Overexpression of Wnt5a in Bone Marrow-Derived Macrophages Promotes Osteoarthritis Progression

A thesis submitted by Wenhui Li

in partial fulfillment of the requirements for the degree of

Master of Science

in

Pharmacology and Drug Development

Tufts University

Graduate School of Biomedical Sciences

May 2024

Advisor: Zeng, Li, PhD

Abstract

Osteoarthritis (OA) is a common disease that affects more than 3 million people in the United States each year and is characterized by synovium inflammation, cartilage degradation, bone remodeling, and osteophyte formation. There is no available disease-modifying medication for the treatment of osteoarthritis. It has been shown that Wnt5a is elevated in human OA synovium. Macrophages, as a source and recipient of Wnt5a, are one of the most abundant immune cells in OA synovium. In this study, we investigated the effects of Wnt5a on macrophages and the function of Wnt5a-overexpressing macrophages on OA. C57BL/6 wild type mice were subjected to anterior cruciate ligament transection (ACLT) surgery and whole knee joints were harvested 12-, 42-, or 70-days post-surgery. Increased numbers of macrophages and Wnt5a positive cells were found in ACLT mice compared to sham mice. A positive correlation between macrophages and Wnt5a positive cells and the colocalization of these two cell types indicated that macrophages are the main source of Wnt5a in OA synovium. After adoptive transfer of Wnt5a-overexpressing macrophages into mouse knee joint, the osteoarthritis mice injected with Wnt5a-overexpressing macrophages developed severer pathological changes compared to the ones injected with control macrophages. In addition, from immunostaining, Wnt5a positive cells tend to be maintained in osteoarthritis mice compared to control mice, without any differences between the level of M1 (iNOS) and M2 (CD206) macrophages, which is consistent with the in vitro results that Wnt5a influenced cytokine expression in macrophages but did not change the phenotype of macrophages. These findings suggest that Wnt5a-overexpressing

macrophages promote osteoarthritis progression without polarization, and this could be a potential therapeutic target for the treatment of OA.

Acknowledgements

I would like to take this opportunity to express my sincere gratitude to those who have supported and guided me throughout the journey of completing this thesis.

First, I extend my deepest appreciation to my advisor, Dr. Li Zeng, for her invaluable support and guidance. Her expertise, guidance, and unwavering support have been invaluable throughout this research endeavor.

I would like to express my sincere gratitude to my mentor, Dr. Sihan Liu. Her patience, encouragement, and willingness to teach me everything in the lab have been foundational to my growth and success.

I am immensely grateful to my lab mates: Jingshu Liu, Anjali R. Mamidwar, Elyse Blank, Keren Chen, Yuelin Zhong, and Dr. Judith M. Hollander. Their friendship, collaboration, and shared experiences have made the research process rewarding and enjoyable.

Furthermore, I would also like to express my gratitude to my second reader, Dr. Heber C. Nielsen, for his constructive feedback and dedication.

Last but not least, I am thankful to the faculty and staff of Pharmacology and Drug Development program for creating a stimulating academic environment conducive to learning and research.

Table of contents

List of Tables

List of Figures

List of Abbreviations

ACLT -- Anterior cruciate ligament transection
ACLT+Mφ-GFP -- ACLT mice injected with Lenti-GFP infected BMDMs
ACLT+Mφ-Wnt5a -- ACLT mice injected with Lenti-Wnt5a infected BMDMs
ANOVA -- Analysis of variance
BMDMs -- Bone marrow derived macrophages
DAPI -- Diaminophenylindole
ECM -- Extracellular matrix
ELISA -- enzyme-linked immunosorbent assay
FLS -- Fibroblast-like synoviocytes
Fz -- Frizzled
IA -- Intraarticular
IF -- Immunofluorescence
IHC -- Immunohistochemistry
IL-1β -- Interleukin-1 beta
IL-6 -- Interleukin-6
iNOS -- Inducible nitric oxide synthase
Lenti-GFP -- GFP lentivirus
Lenti-Wnt5a -- Wnt5a lentivirus
LPS -- lLpopolysaccharides
LRP5/6 -- Lipoprotein-related protein 5/6
MMPs -- Matrix metalloproteinases
MOI -- Multiplicity of Infection
OA -- Osteoarthritis
PCP -- Planer Cell Polarity
PFA -- Paraformaldehyde
ROR2 -- Receptor tyrosine kinase-like orphan receptor 2
RT-PCR, or qPCR -- Real-time polymerase chain reaction
Saf. O -- Safranin O
Sham+Mφ-GFP -- Sham mice injected with Lenti-GFP infected BMDMs
Sham+Mφ-Wnt5a -- Sham mice injected with Lenti-Wnt5a infected BMDMs
TNF -- Tumor necrosis factor

Chapter 1: Introduction

1.1. Background

Osteoarthritis (OA) is the most common type of arthritis, which is characterized by synovium inflammation, cartilage degradation, bone remodeling, and osteophyte formation.[1,2] It occurs most frequently in knee joints, following hips and hand joints.[3] The risk factors of OA include overuse of the joint, aging, overweight, and injury.[4,5] Sex differences of OA were also reported, showing that females were more vulnerable to develop severe knee OA.[3,4,6] Although OA is one of the most common causes for pain and disability, there is no cure except for anti-inflammatory or pain-relief medications.

Cellular interaction plays a major role in the pathogenesis of OA. Single-cell RNA sequencing data showed 12 different cell types in the OA synovium, including different synovial fibroblasts, HLA-DRA+ cells, smooth muscle cells, endothelial cells, T cells, mast cells and proliferating immune cells.[7] Among these cell types, HLA-DRA+ cells, including heterogeneous groups of macrophages, dendritic cells, activated pro-inflammatory fibroblast, and B cells, play an important role in immune responses.[7] In addition, macrophages are the most abundant immune cells in OA synovium. They are the essential component involved in the cellular crosstalk between synoviocytes and chondrocytes, by receiving cytokines, growth factors, matrix metalloproteinases (MMPs) and other factors from activated fibroblast-like synoviocytes (FLS), and by secreting pro-inflammatory mediators, such as interleukin-1 beta (IL-1β), tumor necrosis factor (TNF), and IL-6, which act on FLS and chondrocytes and contribute to extracellular matrix (ECM) degradation.[8]

Except for secreting pro-inflammatory or anti-inflammatory cytokines, macrophage is also a main source of other substances. Wnt proteins, which are highly conserved across species, are both the ligands and the secreted proteins by macrophages.[9,10] Nineteen Wnt proteins have been identified, along with two types of Wnt signaling pathways.[11] The canonical Wnt pathway, or Wnt/ β-catenin pathway, involves the binding of Wnt ligands, such as Wnt1 and Wnt3A, to Frizzled (Fz) receptors and its co-receptors such as low-density-lipoprotein-related protein 5/6 (LRP5/6), following the accumulation and translocation of β-catenin into the nucleus.[12] In the nucleus, β-catenin acts as the co-activator of TCF/LEF transcription factor, which in turn activate the target genes.[11,12] Non-canonical pathway is also known as β-catenin-independent pathway, including the Planer Cell Polarity (PCP) pathway and the Wnt/Ca^{2+} pathway, activated through noncanonical Wnts such as Wnt5A and Wnt11.[13] Both β-catenin-dependent and independent pathways play a crucial role in regulating many developmental processes, such as embryonic development, cell proliferation, differentiation, polarization, and migration.[14,15]

In addition, Wnt signaling plays a critical role in bone homeostasis and joint remodeling.[16] Many pathological processes of OA are regulated by Wnt signaling, including synovitis, cartilage degradation, chondrocyte hypertrophy, and bone sclerosis.[17] Among all the Wnt proteins, it has been studied that the expression level of Wnt5a is elevated in human OA cartilage, where it regulates chondrocyte catabolic activity and promote matrix metalloproteinase expression.[18] Blockade of receptor tyrosine kinase-like orphan receptor 2 (ROR2), one of the receptor for Wnt5a, can promote chondrogenic differentiation and inhibit the expression of the cartilage-degrading enzymes.[19] However,

it is not well understood how Wnt5a affects immune cells, especially macrophages, in OA. Study on the role of Wnt5a in immune cells could lead to a potential therapeutic target for the treatment of OA.

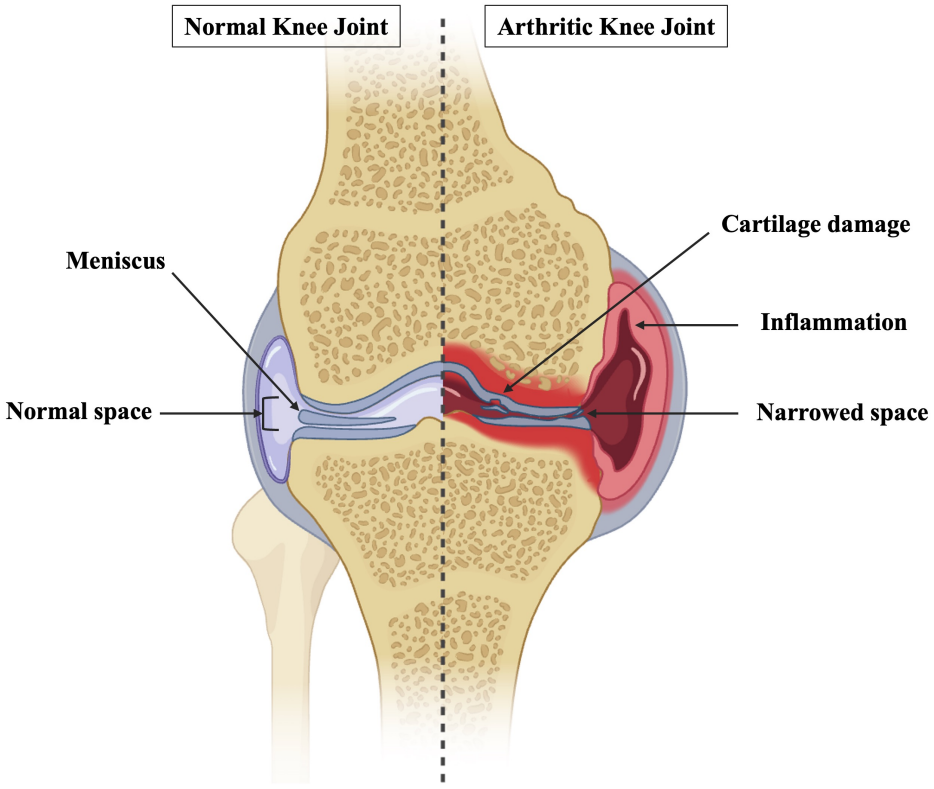

Figure 1.1 Characterizations of arthritic knee joint compared to normal knee joint.

1.2. Hypothesis and Experimental Design

The main purpose of this study is to investigate if overexpression of Wnt5a has effects on the function of macrophages in OA synovium and can promote OA progression.

First, a mouse model of OA was established by subjecting 12-week-old mice to anterior cruciate ligament transection (ACLT) surgery. Physiological changes in the chondrocytes can be induced after surgery, following cartilage degradation and osteophyte formation[20]. After 12, 42, or 70 days of OA development, mouse knee joints were harvested. The OA progression was confirmed using safranin O (Saf. O) staining,

and the numbers of macrophages and Wnt5a positive cells were assessed using immunohistochemistry (IHC) staining. We found a positive correlation between the number of Wnt5a positive cells and macrophages, indicating that macrophages are the main source of Wnt5a in OA synovium. This is further confirmed in the results of immunofluorescence (IF) staining showing the co-localization of macrophages and Wnt5a positive cells in OA synovium.

Second, to investigate the effects of Wnt5a on the function of macrophages, Wnt5a-overexpressing macrophages were established by transducing bone marrow derived macrophages (BMDMs) with lentivirus. Following lentiviral transduction of BMDMs, lipopolysaccharides (LPS) or IL-1β treatment was performed to mimic inflammatory OA environment and activate macrophages. The expression profiles of M1 signature gene iNOS, M2 signature gene Arg1, and the genes of pro-inflammatory or anti-inflammatory cytokines were tested using real-time polymerase chain reaction (RT-PCR, or qPCR). The expression levels of proteins were assessed using Western blotting, ELISA, and cytokine array. After lentiviral transduction, Lenti-Wnt5a infected BMDMs expressed significantly higher level of Wnt5a RNA compared to Lenti-GFP infected controls. This was also observed in Western blotting result showing the elevated level of Wnt5a protein. The expression level of iNOS was elevated in Lenti-Wnt5a infected BMDMs only under IL-1β treated condition, while significantly higher level of Arg1 was observed in Lenti-Wnt5a infected BMDMs under all 3 conditions (non-treated control, LPS treatment, and IL-1β treatment). In addition, ELISA and cytokine array showed that although increased level of Wnt5a promoted cytokine expression in BMDMs, the macrophages did not polarize to M1 or M2. These in vitro results suggest that overexpression of Wnt5a in

macrophages affects the cytokine expression but does not promote macrophage polarization.

Third, Wnt5a-overexpressing BMDMs were adoptively transferred into OA mouse knee joint, and IHC and IF staining were performed to assess the effect on the function of macrophages and OA progression. Saf. O staining and OA scoring showed elevated synovitis and osteophyte formation in mice subjected to ACLT surgery following intraarticular (IA) injection of Wnt5a-overexpressing BMDMs (ACLT+Mφ-Wnt5a). IHC staining and counting results showed larger number of Wnt5a positive cells in ACLT+Mφ-Wnt5a mouse synovium compared to other groups, suggesting that Wnt5a-overexpressing BMDMs tend to be maintained in OA knee joint. Meanwhile, from the results of IF staining, there was no significant difference between the number of M1 and M2 macrophages, which was consistent with the in vitro result, and indicated that the impact of Wnt5a on OA progression was not caused by promoting macrophage polarization.

These findings suggest that increased level of Wnt5a does not change the phenotype of macrophages, but it promotes cytokine expression in macrophages and contributes to severer pathological changes in OA.

1.3. Statement of Contributions

Figure 1.1 is made by Wenhui Li in BioRender.

Chapter 2: Materials and Methods

2.1. Animal models

All animal care and experimental procedures used in this study were approved by the Institutional Animal Care and Use Committees at Tufts University School of Medicine. 12-week-old C57BL/6 wild type mice were anesthetized by isoflurane and subcutaneously injected with sustained release buprenorphine (1mg/kg) before anterior cruciate ligament transection (ACLT) or sham surgery. During the surgery, a small incision was made in the skin over the knee joint to expose the knee joint capsule. Then under dissection microscope, the anterior cruciate ligament (ACL) in the knee joint was transected using scalpels. Once the ACL was transected, the incision was closed with sutures to promote healing and prevent infection. 12-, 42-, or 70-days post ACLT or sham surgery, mice were euthanized using carbon dioxide, and the whole knee joints were harvested.

2.2. Tissue processing and sectioning

Harvested knee joints were fixed in 4% paraformaldehyde (PFA) and incubated in decalcification solution at 4℃ for around 6 days. A series of solutions were used to dehydrate the tissue gradually: 25% ethanol once for 1 hour, 50% ethanol once for 1 hour, 75% ethanol once for 1 hour, 100% ethanol twice for 1 hour each time, and xylene once for 1 hour. Then, the knee joints were submerged in fresh xylene for overnight. Melted paraffin wax was used to completely immerse the tissue for overnight. Knees were embedded into blocks of paraffin and solidified under room temperature. After processing

and embedding, knee samples were sliced at a thickness of 5 μm using LEICA RM2255 microtome and collected onto VWR Superfrost Plus Slide (Avantor).

2.3. Safranin O staining and OA scoring

After being deparaffinized in xylene and rehydrated in a series of ethanol with deceased concentration (100%, 75%, 50%, 25%), joint sections were stained with 1% Safranin O (National Aniline) and 0.02% Fast Green (Aldrich Chemical Company, Inc), by which nuclei were stained by hematoxylin (StatLab), cartilage was stained by Safranin O, and connective tissues were stained by Fast Green.

The OARSI, Articular Cartilage Structure Score (ACS), Synovitis, and Mankin scoring systems have been previously established.[23,24] In short, the scoring system can be summarized as Table 5.1, Table 5.2, and Table 5.3.

2.4. Immunohistochemistry (IHC) staining

Knee joint sections were deparaffinized and rehydrated as described above. Sections were then subjected to antigen retrieval, incubation with 3% hydrogen peroxidase (CVS Health), 10% goat serum (Fisher Scientific), and avidin D and biotin solutions (Vector Laboratories). Sections were subsequently incubated with primary antibodies against mouse F4/80 (Rat, 1:120, Bio-Rad), Wnt5a (Rabbit, 1:75, Invitrogen), Turbo-GFP (Rabbit, 1:200, Fisher Scientific) at 4℃ overnight, followed by incubation with biotin-labeled goat anti-rat (1:100, Vector Laboratories) or biotin-labeled goat anti-rabbit (1:100, Vector Laboratories) antibodies. Avidin-Biotin Complex (Vector Laboratories) -

diaminobenzidine (Vector Laboratories) were used to visualize immunoreactions. 0.5% methyl green (R&D Systems) was used to stain nucleic acids.

2.5. Immunofluorescence (IF) staining

Knee joint sections were deparaffinized and rehydrated as described above. Sections were then subjected to antigen retrieval, incubation with quenching solution, and 10% goat serum (Fisher Scientific). Sections were subsequently incubated with primary antibodies against mouse F4/80 (Rat, 1:120, Bio-Rad), Wnt5a (Rabbit, 1:75, Invitrogen), iNOS (Rabbit, 1:50, Invitrogen), and CD206 (Mouse, 1:50, Santa Cruz) at 4℃ overnight, followed by incubation with Alexa Fluor-conjugated goat anti-rat (AF488, 1:500, Invitrogen), goat anti-rat (AF594, 1:500, Invitrogen), goat anti-rabbit (AF488, 1:500, Invitrogen), goat anti-rabbit (AF594, 1:500, Invitrogen), and goat anti-mouse (AF594, 1:500, Invitrogen) antibodies. Diaminophenylindole (DAPI, 1:1000) was used to stain nucleus. Mouse on Mouse Elite® Immunodetection Kit (Vector Laboratories) was used with mouse raised antibodies.

2.6. Bacterial transformation, proliferation, and plasmids extraction

Competent cells were transformed with Lenti-GFP or Lenti-Wnt5a plasmids (Horizon Discovery) and grown in agar plates. Single colonies were selected and proliferated in Lysogeny broth (LB) medium supplied with ampicillin (200μg/mL). Plasmids were extracted from proliferated bacteria using HiSpeed Plasmid Midi Kit (QIAGEN) and stored in -20℃.

2.7. 293FT transfection and lentiviral generation

293FT cells were seeded in 100 mm dish the day before transfection. 4.5 μg of Lenti-GFP or 3 μg of Lenti-Wnt5a plasmids was mixed with 3 μg of VSVG and 6 μg of Delta-8.91 plasmids. FuGENE 6 (Fugent LLC.) was used as transfection reagent. The mixture was added to 293FT cells with 90% confluency. Cells were incubated at 37 ℃ for 48~72 hours for collecting the first batch of supernatant and then another 48 hours for the second batch of supernatant. Lentivirus were then concentrated by ultracentrifuge.

2.8. Isolation, culture, transduction, and treatment of macrophages

L929 cell supernatant containing M-CSF was prepared by culturing L929 cells with Gibco™ Dulbecco's Modified Eagle Medium (DMEM, Fisher Scientific) supplied with 10% Fetal Bovine Serum (Gibco FBS, Fisher Scientific) and 1% Antibiotic-Antimycotic (Anti-Anti, Fisher Scientific). The supernatant was collected at day 7 to day 10 and filtered through 0.45 μm membrane filters (Pall Corporation).

Bone marrow was isolated from the femur and tibia of wild type mice. Red blood cells were lysed with lysis buffer. Cell pellet was resuspended with differentiation medium (DMEM supplied with 10% FBS, 1% Anti-Anti, and 20% L929 cell supernatant). M0 bone marrow-derived macrophages (BMDMs) were differentiated and collected for following treatment.

For lentiviral transduction of BMDMs, Multiplicity of Infection (MOI) of Lenti-GFP was 5~25, MOI of Lenti-Wnt5a was 50~100. Complete medium without L929 supernatant was changed for BMDMs before transduction. Lentivirus were added to the dishes and incubated for 24-48 hours, then change back to differentiation medium. On the

third day post transduction, BMDMs were treated with LPS (50ng/ml, Sigma-Aldrich) or IL-1β (5ng/ml, Peprotech) for another 24 hours. Then cells were harvested for mRNA or protein analysis.

2.9. Adoptive transfer

Wnt5a-overexpressing BMDMs were induced as described above. 35 days post ACLT or sham surgery, mice were subjected to intraarticular (IA) injection with 5×10^5 lentiviral-infected BMDMs under anesthesia. Whole knees joints were harvested 7 days after injection and processed as described above.

2.10. RNA extraction and quantitative real-time PCR

RNA was isolated from BMDMs using PureLink™ RNA Mini Kit (Invitrogen) according to the manufacturer's instructions. M-MLV Reverse Transcriptase (Invitrogen) and random primers (Promega) were used for reverse transcription. Gene expression was assessed using iTaq Universal SYBR Green Supermix (BioRad) and appropriate primers (IDT). The QuantStudio 6 Pro Real-Time PCR Systems (Fisher Scientific) was used for detection. Data were evaluated using $2^{-\Delta\Delta Ct}$ method, and gene expression was normalized to the internal control GAPDH. The sequences of all primers are listed in Table 5.4.

2.11. Protein extraction and Western blotting

Protein was extracted from BMDMs using RIPA. A BCA assay kit (Bio-Rad) was used to assess the concentrations of proteins. Equal amounts of proteins from each sample were separated with 10% sodium dodecyl sulfate – polyacrylamide gel electrophoresis, and

transferred onto Nitrocellulose Transfer Membrane (Whatman). Ponceau S was used to stain membrane bands. 5% BSA was used to block nonspecific binding for at least 1 hour at room temperature. The membranes were incubated with primary antibodies overnight at 4 ℃, followed by secondary antibodies for 2 hours at room temperature. The following antibodies were used: Wnt5a (Rabbit, 1:500, Invitrogen), GAPDH (Mouse, 1:500, Santa Cruz), peroxidase-conjugated goat anti-rabbit antibody (1:5000, Calbiochem) and peroxidase-conjugated goat anti-mouse antibody (1:5000, Calbiochem).

2.12. Enzyme-Linked Immunosorbent Assay (ELISA)

The level of IL-6 and IL-10 was evaluated in BMDM culture medium by Mouse IL-6 DuoSet ELISA kit and Mouse IL-10 DuoSet ELISA kit (R&D Systems) according to the manufacturer's instructions.

2.13. Cytokine array

The level of cytokines was evaluated by Quantibody™ Mouse Cytokine Array 1 kit (RayBiotech) according to the manufacturer's instructions. Fluorescence detection was performed by RayBiotech.

2.14. Statistical analysis

All results were presented as means ± SEM using Prism 10. Student's t test was used to analyze statistical differences between two groups, and analysis of variance (ANOVA) model with planned contrasts was used to compare data from multiple groups. Cells in at least 6 areas of each tissue were counted by ImageJ. The correlation analysis of cell

number was performed using Linear Regression from built-in analysis in Prism. Differences were considered statistically significant when p values < 0.05.

2.15. Statement of Contributions

Experiments were conducted with the help of Dr. Sihan Liu and guided by Dr. Li Zeng.

Chapter 3: Results

3.1. Macrophages are one of the main sources of Wnt5a in OA synovium.

To investigate OA in mice, we utilized a well-established surgical mouse model, ACLT mouse model, which is mechanically instability-induced osteoarthritis.[21,22]

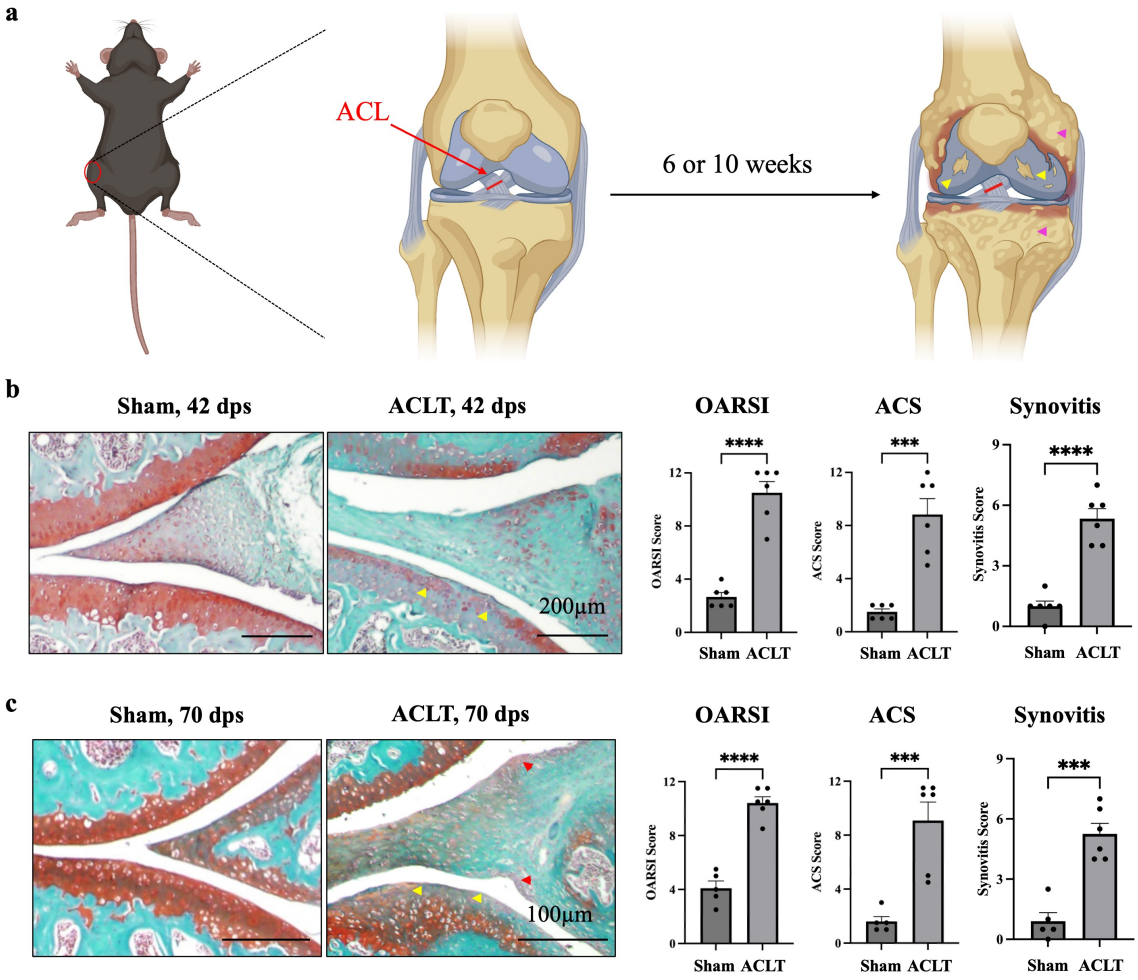

Figure 3.1 Pathological changes are developed in ACLT OA mouse model.
a: The scheme for ACLT surgery. 12-week-old mice were subjected to ACLT or sham surgery. After 6 or 10 weeks, cartilage loss (yellow arrowhead) and osteophyte (purple arrowhead) are developed. b, c: Representative images of Saf. O staining (top) showed severer cartilage loss (yellow arrowhead) and synovitis (red arrowhead) in mice undergone 42 (b) or 70 (c) days post ACLT surgery. Statistical scores (bottom) showed that ACLT mice have higher OARSI, ACS, and synovitis scores. n = 5~6 mice in each group. All scores were assessed by 2 individuals independently. Statistical analysis was conducted using unpaired t test. Error bars, SEM. ***P < 0.001. ****P < 0.0001.

12-week-old male mice were subjected to ACLT surgery, following 6 or 10 weeks of OA development (Figure 3.1 a). Pathological changes are expected to occur, including cartilage loss, structure damage, osteophyte, and synovitis. Sham mice underwent the identical surgical procedure, with the exception that the anterior cruciate ligaments were not transected. The levels of cartilage damage and synovitis were statistically evaluated based on histological assessment system for mouse OA.[23,24] For mice undergone ACLT surgery, much severer cartilage damage and synovitis have been shown in both 42 days post-surgery (dps) and 70 dps groups by Saf. O staining (Figure 3.1 b, c, left). In addition, at both time points, the OARSI and ACS scores are significantly higher in ACLT mice compared to sham mice, which indicates severer cartilage loss and structure damage. The synovitis scores are also elevated in ACLT mice compared to sham mice, suggesting enlarged lining layer, increased cellularity, and dense inflammation infiltrate (Figure 3.1 b, c, right).

The numbers of macrophages and cells expressing Wnt5a have been assessed using IHC staining on the established ACLT model (Figure 3.2 a), including both early (12 dps) and late (42 and 70 dps) stage OA. Compared to sham group, the number of macrophages (F4/80 positive) is significantly increased in ACLT mice at 12 days and 42 days post-surgery, and the level of Wnt5a positive cells is also increased in ACLT OA synovium at all 3 time points (Figure 3.2 b, c). Positive correlations have been found between the number of macrophages and Wnt5a positive cells in both early and late-stage OA synovium, suggesting that Wnt5a may be expressed predominately by macrophages (Figure 3.2 b, c). In addition, compared to the early stage, the number of both macrophages and Wnt5a positive cells was decreased at late stage, but the proportion of

Wnt5a positive macrophages was increased, which implicating a potential that Wnt5a promote OA progression in late stage (Figure 3.2 d, e).

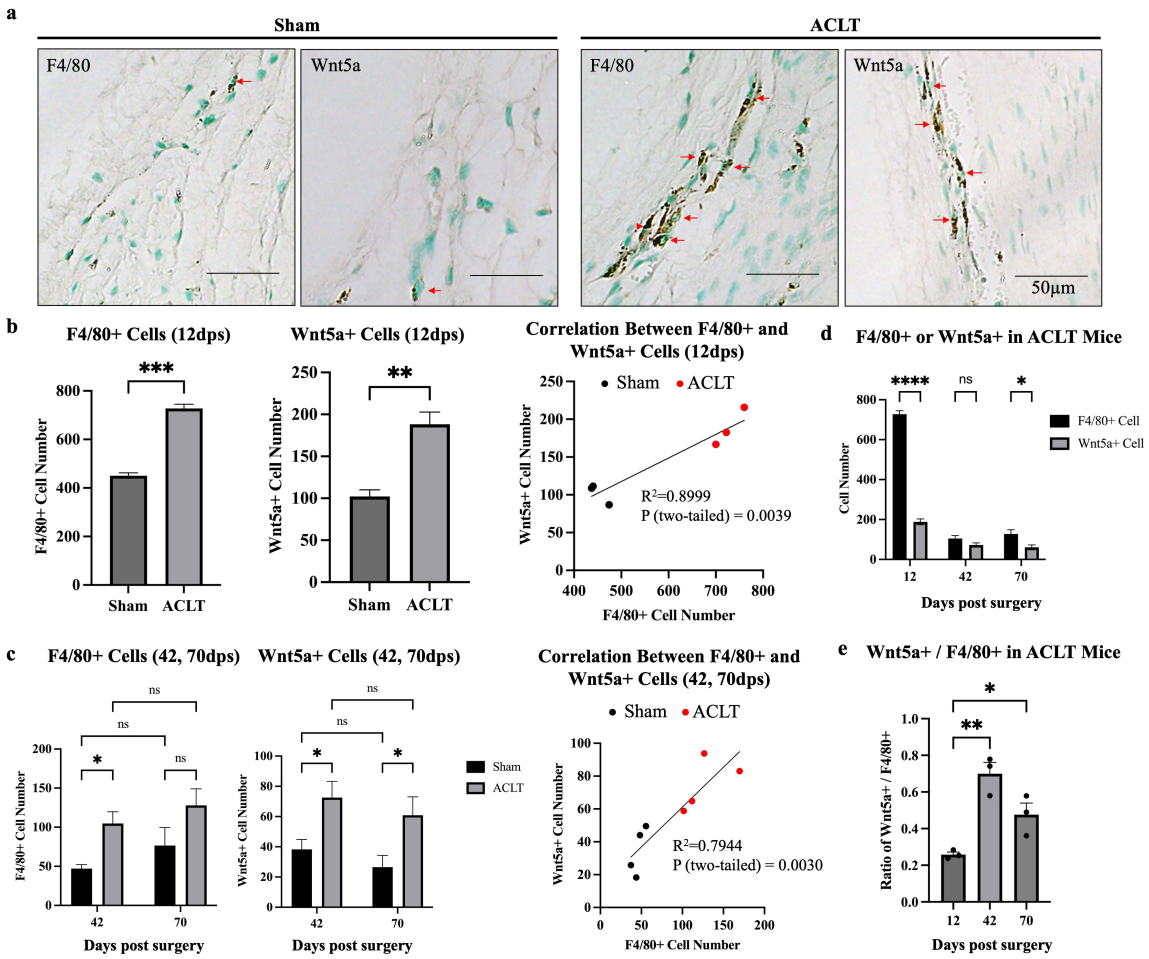

Figure 3.2 Macrophages are the main source of Wnt5a in ACLT OA synovium. a. Representative images of IHC showing the F4/80 and Wnt5a staining in Sham and ACLT knee joints (42 dps). b, c. Quantitative counting results showing the increased number of F4/80 positive cells (left) and Wnt5a positive cells (middle) in 12 dps (b), 42 and 70 dps (c) ACLT OA synovium. Positive correlation (right) has been shown between the number of macrophages and Wnt5a positive cells in 12 dps (b), 42 and 70 dps (c) groups. d. The number of F4/80+ cells and Wnt5a+ cells in ACLT group. n = 3 in each group. Statistical analysis was conducted using unpaired t teat or two-way ANOVA. Error bars, SEM. ns, not significant. *P<0.05. **P<0.01. ***P < 0.001. ****P < 0.0001.

The result of immunofluorescence (IF) staining shows the co-localization of macrophages and Wnt5a positive cells in OA synovium (Figure 3.3). Macrophages exist in OA synovium and some of them express Wnt5a, which showing the heterogeneity of

macrophages in OA. In conclusion, these results suggest that macrophages are the main source of Wnt5a in ACLT OA synovium.

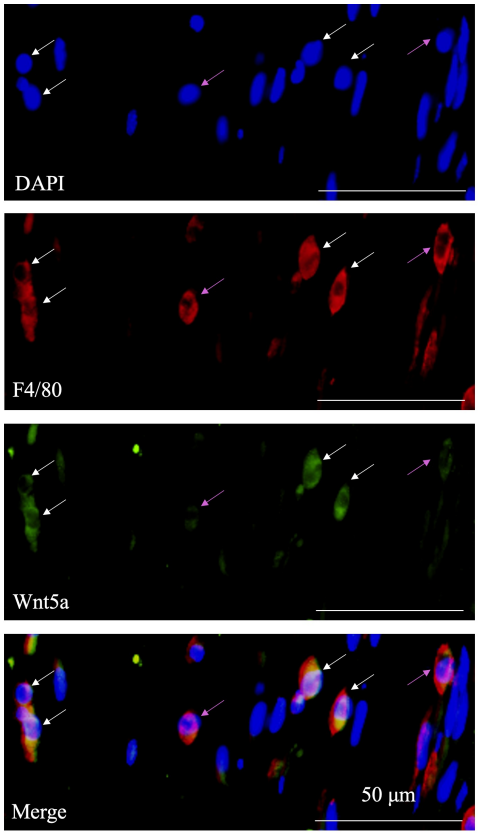

Figure 3.3 Representative images of IF staining showing the colocalization of macrophages and Wnt5a positive cells.
F4/80: macrophage marker, red. Wnt5a: green. White arrows: F4/80+Wnt5a+. Orange arrow: F4/80+Wnt5a-. Scale bar: 50 μm.

3.2. Wnt5a-overexpressing macrophages are established in vitro by lentiviral transduction.

After confirming the elevated macrophages and Wnt5a in ACLT OA synovium, we intended to establish an in vitro overexpression profile of Wnt5a in macrophages. Hematopoietic stem cells are isolated from mouse bone marrow and cultured with macrophage differentiation medium containing L929 cell supernatant (Figure 3.4 a), which contains macrophage colony stimulating factors (M-CSF).[25] Then, BMDMs are

obtained and transduced with GFP or Wnt5a lentivirus (Lenti-GFP / Lenti-Wnt5a, Figure 3.4 d), following with or without LPS or IL-1β treatment.

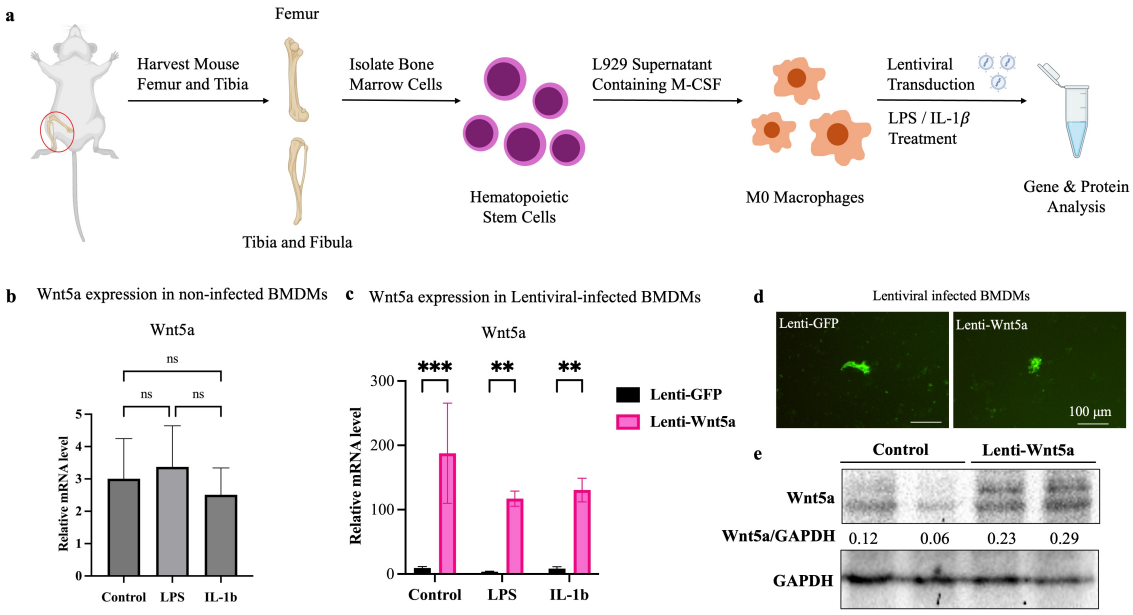

Figure 3.4 Lenti-Wnt5a infected BMDMs express higher level of Wnt5a compared to Lenti-GFP infected BMDMs.
a. The scheme of procedure isolating, differentiating, and transducing BMDMs. b. qPCR data showing that Wnt5a expression is not affected by LPS or IL-1β treatment. n = 3~4 in each group. One-way ANOVA. ns, not significant. c. qPCR data showing the significantly increased expression level of Wnt5a in Lenti-Wnt5a infected BMDMs. n = 3~5 in each group. Two-way ANOVA. ***P = 0.0002. **P = 0.0097 in LPS group, P=0.0071 in IL-1b group. d. Representative images showing the positive cells after lentiviral infection. e. Western blotting data showing the elevated level Wnt5a protein in Lenti-Wnt5a infected BMDMs. Error bars, SEM.

For the M0 macrophages obtained before lentiviral transduction, the expression level of Wnt5a is not affected by the treatment of LPS or IL-1β (Figure 3.4 b). After lentiviral transduction, Lenti-Wnt5a infected BMDMs express higher level of Wnt5a compared to Lenti-GFP infected BMDMs, under all non-treated, LPS-treated, and IL-1β conditions (Figure 3.4 c), and elevated level of Wnt5a protein is also observed by Western blotting (Figure 3.4 e).

3.3. Overexpression of Wnt5a in macrophages in vitro has effects on the cytokine production but not macrophages polarization.

Macrophages can be classified to two phenotypes based on their main functions. M1 macrophages can produce proinflammatory cytokines such as IL-1β, TNF-α, and IL-6, and are mainly involved in inflammation. M2 macrophages are mainly involved in wound healing and tissue repair and producing anti-inflammatory cytokines such as IL-10 and TGF-β.[26,27] Known markers of macrophages include CD86, CD40, and inducible nitric oxide synthase (iNOS) for M1 macrophages, and CD163, CD206, and Arg1for M2 macrophages.[27,28]

It has been previously studied that Wnt5a can induce M2 polarization in tumor-associated macrophages.[29,30] To investigate the effects of Wnt5a overexpression on the function of BMDMs in vitro, RT-PCR (qPCR) analysis was performed to test the expression level of signature genes for M1 and M2 macrophages. The expression level of iNOS was elevated in BMDMs only under IL-1β treatment, whereas Arg1 expression was significantly increased in BMDMs under all non-treated, LPS-treated, and IL-1β treated conditions (Figure 3.5).

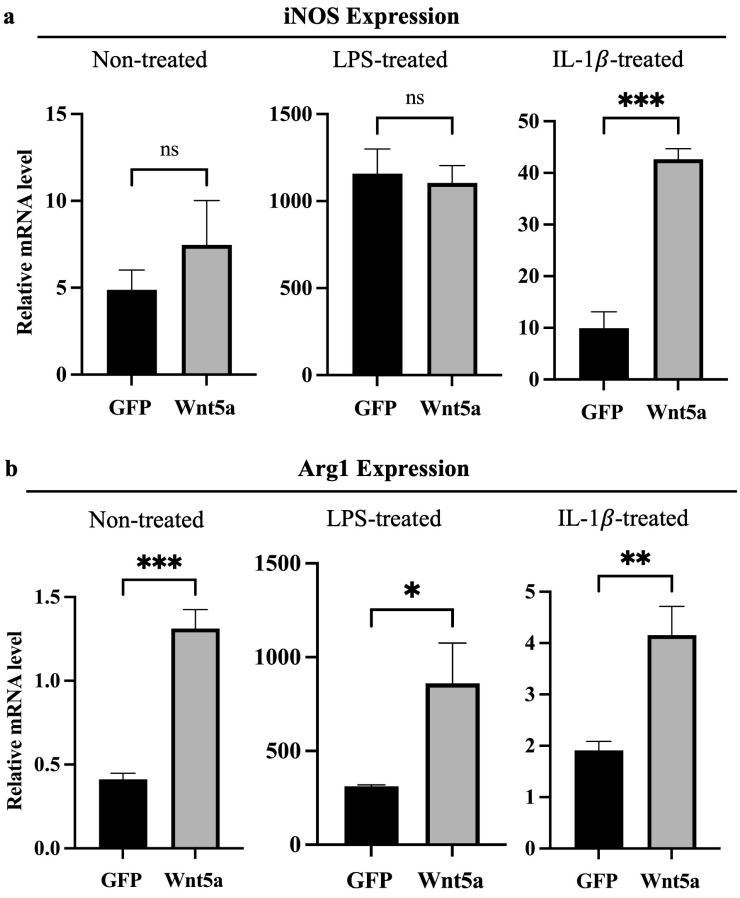

Figure 3.5 The expression profile of M1 and M2 signature genes in Lentiviral-infected BMDMs.
a. qPCR results showing the expression of M1 signature gene iNOS. b. qPCR results showing the expression of M2 signature gene Arg1. n = 3~5 in each group. Statistical analysis was conducted using unpaired t test. Error bars, SEM. ns, not significant. *P<0.05. **P<0.0q. ***P < 0.001.

In addition, the expression level of pro-inflammatory and anti-inflammatory cytokines were also tested. The overall levels of gene expression were not significantly changed, except for the reduced IL-6 expression in Lenti-Wnt5a infected BMDMs under LPS treatment (Figure 3.6). Interestingly, from the results of ELISA and cytokine array, the protein expression levels of both IL-6 and IL-10 were elevated under LPS treated condition (Figure 3.7).

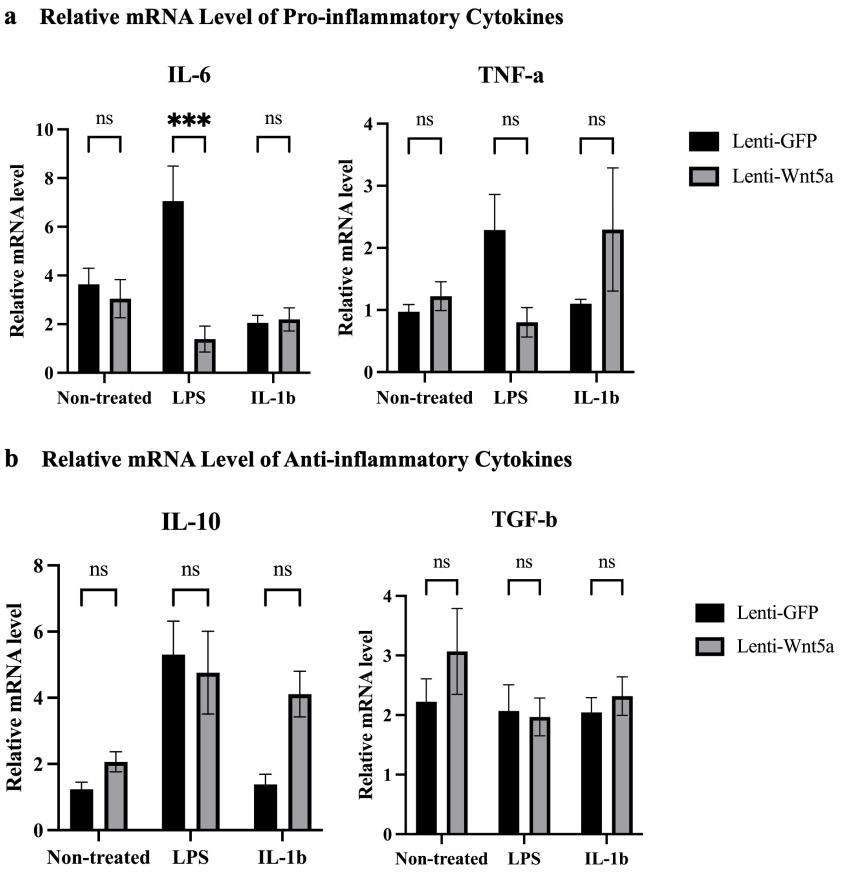

Figure 3.6 Gene expression of pro-inflammatory and anti-inflammatory cytokines tested by qPCR.
a, b. qPCR results showing the relative gene expression of pro-inflammatory cytokines IL-6 and TNFa (a), and anti-inflammatory cytokines IL-10 and TGFb (b). n = 3~5 in each group. Statistical analysis was conducted using two-way ANOVA. Error bars, SEM. ns, not significant. ***P < 0.001.

Except for pro-inflammatory and anti-inflammatory cytokines, Lenti-Wnt5a infection also elevated the expression of RANTES (CCL5), which plays a role in attracting other immune cells, and VEGF, which stimulate angiogenesis and is associate with tumor formation.

These results demonstrate that overexpression of Wnt5a in BMDMs can promote cytokine expression but may not alter the phenotype of macrophages.

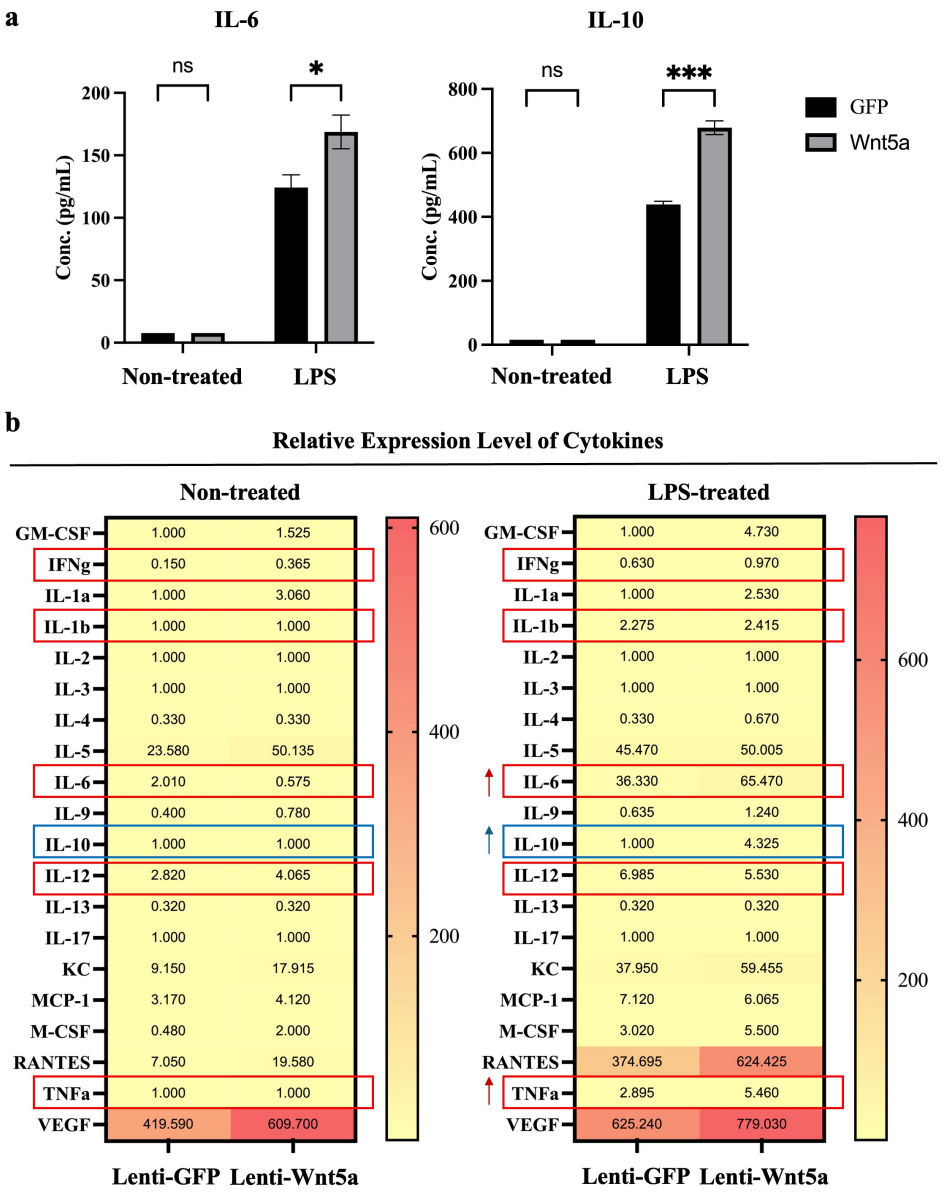

Figure 3.7 Protein expression of pro-inflammatory and anti-inflammatory cytokines tested by ELISA and cytokine array.
a. ELISA data showing the expression of IL-6 and IL-10. b. Cytokine array data showing elevated cytokine production in Lenti-Wnt5a infected BMDMs. Red rectangular indicate the pro-inflammatory cytokines, blue rectangular indicate the anti-inflammatory cytokines. Relative expression level = (Expression level of cytokines in Lenti-GFP or Wnt5a group) / (Expression level of cytokines in medium control). Statistical analysis was conducted using unpaired t test. Error bars, SEM. ns, not significant. **P<0.01.

3.4. After adoptive transfer, Wnt5a-overexpressing macrophages are maintained in OA knee joint and contribute to synovitis and osteophyte.

To further investigate the role of Wnt5a in vivo, adoptive transfer of Wnt5a-overexpressing BMDMs was performed at 35 days post ACLT surgery. Lentiviral-infected BMDMs were intraarticularly injected into mouse knee joints, then 7 days post injection, mice were sacrificed, and knee joints were harvested for histological staining and immunostaining (Figure 3.8 a).

After adoptive transfer of lentiviral-infected BMDMs into ACLT knee joints, severer synovitis and osteophyte formation were developed in ACLT mice injected with Lenti-Wnt5a infected BMDMs compared to the ones injected with Lenti-GFP infected BMDMs (Figure 3.8 b, c). In comparison, Wnt5a did not affect OA progression in sham mice (Figure 3.8 c). Moreover, the number of Wnt5a positive cells was significantly higher in ACLT mice injected with Wnt5a-overexpressing BMDMs compared to all other groups, even though sham mice were also injected with the same amount of Wnt5a-overexpressing BMDMs (Figure 3.9). This is an interesting finding which suggests the potential that Wnt5a-overexpressing BMDMs tend to be maintained in inflammatory OA environment.

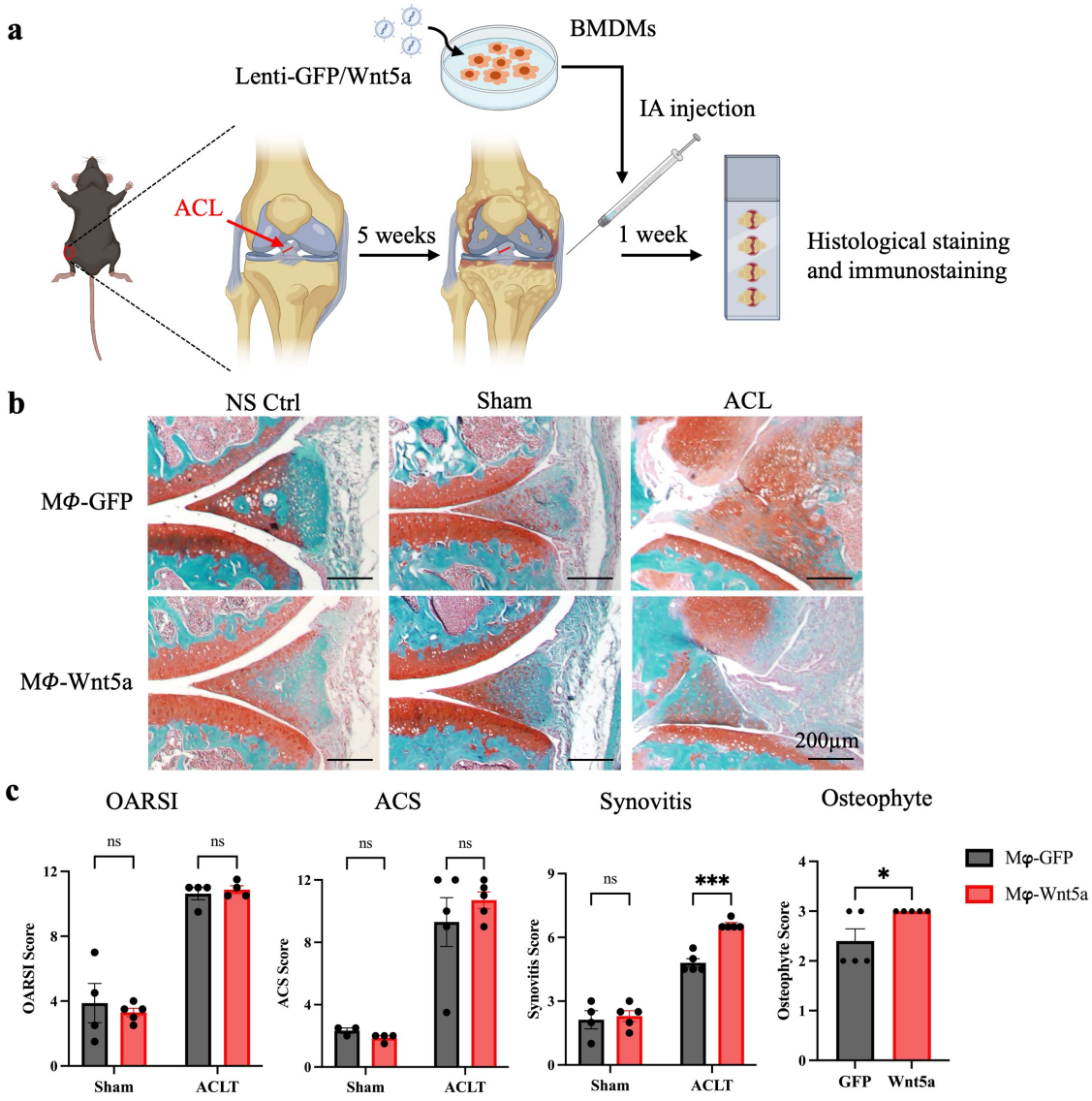

Figure 3.8 Severer pathological changes were observed in ACLT+Mφ-Wnt5a OA synovium.

a. The workflow for adoptive transfer after ACLT surgery. b. Representative images of Saf. O staining showing severer OA progression in ACLT mice. c. OARSI, ACS, Synovitis, and Mankin scores showing elevated cartilage loss, structure damage, synovitis, and osteophyte formation in ACLT+ Mφ-Wnt5a OA synovium. n = 3~5 mice in each group. All scores were assessed by 2 individuals independently. Statistical analysis was conducted using two-way ANOVA or unpaired t test. Error bars, SEM. ns, not significant. *P<0.05. ***P < 0.001.

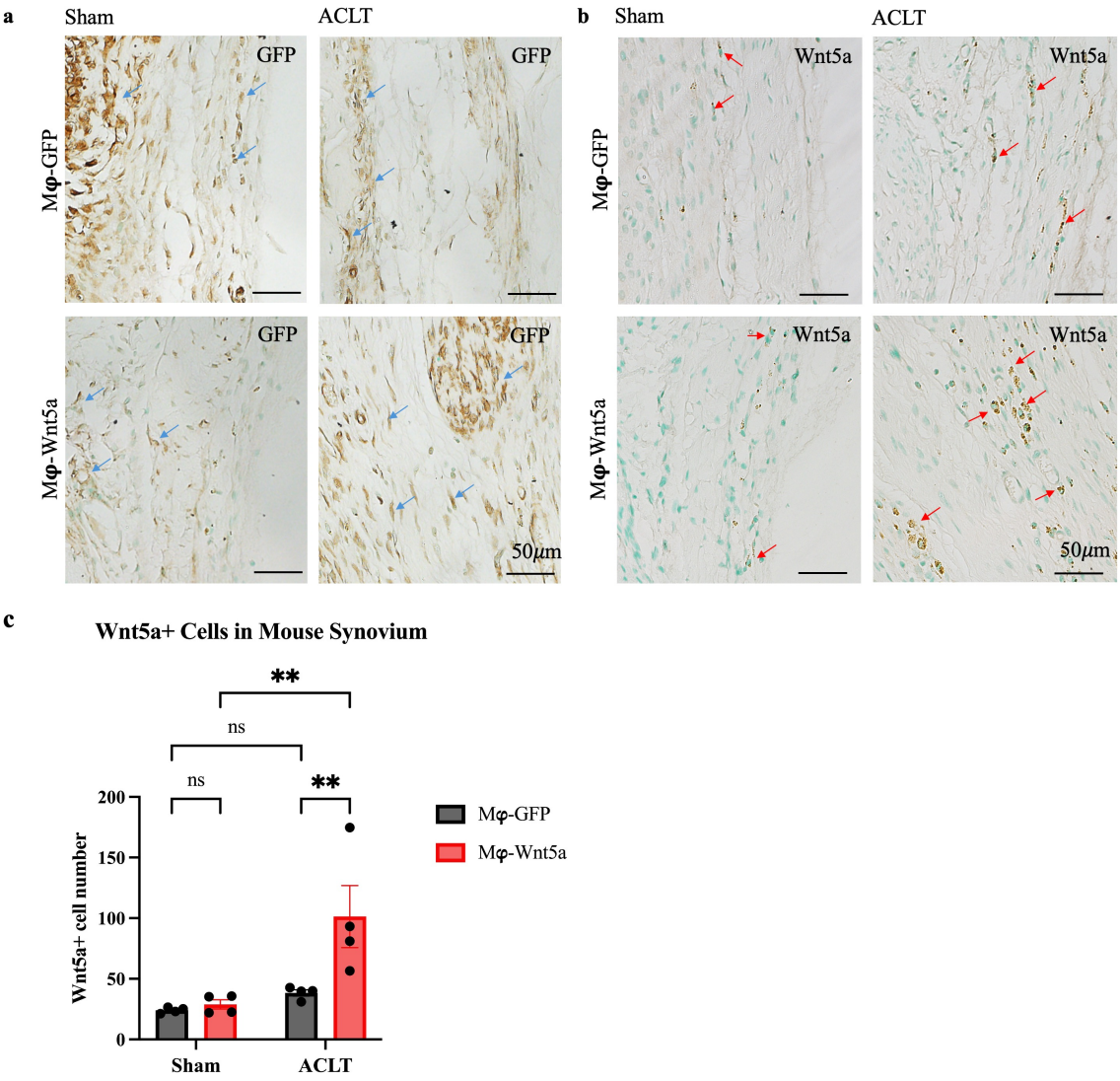

Figure 3.9 GFP and Wnt5a positive cells in mouse synovium after adoptive transfer. a, b. Representative images of IHC for GFP (a, blue arrow) and Wnt5a (b, red arrow) staining in adoptive transferred synovium. c. Counting results of Wnt5a positive cells showing the increased Wnt5a+ cells in ACLT mouse synovium adoptive transferred with Wnt5a-overexpressing BMDMs. n = 4 mice in each group. Statistical analysis was conducted using two-way ANOVA. Error bars, SEM. ns, not significant. **P<0.01.

Furthermore, to access if Wnt5a inflected the phenotype of macrophages in vivo, IF staining was performed on adoptive transferred tissue. The results showed that within the sham groups or ACLT groups, there is no significant differences between the numbers of M1 (F4/80+iNOS+) and M2 (F4/80+CD206+) macrophages (Figure 3.10), which further confirms that Wnt5a do not induce macrophage polarization in vivo.

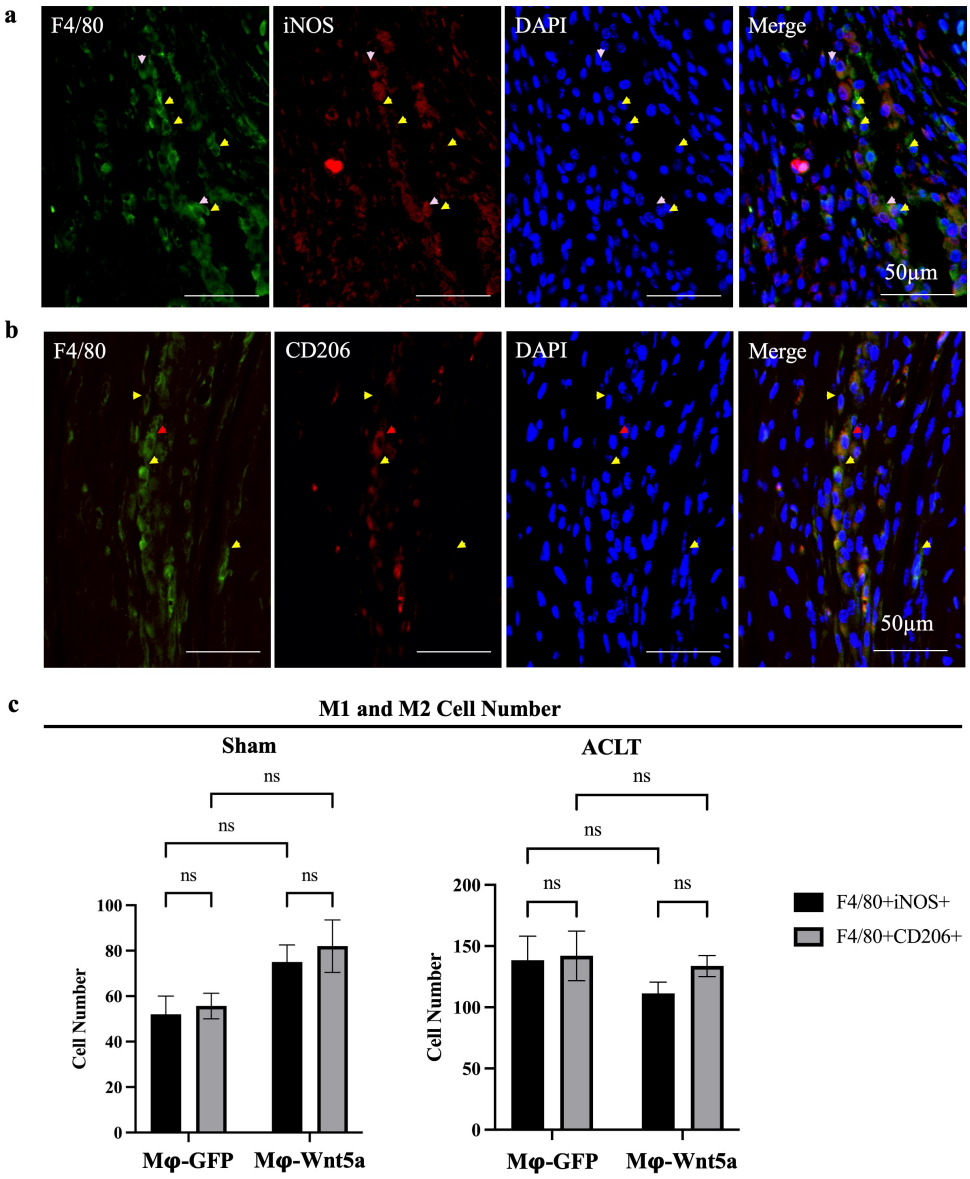

Figure 3.10 Wnt5a-overexpressing macrophages did not change towards M2-like in vivo. a,b. Representative IF images from ACLT+Mφ-Wnt5a mouse showing F4/80+ cells (yellow arrowhead), F4/80+iNOS+ cells (pink arrowhead), and F4/80+CD206+ cells (red arrowhead). c. Quantitative counting results showing the number of M1 and M2 macrophages in Sham or ACLT mice injected with Lenti-GFP infected BMDMs (M**φ**-GFP) or Lenti-Wnt5a infected BMDMs (M**φ**-Wnt5a). n = 3 tissues in each group. Statistical analysis was conducted using two-way ANOVA. Error bars, SEM. ns, not significant.

3.5. Statement of Contributions

Figure 3.1b and the osteophyte score in figure 3.8c was reprinted with permission from Dr. Sihan Liu, unpublished manuscripts. Figure 3.3 was stained by Wenhui Li, imaged by Dr. Sihan Liu. All other figures in this session were made by Wenhui Li.

Chapter 4: Discussion

In this study, we investigated the effects of overexpression of Wnt5a on the function of macrophages and OA progression. The colocalization and positive correlation were found between Wnt5a positive cells and macrophages from ACLT mouse knee joints, which indicate that macrophages are the main source of Wnt5a in OA synovium. In addition, not all the macrophages are expressing Wnt5a, which may implicate the heterogeneity of macrophages in OA.

Lentiviral-induced Wnt5a-overexpressing BMDMs were established to investigate the effects of Wnt5a on the function of macrophages in vitro and in vivo.

For in vitro study, after lentiviral transduction, the expression level of iNOS, a M1 marker, was elevated in Lenti-Wnt5a infected BMDMs only under IL-1β-treated condition, while the expression level of Arg1, a M2 marker, was significantly increased in Lenti-Wnt5a infected BMDMs under all non-treated, LPS-treated, and IL-1β-treated conditions. However, it was found by ELISA and cytokine array that the expression level of both the IL-6 and IL-10 protein are elevated in Lenti-Wnt5a infected BMDMs under LPS condition. In addition to pro- or anti-inflammatory cytokines, the level of CCL5 and VEGF, which are important in immune cell attractant and angiogenesis, was also elevated in Lenti-Wnt5a infected BMDMs. These results suggest that overexpression of Wnt5a in macrophages does not affect the phenotype of macrophages in vitro, but promoting cytokine production.

For in vivo study, Wnt5a-overexpressing BMDMs were adoptively transferred into ACLT OA mouse knee joints. Surprisingly, the ACLT OA mice injected with Wnt5a-overexpressing macrophages developed severer pathological changes, including synovitis

and osteophytes, compared to the ones injected with control macrophages. In addition, after adoptive transfer with Wnt5a-overexpressing BMDMs, higher number of Wnt5a positive cells was found in ACLT mice compared to sham mice, which implicates that Wnt5a-overexpressing macrophages tended to be maintained in osteoarthritis environment. Meanwhile, there was no significant difference on the number of M1 or M2 macrophages between the mice injected with control and Wnt5a-overexpressing macrophages.

From both the in vitro and in vivo results, we conclude that Wnt5a-overexpressing macrophages promote OA progression without changes of the phenotype in vivo, although Wnt5a have the potential to promote macrophages towards a more M2-like state in vitro.

However, whether Wnt5a contributes directly or indirectly to the progression of OA remains to be investigated. As previously discussed, the cellular crosstalk plays a critical role in the microenvironment of diseases.[8] Other immune cells, such as T lymphocytes, are also abundant in OA synovium and contribute to the pathogenesis of disease.[31,32] Does macrophage-expressed Wnt5a affect the function of T cells? What downstream signals does it activate or inhibit? Further studies are still needed on the mechanisms by which Wnt5a affects OA progression.

In summary, our studies indicate that overexpression of Wnt5a in BMDMs promotes OA progression and may be a potential therapeutic target for the treatment of osteoarthritis.

Chapter 5: Appendix

OARSI score:

Type of loss	Depth of loss	Length of loss	Score
Partial loss	≤ 1/2 depth	≤ 1/3 of surface	1
		≤ 2/3 of surface	2
		> 2/3 of surface	3
	> 1/2 depth	≤ 1/3 of surface	4
		≤ 2/3 of surface	5
		> 2/3 of surface	6
Complete loss	≤ 1/2 depth	≤ 1/3 of surface	7
		≤ 2/3 of surface	8
		> 2/3 of surface	9
	> 1/2 depth	≤ 1/3 of surface	10
		≤ 2/3 of surface	11
		> 2/3 of surface	12

Table 5.1 OARSI scoring system

ACS score:

Depth*	Length*	Score
≤ 1/4 depth	≤ 1/3 of surface	1
	≤ 2/3 of surface	2
	> 2/3 of surface	3
≤ 1/2 depth	≤ 1/3 of surface	4
	≤ 2/3 of surface	5
	> 2/3 of surface	6
< Full depth	≤ 1/3 of surface	7
	≤ 2/3 of surface	8
	> 2/3 of surface	9
Full depth	≤ 1/3 of surface	10
	≤ 2/3 of surface	11
	> 2/3 of surface	12

Table 5.2 ACS scoring system.

Score 0: Articular surface is smooth and intact.

*Depth/ Length of fibrillation and/or clefts and/or loss of cartilage

Synovitis score:

Enlargement of synovial lining cell layer	
Lining layer	Score
1 layer	0
2~3 layers	1
4~5 layers	2
More than 5 layers	3
Density of the resident cells	
Cellularity	Score
Normal	0
Slightly increased	1
Moderately increased	2
Greatly increased	3
Inflammatory infiltrate	
Feature	Score
No infiltrate	0
Few lymphocytes or plasma cells	1
Numerous lymphocytes or plasma cells	2
Dense band-line infiltrate	3

Table 5.3 Synovitis scoring system.

The final synovitis score is the summary of scores of three features.

Sequences of primers:

Name	Sequence
Mouse GAPDH Forward	CGT ATT GGG CGC CTG GTC AC
Mouse GAPDH Reverse	ATG ATG ACC CTT TTG GCT CC
Mouse Wnt5a Forward	CTC CTT CGC CCA GGT TGT TAT AG
Mouse Wnt5a Reverse	TGT CTT CGC ACC TTC TCC AAT G
Mouse iNOS Forward	GTT CTC AGC CCA ACA ATA CAA GA
Mouse iNOS Reverse	GTG GAC GGG TCG ATG TCA C
Mouse Arg1 Forward	CTC CAA GCC AAA GTC CTT AGA G
Mouse Arg1 Reverse	AGG AGC TGT CAT TAG GGA CAT C
Mouse IL-6 Forward	GGA AAT TGG GGT AGG AAG GA
Mouse IL-6 Reverse	CCG GAG AGG AGA CTT CAC AG
Mouse IL-10 Forward	TTT GAA TTC CCT GGG TGA GAA G
Mouse IL-10 Reverse	GGG AGA AAT CGA TGA CAG CG
Mouse TNF-α Forward	CAG CCG ATG GGT TGT ACC TT
Mouse TNF-α Reverse	GTG TGG GTG AGG AGC ACG TA
Mouse TGF-β Forward	TGA CGT CAC TGG AGT TGT ACG G
Mouse TGF-β Reverse	GGT TCA TGT CAT GGA TGG TGC

Table 5.4 Sequences of primers used in the study.

Chapter 6: Bibliography

1. He Y, Li Z, Alexander PG, et al. Pathogenesis of Osteoarthritis: Risk Factors, Regulatory Pathways in Chondrocytes, and Experimental Models. Biology (Basel) 2020;9(8). DOI: 10.3390/biology9080194.
2. Hunter DJ, Bierma-Zeinstra S. Osteoarthritis. Lancet 2019;393(10182):1745-1759. DOI: 10.1016/S0140-6736(19)30417-9.
3. Long H, Liu Q, Yin H, et al. Prevalence Trends of Site-Specific Osteoarthritis From 1990 to 2019: Findings From the Global Burden of Disease Study 2019. Arthritis Rheumatol 2022;74(7):1172-1183. DOI: 10.1002/art.42089.
4. Perruccio AV, Young JJ, Wilfong JM, Denise Power J, Canizares M, Badley EM. Osteoarthritis year in review 2023: Epidemiology & therapy. Osteoarthritis Cartilage 2024;32(2):159-165. DOI: 10.1016/j.joca.2023.11.012.
5. Aboulenain S, Saber AY. Primary Osteoarthritis. StatPearls. Treasure Island (FL) ineligible companies. Disclosure: Ahmed Saber declares no relevant financial relationships with ineligible companies.2024.
6. Srikanth VK, Fryer JL, Zhai G, Winzenberg TM, Hosmer D, Jones G. A meta-analysis of sex differences prevalence, incidence and severity of osteoarthritis. Osteoarthritis Cartilage 2005;13(9):769-81. DOI: 10.1016/j.joca.2005.04.014.
7. Chou CH, Jain V, Gibson J, et al. Synovial cell cross-talk with cartilage plays a major role in the pathogenesis of osteoarthritis. Sci Rep 2020;10(1):10868. DOI: 10.1038/s41598-020-67730-y.
8. Sanchez-Lopez E, Coras R, Torres A, Lane NE, Guma M. Synovial inflammation in osteoarthritis progression. Nat Rev Rheumatol 2022;18(5):258-275. DOI: 10.1038/s41584-022-00749-9.
9. Malsin ES, Kim S, Lam AP, Gottardi CJ. Macrophages as a Source and Recipient of Wnt Signals. Front Immunol 2019;10:1813. DOI: 10.3389/fimmu.2019.01813.
10. Nusse R. Wnt signaling in disease and in development. Cell Res 2005;15(1):28-32. DOI: 10.1038/sj.cr.7290260.
11. Komiya Y, Habas R. Wnt signal transduction pathways. Organogenesis 2008;4(2):68-75. DOI: 10.4161/org.4.2.5851.
12. Liu J, Xiao Q, Xiao J, et al. Wnt/beta-catenin signalling: function, biological mechanisms, and therapeutic opportunities. Signal Transduct Target Ther 2022;7(1):3. DOI: 10.1038/s41392-021-00762-6.
13. Buechling T, Boutros M. Wnt signaling signaling at and above the receptor level. Curr Top Dev Biol 2011;97:21-53. DOI: 10.1016/B978-0-12-385975-4.00008-5.
14. Hoppler S, Moon RT. Wnt signaling in development and disease : molecular mechanisms and biological functions. Hoboken, New Jersey: Wiley Blackwell,; 2014:1 online resource.
15. Sharma M, Pruitt K. Wnt Pathway: An Integral Hub for Developmental and Oncogenic Signaling Networks. Int J Mol Sci 2020;21(21). DOI: 10.3390/ijms21218018.
16. Schett G, Zwerina J, David JP. The role of Wnt proteins in arthritis. Nat Clin Pract Rheumatol 2008;4(9):473-80. DOI: 10.1038/ncprheum0881.

17. Lories RJ, Monteagudo S. Review Article: Is Wnt Signaling an Attractive Target for the Treatment of Osteoarthritis? Rheumatol Ther 2020;7(2):259-270. DOI: 10.1007/s40744-020-00205-8.
18. Huang G, Chubinskaya S, Liao W, Loeser RF. Wnt5a induces catabolic signaling and matrix metalloproteinase production in human articular chondrocytes. Osteoarthritis Cartilage 2017;25(9):1505-1515. DOI: 10.1016/j.joca.2017.05.018.
19. Thorup AS, Strachan D, Caxaria S, et al. ROR2 blockade as a therapy for osteoarthritis. Sci Transl Med 2020;12(561). DOI: 10.1126/scitranslmed.aax3063.
20. Fang H, Beier F. Mouse models of osteoarthritis: modelling risk factors and assessing outcomes. Nat Rev Rheumatol 2014;10(7):413-21. DOI: 10.1038/nrrheum.2014.46.
21. Kamekura S, Hoshi K, Shimoaka T, et al. Osteoarthritis development in novel experimental mouse models induced by knee joint instability. Osteoarthritis Cartilage 2005;13(7):632-41. DOI: 10.1016/j.joca.2005.03.004.
22. Lorenz J, Grassel S. Experimental osteoarthritis models in mice. Methods Mol Biol 2014;1194:401-19. DOI: 10.1007/978-1-4939-1215-5_23.
23. McNulty MA, Loeser RF, Davey C, Callahan MF, Ferguson CM, Carlson CS. A Comprehensive Histological Assessment of Osteoarthritis Lesions in Mice. Cartilage 2011;2(4):354-63. DOI: 10.1177/1947603511402665.
24. Krenn V, Morawietz L, Burmester GR, et al. Synovitis score: discrimination between chronic low-grade and high-grade synovitis. Histopathology 2006;49(4):358-64. DOI: 10.1111/j.1365-2559.2006.02508.x.
25. Heap RE, Marin-Rubio JL, Peltier J, et al. Proteomics characterisation of the L929 cell supernatant and its role in BMDM differentiation. Life Sci Alliance 2021;4(6). DOI: 10.26508/lsa.202000957.
26. Yunna C, Mengru H, Lei W, Weidong C. Macrophage M1/M2 polarization. Eur J Pharmacol 2020;877:173090. DOI: 10.1016/j.ejphar.2020.173090.
27. Mushenkova NV, Nikiforov NG, Shakhpazyan NK, Orekhova VA, Sadykhov NK, Orekhov AN. Phenotype Diversity of Macrophages in Osteoarthritis: Implications for Development of Macrophage Modulating Therapies. Int J Mol Sci 2022;23(15). DOI: 10.3390/ijms23158381.
28. Rath M, Muller I, Kropf P, Closs EI, Munder M. Metabolism via Arginase or Nitric Oxide Synthase: Two Competing Arginine Pathways in Macrophages. Front Immunol 2014;5:532. DOI: 10.3389/fimmu.2014.00532.
29. Liu Q, Yang C, Wang S, et al. Wnt5a-induced M2 polarization of tumor-associated macrophages via IL-10 promotes colorectal cancer progression. Cell Commun Signal 2020;18(1):51. DOI: 10.1186/s12964-020-00557-2.
30. Tigue ML, Loberg MA, Goettel JA, Weiss WA, Lee E, Weiss VL. Wnt Signaling in the Phenotype and Function of Tumor-Associated Macrophages. Cancer Res 2023;83(1):3-11. DOI: 10.1158/0008-5472.CAN-22-1403.
31. Sakkas LI, Platsoucas CD. The role of T cells in the pathogenesis of osteoarthritis. Arthritis Rheum 2007;56(2):409-24. DOI: 10.1002/art.22369.
32. Faust HJ, Zhang H, Han J, et al. IL-17 and immunologically induced senescence regulate response to injury in osteoarthritis. J Clin Invest 2020;130(10):5493-5507. DOI: 10.1172/JCI134091.