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Inhibition of CEACAM1 expression in cytokine-activated neutrophils using JAK inhibitors

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Abstract

Objectives Carcinoembryonic-antigen-related cell-adhesion molecule 1 (CEACAM1) is an adhesion molecule that acts as a coinhibitory receptor in the immune system. We previously demonstrated that CEACAM1 is predominantly expressed on peripheral blood neutrophils in patients with RA. The aim of the present study was to investigate the effects of Janus kinase inhibitors (JAKi) on cytokine-activated human neutrophils and CEACAM1 expression.

Methods Peripheral blood neutrophils were obtained from healthy subjects. Isolated neutrophils were stimulated with tumor necrosis factor-alpha (TNF-α) or granulocyte–macrophage colony-stimulating factor (GM-CSF) in the presence or absence of JAKi. The expression of CEACAM1 in peripheral blood neutrophils was analyzed by flow cytometry. Protein phosphorylation of signal transducer and activator of transcription (STAT)1, STAT3, and STAT5 was assessed by western blot using phospho-specific antibodies.

Results We found that TNF-α-induced CEACAM1 expression was marginally suppressed after pretreatment with pan-JAK inhibitor, tofacitinib. Moreover, TNF-α induced STAT1 and STAT3 phosphorylation at the late stimulation phase (4 to 16 h). The expressions of CEACAM1 on neutrophils were markedly up-regulated by GM-CSF not by interleukin (IL)-6 stimulation. All JAKi inhibited GM-CSF-induced CEACAM1 expressions on neutrophils, however, the inhibitory effects of baricitinib were larger compared to those of tofacitinib or filgotinib. Moreover, CEACAM1 was marginally upregulated in interferon (IFN)-γ stimulated neutrophils. Similarly, JAKi inhibited IFN-γ-induced CEACAM1 expressions on neutrophils.

Conclusions We demonstrated that JAKi prevent GM-CSF-induced CEACAM1 expression in neutrophils, and JAKi-induced inhibition depends on their selectivity against JAK isoforms. These findings suggest that JAKi can modulate the expression of CEACAM1 in cytokine-activated neutrophils, thereby limiting their activation.

Keywords Carcinoembryonic-antigen-related cell-adhesion molecule 1, Janus kinase inhibitors, Granulocyte-macrophage colony-stimulating factor neutrophils, Tumor necrosis factor-alpha, Rheumatoid arthritis

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Background

Neutrophils play a pivotal role in innate immunity, especially in host defense against pathogenic infections [1]. Neutrophils are also involved in the progression of a broad range of disorders, including autoimmune and inflammatory diseases [2]. Rheumatoid arthritis (RA) is a complex systemic autoimmune disease mediated by the abnormal activation of various immune cells, and neutrophils have been linked to the onset and progression of RA [3]. In the rheumatoid synovium, neutrophils infiltrating synovial membranes or fluids play an important role in the pathogenesis of RA [4]. We previously reported that carcinoembryonic-antigen-related cell-adhesion molecule 1 (CEACAM1) is selectively expressed in neutrophils not in T cells or monocytes, upregulated in peripheral blood neutrophils isolated from patients with active RA [5]. This observation suggests that inflammatory stimuli induce upregulation of the CEACAM1 gene on neutrophils in patients with active RA. CEACAM1, also known as cluster of differentiation (CD)66a, is a carcinoembryonic antigen involved in intercellular adhesion, cellular growth, differentiation, and immune function [6]. Additionally, CEACAM1 was shown to be an essential molecule in neutrophil activation and serves as a regulator of NETosis [7]. CEACAM1 also plays a role in immune tolerance, acting with the T cell immunoglobulin and mucin domain-3 (TIM-3) regulatory molecule to inhibit immune responses [8]. Although CEACAM1 is a well-known cell surface protein expressed by activated myeloid cells, including neutrophils [9], its expression mechanism in inflammatory processes has not yet been characterized. Recently, it was reported that the expression of CEACAM family in intestinal epithelial cells was suppressed by tofacitinib, a Janus kinase (JAK) inhibitor, in inflammatory bowel diseases [10]. Herein, we explored whether and how CEACAM1 expression is regulated in response to rheumatoid inflammation by stimulating neutrophils from healthy volunteers with inflammatory cytokines. Further, we explored molecular mechanisms by which inflammatory cytokines alter CEACAM1 expression in human neutrophils and the interplay between these mechanisms and the JAK / signal transducer and activator of transcription (STAT) signaling pathway.

Results

Effects of JAK inhibitors (JAKi) on TNF-α-induced CEACAM1 expression

Our previous study showed that TNF-α is an important factor for the induction of CEACAM1 expression on neutrophils. Additionally, Yarilina A et al. showed that the STAT is indirectly activated by TNF [11]. Therefore, we evaluated the CEACAM1 expression on neutrophils to clarify the relationship between the JAK/STAT pathway and CEACAM1 expression. We pretreated neutrophils with a pan-JAKi, tofacitinib, and then stimulated with TNF-α (20 ng/ml) for 24 h. We observed that the level of CEACAM1 increased in response to stimulation with TNF-α. However, tofacitinib pretreatment marginally suppressed the TNF-α-induced CEACAM1 expression (Fig. 1A). The viability after stimulation with TNF-α is shown in Supplemental Fig. 1. There was no difference in the viability between TNF-α stimulated and non-stimulated neutrophils.

TNF-α induces STAT1 and STAT3 phosphorylation

To determine whether TNF-α induces the activation of the JAK/STAT pathway in neutrophils, we assessed the phosphorylation status of JAK/STAT proteins in TNF-α-activated neutrophil cell lysates (Fig. 1B). JAKs or STATs phosphorylation was not detected in neutrophils within 1 h after TNF-α stimulation (data not shown). It was demonstrated the STAT 1/3 phosphorylation is detected in monocytes or macrophages at the late phase (up to 24 h) after TNF stimulation [11]. Therefore, we analyzed the phosphorylation state of the STAT family (STAT1, 3, and 5) up to 16 h after TNF-α stimulation. Although phosphorylation of JAK family (JAK 1, 2, and 3) was not detected (data not shown), STAT1/3 phosphorylation was detected in TNF-α-stimulated neutrophils after 4 to 16 h from the start of stimulation (Fig. 1B). These results may suggest the possibility that TNF-α induces other inflammatory cytokine that activates JAK/STAT pathways. Full-length blots are presented in Supplemental Fig. 2.

GM-CSF induces CEACAM1 expression

We previously reported that IL-6 stimulation did not induce CEACAM1 expression in neutrophils. Therefore, we investigated the effects of other cytokines that use the JAK/STAT pathway in their receptor-mediated signaling. Notably, inflammatory cytokines such as GM-CSF have been reported to be involved in the progression of RA [12]. We explored whether proinflammatory cytokines, GM-CSF modulate CEACAM1 expression in neutrophils. Interestingly, CEACAM1 levels were markedly upregulated by GM-CSF stimulation after 24 h. Moreover, GM-CSF stimulation induced CEACAM1 expression on neutrophils in a dose-dependent manner (Fig. 2A).

Effects of JAKi on GM-CSF-induced CEACAM1 expression

Recently, it was reported that the expression of CEACAM family in intestinal epithelial cells was suppressed by tofacitinib, a JAKi, in inflammatory bowel diseases [10]. To investigate the effects of tofacitinib, baricitinib, and upadacitinib on the level of CEACAM1, we pretreated isolated neutrophils with each JAKi at different

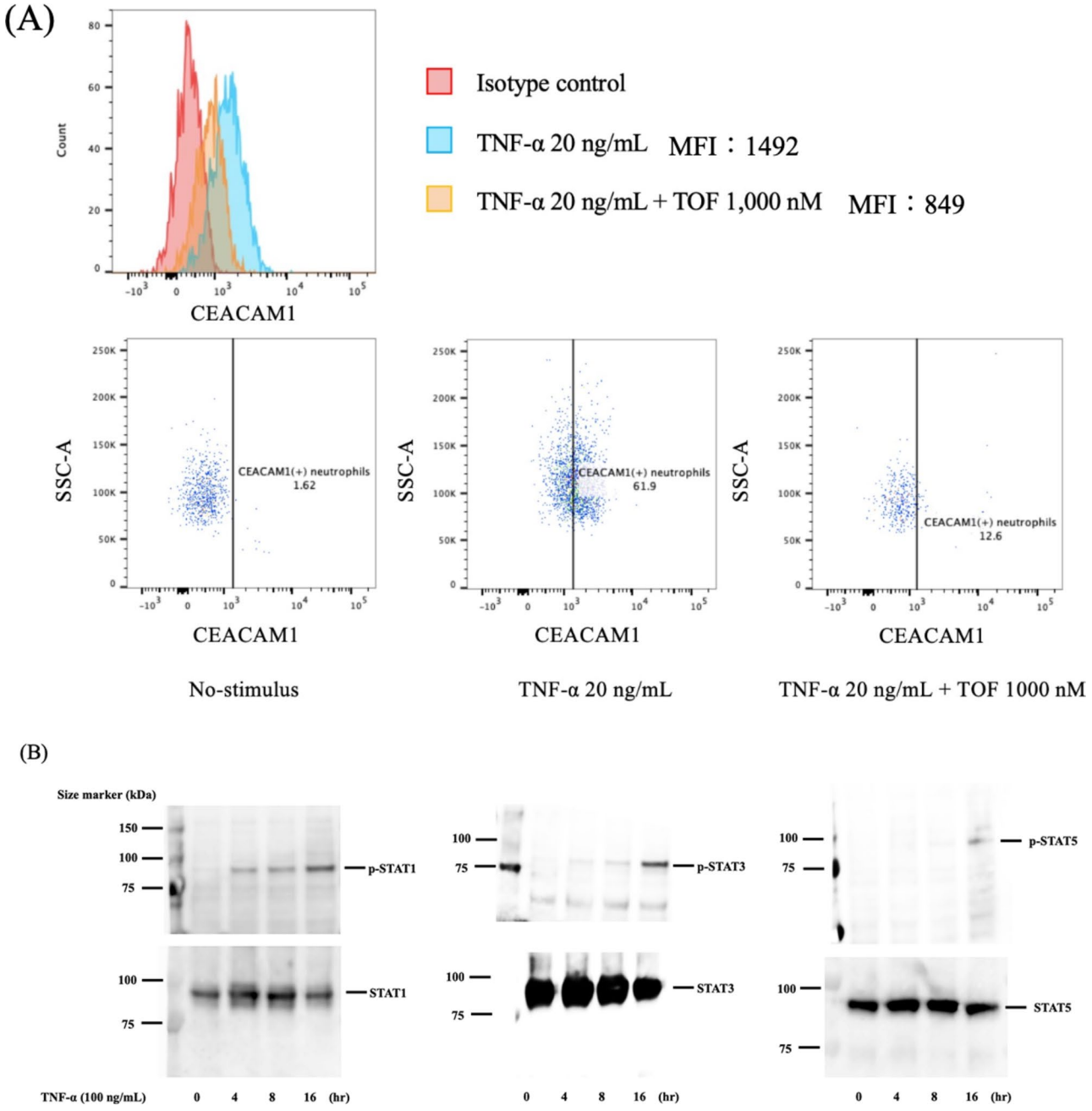

Fig. 1 TNF-α induces CEACAM1 expression, and STAT 1 and STAT3 phosphorylation at the late stimulation phase in neutrophils. (**A**) Flow cytometry overlay histograms for CEACAM1 expression on CD14(-)CD16(+) neutrophils. Representative histograms showing the percentage of CEACAM1(+) neutrophils stimulated with TNF-α with or without tofacitinib pretreatment. CEACAM1 expression was detected using anti–CEACAM1 antibody (blue shaded histogram) or isotype control antibody (red shaded histograms). There were marginal reductions in the number of CEACAM1(+) neutrophils by pretreatments with tofacitinib (orange shaded histograms). Data are representative of three independent experiments. (**B**) Neutrophil lysated were analyzed by western blot using anti-phosphatase STATs (pSTATs) (pSTAT 1, 3, and 5 at upper panel) or anti-STATs (STAT 1, 3, and 5 at lower panel). TNF-α induces STAT1 and STAT3 phosphorylation in neutrophils after 8 h from the stimulation with TNF-α. Whereas TNF-α induced STAT5 phosphorylation was barely observed after TNF-α stimulation. Data are representative of three independent experiments. Full-length blots are presented in Supplemental Fig. 2

concentrations for 1 h and then incubated them with GM-CSF for the next 24 h. The concentration of JAKi for pretreated neutrophils was determined according to the pharmacokinetics in healthy volunteers receiving their JAKi. In neutrophils isolated from healthy volunteers, an almost 100% inhibition rate was confirmed when the concentration of each JAKi was set at 1,000 nM [13], therefore we wet the maximum concentration at 1,000 nM in this study. The expression of CEACAM1 in neutrophils was assessed by flow cytometry. The pretreatment with

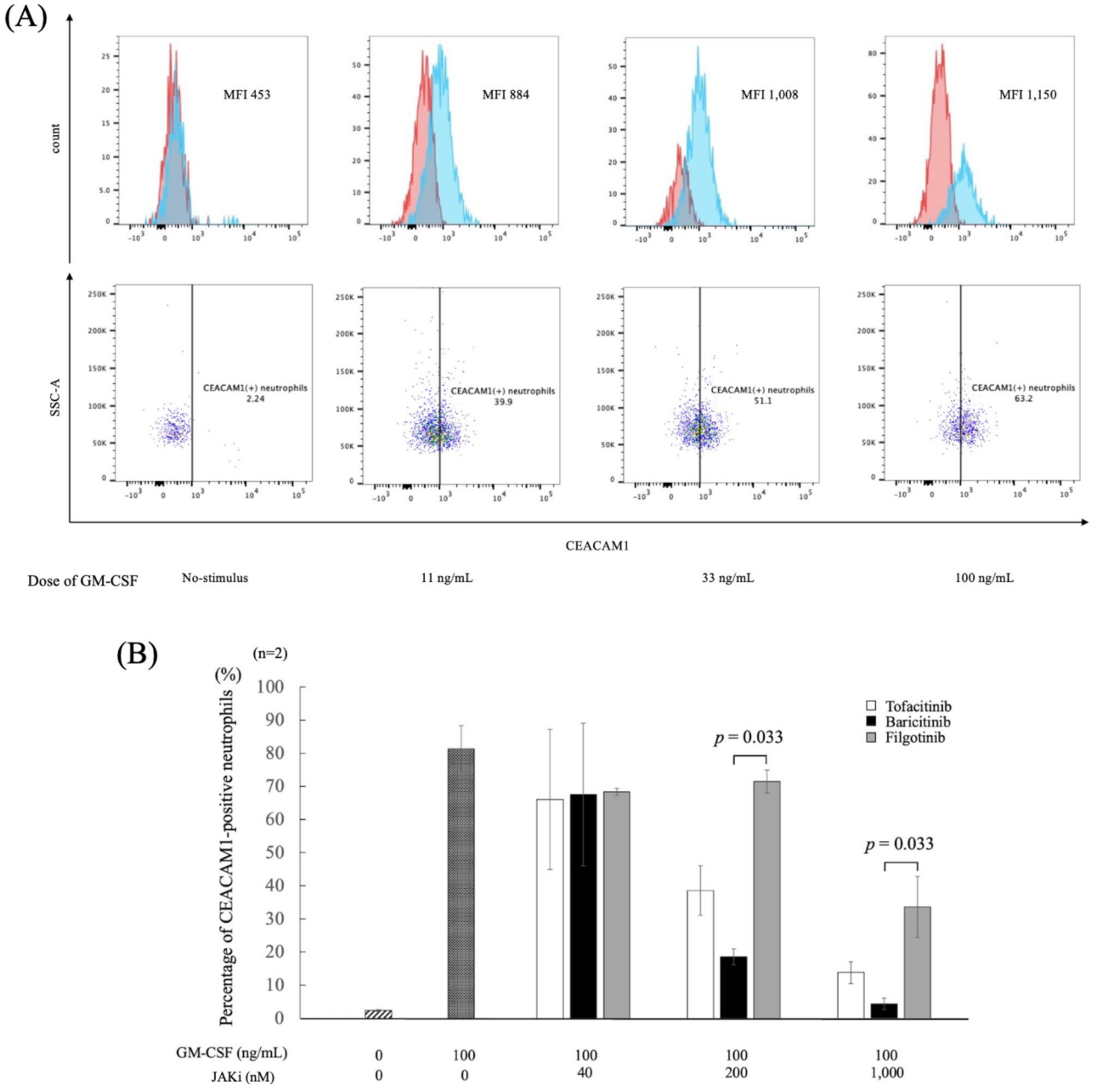

Fig. 2 A GM-CSF induces of CEACMA1 expression on neutrophils in a dose-dependent manner, and JAKi inhibit GM-CSF-induced CEACAM1 expressions on neutrophils. (**A**) CEACAM1 expressions on neutrophils were assessed by flow cytometry. Neutrophils were stimulated with the indicated concentrations of GM-CSF. Flow cytometry overlay histograms for CEACAM1 expression on neutrophils in each culture condition were demonstrated. Data are representative of three independent experiments. (**B**) Neutrophils were pretreated with JAKi (tofacitinib, baricitinib, upadacitinib) at the indicated concentrations for 1 h and then stimulated with GM-CSF (100 ng/ml) for 24 h. CEACAM1 expressions on neutrophils were assessed by flow cytometry. Percentage of CEACAM1-positive neutrophils were evaluated by flow cytometry for CEACAM1 (percentage of positive cells) in GM-CSF-stimulated neutrophils pretreated with the indicated concentrations of JAKi (tofacitinib, baricinib and filgotinib). Statistics were performed using Kruskal-Wallis test. Two independent experiments were combined. Values represent the mean ± SD of two independent experiments

JAKis at low concentrations (40 nM) had only marginal effects on the level of CEACAM1, whereas high concentrations (200–1000 nM) significantly inhibited GM-CSF-induced CEACAM1 expression in neutrophils (Fig. 2B). JAKi showed variable inhibitory effects, and baricitinib caused the most potent suppression of CEACAM1 among JAKi used in this experiment (Fig. 2B).

IFN-γ induced CEACAM1 expression

IFN-γ stimulation induces CEACAM1 expressions in human neutrophils, however, their induction levels

were lower compared to those in GM-CSF or TNF-α-stimulated neutrophils (Fig. 3A). Similarly, IFN-γ induced CEACAM1 expressions were inhibited by JAKi pretreatment and baricitinib most efficiently inhibited IFN-γ induced CEACAM1 expressions (Fig. 3B).

Comparison of CEACAM1 expression by each cytokine

We compared the CEACAM1 expression on neutrophils among inflammatory cytokines (TNF-α, IFN-γ, and GM-CSF), and no statistically significant differences were found, but the stimulation with IFN-γ resulted in the low expression of CEACAM1, as shown in Fig. 4A, B.

Discussion

Our results revealed that TNF-α-induced CEACAM1 expression was marginally suppressed after pretreatment with pan-JAK inhibitor, tofacitinib. Moreover, TNF-α induced STAT1 and STAT3 phosphorylation at the late stimulation phase (4 to 16 h). The expressions of CEACAM1 on neutrophils were markedly up-regulated by GM-CSF not by interleukin (IL)-6 stimulation. All JAKi inhibited GM-CSF-induced CEACAM1 expressions on neutrophils; however, the inhibitory effects of baricitinib were larger compared to those of tofacitinib or filgotinib. CEACAM1 was marginally upregulated in interferon (IFN)-γ stimulated neutrophils, and JAKi inhibited IFN-γ-induced CEACAM1 expressions on neutrophils.

Neutrophil's function is modulated by cytokines during the inflammatory processes [14]. Activated neutrophils express various adhesion molecules and produce inflammatory cytokines and chemokines associated with immune responses [15]. Inflammatory cytokines such as TNF-α and GM-CSF induce neutrophil activation and survival at inflammatory sites [16]. In rheumatoid synovium, these cytokines suppress apoptosis and promote neutrophil activation [17]. Moreover, CEACAM1 is a key signaling molecule that regulates multiple cellular processes, including cell proliferation, tumor growth, apoptosis, angiogenesis, and granulocyte activation [18].

CEACAM1 has been shown to be upregulated in inflammatory responses [9] and is increasingly recognized for its multifaceted role in various autoinflammatory, autoimmune diseases, and cancer in previous reports. In the context inflammatory bowel disease (IBD), alterations in CEACAM1 expression of intestinal epithelial cell can cause mucosal barrier function and inflammatory responses and lead potentially increasing the risk of IBD development [19]. In multiple sclerosis (MS), the imbalance in CEACAM1 expression has also been shown to exacerbate inflammatory T-cell responses and contribute to the inflammatory pathogenesis of MS [20]. Additionally, we previously reported the increased expression levels of CEACAM1 in neutrophils isolated from patients with active RA [5]. In rheumatoid neutrophils, CEACAM1 expression could be induced by inflammatory cytokines, including TNF-α, an essential cytokine in the pathogenesis of RA [21]. Other cytokines that activate the JAK/STAT pathway (e.g., IL-6 and GM-CSF) have been reported to be produced in the rheumatoid synovium [22]. In the present study, we investigated the effects of JAKi on CEACAM1 expression in neutrophils activated by TNF-α, which may not involve in the activation of the JAK/STAT pathway. Unexpectedly, we observed that JAKi marginally downregulate the expression of CEACAM1. Although TNF-α may not directly activate JAK/STAT signaling [23], TNF-α stimulation resulted in the delayed phosphorylation of STAT1 and STAT3 in neutrophils. Therefore, we investigated the mechanisms by which JAKi inhibit cytokine-induced CEACAM1 expression. Among inflammatory cytokines, we focused on GM-CSF because CEACAM1 expression is strongly induced in GM-CSF-treated but not in IL-6-treated neutrophils. Untreated neutrophils barely express CEACAM1 on their cell surface, whereas CEACAM1 is highly upregulated after GM-CSF stimulation.

We determined the effect of JAK inhibition using JAKi (tofacitinib, baricitinib and filgotinib) on CEACAM1 expressions in GM-CSF-activated neutrophils. The GM-CSF receptor is a homodimer complex containing two JAK2 subunits [24]; therefore, it can be efficiently blocked by baricitinib. Indeed, the inhibitory effects of JAKi on CEACAM1 expression were significantly higher in neutrophils pretreated with baricitinib compared to those with tofacitinib or filgotinib. These findings suggest that each JAKi inhibite cytokine-induced CEACAM1 expressions with some variations and the selectivity against JAK2 contribute to the inhibitory properties JAKi on CEACAM1 expressions. Our data demonstrate that JAKi suppress the inflammatory functions by affecting GM-CSF-induced CEACAM1, one of the key molecules during the neutrophil's activation processes. These findings suggest that the suppressive effects against neutrophils may contribute to the therapeutic effect of JAKi in RA.

GM-CSF plays a pivotal role in the rheumatoid cytokine network, and JAK2 is a protein kinase that regulates GM-CSF-induced signal transduction [25]. Baricitinib is a potent and selective inhibitor of JAK1 and JAK2 [26]. Although tofacitinib was developed as a selective JAK3 inhibitor, subsequent studies demonstrated that it also inhibits JAK1 [27]. Moreover, filgotinib was reported as a selective JAK1 inhibitor [28]. In accord to these findings, we observed that GM-CSF induces CEACAM1 expression in neutrophils, and JAK1/2 inhibitor, baricitinib, most efficiently suppresses CEACAM1 expression in neutrophils. It is expected that inhibitory effects depend on the selectivity of JAKi against different JAK isoforms

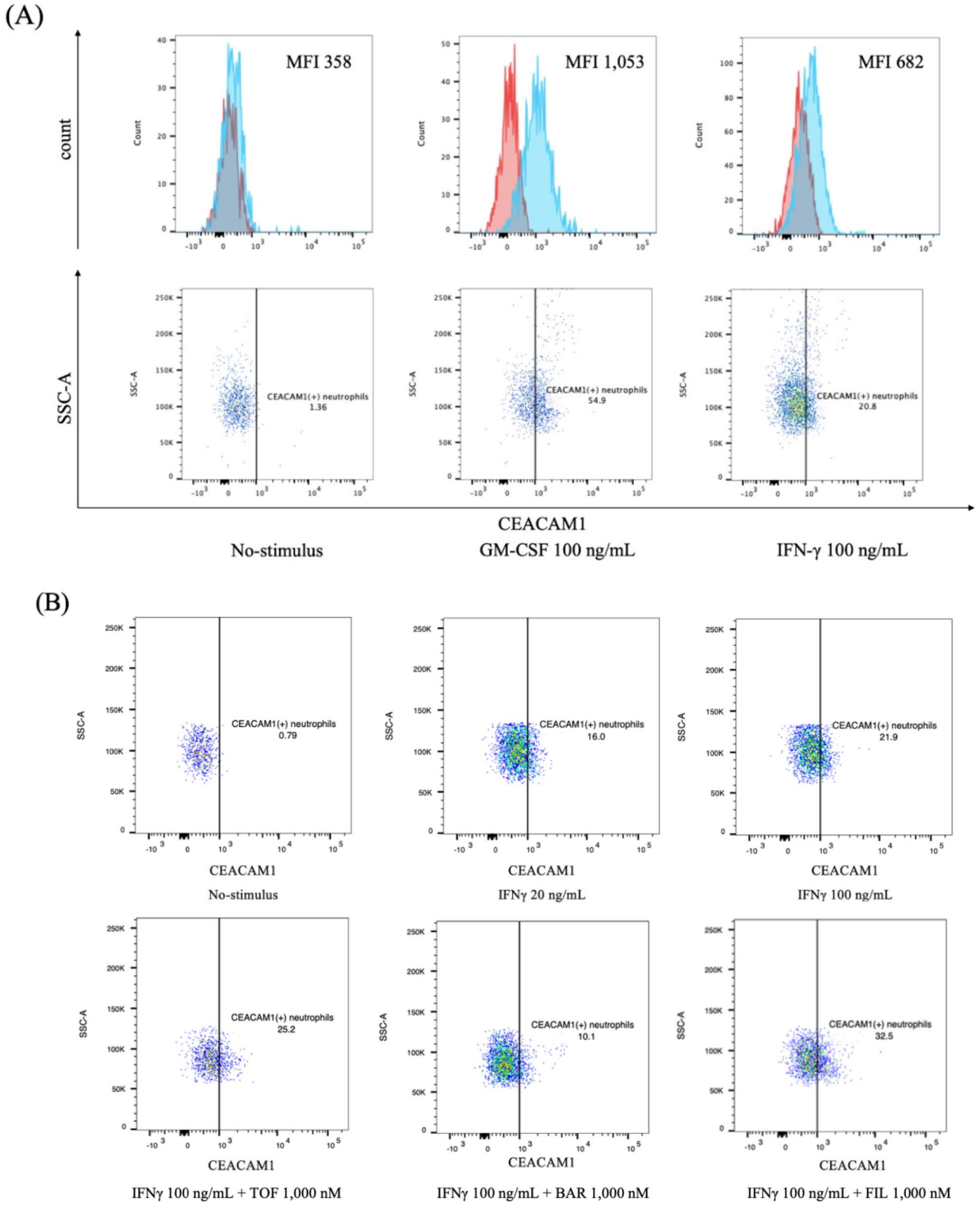

Fig. 3 JAKi inhibited IFN-γ-induced CEACAM1 expressions on neutrophils. (**A**) Neutrophils were stimulated with the indicated concentrations of GM-CSF or IFN-γ and CEACAM1 expressions on neutrophils were assessed by flow cytometry. CEACAM1 expression was induced by IFN-γ, however its induction levels were lower compared to those by GM-CSF. (**B**) Neutrophils were pretreated with JAKi (tofacitinib, baricitinib, upadacitinib) at the indicated concentrations for 1 h and then stimulated with IFN-γ (100 ng/ml) for 24 h. CEACAM1 expressions on neutrophils were assessed by flow cytometry. The percentage of CEACAM1-positive neutrophils was evaluated by flow cytometry. Three experiments were performed by using different neutrophils, and a representative result is shown

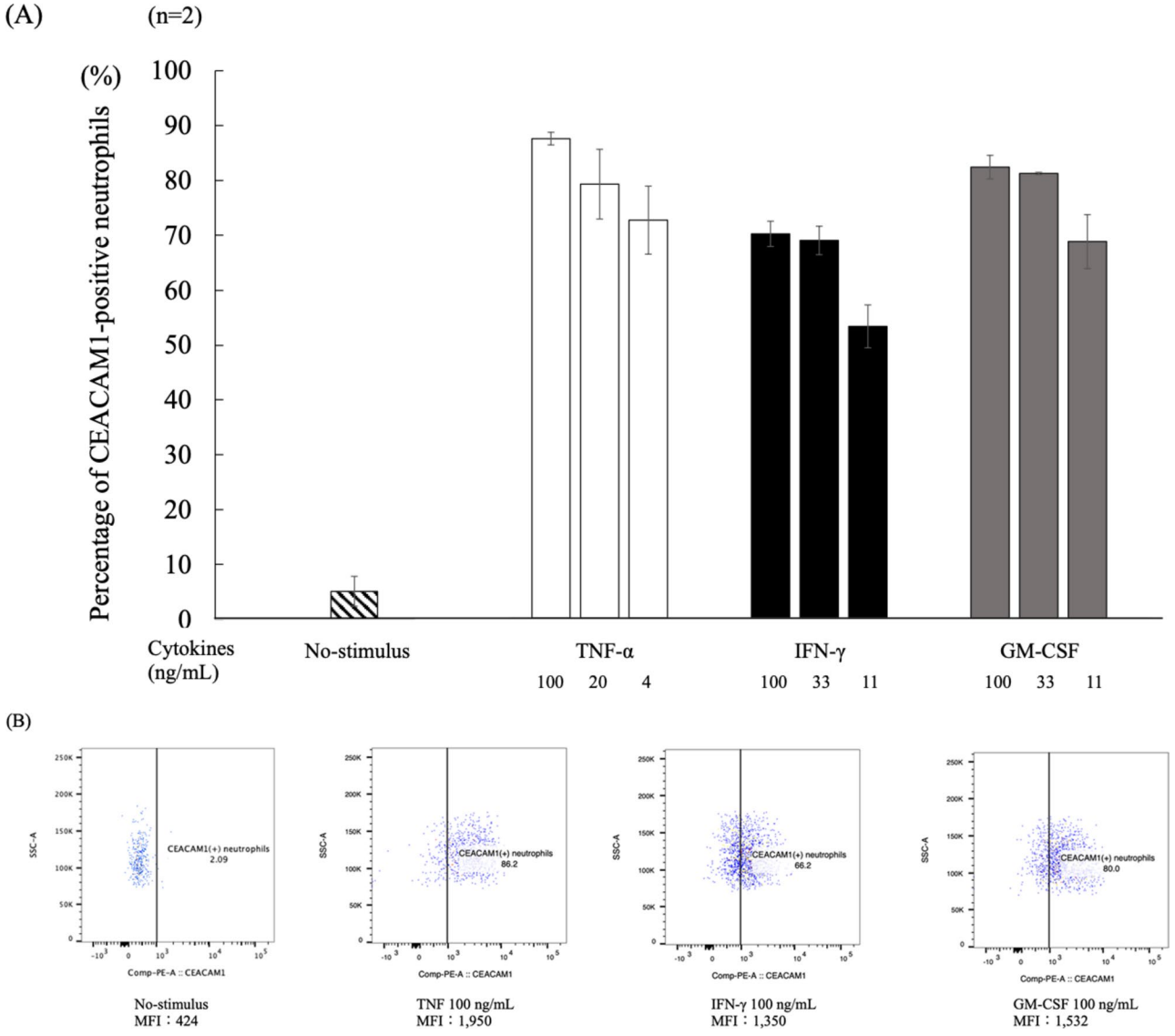

Fig. 4 CEACAM1 expressions on different cytokines-stimulated neutrophils. (**A**) Neutrophils were stimulated with TNF-α, IFN-γ, and GM-CSF for 24 h and CEACAM1 expressions on neutrophils were assessed by flow cytometry. Compared with each cytokine, no statistically significant differences were found, but the stimulation with IFN-γ resulted in the low expression of CEACAM1. Statistics were performed using Kruskal-Wallis test. Two independent experiments were combined. Values represent the mean ± SD of two independent experiments. (**B**) Neutrophils were stimulated with TNF-α, IFN-γ, and GM-CSF for 24 h. Two experiments were performed by using different neutrophils, and representative results of high dose cytokine stimulations were shown

[29]. We hypothesize that JAKi-mediated STAT inactivation decreases CEACAM1 expression in neutrophils. Our results show that the JAK/STAT pathway is associated with cytokine-induced CEACAM1 expression. We first demonstrated that JAKi inhibit CEACAM1 expression in cytokine-stimulated neutrophils, thereby supporting the interplay between CEACAM1 and the JAK/STAT pathway. Further studies are required to dissect the regulatory mechanism of CEACAM1 and its association with the JAK/STAT pathway, as well as to elucidate the role of this protein in RA pathogenesis. Yokoyama K et al. identified pre- and post-treatment dynamics of CEACAM1, 3, 5, and 6-positive classical monocytes in systemic scleroderma with interstitial lung disease [30]. After treatment, the number of CEACAM family-positive classical monocytes decreased, suggesting that this may help to determine the efficacy of treatment in post-treatment. Similarly, the decrease in CEACAM1 expression after treatment with JAKi in our study suggests that it may be a useful parameter for determining treatment efficacy.

Nonetheless, our study has limitations. Several transcription factors, which are identified to bind the promoter region of the CEACAM1 gene (e.g., interferon regulatory factor 1) [31], were not investigated because of the limited number of neutrophils available for study.

These CEACAM1-binding factors may act the downstream of JAK/STAT signaling pathway, thus interfering with our judgment of the results. Both GM-CSF and IL-6 induce JAK/STAT pathways after ligation of their receptors, however, IL-6 did not upregulate CEACAM1 expression in neutrophil. It is presumed that distinct signaling pathways located in the downstream of JAK/STAT also contribute the gene expression of CEACAM1. However, these pathways were not investigated in this study. Various cytokine stimulations may have increased the MFI of CEACAM1 due to differences in the structure of CEACAM1 expressed. CEACAM1 present as monomers and dimers on the cell surface, and the monomeric form is mainly involved in the recognition of the ligand and downstream signaling [32]. On the other hand, CEACAM1 may not function sufficiently in the dimer form. Moreover, microbial ligand interactions can induce CEACAM1 multimerization and activation of CEACAM1-mediated signaling pathways without de-novo induction of monomeric expression [6]. So various cytokines may induce CEACAM1 dimerization of already present on the surface monomers, increasing the overall intensity of staining with the antibodies in the flow cytometry. Kim WM et al. revealed ligands and, respectively, the cellular signals can disrupt the ability of CEACAM1 to participate in trans interactions and undergo multimerization, and this defuses CEACAM1-mediated signaling [6]. So, it is possible that antibodies of flow cytometry are simply binding to the multimerized non-functional CEACAM1. Although it is required to identify these isoforms (monomeric or dimer form) of CEACAM1, it is unknown which isoform is recognized by the antibody used in this study. In our study, STAT phosphorylation was confirmed at four-time points (0, 4, 8, and 16 h) by western blotting, but analysis over a shorter span, response to JAK inhibitors, and mRNA expression at each point should be observed and are subject to further study. This study focused on living neutrophils and did not confirm the expression of CEACAM1 in dead neutrophils. Although CEACAM1 was shown to be a potential activation marker in neutrophils, it has yet to be sufficiently proven as a survival marker, and future studies focusing on dead neutrophils are needed. CEACAM1 expression by stimulation with IFN-γ was suppressed in baricitinib. On the other hand, CEACAM1 expression was not sufficiently suppressed in other JAKis. IFN-γ receptor consists of two subunits: IFNGR1 and IFNGR2. The IFNGR1 subunit is associated with JAK1, and the IFNGR2 subunit is constitutively associated with JAK2 [33, 34]. Baricitinib is a potent and selective inhibitor of JAK1 and JAK2 [26]. The possible reason for this differential response to JAKis is that stimulation of the IFN-γ/JAK1, 2 pathway activated other CEACAM1-activating pathways, which were not fully suppressed by JAKi other than baricitinib. Further studies IFN-γ/JAK1, 2 pathway in the neutrophil are needed.

Conclusions

In conclusion, we demonstrated that a pan-JAKi, tofacitinib, marginally inhibits CEACAM1 expression in TNF-α-stimulated neutrophils. Moreover, GM-CSF markedly upregulates CEACAM1 and baricitinib efficiently inhibits GM-CSF-induced CEACAM1 expression in neutrophils. We hypothesize that the inhibition of CEACAM1 in neutrophils is one of the mechanisms through which JAKi exert their anti-inflammatory effects in inflammatory disorders.

Materials and methods

Reagents

Recombinant human GM-CSF, TNF-α, IL-6 and IFN-γ were purchased from Peprotech (Rocky Hills, NJ). Phospho-specific antibodies against JAK1 (Tyr1022/1023), JAK2 (Tyr1007/1008), JAK3 (Tyr980/981), STAT1 (Tyr701), STAT5 (Tyr701) and STAT3 (Tyr705) were purchased from Cell Signaling Technology (Beverly, MA). Anti-STAT antibodies (STAT1; Cat. No. 9172, STAT3; Rabbit. No. 8768, STAT5; Rabbit. No. 94205) were also purchased from Cell Signaling Technology (Beverly, MA). Tofacitinib, baricitinib and filgotinib were purchased from Selleck Chemicals (Huston TX, USA).

Neutrophil's isolation

Venous peripheral blood was collected from two healthy volunteers (they are men with no pre-existing medical history and no abnormalities on general physical examination, with an average age of 30 years). Written informed consent for blood donation was obtained from each individual. 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. Approximately 1×10^6 neutrophils were used for each sample. The cells were subsequently diluted in complete medium consisting of RPMI-1640. To investigate the effects of JAKi on TNF-α-mediated signaling, freshly isolated neutrophils were pretreated with JAKi for 1 h then stimulated with TNF-α and protein extracts were analyzed by Western blotting. This study conformed to the principles of the Declaration of Helsinki. Ethical approval for this study (No.2021−158) was provided by the Ethics Committee of Fukushima Medical University.

Flow cytometry

Cultured neutrophils were used for flow cytometric analysis of the surface expression of CEACAM1, CD14,

and CD16 using antihuman CEACAM1 (PE; mouse. No. 283340, diluent 2:100; R&D Systems, Minneapolis, MN, USA), antihuman CD14 (FITC; mouse. No.301804, diluent 2:100; Biolegend), and anti-human CD16 antibodies (PerCP; mouse. No. 302029, diluent 2:100; Biolegend), respectively. Dead cells were excluded using LIVE/DEAD™ Fixable Dead Cell Stain Kits. CEACAM1 expressions on the surface of CD14(−) CD16(+) neutrophil populations were analyzed (Supplemental Fig. 1). Anti–CEACAM1–specific mouse monoclonal antibody (clone number 283340) was shown to be non-cross-reactive with other CEACAM families as described previously [10]. Flow cytometry was performed on a FACS–Canto II (BD Biosciences, Franklin Lakes, NJ, USA) and data were analyzed using FlowJo software (BD Biosciences).

Cell lysis and western blotting

Neutrophils were stimulated with TNF-α or 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 min 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 JAK, phospho-STATs, and STATs. Western blots were visualized using the enhanced chemiluminescence system (Amersham, Little Chalfont, UK). Densitometry was done using the automated digitizing software (Image J, NIH, Bethesda, USA). All phosphorylation levels were normalized to the protein expression of β-actin.

Statistical analysis

Between-group differences were examined for statistical significance using Student's t-test. P-values of <0.05 were considered significant. Bonferroni correction was performed for multiple comparisons. The Kruskal-Wallis test was performed to compare two or more samples.

Abbreviations

CD	Cluster of differentiation
CEACAM1	Carcinoembryonic-antigen-related cell-adhesion molecule 1
FSC	Forward scatter
GM-CSF	Granulocyte–macrophage colony-stimulating factor
IFN	Interferon
IL	Interleukin
JAKi	Janus kinase inhibitors
MFI	Mean fluorescence intensity
RA	Rheumatoid arthritis
SD	Standard deviation
SSC	Side scatter
STAT	Signal transducer and activator of transcription
TIM-3	T cell immunoglobulin and mucin domain-3
TNF-α	Tumor necrosis factor-alpha

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Author contributions

HM, RS, YF, KS, YS, SY, JT, NM, TA, SS, ES, KM carried out the molecular biochemical studies, participated in the sequence alignment and drafted the manuscript. HM, KM participated in the sequence alignment and drafted the manuscript. HM participated in the design of the study, performed the statistical analysis. MO, TM contributed as supervisors in this study. All authors discussed the results and commented on the manuscript.

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Data availability

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

Declarations

Ethical approval and consent to participate

This study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of Fukushima Medical University (protocol code No. 2021 – 158). Written informed consent for blood donation was obtained from each individual who agreed to participate.

Consent for publication

Not applicable.

Competing interests

KM has received research grants from Chugai, Pfizer, AbbVie, and Eli Lilly. The rest of the authors declare that they have no competing interests.

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