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Suppression of Pro-Inflammatory Cytokines via Targeting of STAT-Responsive Genes

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1. Introduction

The Janus Kinase/Signal Transducers and Activators of Transcription (JAK/STAT) signaling pathway play a fundamental role in regulating chronic systemic inflammatory responses in rheumatoid arthritis (RA) [1-5], based on compelling evidence that JAK/STAT is activated by many of the pro-inflammatory cytokines such as interleukin-1β (IL-1β), IL-2, IL-3, IL-6, IL-12, IL-17, IL-18, IL-19/IL-20, interferon-α/γ (IFN-α/γ) and oncostatin M (OSM) which are well-known to regulate, in part, immune-mediated inflammation in several autoimmune diseases, including RA [6-10]. However, complicating matters is the fact that some of the anti-inflammatory cytokines, which are known to dampen inflammatory responses induced by pro-inflammatory cytokines, including, IL-4, IL-10 and IL-13 also activate JAK/STAT [11-14]. In this regard, Müller-Ladner *et al*. [15] showed that synovial tissue obtained from RA patients contained significant amounts of constitutively activated IL-4/STAT. Therefore it will be necessary to understand more precisely the extent to which pro- and/or anti-inflammatory cytokine gene expression is deregulated in RA and which of the STAT-responsive genes known to alter immune-mediated inflammation in response to these cytokines may be amenable to therapeutic intervention.

2. JAKs

JAKs are non-receptor tyrosine kinases that are pre-associated with the membrane-proximal site of cytokine receptors [16]. Four mammalian JAK isoforms, JAK1, JAK2 and JAK3 and TYK2 have been described to date mainly from the results of gene structural analysis [17]. All of the JAK isoforms share a common structure known as the JAK homology (JH) do-

main. Leonard and O'Shea [18] identified a proline-rich conserved region in the cytokine receptors, called Box1, that associated with JH7 whereas the catalytic phosphotyrosine kinase site, called YY was determined to correspond to the other JH domains (Figure 1).
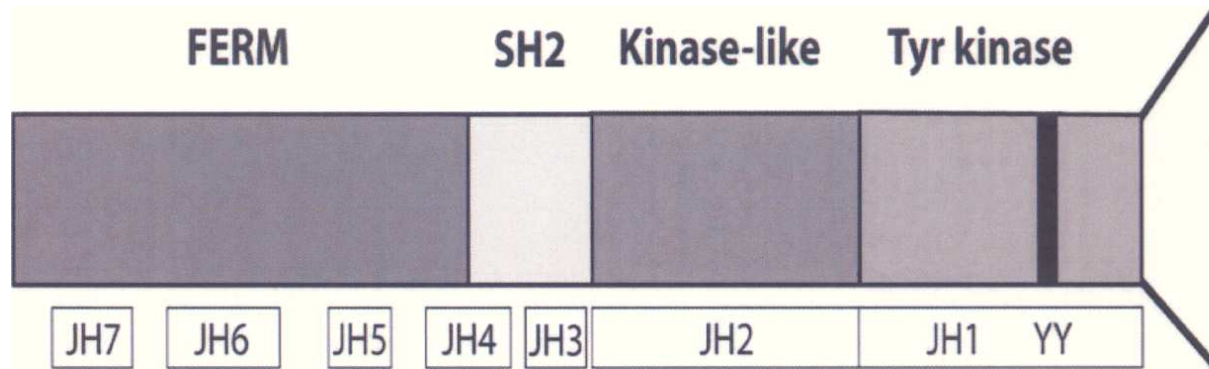

Figure 1. JH domains and phosphorylation sites of JAK3: Structural analysis combined with functional studies of JAK3 showed that the JH4-JH7 region contained band 4.1 also known as the Four-point-one, Ezrin, Radixin, Moesin (FERM) domain. Reprinted by permission from [16].

Additional structural analysis predicted that the JH2 domain was more than likely to be a pseudosubstrate domain [19]. In view of this latter finding the structural requirements for JAK activation was further clarified. Thus, the JH3-JH4 domain which shows a Src-homology-2-like structure had a shared homology with JH2. This finding indicated that the JH4-JH7 domains were, indeed, the critical regions required for regulating the interactions between the various JAK isoforms and other protein kinases. JH4-JH7 were also found to be essential for receptor binding, catalytic function, JAK autophosphorylation and even in some cases, inhibition of JAK activity.

3. Stat proteins

Gene analysis has revealed the existence of at least 6 STAT protein isoforms, namely, JAK1, JAK2, JAK3, JAK5A, JAK5B and JAK6 [20]. In normal homeostasis, phosphorylation of these STAT proteins is achieved via phosphorylation (i.e. activation) of specific JAK isoforms following the interaction of various cytokines and growth factors with their specific receptors [16, 21]. In this manner, cytokine receptor-mediated JAK activation results in the conversion of latent cytoplasmic un-phosphorylated STAT (U-STAT) proteins into phosphorylated STAT (p-STAT) proteins which can form homo- or heterodimers and are then translocated to the nucleus where these activated STAT protein dimers act as potent transcription factors [17-20]. Although phosphorylation of specific STAT-tyrosine residues remains the primary requisite mechanism for p-STAT protein dimer formation, a second phosphorylation site was also recognized at a serine in the C-termini domain of the STAT protein [20, 22].

An amplification loop with potential major clinical significance in RA involves the transcriptional activity of p-STAT proteins which further regulate the expression of pro-inflammato-

ry and anti-inflammatory cytokine genes as well as other genes of significance in cancer and autoimmune diseases [23-28]. In addition, p-STAT proteins can regulate other signaling pathways necessary for lymphocyte development, as well as the aberrant survival of activated dendritic cells, monocytes, lymphocytes and synoviocytes in disorders of the immune system [29-33].

It is noteworthy that during normal homeostasis, activation of STAT proteins induced the expression of Suppressor of Cytokine Signaling (SOCS) and Cytokine-Inducible SH-2 (CIS) proteins and it has been concluded that this is the negative feedback loop that underlies one of the mechanisms responsible for inhibiting JAK-mediated signaling by cytokines [34-38]. Thus, results of recently published experiments with human endothelial cells are germane to this point since the data in this paper provided a direct connection between the silencing of STAT3 with STAT3-specific silencing RNA and the suppression of SOCS3 [39].

The extent to which negative regulation of JAK-mediated signaling by SOCS/CIS may be inactivated in autoimmune diseases is a focus of current studies. In that regard, recent advances in unraveling the details of mechanism(s) governing negative regulation of cytokine signaling by SOCS/CIS proteins have shed additional light on the extent to which SOCS/CIS-mediated down-regulation of pro- and/or anti-inflammatory cytokine JAK/STAT signaling may be compromised in inflammatory arthritis [40]. However, the results of some recent studies with osteoarthritic human cartilage have not clarified this issue. For example, one study showed that the level of SOCS2 and CIS-1, but not SOCS1 and SOCS3, were reduced in femoral head cartilages from subjects with osteoarthritis [41], whilst the results of another study [42] indicated that SOCS3, but not SOCS1 expression, was elevated in chondrocytes obtained from osteoarthritic cartilage compared to chondrocytes from cartilage obtained from patients who had femoral neck fracture.

The status of the activity of certain other negative regulators such as protein tyrosine phosphatases, including SHP-1,-2 [43] and CD45 [44] and the 'Protein Inhibitor of Activated STAT' (PIAS) proteins [16, 45, 46] are also not precisely known in autoimmune diseases. These proteins could very likely suppress the activity of phosphorylated JAKs and p-STAT proteins by dephosphorylation or by interacting with p-STAT proteins in normal cells. However, these pathways may be compromised or markedly suppressed in arthritis.

It is also critical for gaining a further understanding of what alterations may occur in cytokine signaling in RA to recognize the fact that activation of JAK/STAT by any of the relevant cytokines can also activate other intracellular signaling pathways via the "cross-talk" mechanism. Thus, "cross-talk" between JAK/STAT and other signaling pathways [16] can cause activation of the Stress-Activated Protein Kinase/Mitogen-Activated Protein Kinase (SAPK/MAPK) pathway, the Phosphatidylinositol-3-Kinase/Akt/mammalian Target of Rapamycin (PI3K/Akt/mTOR) pathway [47], activation of signaling via Toll-like receptors [47, 48] and immunoreceptor tyrosine-based activation motifs (ITAMs) [49] as well as the NF-κB pathway [50]. These alternative signaling pathways which are all connected to inflammation have also been shown to significantly modulate many of the survival and/or apoptosis-signals required to perpetuate abnormal proliferation and/or to cause the death of activated dendritic cells, lymphocytes, macrophages, synoviocytes and chondrocytes.

Evidence from a genome-wide analysis study (GWAS) of STAT-target genes showed that many of these genes regulated cellular proliferation, angiogenesis and metastasis in cancer cells [51]. These results when coupled with the data from another recent study [52] which highlighted the nature of the several forms of STAT-interacting proteins that bind to DNA suggested that GWAS could be employed to identify pro-inflammatory and/or anti-inflammatory cytokine STAT-target gene structures and potentially additional STAT-interacting proteins present in RA joint tissues. Thus GWAS may be considered the next step in the development of future therapies for RA based on targeting STAT-responsive genes. This could be especially useful depending on the status of the activity of the SOCS/CIS protein family acting on cytokine-receptor-mediated signaling. For example, if SOCS/CIS activity is dampened or deregulated in RA then it would be unlikely that this negative regulator pathway for controlling cytokine signaling would be able to inhibit the amplification of pro-inflammatory cytokine-induced JAK/STAT signaling. To illustrate this point, Isomäki et al. [40] showed that although SOCS-1 and SOCS-2 were up-regulated in T-cells recovered from peripheral blood, that SOCS-3 was found in peripheral blood monocytes and a significant number of synovial tissue macrophages expressed SOCS-1 and SOCS-3 proteins, the majority of T-cells in RA synovium were 'SOCS negative.'

For further discussion, this chapter will focus on the recent progress that has been made in furthering our understanding of how cytokine gene expression is regulated by both U-STAT and p-STAT proteins. The long-term prospect arising from the results of these studies would be to exploit this new knowledge to reduce the level of pro-inflammatory cytokines or to raise the level of anti-inflammatory cytokines in RA. By doing so this could potentially restore the balance between these cytokine families and retard ongoing synovial joint damage whilst also ameliorating RA clinical signs and symptoms.

4. Stat-DNA promoter-binding motifs

Defining transcription factor binding sites was critical for revealing the structure of cis-regulatory motifs that regulated transcriptional activity [53]. However, microarray analysis using different cell types determined that although several hundred genes were potential STAT3-target gene sites, only a small fraction of those STAT3-target gene sites turned out to be true direct STAT3-target genes [54].

As previously indicated, p-STAT proteins do not act independently of one another and U- and p-STAT-protein interactions take various forms which enable them to bind efficiently to DNA [55]. These activated STAT-protein interaction types include, 1) the direct binding of activated STAT homodimers to DNA; 2) the interaction of activated STATs with non-STAT proteins to form activated STAT/non-STAT protein complexes which bind to DNA; and 3) activated STAT proteins interacting with other non-STAT transcription factors or co-activator proteins which bind to DNA [16, 53]. In addition, several novel mechanisms were described for the binding of U-STAT3 and U-STAT1 to DNA [54, 55]. In that regard, Cheon et al. [56] showed that U-STAT3 can drive expression of proteins not induced by p-STAT3, whereas U-STAT1 was shown to extend and up-regulate the expression of a subset of genes initially responsive to p-STAT1 (e.g.,

interferon, IL-6), that result in more prolonged antiviral and/or immune responses. Thus, the results of these studies provided novel information regarding the functional significance for U-STAT1 and U-STAT3 acting as transcriptional activators and organizers of chromatin. These events have been shown to be important cellular mechanisms for regulating gene transcription in the nuclei of cells of the immune system and cancer cells.

The results of DNA sequencing studies originally demonstrated specific DNA-binding sites for STAT1 and STAT3 [57]. Boucheron et al. [58] then demonstrated that specific DNA binding sites existed for STAT5A and STAT5B homodimers despite the fact that STAT5A and STAT5B are evolutionarily conserved and encoded by 2 genes with a 91% homology in amino acid structure [59]. Moreover, targeted gene deletion of STAT5A and STAT5B in mice resulted in distinctive phenotypes [60]. This finding suggested a structural dissimilarity in the DNA-binding motifs for these two STAT proteins. The results of studies reported in [60] were later confirmed using the IL-3-dependent early pre-B cell line, Ba/F3 [61]. Here it was shown that both STAT isoforms bound to all of the promoters tested, but STAT5A and STAT5B bound with differing kinetics [62]. This result suggested that DNA binding activity was likely at the root of any differences in the biological activity of these two STAT protein isoforms.

Ehret et al. [63] compared the specificity of STAT-DNA binding sites in specific STAT gene knockout mice showing distinct phenotypes with the STAT-DNA binding sites in a variety of cultured cells. From the *in vitro* analysis, Ehret et al. [63] also demonstrated that DNA binding site motifs for STAT1, STAT5A, STAT5B and STAT6 were essentially the same with only minor differences in DNA binding site specificity. However, STAT5A DNA-binding specificity was much more similar to STAT6 than was the preferential DNA-binding site for STAT1. Thus, the preferential DNA binding site for STAT6 contained a 4 base pair spacer (i.e. TTCNNNNGAA) (N_4) which was defined as the weak DNA binding site. However, additional analyses showed that STAT6 bound to TTCNNNG-AA (N_3) sites (i.e. the strong binding site) as well [63]. The binding of STAT1 and STAT5 to the N_3 site was distinct from STAT5A which preferred N_4. Of note, most of the STAT6 binding sites were found in IL-4 responsive promoters in the N_4 sites [64-67]. These results reported by Ehret et al. [63] were extended by the findings of Moucadel and Constantinescu [64] who showed that STAT5B bound to chromatin at both the N_3 and the N_4 site.

5. Stat-responsive cytokines genes

This overview covering the specificity of STAT-DNA binding becomes especially important for improving our understanding of which cytokine gene expressional events are altered by p-STAT and U-STAT proteins. This section analyzes our current interpretation of several cytokines relevant to RA and other autoimmune diseases, namely, IL-2, IL-3, IL-4, IL-6, IL-15, IL-17, IL-19 and INF-γ, all of which have been shown to activate the JAK/STAT pathway [16]. Moreover, activation of JAK/STAT signaling by these cytokines was shown to result in altered patterns of transcriptional activity which lead to changes in the expression of the following cytokine or cytokine-related genes, IL-2R, IL-3, IL-4, IL-6, IL-6ST (gp130), IL-10, IL-18R1, INF-γ, oncostatin M (OSM) and TNF-α (Table 1).

STAT Responsive Cytokine/Protein	STAT Activator[1]	Activated STAT(s)[2]	Major Function(s) of STAT-Responsive Genes[3]	Involvement of STAT activator in RA	Representative Reference
IL-2Rα	IL-4	STAT6	Complexes with IL-2Rβ/IL-2Rγ→high affinity IL-2R	Anti-inflammatory Cytokine	[81]
IL-10	IL-2, IL-3	STAT5, STAT5A, STAT5B	Activator of JAK3, I-SRE-4/IL-10 gene	↑ T-Cell Growth ↑ T-Cell Development	[73] [207]
IL-18R1	IL-4	STAT6	↑ T_{reg} Cell Development IL18/IL-18R1	Inducer of TNF-α, GM-CSF, IFN-γ	[127]
IL-6	IL-6/IL-17	STAT3/STAT1	Activator of JAK3	Promotes T_h17 Cell Production;	[9]
	IL-3	STAT5			[174]
	IL-12	STAT4			[140]
	IL-19	STAT3		↑MMP Synthesis	[207]
	IL-10/IL-13	STAT4		↑MMP Synthesis	[140]
IL-6ST(gp130)[4]	IL-6/IL-17/OSM	STAT3/STAT4/STAT5A	Heterodimer between gp130/LIFR[5] forms the OSMR[6]	Promotes T_h17 Cell Production	[100]
IL-4	IL-2	STAT3/STAT4/STAT5A	↑T_h2 Differentiation		[118]
INF-γ	IFN-γ	STAT1/STAT4/STAT5/STAT6	Activator of multiple protein kinases	Inhibitor of Anti-Inflammatory Cytokine IL-4 and IL-10 Production	[86]
	IL-3				[207]
	IL-2	STAT5			[207]
	IL-12	STAT5 STAT4			[113]
OSM	IL-2/IL-3	STAT5 STAT3	Activator of JAK2	↑ Monocyte Trafficking ↑ MMP-2 ↑ VEGF	[122] [125]
TNF-α	IL-3	STAT5	Activator of JAK3;	Activator of NF-κB	[200]
	IL-6/IL-19	STAT3/STAT5			[86]
	IL-15	STAT1/STAT3/STAT5	Activator of p38[7], JNK[8]		[186]
	IL-22				

[1]Cytokines that activate this STAT protein

[2]Activated STAT that becomes a transcription factor for the STAT-responsive cytokine/protein

[3]Function(s) of STAT-responsive cytokine/protein

[4]IL-6 Signal Transducer

[5]Leukemia Inhibitory Factor Receptor

[6]Oncostatin M Receptor

[7]p38 kinase

[8]C-Jun-N-terminal kinase

Table 1. STAT-Responsive Pro-Inflammatory Cytokine Gene Expression

6. T_h1/T_h2 Cells, T_{reg} Cells, IL-2R, and IL-15

Up-regulation of the T_h1 and T_h17 T-cell subsets and reduced levels of human T-regulatory (T_{reg}) cells are known to occur in autoimmune diseases [16, 68]. In addition, T_{reg} cells are a critical contributor to T-cell development in the thymus as well as being the T-cell subset that regulates the genesis and maintenance of immune tolerance [16].

The IL-2Rα/IL-2Rβ subunits in complex with the common IL-2γ subunit make up the high-affinity IL-2 receptor, whereas homodimeric IL-2Rα results in a low-affinity receptor [69]. The functional significance of blocking the high-affinity IL-2R with the small molecule inhibitor (SMI), SP4206 (K_d ~70nM) in response to IL-2 (K_d~10nM) was that JAK/STAT activation was inhibited [70]. This result could provide the impetus for development of the next generation SMI designed to efficiently inhibit the IL-2/IL-2R pathway and this task should be facilitated by employing recently developed technologies based on the principles of protein-protein interactions [71].

As indicated previously, the interaction of IL-2 with the high-affinity IL-2R causes activation of JAK/STAT with STAT5A and STAT5B, the principally activated STAT proteins. However, the eventual change in STAT5-gene responsiveness following IL-2 activation of STAT5 was shown to be dependent on the complexity of the promoter regions of those STAT5-target genes [72]. Interestingly, Tsuji-Takayama et al. [73] showed that IL-2-mediated JAK/STAT activation up-regulated the production of IL-10 by T_{reg} cells. The production of IL-10 arose from the interaction of STAT5 with a STAT5-responsive element within intron 4, designated I-SRE-4 of the IL-10 locus which was considered to be an interspecies conserved enhancer sequence (Table 1). Of note, the clustered CpG regions around I-SRE-4 were under-methylated in IL-10-producing T_{reg} cells, but not in other T-cell subsets. This result confirmed previous results which showed that expression of Foxp3, a member of the forkhead/winged-helix family of transcription factors and a biomarker for the development and function of T_{reg} cells [47, 74] was also IL-2/STAT5-dependent [75]. Thus, development of T_{reg} cells was regulated by the methylation status of CpG residues because methylation of CpG residues suppressed Foxp3 expression [76].

Chen et al. [77] identified a novel set of IL-4/STAT6-target genes in mice that regulated the proliferation of activated T-cells. In addition, these genes were shown to regulate the production of the T_h2 lineage as evinced by the finding that the cells isolated from wild-type mice produced T_h2 whereas cells from STAT6$^{-/-}$ mice did not. Later, Lund et al. [78] showed that the IL-4/STAT6 pathway was also critical for the commitment of naïve T-cells to become either the T_h1 or T_h2 subset. In that regard, the ratio of T_h1 to T_h2 produced from naïve T-cells was found to be dependent on a set of STAT6-responsive genes which included the transcription factors STATB1, Bcl-6, and TCF7 [78, 79]. Moreover, the IL-4/STAT6-mediated pathway was also shown to be a strong modulator of human T_{reg} cell production from either T_h1 or T_h17 cells [80].

Wurster et al. [81] were among the first to demonstrate that IL-4-mediated activation of STAT6 could also up-regulate IL-2Rα gene expression (Table 1). Because IL-2 is the major

growth-promoting cytokine for T-cells [81], elevated production of IL-2Rα in response to activated STAT6 is considered instrumental in facilitating the proliferation of activated T-cells in cancer as well as in several types of autoimmune diseases. In that regard, the high level of expression of IL-2Rα in tumors correlated with a poor prognosis in cancer patients [82]. Thus, it will be interesting to determine if the same relationship holds true for RA patients as well, including what role IL-2Rα polymorphisms [83, 84] might play in determining the level of the expression of IL-2Rα. For example, IL-15, a pro-inflammatory cytokine which interacts with two receptor subunits similar to IL-2Rα/β drives the production of the memory CD8$^+$ T-cell phenotype [85]. Experimental therapies focusing on inhibiting the binding of IL-15 to the IL-2Rα/β receptor complex were a decade ago considered to be a potential target for autoimmune diseases [85]. However, since then considerable evidence has accumulated showing a robust relationship between IL-15/IL-2Rα/β-mediated signaling, osteoclastogenesis and boney erosions in RA joints [3]. In addition, González-Alvaro et al. [86] showed that IL-15 stimulated production of TNF-α by monocytes derived from RA patients including, the induction of the CD69 monocyte biomarker, and synthesis of IFN-γ protein by natural killer (NK) cells. Of note, the results of a clinical study showed that IL-15 expression in RA synovial tissue persisted even after TNF-α blockade, the latter treatment resulting in a positive clinical response and reduced disease activity [87]. However, treating mononuclear cells *in vitro* with HuMax-IL-15 f (ab')$_2$ neutralized the effect of IL-15 on these cells. Furthermore, treatment with HuMax-IL-15 f(ab')$_2$ caused a significant improvement in RA disease activity as measured by the American College of Rheumatology (ACR) clinical response criteria [88]. This finding may be particularly important for future drug development because the results of a recently completed clinical trial showed that high levels of serum IL-15 in patients with early arthritis predicted a more progressive and severe clinical course which may call for early and aggressive drug therapy [89].

6.1. IL-6/gp130/IL-17

The IL-6/IL-6R/gp130 pathway is one of the strongest inducers of STAT3 activation [9] (Table 1) so much so that many studies have been devoted to the activation of the JAK/STAT pathway by IL-6 because IL-6 is critical to the progression of joint damage in RA [16]. In fact, the development of the anti-IL-6R monoclonal antibody, tocilizumab, appears to have been predicated on this emerging evidence such that this drug is now considered useful in the armamentarium of drug therapies for RA [90, 91]. Most compelling was recent evidence that tocilizumab in conjunction with methotrexate retarded the progression of joint damage in RA patients [92], an effect of this drug regimen that was apparently independent of the capacity of tocilizumab to modify several clinical biomarkers of inflammation and concomitant RA disease activity.

Recent results have also emerged which have focused attention on the extent to which other pro-inflammatory cytokines, such as IL-17, activate JAK/STAT and the mechanism by which IL-17 modifies the production of IL-6 and other pro-inflammatory cytokines [9]. In that regard, the results of a study by Jovanovic et al. [93] was extremely informative because it provided evidence that IL-17 was capable of activating additional signaling pathways other

than JAK/STAT which resulted in elevated production of IL-1β and TNF-α. Therefore, it has become obvious that suppressing the activity of IL-17 could bring about a reduction of these pro-inflammatory cytokines as well, although this point must be rigorously reexamined in view of the results from Dragon et al. [94] who showed that IL-17A significantly decreased GM-CSF-induced neutrophil/granulocyte apoptosis by suppressing activation of p38 kinase, extracellular-regulated kinase 1/2 and STAT5B.

Inhibiting aberrant T-cell survival in RA may ultimately hinge on the development of a therapeutic strategy directed specifically at STAT3 since STAT3 was shown to inhibit T-cell proliferation by up-regulating the Class O Forkhead transcriptions factors (Fox) via the binding of STAT3 to FoxO1and FoxO3a promoters [95]. Potentially, STAT3 may also protect T-cells from apoptosis [30, 96] in RA by suppressing IL-2 activity, although the results [95] indicated that STAT3 increased T-cell proliferation and their survival through the up-regulation of OX-40 (CD134), a member of the TNFR-superfamily of receptors and bcl-2 and by suppressing FasL and Bad expression.

Perhaps the most intriguing aspect of the clinical studies with tocilizumab performed in RA patients is the extent to which neutralization of the IL-6/IL-6R/gp130 pathway using this drug together with the putative suppression of IL-6 and gp130 gene expression in response to inhibition of the STAT3 activation rebalances the skewed ratio of T_h17/T_{reg} in favor of T_{reg} [97, 98], the elevated serum levels of T_h17-associated cytokines, IL-17, IL-23, IL-6 and TNF-α, and the depressed level of T_{reg} cells with its associated growth factor, transforming growth factor-β (TGF-β) [99]. What is pertinent to these events are the results of a recent study which showed that treatment of RA patients with tocilizumab in combination with methotrexate resulted in a significant decrease in the percentage of T_h17 cells (from 0.9% at baseline to 0.45%) and a significant increase in T_{reg} cells (from 3.05% to 3.94%) whilst maintaining their functional activity [98].

The extent to which gp130 gene expression is altered in response to inhibition of the JAK/STAT pathway activation is also an area of immense importance because deregulated over-expression of gp130 in RA patients should not be neutralized by anti-IL-6R therapy. Importantly, O'Brien and Manolagas [100] showed that IL-6 or oncostatin M (OSM), a member of the IL-6 protein superfamily, stimulated the activity of the gp130 (Table 1) promoter in which the cytokine response element contained a cis-acting motif for activated STAT complexes, including activated STAT1 and STAT3 homo- and heterodimers. Furthermore, it can be conjectured that other pro-inflammatory cytokine members of the IL-6 protein superfamily, such as ciliary neutrotrophic factor (CNTF), leukemia inhibitory factor (LIF) and cardiotropin-1 which use gp130 as their primary signal transducer protein [9, 101] may provide an alternate mechanism resulting in constitutive JAK/STAT activation. Under those conditions STAT protein activation may not be inhibited any of the anti-IL-6R agents which retain up-regulated gp130 gene expression. Of note, constitutive activation of STAT proteins is one of the signature events in the development and progression of various cancers [102, 103] with a similar phenomenon having been described in RA synovial tissue [15]. Constitutively activated STAT proteins could also be predictive of a more aggressive form of RA [96].

6.2. INF-α/IFN-γ

The interferon protein family in conjunction with the interferon-regulated gene (IRG) pathway plays an important role in RA, SLE and other autoimmune diseases because the IRG pathway is a critical mediator of autoimmune-dependent inflammation [104-106]. INF-γ is known to be one of the strongest activators of JAK/STAT and Tyk2 resulting in IRG-mediated responses [16, 107, 108]. INF-γ has also been shown to play a role in the epigenetic regulation of specific gene activation as evinced by the finding of an association of pJAK2 and IFN-γ receptor in the nucleus with histone H3 in IFN-γ-treated human amnionic (WISH; American Tissue Culture Collection; CCL 25) cells *in vitro* [109]. AG-490 a JAK2 inhibitor, also down-regulated STAT1 gene expression and AG-490 inhibited prolactin-induced IFN-γ, TNF-α, IL-1β and IL-12p40 synthesis in mouse peritoneal macrophages *in vitro* [110]. Of note, inhibition of JNK activity with the SMI, SP600125, also resulted in down-regulating IFN-γ and TNF-α indicating that both the JAK/STAT and MAPK pathways contributed to alterations in the expression of these cytokines. Although the importance of these results in providing a rationale for manipulating signal transduction pathways in human RA remains to be fully elucidated, the fact that the expression of several pro-inflammatory cytokines relevant to RA pathology are potentially controlled by cross-talk between JAK/STAT and MAPK appears to be significant [47, 111, 112].

Three DNA-binding sites related to STAT protein-DNA binding have been recognized within the IFN-γ promoter. These DNA binding sites include an IL-12-mediated STAT4/DNA binding site, an IL-2-induced STAT5/DNA binding site and a CD2-mediated STAT/IFN-γ binding site [113]. Thus, CD2-mediated activation of human peripheral blood mononuclear cells was shown to result in STAT/DNA binding to a 3.6kb DNA motif within the IFN-γ promoter which occurred principally via STAT5A binding and less so by STAT5B, with both being independent of IL-2.

Induction of some of the IFN-regulatory factors (Irfs), including those gene responses brought about by activation of irf9 via IFN-α were found to be STAT protein-independent [114]. In addition, results from other studies showed that Akt activity was also involved in key IFN-α, -γ gene responses [115]. Moreover, regulation of IFN-α, -γ-mediated responses required the direct control of mTOR [116] beginning with the initiation of protein translation [117].

In RA, the depressed level of IL-4 and IL-10 in mononuclear phagocytes is, in part, responsible for the imbalance in T_h1/T_h2 cytokines [3, 16]. The primary model employed to describe the relationship between IFN-γ and IL-4/IL-10 is dependent on several factors. This view was originally proposed by Hamilton et al. [118] as follows; IL-4 was shown to markedly suppress the transcriptional activity of IFN-γ because the promoter sequence between IL-4 and IFN-γ were essentially identical. Proof of this came from the results of experiments that showed that IFN-γ/STAT1 and IL-4/STAT6 both formed complexes at the same regulatory sequence, but whereas activated STAT1 promoted IFN-γ transcription, activated STAT6 did not. However, activated STAT6 was required to suppress the transcriptional up-regulation of IL-4. Thus, in the model, IL-4 appeared to be necessary to reduce IFN-γ gene expression (Table 1) and was related to a competition between activated STAT1 and activated STAT6 for binding to the IFN-γ promoter. In keeping with this model, the expression of IL-10 is also known to be suppressed by INF-γ

[119]. Thus, it was shown that when transfected RPMI 8226.1 B-cells were incubated with IFN-γ followed by lipopolysaccharide (LPS), IFN-γ reduced LPS-induced IL-10 promoter activity which was independent of the irf, but dependent on an activated STAT-motif. Further analyses indicated that IFN-γ down-regulated IL-10 gene expression via displacement of the trans-activated STAT3 by STAT1 induced by IFN-γ.

Experimental strategies could be designed to increase the mononuclear cell expression of IL-4/IL-10 by manipulating the ultra-sensitive INF-γ promoter region with various activated STAT protein types. Another strategy for potentially improving the level of IL-10 in RA would involve manipulating natural T_{reg} cells in a cell-based therapy mode because T_{reg} cells are a rich source of IL-10 [120, 121]. However, as pointed out by Nandakumar et al. [121] one must be mindful that the antigen specificity of natural T_{reg} cells must be carefully regulated to protect against the development of self-reactive effector T-cells or for that matter, T_{reg} cells with inappropriate antigen specificity.

6.3. OSM

Recent advances have assigned OSM, a member of the IL-6 protein superfamily an important role in the pathogenesis and progression of RA and OA [101]. In that regard, one of the more important experimental results involving OSM were reported by Hams et al. [122] who compared the inflammatory responses in wild-type mice to IL-6-deficient and mice deficient in the OSM receptor β (OSMRβ). They showed that the OSMRβ knockout mice showed enhanced trafficking of monocytes to sites of inflammation when these mice were compared to the wild-type or IL-6-knockout mice. However, the OSMRβ knockout mice did not demonstrate any differences in neutrophil or lymphocyte migration to inflamed tissue when compared to their wild-type or IL-6-deficient counterparts. These results suggested that the OSM/OSMRβ-pathway probably regulated chemokine production and chemokine function. Indeed this proved to be the case when the up-regulated chemokine in response to the activation of the OSMRβ-pathway was eventually identified as CCL5. CCL5 has been shown to be a critical chemokine for regulating the recruitment and retention of monocytes in inflamed RA synovial joints [3]. Although the evidence was indirect, these results suggested that a drug with the capacity to neutralize the interaction between IL-6 and IL6R in arthritic joints would not alter OSM/OSMRβ-mediated STAT activation [9]. This view was supported by the results from several previous studies which showed that 1) although the OSMR consisted of a heterodimer of the LIF receptor and gp130, the alternative form of OSMR, namely, OSMRβ, was activated only by OSM and not by LIF [123]; 2) OSM, but neither IL-6 nor LIF induced tyrosine phosphorylation in the Shc adaptor protein p52 and p66 isoforms which in association with growth factor receptor-bound protein 2 (Grb2) were both recruited to OSMR, but not to gp130 [124]; and 3) at least in human or canine osteosarcoma cell lines, treatment with OSM phosphorylated JAK2/STAT3 and Src, each of which was shown to be involved in an OSM dose-dependent-mediated increase in expression of the MMP-2 gene (i.e. 72kDa gelatinase) and vascular endothelial growth factor (VEGF) gene [125]. Of note, the STAT3 SMI, LLL3, inhibited MMP-2 and VEGF gene expression indicating that MMP-2 and VEGF were genes targeted by activated STAT3. Importantly, Clarkson

et al. [126] showed that another one of the activated STAT-responsive genes in mammary epithelial cells was OSMR. This finding was critical for completing the circle which showed that activation of OSMRβ was central to the upstream activation of the OSM-mediated pathway as well as to the downstream increase in the expression of the OSMRβ gene, both events involving STAT proteins.

7. Other cytokines/cytokine receptors

The role played by activated STAT proteins in various aspects of autoimmune diseases and in oncogenesis is best exemplified by the many genes and transcription factors that have been shown to be STAT protein-responsive [51, 127]. Many of these STAT-regulated genes include additional pro-inflammatory cytokine and cytokine receptor genes besides those previously discussed. In this section we will analyze the contributions of these cytokine and cytokine receptor genes to the pathology of RA.

7.1. IL-18

IL-18 is structurally similar to IL-1 and the IL-18 receptor is a member of the IL-1R/TLR protein superfamily [128]. However, the function of IL-18 differs considerably from that of IL-1 and, in fact unlike IL-1, IL-18 is produced by a variety of immune as well as non-immune cells. Although IL-18 in its role as a stimulator of T_h1 responses is well known by its activity as an immune defense cytokine against microbial infection, the over-production of IL-18 can result in autoimmune disease via its capacity to modify and accentuate adaptive immunological responses such as those seen in RA [129-132]. However, paradoxically IL-18 can also stimulate T_h2-related cytokine responses as well [128]. Thus, its putative role in altering the T_h1/T_h2 cytokine repertoire cannot be dismissed.

Particularly important with regard to the role played by IL-18 in RA were results of a study by Gracie et al. [133] who first identified abundant IL-18 in RA synovial tissue. These findings are relevant when coupled with those from other studies by Tanaka et al. [134] who also found elevated IL-18 and the IL-18 receptor α/β in RA synovial tissue. They also demonstrated that IL-18 was a co-factor and regulatory cytokine in stimulating the synthesis of IFN-γ by T-cells in RA synovial tissue, the latter also requiring IL-12, thus implicating the up-regulation of IL-18 gene expression as an important component of RA disease progression.

Activated STAT3 was identified as the JAK/STAT-related transcription factor responsible for the increased synthesis of IL-18 [127]. In that regard, TNF-α was shown to increase IL-18 gene expression in RA synoviocyte cultures suggesting the possibility that TNF-α, a known activator of p38 kinase and JNK may also activate STAT3 in synoviocyte and chondrocyte cultures. Indeed, recent results from our laboratory showed that recombinant human TNF-α activated STAT3 in normal human chondrocyte cultures and TNF-α activated STAT3, p38 kinase and JNK in cultured chondrocytes derived from human osteoarthritic knee cartilage [Malemud et al. submitted]. Thus, it was instructive to learn that treating RA patients with

the combination therapy of infliximab and methotrexate reduced the level of IL-18 in serum whilst the level of the chemokine, CXCL12 was unaltered [135]. Moreover, synovial fluid from these RA patients had higher levels of IL-18 (as well as TNF-α and IL-15) prior to beginning combination therapy with infliximab and methotrexate compared to the level of these cytokines in a patient's sera. In addition, the level of IL-18/TNF-α in synovial fluid was strongly correlated with a patient's high Disease Activity Score-28 [136]. Thus, it may be informative going forward to assess the level of activated STAT3 and IL-18 in the synovial fluid and sera of RA patients before and after treatment with TNF antagonists or other biological drugs that neutralize the activation of JAK/STAT and MAPK pathways to determine the extent to which the level of activated STAT3, p38 kinase or JNK is correlated with IL-18 gene expression by synovium and cartilage *ex vivo*.

7.2. IL-12

IL-12 is made up of 2 disulfide-linked protein subunits, termed IL-12p35 and IL-12p40 linked in a heterodimer configuration [137, 138]. Whilst the IL-12p40 subunit has structural similarities with cytokine receptors, the IL-12p35 component is structurally similar to IL-6 and granulocyte-colony stimulating factor (G-CSF) [139]. Of note, if IL-12p35 and IL-12p40 are produced by the same cell, the bioactive heterodimer is termed, IL-12p70 [140].

IL-12 is synthesized by many cell types of the innate and adaptive immune systems, including, monocytes, macrophages, dendritic cells and neutrophils. IL-12 is a minor product of B-cells [140]. Although IL-12p35 is constitutively expressed at low levels by many of these cells, the expression of IL-12p40 is limited to those phagocytic cells that synthesize IL-12p70.

The connection between IL-12 and activation of the JAK/STAT pathway stems from the finding that IL-12 production was positively regulated by IFN-γ, the latter cytokine which is also induced by IL-12. Thus, IFN-γ regulates IL-12 gene expression and vice versa. By contrast, two of the anti-inflammatory cytokines, namely, IL-10 and IL-13 which also activate JAK/STAT, suppressed IL-12 production [140] (Table 1). In addition, the type I interferon proteins, exemplified by IFN-β, which activates STAT1 [141] was shown to inhibit IL-12 gene expression in mice [142].

The main immune functions of IL-12 involve the regulation of T_h1 differentiation via the activation of STAT4 which induces the synthesis of the T-bet transcription factor [143]. T-bet was shown to regulate IFN-γ expression and CD8$^+$ suppressor T-cell development which had been characterized as principally IFN-γ/STAT1-dependent, and IL-12/STAT4 independent. In fact, expression of T-bet was shown to require activated STAT4 to achieve total IL-12-dependent T_h1 cell-fate determination [143]. However, Yang et al. [144] showed that the effect of IL-12/STAT4 was more complex. Thus, IL-12 -induced activated STAT4 bound to a distant but highly conserved STAT-responsive T-bet enhancer region where it induced IFN-γ-activated STAT1 independent T-bet gene expression in CD8$^+$ cells. Importantly, IL-4-induced STAT6 activation regulates the development and effector functions, not of T_h1 cells, but rather of T_h2 cells in peripheral tissues such as skin, lung and gut [145]. However, T_h2 cell produced in lymph nodes did not require IL-4-mediated activation of STAT6 [145].

In summary, cell-fate determination induced by the IL-12-mediated activation of STAT4, IL-4-mediated activation of STAT6, transforming growth factor-β (TGF-β), IL-6 plus TGF-β and IL-27 activation of STAT3 profoundly influence the balance of T_h1 and T_h2 cells, T_h17 cells and T_{reg} cell production, respectively [80, 146-149]. This conclusion must, however, be tempered by results of recent studies which also showed that formal interplays occurred between IL-4-induced STAT6 phosphorylation, the GATA-binding protein-3 (GATA$_3$) zinc-finger transcription factor [150] and the T_{reg} cell transcription factor, FoxP3 as well. Importantly, GATA$_3$ was revealed as the key transcription factor in this complex interplay because GATA$_3$ could 1) directly inhibit T_h1 differentiation through its capacity to block up-regulation of the IL-12β2 receptor; 2) inhibit the activity of STAT4; and 3) neutralize the activity of runt-related transcription factor 3 (runx3), via its capacity to induce protein-protein interactions [150]. Thus, by modulating the activities of IL-4/STAT6, GATA$_3$/STAT4 and runx3 one could potentially alter the activity of pro-inflammatory and anti-inflammatory cytokines as well as overcome immune tolerance.

7.3. IL-21

IL-21 is a member of the Type I cytokine superfamily of cytokine receptors. In this group, the common γ cytokine receptor complex is the functional component for receptor-mediated signal transduction of IL-2, IL-4, IL-7, IL-9 and IL-15 [151-153]. Although IL-21 has strong structural homology to IL-15, IL-21 interacts with a unique receptor, termed, IL-21Rα, which pairs with the γ-common cytokine receptor chain (i.e. CD132) to form the active IL-21 receptor complex [154].

IL-21-mediated events affect the functions of NK cells, T-cells and B-cells. Although development of T_{reg} cells from the T_h17 lineage is generally considered to require IL-6 because IL-6 reciprocally controls T_h17 and T_{reg} cell development through its ability to inhibit TGF-β-induced FoxP3 and by inducing RORγ, in fact, IL-21 can also induce RORγ and T_h17 development in the absence of IL-6. However, evidence also showed that the number of T_h17 cells, the recruitment of T_h17 cells to inflamed tissues and the development of autoimmune encephalitis and myocarditis did not differ between IL-21R and IL-21 deficient mice compared to their wild-type counterparts [155, 156]. More importantly, IL-6 was the more potent inducer of T_h17 differentiation compared to IL-21 thus calling into question, whether IL-21 was even required for T_h17 development.

Despite the emerging controversies regarding how important IL-21 is in T-cell development and immune responses, a therapeutic intervention designed to limit the responses of immune cells to IL-21 has long been considered for treating cancer and autoimmune diseases [157]. In addition, because the binding of IL-21R induces activation of several of the JAK isoforms [153], it became apparent that it would be necessary to elucidate which cellular events were controlled by STAT proteins activated by phosphorylated JAKs in response to IL-21/IL-21R. Attempting to address this point, Habib et al. [151] found that IL-21 induced proliferation of pro-B-lymphoid cells *in vitro* which was dependent on both γc and the γc-associated JAK3 complex. However, a monoclonal antibody reactive only with γc was effective in limiting the proliferation of BaF3/IL21R α cells [151] indicating that neutralization of γc alone could cause inhibition of JAK activation by IL-21/IL-21R.

Implying a role for IL-21 in the development and progression of RA would also depend on finding an elevated level of IL-21 in human RA tissues and by demonstrating an involvement of IL-21 in the pathogenesis of CIA or inflammatory arthritis in other animal models. Thus the results of a study by Young et al. [158] were noteworthy in this regard for several reasons. First and foremost, treating DBA mice with CIA with an antibody to IL-21R (i.e. IL-21R.Fc) reduced the severity of arthritis. The reduction in hind paw swelling was accompanied by lower levels of IL-6 in the hind paw but also in the sera of mice treated with IL-21R.Fc suggesting that one of the downstream events regulated by IL-21 was IL-6 gene expression. Of note, the level of INF-γ was increased in the hind paws of mice with CIA. Furthermore, the cultured cells from the lymph nodes of mice with CIA treated with IL-21R.Fc showed an increased level of IFN-γ *ex vivo*. These findings (i.e. reduced IFN-γ; increased IL-6) were mirrored *ex vivo* using Type II collagen-specific spleen cells from CIA mice treated with IL-21R.Fc. Most importantly from the perspective of potentially using an anti-IL-21R antibody as a therapeutic agent for RA was the finding that treating Lewis rats with adjuvant –induced arthritis therapeutically with IL-21R.Fc "reversed" the swelling in inflamed joints and tissues from these joints whilst the tissues showed improvement using a well-validated histological scoring system. More recently, Yuan et al. [159] showed that IL-21R mRNA was found in human RA synovial tissue samples. In addition, this group also confirmed the results of the Young et al. study [158] since they showed that an anti-IL-21R antibody ameliorated the severity of arthritis in CIA which was accompanied by reduced cytokine levels in cells derived from the anti-IL-21R antibody-treated mice. Interestingly, IL-21R-deficient K/BxN mice [160] failed to develop arthritis; a result which suggested that IL-21R played a critical role in the pathogenesis of K/BxN serum-induced arthritis.

There now are several lines of evidence that showed that the IL-21/IL-21R pathway plays a functional role in regulating inflammatory responses in autoimmune arthritis. In that regard, anti-IL-21 blockade should also be considered for future drug development for RA. However, what would also be crucial to improving our understanding of the role of IL-21 in RA would be to discover which pro-inflammatory cytokine levels are altered in response to the JAK/STAT activation by IL-21/IL21R. This could provide a novel paradigm for reducing pro-inflammatory cytokine levels in RA.

8. The extended IL-10 cytokine superfamily

IL-19, IL-20, IL-22, IL-24 (melanoma differentiation-associated gene 7; mda-7), and IL-26 (AK155) are all structurally similar to IL-10 and these interleukins constitute members of the extended IL-10 cytokine superfamily [161-163]. Three additional members of the IL-10 cytokine superfamily have recently been added to this list, namely, IL-28A, IL-28B and IL-29 which now comprise the IFN-λ cytokine subfamily [164-166].

IL-19 and IL-20 are α-helical proteins. They have similar cysteine sites; their amino acid sequences are approximately 30% identical. In the human genome, the genes encoding these IL-10 superfamily members are located in two clusters; one cluster comprises the genes for

IL-10, IL-19, IL-20, and IL-24/mda-7 which are located on chromosome 1q31-32 [167]. IL-19 and IL-20 were predominately expressed in monocytes, as well as non-immune cells under inflammatory conditions [168], whereas IL-22 and IL-26 was only produced by T-cells, especially T_h1 cells and NK cells, whilst IL-24 synthesis was restricted to monocytes and T-cells [169].

Both IL-20 and IL-24 bind to the IL-20R complex which is made up of the cytokine receptor family 2-8/IL-20Rα (IL-20R1) [170], although it was previously shown that IL-19/IL-19 receptor binding was similar to IL-20/IL-24 receptor binding [170]. IL-19 was also shown to interact with a DIRS1-like element which is composed of tyrosine recombinase-encoding transposons/IL-20Rβ (IL-20R2) [170-172].

In all cases, the binding of IL-19, IL-20 or IL-24 to these receptors caused activation of STAT3 and activation of a minimal promoter region containing those sequences identified as STAT-binding sites. Importantly, absent either of the R1 proteins in the two types of receptor complexes, IL-20R1/IL-20R2 and IL-22R1/IL-22R2 reduced the affinity of IL-19 or IL-24 for these receptors. Furthermore, IL-20R2, and not IL-20R1, was identified as the high affinity receptor chain for these cytokines [173].

The functional significance of the IL-10-related cytokines, IL-19, IL-20, IL-21, IL-22 and IL-24 in terms of the pathophysiology of RA and other autoimmune diseases is systematically being elucidated. In most cases, the role played by these cytokines has been inferred from measurements in sera of RA patients before and/or after medical therapy.

8.1. IL-19

Sakurai et al. [174] showed that IL-19 was produced by cells of human RA synovial tissue. The majority of IL-19 positive cells were vimentin- and CD68-positive, indicating that fibroblasts and macrophages were the main sources of IL-19 in RA synovium. From a functional perspective, synovial tissue lining and sublining layers were both identified with anti-IL-20R1 and anti-IL-20R2 antibodies.

IL-19 activated synoviocyte STAT3 and, downstream, STAT3 activation caused up-regulation of IL-6 and IL-19 gene expression whilst decreasing synoviocyte apoptosis induced by serum-starvation [174], a change which may predict the role of IL-19 in the development of synovial hyperplasia [30, 96]. However, the role of IL-19 in RA relative to its activation of signal transduction was further complicated by the findings of Alanärä et al. [175] who showed that IL-1β, an activator of the MAPK pathway [176], also increased the level of IL-19 in peripheral blood mononuclear cells *in vitro*. Combined with other data this result showed that in RA joints IL-19 expression was the highest of all of the IL-10 family cytokines. Furthermore, these results suggested that IL-19 played a significant role in synovial tissue inflammation, with the caveat that further consideration of IL-19 as a target for intervention in in RA must focus on the relative level of JAK/STAT activation of JAK/STAT versus activation of the other signaling pathways.

IL-19 was highly expressed in synovial tissue and, in particular, expressed in fibroblasts isolated from rats with collagen-induced arthritis (CIA) [177]. Of note, treating these rats with a anti-IL-19 antibody, 1BB1, reduced arthritis severity which was accompanied by the lower

level of boney erosions and an improvement in the quality of subchondral bone. Moreover, treatment of rats with CIA with 1BB1 reduced the expression of TNF-α, IL-1β, IL-6 and Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL) genes in synovial tissue and also lowered IL-6 levels in serum. Synovial fibroblasts isolated from rats with CIA responded to treatment with IL-19 in a similar fashion seen with synovial tissue *in situ* where increased synthesis of TNF-α, IL-1β, IL-6 and RANKL was detected.

There is now compelling evidence that IL-19-mediated activation of STAT3 was associated with the development and progression of inflammatory arthritis which was characterized by the elevated expression of many of the pro-inflammatory cytokines pertinent to human RA joint destruction. These data also showed that the rat CIA model could be further exploited to determine the extent to which specific dampening or up-regulation of STAT-responsive cytokine genes would ameliorate inflammatory responses associated with CIA.

8.2. IL-20

IL-20 interacts with IL-20R1/IL-20R2 to activate the JAK/STAT pathway [166] and IL-20 has been implicated in the pathogenesis of autoimmune diseases [178]. However, IL-20R2 signaling was shown to blunt mouse CD4 and CD8 T-cell responses to antigen *in vitro* and *in vivo* [179]. Thus, it remains to be determined the extent to which IL-20 promotes or suppresses immune-mediated inflammation.

In the CIA model in the rat, treatment with an anti-IL-20 antibody 7E, either alone, or in combination with the TNF blocker, etanercept was compared to etanercept alone for their capacity to 1) ameliorate cartilage damage; 2) stabilize bone mineral density; and 3) alter cytokine production [180]. In addition, the effect of antibody 7E on expression of various genes implicated in the progression of CIA was evaluated on rat synovial fibroblasts *in vitro*. Treatment with 7E or etanercept or the combination of 7E and etanercept significantly reduced the severity of arthritis as measured by rat hind paw thickness and swelling. These treatments also prevented cartilage degradation and bone loss whilst reducing the level of synovial tissue IL-20, IL-1β, IL-6, RANKL and MMPs. Of note, IL-20 induced the expression of TNF-α in synovial fibroblasts isolated from rats with CIA. Moreover, IL-20 induced RANKL production in synovial fibroblasts, osteoblasts and T_h17 cells. In another study, antibody 7E was shown to inhibit mouse osteoclast differentiation induced by macrophage-CSF and RANKL [181]. These results [181] coupled with results from the CIA model [180] indicated that IL-20 was likely to have promoted the increased bone loss in CIA by promoting osteoclast differentiation and the activity of osteoclast-mediated bone resorption.

Correlative human studies of IL-20-mediated responses in RA are just emerging. However, the results have differed somewhat from those seen in the CIA model. Thus, Kragstrup et al. [182] showed that plasma IL-20 levels were increased in RA compared to OA patients with the elevated level of IL-20 primarily localized to mononuclear cells and neutrophils. Stimulating mononuclear cells isolated from RA synovium with recombinant IL-20 resulted in the increased secretion of the chemoattractant CCL2/MCP-1. However, at variance with findings in the CIA model, recombinant IL-20 did not alter the expression of TNF-α or IL-6 by mononuclear cells *in vitro*.

8.3. IL-22

IL-22 binds to the class II cytokine receptor family, IL-22R and IL-10Rβ [183]. IL-22 was shown to activate STAT-1, -3 and -5 in H4IIE rat hepatoma cells by inducing the phosphorylation of JAK1 and Tyk2, but not JAK1 [184]. However, H4IIE failed to respond to IL-10 via activation of JAK1 and Tyk2 indicating a distinct signaling pathway for IL-22 versus IL-10. IL-22 also failed to inhibit pro-inflammatory cytokine gene expression by monocytes in response to LPS although IL-22 did blunt the inhibitory effects of IL-4 produced from T_h2 cells, a finding distinct from the activity of IL-10.

A role for IL-22 in inflammation was inferred from its involvement as an inducer of pancreatitis-associated protein by pancreatic acinar cells [185] and by the elevated serum levels of IL-22 in patients with active Crohn's disease [186]. With regard to activating various signaling mechanisms, Lejeune et al. [187] showed that IL-22 activated JAK/STAT. However, IL-22 also activated ERK, JNK and p38 kinase indicating that IL-22 could activate all of the 3 major MAPK pathways. Brand et al. [186] then showed that treating intestinal epithelial cells with TNF-α, IL-1β or LPS significantly increased IL-22R1 gene expression without altering IL-10R2 mRNA. IL-22 also activated STAT1/STAT3, Akt, ERK 1/2 and JNK and, most importantly IL-22 increased the expression of SOCS3, TNF-α, IL-8 and human-defensin-2 mRNAs. Because IL-22 was shown to activate several disparate signaling pathways it is conjecture that up-regulation of pro-inflammatory gene mRNAs by IL-22 involves 'cross-talk' between all three pathways. Thus, experiments employing specific SMIs added either individually or together to cells in culture will have to be performed to determine the extent to which any or all of these signaling pathways are involved in regulating TNF-α, IL-8 or IL-1β gene expression in response to IL-22.

IL-22 is elevated in RA synovial tissue with the lining and sublining layers of RA synovium expressing the highest levels of IL-22R1 [188]. Recently, Leipe et al. [189] showed that about 50% of the RA patients studied had elevated serum IL-22 compared to a group of healthy subjects. The level of serum IL-22 closely correlated with the extent of bone erosions as determined from radiographic analysis. However serum IL-22 did not correlate with the presence or absence of either rheumatoid factor (RF) or anti-cyclic citrullinated peptide antibodies nor was IL-22 associated with disease activity. CD4 T-cells were identified as the main source of IL-22 in these RA patients. However, in another study, de Rocha Jr et al. [190] showed that elevated serum IL-22 did correlate with the Disease Activity Score-28 (DAS-28) and the Clinical Disease Activity Index, a positive titer for RF and the extent to which bone was eroded. The findings from this study [190] agreed with the results from another recently published study [191] the latter showing that plasma IL-22 was increased in 30 patients with established RA (i.e. mean disease duration of 10.7 years), even in those patients receiving immunomodulatory therapy. Thus, any discrepancies between the results of these various clinical studies relative to establishing a relationship between IL-22, RA disease activity and RF levels may involve differences in terms of the types and duration of the immunotherapies employed or in the proportion of RA patients who were in the early or late stage of disease. The relationship between IL-22 and the presence of RF could also corre-

late with the immunological status of B-cells since, unlike IL-10, IL-22 does apparently not regulate the induction of Ig by activated B-cells [192].

8.4. IL-24/mda-7

The apoptosis-inducing activity of IL-24/mda-7 has made this unique member of the extend-ed IL-10 cytokine family a target for cancer therapeutics [193-195] in view of the finding that IL-24/mda-7 could kill cancer cells specifically without affecting the vitality off normal cells or tissues [196]. Receptor binding of IL24/mda-7 to IL-20R activates STAT1 and STAT3 al-though additional signaling pathways have been shown to be modulated by cells over-ex-pressing IL-24/mda-7 which did not involve JAK/STAT activation [193]. Besides the interest in IL-24/mda-7 as a tumor suppressor cytokine, mda-7/IL-24 has also been implicated in reg-ulating some of components of RA and psoriasis immunopathology [197]. However, some of the details of the mechanism(s) by which IL-24/mda-7 could alter pro-inflammatory cyto-kine gene expression in RA via JAK/STAT have not been fully elucidated, although epige-netic and other transcriptional factor activity beyond activated STAT proteins have been postulated to play critical roles. Thus, it is of interest that Sahoo et al. [198] recently showed that STAT6 and c-Jun binding to the IL-24 promoter locus in T_h2 cells caused trans-activa-tion of the IL-24 gene. Finding a relationship between the activators of STAT6 and c-Jun that are relevant to RA which leads to IL-24 gene transcription may hold the key to increasing local IL-24/mda-7 levels by T_h2 cells. This, in turn, could help overcome the 'apoptosis-resist-ance' of RA synovium [96].

8.5. IL-3

IL-3 is one of several major cytokines that drive the differentiation of cells of the hemato-poietic lineage. The interaction between IL-3 and its cognate receptor activates several sig-naling pathways, including, JAK/STAT, PI3K/Akt/mTOR and the Ras/Raf/MAPK pathways [199]. Downstream events that are regulated by IL-3 which are germane to RA and autoim-munity, in general, include the findings that depending on the conditions in the microenvir-onment, IL-3 can alter cell proliferation, survival or induce cell death by apoptosis [30].

IL-3 was identified as an activator of JAK2 and STAT5 [200] and the expression of the pro-apoptotic protein, c-myc. This finding provided the initial evidence that cell proliferation and apoptosis was regulated, in part, by activated STAT5. However, a subsequent study by Chaturvedi et al. [201] provided evidence to the contrary in that the interaction of IL-3 with its receptor activated STAT3 via the phosphorylation of tyr^{701}. Moreover, the results of this study [201] also showed that myeloid cell proliferation was regulated by IL-3-activated Src kinase and not by IL-3-actiivated JAK3. This conclusion was based on the following results. Inhibition of c-Src kinase activation using a dominant-negative (dn) Src mutant also blocked STAT3 activation and, this in turn, inhibited proliferation of the 32Dcl3 myeloid cell line in response to IL-3. Moreover, expression of a dn-JAK2 mutation increased apoptosis in 32Dcl3 cells in the absence of IL-3 which also involved the concomitant down-regulation of ERK-2. Taken together these results indicated that Src kinase activation of STAT proteins regulated

myeloid cell proliferation whereas JAKs controlled the activation of ERK-2 and associated anti-apoptotic signals [202].

The results of another study [203] showed that IL-3 played an important role in regulating SOCS3 and PIAS proteins [16, 20, 21] both of which are important in regulating cytokine signaling as well as the fine-tuning of the survival and/or cell death pathways for immune and non-immune cells in general. IL-3 plays a particularly critical role in regulating these events in mast cells [203], plasmacytoid dendritic cells [204], osteoclast-like cells, [205] and osteoclasts [206, in particular. All of these cell types are involved in some aspect of RA pathology.

To further illustrate this point, Gupta et al. [206] showed that osteoclasts treated with IL-3 were diverted to the dendritic cell lineage which may also be related to the finding that that IL-3 dampened human osteoclast-mediated bone resorption. Most recently, Srivastava et al. [207] showed that IL-3 increased the number of functionally active T_{reg} cells by stimulating the production of IL-2 by non-T_{reg} cells the latter being dependent on the dose of IL-3. Of note, treating mice with CIA with IL-3 significantly reduced the severity of arthritis and also increased the frequency of T_{reg} cells found in the thymus, lymph nodes and spleen. Although this study [207] did not directly measure the status of activated STAT proteins in the CIA mice treated with IL-3, these additional results showed that treatment of CIA with IL-3 decreased production of IL-6, IL-17A, TNF-α and IL-1 whilst increasing IFN-γ and IL-10 (Table 1).

8.6. IL-7

IL-7 was shown to be a fundamental contributor to thymocyte development as well as a regulator of T-cell homeostasis in peripheral blood. IL-7 activates both the PI3K/Akt/mTOR and JAK/STAT pathways suggesting that IL-7 regulates the survival and/or death of T-cells [208].

The IL-7 receptor provides an indicator of the biological activity of IL-7. IL-7R is composed of a γC and Rα polypeptide. JAK3 associates with γC. The binding of JAK3 to γC allows IL-7 dimer formation to occur between γC and Rα so that JAK3 can phosphorylate Rα and/or JAK1 [209]. In most cases, activation of JAK3 causes STAT5 to be phosphorylated.

With respect to relationship between IL-7 and RA, Kim et al. [210] showed that the levels of IL-1β and TNF-α found in the synovial fluid of RA patients could typically increase IL-7 production by stromal cells in culture. In addition, IL-7 was also a strong inducer of RANKL production by T-cells, independent of TNF-α [210]. Interestingly, van Roon et al. [211] showed that TNF-α blockade in RA patients reduced IL-7 production. However, high levels of IL-7 persisted in RA patients who failed to respond to antagonists of TNF-α.

Hartgring et al. [212] found significantly higher amounts of IL-7Rα in the synovial fluid of RA patients as well as in synovial fluid from patients with undifferentiated arthritis. IL-7 level strongly correlated with the number of activated CD3^{+} T-cells. IL-7Rα was also identified on B-cells and macrophages from RA patients, but importantly IL-7Rα-expressing T-cells did not co-express, FoxP3. *Ex vivo* studies performed on monocytes collected from RA patients revealed that recombinant human IL-7Rα inhibited IL-7 induced T-cell proliferation

and IFN-γ production suggesting that blockade of IL-7Rα in RA patients reduced the expression of the STAT-responsive gene, IFN-γ.

With respect to the putative role of IL-7 in regulating certain aspects of cartilage responses in arthritis, Yammani et al. [213] reported that IL-7, IL-6 or IL-8 stimulated the production of the Ca^{2+}-binding protein, S100A4, by cultured human articular chondrocytes. Importantly, IL-7 increased the synthesis of S100A4 to a greater extent than either IL-6 or IL-8 with IL-7-stimulated S100A4 resulting from JAK3/STAT3 activation. In that regard, pre-treating chondrocytes with the experimental JAK3, inhibitor, WHI-P154, or with cyclohexamide blocked S100A4 synthesis which also inhibited the production of MMP-13. Because S100A4 has been implicated as significantly contributing to pannus-mediated destruction of cartilage in RA inflammation [214], blockade of IL-7R may be useful for down-regulating the expression of S100A4 and MMP-13 with associated blunting of pannus invasion into cartilage.

The interaction between S100A4 and the tumor suppressor p53 protein was purported to be related to the role of S100A4 as a promoter of cancer metastasis [215]. IL-7 via S100A4 was also shown to induce the expression of MMP-13 as well as MMP-1, MMP-9 and S100A4 was also shown to be involved in the neoangiogenesis and aberrant cell proliferation of rheumatoid synovium [216]. Importantly a selective inhibitor of MMP-13 reduced the level of cartilage destruction in 2 of 3 animal models of RA, including the SCID-mouse co-implantation model and CIA, but not adjuvant arthritis. [217]. Thus, evidence has gradually accumulated to show that up-regulation of S100A4 via activation of STAT3 significantly alters the progression of inflammatory arthritis.

9. Experimental therapies that inhibit activated stat proteins: Is cytokine gene expression altered?

The results of a Phase 2B RA clinical trial have recently been published which showed that the JAK3-specific SMI, tofacitinib (CP690, 550) had clinical efficacy as measured by the ACR response criteria [218]. However, there has been less progress on developing novel strategies to directly inhibit activated STAT proteins or dampen STAT gene responses. Noteworthy have been proof-of-principle studies that activated STAT proteins can be experimentally 'deactivated' which result in the inhibition of STAT/DNA binding. Thus, JNK-mediated phosphorylation of the STAT6 ser^{707} decreased the DNA binding capacity of IL-4-stimulated STAT6 resulting in the inhibition of STAT6-responsive genes [219]. Using immunosuppressive STAT oligodeoxynucleotides (ODN) to inhibit activated STAT proteins have also been relatively successful. These ODN have been shown to interfere with the phosphorylation of STAT1 and STAT4 [220] and STAT1 and STAT3 [221]. Lastly, administration of a single dose of a STAT1 decoy ODN suppressed joint swelling and the histological appearance of acute and chronic adjuvant-induced experimental arthritis in the mouse [222]. Electrophoretic mobility shift analysis of the nuclear extracts from synoviocytes from the STAT1 decoy ODN-treated animals incubated with the STAT-1 decoy ODN inhibited STAT-1 binding to DNA. Of note, STAT-1 decoy ODN

also inhibited the expression of macrophage CD40 suggesting that interference with CD40-mediated signaling by macrophages may be the mechanism responsible for the attenuation of arthritis by the STAT-1 decoy ODN.

10. Conclusion

The medical therapy of RA was revolutionized with the introduction of biological drugs, including TNF antagonists, the IL-6R antagonist, tocilizumab, the T-cell co-stimulatory factor inhibitor, abatacept, the B-cell inhibitor, rituximab and the IL-1 receptor antagonist, anakinra as well as the use of first-line therapy with disease-modifying anti-rheumatic drugs (DMARDs) such as methotrexate and anti-malarial drugs [223]. Nevertheless, the long-term and chronic use of these drugs for treating RA patients is not without potential deleterious consequences for those RA patients who use them. Thus, RA patients prescribed DMARDs and/or biological drugs need to be continuously monitored for changes in liver enzyme levels, ocular and/or kidney toxicities, infections and to a lesser extent malignancies such as lymphoma [224]. Just as important is the fact that some RA patients fail to respond to one or several of these biological drugs or become refractory to their action [225].

Development of JAK-specific SMIs was originally predicated on their use as a treatment for suppressing organ transplant rejection. However, JAK-SMIs were also considered as a potential adjunctive therapy for overcoming issues of long-term use of biological drugs for the therapy of RA [7, 11, 16, 17]. Now only time will tell whether or not the JAK-specific SMI, tofacitinib [218, 225], will be aggressively employed in the treatment of RA, or whether tofacitinib will be used in RA patients who only have exhibited a moderate or inadequate response to biological drugs or DMARDs.

Presently, there has been little attention paid, comparatively speaking, on acquiring data from RA patients in the general population who have been treated over several years with biological drugs to determine the extent to which the pro-inflammatory and/or anti-inflammatory cytokine repertoires have been altered from baseline. In addition, there are hardly any systematic studies, with the exception of some analyses conducted (often as a minor component of an RA clinical trial) with respect to which of several biological drugs restore the imbalance between T_h1 and T_h2 cytokines, suppress the activity of T_h17-producing cytokines, or improve the biological activity of dysfunctional T_{reg} cells [225]. Truly, the possibility exists that treating RA patients with biological drugs only partially inhibit over-expression of the pro-inflammatory cytokines that have been shown to mainly contribute to the progression of RA, namely, IL-6, IFN-γ and TNF-α (Table 1). This 'take-home' point appears to adequately justify a continual search for alternative cellular mechanisms that are active in determining whether clinical remission in RA patients is sustained or not. In conclusion, determining how STAT-responsive cytokine genes are regulated at the molecular and cellular level offers the potential going forward for developing yet another treatment modality designed to suppress the clinical activity and progression of RA pathology.

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