

# JAK inhibitors impair GM-CSF-mediated signaling in innate immune cells

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## Research article

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# Abstract

**Background:** Innate immune cells play a crucial role in the pathophysiology of rheumatoid arthritis (RA) via release of cytokines. Small-molecule inhibitors of Janus kinases (JAKi) are clinically efficacious in patients with RA. However, the isoform-specific action of each JAKi is difficult to assess, since JAKs form heterodimeric complexes with cytokine receptors. We assessed the effects of several JAKi on GM-CSF-primed human innate immune cells.

**Results:** Treatment with JAKi (tofacitinib, baricitinib, upadacitinib) prevented GM-CSF-induced JAK2/STAT5 phosphorylation at higher concentrations (400nM) in THP-1 cells. Whereas compared with baricitinib or upadacitinib, the inhibitory effects of tofacitinib on the GM-CSF-induced JAK2/STAT5 phosphorylation were weak at lower concentrations (< 100nM). All JAKi inhibited GM-CSF-induced IL-1b production by human neutrophils. However, the inhibitory effects of baricitinib on IL-1b production were larger compared to those of tofacitinib or upadacitinib at lower concentrations (< 100nM). Similarly, all JAKi inhibited GM-CSF-induced caspase-1(p20) production by human neutrophils.

**Conclusion:** We conclude that incubation with JAKi prevents GM-CSF-mediated JAK2/STAT5 activation in human innate immune cells. Although baricitinib and upadacitinib almost completely blocked GM-CSF-mediated JAK2/STAT5 signaling, the inhibitory effects of tofacitinib were weaker at lower concentrations suggesting that variation exists among these JAKi in the inhibition of JAK2 signaling pathways.

## Background

Cytokines play an important role in the induction of autoimmune diseases such as rheumatoid arthritis (RA) [1]. Type I/II cytokines transduce signals via the Janus kinase (JAK) / signal transduction activator of transcription (STAT) pathways [2]. Due to their important functions in cytokine signaling, JAKs have been attractive therapeutic targets in inflammatory disorders [3]. Members of JAK family are non-receptor tyrosine kinases comprised of four isoforms: JAK1, JAK2, JAK3 and TYK2 [4]. Activated JAKs lead to phosphorylation of STATs and subsequent gene transcription [5]. Oral JAK inhibitors (JAKis) have been used for treatment of RA, and many JAKi are currently in development [6]. JAKi are presumed to inhibit JAK isoforms with different selectivity; however, the JAK isoform selectivity of each JAKi is relative and not absolute. For example, tofacitinib was originally developed as a JAK3 inhibitor, but subsequent selectivity studies revealed additional inhibitory effects on JAK1 [7]. A new generation of JAKi target single JAK isoforms. However, their precise in vivo specificities remain unclear because most cytokine receptors are equipped with two different JAKs and the complexities associated with sharing of JAKs with cytokine receptors [8]. The in vitro enzymatic and pharmacokinetic properties of JAKis make them unsuitable for examination of in vivo immunological effects [9]. The mechanisms through which JAKis influence cytokine signaling, their specificities and their roles in modulating inflammatory pathways remain poorly understood. Therefore, determining the selectivity profiles of JAKis using human immune cells is necessary to fully realize their potential as anti-inflammatory or anti-immune agents. The effects of JAKis on innate immune cell signaling have also been investigated [10]. Here we focused on GM-CSF-

mediated JAK2 signaling [11] and explored the impact of JAKis. We compared the established JAK1/3 selective, JAK1/2 selective and JAK1 selective JAKis, with the goal of providing insight into JAK2-mediated inflammatory processes. Since GM-CSF signaling is JAK2-dependent and JAK1/3-independent [12], we investigated the effects of JAKis in JAK2-selective inhibition of GM-CSF-stimulated human innate immune cells.

## Results

### Effects of JAKi on GM-CSF-stimulated THP-1 cells.

To investigate whether JAKi alters cytokine production by GM-CSF-stimulated THP-1 cells, IL-1 $\beta$  was quantitated in culture supernatants using ELISA. We found that IL-1 $\beta$  production was induced by GM-CSF stimulation in THP-1 cells (Fig. 1). GM-CSF-induced IL-1 $\beta$  secretion by THP-1 cells was inhibited by JAKi pretreatment in a dose-dependent manner (Fig. 2). To investigate the effects of JAKi on cytokine-mediated signaling, we examined the effects of JAKi on GM-CSF-mediated JAK2/STAT activation using THP-1 cells. GM-CSF stimulation induced JAK2 phosphorylation in THP-1 cells. Although pretreatment with all JAKis blocked GM-CSF-induced JAK2 phosphorylation at high concentrations (400 nM, Fig. 3A), the inhibitory effects of JAKis varied at lower concentrations (Fig. 3B). Baricitinib and upadacitinib inhibited GM-CSF-induced JAK2 phosphorylation even at lower concentrations (25–100 nM). By contrast, tofacitinib only weakly inhibited GM-CSF-induced JAK2 phosphorylation at the same concentrations. In addition, GM-CSF stimulation induced downstream STAT5 phosphorylation, which was presumably induced by activated JAK2. However, GM-CSF stimulation barely induced STAT3 phosphorylation in THP-1 cells (data not shown). Similarly, baricitinib and upadacitinib pretreatment abolished GM-CSF-induced STAT5 phosphorylation completely at high concentrations (Fig. 4A). By contrast, the inhibitory effects of tofacitinib against GM-CSF-induced STAT5 phosphorylation were less pronounced even at high concentrations (Fig. 4A, 4B).

### Effects of JAKi on GM-CSF-stimulated human neutrophils.

None of the JAKi studied were able to completely block IL-1 $\beta$  production by GM-CSF-stimulated THP-1 cells, a human monocyte-lineage tumor cell line (Fig. 2). In order to investigate the effect of each JAKi on human primary innate immune cells, we pretreated freshly isolated neutrophils with various concentrations of JAKi (tofacitinib, baricitinib, upadacitinib) for 1 h then stimulated with GM-CSF for 24 h. We assessed production of IL-1 $\beta$  by GM-CSF-stimulated neutrophils. Consistent with our findings in THP-1 cells, JAKi-pretreated human neutrophils exhibited reduced GM-CSF-induced IL-1 $\beta$  production in a concentration-dependent manner (Fig. 5). Whereas the inhibitory effects of tofacitinib on IL-1 $\beta$  production by GM-CSF-stimulated neutrophils were weak in lower concentrations (25–100 nM) compared to those of baricitinib or upadacitinib (Fig. 5). We next assessed pro-IL-1 $\beta$  mRNA and NLRP3 protein expressions in JAKi-pretreated, GM-CSF-stimulated neutrophils. As shown in Fig. 6, GM-CSF was a potent inducer of pro-IL-1 $\beta$  mRNA expression in neutrophils, and JAKi pretreatment did not affect GM-CSF-induced pro-IL-1 $\beta$  mRNA expression. NLRP3 expression was induced in GM-CSF-stimulated neutrophils. Among these JAKi,

baricitinib was the most potent inhibitor against GM-CSF-induced NLRP3 expression in neutrophils (Fig. 7). We presented data using higher concentrations of JAKi (100–400 nM) since lower concentrations of JAKi (25 nM) had no effect on GM-CSF-induced NLRP3 expression in neutrophils (data not shown). During NLRP3 inflammasome activation, a cleaved form of caspase-1 (p20) is released along with processed IL-1 $\beta$ . Therefore, we analyzed culture supernatants for secretion of caspase-1 using an ELISA detecting the cleaved form of caspase-1 (p20). Consistent with IL-1 $\beta$  production, we found that caspase-1 secretion by GM-CSF-stimulated neutrophils was inhibited by JAKi pretreatment (Fig. 8).

## Discussion

Although there have been significant efforts to develop JAK isoform-selective inhibitors for treatment of RA, only JAK1/3 selective inhibitor (Tofacitinib) and JAK1/2 selective inhibitor (baricitinib) have been used clinically [13]. JAK2 signals in tandem with JAK1 in the heterodimeric context of gp130-family cytokine receptors such as the IL-6 receptor [14]. Therefore, it has been challenging to characterize the relative contributions of both JAK isoforms in transducing signaling from these receptors in the presence of isoform-selective JAKi. An anti-GM-CSF receptor monoclonal antibody showed similar efficacy in a clinical trial of RA patients [15]. GM-CSF may be one of the proinflammatory cytokines which contribute to the pathophysiology of rheumatoid inflammation [11]. Inhibiting JAK2 may produce an anti-inflammatory effect by inhibiting GM-CSF-mediated pathway, since GM-CSF receptor is associated with non-receptor protein tyrosine kinase, JAK2 [16]. We hypothesized that JAKi would differentially affect GM-CSF-mediated signaling based on their selectivity for JAK2. Our results showed that each JAKi inhibited GM-CSF-mediated JAK2/STAT5 activation and subsequent IL-1 $\beta$  production with some variations suggesting that the inhibitory properties of each JAKi against JAK2 could be differed.

Baricitinib, which has shown efficacy in the treatment of RA, is a potent and selective inhibitor of JAK1 and JAK2 [17]. Although tofacitinib had been developed as a JAK3-selective inhibitor, subsequent studies demonstrated that it also potently inhibits JAK1 in biochemical and cellular assays [18]. Recent clinical trials demonstrated the efficacy of upadacitinib, a selective JAK1 inhibitor, in patients with RA [19]. Biochemical assays using recombinant enzyme preparations could provide insufficient information concerning the kinase inhibition profiles of each JAKi [20]. Additionally, the pharmacological properties of JAKi demonstrated using biochemical analyses may be unsuitable for in vivo studies using human immune cells [21]. Due to the cross-inhibition of JAK isoforms forming heterodimeric complexes with various cytokines/growth factor receptors [22], inhibition assays are performed under the influence of JAK heterodimers linked to cytokine receptors in the cellular context. In this study we determined the effects of these JAKi (tofacitinib, baricitinib, upadacitinib) on key aspects of innate immune cell function. These effects are important modulators of the response to inflammatory cytokines and are implicated in an autoinflammatory reaction, IL-1 $\beta$  induction

Our results demonstrated that JAKi modulated IL-1 $\beta$  production from innate immune cells by affecting the GM-CSF-mediated intracellular signaling pathways. This phenomenon was associated with impaired JAK2/STAT5 phosphorylation resulting in the abortive production of IL-1 $\beta$  without affecting the GM-CSF-

induced pro-IL-1 $\beta$  mRNA expression in innate immune cells. These results suggest that JAKi inhibited the GM-CSF-induced inflammasome activation process, such as caspase-1 activation and subsequent IL-1 $\beta$  production, without affecting the inflammasome priming process [23]. Since JAK2 is associated with signaling downstream of GM-CSF [24], inhibition of JAK2 may lead to the impaired the GM-CSF-mediated autoinflammation pathway in innate immune cells [25]. We observed similarities between baricitinib and upadacitinib in the inhibitory effects on the GM-CSF-induced JAK2/STAT5 phosphorylation in innate immune cells. Similar selectivity of baricitinib and upadacitinib against JAK2 kinase may be unexpected because upadacitinib is developed as a novel JAK inhibitor engineered for JAK1 selectivity [26]. Whereas, recent study showed that upadacitinib is the most potent inhibitor among the JAKi (tofacitinib, baricitinib, upadacitinib) on the JAK2-dependent cytokines, IL-3 or GM-CSF-induced STAT5 phosphorylation in the cellular level [27]. These findings may indicate that upadacitinib would also inhibit JAK2-dependent cytokines other than those primarily dependent upon JAK1. By contrast, the suppressive effect of tofacitinib on JAK2/STAT5 phosphorylation was weaker compared to those of baricitinib or upadacitinib at lower concentrations in THP-1 cells. According to previous work using enzymatic assays [28], differential effects of tofacitinib and baricitinib in inhibiting GM-CSF-induced JAK2/STAT5 phosphorylation seem to be expected. In our study, JAKi selectivity was assayed by measuring JAK/STAT phosphorylation and cytokine release using human innate immune cells. Discrepancies may be observed in the impact and isoform selectivity of JAKi depending on the cell lineages used and cytokines measured in different assay systems [29]. Further preclinical investigations and clinical studies will be required to assess the biological impact and clinical benefit of pan-JAK or JAK isoform-selective inhibition.

There is a limitation in our study. We utilized GM-CSF at the relatively high concentration (20 ng/ml), since these concentrations are known to be used for biochemical kinase assays [30]. Whereas the serum levels of GM-CSF in the sera from patients with RA were markedly lower [31] compared to those in our experimental conditions.

## Conclusions

We demonstrated that incubation of innate immune cells with JAKi inhibited the proinflammatory properties of GM-CSF in a dose-dependent manner. However, the inhibitory effects on JAK2/STAT5 signaling and subsequent IL-1 $\beta$  production were influenced by the type of JAKi. This may be due to the fine specificities of each JAKi in modulating JAK2-mediated signaling in innate immune cells. Further work focusing on the ability of each JAKi to modulate key aspects of innate immune activation and JAK2 signaling may explain the anti-inflammatory activities of JAKi for treatment of autoimmune diseases.

## Methods

### Reagents

Recombinant human GM-CSF was purchased from Peprotech (Rocky Hills, NJ). Anti- $\beta$ -actin antibodies were purchased from Santa Cruz Biotechnology Inc (Dallas, USA). Anti-NLRP-3 antibody was purchased from MERCK MILLIPORE (Billerica, MA USA). Human IL-1 $\beta$  and caspase-1 (p20) ELISA kits were purchased from R&D systems (Minneapolis, USA). Phospho-specific antibodies against JAK-2 (Tyr1007/1008), STAT-5 (Tyr701) and STAT-3 (Tyr705) were purchased from Cell Signaling Technology (Beverly, MA). Tofacitinib, baricitinib and upadacitinib were purchased from Sigma-Aldrich (Tokyo, Japan).

### **THP-1 cells**

THP-1 cells were obtained from the American Type Culture Collection (Manassas, VA) and grown according to their instructions. The cells were plated at  $2 \times 10^5$ /ml in LPS-free media and allowed to grow for 2–3 days in tissue culture dishes before stimulation with GM-CSF.

### **Neutrophils isolation**

Venous peripheral blood was collected from healthy volunteers. Written informed consent for blood donation was obtained from each individuals. The blood was layered on a Polymorphprep TM (Axis-Shield, Oslo, Norway) cushion and cells were isolated according to the manufacturer's protocol. Briefly, neutrophils were isolated on the basis of density, washed once in 0.5 N RPMI-1640 to restore osmolality, and then washed once more in RPMI-1640. The cells were subsequently diluted in complete medium consisting of RPMI-1640.

To investigate the effects of JAKi on GM-CSF receptor signaling, freshly isolated neutrophils were pretreated with JAKi for 1 hr then stimulated with GM-CSF and protein extracts or supernatants were analyzed by ELISA or immunoblotting.

### **ELISA**

Cell-free supernatants were collected by centrifugation at 400 g for five minutes and assayed for IL-1 $\beta$  or caspase-1 (p20) using ELISA kits (R&D Systems, Minneapolis, MN, USA). Quantikine human caspase-1 immunoassay (R&D Systems, Minneapolis, MN, USA) ) in which monoclonal antibody specific to the p20 subunit of caspase-1 was pre-coated as captured antibody and be detected by another p20-specific polyclonal antibody.

### **Reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA was extracted using the RNeasy total RNA isolation protocol (Qiagen, Crauley, UK) according to the manufacturer's protocol. First-strand cDNA was synthesized from 1  $\mu$ g of total cellular RNA using an RNA PCR kit (Takara Bio Inc., Otsu, Japan) with random primers. Thereafter, cDNA was amplified using specific primers respectively. The amplification of the IL-1 $\beta$  transcripts was also accomplished on a Light Cycler (Roche Diagnostics, Mannheim, Germany) using specific primers. The housekeeping gene fragment of glyceraldehydes-3-phosphates dehydrogenase (GAPDH) was used for verification of equal loading.

## ***Cell lysis and Western blotting***

Cells were stimulated with GM-CSF for the indicated times in the figure legends and the cells were washed by ice-cold PBS and lysed with RIPA Buffer (Sigma-Aldrich) supplemented with 1.0 mM sodium orthovanadate, 10 µg/mL aprotinin and 10 µg/mL leupeptin) for 20 minutes at 4°C. After 5 min on ice, the cell lysates were centrifuged at 10,000 g for 10 min at 4°C. After centrifugation, cellular lysates (30 µg) were also subjected to 12% SDS-PAGE, followed by western blot with antibodies against human NLRP3, β-actin and phospho-specific anti-JAKs, and STATs antibodies with an ECL Western blotting kit (Amersham, Little Chalfont, UK).

## **Statistical analysis**

Differences between groups were examined for statistical significance using Student t-test. *P* values less than 0.05 were considered statistically significance.

## **Abbreviations**

IL-1:interleukin-1; JAK:Janus kinases; NLRP3:NLR family pyrin domain containing 3; RA: rheumatoid arthritis; STAT:signal transduction activator of transcription

## **Declarations**

### **Ethical Approval and Consent to participate**

Ethical approval for this study (No. 29282) was provided by the Ethics Committee of Fukushima Medical University and written informed consent was obtained from each individual.

### **Consent for publication**

Not applicable

### **Availability of supporting data**

All data generated or analysed during this study are included in this published article

### **Competing interests**

KM has received research grants from Chugai, Pfizer, and AbbVie and Eli Lilly. YK and MT are employees of Eli Lilly Japan K.K. and possesses shares in Eli Lilly and Company.

Rest of the authors declares that they have no competing interests

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## Author Contributions

YF, NM, JT, MYF, TA, SS, HM, HW, KM carried out the molecular biochemical studies, participated in the sequence alignment and drafted the manuscript. HY, AK, KM carried out the genetic assays. AK, KM participated in the sequence alignment and drafted the manuscript. YF, participated in the design of the study, performed the statistical analysis. All authors discussed the results and commented on the manuscript.

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## References

1. McInnes IB, Schett G. Cytokines in the pathogenesis of rheumatoid arthritis. *Nat Rev Immunol*. 2007;7(6):429-42.
2. Schwartz DM, Bonelli M, Gadina M, O'Shea JJ. Type I/II cytokines, JAKs, and new strategies for treating autoimmune diseases. *Nat Rev Rheumatol*. 2016;12(1):25-36.
3. Cohen S, Fleischmann R. Kinase inhibitors: a new approach to rheumatoid arthritis treatment. *Curr Opin Rheumatol*. 2010;22(3):330-5.
4. Ghoreschi K, Laurence A, O'Shea JJ. Janus kinases in immune cell signaling. *Immunol Rev*. 2009;228(1):273-87.
5. Villarino AV, Kanno Y, O'Shea JJ. Mechanisms and consequences of Jak-STAT signaling in the immune system. *Nat Immunol*. 2017;18(4):374-384.
6. Norman P. Selective JAK inhibitors in development for rheumatoid arthritis. *Expert Opin Investig Drugs*. 2014;23(8):1067-77.
7. Thoma G, Drückes P, Zerwes HG. Selective inhibitors of the Janus kinase Jak3—Are they effective? *Bioorg Med Chem Lett*. 2014;24(19):4617-4621.
8. Schwartz DM, Kanno Y, Villarino A, Ward M, Gadina M, O'Shea JJ. JAK inhibition as a therapeutic strategy for immune and inflammatory diseases. *Nat Rev Drug Discov*. 2017;16(12):843-862.
9. Yu V, Pistillo J, Archibeque I, Han Lee J, Sun BC, Schenkel LB, Geuns-Meyer S, et al. Differential selectivity of JAK2 inhibitors in enzymatic and cellular settings. *Exp Hematol*. 2013;41(5):491-500.
10. Kubo S, Nakayamada S, Sakata K, Kitanaga Y, Ma X, Lee S, Ishii A, et al. Janus Kinase Inhibitor Baricitinib Modulates Human Innate and Adaptive Immune System. *Front Immunol*. 2018;9:1510.
11. Wicks IP, Roberts AW. Targeting GM-CSF in inflammatory diseases. *Nat Rev Rheumatol*. 2016;12(1):37-48.
12. Watanabe S, Itoh T, Arai K. Roles of JAK kinases in human GM-CSF receptor signal transduction. *J Allergy Clin Immunol*. 1996;98(6 Pt 2):S183-91.

13. Yamaoka K. Janus kinase inhibitors for rheumatoid arthritis. *Curr Opin Chem Biol.* 2016;32:29-33.
14. Tanner JW, Chen W, Young RL, Longmore GD, Shaw AS. The conserved box 1 motif of cytokine receptors is required for association with JAK kinases. *J Biol Chem.* 1995;270(12):6523-30.
15. Burmester GR, McInnes IB, Kremer J, Miranda P, Korkosz M, Vencovsky J, et al. EARTH EXPLORER 1 study investigators. A randomised phase IIb study of mavrilimumab, a novel GM-CSF receptor alpha monoclonal antibody, in the treatment of rheumatoid arthritis. *Ann Rheum Dis.* 2017;76:1020-1030.
16. Quelle FW, Sato N, Witthuhn BA, Inhorn RC, Eder M, Miyajima A, et al. JAK2 associates with the beta c chain of the receptor for granulocyte-macrophage colony-stimulating factor, and its activation requires the membrane-proximal region. *Mol Cell Biol.* 1994;14:4335-41.
17. Choy EHS, Miceli-Richard C, González-Gay MA, Sinigaglia L, Schlichting DE, Meszaros G, et al. The effect of JAK1/JAK2 inhibition in rheumatoid arthritis: efficacy and safety of baricitinib. *Clin Exp Rheumatol.* [Epub ahead of print]
18. Vijaykrishnan L, Venkataramanan R, Gulati P. Treating inflammation with the Janus kinase inhibitor CP-690,550. *Trends Pharmacol Sci.* 2011;32(1):25-34.
19. Genovese MC, Fleischmann R, Combe B, Hall S, Rubbert-Roth A, Zhang Y, et al. Safety and efficacy of upadacitinib in patients with active rheumatoid arthritis refractory to biologic disease-modifying anti-rheumatic drugs (SELECT-BEYOND): a double-blind, randomised controlled phase 3 trial. 2018 ;391(10139):2513-2524.
20. Choy EH. Clinical significance of Janus Kinase inhibitor selectivity. 2018; 57:2042-2052
21. Moodley D, Yoshida H, Mostafavi S, Asinovski N, Ortiz-Lopez A, Symanowicz P, et al. Network pharmacology of JAK inhibitors. *Proc Natl Acad Sci U S A.* 2016;113:9852-7.
22. Williams NK, Bamert RS, Patel O, Wang C, Walden PM, Wilks AF, et al. Dissecting specificity in the Janus kinases: the structures of JAK-specific inhibitors complexed to the JAK1 and JAK2 protein tyrosine kinase domains. *J Mol Biol.* 2009;387:219-32.
23. Pedra JH, Cassel SL, Sutterwala FS. Sensing pathogens and danger signals by the inflammasome. *Curr Opin Immunol.* 2009;21(1):10-6.
24. Watanabe S, Itoh T, Arai K. Roles of JAK kinase in human GM-CSF receptor signals. 1997 Suppl 3:76-8.
25. Furuya MY, Asano T, Sumichika Y, Sato S, Kobayashi H, Watanabe H, et al. Tofacitinib inhibits granulocyte-macrophage colony-stimulating factor-induced NLRP3 inflammasome activation in human neutrophils. *Arthritis Res Ther.* 2018; 20:196.
26. Parmentier JM, Voss J, Graff C, Schwartz A, Argiriadi M, Friedman M, et al. In vitro and in vivo characterization of the JAK1 selectivity of upadacitinib (ABT-494). *BMC Rheumatol.* 2018;2:23. doi: 10.1186/s41927-018-0031-x.
27. McInnes IB, Byers NL, Higgs RE, Lee J, Macias WL, Na S, Ortmann RA, et al. Comparison of baricitinib, upadacitinib, and tofacitinib mediated regulation of cytokine signaling in human leukocyte subpopulations. *Arthritis Res Ther.* 2019;21:183

28. Clark JD, Flanagan ME, Telliez JB. Discovery and development of Janus kinase (JAK) inhibitors for inflammatory diseases. *J Med Chem.* 2014;57:5023-38
29. Zhan P, Itoh Y, Suzuki T, Liu X. Strategies for the Discovery of Target-Specific or Isoform-Selective Modulators. *J Med Chem.* 2015;58(19):7611-33.
30. al-Shami A, Bourgoin SG, Naccache PH. Granulocyte-macrophage colony-stimulating factor-activated signaling pathways in human neutrophils. I. Tyrosine phosphorylation-dependent stimulation of phosphatidylinositol 3-kinase and inhibition by phorbol esters. *Blood.* 1997;89:1035-44.
31. Osiri M, Wongpiyabovorn J, Sattayasomboon Y, Thammacharoenrach N. Inflammatory cytokine levels, disease activity, and function of patients with rheumatoid arthritis treated with combined conventional disease-modifying antirheumatic drugs or biologics. *Clin Rheumatol.* 2016;35:1673-81.

## Figures

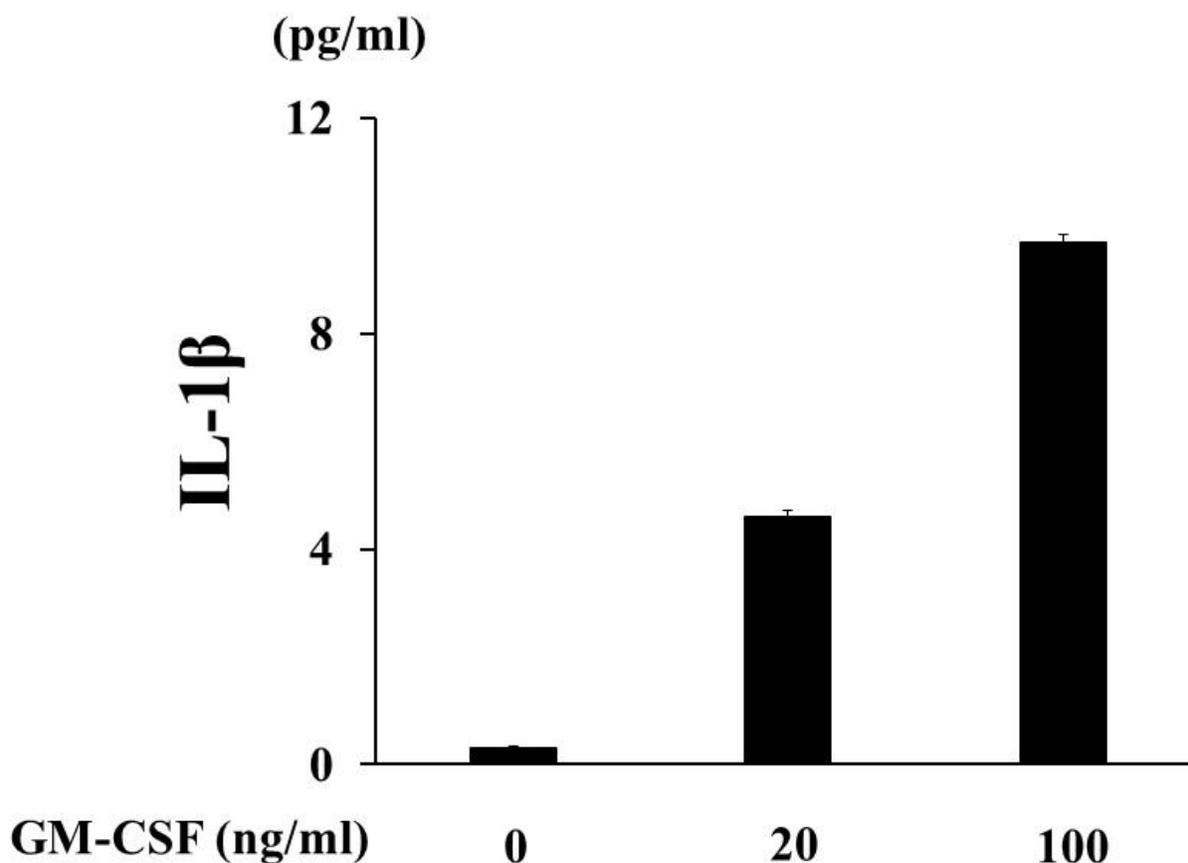
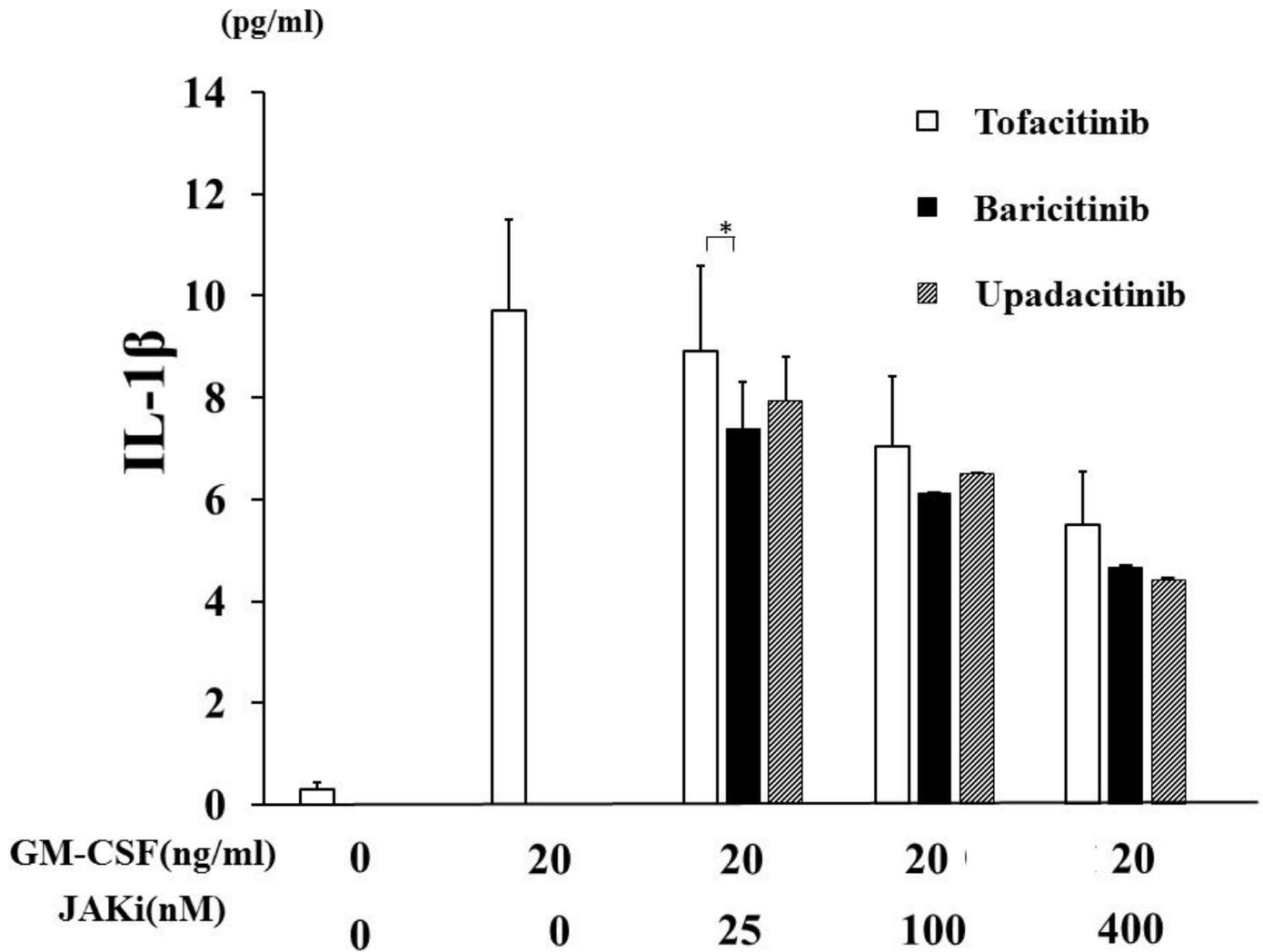


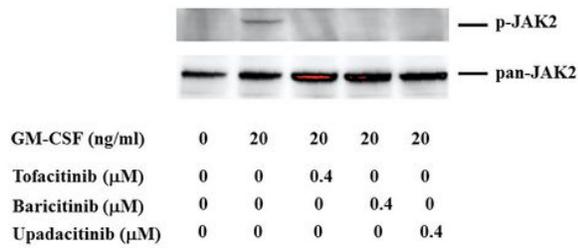
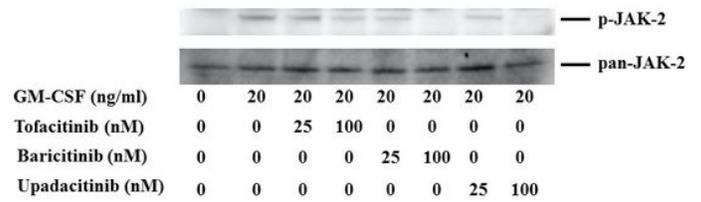
Figure 1

GM-CSF induces IL-1 $\beta$  synthesis from THP-1 cells. THP-1 cells were incubated with the indicated concentrations of GM-CSF for 24 h and supernatants were analyzed for IL-1 $\beta$  production by ELISA. Values represent the mean  $\pm$  SD of two independent experiments.

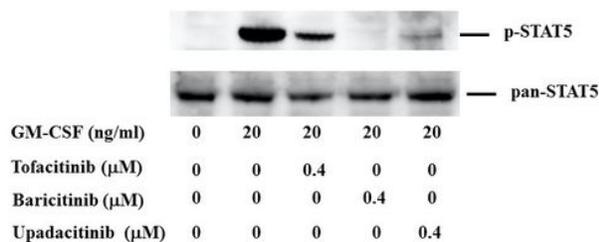


**Figure 2**

JAKi inhibit the IL-1 $\beta$  synthesis from GM-CSF-stimulated THP-1 cells THP-1 cells were stimulated with GM-CSF (20ng/ml) for 24 hr in the presence or absence of the pretreatment JAKi (tofacitinib, baricitinib, upadacitinib for 1 hr) and supernatants were analyzed for IL-1 $\beta$  production by ELISA. Values represent the mean  $\pm$  SD of three independent experiments. \*  $p < 0.01$  baricitinib-pretreated neutrophils versus those with tofacitinib or upadacitinib.

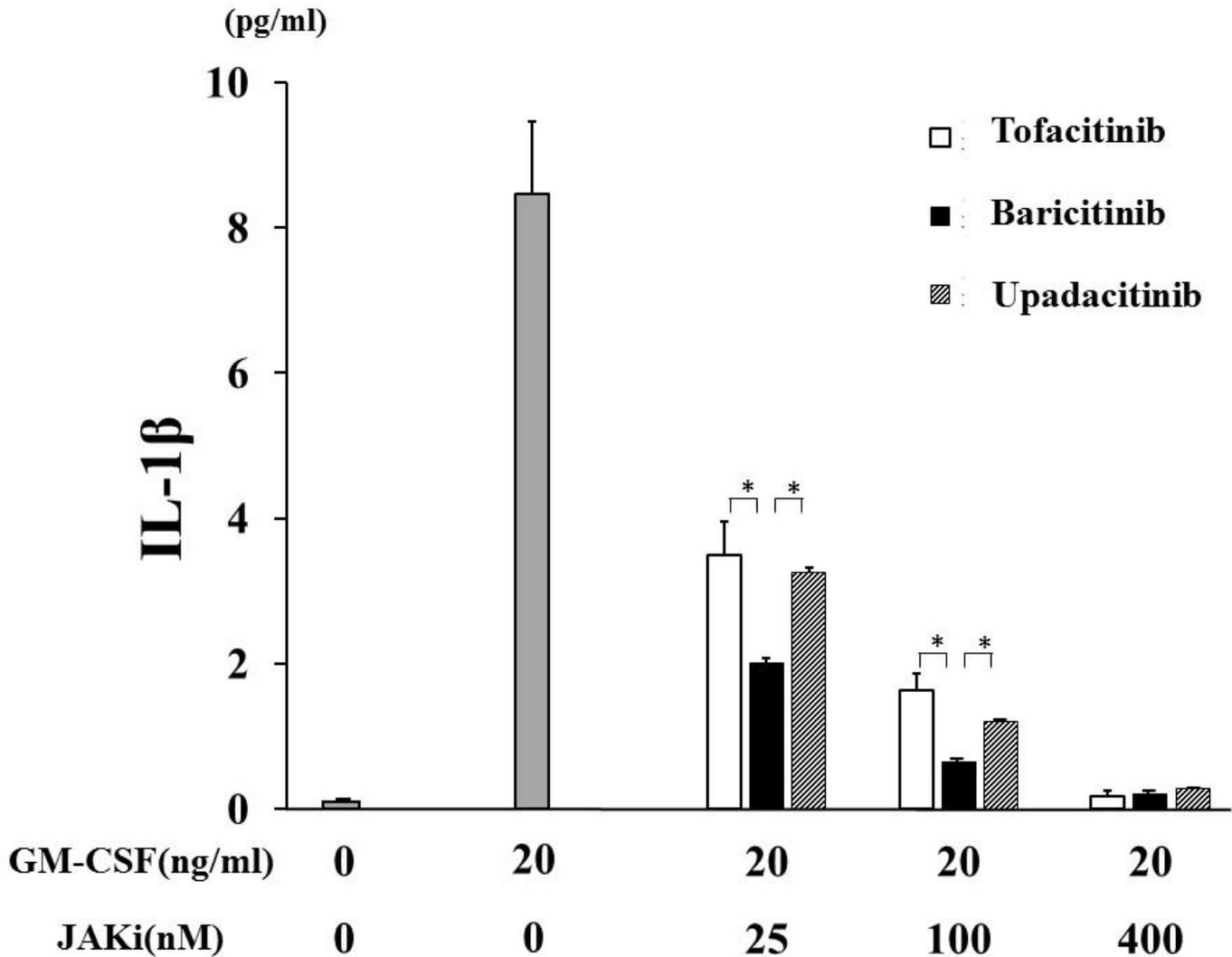
**A****B****Figure 3**

Effects of JAKi on JAK2 phosphorylation in GM-CSF stimulated THP-1 cells A: THP-1 cells were pretreated with JAKi (tofacitinib, baricitinib, upadacitinib) at the indicated concentrations (400nM) for 1 hr and then stimulated with GM-CSF (20ng/ml) for 20 minutes. Phosphorylation of JAK2 was determined by Western blotting using phospho-specific antibodies against JAK2. B: THP-1 cells were pretreated with JAKi (tofacitinib, baricitinib, upadacitinib) at the indicated concentrations (25, 100 nM) for 1 hr and then stimulated with GM-CSF (20ng/ml) for 20 minutes. Phosphorylation of JAK2 was determined by Western blotting using phospho-specific antibodies against JAK2,

**A****B****Figure 4**

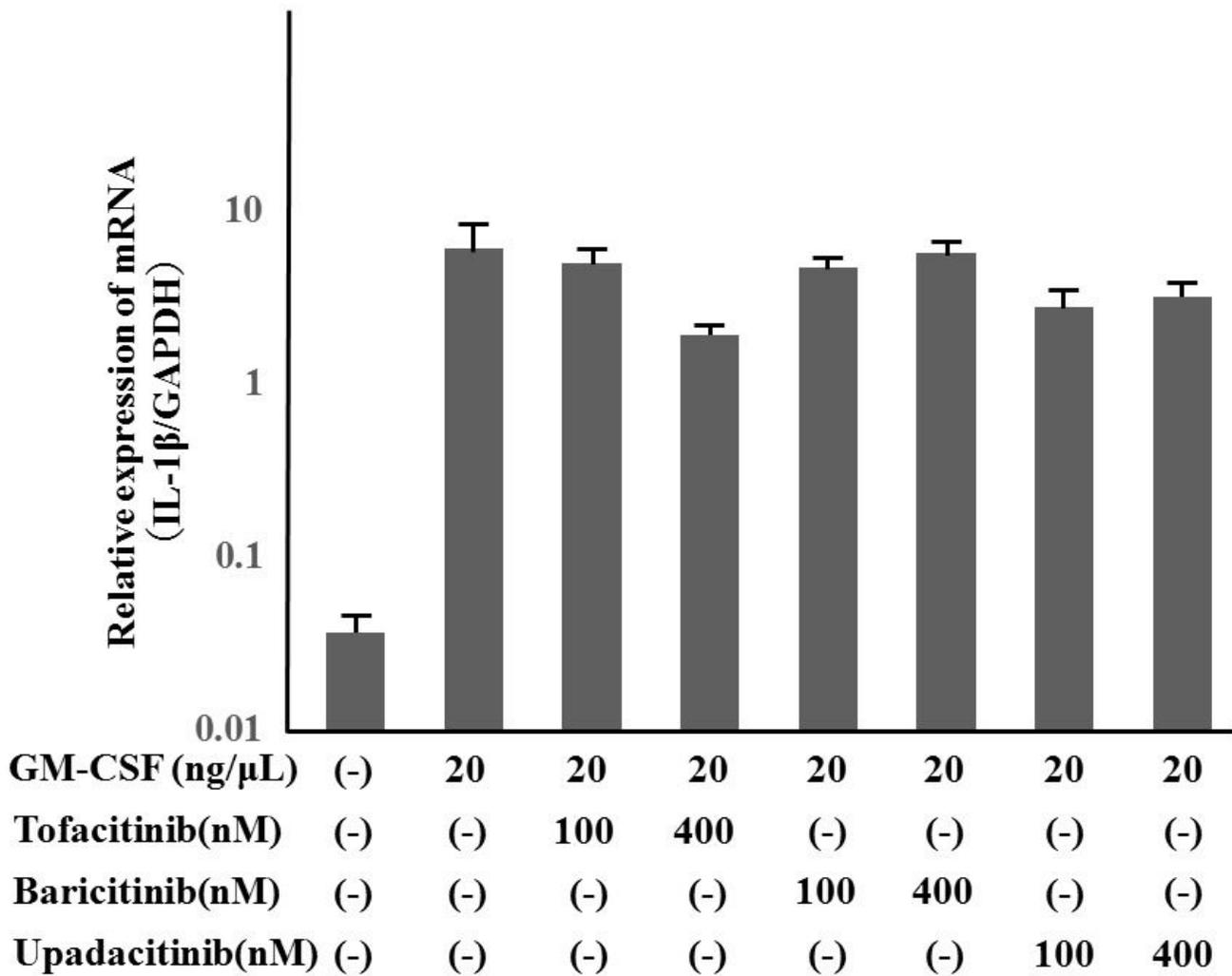
Effects of JAKi on STAT5 phosphorylation in GM-CSF stimulated THP-1 cells A: THP-1 cells were pretreated with JAKi (tofacitinib, baricitinib, upadacitinib) at the indicated concentrations (400nM) for 1 hr and then stimulated with GM-CSF (20ng/ml) for 20 minutes. Phosphorylation of STA5 was determined by Western blotting using phospho-specific antibodies against STAT5. B: THP-1 cells were pretreated with

JAKi (tofacitinib, baricitinib, upadacitinib) at the indicated (25, 100 nM) for 1 hr and then stimulated with GM-CSF (20ng/ml) for 20 minutes. Phosphorylation of STAT5 was determined by Western blotting using phospho-specific antibodies against STAT5.



**Figure 5**

JAKi inhibit the IL-1 $\beta$  synthesis from GM-CSF-stimulated neutrophils Neutrophils ( $1 \times 10^6$ /ml) were stimulated with GM-CSF (20ng/ml) for 24 hr in the presence or absence of the pretreatment JAKi (tofacitinib, baricitinib, upadacitinib for 1 hr) and supernatants were analyzed for IL-1 $\beta$  production by ELISA . Values represent the mean  $\pm$  SD of three independent experiments. \*  $p < 0.01$  baricitinib-pretreated neutrophils versus those with tofacitinib or upadacitinib



**Figure 6**

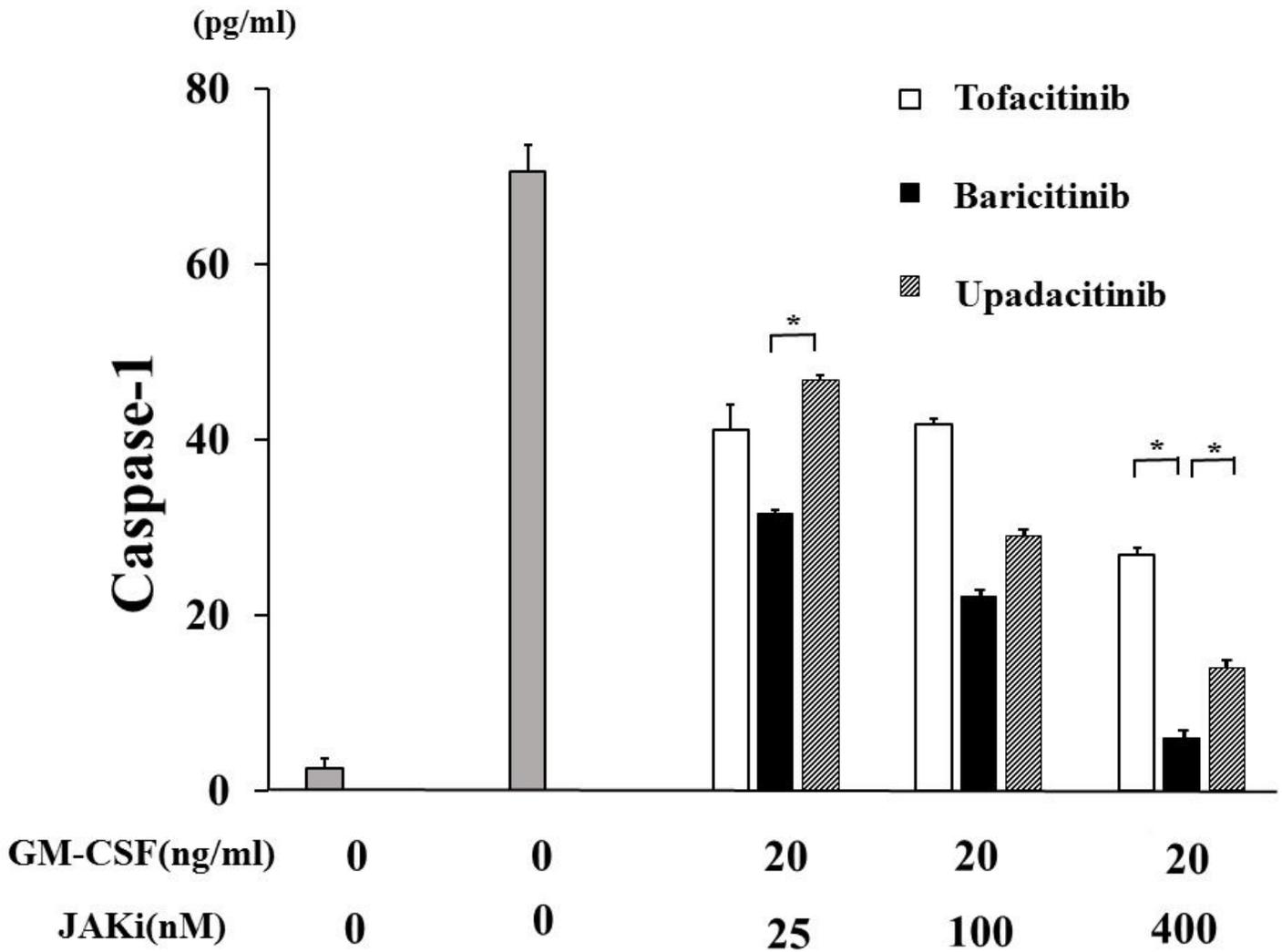
Effects of JAKi on pro-IL-1β mRNA expressions in GM-CSF-stimulated neutrophils. Neutrophils (1×10<sup>6</sup>/ml) were stimulated with GM-CSF (20ng/ml) for 8 hr in the presence or absence of the pretreatment JAKi (tofacitinib, baricitinib, Upadacitinib) for 1 hr. The cells were harvested and analyzed for pro-IL-1β and GAPDH mRNA levels by real-time PCR. Values represent the mean ± SD of two independent experiments.



<b>GM-CSF (ng/ml)</b>	0	20	20	20	20	20	20	20
<b>Tofacitinib (nM)</b>	0	0	100	400	0	0	0	0
<b>Upadacitinib (nM)</b>	0	0	0	0	100	400	0	0
<b>Baricitinib (nM)</b>	0	0	0	0	0	0	100	400

**Figure 7**

NLRP3 expression in neutrophils Neutrophils were stimulated with GM-CSF for 24 hr in the presence or absence of the pretreatment JAKi (tofacitinib, baricitinib, Upadacitinib) for 1 hr. Cellular lysates were analyzed by Western using anti-NLRP3 or anti- $\beta$ -actin antibodies. Three experiments were performed using different neutrophils and a representative result is shown.



**Figure 8**

JAKi inhibit the caspase-1 (p20) release from GM-CSF-stimulated neutrophils. Neutrophils ( $2 \times 10^6$ /ml) were stimulated with GM-CSF (20ng/ml) in the presence or absence of the pretreatment JAKi (tofacitinib, baricitinib, Upadacitinib) for 1 hr and supernatants were analyzed for caspase-1 (p20) by ELISA. Values represent the mean  $\pm$  SD of three independent experiments. \*  $p < 0.01$  baricitinib-pretreated neutrophils versus those with tofacitinib or upadacitinib