Review

The mechanism of action of tofacitinib – an oral Janus kinase inhibitor for the treatment of rheumatoid arthritis

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Key words: tofacitinib, rheumatoid arthritis, Janus kinase, cytokines, immune response

ABSTRACT

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterised by infiltration of immune cells into the affected synovium, release of inflammatory cytokines and degradative mediators, and subsequent joint damage. Both innate and adaptive arms of the immune response play a role, with activation of immune cells leading to dysregulated expression of inflammatory cytokines. Cytokines work within a complex regulatory network in RA, signalling through different intracellular kinase pathways to modulate recruitment, activation and function of immune cells and other leukocytes. As our understanding of RA has advanced, intracellular signalling pathways such as Janus kinase (JAK) pathways have emerged as key hubs in the cytokine network and, therefore, important as therapeutic targets. Tofacitinib is an oral JAK inhibitor for the treatment of RA. Tofacitinib is a targeted small molecule, and an innovative advance in RA therapy, which modulates cytokines critical to the progression of immune and inflammatory responses. Herein we describe the mechanism of action of tofacitinib and the impact of JAK inhibition on the immune and inflammatory responses in RA.

Introduction

Rheumatoid arthritis: a chronic inflammatory disease characterised by a dysregulated and autoimmune response

Rheumatoid arthritis (RA) is a chronic inflammatory, systemic autoimmune disease that damages the joints of the body. An inflamed synovium is the hallmark of RA, characterised by synovial intimal lining hyperplasia with infiltration of immune cells, release of inflammatory cytokines and degradative mediators, and subsequent joint damage (1, 2). Immune and inflammatory responses are the driving forces in RA and transform the synovial membrane into an inflammatory tissue capable of invading and destroying adjacent cartilage and bone (3, 4).

Cytokines: regulators of immune and inflammatory responses

Numerous cytokines, involved in both innate and adaptive immunity, are implicated in the pathogenesis of RA (5). Cytokines are small protein messengers that mediate communication between cells (5). Cytokines regulate a variety of bodily processes, including haematopoiesis, and are particularly important in the regulation of immunity and inflammation (6). Cytokines bind to cell-surface receptors to initiate a cascade of signalling events that transmit information intracellularly and coordinate the cellular response (7). Type I and type II cytokine receptors lack intrinsic kinase activity and rely on associated tyrosine kinases, such as Janus kinases (JAKs), for intracellular signalling (8).

Cytokines are involved in each step of the pathogenesis of RA. They promote autoimmunity, maintain chronic inflammatory synovitis and drive the destruction of joint tissue (2). Key cytokines in RA pathogenesis include: interferon (IFN)α, IFNβ; interleukin (IL)-1, IL-6, IL-7, IL-10, IL-12, IL-15, IL-17, IL-18, IL-21, IL-23; transforming growth factor (TGF)-β; and tumour necrosis factor (TNF) (Fig. 1A) (2, 9). A subset of these cytokines, important to both innate and adaptive immune responses in RA, utilise JAK pathways to transmit signals. These cytokines include: IFNα, IFNβ, IL-6, IL-7, IL-10, IL-12, IL-15, IL-21 and IL-23 (Fig. 1A) (2, 8, 9).

A

Cytokines	JAKs
IL-7	1 3
IL-15	1 3
IL-21	1 3
IL-6*	1 2 T2
Type I IFNs (IFNα/IFNβ)	1 T2
IL-10	1 T2
IL-12	2 T2
IL-23	2 T2
IL-1	-
IL-17	-
IL-18	-
TGFβ	-
TNF	-

Tofacitinib selectivity
JAK1, JAK3 > JAK2, TYK2

JAK1   JAK2   JAK3   TYK2

B

Example of cytokines that signal through JAK/STAT combinations

γ-chain cytokines*	IFNγ	IL-10 IL-22 IFNα/IFNβ	IL-12 IL-23	IL-6¥ IL-11	EPO TPO GM-CSF
JAK1 JAK3	JAK1 JAK2	JAK1 TYK2	JAK2 TYK2	JAK1 JAK2 TYK2	JAK2 JAK2
STAT 1, 3, 5, 6	STAT 1, 3, 5	STAT 1, 3, 5	STAT 3, 4	STAT 1, 3, 5	STAT 5

Fig. 1. Key cytokines in the pathogenesis of RA and cytokine signalling through JAK/STAT combinations. **A**. Key cytokines in the pathogenesis of RA. An important subset of proinflammatory cytokines signal through JAK pathways in RA. *Type II cytokine receptors such as those for the gp130 subunit sharing receptors IL-6 and IL-11 as well as IL-10, IL-19, IL-20 and IL-22 mainly signal through JAK1, but also associate with JAK2 and TYK2. **B**. Cytokine signalling through JAK/STAT combinations. Type I and II cytokine receptors lack intrinsic enzymatic activity and utilise associated JAK combinations to signal. *γ-chain cytokines: IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. ¥Type II cytokine receptors such as those for the gp130 subunit sharing receptors IL-6 and IL-11 as well as IL-10, IL-19, IL-20 and IL-22 mainly signal through JAK1, but also associate with JAK2 and TYK2.
EPO: Erythropoietin; GM-CSF: Granulocyte/macrophage colony stimulating factor; IFN: Interferon; IL: Interleukin; JAK: Janus kinase; RA: Rheumatoid arthritis; STAT: Signal Transducer and Activator of Transcription; TGF: Transforming growth factor; TPP: Thrombopoietin; TYK: Tyrosine kinase.

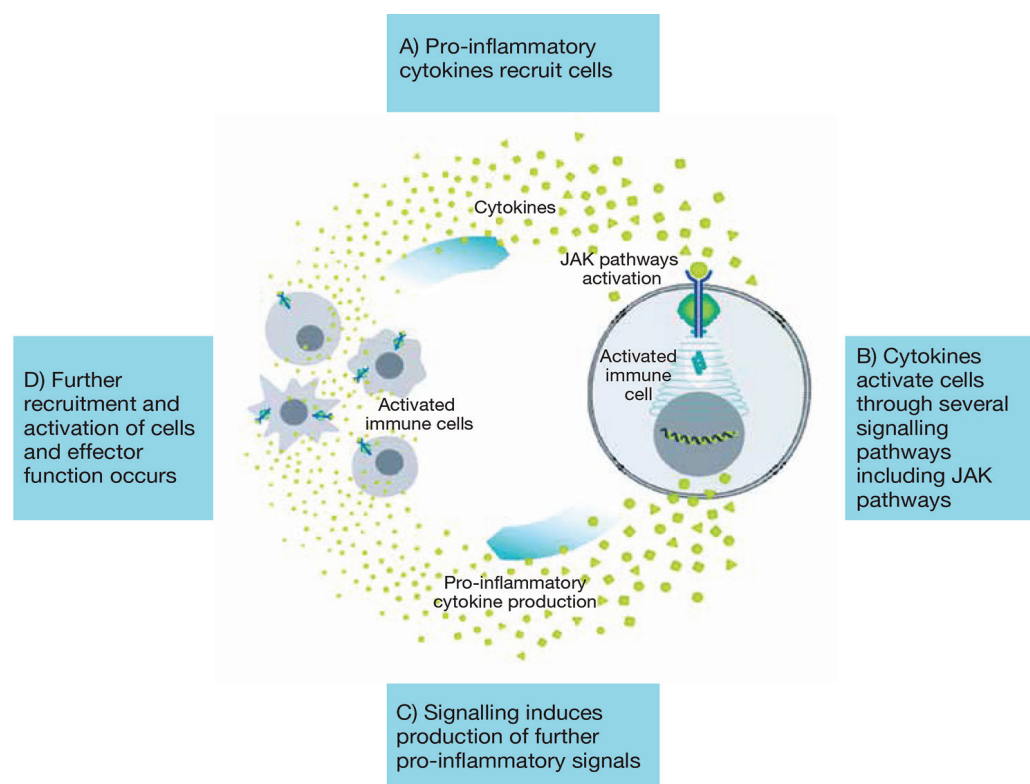## JAK pathways: perpetuating a chronic cycle of inflammation

The JAK family consists of four non-receptor protein tyrosine kinases: JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2) (10). JAK pathways are normally involved in growth, survival, development and differentiation of a variety of cells, but are crucially important for immune and haematopoietic cells.

Each JAK protein has specificity for a different set of cytokine receptors; the function of the JAK protein is thereby linked to the function of the cytokines that bind the receptors (Fig. 1B) (8, 11). Each cytokine receptor requires at least two associated JAKs in order to signal. JAKs may work in pairs of identical JAKs (*e.g.* JAK2/JAK2) or of different JAKs (*e.g.* JAK1/JAK3). JAK3 is the most specific, associating with only the common γ-chain (γc) receptor subunit and JAK1. The γc subunit pairs with a variety of other subunits to form the receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 (8, 11). JAK2 is associated with many receptors, including those for erythropoietin, thrombopoietin, IFNγ, IL-3, IL-5, growth hormone and granulocyte/macrophage colony stimulating factor (GM-CSF) (8, 11). It frequently associates with itself (JAK2/JAK2). JAK1 associates with the receptors for IFNs and IL-10-related cytokines, γc cytokines, and IL-6, as well as other cytokine receptors containing the gp130 subunit (8, 11). It forms pairs with any of the three other JAKs. Finally, TYK2 transmits signalling by type I IFNs (IFNα, IFNβ), IL-12 and IL-23, amongst others (8, 11).

Binding of a cytokine to its receptor activates the receptor-associated JAKs (12). The activated JAKs phosphorylate specific tyrosine residues in the cytoplasmic domains of the cytokine receptor subunits, which then act as docking sites for Signal Transducer and Activator of Transcription (STAT) proteins (12, 13). The STAT family of transcription factors consists of seven proteins: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 (12). After docking to tyrosine-phosphorylated cytokine receptor subunits, the STATs themselves are, in turn, tyrosine-phosphorylated by the receptor-associated JAKs (12). Phosphorylated STATs then dissociate from the receptor subunits, dimerise with each other, and translocate to the cell nucleus. Once in the nucleus, STATs function as transcription factors to regulate gene transcription (12).

In RA**,** B cells, T cells, macrophages and other leukocytes infiltrate the synovium in response to pro-inflammatory cytokines and chemokines, leading to inflammation and tissue destruction (Fig. 2). Cytokine signalling via JAK pathways leads to further induction of inflammatory gene expression, which continues the loop of inflammatory signalling. Inhibiting cytokine signalling by inhibiting the JAK pathways may, therefore, interrupt the cycle of leukocyte recruitment, activation and pro-in-

Fig. 2. Perpetuating the chronic cycle of inflammation in RA: JAK pathways. In response to chemokines and cytokines, B cells, T cells, macrophages and other leukocytes infiltrate the synovium (**A**). These activated immune cells produce cytokines (**B**) which signal through the JAK pathways (**C**) leading to production of new pro-inflammatory mediators such as cytokines (D) propagating a cycle of chronic inflammation.
JAK: Janus kinase;
RA: rheumatoid arthritis.

flammatory cytokine expression at sites of inflammation (5, 14).

Review

Tofacitinib: an oral JAK inhibitor for the treatment of RA

Tofacitinib is an oral JAK inhibitor for the treatment of RA. It is a targeted synthetic small molecule (molecular weight 312.4 Da; 504.5 for the citrate salt), not a biologic. Unlike targeted biologic therapies, which are directed at extracellular targets such as individual soluble cytokines (*e.g.* TNF), cytokine receptors (IL-1R, IL-6R) or other cell-surface receptors (CD20, CD80, and CD86), tofacitinib works at the intracellular level. Tofacitinib possesses high *in vitro* passive permeability properties consistent with intracellular entry by transcellular diffusion.

Tofacitinib is a reversible, competitive inhibitor that binds to the adenosine triphosphate (ATP) binding site in the catalytic cleft of the kinase domain of JAK. The structure of tofacitinib mimics that of ATP without the triphosphate group. As a result of binding to the ATP site, tofacitinib inhibits the phosphorylation and activation of JAK, thereby preventing the phosphorylation and acti-

vation of STATs, and thus the activation of gene transcription (Fig. 3). This leads to decreased cytokine production and modulation of the immune response.

Tofacitinib is a potent inhibitor of the JAK family of kinases with a high degree of selectivity against other kinases in the human kinome. With *in vitro* kinase assays, tofacitinib inhibits JAK1, JAK2, JAK3 and, to a lesser extent, TYK2. In cellular settings, where JAKs signal in pairs, tofacitinib preferentially inhibits signalling by cytokine receptors associated with JAK3 and/or JAK1 with functional selectivity over receptors that signal via pairs of JAK2 (15). Specifically, in human whole blood cellular studies, tofacitinib potently inhibits IL-15-induced phosphorylation of STAT5 with a half maximal inhibitory concentration (IC_{50}) of 56 nM, and IL-6-induced phosphorylation of STAT1 and STAT3 with IC_{50s} of 54 and 367 nM, respectively (15). Both IL-15 and IL-6 are dependent on JAK1, and IL-15 is also dependent on JAK3. In contrast, tofacitinib inhibits GM-CSF-induced phosphorylation of STAT5, a JAK2-mediated signalling event, with lower potency and an IC_{50} of 1377 nM (15).

Tofacitinib: non-clinical studies

Previous studies have established the effects of tofacitinib on murine innate and adaptive immune responses (16). Specifically, tofacitinib inhibited the acute lipopolysaccharide-induced inflammatory response in a murine model of innate immunity which is dependent upon IFNγ and STAT1. Tofacitinib dosed 5 mg/kg inhibited TNF and IL-6 production, whilst the production of the anti-inflammatory cytokine IL-10 was enhanced. These results were interpreted as consistent with inhibition of IFNγ signalling by tofacitinib through blockade of JAK1. In studies of the adaptive immune response, specifically the differentiation of T helper cells, tofacitinib blocked the differentiation of type 1 T helper (Th1) cells and type 2 T helper (Th2) cells, and interfered with the generation of pathogenic type 17 T helper (Th17) cells (16).

These results were extended in studies of tofacitinib *in vivo*. In a murine collagen-induced arthritis model, tofacitinib produced significant dose-dependent attenuation of inflammatory paw swelling and cell influx (16, 17) when dosed before (prophylactically) or after (therapeutically) disease. Plas-
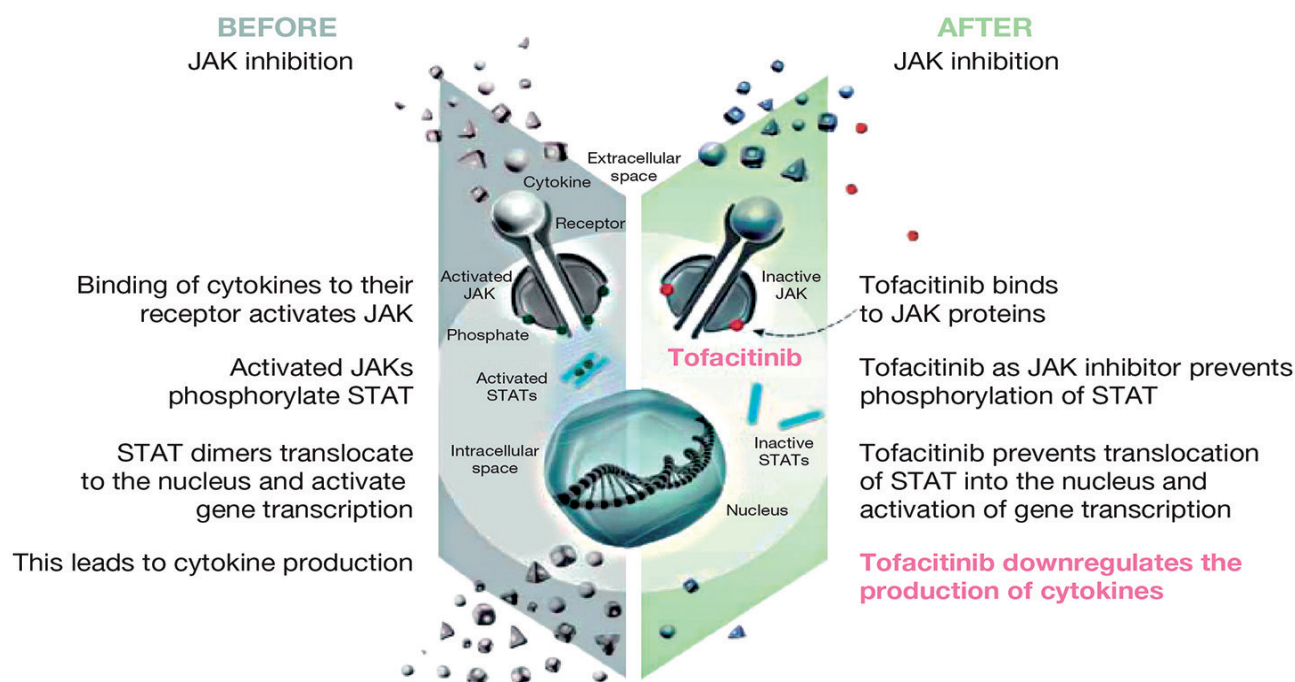
Fig. 3. Tofacitinib mechanism of action. Under normal conditions, binding of a cytokine to its specific cell-surface receptor causes the receptor chains to polymerise and activate the associated JAKs. (**Before**) Activated JAKs phosphorylate specific residues in the cytoplasmic domains of the cytokine receptor chains, which then act as docking sites for STAT proteins. Once they have docked, STATs are phosphorylated by the activated receptor-associated JAKs. Phosphorylated STATs then dissociate from the receptor chains, dimerise with each other, and translocate to the cell nucleus where they activate gene transcription. Tofacitinib binds in the catalytic cleft in the kinase domain of JAK. (**After**) This prevents activation of JAK and thus of STAT phosphorylation and translocation to the nucleus to activate gene transcription.
JAK: Janus kinase; STAT: Signal Transducer and Activator of Transcription.

ma cytokine (*e.g.* IL-6) and chemokine (*e.g.* chemokine [C-X-C motif] ligand 10 [CXCL10]; IFNγ induced protein 10 [IP-10]) mRNA and protein levels were reduced to pre-disease levels but not completely inhibited in tofacitinib-treated mice, demonstrating normalisation of chemokine and cytokine levels. Finally, tofacitinib reduced macrophage and T cell infiltration in inflamed paw tissue of treated mice as assessed by histology and immunohistochemistry (16) as well as bone resorption (18). Similarly, in a rat model of adjuvant-induced arthritis (AIA), tofacitinib was given at the peak of disease and dosed for 1 week (18). Treatment led to a reduction in oedema, in osteoclast-mediated bone resorption, and in monocyte/macrophage and T cell infiltration into the joint and surrounding tissue. The rise in plasma and/or paw tissue levels of several inflammatory cytokines, chemokines and adhesion molecules associated with AIA was inhibited by tofacitinib.

To better understand the mechanism behind reduced bone resorption, ex-periments evaluated the effects of to-facitinib on the production of receptor activator of nuclear factor-kappaB (RANK) and RANK ligand (RANKL). Tofacitinib led to a dose-dependent decrease in human T lymphocyte RANKL production, whereas osteoclast differentiation and function were not directly affected. These data provide a mechanistic explanation for the observed inhibition of structural joint damage by tofacitinib.

Tofacitinib:
clinical studies – overview
The clinical efficacy and safety of to-facitinib 5 mg and 10 mg twice daily (BID) as monotherapy or in combination with conventional synthetic disease-modifying anti-rheumatic drugs (DMARDs) for the treatment of RA have been reported in six Phase 3 clinical studies (19-24). The Phase 3 study populations included patients with RA who previously had an inadequate response to either methotrexate (19, 20), ≥1 biologic DMARD or conventional synthetic DMARD (21, 22), or ≥1 TNF inhibitor (23), and those who were methotrexate-naïve (24). Tofacitinib demonstrated greater efficacy compared with placebo across clinical, functional and structural measures of disease severity (19-24).

The safety profile of tofacitinib was consistent across the Phase 3 studies (19-24). Common adverse events (AEs) with tofacitinib included naso-pharyngitis, upper respiratory tract in-fection, headache and diarrhoea, and the most common serious AEs report-ed were serious infections (including pneumonia, cellulitis, herpes zoster, and urinary tract infection) (25). Malignancies (excluding non-melanoma skin cancer), lymphoma and gastrointestinal perforations have been reported in patients receiving tofacitinib (25). Changes in safety laboratory test values included increases in high-density lipoprotein, low-density lipoprotein, total cholesterol, serum creatinine and liver transaminases (25). Changes in granulocyte and lymphocyte numbers and immune function are described in detail below.

Tofacitinib:
clinical studies – immune impact
The inhibition of JAK pathways is a novel mechanism of action that results in a pattern of partial inhibition of the intracellular effects from several inflammatory cytokines and overall modulation of the immune and inflammatory response. Further characterisation of the impact of immunomodulation by tofacitinib was undertaken. Doses from 1 to 30 mg BID, inclusive, and 20 mg once daily (QD) were studied in Phase 2 of the RA development programme (26-30) and the 5 and 10 mg BID doses were advanced into Phase 3 (19-24).

Neutrophils
A dose-dependent mean decrease in absolute neutrophil counts, which reached a plateau within 3 months of treatment, was observed in tofacitinib-treated RA patients (Fig. 4A; Pfizer unpublished observations). Neutrophil counts largely remained within the normal reference range throughout the duration of tofacitinib treatment. These data are consistent with exposure-response modelling of Phase 2 data predicting a steady state neutrophil nadir by 6 to 8 weeks and a median decrease from baseline of approximately 1.0 and 1.5 x 10^3/L for tofacitinib 5 and 10 mg, respectively (Pfizer unpublished observations). After cessation of tofacitinib, neutrophil counts recovered in a dose-dependent manner by 6 weeks (31).

Modest decreases in neutrophils are expected with efficacious RA treatment. Mean neutrophil count decreases, of a similar magnitude as those with tofacitinib, were also observed in adalimumab-treated patients in one of the Phase 3 studies, which included adalimumab as an active control arm (NCT00853385) (20), with corresponding reductions in acute phase reactants. Therefore, changes in neutrophils may be primarily related to decreasing inflammation, rather than specific to the mechanism of action of tofacitinib.

Lymphocytes
In the Phase 3 studies, mean lymphocyte levels at baseline were below the lower reference range (2 x10^3/mm^3) in all groups (including adalimumab and placebo control groups) and 38% of patients had moderate-severe lymphopenia (0.5–1.5 x10^3/mm^3) (Pfizer unpublished observations). Mean increases from baseline in lymphocyte levels were seen with tofacitinib and adalimumab at Month 1, which persisted for adalimumab, but not tofacitinib (approximate 10% decrease from baseline over 12 months) (Fig. 4B). The occurrence of lymphopenia did not appear to be dose-related and the frequency of lymphopenia in the Phase 3 studies was similar between tofacitinib and placebo patients at Months 3 and 6. The moderate decrease in lymphocyte counts precluded definitive assessment of reversibility after drug discontinuation.

Lymphocyte subsets
The effects of tofacitinib on changes in circulating numbers of lymphocyte subsets including CD3+ cells (pan T lymphocytes), CD4+ cells (helper T cells), CD8+ cells (cytotoxic T cells), CD19+ cells (B cells) and CD16+/CD56+ cells (natural killer [NK] cells) were assessed in early studies in RA patients (Pfizer unpublished observations).
Dose-response plots indicated that changes in CD3+, CD4+ and CD8+ counts were small and variable with no consistent dose-response pattern across studies (Fig. 4C-E). In contrast, NK cell counts showed dose-dependent decreases (Fig. 4F). Model-based characterisation of NK cell counts indicated that reductions were predicted to reach nadir (steady state) by 8 to 10 weeks after initiation of tofacitinib treatment, with approximately 36% and 47% peak mean reductions for 5 and 10 mg doses, respectively (25). Similar changes in lymphocytes subsets were observed in tofacitinib-treated psoriasis patients (32).
Based on the pharmacological properties of tofacitinib, T cell-mediated immunity may be altered by inhibiting cytokine signalling required for T cell development and T cell function after antigen stimulation. T cell development was minimally altered in most patients, based on the findings that circulating T cell counts showed little change with short-term treatment and decreased by less than 20% on average (CD8+ T cells) with long-term treatment (Pfizer unpublished observations). This moderate decrease in T cell counts precluded a definitive assessment of reversibility after drug discontinuation.
The reversibility of tofacitinib's effect on T cell function was assessed in a study of stable kidney transplant recipients (n=8) treated with tofacitinib (30 mg BID) for 29 days (33). Peripheral blood mononuclear cells (PBMC), prepared from blood samples collected on Day 1 (before tofacitinib treatment) and Day 29, were stimulated *in vitro* with allogeneic cells (mixed lymphocyte response). The proliferative response of PBMC from Day 1 was similar to that observed with PBMC from Day 29. Since tofacitinib is washed away during PBMC preparation, this indicates that tofacitinib does not have a residual effect on T cell function at 6-times the approved clinical dose.
B cell counts showed dose-dependent increases in all studies (Fig. 4G; Pfizer unpublished observations). The mechanism and clinical significance of increases in circulating B cell numbers in response to JAK inhibition are unclear. Humans with JAK3 deficiencies (JAK3 -/-) also have increased numbers of circulating B cells but are accompanied by severe reductions in serum immunoglobulin (Ig) levels (34). With tofacitinib, only modest decreases in serum IgG, IgM and IgA levels are observed in RA patients (*see below*) which may be due to changes in disease activity rather than a direct effect on B cell function.
Four Phase 2 studies evaluated serum immunoglobulin levels in tofacitinib-treated RA patients (Pfizer unpublished observations). Decreases in median IgG, IgM and IgA levels (<10 to 20% change from baseline) were observed after 12 (Studies NCT00603512 and NCT00687193 in Japanese patients) or 24 weeks (Studies NCT00413660 and NCT00550446, global studies) of tofacitinib treatment when compared to baseline levels (Fig. 5). The median immunoglobulin concentrations after tofacitinib treatment were within normal reference ranges (35-37). These changes in immunoglobulin levels may be secondary to a decrease in generalised inflammation associated with tofacitinib treatment. It has been reported that
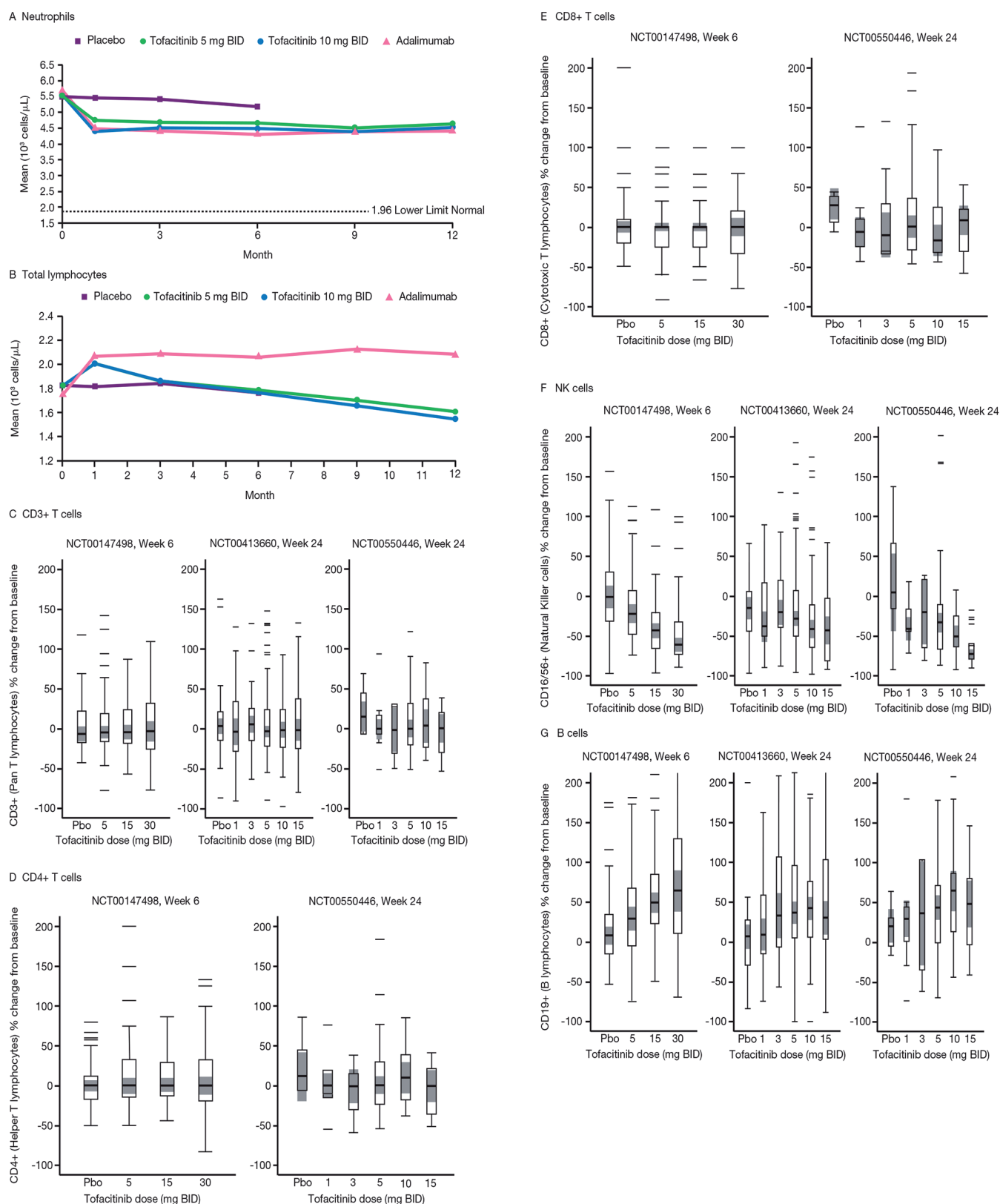
Fig. 4. Immune cells and lymphocyte subsets. **A.** Mean (standard error) neutrophil levels in Phase 3 Studies (0 to 12 Months) **B.** Dose-dependent changes in total lymphocytes were measured in Phase 3 studies (maximum duration of 12 months); lymphocyte subsets were analysed in Phase 2 studies in RA patients after 6 weeks to 24 weeks (6 months) of tofacitinib treatment. Percentage change from baseline in the indicated cell type is depicted for the varying doses of tofacitinib (mg BID). **C.** CD3+ T cells; **D.** CD4+ T cells; **E.** CD8+ T cells; **F.** NK cells; **G.** B cells. Solid horizontal line within each box represents median; lower and upper horizontal lines of each box represent 1st and 3rd quartiles, respectively; whiskers represent 1.5 x inter-quartile range; lines outside the whiskers are outliers (individual data points). Grey shading represents 95% confidence interval of the median. BID: twice daily; NK: natural killer; Pbo: placebo; RA: rheumatoid arthritis.

active RA is associated with increased immunoglobulin levels (38-40).

Additional information on lymphocyte subsets was collected in a substudy of one of the open-label long-term extension studies which enrolled eligible patients from Phase 2 and Phase 3 studies. Subsets were assessed in approximately 150 patients, most of whom had completed tofacitinib treatment in Phase 3 studies and had been treated for a median duration of approximately 22 months. Thus, these patients did not have baseline (prior to treatment initiation) values to formally assess magnitude of change. Nevertheless, a comparison of the distribution of subset levels based on a cross-sectional analysis in different groups of patients suggests that after 6 months of tofacitinib, NK cell counts decrease, with a return to baseline after 22 months of treatment. B cell counts remain elevated after 6 and 22 months of treatment. There were slight decreases in total T cells (10.2%), CD4+ T cells (3.9%) and CD8+ T cells (18.1%) after 22 months of tofacitinib treatment (Fig. 6). The median absolute CD3+ (total), CD4+, and CD8+ T cells, B cells and NK cells in RA patients after long-term tofacitinib exposure are similar to those reported in healthy adult populations from Germany (41) and Switzerland (42), and contrast sharply with measures of CD3+, NK and B cells reported in patients with Severe Combined Immune Deficiency (34).

Effects on immune function: vaccination

The effects of tofacitinib on immune function were primarily addressed by evaluating the antibody responses of tofacitinib-treated RA patients vaccinated with seasonal influenza and pneumococcal polysaccharide vaccines. Antibody responses to non-conjugated pneumococcal polysaccharide vaccine are considered to be T cell-independent whereas the response to influenza vaccine requires T cells to provide help to B cells to produce anti-influenza antibodies (T cell-dependent antibody response).

Two studies were conducted in RA patients: one evaluated vaccine responses over short-term (64 days;
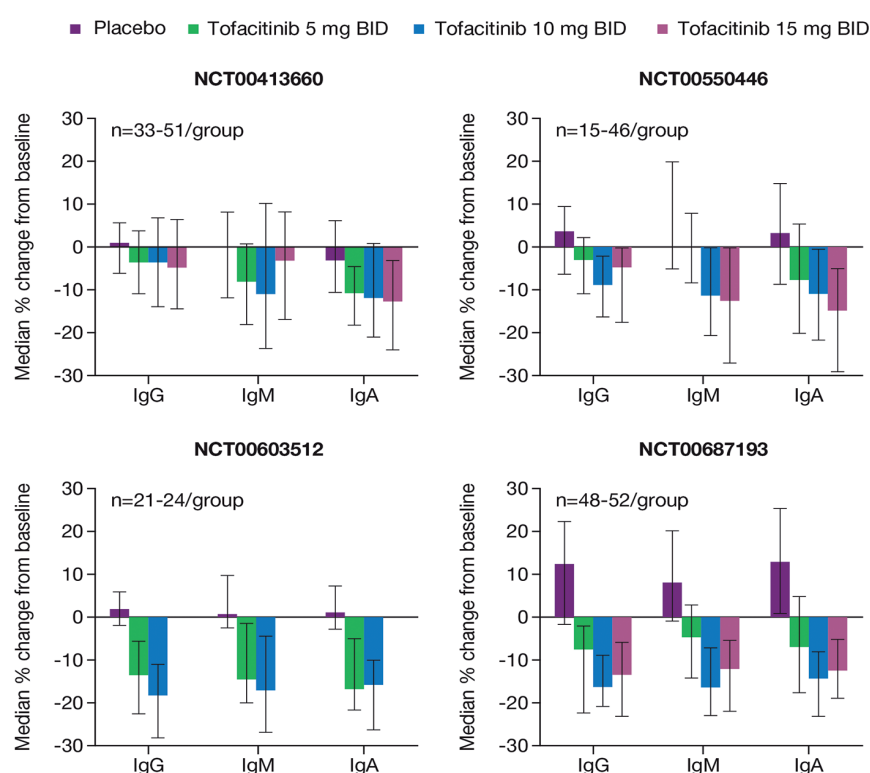
Fig. 5. Serum immunoglobulins. The effect of increasing doses of tofacitinib on serum immunoglobulin levels was evaluated in four Phase 2 studies. Decreases in median IgG, IgM and IgA levels (<10 to 20% change from baseline) after 12 or 24 weeks of tofacitinib treatment were observed when compared to baseline levels.
BID: Twice daily; Ig: Immunoglobulin.
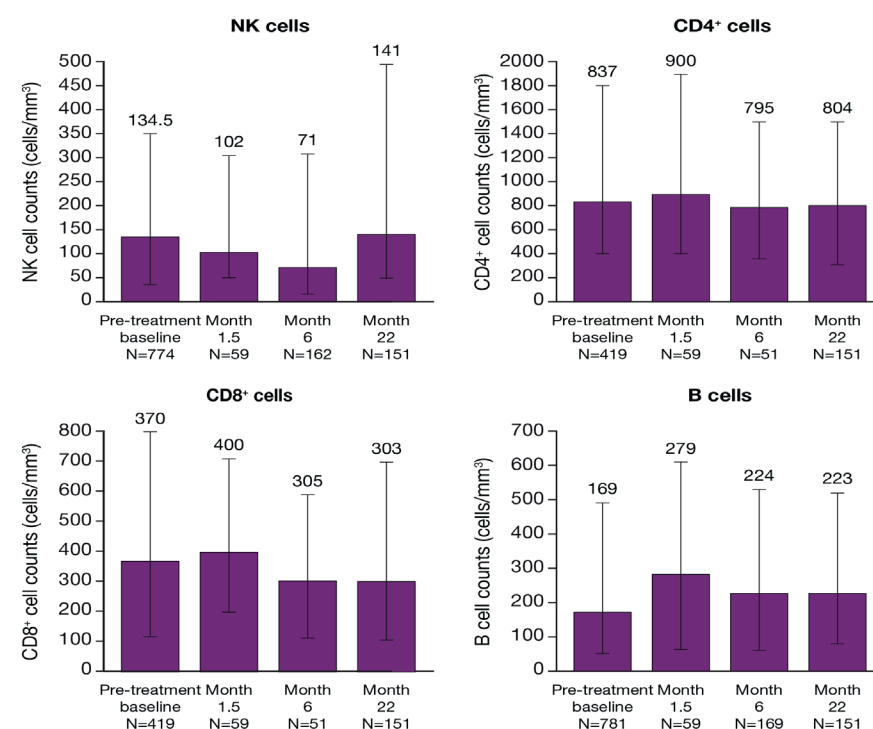
Fig. 6. Lymphocyte subsets long-term. Lymphocyte subsets after short- and long-term tofacitinib treatment: pre-treatment baseline, Month 1.5, Month 6 and Month 22. Baseline, Month 1.5 and Month 6 data are from Phase 2 studies. Month 22 data predominantly represent Phase 3 patients who participated in the long-term extension study, with a median of 22 months' tofacitinib exposure.
NK: Natural killer.

NCT01359150) tofacitinib treatment in patients naïve to tofacitinib, and the other vaccine responses during ongoing long-term (median duration of approximately 22 months; NCT00413699 vaccine substudy) tofacitinib therapy. In both studies tofacitinib was administered with or without background methotrexate (MTX) therapy (43-45). Naïve patients treated with tofacitinib were vaccinated 4 weeks later and antibody responses measured 35 days post-vaccination. The percentage of patients achieving a satisfactory response to influenza vaccine (≥4-fold increase above baseline in antibody titers for 2/3 antigens) was similar between the tofacitinib 10 mg BID and placebo groups after 64 days of treatment. The percentage of patients achieving a satisfactory response to pneumococcal vaccine (≥2-fold increase above baseline in antibody titers for 6/12 serotypes) was similar between tofacitinib 10 mg BID monotherapy and MTX alone groups. However, the tofacitinib response was diminished in patients taking MTX in combination with tofacitinib (45). Similarly, in patients with ongoing tofacitinib treatment (median duration ~22 months) greater than 60% of patients treated with tofacitinib (with or without MTX) had satisfactory responses to influenza and pneumococcal vaccines. However, pneumococcal responses were again lower in patients receiving MTX background therapy (44). Overall, the data from the vaccine studies support that tofacitinib treatment (short- or long-term) does not have a major effect on B cell and T cell function required for humoral immune responses to vaccines.

Tofacitinib pharmacodynamic profile: biomarkers of inflammation
Many of the pro-inflammatory cytokines involved in the pathogenesis and maintenance of RA signal through JAK and, therefore, may be modulated by tofacitinib therapy. Studies *in vitro* and *in vivo* in RA patients have investigated these effects.

Cytokine and chemokine effects
Tofacitinib therapy for a period of 12 to 24 weeks in Phase 2 studies re-sulted in decreased levels of circulating IL-6 in RA patients (Studies NCT00413660 and NCT00550446). However, circulating levels of IL-8, CXCL10 (IP-10), TNF, or IL-10 were variable and inconsistent. In another study (NCT00976599), levels of the circulating chemokine, CXCL10 (IP-10) and acute phase proteins serum amyloid A (SAA) and C-reactive protein (CRP) were decreased after 4 weeks of tofacitinib (10 mg BID) treatment. In addition, in a study that evaluated early changes in CRP levels, decreases were observed within 1 to 2 weeks of tofacitinib treatment (NCT00147498) (Pfizer unpublished observations).

The mechanistic basis for the observed effects with tofacitinib was evaluated *in vitro* in human cells. *In vitro* tofacitinib did not directly alter IL-6 and IL-8 production by synovial fibroblasts from RA patients and monocytes (46). However, tofacitinib decreased the production of IL-6 and IL-8 by synovial fibroblasts and monocytes stimulated with supernatant from anti-CD3 stimulated CD4$^+$ T cells, suggesting that decreases may, in part, be indirectly mediated by inhibiting T cell production of cytokines that stimulate IL-6 and IL-8 production. T cell cytokines such as IL-17 and IFNγ are known to stimulate IL-6 production, and production of IL-17 and IFNγ by CD4$^+$ T cells from RA synovium and peripheral blood were decreased with *in vitro* tofacitinib exposure. This may, in turn, be due to inhibition of IL-2 autocrine stimulation of T cells and is consistent with findings that tofacitinib decreases the proliferative responses and cytokine production (IL-4, IL-17, IL-22 and IFNγ) of anti-CD3 stimulated human peripheral blood CD4$^+$ T cells from healthy donors (47). Decreased circulating IL-6 levels with tofacitinib may also be associated with the inhibition of oncostatin M (OSM) stimulated IL-6 production by fibroblast-like synoviocytes (48). OSM is a member of the IL-6 family of cytokines produced by macrophages and T cells and signals through JAK.

TNF-induced production of the chemokines, CXCL10, CCL5 and CCL2 by synovial fibroblasts was inhibited by *in vitro* tofacitinib exposure, through an indirect mechanism involving IFNβ. Specifically, TNF induces the production of IFNβ, which stimulates the production of various chemokines, and tofacitinib inhibits IFNβ signalling (49). Inhibition of TNF-induced chemokine and IL-6 production by synovial macrophages with tofacitinib *in vitro* was also mediated by inhibiting the IFN-β feedback loop (50).

In summary, the mechanism for the decrease in circulating levels of IL-6 and chemokines with tofacitinib may be due to an indirect effect on T cells that produce cytokines that stimulate IL-6 production and the inhibition of signalling by the IL-6 family of cytokines and/or IFNα/β through a feedback loop. In contrast, the reductions in acute phase proteins CRP and SAA are likely mediated by a direct effect of tofacitinib on IL-6 signalling. This is supported by the findings that IL-6 stimulation of expression of SAA by fibroblast-like synoviocytes from RA patients and human hepatocytes was inhibited with *in vitro* tofacitinib exposure (48).
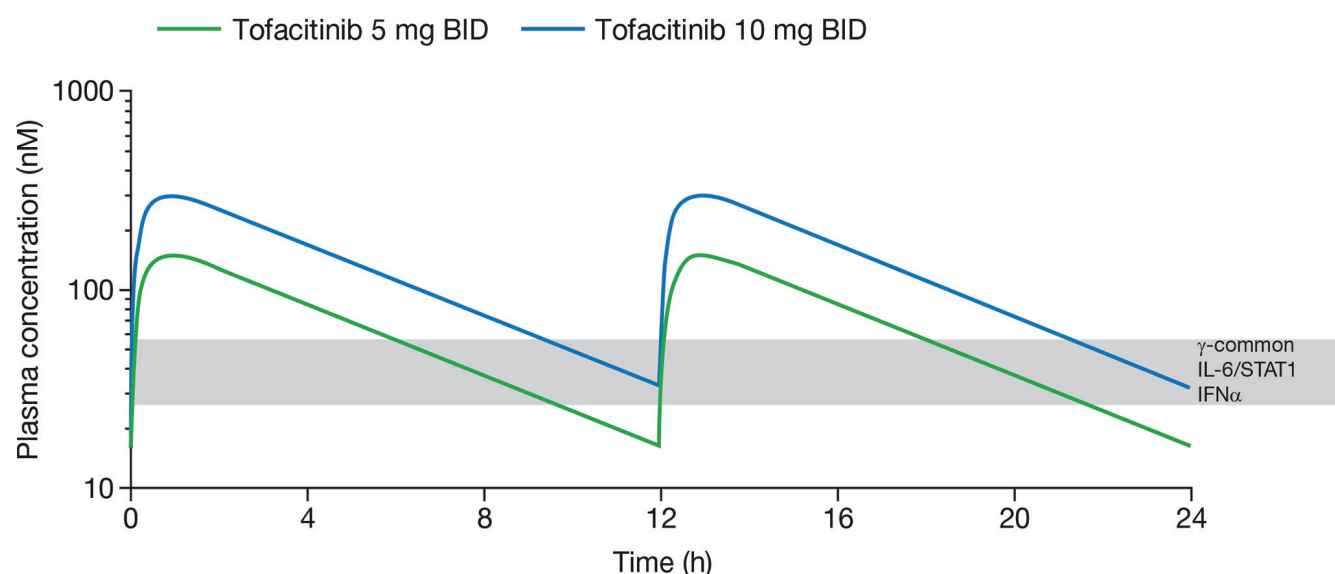
Tofacitinib:
partial and reversible inhibition
Tofacitinib is a partial and reversible inhibitor of JAKs with a short pharmacokinetic (PK) half-life. The PK profile of tofacitinib in humans is characterised by rapid absorption and elimination, with a time to peak concentration (Tmax) of approximately 1 hour and a half-life (t½) of approximately 3 hours (Fig. 7A). At the 5 mg BID dose, the average inhibition of preferentially inhibited cytokines is partial (50–60%) and declines to 10–30% at the trough or Cmin values in each dosing period. The clearance mechanisms for tofacitinib in humans appear to be both non-renal (hepatic metabolism, 70%) and renal (30%) excretion of the parent drug. The metabolism of tofacitinib is primarily mediated by cytochrome P450 3A4 (CYP3A4; 53%) with a minor contribution from CYP2C19 (17%) (51).
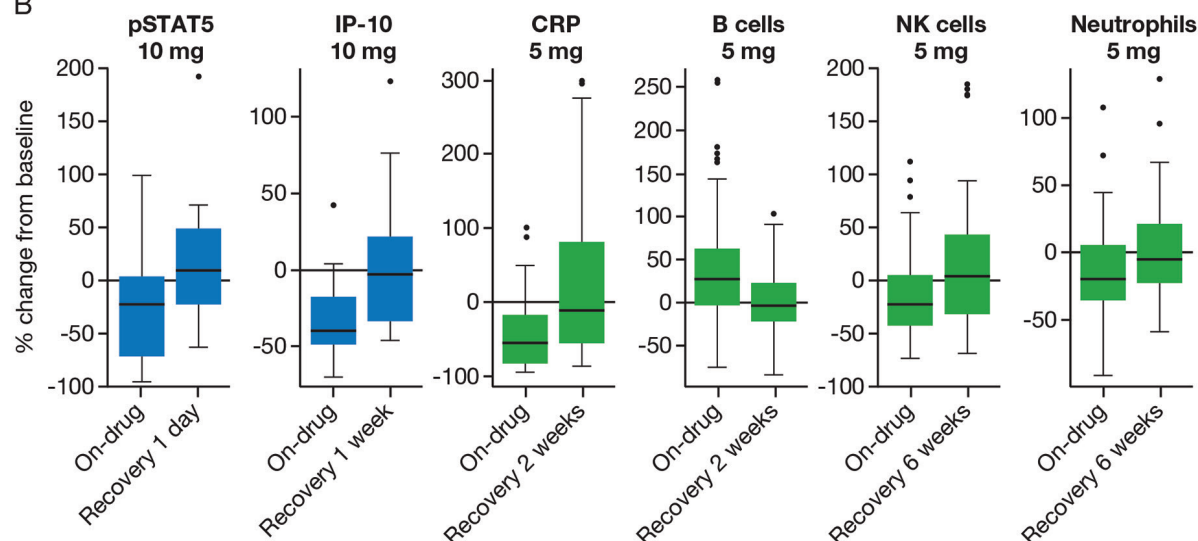
Since tofacitinib is metabolised by CYP3A4, interaction with drugs that inhibit or induce CYP3A4 is likely. Tofacitinib exposure is increased approximately 2 fold when co-administered with potent CYP3A4 inhibitors (*e.g.* ketocona-

A

B

Fig. 7. Partial and reversible inhibition; biochemical targets and immune cells.

A. Tofacitinib partially and reversibly inhibits multiple JAK-dependent cytokine signalling pathways. Maximum plasma concentrations (Cmax) at 5 and 10 mg BID in RA patients occur approximately 1 hour after administration of tofacitinib. At Cmax, the inhibition of the preferentially inhibited cytokines is ~80–90% for 5 mg BID and ~89–95% for 10 mg BID. At Cmin, the inhibition is ~26–43% for 5 mg BID and ~42–60% for 10 mg BID. The average inhibition for the preferentially inhibited cytokines during the dosing period is ~60–76% for 5 mg BID and ~75–86% for 10 mg BID.

B. Tofacitinib is a partial and reversible inhibitor of JAK activity. Rapid reversibility (1 day to 2 weeks) of biochemical targets (left) and reversibility of immune cells within 2 to 6 weeks (right). pSTAT5: IL-15 stimulated pSTAT5 in CD8^{+} T cells. Solid horizontal line within each box represents median; lower and upper horizontal lines of each box represent 1st and 3rd quartiles, respectively; whiskers represent 1.5 x inter-quartile range; dots outside the whiskers are outliers (individual data points). Cmax: Maximum plasma concentration; Cmin: Minimum plasma concentration; CRP: C reactive protein; IP-10: Interferon gamma induced protein 10; JAK: Janus kinase; NK: Natural killer; pSTAT: Phosphorylated Signal Transducer and Activator of Transcription.

zole) or when administration of one or more concomitant medications results in moderate inhibition of CYP3A4 and potent inhibition of CYP2C19 (*e.g.* fluconazole). Tofacitinib exposure is decreased when co-administered with potent CYP3A4 inducers (*e.g.* rifampin). Inhibitors of CYP2C19 or P-glycoprotein are unlikely to alter the PK of tofac-

itinib. At clinical exposures, tofacitinib itself does not significantly inhibit the major CYP450 or uridine-diphosphate-glucuronosyltransferase (UGT) hepatic enzymes or a number of transporters, including P-glycoprotein, breast cancer resistance protein (BCRP), multidrug resistance associated protein (MRP), organic anion transporting polypep-

tide (OATP), organic anion transporter (OAT), organic cation transporter (OCT), or multidrug and toxic compound extrusion (MATE). The potential for tofacitinib to induce CYP3A4, 1A2 or 2B6 enzymes was also low at clinically relevant exposures.

The relationship between plasma concentrations and pharmacodynamic (PD)

effects, as reflected in changes in bio-markers, may be indirect, resulting in longer duration of PD activity relative to a short PK half-life. PD reversibility was evaluated over a range of bio-markers, from STAT phosphorylation, a direct read-out of JAK inhibition, to changes reflecting downstream events, such as levels of circulating chemokine CXCL10 and CRP levels, and lymphocyte subset and neutrophil counts (Fig. 7B) (31).

Phosphorylated STAT5 levels in *ex vivo* IL-15 stimulated CD8$^+$ T cells, serum CXCL10 levels, CRP levels and B cell counts reverse to baseline levels over a 2-week washout period in RA patients who received tofacitinib for 4 to 12 weeks. Reversibility in STAT phosphorylation is seen as early as 24 hours after cessation of tofacitinib treatment. NK cell and neutrophil counts partially reverse to baseline over 2–6 weeks (31). This reversibility in RA patients may reflect the effects of inflammatory cytokines and chemokines in elevating baseline NK cell and neutrophil counts, rather than residual PD effects of to-facitinib.

The totality of these PD data demonstrates reversibility of PD effects, generally within 14 days of tofacitinib discontinuation, after both short- and long-term treatment. This is consistent with tofacitinib being a reversible inhibitor of JAK, as demonstrated by *in vitro* assays, and having a short plasma elimination half-life. This also provides a scientific rationale for a 14-day discontinuation of tofacitinib while managing adverse events such as infections and laboratory abnormalities.

Conclusions

RA is a destructive, chronic inflammatory disease mediated by multiple cytokines and cell types of the innate and adaptive immune system. Tofacitinib is an oral JAK inhibitor for the treatment of RA. Tofacitinib is a targeted small molecule that works intracellularly to partially and reversibly inhibit JAK-dependent cytokine signalling. Unlike biological treatments, that target one cytokine pathway in the inflammatory network, JAK inhibition results in a pattern of partial inhibition of the intracellular effects from several inflam-matory cytokines and overall modulation of the immune and inflammatory response. Tofacitinib also indirectly modulates the production of inflammatory cytokines through autocrine and paracrine feedback loops. As a consequence of these mechanisms, effects on the immune system such as moderate decreases in total lymphocyte counts, dose-dependent decreases in NK cell counts, and increases in B cell counts have been observed. Despite these observations, and modest decreases in serum immunoglobulin levels, the immune response as measured by vaccination to both T cell-independent and -dependent antigens was intact and PD effects of short- or long-term tofacitinib treatment were reversible after approximately 2 weeks. Therefore tofacitinib provides a novel, innovative approach to modulating the immune and inflammatory response in RA.

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