Molecular aspects in inflammatory events of temporomandibular joint: Microarray-based identification of mediators

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Osteoarthritis;
Synoviocytes;
IL-1β;
TNF-α;
Microarray analysis

Summary Synovial inflammation (synovitis) frequently accompanies intracapsular pathologic conditions of the temporomandibular joint (TMJ) such as internal derangement (ID) and/or osteoarthritis (OA), and is suggested to be associated with symptom severity. To identify the putative factors associated with synovitis, we investigated interleukin (IL)-1β- and/or tumor necrosis factor (TNF)-α-responsive genes of fibroblast-like synoviocytes (FLS) from patients with ID and/or OA of TMJ using microarray analysis. In this review, we first summarize the FLS of TMJ and the signaling pathways of IL-1β and TNF-α. Next, we show the up-regulated genes in FLS after stimulation with IL-1β or TNF-α, and summarize the gene functions based on recent studies. Among the top 10 up-regulated factors, molecules such as IL-6 and cyclooxygenase-2 have been well characterized and investigated in the inflammatory responses and tissue destruction associated with joint diseases such as RA and OA, but the roles of some molecules remain unclear. The FLS reaction can lead to the synthesis and release of a wide variety of inflammatory mediators. Some of these mediators are detected in joint tissues and synovial fluids under intracapsular pathologic conditions, and may represent potential targets for therapeutic interventions in ID and/or OA of TMJ.

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Inflammatory mediators in TMJ

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1. Introduction

The temporomandibular joint (TMJ) is one of the most complex and active joints in the human body, playing an important role in functions such as speaking, chewing and swallowing. Patients with temporomandibular disorders (TMD) most frequently present with pain, limited mandibular motion and TMJ sounds. However, TMD are defined as a subgroup of craniofacial pain problems that involve the TMJ, masticatory muscles and associated head and neck musculoskeletal structures. The details and/or taxonomy of TMD have been reported in other review papers [1–3]. It is necessary to carry out systematic investigations into the development and progression of this disease. We have investigated the crucial factors in the intracapsular pathologic condition of TMJ.

Recently, the accumulation of arthroscopic [3–6] and magnetic resonance imaging findings [7,8], histological studies [4,9] and synovial fluid analysis [10–13] have led to an increase in knowledge that may contribute to understanding the intracapsular pathologic conditions of TMJ, such as internal derangement (ID), disc displacement (DD), fibrous adhesions, synovial inflammation (synovitis) and osteoarthritis (OA). These entities are not mutually exclusive and may coexist. Synovitis frequently accompanies TMJ ID and/or OA [5,6]. In orthopedics, synovitis has been suggested to be a key feature of OA [14], although the relationship between synovitis and OA is not well defined. On the other hand, numerous mediators contribute to both inflammatory and degradative pathways associated with the progression of the pathologic condition in the joints such as OA and rheumatoid arthritis (RA) [15–17].

Inflammatory factors have been detected in the synovial fluids and/or tissues from patients with ID and/or OA of TMJ [10–13] and from animal TMJ inducing inflammation using antigens such as ovalbumin [18]. These studies have suggested that over-production of inflammatory molecules is a crucial event in mediating the acute and chronic inflammation and tissue destruction in intracapsular pathological conditions, but little is known about the molecular mechanisms that underlie the development of the pathologic condition. To understand the molecular mechanisms underlying the role of these factors in the pathologic condition, it is necessary to elucidate the signaling pathways and molecular network.

In order to identify the putative factors associated with the intracapsular pathologic condition, we investigated interleukin (IL)-1β- and/or tumor necrosis factor (TNF)-α-responsive genes of synoviocytes from patients with ID and/or OA of TMJ [19–26]. According to microarray analysis, a large number of IL-1β- and/or TNF-α-responsive genes of synoviocytes were detected [19–21]. All of the responsive genes cannot be introduced and discussed in this review. Therefore, this review will address the top 10 genes up-regulated in synoviocytes after treatment with IL-1β- or TNF-α, and will then summarize current knowledge regarding the functional roles of these inflammatory signaling networks.

2. Synovium and synoviocytes

The articular surfaces involved in the TMJ are the articular eminence of the temporal bone and mandibular condyle [2,27]. The articular surface of joints such as the knee is covered by hyaline cartilage, whereas in TMJ, the articular lining consists of fibrocartilage. The TMJ is covered with a fibrous capsule known as the synovial membrane, synovial tissues or synovium. Synovial membrane, which consists of the synovial lining layer and the connective sublining layer, lines all intra-articular non-loaded structures, except for articular cartilage of the eminence, fossa and mandibular condyles, and the articular disc [27,28].

The synovial membrane comprises synoviocytes for the production/secretion and resorption of synovial fluids. There are two different types of cell in the synovial lining
of the knee; macrophage-like type cells and fibroblast-like type cells [29]. The synovial membrane in TMJ also consists of macrophage-like type A cells and fibroblast-like type B cells [30–32]. It has been reported that the macrophage-like type A cells are ultrastructurally characterized by dense filopodia-like surface folds, numerous vesicles, vacuoles and lysosomes, and are regarded as being of a macrophage lineage with phagocytic activity [30–33]. It is plausible that macrophage-like type A cells are derived from a monocyte-lineage and have high migratory and phagocytic activity. In contrast, fibroblast-like type B cells are ultrastructurally characterized by the presence of a well-developed rough endoplasmic reticulum and dense secretory granules [30–32]. It has been suggested that fibroblast-like type B cells produce and secrete both synovial fluids and extracellular matrix components, such as collagen, as well as a number of putative mediators of inflammation. Both macrophage-like type A and fibroblast-like type B cells have important roles in homeostasis and pathologic conditions in intracapsular TMJ. However, little is known about the function and response of these synoviocytes in TMJ.

3. In vitro model for synovitis

Fibroblast-like synoviocytes (FLS) that were positive for propyl 4-hydroxylase and vimentin can be isolated from synovium as biopsy specimens by arthroscopic surgery [25]. We investigated IL-1β- or TNF-α-responsive genes in FLS using microarray analysis to identify the putative factors associated with synovitis in TMJ. IL-1β and TNF-α, which are pleiotropic cytokines, have been shown to play important roles in both immune reactions and inflammation, as well as in pain response and cartilage/bone remodeling, particularly cartilage degradation and bone resorption [33–36]. These cytokines have been widely studied in the development of joint pathologic conditions such as OA and rheumatoid arthritis (RA) [14,37]. It has also been reported that the levels of IL-1β and TNF-α are significantly higher in synovial fluids from patients with ID and OA in TMJ, as compared to healthy TMJ [10,12]. The signal transduction pathways of IL-1β and TNF-α are shown in Figs. 1 and 2.

3.1. IL-1β

IL-1 comprises IL-1α and IL-1β, and is produced by various cell types, such as macrophages, fibroblasts, osteoblasts and synoviocytes [38]. The details of the IL-1 signaling pathways have been reported [39,40]. As shown in Fig. 1, the activating IL-1 receptor is a complex of two chains, IL-1 receptor type 1 (IL-1R1) and IL-1 receptor accessory protein (IL-1RaCP). The binding of IL-1 to IL-1R complex leads to the recruitment of myeloid differentiation primary response gene 88 (MyD88), an adaptor protein that in turn recruits IL-1 receptor associated kinases (IRAKs). The subsequent dissociation of these kinases from the receptor complex and recruitment of downstream signaling molecules, ultimately results in activation of the nuclear factor kappa B (NFκB) and MAPK (p38 JNK, and ERK) pathways. IL-1RII does not possess a signal transduction domain, and the resulting heterodimeric receptor complex is non-signaling. Hence, sequestration of IL-1 and the co-receptor IL-1RaCP negatively regulates IL-1RI-mediated signaling. IL-1 receptor antagonist (IL-1Ra) also inhibits IL-1 signaling.

Both IL-1α and IL-1β were detected in synovial fluids from patients with ID and OA of TMJ [41], but IL-1β is typically reported. On microarray analysis, IL-1β was predominantly expressed in FLS when compared to IL-1α (data not shown). In addition, expression of IL-1α and IL-1β were up-regulated in FLS by treatment with IL-1β, which may be mediated by NFκB activation (data not shown).

3.2. TNF-α

TNF-α is produced in response to pathological conditions such as inflammation and infection, mainly by activated macrophages and T lymphocytes, but also by several cell types including natural killer (NK) cells, mast cells and fibroblasts. Macrophages are the primary source of TNF in inflamed synovial tissue [41], and TNF-α is related to both macrophage migration and pain in inflammatory joints [42].

The details of the TNF-α signaling pathway have also been reported in other review papers [43–46]. As shown in Fig. 2, binding of ligand TNF-α to its receptor TNFR1 leads to the recruitment of TNFR-associated death domain (TRADD), TRADD also interacts with TNF receptor associated factor (TRAF) 2, followed by sequential recruitment of receptor interacting protein (RIP), and then subsequent kinase activation. TNF-α triggers several signaling cascades such as apoptotic pathways, activation of NFκB and MAPKs (p38 MAPK, ERK and JNK). TRADD directly binds to Fas-associated death domain protein (FADD) and activates apoptosis via caspase cascade. On the other hand, cell signaling associated with TNFR2 is poorly understood. TNFR2 lacks a death domain, despite interacting with TRAF2, through which it can activate the transcription factors NFκB and AP-1.

4. IL-1β- or TNF-α-responsive genes

FLS were treated with or without 0.1 ng/ml IL-1β or 10 ng/ml TNF-α for 4 h, and total RNA was then extracted for microarray analysis. Expression of 8,793 genes on the Human Genome Focus Array (Affymetrix) in control and IL-1β- or TNF-α-stimulated cells was then compared. A total of 212 genes showed greater than 2-fold up-regulation by IL-1β, while 239 genes were up-regulated genes by at least 2-fold in the presence of TNF-α [20]. Table 1 lists the top 10 up-regulated genes in FLS treated with IL-1β or TNF-α. There were five genes that overlapped between the two treatments, and MIP-3α, which is a member of the chemokine family, was found to be the most strongly up-regulated gene by both IL-1β and TNF-α. In addition, MIP-3α production was decreased by treatment with inhibitors against MAPK and NFκB signaling in both IL-1β- and TNF-α-stimulated cells (Table 2).

4.1. Chemokines

As shown in Table 1, numerous chemokine family members are listed among the top 10 up-regulated genes in FLS treated with pro-inflammatory cytokine; six chemokines with IL-1β-stimulation and five chemokines with
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Figure 1  IL-1 signaling. The IL-1 signaling complex is assembled when IL-1 binds to its receptor, IL-1RI. The heterodimeric receptor complex consisting of IL-1RI and IL-1RacP leads to the recruitment of MyD88 and IRAKs. Subsequently, IRAK4 phosphorylates IRAK1. These events lead to the activation of the NFκB pathway by providing a platform for the recruitment of TGF-β-activated kinase 1 (TAK1, a MAPK kinase kinase) and the IKK complex. TAK1 activates IKK2, which in turn phosphorylates IkBα, thereby freeing NFκB. On the other hand, TAK1 also phosphorylates MAPK kinases such as MKK3/6 and MKK4/7, and then phosphorylate MAPKs. MAPKs also activate several transcription factors, such as AP-1.

Table 1  Up-regulated genes after treatment with IL-1β or TNF-α.

<table>
<thead>
<tr>
<th>Rank</th>
<th>Gene</th>
<th>GenBank</th>
<th>Fold</th>
<th>Gene</th>
<th>GenBank</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CCL20 (MIP-3α)</td>
<td>NM_004591</td>
<td>429.9</td>
<td>CCL20 (MIP-3α)</td>
<td>NM_004591</td>
<td>322.2</td>
</tr>
<tr>
<td>2</td>
<td>CXCL3 (GRO-γ)</td>
<td>NM_002090</td>
<td>150.4</td>
<td>IL-8 (CXCL8)</td>
<td>AF043337</td>
<td>76.7</td>
</tr>
<tr>
<td>3</td>
<td>CSF2</td>
<td>M11734</td>
<td>107.4</td>
<td>CSF2</td>
<td>M11734</td>
<td>37.9</td>
</tr>
<tr>
<td>4</td>
<td>IL-8 (CXCL8)</td>
<td>AF043337</td>
<td>89.8</td>
<td>ICAM1</td>
<td>NM_000201</td>
<td>32.1</td>
</tr>
<tr>
<td>5</td>
<td>CXCL1 (GRO-α)</td>
<td>NM_001511</td>
<td>59.5</td>
<td>CXCL3 (GRO-γ)</td>
<td>NM_002090</td>
<td>31.1</td>
</tr>
<tr>
<td>6</td>
<td>CXCL2 (GRO-β)</td>
<td>M57731</td>
<td>50.1</td>
<td>CXCL10 (IP10)</td>
<td>NM_001565</td>
<td>27.8</td>
</tr>
<tr>
<td>7</td>
<td>IL-6</td>
<td>NM_000600</td>
<td>40.1</td>
<td>BCL2A1</td>
<td>NM_004049</td>
<td>24.5</td>
</tr>
<tr>
<td>8</td>
<td>PTGS2 (COX-2)</td>
<td>NM_000963</td>
<td>37.8</td>
<td>GCH1</td>
<td>NM_000161</td>
<td>21.9</td>
</tr>
<tr>
<td>9</td>
<td>BCL2A1</td>
<td>NM_004049</td>
<td>37.3</td>
<td>IL17RB</td>
<td>NM_019583</td>
<td>21.9</td>
</tr>
<tr>
<td>10</td>
<td>CXCL10 (IP10)</td>
<td>NM_001565</td>
<td>28.7</td>
<td>CX3CL1 (flactalkine)</td>
<td>U84487</td>
<td>21.6</td>
</tr>
</tbody>
</table>

Source: Akutsu et al. [20].
Rank; ranking of up-regulated genes with IL-1β or TNF-α. Fold; average normalized intensity of stimulated FLS/average normalized intensity of control FLS. Three FLSs, which were isolated from three patients, were experimented independently.
Figure 2  TNF-α signaling. Binding of TNF-α to TNFRI results in activation of its receptors, followed by recruitment of adaptor proteins (TRADD, FADD, TRAF and RIP) in a sequential manner that activates several signaling cascades leading to the activation of transcription factors NFκB, AP-1 and/or caspase cascades. TNFRI has a so-called death domain region, which is essential for induction of apoptosis through the activation of caspase cascades. TNFRII does not have the death domain. As compared with TNF-R1, the function of TNFRII is less well understood.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IL-1β</th>
<th>% inhibition</th>
<th>TNF-α</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>118.0 ± 27.2</td>
<td>0</td>
<td>482.1 ± 223.2</td>
<td>0</td>
</tr>
<tr>
<td>PD98059 (ERK1/2)</td>
<td>43.8 ± 15.2</td>
<td>63</td>
<td>142.7 ± 23.2</td>
<td>70</td>
</tr>
<tr>
<td>SB203580 (p38)</td>
<td>19.7 ± 4.0</td>
<td>83</td>
<td>115.8 ± 17.9</td>
<td>76</td>
</tr>
<tr>
<td>SP600125 (JNK)</td>
<td>14.6 ± 8.5</td>
<td>88</td>
<td>80.8 ± 11.9</td>
<td>83</td>
</tr>
<tr>
<td>APDC (NFκB)</td>
<td>15.3 ± 1.9</td>
<td>87</td>
<td>17.2 ± 3.8</td>
<td>96</td>
</tr>
</tbody>
</table>

Source: Akutsu et al. [20].
FLSs were pre-treated with 40 μM PD98059, 10 μM SB203580, 10 μM SP600125 and 10 μM APDC for 15 min, and were then treated with 0.1 ng/ml IL-1β or 10 ng/ml TNF-α for 3 h. Results are expressed as means ± S.D. (n = 4).

TNF-α-stimulation. Chemokines are small, chemoattractant cytokines that play key roles in the accumulation of inflammatory cells at the site of inflammation [47–49]. Chemokines are classified into four groups based on the motif displayed by the first two cysteine residues near the N-terminus (CC, CXC, C and CX3C), and their receptors are respectively classified as CCR, CXCR, CR and CX3CR [47–49]. Chemokines act through seven transmembrane G-protein coupled receptors that are expressed selectively on the surface of specific leukocyte and lymphocyte subsets [47]. The CXC chemokines mainly act on neutrophils and lymphocytes, whereas the CC chemokines mainly act on monocytes and
lymphocytes [48,49]. Chemokines are considered key players in the diapedesis of leukocytes from the vasculature into tissues in inflammatory diseases. In TMJ, inflammatory cells were also increased in synovial tissues from not only RA and OA [50], but also ID [51].

It has been shown that chemokines such as IL-8/CXCL8, MCP-1/CCL2 and RANTES/CCL5 are expressed by human chondrocytes and synoviocytes isolated from patients with OA and RA [52,53]. Several chemokines, such as IL-8/CXCL8, GRO-a/CXCL1, RARES/CCL5 and MIP-1α/CCL3, are increased in synovial tissue and synovial fluid from RA and OA, as compared to healthy controls [49,54,55]. IL-8/CXCL8 and MCP-1/CCL2 are also elevated in synovial fluids from patients with ID and/or OA of TMJ [12,21,41].

Inflammatory arthropathies are characterized histologically by infiltration of inflammatory cells and enlargement of the synovial lining layer [55]. Accumulation of neutrophils and macrophages in inflamed synovial tissues may lead to significant structural damage to joints with arthritis [52,55]. Inflammatory cells have also been detected in synovial tissue and in fluid from patients with intracapsular pathologic condition of the TMJ [50,56]. The mechanisms leading to cellular infiltration of the synovium and joint degeneration have been elucidated to a degree by studying the release of degradative enzymes, various products of oxidative metabolism and inflammatory cytokines. The following sequence of events is consistent with these findings: (1) chemokines produced by FLS stimulate chemotaxis of neutrophils, macrophages and T-lymphocytes; (2) these inflammatory cells produce inflammatory cytokines such as IL-1β, matrix degradative enzymes and various products of oxidative metabolism; (3) enzymes and oxidative metabolites cause degradation of the extracellular matrix; and (4) inflammatory cytokines stimulate FLS to produce more chemokines. Furthermore, the subset of CXC-type chemokines that contain the sequence Glu-Leu-Arg (the “ELR” motif) are also thought to promote angiogenesis [57]. Thus, induction of ELR-CXCs such as CXCL1, 2, 3, 6, and 8 may lead to both recruitment of inflammatory cells and new small vessels in synovial tissues. Although chemotaxis is a necessary function of homeostasis, inappropriate infiltration of inflammatory cells may lead to joint degeneration.

4.2. IL-6

IL-6 was ranked 7 among the top 10 up-regulated genes in FLS treated with IL-1β (Table 1). In contrast, IL-6 was not found among the top 10 up-regulated genes with TNF-α (it was ranked 16; data not shown). IL-6, which is produced by T-cells, B-cells, monocytes, fibroblasts, endothelial cell and FLS, is considered to play a central role in chronic inflammation and is expressed in excess at sites of inflammation [58,59]. IL-6 plays an important role in RA inflammation. IL-6 levels are markedly elevated in the serum and in the synovial fluid of RA patients, and this elevation has been directly correlated with clinical indices of disease activity [60,61]. In addition, high levels of soluble IL-6 receptor (sIL-6R) have been shown to correlate with the degree of joint destruction, particularly in advanced stages of RA [62]. In ID and OA of TMD, IL-6 is detected in the synovial fluids from patients, and IL-6 levels are positively correlated with the degree of synovitis [10,63]. IL-6 levels are an indicator of unsuccessful outcome of TMJ irrigation by arthrocentesis [64]. In addition, sIL-6R was also detected in synovial fluids from patients with ID or OA [65].

IL-6 exerts its biological activity through two molecules: type I transmembrane IL-6 receptor (IL-6R) and transmembrane signal transducer protein gp130 (gp130) [59]. IL-6R is important for ligand binding and is able to play only a minor role in signal transduction. gp130 contains several potential motifs for intracellular signaling for JAK/STAT and ERK [66]. The soluble type receptor (sIL-6R) binds to its ligand IL-6, forming a complex with gp130 [59]. The complex of IL-6/sIL-6R/gp130 is able to induce signal transduction in target cells, although other soluble receptors, such as the receptors for IL-1 or TNF, are known to inhibit the effects of their ligands [67]. Therefore, the population of potential IL-6 target cells is strongly increased by the presence of sIL-6R. Activation of cells that only express gp130 via the IL-6/sIL-6R complex is known as trans-signaling, whereas activation of cells via membrane-bound IL-6R in complex with IL-6 is known as classic signaling.

IL-6 elicits the development of specific cellular and humoral immune responses. IL-6 is identified as a B-cell differentiation factor [68]. IL-6 enhances the proliferation and activation T-cells [69]. Th17 helper cells of the T-cell subset, which produce IL-17 in autoimmune pathology, differentiate from naïve CD4+ T-cells by IL-6 stimulation [70]. IL-6 also induces the transmigration of neutrophils to inflammatory sites in arthritis by stimulating chemokine production in various cell types [71]. There is substantial evidence that IL-6 plays an important role in rheumatoid inflammation [58].

In addition, IL-6 is suggested to have a pathogenetic role in abnormal bone resorption in RA [72]. IL-6 induces abnormal osteoclastogenesis in the inflamed joints of RA via the induction of RANKL expression in osteoblasts and synoviocytes [72,73]. With regard to joint cartilage, matrix metalloproteinase (MMP) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) are thought to play crucial roles in cartilage matrix degradation. IL-6 induces the production of MMPs (MMP-1, MMP-3 and MMP-13) and ADAMTS-4 from chondrocytes [74]. These findings suggest that IL-6 plays a number of critical roles in the pathogenesis of joint diseases.

We have shown that FLS constitutively expresses gp130, but not IL-6R (Fig. 3) [75]. In contrast, sIL-6R was detected in synovial fluids from patients with ID and/or OA of TMJ [65]. In the intercapsular pathologic condition of TMJ, MMP production may be enhanced in FLS through the IL-6/sIL-6R/gp 130 complex, as sIL-6R exists in synovial fluids and IL-6 is produced by FLS in the presence of IL-1β and/or TNF-α.

4.3. COX-2 (PTGS2)

Cyclooxygenase (COX) -2, also known as prostaglandin-endoperoxide synthase 2 (PTGS2), was ranked 8 among the top 10 up-regulated genes in FLS treated with IL-1β (Table 1). In contrast, COX-2 was not observed among the top 10 up-regulated genes with TNF-α, although it was rank 17 (data not shown). COXs are the rate-limiting enzyme in the synthesis of biological mediators known as prostanoids, consisting of
prostaglandin (PG) D₂, PGE₂, PGF₂α, prostacyclin PG₁₂ and thromboxane A₂ [76]. Following cellular activation, arachidonic acid (AA) is released from membrane phospholipids by phospholipase A₂ and is then converted to the PGs intermediate PGH₂ by COXs. Short-lived PGH₂ is then quickly converted to five prostanooids by each prostanoid synthase. COX-1 is a constitutive enzyme in the majority of cells. In contrast, COX-2 is inducible in response to inflammation, its gene expression can be induced by multiple cytokines and growth factors, via activation of transcriptional regulatory proteins that act on the promoter sites, such as NFKB, AP1 and C/EBP [77,78]. COX-3 is a splice variant of COX-1 [79].

PGE₂ is the most studied member of the eicosanoid family and is generated from unsaturated 20-carbon fatty acids such as AA, playing a pivotal role in inflammation by mediating local dilatation of blood vessels, increasing blood vessel permeability and sensitizing peripheral nociceptors [80]. PGE₂ exerts a range of biological activities that include stimulation of inflammation-associated bone resorption [81]. The major effect of PGE₂ on resorption is generally considered to occur indirectly via up-regulation of RANKL expression and by inhibition of OPG expression in osteoblastic cells, although it has been reported that PGE₂ has both stimulatory and inhibitory effects on RANKL-stimulated osteoclast formation [81].

PGE₂ contributes to the pathogenesis of several chronic inflammatory conditions, including periodontitis, RA and cardiovascular inflammatory disease. High concentrations of PGE₂ have been detected in the synovial fluid of patients with OA and RA [82,83]. PGE₂ has also been found in TMJ synovial fluids from patients with ID, and is associated with synovitis index based on synovial membrane hyperemia as the diagnostic criteria for TMJ arthroscopy [84,85]. It has been reported that cytokine-activated cells, such as synovial cells, chondrocytes and macrophages/monocytes, are the primary source of PGE₂ in arthritic joints. PGE₂ production at sites of inflammation coincides with the up-regulation of COX-2 expression in articular cells activating pro-inflammatory factors [86].

The existence of four EP receptor subtypes (EP1–4) encoded by distinct genes also contributes to the diversity of the biological activity of PGE₂ [87]. EP1 acts largely by increasing calcium flux but perhaps also via protein kinase C (PKC) [88]. Although it might be coupled to Gq, the absence of a phosphatidylinositol response has led to speculation that it is coupled to an as yet unidentified G protein [87]. Both EP2 and EP4 are coupled to Gs and stimulate cyclic 3,5-adenosine monophosphate (cAMP) formation [89,90]. EP3 is coupled to Gi and acts largely by inhibiting cAMP production [91]. FLS from patients with RA and OA mainly expressed EP2 and EP4 [92]. A previous study has shown that PGE₂ increased the production of pro-inflammatory cytokines such as IL-6 and VEGF in FLS with RA and OA through the activation of EP2 and EP4, and with increases in cAMP [92]. These findings suggest that elevated PGE₂ production is associated with inflammatory joint diseases, and can lead to bone loss.

On microarray analysis, EP2 and EP4 were expressed in FLS from patients with ID TMJ, and the expression of EP2 and EP4 was slightly elevated in FLS treated with IL-1β or TNF-α (Table 3). The agonists of EP2 and EP4, and PGE₂ stimulate the production of IL-6 in FLS (Fig. 4) [19]. EP4 receptor was partially effective with regard to IL-6 production in FLS mediated by PGE₂, as indicated by treatment

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Expressions of EP receptor (EP1–4) genes in FLS by microarray.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normalized intensity</td>
</tr>
<tr>
<td>Control</td>
<td>A</td>
</tr>
<tr>
<td>IL-1β</td>
<td>A</td>
</tr>
<tr>
<td>TNF-α</td>
<td>A</td>
</tr>
<tr>
<td>N = 3; A, absent.</td>
<td></td>
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</tbody>
</table>

Figure 3 Gene expression of IL-6 and IL-6 receptors. The gene expression of IL-6 and IL-6R, and gp 130 was examined in FLS treated with or without IL-1β and/or TNF-α for 4 h by RT-PCR. C, control; I, IL-1β; T, TNF-α; I + T, IL-1β + TNF-α. Source: Kawashima et al. [75].

Figure 4 Effects of EP agonists on IL-6 production. Time course of IL-6 protein production in the conditioned medium of FLS was determined by ELISA. Cells were cultured with or without PGE2 or EP receptor agonists, and were incubated for 4, 8, 12 or 24 h. (●) None, (●) PGE2, (▲) EP1 agonist (ONO-DI-004), (●) EP2 agonist (ONO-AE-259-1), (●) EP3 agonist (ONO-AE-248), (●) EP4 agonist (ONO-AE-329), *P < 0.05, **P < 0.01, ***P < 0.005. Source: Kawashima et al. [19].
with EP4 agonist. Expression of EP4 receptor was at a lower level than that of the EP2 receptor (Table 3), and was transiently enhanced by IL-1β [19]. PGE2 affects IL-6 production through EP2 and EP4 in the FLS. In addition, COX inhibitors (indomethacin or celecoxib) decreased the expression of IL-6 in FLS stimulated with IL-1β (Fig. 5) [19]. Our results suggest that COX inhibitors as non-steroidal anti-inflammatory drugs (NSAIDs) are useful for treating synovitis in TMJ through the suppression of both PGE2 and inflammatory mediators such as IL-6.

4.4. GM-CSF (CSF2)

Granulocyte macrophage colony stimulating factor (GM-CSF), also known as colony stimulating factor-2 (CSF2), was observed among the top 10 up-regulated genes in FLS with inflammatory stimulation, and ranked 3 with both IL-1β-stimulation and TNF-α-stimulation (Table 1). GM-CSF was originally defined as a hemopoietic growth factor [93]. However, it is able to act on mature myeloid cells and has other functions, acting as a pro-inflammatory cytokine [94]. GM-CSF influences dendritic cells and macrophage recruitment into inflammatory sites [95]. GM-CSF is also important for effective antigen processing and presenting function in antigen present cells (APC) [96]. Recent studies have highlighted a surprising role for GM-CSF responsive monocyte-derived dendritic cells in the pathogenicity of Th17 cells, which are important mediators for inflammatory diseases [97]. Moreover, IL-17 produced by activated Th17 induced the GM-CSF production from macrophages [98].

GM-CSF is produced by a wide variety of cell types, including activated T-cells, B-cells, macrophages, endothelial cells and fibroblasts, and its production is increased by inflammatory factors such as IL-1, TNF and PGE2. GM-CSF is detected abundantly in RA synovial fluid and is scarcely detected in non-inflammatory synovial fluid [99]. GM-CSF was also detected in synovial fluids from patients with ID and/or OA of TMJ, but was not detected in normal controls [100].

GM-CSF was detected in the erosive inflammatory reaction around orthopedic implants [101] and in osteolytic bone metastasis from malignant tumors [102]. However, studies examining the effects of GM-CSF on osteoclastogenesis have reported contradictory results. It has been reported that GM-CSF induced the fusion of prefusion osteoclasts to form multinucleated osteoclasts, making the osteoclast capable of bone resorption [103]. In contrast, another study demonstrated that GM-CSF abolished monocyctic differentiation into osteoclasts while inducing DC differentiation even in the presence of M-CSF and RANK ligand [104]. Although the basis for these apparently contradictory results reached by these different approaches is unclear, this uncertainty as to the effects of GM-CSF on osteoclast differentiation may be related to the stages for osteoclast differentiation and/or the potential significance of its increased production at sites of inflammation.

4.5. BCL2A1

B-cell lymphoma 2-related protein A1 (BCL2A1), also known as Bcl-2-related gene expressed in fetal liver (Bf-1), is among the top 10 up-regulated genes in FLS with inflammatory stimulation, ranked 9 with IL-1β-stimulation and ranked 7 with TNF-α-stimulation (Table 1). BCL2A1 is a member of the B-cell lymphoma 2 (BCL2) protein family, which are important cell death regulators [105]. The BCL2 family comprises both pro- and anti-apoptotic proteins [106]. BCL2A1 is an anti-apoptosis protein of the BCL2 family whose main function is binding to pro-apoptosis BCL2 members such as BAK, and then inhibiting BAK function [106, 107]. Pro-apoptosis proteins induce the release of cytochrome c from mitochondria, caspase activation and apoptosis [106,107]. Therefore, BCL2A1, similarly to BCL2, is an anti-apoptotic BCL2 protein to control the release of cytochrome c from mitochondria in the intrinsic apoptotic pathway. In addition, BCL2A1 is a highly regulated NFκB target gene that exerts important pro-survival functions [108].

In a physiological context, BCL2A1 is mainly expressed in the hematopoietic system, where it facilitates survival of selected leukocytes subsets and inflammation [106]. In RA, the synovium is infiltrated by chronic inflammatory cells, such as macrophages, dendritic cells, and lymphocytes [109]. The resident fibroblasts adopt a quasi-malignant phenotype with up-regulation of oncogenes, inhibition of
apoptosis, and secretion of cytokines, chemokines, and enzymes, which reinforce inflammation and catalyze joint destruction. This suggests the necessity of further evaluation of the role of BCL2 in RA and, in particular, a potentially overlooked role for long-term survival of inflammatory cells [106]. To the best of our knowledge, there have been no reports of BCL2A1 being detected in synovium or being expressed in FLS from inflammatory joint diseases.

On microarray expression of BCL2A1 was elevated in FLS-treated IL-1β. We also found that the intimal layer of synovial tissue was hypertrophic in rat TMJ after in vivo injection of IL-1β. This suggests that BCL2A1 is associated with hypertrophy of the synovial layer, although there have been no reports of BCL2A1 detection in the synovial tissue of ID and OA TMJ.

4.6. ICAM

Intercellular Adhesion Molecule 1 (ICAM1) was ranked 4 among the top 10 up-regulated genes in FLS treated with TNF-α (Table 1). In contrast, ICAM1 was not observed among the top 10 up-regulated genes with IL-1β (it was ranked 12; data not shown). ICAM1 is a member of the immunoglobulin superfamily of adhesion molecules mediating the contact between two cell types, or between cells and the extracellular matrix [110]. ICAM1 is expressed in numerous cell types, including leukocytes, macrophages, dendritic cells, fibroblasts, endothelial and epithelial cells. ICAM1 is scarcely detectable in normal cells, but its expression is enhanced in FLS, chondrocytes and endothelial cells in response to inflammatory cytokines such as TNF-α, IL-1β and IFN-γ [111]. ICAM-1 was detected in synovium and cartilage from RA patients [112]. ICAM-1 also mediates the infiltration of leucocytes by recognition with ligand lymphocyte function-associated antigen-1 (LFA-1) [113]. It has been suggested that activation of RA synovial fibroblasts with inflammatory cytokines stimulates the synthesis and expression of adhesion molecules such as ICAM-1, which facilitate recruitment and retention of inflammatory cells in the synovium resulting in joint inflammation.

A fragment of ICAM-1 found in the circulation (sICAM-1) is thought to be cleaved from the surface of ICAM-1-expressing cells [110]. This adhesion molecule plays critical roles in several different inflammatory and immunological processes. Synovial fluids from patients with RA and other inflammatory arthritides had significantly higher sICAM-1 levels when compared with osteoarthritis (OA) synovial fluids [114]. Synovial fluid sICAM-1 levels were significantly and positively correlated with synovial fluid leukocyte counts. Soluble level of ICAM1 in sera and synovial fluid (SF) are correlated with some clinical parameters and synovial tissue expression of ICAM1 in RA [115]. It has been shown that sICAM1 is able to bind to LFA-1 and competitively inhibit ICAM-1/LFA-1-mediated cell—cell interaction in vitro [116], albeit at concentrations much greater than those found in plasma. As a consequence, it is unlikely that sICAM1 antagonizes ICAM1/LFA-1-mediated cellular events in vivo. To the best of our knowledge, there have been no reports of the detection of ICAM1 and sICAM1 in ID and OA TMJ.

4.7. GCH1

Guanosine triphosphate (GTP) cyclohydrolase I (GCH1) was ranked 8 among the top 10 up-regulated genes in FLS treated with TNF-α (Table 1). In contrast, GCH1 was not observed among the top 10 up-regulated genes with IL-1β (it was ranked 15; data not shown). GCH1 catalyzes the conversion of GTP to D-erythro-β,β-dihydronopterin triphosphate, the first and rate-limiting step in tetrahydrobiopterin (BH4) biosynthesis [117]. It has been demonstrated that GCH1 is a key modulator of peripheral neuropathic and inflammatory pain. BH4 represents an essential cofactor for the production of catecholamines, serotonin and nitric oxide [118], all of which are heavily implicated in the pathogenesis of migraines [119]. After axonal injury, concentrations of BH4 rose in primary sensory neurons, owing to up-regulation of GCH1. After peripheral inflammation, BH4 also increased in dorsal root ganglia, owing to enhanced GCH1 enzyme activity. Recently, it has been shown that carriers of a particular haplotype of GCH1 had decreased sensitivity to some experimental mechanical pain stimuli [120]. While recent data suggest a “protective” (less pain) haplotype in the GCH1 gene, other research has failed to confirm this association. To the best of our knowledge, although there have been no reports for GCH1 in joint diseases, GCH1 may be associated with pain in joint diseases. Further studies on GCH1 in joint diseases such as RA and OA are therefore necessary.

4.8. IL17RB

IL-17 receptor B (IL17RB) was ranked 9 among the top 10 up-regulated genes in FLS treated with TNF-α (Table 1). In contrast, IL17RB was not among the top 10 up-regulated genes with IL-1β (it was ranked 16; data not shown). IL17RB is one of IL-17 receptor family members that now consist of 5 members (IL17RA, IL17RB, IL17RC, IL17RD and IL17RE). In contrast, the IL-17 ligand family comprises 6 members; IL17A, IL17B, IL17C, IL17D, IL17E (also called IL-25) and IL17F [121]. IL-17 typically refers to IL-17A. IL-17A is well characterized as a signature cytokine that participates in both acute and chronic inflammatory responses, while the other forms have not been widely studied [122]. IL-17A, which is produced by Th1 helper cells, plays a pathological role in inflammatory and autoimmune diseases. High IL-17A levels have been observed in patients with autoimmune diseases such as RA [123]. Previous studies have reported that IL-17A stimulation leads to TRAF 6 recruitment to the IL17RA-IL17RC receptor heterodimer complex, and induces NFκB activation [121].

Recent reports have shown that IL-17E (IL-25) and IL-17B have an affinity for IL17RB [121,124]. IL-17E signaling through the IL17RA-IL17RB receptor heterodimer complex induces Th2 responses by activating the MAPK and NFκB pathways. IL-17E is produced by eosinophils, mast cells and airway epithelial cells and stimulates asthma-like, allergic inflammation [121,125]. On the other hand, IL-17B, which is expressed in chondrocytes [126], has been shown to interact with IL-17RB [121]; however, its biological function remains unclear.

The IL-17 cytokine family, derived from a wide array of cell types, coupled to the differential expression of their
receptors on various cells and tissues, illustrate the complexity of the cytokine family network in modulating the immune response and inflammation. Further studies into the role of IL17RB in joint diseases such as RA and OA remain necessary.

4.9. Other molecules

We have reported several IL-1β-responsive genes other than the top 10 genes up-regulated in FLS by IL-1β and/or TNF-α. The expression and the production of monocyte colony stimulating factor (M-CSF), also known as CSF1, which was ranked 26 with IL-1β-stimulation and ranked 22 with TNF-α-stimulation (data not shown), was increased in FLS treated with IL-1β (Fig. 6), and was detected in the synovial tissues of the IL-1β-injected TMJ in rats [127]. M-CSF is critical for the proliferation and survival of macrophages and osteoclast precursors [128,129].

In contrast, gene expressions of inhibitor kBα (IkBα), TNF-α-induced protein 3, TNFAIP3, also known as A20; TNFAIP3-interacting protein 1 (TNIP1, also known as ABIN1; A20-binding inhibitor of NFκB1), and cellular inhibitors of apoptosis (c-IAP, also known as BIRC; baculoviral IAP repeat-containing protein), which inhibit NFκB activation, were also increased after IL-1β treatment in FLS (Fig. 7) [130]. IkBα was ranked 64 with IL-1β-stimulation and ranked 42 with TNF-α-stimulation. TNFAIP3 was ranked 27 with IL-1β-stimulation and ranked 19 with TNF-α-stimulation. TNIP1 was ranked 78 with IL-1β-stimulation and ranked 55 with TNF-α-stimulation. c-IAP2 was ranked 25 with IL-1β-stimulation and ranked 28 with TNF-α-stimulation. The gene expression of these inhibitors of NFκB activation was rapidly induced by NFκB in a negative feedback loop that may maintain a transient NFκB response.

5. Conclusion

This review focused on the molecules that are enhanced in FLS treated with IL-1β and/or TNF-α based on microarray analysis, and summarized their functions based on recent studies. It is notable that the expression of chemokines and adhesion molecules is up-regulated in FLS after stimulation with IL-1β or TNF-α. Previous reports have shown that synovitis is characterized by infiltration of inflammatory cells, and increases in new capillaries and small vessels. Similarly, several molecules categorized as inflammatory factors, catabolic enzymes, osteoclast differentiation factors,
Figure 8  Schema of synovitis progression. When levels of IL-1β and/or TNF-α are increased in synovial fluids, FLS increase production of chemokines, adhesion molecules and CFSs. These factors produced by FLS stimulate chemotaxis of neutrophils, macrophages and T-lymphocytes, and these inflammatory cells produce inflammatory cytokines such as IL-1β, matrix degradative enzymes and various products of oxidative metabolism. These enzymes and oxidative metabolites cause degradation of extracellular matrix, and inflammatory cytokines stimulate FLS to produce more pro-inflammatory cytokines, such as chemokines, PGE2 and catabolic enzymes.

nociceptors and signal transducers were also up-regulated in FLS on microarray analysis. Previously, the gene expression of catabolic enzymes such as MMP-1, -2 and -3, and ADAMTS-4 and -5 was confirmed to be up-regulated in FLS after mechanical stress [131]. This suggests that excess mechanical compressive stress such as clenching or bruxism up-regulates the mRNA expression of MMPs and ADAMSTS in FLS, and induces inflammation and tissue degradation in synovium, and may then promote osteoarthritis of the TMJ [131]. In addition, M-CSF, which is critical for the proliferation and survival of macrophages and osteoclast precursors, was up-regulated in FLS treatment with IL-1β and TNF-α. These regulated genes may be associated with the pathology of painful and dysfunctional ID or OA in TMJ. Our data support the notion that one of the reasons behind synovitis induction is the increased levels of IL-1β and/or TNF-α in the synovial fluids of patients (Fig. 8).

Among the top 10 up-regulated factors, most molecules are well characterized and have been investigated in the inflammatory responses and tissue destruction associated with joint diseases such as RA and OA, but some molecules such as GCH1 remain unclear. Recently, numerous molecules have been detected by in silico identification using genetic databases. For instance, the IL-1 family has grown impressively in size, complexity and division of labor. IL-1 family ligands include seven molecules with agonist activity (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β and IL-36γ), three receptor antagonists (IL-1Ra, IL-36Ra and IL-38) and an anti-inflammatory cytokine (IL-37) [132]. These new cytokines and their associated signaling pathways have been implicated in the pathogenesis of RA, and could be targeted to offer new therapeutic options for RA therapy. Currently, numerous underlying pathways remain unknown, but recent efforts have begun to increase our understanding. Nevertheless, better understanding of these interactions and signaling in inflammatory and dysfunctional settings will be crucial for the discovery of new therapeutic targets that will enable us to design more suitable treatments for patients who do not respond to conventional therapies.

Conflict of interest statement

There are no conflicts of interest associated with this review.

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Inflammatory mediators in TMJ


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