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A Restricted Role for TYK2 Catalytic Activity in Human Cytokine Responses Revealed by Novel TYK2-Selective Inhibitors

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TYK2 is a JAK family protein tyrosine kinase activated in response to multiple cytokines, including type I IFNs, IL-6, IL-10, IL-12, and IL-23. Extensive studies of mice that lack TYK2 expression indicate that the IFN-α, IL-12, and IL-23 pathways, but not the IL-6 or IL-10 pathways, are compromised. In contrast, there have been few studies of the role of TYK2 in primary human cells. A genetic mutation at the tyk2 locus that results in a lack of TYK2 protein in a single human patient has been linked to defects in the IFN-α, IL-6, IL-10, IL-12, and IL-23 pathways, suggesting a broad role for TYK2 protein in human cytokine responses. In this article, we have used a panel of novel potent TYK2 small-molecule inhibitors with varying degrees of selectivity against other JAK kinases to address the requirement for TYK2 catalytic activity in cytokine pathways in primary human cells. Our results indicate that the biological processes that require TYK2 catalytic function in humans are restricted to the IL-12 and IL-23 pathways, and suggest that inhibition of TYK2 catalytic activity may be an efficacious approach for the treatment of select autoimmune diseases without broad immunosuppression. The Journal of Immunology, 2013, 191: 2205–2216.
to other cytokine receptors beyond the type I IFNR. In this regard, it has been suggested that TYK2 plays a similar role in the surface expression of the human IL-10R (11). The overall contribution of TYK2 catalytic activity to cytokine responses is still poorly understood, although a few studies have assessed the role of TYK2 catalytic activity in IFN signaling by reconstituting TYK2-deficient cell lines with mutant versions of TYK2 (12–16) or by generating mice that express a catalytically inactive TYK2 protein (17).

Although small-molecule inhibitors of JAK family kinases have been previously generated, most of these inhibitors are pan-JAK kinase inhibitors, because it has been difficult to generate small-molecule inhibitors that can discriminate among the few sequence differences in the JAK1, JAK2, JAK3, and TYK2 kinase pockets. Our work on small-molecule inhibitors of JAK family kinases has resulted in the identification of novel compounds that possess a broad range of selectivity against TYK2, JAK2, and JAK1. In this study, we have used these inhibitors to determine the contribution of TYK2 catalytic activity to human cytokine responses that are known to associate with and activate TYK2, particularly pertaining to inflammation. Specifically, we evaluated the IL-12 and IL-23 pathways, which activate JAK2 and TYK2, and the type I IFN, IL-6, and IL-10 pathways, which activate TYK2 and JAK1.

Materials and Methods

JAK biochemical assays

In vitro kinase activities of purified JAK1, JAK2, and TYK2 JH1 kinase domains and the K_{i} values for ATP-competitive inhibition were determined as described previously (18).

JAK reference cell assays

JAK reference cell assays for TYK2 (IL-12 phospho-STAT4 NK92), JAK1 (IL-6 phospho-STAT3 TF-1), and JAK2 (EPO phospho-STAT5 TF-1) were performed as follows. TF-1 cells (American Type Culture Collection, Manassas, VA) were starved overnight in OptimEM medium with 0.5% charcoal/dextran stripped FBS, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and without phenol red. NK92 cells (American Type Culture Collection, Manassas, VA) were starved overnight in RPMI 1640 with 10% charcoal/dextran stripped FBS. Cells were then pretreated with compounds for 20 min before the addition of activating cytokines (30 ng/ml IFN-γ, 10 U/ml EPO, or 30 ng/ml IL-12 final concentration). Cytokines were obtained from the following sources and were used at concentrations that generated ∼50–90% of the maximal cytokine-induced signal in each assay (i.e., EC_{50–90} of the cytokine for each assay readout): IL-12 (R&D Systems), IL-6 (R&D Systems), and EPO (Life Technologies). Compounds were serially diluted in DMSO and added to 100,000 cells in RPMI 1640 per well in 384-well microtiter plates to a final concentration of 0.2% DMSO. Phosphorylation of STAT1 (IL-6), STAT3 (EPO), or STAT4 (IL-12) was measured in cell lysates using Meso Scale Discovery technology (Gaithersburg, MD) using the manufacturer’s protocol. The curve fit was generated, and statistical analysis was performed using Geneda Screenware software (Genedata, Basel, Switzerland). IC_{50} values were defined as the compound concentration at which the response level was reduced to half of its maximum relative to a DMSO control.

Human primary cell assays

For all assays, cells were pretreated with compounds for 30 min before the addition of activating cytokines. For each compound, a 10-point titration was performed by serial dilution in cell culture media before addition to the cells. The curve fit was generated and statistical analysis was performed using Prism (GraphPad Software, San Diego, CA) or Excel (Microsoft, Redmond, WA) software. IC_{50} values were defined as the compound concentration at which the response level was reduced to half its maximum. In cases where the curve fit was deemed ambiguous, the concentration of the compound that induced 50% of the maximal response was calculated by interpolation. Blood samples were taken from donors from whom informed consent was obtained.

IL-12 assay. Recombinant human IL-12 (R&D Systems) was used at a concentration that generated ∼50–90% of the maximal IL-12–induced signal in the assay (i.e., EC_{50–90} of IL-12). PBMCs were incubated overnight at 37°C with 7.5 pg/ml plate-bound anti-CD3 (BD Biosciences) and 0.1 μg/ml anti-CD28 (BD Biosciences). Cells were then stimulated with recombinant human IL-12 at 12.5 ng/ml for 15 min and lysed, and phospho-STAT4 was measured in cell lysates using Meso Scale Discovery technology (Gaithersburg, MD). For the IFN-γ readout, PBMCs were stimulated with recombinant human IL-18 (MBL Laboratories) at 12.5 ng/ml and IL-12 at 0.5 ng/ml in the absence of TCR cross-linking for 24 h. Cell supernatants were collected and analyzed for IFN-γ using Meso Scale Discovery technology (Gaithersburg, MD).

IL-23 assay. IL-23 was obtained from R&D Systems and was used at a concentration that generated ∼50–90% of the maximal IL-23–induced signal in the assay (i.e., EC_{50–90} of IL-23). CD4 CD45RO+ T cells obtained from AllCells (Emeryville, CA) were cultured in Yssel’s T cell Medium containing 10 ng/ml recombinant human IL-1β (MBL Laboratories), IL-23 (R&D Systems, Minneapolis, MN), and TGFB-β1 (eBioscience, San Diego, CA) and 50 ng/ml IL-6 (R&D Systems) together with anti-CD3/CD28 Dynabeads (Invitrogen), 1 wk to induce expansion of Th17 cells. These in vitro–expanded Th17 cells were restimulated overnight in Yssel’s T Cell Medium and 1% human serum without cytokines, followed by serum starvation for 4 h in RPMI 1640 without any supplements. They were then resuspended and plated in 384-well cell culture–treated assay plates (Corning) at 200,000 cells/well in RPMI 1640. Compounds dilutions were made in DMSO using 1/2 log dilution steps, 10 points, using a Pre-cession 2000 (BioTek). After preincubation with the compounds, cells were stimulated for 15 min with recombinant human IL-23 at 12.5 ng/ml lysed using 10X Cell Lysis Buffer (Cell Signaling), and the phospho-STAT3 level was measured using Meso Scale Discovery technology (Gaithersburg, MD). For the IL-17F readout, cells were resuspended in primary cell growth medium (RPMI 1640, 10% FBS, sodium pyruvate, nonessential amino acids, HEPES pH 7.4, 10 mM 2-ME, 1% human serum without cytokines, and 50 ng/ml IL-18), plated at 200,000 cells/well in 96-well assay plates, preincubated with compounds for 30 min at 37°C, followed by stimulation with recombinant human IL-23 at 6.25 ng/ml (eBioscience) and recombinant human IL-1β at 0.1 ng/ml (R&D Systems) for 48 h. Supernatants were removed and evaluated for the IL-17F levels using an in-house–designed Meso Scale Discovery (Gaithersburg, MD) assay specific for IL-17F.

IFN-α assay. IFN-α2A was obtained from PBL IFN Source and was used at a concentration that generated ∼50–90% of the maximal IFN-α–induced signal in the assay (i.e., EC_{50–90} of IFN-α). The IFN-α response was measured using commercially available primary human fibroblasts (Invi- trogen, Grand Island, NY, and Lonza, Walkersville, MD) that had been seeded at 10,000 cells/well in 96-well flat-bottom plate, pretreated with compounds for 30 min, and incubated with recombinant human IFN-α2A (2 ng/ml; 3.4 × 10^{10} U/ml). For the phospho-STAT1 readout, cells were lysed in lysis buffer (supplied with the ELISA kit) at 30 min after IFN-α stimulation and analyzed by phospho-STAT1 (Y701) ELISA kit per manufacturer’s instructions (Cell Signaling Technology, Danvers, MA). Ms4a mRNA levels were determined at 2 h after stimulation by first converting the cells to cDNA using the Cells-to-cDNA II kit (Applied Biosystems, Foster City, CA) and then performing TaqMan analysis for Ms4a and normalizing against GAPDH as an internal control. The se- quence for Ms4a was as follows: forward primer, 5'-TGG AAG GAT GCT GTC TTC GTT-3'; reverse primer, 5'-GCA AGG TGG AGC GAT TTC-3'; and probe, 5'-CTG GGA AGG GAA TTT TCA GCC CTC-3'. The sequence for RPL19 was as follows: forward primer, 5'-AGC GAG TTC TCA TGG AAC A-3'; reverse primer, 5'-CTG GTC AGC CAG GAG CTT-3'; and probe, 5'-TCC ACA AGC TGA AGG CAG ACA AGG-3'. The relative quantity (RQ) values were calculated as the ratio of normalized change in cycle threshold (delta cycle threshold [dCT]) values of IFN-α–stimulated versus unstimulated samples.

IL-6 assay. IL-6 was obtained from R&D Systems and was used at a concentration that generated ∼50–90% of the maximal IL-6–induced signal in the assay (i.e., EC_{50–90} of IL-6). The IL-6 assay was performed with primary human hepatocytes (Celsys) that were seeded at 50,000/well in collagen-coated plates (Becton-Dickinson) before stimulation with human recombinant IL-6 at 10 ng/ml in serum-free media. Phospho-STAT3 signal was measured at 30 min after stimulation by ELISA (Cell Signaling Technologies). For the human C-reactive protein (CRP) readout, the culture supernatants were collected at 18 h after stimulation and ana- lyzed by human CRP ELISA (R&D Systems).

IL-10 assay. IL-10 was obtained from R&D Systems and was used at a concentration that generated ∼50–90% of the maximal IL-10–induced signal in the assay (i.e., EC_{50–90} of IL-10). The IL-10 assay was performed with human monocyte-derived dendritic cells (MDDCs), differentiated from monocytes isolated from buffy coat and cultured in vitro in the presence of IL-4 (20 ng/ml) and GM-CSF (10 ng/ml) for 5 d. For phospho-STAT3 readout, cells were seeded at 50,000/well in 96-well plates and

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stimulated with recombinant human IL-10 at 3 ng/ml for 30 min after pretreatment with the compounds, lysed, and analyzed by phospho-STAT3 ELISA. For the TNF-\(\alpha\) readout, IL-10 stimulation was prolonged to 24 h, followed by LPS treatment at 50 ng/ml for an additional 24 h, and the culture supernatants were harvested and analyzed by ELISA (R&D Systems). PD-L1 expression was analyzed by FACS on MDCCs that had been stimulated overnight with IL-10 (50 ng/ml), stained with anti–PD-L1 Ab conjugated to PE (eBioscience), and fixed in 1% paraformaldehyde. The percent inhibition was calculated using the following formula: % inhibition = (max – experimental)/(max – min) \(\times 100\), where max = % PD-L1+ in IL-10–stimulated cells without the compound and min = % PD-L1+ in unstimulated cells (no compound).

**IL-22 assay.** IL-22 was obtained from R&D Systems and was used at a concentration that generated ~50–90% of the maximal IL-22–induced signal in the assay (i.e., EC_{50–90} of IL-22). The IL-22 assay was performed with commercially purchased primary human keratinocytes (Lonza) that were seeded at 25,000/well in 96-well plates, stimulated with recombinant human IL-22 at 100 ng/ml for 30 min, lysed, and analyzed using the phospho-STAT3 ELISA kit.

**Phospho-STAT and total STAT immunoblots**

Primary human fibroblast (for IFN-\(\alpha\)2A), hepatocytes (for IL-6), or monocyte-derived DCs (for IL-10) were rested in serum-free media for 2 h, treated with the indicated compounds for 30 min, stimulated with appropriate cytokines for another 30 min, and immediately lysed in lysis buffer (Cell Signaling Technology). For each compound, two concentrations (0.04 and 3\(\mu\)M) were tested based on the values that approximate IC_{50} and IC_{\text{50}} values for INCB018424 in human PBMC IL-6 pSTAT3 assay (data not shown), respectively. For IL-12 phospho-STAT4 immunoblot, purified CD4 T cells were preactivated with anti-CD3/CD28 activation beads (Invitrogen) for 2 d, detached from the beads, and rested for 2 h before IL-12 stimulation. Equivalent amounts of lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and blotted with anti-pSTAT1 (Cell Signaling Technology), anti-pSTAT3 (Cell Signaling Technology), anti-pSTAT4 (BD Biosciences Pharmingen), or anti-total STAT1 (Cell Signaling Technology), STAT3 (Cell Signaling Technology), or STAT4 (R&D Systems) Abs, probed with HRP-conjugated secondary Ab (Cell Signaling Technology), and developed by ECL Plus Western blotting Detection Reagents (GE Healthcare Life Sciences).

**In vitro mouse cell assays**

For mouse IL-6 and IL-10 assays, bone marrow cells from B10.Q/Ai tyk2 wild-type strain, Taconic) or B10.D1-H2q/SgJ (tyk2 knockout strain; Jackson Laboratory) mice were cultured in the presence of recombinant mouse M-CSF (R&D Systems) at 20 ng/ml for 6 d before initiating IFN-\(\gamma\) treatment. The general format of the JAK reference cell line assays is the inhibition of a JAK kinase-dependent phospho-STAT signal in a transformed human cell line upon stimulation with a specific cytokine. Previous studies have implicated JAK1 in the phosphorylation of STAT3 downstream of IL-6 stimulation (19), JAK2 in the phosphorylation of STAT5 downstream of EPO stimulation (20), and TYK2 in the phosphorylation of STAT4 downstream of IL-12 stimulation (21, 22). Therefore, we developed and tested JAK1, JAK2, and TYK2 reference cell assays consisting of IL-6–induced phospho-STAT3 in TF-1 cells, EPO-induced phospho-STAT5 in TF-1 cells, and IL-12–induced phospho-STAT4 in NK92 cells, respectively. By using a phospho-STAT readout that is directly downstream of a JAK family kinase in a cell line, we were able to minimize assay variation and could determine robust cellular IC_{50} values for each of the small-molecule compounds in our reference panel. We confirmed that the IC_{50} values for the reference compounds in each JAK kinase reference cell assay correlated well with the IC_{50} values for the same JAK kinase in a biochemical assay, but not with the IC_{50} values for the other JAK kinases in biochemical assays (Fig. 1). Thus, the IC_{50} values determined for small molecule compounds in our reference cell assays can be used as benchmark cellular potencies for each of the JAK family kinases JAK1, JAK2, and TYK2.

**Effects of TYK2 versus JAK2 inhibition on IL-12 and IL-23 signaling**

The IL-12R and IL-23R share a common subunit, IL-12R\(\beta\)1, which associates with TYK2. Their unique subunits, IL-12R\(\beta\)2 and IL-23R, respectively, associate with JAK2 (23–25). To determine the relative contributions of TYK2 and JAK2 to aspects of IL-12R and IL-23R signaling, we evaluated the ability of a second panel of specific JAK kinase inhibitors with varying selectivity for TYK2 and JAK2 to suppress IL-12 and IL-23 responses. The 30 compounds in this TYK2/JAK2 kinase panel represent 6 chemically distinct series and cores, are selective against other kinases in the human genome as determined by profiling of a subset of representative compounds against panels of 28–286 human kinases, and include potent inhibitors of TYK2 kinase activity as determined in biochemical assays (Supplemental Table II). In addition, the cellular IC_{50} values for these compounds in the JAK kinase reference cell line assays correlated well with the biochemical IC_{50} values determined for our 30 compounds (log[TYK2 IC_{50}] versus log[JAK2 IC_{50}], \(R^2 = 0.82;\) data not shown). Calculated cellular JAK2:TYK2 selectivity indices of the compounds range from 25-fold enhanced potency for JAK2 over TYK2 to 139-fold enhanced potency for TYK2 over JAK2 (Supplemental Table II). A pan-JAK inhibitor INCB018424 (26) was included as a control compound. Although highly selective against other kinases in the human genome, INCB018424 is relatively nonselective against JAK kinase family members, exhibiting similar cellular potencies against JAK1, JAK2, and TYK2 (Supplemental Table II).

We next tested the TYK2/JAK2 compound panel for its activities on IL-12 and IL-23 responses in primary human cells. We measured a receptor-proximal phospho-STAT readout and a downstream biological readout of IL-12 and IL-23 signaling, to confirm that inhibition of the JAK-STAT module translated to suppression of relevant biological responses. For the IL-12 pathway, we measured phospho-STAT4 and IFN-\(\gamma\) production (21, 22, 27, 28) in PBMCs from multiple donors (Fig. 2, Supplemental Table III). As shown in Fig. 2A and 2B, compound IC_{50} values in this primary cell assay show significantly better correlation with TYK2 reference cell line IC_{50} values than JAK2 reference cell line IC_{50}.
values ($R^2 = 0.63$ versus 0.28 for TYK2 versus JAK2 for the phospho-STAT4 readout, Fig. 2A; and $R^2 = 0.82$ versus 0.042 for TYK2 versus JAK2 for the IFN-γ readout; Fig. 2B). Moreover, in the PBMC assay, the phospho-STAT4 IC$_{50}$ values of the compounds showed a correlation with the IFN-γ IC$_{50}$ values ($R^2 = 0.60$, Fig. 2C), confirming that IL-12–driven STAT4 phosphorylation and activation is linked to the induction of the ifng gene. As a control, we also confirmed that INCB018424 and the TYK2-selective compound 26, but not the JAK1/2 compound 1, inhibited IL-12–induced phospho-STAT4 by Western blot analysis (Fig. 2D). These data suggest that in primary human cells, IL-12–induced phosphorylation of STAT4 and IFN-γ production is driven by TYK2 rather than JAK2 catalytic activity, because inhibition of both events is well correlated with TYK2 catalytic inhibition but is poorly correlated with JAK2 catalytic inhibition.

For the IL-23 pathway, we measured phospho-STAT3 and IL-17F production (24, 29) in primary human memory CD4 T cells from multiple donors that had been expanded under conditions that support the maintenance of Th17 cells (Fig. 3, Supplemental Table III). The IC$_{50}$ values of the compounds in the Th17 phospho-STAT3 and Th17 IL-17F assays showed a slightly better correlation with the IFN-γ IC$_{50}$ values ($R^2 = 0.62$ for the phospho-STAT3 readout and $R^2 = 0.77$ for the IL-17F readout) compared with TYK2 reference cell line IC$_{50}$ values ($R^2 = 0.43$ for the phospho-STAT3 readout and $R^2 = 0.40$ for the IL-17F readout; Fig. 3A, 3B). The modest correlation of IL-23–driven phosphorylation of STAT3 and IFN-γ production is regulated by TYK2 catalytic activity, consistent with previous studies of TYK2-deficient mice and a TYK2-deficient human patient. However, in contrast with the IL-12 pathway readouts, JAK2 catalytic activity may also contribute to the IL-23 pathway readouts.

Effects of TYK2 versus JAK1 inhibition on type I IFN signaling

In addition to having impaired responses to IL-12 and IL-23, cells from a TYK2-deficient human patient did not respond to stimulation with type I IFNs, IL-6, and IL-10 (9, 30). In contrast, mice deficient in TYK2 protein show reduced responsiveness to type I IFNs and normal responses to IL-6 and IL-10 (6, 7). We therefore investigated whether inhibition of TYK2 catalytic activity was sufficient to block responses to these cytokines in primary human cells. Because the receptors for type I IFNs, IL-6, and IL-10 associate with and activate TYK2 and JAK1 (31–34), rather than TYK2 and JAK2, we used a group of six compounds with clear selectivity profiles for JAK1 versus TYK2 or vice versa (Supplemental Table II). All six JAK1- and TYK2-selective compounds were selective against other kinases in the human genome as determined by profiling against panels of 28–286 human kinases. INCB018424 was included as a pan-JAK control compound.

The JAK1-selective compounds were 34- to 80-fold selective for JAK1 and were potent inhibitors of the JAK1 reference cell assay (Supplemental Table II) but did not significantly inhibit IL-12–
induced IFN-γ in PBMCs (Supplemental Table III). One of the JAK1-selective compounds (compound 36) also did not significantly inhibit IL-12–induced phospho-STAT4 in PBMCs, although the other two compounds had some inhibitory effects (Supplemental Table III). The TYK2-selective compounds were >100-fold selective for TYK2 and were potent inhibitors of the TYK2 reference cell assay (Supplemental Table II), as well as IL-12–induced phospho-STAT4 and IFN-γ in PBMCs (Supplemental Table III). These TYK2/JAK1 compounds enabled us to evaluate whether inhibition of TYK2 and/or JAK1 catalytic activity affected type I IFN, IL-6, and IL-10 signaling, and were grouped into three pairs, with each pair containing a TYK2-selective compound and a JAK1-selective compound that had similar inhibitory potencies against TYK2 and JAK1 in reference cell assays. By pairing the JAK1- and TYK2-selective compounds in this way, we could assess whether both JAK1 and TYK2 kinase activities contributed to a signaling readout by comparing the inhibitory activity of each individual compound with that of a combined treatment with both compounds. If both JAK1 and TYK2 kinase activities contribute to a signaling readout, the IC_{50} for inhibition using a combined treatment with both inhibitors should be shifted to lower a concentration compared with the IC_{50}s for inhibition by each individual inhibitor.

Type I IFNs promote antiviral activities through a heterodimeric receptor complex composed of IFNAR1 and IFNAR2 (35). We measured the phosphorylation of STAT1 and the induction of MxA mRNA levels, as a surrogate for antiviral responses (36), in primary human fibroblasts stimulated with IFN-α (Fig. 4, Supplemental Table IV). As shown in Fig. 4A, the pan-JAK control compound INCB018424 blocks IFN-α–induced phospho-STAT1 and MxA in a concentration-dependent manner, confirming the requirement of JAK kinases in IFN-αR signaling. However, in each of the three different pairs of TYK2- or JAK1-selective compounds we tested, only the JAK1-selective and not the TYK2-selective compounds inhibited the response (Fig. 4B–D). Moreover, combination treatment with JAK1- and TYK2-selective compounds did not lead to more potent inhibition compared with the JAK1-selective compound alone, suggesting that IFN-α-in-

**FIGURE 2.** Inhibition of primary human PBMC responses to IL-12 by TYK2- and JAK2-selective compounds. Log(IC_{50}) values for compound inhibition of (A) phospho-STAT4 and (B) IFN-γ production in IL-12-stimulated PBMCs are plotted against log(IC_{50}) values for inhibition of TYK2 or JAK2 reference cell assays. (C) Log(IC_{50}) values for compound inhibition of phospho-STAT4 are plotted against log(IC_{50}) values for compound inhibition IFN-γ production in IL-12–stimulated human PBMCs. (D) Western blot analysis of INCB018424, JAK1/2-selective compound 1, and TYK2-selective compound 26 inhibition of IL-12–induced phospho-STAT4 in primary human CD4 T cells.
duced phosphorylation of STAT1 and MxA transcription is driven by JAK1 and not by TYK2 catalytic activity. Western blot analysis confirmed the inhibition of IFN-α–induced phospho-STAT1 by INCB018424 and JAK1-selective compound 35, but not TYK2-selective compound 31 (Fig. 4E).

**Effects of TYK2 versus JAK1 inhibition on IL-6 signaling**

IL-6 mediates an acute inflammatory response through its receptor complex, which consists of gp130 and IL-6Rα (37, 38). Activation of the IL-6R induces phosphorylation of JAK1, JAK2, and TYK2 in human cells (19). We measured IL-6–dependent phospho-STAT3 responses and CRP production by primary human hepatocytes (39) and evaluated the effect of JAK1- or TYK2-selective compounds on these responses (Fig. 5, Supplemental Table IV). As shown in Fig. 5A, INCB018424 inhibited phospho-STAT3 and CRP induction, as did all three JAK1-selective compounds (Fig. 5B–D). In contrast, the TYK2-selective compounds showed much weaker activities and failed to completely inhibit phospho-STAT3 or CRP induction in the concentration range tested (Fig. 5B–D). Combination treatment with JAK1- and TYK2-selective compounds did not potentiate the JAK1-selective compound-mediated inhibition, suggesting that IL-6–induced phosphorylation of STAT3 and CRP production is driven by JAK1 and not TYK2 catalytic activity. Western blot analysis confirmed the inhibition of IL-6–induced phospho-STAT3 by INCB018424 and JAK1-selective compound 35, but not TYK2-selective compound 31 (Fig. 5E).

**Effects of TYK2 versus JAK1 inhibition on IL-10 cytokine family signaling**

IL-10 mediates anti-inflammatory responses through a heterotetrameric receptor composed of two molecules each of IL-10R1, which associates with JAK1, and IL-10R2, which associates with TYK2 (40). The main biological effects of IL-10 are mediated via phosphorylation of STAT3 (41), which is required for suppression of LPS-induced TNF-α production in myeloid cells (40, 42). We evaluated the effects of our inhibitors on IL-10 responses by measuring inhibition of the phospho-STAT3 signal and restoration.
of LPS-induced TNF-α production in primary human MDDCs (Fig. 6, Supplemental Table IV). As shown in Fig. 6A, INCB018424 inhibited IL-10–induced phospho-STAT3 and IL-10–mediated suppression of LPS-induced TNF-α induction, leading to a restoration of TNF-α production. In each of the JAK1- and TYK2-selective compound pairs, only the JAK1 compounds affected IL-10-induced readouts, and combination treatment with both JAK1- and TYK2-selective compounds did not lead to more potent inhibition of the IL-10-induced readouts compared with treatment with the JAK1-selective compounds alone (Fig. 6B–D). Western blot analysis confirmed the inhibition of IL-10–induced phospho-STAT3 by INCB018424 and JAK1-selective compound 35, but not TYK2-selective compound 31 (Fig. 6E).

IL-10 also induces surface expression of immune-regulatory molecules such as PD-L1, which suppresses T cell activation via interaction with the inhibitory receptor PD1 (43). We further assessed the activity of our compounds on IL-10–induced PD-L1 induction in MDDCs (Fig. 6F; 6G), choosing two concentrations of compound treatments that had resulted in half-maximal and near-complete inhibition of IL-10–induced phospho-STAT3 for the JAK1-selective compounds. Based on the percentages of cells that upregulated PD-L1 after IL-10 stimulation with or without compound treatment, we calculated a percent inhibition of IL-10–induced PD-L1 upregulation achieved by the JAK1-selective, TYK2-selective, or a combination of both JAK1- and TYK2-selective compounds (Fig. 6F). Consistent with the results from the phospho-STAT3 and TNF-α readouts of IL-10 signaling, JAK1-selective inhibitors suppressed IL-10–mediated PD-L1 induction, whereas TYK2-selective inhibitors had minimal effects. No additional effects were observed upon combining JAK1- and TYK2-selective compounds. Our results indicate that JAK1, but not TYK2, catalytic activity is the main driver of IL-10–induced phosphorilation of STAT3, suppression of LPS-induced TNF-α production, and PD-L1 upregulation in primary human myeloid cells.

IL-22 is another member of the IL-10 cytokine family that acts on multiple cell types to promote inflammation and tissue repair at barrier surfaces (44, 45). The receptor for IL-22 consists of the IL-10R2 subunit of the IL-10R and a specific receptor IL-22R1, which associate with TYK2 and JAK1, respectively (46–48). We tested the effects of our compounds on IL-22–induced phosphorilation of STAT3, suppression of LPS-induced TNF-α production, and PD-L1 upregulation in primary human myeloid cells.

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TYK2-selective compounds failed to further enhance the inhibitory effects of the JAK1-selective compounds. Thus, similar to the IL-10 pathway, JAK1, but not TYK2, catalytic activity appears to mediate IL-22–induced phosphorylation of STAT3.

Effects of TYK2 and JAK1 compounds on mouse IL-6 and IL-10 signaling

We confirmed that, similar to its effect on primary human cells, the TYK2-selective compound 31 did not inhibit IL-6– or IL-10–induced phospho-STAT3 in bone marrow–derived macrophages from TYK2 wild-type mice (Fig. 8). In addition, the JAK1-selective compound 35 inhibited both IL-6– and IL-10–induced phospho-STAT3 (green), and a combination of compounds 33 and 36 (black). Data are mean ± SEM of duplicate samples and are representative of four independent experiments, each using a different donor. (E) Western blot analysis of INCB018424, JAK1-selective compound 35, and TYK2-selective compound 31 inhibition of IL-6–induced phospho-STAT3 in primary human hepatocytes.

Discussion

To our knowledge, our studies are the first to assess the role of TYK2 catalytic function in the signaling of IL-12, IL-23, IFN-α, IL-6, IL-10, and IL-22 in primary human cells. Using a panel of novel small-molecule inhibitors of TYK2 with varying selectivities against JAK1 or JAK2, we have dissected the contribution of TYK2, JAK1, and JAK2 catalytic activity to these cytokine pathways. In our assays, we find that TYK2 catalytic activity is required for major signaling events downstream of IL-12 and IL-23, but does not appear to contribute significantly to signaling events downstream of IFN-α, IL-6, IL-10, and IL-22, which depend on JAK1 catalytic activity instead. Interestingly, JAK2 catalytic activity may contribute to IL-23, but not IL-12, signaling in our assays, because the inhibitor activity of our compounds against IL-23–induced phospho-STAT3 and IL-17F production correlates with their inhibitory activity against both TYK2 and JAK2 kinase activity in reference cell assays. In contrast, the inhibitory activity of our compounds against IL-12–induced phospho-STAT4 and IFN-γ production correlates strongly with their inhibitory activity against TYK2 kinase activity, but very poorly with their inhibitory activity against JAK2 kinase activity in reference cell assays. Importantly, we performed all of our experiments on primary human cells from multiple donors and assessed both proximal end points (phosphorylation of STAT proteins) and downstream biological end points associated with each cytokine signaling pathway. In addition, although others have generated pan-JAK inhibitors, the broad range and differences in selectivities of our compounds against the various members of the JAK kinase family have enabled us to specifically assess the contributions of TYK2 kinase activity (versus other JAK family kinases) to various cytokine signaling pathways. Thus, our compounds may be more broadly useful for elucidating the contributions of the kinase activity of the various JAK family kinases to other cytokine signaling pathways.

In our studies, we chose to assess the major STAT protein and a significant and commonly studied downstream biological re-
response for each of the cytokine pathways, but we did not determine the effects of our compounds on the phosphorylation of all STAT proteins or all biological responses downstream of IL-12, IL-23, IFN-α, IL-6, IL-10, and IL-22 signaling. In addition,
we assessed the effects of our compounds at a single point in time and did not perform a comprehensive kinetic analysis of each assay. Thus, it remains possible that JAK2 catalytic activity may contribute to some aspects of IL-12 signaling that we have not assessed, and TYK2 catalytic activity may contribute to some aspects of IFN-α, IL-6, IL-10, and/or IL-22 signaling that we have not assessed.

Our studies of the role of TYK2 catalytic activity in various human cytokine signaling pathways are consistent with studies of TYK2-deficient mice, but differ from the roles for TYK2 deduced from studies of a single TYK2-deficient human patient. TYK2 deficiency results in pathway-specific impairment of immune responses in mice, characterized by a reduced ability of IFN-α to induce type I IFN-responsive genes (6) and antiviral responses (7), an inability of IL-12 to induce IFN-γ production from T cells and NK cells (6, 7), and an inability of IL-23 to induce IL-17 production from T cells (49). However, the IL-10 responses of T cells and myeloid cells (6, 7) and the IL-6 responses of T cells (7) remain intact in TYK2-deficient mice. In humans, TYK2 deficiency has also been associated with the abrogation of IL-12 and IL-23 responses in a single patient who exhibited hyper-IgE syndrome (9). However, this patient was also shown to have additional defects in type I IFN, IL-6, and IL-10 signaling (30), suggesting that TYK2 deficiency may affect a broader group of cytokines in humans than in mice.

Possible explanations for the discrepancy between our studies and those of the TYK2-deficient hyper-IgE syndrome patient include a difference in scaffold versus catalytic function of TYK2, developmental alterations in cytokine signaling pathways in the TYK2-deficient patient, and/or additional unknown mutations in the TYK2-deficient patient that confer broader effects than deficiency of TYK2 alone. A scaffold function for TYK2 has previously been demonstrated for type I IFN and IL-10Rs in human cell lines, where full-length TYK2 protein was required, but the catalytic activity of TYK2 was dispensable for cell-surface receptor expression (11). In addition, a study of two rare disease-associated human TYK2 variants found that although these variants lack catalytic activity, STAT protein phosphorylation downstream of IL-6, IFN-α, and IL-10 signaling is intact in a human cell line reconstituted with the mutant TYK2 proteins and in EBV-transformed B cells from patients harboring the mutant TYK2 proteins (16). In contrast, a study of mice engineered to express a catalytically inactive TYK2 protein found impaired IFN-α signaling similar to that of TYK2-deficient mice; however, this was likely a consequence of a significant reduction in the total level of TYK2 protein in these mice (17). Interestingly, a recent report of a second TYK2-deficient patient described a clinical phenotype that differs from that of the first TYK2-deficient patient (10), suggesting that at least one of the TYK2-deficient patients may have additional mutations that impact on their
phenotypes in addition to their TYK2 deficiency. Finally, we find that a JAK1-selective compound, but not a TYK2-selective compound, inhibits IL-6–induced phospho-STAT3 and IL-10–induced phospho-STAT3 in primary mouse cells and confirm that these readouts are also not affected in TYK2-deficient mice (Fig. 8). This is consistent with the previous studies of TYK2-deficient mice and further validates these compounds as JAK1-selective and TYK2-selective inhibitors. The effects and IC₅₀ values of these compounds in primary mouse assays are comparable with those observed in primary human cells (compare Fig. 8A and 8B versus Figs. 5B and 6B), further supporting our conclusion that these pathways are dependent on the catalytic activity of JAK1, but not TYK2, in human cells.

In our studies of IFN-α, IL-6, IL-10, and IL-22 signaling, the IC₅₀ values for the inhibition of STAT protein phosphorylation by the JAK1-selective compounds in our primary human cell assays are similar to the IC₅₀ values for inhibition of STAT protein phosphorylation in the JAK1 reference cell assay. Together with a lack of significant inhibitory activity of the TYK2-selective compounds in the primary cell assays and the superposition of the inhibitory curves for combined treatment with JAK1- and TYK2-selective compounds together versus treatment with the JAK1-selective compound alone, these results indicate that JAK1 kinase activity and not TYK2 kinase activity is important for these signaling events. However, in our IFN-α, IL-6, IL-10, and IL-22 assays, we sometimes observe a partial inhibition of readouts by our TYK2-selective compounds, resulting in up to 50% inhibition of assay readouts at the highest compound concentrations. These effects occur at concentrations of the TYK2-selective compounds that are much higher than their cellular potencies against TYK2 in the TYK2 reference cell assay. This inhibition of assay readouts at very high concentrations of the TYK2-selective compounds may thus be caused by partial inhibition of JAK1 kinase activity and/or off-target effects of the compounds at these concentrations.

We also observe a significant shift in the IC₅₀ values for the inhibition of proximal phosphorylation of STAT proteins versus downstream biological end points by the JAK1-selective compounds in our IFN-α and IL-10 assays, but not in our IL-6 assay. This shift in the IC₅₀ values between the JAK kinase-proximal and -distal end points may be because of signal amplification and/or redundancy of signaling inputs to the downstream biological end points, as compared with the proximal phospho-STAT end points, as has been discussed previously (50). For the IL-10 assay, given the significant differences between IC₅₀ values for STAT3 phosphorylation versus suppression of LPS-induced TNF-α production, we also assessed compound effects on IL-10–induced PD-L1 upregulation and found that this end point was inhibited at lower concentrations of compounds and, similar to suppression of LPS-induced TNF-α production, was dependent on JAK1 kinase activity. Importantly, all of the IL-10– and IFN-α–induced biological end points that we assessed were inhibited by the JAK1-selective but not by the TYK2-selective compounds, regardless of shifts in IC₅₀ values.

The requirement of TYK2 catalytic activity in IL-12 and IL-23 signaling, but not in IFN-α, IL-6, IL-10, or IL-22 signaling, indicates that small-molecule inhibitors of TYK2 catalytic activity may exert selective inhibitory effects on IL-12 and IL-23 signaling without broad-based immunosuppression. Genetic studies implicate IL-12 and IL-23 in the pathogenesis of psoriasis (51) and inflammatory bowel disease (IBD) (52, 53), and increased activity of IL-12 and IL-23 is observed in patients with these diseases (54–56). Moreover, inhibition of IL-12 and/or IL-23 is efficacious in preclinical models of psoriasis and IBD (55, 57), and mAbs against the shared p40 subunit of IL-12 and IL-23 are efficacious for the treatment of psoriasis (58–60) and have shown some clinical efficacy in the treatment of IBD (61, 62). Thus, our studies indicate that selective TYK2 small-molecule inhibitors may be used as promising therapies for the treatment of psoriasis and IBD.

Acknowledgments

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Disclosures

S.J.S., K.B., A.V.A., C.C., P.B.K., H.K.S., J.S., Y.L., A.Z.B., N.L.K.-H.S., D.A.R.J., N.G.M., J.Z.L., W.S.B., S.M., and L.C.W. are or were fully employed by Genentech during the course of these studies and hold equity in the Roche group. W.Y., K.W., C.M., C.A.H., and J.J.K. are employed by ChemPartner or Argenta and synthesized and delivered materials to Genentech under an arrangement funded by Genentech.

References


**Supplementary Table I. Biochemical Kᵢ values and biochemical JAK kinase selectivity for 51 reference JAK kinase inhibitors.**

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\( ^a \)Values represent individual Ki values or mean Ki values from 2-11 experiments.

\( ^b \)Approximate biochemical JAK enzyme selectivities are designated. Selectivity for a particular JAK kinase is defined as \( \geq 5x \) decrease in potency for the remaining two JAK kinases.
## Supplementary Table II. Biochemical $K_i$ values, cellular $IC_{50}$ values, and cellular JAK2:TYK2 or JAK1:TYK2 selectivity indices for 36 JAK kinase-specific inhibitors and control inhibitor INCB018424.

<table>
<thead>
<tr>
<th>Compound</th>
<th>JAK2 $K_i$ (μM)</th>
<th>JAK1 $K_i$ (μM)</th>
<th>TYK2 $K_i$ (μM)</th>
<th>JAK2 reference cell $IC_{50}$ (μM)</th>
<th>JAK1 reference cell $IC_{50}$ (μM)</th>
<th>TYK2 reference cell $IC_{50}$ (μM)</th>
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<td>IC50 (μM)</td>
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The JAK2:TYK2 cellular selectivity index is the IC₅₀ value for the JAK2 reference cell assay divided by the IC₅₀ value for the TYK2 reference cell assay.

The JAK1:TYK2 cellular selectivity index is the IC₅₀ value for the JAK1 reference cell assay divided by the IC₅₀ value for the TYK2 reference cell assay.
Supplementary Table III. Reference cell line JAK2 and TYK2 IC\textsubscript{50} values and primary cell IC\textsubscript{50} values for IL-12-induced phospho-STAT4, IL-12-induced IFN\textgamma, IL-23-induced phospho-STAT3, and IL-23-induced IL17F for 30 JAK kinase-specific inhibitors.

<table>
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<th>Compound</th>
<th>JAK2 reference cell IC\textsubscript{50} (\textmu M)</th>
<th>TYK2 reference cell IC\textsubscript{50} (\textmu M)</th>
<th>IL-12-induced PBMC pSTAT4 IC\textsubscript{50} (\textmu M)</th>
<th>IL-12-induced PBMC IFN\textgamma IC\textsubscript{50} (\textmu M)</th>
<th>IL-23-induced Th17 pSTAT3 IC\textsubscript{50} (\textmu M)</th>
<th>IL-23-induced Th17 IL17F IC\textsubscript{50} (\textmu M)</th>
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<td>0.36</td>
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<td>≥10</td>
<td>≥10</td>
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<td>0.23</td>
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<td>JAK1 reference cell IC₅₀ (μM)</td>
<td>TYK2 reference cell IC₅₀ (μM)</td>
<td>IL-12-induced PBMC pSTAT4 IC₅₀ (μM)</td>
<td>IL-12-induced PBMC IFNγ IC₅₀ (μM)</td>
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<td>----------</td>
<td>-----------------------------</td>
<td>-------------------------------</td>
<td>------------------------------------</td>
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*Compounds that are shown in Figure 3C.*
Supplementary Table IV. Reference cell line JAK1 and TYK2 IC$_{50}$ values and primary cell IC$_{50}$ values for IFNα, IL-6, IL-10, and IL-22 assays for three JAK1-selective inhibitors, three TYK2-selective inhibitors, and control inhibitor INCB018424. Donors are not the same across different assays.

<table>
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<th>Compound</th>
<th>JAK1 reference cell IC$_{50}$ (μM)</th>
<th>TYK2 reference cell IC$_{50}$ (μM)</th>
<th>Donor</th>
<th>IFNα-induced pSTAT1 IC$_{50}$ (μM)</th>
<th>IFNα-induced MxA IC$_{50}$ (μM)</th>
<th>IL-6-induced pSTAT3 IC$_{50}$ (μM)</th>
<th>IL-6-induced CRP IC$_{50}$ (μM)</th>
<th>IL-10-induced pSTAT3 IC$_{50}$ (μM)</th>
<th>IL-10-induced TNFα IC$_{50}$ (μM)</th>
<th>IL-22-induced pSTAT3 IC$_{50}$ (μM)</th>
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<td>Ruxolitinib (INCB018424)</td>
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<td>0.05715 ± 0.03708</td>
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<td>&gt;10</td>
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<td>0.2485 ± 0.1566</td>
<td>0.3086 ± 0.2422</td>
<td>1.746 ± 0.604</td>
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N.D., no data (i.e., IC<sub>50</sub> cannot be determined due to poor data quality).

<sup>a</sup>Manual determination of IC<sub>50</sub> (the concentration of compound at which the response is half-maximal), instead of by curve fit.