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Research Communication

Anti-inflammatory effects of the petasin phyto drug Ze339 are mediated by inhibition of the STAT pathway

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Ze339, an herbal extract from *Petasites hybridus* leaves is effective in treatment of allergic rhinitis by inhibition of a local production of IL-8 and eicosanoid LTB_4 in allergen-challenged patients. However, the mechanism of action and anti-inflammatory potential in virally induced exacerbation of the upper airways is unknown. This study investigates the anti-inflammatory mechanisms of Ze339 on primary human nasal epithelial cells (HNECs) upon viral, bacterial and pro-inflammatory triggers. To investigate the influence of viral and bacterial infections on the airways, HNECs were stimulated with viral mimics, bacterial toll-like-receptor (TLR)-ligands or cytokines, in presence or absence of Ze339. The study uncovers Ze339 modulated changes in pro-inflammatory mediators and decreased neutrophil chemotaxis as well as a reduction of the nuclear translocation and phosphorylation of STAT molecules. Taken together, this study suggests that phyto drug Ze339 specifically targets STAT-signalling pathways in HNECs and has high potential as a broad anti-inflammatory drug that exceeds current indication. BioFactors, 43(3):388–399, 2017

Keywords: *human nasal epithelial cells; microbial patterns; petasin; viral inflammation; cytokines; STAT signalling*

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1. Introduction

Ze339 is an extract obtained from leaves of *Petasites hybridus,* ((L.) Gaertn., B. Mey. et Scherb.), a plant that has been exploited for the treatment of gastrointestinal colics, spasms of the urogenital tract, asthma, cough, and dysmenorrhea for more than 2000 years [1]. In a recent placebo-controlled double-blinded randomized clinical trial, Ze339 treatment after allergen challenge led to faster recovery from nasal obstruction and decreased the local production of IL-8 and LTB_4 as measured in nasal lining fluid of allergic patients [2]. Ze339 is currently approved only for the treatment of allergic rhinitis in Switzerland and other countries.

Since Ze339 is a complex herbal extract composed of various compounds, among them three different petasins, we tested a mix of the petasin isoforms (petasin mix; PM) in Ze339-matched concentrations. It was previously suggested that petasins decrease intracellular Ca^{2+} transients and inhibit the activation of 5-lipoxygenase in neutrophils, eosinophils, and basophils, thereby limiting their leukotriene B_4 synthesis [2–4].

Notably, the pro-inflammatory actions of IL-8 and LTB_4 are also known to be relevant in acute immune responses to microbial pathogens and environmental stressors [5–7].

Nasal epithelial cells express an array of pattern recognition receptors [8] and sense multiple pathogen-associated microbial patterns (PAMPs) and metabolites [9,10]. Thereby, they contribute to the initiation and control of innate immune responses in the upper airways [11]. These microbial interactions are also relevant for epithelial cells which respond by the release of growth factors and pro-inflammatory mediators leading to inflammatory cell recruitment and modulation of dendritic cell-primed T helper cell responses [12]. In the context of inflammation, immune cell-derived cytokines, such as IL-4, IFN-γ, and IL-6 act on activated epithelia via their respective receptors, providing a feedback mechanism for the adjustment of epithelial immune responses [13,14].

The downstream signalling elicited by these type-I and -II cytokine receptors is mediated by Janus kinase (JAK) and signal transducer and activators of transcription (STAT) molecules [15]. Interaction of cytokines with their respective receptors activates JAK, which in turn undergoes autophosphorylation enabling STAT recruitment. Subsequently, STATs undergo JAK-mediated tyrosine phosphorylation, leading to hetero- or homodimerization, nuclear translocation, and interaction with target promoter elements. This interaction in turn triggers the expression of pro-inflammatory cytokines and chemokines [16]. In the context of pre-existing inflammation, cytokine signalling drives innate and adaptive immune responses against microbes and allergens [15,17,18].

In this study, we investigated the immune modulatory mechanisms of Ze339 on human nasal epithelial cells in different inflammatory settings, such as viral, bacterial, and cytokine stimulation. Additionally, the effect of Ze339 on cytokine-induced phosphorylation and intracellular localization of STAT1, STAT3, and STAT6 was assessed. This study reveals the potential of Ze339 for the treatment of allergic airway disease as well as non-allergic rhinitis and sinusitis.

2. Materials and Methods

2.1. Cell culture

Primary human nasal epithelial cells were isolated from inferior turbinates of patients (median age 31 years), who underwent surgical turbinoplasty. Written informed consent was obtained from all participants. Single cell suspensions were generated from the biopsies and seeded into tissue culture plates in supplemented Airway Epithelial Cell Growth Medium (Promocell, Heidelberg, Germany) plus antibiotics/antimycotics. Cells from passage 0-2 were used.

2.2. Ethics statement

The ethical committee of the Technical University of Munich approved the study, and volunteers were enrolled after written informed consent.

2.3. Nasal epithelial cell stimulation

Cells were cultured for 24 h in basal medium or stimulated with 10 μg/mL Poly(I:C), 4 μg/mL Poly(I:C)-LyoVec, 10 μg/mL R-848, 1 μM CpG oligonucleotide 5′-tcgtcgttttgtcgttttgtcgtt-3′, 5 μg/mL flagellin from *Pseudomonas aeruginosa*, 200 ng/mL synthetic triacylated lipoprotein Pam3CSK4 (Invivogen, San Diego), 15 ng/mL IFN-γ, 50 ng/mL Interleukin (IL)−4, 800 U/mL IL-6 (Promocell) with or without addition of Ze339 (3 μg/mL). The subcritical carbon dioxide (CO_2) extract of *Petasites hybridus* leaves (Ze339) and its isolated active compounds, petasin, neopetasin, and isopetasin, were provided by Max Zeller Söhne AG, Romanshorn, Switzerland.

2.4. Lactate dehydrogenase assay

Cells were cultured for 24 h in basal medium or stimulated with the indicated concentrations of Ze339 in the absence or presence of PolyI:C (10 μg/mL). To assess cytotoxicity of the stimulants, lactate dehydrogenase (LDH) activity was quantified in supernatants using the Cytotoxicity Detection Kit (Roche, Mannheim, Germany). Cells lysed with Triton X-100 (Sigma, Taufkirchen, Germany) were used as positive control for dead cells.

2.5. Neutrophil migration assays

Neutrophil migration assays were performed in 96-well format using ChemoTX transwell plates (Neuro Probe, Inc., Gaithersburg). Freshly isolated human neutrophils from non-atopic donors were added to the top chambers, supernatants of pretreated HNECs were added to the bottom chambers of the transwells. After 1 h, transmigrated cells were acquired and counted by LSR II Fortessa flow cytometer (BD-Bioscience, Heidelberg, Germany).

2.6. Measurement of cytokines and chemokines

Levels of IL-8 and CCL-5 in HNEC supernatants were measured by ELISA (BD and R&D Systems, Abingdon, U.K.). Levels

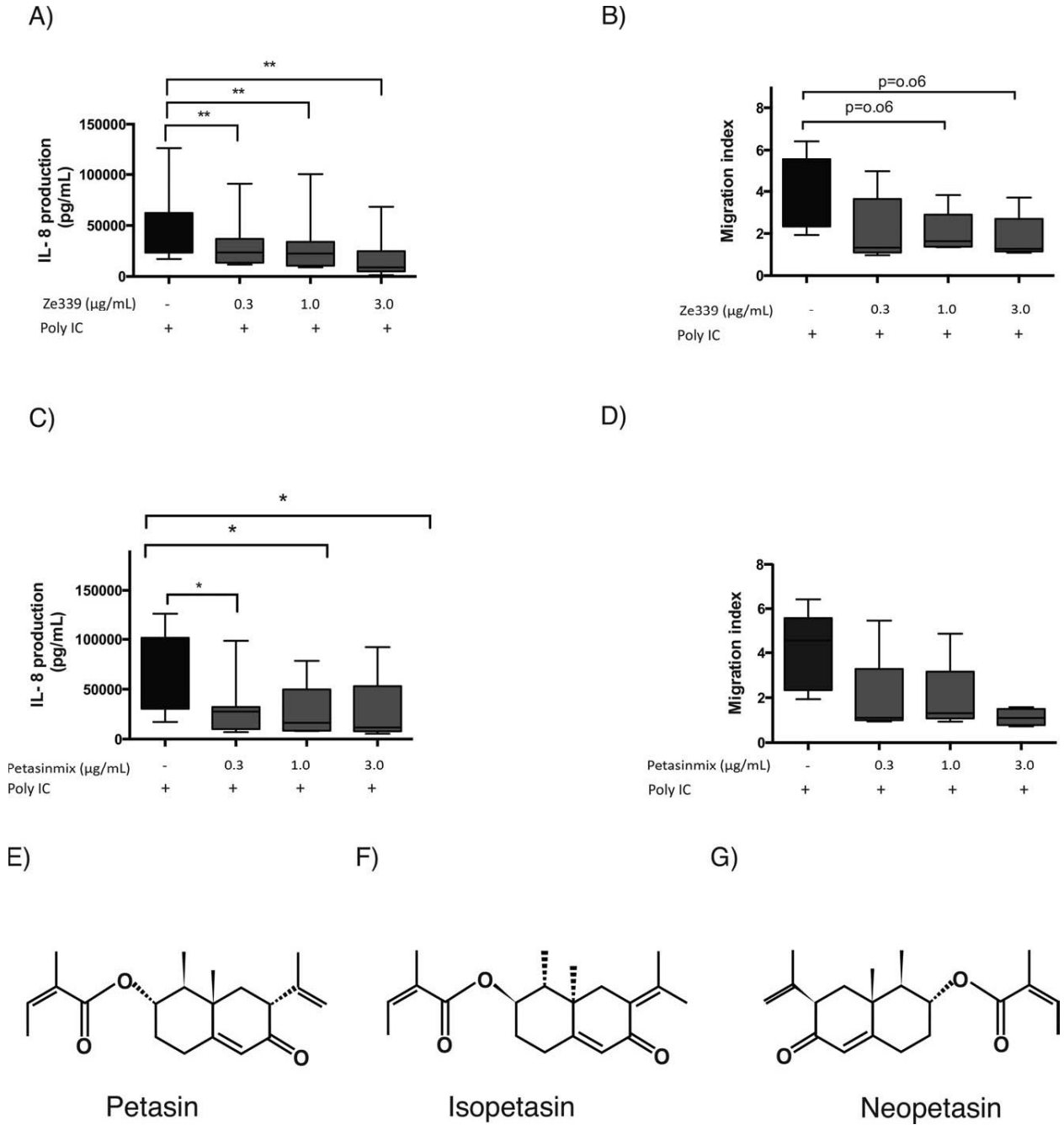

FIG 1

Ze339 and Petasins decrease the PolyIC induced IL-8 expression and neutrophil chemotaxis. HNECs were stimulated with PolyIC in the absence or presence of indicated concentrations of Ze339 or an isomeric mixture of petasins. After 24 h, supernatants were removed and analyzed for the presence of IL-8 by ELISA. In chemotaxis assays, neutrophil migration towards the HNEC supernatants was assessed in transwell chambers. (A) IL-8 in supernatants of HNEC ($n = 7$ *genetically independent donors) treated with medium (unstimulated), PolyIC (10 µg/mL) or Ze339 plus indicated concentrations of Ze339. (B) Neutrophil migration toward HNEC supernatants (*$n = 5$ *genetically independent donors) treated with medium, PolyIC or PolyIC plus indicated concentrations of Ze339. (C) IL-8 in supernatants of HNEC treated with medium, PolyIC or PolyIC plus indicated concentrations of petasin-mix (*$n = 7$ *genetically independent donors). (D) Neutrophil migration towards HNEC supernatants treated with medium, PolyIC or PolyIC plus indicated concentrations of petasin-mix (*$n = 5$ *genetically independent donors). *:* $P < 0.05$*; **:* $P < 0.01$*, Wilcoxon test. Chemical structures are shown for Petasin isoforms (E) Petasin, (F) Isopetasin, and (G) Neopetasin.*

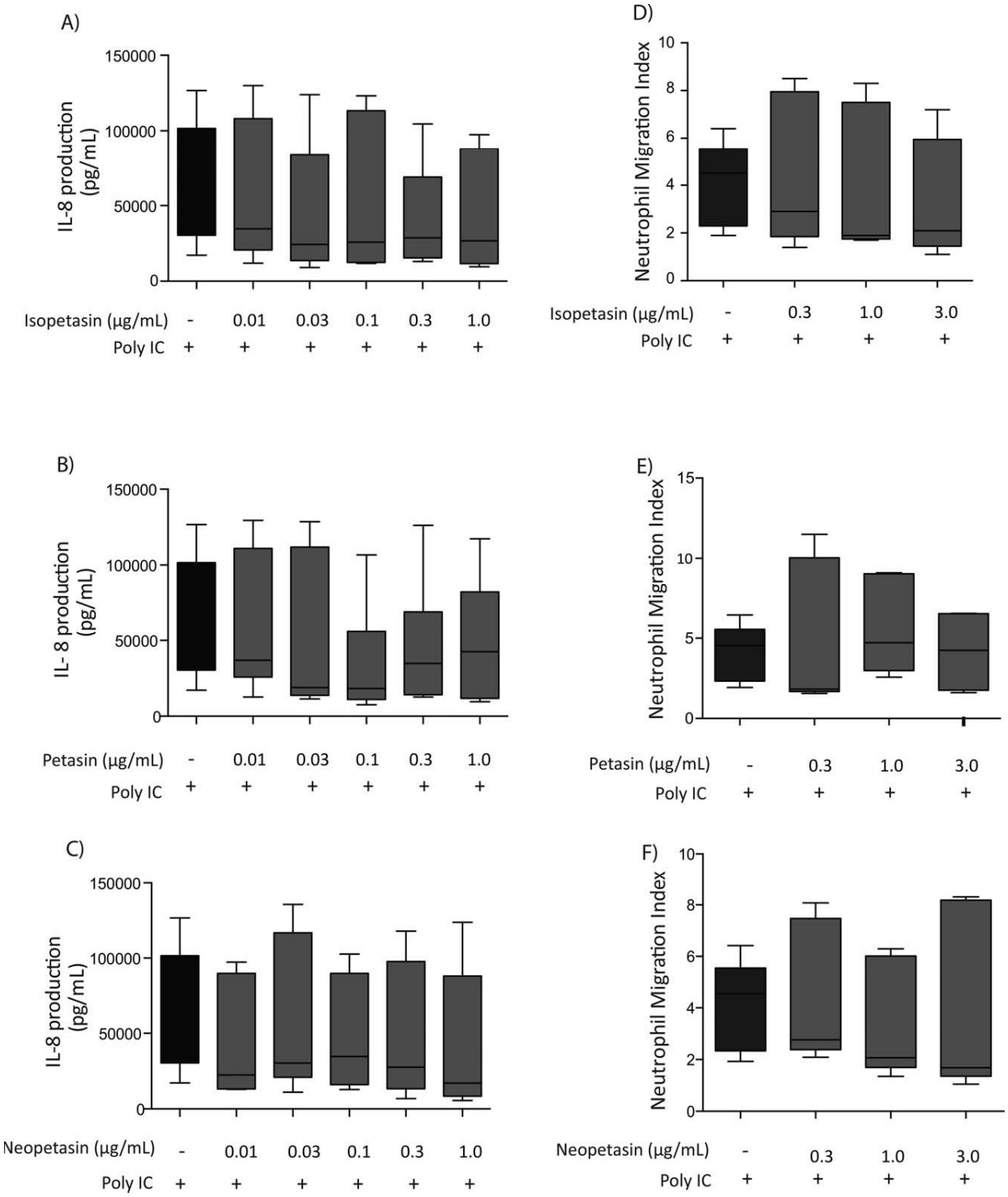

FIG 2

Anti-inflammatory potential of Ze339 is not mediated by the single petasin isoforms. HNECs were stimulated with PolyIC in the absence or presence of indicated concentrations of petasin isoforms. After 24 h, supernatants were removed and analyzed for the presence of IL-8 by ELISA. In chemotaxis assays, neutrophil migration towards the HNEC supernatants was assessed in transwell chambers. IL-8 in supernatants of HNEC stimulated with medium, PolyIC or PolyIC plus indicated concentrations of petasin (A), neopetasin (B), or isopetasin (C). Neutrophil migration towards supernatants of HNEC treated with medium, PolyIC or PolyIC plus indicated concentrations of petasin (D), neopetasin (E), or isopetasin (F). For each experiment, a number of 5 genetically independent donors was used.

of CCL-3, CCL-4, MCP-1, CXCL-10, IL-1α, IL-6, G-CSF, TNF-α were measured using a multiplex assay (MILLIPLEX-MAP). The magnetic bead assay was performed according to the manufacturers' instructions. Data were acquired on a validated and calibrated Bio-Plex-200 system and analyzed with Bio-Plex Manager 6.0 software (Bio-Rad).

2.7. Immunoblotting

HNECs were stimulated for 30 min (STAT1 and STAT3) and 60 min (STAT6) with medium, 800 U/mL IL-6, 15 ng/mL IFN-γ, or 50 ng/mL IL-4 (Promocell) in the absence or presence of 3 μM Ze339. As PEG was used for solubilizing Ze339, it was added to all wells at a dilution of 1:10,000. Total extracts and subcellular fractions were prepared from cells using Cell Fractionation Kit (Cell Signaling Technology, Danvers). Total protein extracts were subjected to SDS-PAGE under reducing conditions and transferred to a PVDF membrane by Western Blotting. To detect phospho-STATs and housekeeping proteins, the membrane was divided into two parts: the upper part was incubated with monoclonal anti-phospho-tyrosine-STAT3, polyclonal anti-phospho-tyrosine-STAT6 and monoclonal anti-phospho-tyrosine-STAT1 (Cell Signaling Technology; dilution 1:1000), the lower part was incubated with anti-β-actin monoclonal antibody (Santa Cruz Biotechnology, Dallas) or polyclonal anti-Histone H3 (Cell Signaling Technology). HRP-conjugated secondary antibodies were used (Santa Cruz Biotechnology, Dallas). Signals were quantitated with the ChemoStar™ software (Intas, Goettingen, Germany). The expression of the STATs was reported as relative to β-actin or histone signal.

2.8. Immunofluorescence

HNECs were seeded on chamber glass slides (Ibidi GmbH, Martinsried, Germany) in complete medium and grown to 80% confluence. Cells were stimulated with medium control, IFN-γ, IL-6, or IL-4 ± Ze339 for 30 min (INF-γ, IL-6) or 60 min (IL-4). Staining of total and phospho-STATs was performed using anti-phospho-tyrosine-STAT6, anti-phospho-tyrosine-STAT3, anti-phospho-tyrosine-STAT1 (Cell Signaling Technology). Secondary fluorescent dye antibodies Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 568 goat anti-rabbit IgG (Life Technology Inc.) were used. Images were acquired on a Confocal Leica SP5 microscope (Leica Microsystems, Wetzlar, Germany).

2.9. Statistics

Data are presented as mean ± SD (bar charts) or medians ± 5-95 percentiles (box plots). To detect differences between treatment groups, samples were compared by Wilcoxon test. Differences between treatment groups were considered significant if $P < 0.05$.

3. Results

3.1. Ze339 and petasin isoforms decrease the PolyIC-induced IL-8 expression and neutrophil chemotaxis

We first evaluated the effect of Ze339 on primary human nasal epithelial cells (HNECs) which were isolated from inferior turbinates of patients, who underwent surgical turbinoplasty. After seeding and culturing single cell suspensions which were generated from the biopsies we stimulated the HNECs with the TLR3 ligand PolyIC. TLR3 ligation led to an increase in IL-8 secretion as compared to medium control. Ze339 significantly reduced this PolyIC-induced IL-8 production in a dose-dependent manner (Fig. 1A). This effect was independent of atopy-status of the HNEC donor (see Supporting Information Fig. S1).

To address whether reduction of the IL-8 response translates into reduced recruitment of inflammatory cells, we performed transwell-based neutrophil migration assays. We observed enhanced neutrophil migration toward supernatants of PolyIC-stimulated HNECs as compared to supernatants of medium-treated HNECs. Supernatants of cells stimulated with a combination of PolyIC and Ze339 were less potent in attracting neutrophils than supernatants of cells treated with PolyIC only (Fig. 1B; $P = 0.06$). We tested a mix of the petasin isoforms (petasin mix; PM) in Ze339-matched concentrations which were sufficient to inhibit the PolyIC-induced IL-8 response of HNECs (Fig. 1C). In line with previous findings, HNEC supernatants stimulated with PolyIC and PM were less potent in mediating neutrophil chemotaxis than supernatants of cells stimulated with PolyIC alone (Fig. 1D).

3.2. Additive effects of three petasin isoforms mediate the anti-inflammatory potential of Ze339

To assess which of the three petasin isoforms (Figs. 1E–1G) present in Ze339 mediates the anti-inflammatory effects, we first tested the effects of single petasin isoforms. None of the single petasin isoforms mediated any inhibition of PolyIC-induced IL-8 production in HNECs (Figs. 2A–2C). Concurrently, supernatants of HNECs stimulated with PolyIC plus the single petasin isoforms mediated neutrophil chemotaxis to the same extent as supernatants stimulated with PolyIC only (Fig. 2D–2F). Ze339 and petasins, alone or in combination with PolyIC, did not mediate cytotoxicity at the concentrations tested (Supporting Information Figs. S2 and S3).

3.3. Ze339 inhibits pro-inflammatory cytokine and chemokine response to viral mimics

Based on the observation that Ze339 inhibits the IL-8 expression following PolyIC stimulation, we also investigated if this effect is also present in a setting with various other viral PAMPs such as a RIG-I/MDA-5 ligand (PolyIC-LyoVec) or a TLR7/8 ligand (R-848). Therefore, HNECs were separately treated with these viral stimulants in the absence or presence of Ze339. After 24 h, supernatants were collected and tested by specific ELISAs for a panel of pro-inflammatory cytokines and chemokines (IL-8, CCL-2/MCP-1, CCL-3/MIP-1α, CCL-4/

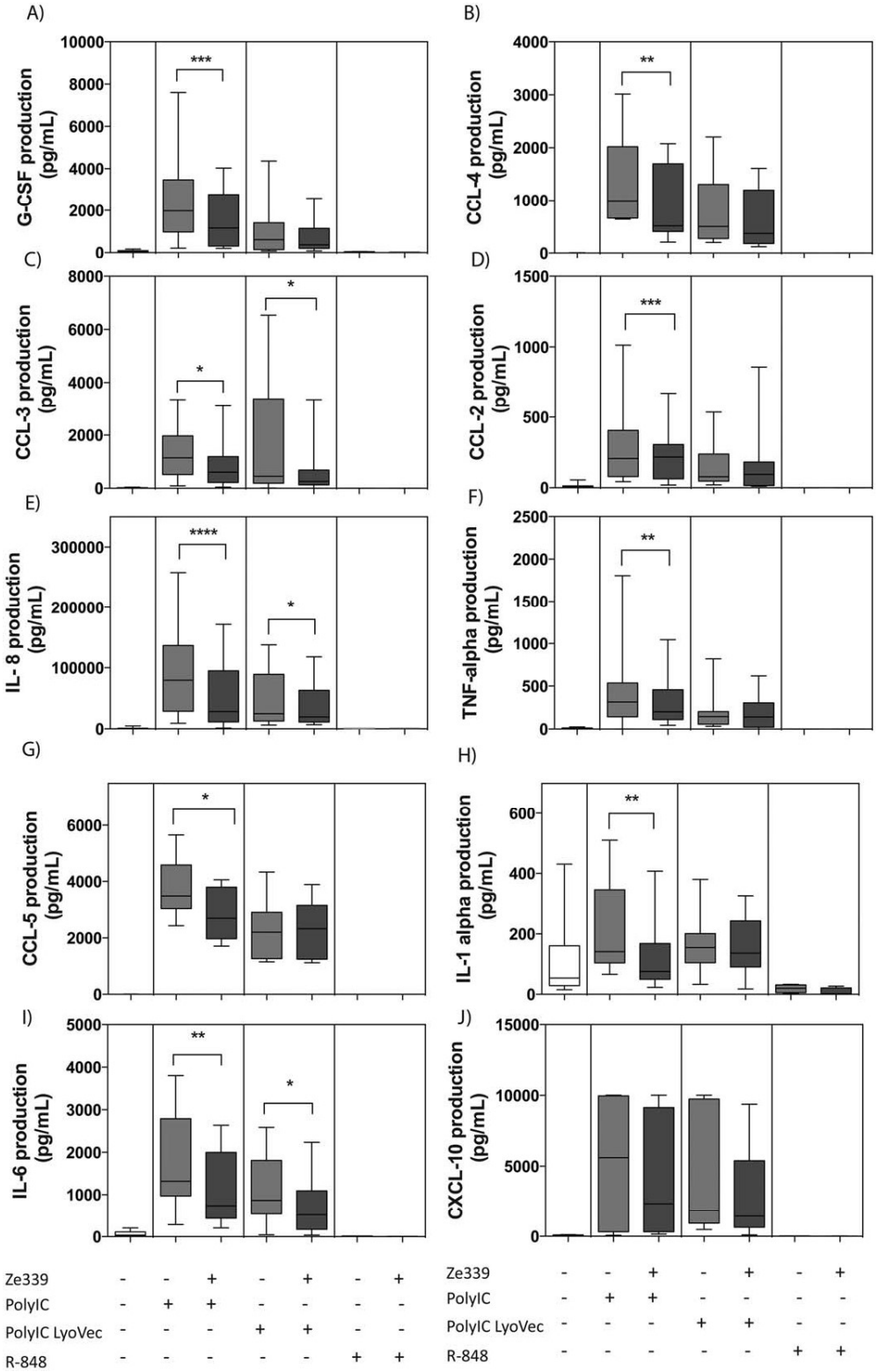

FIG 3

*Ze339 decreases the pro-inflammatory cytokine and chemokine response to viral stimuli. HNEC were cultured for 24 h with medium or with different stimulatory viral mimics PolyIC (TLR-3; 10 μg/mL), PolyIC-LyoVec (RIG-I/MDA-5; 4 μg/mL), and R-848 (TLR-7/-8; 10 μg/mL), in the absence or presence of Ze339 (3 μg/mL). After 24 h, supernatants were collected and tested for the pro-inflammatory cytokines and chemokines by Bioplex analysis: IL-8 (A), CCL-5 (B), CCL-2 (C), CCL-3 (D), CCL-4 (E), G-CSF (F), TNF-α (G), IL-6 (H), IL-1α (I), CXCL-10 (J). For each experiment, a number of 13 genetically independent donors was used; except for "medium" and "PolyIC" in (A) where a number of 34 genetically independent donors was used. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$, Wilcoxon test.*

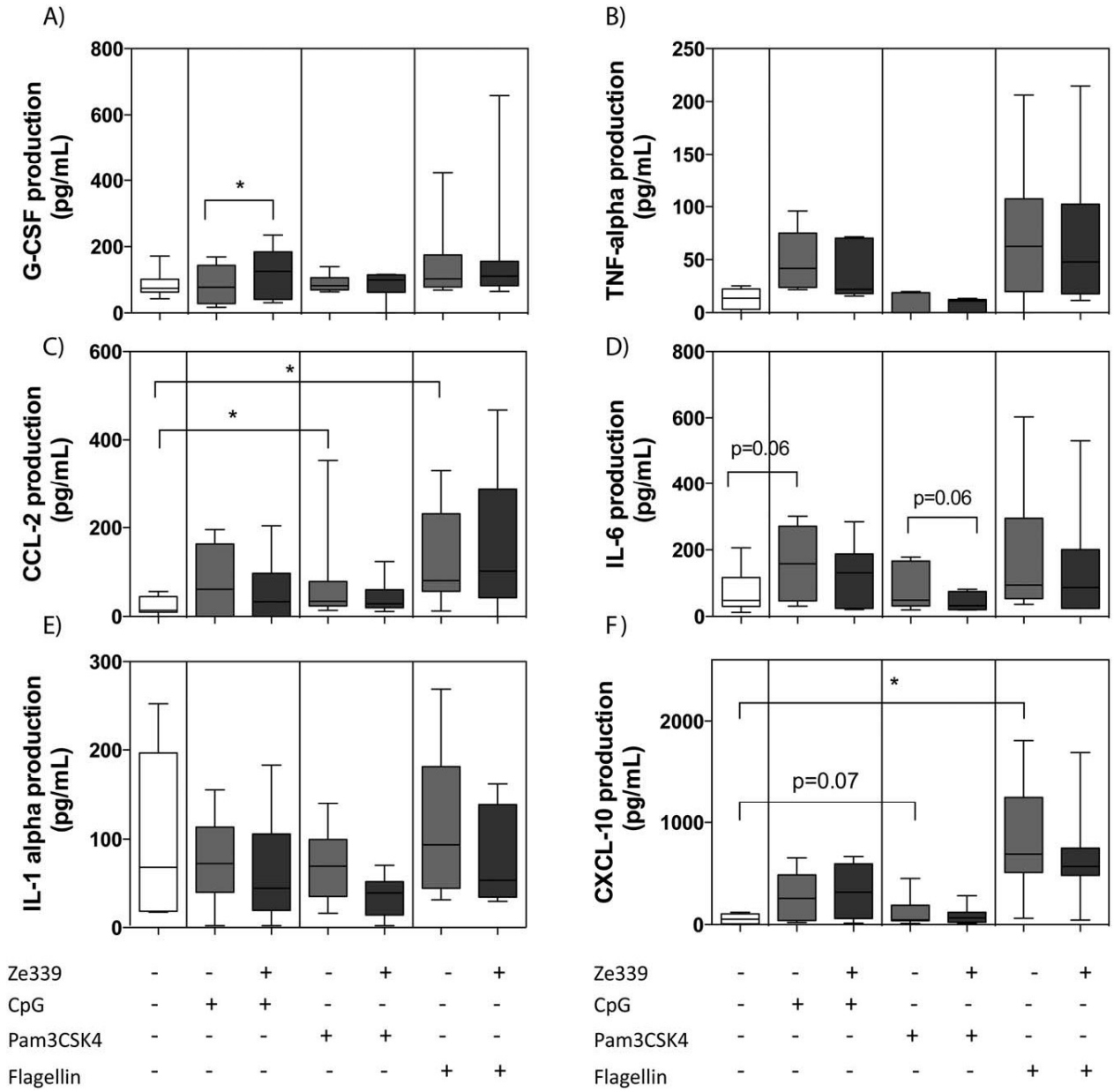

FIG 4

Ze339 does not inhibit the pro-inflammatory cytokine/chemokine response to bacterial stimuli. HNEC were cultured for 24 h with medium or with stimulatory different bacterial mimics CpG-ODN 2006 (TLR-9; 5 μg/mL), Pam3CSK4 (TLR-1/-2, TLR-2/-6; 200 ng/mL), and Pseudomonas aeruginosa *flagellin (TLR-5; 5 μg/mL), in the absence or presence of Ze339 (3 μg/mL). After 24 h, supernatants were collected and tested for the pro-inflammatory cytokines and chemokines G-CSF (A), CCL2 (B) IL-1α (C), TNF-α (D), IL-6 (E), CXCL-10 (F). *:* $P < 0.05$*, Wilcoxon test.*

MIP-1β, CCL-5/RANTES, CXCL-10/IP-10, G-CSF, TNF-α, IL-1α, IL-6). Stimulation with PolyIC and PolyIC-LyoVec led to the induction of a pro-inflammatory response in HNECs, whereas the TLR7/8 ligand R-848 did not induce any changes for G-CSF, CCL-2, CCL-3, CCL-4, CCL-5, IL-1α, IL-6, IL-8, TNF-α, or CXCL-10 (Figs. 3A–3J). Ze339 significantly reduced PolyIC-induced the response of most cytokines and chemokines except IP-10 (Fig. 3J). Ze339-mediated pro-inflammatory inhibition was stronger in PolyIC-stimulated compared to PolyIC-LyoVec-stimulated cells. Ze339 decreased only the PolyIC-LyoVec-induced production of IL-8, CCL-3, and IL-6 (Figs. 3A, 3D, 3H), whereas the production of other cytokines and chemokines remained unaffected (Figs. 3B, 3C, 3E, 3F, 3G, 3I, 3J).

3.4. Pro-inflammatory cytokine/chemokine response to bacteria is not inhibited by Ze339

To define whether Ze339 exerts an anti-inflammatory effect on HNECs exposed to bacterial PAMPs, we incubated the cells

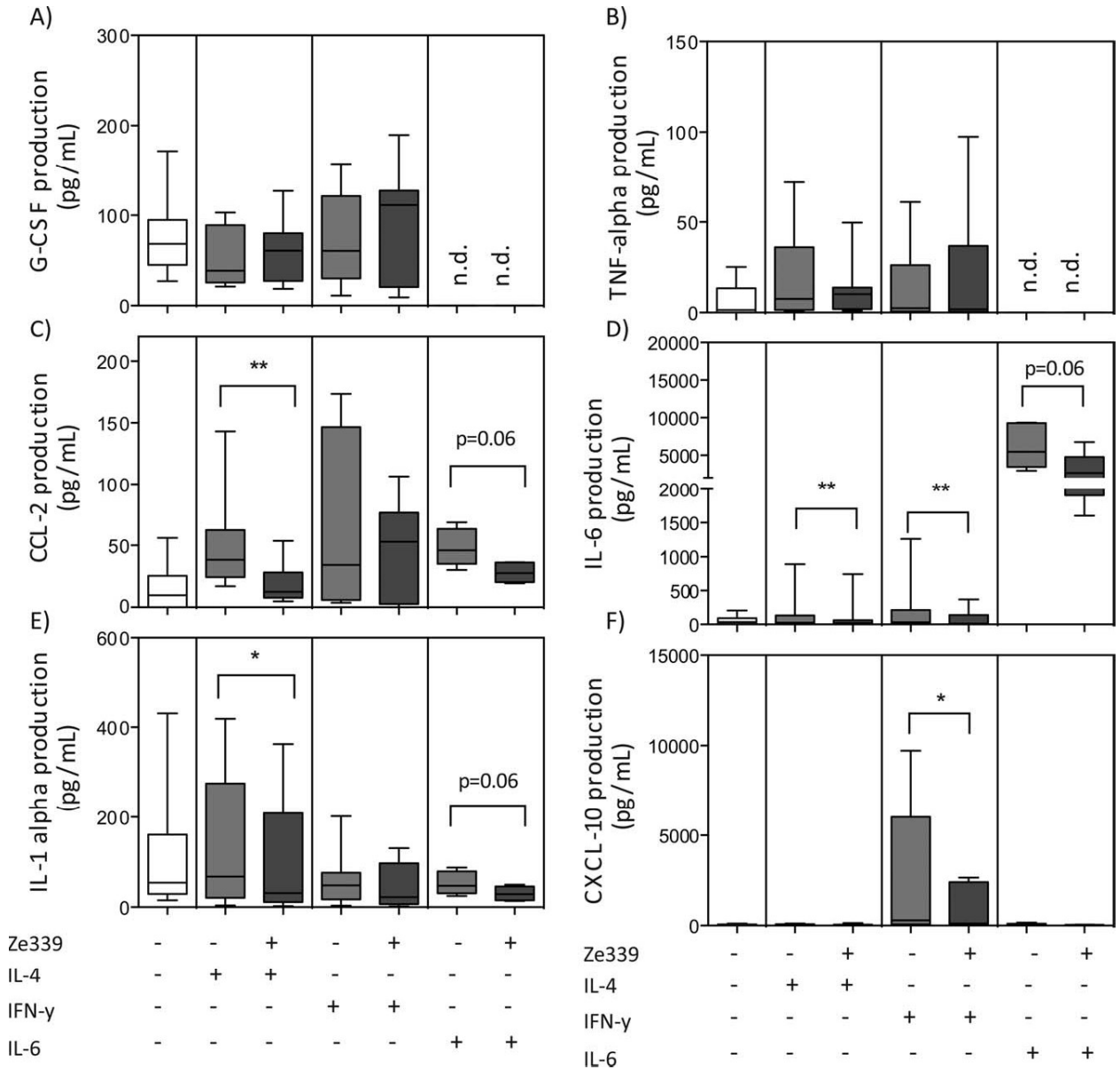

FIG 5

*Ze339 modulates cytokine-induced pro-inflammatory cytokine and chemokine responses in HNEC. HNEC were cultured for 24 h with basal medium or with stimulatory cytokines IL-4 (Th2 model; 50 ng/mL), IFN-γ (Th1 model; 15 ng/mL), and IL-6 (pro-inflammatory model; 800 U/mL), in the absence or presence of Ze339 (3 μg/mL). After 24 h, supernatants were collected and tested for the pro-inflammatory cytokines and chemokines G-CSF (A), CCL2 (B), IL-1a (C), TNF-a (D), IL-6 (E) and CXCL-10 (F). *: $P < 0.05$; **: $P < 0.01$, Wilcoxon test.*

with ligands for TLR9 (CpG ODN 2006), TLR2 (Pam3CSK4), and TLR5 (flagellin) with or without addition of Ze339. After 24 h, supernatants were collected to assess pro-inflammatory cytokines and chemokines. Stimulation of HNECs with CpG, Pam3CSK4 and flagellin resulted in increased production of CCL-2, TNF-α, IL-6, and IP-10 (Figs. 4B, 4D, 4E, 4F). G-CSF and IL-1α were not induced by the bacterial PAMPs tested. Ze339 did not mediate inhibition of pro-inflammatory mediator production in response to the bacterial stimuli (Figs. 4A–4F). In contrast, Ze339 significantly increased the CpG-induced production of G-CSF (Fig. 4A).

3.5. Modulation of IL-4-, IFN-γ-, and IL-6-induced production of pro-inflammatory mediators by Ze339

We further investigated whether the anti-inflammatory effect of Ze339 is also evident in a model of HNECs under Th1-, Th2-, or pro-inflammatory cytokine exposure. Therefore, we stimulated HNECs with IL-4 (Th2 condition), IFN-γ (Th1

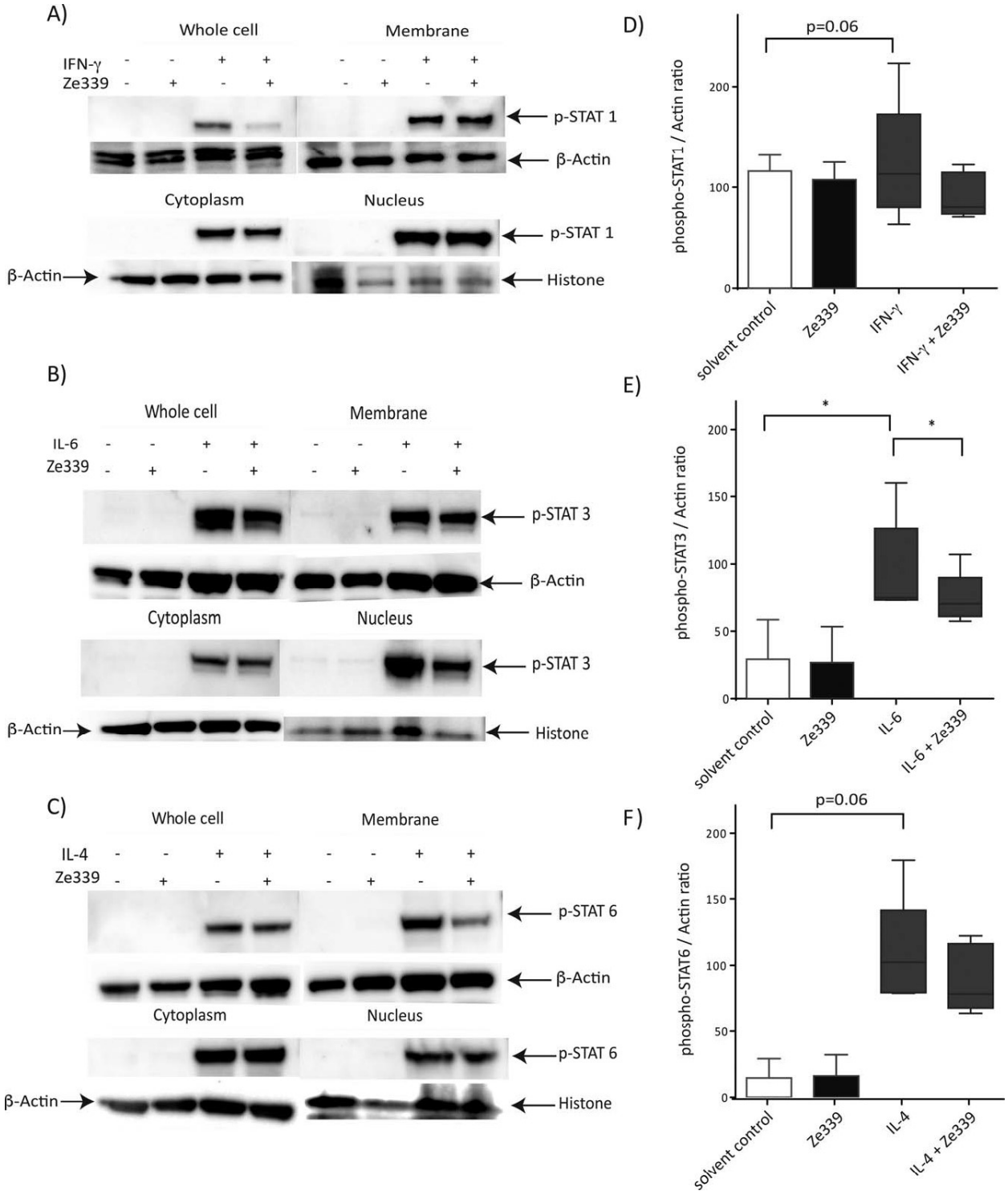

FIG 6

Ze339 reduces cytokine-induced signalling of STAT1, STAT3 and STAT6. HNEC were treated for 30 min with IFN-γ (A) and IL-6 (B) and for 60 min with IL-4 (C). Total cell lysates or subcellular fractions were subjected to reducing SDS-PAGE, and proteins were transferred on to a PVDF membrane by Western blotting. Total and tyrosine-phosphorylated forms of STATs were detected on the membrane using specific primary and HRP-conjugated secondary antibodies. Right panels: Relative expression levels of STATs in total cell lysates relative to β-Actin levels, statistics: Wilcoxon test. Representative images of n = *5 experiments are shown.*

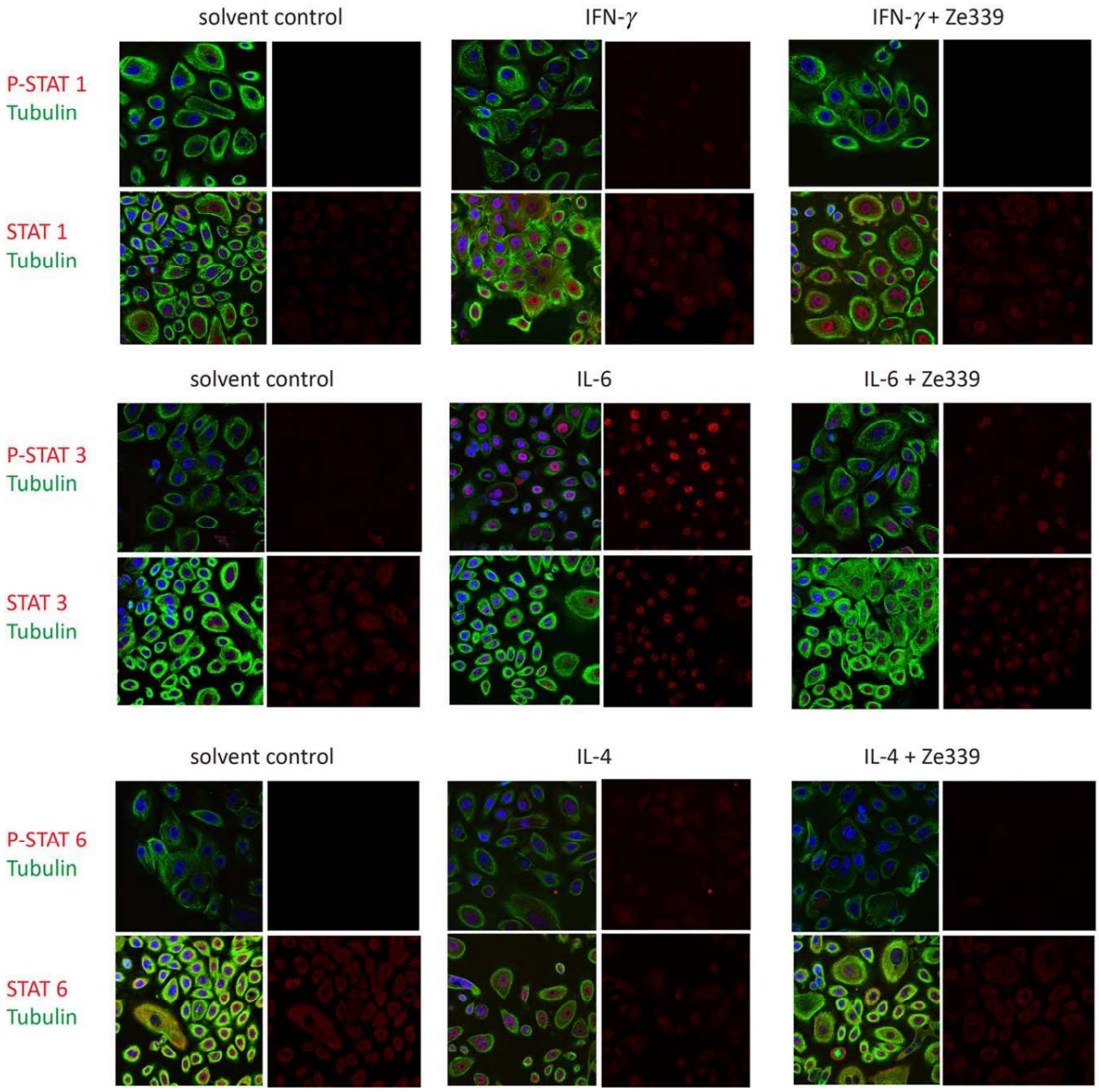

FIG 7

Ze339 inhibits cytokine-induced nuclear translocation of phospho-STATs. HNEC were grown on glass chamber slides. At 80% confluence, cells were washed, stimulated and stained with PE-conjugated antibodies against total or tyrosine-phosphorylated STATs. FITC-conjugated anti-α-tubulin and DAPI blue were used as counter-stains for cytoskeleton and nucleus, respectively. As polyethylene glycol (PEG, 0.1‰) served as solvent control for Ze339, it was added to all wells. (A) Cells incubated for 30 min with medium (unstim.), IFN-γ (15 ng/mL) or IFN-γ (15 ng/mL) + Ze339 (3 μg/mL). (B) Cells incubated for 30 min with medium (unstim.), IL-6 (800 U/mL) or IL-6 plus Ze339 (3 μg/mL). (C) Cells were stimulated for 60 min with medium (unstim.), IL-4 (50 ng/mL) or IL-4 plus Ze339 (3 μM). Red: STATs/phospho-STATs; green: α-tubulin; blue: DAPI; yellow: overlay of STATs and α-tubulin. Representative images of experiments with three genetically independent donors are shown.

condition), or IL-6 (pro-inflammatory condition) for 24 h and assessed the pro-inflammatory cytokine and chemokine response on protein levels. IL-4 induced low-level secretion of CCL-2 ($P < 0.01$) and IL-6 ($P = 0.06$), and Ze339 significantly inhibited this response (Figs. 5B and 5E). IFN-γ stimulation, in contrast, led to a significant induction of IP-10, which was decreased by Ze339 (Fig. 5F). Under IL-6 stimulation, Ze339 decreased the expression levels of CCL-2, IL-1α, and IL-6, however, the effect did not reach statistical significance (Figs. 5B, 5C, 5E).

3.6. Inhibition of cytokine-induced STAT3 and STAT6 signalling and cytokine-induced nuclear translocation of STAT molecules by Ze339

To assess whether Ze339 interferes with cytokine-induced STAT signalling, HNECs were stimulated with IFN-γ, IL-6, and IL-4 (Fig. 6). Western blots were performed of whole cell lysates and subcellular fractions. Ze339 reduced the cytokine-induced phosphorylation of STATs. STAT3 phosphorylation was significantly decreased (IL-6 vs. IL-6 + Ze339: $P = 0.05$; Fig. 6B).

To visualize the effect of Ze339 on STAT signalling, we performed immunofluorescence microscopy of HNECs stimulated with the cytokines IL-4, IFN-γ, and IL-6. Stimulation of HNECs with all three cytokines led to the induction of several STAT molecules and to nuclear translocation of their phosphorylated forms (Fig. 7). In IL-6-stimulated cells, Ze339 led to a reduction of total STAT3 protein expression as well as to a decrease of nuclear translocation of phospho-STAT3. In IL-4-stimulated cells, Ze339 inhibited nuclear translocation of both, total and phospho-STAT6. Likewise, Ze339 reduced the IFN-γ-induced nuclear translocation of phospho-STAT1 (Fig. 7).

4. Discussion

The efficacy and safety of Ze339 for the treatment of seasonal allergic rhinitis has been demonstrated in several clinical trials [19,20]. When compared with desloratadine and placebo, a recently published placebo-controlled double-blinded randomized clinical trial showed that the phyto drug Ze339 treatment led to faster recovery from nasal obstruction and decreased the local production of IL-8 and LTB_4 [2]. In this study, we show that Ze339 decreases the release of IL-8 from human nasal epithelial cells and impedes neutrophil chemotaxis toward cells that encountered viral RNA. The inhibitory effect on IL-8 and neutrophil chemotaxis was also observed when cells were treated with a mixture of petasin isoforms. Therefore, we could identify the petasins as the active components of the Ze339 extract, while single petasin isoform treatment did not exhibit the full effect on cytokines compared when all three petasin isoforms acted synergistically. This additive effect suggests a simultaneous petasin recognition by several receptors or a hetero-multimeric one. Of note, several anti-inflammatory sesquiterpenes are ligands for the nuclear receptors peroxisome proliferator-activated receptor (PPAR)-γ [21], retinoid x receptor (RXR)-α [22], or farnesoid X receptor (FXR) [23], classically acting as heterodimers in anti-inflammatory signalling.

Petasins were previously shown to act on human granulocytes, inhibiting cysteinyl-leukotriene and leukotriene B_4 (LTB_4) synthesis and intracellular calcium transient induction by platelet activating factor (PAF) and C5a [4]. These effects may be related to the reduction of local LTB_4 release into nasal lining fluids of allergic patients under treatment with Ze339 as observed before [2]. The same human allergen challenge study also revealed reduced local nasal IL-8 levels in Ze339 treated individuals suggesting an inhibition of epithelial-derived IL-8.

The inhibitory effect of Ze339 on IL-8 production may explain the finding that Ze339 treatment ex-vivo impedes neutrophil migration towards PolyIC-stimulated HNEC. Additionally, in this setting Ze339 decreased the induced secretion of further pro-inflammatory chemokines, that is, G-CSF, CCL-2, and CCL-3, all of which are implicated in neutrophil chemotaxis [24,25]. The inhibitory effects of Ze339 on neutrophil chemotaxis render it a promising drug candidate in multiple conditions involving neutrophilic inflammatory processes [26–28].

In addition, Ze339 led to a clear reduction of the pro-inflammatory cytokine and chemokine response to TLR3 stimulation, whereas the inhibitory effect on cytosolic RIG-I- and MDA-5-ligand-induced responses was less pronounced. This finding suggests that Ze339 might act on the endosomal pathway or might be taken up by endosomes. Being a CO_2 extract of a specific chemovariety of butterbur leaves, Ze339 is mainly composed of hydrophobic compounds, among them terpenoids, lipids and glycolipids [4]. Therefore, it could be incorporated into cellular membranes, which might decrease its bioavailability in the cytosol.

In contrast to the inhibiting effect in viral models, Ze339 failed to attenuate bacterial stimulation by PAMPs. Selected bacterial ligands were chosen on the basis of data on TLR expression profiles of HNECs [29,30], however, the TLR4 ligand LPS did not induce any of the cytokines/chemokines tested (data not shown). A previous study demonstrated presence of TLR4 mRNA expression on HNECs [29], while our results failed to demonstrate any functionally active TLR4 in HNECs. The finding that Ze339 did not inhibit bacterial TLR ligand-induced responses highlights Ze339 as a promising candidate compound for specific treatment of acute viral rather than bacterial infection.

Under stimulation with the cytokines IL-4, IL-6, and IFN-γ, Ze339 decreased the release of pro-inflammatory cytokines and chemokines from HNECs. The receptors for IL-4, IL-6, and IFN-γ act via STATs, and phosphorylation of STATs is a prerequisite for nuclear translocation and activity. Synthetic JAK/STAT inhibitors such as Tofacitinib were developed as immunosuppressants and have been licensed for treatment of autoimmune diseases. STAT signalling pathways are key regulators of various allergic and non-allergic inflammatory diseases [17,31]. The finding that Ze339 inhibits cytokine-induced nuclear translocation of all analyzed phospho-STATs suggests that the compound interferes with a common upstream signalling step. Cytokine receptor signalling via STATs requires the assembly of a multi-protein complex in lipid rafts. Hydrophobic substances within Ze339, including petasins, could be retained in the plasma membrane or selectively enriched in lipid rafts where they might interfere with assembly of the JAK/STAT signalling complex.

Ze339 exerted the most pronounced effects on the IL-6-induced nuclear translocation of phospho-STAT3 in HNECs. In naïve T lymphocytes, IL-6 receptor signalling is critical for the induction of T helper cell (Th) 17 differentiation. Current

studies are, therefore, focusing on the impact of Ze339 on the priming of naïve T cells in the process of Th17 differentiation and Th17-mediated immune reactions.

Taken together, our study on human nasal epithelial cells implies that Ze339 has broader mechanism of action as previously anticipated and might, therefore, be applied for treatment of acute viral infections in the airways. In addition to its established anti-inflammatory characteristics in the treatment of seasonal allergic rhinitis and the anti-chemotactic quality in a viral stimulation model, Ze339 appears to exert broad anti-cytokine effects by interfering with nuclear translocation of STAT. Therefore, Ze339 constitutes a candidate compound in the treatment of immune deviations associated with continuous cytokine-induced inflammation and of inflammatory diseases such as sinusitis.

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Disclosure

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