Mini Review

JAK inhibition and modulation of T cell function

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Cytokines play key roles in the pathogenesis of autoimmune diseases. They activate intracellular signaling pathways via receptors on the cell membrane, and tyrosine kinases are the primary mediators of this stimulation. In particular, members of the Janus kinase (JAK) family are important for signaling pathways and have been implicated in the pathogenesis of autoimmune diseases. Helper T cell development is regulated by various cytokines and the balance between Th1, Th2, and Th17 development is primarily determined by the cytokine milieu and by signals transduced by cytokines. Tofacitinib (CP-690,550), a selective inhibitor of JAKs, has been shown to suppress the production of IL-17 and IFN-γ from human CD4+ T cells. Furthermore, JAK inhibition may confer a regulatory phenotype on CD4+ T cells. JAK3 is exclusively expressed in hematopoietic cells and is essential for signaling through cytokine receptors that use a common γ-chain. Activated JAK3-deficient CD4+ T cells produce IL-10 and display an immune-regulatory phenotype. In this review, we discuss the effect of JAK inhibition on effector T cell function as well as the possibility of the induction of a regulatory phenotype by tofacitinib.

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Introduction

Cytokines play key roles in the pathogenesis of autoimmune diseases. They activate intracellular signaling pathways via receptors on the cell membrane, and tyrosine kinases are the primary mediators of this stimulation. In particular, members of the Janus kinase (JAK) family are important for signaling pathways and have been implicated in the pathogenesis of autoimmune diseases. Helper T cell development is regulated by various cytokine signals. JAK family proteins are non-receptor type tyrosine kinases consisting of JAK1, JAK2, JAK3, and TYK2, which transduce various cytokine signals by activating cytoplasmic Signal Transducers and Activators of Transcription (STAT)s via tyrosine phosphorylation on a specific tyrosine residue close to the Src homology domain. Recently, JAK inhibition has become an emergent strategy to control immune mediated diseases. Tofacitinib selectively inhibits JAK1, JAK2, and JAK3 *in vitro*, with functional cellular specificity for JAK1 and JAK3 over JAK2. The phenotypes of each JAK gene observed in null mutants differ, indicating that each JAK

has a unique function. Firstly, we will look at the phenotype of JAK-deficient mice to understand the possible effect of JAK inhibition.

JAK1 deficiency

Rodig et al. generated JAK1-deficient mice[1]. Jak1-deficient mice die perinatally. JAK1 deficiency in mice led to a severely reduced number of thymocytes, pre-B cells, and mature T and B cells, but did not lead to alterations in the development of other hematopoietic cells. Thymi from JAK1-deficient mice show severe reductions in size. IL-2, IL-4, and IL-7 induced proliferation is impaired in JAK1-deficient thymocytes, indicating that JAK1 deficiency leads to the ablation of lymphopoiesis in response to many cytokine receptors that utilize γc. In addition, JAK1-deficiency in fibroblasts results in unresponsiveness to IFN-α and IFN-γ. STAT3 activation was severely reduced but detectable in JAK1-deficient cells stimulated with IL-6 and leukemia inhibitory factor. Therefore, JAK1-deficient cells do not respond to cytokines that utilize γc-subunit, class II cytokine receptors, and the gp130 subunit. In terms of signal transduction via the γc-subunit, JAK1 could be the obligate partner for JAK3 in various immune responses. Patients with a deficiency in JAK1 have not been reported.

JAK2 deficiency

JAK2 plays an essential role in cytokine signaling during hematopoiesis. JAK2 deficiency is lethal. The phenotype of JAK2-deficient embryos is very similar to that of erythropoietin (EPO) or EPO receptor-deficient mice[2]. Responses to other cytokines including thrombopoietin (TPO), GM-CSF, IL-5, and IL-3 are also impaired in JAK2-deficient mice. GM-CSF, IL-5, and IL-3 utilize a common cytokine receptor, termed the common β chain, which has been reported to be associated with JAK2. Individuals with a deficiency in JAK2 have not been described.

JAK3 deficiency

JAK3 is basically expressed in hematopoietic cells, associates with the γc chain, and is activated by the cytokines IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 that utilize the γc chain. JAK3 plays a crucial role in immune system development and function, supported by the fact that mutations of the γc chain and JAK3 are associated with human X-linked severe combined immunodeficiency (XSCID)[3].

Mice deficient in JAK3 have several immunological abnormalities, including a block in B, NK, and $\gamma\delta$ T cell development[4]. In these mice, the cell number of the thymus is greatly reduced because of a deficiency in the number of thymic progenitor cells. In spite of this reduced thymocyte cellularity, $\alpha\beta$ T cells develop normally in JAK3-deficient mice, and adult JAK3-deficient mice have normal numbers of peripheral T cells. These T cells are mostly CD4+ T cells and show a surface phenotype similar to memory T cells. JAK3-deficient T cells fail to proliferate in response to TCR stimulation. Mayack et al. reported that JAK3-deficient CD4+CD44^{high} T cells express increased levels of programmed death-1 (PD-1), lymphocyte activation gene-3 (LAG-3), IL-10, and TGF-β1 mRNA, indicating an increase in inhibitory molecule expression[5]. Although JAK3-deficient T cells do not express CD25 or Foxp3, they modestly suppress the proliferation of wild-type T cells. JAK3-deficient T cells secrete substantially higher levels of IL-10, IFN-γ, and TGF-β1 than JAK3-sufficient controls, with no detectable IL-2 and low levels of IL-5 and IL-4. These observations indicated that JAK3-deficient CD4+CD44^{high} T cells are not conventional Th1 or Th2 cells, but instead show a T regulatory type-1 (Tr1)-like phenotype. Therefore, JAK3 inhibition may induce a unique T cell population with regulatory activity.

TYK2 deficiency

TYK2 is ubiquitously expressed, while the expression of JAK3 is restricted to hematopoietic cells. TYK2 is a molecule initially identified as an essential molecule for IFN-α and IL-6 signaling[6]. IL-10, IL-23, IL-27 and IL-12 signaling also phosphorylates TYK2[7, 8]. Shimoda et al. reported that although TYK2-deficient mice show no developmental abnormalities, they show an impaired responsiveness to a small amount of IFN-α[9]. Moreover, IL-12 induced T cell function is defective in these mice. A deficiency in TYK2 greatly inhibited IFN-γ production from T cells due to the abolishment of IL-12 signaling. TYK2 deficiency in mice resulted in impaired immune responses to pathogens and enhanced lung inflammation. A patient with autosomal recessive hyperimmunoglobulin E was reported to have mutated Tyk2[10, 11]. The patient was vulnerable to multiple infections. Shaw et al. demonstrated that tyrosine phosphorylation of both STAT1 and STAT3 proteins following IL-10 stimulation was attenuated by TYK2 deficiency[12]. In a *Toxoplasma gondii* challenge, TYK2 indirectly controls IL-10 reactivation in CD4+ T cells by maximal IFN-γ se-

cretion. Thus, TYK2 is required for IL-10 reactivation by IFN-γ during the Th1 response, which represents a classic feedback loop.

The use of tofacitinib in rheumatoid arthritis

Based on the phenotype of mice deficient for various JAK family molecules described above, JAK inhibition may result in broad suppression of immunological and hematopoietic cells.

Tofacitinib (CP-690,550), a selective inhibitor of JAKs, is being developed as an immunosuppressive agent. The *in vivo* effect of tofacitinib was first investigated in animal models of organ allograft rejection. Tofacitinib prolonged survival after heart or kidney transplantation without the metabolic abnormalities or severe adverse effects associated with immunosuppression[13]. Tofacitinib also suppressed cartilage damage in a model of collagen-induced arthritis in mice and adjuvant-induced arthritis in rats[14]. Tofacitinib is under investigation in clinical trials for the therapy of rheumatoid arthritis (RA) with satisfactory effects and safety. Tofacitinib administration resulted in a dose-dependent decrease in peripheral blood neutrophil counts in active RA patients, but not in psoriasis patients or normal volunteers[15]. Kremer et al. reported a phase clinical II trial to characterize the efficacy and safety dose-response profile of tofacitinib[16]. In patients with active RA who exhibited inadequate responses to MTX, the addition of tofacitinib at a dosage >3 mg twice daily showed significant efficacy. At week 12, ACR20 response rates for patients receiving all tofacitinib dosages >3 mg twice daily (52.9% for 3 mg twice daily, 50.7% for 5 mg twice daily, 58.1% for 10 mg twice daily, 56.0% for 15 mg twice daily, and 53.8% for 20 mg/day) were significantly ($p < 0.05$) greater than those for placebo (33.3%). Greater ACR50 response rates were observed in all tofacitinib groups relative to placebo. At weeks 12 and 24, the ACR50 response rate was significantly greater for patients receiving tofacitinib at dosages of 15 mg twice daily and 20 mg/day than that for placebo ($p < 0.05$); at week 12, patients receiving 5 mg of tofacitinib twice daily exhibited a significantly greater response than that of patients receiving placebo ($p < 0.05$). Improvements were sustained at week 24 for ACR20, ACR50, and ACR70 responses for scores of the Health Assessment Questionnaire disability index (HAQ-DI), and DAS28. Tofacitinib administered on MTX demonstrated an acceptable safety and tolerability profile over 24 weeks in patients with active RA. The most common treatment-associated adverse events occurring in >10% of patients in any tofacitinib group included diarrhea, upper respiratory tract infections, and headaches. Increases in transaminase levels, cholesterol, and serum creatinine levels, and decreases in neutrophil and hemoglobin levels were reported. Although all dosages of tofacitinib were associated with adverse events, the majority were mild in severity. Recently, a 12-month, phase 3 trial of tofacitinib, the ORAL Standard trial, has been reported[17]. A total of 717 RA patients who were under stable doses of methotrexate were randomly assigned to 5 mg of tofacitinib twice daily, 10 mg of tofacitinib twice daily, 40 mg of adalimumab once every 2 weeks, or placebo. At month 6, ACR20 response rates were higher among patients receiving 5 mg or 10 mg of tofacitinib (51.5% and 52.6%, respectively) and among those receiving adalimumab (47.2%) than among those receiving placebo (28.3%). Greater reductions were also observed in HAQ-DI scores at month 3. This study indicated that tofacitinib was significantly superior to placebo and was similar to adalimumab in clinical efficacy. The percentage of patients, who reported serious adverse events, and either temporary or permanent discontinuations in both tofacitinib groups, was similar to that in the adalimumab group. Freishmann et al. reported on a phase III study for tofacitinib monotherapy at a dose of 5 mg or 10mg twice daily[18]. Both doses were associated with reductions in the signs and symptoms of RA and improvements in the physical function of patients who showed an inadequate response to DMARDs. In addition to tofacitinib, other JAK inhibitors were studied in RA; the JAK3 inhibitors VX-509, the JAK3/Spleen tyrosine kinase inhibitor R348, the JAK1/JAK2 inhibitor baricitinib, and the JAK1 inhibitor GLPG-

Table 1. JAK inhibitors in development for RA

Agent	Targeted JAK	Development Stage
Tofacitinib	3,1,2	FDA approval
VX-509	3	Phase II trial
R-348	3	Phase I trial
Baricitinib (formerly INCB-28050)	1,2	Phase II trial
GLPG-0634	1,2,Tyk2	Phase II trial
ASP015K	1,2,3,Tyk2	Phase II trial
AC-430	2	Preclinical
CEP-33779	2	Preclinical

0634 (Table 1)[19, 20]. Based on the finding in JAK2-deficient mice, JAK2 may have side effects such as anemia, while selective JAK1 inhibition could maintain the anti-inflammatory potency. Lipid elevation observed in tofacitinib and VX-509 could be attributed to the inhibition of gp130-JAK1 function, because IL-6-gp130 signaling suppresses blood lipid levels[21].

The mode of action of tofacitinib

In spite of the clear efficacy of tofacitinib in RA, the mode of action of tofacitinib remains to be clarified. Walker et al. reported that JAK3, STAT1, STAT4, and STAT6 were highly expressed in the synovium of patients with RA, although their expression was limited to the synovium of healthy controls and patients with osteoarthritis and spondyloarthritis[22]. Their observation that CD1a positive dendritic cells intensely express JAK3, STAT4, and STAT6 in seropositive RA tissue suggested that JAK3 inhibition may prevent dendritic cell maturation in RA and may possibly become a hopeful therapeutic strategy. However, Maeshima et al. reported that tofacitinib did not affect IL-6 and IL-8 production by RA synovial fibroblasts and $CD14^+$ monocytes in SCID-HuRAg mice, an RA animal model using SCID mice implanted with synovium and cartilage from RA patients. In contrast, tofacitinib directly suppressed the production of IL-17 and IFN-γ and proliferation of human $CD4^+$ T cells, associated with the inhibition of IL-6 production by RA synovial fibroblasts and IL-8 production by $CD14^+$ cells[23]. The concentration of IL-2 in the culture supernatant of $CD4^+$ T cells stimulated with anti-CD3/anti-CD28 antibodies was increased when tofacitinib was added to the culture. In contrast, tofacitinib significantly decreased IL-2 — induced production of IL-17 and IFN-γ by peripheral blood $CD4^+$ T cells[24]. Therefore, tofacitinib could inhibit the IL-2 mediated JAK/STAT signaling pathway in human T cells. Ghoreschi et al. examined the consequences of tofacitinib treatment on helper T cell differentiation in naïve murine $CD4^+$ T cells[19]. Expression of the IL-23 receptor and Th17 cytokines IL-17, IL-17F, and IL-22 were inhibited under naïve T cell stimulation with IL-6 and IL-23. On the other hand, IL-17 production was enhanced when Th17 cells were differentiated in the presence of TGF-β1. Moreover, tofacitinib also prevented the activation of STAT1, induction of T-bet, and subsequent development of Th1 cells. Tofacitinib rapidly improved the arthritis model by inhibiting the production of inflammatory mediators and suppressing STAT1-dependent genes in joint tissue. Thus, tofacitinib may improve autoimmunity by suppressing the differentiation of pathogenic Th1 and Th17 cells. Tofacitinib and Pyridone 6, a pan-JAK inhibitor, could enhance Th17 differentiation, while inhibiting Th1 and Th2 development in mice[25, 26]. *In vitro,* tofacitinib promoted Th17 differentiation at low concentrations. A low dose of tofacitinib promoted the onset of experimental autoimmune encephalomyelitis (EAE) at a concentration that suppressed the arthritis model[25]. On the other hand, Migita et al. reported that tofacitinib suppressed IL-17 production by human $CD4^+$ T cells at a low concentration[27]. There is the possibility that the sensitivity of Th17 to tofacitinib depends on the inducing condition and the fate of Th17 in tofacitinib-treated RA patients should be investigated. The importance of $CD4^+$ T cells in the pathogenesis of autoimmunity is generally accepted based on the fact that certain HLA-DR alleles such as HLA-DR*0401 confer strong susceptibility to RA, and it is reasonable to speculate that the efficacy of tofacitinib is associated with modulation of the function of $CD4^+$ T cells. A recent study revealed that tofacitinib effectively inhibited effector T cell (Teff) function but preserves the suppressive activity of $CD4^+CD25^{bright}$ regulatory T cells (Treg) in kidney transplant patients[28]. The baseline of IL-2-induced intracellular STAT5-phospholylation (pSTAT5) was significantly higher for $CD4^+CD25^{bright}$ Treg than for $CD4^+CD25^{neg}$ Teff. In the presence of tofacitinib, IL-2-induced pSTAT5 was reduced by half in $CD4^+CD25^{bright}$ Treg, while reduced by 84% in Teff. Teff might be more sensitive to the effect of tofatinib than Treg. In macrophage, TNF-α-induced NFAT nuclear translocation is enhanced by tofacitinib[29]. In T cells, TCR engagement in the absence of costimulatory signals leads to the activation of NFAT, but with poor concomitant activation of AP1. This results in the initiation of a transcriptional program that leads to T cell anergy. We suppose that tofacitinib may enhance TCR-induced NFAT nuclear translocation in CD4 T cells to induce T cell anergy and regulatory response. The mechanism how JAK inhibition works on T cell function should be investigated further.

Tofacitinib suppresses JAK1 and JAK3

Meyer et al. extensively evaluated the effect of tofacitinib for each JAK family molecule[15]. They measured the potency of tofacitinib against JAK1, JAK2, JAK3, and TYK2 using recombinant human kinase domains. Tofacitinib po-

tently inhibits JAK3 (IC50, 1.6±0.2 nM) and JAK1 (IC50, 3.2±1.4 nM) and to lesser extent JAK2(IC50, 4.1±0.4 nM)15). It has little effect on Tyk2 (IC50, 34.0±6.0 nM). However, tofacitinib potently inhibited signaling through JAK1 and JAK3 with 5-100 fold selectivity over JAK2 in cellular assays. Interestingly, the plasma concentration of tofacitinib at clinical doses was above the *in vitro* whole blood IC50 of JAK1 and JAK3 inhibition, but not JAK2. Therefore, tofacitinib may be a potent inhibitor of JAK1 and JAK3 with reduced cellular and *in vivo* potency for JAK2. Because not only JAK3 inhibition but also JAK1 inhibition suppresses signal transduction via the γc-subunit, tofacitinib may strongly inhibit γc-subunit-mediated signaling. Although this result is consistent with a number of reports, increases in hemoglobin concentrations were observed only in a few patients receiving higher doses of tofacitinib in the phase II trial despite improvements in disease activity[16]. This result suggests that tofacitinib may be related to the inhibition of JAK2 signaling *in vivo*.

As mentioned above, JAK3 deficiency results in regulatory cytokine production in T cells[5] and tofacitinib induces preferential production of IL-10[19]. VanDeusen et al. reported STAT1-mediated repression of IL-10[30] and suppression of STAT1 may be associated with the promotion of IL-10 production by tofacitinib. In addition, the relatively low suppression of TYK2 by tofacitinib may also be associated with regulatory cytokine production. Shaw et al. reported that TYK2 is critical for optimal IL-10-mediated signaling and suppressor function[12]. Tyrosine phosphorylation of STAT3 following IL-10 stimulation was reduced by a deficiency in TYK2. A nearly 100-fold higher concentration of IL-10 was required to achieve a comparable intensity of STAT3 activation in TYK2-deficient macrophages. Thus, relative dominance of TYK2 in the presence of tofacitinib may facilitate IL-10 signaling. The link between JAK1 and JAK3 inhibition with tofacitinib and the induction of the regulatory phenotype in CD4+ T cells should be investigated further.

Conclusion

Accumulating studies revealed that JAK inhibition with tofacitinib in patients with RA results in a prompt and satisfactory clinical effect comparable to that of TNF inhibitors. However, the mode of action of tofacitinib remains to be clarified. Although suppression of IFN-γ and IL-17 production from CD4+ T cells has emerged as one of the important mechanisms of tofacitinib efficacy in RA, there is a possibility that tofacitinib induces dominant regulatory systems in CD4+ T cells. Further study of tofacitinib may provide some clues that elucidate the key pathway in RA pathogenesis.

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