Abstract
Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic systemic inflammation causing progressive joint damage that can lead to lifelong disability. The pathogenesis of RA involves a complex network of various cytokines and cells that trigger synovial cell proliferation and cause damage to both cartilage and bone. Involvement of the cytokines TNF-α and IL-6 is central to the pathogenesis of RA, but recent research has revealed that other cytokines such as IL-17, IL-23, GM-CSF, IL-33, and IL-2 also play a role. Clarification of RA pathology has led to the development of therapeutic agents such as biological disease-modifying anti-rheumatic drugs (DMARDs) and Janus kinase (JAK) inhibitors, and further details of the immunological background to RA are emerging. This review covers existing knowledge regarding the roles of cytokines, related immune cells and the immune system in RA, manipulation of which may offer the potential for even safer and more effective treatments in the future.

Key words: rheumatoid arthritis, TNF-α, IL-6, IL-17, IL-23, GM-CSF, IL-33, IL-2
Introduction

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease causing progressive joint damage that can lead to lifelong disability [1]. Although RA itself is not life-threatening, it may cause secondary amyloidosis, which carries a risk of organ failure and death [2]. This systemic disease is characterized by synovial inflammatory cell infiltration, synovial hyperplasia, angiogenesis, and cartilage damage, which in turn can lead to bone destruction [3]. Various autoantibodies such as rheumatoid factor (RF) and anti-cyclic citrullinated peptide (CCP) antibody (ACPA) are important serological markers for not only RA diagnosis but also prediction of treatment responsiveness and progression of bone destruction [4]. The level of ACPA is elevated with high specificity in RA, and clinically it is widely used for diagnosis [5]. The production of ACPA is thought to be correlated with certain genetic background factors such as HLA-DR [6], other existing conditions such as periodontal disease [7], and smoking [8]. ACPA and RF form immune complexes with citrullinated proteins and activate macrophages, triggering the release of inflammatory cytokines such as TNF-α and IL-6 [9]. This cytokine-mediated pathway is central to the pathogenesis of RA. At the affected joint, infiltrating immune cells are associated with the release of a variety of cytokines, which are important mediators of cell differentiation, inflammation, immune pathology, and immune response [10]. As further details of RA pathogenesis have emerged, biological disease-modifying anti-rheumatic drugs (DMARDs) and Janus kinase (JAK) inhibitors have been developed as therapeutic agents, and the underlying immunological conditions are being further clarified. It has become evident that numerous cytokines are associated with the pathophysiology of RA, and that some of them might become targets for future therapeutics. Here we review several of these cytokines, including TNF-α, IL-6, IL-17, IL-23, IL-33, GM-CSF, and IL-2, which are known to be active from the acute to the chronic stage of RA, and may have potential for therapeutic targeting.

1. TNF and RA

Based on the rationale that TNF-α plays a central role in the regulation of RA-related molecules, anti-TNF drugs were the first biological agents to be introduced for treatment of RA, starting with infliximab, a chimeric anti-TNF-α monoclonal antibody (mAB), in 1999 [11]. Since then, it has become clear that TNF regulates the production of IL-6, IL-8, MCP-1, and VEGF, recruitment of immune and inflammatory cells into joints, angiogenesis, and the blood levels of matrix metalloproteinases (MMP) 1 and 3 [12]. TNF was originally named by Carswell et al. on the basis of their observation that an endotoxin caused host macrophages to release a substance that induced necrosis of tumor cells [13].

Human TNF-α is a 17-kDa secreted protein comprising 157 amino acids and is produced mainly by macrophages [14]. Primarily, the TNF-α precursor molecule is produced as a transmembrane protein (memTNF), which is then cleaved by a metalloproteinase, such as TNF-α converting enzyme (TACE), leading to the release of soluble TNF (sTNF). sTNF then forms a homotrimer and binds to its receptor. There are two known receptor isoforms – TNF receptors 1 (TNFR1) and TNFR2 – both of which form a homotrimer. TNFR1 possesses the intracellular DEATH domain, which regulates apoptosis through activation of caspases, whereas TNRF2 does not.

MemTNF activates both TNFR1 and TNFR2, whereas sTNF activates mainly TNFR1 [15]. TNFR2 can only be activated properly by memTNF, because activation of TNFR2-associated signaling pathways requires subsequent stabilization of TNF-TNFR2 homotrimer complexes. This subsequent stabilization is achieved only by memTNF [16], perhaps because of the memTNF costrain physical distance of the inter-TNF-TNFR2 complex. TNFR1 recruits the adaptor protein, TNF receptor-associated death domain (TRADD), through its DEATH domain. Usually NF-κB binds IκBα and is localized in the cytosol. Activated TNFR1 recruits TRADD, TNF receptor-associated factor 2 (TRAF2), receptor-interacting protein kinase 1 (RIPK1), and cell inhibitor of
apoptosis protein-1/2 (cIAP1/2) to form Complex I. A precisely controlled multistep ubiquitination process activates the I kappa B kinase (IKK) complex through the NF-κB essential modulator (NEMO). The IKK phosphorylates IκBα, and then IκBα is K48-ubiquitinated, leading to its degradation. Finally, NF-κB becomes detached from IκBα and translocates to the nucleus. TNFR1 signaling also activates c-Jun N-terminal kinase (JNK) and p38MAPK through TGF-β-activated kinase 1 (TAK1) and caspase-8, via FADD. Although TNFR2 signaling also activates NF-κB and p38MAPK through a TRAF2-dependent mechanism, this process is dependent on NF-κB inducing kinase (NIK), and independent of NEMO. In addition, recruitment of TRAF2 by TNFR2 leads to depletion of cytosolic TRAF2 and perturbation of TNFR1-mediated signaling [14].

Although TNF inhibitors have advanced the management of RA and led to dramatic improvements in disease status [1,17], the cellular source of TNF and its relationship to the complex pathogenesis of RA have remained unclear. Kruglov et al. performed a series of studies using mice with conditional TNF knockout [18]. These revealed that memTNF has a protective function against arthritis, and suppresses autoreactive T cells. It was also shown that TNF produced by myeloid cells controls arthritis onset by regulating the activation of synovial fibroblasts, that B cell-derived TNF regulates the severity of arthritis through induction of autoantibodies, and that T cell-derived TNF exerts a protective action by regulating the development of autoreactive T cells.

TNF also regulates the function and differentiation of T regulatory (Treg) cells, which are essential for maintenance of immune homeostasis and prevention of autoimmunity, and whose differentiation is regulated by FoxP3 [19]. While signaling via TNFR1 promotes the pathogenesis of arthritis, TNFR2 signaling exerts protective functions [20, 21, 22]. Several lines of evidence suggest that interaction of TNF with TNFR2 promotes Treg function [23]. For example, the TNF-TNFR2 axis expands the number and function of mCD4 CD25 Tregs [24]. Human peripheral blood CD4 CD25 TNFR2 cells have been shown to markedly inhibit proliferation of, and cytokine production by, co-cultured T-responder cells, whereas CD4 CD25 TNFR2 cells do not [25]. In a study using TNFR2-knockout mice, FoxP3 gene methylation in Treg cells was shown to be greater than in wild-type mice, and Treg cells differentiated into pro-inflammatory Th17-like cells. Thus, TNFR2 signaling appears to block methylation of the promoter region of FoxP3, thus maintaining FoxP3 transcription and preventing pathogenic conversion of Tregs to Th17-like cells [26]. Furthermore, transfer of TNFR2-expressing Treg cells has been shown to ameliorate inflammation in an experimental arthritis model [27]. On the other hand, TNFR1 deficiency increases Treg activity, suggesting that TNFR1 signaling mediates the disease exacerbation attributable to Tregs. TNF-TNFR1 signaling activates NF-κB, leading to transcription of protein phosphatase 1 (PP1), which dephosphorylates Ser418 of FoxP3. Because dephosphorylated FoxP3 is unable to bind the target sequence, Treg differentiation is blocked [28].

During the course of RA, from the very early preclinical stage to the established disease stage, the DNA methylation status of RA synovial fibroblasts changes dynamically [29]. TNF controls gene expression by regulating methylation and acetylation. It has been shown that hypomethylation is associated with increased gene expression, and that many genes involved in RA pathogenesis, including signal transducer and activator of transcription 3 (STAT3) and TRAF2, are hypomethylated in synovial fibroblasts derived from RA patients [30]. Cytokines such as TNF and IL-1β can inhibit the expression of DNA methyltransferase (DNMT), leading to reduced DNA methylation and increased pathogenic gene expression [31]. Acetylation is another mechanism by which TNF can regulate gene expression. Histone deacetylases (HDAC) are a class of enzymes that remove acetyl groups from lysine on histone, allowing histones to wrap DNA more tightly. DNA expression is controlled through a balance between acetylation and deacetylation. HDAC5 is known to exert an anti-inflammatory role. TNF and IL-1β suppress HDAC5 and upregulate the production of inflammatory cytokines and chemokines [32]. Loh et
al. investigated genome-wide changes in gene expression and chromatin remodeling induced by TNF in synovial fibroblasts and macrophages. They stimulated synovial fibroblasts from RA patients and human CD14+ monocyte-derived macrophages with TNF ex vivo, and identified 280 TNF-inducible arthritogenic genes, including IL-6, CXCL8, CXCL10, and MMP-19. These genes were expressed transiently in macrophages, but their expression was sustained in synovial fibroblasts. They further identified 80 of these genes that lost their reactivity with TNF when stimulated repeatedly in macrophages, but which retained their reactivity in synovial fibroblasts. Use of Assay for Transposase-Accessible Chromatin using SEQuencing (ATAC-seq), which can identify open chromatin, and chromatin immunoprecipitation coupled with high-throughput sequencing (ChIP-seq) for Histone 3 lysine 27 acetylation (H3K27ac) of these genes in macrophages and synovial fibroblasts demonstrated persistent TNF regulation of H3K27 acetylation and increased chromatin accessibility in the regulatory elements of arthritogenic genes in TNF-stimulated synovial fibroblasts [33]. Not only TNF, but also other cytokines contribute to epigenetic modification. When stimulated with a mixture of 8 cytokines, including IL-1β, IFN-α, and IFN-γ, the genomic structure of synovial fibroblasts changes dramatically, with formation of chromatin loops that can be detected by high-throughput chromosome conformation capture (Hi-C) analysis. The transcription factors metal-regulatory transcription factor-1 (MTF1) and runt-related transcription factor-1 (RUNX1) could be key regulators of chromatin remodeling for expression of pathogenic molecules in fibroblasts [34]. Table 1 summarizes the RA-related cytokines, signaling pathways, and clinical assessments described in this article.

**Tabel 1. Representative cytokines, signal transduction, and their pathogenic roles in rheumatoid arthritis. Abbreviations,**

<table>
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<tr>
<th>Cytokines-Receptor-major signaling molecules</th>
<th>Proposed roles in rheumatoid arthritis</th>
<th>Clinical application</th>
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<tr>
<td>TNF - TNFR 1/2- NFkB, MAPKs, PI3K</td>
<td>Osteoclastogenesis, Pro-inflammatory cytokine production TNFR1; Treg inhibition TNFR2; Treg activation Epigenetic modification (Acetylation/Methylation) memTNF; Protective for arthritis</td>
<td>Widely used</td>
</tr>
<tr>
<td>IL-6 - IL-6R-gp130, JAK1/2, Tyk2, STAT1/3, PI3K, SHP-2, ERK</td>
<td>Osteoclastogenesis Pro-inflammatory cytokine production Auto-antibody production Th17 differentiation, Treg inhibition</td>
<td>Widely used (anti-receptor antibody)</td>
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<td>IL-33 - ST2- IL1-RacP, MyD88, IRAKs, TRAF6, MAPKs, NFKB, AP1</td>
<td>Pro-inflammatory cytokine production Activation of mast cell, Treg, Th2, and ILC2</td>
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<tr>
<td>IL-23 – IL-12Rβ1/IL-23R-</td>
<td>Activation of Th17, NKT, and ILC3 cells</td>
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<td>Protein Complex</td>
<td>Cytokine Production</td>
<td>Effects</td>
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<tr>
<td>Tyk2, JAK2, STAT3</td>
<td>Cytokine production (IL17, TNF-α, GM-CSF)</td>
<td>Did not meet primary endpoint</td>
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<tr>
<td>IL-17 - IL17R - ACT1, TRAF6, NFKB, MAPKs</td>
<td>Pro-inflammatory cytokine production Osteoclastogenesis</td>
<td></td>
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<tr>
<td>GM-CSF - GM-CSF-R- JAK2, STAT3/5</td>
<td>Macrophage activation Pro-inflammatory cytokine production</td>
<td>Phase II a</td>
</tr>
<tr>
<td>IL-2 - IL2R- JAK1/3, STAT3/5, SHC-1, ERK</td>
<td>Late phase: arthritogenic Activation of ILC2, NK cell, Th17 cell, IL-33 production, Early phase: Anti-arthritogenic via IFN-γ</td>
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2. IL-6 and RA

IL-6, another key regulator of RA, is a 26-kDa secreted protein that was originally identified in 1986 as a secreted factor that induced immunoglobulin production [35]. IL-6 is involved in a wide range of physiological processes, such as the immune response, inflammation, and bone metabolism, and has also been implicated in the pathogenesis of RA. Anti-IL-6 receptor antibody was first approved for RA in 2008, and is now used for treatment of various rheumatic diseases [36].

IL-6 activates a signal cascade via three modes of receptor-ligand interaction, the first of which is classical signaling whereby IL-6 binds to its receptor, IL-6Rα, which is expressed on cells of lymphoid or myeloid lineage, thus activating an intracellular signal transduction pathway via gp130 dimerization. The second mode is trans-signaling. IL-6R also exists as a secreted form (sIL-6Ra), unlike the membrane-bound form, IL-6Ra, which is cleaved by proteases such as TACE. sIL-6Ra binds to IL-6, and the resulting complex binds to gp130 on endothelial cells and synovial fibroblast, which usually do not express IL6R. In the third mode, so-called transpresentation, circulating IL-6 binds to IL-6Rα expressed on dendritic cells (DC), and then the IL-6/IL-6Ra complex binds to gp130 expressed on CD4+ T-cells. This mode of interaction is required for priming of Th17 cells [37].

The IL-6/IL-6Ra complex binds gp130 to form a gp130 homodimer, thus activating Janus kinase (JAK). JAK activates the STAT family transcription factors, mainly STAT3, and src homology region 2 domain-containing phosphotase 2 (SHP-2). JAK phosphorylates STAT3 to form a homodimer, which then translocates to the nucleus where it functions as a transcription factor. JAK also activates the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway through SHP-2. STAT3 induces suppressor of cytokine signaling1 (SOCS1) and SOCS3; SOCS1 inhibits JAK directly, and SOCS3 inhibits gp130 [36].

Sustained production of IL-6 after TNF-α stimulation in synovial fibroblasts is one of the features of RA [38]. F759 mice, which carry the Y759F mutation in gp130 and lack the negative feedback loop mediated by SOCS3, develop arthritis spontaneously [39]. Genetic experiments have shown that F759 mice develop arthritis only if mutated gp130 functions in non-immune cells. Detailed experiments have shown that accumulation of IL-6 secreted from synovial fibroblasts leads to proliferation of CD4+ T-cells, differentiation of Th17 cells, and subsequent development of arthritis [40, 41]. This suggests that the pathogenesis of RA requires coordinated interaction of TNF, IL17, and IL-6. In fibroblasts, TNF induces various types of cytokines and chemokines, whereas IL-17A alone does not induce cytokines and/or chemokines significantly. Slowikowski et al. reported that TNF induced the expression of 370 genes, but that the expression of these genes was unaffected by addition of IL-17A. They also identified 26 genes whose expression was induced only upon co-stimulation with TNF and IL17-A, and 25 genes whose expression was induced by TNF and dose-dependently amplified by IL-17A; these included C-X-C motif chemokine ligand 1 (CXCL1), CXCL2, CXCL3, IL-6, IL-8, and MMP-3. This synergistic control
is regulated by atypical IκB factor IκBζ, which is induced in proportion to IL17A concentration and functions in a dose-dependent manner. Synergistic induction of IL-6 is canceled when IκBζ is repressed by siRNA [42].

IL-6 also regulates the differentiation of Treg, Th17, and Tfh cells. The IL-6-STAT3 pathway is required for Th17 cell development, and enhancement of the IL6-STAT3 signaling axis causes IL17A-dependent autoimmune arthritis in mice [43]. The importance of STAT3 in the differentiation of Th17 cells has also been demonstrated in humans [44]. Furthermore, IL-6 downregulates Foxp3 expression through STAT3 and induces the genetic reprogramming of Treg cells to Th17-like cells [45]. Thus, IL-6 regulates the Treg vs Th17 cell balance. Conversion of Treg cells to Th17 cells is induced by IL-6 derived from synovial fibroblasts, and the converted Th17 cells are more osteoclastogenic than conventional Th17 cells [46].

T follicular helper (Tfh) cells have been identified as CXCR5+ PD-1+ CD4+ T cells, which regulate B cell differentiation into plasma cells and memory B cells by mediating class switching and affinity maturation of antibodies in germinal centers [47]. In mice, Bcl6+ CXCR5+ Tfh differentiation is severely impaired in the absence of IL-6, suggesting that IL-6 is an essential factor for the development of murine Tfh cells [48]. In humans, however, Tfh differentiation might be less affected by IL-6, and TGF-β together with IL-12 or IL-23 induces various Tfh markers on CD4+ T cells [49].

IL-6 signaling regulates osteoclastogenesis in combination with TNF. Extensive analysis of osteoclast differentiation has identified various osteoclast types. Osteoclasts regulate bone absorption and bone mineral density (BMD), and cause bone erosion in both RA patients and model mice [50]. Differentiation of osteoclasts depends on TNF and receptor activator of nuclear factor-κB ligand (RANKL), IL-6, and IL-17.

3. Synovial structure, inflammation, and pathogenesis of RA
The synovium of humans is composed of a lining and a sublining layer [51]. The synovium is the primary site of inflammation in RA, and undergoes marked hyperplasia, becoming inflamed and invasive, thus destroying the cartilage and bone. Fibroblasts are the major component of the synovium, and those in the sublining layer undergo major expansion in RA. Synovial tissue is also composed of lymphocytes, macrophages, and dendritic cells. Recently, the molecular characteristics of these cells, as well as their mechanism of differentiation and contribution to RA development have been revealed at high resolution using modern techniques such as single-cell (sc) RNA sequencing, bulk RNA sequencing, and mass cytometry.

Fibroblasts are key players in synovial inflammation in RA. Stephenson et al. have identified Thy1+ fibroblasts in the sublining, and CD55+ fibroblasts in the lining. Thy1+ fibroblasts are rich in modules associated with metalloproteinase activity, while CD55+ fibroblasts express functional modules associated with endothelial cell proliferation [52]. Mizoguchi et al. have classified RA-related synovial fibroblasts into at least three subpopulations: a CD34+Thy1+ population in the sublining, especially the perivascular area, a CD34+Thy1+ population in the lining area, and a CD34+ population. The CD34+Thy1+ population expresses a higher level of RANKL, and is expanded three-fold in RA patients relative to patients with osteoarthritis (OA), whereas the CD34+Thy1+ population expresses a higher level of MMP-3 [53]. Zhang et al. have identified Thy1+HLA-DR+ synovial fibroblasts in the sublining layer as the origin of IL-6 [54]. Croft et al. have identified FAPα+Thy1+fibroblasts as driving joint injury, and FAPα+Thy1+ sublining fibroblasts as driving inflammation [55]. Wei et al. have shown that differentiation of Thy1+ lubricin+ lining fibroblasts and Thy1+ sublining fibroblasts is regulated by the vascular endothelium expressing Jagged1 through fibroblasts expressing its receptor, Notch3. Blocking of the Jagged1-Notch3 pathway inhibits Thy1+positive sublining fibroblasts and blocks joint destruction [56]. Orange et al. have reported that a CD45 CD31 PDPN+ population emerges in the blood one week before relapse of arthritis, and have named this population preinflammatory mesenchymal (PRIME) cells. These cells show an expression pattern similar to that of
Thy1+HLA-DR+ synovial fibroblasts, and are considered to be one of the origins of sublining inflammatory fibroblasts [57]. Although several researchers have reported a variety of fibroblast subsets to date, those subsets and the functional molecules they express as well as their proposed cellular functions have been divergent depending on the experimental design used.

Macrophages also play a crucial role in the pathogenesis of RA. Traditionally, macrophages were identified as "phagocytes", and were believed to be involved only in the clearance of debris or pathogens. However, recent studies have revealed diverse biological functions of macrophages in various tissues. Macrophages not only scavenge tissue debris or pathogens, but also regulate the immune system, functioning as drivers of tissue inflammation or key regulators of tissue repair. Furthermore, in some instances they help to maintain tissue homeostatic balance. For example, osteoclasts, which are bone-related macrophages, destroy old bone tissue and promote its reconstruction [50, 58].

Macrophages that migrate via peripheral blood are often pathogenic. Hasegawa et al. have identified CX3CR1hi, Ly6Cint, F4/80hi, MHCII+ macrophages in the joints of CIA model mice. In ex vivo culture, these macrophages differentiate into osteoclasts. This population has been referred to as arthritis-associated osteoclastogenic macrophages (AtoM), and express molecular markers of osteoclasts, as well as the transcription factor FoxM1. Inhibition of FoxM1 blocks the differentiation of AtoM cells into osteoclasts [59]. Another macrophage type, tissue-intrinsic macrophages, colonize sites during the embryonic period and exert a tissue protective function. Culemann et al. have found that CX3CR1+ lining macrophages form a barrier on the synovium and protect the joint by maintaining tight junctions. Molecules such as TREM2, MERTK, AXL and claudin function to maintain these tight junctions, and silencing of these molecules causes severe joint inflammation [60].

MerTK+CD206+ synovial macrophages have also been recently identified as having a protective function [61]. They are abundant in the synovial lining layer of patients with OA or RA in remission. On the other hand, MerTK CD206+ synovial macrophages are abundant in the synovial sublining layer in patients with active RA. MerTK+CD206+ synovial macrophages express pro-resolving mediators such as CD163, which functions as a scavenger receptor, resolvin D1 and IL10. These molecules play a role in suppressing inflammation [62,63].

Tu et al. have also identified two types of synovial macrophage in mouse: one is an intrinsic macrophage present in the joint synovium since their birth, and the other is an extrinsic macrophage derived from bone marrow. Intrinsic macrophages express anti-inflammatory cytokines such as IL-4 and IL-10, whereas extrinsic macrophages express pro-inflammatory cytokines such as IL1-β and TNF-α. They also identified similar cells in patients with RA [64].

In lymph nodes, CXCL13 recruits CXCR5+ Tfh cells and Tfh cells regulate lymphoid follicle formation. Tfh cells function to assist B cell maturation, and have a PD-1hiCXCR5+ CD4+ T cell phenotype [47]. The synovium in RA is rich in lymphoid follicles. However, RA synovial tissue contains fewer PD-1hiCXCR5+ Tfh cells, and almost 85% of PD-1hiCD4+ cells in synovial tissue lack CXCR5 expression [65]. PD-1hiCXCR5CD4+ cells express functional molecules such as IL-21, CXCL13, ICOS, and MAF, which are known to be expressed in Tfh cells, but do not express CXCR5 and BCL-6, which are important for Tfh function in the germinal centers of lymph nodes. In terms of function, these cells assist the maturation of B cells, as do Tfh cells, and have therefore been named T peripheral helper (Tph) cells. Because CXCR5 is required for migration into the germinal center, Tph cells cannot do so. Instead, they express chemokine receptors, such as CCR2, CX3CR1, and CCR5, which allow Tph cells to migrate into sites of inflammation. Because Tph cells in peripheral blood do not express CXCL13, whereas those in joints affected by RA express CXCL13 strongly, it has been proposed that the TGF-β-abundant, IL-2-limiting inflammatory milieu in RA joints induces the transcriptional factor Sox4 in Tph cells, which migrate from the peripheral blood, and that Sox4 then upregulates the expression of CXCL13. The CXCL13-expressing Tph cells then attract B cells, leading to the formation of ectopic lymphoid-like structures [66]. These findings indicate that tissue-localized T-B interactions might play an
important role in the development of RA.

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5. IL-17 and RANKL elicit bone resorption by driving the function of osteoclasts

Several T-cell subsets and their complex interactions likely contribute to RA pathology. It is largely accepted that regulatory T cells accumulate in RA synovial fluid and that the equilibrium between them and effector cells is a key factor in controlling the inflammatory processes involved in RA [67]. Th17 cells are known to comprise a third T-cell subset since Th1/Th2 cells, and are induced by the cytokines IL-6, IL-1$\beta$, IL-21, TGF-$\beta$, and IL-23. They are present in synovial joints [68, 69] and secrete IL-17, which is a pro-inflammatory cytokine contributing to osteoclastogenesis along with TNF and IL-6.

Bone erosion by osteoclasts is one of the most important pathologic features of RA [70, 71]. Receptor activator of nuclear factor kappa-B ligand (RANKL) is a cytokine belonging to the TNF superfamily and mainly stimulates osteoclast differentiation. It binds to receptor activator of nuclear factor kappa-B (RANK) on osteoclasts and osteoclast precursors and promotes osteoclast differentiation and activation. Osteoprotegerin (OPG) is a soluble decoy receptor of RANKL, and upon binding to RANKL inhibits the activation of RANK. The inflamed synovium and pannus in RA produce significantly higher levels of RANKL and lower levels of OPG in comparison to healthy synovium [72-74]. The cells responsible for the increased expression of RANKL in the inflamed synovial membrane are fibroblast-like synoviocytes (FLS) and T lymphocytes. The increased RANKL/OPG ratio results in increased osteoclast differentiation and activation at the synovium-bone interface and the development of bone erosions in RA [74, 75].

RANKL on T cells or fibroblasts, which are activated by a combination of IL-6, IL-17, and...
TNF, regulates the differentiation of osteoclasts. IL-6 signaling induces RANKL expression in RA-FLS through expression of NFATc1 and TRAP5b mRNA in co-cultures of RA-FLS and osteoclast precursor cells [76].

IL-17 increases RANKL expression in adjuvant-induced arthritis-derived synovial fibroblasts, leading to increased osteoclastogenesis in vitro. RANKL expression and osteoclastogenesis are dependent on IL-17R and STAT-3, as both are reduced by blocking these molecules [77]. Furthermore, IL-17 modulates osteoclast precursor cells. Raw264.7 cell culture experiments have shown that a low level of IL-17A promotes the RANKL-RANK system by mediating the JNK signaling pathway and activating autophagy and osteoclastogenesis in induced osteoclast precursor cells. However, a high level of IL-17A inhibits osteoclastogenesis [78].

A RANKL-independent osteoclast differentiation pathway has also been reported. TNF induces differentiation of osteoclasts from mouse bone marrow myeloid cells (mBMM) and human peripheral blood monocytes (PBMC) independently of RANKL function [79]. Tartrate-resistant acid phosphatase (TRAP)-positive cells induced by TNF lack bone absorption ability, but gain it upon treatment with a combination of IL-6 and TNF. Thus IL-6 is required for the bone-resorbing activity of TRAP-positive cells induced by TNF. Interestingly, whereas bone absorption activity cannot be abolished in mice with STAT3 conditional knockout, treatment with JAK inhibitor or MEK inhibitor achieves this [80]. Furthermore, the number of osteoclasts induced ex vivo from RA patient PBMC with TNF and IL-6 is positively correlated with the host patient modified Total Sharp score [81].

In RA patients, reactive oxygen species (ROS) are highly expressed in neutrophils and synovium [82]. Oxidative stress is one of the main mechanisms responsible for destructive proliferative synovitis [83], and ROS activate osteoclasts and drive bone resorption. RANKL itself induces nitric oxide synthase and N-acetyl cysteine (NAC). NOS and NAC inhibit RANKL-induced ROS production and inhibit the differentiation of osteoclasts from bone marrow monocyte-macrophage lineage cells [84].

In RA synovial fibroblasts, NAC dose-dependently attenuates the induction of RANKL mRNA by IL-17 [85]. NAC inhibits both ROS and MMP-3 mRNA by interfering with the JNK signaling pathway [86]. Thus, JNK has potential as a target of intervention for RA.

AtoM cells are another type of osteoclast that develop when macrophages derived from peripheral blood are stimulated with TNF and RANKL [54].

6. Biological DMARDs, JAK/STAT inhibitors and osteoclastogenesis

A previous study using synovial samples from 18 RA patients has evaluated the expression of OPG and RANKL protein by immunohistochemistry. After 8 weeks of treatment, infliximab and etanercept were found to increase the expression of osteoprotegerin (OPG) in synovial tissue, but neither influenced the expression of RANKL. In TNF inhibitor-treated groups of patients, the RANKL:OPG ratio decreased following therapy, suggesting that TNF inhibitors in RA modulate the OPG/RANKL system, possibly explaining the retardation of radiographically evident damage following anti-TNF therapy [87].

In another study, BMD in the spine, hip, and hand, as well as serum RANKL and RANKL/OPG, were monitored for 12 months in 102 RA patients receiving infliximab. BMD in the spine and hip showed no significant change, but BMD in the hand showed a significant decrease. The serum RANKL and RANKL/OPG ratio were significantly decreased by infliximab treatment [88]. On the other hand, infliximab directly promotes the differentiation of osteoclast precursor cells in PBMC and lacunar resorption induced by RANKL. Addition of infliximab has been shown to markedly increase the number of TRAP-positive multinucleated cells (TRAP+ MNCs) and the extent of lacunar resorption in comparison with control cultures [89].

IL-6 and soluble IL-6 receptor (sIL-6R) induce RANKL expression in RA-FLS. Although IL-17 and TNF-alpha stimulate cell growth and IL-6 production in RA-FLS, they do not induce RANKL expression. IL-6 and sIL-6R induce the expression of NFATc1 and TRAP5b mRNA in cocultured
RA-FLS and osteoclast precursor cells [76].

In mouse calvarial osteoblasts, IL-6 and sIL-6R induce bone resorption, and this is decreased by osteoclast inhibitors. Thus, IL-6 signaling influences osteoclastogenesis [90]. Tocilizumab significantly ameliorates bone erosion in metacarpal bones of patients with RA when injected subcutaneously weekly for 52 weeks, whereas adalimumab and methotrexate do not ameliorate bone erosion to any significant degree [91]. Tocilizumab suppresses the number of histologically evident osteoclasts and the degree of RANKL-induced bone erosion in the metacarpal bones of model monkeys with collagen-induced arthritis, indicating that IL-6/IL-6R is involved in subchondral bone and bone marrow change in RA patients [92].

Abatacept, a chimeric molecule consisting of the extracellular domain of the co-inhibitory molecule CTLA-4 fused to the Fc portion of a human IgG1 antibody, neutralizes binding of the CTLA-4 part to either CD80 or CD86 on the surface of activated antigen-presenting cells [93,94]. Abatacept dose-dependently inhibits RANKL-mediated osteoclast formation in monocytes, exerting an anti-bone resorbing effect [95].

Recent studies have clarified the action of abatacept on osteoclastogenesis in more detail. Bozec et al. have described a mechanism for control of bone resorption by the adaptive immune system and showed that CD80/86 negatively regulated the generation of bone-resorbing osteoclasts. In CD80/86-deficient mice with osteopenia due to increased osteoclast differentiation, inhibition of CD80/86 by administration of CTLA-4 activated the enzyme indoleamine 2,3-dioxygenase (IDO) in osteoclast precursors, causing degradation of tryptophan and promotion of apoptosis [96]. CTLA4-Ig inhibits osteoclast differentiation and reduces the expression of nuclear factor of activated T cells1 (NFATc1) in bone marrow macrophages. It also suppresses calcium oscillations dependently on FCgammaR [97].

The JAK/STAT signaling pathway appears to be related to bone homeostasis. JAK protein associated with the receptor after formation of the ligand-receptor complex is activated by transphosphorylation. JAK activation induces the phosphorylation of tyrosine on a cytoplasmic tail subunit of the receptor at docking sites for STAT proteins. The STAT proteins then undergo phosphorylation and dimerization, and the dimer translocates into the nucleus, where it binds to DNA and activates the transcription of targeted genes that affect cell behavior. Suppressors of cytokine signaling (SOCS) provide negative feedback to the receptor and prevent continuous signaling. JAK1 and STAT3 signaling is mediated by IL-6 family cytokines, which bind to the glycoprotein 130 (gp130) IL-6 receptor subunit and are indispensable for normal skeletal development in mice and humans [98]. IL-6 family cytokines such as IL-6, IL-11, oncostatin M, cardiotrophin 1, leukemia inhibitory factor, and ciliary neurotrophic factor are needed for normal levels of bone formation but also induce pathological bone formation such as heterotopic ossification. They also stimulate osteoclast formation by acting indirectly on osteoblasts through STAT3 signaling [99]. The JAK1/STAT3/SOC3 pathway is also activated by G-CSF, thus inhibiting bone formation and promoting osteoclast formation [100-102]. Tofacitinib (a JAK1/JAK3 inhibitor) dose-dependently reduces RANKL expression in cultured T cells [103]. In a rat adjuvant-induced arthritis model, tofacitinib has been reported to increase bone cortical and trabecular hardness, but it does not reverse the effects of arthritis on cortical and trabecular bone structure [104].

Stattic, a STAT3-specific inhibitor, suppresses STAT3 and NF-kB, resulting in inhibition of RANKL-mediated osteoclastogenesis in RANKL-induced Raw264.7 cells. Stattic also inhibits bone loss caused by ovariectomy [105]. Baricitinib (a Jak1/Jak2 inhibitor) exerts an inhibitory effect on osteoclastogenesis. It suppresses RANKL expression in murine calvaria-derived osteoblasts. Furthermore, shRNA-mediated knockdown of Jak1 or Jak2 suppresses RANKL expression in osteoblasts and inhibits osteoclastogenesis. Therefore it has been suggested that Jak1 and Jak2 represent novel therapeutic targets for osteoporosis as well as RA [106].

In RA patients, ROS are highly expressed in neutrophils and synovium [82]. Oxidative stress is one prominent mechanism causing destructive proliferative synovitis [83]. RANKL itself induces
nitric oxide synthase, and N-acetyl cysteine (NAC) inhibits RANKL-induced ROS production and differentiation of osteoclasts in bone marrow monocyte-macrophage lineage cells [84]. Osteoclasts are activated by ROS to drive bone resorption [107]. In RA synovial fibroblasts, NAC attenuates the expression of RANKL mRNA and production of soluble RANKL in an IL-17 dose-dependent manner. IL-17 enhances the phosphorylation of mammalian target of rapamycin (mTOR), c-Jun N-terminal kinase (JNK), and inhibitor of kappaB alpha (IκK-α) [85]. NAC inhibits both ROS and MMP-3 mRNA by interfering with the JNK signaling pathway [86]. Thus, JNK may have potential as a target for intervention in RA patients.

5. GM-CSF and the pathogenesis of RA
Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a well-known hematopoietic growth factor produced mainly by T-cells and stromal cells. It is also essential for regulating the functions of mature myeloid cells such as macrophages. As well as its stimulating effects on mature granulocytes, it induces the expression of HLA class II-antigen on synovial tissue-cells in patients with RA [108]. GM-CSF levels are increased in the sera, synovial fluid and bone marrow of patients with RA, especially at the chronic stage [109, 110]. IL-2, IL-7, and IL-33 are known to regulate the function of type 2 innate lymphoid cells [111]. These cytokines are involved in the production of GM-CSF by innate lymphoid cells of the synovium. In addition, the production of IL-33 is significantly increased in inflamed joints relative to healthy controls, and IL-2 is supplied from activated Th17 cells [112]. When the innate lymphoid cells of the synovium are stimulated with IL-2, IL-7, or IL-33 alone, GM-CSF is produced. Additionally, the production is synergistically increased when IL-33 and IL-2 are applied in combination [112].

Myeloid cells, T and B cells, and tissue resident cells can secrete GM-CSF, which in turn is capable of polarizing the function of macrophages into M1-like production of inflammatory cytokines. These cytokines induce the recruitment of inflammatory cells and activation of tissue resident cells. GM-CSF induces the production of IL-6 and IL-23 by antigen-presenting cells. IL6 and IL-23 cause activation of T cells and differentiation to Th17 cells, which in turn secrete GM-CSF and IL-17, thus maintaining the cycle. GM-CSF produced by Th17 cells also induces inflammation by activating the monocyte-macrophage system and neutrophils [113]. Macrophage populations in synovial tissue are associated with articular damage [114]. GM-CSF receptor activation leads to downstream involvement of Janus kinase-signal transducer and activator of transcription-suppressor of cytokine signaling (JAK-STAT-SOCS) as well as other pathways involving mitogen-activated protein kinases (MAPK), phosphatidylinositol 3 kinase (PI3K), and NF-kB [115, 116].

Clinical trials of agents targeting the GM-CSF pathway in RA have been reported. Mavrilimumab is a human monoclonal antibody that inhibits the human GM-CSF receptor [117]. A phase 2a trial of mavrilimumab at doses of up to 100 mg found that 55.7% of the subjects met the primary endpoint of a ≥1.2 decrease from baseline disease activity scores at week 12 (vs. only 34.7% of subjects given a placebo) [118]. Theoretically, the signal of GM-CSF could be controlled by blocking the signal transduction pathway, which involves JAK-STAT and MAPK. Jak2 inhibits the GM-CSF signaling pathway by acting on JAK-STAT. For the treatment of RA, peficitinib is an oral JAK inhibitor that targets JAK family enzymes (JAK1, JAK2, JAK3, TYK2) and blocks the signal transduction of various cytokines including GM-CSF [119]. Baricitinib is another inhibitor exerting similar effects [120]. Since these two drugs also inhibit Jak1 and block the IL-6 signaling pathway, any inhibitory effect on GM-CSF alone remains unclear.

6. IL-33 and the pathogenesis of RA
IL 33 is a member of the IL 1 family and recognized as a ligand for the ST2 receptor [121]. IL-33 binds to a specific heterodimeric cell surface receptor, which is a dimer of IL1Receptor associated protein (IL-1RaCP) and ST2. ST2 exists in two forms as splice variants: a soluble form (sST2), which acts as a decoy receptor, sequesters free IL-33, and does not signal, and a
membrane-bound form (ST2), which activates the NF-κB and MAP kinase signaling pathway to enhance the functions of mast cells, Th2 regulatory T cells (Tregs), and innate type 2 lymphoid cells [122]. The IL-33 protein is expressed mainly in epithelial and endothelial cells, particularly in high endothelial venules in human synovial tissue, and in cultured human RA fibroblasts [123]. IL-33 expression is strongly induced by IL-1 and/or TNF-α. Furthermore, IL-33 is highly expressed and its mRNA also detected in the joints of mice with collagen-induced arthritis (CIA) and increased during the early phase of the disease. In mice with CIA, and in arthritic mouse joints, inhibition of IL-33 signaling attenuates the severity of experimental arthritis. Administration of a blocking anti-ST2 antibody at the onset of disease has been reported to attenuate the severity of CIA and reduced joint destruction. In addition, anti-ST2 antibody treatment is associated with a marked decrease in interferon production as well as with a more limited reduction of IL-17 production by ex vivo-stimulated draining LN cells, and levels of RANKL mRNA in the joint are reduced [123]. IL-33 levels are increased in the sera and synovial fluid of patients with RA [124,125]. Increased levels of IL-33 in sera and synovial fluid are associated with disease activity [126] and bone erosion [127] in RA patients. Since it has been suggested that IL-33 is strongly associated with the pathogenesis of RA, several attempts to neutralize IL-33 have been conducted in model CIA mice. One experiment demonstrated that anti-IL-33 treatment also significantly decreased the serum levels of IFN-γ, IL-6, IL-12, IL-33, and TNF-α [128]. It is widely known that suppression of IL-6 and TNF-α directly prevents joint destruction. Despite promising findings from animal models, no clinical trials have yet been conducted in humans. The network of the IL-33 signaling pathway was recently revealed, and the findings suggest that it may affect a wide variety of diseases, although it is complex [129].

The activity of IL-33 is mediated by binding to the IL-33 receptor complex (IL-33R) and activation of NF-κB signaling via the classical MyD88/IRAK/TRAF6 module. IL-33 also induces phosphorylation and activation of the ERK1/2, JNK, p38 and PI3K/AKT signaling modules, resulting in the production and release of proinflammatory cytokines [129]. Theoretically, the IL-33 signal should be controllable by blocking the transduction pathway. In reality, however, inhibition of pathways other than the Jak-Stat system is not considered to be easy due to the problem of crosstalk in which signals enter from other pathways. Nevertheless, intervention with anti-IL-33 antibody and T2 / IL-33 signaling might be an effective therapeutic option for RA.

7. IL-2 and the pathogenesis of RA

IL-2 is a Th1 lymphocyte-derived cytokine, and the principal autocrine growth factor that promotes T-cell activation and proliferation [130]. Clinical studies have shown that the serum IL-2 level is correlated with disease activity in RA [131, 132]. We have also shown that the levels of IL-2 in serum and bone marrow are correlated with disease activity in mutilans-type RA [110]. The effects of IL-2 are mediated by cell surface receptors (IL-2 R) expressed on activated T cells. The serum sIL-2 R level in RA reflects disease activation, and a rising level may also predict exacerbation of disease activity [133]. On the other hand, most studies of cytokines in RA have failed to detect IL-2 protein in RA synovial fluid [134]. In the CIA animal model, IL-2 was reported to exert two opposite effects, namely a direct stimulatory effect and an indirect suppressive effect. It was demonstrated that administration of recombinant human IL-2 (rhIL-2) at or just before disease onset exacerbated the disease (days 21–28 after the first immunization), whereas rhIL-2 administered before disease onset (days 14–21 after the first immunization) inhibited the CIA. It was concluded that the indirect suppressive effect was mediated by IFN-γ because in mice treated with an anti-IFN-γ Ab, both early and late IL-2 administration exacerbated the CIA [135]. In addition, IL-2/anti-IL-2 monoclonal antibody immune complexes have been reported to inhibit murine CIA. Histopathological examination of joints revealed inhibition of synovial cell proliferation and lower levels of IL-17, IL-6, and TNF-α [136]. Although these data may reflect the usage of different types of IL-2, they may also reflect
a change in the cytokine cascade in the mouse CIA model between the onset stage and the later stage. IL-2, IL-7, and IL-33 are known to regulate the function of type 2 innate lymphoid cells [137]. Stimulation with IL-33 caused GM-CSF production, and stimulation with a combination of IL-2 and IL-33 increased it. GM-CSF activates monocyte-macrophages and leads to joint damage. In RA patients, serum IL-2 levels are not only correlated with disease activity and autoantibody levels, but also impact their Th17/Treg immune imbalance. Also, in patients with active RA, levels of NK cells are abnormally elevated, possibly due to high serum levels of IL-2 [132]. IL-2-stimulated NK cells, which produce GM-CSF, induce monocyte-macrophage activation leading to joint damage. As mentioned in the section on GM-CSF, the network in which IL-2 is produced from Th-17 lymphocytes acts on NK cells and macrophages via GM-CSF. These networks characterize RA in the chronic phase.

Conclusions
Recent advances in molecular biology have greatly expanded our understanding of the pathophysiology of RA. We have overviewed the cytokines and molecules involved, starting from RF and ACPA, which represent the cornerstone autoantibodies operating in RA, and examined cytokines such as TNF-α, IL-6, IL-17, IL-23, IL-33, GM-CSF, and IL-2, which also play diverse roles. TNF and IL-6 inhibitors have already been used in many patients with remarkable clinical effects. GM-CSF inhibitors are also likely to be used as therapeutic agents. Jak inhibitors can suppress multiple molecules at the same time, but for RA, the Jak-Stat pathway is currently suitable for inhibition. Other possibilities for signaling inhibitors should be considered. Other cytokines may also have potential in future treatment avenues.

![Figure 1. Possible mechanism of joint inflammation and destruction.](image)

Interleukin (IL)-23 secreted by T helper (Th) 23 cells activate Th17, which in turn secretes IL-17 that activates group 2 innate lymphoid cells (ILC2) and natural killer (NK) cells. IL-2 and IL-33 released by Th17 cells, as well as NK, ILC2, and synovial fibroblast activates macrophages via the granulocyte-macrophage colony-stimulating factor (GM-CSF). Lastly, activated macrophages will secrete proinflammatory cytokines.

Cytokines, such as IL-6, tumor necrosis factor (TNF), and IL-17, activate synovial fibroblasts. Recent single-cell transcriptomics and mass cytometry analysis suggested the existence of various types of synovial fibroblasts, among which thymocyte differentiation antigen 1 (Thy1)-positive and -negative synovial fibroblasts in the lining and sublining layers, respectively, are the major subsets.
Cell identity between Thy1+ and Thy1− is controlled by Jagged1 on the vascular endothelium and by Notch3 on synovial fibroblasts.

Macrophages can migrate from extra-synovial tissues and function as pathogenic cells by secreting proinflammatory cytokines. In contrast, tissue-intrinsic macrophages function as anti-arthritisogenic, expressing c-mer tyrosine kinase (MerTK), claudin, resolvin D1, and IL-10.

Osteoclasts differentiate from extrinsic or intrinsic macrophages, through a process regulated by the receptor activator of nuclear factor-kappa B ligand (RANKL), TNF, and IL-6. In turn, RANKL expression in synovial fibroblasts is regulated by TNF-α, IL-6, and IL-17.

TNF receptor (TNFR) 2 and IL-6 signals regulate the balance between regulatory T-cells (Treg) and Th17, whereas peripheral Th cells (Tph) contribute to lymphoid follicle formation.

Conflict of Interest
The authors declare that they have no conflicts of interest in relation to this work.

Author Contributions
N.K., T.K., and D.K. contributed to the design and theoretical framework, to analysis of the results, and to writing of the manuscript.

Acknowledgements
This work was supported by a grant for JSPS KAKENHI (Grant Number 17K09973) from the Ministry of Health, Labour and Welfare, Japan.

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