Supplemental Materials to:

Traves PG, Murray B, Campigotto F, et al.
JAK Selectivity and the Implications for Clinical Inhibition of Pharmacodynamic Cytokine Signaling by Filgotinib, Upadacitinib, Tofacitinib, and Baricitinib

Contents

Detailed Methods

PBMC and WB Preparation and Experimental Design

Figure 2 illustrates the experimental and data analysis procedures. Whole blood samples from healthy donors (N=10) were collected by venipuncture in sodium heparin and processed within 24 hours. PBMCs were harvested from matched whole blood (200mL) by Ficoll gradient. PBMCs or WB were plated into 96-well flat bottom plates and incubated with each JAKinib. Filgotinib (GS-6034) and its active metabolite (MET, GS-829845) were synthesized at Gilead Sciences, Inc. (Foster City, CA). Baricitinib (GS-623452) was purchased from AstaTech, Inc. (Bristol, PA), tofacitinib (GS-493645) was synthesized by Advanced Chemblocks, Inc. (Burlingame, CA), and upadacitinib (GS-942618) was synthesized by MedChemExpress (Monmouth Junction, NJ). Baricitinib, tofacitinib, and upadacitinib were prepared as 10mM stocks and filgotinib and MET as 40mM or 160mM stocks in dimethyl sulfoxide (DMSO). JAKinibs were applied using 8-point dose-titrations ranging from 0.6–10,000nM (baricitinib, tofacitinib, and upadacitinib) or either 2.4–40,000nM or 9.7–160,000nM (filgotinib and MET) (four-fold dilutions) in duplicate, for 60 min prior to cytokine stimulation for 15 min at 37°C.

Whole blood samples from rheumatoid arthritis donors (n=3; patient details are provided in **Appendix 1**) and matched healthy donors (n=2) were collected by venipuncture in sodium heparin, shipped ON at 4C and processed within 36 hours. RA and HD samples were gender and aged matched. RA blood samples were obtained from BioIVT (Hicksville, NY) for experimental use. The inclusion criteria for RA patients included patients able to provide written informed consent, with confirmed diagnosis of RA, with active moderate to severe RA, who have not taken an investigational drug in the last year and who have not been treated with any JAK inhibitor.

Cytokine EC_{80} Determination

Cytokine concentrations were optimized in PBMCs and WB (N=3) to achieve 80% maximal pSTAT signals in each cell population. Final concentrations were: PBMCs; GM-CSF (9pg/ml), G-CSF (4ng/mL), IFN-γ (0.2ng/ml), IFN-α (6ng/ml), IL-2 (5ng/ml), IL-4 (0.2ng/ml), IL-6 (4ng/ml), and IL-15 (0.4ng/ml). Blood; GM-CSF (9pg/ml), G-CSF [10-25ng/mL (10ng/ml donors 1 and 2, 25ng/ml donors 4-6)], IFN-γ (8ng/ml), IFN-α (6ng/ml), IL-2 (5ng/ml), IL-4 (0.3ng/ml), IL-6 (10ng/ml), IL-12 (10ng/ml), IL-23 (100ng/ml) and IL-15 (1ng/ml).

Flow Cytometry

Cells were lyse/fixed, permeabilized and frozen at -80°C. STAT phosphorylation (pSTAT1, pSTAT3, pSTAT4, pSTAT5 and pSTAT6) was detected and quantified by multicolor flow cytometry in the phenotypically gated leukocyte subpopulations defined as CD19+ (B cells), CD3+CD4+ (CD4+ T cells), CD3+CD8+ (CD8+ T cells), CD3+CD8+CD45RA- (CD8+ memory T cells), CD3-CD56+ (NK cells), CD15+ (neutrophils), and CD14+ (monocytes) (See **Appendix 2** for reagents and compounds and **Appendix 3** for gating strategies). The mean fluorescent intensity (MFI) for the unstimulated versus stimulated conditions was analyzed and reported when >1.4 and >200 fluorescent units. The raw flow cytometry data are shown in **Appendix 4**.

Curve-Fitting Parameters for Concentration Response Curves

For a given response [cell source (PBMC or WB), stimulation (IL-2, IL-15, IL-4, IL-12, IL-23, IFNα, IFNγ, IL-6, G-CSF, and GM-CSF), cell type (B, CD4+, CD8+, CD8+ memory T cells, NK, monocytes, and neutrophils), and pSTAT (pSTAT1, 3, 4, 5, 6)] the half maximal (IC_{50}) values for each JAKinib was determined by inhibition of MFI. A 4-parameter logistic curve fit was reported as % pSTAT inhibition using GraphPad Prism 8.1.2. The IC_{50} value from a curve was accepted if the following conditions were met: (1) the R^2 was >0.7, and (2) the standard error

of the slope was <6. The arithmetic mean IC_{50} value for each case was estimated in PBMCs (N=3-5) and WB (N=6–10) and compared among JAKinibs.

Pharmacokinetic (PK) Profiles

Human steady-state population PK curves in RA subjects for baricitinib once-daily dosing at 2 or 4mg,[1] TOFA with twice-daily 5 and 10mg,[2], and UPA with once-daily dosing at 15 and 30mg,[3] were used as previously reported. The PK profile of FIL was simulated based on a 2-compartment model, including a first-order absorption with lag time. For the metabolite, the PK was obtained based on a 1-compartment model, including a zero-order input, followed by first-order absorption model.

Estimation and Comparison of Time Above IC_{50} and Daily Percent Inhibition

The mean IC_{50} values obtained from duplicate dose-response curves and human population PK curves were used to model time above IC_{50} and average daily percent inhibition for each cytokine/STAT pair in individual cell types. In vitro IC_{50} value in PBMCs were adjusted for protein binding by dividing the IC_{50} value for each donor by the experimentally assessed unbound fraction (N=3). Measured protein binding were: baricitinib 53%, filgotinib 64%, GS-829845 69%, tofacitinib 67%, and upadacitinib 50% (FDA Center for Drug Evaluation and Research NDA 211675). No adjustments were made to whole blood values. The time above IC_{50} was defined as the total time the PK concentration was above the IC_{50} values. The average daily percent inhibition was obtained by entering the steady state PK concentrations into the concentration response curves, computing the area under the curve (AUC), and dividing it by 24 hours.

Statistical Analysis

Comparisons between filgotinib and the other treatment groups were performed using a two-way mixed effect Analysis of Variance (ANOVA) to model the time above IC_{50} and average daily percent inhibition with fixed effect for compound for each stimulation, cell type, and pSTAT combination and random effect for donor with Dunnett's multiplicity correction for multiple comparison adjustments. Filgotinib 200mg was considered the reference group for the comparisons. The same mixed effect model was used to fit the log(IC_{50}) values and estimate and compare mean IC_{50} values. Reported time above IC_{50}, average daily percent inhibition, and IC_{50} values corresponded to the least squares means of the compound effects from those models. Comparisons were considered significant at a two-sided alpha level of 0.05. Analyses were performed using GraphPad Prism version 8.1.2 and SAS version 9.4. Analysis between protein-adjusted PBMC values and WB were performed using Pearson's correlation. Sample size (N=10) was based on feasibility and internal pilot data.

Clinical study: Ex Vivo Stimulation of Phase 1 Samples

The phase 1 study in human volunteers was a randomized, placebo-controlled study: (study identifier: NCT01179581). Subjects received filgotinib 200mg once daily or placebo. Blood was collected from healthy volunteers (N=10) into lithium heparin vacutainer tubes, following their informed consent. Blood was equilibrated at 37^{o}C for 30 min and triggered with either recombinant human IL-6 (10 ng/mL; R&D Systems) or recombinant human GM-CSF (20 pg/mL; PeproTech) at 37^{o}C for 20 min and treated with prewarmed 1X lysis/fix buffer (BD Biosciences) to lyse RBCs and fix leukocytes. Cells were permeabilized with 100% methanol and incubated with anti-pSTAT1 (#612564) and anti-CD4 (#555349) or anti-pSTAT5 (#612567) and anti-CD33 (#345800) antibodies (BD Biosciences) at 4^{o}C for 30 min, washed once with PBS 1X, and analyzed on a FACSanto II flow cytometer. Full details have been published previously.[4]

References

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Appendix 1: Characteristics of RA Donors

	RA Donor 1	**RA Donor 2**	**RA Donor 3**
RA Diagnosis Date	1996	2014	2017
Gender	Female	Female	Female
Age	52 years	40 years	56 years
Ethnicity	Caucasian	Caucasian	Caucasian
Other Conditions	Celiac Disease, Lupus, Sjogren's Syndrome, Psoriasis	None	None
Treatment	Hydrocodone	Gabapentin, Hydroxychloroquine (Plaquenil), Meloxicam, Tramadol	Humira Injection
Disease Severity	Severe	Moderate	Moderate
Receiving Treatment for Other Pathologies?	Yes; Synthroid, Tizanidine, Prednisone, Doxycycline, Testosterone Cream	No	No
Pregnant or nursing?	No	No	No
Taking an investigational product in the last year?	No	No	No

Appendix 2: Reagents and Compounds

EC_{80} Values

Whole Blood Cytokine EC80s			
Cytokine	**Vendor**	**Catalog No.**	**EC80**
GMCSF	Peprotech	300-03	9pg/ml
GCSF*	Peprotech	300-23	Donor1-2: 10ng/ml Donor 4-6: 25ng/ml
IFNγ	Peprotech	300-02	8ng/ml
IFNα	Sigma	H6041	6ng/ml
IL-2	Gibco	PHC0021	5ng/ml
IL-4	Peprotech	200-04	0.3ng/ml
IL-6	Peprotech	200-06	10ng/ml
IL-15	Peprotech	200-15	1ng/ml
IL-12	R&D systems	219-IL	10ng/ml
IL-23	Proteintech	HZ-1254	100ng/ml
PBMC Cytokine EC80s			
Cytokine	**Vendor**	**Catalog No.**	**EC80**
GMCSF	Peprotech	300-03	9pg/ml
GCSF	Peprotech	300-23	4ng/ml
IFNγ	Peprotech	300-02	0.2ng/ml
IFNα	Sigma	H6041	6ng/ml
IL-2	Gibco	PHC0021	5ng/ml
IL-4	Peprotech	200-04	0.2ng/ml
IL-6	Peprotech	200-06	4ng/ml
IL-15	Peprotech	200-15	0.4ng/ml

*EC_{80} value of GCSF on whole blood was increased by 2.5x after the first 2 donors were treated.

Staining Panels

Conjugated STAT Antibodies (Post MeOH Perm) All Donors					
Cytokines	**Antibody**	**Vendor**	**Dilution**	**Catalog No.**	**Clone**
IFNγ, IL-6, IFNα	STAT1	CST	50	8009S	58D6/P-STAT1 (Tyr701)
G-CSF, IL-6, IFNα, IL-23	STAT3	BD	20	562071	4/P-STAT3 (pY705)
GM-CSF, IL-2, IFNα, IL-15	STAT5	BD	20	562076	47/P-STAT5 (pY694)
IL-4	STAT6	BD	20	562079	18/P-STAT6 (pY641)
IL-12	STAT4	Miltenyi	20	130-114-492	REA855/P-STAT4 (pY693)

Panel 1: Donor 1 WB						
Antigen	**Color**	**Titer**	**Catalog No.**	**Clone**	**Vendor**	**Pre vs Post**
CD8	UV805	50	612889	SK1	BD	Pre-Perm
CD4	UV737	50	612748	SK3	BD	Pre-Perm
CD14	UV395	50	563561	MphiP9	BD	Pre-Perm
CD19	BV785	50	563325	SJ25C1	BD	Pre-Perm
CD56	BV711	100	318336	HCD56	Biolegend	Pre-Perm
CD16	BV711	100	302044	3G8	Biolegend	Pre-Perm
CD3	APC Fire	200	300470	UCHT1	Biolegend	Post-Perm

Panel 2: Donor 1 PBMC, Donor 2 WB+PBMC						
Antigen	**Color**	**Titer**	**Catalog No.**	**Clone**	**Vendor**	**Pre vs Post**
CD8	UV737	50	612754	SK1	BD	Pre-Perm
CD4	UV805	50	612887	SK3	BD	Pre-Perm
CD14	UV737	25	612763	M5E2	BD	Pre-Perm
CD19	BV785	50	563325	SJ25C1	BD	Pre-Perm
CD56	BV711	100	318336	HCD56	Biolegend	Pre-Perm
CD16	BV711	100	302044	3G8	Biolegend	Pre-Perm
CD3	PE	50	300441	UCHT1	Biolegend	Post-Perm
CD15	PECY7	100	323030	W6D3	Biolegend	Post-Perm

Panel 3: Donor 4 WB+PBMC						
Antigen	**Color**	**Titer**	**Catalog No.**	**Clone**	**Vendor**	**Pre vs Post**
CD8	UV737	50	612754	SK1	BD	Pre-Perm
CD4	UV805	50	612887	SK3	BD	Pre-Perm
CD14	UV395	25	563561	MphiP9	BD	Pre-Perm
CD19	BV785	50	563325	SJ25C1	BD	Pre-Perm
CD56	BV711	100	318336	HCD56	Biolegend	Pre-Perm
CD16	BV711	100	302044	3G8	Biolegend	Pre-Perm
CD3	PE	50	300441	UCHT1	Biolegend	Post-Perm
CD15	Percpcy5.5	200	323030	W6D3	Biolegend	Post-Perm

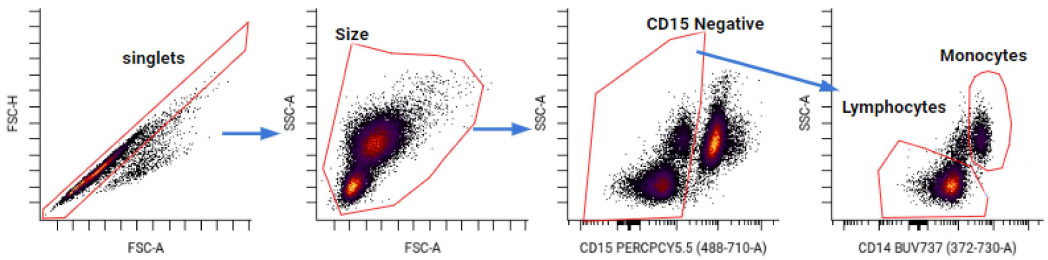
Panel 4: Donor 5 WB+PBMC, Donor 6 WB+PBMC						
Antigen	**Color**	**Titer**	**Catalog No.**	**Clone**	**Vendor**	**Pre vs Post**
CD8	AX700	100	557945	RPA-T8	BD	Pre-Perm
CD4	UV805	50	612887	SK3	BD	Pre-Perm
CD14	UV737	25	612763	M5E2	BD	Pre-Perm
CD19	BV785	50	563325	SJ25C1	BD	Pre-Perm
CD56	BV711	100	318336	HCD56	Biolegend	Pre-Perm
CD16	BV711	100	302044	3G8	Biolegend	Pre-Perm
CD3	BUV395	50	563546	UCHT1	BD	Pre-Perm
CD15	Percpcy5.5	200	323030	W6D3	Biolegend	Post-Perm

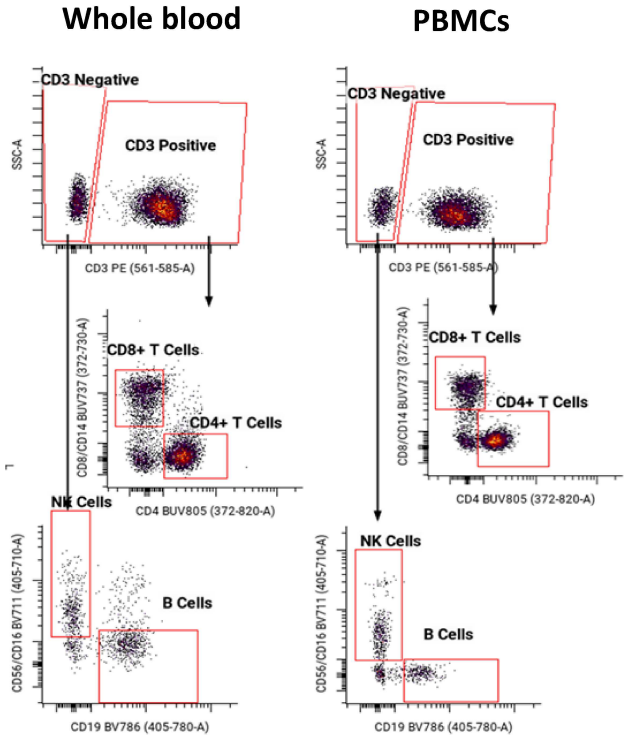
Panel 5: IL-12 and IL-23 Donors 1-10 WB						
Antigen	**Color**	**Titer**	**Catalog No.**	**Clone**	**Vendor**	**Pre vs Post**
CD8	BUV805	100	612889	SK1	BD	Pre-Perm
CD4	BUV737	50	612748	SK3	BD	Pre-Perm
CD45RA	PE-Cy7	25	560675	HI100	BD	Pre-Perm
CD19	BV786	50	563325	SJ25C1	BD	Pre-Perm
CD56	BV711	100	318336	HCD56	Biolegend	Pre-Perm
CD16	BV711	100	302044	3G8	Biolegend	Pre-Perm
CD3	PE	50	552127	SP34-2	BD	Post-Perm
CD15	PE-Cy7	200	323030	SSEA-1	Biolegend	Post-Perm

Appendix 3: Gating Strategies

A) Gating strategy for common whole blood and PBMC cell populations (monocytes, CD4+, CD8+, NK cells, and B cells)

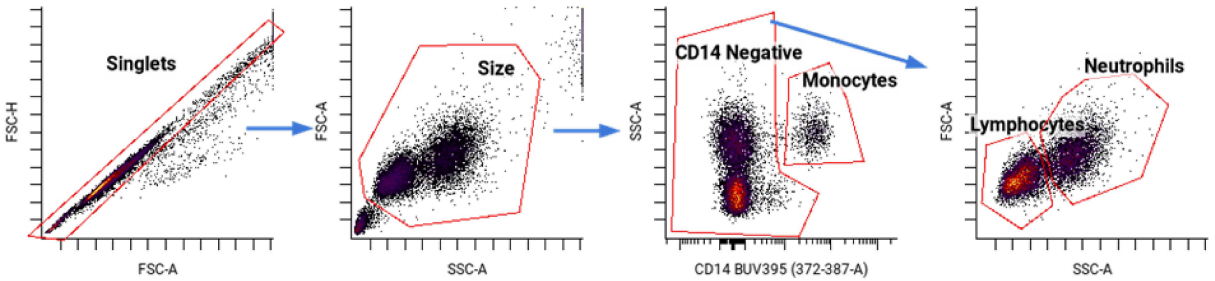
Initial phases of the gating strategy were common for whole blood and PBMCs



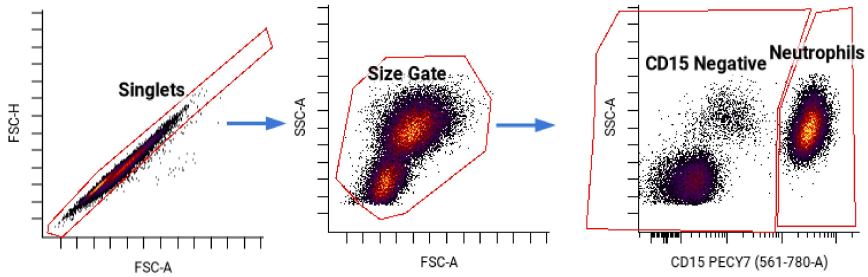


B) Gating strategy for neutrophils (whole blood). There were 3 different gating strategies depending on the antibody cocktail used.

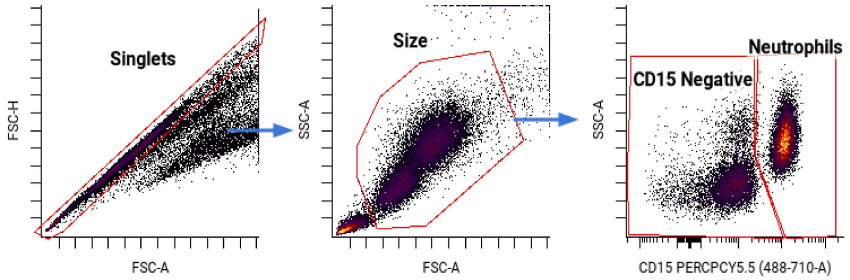
Strategy 1. No CD15 marker included.



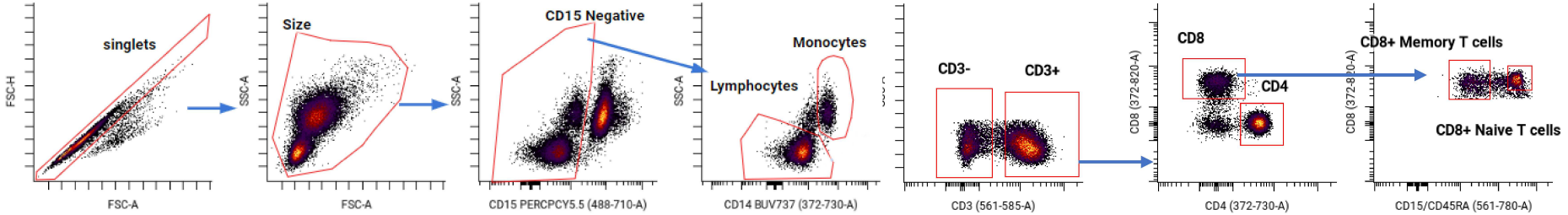
Strategy 2. CD15 PE-Cy7 marker.



Strategy 3. CD15 Percp-Cy5.5 marker.



C) Gating strategy for CD8+ memory T cells (IL-23 stimulation; Whole blood)



Appendix 4: Raw Flow Cytometry Data

Raw flow cytometry data is provided separately as an Excel document. Datasets are described below:

- Dataset 1: Donors 1-5. Whole blood and PBMCs data. Monocytes, B cells, CD4 T cells, CD8 T cells and NK cells Dataset 1-Neutrophils. Donors 1-5. Whole blood. Neutrophils
- Dataset 2: Donors 6-10. Whole blood data. Monocytes, B cells, CD4 T cells, CD8 T cells, NK cells and neutrophils
- Dataset 3: Donors 1-10. Whole blood data. IL-12 and IL-23 stimuli. NK cells and CD8+ memory T cells
- Dataset 4: Whole blood data. RA donors and matching healthy donors

Supplemental Table 1. Clinical Efficacy and Safety Findings From Phase 3 Studies of JAKinibs.

	Filgotinib	Tofacitinib	Upadacitinib	Baricitinib	
	200 mg	5 mg, bid	15 mg	2 mg	4 mg
MTX-IR ACR20/50/70/DAS28CR[a]	19/25/21/32 [1]	25/26/15/5 [2]	31/33/25/32 [3]	NR	37/32/22/33[b] [4]
Biologic-IR ACR20/50/70/ DAS28CR[c]	35/28/15/14 [1]	17/18/12/4 [2]	37/22/5/20 [3]	22/12/11/9[d] [4]	28/20/9/8[d] [4]
Hemoglobin (g/dL)	+0.2 [5]	+0.08 [6]	–0.8 [7]	–0.28 [8]	–0.2 [8]
Herpes zoster (E/100PY)	0.1% (0.3% placebo) [1]	1-10% [2]	0.7% (0.2% placebo) [3]	1.4% (0.4% placebo) [4]	4.3% (1% placebo) [9]
Infections (E/100PY)	26.5 [1]	43.8 [2]	93.7 [3]	101 [4]	
Serious infection (E/100PY)	1.7 [1]	2.4 [2]	3.8 [3]	3.2 [4]	
Opportunistic infections (E/100PY)	0.1 [1]	<0.1 [2]	0.6 [3]	0 [9]	0.5 [9]
VTE (E/100PY)	0.2 [10]	0.27 [2]	0.5 [7]	0.6 [11]	0.8 [11]

[a] MTX-IR response rates at week 24/26 (placebo-corrected); all patients on background MTX.
[b] DAS28-hsCRP ≤ 3.2.
[c] Biologic-IR response rates at week 12 (placebo corrected); DAS28 CR, complete remission is defined as DAS28<2.6; tofacitinib, baricitinib uses DAS28-ESR and other JAKinibs use DAS28-CRP⬇, dose-dependent decrease; E/100 PY, events per 100 patient years; JAK, Janus kinase; JAKinib; Janus kinase inhibitor; NK, natural killer; NR, not reported; VTE, venous thromboembolism.
[d] DAS28-ESR ≤ 3.2.

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Supplemental Table 2. JAKinib IC_{50} Values in CD4+ T Cells, Monocytes, and NK Cells From PBMC Assays.

	CD4+ T Cells					Monocytes					NK Cells				
	BARI	**FIL**	**MET**	**TOFA**	**UPA**	**BARI**	**FIL**	**MET**	**TOFA**	**UPA**	**BARI**	**FIL**	**MET**	**TOFA**	**UPA**
Stimulation/pSTAT	**IC_{50}, nM**														
JAK2/2 or JAK2/TYK2-dependent cytokines															
G-CSF/pSTAT3	NS					13	985	11,630	67	13	NS				
GM-CSF/pSTAT5	NS					51	5012	59,031	240	24	NS				
JAK1/JAK2/TYK2-dependent cytokines															
IFNα/pSTAT1	20	618	9539	44	8	47	1440	34,045	108	19	53	1360	23,053	122	40
IFNα/pSTAT3	17	511	7720	39	7	8	275	4511	25	4	NS				
IFNα/pSTAT5	11	338	5227	23	5	8	224	3478	17	3	NS				
IFNγ/pSTAT1	NS					48	2785	41,945	165	26	NS				
IL-6/pSTAT1	18	512	5746	40	9	16	547	5457	46	6	NS				
IL-6/pSTAT3	NS					53	1556	20,908	157	25	NS				
JAK1/3-dependent cytokines															
IL-2/pSTAT5	16	447	7316	18	5	NS					27	875	11,473	31	13
IL-4/pSTAT6	29	744	20,257	38	11	20	604	17,497	52	9	19	388	10,974	29	8
IL-15/pSTAT5	14	453	7222	18	5	NS					29	978	13,072	34	14

G-CSF, GM-CSF, IFNα, IFNy, IL-6, IL-2, IL-4, and IL-15, reported IC_{50} values are based on the average of duplicate measures (N=3–5 healthy donors). Protein binding was not accounted for in the IC_{50} calculations reported.

BARI, baricitinib; FIL: filgotinib; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte macrophage colony stimulating factor; IC_{50}, half maximum inhibitory concentration; IFN, interferon; IL, interleukin; JAK, Janus kinase; MET, GS-829845 (filgotinib metabolite); NK, natural killer; NS, not sampled; PBMC, peripheral blood mononuclear cells; pSTAT, phosphorylated signal transducer and activator of transcription; TOFA, tofacitinib; TYK, tyrosine kinase; UPA, upadacitinib.

Supplemental Table 3. JAKinib IC_{50} Values in B Cells, and CD8+ T Cells from PBMC Assays.

	B Cells					CD8+ T Cells				
	BARI	**FIL**	**MET**	**TOFA**	**UPA**	**BARI**	**FIL**	**MET**	**TOFA**	**UPA**
Stimulation/pSTAT	**IC_{50}, nM**									
JAK1/3-dependent cytokines										
IL-2/pSTAT5	NS					13	395	5377	16	5
IL-4/pSTAT6	106	2872	82,459	172	43	19	470	11,836	29	9
IL-15/pSTAT5	NS					20	685	10,370	25	8
JAK1/JAK2/TYK2-dependent cytokines										
IFNα/pSTAT1	56	1548	30,698	136	30	23	646	9465	45	10
IFNα/pSTAT3	13	380	6106	31	6	16	453	6628	36	8
IFNα/pSTAT5	9	255	4083	21	4	11	302	4236	21	5
IFNγ/pSTAT1	11	629	9303	38	9	NS				

IFNα, IFNγ, IL-2, IL-4, and IL-15, reported IC_{50} values are based on the average of duplicate measures (N=3–5 healthy donors). Protein binding was not accounted for in the IC_{50} calculations reported.

BARI, baricitinib; FIL, filgotinib; IC_{50}, half maximum inhibitory concentration; IFN, interferon; IL, interleukin; JAK, Janus kinase; MET, GS-829845 (filgotinib metabolite); NS, not sampled; PBMC, peripheral blood mononuclear cells; pSTAT, phosphorylated signal transducer and activator of transcription; TOFA, tofacitinib; TYK2, tyrosine kinase 2; UPA, upadacitinib.

Supplemental Table 4. JAKinib Potency Differences Based on Cell Type.

Stimuli	pSTAT	Cell type A	Cell type B	BARI	FIL	MET	TOFA	UPA
G-CSF	STAT3	neutrophils	Monocytes	***	***	***	***	***
GM-CSF	STAT5	Monocytes	neutrophils	***	***	***	***	***
IFNα	STAT1	B cells	CD4+ cells	***	***	***	***	***
		B cells	CD8+ cells	***	***	***	***	***
		Monocytes	B cells		**	**		
		B cells	NK cells					
		CD8+ cells	CD4+ cells	*	*		*	**
		Monocytes	CD4+ cells	***	***	***	***	***
		NK cells	CD4+ cells	***	***	***	***	***
		Monocytes	CD8+ cells	***	***	***	***	***
		NK cells	CD8+ cells	***	***	***	***	***
		Monocytes	NK cells	*	***	***	*	*
	STAT3	CD4+ cells	B cells		**			
		CD8+ cells	B cells					
		Monocytes	B cells		***	**		
		NK cells	B cells					
		neutrophils	B cells	**	*	**		
		CD4+ cells	CD8+ cells					
		Monocytes	CD4+ cells					
		CD4+ cells	NK cells		**	*		
		neutrophils	CD4+ cells					
		Monocytes	CD8+ cells			*		
		CD8+ cells	NK cells					
		neutrophils	CD8+ cells			*		
		Monocytes	NK cells		***	***		*
		neutrophils	Monocytes					
		neutrophils	NK cells	*	*	***		*
	STAT5	CD4+ cells	B cells		**			
		CD8+ cells	B cells		*			
		Monocytes	B cells		*			*
		NK cells	B cells	*				**
		CD4+ cells	CD8+ cells					
		CD4+ cells	Monocytes					**
		NK cells	CD4+ cells					*
		CD8+ cells	Monocytes					**
		NK cells	CD8+ cells					*
		NK cells	Monocytes					***
IFNγ	STAT1	Monocytes	B cells	***	***	***	***	***
		neutrophils	B cells	***	***	***	***	***
		Monocytes	neutrophils				**	

IL-15	STAT5	CD8+ cells	CD4+ cells	**	***	*	**	**
		NK cells	CD4+ cells	***	***	***	***	***
		NK cells	CD8+ cells	*	*		**	***
IL-2	STAT5	CD4+ cells	CD8+ cells					
		NK cells	CD4+ cells	***	***	***	***	***
		NK cells	CD8+ cells	***	***	***	***	***
IL-4	STAT6	B cells	CD4+ cells	***	***	***	***	***
		B cells	CD8+ cells	***	***	***	***	***
		B cells	Monocytes	***	***	***	***	***
		B cells	NK cells	***	***	***	***	***
		B cells	neutrophils	***	***	***	***	***
		CD4+ cells	CD8+ cells	*		*	*	
		CD4+ cells	Monocytes				**	
		CD4+ cells	NK cells	***	***	***	**	
		CD4+ cells	neutrophils					
		Monocytes	CD8+ cells				***	
		CD8+ cells	NK cells					
		neutrophils	CD8+ cells				***	
		Monocytes	NK cells		*	***	***	
		neutrophils	Monocytes					
		neutrophils	NK cells	**		**	***	
IL-6	STAT1	Monocytes	CD4+ cells	*	*	***	*	
	STAT3	CD4+ cells	Monocytes	***	***	*	***	***

Comparisons between mean IC_{50} values of different cell types in response to the same stimuli were performed using a 2-way mixed effect Analysis of Variance (ANOVA). Analysis based on in vitro whole blood measurements in healthy donors.
N=7–10 donors. **P*<0.05, ***P*<0.01, ****P*<0.001
BARI, baricitinib; FIL, filgotinib; IFN, interferon; IL, interleukin; JAK, Janus kinase; JAKinib; Janus kinase inhibitor; MET, GS-829845 (filgotinib metabolite); NK, natural killer; pSTAT, phosphorylated signal transducer and activator of transcription; TOFA, tofacitinib; UPA, upadacitinib.

Supplemental Table 5. JAKinib Potency Differences Based on STAT Substrate.

Stimuli	Cell type	STAT A	STAT B	BARI	FIL	MET	TOFA	UPA
IFNα	B cells	STAT1	STAT3	***	***	***	***	***
	B cells	STAT1	STAT5	***	***	***	***	***
	B cells	STAT3	STAT5				*	
	CD4+	STAT1	STAT3					
	CD4+	STAT1	STAT5	**	**	**	***	**
	CD4+	STAT3	STAT5	*			**	*
	CD8+	STAT1	STAT3	***	**	**	**	***
	CD8+	STAT1	STAT5	***	***	***	***	***
	CD8+	STAT3	STAT5				**	*
	NK cells	STAT1	STAT3	***	***	***	***	***
	NK cells	STAT1	STAT5	***	***	***	***	***
	NK cells	STAT3	STAT5					
	monocytes	STAT1	STAT3	***	***	***	***	***
	monocytes	STAT1	STAT5	***	***	***	***	***
	monocytes	STAT3	STAT5	**	*	*	**	**
IL-6	CD4+	STAT3	STAT1	***	***	***	***	***
	monocytes	STAT3	STAT1	***	***	***	***	***

Comparisons between mean IC_{50} values of different STATs activated in the same cell time and in response to the same stimuli were performed using a 2-way mixed effect ANOVA. Analysis based on in vitro whole blood measurements in healthy donors.
N=7–10 donors. **P*<0.05, ***P*<0.01, ****P*<0.001
BARI, baricitinib; FIL, filgotinib; IFN, interferon; IL, interleukin; JAK, Janus kinas; JAKinib; Janus kinase inhibitor; MET, GS-829845 (filgotinib metabolite); NK, natural killer; STAT, signal transducer and activator of transcription; TOFA, tofacitinib; UPA, upadacitinib.

Supplemental Table 6. JAKinib Potencies Impacted by Stimuli Utilizing the Same JAK Pair.

Cell type	STAT	Stimuli A	Stimuli B	BARI	FIL	MET	TOFA	UPA
Monocytes	STAT1	IFNγ	IL-6	**	***	***	***	***

Comparisons between mean IC_{50} values observed for monocytes in response to 2 different stimuli signaling through the same JAK/STAT pair (JAK1/JAK2) were performed using a 2-way mixed effect ANOVA. Analysis based on in vitro whole blood measurements in healthy donors.

N=7–10 donors. ***P*<0.01, ****P*<0.001

BARI, baricitinib; FIL, filgotinib; IFN, interferon; IL, interleukin; JAK, Janus kinas; JAKinib; Janus kinase inhibitor; MET, GS-829845 (filgotinib metabolite); NK, natural killer; STAT, signal transducer and activator of transcription; TOFA, tofacitinib; UPA, upadacitinib.

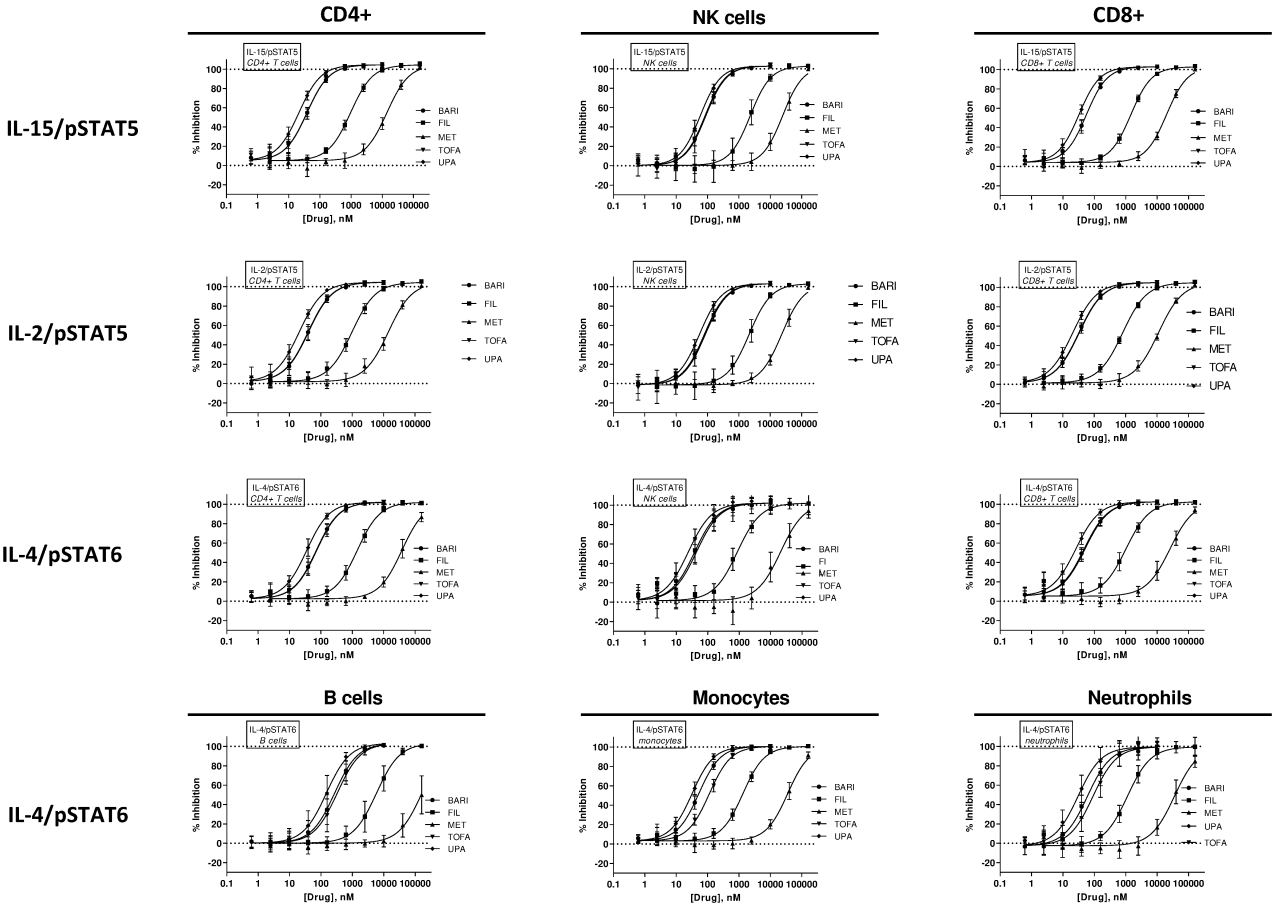
Supplemental Table 7. Heatmap of Time Above 50% Inhibition at Clinical Doses of JAKinibs.

Signaling Pair	Cell Type	Stimuli Readout	Filgotinib[M] 100mg	Filgotinib[M] 200mg	Tofacitinib 5mg	Tofacitinib 10mg	Upadacitinib 15mg	Upadacitinib 30mg	Baricitinib 2mg	Baricitinib 4mg
JAK1/TYK2	B cells	IFNα/pSTAT5	7†	20	16†	23	16†	24*	9†	19
	NK	IFNα/pSTAT5	7†	20	14†	22	12†	18	5†	13†
	CD8+ T cells	IFNα/pSTAT5	6†	17	15	22*	15	23*	8†	16
	NK	IFNα/pSTAT3	6†	17	13†	20*	11†	18	7†	16
	monocytes	IFNα/pSTAT5	5†	12	14	21*	21*	24*	8†	18*
	CD4+ T cells	IFNα/pSTAT5	5†	12	14*	22*	15*	23*	7†	16*
	B cells	IFNα/pSTAT3	5†	13	12	19*	13	21*	7†	16*
	CD8+ T cells	IFNα/pSTAT3	4†	10	10	17*	12	19*	5†	13*
	neutrophils	IFNα/pSTAT3	4†	8	12*	19*	15*	22*	3†	10
	CD4+ T cells	IFNα/pSTAT3	3†	7	9	16*	12*	19*	4†	12*
	monocytes	IFNα/pSTAT3	3†	7	10*	17*	16*	23*	4	12*
	CD4+ T cells	IFNα/pSTAT1	3†	7	7	15*	11*	17*	2†	9
	CD8+ T cells	IFNα/pSTAT1	2†	6	5	12*	8	14*	1†	7
	NK	IFNα/pSTAT1	0	1	0	2	1	3	0	0
	B cells	IFNα/pSTAT1	0	1	0	2	2	6*	0	0
	monocytes	IFNα/pSTAT1	0	0	0	1	4*	8*	0	0
JAK1/JAK2	CD4+ T cells	IL-6/pSTAT1	11†	24	13†	20†	12†	18†	9†	17†
	monocytes	IL-6/pSTAT1	5†	15	9†	17	13	21*	5†	13
	monocytes	IL-6/pSTAT3	0	0	0	2	3*	8*	0	0
	B cells	IFNγ/pSTAT1	4†	9	13*	20*	13*	21*	12*	21*
	neutrophils	IFNγ/pSTAT1	0	1	3	11*	8*	14*	1	5*
	monocytes	IFNγ/pSTAT1	0	0	0	6*	6*	11*	0	5*
JAK1/JAK3	CD8+ T cells	IL-2/pSTAT5	2†	5	14*	21*	11*	18*	2†	19*
	CD4+ T cells	IL-2/pSTAT5	3†	7	17*	23*	14*	21*	4†	11*
	NK	IL-2/pSTAT5	0	2	8*	16*	6*	11*	0	4
	CD4+ T cells	IL-15/pSTAT5	3†	9	19*	24*	15*	23*	5†	13*
	CD8+ T cells	IL-15/pSTAT5	0†	4	13*	21*	10*	16*	1†	7*
	NK	IL-15/pSTAT5	0	2	8*	16*	6*	11*	0	3
	NK	IL-4/pSTAT6	3†	6	15*	22*	12*	18*	4	12*
	CD8+ T cells	IL-4/pSTAT6	4†	9	19*	24*	13*	21*	6†	14*
	neutrophils	IL-4/pSTAT6	1	4	7*	15*	10*	16*	1	7
	monocytes	IL-4/pSTAT6	1†	4	5	13*	10*	16*	1	8*
	CD4+ T cells	IL-4/pSTAT6	0†	3	10*	17*	9*	15*	0†	5
	B cells	IL-4/pSTAT6	0	0	1	3*	2	5*	0	1
JAK2/TYK2	monocytes	G-CSF/pSTAT3	0	0	1	4*	4*	9*	1	4*
	neutrophils	G-CSF/pSTAT3	0	0	0	0	0	1	0	0
	NK	IL-12/pSTAT4	0	0	0	0	0	0	0	0
	CD8+ m T cells	IL-23/pSTAT3	0	0	0	0	0	0	0	0
JAK2/JAK2	neutrophils	GM-CSF/pSTAT5	0	1	3	11*	14*	21*	0	6*
	monocytes	GM-CSF/pSTAT5	0	0	0	0	5*	9*	0	0

Heatmap represents time (hours) above 50% target inhibition by JAKinibs at clinical doses over a 24-hour dose period (based on in vitro WB measurements, N=6–10). FIL includes the metabolite exposure/activity calculation.
*P<0.05 higher vs FIL 200 mg; †P<0.05 lower vs FIL 200 mg

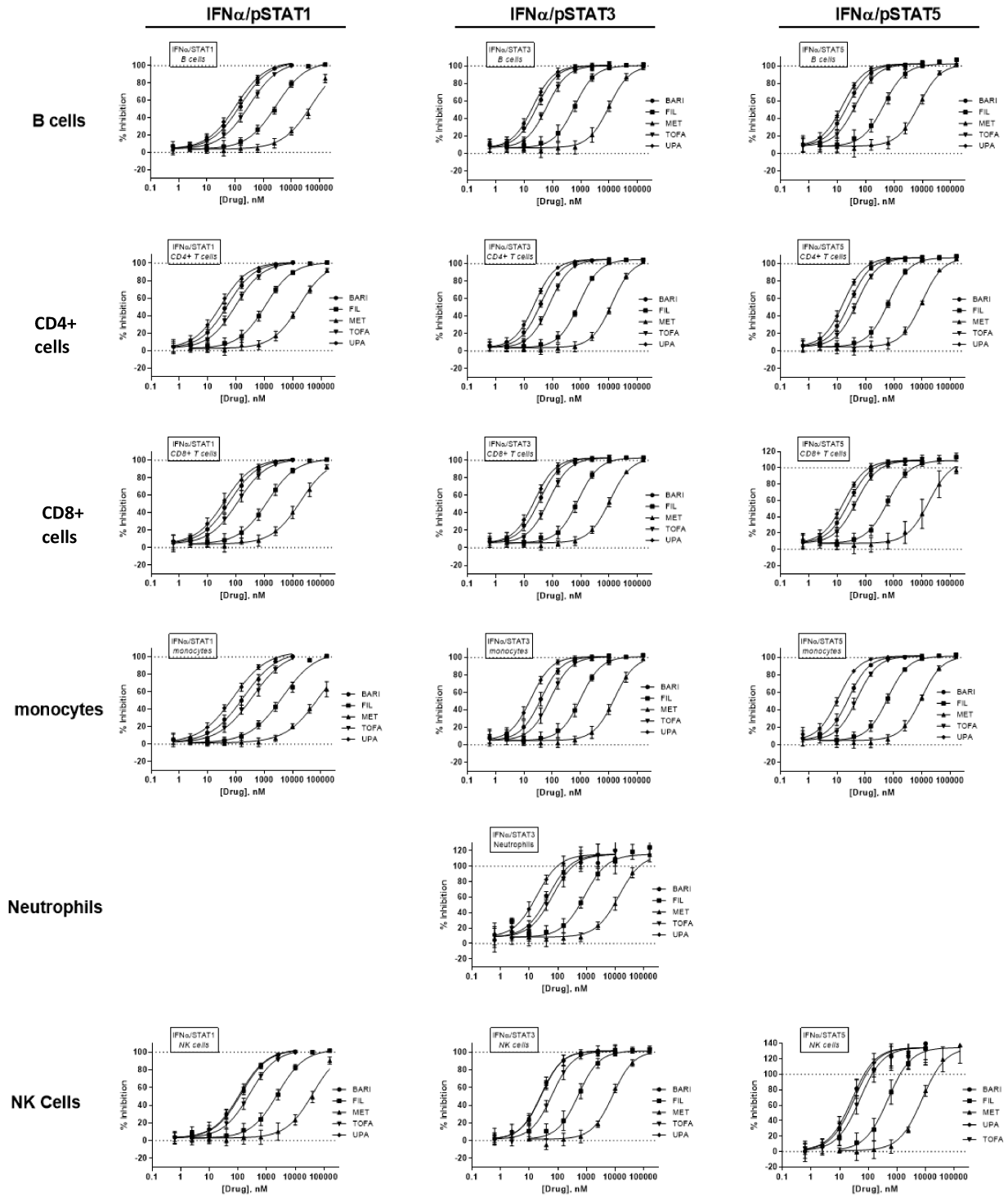
IFN, interferon; IL, interleukin; JAK, Janus kinase; JAKinib; Janus kinase inhibitor; NK, natural killer; pSTAT, phosphorylated signal transducer and activator of transcription; TYK2, tyrosine kinase 2.

Supplemental Figure 1.



JAKinib dose-response curves for STAT phosphorylation in response to JAK1/JAK3 dependent cytokines IL-2(pSTAT5), IL-4(pSTAT6) and IL-15(pSTAT5). Graphs represent percent pSTAT inhibition in whole blood from n=7–10 healthy volunteers. The percent inhibition values were calculated from duplicate wells of an 8-point dose response of each JAKinib and normalized to the difference in MFI measurements of cytokine stimulated minus unstimulated samples. Error bars represented SD. A four-parameter logistic fit model with shared upper and lower asymptotes and hillslope were used to fit the composite data.

Supplemental Figure 2.

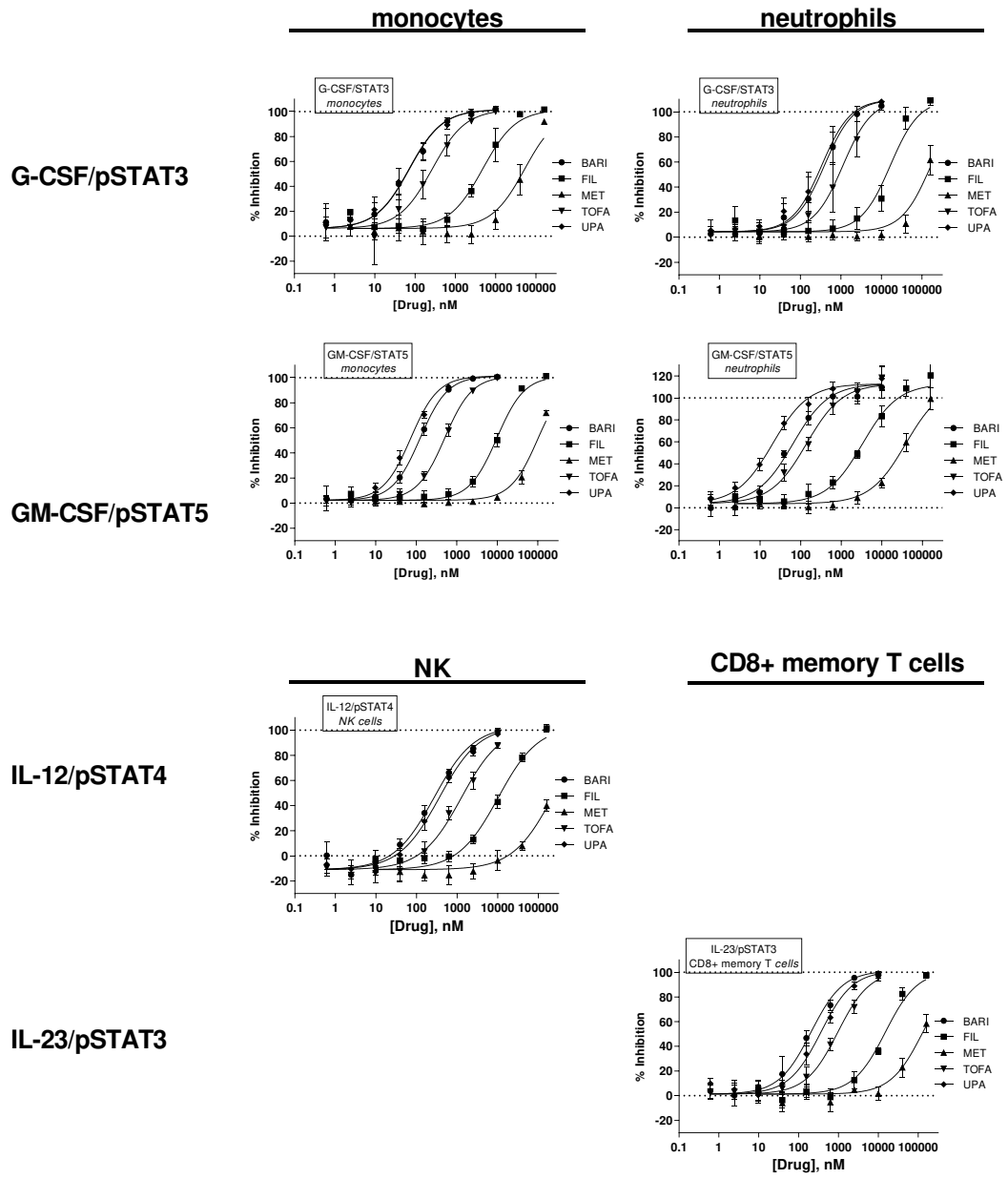


JAKinib dose-response curves for STAT (pSTAT1, pSTAT3, pSTAT5) phosphorylation in response to JAK1/TYK2 dependent cytokine IFNα. Graphs represent percent pSTAT inhibition in whole blood from n=9–10 healthy volunteers. In this figure, as well as

Supplemental Figures 3 and 4, the percent inhibition values were calculated from duplicate wells of an 8-point dose response of each JAKinib and normalized to the difference in MFI measurements of cytokine stimulated minus unstimulated samples. Error bars represented SD. A 4-parameter logistic fit model with shared upper and lower asymptotes and hillslope were used to fit the composite data.

BARI, baricitinib; FIL, filgotinib; IL, interleukin; JAK, Janus kinase; JAKinib; Janus kinase inhibitor; MET, GS-829845 (filgotinib metabolite); NK, natural killer; pSTAT, phosphorylated signal transducer and activator of transcription; TOFA, tofacitinib; TYK2, tyrosine kinase 2; UPA, upadacitinib.

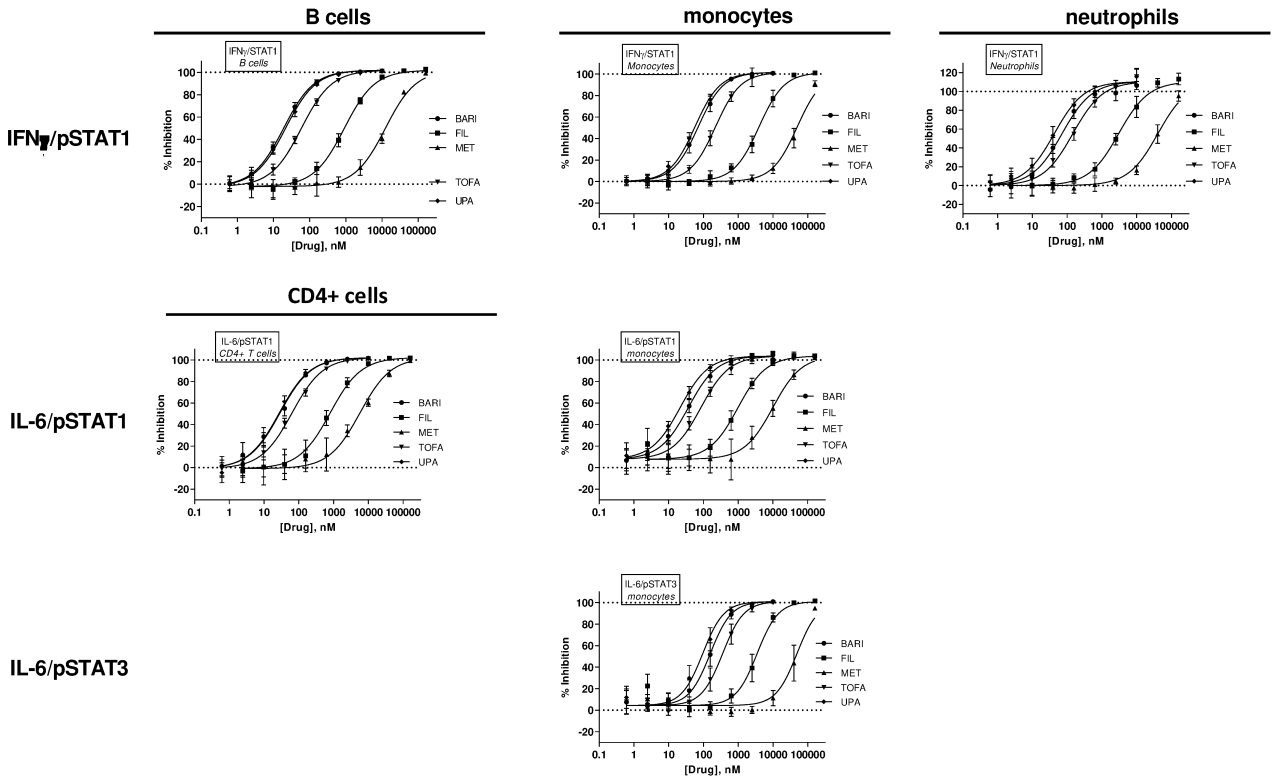
Supplemental Figure 3.



JAKinib dose-response curves for STAT phosphorylation in response to JAK2/TYK2-dependent cytokines G-CSF (pSTAT3), IL-12 (pSTAT4), and IL-23 (pSTAT3) and JAK2/JAK2-dependent GM-CSF (pSTAT5). Graphs represent percent pSTAT inhibition in whole blood from n=6–10 healthy volunteers.

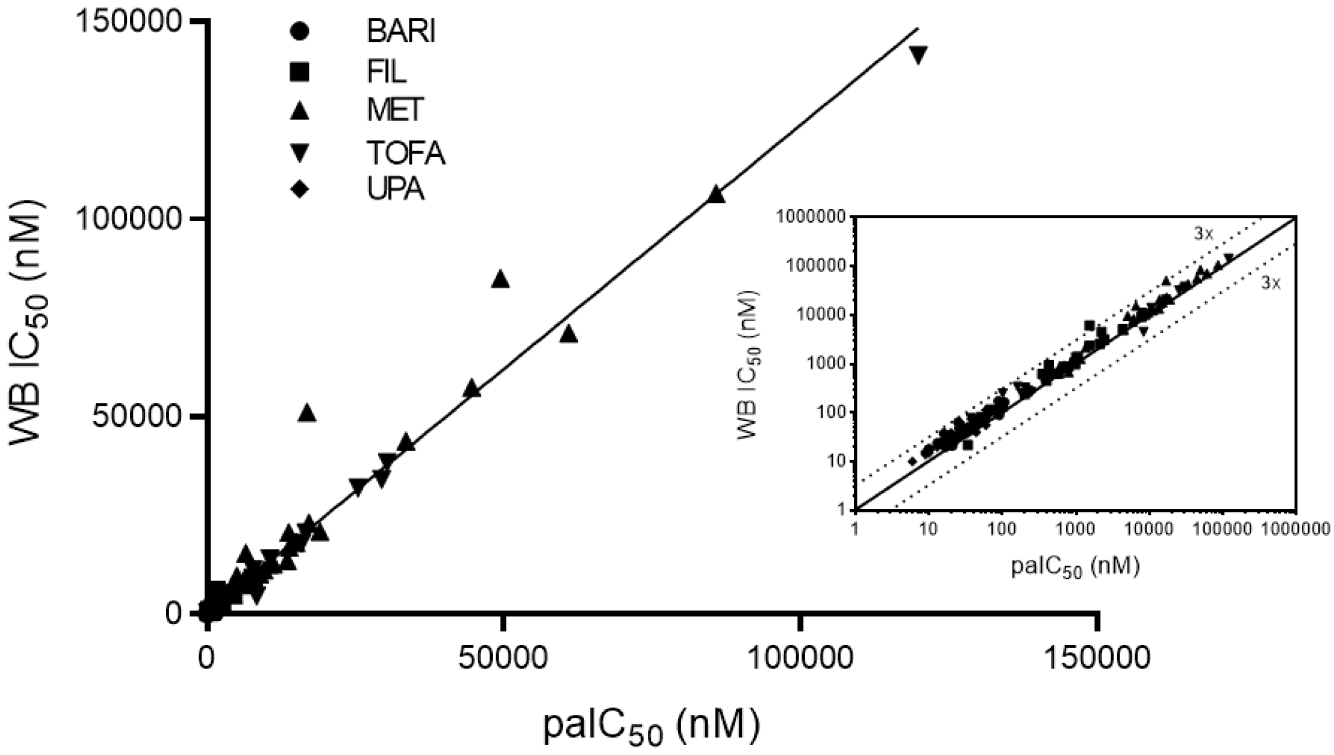
BARI, baricitinib; FIL, filgotinib; IL, interleukin; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte macrophage-CSF; JAK, Janus kinase; JAKinib; Janus kinase inhibitor; MET, GS-829845 (filgotinib metabolite); NK, natural killer; pSTAT, phosphorylated signal transducer and activator of transcription; TOFA, tofacitinib; TYK2, tyrosine kinase 2; UPA, upadacitinib.

Supplemental Figure 4.



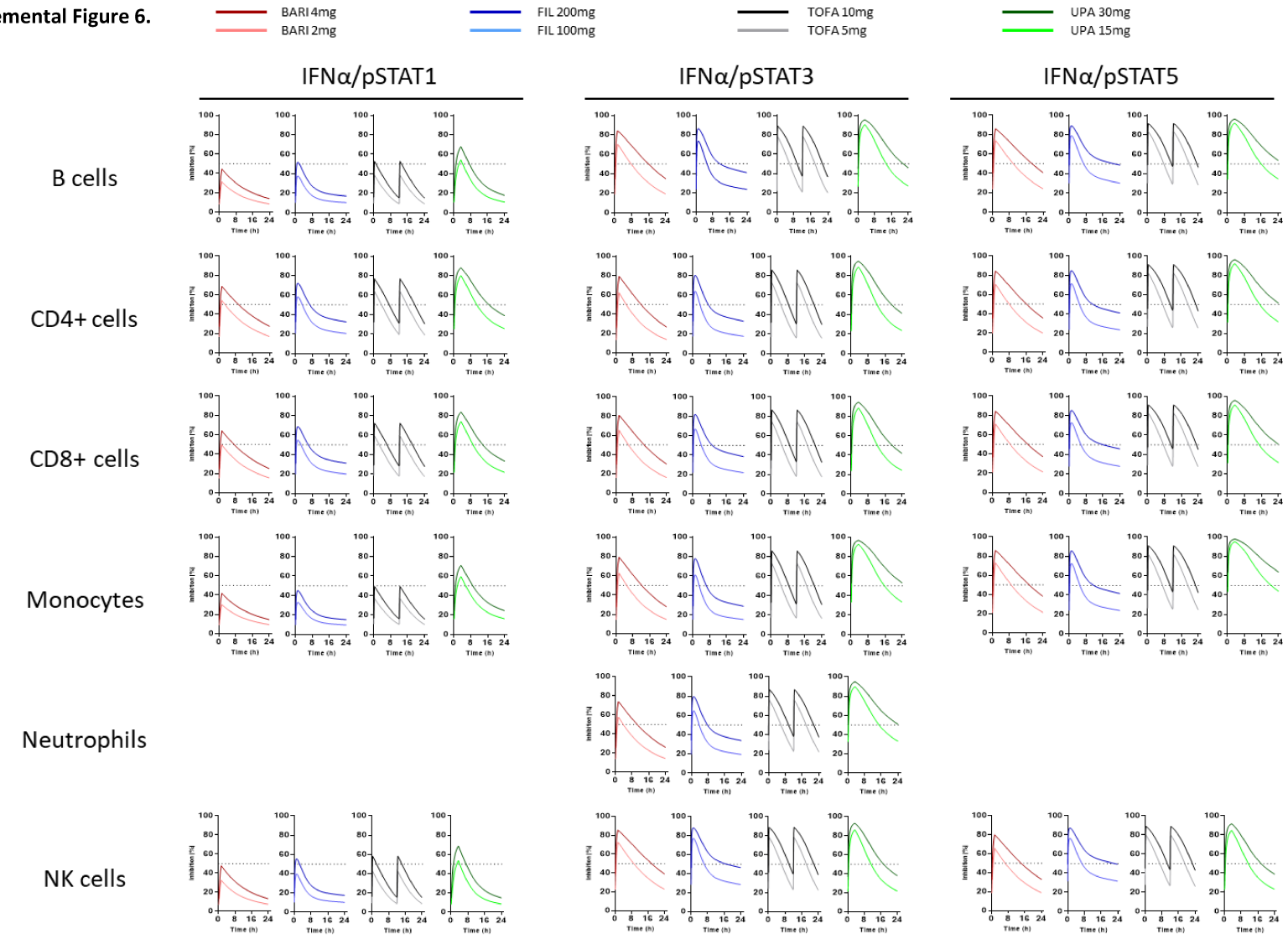
JAKinib dose-response curves for STAT phosphorylation in response to JAK1/JAK2 dependent cytokines IFNγ (pSTAT1) and IL-6 (pSTAT1, pSTAT3). Graphs represent percent pSTAT inhibition in whole blood from n=7–10 healthy volunteers.
BARI, baricitinib; FIL, filgotinib; IL, interleukin; INF, interferon; JAK, Janus kinase; JAKinib; Janus kinase inhibitor; MET, GS-829845 (filgotinib metabolite); pSTAT, phosphorylated signal transducer and activator of transcription; TOFA, tofacitinib; TYK2, tyrosine kinase 2; UPA, upadacitinib.

Supplemental Figure 5.



$palC_{50}$ values from PBMCs (n=5 donors) were compared to IC_{50} values in whole blood from the same donors (n=5). Pearson's analysis indicated a coefficient of determination r^2=0.98.
BARI, baricitinib; FIL, filgotinib; ; IC_{50}, half maximum inhibitory concentration; MET, GS-829845 (filgotinib metabolite); NK, natural killer; $palC_{50,}$ plasma binding-adjusted IC_{50}; PBMCs, peripheral blood mononuclear cells; TOFA, tofacitinib; UPA, upadacitinib; WB, whole blood.

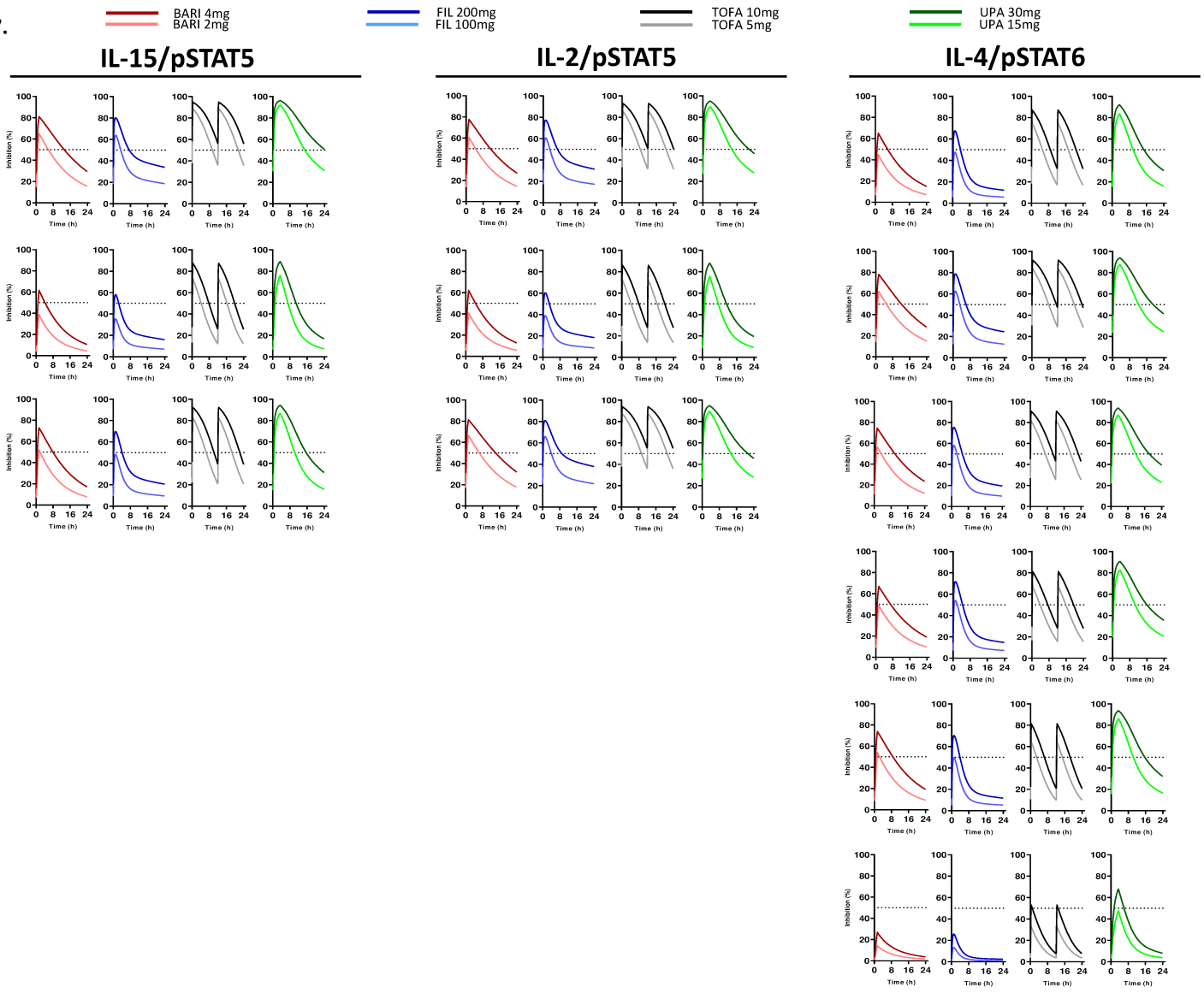
Supplemental Figure 6.



Inhibition curves of IFNα-induced STAT phosphorylation by JAKinibs (24-hour dose interval) at clinical doses (calculated based on in vitro WB measurements) from healthy volunteers) n=9–10 donors. In this figure, as well as Supplemental Figures 7-9, filgotinib takes into consideration the presence of the active human metabolite, GS-829845, and its potency. Curves above the dashed line (at 50%) indicates the time above 50% target coverage of each JAKinib during the dose interval.

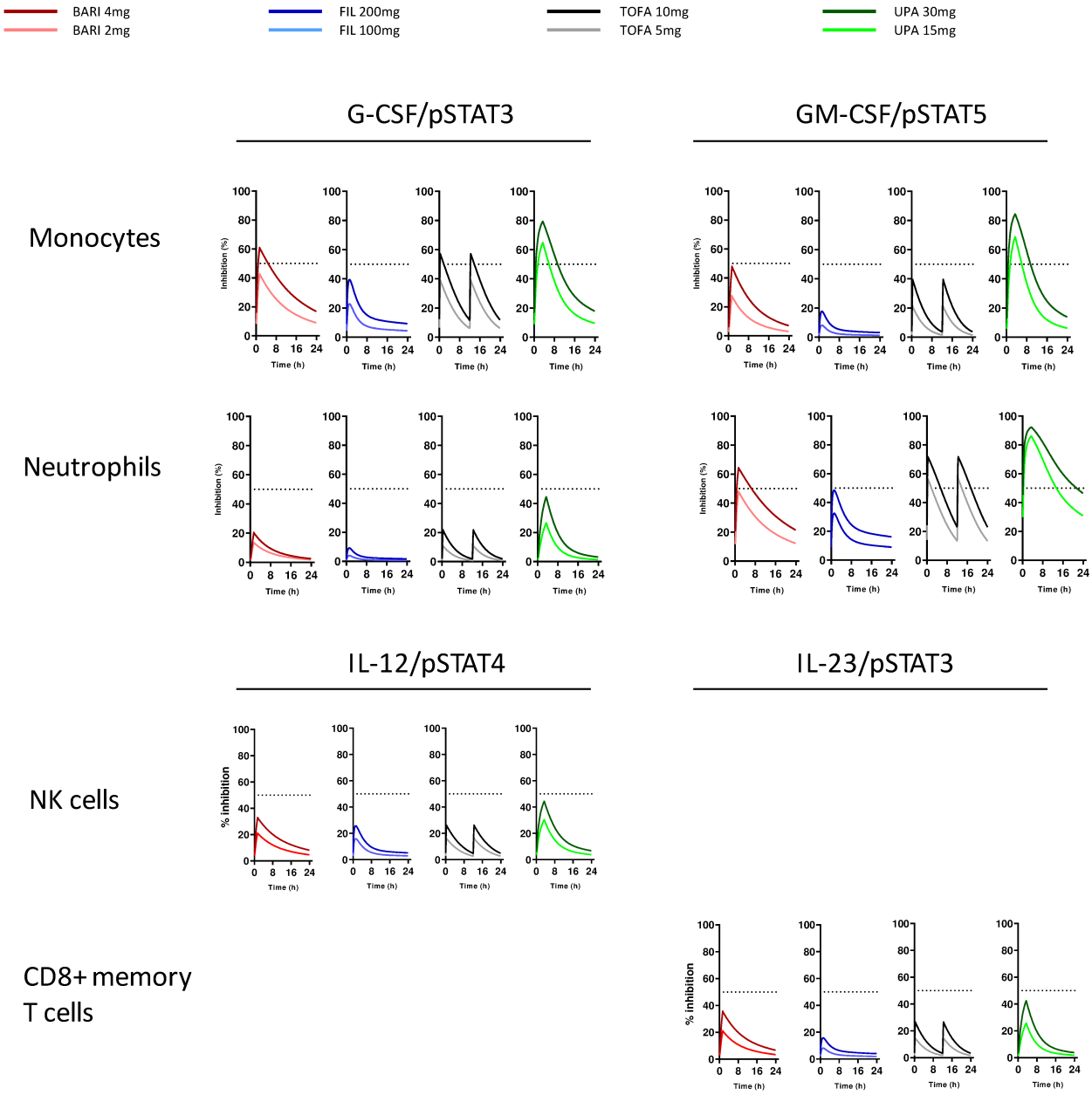
BARI, baricitinib; FIL, filgotinib; IFN, interferon; JAK, Janus kinase; JAKinib; Janus kinase inhibitor; NK, natural killer; pSTAT, phosphorylated signal transducer and activator of transcription; TOFA, tofacitinib; UPA, upadacitinib; WB, whole blood.

Supplemental Figure 7.



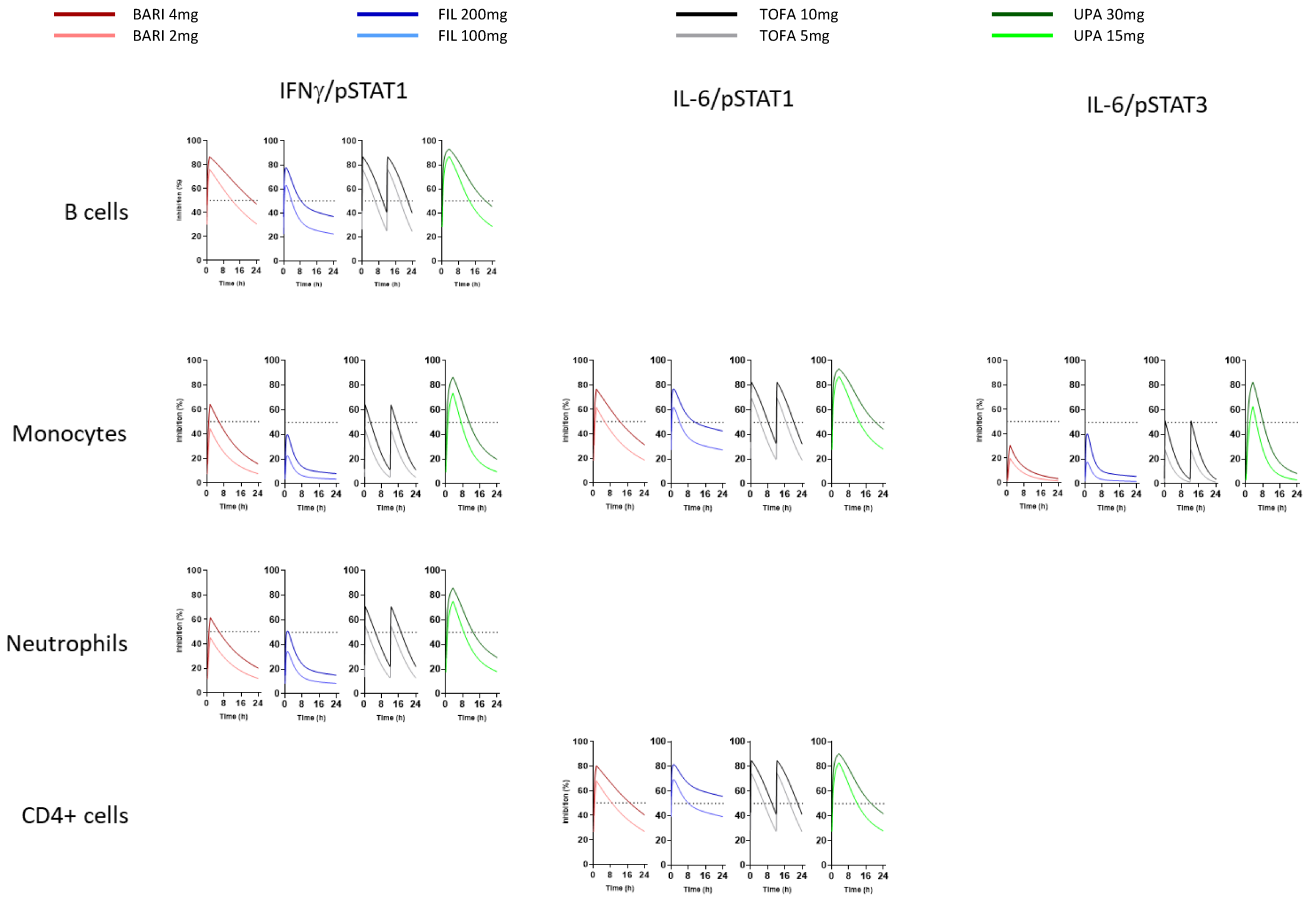
Inhibition curves of IL-2, IL-4 and IL-15-induced STAT phosphorylation by JAKinibs (24 h dose interval) at clinical doses (calculated based on in vitro WB measurements) from healthy volunteers). N=7–10 donors. BARI, baricitinib; FIL, filgotinib; IL, interleukin; JAK, Janus kinase; JAKinib; Janus kinase inhibitor; NK, natural killer; pSTAT, phosphorylated signal transducer and activator of transcription; TOFA, tofacitinib; UPA, upadacitinib; WB, whole blood.

Supplemental Figure 8.



Inhibition curves of G-CSF, IL-12, IL-23 and GM-CSF-induced STAT phosphorylation by JAKinibs (24-hour dose intervals) at clinical doses (calculated based on in vitro WB measurements from healthy volunteers). N=6–10 donors.
BARI, baricitinib; FIL, filgotinib; IL, interleukin; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte macrophage-CSF; JAK, Janus kinase; JAKinib; Janus kinase inhibitor; NK, natural killer; pSTAT, phosphorylated signal transducer and activator of transcription; TOFA, tofacitinib; UPA, upadacitinib; WB, whole blood.

Supplemental Figure 9.



Inhibition curves of IFNγ and IL-6-induced STAT phosphorylation by JAKinibs (24-hour dose intervals) at clinical doses (calculated based on in vitro WB measurements from healthy volunteers). N=7–10 donors.
BARI, baricitinib; FIL, filgotinib; IL, interleukin; INF, interferon; JAKinib; Janus kinase inhibitor; NK, natural killer; pSTAT, phosphorylated signal transducer and activator of transcription; TOFA, tofacitinib; UPA, upadacitinib; WB, whole blood.