A comprehensive review of inflammatory cell migration in rheumatoid arthritis

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Abstract

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease that primarily affects the joints. Self-reactive B and T lymphocytes cooperate to promote antibody responses against self proteins and are major drivers of disease. T lymphocytes also promote RA independently of B lymphocytes mainly through the production of key inflammatory cytokines, such as IL-17, that promote pathology. While the innate signals that initiate self-reactive adaptive immune responses are poorly understood, the disease is predominantly caused by inflammatory cellular infiltration and accumulation in articular tissues, and by bone erosions driven by bone-resorbing osteoclasts. Osteoclasts are giant multinucleated cells formed by the fusion of multiple myeloid cells that require short-range signals, such as the cytokines MCSF and RANKL, for undergoing differentiation. The recruitment and positioning of osteoclast precursors to sites of osteoclast differentiation by chemoattractants is an important point of control for osteoclastogenesis and bone resorption. Recently, the GPCR EBI2 and its oxysterol ligand 7a, 25 dihydroxycholesterol were identified as important regulators of osteoclast precursor positioning in proximity to bone surfaces, and of osteoclast differentiation under homeostasis. In chronic inflammatory diseases like RA, osteoclast differentiation is also driven by inflammatory cytokines such as TNFa and IL-1, and can occur independently of RANKL. Finally, there is growing evidence that the chemotactic signals guiding osteoclast precursors to inflamed articular sites contribute to disease and are of great interest. Furthering our understanding of the complex osteoimmune cell interactions should provide new avenues of therapeutic intervention for RA.

Index entries

Rheumatoid arthritis; osteoclasts; osteoclast precursors; cell migration

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Introduction

Risk for inflammatory autoimmune diseases, like rheumatoid arthritis (RA), is influenced by genetic variation and environment. Genetic risk loci for autoimmune diseases have been identified by GWAS [1] and recently genetic fine mapping studies coupled with epigenomic analyzes have begun to identify putative causal variants [2,3]. Multiple disease-associated single nucleotide polymorphisms (SNPs) were mapped to genes and regulatory regions that are active in a cell-specific manner. These analyses revealed RA-associated SNP enrichment in genes and regulatory regions active in B cells, T cells, and to a lesser extent, monocytic cells [2,3]. This work provides a contextual framework for understanding the genesis of human autoimmune disease that can be applied to present and future studies in animal models. It also places the adaptive immune system front and center in the pathogenesis of RA.

Pathogenesis of RA

Rheumatoid arthritis is a chronic inflammatory disease characterized by an accumulation of inflammatory leukocytes in the synovium and articular tissues, and autoantibodies (rheumatoid factor and anti-citrullinated protein antibodies (ACPA)). The disease is also comprised of bone and cartilage tissue destruction and by other systemic features presumably caused by flares of inflammation. Even though RA is a common autoimmune disease that has been studied for several decades now, it is still unclear why articular tissues are predominantly attacked. The cellular components of the articular space include cells of the innate and adaptive immune system, chondrocytes, fibroblast-like synoviocytes, and bone remodeling cells, i.e. osteoclasts and osteoblasts. The accumulation of inflammatory leukocytes in articular tissues is a hallmark feature of RA, but this accumulation is thought to occur primarily because of cellular recruitment into inflamed tissues rather than as a consequence of local leukocyte proliferation [4].

The adaptive immune response in RA has been a focus of intense research over the last several decades. The strongest genetic link to RA involves SNPs in the Human Leukocyte Antigen (HLA)-DRB1 gene that likely alter T cell repertoire selection and antigen presentation, amongst other possibilities. The B cell repertoire is also constrained in RA patients, in which defects in early B cell tolerance checkpoints have been described [5], but for which there is still little mechanistic insight. Numerous antigens have been described in RA patients and in RA mouse models, and they include antibodies against the immunoglobulin Fc region (rheumatoid factor) and against citrullinated proteins (ACPA). Citrullination is a post-translational modification in which arginines are deiminated by peptidylarginine deiminases. ACPA are not only prevalent biomarkers for RA diagnosis, they also predict the development of the more debilitating erosive aspects of the disease. To date, it still remains unclear if specific autoantigen epitopes are key for the pathogenesis of RA, but autoantigens are clearly essential for the adaptive immune response in the pathogenesis of RA.

Numerous citrullinated polypeptides have been detected in the synovium of RA patients, populating a "citrullinome" that is comprised of both intracellular and extracellular antigens

ranging in size from ~14-100 KDa [6]. ACPAs can be detected years before disease onset and are likely important for shaping the development of disease [7–9]. In support of this possibility it was recently shown that immune complexes with citrullinated histones are potent activators of myeloid cells (macrophages and neutrophils) and induce TNFα secretion [10].

Evidence from several mouse models of inflammation in the joint and of inflammatory arthritis provides valuable insight into the spatio-temporal dynamics of the antibody response [11]. The K/BxN transgenic mouse is a model that allows studying the mechanisms whereby autoantibodies drive inflammation in the joint. In K/BxN transgenic mice there is spontaneous inflammation in the articular space that presents synovial pannus formation and bone and cartilage erosions [12]. K/BxN mice spontaneously produce autoantibodies against glucose 6-phosphate isomerase (GPI) that selectively deposit in the joints as immune complexes and trigger complement fixation [13]. Transfer of serum containing arthritogenic anti-GPI antibodies from K/BxN transgenic mice into naïve wild-type mice leads to the development of inflamed joints clinically and pathologically similar to what is observed in K/BxN transgenic mice [14]. Given that the generation of autoantibodies in the K/BxN model is crucial for disease development, effort has been made to identify the anatomical location(s) and kinetics of anti-GPI antibody production. The anti-GPI antibody response initially localizes to draining LNs and persists until later stages of the disease [15]. This indicates that draining LNs are involved in the initiation and perpetuation of the inflammatory arthritis antibody response in this model. Evidence in the clinical setting for LN involvement in RA patients has long been appreciated, including data showing that immune responses develop in draining LNs prior to the appearance of arthritis symptoms [16]. Importantly, B cell depletion from the draining LNs (popliteal) ameliorates inflammatory arthritis [17,18], and B cell depletion therapies significantly reduce RA symptoms in humans [19].

The activation of the B cell antibody response in the LN requires two antigen-driven events that are segregated in distinct temporal and anatomical locations: 1.) antigen recognition by B cells in follicles 2.) antigen presentation by T-zone dendritic cells and subsequent T cell activation in the T cell zone. Relatively small soluble antigens <70 kDa can gain access to B and T cell areas of the LN by free diffusion via the follicular conduit network [20–22]. Larger antigens are transported into follicles through their initial capture by CD169+ subcapsular sinus macrophages from the incoming lymph, and relay to B cells in follicles in a complement receptor-dependent manner. These large immune complexes become subsequently deposited on follicular dendritic cells (FDCs) where they persist for long periods of time [23–26]. The molecular sizes of autoantigens uncovered in arthritis patients suggests both mechanisms of antigen delivery to T and B cell niches in secondary lymphoid organs are likely to operate in RA. But, T cells can also be activated by DCs that may sample antigens in inflamed articular spaces and migrate to the draining LN for T cell priming. In RA patients, both mature and immature DCs have long been identified in synovium and synovial tissue by histology [27–32]. Plasmacytoid DCs have also been identified in rheumatoid synovium and synovial fluid [33–35]. Whether various DC subsets are recruited to the joints during the earliest stages of synovial inflammation remains unknown.

Shortly after antigen-engagement, activated B cells increase the expression of the T-zone homing chemokine receptor CCR7, and migrate to the T-B border where they interact with a subset of activated (cognate) T cells [36]. At this stage, activated B cells receive further differentiation signals from cognate T cells mostly through CD40/CD40L interactions. Importantly, SNPs in CD40 have been linked to RA [4]. Within 1–2 days after antigen engagement and CD40 signaling B cells increase EBI2 (GPR183) expression [37–40] and together with activated T cells, migrate to outer and inter-follicular areas in an EBI2-dependent manner. The oxysterol ligands of EBI2 (mostly 7α, 25-hydroxycholesterol, (7α, 25-OHC)) are presumably abundant in the follicle perimeter, including interfollicular regions [37–40]. Dendritic cells also express EBI2 and migrate to inter-follicular niches, and signals or cellular interactions occurring at these sites contribute significantly to the development of primary antibody responses by promoting plasmablast differentiation [41,42]. Later in the immune response a subset of activated T and B cells initiates germinal center responses where antibody affinity maturation, isotype switching, and long-lived plasma cell differentiation take place.

The GC reaction is thought to play an important role in RA. Follicular dendritic cells (FDCs) are critical for the formation of autoreactive GC reactions and development of joint inflammation in the K/BxN model [43]. In RA patients, T and B cells form aggregates in the articular space and can form ectopic GCs in about 40% of patients [44]. Given that anti-B cell therapy (Rituximab) is inefficient at depleting antibody-secreting plasma cells, and variably affects antibody titers [19], its efficacy may be associated with other roles B cells play in the organization of ectopic lymphoid follicles and GCs, and in T cell activation through cytokine secretion and autoantigen presentation. These tertiary lymphoid structures do not form in most experimental mouse models of inflammatory arthritis, the exception being in the collagen-induced arthritis (CIA) model [45]. The fact that CIA displays many similar histophathological features with human RA has made it one of the most widely studied models of inflammatory arthritis [46]. In this model, type II collagen (an abundant protein in cartilage), is used as an antigen for immunizing mice, which leads to the development of inflammatory arthritis. CIA is often used to evaluate therapeutic interventions.

Inflammation in the synovial compartment

Despite many decades of intense research very little is still known about the kinetics of cell recruitment into synovial tissues in RA patients and in experimental inflammatory arthritis. In a permutation of the K/BxN inflammatory arthritis model, LaBranche et al. transferred autoreactive KRN TCR transgenic T cells into T cell-deficient mice (B6.TCR.c$\alpha^{-/-}$H-2$^{b/g7}$) and were able to induce arthritic lesions that were synchronized and chronic in nature [47]. Interestingly, T cells were not detected in the joint but were present in the draining popliteal LN. Instead, monocyte/macrophage and neutrophil infiltration occurred early in disease progression, and correlated with bone and articular erosions. The influx of monocytes and macrophages, whether by recruitment or local differentiation and activation, highly correlates with inflammation and tissue damage in RA patients [48–50]. Given that monocytes and macrophages secrete numerous chemokines and cytokines, they also could be involved in the recruitment of other inflammatory cells into the joint, such as neutrophils.

Neutrophils are abundant in synovial tissues and fluid, and mouse models provide clear evidence for neutrophil involvement in disease. Neutrophil depleted mice are completely resistant to the K/BxN serum transfer model of inflammatory arthritis [51]. A study in an anti-type II collagen antibody induced inflammatory arthritis model reported that the initial cellular infiltrate to the synovium is mainly composed of neutrophils and macrophages [52], and depletion of neutrophils attenuates disease progression [52,53]. However, the mechanisms controlled by neutrophils that exacerbate disease remain poorly understood. Furthermore, whether macrophages also contribute to the onset of disease remains unclear.

Recruitment of innate immune cells

In the synovium and synovial fluid of RA patients numerous homeostatic and inflammatory chemokines with the potential to influence the trafficking of leukocytes into the tissue are upregulated. CCR7^{+} mature DC (CD83^{+} and DC-LAMP^{+}) present within synovial infiltrates of RA patients were found in close association with CCR7-ligand (CCL19 and CCL21) expressing cells, suggesting these chemokine pairs may play a role in the recruitment of DCs to the synovium [32]. In this study immature DC (CD1a^{+}) expressed CCR6 and were in close association with CCL20-producing synovial cells [32]. In humans it was also recently reported that pDCs expressed CXCR3 and CXCR4 and accumulated in the inflamed joints of RA patients, [34]. Furthermore, the levels of CXCR3 ligands CXCL10, CXCL11, and of the CXCR4 ligand CXCL12 in the synovial fluid of RA patients were significantly increased when compared to osteoarthritis (OA) patients [34], suggesting the involvement of CXCR3 and CXCR4 in pDC homing or retention in inflamed joints. Besides pDCs, other DC subsets express functional CXCR4 [54], and CXCL12 was found to be highly expressed in synovial tissues [55,56] and synovial fibroblasts from RA patients [57].

In the K/BxN serum transfer model, neutrophils are absolutely required for disease progression [51]. Neutrophils express CXCR2 and its ligand CXCL2 is abundant in arthritic tissues including CXCL2 [58]. Furthermore, CXCR2 was crucial for disease progression in this model [58]. Importantly, blockade of CXCR2 in other animal models of arthritis quantitatively reduces disease severity and the recruitment of neutrophils [59–61]. Given that CXCR2 promotes neutrophil egress from bone marrow cavities [62], it is unclear if CXCR2 deficiency reduced inflammatory arthritis by impairing neutrophil recruitment to the synovium or by reducing their egress from bone marrow, or both. Using intravital 2-photon imaging of LysM-GFP mice and the K/BxN serum transfer model of arthritis, Wang and colleagues were able to visualize neutrophil recruitment to the inflamed synovial space [63]. Intriguingly, they observed that neutrophils were often associated with monocytes at sites of extravasation, and global depletion of monocytes arrested neutrophil extravasation [63].

Recruitment of lymphocytes

Th1, Th17, and Treg cell types are present in inflamed articular tissues in RA patients, with Th1 cells being the most prevalent T helper cell subset [64,65]. Studies from mouse models in combination with data obtained from RA patients have provided some insight into the trafficking mechanisms for T cells in arthritis. CXCR3 and CCR5 are abundantly expressed by T cells isolated from the synovial fluid and synovial tissues of RA patients, and both

receptors are preferentially expressed on Th1 cells [66–68]. In adoptive transfer experiments in a mouse adjuvant arthritis model, T cells deficient in CXCR3 were poorly recruited to inflamed joints, and CXCR3 blockade using antibodies inhibited T cell recruitment and reduced disease severity [69]. Consistent with these findings, the CXCR3 ligands CXCL10 and CXCL9 are abundant in the synovial fluid of RA patients [66] and, interestingly, myeloid DCs isolated from the synovial fluid of RA patients express higher CXCL9 and CXCL10 amounts than myeloid DCs isolated from peripheral blood [70]. CCR5 has also been suggested to contribute to the recruitment of T cells to the synovium. Several studies have reported that a single nucleotide polymorphism (SNP) resulting in a non-functional CCR5 allele was negatively associated with RA [71–74], but this finding was not confirmed in other studies [75,76]. In animal models CCR5 deficiency had no effect on disease outcome [77].

In RA patients, CXCR4 is expressed on memory $CD4^+$ T cells ($CD45RO^+$) infiltrating the synovium [68]. In mice, T cell specific deletion of CXCR4 impaired their infiltration into the joints and decreased the severity of collagen-induced arthritis [78].

CX_3CR1 is expressed on T cell subsets, and in RA patients the CX_3CR1^+ fraction of T cells is increased when compared to healthy controls [79]. The ligand for CX_3CR1, fractalkine (CX_3CL1), is expressed by synoviocytes and endothelial cells and is elevated in RA patients compared to OA patients [80,79,81]. In the collagen induced mouse model of arthritis, CX_3CR1 is also expressed on T cells infiltrating the synovium and CX_3CL1 is also upregulated [82]. Given that the depletion of CX_3CL1 in synovial fluid in animal models of inflammatory arthritis reduced overall chemotactic potency [83], this chemokine receptor pair likely guides the recruitment of T cells to inflamed synovium.

Th17 cells play a key role in the pathogenesis of RA and these cells express CCR6 [84]. CCR6-expressing cells were identified in synovial tissue from RA patients several years ago [85], and the CCR6 ligand CCL20 is robustly secreted by synovial fibroblasts isolated from arthritic joints (but not secreted by granulocytes and monocytes) [84,85]. CCR6 blockade suppressed the onset and severity of disease and reduced the number of $CD4^+$ T cells that infiltrated the joint [84], although the numbers of Th17 cells were not specifically reported.

Administration of FTY720, a high affinity agonist for sphingosine 1-phosphate (S1P) receptors that induces receptor internalization and renders targeted cells unresponsive to chemotactic gradients of S1P, was protective in mouse models of inflammatory arthritis by reducing lymphocytic infiltration into the joints and bone erosions [86–88]. Lymphocytes (including T cells) [86–88] and monocytes [87] were sequestered in secondary lymphoid organs and thus, were unavailable to egress into the circulation and migrate to arthritic tissues. Although bone marrow populations were not examined in either study, it highlights the importance of this pathway in lymphocyte circulation and trafficking in inflammatory arthritis. S1P levels are elevated in the synovium and synovial fluid of RA patients (even higher than serum levels) and could contribute to cellular recruitment and retention [89,90].

An essential component of the pathogenesis of RA is B cells, highlighted by the efficacy of B cell depletion therapy [91,92]. In RA, B cell production of ACPA is strongly predictive of

erosive bone disease [93]. B cells can be found in abundant numbers organized into follicular structures in the synovium, and in ACPA+ RA patients about 25% of IgG+ B cells isolated from articular space encode antigen receptors that are specific for citrullinated antigens [94]. Whether self-reactive B cells differentiate within ectopic follicles and germinal centers in the synovium, or are recruited from draining LNs is not entirely clear.

The chemokine expression pattern on B cells isolated from the synovium of RA patients includes CCR5, CCR6, CCR7, CXCR3, CXCR4, and CXCR5 [95]. The levels of CCL19, CXCL12, and CXCL13 are all increased in the serum of RA patients [96], CCL19 is increased in the synovium of RA patients [97], and CXCL13 is expressed in ectopic germinal centers in the synovium of RA patients [98]. This combination of homeostatic and inflammation-associated chemoattractant receptors suggests an active involvement in B lymphocyte recruitment and cell organization in synovial spaces.

Inflammation in the subchondral bone marrow

The synovium, and more recently LNs, represent the main focus of the inflammatory response in RA, but nearby compartments also develop inflammation including the subchondral bone marrow. The subchondral bone marrow is located under the subchondral bone that separates the articular cartilage from the bone marrow cavity. The bone marrow cavity is an important site of hematopoiesis but the subchondral bone marrow is populated by adipocytes and lacking abundant hematopoietic activity under homeostatic conditions. However, evidence indicates that subchondral bone marrow participates in the inflammatory response in RA. Magnetic resonance imaging data (MRI) from RA patients identifies edema throughout the bone marrow, even at stages where there is a low level of synovial inflammation [99,100]. Indeed, like synovitis, bone marrow edema is also predictive of subsequent pathologic bone erosion [101,102]. Subchondral bone marrow inflammation, including ectopic germinal center-like structures, has been found in the CIA mouse model [45,103,104]. The subchondral bone marrow undergoes significant changes during the course of RA, including infiltrating leukocytes [105–108] and development of bone resorbing osteoclasts (OC) on the subchondral surface [109,110]. Collectively, these studies indicate that the bone marrow compartment may be important for the pathogenesis of RA, but this compartment has not yet been fully integrated into disease models.

Bone erosion in RA

Periarticular bone erosions are highly prevalent in RA and lead to joint deformities and impaired function [111]. As the only cell capable of bone resorption in the body, osteoclasts have a critical role in inflammatory disorders associated with bone erosions and focal bone loss, including RA [112]. In mouse models of inflammatory arthritis numerous studies have demonstrated that osteoclasts are absolutely required for bone erosions to occur [113–118]. Osteoclasts form early in disease progression in animal models and initially appear at the junction of synovial membrane and periosteal lining and within subchondral bone [119]. Even though osteoclasts can be detected on both the synovial and subchondral bone marrow sides of the joints, osteoblasts (bone synthesizing cells) are only detected on the subchondral

side [110,120–122]. Therefore the mechanisms of osteoclast differentiation from these two sides may be distinct.

Osteoclast differentiation under homeostasis

Osteoclasts are hematopoietic cells that differentiate from monocyte progenitors through sequential developmental stages. Although the exact identity of osteoclast precursors still remains unclear, studies have found that cells with osteoclastogenic potential in the bone marrow do not express lymphoid cell surface proteins or the aM integrin (CD11b/MAC1), and instead express the macrophage colony stimulating factor-1 receptor (Cfms, CSF1R, CD115) and the stem cell factor receptor (cKit). These cells overlap with the macrophage/ dendritic cell precursor (MDP) subset [123] and can also differentiate into monocytes, macrophages, and dendritic cells [124–127]. More recently, a clonogenic, monocyte- and macrophage-restricted progenitor cell (named cMoP) derived from the MDP was identified [128], but whether the cMoP subset retains osteoclast differentiation potential has not been defined.

The dominant cytokine that governs osteoclast differentiation in vitro and in vivo is RANKL, which signals through its receptor RANK [129–131]. Osteoblasts are key sources of MCSF (a cytokine required for cell survival and osteoclastogenesis) and RANKL [132], and RANKL expressed by osteocytes was recently shown to be critical for osteoclast differentiation in vivo [133]. RANKL is a type II transmembrane receptor and as such requires cell-cell interactions for engaging its receptor RANK expressed on osteoclast precursors. This predicts that positional cues are involved in the recruitment of osteoclast precursors to interact with RANKL^{+} cells. For many years it has been appreciated that resorbing bone is chemotactic for osteoclast precursors [134]. Recently, osteoblasts were reported to express oxysterol-synthesis enzymes, namely cholesterol 25 hydroxylase (gene name *Ch25h*) and 25-hydroxycholesterol 7-alpha-hydroxylase (gene name *Cyp7b1*) [135]. They also secrete the oxysterol 7a, 25 dihydroxycholesterol (7a, 25-OHC), which acts as a potent chemotractant for the pertussis toxin sensitive Gai protein coupled receptor EBI2 [135].

Furthermore, EBI2 is abundantly expressed in murine osteoclast precursors, and its expression increases during osteoclast differentiation [135]. It was reported that EBI2 directs the migration of osteoclast precursors to bone endosteal niches and promotes osteoclastogenesis in vivo [135]. Besides its role in cell positioning at the endosteum, EBI2 signaling also contributes to promote osteoclast precursor motility within bone marrow parenchyma [135], possibly in synergy with CX3CR1 [136]. Importantly, deficiencies in EBI2 or CH25H resulted in similar decreases in osteoclast numbers and increased bone mass in vivo [135], indicating that 7a,25OHC is the main EBI2 ligand controlling osteoclastogenesis and bone mass homeostasis. In summary, this study provided direct in vivo evidence for EBI2 and oxysterols in osteoclast precursor recruitment to endosteal surfaces, and consequently in osteoclast differentiation [135].

The chemokine monocyte chemoattractant protein-1 (MCP-1) is also expressed by osteoblasts in vitro when cultured in inflammatory conditions [137–139] and the MCP-1

receptor, CCR2, is expressed on mouse and human osteoclast precursors [140,141]. These findings led to a model where bone proximal MCP-1, produced by osteoblasts, attracts osteoclast precursors to sites of osteoclast differentiation [142]. In support of this model, CCR2 deficient mice display a mild increase in bone mass and stability, likely due to a reduction in osteoclast differentiation [143]. In humans, a polymorphism in CCR2 (V64I) was associated with increased bone mineral density in middle aged men and postmenopausal women [144], but whether this polymorphism alters CCR2 signaling is unclear [145]. Altogether, the bone mass phenotype reported in CCR2 deficient mice is mild and contrasts with the essential role of CCR2 in promoting monocyte egress from bone marrow, both in homeostasis and during systemic acute inflammation [146–148].

CCR1 and CCR5 have also been reported as abundantly expressed on human, mouse and rat osteoclast precursors, upregulated on osteoclasts, and promoting their chemotaxis in response to several of their ligands, as reviewed elsewhere [149]. Furthermore, CCR1 and its ligands significantly promote osteoclast differentiation in vitro and in vivo (particularly CCL9) and CCR1 deficient mice are mildly osteopenic [150,149]. Several CCR1 ligands are shared by CCR5, but no bone mass phenotypes in CCR5 deficient mice have been reported.

The chemokine receptor pair CX_3CR1 and its ligand CX_3CL1 are also functionally expressed during osteoclast differentiation, as reviewed elsewhere [149]. Interestingly, CX_3CL1 is produced by mouse osteoblasts [151] and CX_3CR1 deficient mice have a reduction in osteoclasts that results in a mild increase in bone mass [152]. Furthermore, functional inhibition of CX_3CR1 signaling provided protection from bone loss induced by irradiation [153], perhaps by disrupting CX_3CR1-mediated migration and retention in the bone marrow [136]. In summary, while these studies suggest that synergy between multiple chemokine receptors controls osteoclastogenesis, only EBI2 was shown to be directly required for osteoclast precursor positioning in bone endosteal niches (Figure 1).

In contrast to chemoattractants derived from bone-proximal niches, chemoattractants emanating from bone-distal niches can also influence the positioning of osteoclast precursors in the bone marrow. Furthermore, the balance between bone marrow retention and egress cues is likely an important point of control for osteoclast precursor positioning and differentiation. In favor of such a model is the fact that osteoclasts are predominantly in close proximity to blood vessels in bone marrow thus making it likely that osteoclast precursors encounter bone and blood vessel chemoattractants (Figure 2) [149].

Emerging evidence has provided insight into leukocyte migration and dynamic behavior in steady-state conditions, including the mechanisms controlling their egress from bone marrow. Leukocytes are thought to egress from bone marrow through a vast network of blood vessels often referred to as bone marrow sinusoids. The majority of bone marrow sinusoids are characterized by a single layer of endothelial cells often separated by small gaps and without a basement membrane. Several hematopoietic cells exit bone marrow parenchyma by moving towards bone marrow sinusoids in a chemoattractant-dependent manner. For example, natural killer cells, eosinophils and B lymphocytes express S1P receptors and migrate towards high concentrations of S1P within sinusoids [154–157]. Other hematopoietic cells, such as monocytes and neutrophils depend on chemokine receptors,

namely CCR2 and CXCR2, for exiting bone marrow parenchyma, and CCR2 ligands are abundant around sinusoids [147,62,146]. Osteoclast precursors have also been shown to use S1P receptors for exiting bone marrow, and defects in S1PR signaling alter osteoclast precursor differentiation [158,159]. CXCR4 is also expressed in osteoclast precursors and directs their migration towards CXCL12 in vitro [160,161]. CXCR4, and its ligand CXCL12, have a well-described role in the retention of multiple hematopoietic cell subsets in bone marrow, including hematopoietic stem and progenitor cells [162]. Even though CXCR4 is one of the most studied chemokine receptors, its role in steady-state osteoclastogenesis in vivo remains controversial. Several studies reported that osteoblasts express CXCL12 in vivo [163,164] and it was proposed that CXCR4 promotes osteoclast precursor migration towards sites of osteoclast differentiation in vivo [158,165]. However, studies using CXCR4 deficient and CXCL12 reporter mouse strains provided results that are not compatible with this model. For example, CXCL12 expression is highest in rare mesenchymal stromal cells capable of multilineage differentiation (osteoblasts, chondrocyte, and adipocytes) distributed in parenchyma and some adjacent to blood vessels [166–168]. Furthermore, CXCL12 expression is reduced by nearly 100-fold in osteoblasts in vivo [169], making it unlikely that CXCR4 would direct osteoclast precursors towards osteoblasts. In agreement with this possibility, osteoclast differentiation and bone resorption were increased when hematopoietic cells lack CXCR4 expression, [170], which favors a model where the balance between responsiveness to bone chemoattractants and to other chemoattractants influences osteoclast development in vivo (Figure 1).

Cannabinoid receptor-2 (CB2) is abundantly expressed in various hematopoietic cells, including monocytes/osteoclast precursors and it promotes cell migration to its ligand 2-arachidonoylglycerol (2-AG) [171]. In vivo, 2-AG activity is likely high in (or near) bone marrow sinusoids given that CB2 promotes immature B lymphocyte positioning in these locations, a process that is also dependent on $\alpha 4\beta 1$ adhesion to VCAM-1 [172]. It is, thus, conceivable that monocytes and osteoclast precursors are similarly attracted and/or retained within bone marrow sinusoids via CB2 chemotactic activity. In support of this hypothesis, CB2 deficient mice exhibit low trabecular bone mass, and CB2 antagonists inhibit osteoclast differentiation in vitro [173,174]. Furthermore, in humans a single nucleotide polymorphism in *CNR2* (encoding CB2) was significantly associated with osteoporosis [175–177].

Leukocytes can exit bone marrow through mechanisms that are independent of pertussis toxin-sensitive Gαi protein coupled receptors, and presumably independent of chemoattractant gradient sensing and cell intrinsic motility [178]. B-lineage lymphocytes enforced to express pertussis toxin or deficient in CXCR4 expression were found to be largely non-motile within bone marrow cavities of live mice, and were rapidly mobilized from bone marrow parenchyma into blood [178]. It was also noted that the bone marrow parenchyma is under shear stress induced by plasma perfusion and interstitial fluid flow [178]. It is plausible that the highly fenestrated nature of the sinusoidal network in combination with plasma and interstitial fluid flow back to collecting sinusoids allows non-motile cells (e.g. red blood cells) to exit the bone marrow in a passive manner, and that such unconventional exit routes are used by essentially all leukocytes, including osteoclast precursors.

Osteoclast differentiation within inflamed synovial space

The identity of osteoclast precursors in arthritis may be distinct from steady-state osteoclast precursors, but presumably belongs to the myeloid cell compartment. Myeloid cells are accumulated in synovial tissue and synovial fluid in RA [48,49]. Some studies have interrogated the phenotype of osteoclast precursors in inflammatory arthritis models. The hTNFα transgenic strain develops synovial hyperplasia and lymphocytic infiltrate, pannus formation, articular cartilage destruction, and osteoclast driven bone erosion [179]. In this model, a cell population expressing the aM integrin CD11b but not Gr-1 displayed osteoclastogenic potential, and this population was increased in the bone marrow and blood of hTNFα transgenic mice [180]. Another study utilizing the SKG model of spontaneous inflammatory arthritis identified a population of cells with osteoclastogenic potential that had low to negative expression for CD11b and expressed high levels of Ly6C [125], and these cells may overlap with cMoPs (Nevius and Pereira unpublished observations). Dendritic cells have also been reported to contain osteoclast differentiation potential. Specifically, immature DCs were able to form osteoclasts in response to MCSF and RANKL, and unidentified soluble factors in human synovial fluid increased the DC differentiation into osteoclasts. These findings indicate that DCs may contribute to arthritis not only by acting as antigen-presenting cells and promoting T cell activation, but also by their potential to differentiate into bone-resorbing osteoclasts [181,182]. Collectively these studies suggest that multiple myeloid cell populations contain osteoclast differentiation potential (Figure 3).

RANKL is expressed on activated T cells, B cells, DCs, and synovial fibroblasts, besides bone-producing cells, RANKL expressed on T cells [133,183] and B cells [184] is dispensable for osteoclast differentiation and skeletal development and maintenance under homeostatic conditions. However, in mouse models of inflammatory arthritis, and in RA patients, the expression of RANKL on T cells and synovial fibroblasts is robust [113,185–187]. In murine inflammatory arthritis it has been established that synovial fibroblasts support the conversion of FOXP3$^+$ Tregs into pathogenic Th17 cells, which express higher RANKL amounts than other CD4$^+$ T cell subsets [188]. Furthermore, Th17-derived RANKL is able to support osteoclastogenesis in vitro [188,189]. That the cytokine IL-17A is an important driver of osteoclastogenesis in arthritis has been appreciated for some time now [190], but the mechanisms of its action are still being elucidated. Interestingly, IL-17A has been shown to induce RANK on human and murine osteoclast precursors and promotes osteoclastogenesis [191–193]. In addition, some evidence suggests IL-17A may increase RANKL expression on fibroblastic synoviocytes and/or mesenchymal-derived cells [189]. A recent study has dissected the relative contributions of RANKL derived from T cells and synovial fibroblasts and found that synovial fibroblasts provide the relevant sources of RANKL in models of inflammatory arthritis [194]. In humans, RANKL blockade with denosumab reduces bone erosion possibly through inhibition of osteoclast differentiation and function [195]. In mice, RANKL-expressing Th17 cells have been reported to interact directly with osteoclasts in vivo. By fluorescently labeling osteoclasts and Th17 cells it was observed that Th17 cells preferentially located in contact with mature osteoclasts and this interaction was partially dependent on RANKL [196]. Finally, by using a pH sensitive

fluorescent probe and an indicator of local pH decreases associated with osteoclast H^+ secretion and bone resorption it was observed that Th17 and osteoclast interactions occurred in sites of active bone resorption [196]. Collectively through these studies a picture emerges in which Th17 derived IL-17A regulates the expression of RANKL on synovial fibroblasts, enhances the differentiation of osteoclasts in the inflamed joint, and exacerbates bone resorption in vivo (Figure 3).

As mentioned previously, MCSF and RANKL are critical cytokines for osteoclastogenesis, and while MCSF is a soluble cytokine, RANKL is a type II membrane protein. Therefore osteoclast precursors in the joint presumably receive RANK signaling directly from fibroblastic synoviocytes, and possibly from T cells, osteoblasts, or from interaction with osteocyte processes at the bone surface (Figure 3). Given that osteoclasts can form bi-directionally in the joint (synovium and bone marrow), the specific cell type presenting RANKL (and other cytokines) on the synovial or bone marrow side may differ, and may be under the influence of distinct cell recruitment mechanisms.

RANKL independent osteoclast differentiation in RA

It is now appreciated that in inflammatory settings osteoclast differentiation can occur independently of RANKL signaling [197]. Inflammatory cytokines such as TNFα [198], TNFα with IL-6 [199], and IL-1α [200] can induce osteoclast differentiation when in the presence of MCSF, but non-inflammatory cytokines like TGFβ have also been shown to trigger osteoclast differentiation [201]. Collectively these data identify cytokine-driven alternative pathways of osteoclast differentiation not dependent on the RANK/RANKL signaling pathway. Many of these pro-osteoclastogenic cytokines can be produced by macrophages, as reviewed elsewhere [197], and synovial macrophage numbers correlate with severity of inflammatory activity in RA [202]. Although pro-osteoclastogenic cytokines are not exclusively produced by synovial macrophages, these cytokines are present in the synovial milieu and may contribute to alternative pathways of osteoclast differentiation. Recent work from Harre and colleagues found that ACPAs enhanced the differentiation of osteoclasts in vitro [203]. They further showed that administration of ACPAs to lymphocyte deficient Rag1$^{-/-}$ mice was able to increase osteoclast numbers and reduce bone mass in vivo [203]. The mechanism by which ACPAs promote osteoclast differentiation is in part dependent on the Fc sialylation state of IgG in immune complexes [204]. Alternative pathways of osteoclast differentiation, driven by the unique inflammatory cytokine milieu (e.g. TNFα) and pathogenic autoantibody responses likely synergize with the dominant RANK/RANKL signaling pathway to induce bone erosions associated with RA. Indeed, TNFα induces RANKL expression on stromal cells via IL-1α signaling and enhance RANKL-dependent pathways of osteoclast differentiation [205].

Recruitment of osteoclast precursors in RA

It is clear in murine inflammatory arthritis and human RA that osteoclast precursors populate the synovium and synovial fluid. Conceptually, osteoclast differentiation in RA is likely controlled by multiple guidance cues balancing osteoclast precursor retention in bone marrow, migration from the bone marrow to egress sites, and migration into tissues, i.e. the

synovium (Figure 3). The chemotactic signals guiding cells to these sites are incompletely defined and are of great interest. Some studies indicate that the cytokine TNFα, which is abundant in synovial tissue, can expand osteoclast precursors in inflammatory arthritis models and can also induce the mobilization of osteoclast precursors from the bone marrow into the periphery [206,207,180]. Given that in RA patients S1P levels are elevated in the synovium and synovial fluid (even higher than serum levels) [89,90], S1P receptor signalling could contribute to monocyte recruitment and retention in the joints. Consistent with this hypothesis, blockade of S1P signaling by administration of FTY720 reduces lymphocyte [86–88] and monocyte [87] migration to the joints.

Human and mouse monocytes/osteoclast precursors and osteoclasts also express functional CCR1, CCR5, and CCR2. The CCR2 and MCP-1 chemokine receptor pair not only promotes monocyte egress from bone marrow under homeostatic and inflammatory conditions [146–148], but MCP-1 expression is increased in inflamed synovial fluids [208,209]. Therefore, these collective reports suggest that CCR2 attracts monocytes/ osteoclast precursors from blood circulation to inflamed joints. It remains to be completely dissected the relative contribution of CCR2 in the egress of monocytic cells and in the recruitment of those cells into tissues like the inflamed synovium. Potential sources for MCP-1 include osteoblasts and mesenchymal stromal cells.

Early studies in mouse models showed that the severity of inflammatory arthritis is mitigated by administration of receptor antagonists to CCR2 and CCR5 [210,211]. It was subsequently elucidated that the kinetics of CCR2 blockade are crucial for a beneficial outcome [212]. However, genetic deletion of these receptors had an opposite effect. Mice genetically deficient in CCR2 and CCR5 displayed either enhanced severity of inflammatory arthritis (including enhanced monocyte recruitment) or had no effect on disease outcome, respectively [77]. More recently, inflammatory arthritis induced in CCR2 deficient mice were described to have increased Th17 cells, which may partially explain the disease exacerbation [213]. The blockade of another GPCR, CCR1, in inflammatory arthritis mouse models showed both inhibition of chemotaxis and reduction of inflammation [214,215,211,216].

The promising data from mouse models prompted the development and testing of CCR1, CCR2, and CCR5 antagonists as a therapeutic approach for the treatment of RA [217–221]. However, the results of the clinical trials were variable and did not show an overt efficacy in the treatment of RA patients. This could be in part due to the redundancy of the chemokine receptor and chemokine system. For example, CCR1 has 13 distinct chemokine ligands, some of which are shared ligands for CCR2, CCR3, CCR5, and CCR10. There are additional chemokine receptors that have been suggested to regulate the recruitment of monocytes/osteoclast precursors in RA.

Other chemokine receptor pairs that may regulate the recruitment of osteoclast precursors to the bone surfaces in homeostasis may also play a role in inflammatory conditions, like RA. For instance, CXCL12 is highly expressed in synovial tissues [55,56] and synovial fibroblasts from RA patients [57] and the migration of monocytes to supernatants from synovial fibroblasts was significantly decreased after neutralization of CXCL12 [222]. In the

context of RA, CB2 is more abundantly expressed in the synovial tissues of RA patients, and administration of a selective CB2 antagonist in a mouse model ameliorated inflammatory arthritis and bone destruction [223]. Although the distribution of 2-AG within bone marrow has not been exactly defined, the fact that CB2 signaling occurs in the vicinity of (or within) bone marrow sinusoids under normal homeostatic conditions, and that it can be detected in synovial tissues, suggests that 2-AG may act as a chemoattractant or a retention signal. Finally, the neutralization of the CX_3CR1 ligand, CX_3CL1 (fractalkine), in synovial fluid in murine inflammatory arthritis reduced overall chemotactic potency [83]. Blockade of CX_3CR1 in a murine model decreased the recruitment of monocytic cells to synovial tissues and decreased disease incidence and severity [82]. CX_3CL1 is elevated in RA patients compared to OA patients [81,80] and CX_3CL1 has been shown to be upregulated in mouse bone marrow endothelial cells in inflammatory conditions, such as full body irradiation [153].

CXCR2 and CXCL2 have also been implicated in inflammatory bone remodeling [224]. In osteoclast precursors, CXCL2 is induced by RANKL and promotes their chemotaxis to CXCL2 [224]. The expression of CXCL2 was increased in the synovial fluid of RA patients and CXCL2 significantly enhanced bone resorption in vivo [224]. Therapeutic neutralization of this chemokine receptor pair had positive results in animal models of arthritis [225,60,226], possibly owing to the inhibition of CXCL2-mediated recruitment of osteoclast precursors to inflamed joints. In the K/BxN serum transfer model, the relevant chemokine receptor for arthritis progression was shown to be CXCR2, specifically for the recruitment of neutrophils [58]. But this study does not preclude an important role for CXCR2 in the recruitment of osteoclast precursors [58].

Under homeostasis, EBI2 and CH25H are required for osteoclast precursor recruitment and osteoclast differentiation. But, EBI2 and CH25H are also required for the generation of antibody responses, and have also been implicated in a variety of inflammatory diseases. SNPs in Ebi2 or in regulatory elements of *Ebi2* expression are associated with cardiac inflammation, type I diabetes and inflammatory bowel disease [227–229]. Furthermore, *Ch25h* is one of the most induced interferon-stimulated genes [230]. Future studies should determine if CH25H activity is increased in the inflamed articular space, and if EBI2 ligands play roles in inflammatory cell recruitment and osteoclast differentiation in inflamed joints.

While many studies interrogated functional roles for chemoattractant receptors in osteoclast differentiation and bone erosions in RA patients, and mouse models of RA, little remains understood about the manner in which they contribute to disease. It is likely that osteoclast precursor differentiation within inflamed synovium requires sequential chemotactic signals: at the first stage cells must be captured from blood circulation and extravasate into the synovium. Subsequently, osteoclast precursors likely follow chemotactic gradients that not only promote retention within the articular space but may also direct their migration towards sites of osteoclast differentiation. It is possible that redundancy between chemokine and other chemoattractants control such multi-step process of osteoclast differentiation.

Inflammation induced changes in bone homeostasis

Hematopoiesis has been proposed to be regulated locally by cytokines [231]. Cytokines are capable of instructing hematopoietic lineage choice by acting on 1) HSC, 2) Hematopoietic progenitor cells (HPC), and 3) mesenchymal stromal cells that support HSC. Furthermore, inflammatory cytokines, such as type I interferons may also function as rheostats of HSC proliferation [232,233]. During stress response, HSC enter in cycle and self-renew but when homeostasis is reestablished, HSC return to a quiescent state [234].

The inflammatory cytokine signature of RA includes several cytokines that have profound effects in hematopoiesis by biasing cell lineage choices. Two of the most notorious are TNFα and IL-1, and these cytokines can induce hematopoietic shifts that favor the production of inflammatory, innate-type cells, such as neutrophils and monocyte-lineage cells, at the expense of lymphopoiesis [235,236]. Of note, systemic TNFα increases the number of CD11b^{high} osteoclast precursors in circulation [237], and this effect may in part be due to an increase in osteoclast precursor proliferation and differentiation in bone marrow [180]. Such inflammation-induced shifts in hematopoiesis are most likely dependent on local G-CSF production, although it is possible that other inflammatory signals contribute to this effect [238]. In experimental mouse models of RA, genetic and pharmacologically induced G-CSF deficiency prevented acute and chronic arthritis [239], possibly because it reduced myeloid cell differentiation in bone marrow.

Concluding remarks

It is becoming increasingly understood that a variety of processes controlled by the adaptive immune system are the major determinants of RA. But, adaptive immunity requires instructive signals provided by innate immune effector mechanisms, thus leaving open the question of which is/are the triggers that inadvertently fire self-reactive lymphocytes. Furthermore, even though RA does not develop without lymphocytes, disease only manifests when innate immune effector cells infiltrate articular spaces and cause pathology. Of note, the resorptive activity of synovial osteoclasts is a hallmark feature of chronic, advanced stage RA and is a major contributor to articular damage.

Considering the multiple pathways EBI2 signaling is involved in (namely osteoclast development and the generation of adaptive immune responses), which are central to the development of inflammation in articular spaces, antagonists of EBI2 signaling may provide significant protection against RA. Understanding the multitude of osteoclast differentiation pathways and mechanisms of osteoclast precursor recruitment in homeostasis and during inflammation is thus of utmost importance.

Acknowledgments

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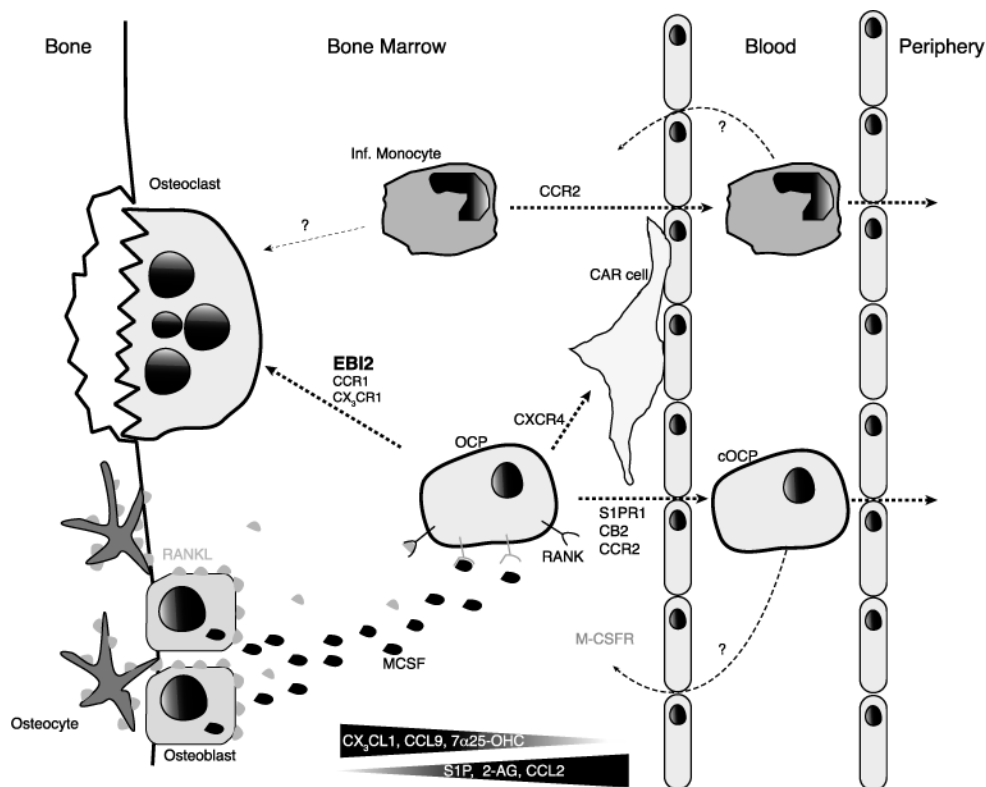
Fig. 1.

Trafficking of osteoclast precursors in the bone marrow (BM), blood, and peripheral tissues. Monocytic osteoclast precursor cells (OCP) and inflammatory monocytes (Inf Mono) migrate into osteoclastogenic niches where osteoblasts (OB) and osteocytes provide important signals for osteoclast differentiation (MCSF and RANKL). EBI2 directs the migration of OCP to the endosteum, where the EBI2 ligand 7α, 25-OHC is presumably abundant. CX_3CR1 promotes the retention of OCP in BM parenchyma, and possibly directs cells to the endosteum in response to CX_3CL1 produced by OB. OB also express CCR1 ligands (e.g. CCL9) and CCR1 may also direct OCP to sites of osteoclast differentiation. S1PR1 is essential for the egress of monocytic cells in the bone marrow as they follow S1P gradients into the circulation and inflammatory monocytes egress via CCR2. CXCR4 is likely to guide OCP away from sites of osteoclastogenesis given that its ligand CXCL12 is abundant parenchymal and perivacular mesenchymal stromal cells, while it is reduced in OBs. The CB2 ligand 2-AG is likely abundant in the BM sinusoids, and may guide OCP away from osteoclastogenic niches. Both circulatory OCP and inflammatory monocytes re-enter into BM by unknown mechanisms.
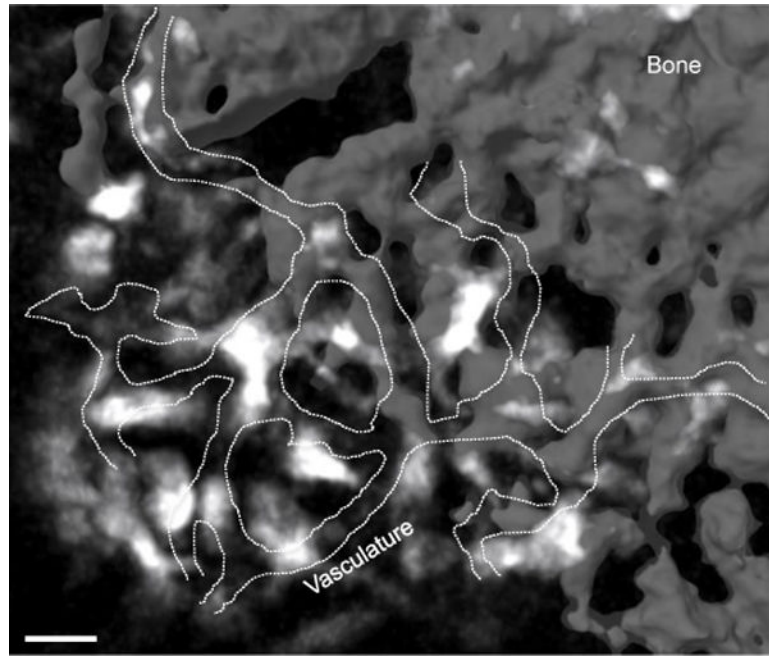
Fig. 2.
Osteoclasts are located in close proximity to blood vessels in the bone marrow. Image acquired using two-photon microscopy of calvaria bone marrow of TRAP[Red] reporter mouse. Osteoclasts (white); blood vessels (dashed line) were visualized with injection of dextran-FITC; and bone (gray). Scale bar= 30μm

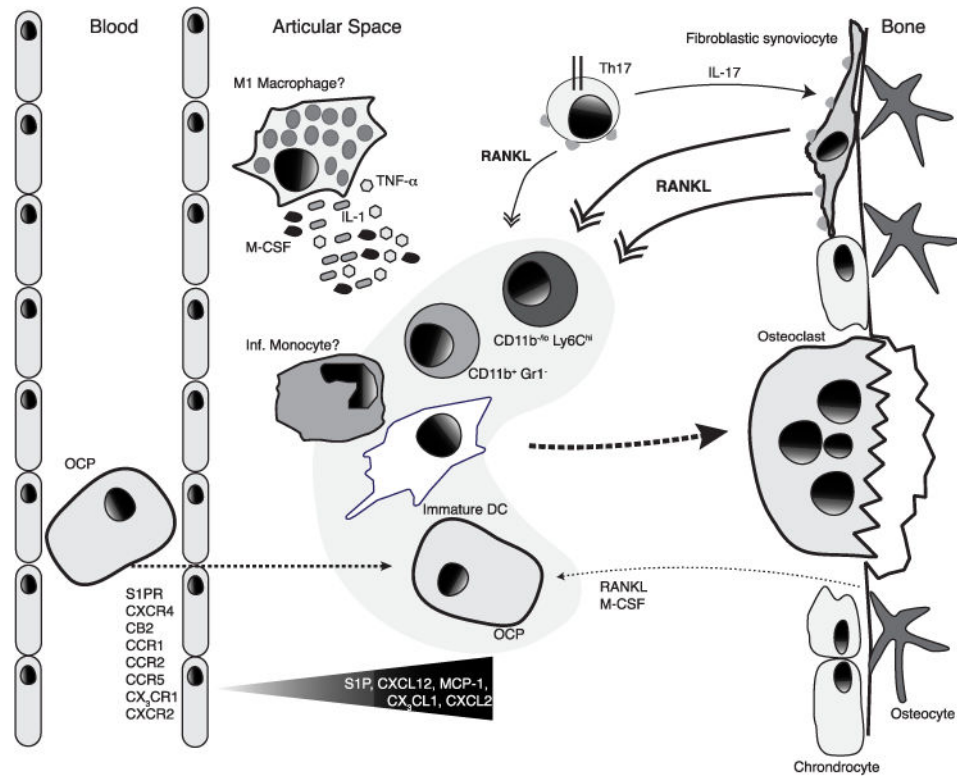**Fig. 3.**
Trafficking of monocytic osteoclast precursors (OCP) into inflamed joints. Cells with osteoclastogenic potential include CD11b$^{-/lo}$Ly6C^{hi}, CD11b^{+}GR-1^{-}, and DCs. In RA, sinusoidal fibroblastic cells provide RANKL, which can be induced by IL-17 provided by Th17 cells. The cytokines TNF-α, IL-1, and IL-6, which may be locally secreted by macrophages also promote osteoclast differentiation under inflammatory conditions. S1P receptor expression on OCPs possibly directs cells into the synovial tissue where S1P is upregulated during inflammation. CXCR4 also likely directs cells into the synovial tissue with fibroblasts, and possibly other cells, express high levels of CXCL12. Selective antagonism of CB2 inhibits the migration of monocytes into the synovium, indicating that 2-Ag levels may be present in synovial fluid. CXCR2, CX$_3$CR1, CCR1, CCR2, and CCR5 are also implicated in inflammatory cell recruitment into the inflamed articular space.