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Regulation of Inflammatory Responses in Tumor Necrosis Factor - Activated and Rheumatoid Arthritis Synovial Macrophages by Janus Kinase Inhibitors

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Abstract

Objective—Inhibitors of the Janus kinases (JAKs) have been developed as anti-inflammatory and immunosuppressive agents and are currently undergoing testing in clinical trials. The JAK inhibitors CP-690,550 (tofacitinib) and INCB018424 (ruxolitinib) have demonstrated clinical efficacy in rheumatoid arthritis (RA). However, the mechanisms that mediate the beneficial actions of these compounds are not known. In this study, we examined the effects of both JAK inhibitors on inflammatory and tumor necrosis factor (TNF) responses in human macrophages (MΦs).

Methods—In vitro studies were performed with peripheral blood MΦs from healthy donors treated with TNF and synovial fluid MΦs from patients with RA. Levels of activated signal transducer and activator of transcription (STAT) proteins and other transcription factors were detected by Western blot, and gene expression was measured by real-time polymerase chain reaction. In vivo effects of JAK inhibitors were evaluated in the K/BxN serum-transfer model of arthritis.

Results—JAK inhibitors suppressed activation and expression of STAT1 and downstream inflammatory target genes in TNF-stimulated and RA synovial macrophages. In addition, JAK inhibitors decreased nuclear localization of NF-κB subunits in TNF-stimulated and RA synovial macrophages. CP-690,550 significantly decreased *IL6* expression in synovial MΦs. JAK inhibitors augmented nuclear levels of NFATc1 and cJun, followed by increased formation of osteoclast-like cells. CP-690,550 strongly suppressed K/BxN arthritis that is dependent on macrophages but not on lymphocytes.

Conclusion—Our findings demonstrate that JAK inhibitors suppress macrophage activation and attenuate TNF responses, and suggest that suppression of cytokine/chemokine production and innate immunity contributes to the therapeutic efficacy of JAK inhibitors.

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Keywords

macrophages; TNF; STAT1; rheumatoid arthritis; JAK inhibitors

Rheumatoid arthritis (RA) is a chronic inflammatory disease that preferentially targets synovial tissue, cartilage and bone. Multiple cytokines produced by innate and adaptive immune cells are implicated in pathogenesis of RA (1). Imbalance between pro- and anti-inflammatory cytokines leads to autoimmunity, chronic inflammation and tissue destruction. Several biologics developed against specific cytokines and their receptors, with tumor necrosis factor (TNF) inhibitors leading the pack, demonstrate clinical efficacy in chronic inflammatory diseases, including RA (2, 3). However, resistance to therapy in subpopulations of patients, increased infection rates, high treatment costs, difficulty in titrating dosage, and injection-related complications have prompted the search for orally active small molecule compounds that can selectively interfere with molecular mediators of cytokine signaling. Recently, Janus kinase (JAK) family of nonreceptor tyrosine kinases that plays a critical role in mediating inflammatory and immune responses has gained significant interest as a therapeutic target (4–6).

The JAK family is comprised of four enzymes (JAK1, JAK2, JAK3 and TYK2) that control signaling by numerous cytokines important for acquired and innate immunity and hematopoiesis (4, 7). In resting cells JAKs associate with the intracellular domains of type I and II cytokine receptors. Upon ligation of cytokine receptors, JAKs transactivate each other and phosphorylate tyrosine residues on the receptor cytoplasmic domain, leading to the recruitment and phosphorylation of signal transducers and activators of transcription (STATs) that culminates in STAT dimerisation, translocation to the nucleus and activation of gene transcription (7–9). Studies in mice and humans with deleted or mutated JAKs revealed their specific role in regulation of cytokine signaling. JAK1, JAK2 and TYK2 regulate signaling triggered by activation of both type I and II cytokine receptors whereas JAK3 is specifically associated with IL-2 receptor common γ chain (γ_c) shared by the receptors for cytokines important for development and function of T, B and NK cells (10–14).

Several small-molecule JAK inhibitors are currently in clinical development for the treatment of transplant rejection, hematopoietic disorders, and autoimmune and inflammatory diseases, including RA (4, 6). Among them, CP-690,550 (tofacitinib) demonstrated significant efficacy in RA (5, 15). CP-690,550 was initially developed as a selective JAK3 inhibitor, however, recent studies demonstrated that in cell culture, it suppressed cytokine signaling mediated by JAK1/3, JAK1/2 and JAK1/TYK2 with much less activity against JAK2 homodimers important for the signaling by hematopoietic factors (16, 17). INCB18424 (ruxolitinib) has higher specificity against JAK1, JAK2 and TYK2, and also demonstrated clinical efficacy in RA clinical trials (6, 18). Despite the successful results of clinical trials and efficacy in animal models of arthritis, the precise mechanism of action by CP-690,550 and INCB018424 that suppresses disease activity in RA is not clear. Consistent with effective inhibition of γ_c cytokines required for lymphocyte proliferation and function, several in vivo and in vitro studies of CP-690,550 demonstrate suppression of lymphocyte activation and proliferation in various animal models (15, 19–21). Also, CP-690,550 interferes with Th1 and Th2 differentiation and impairs the production of inflammatory Th17 cells (17). Recently, it has been suggested that CP-690,550 can also target innate immunity in vivo (17); underlying mechanisms are completely unknown as JAKs do not play a direct role in signaling by many receptors important for innate immune responses, such as TNF, IL-1 or Toll-like receptors.

Macrophages (MΦs) are innate immune cells that play an important role in synovial inflammation and tissue destruction in RA (22). Macrophages contribute to RA pathogenesis in part by producing key inflammatory cytokines, such as TNF, IL-1 and IL-6, and chemokines, such as IL-8 and CXCL10 (IP-10). RA synovial macrophages express high levels of STAT1 and an 'IFN-STAT1 signature' that reflects activation by synovium-expressed cytokines that signal via JAK1, JAK2 and TYK2, likely including IFN-β, IFN-γ and IL-6 (1, 8, 12, 13, 23, 24). Importantly, JAK-STAT signaling in macrophages can be indirectly activated by innate immune receptors such as Toll-like receptors (TLRs) and TNF receptors via induction of an autocrine loop mediated by cytokines such as IFN-β and IL-6 (1, 25, 26). Jak-STAT signaling in macrophages augments production of multiple inflammatory cytokines and chemokines (27), and the importance of an TNF-IFN-β-JAK-STAT1 autocrine loop in cell activation and inflammatory gene expression has been recently established (25, 28). This suggests that JAK inhibitors may also target macrophages to suppress inflammatory cytokine and chemokine production. Thus, we examined effects of JAK inhibition on inflammatory responses in human blood-derived and RA synovial MΦs, with a focus on the key pathogenic cytokine TNF that activates JAK-STAT signaling indirectly and with delayed kinetics. JAK inhibitors abrogated expression of STAT-dependent cytokines such as CXCL10. Unexpectedly, JAK inhibitors also decreased nuclear localization of NF-κB subunits and CP-690,550 significantly decreased *IL6* expression in synovial fluid MΦs. Both JAK inhibitors augmented nuclear levels of NFATc1 and cJun, followed by increased formation of osteoclast-like cells. Lastly, CP-690,550 effectively suppressed K/BxN arthritis, a model that is solely dependent upon innate immune mechanisms. Our data demonstrate that JAK inhibitors suppress inflammatory functions of macrophages, in part by altering cell responses to the key pathogenic cytokine TNF. These findings suggest that suppression of macrophages and innate immunity may contribute to the therapeutic efficacy of Jak inhibitors in RA.

MATERIALS AND METHODS

Cell culture and media

Synovial fluids were obtained using a protocol approved by the Hospital for Special Surgery Institutional Review Board from RA patients by their physicians as a part of standard medical care and de-identified specimens that would otherwise have been discarded were used in this study. Peripheral blood mononuclear cells (PBMC) were isolated from blood leukocyte preparations (NYC Blood Center) or synovial fluids by density gradient centrifugation and CD14⁺ cells were purified using anti-CD14 magnetic beads (Miltenyi Biotec). Human monocytes were cultured overnight in α-MEM medium (Invitrogen Life Technologies) supplemented with 10% FBS (HyClone), 100 U/ml penicillin/streptomycin (Invitrogen Life Technologies), 2 mM L-glutamine (Invitrogen Life Technologies) and 20 ng/ml of human macrophage colony-stimulating factor (M-CSF, Peprotech). The following reagents were added to cell cultures as indicated: recombinant human TNF, 40 ng/ml (Peprotech), recombinant universal type IFNα A/D, 5000 U/ml (PBL Interferon Source), human recombinant IFNγ, 100 U/ml (Roche Applied Science), CP-690,550 0.1–10 μM and INCB18424 0.1–1 μM (Active Biochemicals Co. Limited).

Multinuclear cell/osteoclast differentiation

Human CD14⁺ cells (0.25×10^6 cells/ml) were incubated in α-MEM supplemented with 10% FBS, 20 ng/ml of M-CSF and 40 ng/ml of human TNF for various times in the presence or absence of JAK inhibitors. Cytokines were replenished every 3 days. At the end of culture period cells were stained for tartrate-resistant acid phosphatase (TRAP) activity, according to manufacturer's instructions (Sigma). Multinucleated (>3 nuclei), TRAP-positive cells were counted in triplicate wells of 96-well plates. For bone resorption assays,

cells were cultured as described above on Corning® Osteo Assay Surface 96-well plates for 25 days. Cells were removed by incubation for 10 min with 10% bleach and resorption area was quantified using IPLab™ imaging software (BD Biosciences).

Quantitative real time PCR (qRT-PCR)

Total RNA was extracted using an RNeasy mini kit (Qiagen) with DNase treatment, and 0.5 µg of total RNA was reverse transcribed using a First Strand cDNA Synthesis kit (Fermentas). qPCR was performed using the Fast SYBR® green Master Mix and 7500 Fast Real-time PCR System (Applied Biosystems). Expression of the tested genes was normalized relative to levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Immunoblotting

Cytoplasmic and nuclear cell extracts were obtained, and equal amounts of total protein were fractionated on 7.5% polyacrylamide gels using SDS-PAGE, transferred to polyvinylidene fluoride membranes (Millipore), incubated with specific antibodies (Abs) recognizing NFATc1, STAT2 (BD Biosciences), RelB, NF-κB p100/p52, phospho-NF-κB p65 (Ser536), c-Jun, Akt and phospho-STAT1(Tyr701) (Cell Signaling Technology), phospho-STAT2 (Tyr689) (Millipore), Lamin B1 (Abcam) and p38 (Santa Cruz Biotechnology) and horseradish peroxidase-conjugated secondary Abs were used for detection with ECL (Amersham). The signal intensities of bands specific for transcription factors were quantified using IPLab™ imaging software (BD Biosciences) and normalized relative to the intensity of loading control Lamin B1.

Mouse arthritis model

We used C57BL/6 mice (Jackson Laboratory) in our study. Animals were maintained in the Animal Facility of the Hospital for Special Surgery (New York, NY), and protocols were approved by the Institutional Animal Care and Use Committee. K/BxN serum pools were prepared as described (29). Arthritis was induced by i.p. injection of 100 µl of K/BxN serum (experimental group, n=10) or PBS (control group, n=10) i.p. on days 0 and 2. Control and arthritic animals were divided into two additional groups and administered vehicle (0.5% methylcellulose/0.025% Tween 20 (Sigma-Aldrich)) or 50 mg/kg CP-690,550 resuspended in 0.5% methylcellulose/0.025% Tween 20 twice daily from day 1 by oral gavage. The severity of arthritis was monitored by measuring the thickness of both wrist and ankle joints using a dial type caliper (Bel-Art Products). For each animal, the joint thickness was calculated as a sum of measurements of two wrists and two ankles. The joint thickness was represented as an average for every group of treatment. For histopathology, mice were sacrificed at day 9 after first serum injection and fore and hind paws were harvested and fixed in 10% neutral buffered formalin for 24 h.

Histopathology

Fixed paws were decalcified with 10% neutral buffered EDTA (Sigma-Aldrich) for 7 days, and embedded in paraffin. To assess inflammation, sections 5 µm thick were stained with hematoxylin (Sigma-Aldrich), fast green (Acros Organics) and safranin O (Sigma-Aldrich) using standard techniques.

Statistical analysis

Statistical analysis was performed using GraphPad Prism Analytical Software Version 5.03 for Windows (GraphPad Software, Inc.). Statistical tests included nonparametric Wilcoxon matched-pairs signed-ranks test, two- and one-way analysis of variance with post hoc tests for multiple comparisons. P<0.05 was considered to denote significance.

RESULTS

JAK inhibitors CP-690,550 and INCB018424 inhibit IFN-induced STAT activation

The efficacy of JAK inhibitors can vary according to cytokine receptor, associated JAKs, and cell type and we wished to test and compare the effects of CP-690,550 and INCB018424 on signaling by cytokines that activate STAT1 in human MΦs and can contribute to the 'STAT1 signature' observed in RA (30, 31). We stimulated primary human MΦs for 15 minutes with IFN α (Figure 1A) which activates JAK1/TYK2 or IFN γ (Figure 1B) that signals through JAK1/2 (23, 32). IFN α activates STAT1 and STAT2 while IFN γ activates primarily STAT1 and we prepared nuclear extracts and analyzed STAT activation by measuring nuclear translocation and tyrosine phosphorylation. CP-690,550 and INCB018424 blocked IFN α - and IFN γ -induced STAT1 and STAT2 nuclear translocation and tyrosine phosphorylation in a dose-dependent manner and strong inhibition was observed at nanomolar concentrations of JAK inhibitors (Figure 1A and 1B). IFN γ signaling was inhibited more effectively than IFN α signaling, which is most likely explained by lower efficacy of TYK2 inhibition by these compounds (5, 6, 33, 34). Overall, INCB018424 inhibited IFN signaling at lower concentrations than did CP-690,550, which is consistent with differential potency of these compounds in suppressing JAK1 and JAK2 (6, 33). These findings demonstrate that CP-690,550 and INCB018424 can inhibit IFN-JAK-STAT signaling in primary human MΦs at concentrations similar to those reported for other cell types (5, 6, 16, 17, 28), and show modestly different dose-dependent effects of these JAK inhibitors.

JAK inhibitors decrease TNF-dependent STAT1 activation, STAT1 expression, and induction of IFN-dependent genes

We wished to test the effects of JAK inhibitors on MΦ responses to the key pathogenic cytokine TNF. We recently demonstrated that in MΦs, TNF-induced expression of key T cell chemokines such as CXCL10 and CXCL11 is dependent on synergy between canonical TNF signaling and a TNF-induced IFN β -JAK-STAT-mediated autocrine loop, which also activates the delayed expression of classic IFN-response genes such as IFIT1 and IRF7 (25). We found that both CP-690,550 and INCB018424 inhibited TNF-induced expression of CXCL10 and IFIT1 in a dose-dependent manner (Figure 2A). TNF-induced IFN β expression can be detected within 2 hours after stimulation, reaches a maximum at 6 hours, returns to baseline after 24 hours of culture, and leads to sustained STAT1 activation and related gene expression for several days (25, 35). Therefore, we performed a time course analysis of the effects of JAK inhibition on the expression of chemokines and IFN-response genes (Figure 2B and 2C, note logarithmic scale on ordinate) from 3 to 48 hours after TNF stimulation. CP-690,550 and INCB018424 strongly suppressed TNF-mediated induction of the CXCL10 and CXCL11 chemokine genes and of the IFIT1 and IRF7 IFN response genes over the entire time course (Figure 2B and C). Multiple TNF-induced intermediate response genes (regulated similarly as CXCL10 and CXCL11) and classical IFN response genes were inhibited by CP-690,550 and INCB018424 without significant effect on cell viability; TNF-induced IFN β expression was not affected by Jak inhibitors (data not shown). Thus, inhibition of JAKs resulted not only in the expected suppression of IFN response genes but also strongly suppressed inflammatory chemokine genes. This suggests that canonical NF- κ B signaling is not sufficient to fully induce expression of these chemokine genes and that JAK inhibitors broadly suppress TNF responses. Next, we examined TNF-activated STAT1 signaling and found that CP-690,550 and INCB018424 abrogated tyrosine phosphorylation that regulates transcriptional activity of STAT1 and suppressed nuclear translocation of STAT1 (Figure 2D, upper panel, compare lanes 4 versus 5, 6; 10 versus 11, 12; 16 versus 17, 18). JAK inhibitors suppressed TNF-induced STAT1 activation at both early and late time points (Figure 2D) and this inhibition correlated with suppression of TNF-induced gene

expression (Figure 2A–C). STAT1 itself is a target of JAK/STAT signaling and is highly expressed in RA synovium (30). Inhibition of JAKs decreased total STAT1 protein and RNA expression in TNF-treated MΦs at 24 and 48 hours (Figure 2D, lanes 16 – 18 and 22 – 24, and data not shown). Taken together, our results demonstrate that JAK inhibitors abrogate TNF-activated IFN-STAT1 signaling and suppress STAT1 expression in human MΦs, which in turn leads to decreased expression of pro-inflammatory chemokines and suppression of IFN-regulated genes.

JAK inhibitors increase TNF-induced NFATc1 activation and formation of osteoclast-like cells

We recently found that prolonged exposure (days) of human MΦs to TNF activates an NFATc1-mediated gene program important for cell fusion and osteoclastogenesis (35). Activation of NFAT transcription factors requires dephosphorylation, which allows nuclear translocation and transcription of target genes (36). We examined TNF-induced NFATc1 activation in the presence of JAK inhibitors (Figure 3A) and found that CP-690,550 and INCB018424 strongly increased nuclear expression of NFATc1 starting at 24 hours of culture (Figure 3A, lanes 4–6 and 10–12). This finding with TNF is consistent with previous reports showing IFN-STAT signaling can also inhibit RANKL-induced NFATc1 activation and osteoclastogenesis (37, 38). In human MΦs, cJun member of AP-1 family is important for TNF-mediated activation of NFATc1 (35). CP-690,550 and INCB018424 treatment increased cJun nuclear expression at 24 hours after TNF stimulation (Figure 3B, lanes 4–6; right panel shows densitometric quantitation of cJun induction in five independent experiments) which correlated with upregulation of NFATc1 nuclear levels (Figure 3A, lanes 4–6). Next, we examined effect of JAK inhibition on TNF-induced osteoclastogenesis and found that CP-690,550 treatment significantly increased formation of TRAP+ multinuclear cells in 90% of experiments (Figure 3C) and strongly enhanced resorptive activity of osteoclasts (Figure 3D). INCB018424 treatment had variable effects with increased cell fusion in 70% of experiments (Figure 3C and data not shown) without increasing resorptive activity (Figure 3D). Moreover, cell fusion was observed more rapidly in the presence of JAK inhibitors (data not shown). Overall, the results show that JAK inhibitors can enhance aspects of TNF-induced cell fusion and osteoclast differentiation.

JAK inhibitors attenuate the late phase of TNF-induced NF-κB activation and affect expression of inflammatory cytokine genes

CP-690,550 and INCB018424 can decrease plasma levels of inflammatory cytokines (6, 16, 17). However, the cellular basis of this phenomenon is not known. Cytokine induction in response to inflammatory stimuli such as LPS and TNF occurs rapidly and declines after several hours. On the other hand, late expression of inflammatory cytokines in response to TNF has not been explored. Therefore we analyzed expression of *IL1B*, *TNF* and *IL6* in human MΦs stimulated with TNF for 1 to 48 hours in the presence or absence of JAK inhibitors (Figure 4A, B). Expression of *TNF* and *IL6* followed the expected transient expression pattern described above (Figure 4A, top and bottom panels). Surprisingly, *IL1B* expression demonstrated a second wave of increase with a second peak at 24 hours post-TNF stimulation (Figure 4B, upper panel). CP-690,550 and INCB018424 did not affect the early expression of pro-inflammatory cytokines (Figure 4A and B, upper panel), but in contrast, suppressed the late wave of *IL1B* induction, with significant inhibition by CP-690,550 (Figure 4B, lower panel, note logarithmic scale on ordinate). To explain the suppression of the late *IL1B* expression, we analyzed the effects of JAK inhibitors on the late phase of TNF-induced signaling. We previously demonstrated that TNF induces nuclear accumulation of components of both canonical and noncanonical NF-κB pathways with biphasic kinetics characterized by sustained nuclear expression of phospho(Ser536)-p65 (RelA), p52, and RelB at 24–72 h after TNF stimulation (35). JAK inhibition affected TNF-

induced nuclear accumulation of NF- κ B subunits at 24 and 48 hours after TNF stimulation, with the most prominent inhibitory effect for RelB and p52 at the 48 hour time point (Figure 4C, lanes 4 – 6 and 10 – 12; densitometric quantitation of results from five independent experiments is shown in bottom panel). However, RNAi-mediated knockdown of RelB or upstream kinase IKK α had minimal effects on the late phase of *IL1B* expression (data not shown), suggesting an alternative JAK/STAT-dependent mechanism contributes to the late phase of *IL1B* regulation.

Effects of JAK inhibitors on RA synovial macrophages

Next, we investigated the direct pathophysiological importance of our findings by testing the effects of JAK inhibitors on the inflammatory phenotype of RA synovial MΦs. RA synovium and synovial MΦs demonstrate an 'IFN signature' as evidenced by increased expression of IFN-regulated genes, including STAT1 and the chemokine and IFN response genes analyzed in this study. However, the function of the 'IFN/STAT1 signature' in synovial MΦs is not well understood (39). We used JAK inhibitors to test the role of JAK-STAT signaling in RA synovial MΦs. As shown on Figure 5A, CP-690,550 and INCB018424 strongly and significantly suppressed expression of CXC chemokines, IFN response genes, and STAT1 in RA synovial MΦs. Interestingly, CP-690,550 also significantly decreased *IL6* expression (Figure 5A, top panel); whereas INCB018424 displayed variable effects on *IL6* expression in synovial MΦs samples (Figure 5A, bottom panel). In accord with these results, CP-690,550 and INCB018424 decreased nuclear expression of tyrosine-phosphorylated STAT1, total STAT1, RelA and RelB in RA synovial MΦs (Figure 5B). We previously demonstrated that NFATc1 is expressed in synovial macrophages from patients with inflammatory arthritis (35). JAK inhibitors further increased nuclear expression of NFATc1 in RA synovial MΦs (Figure 5B). These results show that JAK inhibitors suppress the inflammatory phenotype of RA synovial MΦs, while augmenting NFATc1 expression.

CP-690,550 ameliorates joint inflammation in the K/BxN serum-induced arthritis model

We evaluated the effect of JAK inhibition in the K/BxN serum transfer model of arthritis that is driven by innate immunity and inflammatory cytokines including TNF and IL-1 β (29, 40). K/BxN arthritis is mediated by innate immune cells including MΦs and does not require T and B cells that express IL-2 receptor common γ chain sensitive to JAK3 inhibition (40, 41). Arthritis was induced by intraperitoneal injection of pooled K/BxN serum at days 0 and 2 and CP-690,550 or vehicle control treatment was started from day 1. As expected, arthritis developed rapidly in mice injected with K/BxN serum and vehicle control (Figure 6A). CP-690,550 treatment nearly completely and significantly suppressed development of arthritis as assessed by measuring joint thickness (Figure 6A) and histology of ankle joints (Figure 6B). Histological analysis revealed that CP-690,550 suppressed synovial hyperplasia, with decreased numbers of synovial lining cell layers and decreased synovial thickness (Figure 6B). Thus, inhibition of JAKs effectively suppressed the effector phase of arthritis that depends solely on innate immune mechanisms.

DISCUSSION

Several small-molecule JAK inhibitors are currently in development for therapy of RA, with CP-690,550 being in advanced stage of clinical trials. Results of multiple studies suggest that beneficial as well as adverse effects of JAK inhibitors are related to inhibition of multiple JAKs in different cell types. However, the inhibition of JAK signaling in T cells has been the main focus of research and little is known about effects of JAK inhibitors on cells of innate immune system. In this study, we demonstrated that JAK inhibitors CP-690,550 and INCB018424 can effectively suppress activation of blood-derived and RA

synovial MΦs, including a subset of inflammatory responses induced by the pathogenic cytokine TNF. In addition to interrupting an IFN-mediated autocrine loop and STAT1 that promote inflammatory chemokine production, JAK inhibitors unexpectedly suppressed late phases of NF-κB activation and of inflammatory cytokine production, while augmenting TNF-mediated induction of c-Jun and NFATc1. CP-690,550 effectively suppressed K/BxN serum transfer arthritis, which is entirely dependent on innate immune cells. Overall, our findings demonstrate that JAK inhibitors such as CP-690,550 and INCB018424 effectively inhibit human MΦs, thus identifying another cellular target for JAK inhibitory therapy. The results also suggest that inhibition of JAK-STAT signaling in innate immune cells, and attenuation of TNF responses, contributes to the efficacy of JAK inhibitors in the treatment of RA.

A key question is inhibition of which cell types and which cytokines is responsible for the therapeutic effectiveness of JAK inhibitors. Previous reports have suggested a role for inhibition of T cells and fibroblasts (16, 17, 28), and now we have added macrophages to this list. It is possible that inhibition of other innate immune cell types, such as neutrophils and mast cells, may contribute to the efficacy of CP-690,550 in K/BxN arthritis, although these cell types are not prominently regulated by JAK-STAT signaling cytokines. In terms of explaining efficacy based on which cytokine is being targeted, it is likely that inhibition of T cell γ c cytokine-JAK3 signaling contributes to the efficacy of CP-690,550, although perhaps less so with INCB018424 that is more selective for JAK1 and JAK2. Many cytokines expressed in RA synovium that act on macrophages and innate immune cells are implicated in RA pathogenesis, including IL-6, IL-15, GM-CSF, type I IFNs (IFN- α/β) and IFN- γ . Of these, IL-6 is an attractive candidate target for explaining efficacy of JAK inhibitors, as IL-6 blockade is an effective therapy for RA. However, inhibition of K/BxN arthritis, which is independent of IL-6 (41) by CP-690,550 indicates that inhibition of signaling by other cytokines contributes to the clinical efficacy of JAK inhibitors on the effector phase of arthritis. Our results raise the possibility that inhibition of TNF and IFN signaling helps explain the therapeutic efficacy of JAK inhibitors.

IFN-STAT1 signaling, as evidenced by high expression of STAT1 and IFN-target genes known as an 'IFN signature', occurs in RA synovial cells (30, 31, 42). This IFN signature is induced in RA synovial macrophages at least in part by TNF (25, 43) and may contribute to pathogenesis. One mechanism by which an IFN signature can contribute to synovitis is expression of IFN-inducible genes that promote inflammation, such as the chemokines CXCL10 and CXCL11 that were shown to be sensitive to JAK inhibitors in this study. In addition, IFN-stimulated cells and cells that express high levels of STAT1 respond more strongly to inflammatory stimuli, such as TLRs and inflammatory cytokines, and increased cytokine production associated with such enhanced responses likely contributes to disease pathogenesis (23, 30, 44). On the other hand, type I IFN has a protective role in animal models of arthritis, possibly related to inhibition of stromal and endothelial cells (39, 45–47). In most arthritis models, IFN γ also can be protective, depending on timing and context (1, 27). Thus, inhibiting IFN signaling using JAK inhibitors can have both beneficial and harmful effects relevant for RA pathogenesis. The balance between these effects, and thus the functional outcome, will be determined by the timing, context, and cell type in which JAKs are inhibited; to date it appears that JAK inhibition is overall strongly beneficial for suppressing disease activity. Interestingly, our findings showed that JAK inhibitors also partially suppressed macrophage responses to TNF, a cytokine that is clearly pathogenic in RA (1–3). This raises the question of how JAK inhibitors block cell responses to TNF, which does not signal directly by the JAK-STAT pathway. In part, JAK inhibitors worked by suppressing a TNF-IFN β -JAK-STAT1 autocrine loop that we previously described (25) and likely is operative in RA synovial macrophages (43). Among TNF-induced STAT1-target genes suppressed by JAK inhibitors, the CXCL9, 10 and 11 group of chemokines that

interacts with CXCR3 receptors on T cells has been related to pathogenesis of arthritis (31, 48). Moreover, the genes encoding these chemokines were among the genes most strongly suppressed by JAK inhibitors in RA synovial macrophages. In addition, JAK inhibitors had unexpected inhibitory effects on TNF responses, namely suppression of late phase of NF- κ B signaling and in parallel suppression of inflammatory cytokines production including IL-1 and IL-6. The suppression of *IL6* expression was especially notable in RA synovial macrophages. Thus, the efficacy of JAK inhibitors in RA may be partially explained by inhibition of innate immune cytokine production by synovial macrophages. The most likely mechanism is inhibition of a JAK-dependent priming effects that elevate STAT1 and augment inflammatory cytokine production in response to various macrophage-activating factors (44).

Our results also reveal that inhibition of JAKs, resulted in increased TNF-mediated induction of c-Jun and NFATc1, and a parallel increase in osteoclastogenesis. These results are consistent with reports that JAK-STAT signaling can inhibit osteoclastogenesis (37, 38). The results raise a cautionary note that JAK inhibition may lead to increased bone resorption in certain settings. Arguing against this possibility, results of clinical studies and animal experiments have shown a protective role of CP-690,550 against joint destruction (5, 15). This is most likely because JAK inhibitors so effectively suppress inflammation (as also observed in our experiments with the K/BxN arthritis model) that inflammation-induced factors that drive synovial osteoclastogenesis, such as RANKL, are suppressed. Alternatively, JAK inhibitors can suppress bone erosion by suppressing osteoclastogenic Th17 cells (17), and may also promote osteoblast function (49). However, the increase in NFATc1 observed in synovial macrophages treated with JAK inhibitors suggests that increased osteoclast formation might be a potential problem and careful monitoring of bone resorption is probably warranted for patients on JAK inhibitor therapy.

In conclusion, together with previous findings in T cells and synovial fibroblasts, our results indicate that JAK inhibition can affect multiple steps of RA pathogenesis by targeting cytokine and chemokine production and affecting function of innate and acquired immune cells. Suppression of expression of STAT1 and STAT1-dependent chemokines, of inflammatory cytokine production by synovial macrophages, and of TNF responses likely contributes to the beneficial effects of JAK inhibitors in RA.

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Figure 1.
Dose-dependent effect of JAK inhibitors on STAT signaling in human macrophages. Human MΦs were stimulated with IFN α (A) or IFN γ (B) or left untreated in the presence of increasing concentrations of JAK inhibitors CP-690,550 or INCB018424 for 15 min and nuclear extracts were analyzed by immunoblotting for expression and tyrosine phosphorylation of STAT proteins. Data are representative of two independent experiments.

Figure 2.

JAK inhibitors downregulate TNF-induced STAT1 activation and expression of STAT1-dependent genes. (A) Human MΦs were stimulated with TNF in the presence of increasing concentrations of CP-690,550 or INCB018424 for 6 h and expression of STAT1 dependent genes was analyzed by qPCR. Data are representative of two independent experiments. (B and C) MΦs were stimulated with TNF for indicated times in the presence or absence of JAK inhibitors and mRNA expression of STAT1-dependent chemokines (B) and interferon response genes (C) was measured by qPCR. Target gene expression is presented relative to expression in untreated cells 1h after TNF stimulation (set as 1). Results of six (CP) and five (INCB) independent experiments (mean $\pm$ SD) are shown. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (repeated-measures two-way ANOVA with Bonferroni post hoc test for multiple comparisons). (D) Human MΦs were stimulated with TNF (+) or left untreated (-) in the presence (+) or absence (-) of CP-690,550 or INCB018424 for indicated times and tyrosine phosphorylated- and total STAT1 in nuclear (upper panels) and cytoplasmic (lower panels) extracts was analyzed by immunoblotting. Data are representative of five independent experiments.

Figure 3. Jak inhibitors augment TNF-induced expression of NFATc1 and cJun, and increase formation of osteoclast-like cells. Human macrophages were stimulated with TNF (+) or left untreated (-) in the presence (+) or absence (-) of CP-690,550 or INCB018424. Nuclear extracts were analyzed for expression of NFATc1 (A) and cJun (B, left). Data are representative of 5 independent experiments. (B) Right, the intensity of cJun bands at 24 hours was quantified by densitometry. Results of five independent experiments (mean $\pm$ SEM) are shown. (C) Quantification of TNF-induced TRAP+ multinuclear cells. Cells were fixed and stained for TRAP activity after 8–12 days of culture. Each symbol indicates an independent experiment, n=11; small horizontal lines, mean; **, p<0.01 (one-way ANOVA with Dunn post hoc test). (D) Quantification of TNF-induced resorption. Results of four independent experiments (mean $\pm$ SEM) are shown; *, p<0.05 (one-way ANOVA with Dunn post hoc test).

Figure 4.

Effect of JAK inhibition on the expression of inflammatory cytokines and NF- κ B signaling. Human M Φ s were stimulated with TNF (+) or left untreated (-) in the presence (+) or absence (-) of CP-690,550 or INCB018424 for the indicated times. Data are representative of six (CP) and five (INCB) independent experiments. (A) and (B, upper panel) TNF, IL-6 and IL-1 β mRNA levels were measured by qPCR. Gene expression is presented relative to expression in untreated cells 1h after TNF stimulation (set as 1). (B) Lower panel, effects of CP-690,550 and INCB018424 on IL-1 β mRNA expression 48 h after TNF stimulation. Each symbol indicates an independent experiment; n=5; *, p<0.05 (Wilcoxon matched-pairs signed rank test). (C) Nuclear extracts were analyzed by immunoblotting for expression of NF- κ B subunits (upper panel). Data are representative of three (p-p65) and five (RelB and p52) independent experiments. The intensity of RelB and p52 bands was quantified by densitometry (lower panel). Results of five independent experiments (mean $\pm$ SEM) are shown; *, p<0.05 (one-way ANOVA with Dunn post hoc test).

Figure 5. Effects of JAK inhibitors on synovial macrophages isolated from patients with RA. CD14+ cells were isolated from synovial fluids of patients with RA and were cultured for 24 h with (+) or without (-) CP-690,550 or INCB018424. (A) Gene expression in JAK inhibitor-treated cells shown as percent of expression in untreated cells (set at 100%, shown as dotted line). Results of seven (CP) and six (INCB) independent experiments are shown (mean ± SEM). *, p<0.05 (Wilcoxon matched-pairs signed rank test). (B) Synovial MΦs isolated from patients with RA (lanes 3 –7) were treated as in (A) and nuclear extracts were analyzed by immunoblotting for expression of transcription factors. Human donor MΦs (HD) cultured with TNF (+) for 24 hours (lane 2) or 72 hours (lane 8) served as a positive control, whereas cells cultured without TNF (-) for same time (lanes 1 and 9) represent a negative control.

Figure 6.

CP-690,550 attenuates K/BxN serum induced arthritis. Arthritis was induced in C57BL/6 mice as described in *Methods*. (A) Time course of arthritis development with or without CP-690,550 treatment. Data represents means $\pm$ SEM from 5 mice in each group. *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$ (repeated-measures two-way ANOVA with Bonferroni post test for multiple comparisons). (B) Representative histology of ankle joints. Animals were sacrificed on day 9 after first serum injection, hind legs were fixed and decalcified, and ankle joint sections were stained with hematoxylin, fast green and safranin O. Original magnification, 40 $\times$. Bigger arrows, synovium; asterisks, bone; smaller arrows, cartilage.