



Inhibition of Bruton's tyrosine kinase and IL-2 inducible T-cell kinase suppresses both neutrophilic and eosinophilic airway inflammation in a cockroach allergen extract-induced mixed granulocytic mouse model of asthma using preventative and therapeutic strategy

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ABSTRACT

Asthma is a complex airways disease with a wide spectrum which ranges from eosinophilic (Th2 driven) to mixed granulocytic (Th2/Th17 driven) phenotypes. Mixed granulocytic asthma is a cause of concern as corticosteroids often fail to control this phenotype. Different kinases such as Bruton's tyrosine kinase (BTK) and IL-2 inducible T cell kinase (ITK) play a pivotal role in shaping allergic airway inflammation. Ibrutinib is primarily a BTK inhibitor, however it is reported to be an ITK inhibitor as well. In this study, we sought to determine the effect of Ibrutinib on Th1, Th17 and Th2 immune responses in a cockroach allergen extract (CE)-induced mixed granulocytic (eosinophilic and neutrophilic) mouse model in preventative mode. Ibrutinib attenuated neutrophilic inflammation at a much lower doses (25–75 µg/mouse) in CE-induced mixed granulocytic asthma whereas Th2/Th17 immune responses remained unaffected at these doses. However, at a much higher dose, i.e. 250 µg/mouse, Ibrutinib remarkably suppressed both Th17/Th2 and lymphocytic/neutrophilic/eosinophilic airway inflammation. At molecular level, Ibrutinib suppressed phosphorylation of BTK in neutrophils at lower doses and ITK in CD4 + T cells at higher doses in CE-treated mice. Further, effects of Ibrutinib were compared with dexamethasone on CE-induced mixed granulocytic asthma in therapeutic mode. Ibrutinib was able to control granulocytic inflammation along with Th2/Th17 immune response in therapeutic mode whereas dexamethasone limited only Th2/eosinophilic inflammation. Thus, Ibrutinib has the potential to suppress both Th17/Th2 and neutrophilic/eosinophilic inflammation during mixed granulocytic asthma and therefore may be pursued as alternative therapeutic option in difficult-to-treat asthma which is resistant to corticosteroids.

1. Introduction

Asthma is a chronic disorder of the airways in which there is airway inflammation and reversible airflow obstruction. Airway inflammation is characterized by increased expression of a number of cytokines, chemokines, and various other inflammatory mediators which together cause remodeling of the airways through resident/infiltrating immune cells such as dendritic cells (DCs) and neutrophils [1,2]. Numerous research documentations have pointed out that classical asthma is predominantly a Th2-mediated inflammatory disorder, however other

inflammatory cytokines such as IL-6, IL-17A, and TNF-α play a significant role in mixed granulocytic asthma [1–4].

Bruton's Tyrosine Kinase (BTK) and IL-2 inducible T cell kinase (ITK) are well known members of Tec tyrosine kinase family which participate in B cell receptor (BCR)/toll-like receptor (TLR), and T cell receptor (TCR) signaling respectively. BTK and ITK are present in B cells/myeloid cells and T cells respectively [5–7]. The activation of BCR/TCR results in the phosphorylation of BTK/ITK with the aid of other kinases. BTK and ITK in their inactive forms are present in the cytoplasm but on activation, they migrate to the cell membrane [6,7].

Abbreviations: BAL, Bronchoalveolar lavage; BTK, Bruton's tyrosine kinase; CE, Cockroach extract; i.n., Intranasal; ITK, IL-2 inducible T cell kinase; STAT3, signal transducer and activator of transcription 3

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Upon activation, BTK/ITK gets phosphorylated thereby causing induction of downstream events like the proliferation/differentiation in innate (neutrophils) and adaptive (B/T cells) immune cells through numerous transcription factors, e.g., NF κ B, NFAT, STAT3, etc. [6,8–10]. On this basis, both of these kinases are important biological targets for therapeutic intervention in many disorders which include inflammatory diseases and B cell malignancies. For example, BTK and ITK inhibitors have shown amelioration of inflammatory pathways associated with pathogenesis of rheumatoid arthritis, systemic lupus erythematosus and acute lung injury [11–15]. However, role of combined inhibition of BTK and ITK in innate and adaptive immune cells respectively in the context of mixed granulocytic asthma has not been explored earlier.

Corticosteroids are well established and most effective drugs to control the symptoms of Th2-driven asthma [16–18]. However, there are other forms of asthma which may be resistant to corticosteroids which include neutrophilic as well as mixed granulocytic asthma phenotypes. Both neutrophils and eosinophils release a myriad set of inflammatory mediators that are responsible for the development of mixed granulocytic asthma [19,20]. All forms of asthma exacerbation and corticosteroid-resistant asthma are connected to the growing numbers of neutrophils along with concomitant presence of eosinophils in bronchoalveolar lavage fluid, sputum or lung biopsies from asthma patients [4,20]. This is supposedly due to generation of oxidants by neutrophils which are thought to be responsible for corticosteroids insensitivity [21–23]. Therefore, there is a need for alternative therapies that can attenuate both neutrophilic and eosinophilic airway inflammation concomitantly. In this context, combined inhibition of ITK/BTK may be an attractive therapeutic approach to treat mixed granulocytic asthma.

Ibrutinib is a well-known inhibitor of BTK, however it has also been reported to cause inhibition of ITK as well at a relatively higher concentration. Ibrutinib has been used against B cell malignancies as well as inflammatory/autoimmune disorders such as rheumatoid arthritis, lupus, acute lung injury [24–27]. However, its mechanism of action was mostly reported to be based on modification of signaling functions either in neutrophils or B cells. In this study, we wanted to assess the effect of Ibrutinib on both innate neutrophils and T cells (Th1/Th17) during mixed granulocytic asthmatic inflammation.

In order to understand the relevance of Ibrutinib in mixed granulocytic asthma, we employed cockroach allergen extract-induced mouse model which exhibits both Th2/Th17 endotype and mixed granulocytic inflammation, i.e. neutrophils/eosinophils [28]. Our results point to an inhibitory effect of Ibrutinib on both BTK and ITK signaling in neutrophils and CD4 + T cells respectively, thereby causing suppression of Th17/Th2 as well as neutrophilic/eosinophilic inflammation in airways. These results suggest that the combined inhibition of BTK/ITK by Ibrutinib may be a potential alternative therapy against mixed granulocytic asthma.

2. Methods

2.1. Animals

Male Balb/c mice, 8 to 10 weeks of age (20–25 g), free of specific pathogens, were used in the experiments. These mice were obtained from Experimental Animal Care Center, College of Pharmacy, King Saud University. The mice were housed under standard laboratory conditions of 12-h light-dark cycle and 24–26 °C ambient temperature. All experimental/ethical protocols were approved by the Animal Care and Research Committee of College of Pharmacy, King Saud University.

2.2. Cockroach allergen extract (CE)-induced mixed granulocytic asthma model

Cockroach extract (CE)-induced airway inflammation having both eosinophilic as well as neutrophilic inflammation was developed as

described earlier [28]. Briefly, mice were sensitized intranasally (i.n.) with 50 μ g whole-body German cockroach (*Blattella germanica*) extract (CE) from Greer Laboratories (Lenoir, NC) under light anesthesia through days 1–5. After 10 days of 1st sensitization, the mice were instilled 50 μ g CE/20 μ l saline twice daily (morning and evening) for 7 days (from day 11 to 17). Non-sensitized control animals received only saline with the same volumes.

To assess the role of Ibrutinib on BTK/ITK signaling and CE-induced airway immune responses in preventative mode, Ibrutinib (Tocris, UK) was administered i.n. once every day before morning CE challenge at a concentration of 25–250 μ g/mouse (dissolved in 20 μ l dimethylsulfoxide) for 7 consecutive days starting day 11.

For comparative purpose in therapeutic mode, Ibrutinib and dexamethasone were administered, i.n. at 250 μ g/mouse and 25 μ g/mouse respectively for 5 consecutive days starting day 13 once daily before morning CE challenge.

2.3. Bronchoalveolar lavage (BAL)

The mice were sacrificed by deep isoflurane anesthesia after 12 h of last allergen challenge followed by BAL procedure through cannulation of trachea as stated elaborately before [28]. Recovered BAL cells were counted in a hemocytometer chamber followed by differential count of at least 300 cells on cytocentrifuged Diff-Quik stained slides. Data are expressed as mean $\pm$ SEM/ml for each group.

2.4. Flow cytometry

For detection Th1/Th2/Th17/neutrophilic markers in different pulmonary immune cells, single-cell suspensions of lung were prepared as detailed earlier [28,29]. Cells were first labeled with antibodies against cell surface markers such as CD4, Ly6G conjugated to FITC/PE/APC (BioLegend, USA). After wash, cells were then fixed/permeabilized for intracellular staining as per manufacturer's protocol (BioLegend, USA). Specific antibodies against intracellular proteins such as, IL-5, IFN- γ , GATA-3, IL-17A, p-STAT3, IL-23R, iNOS, nitrotyrosine, p-BTK, p-ITK/p-BTK (p-ITK), conjugated to PE or FITC or APC (BioLegend/SantaCruz Biotech/BD Biosciences, USA) were utilized. At least 10,000 immunostained cells were acquired on a flow cytometer (Beckman Coulter, USA) and analyzed for the expression of phenotypic markers using Cytomics FC 500 software as described earlier [28,29].

2.5. Real-time PCR

Total RNA was isolated from the lungs followed by its conversion (0.5 μ g) into cDNA using High Capacity cDNA archive kit (Applied Biosystems, USA) as per to the manufacturer's protocol as detailed earlier [28,29]. mRNA levels were measured by real-time PCR analysis using the ABI PRISM 7500 sequence detection system (Applied Biosystems) using Taqman inventoried assays-on-demand probes for IL-5, GATA-3, STAT3, IL-17A, and iNOS (Applied Biosystems, USA). The fold difference in expression of target cDNA was determined using the comparative CT method as stated before [30].

2.6. Antigen recall assay in lung draining lymph nodes

Single cell suspensions were prepared from lung-draining lymph nodes at the end of the experiment, i.e. on day 18 from the groups treated preventively with Ibrutinib. Briefly, single cell suspensions were cultured in RPMI-1640 containing penicillin-streptomycin as described earlier [28]. The cells were then re-stimulated *in vitro* with CE (10 μ g/ml) for 2 days, followed by measurement of IL-17A, IL-5, and IFN- γ in the culture supernatant as described below.

2.7. Measurement of cytokines levels by ELISA

Protein levels of Th1/Th2/Th17 cytokines in the lung samples/ lymph node cell cultures were assayed using ELISA kits from Biolegend (USA), and R&D Systems (USA).

2.8. Evaluation of myeloperoxidase activity

Myeloperoxidase (MPO) activity in the pulmonary supernatants as a marker of neutrophil inflammation was carried out as detailed earlier [29].

2.9. Lung histology

Lungs were dissected from the thoracic cavity at the of the experiment and fixed in 10% phosphate buffered formalin. Thereafter, sectioning of fixed lungs (5 µm thick sections) was carried out for assessment of general inflammation and mucus production by using haematoxylin and eosin (H&E), and Periodic Acid Schiff (PAS) stains respectively. Stained sections were inspected under bright-field microscopy.

2.10. Chemicals

All chemicals were procured from Sigma Chemicals (USA) unless stated otherwise and were of the highest quality.

2.11. Statistical analysis

The data were expressed as mean $\pm$ SEM and derived from two independent experiments. Comparisons among different groups were analyzed by one-way ANOVA (analysis of variance) followed by Tukey's multiple comparison tests. A 'P' value of less than 0.05 was considered significant for all statistical tests. All the statistical analyses were performed using Graph Pad Prism statistical package.

3. Results

3.1. Effect of Ibrutinib on CE-induced mixed granulocytic airway inflammation and related parameters employing preventive strategy

As Ibrutinib is known to inhibit both BTK and ITK which are important regulators of both innate and adaptive immunity respectively, therefore we first wanted to evaluate its effects on lymphocytic, neutrophilic and eosinophilic airway inflammation in mixed granulocytic asthma model in mice. Our data show that Ibrutinib blocked neutrophilic inflammation/MPO activity at almost all doses (25–250 µg), but had limited effect on eosinophilic/ lymphocytic inflammation at lower doses, i.e. 25–75 µg (Fig. 1A–E). However, at a dose of 250 µg, Ibrutinib also remarkably attenuated eosinophilic and lymphocytic airway inflammation (Fig. 1D–E). Reduction in lymphocytic, neutrophilic and eosinophilic inflammation at the highest dose was associated with a reduction in overall inflammation as reflected by BAL counts, H&E and PAS staining (Fig. 1A, F & G). There was no significant difference in mixed airway inflammation other inflammatory markers between Ibrutinib-treated control and vehicle-treated control groups (Fig. 1A–G); therefore, Ibrutinib-treated control group was not included in further experiments. Overall, these data propose that Ibrutinib has the potential to suppress both neutrophilic and eosinophilic airway inflammatory changes during mixed granulocytic asthma model.

3.2. Effect of Ibrutinib on ITK phosphorylation and Th17/ Th2/Th1 markers during mixed granulocytic inflammation employing preventive strategy

Since Ibrutinib attenuated both neutrophilic and eosinophilic

inflammation, we next determined its effect on ITK phosphorylation during mixed granulocytic inflammation as ITK is known to control Th2 and Th17 immune responses. Our data show that Ibrutinib caused significant attenuation of CE-induced phosphorylated ITK in pulmonary CD4 + T cells at the highest dose, 250 µg but not at 25/75 µg dose (Fig. 2A & D). We next asked the question whether Ibrutinib was able to cause attenuation of Th2/Th17 immune responses. Our results reveal that Ibrutinib caused reduction in Th2 as well as Th17 cells and related markers at the highest dose, i.e. 250 µg. This was evidenced by downregulation of CE-induced GATA3, and IL-5 expression in Th2 cells by Ibrutinib at 250 µg (Fig. 2B–C & F–G). Similarly, Ibrutinib caused downregulation of CE-induced p-STAT3, IL-17A, and IL-23R expression in Th17 cells at 250 µg (Fig. 3A–C and E–G). Ibrutinib also caused marked attenuation of both IL-5 and IL-17A protein levels in lung homogenates of CE-treated mice (Fig. 2 D and 3D). We also measured Th1 related marker in the lungs of different groups (Vehicle, CE, and CE + Ibrutinib treated mice). Although, CE administration led to significant upregulation of Th1 responses as depicted by increased % of IFN- γ + CD4 + T cells and IFN- γ protein levels in pulmonary compartment; however, Ibrutinib at any dose did not cause any significant attenuation of CE-induced upregulation of IFN- γ levels (Fig. 4A and B). These data show that neutrophilic and eosinophilic inflammation at the highest dose could be a result of inhibition of Th2 and Th17 related markers which is probably due to ITK inhibition.

3.3. Effect of Ibrutinib on Th17/ Th2/Th1 related cytokines in antigen recall assay employing preventive strategy

Additionally, we explored if Th17/Th2/Th1 related markers are affected during antigen recall assay in mice preventively treated with Ibrutinib. Our data show that CE-restimulated lung draining lymph node cells from CE-sensitized and challenged mice had increased release of Th17/Th2/Th1 related cytokines, i.e. IL-17A, IL-5 and IFN- γ respectively, however Ibrutinib treatment only at the highest dose, i.e. 250 µg caused significant attenuation of IL-17A and IL-5 but not IFN- γ (Fig. 4C–E). These data further confirm *in vivo* findings and demonstrate that Ibrutinib treated allergic mice show considerable reduction in Th2 and Th17 related cytokines even during *in vitro* re-stimulation with the CE allergens.

3.4. Effect of Ibrutinib on BTK phosphorylation and oxidative stress in neutrophils during mixed granulocytic inflammation employing preventive strategy

Since there was an inhibitory effect of Ibrutinib on neutrophilic inflammation at the lower doses, i.e. 25/75 µg as well, we further evaluated its effect on BTK phosphorylation (p-BTK) and oxidative stress markers in pulmonary neutrophils. Our data show that there was remarkable upregulation of phosphorylated BTK expression in pulmonary neutrophils of CE-treated mice as reflected by an increase in p-BTK + GR-1 + cells (Fig. 5A & D). Ibrutinib caused downregulation of p-BTK levels at much lower levels (25/75 µg) than what was observed for p-ITK in CD4 + T cells (Fig. 5A & D). Next we examined the effect of Ibrutinib on iNOS/nitrotyrosine levels in neutrophils. Ibrutinib caused downregulation of iNOS/nitrotyrosine expression at much lower levels (25/75 µg) similar to inhibition of p-BTK (Fig. 5B–C & E–F). These data reveal that downregulation of p-BTK along with decrease in oxidative stress in neutrophils could be responsible for attenuated neutrophilic inflammation at lower doses of Ibrutinib during CE-induced granulocytic asthma.

3.5. Comparison of Ibrutinib with dexamethasone on mixed granulocytic airway inflammation and related parameters employing therapeutic strategy

Further to reflect the scenario of clinical situation where asthma is treated post development of airway inflammation, effect of ibrutinib

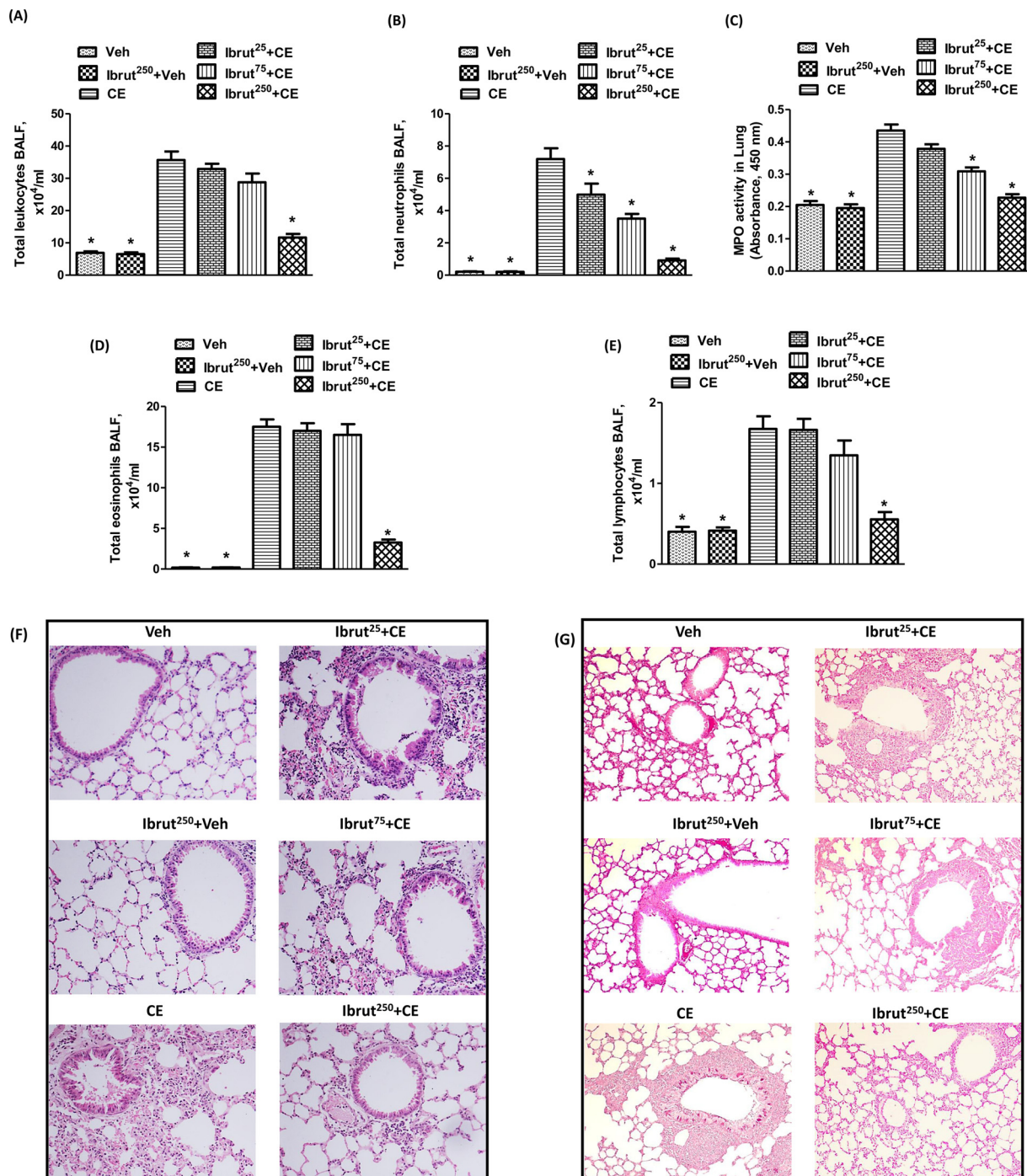


Fig. 1. Effect of different doses of Ibrutinib (BTK/ITK inhibitor) on CE-induced mixed granulocytic airway inflammation in preventive mode. (A) Total leukocytes count in BALF, (B) Neutrophilic count in BALF, (C) Pulmonary MPO activity, (D) Eosinophilic count in BALF, (E) Lymphocyte count in BALF, (F) H&E staining of the lung sections, and (G) PAS staining of the lung sections. Values are expressed as mean $\pm$ SE, $n = 9/\text{group}$ from three independent experiments. * $P < 0.05$, vs. CE group.

was compared with dexamethasone on mixed airway inflammation in therapeutic mode. Our data exhibit that Ibrutinib was able to control both neutrophilic and eosinophilic inflammation even in therapeutic model whereas dexamethasone failed to inhibit neutrophilic inflammation but was successful in limiting eosinophilic inflammation up to some extent (Fig. 6). This was evident from total leukocytic counts, neutrophilic counts, eosinophilic counts, and H&E staining (Fig. 6A–D). This confirms our above discussed observations that Ibrutinib is a potential candidate for controlling both neutrophilic and eosinophilic inflammation during mixed granulocytic asthma.

3.6. Comparison of Ibrutinib with dexamethasone on CE-induced Th17 and Th2 markers during mixed granulocytic inflammation employing therapeutic strategy

Next, we wanted to confirm if dexamethasone was able to control Th2 and Th17 immune responses similar to Ibrutinib. Our data reveal that dexamethasone controlled CE-induced Th2 immune responses but failed to control Th17 immune responses. However, Ibrutinib controlled both Th2 as well as Th17 immune responses even in the therapeutic mode (Fig. 7A–F). This was evident from reduction in CE-induced elevation in IL-5 and IL-17A protein levels by Ibrutinib (Fig. 7). Th2

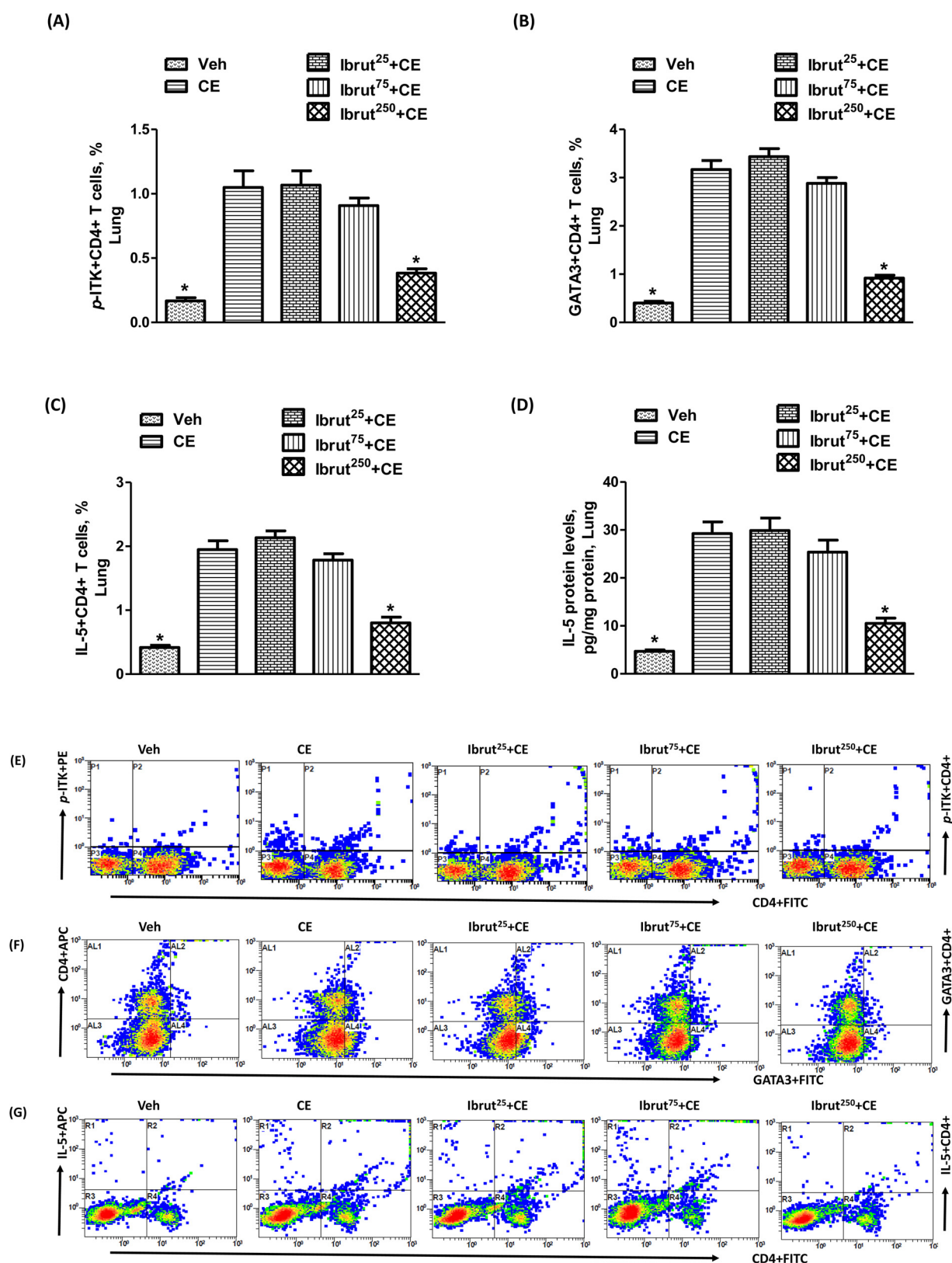


Fig. 2. Effect of different doses of Ibrutinib (BTK/ITK inhibitor) on CE-induced ITK phosphorylation and Th2 markers in pulmonary CD4+ T cells in preventive mode. (A) % p-ITK + CD4 + T cells in the lung, B) % GATA-3 + CD4 + T cells in the lung, C) % IL-5 + CD4 + T cells in the lung, D) IL-5 protein levels in the lung, E) Representative flow plot exhibiting p-ITK expression in CD4 + T cells in the lung, F) Representative flow plot exhibiting GATA3 expression in CD4 + T cells in the lung, and G) Representative flow plot exhibiting IL-5 expression in CD4 + T cells in the lung. Values are expressed as mean $\pm$ SE, $n = 6-9$ /group from three independent experiments. * $P < 0.05$, vs. CE group.

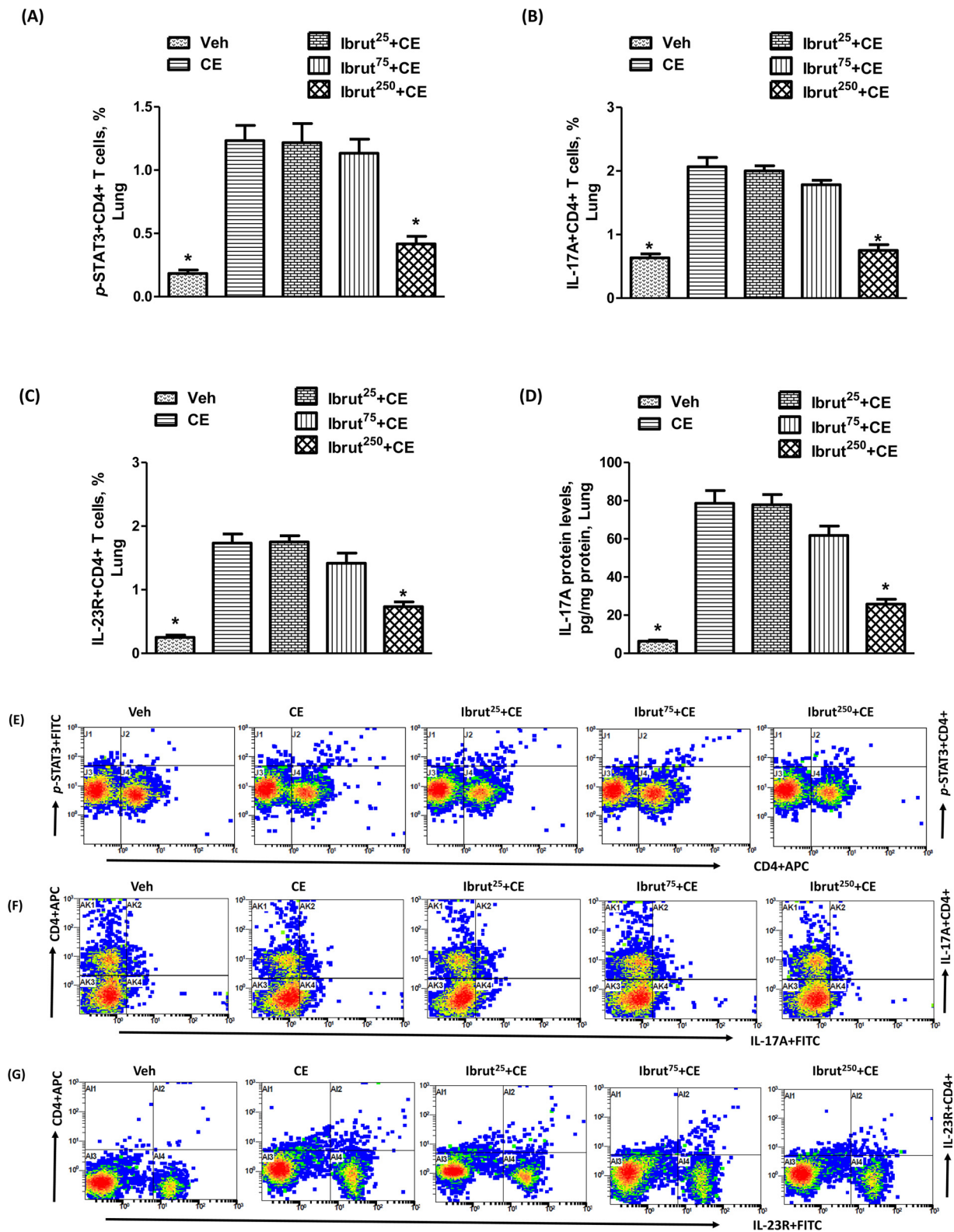


Fig. 3. Effect of different doses of Ibrutinib (BTK/ITK inhibitor) on CE-induced Th17 markers in pulmonary CD4+ T cells in preventive mode. (A) % p -STAT3+CD4+ T cells in the lung, B) % IL-17A + CD4+ T cells in the lung, C) % IL-23R + CD4+ T cells in the lung, D) IL-17A protein levels in the lung, E) Representative flow plot exhibiting p -STAT3 expression in CD4+ T cells in the lung, F) Representative flow plot exhibiting IL-17A expression in CD4+ T cells in the lung, and G) Representative flow plot exhibiting IL-23R expression in CD4+ T cells in the lung. Values are expressed as mean $\pm$ SE, $n = 6-9$ /group from three independent experiments. * $P < 0.05$, vs. CE group.

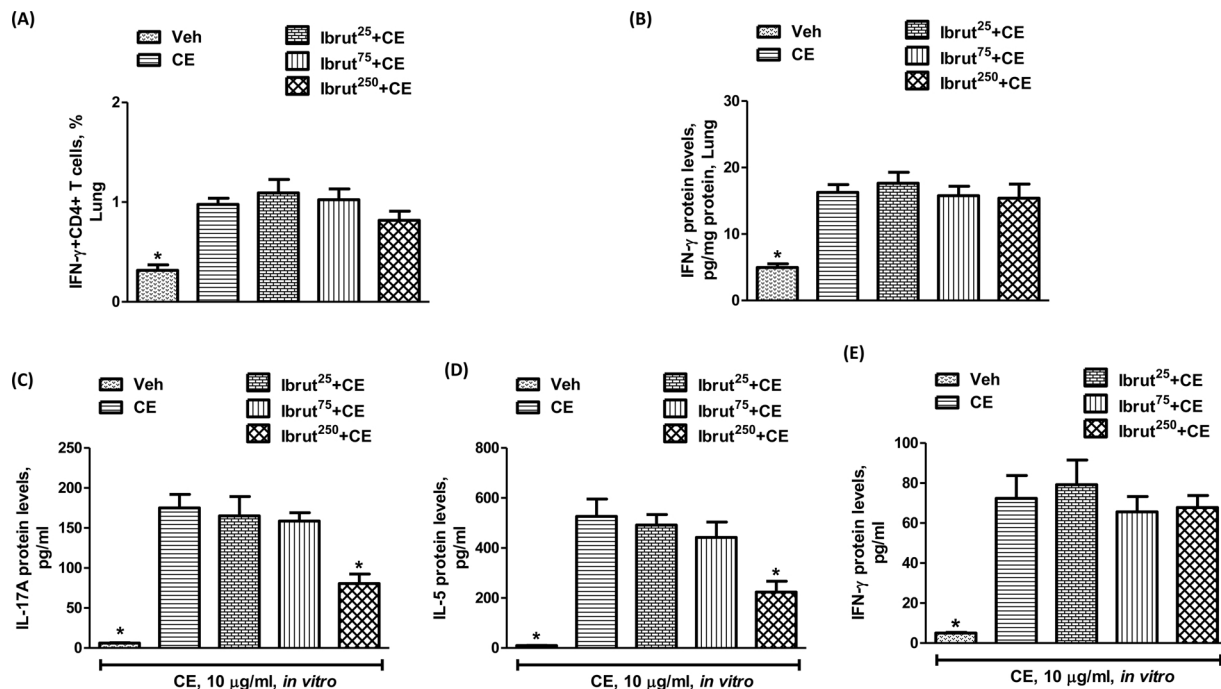


Fig. 4. Effect of different doses of Ibrutinib (BTK/ITK inhibitor) using preventive strategy on CE-induced Th1 marker in the lung (*in vivo*), and Th17/Th2/Th1 related cytokines in lung draining lymph node cells (LDNCs) during antigen recall assay (*in vitro*). (A) % IFN-γ + CD4 + T cells in the lung, (B) IFN-γ protein levels in the lung, (C) IL-17A protein levels in culture supernatant from CE-treated LDNCs, (D) IL-5 protein levels in culture supernatant from CE-treated LDNCs, and (E) IFN-γ protein levels in culture supernatant from CE-treated LDNCs. Values are expressed as mean ± SE, *n* = 6/group from three independent experiments. **P* < 0.05, vs. CE group.

(GATA3/IL-5) and Th17 (STAT3/IL-17A) related markers at mRNA level were also greatly attenuated by therapeutic treatment with Ibrutinib (Fig. 7C and F). This further shows a greater potential of Ibrutinib in controlling both Th2 and Th17 immune responses than corticosteroids during mixed granulocytic asthma.

3.7. Comparison of Ibrutinib with dexamethasone on CE-induced neutrophilic oxidative stress during mixed granulocytic inflammation employing therapeutic strategy

Lastly, we wanted to compare the effect of dexamethasone with Ibrutinib on neutrophilic oxidative stress which is a contributing factor for corticosteroid insensitivity. Our data reveal that dexamethasone failed to significantly attenuate CE-induced neutrophilic oxidative stress, whereas Ibrutinib markedly attenuated oxidative markers both in the neutrophils/lung in the therapeutic mode. This was evident from reduction in CE-induced elevation in iNOS and nitrotyrosine levels by Ibrutinib (Fig. 8A–C). This confirms that corticosteroids may be poor controllers of mixed granulocytic asthma due to lack of attenuation of neutrophilic inflammation. Further, Ibrutinib may be a successful alternative therapy to treat mixed granulocytic asthma due to down-regulation of both neutrophilic and eosinophilic inflammation.

4. Discussion

Mixed granulocytic inflammation is predominant in patients with severe asthma. Previous studies demonstrated that both eosinophils and neutrophils play a regulatory role in mixed granulocytic asthma, since they synthesize and release an array of inflammatory mediators that trigger the development of severe asthma [19,20,31]. The mechanisms behind these diverse inflammatory profiles are quite complex in asthma, but a Th17/neutrophilic presence might be indicative of a disease pathogenesis that is not driven by Th2 cells alone. Neutrophilic presence also contributes to the amount of oxidative stress in the airways which accounts for corticosteroid insensitivity in patients with

severe asthma [18,21,28]. Our study shows that Ibrutinib has the potential to regulate neutrophilic and eosinophilic airway inflammation thorough differential regulation of BTK and ITK in different immune cells during mixed granulocytic asthma both in preventative and therapeutic mode.

Corticosteroids are used as mainstream anti-inflammatory drug treatment in asthma. Corticosteroids predominantly dampen inflammation in the lungs by repressing the transcription of inflammatory mediators through several mechanisms [17,32]. Generally, symptoms of classical asthma in most patients can be very well managed by corticosteroids, however a subset of patients are resistant to the anti-inflammatory actions of corticosteroids (steroid resistant asthma). Approximately 10–25% of asthmatics are steroid resistant, which could be due to multiple factors [16,23,28]. A number of studies reveal that steroid resistant asthma in humans and mice is characterized by huge presence of neutrophils and Th17 cells in the airways along with Th2 cells and eosinophils. Neutrophils and Th17 cells have been found to be elevated in the bronchial tissues, sputum, serum, and BALF in such patients [21,28,33–35]. Our study also exhibited only limited potential of dexamethasone in controlling mixed granulocytic airway inflammation. Therefore, alternative therapies are required can control both Th2/Th17 and eosinophilic/neutrophilic inflammation during mixed granulocytic asthma.

BTK has a crucial role in BCR signaling during normal B cell development. Aberrant BCR signaling is associated with autoimmune diseases and B cell malignancies, such as rheumatoid arthritis and chronic lymphocytic leukemia respectively [10,15,36]. In addition, BTK is also expressed in myeloid cell populations, including neutrophils, monocytes/macrophages, and mast cells. These innate cells infiltrate the tissue and produce inflammatory cytokines, aggravating inflammatory symptoms. In myeloid cell populations, BTK functions downstream of innate receptors such as TLRs or Fcγ receptors or Fcε receptors. In the absence of BTK, oxidant/cytokine pathways are impaired due to hampering in innate receptor signaling [26,37,38]. Collectively, these data suggest that BTK signaling plays an important role

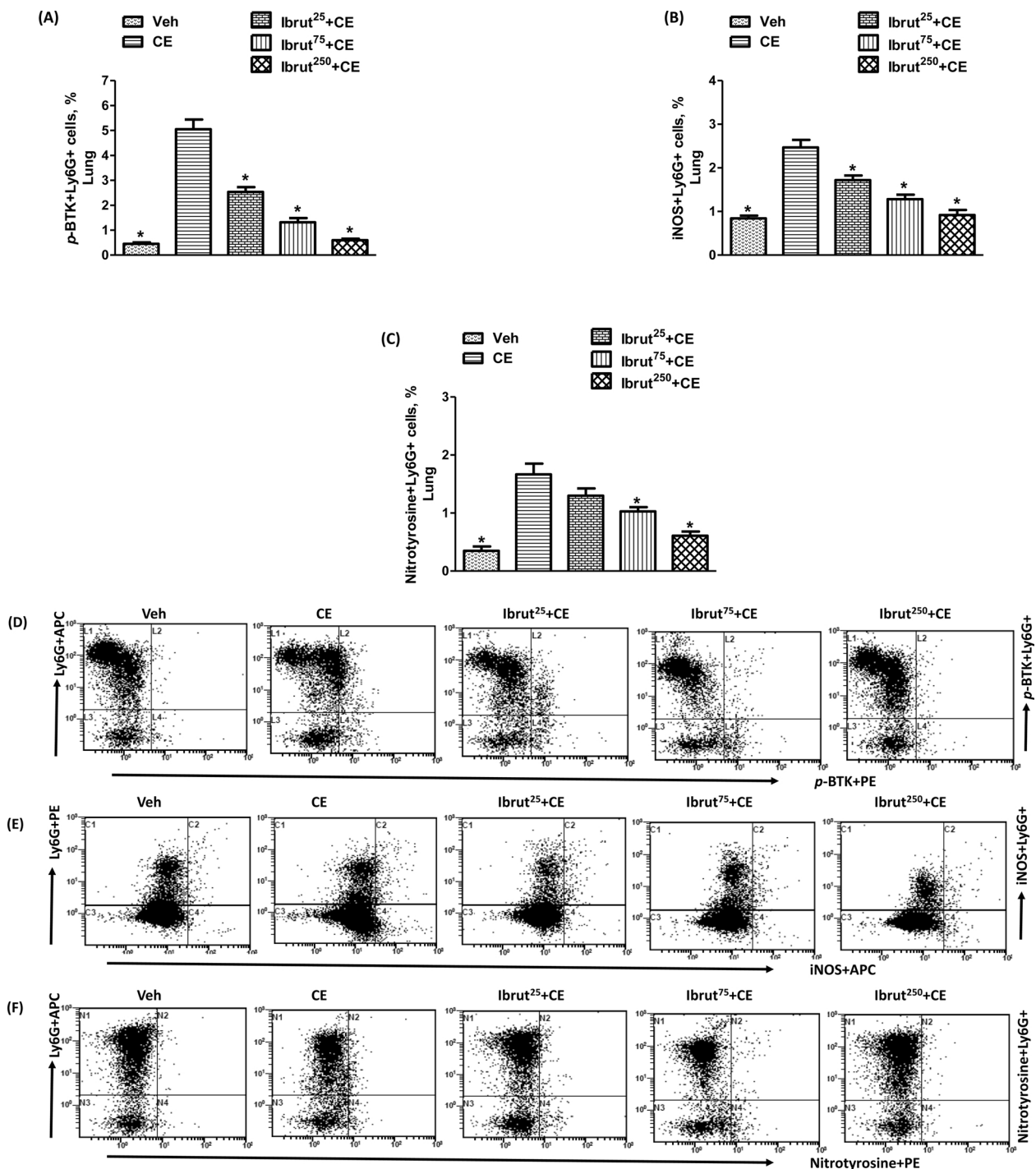


Fig. 5. Effect of different doses of Ibrutinib (BTK/ITK inhibitor) on CE-induced BTK phosphorylation and oxidative stress in the pulmonary neutrophils in preventive mode. (A) % p-BTK + Ly6G + cells in the lung, B) % iNOS + Ly6G + cells in the lung, C) % Nitrotyrosine + Ly6G + cells in the lung, D) Representative flow plot exhibiting p-BTK expression in Ly6G positive cells in the lung, E) Representative flow plot exhibiting iNOS expression in Ly6G positive cells in the lung, and F) Representative flow plot exhibiting nitrotyrosine expression in Ly6G positive cells in the lung. Values are expressed as mean $\pm$ SE, $n = 6$ /group from three independent experiments. * $P < 0.05$, vs. CE group.

in different inflammatory processes in innate immune cells. Our study further shows that BTK signaling serves a crucial function in development of neutrophilic inflammation during mixed granulocytic asthma.

BTK plays a crucial role in neutrophils as its activation causes induction of multiple pathways which are necessary for neutrophil recruitment/ infiltration during inflammation [39,40]. BTK activation along with inflammatory mediators and pulmonary inflammation are increased in murine models of acute lung injury such as those induced

by trauma-hemorrhagic shock or LPS [11,41]. Knocking down BTK in neutrophils or its pharmacological inhibition in the lung cause reduction in pulmonary neutrophilic inflammation, microvascular permeability, pulmonary edema and other inflammatory markers such as iNOS/NF κ B expression in these lung injury models [11,41,42]. Other models involving organ injury such as liver damage following reperfusion injury also show amelioration of disease symptoms by BTK inhibition due to attenuation of neutrophilic recruitment and activation

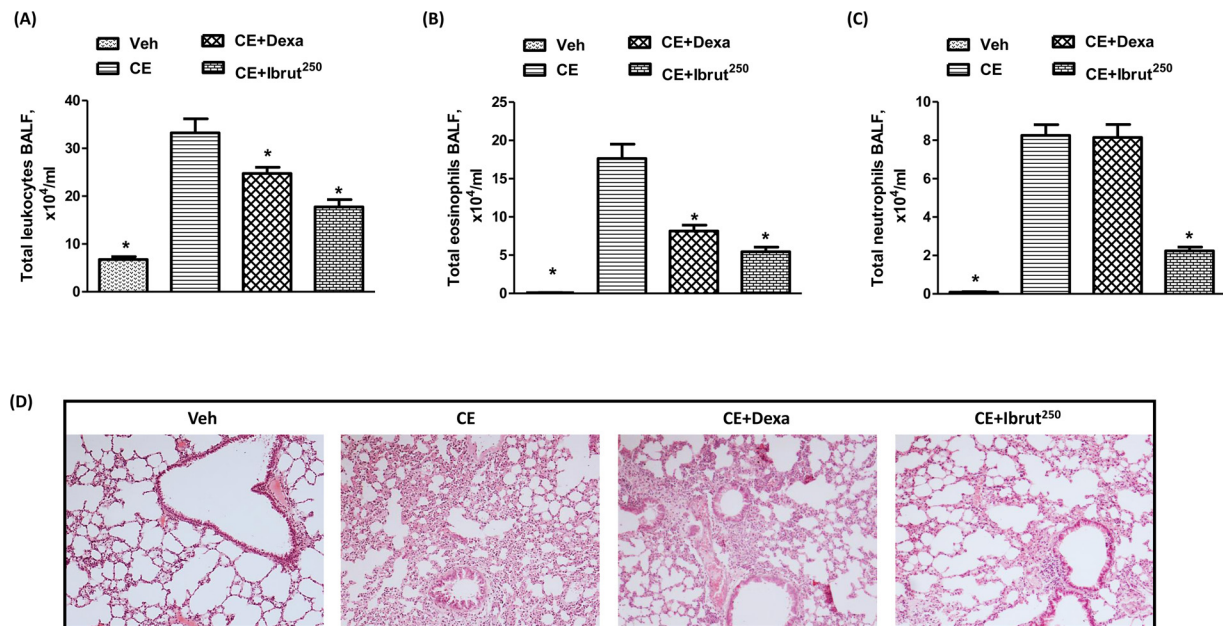


Fig. 6. Comparison of Ibrutinib (BTK/ITK inhibitor) and dexamethasone on CE-induced mixed granulocytic airway inflammation in therapeutic mode. (A) Total leukocytes count in BALF, (B) Eosinophilic count in BALF, (C) Neutrophilic count in BALF, and (D) H&E staining of the lung sections. Values are expressed as mean $\pm$ SE, $n = 6-9$ /group from three independent experiments. * $P < 0.05$, vs. CE group.

[43]. Our study further reveals that Ibrutinib has the potential to cause significant reduction in neutrophilic inflammation through down-regulation of BTK in neutrophils at doses which did not affect ITK phosphorylation in CD4 + T cells.

ITK signaling has also been shown to be involved in multiple inflammatory pathways in different disease models. Therefore, deficiency of ITK either by knock-out approach or pharmacological inhibition has shown potential benefit against various inflammatory conditions such as multiple sclerosis, asthma and rheumatoid arthritis [5,13–15,44,45]. Recently, it has been shown that Ibrutinib not only inhibits BTK but also

ITK, however at a relatively higher concentration [24,27,36]. Our study also shows that Ibrutinib could differentially affect BTK and ITK signaling in neutrophils and CD4 + T cells respectively. It inhibits BTK in neutrophils at much lower concentration than ITK in CD4 + T cells, thus potentiating the overall efficacy of this compound on mixed granulocytic airway inflammation. It is obvious from marked inhibition of both Th2/Th17 and eosinophilic/neutrophilic inflammation at the highest dose used in this study; whereas at lower doses it only caused downregulation of neutrophilic inflammation during mixed granulocytic asthma.

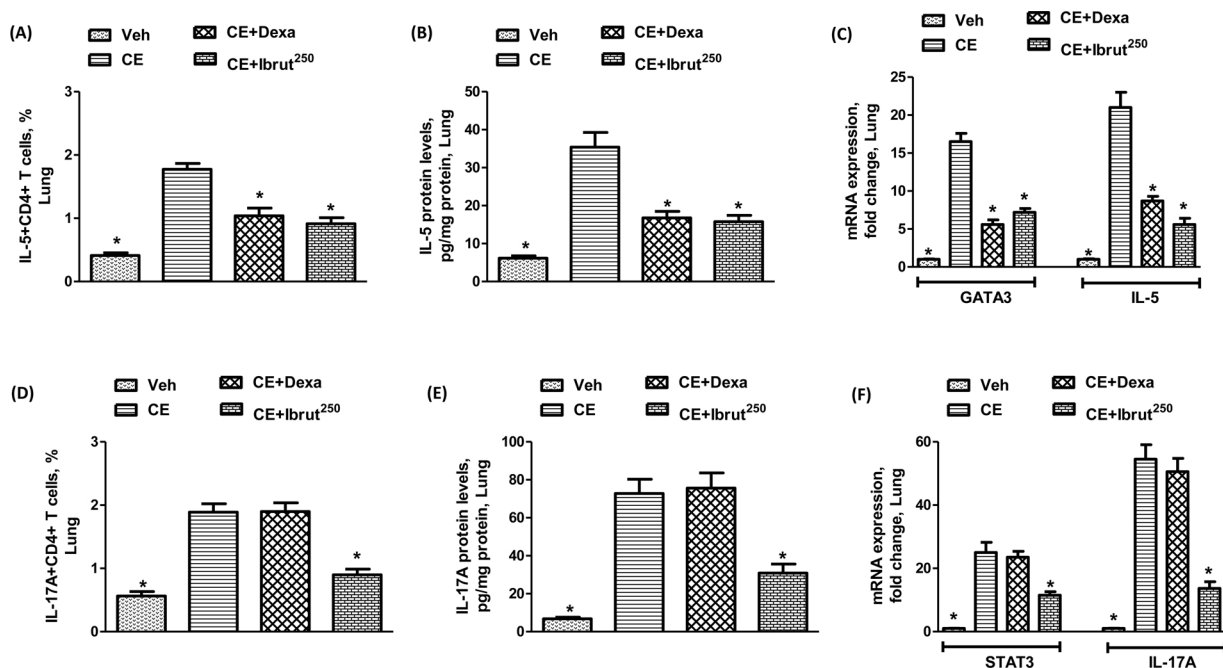


Fig. 7. Comparison of Ibrutinib (BTK/ITK inhibitor) and dexamethasone on CE-induced Th2/Th17 markers in pulmonary CD4 + T cells in therapeutic mode. (A) % IL-5 + CD4 + T cells in the lung, (B) IL-5 protein levels in the lung, (C) mRNA levels of GATA3 and IL-5 in the lung, (D) % IL-17A + CD4 + T cells in the lung, (E) IL-17A protein levels in the lung, and (F) mRNA levels of STAT3 and IL-17A in the lung. Values are expressed as mean $\pm$ SE, $n = 6-9$ /group from three independent experiments. * $P < 0.05$, vs. CE group.

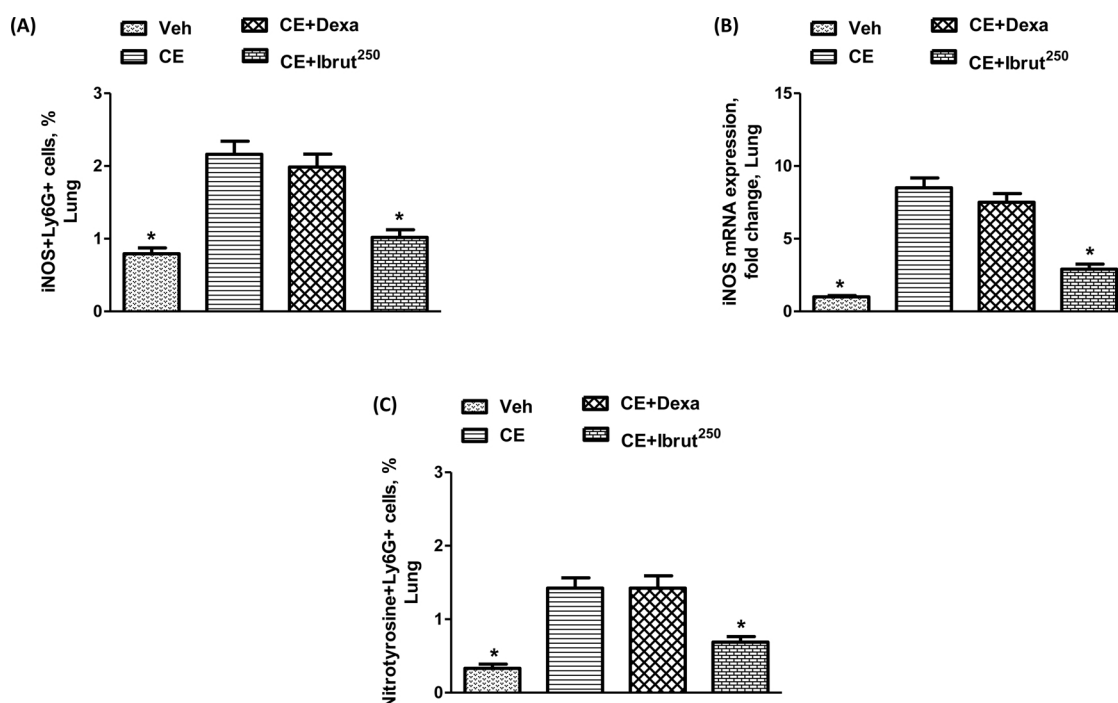


Fig. 8. Comparison of Ibrutinib (BTK/ITK inhibitor) and dexamethasone on CE-induced neutrophilic oxidative stress in therapeutic mode. A) % iNOS + Ly6G + cells in the lung, B) iNOS mRNA levels in the lung, C) % Nitrotyrosine + Ly6G + cells in the lung. Values are expressed as mean $\pm$ SE, $n = 6-9$ /group from three independent experiments. * $P < 0.05$, vs. CE group.

ITK signaling is needed for Th2/Th17 immune responses through activation of various downstream pathways in CD4 + T cells. ITK has been shown to regulate GATA3 which is an essential transcription factor for Th2 cell differentiation [46,47]. ITK signaling has been also reported cause differentiation of Th17 cells through STAT3 dependent and independent mechanisms [47–49]. On the other hand, both Th2 and Th17 related markers have shown to be affected by ITK inhibition/deficiency [47,48,50,51]. Further, ITK deficiency also causes suppression of Th2/Th17 immune responses in animal models of asthma; whereas ITK deficiency in a mouse model of allergic asthma or ITK inhibition by Ibrutinib in mouse model of cutaneous leishmaniasis do not cause downregulation of Th1 related markers [13,14,27,44,45,48]. Ibrutinib, also led to marked attenuation of CE-induced upregulation in Th2/Th17 responses, whereas it failed to inhibit CE-induced Th1 response in the lung. This could be due to suppression of *p*-ITK expression in CE-treated CD4 + T cells by Ibrutinib. This suggests that Ibrutinib has the potential to control Th2/Th17 immune responses while sparing Th1 immune response during mixed granulocytic airway inflammation.

Ibrutinib is known to be quite efficacious in mice models of arthritis, lupus, brain ischemia/reperfusion model, and graft-versus host disease [24,26,52]. Ibrutinib has also been useful earlier in amelioration of allergy. Mast cells isolated from BTK/ITK double knockout mice or ibrutinib-treated basophils have defective degranulation and cytokine secretion responses downstream to FcεR signaling [53–55]. However, our study revealed its potential in combating mixed granulocytic airway inflammation having Th2/Th17 immune responses in an allergen driven mouse model of asthma for the first time.

Based on our results, Ibrutinib may have the potential as an alternative therapy for mixed granulocytic airway inflammation which is resistant to corticosteroids. As evident from the current data, it leads to downregulation of neutrophilic inflammation both in preventive and therapeutic mode. Neutrophilic inflammation and oxidative stress are known perpetrators for the development of corticosteroid resistance [16,21,28]. Our study shows that Ibrutinib attenuates neutrophilic oxidative stress through inhibition of BTK signaling. Therefore, inhibition of both neutrophilic and eosinophilic inflammation by

Ibrutinib may confer a better protection against mixed granulocytic airway inflammation which is not the case with corticosteroids.

Overall, our study shows that Ibrutinib, through differential inhibition of two different kinases, *i.e.* BTK and ITK can be a useful compound to inhibit both Th17/Th2 and neutrophilic/eosinophilic immune responses during mixed granulocytic asthma. Therefore, Ibrutinib may be an alternative therapy to manage difficult-to-treat asthma phenotypes having mixed granulocytic inflammation.

Declaration of Competing Interest

All authors declare no conflicts of interest.

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References

- [1] S.T. Holgate, Innate and adaptive immune responses in asthma, *Nat. Med.* 18 (2012) 673–683.
- [2] C. Page, B. O'Shaughnessy, P. Barnes, Pathogenesis of COPD and asthma, *Handb. Exp. Pharmacol.* 237 (2017) 1–21.
- [3] P. Licona-Limón, L.K. Kim, N.W. Palm, R.A. Flavell, TH2, allergy and group 2 innate lymphoid cells, *Nat. Immunol.* 14 (2013) 536–542.
- [4] S. Svenningsen, P. Nair, Asthma endotypes and an overview of targeted therapy for asthma, *Front. Med. (Lausanne)* 4 (2017) 158.
- [5] M. Kaur, M.S. Bahia, O. Silakari, Inhibitors of interleukin-2 inducible T-cell kinase as potential therapeutic candidates for the treatment of various inflammatory disease conditions, *Eur. J. Pharm. Sci.* 47 (2012) 574–588.
- [6] N. Boucheron, W. Ellmeier, The role of Tec family kinases in the regulation of T-helper-cell differentiation, *Int. Rev. Immunol.* 31 (2012) 133–154.
- [7] U. Schmidt, N. Boucheron, B. Unger, W. Ellmeier, The role of Tec family kinases in myeloid cells, *Int. Arch. Allergy Immunol.* 134 (2004) 65–78.
- [8] K.G. Lee, S. Xu, Z.H. Kang, J. Huo, M. Huang, D. Liu, et al., Bruton's tyrosine kinase phosphorylates Toll-like receptor 3 to initiate antiviral response, *Proc. Natl. Acad. Sci. U. S. A.* 109 (2012) 5791–5796.
- [9] C.A. Jefferies, L.A. O'Neill, Bruton's tyrosine kinase (Btk)-the critical tyrosine kinase in LPS signalling? *Immunol. Lett.* 92 (2014) 15–22.

- [10] S. Pal Singh, F. Dammeijer, R.W. Hendriks, Role of Bruton's tyrosine kinase in B cells and malignancies, *Mol. Cancer* 17 (2018) 57.
- [11] A. Krupa, M. Fol, M. Rahman, K.Y. Stokes, J.M. Florence, I.L. Leskov, et al., Silencing Bruton's tyrosine kinase in alveolar neutrophils protects mice from LPS/immune complex-induced acute lung injury, *Am. J. Physiol. Lung Cell Mol. Physiol.* 307 (2014) L435–48.
- [12] Y.Y. Kim, K.T. Park, S.Y. Jang, K.H. Lee, J.Y. Byun, K.H. Suh, et al., HM71224, a selective Bruton's tyrosine kinase inhibitor, attenuates the development of murine lupus, *Arthritis Res. Ther.* 19 (2017) 211.
- [13] A. Kannan, Y. Lee, Q. Qi, W. Huang, A.R. Jeong, S. Ohnigian, et al., Allele-sensitive mutant, Itkas, reveals that Itk kinase activity is required for Th1, Th2, Th17, and iNKT-cell cytokine production, *Eur. J. Immunol.* 45 (2015) 2276–2285.
- [14] A.K. Kannan, D.G. Kim, A. August, M.S. Bynoe, Itk signals promote neuroinflammation by regulating CD4+ T-cell activation and trafficking, *J. Neurosci.* 35 (2015) 221–233.
- [15] L. Vargas, A. Hamasy, B.F. Nore, C.I. Smith, Inhibitors of BTK and ITK: state of the new drugs for cancer, autoimmunity and inflammatory diseases, *Scand. J. Immunol.* 78 (2013) 130–139.
- [16] D.C. Cowan, J.O. Cowan, R. Palmay, A. Williamson, D.R. Taylor, Effects of steroid therapy on inflammatory cell subtypes in asthma, *Thorax* 65 (2010) 384–390.
- [17] H.P. Hauber, M. Gotfried, K. Newman, R. Danda, R.J. Servi, P. Christodouloupoulos, et al., Effect of HFA-flunisolide on peripheral lung inflammation in asthma, *J. Allergy Clin. Immunol.* 112 (2003) 58–63.
- [18] S.E. Wenzel, Complex phenotypes in asthma: current definitions, *Pulm. Pharmacol. Ther.* 26 (2013) 710–715.
- [19] G. Pelaia, A. Vatrella, M.T. Busceti, L. Gallelli, C. Calabrese, R. Terracciano, et al., Cellular mechanisms underlying eosinophilic and neutrophilic airway inflammation in asthma, *Mediators Inflamm.* 2015 (2015) 879783.
- [20] J. Monteseirin, Neutrophils and asthma, *J. Investig. Allergol. Clin. Immunol.* 19 (2009) 340–354.
- [21] D.C. Newcomb, R.S. Peebles, Th17-mediated inflammation in asthma, *Curr. Opin. Immunol.* 25 (2013) 755–760.
- [22] A. Manda-Handzik, U. Demkow, Neutrophils: the role of oxidative and nitrosative stress in health and disease, *Adv. Exp. Med. Biol.* 857 (2015) 51–60.
- [23] D.Y. Leung, J.W. Bloom, Update on glucocorticoid action and resistance, *J. Allergy Clin. Immunol.* 111 (2003) 3–22.
- [24] S.D. Schutt, J. Fu, H. Nguyen, D. Bastian, J. Heinrichs, Y. Wu, et al., Inhibition of BTK and ITK with ibrutinib is effective in the prevention of chronic graft-versus-host disease in mice, *PLoS One* 10 (2015) e0137641.
- [25] J.M. Florence, A. Krupa, L.M. Booshehri, S.A. Davis, M.A. Matthey, A.K. Kurdowska, Inhibiting Bruton's tyrosine kinase rescues mice from lethal influenza-induced acute lung injury, *Am. J. Physiol. Lung Cell Mol. Physiol.* 315 (2018) L52–L58.
- [26] L.A. Honigberg, A.M. Smith, M. Sirisawad, E. Verner, D. Loury, B. Chang, et al., The Bruton tyrosine kinase inhibitor PCI-32765 blocks B-cell activation and is efficacious in models of autoimmune disease and B-cell malignancy, *Proc. Natl. Acad. Sci. U. S. A.* 107 (2010) 13075–13080.
- [27] J.A. Dubovsky, K.A. Beckwith, G. Natarajan, J.A. Woyach, S. Jaglowski, Y. Zhong, et al., Ibrutinib is an irreversible molecular inhibitor of ITK driving a Th1-selective pressure in T lymphocytes, *Blood* 122 (2013) 2539–2549.
- [28] A. Nadeem, S.F. Ahmad, N.O. Al-Harbi, N. Siddiqui, K.E. Ibrahim, S.M. Attia, Inhibition of BET bromodomains restores corticosteroid responsiveness in a mixed granulocytic mouse model of asthma, *Biochem. Pharmacol.* 154 (2018) 222–233.
- [29] A. Nadeem, N.O. Al-Harbi, M.A. Ansari, M.M. Al-Harbi, A.M. El-Sherbeeny, K.M. Zoheir, et al., Psoriatic inflammation enhances allergic airway inflammation through IL-23/STAT3 signaling in a murine model, *Biochem. Pharmacol.* 124 (2017) 69–8.
- [30] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, *Methods* 25 (2001) 402–408.
- [31] A.R. Koczulla, C.F. Vogelmeier, H. Garn, H. Renz, New concepts in asthma: clinical phenotypes and pathophysiological mechanisms, *Drug Discov. Today* 22 (2017) 388–396.
- [32] T. Joshi, M. Johnson, R. Newton, M. Gienbycz, An analysis of glucocorticoid receptor-mediated gene expression in BEAS-2B human airway epithelial cells identifies distinct, ligand-directed, transcription profiles with implications for asthma therapeutics, *Br. J. Pharmacol.* 172 (2015) 1360–1378.
- [33] C. Doe, M. Bafadhel, S. Siddiqui, D. Desai, V. Mistry, P. Rugman, et al., Expression of the T helper 17-associated cytokines IL-17A and IL-17F in asthma and COPD, *Chest* 138 (2010) 1140–1147.
- [34] D.M. Bullens, E. Truyen, L. Coteur, E. Dilissen, P.W. Hellings, L.J. Dupont, et al., IL-17 mRNA in sputum of asthmatic patients: linking T cell driven inflammation and granulocytic influx? *Respir. Res.* 7 (2006) 1–9.
- [35] I. Agache, C. Ciobanu, C. Agache, M. Anghel, Increased serum IL-17 is an independent risk factor for severe asthma, *Respir. Med.* 104 (2010) 1131–1137.
- [36] P. Kokhaei, F. Jadidi-Niaragh, A. Sotoodeh Jahromi, A. Osterborg, H. Mellstedt, M. Hojati-Farsangi, Ibrutinib-A double-edge sword in cancer and autoimmune disorders, *J. Drug Target* 24 (2016) 373–385.
- [37] J.A. Wang, B.Y. Chang, Bruton's tyrosine kinase inhibitors for the treatment of rheumatoid arthritis, *Drug Discov. Today* 19 (2014) 1200–1204.
- [38] L.M. Hartkamp, J.S. Fine, I.E. van Es, M.W. Tang, M. Smith, J. Woods, et al., Btk inhibition suppresses agonist-induced human macrophage activation and inflammatory gene expression in RA synovial tissue explants, *Ann. Rheum. Dis.* 74 (2015) 1603–1611.
- [39] H. Mueller, A. Stadtmann, H. Van Aken, E. Hirsch, D. Wang, K. Ley, et al., Tyrosine kinase Btk regulates E-selectin-mediated integrin activation and neutrophil recruitment by controlling phospholipase C (PLC) gamma2 and PI3Kgamma pathways, *Blood* 115 (2010) 3118–3127.
- [40] S. Volmering, H. Block, M. Boras, C.A. Lowell, A. Zarbock, The neutrophil Btk signalosome regulates integrin activation during sterile inflammation, *Immunity* 44 (2016) 73–87.
- [41] X. Liu, J. Zhang, W. Han, Y. Wang, Y. Liu, Y. Zhang, et al., Inhibition of BTK protects lungs from trauma-hemorrhagic shock-induced injury in rats, *Mol. Med. Rep.* 16 (2017) 192–200.
- [42] P. Zhou, B. Ma, S. Xu, S. Zhang, H. Tang, S. Zhu, et al., Knockdown of Bruton's tyrosine kinase confers potent protection against sepsis-induced acute lung injury, *Cell Biochem. Biophys.* 70 (2014) 1265–1275.
- [43] T. Palumbo, K. Nakamura, C. Lassman, Y. Kidani, S.J. Bensinger, R. Busuttill, et al., Bruton tyrosine kinase inhibition attenuates liver damage in a mouse warm ischemia and reperfusion model, *Transplantation* 101 (2017) 322–331.
- [44] T. Ferrara, C. Mueller, N. Sahu, A. Ben-Jebria, A. August, Reduced airway hyperresponsiveness and tracheal responses during allergic asthma in mice lacking tyrosine kinase inducible T-cell kinase, *J. Allergy Clin. Immunol.* 117 (2006) 780–786.
- [45] J.D. Harling, A.M. Deakin, S. Campos, R. Grimley, L. Chaudry, C. Nye, et al., Discovery of novel irreversible inhibitors of interleukin (IL)-2-inducible tyrosine kinase (Itk) by targeting cysteine 442 in the ATP pocket, *J. Biol. Chem.* 288 (2013) 28195–28206.
- [46] A. Kannan, N. Sahu, S.S.M. Mohanan, A. August, Itk modulates allergic airway inflammation by suppressing IFN γ in naïve CD4+ T-cells, *J. Allergy Clin. Immunol.* 132 (2013) 811–820.
- [47] N. Hermann-Kleiter, G. Baier, NFAT pulls the strings during CD4+ T helper cell effector functions, *Blood* 115 (April (15)) (2010) 2989–2997.
- [48] J. Gomez-Rodriguez, N. Sahu, R. Handon, T.S. Davidson, S.M. Anderson, M.R. Kirby, A. August, P.L. Schwartzberg, Differential expression of interleukin-17A and -17F is coupled to T cell receptor signaling via inducible T cell kinase, *Immunity* 31 (2009) 587–597.
- [49] A. Eken, M. Cansever, I. Somekh, Y. Mizoguchi, N. Zietara, F.Z. Okus, et al., Genetic deficiency and biochemical inhibition of ITK affect human Th17, Treg, and innate lymphoid cells, *J. Clin. Immunol.* 39 (2019) 391–400.
- [50] D.J. Fowell, K. Shinkai, X.C. Liao, A.M. Beebe, R.L. Coffman, D.R. Littman, et al., Impaired NFATc translocation and failure of Th2 development in Itk-deficient CD4+ T cells, *Immunity* 11 (October (4)) (1999) 399–409.
- [51] J. Gomez-Rodriguez, E.A. Wohlfert, R. Handon, F. Meylan, J.Z. Wu, S.M. Anderson, et al., Itk-mediated integration of T cell receptor and cytokine signaling regulates the balance between Th17 and regulatory T cells, *J. Exp. Med.* 211 (2014) 529–543.
- [52] M. Ito, T. Shichita, M. Okada, R. Komine, Y. Noguchi, A. Yoshimura, et al., Bruton's tyrosine kinase is essential for NLRP3 inflammasome activation and contributes to ischaemic brain injury, *Nat. Commun.* 6 (2015) 7360.
- [53] A.S. Iyer, J.L. Morales, W. Huang, F. Ojo, G. Ning, E. Wills, et al., Absence of Tec family kinases interleukin-2 inducible T cell kinase (Itk) and Bruton's tyrosine kinase (Btk) severely impairs Fc epsilonRI-dependent mast cell responses, *J. Biol. Chem.* 286 (2011) 9503–9513.
- [54] D. MacGlashan Jr., L.A. Honigberg, A. Smith, J. Buggy, J.T. Schroeder, Inhibition of IgE-mediated secretion from human basophils with a highly selective Bruton's tyrosine kinase, Btk, inhibitor, *Int. Immunopharmacol.* 11 (2011) 475–479.
- [55] J.A. Regan, Y. Cao, M.C. Dispenza, S. Ma, L.I. Gordon, A.M. Petrich, et al., Ibrutinib, a Bruton's tyrosine kinase inhibitor used for treatment of lymphoproliferative disorders, eliminates both aeroallergen skin test and basophil activation test reactivity, *J. Allergy Clin. Immunol.* 140 (2017) 875–879.