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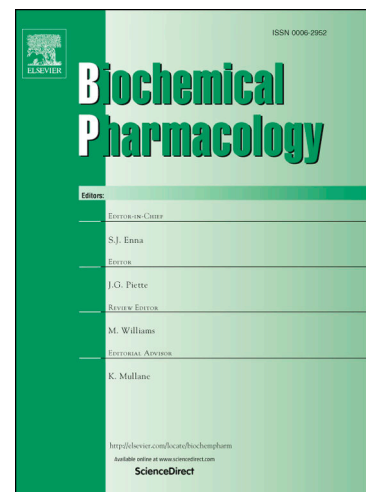
The JAK1/2 inhibitor baricitinib suppresses eosinophil effector function and restricts allergen-induced airway eosinophilia

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The JAK1/2 inhibitor baricitinib suppresses eosinophil effector function and restricts allergen-induced airway eosinophilia.

Running title: Baricitinib suppresses eosinophil effector function.

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Abstract

Background. Eosinophilic asthma is increasingly recognized as one of the most severe and difficult-to-treat asthma subtypes. The JAK/STAT pathway is the principal signaling mechanism for a variety of cytokines and growth factors involved in asthma. However, the direct effect of JAK inhibitors on eosinophil effector function has not been addressed thus far.

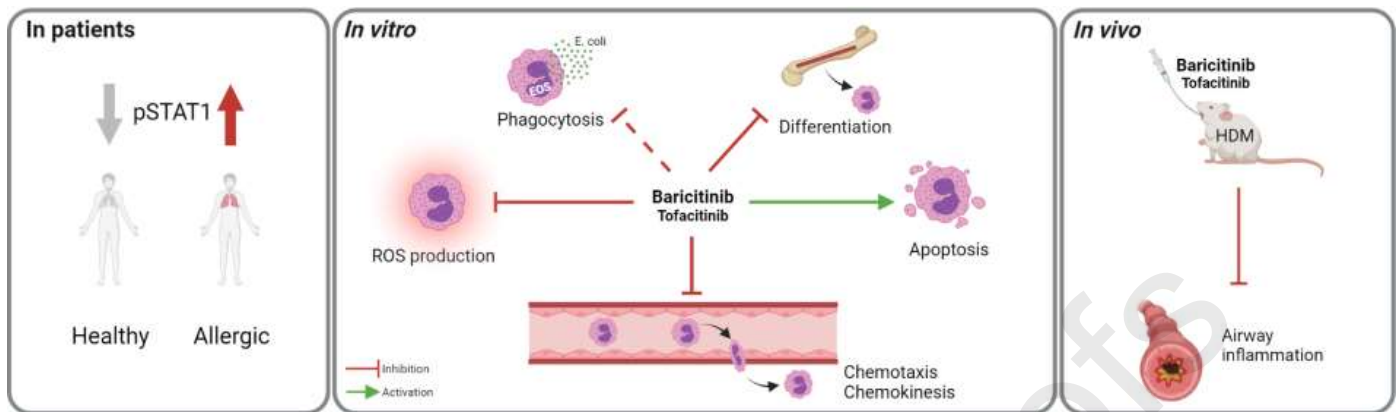
Objective. Here we compared the effects of the JAK1/2 inhibitor baricitinib and the JAK3 inhibitor tofacitinib on eosinophil effector function *in vitro* and *in vivo*.

Methods. Differentiation, migratory responsiveness, respiratory burst, phagocytosis and apoptosis of human peripheral blood eosinophils were assessed *in vitro*. *In vivo* effects were investigated in a mouse model of acute house dust mite-induced airway inflammation in BALB/c mice.

Results. Baricitinib more potently induced apoptosis and inhibited eosinophil chemotaxis and respiratory burst, while baricitinib and tofacitinib similarly affected eosinophil differentiation and phagocytosis. Of the JAK inhibitors, oral application of baricitinib more potently prevented lung eosinophilia in mice following allergen challenge. However, both JAK inhibitors neither affected airway resistance nor compliance.

Conclusion. Our data suggest that the JAK1/2 inhibitor baricitinib is even more potent than the JAK3 inhibitor tofacitinib in suppressing eosinophil effector function. Thus, targeting the JAK1/2 pathway represents a promising therapeutic strategy for eosinophilic inflammation as observed in severe eosinophilic asthma.

Graphical Abstract



Key words: JAK inhibitor; Allergy; Eosinophils; House dust mite-induced airway inflammation.

Abbreviations: BAL, bronchoalveolar lavage; CD, cluster of differentiation; EDTA, ethylenediamine tetraacetic acid; EPO, eosinophil peroxidase; FLT3L, FMS-like tyrosine kinase 3 ligand; HDM, house dust mite; Ig, immunoglobulin; IL, interleukin; JAK, janus kinase; OVA, ovalbumin; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PG, prostaglandin; PMNL, polymorphonuclear leukocytes; ROS, reactive oxygen species; SCF, stem cell factor; STAT, signal transducer and activator of transcription; TSLP, thymic stromal lymphopoietin; TYK2, tyrosine kinase 2;

1. Introduction

Asthma is a long-term inflammatory disease of the airways which can vary in severity and persistence. Severe asthma is characterized as a disease that requires treatment with high-dose inhaled corticosteroids plus a second controller or systemic corticosteroids [1]. Severe asthma is often associated with airway and blood eosinophilia that is driven by a distinct pathophysiological process involving the abnormal production of type 2 cytokines from Th2 and type 2 innate lymphoid cells [2, 3]. Sputum eosinophilia is found in slightly less than half of all patients with asthma and both blood and sputum eosinophilia are associated with more severe disease, worse control, and worse prognosis [4]. In patients who died from asthma, significantly more eosinophils were found in large and small airways, as compared to biopsies from patients with milder exacerbations [5]. The prevalence of severe asthma is estimated to be around 5% to 10% of the total asthmatic population [6-8], but is difficult to determine as the lack of asthma control depends on a variety of factors, including the patient's adherence and inhalation technique, persistent trigger exposure or untreated co-morbidities. Despite major progresses such as the implementation of anti-IL-5 antibodies, half the patients with severe eosinophilic asthma continue to experience exacerbations and suboptimal control [9-11]. Thus, there remain significant unmet needs in treating patients with severe eosinophilic asthma.

The Janus kinase (JAK) family of cytoplasmic tyrosine kinases comprises four isoforms, JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2). Different interactions of JAK isoforms are used by many cytokine-receptor complexes, transducing cytokine-mediated signals through the association and activation of STAT (signal transducer and activator of transcription) proteins [12]. Seven different STAT members (STAT1-4, STAT5A, STAT5B and STAT6) have been described so far [13, 14]. The most validated cytokines in Th2-driven asthma such as interleukin (IL)-4, IL-5, IL-13 (reviewed in [15]) and TSLP [16], all signal through JAK1 and/or JAK2,

hence targeting these JAK isoforms might be a beneficial treatment approach especially for patients with severe eosinophilic asthma.

Several JAK inhibitors are under development for a variety of inflammatory disease. As different JAK inhibitors have different specificities for JAK isoforms or TYK2, there is ongoing discussion regarding which profile of JAK inhibition would be optimal for which specific disease. Tofacitinib is a JAK3 inhibitor with less affinity for JAK1 and JAK2, which has been approved for the treatment of rheumatoid and psoriatic arthritis as well as moderate to severe ulcerative colitis. Other indications such as Crohn's disease [17] are under clinical investigation. The JAK1/2 inhibitor baricitinib is approved as a second-line therapy for moderate to severe active rheumatoid arthritis in adults, either alone or in combination with methotrexate [18] while other indications such as lupus erythematosus and atopic dermatitis are still being investigated in clinical studies [19, 20].

During the last decade, promising experimental data from JAK inhibitors have also been published in asthma research. For instance, Southworth et al. demonstrated that tofacitinib suppresses T-cell receptor (TCR) stimulated interferon- γ , IL-13 and IL-17 production in BAL cells from asthmatic and healthy donors *in vitro* [21]. Another study showed that mice which were dosed with tofacitinib during the period of ovalbumin (OVA)-sensitization and boosting, exhibit marked reductions in eosinophil counts and IL-13 and CCL11 levels in BAL fluid [22]. More recently, LAS194046, a novel pan-JAK inhibitor, has been shown to reduce allergen-induced airway inflammation, late asthmatic response and pSTATs activation in rats when applied intratracheally [23]. Furthermore, the non-selective inhaled JAK1 inhibitor iJAK-381 suppressed ovalbumin-induced lung inflammation in both murine and guinea pig asthma models and improved allergen-induced airway hyperresponsiveness in mice [24].

The airway epithelium represents the first line of our host defense against inhaled allergens, pathogens, and particles and is actively involved in the initiation, perpetuation, and

chronification of asthma [25, 26]. Of note, previous studies revealed an inhibitory effect of baricitinib, tofacitinib, and gandotinib on the production of cytokines such as CCL26 in airway epithelial cells [27, 28]. Moreover, baricitinib and ruxolitinib have been shown to mitigate interferon-induced expression of the *ACE2* gene in the airway epithelium, when used therapeutically in COVID-19 patients [29].

However, the direct effects of JAK inhibitors on specific eosinophil effector functions have not been addressed so far. Thus, in this study we aimed to advance the discussion about the relevance of JAK inhibitors for the treatment of eosinophilic diseases. We therefore compared the efficacy of the approved oral JAK inhibitors baricitinib (JAK1/2) and tofacitinib (JAK3) at inhibiting eosinophil effector function *in vitro* and in a mouse model of allergic eosinophilic lung inflammation *in vivo*. Here, we show for the first time that baricitinib is more potent than tofacitinib in inhibiting eosinophil effector function. Thus, targeting the JAK1/2 pathway represent a promising therapeutic strategy for the treatment of eosinophilic inflammation.

2. Materials and Methods

2.1. Reagents

All reagents were from Sigma-Aldrich (St. Louis, USA), unless specified. Assay buffer was made from Dulbecco's modified PBS (with 0.9 mmol/L Ca^{2+} and 0.5 mmol/L Mg^{2+} ; Pan Biotech, Aidenbach, Germany), 0.1% BSA, 10 mmol/L HEPES, and 10 mmol/L glucose, pH 7.4. Eosinophil isolation kit was from (Miltenyi Biotech, Bergisch Gladbach, Germany). Fix & Perm was from Nordic MUBio (Susteren, The Netherlands). Baricitinib and tofacitinib were from TargetMol (Wellesley Hills, USA). Human CCL11, IL-5 and IL-8 were from ImmunoTools (Friesoythe, Germany). PGD₂ was from Cayman Chemical (Tallinn, Estonia). Complement 5a, recombinant mouse FLT3-L and recombinant mouse SCF were from Bio-Techne (Minneapolis, USA). Recombinant mouse IL-5 was from R&D Systems (Minneapolis, USA). PhagostestTM, FITC annexin-V Apoptosis Detection Kit I, AlexaFluor-647 anti-human STAT1 antibodies, CellFix and FACS-Flow were from Becton Dickinson (New Jersey, USA). PE anti-human phospho-STAT1, PE anti-mouse CCR3 (CD193), APC anti-mouse CD45, BV421 anti-mouse Siglec F (CD170), PE/Cy5.5 anti-mouse CD11c, APC/Cy7 anti-mouse CD11b and PE anti-mouse Ly6G were from Biolegend (San Diego, USA). FITC anti-mouse phospho-JAK1 and JAK2 were from Biorbyt (Cambridge, UK). GibcoTM Hepes, GibcoTM HBSS, GibcoTM RPMI 1640, Cytiva HyCloneTM Fetal Bovine Serum, FalconTM cell strainer, Texas Red Phalloidin and dihydrorhodamine-123 were from Fisher Scientific (Hampton, USA). VECTASHIELD® Antifade Mounting Medium with DAPI was from Vector Laboratories (Burlingame, USA). House dust mite extract was from Greer Laboratories (Lenoir, USA). CellFix and FACS-Flow were from Becton Dickinson (New Jersey, USA). PVP-free polycarbonate filters were from Neuro Probe (Gaithersburg, USA).

Fixative solution was prepared by adding 9 mL of distilled water and 30 mL of FACS-Flow to 1 mL of CellFix.

2.2. Blood donors

Blood was collected according to a protocol approved by the Ethics Committee of the Medical University of Graz (17-291 ex 05/06). For functional assays, blood was collected on a daily basis from healthy non-allergic donors. For STAT1 staining, blood was collected from age- and sex-matched healthy non-allergic and allergic volunteers with allergic symptoms to aeroallergens, mainly house dust, tree and grass pollen (Table 1). Allergic sensitization was confirmed by detecting specific and total IgE antibodies in serum by ImmunoCAP 250 (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions. Specific IgE values greater than 0.35 kU/L were considered positive.

Table 1. Allergic patients and controls enrolled for STAT1 measurements.

Variable	Controls (n=12)	Patients (n=10)
Demographics		
Age, years (mean (SD))	32.81 (8.47)	31.20 (5.07)
Gender (% females)	66.67 %	60.00 %
IgE reactivity, mean kU/L (SD)		
Total IgE	69.03 (54.03)	125.53 (104.36)
Grass	-	4.51 (4.54)
Birch	-	10.49 (12.79)
House dust mite	-	7.15 (9.28)
Cat	-	1.87 (1.00)

2.3. Preparation of human peripheral blood leukocytes

In brief, erythrocytes were removed from citrated whole blood by dextran sedimentation for 30 min at room temperature. Polymorphonuclear leukocytes (cell pellet) were separated from mononuclear cells (buffy coat) by density gradient centrifugation (Histopaque 1077, Sigma–Aldrich) for 20 min at room temperature at 400g. Eosinophils were separated from neutrophils in the polymorphonuclear leukocyte fraction by negative magnetic selection using the MACS® cell separation system (Eosinophil Isolation Kit, Miltenyi Biotech). Therefore, non-eosinophils were indirectly magnetically labeled with a cocktail of biotin-conjugated monoclonal antibodies against CD2, CD14, CD16, CD19, CD56, CD123, and CD235a, as primary labeling reagent, and anti-biotin monoclonal antibodies conjugated to MACS® MicroBeads (Miltenyi Biotech), as secondary labeling reagent. The magnetically labeled non-eosinophils were depleted by retaining them on a MACS® column (Miltenyi Biotech) in the magnetic field of a separator, while unlabeled eosinophils passed through the column with a resulting purity of typically $\geq 98\%$.

2.4. Flow cytometric staining of total and phospho-STAT1

Purified eosinophils from allergic and healthy donors were processed with Fix & Perm (Nordic MUBio) following the manufacturer's instructions, blocked in PBS/2% FBS (Fisher Scientific) for 15 min and stained with isotype control or specific antibodies against total-STAT1 (Beckton Dickinson) and phospho-STAT1 (Biolegend) for 30 min at 4 °C. In some experiments eosinophils from healthy donors were pretreated with vehicle, baricitinib (200 nM; TargetMol)) or tofacitinib (200 nM; TargetMol)) for 30 min at 37 °C and stimulated with vehicle or IL-5 (100 pM) for 10 min at 37 °C prior to the staining. Samples were measured by flow cytometry

(FACSCanto II) and analyzed by FlowJo 10.7.1 software. Data were calculated as fold increase in fluorescence intensity with respect to the isotype control and reported as phospho-STAT1/total-STAT1 ratios (mean $\pm$ SEM).

2.5. Generation of bone marrow-derived eosinophils (bmEos)

Bone marrow-derived eosinophils (bmEos) were differentiated *ex vivo* from unselected bone marrow progenitors using a well-defined cytokine regimen [30, 31] in the absence or presence of baricitinib (200 nM) or tofacitinib (200 nM). In brief, bone marrow cells were collected from the femurs and tibiae of three female BALB/c mice and cultured at 10^6 cells/mL in media supplemented with 100 ng/mL SCF and 100 ng/mL FLT3-ligand (both Biotechne) from day 0 to day 4. On day 4, the media was replaced with media containing 10 ng/mL IL-5 (R&D Systems) with or without baricitinib (200 nM) or tofacitinib (200 nM). Baricitinib has a biological half-life of 12.5 h whereas the half-life of tofacitinib is only 3-4 h. Thus, for patients it is recommended to take baricitinib once a day and tofacitinib twice a day. However, the stability of baricitinib and tofacitinib in a cellular system has not been described yet. Here, we decided to add baricitinib (200 nM) once a day and tofacitinib (200 nM) twice a day to the differentiation media. Fresh IL-5 medium was added every second day. On the indicated days, cells were enumerated, stained with anti-Siglec F and anti-CCR3 antibodies (both Biolegend) at 4 °C for 30 min, evaluated by flow cytometry (BD FACSCanto II) and analyzed by FlowJo 10.7.1 software. On day 12, cells were processed with Fix & Perm (Nordic MUBio) and additionally stained with FITC anti-mouse phospho-JAK1 and JAK2 antibodies (both Biorbyt), evaluated by flow cytometry (BD FACSCanto II) and analyzed by FlowJo 10.7.1 software.

2.6. F-actin staining

Purified human eosinophils were seeded on poly-L-lysine coated chamber slides and pre-treated with baricitinib (200 nM), tofacitinib (200 nM) or vehicle for 30 min at room temperature. F-actin polymerization was induced by stimulation with IL-5 (1 nM) at 37 °C for 15 min. Adherent cells were fixed with 3.7% formaldehyde at room temperature for 10 min, permeabilized with 0.1% Triton X-100 at room temperature for 5 min and blocked in PBS/1% BSA at room temperature for 25 min. F-actin staining was performed with Phalloidin-Texas Red (Fisher Scientific) at room temperature for 20 min and slides were mounted with VECTASHIELD® Antifade Mounting Medium with DAPI (Vector Laboratories). Fluorescence images were taken by an Olympus IX70 connected with an Olympus MT20 light source and a Hamamatsu ORCA-ER digital camera (Hamamatsu Photonics K.K., Japan). Olympus xcellence® imaging analysis software 1.1 was used for acquiring images.

2.7. Chemotaxis

Purified eosinophils were used to study eosinophil chemotaxis, whereas neutrophil chemotaxis was performed separately with polymorphonuclear leukocyte (PMNL) fractions. Cells were pre-treated with baricitinib (200 nM or 500 nM), tofacitinib (200 nM or 500 nM) or vehicle for 30 min at 37 °C, placed into the top wells of a 48-well micro-Boyden chemotaxis chamber (Neuro Probe) and were allowed to migrate towards the respective chemoattractant for 1 hour at 37 °C. PVP-free polycarbonate filter membranes with a pore size of 5 µm (Neuro Probe) were used. Migrated eosinophils or neutrophils in the bottom wells were collected and then enumerated by flow cytometry (BD FACSCanto II, acquisition time: 30 sec, high flow rate) and analyzed by FlowJo 10.7.1 software. Therefore, eosinophils and neutrophils were gated by their forward and side scatter properties and by autofluorescence.

2.8. Respiratory burst

Purified human eosinophils were pre-treated with baricitinib (500 nM), tofacitinib (500 nM) or vehicle for 30 min at 37 °C and stimulated with serial dilutions of the respective chemoattractant in the presence of 1 μ M dihydrorhodamine-123 (Fisher Scientific) for 20 min at 37 °C as described previously [32]. Production of ROS was quantified by flow cytometry (BD FACSCanto II) as the increase of fluorescence in the B530/30 channel due to oxidation of nonfluorescent dihydrorhodamine-123 into fluorescent rhodamine-123 and analyzed by FlowJo 10.7.1 software. Responses were expressed as geometric mean fluorescence intensity (MFI).

2.9. Phagocytosis assay

Eosinophil phagocytosis was determined using the Phagotest™ kit (BD Biosciences). Therefore, purified eosinophils were pre-treated with vehicle, baricitinib or tofacitinib (500 nM and 2.5 μ M) for 30 min at 37 °C and Phagotest™ was performed following the manufacturers' instructions. In brief, samples were incubated with opsonized *Escherichia coli*-FITC and the percentage of *E. coli*-FITC-positive eosinophils was determined by flow cytometry (BD FACSCanto II) and analyzed by FlowJo 10.7.1 software.

2.10. Eosinophil degranulation

To determine the release of eosinophil peroxidase (EPO) from purified eosinophils, cells were resuspended in assay buffer at 1×10^6 cells/mL and mixed with cytochalasin B (10 μ g/mL). Thereafter cells were pretreated with vehicle, baricitinib or tofacitinib (500 nM) for 30 min at 37 °C, transferred to a microplate and subsequently stimulated with various concentrations of complement 5a for 20 min at 37 °C. Then, H₂O₂ (1 mM) was added to each sample to start the peroxidase reaction. To detect the reaction, 2.8 mM tetramethylbenzidine was used. Following

incubation for 1 min at room temperature, the peroxidase reaction and the color development were terminated with 4 M acetic acid. Microplates were analyzed on a bench reader at a wavelength of 630 nm. Data were expressed as the percentage of the vehicle response (unstimulated sample, 100 %).

2.11. Apoptosis assay

Purified eosinophils were cultured in RPMI 1640 (Fisher Scientific) supplemented with IL-5 (ImmunoTools) (100 pM), 1% FBS and Penicillin/Streptomycin in the absence or presence of baricitinib (500 nM) or tofacitinib (500 nM). After 18 hours, cells were washed twice in PBS and resuspended in binding buffer. Eosinophils were stained with FITC-annexin-V (1/100) and propidium iodide (PI) (1/50) in the dark for 10 min at room temperature according to the manufacturer's protocol (annexin V-FITC Apoptosis Detection Kit I, BD Pharmingen) and immediately evaluated by flow cytometry (BD FACSCanto II) and analyzed by FlowJo 10.7.1 software. Each sample was acquired for 1 min, and the total number of eosinophils gated on a forward scatter/side scatter plot and the percentage of live cells (annexin-V negative/propidium iodide negative), early apoptotic cells (annexin-V positive/propidium iodide negative), late apoptotic cells (annexin-V positive/propidium iodide positive) and necrotic cells (annexin-V negative/propidium iodide positive) was recorded.

2.12. House dust mite (HDM)-induced allergic lung inflammation

The HDM model was performed as previously described [33]. Eight-week-old female BALB/c mice (Charles River Laboratories, Sulzfeld, Germany) were used in this study. Mice were housed in individually ventilated cages (5 per cage) under controlled conditions of temperature (21°C), air humidity (50%) and a 12 h light/dark cycle (lights on at 6:00 a.m.). Standard chow

(altromin 1324 FORTI, Altromin, Lage, Germany) and water were provided *ad libitum*. The experimental procedure used in this study was approved by the Austrian Federal Ministry of Science, Research and Economy (protocol number: BMWWF-66.010/0034-WF/V/3b/2017) conform to Directive 2010/63/EU, and was performed in accordance with national and international guidelines. Mice were randomly assigned to the different treatments. On day 1 mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) via intraperitoneal injection and sensitized intranasally with 1 µg HDM extract (Greer Laboratories) in 40 µL PBS. From day 7 to day 12, mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) via intraperitoneal injection and challenged by intranasal application of 10 µg HDM protein in 40 µL PBS. From day 8 to day 14, mice received 200 µL vehicle or 50 µmol/200 µL PBS of baricitinib or tofacitinib via oral gavage (six mice per group). Baricitinib has a biological half-life of 12.5 h whereas the half-life of tofacitinib is only 3-4 h. Therefore, baricitinib was given once a day and tofacitinib was applied twice a day. On day 15, mice were again anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) via intraperitoneal injection and either airway hyperresponsiveness to methacholine was recorded or lungs, spleens and bone marrow were collected and single cell suspensions were prepared as described previously [34-36].

2.13. Single cell suspensions of lung, spleen and bone marrow

Lung, spleen and bone marrow single cell suspensions were prepared as described previously [34-36]. In brief, lungs were perfused by injecting the right ventricle with 8 mL of perfusion media (PBS with 10 mM EDTA). Lungs were minced and incubated in 8 mL digestion media: RPMI 640 supplemented with 20 µg/mL DNase I, 5% FBS and 2 mg/mL collagenase D. The contents were stirred for 45 min at 37 °C, then 3 mL of fresh digestion media were added and the contents were incubated for another 45 min. After incubation, EDTA was added to a final

concentration of 10 mM and samples were incubated for 5 min at room temperature. The contents of the tubes were strained through a 70 µm cell strainer (Fisher Scientific). The strainer was washed with 5 mL Wuerzburg buffer (PBS/0.3% BSA/5 mM EDTA/20 µg/mL DNase I), cells were spun and red blood cells were subjected to hypotonic lysis. Wuerzburg buffer was added and cells were washed with HBSS.

Spleens were cut into small pieces in HBSS (Fisher Scientific) with 1% fetal bovine serum and 10 mM HEPES (Fisher Scientific). Fragments were passed through a 70 µm strainer followed by a 40 µm strainer (Fisher Scientific). The red blood cells were subjected to hypotonic lysis, and the remaining cells were suspended in HBSS [36].

For collecting bone marrow, the femurs and tibiae were flushed with RPMI media, the red blood cells were subjected to hypotonic lysis, and the remaining cells were suspended in HBSS with 1% fetal bovine serum and 10 mM HEPES [36].

2.14. Flow cytometric analysis of lung, spleen and bone marrow single cell suspensions

Single cells collected from lungs, spleens and bone marrow were subsequently subjected to a staining protocol. In brief, after a washing step, non-specific binding sites were masked using TruStain fcX™ (anti-mouse CD16/32) antibody (Biolegend, 1:100) for 15 min on ice. Followed by incubation with specific antibodies (all anti mouse): Siglec F (1:100), CD11b (1:200), CD11c (1:200), and Ly6G (1:500) (all from Biolegend) for 30 min at 4 °C. Cells were washed, centrifuged (400 x g, 7 mins), resuspended in 200 µl fixative solution, measured by a BD FACS Canto II flow cytometer and analyzed by FlowJo 10.7.1 software.

Lungs: after single cell and CD45^{pos} gating, eosinophils were identified as CD11c^{neg}/Siglec F^{pos} and alveolar macrophages as CD11c^{pos}/Siglec F^{pos} cells. CD11c/Siglec F double negative cells were characterized as CD11b^{pos}/Ly6G^{pos} neutrophils, CD11b^{pos}/Ly6G^{neg} monocytes and

FSC/SSC^{low} CD11b^{neg}/Ly6G^{neg} lymphocytes. The gating strategy is illustrated in **Figure 8B**. For spleen and bone marrow single cell suspensions a similar gating strategy was used: after single cell and CD45^{pos} gating, eosinophils were identified as CD11c^{neg}/Siglec F^{pos} cells. CD11c/Siglec F double negative cells were characterized as CD11b^{pos}/Ly6G^{pos} neutrophils, CD11b^{pos}/Ly6G^{neg} monocytes and FSC/SSC^{low} CD11b^{neg}/Ly6G^{neg} lymphocytes.

2.15. Lung function

On day 15, mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) by intraperitoneal injection, the trachea was cannulated and mice were connected to a miniature computerized flexiVent apparatus (SCIREQ, Montreal, QC, Canada). Mice were ventilated with a tidal volume of 10 mL/kg at a rate of 150 breaths/min. Nebulized methacholine (1-100 µg/mL) was used to measure airway responsiveness by means of respiratory system resistance (Rrs) and compliance (Crs) [33, 37].

2.16. Statistical analysis

All data are shown as mean±SEM for n observations. Statistical analyses were performed using GraphPad Prism software 6.0 (La Jolla, CA; USA). Groups were compared by one-way or two-way ANOVA followed by Tukey multiple comparison test or Student's t-test. Probability values of $P < 0.05$ were regarded as statistically significant and are indicated as * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.005$.

3. Results

3.1. Differential expression and phosphorylation of STAT1 in eosinophils from allergic and healthy non-allergic donors

Dysregulation of the JAK/STAT pathway is known to be involved in the pathogenesis of allergic inflammation [38]. Thus, we compared the total expression and phosphorylation of STAT molecules in purified eosinophils from allergic donors and healthy non-allergic controls. As illustrated in **Figure 1A**, among all STAT members only STAT1 showed differential total expression and phosphorylation between the two groups. Phospho-STAT1 levels were significantly increased in allergic donors, which is reflected by a mean phospho-STAT1/total-STAT1 ratio close to 1. Moreover, stimulation of purified eosinophils from non-allergic controls with IL-5 (300 pM) increased STAT1 phosphorylation (**Fig. 1B**). Eosinophils pre-incubated with the JAK1/2 inhibitor baricitinib (**Fig. 1C**) or with the JAK3 inhibitor tofacitinib (**Fig. 1D**) displayed significantly less STAT1 phosphorylation (**Fig. 1C**). Both, baricitinib and tofacitinib had no effect on STAT1 phosphorylation in vehicle-treated cells.

3.2. Effects of the JAK inhibitors baricitinib and tofacitinib on eosinophil differentiation

Besides other signalling cascades involving Lyn and ERK/MAPK activity, the JAK/STAT pathway is essential for eosinophil differentiation [39, 40]. Hence, we next investigated the impact of the JAK1/2 inhibitor baricitinib and the JAK3 inhibitor tofacitinib on eosinophil differentiation using a well-established *ex vivo* culture system [30]. In brief, bone marrow cells were collected from the femurs and tibiae of three female BALB/c mice and cultured at $10^6/\text{mL}$ in media supplemented with 100 ng/mL SCF and 100 ng/mL FLT3-ligand from day 0 to day 4. On day 4, media were replaced with media containing 10 ng/mL IL-5 with or without baricitinib (200 nM) or tofacitinib (200 nM) and cells were cultured until day 12. Cells were enumerated,

stained with anti-Siglec F and anti-CCR3 antibodies and analyzed by flow cytometry (BD FACSCanto II) on the indicated days. Of note, baricitinib and tofacitinib similarly inhibited eosinophil differentiation as observed by a significant reduction of total cell count (**Fig. 2A**) and suppression of Siglec F (**Fig. 2B**) and CCR3 (**Fig. 2C**) positive cells. Differentiation in IL-5 medium led to JAK1 (**Fig. 2D**) and JAK2 (**Fig. 2E**) phosphorylation, which was reduced to a similar extent in Siglec F^{pos}/CCR3^{pos} eosinophils that were differentiated in the presence of baricitinib (200 nM) or tofacitinib (200 nM) in the culture media.

3.3. Differential effects of baricitinib and tofacitinib on eosinophil migratory responsiveness

The production and release of eosinophil-specific chemotactic factors is a pivotal event during allergic responses resulting in eosinophil accumulation. When encountering such a chemokinetic or chemotactic factor, eosinophils immediately prepare for diapedesis through the endothelium by rearranging their cytoskeleton leading to shape change. As illustrated in **Figure 3A**, IL-5 (1 nM) elicited filamentous (f)-actin polymerization which was similarly prevented by baricitinib (200 nM) and tofacitinib (200 nM) pretreatment for 30 min.

For further evaluation of eosinophil migration, we performed chemotaxis experiments using a 48-well micro-Boyden chamber. Purified eosinophils were pretreated with vehicle, baricitinib (200 nM or 500 nM) or tofacitinib (200 nM or 500 nM) for 30 min at 37 °C and were allowed to migrate towards a variety of eosinophil chemoattractants for 60 min at 37 °C: IL-5 (300 pM), CCL11 (10 nM), complement 5a (10 nM) and prostaglandin (PG) D₂ (30 nM). Baricitinib (200 nM) significantly reduced the number of migrated eosinophils towards IL-5, CCL11 and PGD₂ by 58.6% (from 157 to 65), 38.8% (from 170 to 104) and 34.0% (from 681 to 449) respectively but did not affect complement 5a-induced responses (**Fig. 3B**). By comparison, tofacitinib (200

nM) had no impact on complement 5a-, CCL11- and PGD₂-induced responses but decreased IL-5-induced migration by 47% (from 194 to 103) (**Fig. 3C**). When applied at higher concentration, baricitinib (500 nM) not only blocked eosinophil migration towards IL-5 by 68.7% (from 179 to 56), but also significantly abrogated eosinophil chemotaxis towards complement 5a by 43.5% (from 315 to 178), towards CCL11 by 41.9% (from 229 to 133) and towards PGD₂ by 33.1% (from 242 to 162) (**Fig. 3D**), while tofacitinib significantly affected eosinophil chemotaxis towards IL-5 by 66.7% (from 189 to 63) and towards complement 5a by 43.0% (from 535 to 305) (**Fig. 3E**). Responses to CCL11 and PGD₂ were decreased by 23.4% (from 462 to 354) and 32.1% (from 722 to 490), respectively, but did not reach significance.

Apart from eosinophils, also neutrophils play a crucial role in the onset of inflammatory diseases such as asthma. For instance, the percentage of neutrophils in the sputum in uncontrolled asthma is higher compared to controlled asthma [41, 42]. Hence, we also tested the efficacy of both JAK inhibitors on neutrophil chemotaxis. Therefore, peripheral blood polymorphonuclear leukocytes were pretreated with vehicle, baricitinib (500 nM) or tofacitinib (500 nM) for 30 min at 37 °C and were allowed to migrate towards the neutrophil chemoattractants IL-8 (10 nM) and complement 5a (10 nM) for 60 min at 37 °C. As illustrated in Figure S2, baricitinib blocked neutrophil chemotaxis towards complement 5a by 45.6% (**Fig. 4A**), whereas tofacitinib inhibited both, neutrophil migration towards complement 5a by 49.4% and IL-8 by 35.4% (**Fig. 4B**).

3.4. Effects of baricitinib and tofacitinib on eosinophil ROS production by respiratory burst

In addition to increased eosinophil differentiation in the bone marrow and recruitment to sites of inflammation, eosinophil respiratory burst is another important tissue-damaging process in

asthma and related inflammatory disorders. Consequently, we explored whether baricitinib and tofacitinib also affect eosinophil ROS production in response to several stimuli. In the course of this, purified eosinophils were pretreated with vehicle, baricitinib (500 nM) or tofacitinib (500 nM) for 30 min at 37 °C and respiratory burst was stimulated with serial dilutions of IL-5, complement 5a, CCL11 and PGD₂. Similar to our results from the chemotaxis assay, the JAK1/2 inhibitor baricitinib seems to be more effective in preventing eosinophil respiratory burst than the JAK3 inhibitor tofacitinib (**Fig. 5**). Both JAK inhibitors reduced ROS production in response to IL-5 (**Fig. 5A**), while only baricitinib inhibited eosinophil respiratory burst in response to complement 5a (**Fig. 5B**), CCL11 (**Fig. 5C**) and PGD₂ (**Fig. 5D**). The maximal inhibition by tofacitinib in response to IL-5 was 54.2% (**Fig. 5A**), while the extent of inhibition by baricitinib was 65.2% (**Fig. 5A**), 39.5% (**Fig. 5B**), 50.2% (**Fig. 5C**) and 38.7% (**Fig. 5D**) in response to IL-5, complement 5a, CCL11 and PGD₂, respectively.

3.5. Effect of baricitinib and tofacitinib on eosinophil phagocytosis and peroxidase release

Besides their role in allergic inflammation, eosinophils are also part of our first-line host defense and have been shown to phagocytose and destroy parasites such as *Trypanosoma dionisii* [43] and bacteria such as *Staphylococcus aureus* and *Escherichia coli* [44]. Thus, we evaluated the impact of baricitinib and tofacitinib on these eosinophil host defense mechanisms by means of phagocytosis and EPO release. Using the Phagotest™ kit, we observed that phagocytosis by eosinophils is similarly impaired by baricitinib or tofacitinib, when treated for 30 min prior to the assay. However, only with a high concentration of 2.5 µM we observed a significant reduction in phagocytic eosinophils by 13.7 % (baricitinib) and by 14.5 % (tofacitinib), respectively (**Fig. 6A and B**). As eosinophil phagocytosis is accompanied by degranulation and the release of eosinophil cytotoxic enzymes, we next investigated the effects of baricitinib and tofacitinib on the release of eosinophil peroxidase (EPO). As shown in **Figure**

6C, neither pre-treatment with baricitinib nor tofacitinib significantly affected EPO release in eosinophils.

3.6. Effects of baricitinib and tofacitinib on eosinophil apoptosis

Having confirmed that STAT1, which is a member of the IL-5-induced JAK2/STAT1/STAT3/STAT5 pro-survival pathway, is phosphorylated in allergic individuals, and that IL-5 induced STAT1 phosphorylation is decreased in the presence of baricitinib and tofacitinib, we wished to establish which JAK inhibitor interferes best with the pro-survival function of IL-5. Therefore, purified human eosinophils from healthy donors were cultured in the presence or absence of IL-5 (100 pM) with or without the JAK1/2 inhibitor baricitinib (500 nM) or the JAK3 inhibitor tofacitinib (500 nM) for 18 hours. Apoptosis was assessed by flow cytometry using a FITC-annexin-V/propidium iodide assay. Both JAK inhibitors counteracted the pro-survival effect of IL-5 as reflected by significantly decreased live cells compared to vehicle-treated cells (**Fig. 7A**). Of note, only baricitinib also significantly increased the percentage of early apoptotic cells (**Fig. 7B**). Interestingly, in vehicle treated cells we observed a contrary effect of JAK inhibition, as both baricitinib and tofacitinib decreased the percentage of vehicle-treated late apoptotic cells (**Fig. 7C**) and baricitinib also reduced the percentage of vehicle-treated necrotic cells (**Fig. 7D**).

3.7. Effect of baricitinib and tofacitinib on allergen-induced eosinophilic airway inflammation in mice

It has been shown previously that tofacitinib reduced the number of eosinophils in the BAL fluid of allergen-challenged mice when applied either systemically [22] or per inhalation [45]. To date, however, the efficacy of the JAK1/2 inhibitor baricitinib in allergen-induced

eosinophilic airway inflammation has not been evaluated. Therefore, BALB/c mice were sensitized with HDM on day 1 and challenged from day 7-12 leading to a mixed (eosinophilic and neutrophilic) lung inflammation. In addition, mice were either treated by oral gavage with vehicle, baricitinib (50 μ mol; once a day) or tofacitinib (50 μ mol; twice a day) from day 8-14. On day 15 the lungs were flushed with 10 mL PBS via the right ventricle and single cell suspensions were prepared (**Fig. 8A**).

As depicted in **Figure 8C**, baricitinib strongly repressed the infiltration of total immune cells, mainly eosinophils, into the lung tissue whereas tofacitinib was less effective. Treatment with baricitinib for 7 days protected mice from lung eosinophilia as reflected by a 63 % reduction in eosinophil counts in the lung single cell suspension of baricitinib treated mice compared to vehicle treated controls. Moreover, baricitinib decreased the number of infiltrated monocytes by 74 %. Of note, in tofacitinib treated mice, eosinophil and monocyte numbers were reduced by 13 and 52 % respectively, however these differences did not reach significance. Both, baricitinib and tofacitinib did not affect counts of lung alveolar macrophages, neutrophils and lymphocytes.

In addition, baricitinib treatment led to a reduction of total immune cells in the spleen (**Fig. 8D**) and both baricitinib and tofacitinib protected from systemic eosinophilia as reflected by a significant reduction of eosinophil counts in spleen tissue by 72 and 58 %, respectively. Tofacitinib treatment also reduced neutrophil counts in the spleen by 90 %, whereas, baricitinib reduced lymphocyte counts by 52 %. Neither baricitinib nor tofacitinib were able to reduce the number of monocytes in the spleen. Moreover, baricitinib also significantly reduced total immune cells in the bone marrow (**Fig. 8E**) and decreased eosinophil counts by 60 %. The numbers of neutrophils, lymphocytes and monocytes in the bone marrow were neither affected by baricitinib nor by tofacitinib.

3.8. Effect of baricitinib and tofacitinib on allergen-induced airway hyperresponsiveness

Airway hyperresponsiveness, one of the hallmarks of human asthma, is defined as an exaggerated response of the airways to various stimuli resulting in airway obstruction. Having shown that baricitinib prevents the influx of eosinophils into the lungs, we further assessed the impact of baricitinib and tofacitinib on airway hyperresponsiveness. Therefore, HDM challenged/sensitized mice were either treated by oral gavage with vehicle, baricitinib (50 μmol ; once a day) or tofacitinib (50 μmol ; twice a day) from day 8-14. On day 15, airway hyperresponsiveness to methacholine was recorded by spirometric measurements using the flexiVent system. Unexpectedly, neither baricitinib nor tofacitinib significantly affected methacholine-induced increases in resistance and decreases in compliance (**Fig. 8F**). Although, baricitinib reduced resistance by 25%, this effect did not reach significance.

4. Discussion

In the present study we demonstrate that targeting JAK1/2 kinases represents a promising therapeutic approach for the treatment of eosinophilic inflammation as observed in severe eosinophilic asthma. Our data imply that the JAK 1/2 inhibitor baricitinib bears potent anti-inflammatory properties *in vitro* and *in vivo* with respect to eosinophilic inflammation, whereas the JAK3 inhibitor tofacitinib seems to be less effective.

First, we found that active phospho-STAT1 is increased in eosinophils from allergic individuals. This observation is in line with a previous report from Gernez et al., who detected increased phospho-STAT1 levels in CD4^{pos} CD161^{pos} T cells from asthmatic patients [46]. Binding of IL-5 to the IL-5 receptor activates JAK1 and JAK2, whereby JAK2 is believed to phosphorylate STAT1/5 in eosinophils [47-49]. Interestingly, we observed that stimulation with IL-5 induced STAT1 phosphorylation in eosinophils from healthy donors, which was prevented in both baricitinib- and tofacitinib-treated cells. These findings affirm that the STAT1 pathway is activated in eosinophils from allergic donors and that targeting JAK1 and JAK2 with both the JAK1/2 inhibitor baricitinib and also with the JAK3 inhibitor tofacitinib, which has less affinity for JAK1 and JAK2, satisfactorily prevents IL-5-induced eosinophil activation.

Human eosinophil progenitors pursue their maturation and proliferation in response to transcription and growth factors, most notably through IL-5. Thus, as a next step we compared the effects of baricitinib and tofacitinib on IL-5-driven differentiation of mouse eosinophils from unselected bone marrow progenitors and observed that both JAK inhibitors inhibited eosinophil differentiation with comparative potency. Unexpectedly, in eosinophils that differentiated in spite of the presence of baricitinib or tofacitinib, JAK1 and JAK2 phosphorylation was similarly impaired. Thus, our data indicate that both JAK1 and JAK2 are involved in mouse eosinophil differentiation and that long-term treatment not only with the

JAK1/2 inhibitor baricitinib but also the JAK3 inhibitor tofacitinib which has a lower affinity for JAK1 and JAK2 is sufficient to prevent IL-5-mediated eosinophil differentiation.

The production and release of eosinophil-specific chemotactic factors during allergic responses is a pivotal event resulting in eosinophil accumulation in the airways and lung tissue. It is already known that treatment with tofacitinib prevents the production of cytokines that stimulate eosinophil activation and recruitment such as IL-13 and CCL11 in a mouse model of ovalbumin (OVA)-induced airway inflammation [22]. However, thus far it has not been investigated how blocking the JAK/STAT pathway in eosinophils interferes with their chemotactic responsiveness. Here we show for the first time that the JAK1/2 inhibitor baricitinib decreased eosinophil migration not only towards IL-5 but also towards other chemoattractants such as complement 5a, CCL11 and PGD₂, whereas the JAK3 inhibitor tofacitinib inhibited IL-5 and complement 5a responses with a similar potency, but had no significant effect on CCL11 and PGD₂ responses. In contrast, neutrophil chemotaxis towards IL-8 was inhibited by tofacitinib but not baricitinib which complies with the finding of Henkels et al. that JAK3 is a mediator of IL-8-induced chemotaxis in neutrophils [50]. Baricitinib but not tofacitinib significantly reduced the chemotaxis towards CCL11 and PGD₂ in eosinophils, underlining the involvement of JAK2 in this process. Interestingly, PGD₂ has been shown to inhibit activation and phosphorylation of JAK2 and STAT3 in the human gastric cancer cell line SGC-7901 and in HEK293T cells [51]. Moreover, Kong et al. stated that PGD₂/DP1-induced M2 polarization facilitates resolution of inflammation through a cAMP-dependent protein kinase type II- α regulatory subunit-mediated suppression of JAK2/STAT1 signaling [52]. Thus, our data and previous reports indicate that PGD₂ signaling interferes with the JAK2 pathway, however, the exact mechanism still needs to be elucidated. The role of JAK kinases in complement 5a signaling is still unclear. In 2018 An et al. showed that complement 5a induces STAT3 phosphorylation in microglial cells, which was prevented by the JAK2/3

inhibitor AG490 [53]. Correspondingly, we demonstrated that both baricitinib and tofacitinib prevent complement 5a-induced chemotaxis in eosinophils and neutrophils.

During respiratory burst, eosinophils produce reactive oxygen species, such as superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) in response to proinflammatory mediators. These mediators have been implicated in bronchial tissue damage and persistent inflammation in the airways. Similar to our chemotaxis results we observed that baricitinib significantly reduced ROS production in response to IL-5, complement 5a, CCL11 and PGD_2 . However, tofacitinib inhibited ROS production in response to IL-5 and slightly but not significantly in response to CCL11 and PGD_2 but had no effect on complement 5a elicited ROS. Thus, we speculate that ROS production in eosinophils is mainly mediated through JAK2. The crucial role of JAK2 in ROS production has previously been highlighted in neutrophils from patients with myeloproliferative disorders with a hyperactive JAK2 (V617F) mutation, who exhibit dramatically increased ROS production [54].

Apoptosis and subsequent clearance of tissue eosinophils by macrophages and dendritic cells is an important mechanism to resolve local inflammation [55] which is clearly attenuated in asthma. IL-3, IL-5 and GM-CSF are key pro-survival cytokines for eosinophils that are derived from paracrine (T cells, macrophages and epithelial cells) and autocrine (eosinophils) sources. The intracellular pathways that are important in eosinophil apoptotic decisions include Lyn, JAK2, FAS-associated death domain protein, phosphatidylinositol 3-kinase/protein kinase B, proto-oncogene serine/threonine-protein kinase, and mitogen-activated protein kinases [56-58]. Accordingly, in an annexin-V/PI-based apoptosis assay we observed that the JAK1/2 inhibitor baricitinib more efficiently mitigated the anti-apoptotic effect of IL-5 than the JAK3 inhibitor tofacitinib which has less affinity for JAK1 and JAK2. This data is supplementing our existing knowledge that primarily JAK2, together with Lyn and Raf-1 kinases, contributes to the pro-survival effect of IL-5 [49, 59].

Several reports have implied a higher susceptibility for treatment-emerged infections in JAK inhibitor users, including pneumonia [60], herpes zoster [61] and opportunistic fungal infections [62], likely through antagonizing PMNL effector function. As eosinophils contribute to our host defense against helminth parasites and bacteria via their phagocytic capabilities and the release of cytotoxic enzymes from their granules, we evaluated the effects of baricitinib and tofacitinib on eosinophil phagocytosis and EPO release. Baricitinib and tofacitinib similarly reduced eosinophil phagocytosis whereby a significant effect was only observed at a very high concentration of 2.5 μ M. Neither baricitinib nor tofacitinib had a significant impact on eosinophil EPO release. Thus, our data suggests that although eosinophil differentiation, migration and apoptosis is affected by JAK inhibition, eosinophil phagocytosis and degranulation, two important functions in host defense, are hardly hampered by treatment with JAK inhibitors.

To confirm the *in vivo* relevance of the observed anti-eosinophilic activities of baricitinib and tofacitinib, we performed a well-established mouse model of HDM-induced airway inflammation [33]. We could show that daily oral treatment with baricitinib for seven days reduced eosinophil counts in lung tissue, spleen and bone marrow of HDM-challenged mice, whereas tofacitinib given twice a day for seven days only reduced eosinophil counts in the spleen, although tofacitinib also prevented IL-5-stimulated eosinophil differentiation *in vitro*. Consequently, these findings emphasize that baricitinib is the more effective repressor of eosinophilic inflammation *in vivo*. So far, it has been shown that tofacitinib significantly lowered eosinophil numbers and CCL11 and IL-13 levels in BAL fluid in an OVA-model of allergic lung inflammation [22]. However, in the mentioned study, tofacitinib was administrated s.c. via an osmotic minipump throughout the period of sensitization and challenge, which does not mimic a possible treatment regimen for human asthma patients. In contrast, recent findings of Wagh et al., who investigated the effects of tofacitinib (30 mg/kg)

in an acute OVA model, are in clear concordance with our *in vivo* data. The study did neither find decreased eosinophil cell counts in the BAL fluid nor reduced IL-5 levels in lung homogenates, but a significant reduction in neutrophil counts in the BAL fluid [63]. Interestingly, when formulated as an aerosol and applied on three consecutive days over three weeks, tofacitinib was able to reduce eosinophils and total protein content in the BAL fluid of HDM-treated mice [45]. Further, in our model, oral application of both baricitinib and tofacitinib during the HDM-challenge phase failed to significantly suppress airway hyperresponsiveness to methacholine. This observation is in line with a previous report from Matsunaga et al., who found that i.p. application of the pan-JAK inhibitor pyridine 6 suppressed eosinophilia in BAL fluid but did not affect airway hyperresponsiveness in OVA-treated mice [64]. Otherwise, the inhaled JAK1 inhibitor JAK-381 has been shown to reduce eosinophilia in bronchoalveolar lavage fluid and airway resistance in an OVA-model in mice [24].

Taken together, here we evaluated the anti-eosinophilic efficacy of the two already approved oral JAK inhibitors baricitinib and tofacitinib in well-established *in vitro* assays and in a mouse model of HDM-lung inflammation. We show for the first time that the JAK1/2 inhibitor baricitinib is more effective in combating eosinophilic inflammation *in vitro* and *in vivo* than the JAK3 inhibitor tofacitinib, although both inhibitors prevented IL-5-induced functions to a similar extent. Thus, targeting JAK1 and JAK2 might represent a promising novel approach for the pharmacotherapy of eosinophilic inflammation such as observed in severe eosinophilic asthma.

Most cytokine receptors are associated with a combination of different JAKs, which interferes with the therapeutic strategy of targeting only a single JAK subtype and favors the use of pan-JAK inhibitors. However, toxicity and anticipated side effects may limit the usefulness of a comprehensive JAK blockade. Moreover, our and other studies clearly indicate that selectivity,

dosage, treatment duration, formulation, and application route of JAK inhibitors have a major influence on their therapeutic outcome.

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References

- [1] Global Strategy for Asthma Management and Prevention., Global Initiative for Asthma (GINA), 2021.
- [2] K.F.Chung, Targeting the interleukin pathway in the treatment of asthma, *Lancet* 386(9998) (2015) 1086-96.
- [3] K.F. Chung, S.E. Wenzel, J.L. Brozek, A. Bush, M. Castro, P.J. Sterk, I.M. Adcock, E.D. Bateman, E.H. Bel, E.R. Bleeker, L.P. Boulet, C. Brightling, P. Chanez, S.E. Dahlen, R. Djukanovic, U. Frey, M. Gaga, P. Gibson, Q. Hamid, N.N. Jajour, T. Mauad, R.L. Sorkness, W.G. Teague, International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma, *Eur Respir J* 43(2) (2014) 343-73.
- [4] F.N. Schleich, A. Chevrement, V. Paulus, M. Henket, M. Manise, L. Seidel, R. Louis, Importance of concomitant local and systemic eosinophilia in uncontrolled asthma, *Eur Respir J* 44(1) (2014) 97-108.
- [5] N. Carroll, C. Cooke, A. James, The distribution of eosinophils and lymphocytes in the large and small airways of asthmatics, *Eur Respir J* 10(2) (1997) 292-300.
- [6] P.J. Barnes, A.J. Woolcock, Difficult asthma, *Eur Respir J* 12(5) (1998) 1209-18.
- [7] W.W. Busse, S. Banks-Schlegel, S.E. Wenzel, Pathophysiology of severe asthma, *J Allergy Clin Immunol* 106(6) (2000) 1033-42.
- [8] P.M. O'Byrne, N. Naji, G.M. Gauvreau, Severe asthma: future treatments, *Clin Exp Allergy* 42(5) (2012) 706-11.
- [9] H.G. Ortega, M.C. Liu, I.D. Pavord, G.G. Brusselle, J.M. FitzGerald, A. Chetta, M. Humbert, L.E. Katz, O.N. Keene, S.W. Yancey, P. Chanez, M. Investigators, Mepolizumab treatment in patients with severe eosinophilic asthma, *N Engl J Med* 371(13) (2014) 1198-207.
- [10] E.R. Bleeker, J.M. FitzGerald, P. Chanez, A. Papi, S.F. Weinstein, P. Barker, S. Sproule, G. Gilmartin, M. Aurivillius, V. Werkstrom, M. Goldman, S.s. investigators, Efficacy and

safety of benralizumab for patients with severe asthma uncontrolled with high-dosage inhaled corticosteroids and long-acting beta2-agonists (SIROCCO): a randomised, multicentre, placebo-controlled phase 3 trial, *Lancet* 388(10056) (2016) 2115-2127.

[11] J.M. FitzGerald, E.R. Bleeker, P. Nair, S. Korn, K. Ohta, M. Lommatzsch, G.T. Ferguson, W.W. Busse, P. Barker, S. Sproule, G. Gilmartin, V. Werkstrom, M. Aurivillius, M. Goldman, C.s. investigators, Benralizumab, an anti-interleukin-5 receptor alpha monoclonal antibody, as add-on treatment for patients with severe, uncontrolled, eosinophilic asthma (CALIMA): a randomised, double-blind, placebo-controlled phase 3 trial, *Lancet* 388(10056) (2016) 2128-2141.

[12] K. Yamaoka, P. Saharinen, M. Pesu, V.E. Holt, 3rd, O. Silvennoinen, J.J. O'Shea, The Janus kinases (Jaks), *Genome Biol* 5(12) (2004) 253.

[13] K. Shuai, B. Liu, Regulation of JAK-STAT signalling in the immune system, *Nat Rev Immunol* 3(11) (2003) 900-11.

[14] P.J. Murray, The JAK-STAT signaling pathway: input and output integration, *J Immunol* 178(5) (2007) 2623-9.

[15] A.B. Pernis, P.B. Rothman, JAK-STAT signaling in asthma, *J Clin Invest* 109(10) (2002) 1279-83.

[16] Y. Rochman, M. Kashyap, G.W. Robinson, K. Sakamoto, J. Gomez-Rodriguez, K.U. Wagner, W.J. Leonard, Thymic stromal lymphopoietin-mediated STAT5 phosphorylation via kinases JAK1 and JAK2 reveals a key difference from IL-7-induced signaling, *Proc Natl Acad Sci U S A* 107(45) (2010) 19455-60.

[17] L. Vuitton, S. Koch, L. Peyrin-Biroulet, Janus kinase inhibition with tofacitinib: changing the face of inflammatory bowel disease treatment, *Curr Drug Targets* 14(12) (2013) 1385-91.

[18] E.M. Agency, Olumiant® EPAR – Product Information, (2017).

[19] D.J. Wallace, R.A. Furie, Y. Tanaka, K.C. Kalunian, M. Mosca, M.A. Petri, T. Dorner, M.H. Cardiel, I.N. Bruce, E. Gomez, T. Carmack, A.M. DeLozier, J.M. Janes, M.D. Linnik, S.

- de Bono, M.E. Silk, R.W. Hoffman, Baricitinib for systemic lupus erythematosus: a double-blind, randomised, placebo-controlled, phase 2 trial, *Lancet* 392(10143) (2018) 222-231.
- [20] E. Guttman-Yassky, J.I. Silverberg, O. Nemoto, S.B. Forman, A. Wilke, R. Prescilla, A. de la Pena, F.P. Nunes, J. Janes, M. Gamalo, D. Donley, J. Paik, A.M. DeLozier, B.J. Nickoloff, E.L. Simpson, Baricitinib in adult patients with moderate-to-severe atopic dermatitis: A phase 2 parallel, double-blinded, randomized placebo-controlled multiple-dose study, *J Am Acad Dermatol* 80(4) (2019) 913-921.
- [21] T. Southworth, J. Plumb, V. Gupta, J. Pearson, I. Ramis, M.D. Lehner, M. Miralpeix, D. Singh, Anti-inflammatory potential of PI3Kdelta and JAK inhibitors in asthma patients, *Respir Res* 17(1) (2016) 124.
- [22] E. Kudlacz, M. Conklyn, C. Andresen, C. Whitney-Pickett, P. Changelian, The JAK-3 inhibitor CP-690550 is a potent anti-inflammatory agent in a murine model of pulmonary eosinophilia, *Eur J Pharmacol* 582(1-3) (2008) 154-61.
- [23] M. Calbet, I. Ramis, E. Calama, C. Carreno, S. Paris, M. Maldonado, A. Orellana, E. Calaf, M. Pauta, J. de Alba, J. Bach, M. Miralpeix, Novel inhaled pan-JAK inhibitor, LAS194046, reduces allergen-induced airway inflammation, late asthmatic response and pSTATs activation in Brown Norway rats, *J Pharmacol Exp Ther* 370(2) (2019) 137-147.
- [24] H.S. Dengler, X. Wu, I. Peng, C.H. Rinderknecht, Y. Kwon, E. Suto, P.B. Kohli, M. Liimatta, K. Barrett, J. Lloyd, G. Cain, M. Briggs, S. Addo, G. Salmon, S. Ubhayakar, G. Deshmukh, S.K. Shahidi-Latham, C.M. Quiason-Huynh, J. Jackman, J. Liu, N.C. Ray, S.C. Goodacre, A. Johnson, B.S. McKenzie, W.P. Lee, M. Zak, J.R. Kenny, N. Ghilardi, Lung-restricted inhibition of Janus kinase 1 is effective in rodent models of asthma, *Sci Transl Med* 10(468) (2018) 2151.
- [25] B. Alashkar Alhamwe, S. Miethe, E. Pogge von Strandmann, D.P. Potaczek, H. Garn, Epigenetic Regulation of Airway Epithelium Immune Functions in Asthma, *Front Immunol* 11 (2020) 1747.

- [26] D.P. Potaczek, S. Miethe, V. Schindler, F. Alhamdan, H. Garn, Role of airway epithelial cells in the development of different asthma phenotypes, *Cell Signal* 69 (2020) 109523.
- [27] A. Bujak, P. Nejman-Gryz, P. Gunerka, M. Lamparska-Przybysz, M. Wieczorek, R. Chazan, A. Stanczak, Inhibition of JAK/STAT pathway in airway epithelium as a potential targeted therapy of asthma, *European Respiratory Journal* 44(Suppl 58) (2014) 3413.
- [28] P.S. Fenwick, P. Macedo, I.C. Kilty, P.J. Barnes, L.E. Donnelly, Effect of JAK Inhibitors on Release of CXCL9, CXCL10 and CXCL11 from Human Airway Epithelial Cells, *PLoS One* 10(6) (2015) e0128757.
- [29] H.K. Lee, O. Jung, L. Hennighausen, JAK inhibitors dampen activation of interferon-stimulated transcription of ACE2 isoforms in human airway epithelial cells, *Commun Biol* 4(1) (2021) 654.
- [30] K.D. Dyer, J.M. Moser, M. Czapiga, S.J. Siegel, C.M. Percopo, H.F. Rosenberg, Functionally competent eosinophils differentiated ex vivo in high purity from normal mouse bone marrow, *J Immunol* 181(6) (2008) 4004-9.
- [31] M. Kienzl, C. Hasenoehrl, P. Valadez-Cosmes, K. Maitz, A. Sarsembayeva, E. Sturm, A. Heinemann, J. Kargl, R. Schicho, IL-33 reduces tumor growth in models of colorectal cancer with the help of eosinophils, *Oncoimmunology* 9(1) (2020) 1776059.
- [32] E.M. Sturm, G.P. Parzmair, B. Radnai, R.B. Frei, G.J. Sturm, A. Hammer, R. Schuligoi, I.T. Lippe, A. Heinemann, Phosphoinositide-dependent protein kinase 1 (PDK1) mediates potent inhibitory effects on eosinophils, *Eur J Immunol* 45(5) (2015) 1548-59.
- [33] D. Roula, A. Theiler, P. Luschnig, G.J. Sturm, P.V. Tomazic, G. Marsche, A. Heinemann, E.M. Sturm, Apolipoprotein A-IV acts as an endogenous anti-inflammatory protein and is reduced in treatment-naïve allergic patients and allergen-challenged mice, *Allergy* 75(2) (2020) 392-402.

- [34] E.M. Sturm, K.D. Dyer, C.M. Percopo, A. Heinemann, H.F. Rosenberg, Chemotaxis of bone marrow derived eosinophils in vivo: a novel method to explore receptor-dependent trafficking in the mouse, *Eur J Immunol* 43(8) (2013) 2217-28.
- [35] K.D. Dyer, K.E. Garcia-Crespo, C.M. Percopo, E.M. Sturm, H.F. Rosenberg, Protocols for identifying, enumerating, and assessing mouse eosinophils, *Methods Mol Biol* 1032 (2013) 59-77.
- [36] K.D. Dyer, K.E. Garcia-Crespo, K.E. Killoran, H.F. Rosenberg, Antigen profiles for the quantitative assessment of eosinophils in mouse tissues by flow cytometry, *J Immunol Methods* 369(1-2) (2011) 91-7.
- [37] R.B. Frei, P. Luschnig, G.P. Parzmair, M. Peinhaupt, S. Schranz, A. Fauland, C.E. Wheelock, A. Heinemann, E.M. Sturm, Cannabinoid receptor 2 augments eosinophil responsiveness and aggravates allergen-induced pulmonary inflammation in mice, *Allergy* 71(7) (2016) 944-56.
- [38] D. Sampath, M. Castro, D.C. Look, M.J. Holtzman, Constitutive activation of an epithelial signal transducer and activator of transcription (STAT) pathway in asthma, *J Clin Invest* 103(9) (1999) 1353-61.
- [39] S. Stafford, C. Lowell, S. Sur, R. Alam, Lyn tyrosine kinase is important for IL-5-stimulated eosinophil differentiation, *J Immunol* 168(4) (2002) 1978-83.
- [40] L.X. Xia, W. Hua, Y. Jin, B.P. Tian, Z.W. Qiu, C. Zhang, L.Q. Che, H.B. Zhou, Y.F. Wu, H.Q. Huang, F. Lan, Y.H. Ke, J.J. Lee, W. Li, S.M. Ying, Z.H. Chen, H.H. Shen, Eosinophil differentiation in the bone marrow is promoted by protein tyrosine phosphatase SHP2, *Cell Death Dis* 7 (2016) e2175.
- [41] A. Jatakanon, C. Uasuf, W. Maziak, S. Lim, K.F. Chung, P.J. Barnes, Neutrophilic inflammation in severe persistent asthma, *Am J Respir Crit Care Med* 160(5 Pt 1) (1999) 1532-9.

- [42] W.C. Moore, A.T. Hastie, X. Li, H. Li, W.W. Busse, N.N. Jarjour, S.E. Wenzel, S.P. Peters, D.A. Meyers, E.R. Bleecker, L. National Heart, P. Blood Institute's Severe Asthma Research, Sputum neutrophil counts are associated with more severe asthma phenotypes using cluster analysis, *J Allergy Clin Immunol* 133(6) (2014) 1557-63.
- [43] K.J. Thorne, A.M. Glauert, R.J. Svvnnssen, D. Franks, Phagocytosis and killing of *Trypanosoma dionisii* by human neutrophils, eosinophils and monocytes, *Parasitology* 79(3) (1979) 367-79.
- [44] M.J. Cline, J. Hanifin, R.I. Lehrer, Phagocytosis by human eosinophils, *Blood* 32(6) (1968) 922-34.
- [45] U.S. Younis, E. Vallorz, K.J. Addison, J.G. Ledford, P.B. Myrdal, Preformulation and Evaluation of Tofacitinib as a Therapeutic Treatment for Asthma, *AAPS PharmSciTech* 20(5) (2019) 167.
- [46] Y. Gernez, R. Tirouvanziam, K.D. Nguyen, L.A. Herzenberg, A.M. Krensky, K.C. Nadeau, Altered phosphorylated signal transducer and activator of transcription profile of CD4⁺CD161⁺ T cells in asthma: modulation by allergic status and oral corticosteroids, *J Allergy Clin Immunol* 120(6) (2007) 1441-8.
- [47] K. Pazdrak, S. Stafford, R. Alam, The activation of the Jak-STAT 1 signaling pathway by IL-5 in eosinophils, *J Immunol* 155(1) (1995) 397-402.
- [48] N. Ogata, Y. Kikuchi, T. Kouro, M. Tomonaga, K. Takatsu, The activation of the JAK2/STAT5 pathway is commonly involved in signaling through the human IL-5 receptor, *Int Arch Allergy Immunol* 114 Suppl 1 (1997) 24-7.
- [49] T. van der Bruggen, E. Caldenhoven, D. Kanters, P. Coffier, J.A. Raaijmakers, J.W. Lammers, L. Koenderman, Interleukin-5 signaling in human eosinophils involves JAK2 tyrosine kinase and Stat1 alpha, *Blood* 85(6) (1995) 1442-8.

- [50] K.M. Henkels, K. Frondorf, M.E. Gonzalez-Mejia, A.L. Doseff, J. Gomez-Cambronero, IL-8-induced neutrophil chemotaxis is mediated by Janus kinase 3 (JAK3), *FEBS Lett* 585(1) (2011) 159-66.
- [51] B. Zhang, Q. Bie, P. Wu, J. Zhang, B. You, H. Shi, H. Qian, W. Xu, PGD2/PTGDR2 Signaling Restricts the Self-Renewal and Tumorigenesis of Gastric Cancer, *Stem Cells* 36(7) (2018) 990-1003.
- [52] D. Kong, Y. Shen, G. Liu, S. Zuo, Y. Ji, A. Lu, M. Nakamura, M. Lazarus, C.A. Stratakis, R.M. Breyer, Y. Yu, PKA regulatory $\text{H}\alpha$ subunit is essential for PGD2-mediated resolution of inflammation, *J Exp Med* 213(10) (2016) 2209-26.
- [53] X.Q. An, W. Xi, C.Y. Gu, X. Huang, Complement protein C5a enhances the beta-amyloid-induced neuro-inflammatory response in microglia in Alzheimer's disease, *Med Sci (Paris)* 34 Focus issue F1 (2018) 116-120.
- [54] M. Hurtado-Nedelec, M.J. Csillag-Grange, T. Boussetta, S.A. Belambri, M. Fay, B. Cassinat, M.A. Gougerot-Pocidalo, P.M. Dang, J. El-Benna, Increased reactive oxygen species production and p47phox phosphorylation in neutrophils from myeloproliferative disorders patients with JAK2 (V617F) mutation, *Haematologica* 98(10) (2013) 1517-24.
- [55] G.M. Walsh, Eosinophil apoptosis: mechanisms and clinical relevance in asthmatic and allergic inflammation, *Br J Haematol* 111(1) (2000) 61-7.
- [56] G.M. Walsh, Advances in the immunobiology of eosinophils and their role in disease, *Crit Rev Clin Lab Sci* 36(5) (1999) 453-96.
- [57] H.U. Simon, Molecules involved in the regulation of eosinophil apoptosis, *Chem Immunol Allergy* 91 (2006) 49-58.
- [58] H.U. Simon, S. Yousefi, B. Dibbert, H. Hebestreit, M. Weber, D.R. Branch, K. Blaser, F. Levi-Schaffer, G.P. Anderson, Role for tyrosine phosphorylation and Lyn tyrosine kinase in fas receptor-mediated apoptosis in eosinophils, *Blood* 92(2) (1998) 547-57.

- [59] K. Pazdrak, B. Olszewska-Pazdrak, S. Stafford, R.P. Garofalo, R. Alam, Lyn, Jak2, and Raf-1 kinases are critical for the antiapoptotic effect of interleukin 5, whereas only Raf-1 kinase is essential for eosinophil activation and degranulation, *J Exp Med* 188(3) (1998) 421-9.
- [60] K.L. Winthrop, M. Harigai, M.C. Genovese, S. Lindsey, T. Takeuchi, R. Fleischmann, J.D. Bradley, N.L. Byers, D.L. Hyslop, M. Issa, A. Nishikawa, T.P. Rooney, S. Witt, C.L. Dickson, J.S. Smolen, M. Dougados, Infections in baricitinib clinical trials for patients with active rheumatoid arthritis, *Annals of the Rheumatic Diseases* 79(10) (2020) 1290-1297.
- [61] K.L. Winthrop, A. Wouters, E.H. Choy, C. Chen, P. Biswas, L. Wang, K. Soma, E. Needle, H. Valdez, W.F. Rigby, Long-term effectiveness of live herpes zoster vaccine in patients with rheumatoid arthritis subsequently treated with tofacitinib, *Ann Rheum Dis* 79(5) (2020) 669-671.
- [62] Y. Chen, F.Y. Gong, Z.J. Li, Z. Gong, Z. Zhou, S.Y. Ma, X.M. Gao, A study on the risk of fungal infection with tofacitinib (CP-690550), a novel oral agent for rheumatoid arthritis, *Sci Rep* 7(1) (2017) 6779.
- [63] A.D. Wagh, M. Sharma, J. Mahapatra, A. Chatterjee, M. Jain, V. Addepalli, Investigation into the Role of PI3K and JAK3 Kinase Inhibitors in Murine Models of Asthma, *Front Pharmacol* 8 (2017) 82.
- [64] Y. Matsunaga, H. Inoue, S. Fukuyama, H. Yoshida, A. Moriwaki, T. Matsumoto, K. Matsumoto, Y. Asai, M. Kubo, A. Yoshimura, Y. Nakanishi, Effects of a Janus kinase inhibitor, pyridone 6, on airway responses in a murine model of asthma, *Biochem Biophys Res Commun* 404(1) (2011) 261-7.

Figure legends

Figure 1. Differential expression and phosphorylation of STAT1 in eosinophils from allergic and healthy non-allergic donors. (A) Purified eosinophils from allergic ($n=10$) and healthy donors ($n=12$) were fixed and permeabilized, stained with the respective isotype control or specific antibodies against total-STAT1 and phospho-STAT1 and measured by flow cytometry. Data were analyzed as fold increase in fluorescence intensity with respect to the isotype control and reported as phospho-STAT1/total-STAT1 (mean $\pm$ SEM). ** $P<0.01$; student's t-test. (B-D) Purified eosinophils from healthy donors were pretreated with (B) vehicle ($n=7$), (C) baricitinib (BARI, 200 nM, $n=6$) or (D) tofacitinib (TOFA, 200 nM, $n=6$) for 30 min at 37 °C, stimulated with vehicle or IL-5 (300 pM) for 10 min at 37 °C, fixed and permeabilized, stained with isotype control or a phospho-STAT1 antibody and analyzed by flow cytometry. Data are expressed as fold increase in fluorescence intensity with respect to the isotype control (mean $\pm$ SEM). * $P<0.05$; Student's t-test.

Figure 2. Baricitinib and tofacitinib similarly inhibit eosinophil differentiation and JAK1/2 activation. Bone marrow cells were collected from the femurs and tibiae of three female BALB/c mice and cultured at 10^6 /mL in media supplemented with 100 ng/mL SCF and 100 ng/mL FLT3-Ligand from day 0 to day 4. On day 4, media were replaced with media containing 10 ng/mL IL-5 supplemented with or without baricitinib (BARI, 200 nM) or tofacitinib (TOFA, 200 nM). (A) Total cells, (B) Siglec F positive cells and (C) CCR3 positive cells were enumerated by flow cytometry on the indicated days. On day 12 (D) JAK1 and (E) JAK2 phosphorylation was measured by flow cytometric intracellular staining. Data are shown as mean $\pm$ SEM from 3 independent experiments, ** $P<0.01$, *** $P<0.005$; (A-C) two-way ANOVA and (D and E) one-way ANOVA.

Figure 3. Effects of baricitinib and tofacitinib on eosinophil migratory responsiveness. (A)

Purified eosinophils were seeded on L-lysine coated chamber slides and pre-treated with baricitinib (200 nM), tofacitinib (200 nM) or vehicle for 30 min at room temperature. F-actin polymerization was induced by stimulation with IL-5 (1 nM) at 37 °C for 15 min. Adherent cells were fixed, permeabilized and blocked prior to f-actin staining with Phalloidin-Texas Red. Slides were mounted with mounting media with DAPI and fluorescence images were taken by an Olympus IX70 microscope connected with a Hamamatsu ORCA-ER digital camera. Olympus xcellence® imaging analysis software 1.1 was used for acquiring images. Representative pictures of 4 independent experiments are shown. (B-E) Purified eosinophils ($n=4-16$) were pretreated with vehicle, baricitinib (BARI, 200 nM or 500 nM) (B and D) or tofacitinib (TOFA, 200 nM or 500 nM) (C and E) for 30 min at 37 °C and were allowed to migrate towards eosinophil chemoattractants: IL-5 300 pM, complement 5a 10 nM, CCL11 10 nM and PGD₂ 30 nM for 60 min in a 48-well micro-Boyden chamber. Migrated cells were enumerated by flow cytometry (BD FACSCanto II, high flow-rate for 30 seconds). Data are shown as mean±SEM. * $P<0.05$, ** $P<0.01$, *** $P<0.005$; Student's t-test.

Figure 4. Effects of baricitinib and tofacitinib on neutrophil chemotaxis. PMNL ($n=14$)

were pretreated with vehicle, baricitinib (BARI, 500 nM) or tofacitinib (TOFA, 500 nM) for 30 min at 37 °C and were allowed to migrate towards neutrophil chemoattractants: IL-8 (10 nM) and complement 5a (10 nM) for 60 min in a 48-well micro-Boyden chamber. Migrated cells were enumerated by flow cytometry (BD FACSCanto II, high flow-rate for 30 minutes). Data are shown as mean±SEM. * $P<0.05$; Student's t-test.

Figure 5. Effects of baricitinib and tofacitinib on eosinophil ROS production. Purified

eosinophils were pretreated with vehicle, baricitinib (BARI, 500 nM) or tofacitinib (TOFA, 500

nM) for 30 min at 37 °C and ROS production was induced by IL-5 (A, $n=4-8$), complement 5a (B, $n=6$), CCL11 (C, $n=5-8$) and PGD₂ (D, $n=7-9$). ROS production was detected by flow cytometry using a dihydrorhodamin-123 based assay. Data are expressed as geometric mean fluorescence intensity (MFI) and shown as mean $\pm$ SEM. * $P<0.05$, ** $P<0.01$, *** $P<0.005$; two-way ANOVA.

Figure 6. Effects of baricitinib and tofacitinib on eosinophil phagocytosis and degranulation. Purified eosinophils were pre-treated with vehicle, baricitinib (500 nM and 2.5 μ M) (A, $n=4$) or tofacitinib (500 nM and 2.5 μ M) (B, $n=4$) for 30 min at 37 °C, incubated with opsonized *Escherichia coli*-FITC and analyzed by flow cytometry. Phagocytosis is presented as the percentage of *E. coli*-FITC-positive eosinophils. Data are shown as mean $\pm$ SEM. * $P<0.05$, ** $P<0.01$; One-way ANOVA. (C) Purified eosinophils were pretreated with vehicle, baricitinib or tofacitinib (500 nM) for 30 min at 37 °C and then stimulated with complement 5a for 30 min at 37 °C. The release of EPO activity into the supernatant was determined by photometry. Data were expressed as a percentage of the vehicle response (unstimulated) and are shown as the mean $\pm$ SEM; $n=14$. * $P<0.05$, two-way ANOVA.

Figure 7. Effects of baricitinib and tofacitinib on eosinophil apoptosis. Purified eosinophils were cultured in vehicle or IL-5 100 pM-treated medium, supplemented with or without baricitinib (BARI, 500 nM) or tofacitinib (TOFA, 500 nM) for 18 h. Apoptosis and necrosis were detected by flow cytometry using FITC-annexin-V/propidium iodide (PI). annexin/PI double-negative cells were identified as live cells (A), annexin positive/PI negative cells as early apoptotic cells (B), annexin/PI double-positive cells as late apoptotic cells and annexin negative/PI positive cells as necrotic cells (D). Data are expressed as % of total cells and shown

as mean $\pm$ SEM of 11 independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$; two-way ANOVA.

Figure 8. Effects of baricitinib and tofacitinib on HDM-induced lung inflammation and airway hyperresponsiveness. (A) On day 1, eight-week-old female BALB/c mice were sensitized intranasally with 1 μ g HDM and challenged by intranasal application of 10 μ g HDM per day from day 7 to 11. From day 8 to 14 mice received intragastric applications of tofacitinib (TOFA, 50 μ mol, twice a day) and baricitinib (BARI, 50 μ mol, once a day). (B) Gating strategy for lung single cell suspensions. After single cell and CD45^{pos} gating, eosinophils were identified as CD11c^{neg}/Siglec F^{pos} and alveolar macrophages as CD11c^{pos}/Siglec F^{pos} cells. CD11c/Siglec F double negative cells were characterized as CD11b^{pos}/Ly6G^{pos} neutrophils, CD11b^{pos}/Ly6G^{neg} monocytes and FSC/SSC^{low} CD11b^{neg}/Ly6G^{neg} lymphocytes. On day 15, (C) lungs, (D) spleens, and (E) bone marrow were taken from six mice per group and inflammatory cells were analyzed by flow cytometry: Total CD45^{pos} cells, Siglec F^{pos}/CD11c^{neg} eosinophils, CD11c^{neg}/CD11b^{pos}/Ly6G^{neg} monocytes, Siglec F^{pos}/CD11c^{pos} alveolar macrophages, CD11c^{neg}/CD11b^{pos}/Ly6G^{pos} neutrophils, and SSC/SSC^{low} CD11b^{neg}/CD11c^{neg}/Siglec F^{neg} lymphocytes. Data are shown as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$; one-way ANOVA. (F) On day 15, respiratory system resistance and compliance in response to methacholine (1-100 μ g/mL) were recorded in 7-10 mice per group using the flexiVent system.

Figure 1

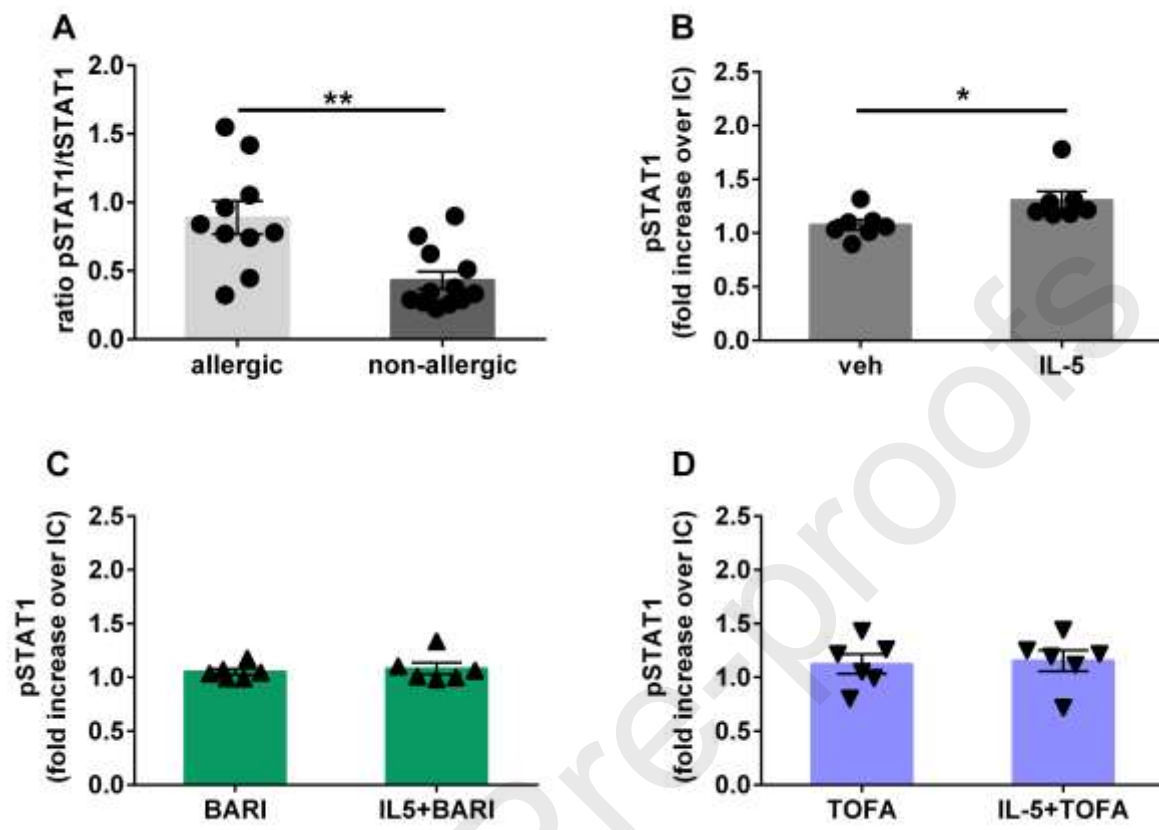


Figure 2

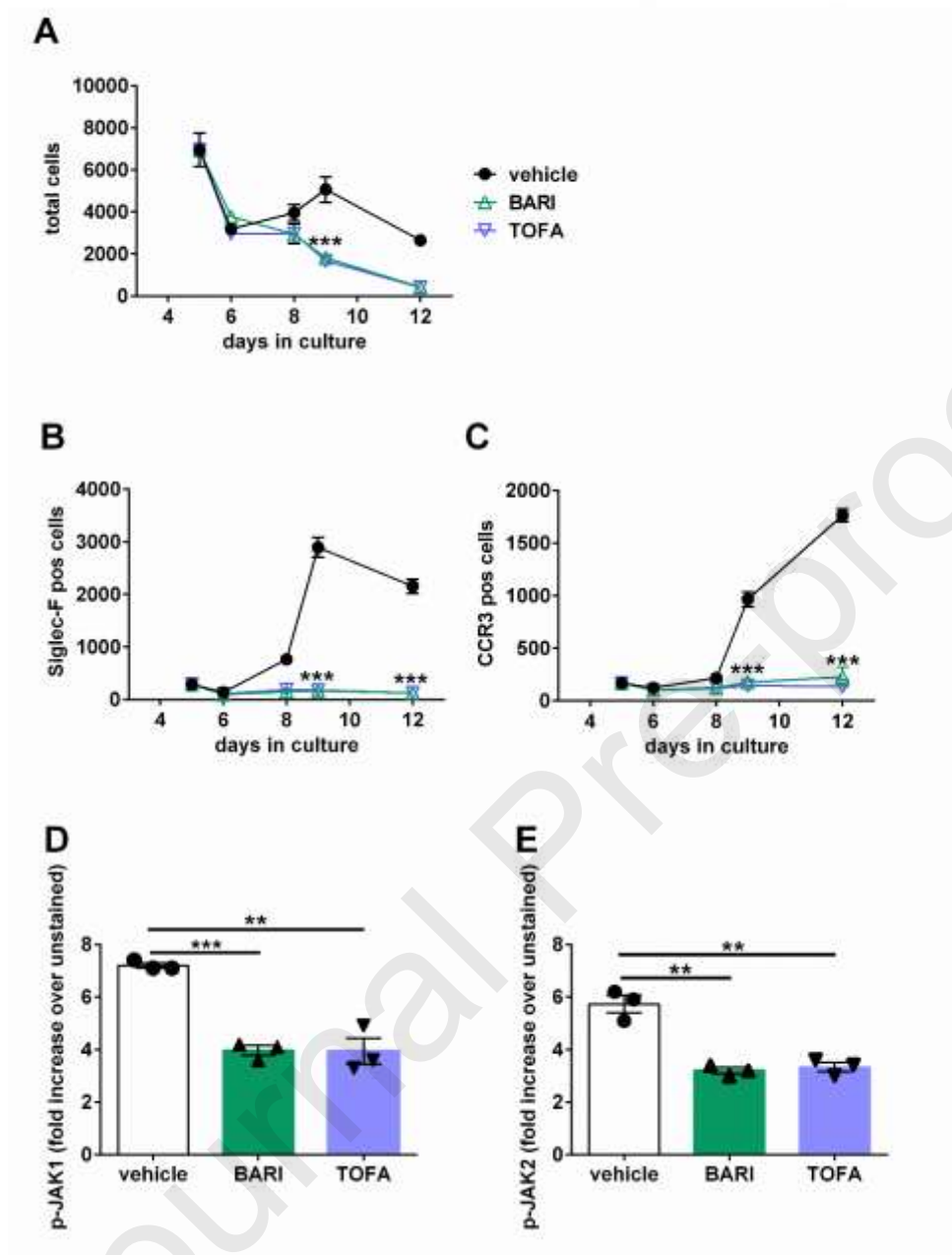


Figure 3

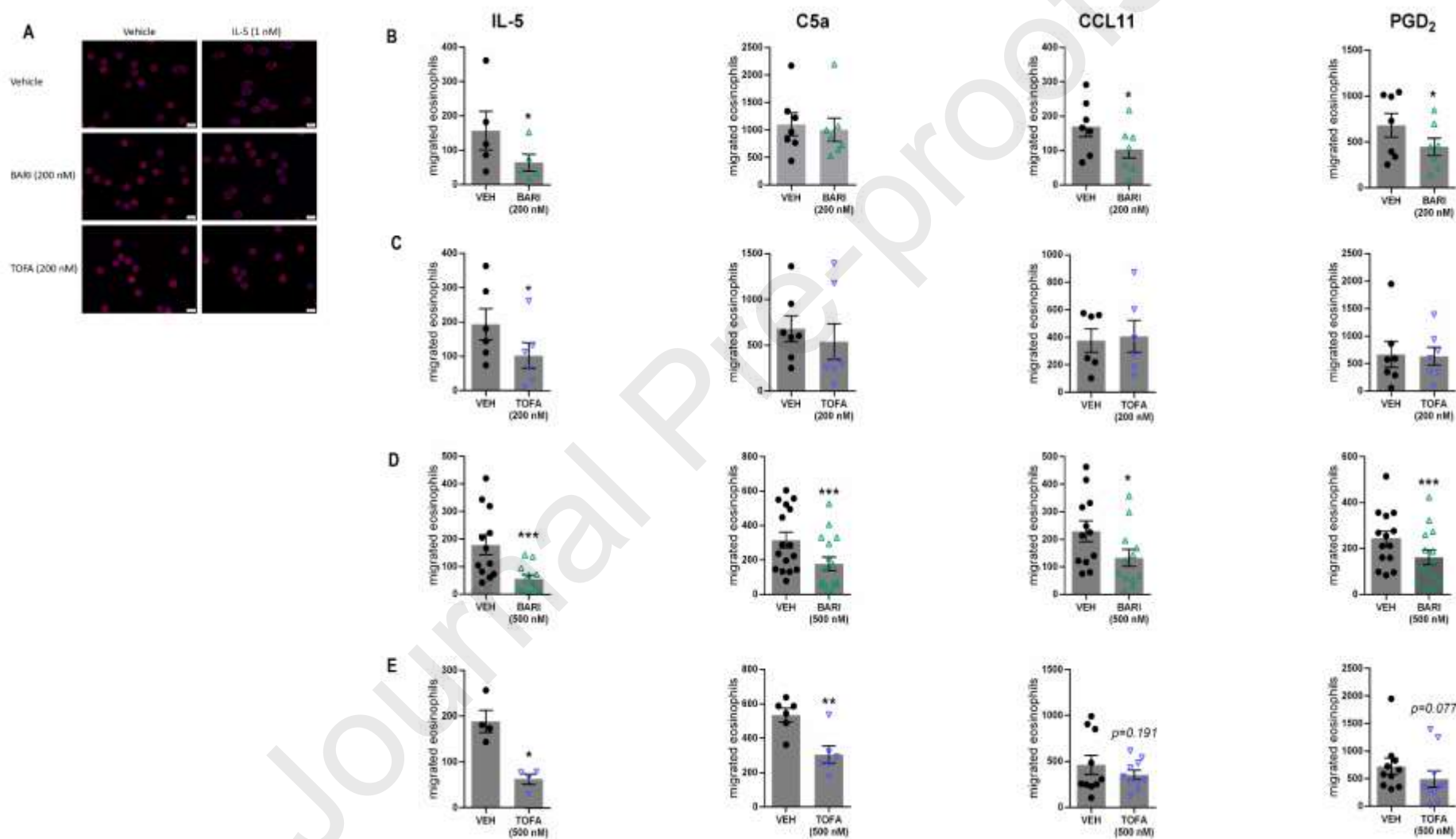


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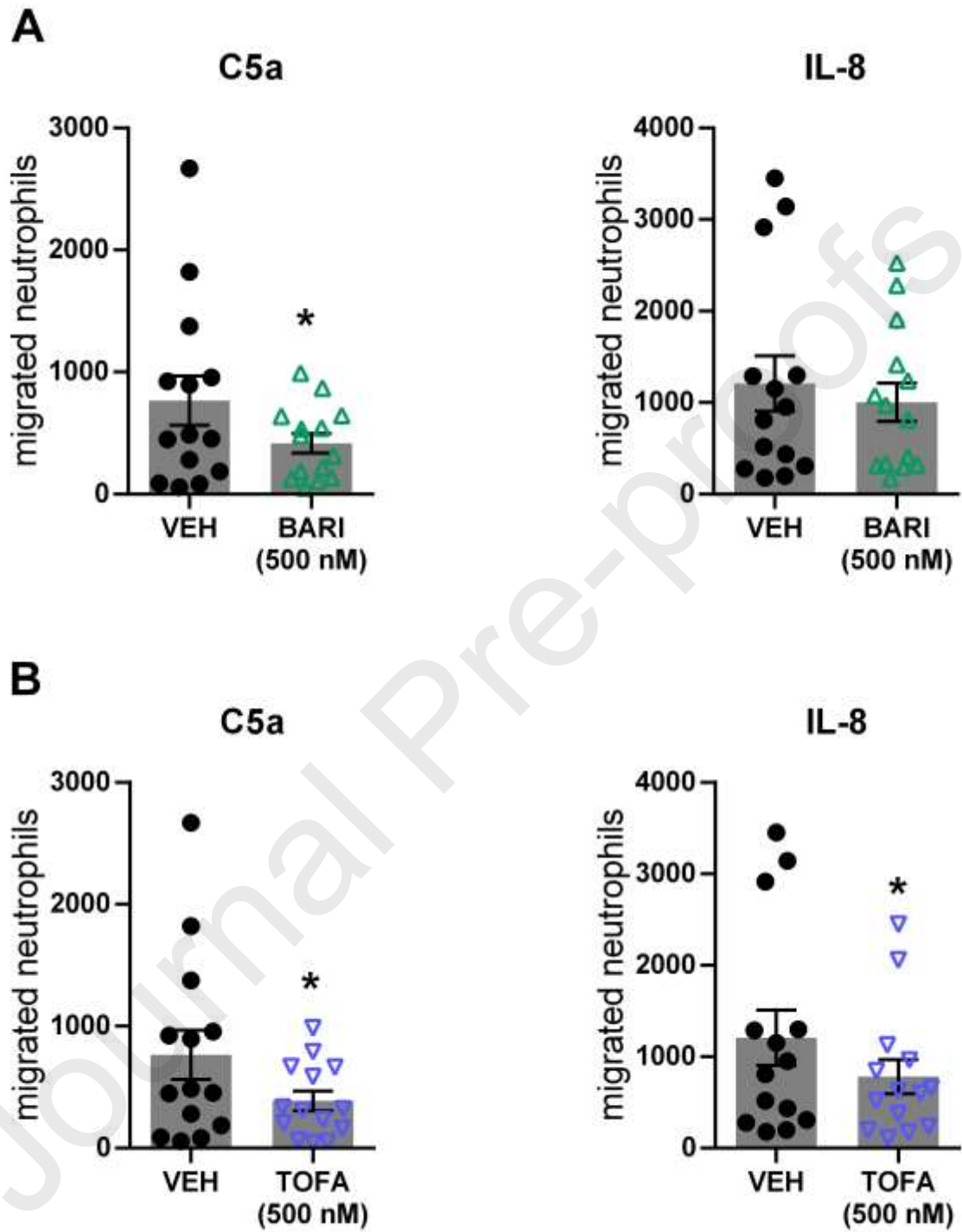


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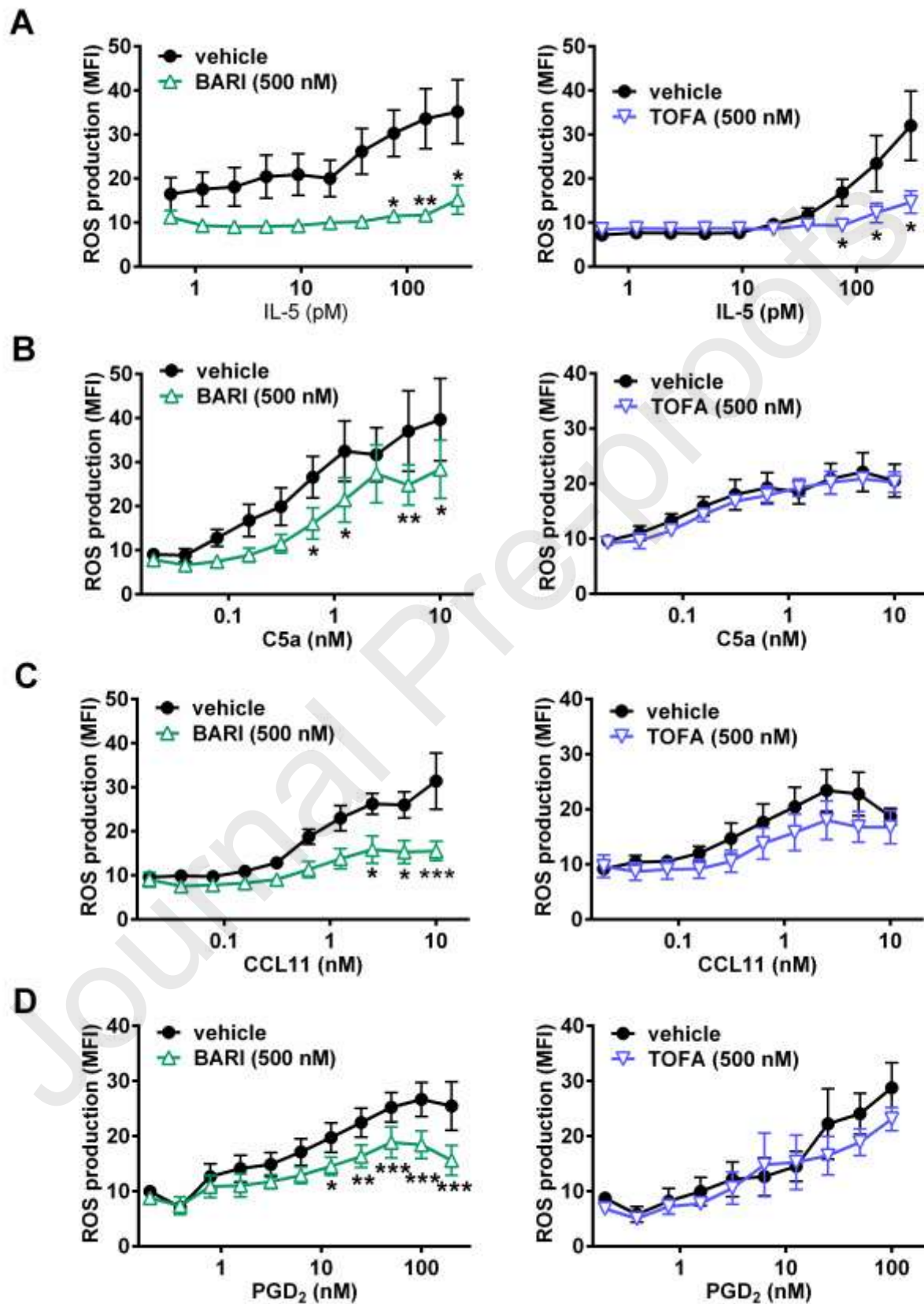


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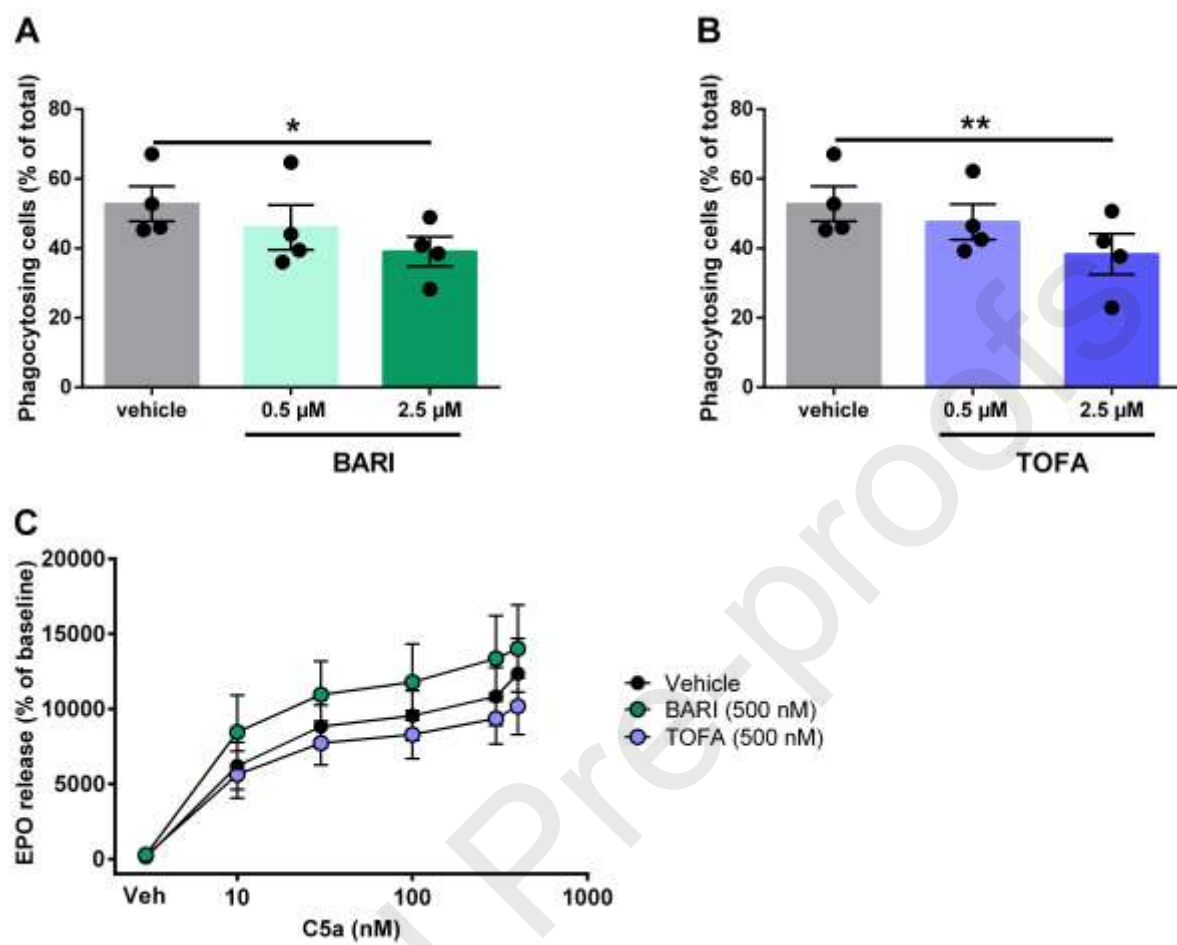


Figure 7

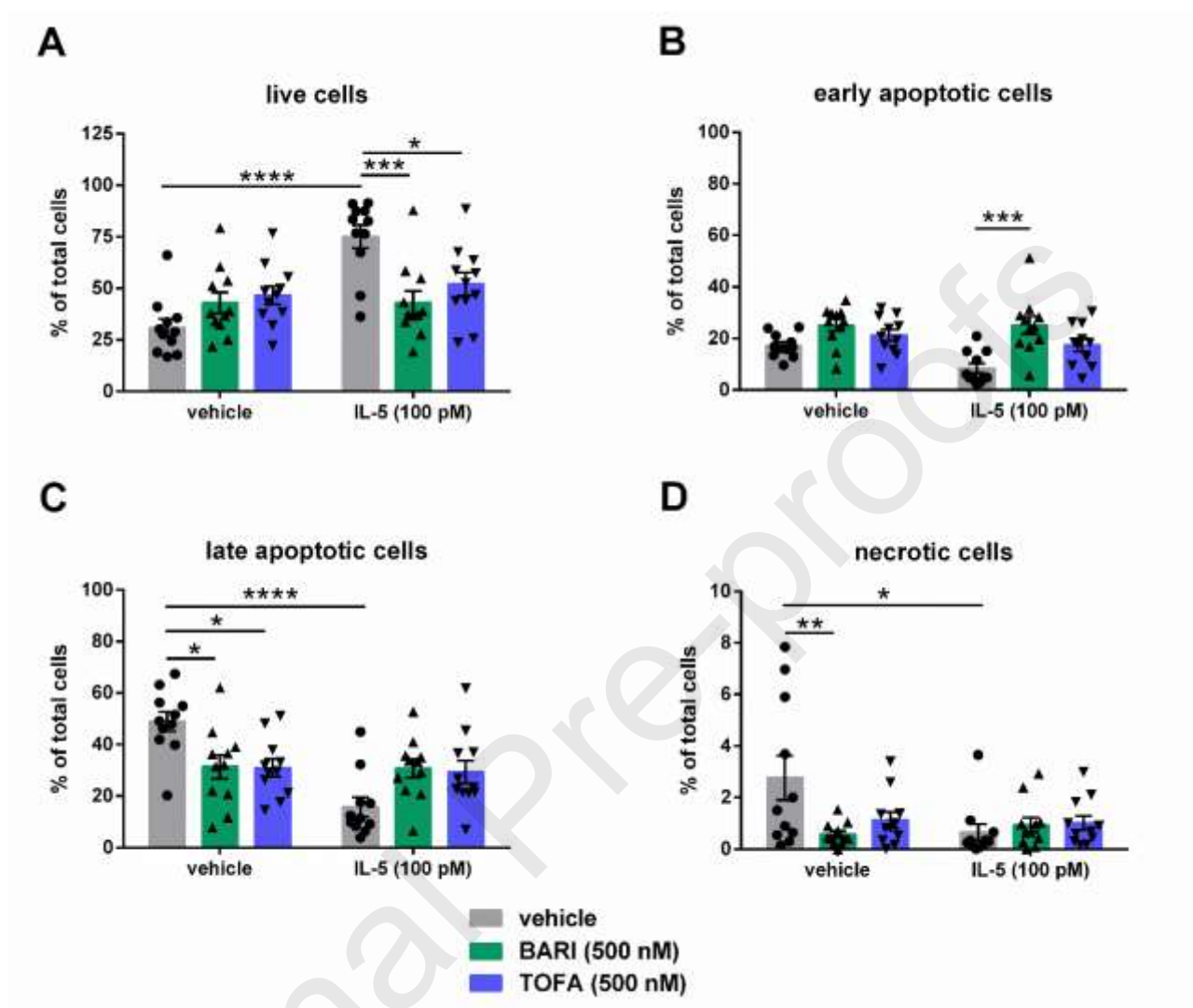
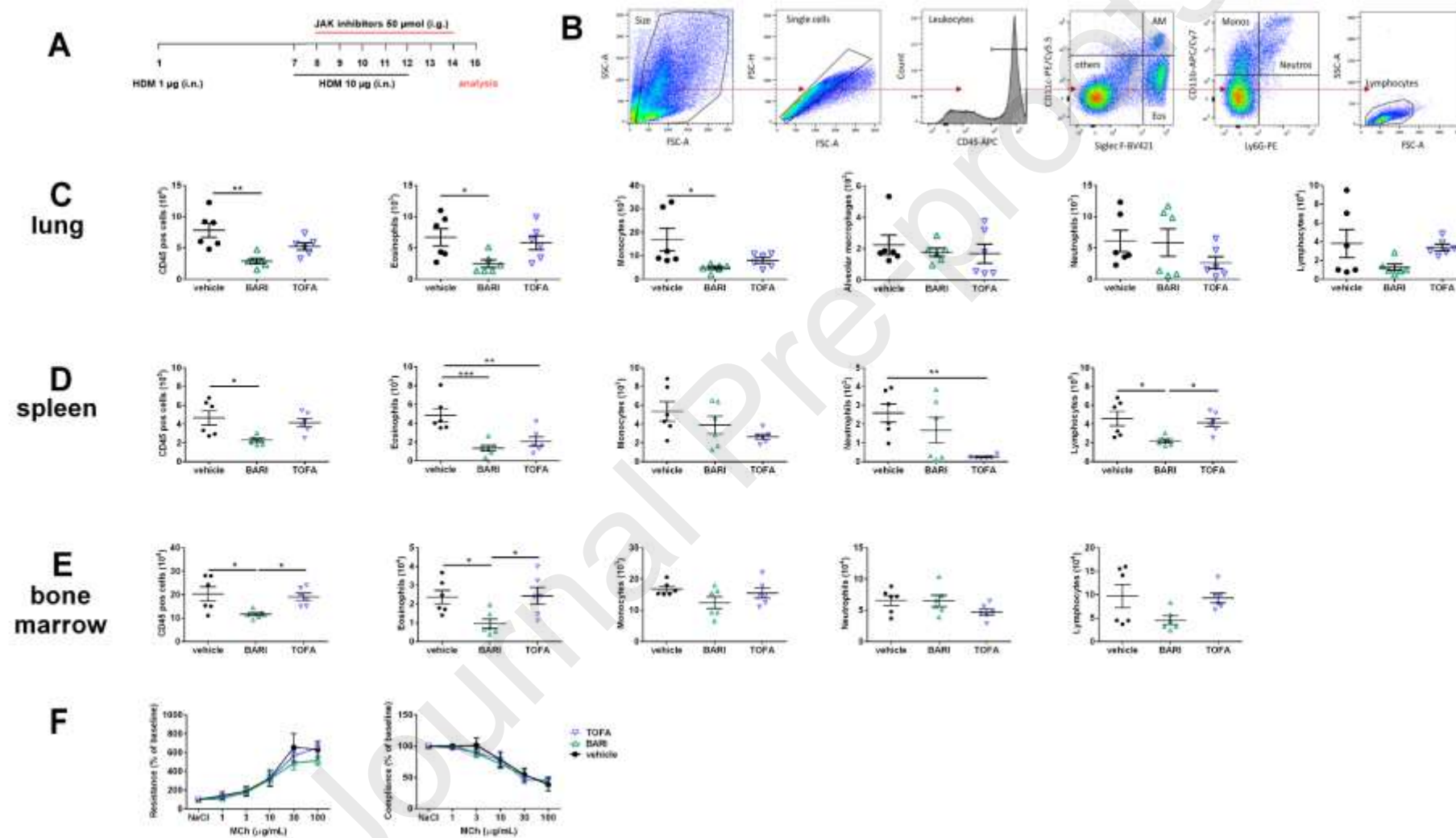
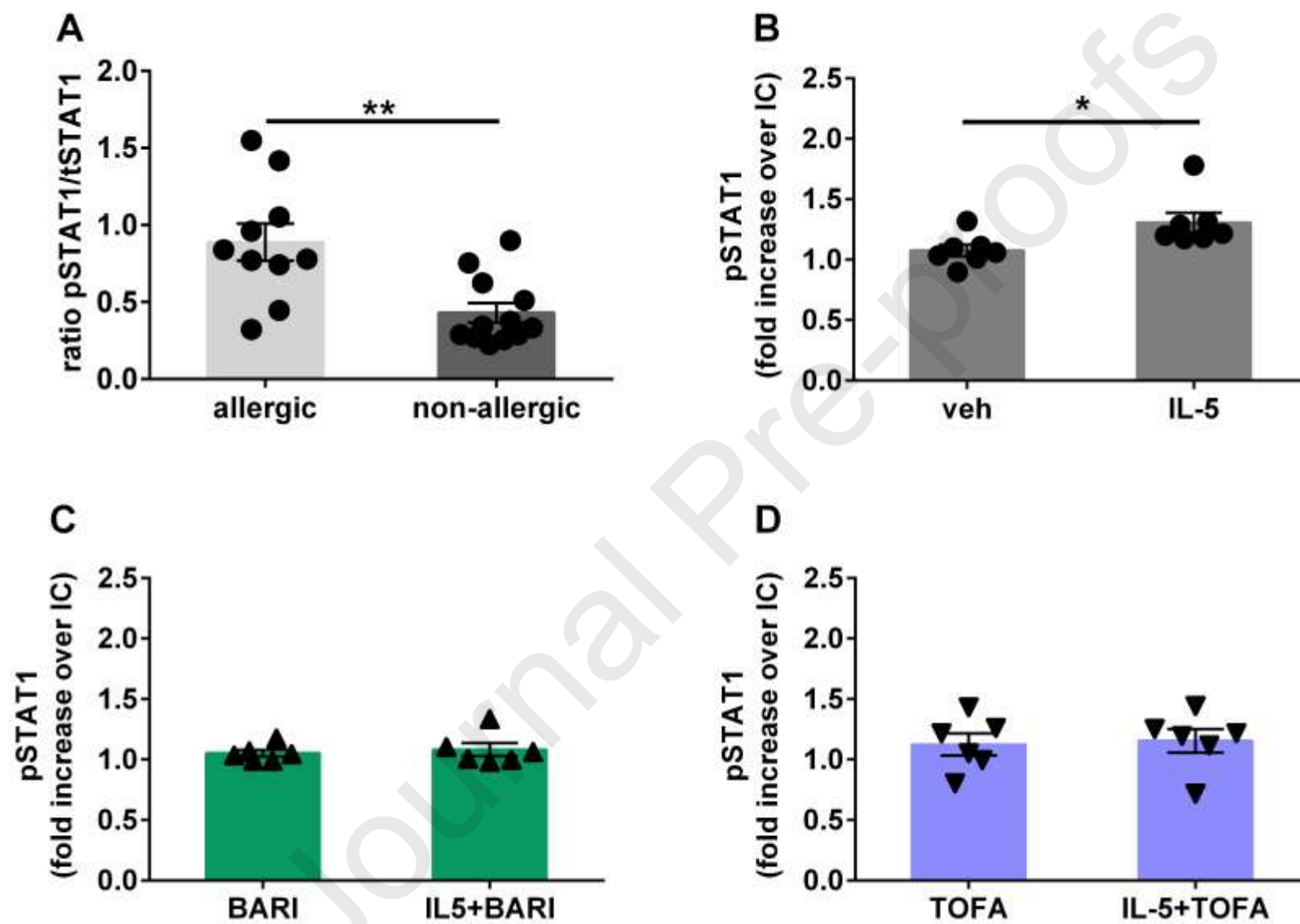
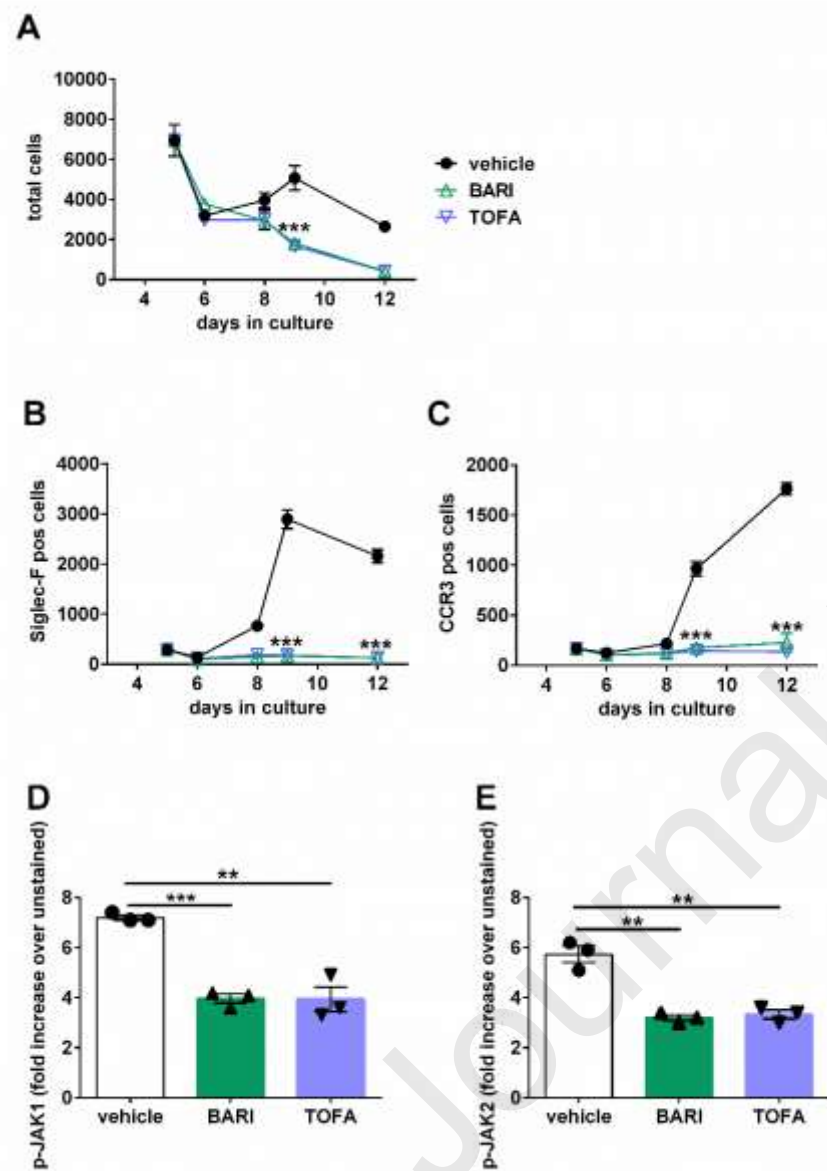
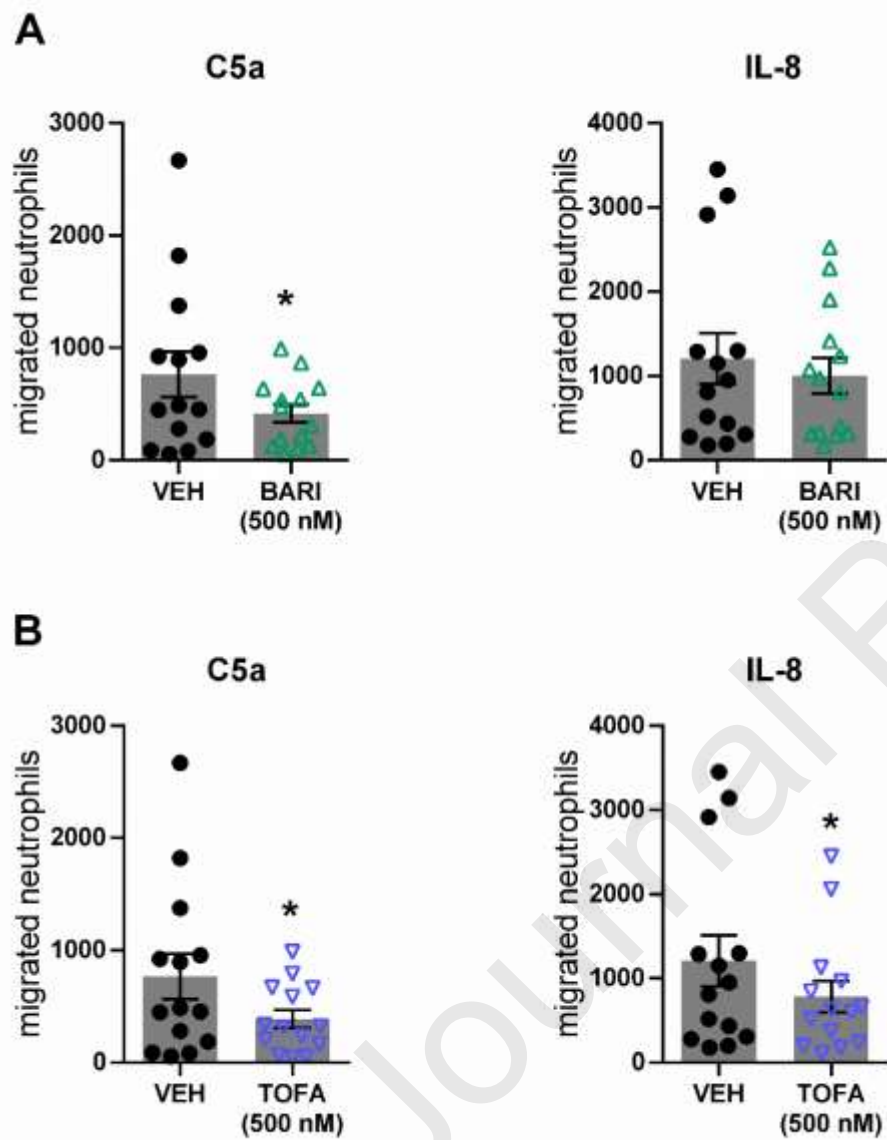


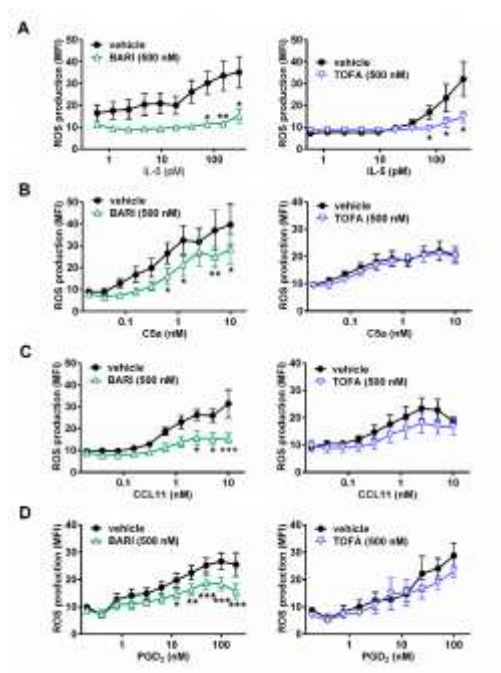
Figure 8

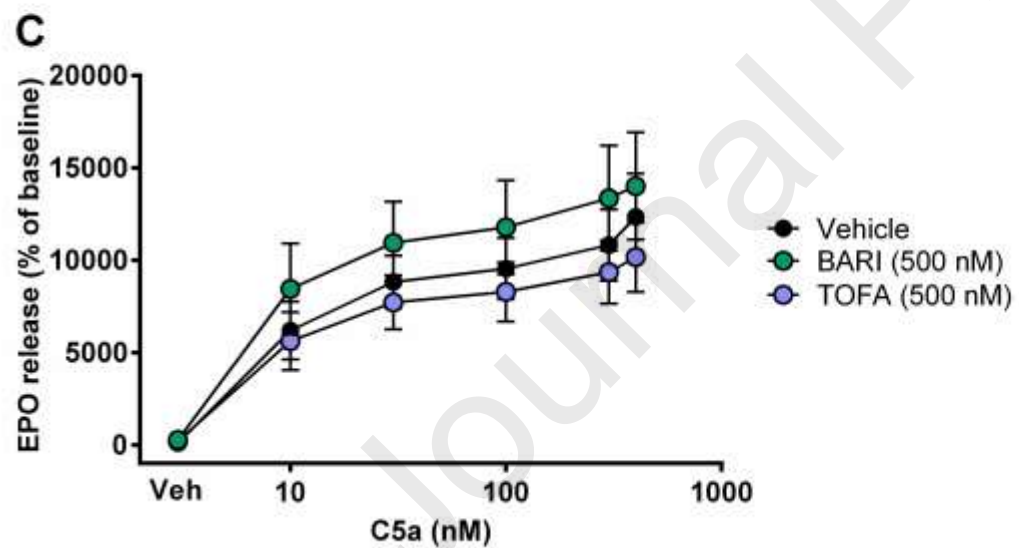
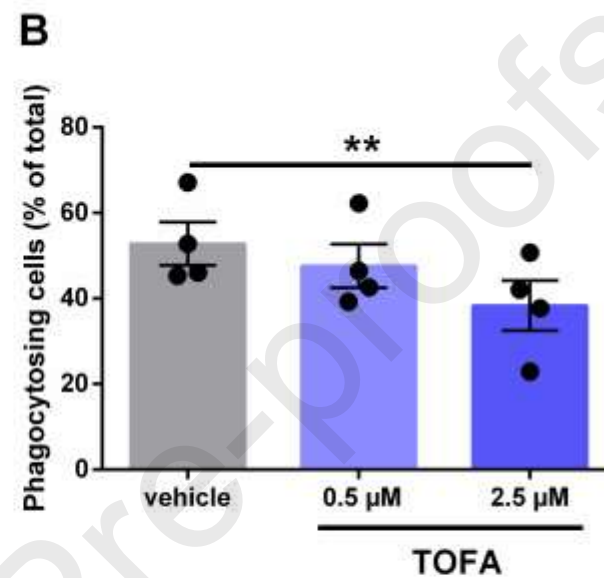
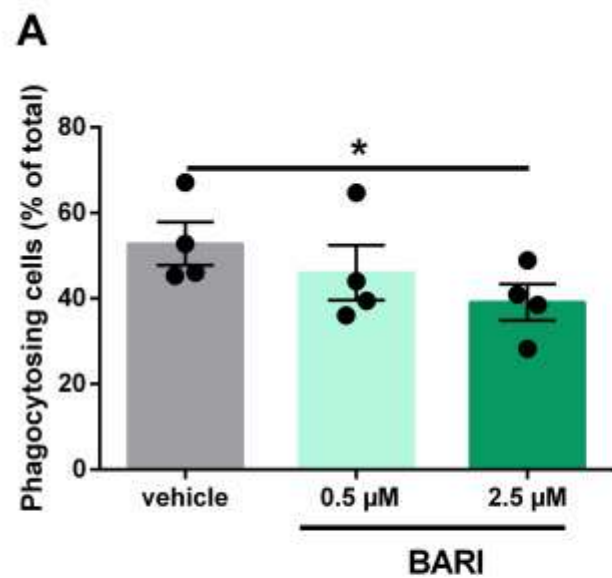


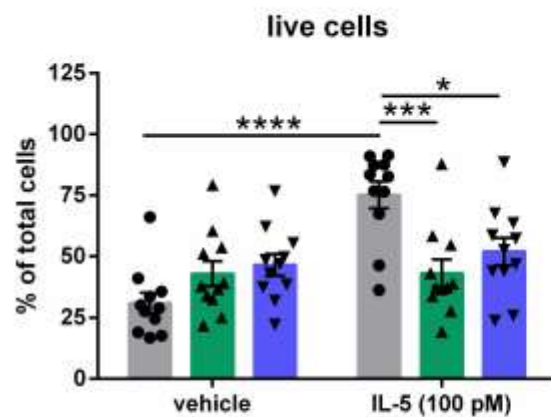
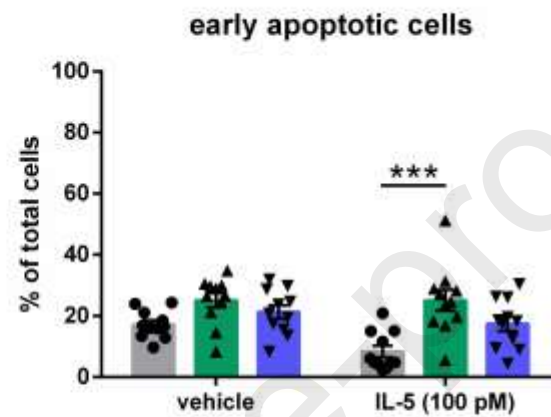
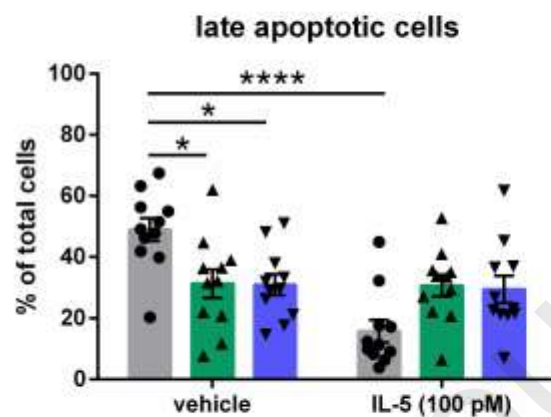
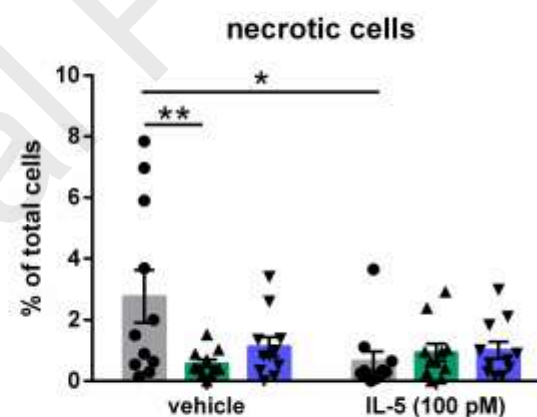




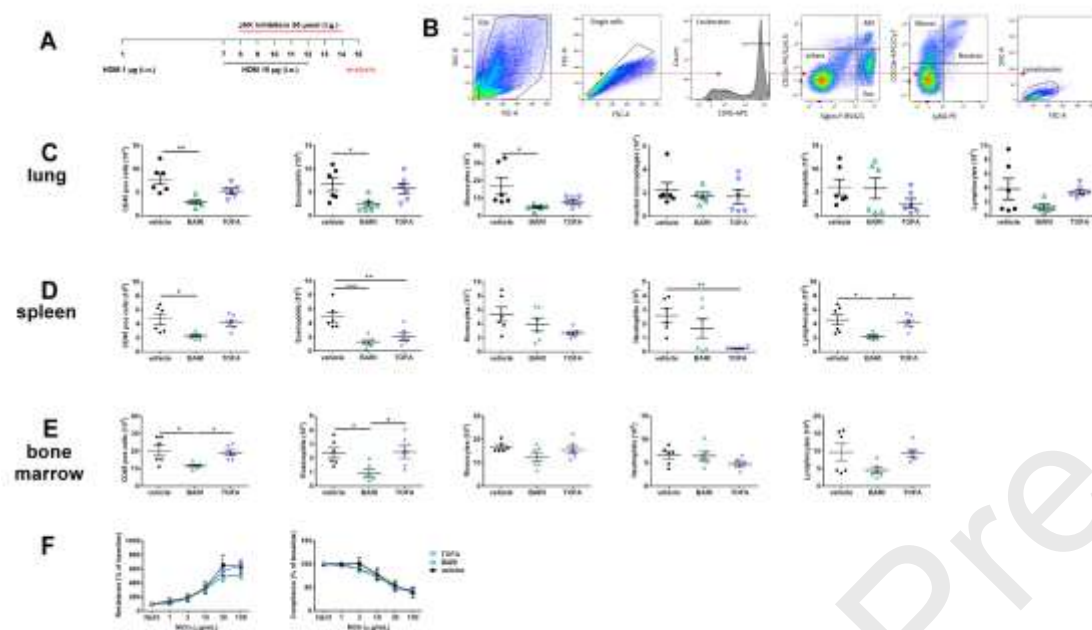


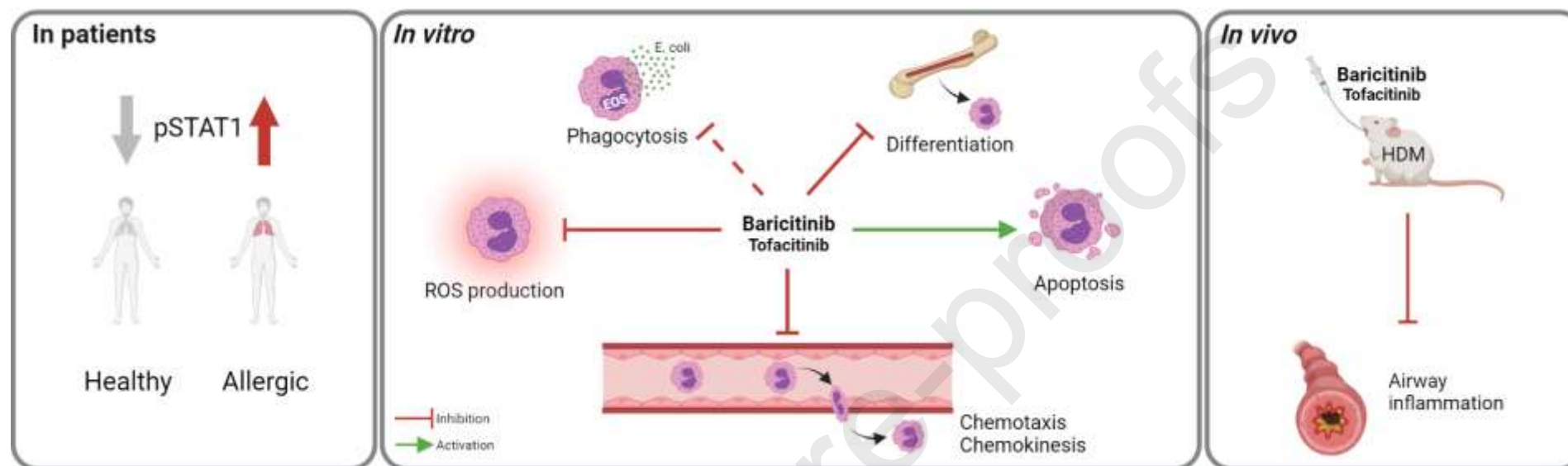




A**B****C****D**

vehicle
BARI (500 nM)
TOFA (500 nM)





Credit author statement

Petra Luschnig: Methodology, Investigation, Writing- Reviewing and Editing. **Melanie Kienzl:** Investigation, Formal Analysis, Writing- Reviewing and Editing. **David Roula:** Investigation, Writing- Reviewing and Editing. **Johannes Pilic:** Investigation, Writing- Reviewing and Editing. **Reham Atallah:** Investigation, Writing- Reviewing and Editing. **Akos Heinemann:** Funding Acquisition, Supervision, Writing- Reviewing and Editing. **Eva M. Sturm:** Conceptualization, Methodology, Investigation, Validation, Formal Analysis, Funding Acquisition, Supervision, Writing- Original draft, Writing- Reviewing and Editing.

Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Akos Heinemann received consultancy fees from AstraZeneca and Bayer. All other authors declare no conflicts of interest.