Osteoclasts have a unique capacity to destroy bone, playing key roles in physiological bone remodeling and arthritic bone erosion. It is not known whether the osteoclast populations in different tissue settings arise from similar monocytoid precursors. The rapid progress in the next-generation sequencing technologies has provided many valuable insights into the field of osteoimmunology, and single-cell RNA sequencing (scRNA-Seq) can elucidate cellular heterogeneity within the synovial microenvironment. The application of scRNA-Seq to the defined osteoclast precursor (OP)-containing population enabled the identification of individual cells differentiating into mature osteoclasts in the inflamed synovium, which were distinct from conventional OPs in the bone marrow. In addition, an intravital imaging system using multi-photon microscopy has been applied to the synovial tissues of arthritic mice to observe the real-time dynamics of osteoclasts and immune cells in the pannus. These technologies have contributed to elucidate the transcriptomics and dynamics of specific cells involved in pathological osteoclastogenesis, improving our understanding of the pathophysiology of inflammatory osteolytic diseases. Here, we review how novel technologies such as scRNA-Seq and intravital imaging help to better understand the pathogenesis of bone erosion and we introduce recent studies that have identified and directly visualized pathological OPs in inflamed synovium.

**1. Introduction**

Macrophages follow distinct developmental pathways in response to environmental stimuli in each organ and differentiate into osteoclasts in the bone marrow (BM) cavity. Osteoclasts are myeloid lineage multinucleated cells that have a unique bone-destructing capacity. Macrophage-colony stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) are essential for their differentiation [1–3], and osteoclasts support steady-state bone remodeling in the BM together with osteoblasts. In contrast, osteoclasts are also involved in pathological joint destruction in patients with rheumatoid arthritis (RA). Synovial inflammation induced by autoimmunity affects the outer surface of articular bone, eventually causing permanent joint destruction. Although the osteoclast precursor (OP)-containing population has been studied extensively in the BM of arthritic mice [4,5], no precise analysis of OPs has been performed in the inflamed synovium, the site of bone erosion in arthritis. Many questions remain regarding pathological osteoclastogenesis in arthritis. Is there a specific subpopulation within OP-containing population that actually differentiates into mature pathological osteoclasts in the synovium? Does the manner of bone resorption by pathological osteoclasts in the joint differ from that of osteoclasts in the BM? Are the osteoclasts formed at the pannus–bone interface derived from BM cells or resident synovial macrophages?

Advances in single-cell analysis have enabled the identification of small, rare subsets in heterogeneous cell populations, which cannot be detected by conventional bulk analysis due to the dilution of cell-specific information. Furthermore, the development of intravital imaging system using multi-photon microscopy has contributed immensely to the direct analysis of the dynamics of immune cells and osteoclasts in living mice. The combination of these two techniques facilitates the characterization of the transcriptomics and dynamics of specific cells involved in pathological osteoclastogenesis in the synovial microenvironment, improving our understanding of the pathogenesis of arthritic bone destruction.
This review introduces recent studies that used single-cell RNA sequencing (scRNA-seq) and intravital imaging techniques to identify and visualize pathological OPs in inflamed synovium.

**2. Differentiation trajectory of pathological osteoclasts in arthritis**

The precise analysis of synovial tissue is difficult due to the tiny amount of synovial tissue in arthritic mice. Therefore, we first established a protocol to purely isolate inflamed synovium of the knee joints in a collagen-induced arthritis model (CIA) without contaminating BM cells by removing the quadriceps femoris muscle, patella, and patellar ligament together under a stereoscopic microscope [6].

*Ex vivo* culture system of the inflamed synovium showed that OPs are included in the CX3CR1⁺ cells and *in vitro* analysis comparing several monocyte lineage cells within the CX3CR1⁺ cells revealed that CX3CR1hiLy6CintF4/80⁺I-A/I-E⁺macrophages had a high capacity to differentiate into osteoclasts, which we designated as arthritis-associated osteoclastogenic macrophages (AtoM) [6]. To elucidate the origins of AtoMs and pathological osteoclasts at the pannus–bone interface, BM chimeric models and a parabiosis model were used with CX3CR1-EGFP/TRAP-tdTTomato double transgenic mice, which label AtoMs with EGFP and mature osteoclasts with tdTomato. This showed that AtoMs and pathological osteoclasts are derived from BM cells and not from synovium-resident macrophages (Figure 1). AtoMs most efficiently differentiate into osteoclasts upon simultaneous stimulation with RANKL and TNF-α. These results support the clinical efficacy of TNF inhibitors against not only joint inflammation, but also pathological osteoclast formation. Although IL-6 did not have a direct effect on the osteoclastogenesis of AtoMs, IL-6 inhibitors clearly suppress bone erosion in RA, and IL-6 may function indirectly in osteoclastogenesis by promoting secretion of cytokines involved in osteoclastogenesis. In addition, experiments were performed with samples from a CIA model, in which the therapeutic effects of IL-6 inhibitors are relatively small compared to their effects in RA. Therefore, the effect of IL-6 may be underestimated.

A detailed analysis of the differentiation trajectory of pathological osteoclasts showed that CX3CR1LoLy6Chi cells in the blood (R1) transmigrate into the synovium (R2) and produce chemokines, inflammatory cytokines, and VEGFA. Some of these cells differentiate into osteoclast precursor cells (R3), arthritis-associated osteoclastogenic macrophages (AtoMs), via abundant M-CSF in the inflamed synovium. The combination of RANKL and TNF-α promotes pathological osteoclast differentiation at the pannus–bone interface, leading to devastating bone destruction in arthritis.

![Figure 1. Differentiation trajectory of pathological osteoclasts in the inflamed synovium. BM-derived CX3CR1LoLy6Chi cells (R1) ingress into the synovium (R2) and produce chemokines, inflammatory cytokines, and VEGFA. Some of these cells differentiate into osteoclast precursor cells (R3), arthritis-associated osteoclastogenic macrophages (AtoMs), via abundant M-CSF in the inflamed synovium. The combination of RANKL and TNF-α promotes pathological osteoclast differentiation at the pannus–bone interface, leading to devastating bone destruction in arthritis.](image-url)
strongly express CX3CR1, inhibition of CX3CL1 (fractalkine) may suppress the chemotactic activity of AtoMs, thereby inhibiting pathological osteoclast formation. Indeed, an anti-fractalkine monoclonal antibody clinical trial demonstrated an effective clinical response in active RA patients [7]. M-CSF and Foxm1 are other possible candidates for targeting pathological osteoclastogenesis.

In contrast to the OPs in the BM, AtoMs express cell surface molecules for antigen presentation, such as MHC class II and CD80/86. Because several studies have shown that immature dendritic cells (DCs) can differentiate into osteoclasts [8,9] and inflammatory osteoclasts can induce TNF-producing CD4+ T cells [10], AtoMs and pathological osteoclasts in arthritic joints may be involved in antigen presentation in the synovial microenvironment. AtoMs possess characteristics of macrophages, such as F4/80 expression and the presence of vacuolar cytoplasm, and DCs, such as CD11c and MHC class II expression, implying that these synovial OPs may share functional characteristics with both macrophages and DCs. This should be investigated further.

The physiological function of AtoMs remains unclear. Because articular bone destruction is also induced in pyogenic arthritis, AtoMs may function as a biological defense against bacterial invasion of the synovium by removing the bone tissue, which can serve as a scaffold for bacterial proliferation. However, this remains to be clarified.

3. ScRNA-Seq analysis of osteoclast precursors in the inflamed synovium

Many studies have identified OP-containing populations in various organs, including the BM, spleen, and blood. However, the number of cells in these OP populations is large compared to the actual number of mature osteoclasts in vivo, and the precise number and subpopulation responsible for differentiating into mature osteoclast in situ remains unclear. Therefore, we used an scRNA-Seq technique to precisely analyze the OP population in the

Figure 2. Intravital imaging of the inflamed synovium using two-photon microscopy. (A) Experimental procedures of intravital imaging of the inflamed synovium. CTLA-4 Ig is labeled with Alexa Fluor 647 (AF647) and intravenously injected into the collagen-induced arthritis (CIA) model after the onset of arthritis. (B) Tile scan images of the inflamed synovium of CX3CR1-EGFP transgenic mice. Inverted two-photon microscopy was used. CTLA-4 Ig labeled with AF647 extravasates and binds to CX3CR1+ cells as indicated by arrowheads. Second harmonic fluorescence generated via two-photon excitation visualized collagen fibers. Scale bar: 200 μm. The maximum intensity projections of two-dimensional image stacks of vertical synovial slices are shown.
synovium with high resolution of cellular differences. This revealed that about 10% of AtoMs (approximately 1000 cells in the knee joints per mouse) differentiate into mature osteoclasts at the pannus–bone interface [6]. Our future study will focus on further characterizing this small population of real OPs differentiating into mature osteoclasts in situ.

4. Development of an intravital imaging system for synovial tissue

4.1 Multi-photon microscopy

Over the past two decades, an intravital imaging system using multi-photon microscopy and a variety of reporter mouse strains have provided a number of valuable insights into the intravital behavior of immune cells in the lymph node, skin, lungs, kidneys, and BM cavity [11–14]. Two-photon microscopy uses two near-infrared photons to excite the fluorescent molecule and this technique has several advantages over single-photon confocal microscopy. First, near-infrared lasers can penetrate deeper tissues (100–1000 µm) to observe the cellular dynamics of organs in vivo. Second, minimal photobleaching and phototoxicity of the near-infrared lasers are suitable for intravital imaging for extended periods of time. Third, a nonlinear optical process called second-harmonic generation (SHG) can be used to visualize collagen fibers and bone matrix, which is indispensable in osteoimmunology research. In contrast, confocal microscopy can record multicolor images with high spatial and temporal resolutions if the depth of tissue is less than 50 µm. Therefore, single-photon confocal microscopy cannot be totally replaced by multi-photon microscopy. In fact, we combine the single-photon and two-photon images to simultaneously acquire a wide variety of fluorescence signals together with SHG signals to visualize fibrous tissues [6].

4.2 Intravital imaging of the synovial tissue

Although the intravital imaging of the bone and immune cells in the BM cavity has been extensively performed, visualization of pathological osteoclasts at the pannus–bone interface has been difficult for several reasons. First, the red blood cells in the hypervascular pannus tissue scatter light and impede the deep tissue imaging. Second, the hypertrophied synovial tissue is composed of multiple layers with various refraction indexes, which limits the observation depth. Third, the skeletal system is directly connected throughout the body and respiratory movement causes the visual field of the joint tissue to drift. Hence, we exposed relatively small joints of the arthritic mice, such as the wrist and metacarpophalangeal joints, and used inverted microscopy to minimize the visual field drift by fixing the region of interest to a cover glass.

A wide field of view can be achieved by tile scan imaging, and living immune cells, blood vessels, and collagen fibers were directly visualized in the synovial microenvironment of the CIA model in vivo (Figure 2) [15]. Combined with fluorescence labeling of CTLA-4 Ig, a biological agent used to treat RA, we tracked the intravital distribution of CTLA-4 Ig under arthritic conditions and revealed that CTLA-4 Ig predominantly distributes within the inflamed synovium, binding to CX₃CR1⁺ macrophages and CD140a⁺ fibroblasts immediately after intravenous injection (Figure 2). Further investigations are required to elucidate whether CTLA-4 Ig binding induces any change in the dynamics,

Figure 3. Intravital imaging of pannus–bone interface in arthritis. (A, B) Intravital images of the pannus-bone interface of CX₃CR1-EGFP/TRAP-tdTomato double transgenic mice taken by two-photon microscopy. Arthritis-associated osteoclastogenic macrophages (AtoMs) expressing CX₃CR1-EGFP signals, mature osteoclasts expressing TRAP-tdTomato signals, and second harmonic generation (SHG) defining the bone matrix. Some of AtoMs merge with TRAP-tdTomato, indicating that these cells are in transition from osteoclast precursors to mature osteoclasts. Scale bars: 100 µm. The maximum intensity projections (MIPs) of two-dimensional image stacks of vertical synovial slices are shown.
transcriptomics, or immunological function of immune cells in vivo.

Mature osteoclasts resorbing bone at the pannus–bone interface can be observed with multi-photon microscopy at the third metacarpophalangeal joint of the front paw [15]. We used mice expressing tdTomato in the cytosol of osteoclasts (TRAP-tdTomato mice) [16] and visualized bone tissue by SHG. Pathological osteoclasts were observed making small 50-μm-diameter resorption pits [15], which was unique to arthritic joints (Figure 3). Intravital images of the pannus—bone interface of CX3CR1-EGFP/TRAP-tdTomato double transgenic mice showed that some of AtoMs labeled with CX3CR1-EGFP merge with TRAP-tdTomato, indicating that these cells are in transition from OPs to mature osteoclasts (Figure 3).

To confirm that these osteoclasts are resorbing the bone matrix, we used a pH-sensing chemical fluorescent probe that detects the acidic region caused by functional osteoclasts in vivo [17]. This probe is composed of a bisphosphonate group and boron-dipyrromethene (BODIPY) dye, which has large molar absorption coefficients, high environmental stability, and environment-independent fluorescence quantum yields. After subcutaneous injection of this probe, intravital imaging of the pannus showed that pathological osteoclasts are actively resorbing the bone matrix in the tiny resorption pits without migrating on the bone surface [15]. This contrasts with the physiological osteoclasts in the BM, which are in close contact with osteoblasts and slowly migrate on the bone surface [18]. These results imply that the manner of bone resorption differs between the osteoclast populations in the BM and synovial microenvironment. Although the determinants of this unique bone-destroying capacity of pathological osteoclasts in the pannus are unclear, the cell components and cytokine milieu of the two tissues are quite different and the presence of fibroblast-like cells instead of osteoblasts in the synovium may help to answer this question. Overall, intravital imaging of the synovium can serve as a platform for exploring the dynamics of osteoclasts, immune cells, and biological agents involved in pathological osteoclastogenesis in vivo.

5. Conclusion

Advances in next-generation sequencing technologies and intravital imaging system have contributed considerably to clarify the reciprocal interplay between bone and immune cells in arthritis. scRNA-Seq analysis of the OP-containing population in the inflamed synovium succeeded in specifying the individual cells differentiating into pathological osteoclasts in the pannus. Contrasting the physiological OP-containing population in the BM, synovial OPs, called AtoMs, express surface markers related to antigen presentation and may share functional characteristics with both OPs and DCs, which can aggravate inflammation to promote osteoclastogenesis by themselves. AtoMs are derived from the circulation, and not from synovial resident macrophages, suggesting that the chemokines involved in synovial ingress of these cells are a new target for suppressing pathological bone destruction.

The state-of-the-art intravital imaging using multi-photon microscopy enabled the direct visualization of immune cells in the bone tissue, leading to characterize the molecular regulations and real-time dynamics of individual cells involved in the pathological osteoclastogenesis. In contrast to the physiological osteoclasts that migrate slowly on the bone surface, pathological osteoclasts stay in one position and make small resorption pits. Since inflammatory osteolysis is originally induced in pyogenic arthritis, this mode of resorption may be important for removing the scaffold needed for bacterial proliferation.

New fluorescent probes and optogenetic techniques, such as pH-sensing probe [17], photoconvertible fluorescent proteins (Kaede and KikGR), cell-cycle indicators (Fucci), and caspase-3 indicators (SCAT3.1) [19], are continuously developed to elucidate the unbiased spatiotemporal information on the complex biological phenomena in vivo. Future applications of these novel technologies will further reveal the relationships between the synovial microenvironment and functions of immune cells in situ, leading to the development of novel therapeutic approaches to inflammatory osteolytic diseases, including RA, crystal arthropathies, histiocytosis, spondyloarthritis, and sarcoidosis.

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