

p38 MAPK inhibition suppresses the TLR-hypersensitive phenotype in FANCC- and FANCA-deficient mononuclear phagocytes

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Fanconi anemia, complementation group C (FANCC)-deficient hematopoietic stem and progenitor cells are hypersensitive to a variety of inhibitory cytokines, one of which, TNF α , can induce BM failure and clonal evolution in *Fancc*-deficient mice. FANCC-deficient macrophages are also hypersensitive to TLR activation and produce TNF α in an unrestrained fashion. Reasoning that suppression of inhibitory cytokine production might enhance hematopoiesis, we screened small molecules using TLR agonist-stimulated FANCC- and Fanconi anemia, complementation

group A (FANCA)-deficient macrophages containing an NF- κ B/AP-1-responsive reporter gene (*SEAP*). Of the 75 small molecules screened, the p38 MAPK inhibitor BIRB 796 and dasatinib potently suppressed TLR8-dependent expression of the reporter gene. Fanconi anemia (FA) macrophages were hypersensitive to the TLR7/8 activator R848, overproducing *SEAP* and TNF α in response to all doses of the agonist. Low doses (50nM) of both agents inhibited p38 MAPK-dependent activation of MAPKAPK2 (MK2) and suppressed MK2-dependent TNF α produc-

tion without substantially influencing TNF α gene transcription. Overproduction of TNF α by primary FA cells was likewise suppressed by these agents and involved inhibition of MK2 activation. Because MK2 is also known to influence production and/or sensitivity to 2 other suppressive factors (MIP-1 α and IFN γ) to which FA hematopoietic progenitor cells are uniquely vulnerable, targeting of p38 MAPK in FA hematopoietic cells is a rational objective for preclinical evaluation. (*Blood*. 2012;119(9):1992-2002)

Introduction

BM failure is a nearly universal complication of Fanconi anemia (FA), an inherited disease caused by biallelic inactivating mutations of any one of 15 genes.¹⁻⁴ FA gene products collectively facilitate responses to DNA damage,¹ and therefore it is often presumed (notwithstanding a lack of direct evidence supporting the idea) that hematopoietic defects simply reflect attrition of hematopoietic stem cells (HSCs) that have specifically suffered excessive DNA damage. An alternative explanation is that the FA proteins are multifunctional and play a direct role in stem cell maintenance, and therefore, DNA damage in FA HSCs is not necessarily required to suppress their function.⁵⁻⁹ In normal cells, for example, Fanconi anemia, complementation group C (FANCC) modulates the hematopoietic inhibitory effects of TNF α , IFN γ , and MIP-1 α , each of which normally function to suppress hematopoiesis.^{6,10-12} FANCC influences TNF α responsiveness at least in part by modulating the activation state of the IFN-inducible double-stranded RNA activated protein kinase.¹³ FANCC also suppresses the activation potential of certain TLR pathways in normal mononuclear phagocytes.¹⁴ Therefore, in hematopoietic tissues, FANCC deficiency results in a TLR-dependent overproduction of TNF α , one of the cytokines to which the stem-cell pool is uniquely intolerant.^{10,15-19} These abnormalities are important elements in the pathogenesis of BM failure.^{6,20} There is also experimental evidence that this TNF α -inhibitory loop is a selective pressure that enhances the ultimate emergence of TNF-resistant leukemic and preleukemic

clones.²¹⁻²³ Therefore, interdiction of TNF α -induced BM failure, particularly in ways that might have an additional favorable influence on IFN γ - and MIP-1 α -activated signaling pathways, may improve BM function and reduce the likelihood of clonal evolution by improving the fitness landscape and altering the coefficient of selection.²¹

Seeking small molecules with these attributes, in the present study we exploited the TLR-hypersensitive phenotype as a screening tool to identify therapeutic agents that might suppress that pathway in FA cells. Using a TLR8-hypersensitive, FANCC-deficient mononuclear phagocyte cell line that we described previously,¹⁴ we screened 75 small molecules, approximately 50 of which were kinase inhibitors. We identified 2 inhibitors, BIRB 796 and dasatinib, that functioned to suppress the TLR-dependent overproduction of TNF α in FANCC- and Fanconi anemia, complementation group A (FANCA)-deficient macrophages. We also determined that both agents functioned to suppress TNF α gene expression posttranscriptionally by inhibiting p38 MAPK activation of MAPKAPK2 (MK2). Others have demonstrated that short-term p38 MAPK inhibition enhances the repopulating capacity of *Fancc*-deficient HSCs,²⁴ that p38 MAPK modulates responses to IFN γ in some cell types,²⁵ and that MK2 controls expression of not only TNF α , but also IFN γ and MIP-1 α production.^{26,27} Therefore, preclinical evaluation of p38 MAPK inhibitors is clearly warranted using murine models of FA.

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Table 1. Small molecules used in screening

17-AAG	CHIR258	JAK1i	Nilotinib	SB-43152
8 Azaguanine	CHIR265	JAK3i	P38i	SB203580
ABT-864	CI-1033	JNK2i	Pan-JAKi	Sorafenib
Adiphenine	CP-690550	JNK3i	PD153035	SRCi
AG490	CYC-202	KN92	PD325901	Staurosporine
AKTIV	Dasatinib	KN93	PD98059	STO609
AKTX	EGFRi	Lapatinib	PI-103	Sunitinib
AMPK	Erlotinib	LSD1	Piperine	SYKi
Anisomycin	Estradiol	LY294002	PKC412	Thapsigargin
AZD-1152	Gefitinib	LY333531	PP2	Trichostatin A
Azithromycin	Go6976	MEK1/2	Proguanil	Tunicamycin
BAY-11	GW-786034	Menadione	PTK787	Vorinostat
BIRB-796	H-89	MLN-8054	R1881	VX-680
Boldine	Harmaline	MLN518	Resveratrol	Zardaverine
cAMP	Imatinib	QNZ	SB-202190	ZD-6474

For each molecule, at least 3 doses were tested. Cell viability was quantified using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium assay.

Methods

Abs and reagents

Anti-acetyl-NF- κ B p65 (Lys310), anti-phospho-NF- κ B p65 (Ser536), anti-MAPKAPK2 (MK2), anti-phospho-MAPKAPK2 (Thr334), anti-p38 MAPK, anti-phospho-p38 MAPK (Thr 180/Tyr182), and anti-phospho-c-Jun (Ser73) rabbit mAbs were all purchased from Cell Signaling Technology. Anti-c-Jun (H-79) and anti-NF- κ B p65 rabbit polyclonal Abs and anti-FANCD2 mouse mAb were purchased from Santa Cruz Biotechnology. BIRB 796 (doramapimod) was obtained from Axon Medchem. Dasatinib was obtained from LC Laboratories. Other inhibitors (Table 1) were obtained from LC Laboratories, Axon Medchem, and Sigma-Aldrich. MMP V Inhibitor was obtained from Santa Cruz Biotechnology. MMP Inhibitor II, PP-2, GM-6001, and the negative control for GM-6001 were obtained from EMD Chemicals. The pNiFty plasmid, QUANTI-Blue, and lipopolysaccharide (LPS) were obtained from InvivoGen. R848 was obtained from Enzo Life Sciences. Actinomycin D and mitomycin C were obtained from Sigma-Aldrich.

Cell lines and primary cells

THP-1 cells were obtained from the American Type Culture Collection and THP1-Blue cells were obtained from InvivoGen. The development and culture conditions of the cell lines T-shNT and T-shFC were described previously.¹⁴ The EBV-transformed patient-derived lymphoblast cell line HSC536N (FANCC-deficient) and isogenic cells complemented with normal human FANCC cDNA (HSC536N/FANCC) were used as described previously.²⁸ BM-derived macrophages were prepared from low-density (Ficoll-Paque PLUS; GE Healthcare) BM mononuclear cells obtained from 2-month-old wild-type or Fanc-deficient mice using published methods.²⁹⁻³¹ Specifically, mononuclear cells were cultured for 8 days in IMDM (Life Technologies), 20% FBS (Hyclone), and 40 ng/mL of recombinant murine GM-CSF (R&D Systems), followed by a 2-day treatment in serum-free medium. Macrophages were replated into 96-well dishes (10 000 cells/well) and treated with 1 μ g/mL of LPS for 24 hours. Conditioned media were then harvested for TNF α quantification using the Quantikine ELISA Kit (R&D Systems). All murine studies were approved by the Portland Veterans Administration Institutional Animal Care and Use Committee. Primary CD14⁺ cells were prepared from peripheral blood mononuclear leukocytes (isolated using Ficoll-Paque Plus) using magnetic microbeads (EasySep; StemCell Technologies). Cells were then cultured at a concentration of 10 000 cells/200 μ L for 24 hours in RPMI medium and 10% FBS in the presence of multiple doses of R848. Conditioned media were harvested for TNF α quantification using the Quantikine ELISA Kit (R&D Systems). Cells from healthy volunteers and from patients were obtained after having obtained informed consent from either the subject or

the subject's parents on a clinical study approved by the Institutional Review Board of G. Gaslini Children's Institute.

Stable suppression of FANCC and FANCA with lentiviral shRNA

THP-1 Blue cells were transduced with lentiviral particles expressing shRNA targeting FANCC, as we described previously.¹⁴ A similar strategy was used to suppress FANCA. Specifically, 5 lentiviral shFANCA MISSION vectors (TRCN0000118982, TRCN0000118983, TRCN0000118984, TRCN0000118985, and TRCN0000118986; Sigma-Aldrich) were tested. shRNA-expressing cells were selected with 0.6 μ g/mL of puromycin for 2-3 weeks, after which time the cells were maintained in medium containing 0.3 μ g/mL of puromycin. Knockdown was quantified using immunoblots for FANCA and FANCD2. TRCN0000118982 (a coding region target sequence of GCCGACCTCAAGGTTTCTATA) provided the greatest knockdown of FANCA and FANCD2L (supplemental Figure 1A-B, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article) and was therefore used in all subsequent experiments.

Drug-screening assay

Recently, we reported the development of an shRNA-transduced, FANCC-deficient THP1-Blue cell line (T-shFC) in which the TLR7/8 agonist R848 induced increased TNF α production compared with the control cell line (T-shNT, transduced with a nontargeted shRNA).¹⁴ Likewise, treatment with R848 induced the expression of the NF- κ B- and AP-1-dependent reporter gene and secreted embryonic alkaline phosphatase (SEAP), the activity of which was quantified using the QUANTI-Blue reagent, as described previously.¹⁴ In the present study, we used T-shFC cells in a functional screening assay to identify molecules that suppressed or induced SEAP. We tested 75 inhibitors (Table 1). For each of the inhibitors tested, at least 3 doses were analyzed and cell viability was assessed with the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium assay. The requirements for considering any screening result to be positive were: (1) \geq 70% suppression of SEAP expression in R848-stimulated cells; and (2) greater than 80% viability of the cell population at the end of the 24-hour culture period. Studies using the SEAP reporter driven only by 5 NF- κ B-binding sites (and lacking the AP-1 sites) were accomplished by transfecting the pNiFty plasmid into THP-1 cells using Nucleofector II with Program X-001 (Amaxa). The cells were selected in vitro with Zeocin (200 μ g/mL; Life Technologies).

TNF α and MMP7 assays

A total of 100 000 cells/200 μ L were cultured in 96-well plates and either maintained in culture medium (HSC536N and HSC536N/FANCC) or treated with 30 μ M R848 for 24 hours (T-shNT and T-shFAC), after which time culture supernatants were assayed for TNF α or MMP7 using

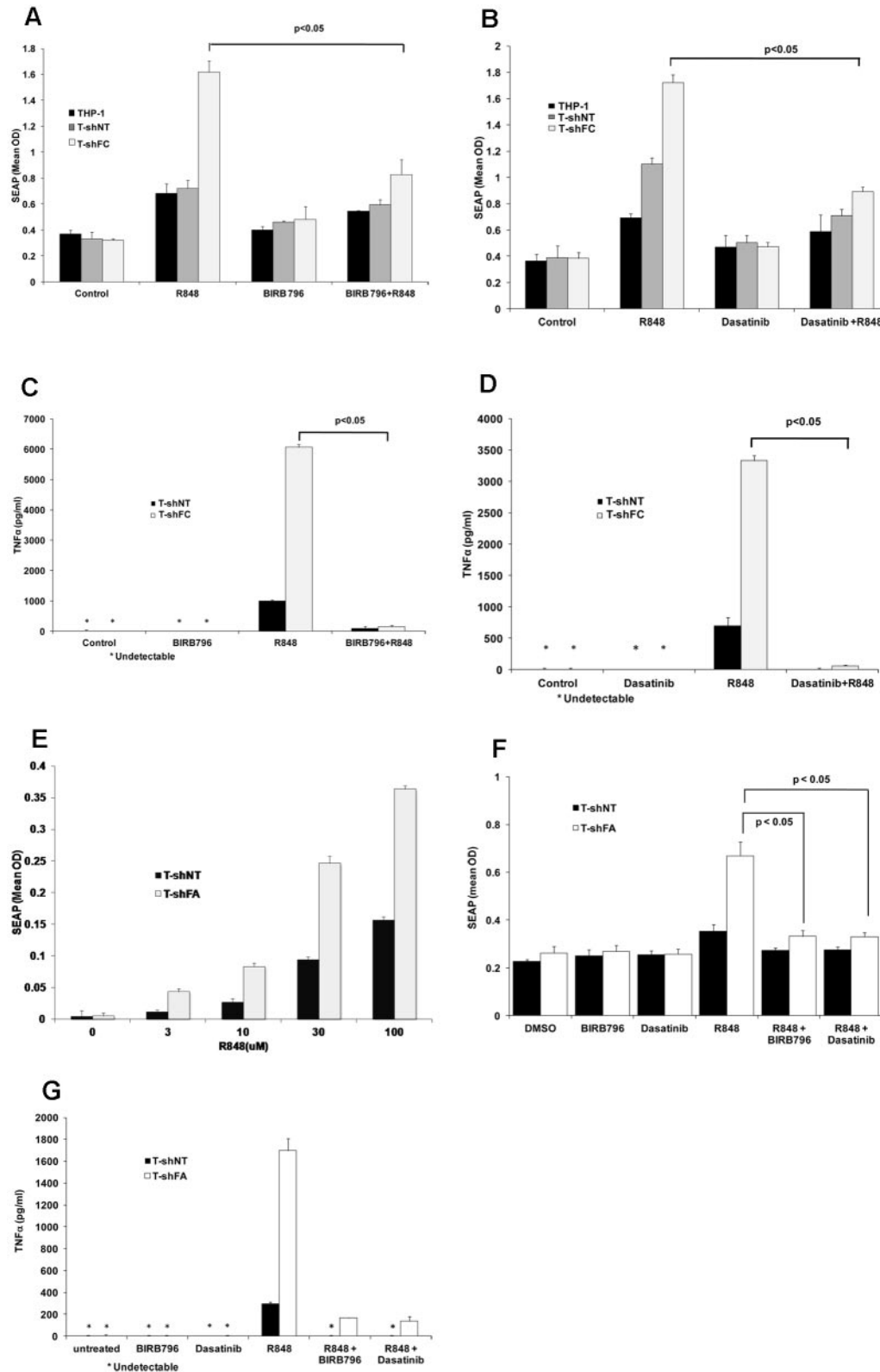


Figure 1. BIRB 796 and dasatinib inhibit NF- κ B/AP-1-dependent SEAP production in T-shFC cells. Mean values \pm SD are shown. All *P* values were calculated using the Student *t* test. (A) BIRB 796 inhibits R848-induced SEAP gene expression in FANCC-deficient cells. THP1-Blue cells (THP1) or THP1-Blue cells stably expressing either control (nontarget) shRNA (T-shNT) or shRNA targeting FANCC (T-shFC) were cultured in medium alone (control) or medium with the TLR7/8 agonist R848 (30 μ M) alone, BIRB 796 (500 nM) alone, or R848 (30 μ M) alone after a 6-hour pretreatment with BIRB 796 (500 nM). Conditioned media were collected after 24 hours of R848 exposure and SEAP was quantified colorimetrically using QUANTI-Blue reagent. (B) Dasatinib inhibits R848-induced SEAP expression in FANCC-deficient cells. The experimental design was identical to that of 1A except that dasatinib (500 nM) was used as the inhibitor. (C) BIRB 796 inhibits TNF α secretion in THP-1 cells. T-shNT and T-shFC were treated as in panel A with BIRB 796 (500 nM) and/or R848 (30 μ M). Conditioned media were collected after 24 hours of incubation with R848 and TNF α was quantified by ELISA. Untreated cells or cells treated only with BIRB 796 did not produce detectable TNF α (indicated with an asterisk). (D) Dasatinib inhibits TNF α secretion in THP-1 cells. T-shNT and T-shFC cells were treated as in panel B with R848 (30 μ M) and dasatinib (500 nM). Untreated cells or cells treated with dasatinib alone did not produce detectable TNF α (indicated with

Quantikine ELISA Kits (R&D Systems). In some experiments with T-shNT and T-shFAC cells, cultures were pretreated with MMP inhibitors for 6 hours before the addition of 30 μ M R848 for 24 hours and subsequent medium collection.

Immunoblotting

Immunoblot analyses were carried out as described previously.^{6,14}

Real-time qRT-PCR

For real-time quantitative RT-PCR (qRT-PCR), total RNA was prepared from $1-5 \times 10^6$ cells using the RNeasy Mini kit (QIAGEN). Complementary DNA synthesis and real-time PCR were performed as described previously.¹⁴ Primer and probe sets for *TNF α* (Hs00174128_m1) and *MMP7* (Hs01042795_m1) were purchased as TaqMan Gene Expression Assays (Life Technologies). Primers and TaqMan probes for *SEAP* detection were designed using Primer Express Version 3.0 software (Life Technologies) and were as follows: probe 6-FAM-ACACGCGGCAACG-MGB; 5' primer CCGCTTTAACCAGTGCAACA; 3' primer CCCGATTCATCACGGAGATG. Primers were purchased from IDT and probes from Life Technologies.

Quantifying mRNA decay

Actinomycin D (5 μ g/mL) was added to cells pretreated for 24 hours with 30 μ M R848. Total RNA was prepared at regular time intervals thereafter. *TNF α* mRNA was quantified using real-time qRT-PCR.

siRNA

SMARTpool siRNA targeting *MK2* and control siRNA were purchased from Thermo Scientific Dharmacon. The pool consisted of 4 distinct sequences: GAACCACCCUUGGAUCAUG, GAAUGACCAUCAC-CGAGUU, CGAAUGGGCCAGUAUGAAU, and UGAUUGUCAUG-GAAUGUUU. Cells were transfected with siRNA using Nucleofector II (Amaxa), as described previously.¹⁴ After transfection, the cells were cultured for 72 hours, after which time *MK2* expression was maximally suppressed.

Statistical analysis

All statistical analyses were performed using a 2-tailed Student *t* test. Unless otherwise stated, data are presented as mean values \pm SD of 3 or more independent experiments. *P* < .05 was considered statistically significant.

Results

Agents that inhibit *TNF α* gene expression in FANCC- and FANCA-deficient cells

THP1-Blue cells report TLR-induced NF- κ B/AP-1 activation by expressing SEAP. Using our inhibitor-screening assay, we tested small molecules for their capacity to suppress the TLR-dependent expression of SEAP. Two of the 75 small-molecule inhibitors initially screened (Table 1), BIRB 796, a specific p38 MAPK inhibitor, and dasatinib, a dual Abl/Src kinase inhibitor, were among the most potent inhibitors of SEAP production. BIRB 796 and dasatinib effectively suppressed R848-induced SEAP production in both T-shFC and T-shNT cells to the same level as

that observed in controls (Figure 1A-B). Both agents also inhibited *TNF α* production (as quantified by ELISA) by R848-stimulated T-shNT and T-shFC cells (Figure 1C-D). Compared with T-shNT cells, FANCA-deficient THP-1 cells (T-shFA) were also hypersensitive to R848. Specifically, T-shFA cells produced more SEAP at all 4 doses of R848 tested (Figure 1E), and the overproduction of both SEAP (Figure 1F) and *TNF α* (Figure 1G) by these cells was suppressed by both BIRB 796 and dasatinib.

BIRB 796 and dasatinib inhibit *TNF α* overproduction in a patient-derived cell line and in primary FANCC- and FANCA-deficient macrophages

The B-cell line HSC536N, derived from a FANCC-deficient patient, produces *TNF α* constitutively, and *TNF α* production in these cells is completely suppressed by complementation with wild-type FANCC cDNA.¹⁴ Both BIRB 796 and dasatinib fully suppressed *TNF α* production in HSC536N cells, matching *TNF α* levels produced by the complemented isogenic cell line (HSC536N/FANCC; Figure 2A). Because LPS induces *TNF α* overproduction and *TNF α* -dependent BM failure in *Fancc*^{-/-} mice,¹⁸ we tested the effectiveness of BIRB 796 and dasatinib as inhibitors of *TNF α* production by LPS-treated BM-derived murine macrophages from wild-type and *Fancc*^{-/-} mice. Both BIRB 796 and dasatinib suppressed *TNF α* production effectively (Figure 2B). As was the case with T-shFA cells (Figure 1G), primary peripheral blood mononuclear phagocytes from a Fanconi group A patient (FANCA-deficient) were hypersensitive to both R848 and LPS compared with an age-matched control sample studied in parallel. Moreover, both small molecules suppressed *TNF α* gene expression induced by both TLR agonists (Figure 2C-D).

High doses of BIRB 796 and dasatinib inhibit *TNF α* gene transcription

We quantified *TNF α* mRNA in R848-stimulated and unstimulated T-shFC and T-shNT cells with and without exposure to each drug. High doses of BIRB 796 and dasatinib (500nM) suppressed *TNF α* mRNA, although suppression by dasatinib was more complete (Figure 3A). Both drugs likewise inhibited *SEAP* mRNA (Figure 3B). Because neither BIRB 796 nor dasatinib reduced the *TNF α* mRNA half-life (Figure 3C-D), inhibition of both *SEAP* mRNA and *TNF α* mRNA reflects transcriptional suppression by these 2 agents at 500nM doses.

BIRB 796 and dasatinib differentially influence c-Jun activation

The reporter we used in our screens was responsive to a promoter containing both NF- κ B and AP-1 sites. To determine whether the AP-1 site was required for SEAP and *TNF α* gene expression in THP1-Blue cells, we transfected THP-1 cells with the pNiFty plasmid featuring only 5 NF- κ B sites (and no AP-1 site)³² that governs the expression of SEAP. pNiFty-transfected cells did not produce SEAP, but did produce *TNF α* in response to R848 (supplemental Figure 2A-B), indicating that additional elements in the native *TNF α* promoter are required and that NF- κ B activation alone is insufficient to account for the R848-induced *TNF α* gene

Figure 1. (continued) an asterisk). (E) FANCA-deficient THP-1 cells are hypersensitive to R848. THP1-Blue cells stably expressing shRNA targeting FANCA (T-shFA) and T-shNT cells were exposed to R848 (0-100 μ M) for 24 hours. Supernatant media were collected and SEAP was quantified colorimetrically using QUANTI-Blue reagent. At each dose of R848, T-shFA cells produced more SEAP than did T-shNT cells (*P* < .05). (F) BIRB 796 and dasatinib suppress the production of SEAP in both T-shFA and T-shNT cells. The design of these studies on T-shFA cells was identical to that of studies on T-shFC cells (A-B). (G) BIRB 796 and dasatinib suppress *TNF α* production in R848-stimulated T-shFA and T-shNT cells. The design of these studies on T-shFA cells was identical to that used with T-shFC cells (C-D).

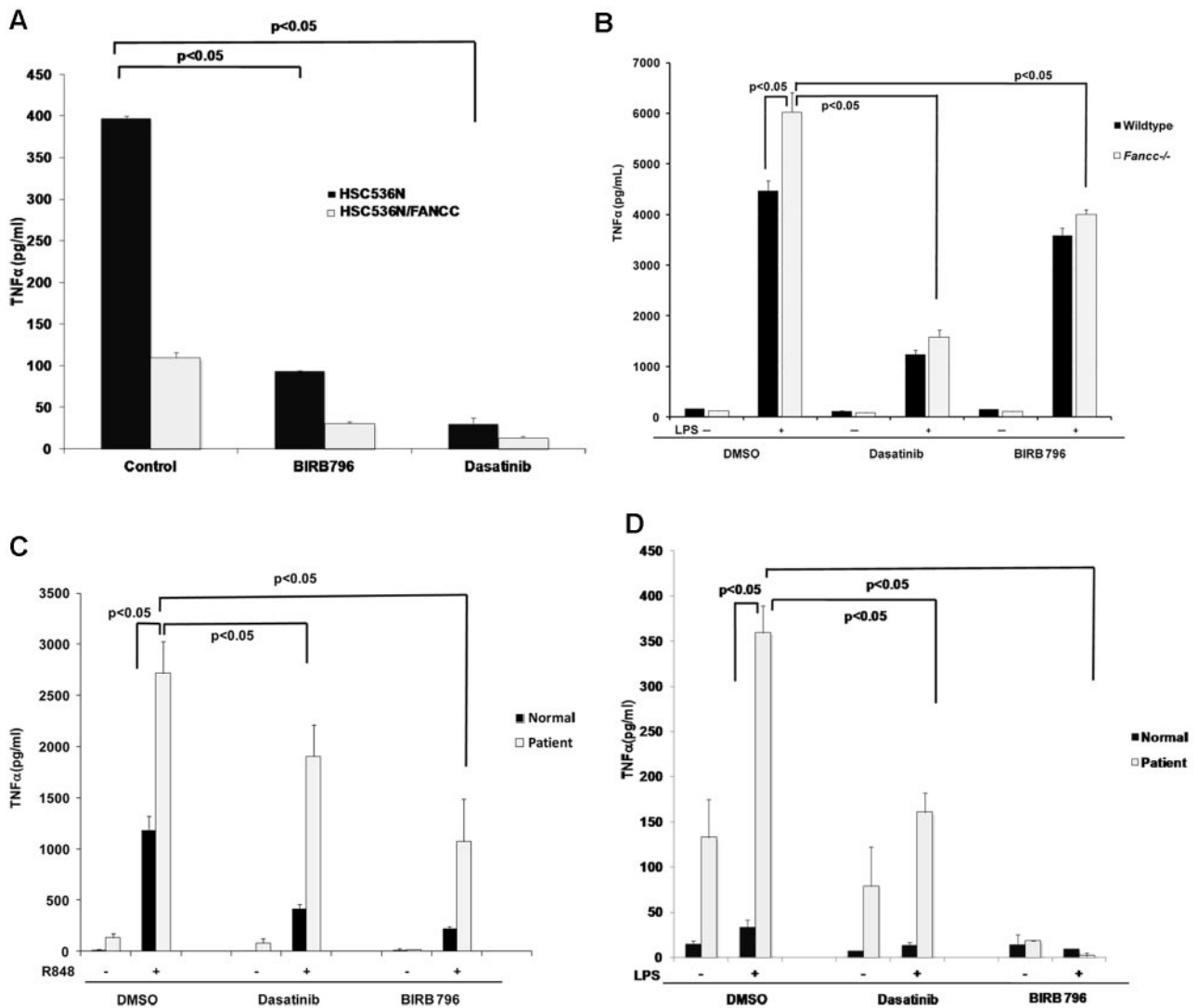


Figure 2. BIRB 796 and dasatinib suppress TLR-activated TNF α gene expression in primary and patient-derived FA cells. Means \pm SD are shown. All *P* values were calculated using the Student *t* test. (A) BIRB 796 and dasatinib inhibit constitutive TNF α secretion in the patient-derived FANCC^{-/-} lymphoblast cell line HSC536N. HSC536N and HSC536N/FANCC (complemented with normal FANCC cDNA) were cultured in medium alone (control) or medium treated with BIRB 796 (500nM) or dasatinib (500nM) for 6 hours. After 6 hours, supernatant media were collected and TNF α measured by ELISA. (B) BIRB 796 and dasatinib inhibit LPS-induced TNF α production in FANCC-deficient murine macrophages. Primary murine BM-derived macrophages obtained from FANCC^{-/-} and wild-type mice were maintained in control medium (plus DMSO) or treated for 6 hours with BIRB 796 (500nM) or dasatinib (500nM), followed by stimulation with LPS (1 μ g/mL) for 24 hours. TNF α in conditioned media was measured by ELISA. (C-D) BIRB 796 and dasatinib inhibit R848- and LPS-induced TNF α production in FANCA-deficient human macrophages. Primary human CD14⁺ macrophages obtained from a normal age-matched volunteer and a FANCA-deficient patient were cultured for 6 hours in control medium (plus DMSO), BIRB 796 (500nM), or dasatinib (500nM), before stimulation for 24 hours with R848 (1 μ M; C) or LPS (0.1 ng/mL; D). TNF α in conditioned media was measured by ELISA.

expression. Indeed, results of studies on the states of activation of NF- κ B p65 and c-Jun supported the requirement for both factors in FANCC-deficient and wild-type cells. Transcription of *TNF α* relies both on phosphorylation (Ser536)²⁴ and acetylation (Lys310)²⁵ of p65. Both posttranslational modifications are overrepresented in R848-exposed T-shFC cells, but only BIRB 796 suppressed p65 acetylation and phosphorylation (Figure 4A) and only dasatinib suppressed c-Jun activation (Figure 4B). Moreover, the degree to which the activation of these proteins was suppressed by either agent was minimal and was not observed at low (50nM) doses of these agents (Figure 5D).

BIRB 796 and dasatinib suppress TNF α expression posttranscriptionally

Because both agents were equally potent in suppressing TNF α production, we reasoned that they might function more effectively

to suppress posttranscriptional steps in TNF α gene expression. Therefore, in light of the known suppressive effects of BIRB 796 on p38 MAPK function, we tested the capacity of both agents to inhibit MK2, a major effector of *TNF α* mRNA translation and canonical p38 MAPK substrate.^{33,34} Specifically, when phosphorylated on Thr334 in some cell types, MK2 enhances TNF α production by inhibiting *TNF α* mRNA degradation³⁵ and enhancing *TNF α* mRNA translation.^{34,36} Indeed, exposure of FANCC-deficient THP-1 cells to R848 resulted in robust phosphorylation of MK2, and both BIRB 796 and dasatinib inhibited MK2 phosphorylation profoundly (Figure 4C). We also quantified activation of p65, c-Jun, p38, and MK2 in FANCA-deficient THP-1 cells and determined that: (1) only MK2 and p38 were overactivated in FANCA-deficient cells; and (2) BIRB 796 profoundly suppressed the phosphorylation of both (Figure 4D).

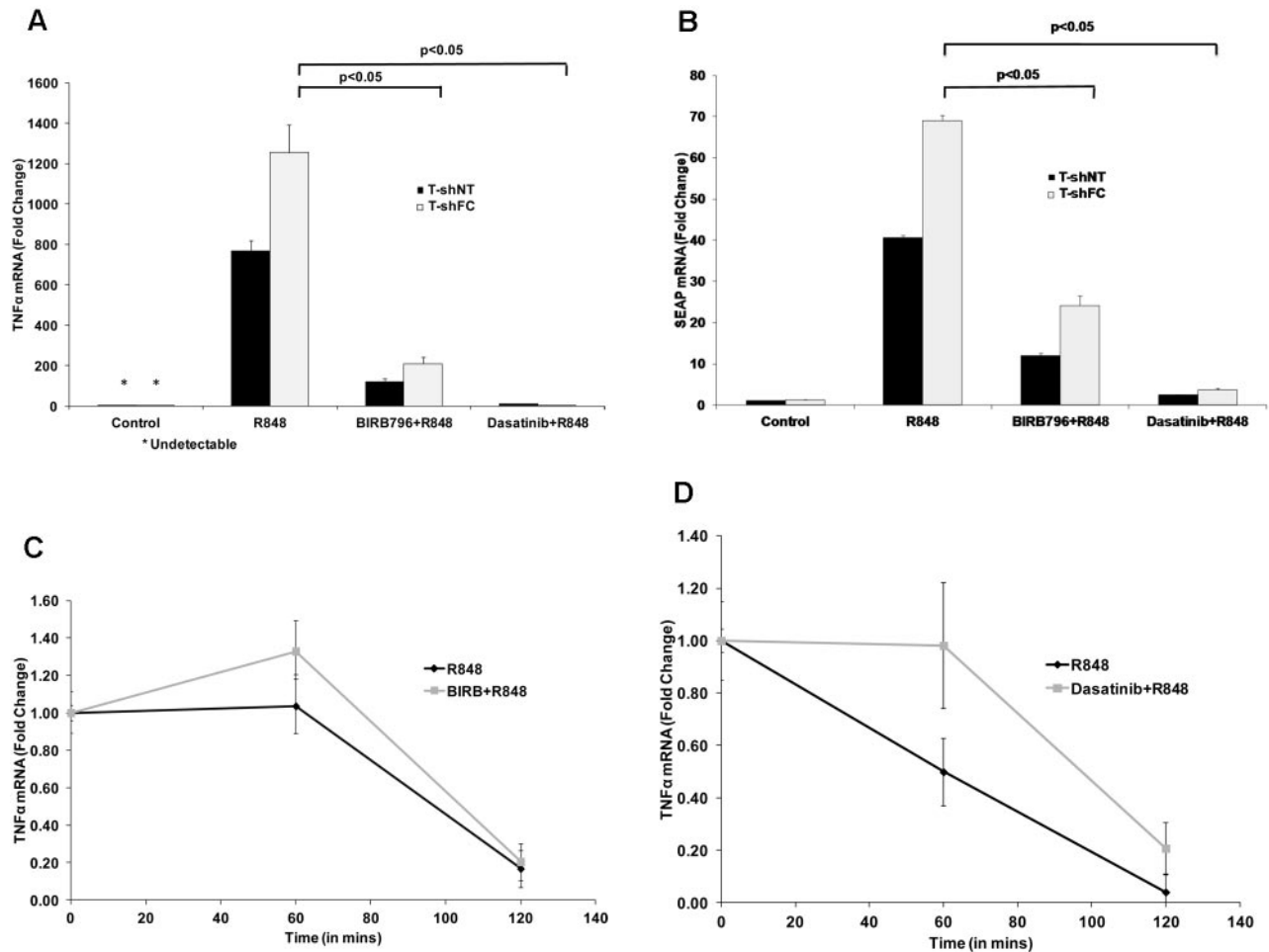


Figure 3. BIRB 796 and dasatinib suppress the production of *TNF α* and *SEAP* transcripts. T-shNT and T-shFC were maintained in medium alone (control), or medium treated with R848 (30 μ M) for 24 hours after 6 hours of pretreatment with either BIRB 796 (500nM) or dasatinib (500nM). Total RNA was prepared and *TNF α* (A) and *SEAP* (B) mRNAs quantified using real-time qRT-PCR. Data are expressed as the mean \pm SD -fold change relative to transcripts in unexposed (control) cells and based on 3 technical replicates normalized to 18S rRNA. Dasatinib suppressed *TNF α* mRNA levels to a greater degree than did BIRB 796. (C-D) Neither BIRB 796 nor dasatinib enhanced *TNF α* mRNA degradation. T-shFC cells were either cultured in medium alone or treated with BIRB 796 (500nM; C) or dasatinib (500nM; D) for 6 hours, after which time all cells were stimulated with R848 (30 μ M) for 24 hours. After adding actinomycin D (5 μ g/mL), total RNA was collected at 0, 60, and 120 minutes and *TNF α* mRNA was measured by real-time qRT-PCR. For each drug, 1 representative of 2 experiments with identical results is shown.

To confirm that BIRB 796 functions most potently to suppress *TNF α* gene expression largely at a posttranscriptional control point, we took advantage of the fact that this agent inhibits *TNF α* release completely at doses as low as 50nM (Figure 5A). We first determined that *TNF α* mRNA was minimally suppressed in R848-stimulated shFC cells by this dose of BIRB 796 (Figure 5B) and then that this dose effectively suppressed MK2 phosphorylation without inhibiting either p65 or c-Jun activation (Figure 5C-D). This indicates that the primary effect of this agent in FANCC- and FANCA-deficient cells is to suppress translation of *TNF α* mRNA. Likewise, 50nM dasatinib suppressed *SEAP* expression and *TNF α* mRNA only marginally (supplemental Figure 3A-B), but inhibited *TNF α* production by more than 3-fold (supplemental Figure 3C). To confirm that MK2 hyperactivation plays a role in the FA phenotype, we suppressed MK2 gene expression in T-shFC and T-shNT cells using MK2 RNAi. We confirmed that the siRNA we used suppressed MK2 gene expression by approximately 50% (Figure 6A) and that *TNF α* protein production was suppressed in both T-shNT and T-shFC cells exposed to R848 by approximately 50% (Figure 6B). MK2 RNAi did not have an equivalent effect on either *SEAP* expression (Figure 6C) or *TNF α* mRNA (Figure 6D). Therefore, *TNF α*

overproduction in FANCC-deficient THP-1 cells is dependent on MK2 phosphorylation by p38 MAPK. Moreover, because neither BIRB 796 nor dasatinib suppresses *TNF α* mRNA half-life (Figure 3), and taking into account the role of MK2 in facilitating *TNF α* mRNA translation,^{34,36} the control point for this agent is likely suppression of *TNF α* mRNA translation.

Dasatinib's effect reflects its Src-kinase inhibitory activity

Dasatinib is a well-characterized Src kinase inhibitor but also targets other non-Src-family kinases involved in innate immune signaling.³⁷ Therefore, to determine whether the Src kinase inhibitory activity suppressed p38 activation, we used pyrazolopyrimidine (PP2), a highly specific Src kinase inhibitor known to inhibit *TNF α* gene expression in endotoxin-stimulated human macrophages.^{38,39} PP2 not only inhibited *TNF α* in R848-induced macrophages in our studies (supplemental Figure 4A), but also suppressed the activation of both p38 MAPK and its substrate, MK2 (supplemental Figure 4B). Therefore, it is likely that the effectiveness of dasatinib in posttranscriptionally suppressing p38-dependent *TNF α* gene expression depends on its activity as an inhibitor of Src family kinases.

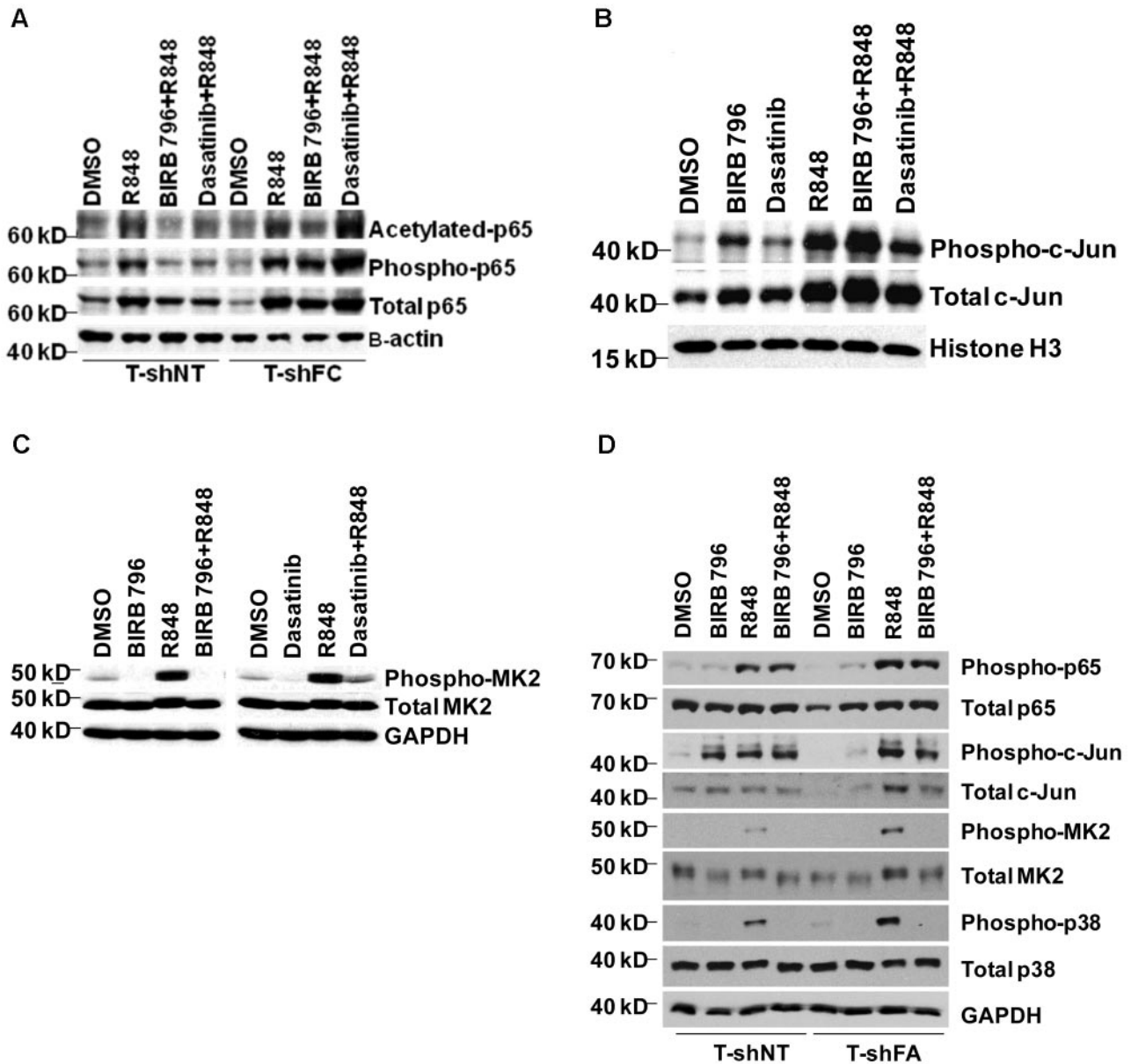


Figure 4. Dasatinib and BIRB 796 do not inhibit R848-induced NF- κ B p65 or c-Jun levels in FANCC- or FANCA-deficient mononuclear phagocytes, but do inhibit MK2 phosphorylation. T-shNT cells and T-shFC cells were treated with BIRB 796 (500nM) or dasatinib (500nM) for 6 hours and then stimulated with R848 (30 μ M) for 24 hours. (A) Western blot analyses of nuclear extracts were performed using Abs for total p65, phospho-p65(Ser536), and acetylated p65(Lys310). Whereas both BIRB 796 and dasatinib suppressed p65 phosphorylation and acetylation in T-shNT cells, neither agent suppressed p65 phosphorylation in T-shFC cells, and only BIRB 796 inhibited p65 acetylation. One representative example of 3 identical experiments is shown. (B) Western blot analyses of nuclear extracts were performed as above, but Abs to total c-Jun and phosphorylated (Ser73) c-Jun were used. BIRB 796 did not suppress c-Jun phosphorylation and the suppressive effect of dasatinib was minimal (lane 6 vs lane 4). One representative example of 3 identical experiments is shown. (C) Immunoblot analysis of whole-cell lysates of shFC cells exposed to R848 with and without either dasatinib or BIRB 796 demonstrated marked suppression of MK2 phosphorylation by both small molecules (lanes 4 and 8 vs lanes 3 and 7). One representative example of 3 identical experiments is shown. (D) T-shFA and T-shNT cells were exposed to R848 alone for 24 hours or to BIRB 796 for 6 hours, followed by R848 for 24 hours. Whole-cell lysates were used in immunoblot assays for total and phosphorylated p65, c-jun, p38, and MK2. BIRB 796 had no influence on p65 or c-jun phosphorylation, but profoundly suppressed p38 and MK2 phosphorylation. One representative experiment of 2 is shown.

MMPs are not involved in regulating TNF α gene expression in THP-1 cells

Others have reported alternative p38 MAPK-dependent control points for TNF α gene expression in FANCC-deficient lymphoblasts (a few of which produce TNF α constitutively). p38-activated MMP7 was found to enhance the secretion of TNF α from these cell lines.⁴⁰ Although we did detect a significant amount of MMP7 mRNA and secreted matrix metalloproteinase 7 (MMP7) in FANCC-deficient lymphoblasts (supplemental Figure 5A-B), we detected no secreted MMP7 protein or mRNA in THP-1 cells (not

shown). We were also surprised to find that an MMP7 inhibitor had no impact on TNF α production by FANCC-deficient lymphoblasts (supplemental Figure 5C). We therefore tested broader-spectrum MMP inhibitors, including GM-6001 (an inhibitor of MMPs 1, 2, 3, 8, and 9) and MMP inhibitor V (which inhibits MMPs 2, 3, 8, 9, 12, and 13) and also found no inhibition of TNF α secretion at multiple doses in either FANCC-deficient lymphoblasts (not shown) or R848-stimulated, FANCC-deficient THP-1 cells (supplemental Figure 5D-E). This result indicates that MMPs do not play a role in controlling TNF α secretion in FANCC-deficient monocytes.

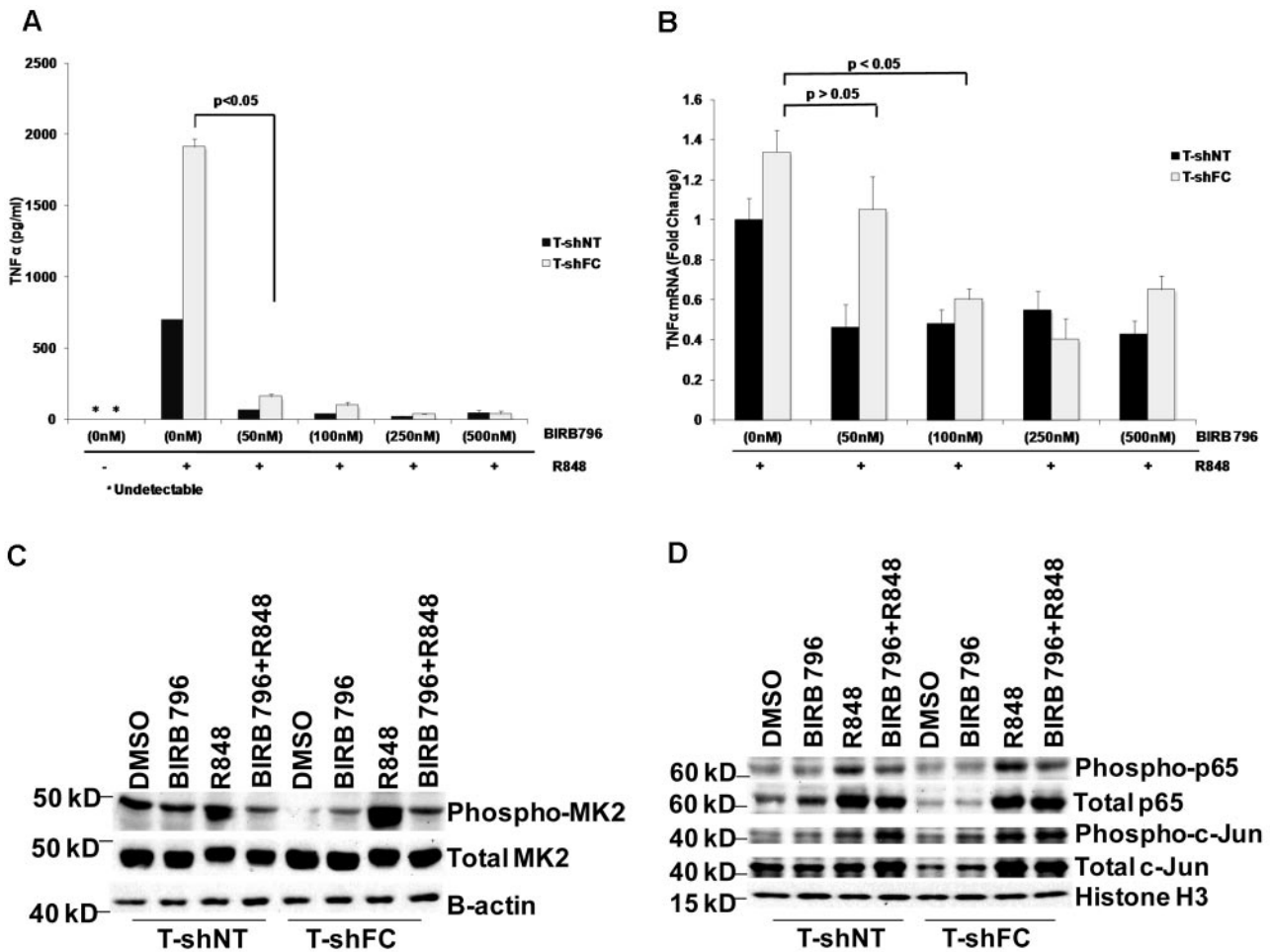


Figure 5. BIRB 796 and dasatinib influence TNF α gene expression posttranscriptionally. Means \pm SD are shown. All *P* values were calculated using the Student *t* test. (A) BIRB 796 effectively inhibited secreted TNF α in monocytes. T-shNT and T-shFC cells were treated with BIRB 796 (0–500nM) for 6 hours, followed by treatment with R848 (30 μ M) for 24 hours. Supernatant media were then collected and TNF α was quantified by ELISA. Low doses of BIRB 796 (50nM) suppressed secreted TNF α protein by 20-fold. (B) T-shNT and T-shFC cells were treated with BIRB 796 (0–500nM) for 6 hours, followed by treatment with R848 (30 μ M) for 24 hours. mRNA was then prepared and TNF α transcripts measured by real-time qRT-PCR. Data are expressed as the fold change normalized to 18S rRNA and are relative to mRNA levels in unexposed (control) T-shNT cells. TNF α mRNA is reduced modestly in the presence of BIRB 796 and minimally at the 50nM dose that was fully suppressive of TNF α protein production (A). One of 2 identical experiments is shown. (C) Low doses of BIRB 796 (50nM) suppress R848-induced MK2 phosphorylation in whole-cell lysates from T-shFC and T-shNT cells. One of 2 identical experiments is shown. (D) Immunoblot analysis for p65 and c-Jun phosphorylation in nuclear extracts of T-shFC and T-shNT cells revealed that low doses of BIRB 796 had no effect on p65 or c-Jun phosphorylation in R848-induced T-shFC and T-shNT cells. One representative immunoblot of 3 identical experiments is shown.

Discussion

Seeking small molecules that might suppress aberrant activation of the TLR pathway in FA cells, we used FANCC- and FANCA-deficient cell lines (T-shFC and T-shFA, respectively) and a control cell line (T-shNT), each of which has a stably integrated TLR pathway-responsive reporter gene under the control of NF- κ B and AP-1 response elements.¹⁴ Using these cell lines to screen 75 small molecules (Table 1), we identified 2, BIRB 796 and dasatinib, that potently suppressed expression of the reporter gene (SEAP). We then confirmed that these agents also suppressed the TLR-dependent expression of TNF α in the T-shFC and T-shFA cells (Figure 1C-D) in a Fanconi group C patient-derived cell line (Figure 2A), in primary murine *Fancc*-deficient mononuclear phagocytes (Figure 2B), and in primary human FANCA-deficient mononuclear phagocytes (Figure 2C-D).

Whereas both agents are known to suppress TLR-induced expression of TNF α ,^{41,42} they are in different functional classes. Therefore, we sought pathways influenced by both of them using

TNF α gene expression. Interestingly, although we identified these agents using a screening tool that relied on NF- κ B- and c-Jun-dependent transcription of a reporter gene, our mechanistic studies indicate unambiguously that both agents have a greater effect on TNF α gene expression at a posttranscriptional control point, likely by suppressing TNF α mRNA translation. To confirm that BIRB 796 functions most potently to suppress TNF α gene expression largely at a posttranscriptional control point, we took advantage of the fact that this agent inhibits TNF α release completely at doses as low as 50nM (Figure 5A). We first determined that TNF α mRNA was minimally suppressed in R848-stimulated shFC cells by this dose of BIRB 796 (Figure 5B), and then that this dose effectively suppressed MK2 phosphorylation without inhibiting either p65 or c-Jun activation (Figure 5C-D). This result indicates that the primary effect of this agent in FANCC- and FANCA-deficient cells is to suppress translation of TNF α mRNA or secretion of the protein. Likewise, 50nM dasatinib suppressed SEAP expression and TNF α mRNA only marginally (supplemental Figure 3A-B), but inhibited TNF α production by more than 3-fold (supplemental Figure 3C). To confirm that MK2 hyperactivation plays a role in the

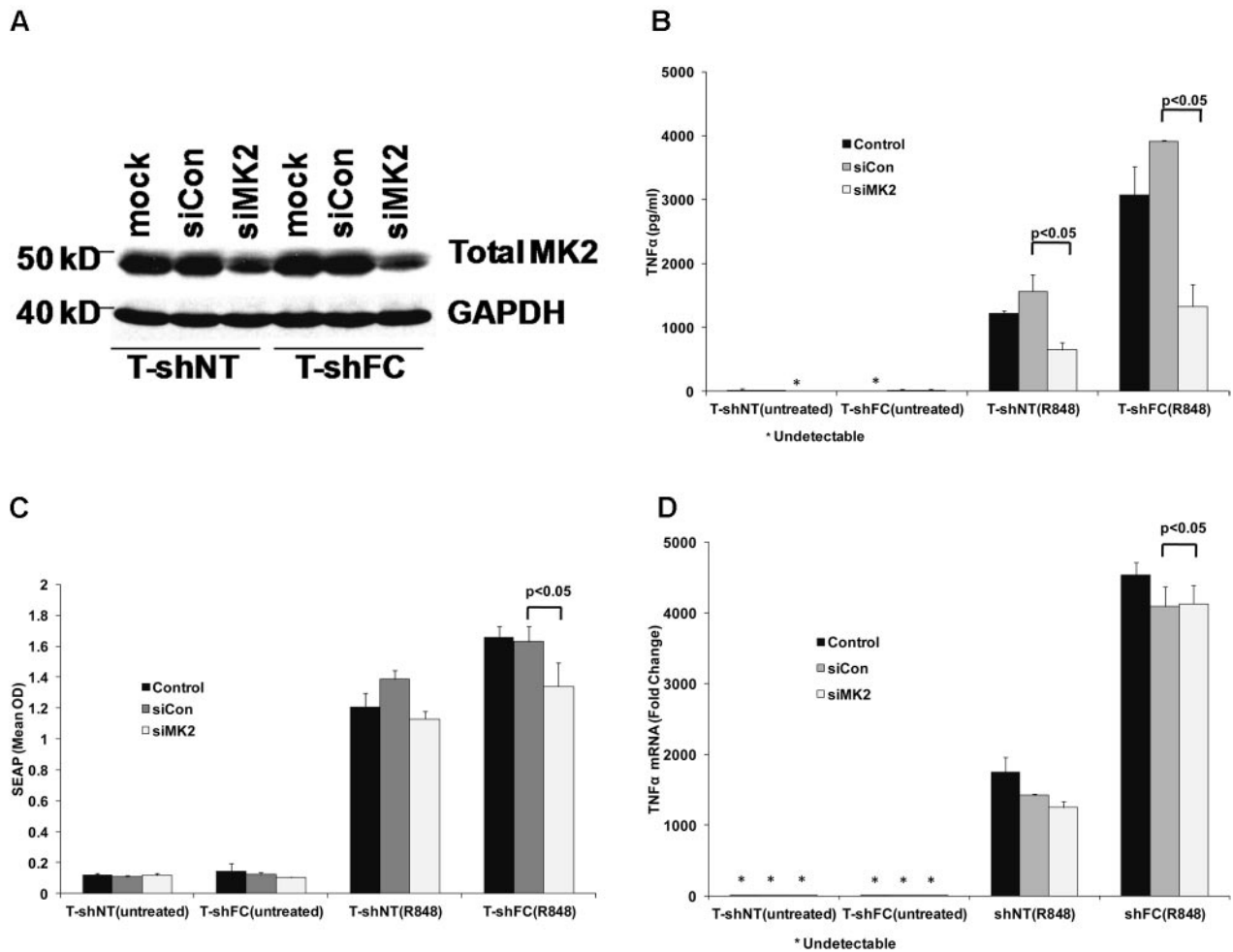


Figure 6. MK2 regulates TNF α production posttranscriptionally. T-shFC and T-shNT cells were transfected with nontargeted siRNAs (siCon) or siRNA targeting MK2 (siMK2) by nucleofection. Cells were also subjected to nucleofection without siRNA present (mock). At the time of maximal MK2 knockdown (72 hours), the cells were stimulated with R848 (30 μ M) for 24 hours or were left untreated. (A) MK2 suppression (lanes 3 and 6) was confirmed by Western blot of whole-cell extracts 72 hours after nucleofection. (B) TNF α protein in supernatant media was quantified by ELISA 24 hours after R848 stimulation. siMK2 suppressed TNF α levels produced by both T-shFC and T-shNT cells and the degree of suppression matched the degree of MK2 knockdown with RNAi (6A). (C) SEAP expression, as quantified by QUANTI-Blue, was minimally suppressed by siMK2. (D) TNF α mRNA measured 24 hours after R848 stimulation by real-time qRT-PCR revealed no suppression by treatment of cells with siMK2. Therefore, MK2 suppression inhibited TNF α production and/or release, but not SEAP or TNF α gene transcription in T-shFC cells.

FA phenotype, we suppressed MK2 gene expression in T-shFC and T-shNT cells using MK2 RNAi. We confirmed that the siRNA we used suppressed MK2 gene expression by approximately 50% (Figure 6A), and that TNF α protein production was suppressed in both T-shNT and T-shFC cells exposed to R848 by approximately 50% (Figure 6B). MK2 RNAi did not have an equivalent effect on either SEAP expression (Figure 6C) or TNF α mRNA (Figure 6D). Therefore, TNF α overproduction in FANCC-deficient THP-1 cells is dependent on MK2 phosphorylation by p38 MAPK. Moreover, because neither BIRB 796 nor dasatinib suppresses TNF α mRNA half-life (Figure 3), and taking into account the role of MK2 in facilitating TNF α mRNA translation,^{34,36} the control point for this agent is likely suppression of TNF α mRNA translation.

The effect of BIRB 796, an inhibitor of all 4 isoforms of p38, is not unexpected,⁴³ but the influence of dasatinib on p38 activation was less anticipated, in large part because wholesale deficiency of the 3 most highly expressed Src kinases in murine macrophages (Hck, Lyn, and Fgr) impairs neither p38 and NF- κ B activation nor TNF α production.⁴⁴ Nonetheless, it has been reported that this agent does suppress both LPS-induced TNF α gene expression in mice,^{41,45} and Tlr4- and Tlr9-dependent TNF α expression in rodent

macrophages.⁴⁵ Our finding that a highly specific Src kinase inhibitor suppressed R848-induced TNF α gene expression and suppressed p38 MAPK, MK2, and c-Jun activation but not p65 phosphorylation (supplemental Figure 4A-B), a profile identical to that which we describe for dasatinib, is compatible with the notion that dasatinib's effect in our studies reflects its Src kinase-inhibitory activity.

Both BIRB 796 and dasatinib inhibit p38 MAPK activity, inhibit phosphorylation of its canonical substrate MK2, and suppress TNF α protein production. The importance of MK2 suppression by these agents as a key mechanism is emphasized by studies showing that LPS does not induce expression of TNF α in *Mk2*-deficient mice,³³ and that MK2 plays a role in modulating the production^{26,27} of 2 other key cytokines to which FA hematopoietic progenitors and stem cells are hypersensitive,^{10,11,28,46} namely IFN γ and MIP-1 α . Whereas p38 MAPK-activated MK2 is known to enhance the half-life and translation of TNF α mRNA in some cell types,³⁴ in our experiments BIRB 796 and dasatinib suppressed TNF α production without reducing TNF α mRNA half-life, (Figure 3C-D), suggesting that the effects of MK2 activation were exclusively translational. Others have reported that an important p38

MAPK-dependent control point for TNF α gene expression in FA cells is the expression of MMP7, which functions to enhance TNF α secretion.⁴⁰ However, we detected no secreted MMP7 protein or mRNA in THP-1 cells (not shown) and found that neither MMP7 specific nor more general MMP inhibitors influenced R848-induced TNF α gene expression in either FANCC-deficient or -proficient cells (supplemental Figure 4C-E).

The results of the present study show that both FANCA- and FANCC-deficient mononuclear phagocytes exhibit the TLR-hypersensitive phenotype, that the phenotype is effected by hyperactivation of p38 MAPK and its substrate MK2, and that 2 effective inhibitors of the phenotype act by suppressing the activation states of these 2 kinases. At higher doses, these agents also function to slightly suppress activation of either NF- κ B or c-Jun; otherwise we would not have been able to ascertain them in our screening assay. This has persuaded us that the screening method we have used may give rise to false negative results in future screening experiments and that quantification of cytokine production would likely be a more reliable approach.

The precise biochemical mechanisms by which FANCA and FANCC influence TLR pathways are not yet clearly defined. Nor have we yet determined that other FA genes influence precisely the same pathways. However, the discovery that inhibition of p38 MAPK is a key mechanism underlying the effects of these 2 agents helps to better focus future mechanistic studies of FANCA and FANCC on pathways that modulate p38 and MK2 activation. For example, of the 2 well-known p38 activators, MEKK1 and ASK1,^{47,48} ASK1 is of substantial interest. Because its activity is inhibited by proteins⁴⁹⁻⁵¹ known to interact with^{6,8} or to be stabilized by^{52,53} FA proteins, it has been implicated in aberrant TNF α responses in *Fancc*-deficient murine embryonic fibroblasts.^{20,54}

FANCA and FANCC mutations account for at least 65% of patients with FA worldwide. Therefore, regardless of the mechanisms by which these 2 FA proteins modulate TLR-dependent p38 MAPK activation and whether these mechanisms are shared with other FA complementation groups, the control of p38 MAPK and MK2 with small molecules has substantial appeal. In light of the capacity of p38 inhibition to suppress IFN γ and TNF α responsiveness,^{20,25,54} and because inhibition of MK2 suppresses inducible production of MIP-1 α , TNF α , and IFN γ ,^{26,27} p38 MAPK and MK2 play a central role in the FA hematologic phenotype, at least in 2 of the most common FA complementation groups, A and C. Preclinical

studies of new-generation p38 MAPK inhibitors are clearly warranted and should be designed in a way that tests their capacity in murine models of FA to safely correct the aberrant hematopoietic phenotype (both cytokine overproduction and hypersensitivity) without enhancing the evolution of neoplastic clones.

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Authorship

Contribution: P.A. and G.C.B. designed the project, performed the experiments, analyzed all of the primary data, developed the new assays, and wrote the manuscript; J.E.Y. conducted RT-PCR and immunoblot experiments and proofread and revised the final manuscript and figures; M.R.G. performed ELISA and immunoblot experiments and helped with manuscript revisions; S.V. helped to design and perform the inhibitor screening and conducted the T-shFA experiments; W.K. maintained all cell lines; K.R. and L.E.H. developed the murine BM macrophage and murine splenocyte experiments; J.W.T. helped to design, perform, and analyze the inhibitor screening; J.S., E.C., and C.D. designed and performed all experiments using human samples and helped with manuscript revisions; and J.S. performed helpful studies on *Fancc*-deficient mice.

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