblast**. Molecular immunology *150*, 29-38, doi: 10.1016/j.molimm.2022.07.007.

Yatim, K. M. and Lakkis, F. G. (2015). **A brief journey through the immune system**. Clinical journal of the American Society of Nephrology : CJASN *10 (7)*, 1274-1281, doi: 10.2215/CJN.10031014.

Yu, P., Zhang, X., Liu, N., Tang, L., Peng, C. and Chen, X. (2021). **Pyroptosis: mechanisms and diseases**. Signal transduction and targeted therapy *6 (1)*, 128, doi: 10.1038/s41392-021-00507-5.

Zehender, A., Li, Y.-N., Lin, N.-Y., Stefanica, A., Nüchel, J., Chen, C.-W., Hsu, H.-H., Zhu, H., Ding, X., Huang, J., Shen, L., Györfi, A.-H., Soare, A., Rauber, S., Bergmann, C., Ramming, A., Plomann, M., Eckes, B., Schett, G. and Distler, J. H. W. (2021). **TGFβ promotes fibrosis by MYST1-dependent epigenetic regulation of aut ophagy**. Nature communications *12 (1)*, 4404, doi: 10.1038/s41467-021-24601-y.

Zhang, L., Xing, R., Huang, Z., Ding, L., Zhang, L., Li, M., Li, X., Wang, P. and Mao, J. (2021). **Synovial Fibrosis Involvement in Osteoarthritis**. Frontiers in medicine *8*, 684389, doi: 10.3389/fmed.2021.684389.

Zhang, L., Xing, R., Huang, Z., Zhang, N., Zhang, L., Li, X. and Wang, P. (2019). **Inhibition of Synovial Macrophage Pyroptosis Alleviates Synovitis and Fibrosis in Knee Osteoarthritis**. Mediators Inflamm *2019*, 2165918,

doi: 10.1155/2019/2165918.

Zhao, Y., Zou, W., Du, J. and Zhao, Y. (2018). **The origins and homeostasis of monocytes and tissue-resident macrophag es in physiological situation**. Journal of cellular physiology *233 (10)*, 6425-6439, doi: 10.1002/jcp.26461.

8 Personal Publications

I hereby declare that all work in this thesis is my own original work unless otherwise stated.

Curriculum Vitae

PERSONAL INFORMATION

Name:	Jia Li
Date of Birth:	Oct 07, 1991
Place of Birth:	Shanxi, China
Nationality:	Chinese
Marital Status:	Married

PRIMARY AND SECONDARY SCHOOLS

(1998) – (2004)	Taiyuan Xishan 2nd Primary School
(2004) – (2007)	Taiyuan Xishan 6th Junior High School
(2007) – (2010)	Shanxi Modern Bilingual School
(Jun, 2010)	National College Entrance Examination

UNIVERSTIY AND PROFESSIONAL CAREER

(2010) – (2015)	Begin of undergraduate studies with Shaanxi University of Chinese Medicine
(2014) – (2015)	Internship in Shaanxi Hospital of Chinese Medicine
(Jun, 2015)	Undergraduate medical examination and awarded Bachelor of Medicine
(Dec, 2015)	National Postgraduate Entrance Examination
(2016) – (2019)	Postgraduate studies in Shaanxi University of Chinese Medicine
(Feb, 2016)	Passed Medical License Examination (TCM) and registered as a clinical resident
(2016) – (2019)	Period of standardized training for resident surgeons
(June, 2019)	Passed the final approbation and awarded Master of Medicine in Integrated Chinese Medicine and Western Medicine

Acknowledgments

I would like to express my deepest gratitude to Prof. Dr. med. Hanns-Martin Lorenz for providing me with the opportunity to work on this project and allowing me to achieve academic success. I am also indebted to Dr.rer.nat Theresa Tretter, my project leader, for her guidance and encouragement throughout my nearly three years of doctoral research, and for her invaluable inspiration when I encountered difficulties.

I would like to extend my sincere thanks to all the scientists at the Rheumatology Department of Internal Medicine V of Heidelberg University Hospital, and to the doctoral students who provided me with many useful suggestions and ideas during our lab meetings and other exchanges. I am especially grateful to Dr. med. Yanrong Cai and Ms. Xin Guan, who provided me with significant help both in life and in learning.

I would like to thank my parents for their constant support and encouragement. Without them, I would not be where I am today. I would also like to express my special thanks to my beloved wife, who has brought me immeasurable happiness in life and allowed me to focus more on my academic research.

I would also like to acknowledge the support of the China Scholarship Council that enabled me to complete my doctoral degree in Germany. Last but not least, I would like to thank Prof. Yuan, Puwei, Prof. Liu, Deyu, and Prof. Li, Kanyin for their important mentorship in my clinical facility and academic thinking.

I am grateful to everyone who has helped and supported me during my doctoral journey. Your unwavering support and guidance have been invaluable to my success. I am honored to have had the opportunity to work with such remarkable individuals and institutions, and I will always be grateful for the knowledge and skills I have gained through this experience. Thank you all.

Eidesstattliche Versicherung

1. Bei der eingereichten Dissertation zu dem Thema

   Bidirectional Interaction of Differently Activated and Polarized Monocytes/Macrophages and Stromal Cells in Context of Tissue Remodeling in Chronic Inflammatory Disease

   handelt es sich um meine eigenständig erbrachte Leistung.

2. Ich habe nur die angegebenen Quellen und Hilfsmittel benutzt und mich keiner unzulässigen Hilfe Dritter bedient. Insbesondere habe ich wörtlich oder sinngemäß aus anderen Werken übernommene Inhalte als solche kenntlich gemacht.

3. Die Arbeit oder Teile davon habe ich bislang nicht an einer Hochschule des In- oder Auslands als Bestandteil einer Prüfungs- oder Qualifikationsleistung vorgelegt.

4. Die Richtigkeit der vorstehenden Erklärungen bestätige ich.

5. Die Bedeutung der eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unrichtigen oder unvollständigen eidesstattlichen Versicherung sind mir bekannt. Ich versichere an Eides statt, dass ich nach bestem Wissen die reine Wahrheit erklärt und nichts verschwiegen habe.

Place, Date doctoral candidate’s signature