



Inhibition of BET bromodomains restores corticosteroid responsiveness in a mixed granulocytic mouse model of asthma

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ABSTRACT

Asthma is a heterogeneous disease characterized by different endotypes/phenotypes. Th2/Th17 driven mixed granulocytic asthma is one of them and shows resistance to corticosteroid therapy. Bromodomain and extra-terminal (BET) proteins are required for differentiation of Th17 cells which play a pivotal role in neutrophilic inflammation. Therefore, we sought to characterize the differential effects of BET inhibitor versus corticosteroids, and their potential synergism in cockroach allergen extract (CE)-induced mixed granulocytic (eosinophilic and neutrophilic) mouse model of asthma having Th2/Th17 endotype. Effects of BET inhibitor, (+)JQ-1 alone and in combination with dexamethasone (Dexa) were assessed on airway inflammation as well as Th2/Th17 related airway immune responses in CE-induced mixed granulocytic asthma. Markers of steroid resistance [histone deacetylase 2 (HDAC2), and oxidative stress] were also assessed in the lungs of mice and primary human bronchial epithelial cells (HBECs). BET inhibitor, (+)JQ-1 abolished Th17 driven neutrophilic inflammation in CE-induced mixed granulocytic asthma. Dexa had limited effect on overall airway inflammation despite having significant reductions in Th2 driven immune responses. However, combination of (+)JQ-1 with Dexa completely blocked both Th2 and Th17 driven immune responses in the lung which led to significant reductions in eosinophils, neutrophils, and mucin secretion. (+)JQ-1 also reversed CE- and IL-17A-induced decrease in HDAC2 expression in murine and human airway epithelial cells respectively.

1. Introduction

Asthma is a chronic lung disease that occurs due to inflammation of the airways and is associated with airway remodeling, mucous cell metaplasia, and increased airway reactivity [1,2]. Indoor allergens originating from cockroach and house dust mite contribute significantly towards the pathogenesis of asthma through mucosal sensitization [3–5]. The prevalence of asthma has increased significantly over the past few decades globally and numbers suggest that it afflicts more than 300 million people worldwide which could go up to 400 million by 2025 due to increase in urbanization [6,7].

Asthma manifests itself with different patterns of airway inflammation that cannot be explained by the Th2 immune response alone. Recent studies in asthmatic patients and animal models have shown that in response to an allergen, there is development of not only Th2 immune response but also Th17 immune response in the lung, both

of which contribute significantly towards the development of airway inflammation [8–11]. Th2-driven airway inflammation is mostly associated with eosinophils, whereas Th17 driven airway inflammation is usually associated with neutrophils [9,12,13]. Based on this, asthma has been classified into different endotypes/phenotypes depending upon the infiltration of leukocytes into the lung, e.g. Th2/eosinophilic, Th17/neutrophilic, and Th2/Th17 with mixed neutrophils and eosinophils [11,14–16]. The lowest lung function is found in asthmatic patients who have increased mixed granulocytes (both eosinophils and neutrophils) in their airways. They also have worse control of asthma and increased exacerbations [1,15–18]. Cockroach allergen extract (CE)-induced asthma has been shown to have mixed granulocytic airway inflammation which is c cytokines such as IL-4 and IL-17A respectively [8,10,19].

Post-translationally acetylated histones can affect the inflammatory gene expression by altering the chromatin structure through

Abbreviations: BAL, bronchoalveolar lavage; BET, bromodomain and extra-terminal; CE, cockroach extract; i.n., intranasal; STAT3, signal transducer and activator of transcription 3; RORC, retinoic acid receptor-related orphan receptor C; HDAC, histone deacetylase

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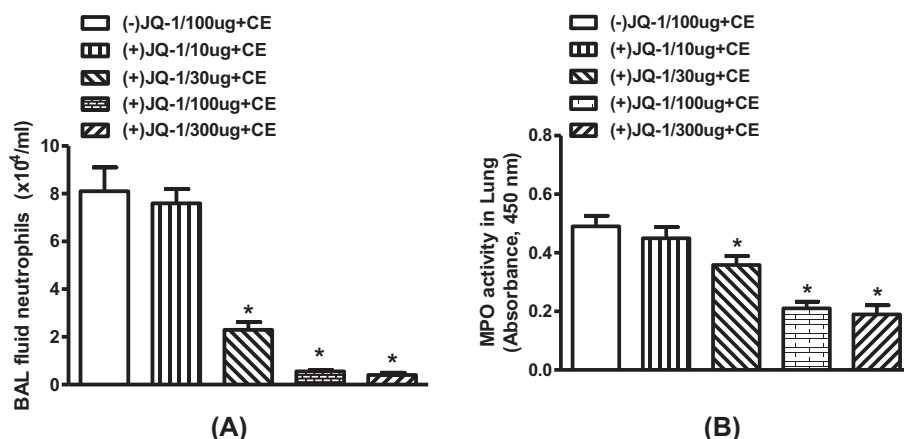


Fig. 1. Effect of BET inhibitor, (+)JQ-1 on CE-induced (A) neutrophilic influx in BAL, (B) lung MPO activity. Values are expressed as mean $\pm$ SEM, $n = 10$ /group from two independent experiments. * $P < 0.05$, vs. (–)JQ-1/100 + CE group.

employment of histone modifiers known as readers of histone code/language [20–21]. One such family of readers for the acetylated histones is grouped under bromo- and extra-terminal (BET) bromodomain family. This family consists of Brd2, Brd3, Brd4, and BRDT members. BET family controls the assembly of chromatin complexes which are dependent on histone acetylation for inflammatory gene transcription. BET inhibitors such as (+)JQ-1/IBET-762 initially showed efficacy in cancer and sepsis models [22,23]. However, recent studies have shown that inhibition of BET proteins in autoimmune/inflammatory disease models may also be an effective treatment option [24–27].

The discovery of Th17 cells whose differentiation is dependent on BET proteins, has added an additional layer of complexity to the regulation of airway inflammation [27]. In asthmatic patients, IL-17 expression is increased in the lungs, sputum, bronchoalveolar lavage (BAL) fluids, or sera, and the severity of airway hypersensitivity in patients correlates with IL-17 expression level [28,29]. Th17-derived cytokines, such as IL-17A not only regulate neutrophilic inflammation, but also enhances Th2 airway responses [30,31]. Usually Th17 driven airway inflammation is resistant to steroid therapy, however recent studies have shown efficacy of BET inhibitors in steroid resistant asthma [32,33]. Other studies have shown *in vitro* that BET inhibition could be an effective strategy in combating airway remodeling of asthmatic patients through down-regulation of inflammatory gene expression [34]. However, the effect of BET inhibition on CE-induced mixed granulocytic airway inflammation has not investigated until now.

In the current study, we hypothesized that BET inhibition by blocking Th17 driven airway inflammation would be able to restore corticosteroid responsiveness in mixed granulocytic asthma. To test this hypothesis, we utilized CE-induced mixed granulocytic mouse model of asthma which has some features of difficult-to-control human asthma, i.e. mixed Th2/Th17 endotype and mixed neutrophilic/eosinophilic phenotype [8,11,15,35]. Using this model, we show for the first time that BET inhibitor, (+)JQ-1 abolishes CE-induced Th17 immune responses in the lung with concomitant reductions in airway inflammation, and mucus secretion. Further, BET inhibition restores corticosteroid sensitivity through blockade of Th17-driven airway inflammation. Our study suggests that a combined therapy of BET inhibitor with corticosteroids might be beneficial in difficult-to-treat mixed granulocytic airway inflammation.

2. Materials and methods

2.1. Animals

The current study utilized male Balb/c mice (8–10 weeks of age;

20–25 g), free of specific pathogens. These mice were housed under standard laboratory conditions such as 12-h light-dark cycle and surround temperature of 24–26 °C. The mice utilized in this study were provided by The Experimental Animals Center, College of Pharmacy, King Saud University. The research protocol for usage of mice in this study was 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

Sensitization was performed according to the protocol described earlier by us with some modifications [3,10,19]. Mice were sensitized on days 1, 2, 3, 4, and 5 with intranasal (i.n.) administrations of 50 μ g whole-body German cockroach (*Blattella germanica*) extract (CE) from Greer Laboratories (Lenoir, NC) under light anesthesia. Ten days after 1st sensitization, the mice were challenged i.n. under light anesthesia with 50 μ g CE in 20 μ l saline twice daily (morning and evening) on days 11, 12, 13, 14, 15, 16, and 17. Non-sensitized control animals received only saline with the same volumes.

To assess the role of BET bromodomains on CE-induced airway immune responses, a BET bromodomain inhibitor, (+)JQ-1 (Tocris, UK) was administered i.n. once every day before morning CE challenge at a concentration of 100 μ g/mouse (dissolved in 10 μ l dimethylsulfoxide) for 7 days. (–)JQ-1 (Tocris, UK) was used as an optically inactive enantiomer as a background control at a concentration of 100 μ g/mouse (Fig. 2A). To assess the efficacy of corticosteroid therapy in this model, dexamethasone (Dexa) was also administered i.n. once every day alone or in combination with (+)JQ-1 before morning CE challenge at a concentration of 25 μ g/mouse for 7 days (Fig. 4A).

Effect of IL-17A and neutrophils on histone deacetylase 2 expression in CE mixed granulocytic asthma was assessed by their respective depletion using antibodies against them. Mice were injected i.p. with rat anti-mouse Ly6G mAb, clone 1A8 (250 μ g/200 μ l/mouse, Biolegend, USA) or anti-IL-17A antibody (150 μ g/200 μ l/mouse, Biolegend, USA) or isotype control antibody (Biolegend, USA), 1 hr before administration of intranasal CE or saline on alternate days, i.e. on day 11, 13, 15 and 17.

2.3. Bronchoalveolar lavage (BAL)

The mice were sacrificed by isoflurane anesthesia and the trachea was cannulated to perform BAL 12 h after the final allergen challenge. A differential count of at least 300 cells was carried out as described previously [3,10,19].

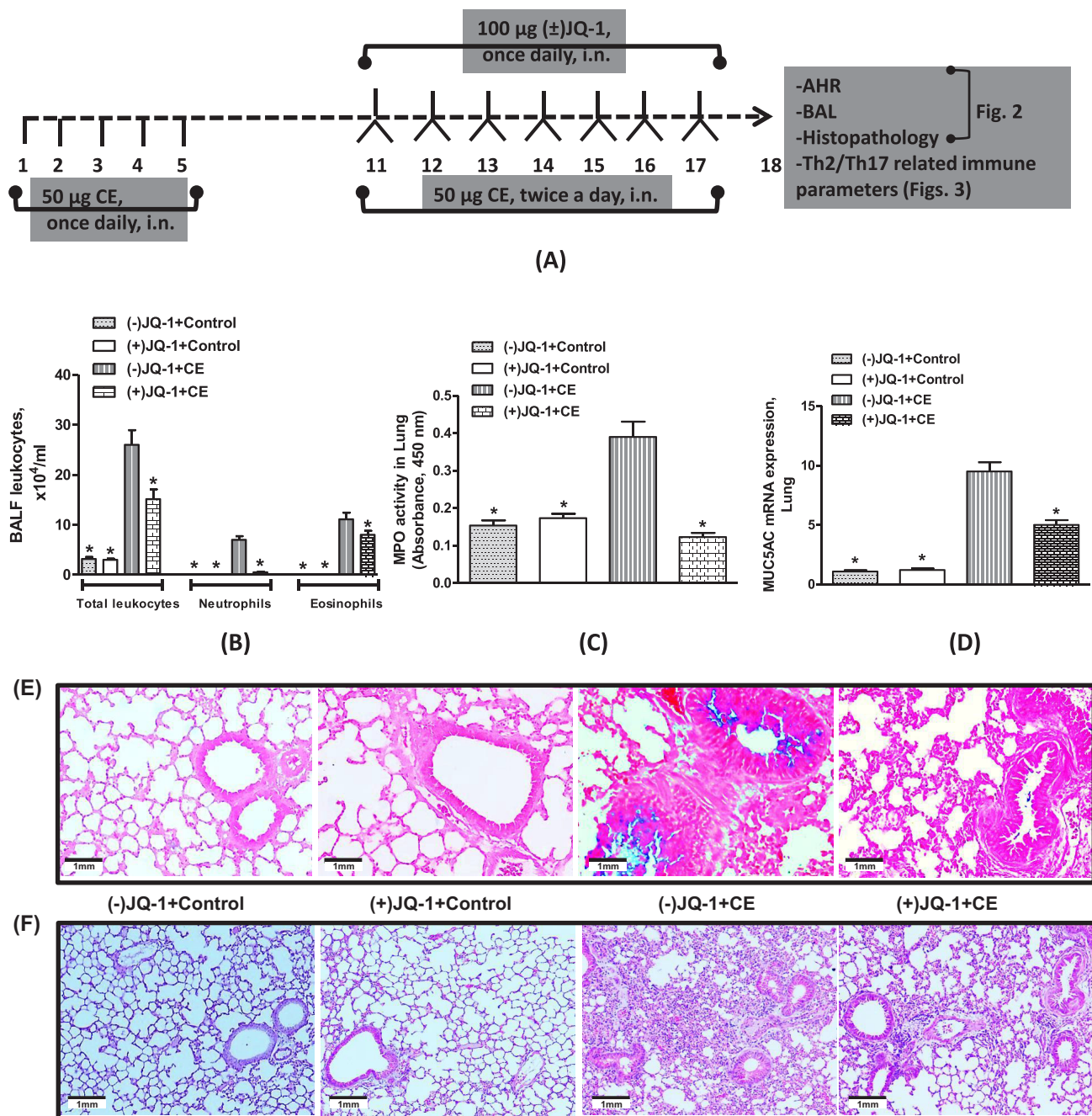


Fig. 2. Effect of BET inhibitor, (+)JQ-1 on CE-induced mixed granulocytic airway inflammation and related parameters in the mice. A) Study design for the effect of (+)JQ-1 in CE-induced mixed granulocyte asthma model, B) Total leukocytes, neutrophils, and eosinophils in BALF, C) Lung MPO activity, D) Lung MUC5AC mRNA expression, E) Lung sections stained with Alcian blue (upper horizontal panel; 200X), and F) H&E (lower horizontal panel; 100X). Each photomicrograph is a representative image from every group. BAL was performed 12 h after last CE challenge. Values are expressed as mean $\pm$ SEM, $n = 10$ –12/group from three independent experiments. * $P < 0.05$, vs. (–)JQ-1 + CE group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.4. Flow cytometry

For detection Th2/Th17 related markers in CD4⁺ T cells of the lung, single-cell suspensions of lung were obtained by dispersion of chopped lung pieces in 0.2% collagenase and 0.1% DNase I in RPMI 1640 (Invitrogen, USA) at 37°C for 45 min as described in detail earlier [10]. Briefly, cells were stimulated with lymphocyte activator mixture [PMA (10 ng/ml)/ionomycin (400 ng/ml)/brefeldin A (5 µg/ml)] for 5 h and labeled with surface markers FITC/PE/APC anti-CD4 mAb (BioLegend, USA). Cells were then stained intracellularly with specific monoclonal antibodies against IL-4, GATA-3, IL-17A, IL-23R and RORC,

conjugated to PE or FITC or APC (BioLegend, USA, and BD Biosciences).

Single cell suspensions obtained from tracheal lumen brushings or HBECs (as described below) were labeled with APC-conjugated anti-CD326 (Ep-CAM; human/mice) antibody (BioLegend, USA) for surface labeling followed by FITC/PE-conjugated anti-HDAC2/nitrotyrosine monoclonal antibody (human/mice; Santa cruz Biotech, USA) using immunostaining methods described above. The stained 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 by us [10,25].

2.5. Isolation of lung CD4+ T cells

For mRNA expression in pulmonary CD4+ T cells, lungs were processed as described above. CD4+ T cells were then purified using Dynabeads® Untouched Mouse CD4+ T Cell isolation negative selection kit (Invitrogen, USA).

2.6. Ex vivo restimulation of splenocytes

Spleens collected on day 18 were disrupted to create single cell suspensions as described earlier [10]. Splenic cells were then restimulated with CE (10 µg/ml) in complete RPMI 1640 having 10% FCS and 1% Pen/Strep in 24 well-culture plates for 2 days. After 2 days of culture, supernatants were harvested for cytokine measurements.

2.7. Real-time PCR

Total RNA was isolated from the lungs/CD4+ T cells of different groups. This was followed by conversion of 0.5 µg of total RNA into cDNA using High Capacity cDNA archive kit (Applied Biosystems, USA) according to the manufacturer's instructions as described earlier by us [3,10]. 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-4, GATA-3, RORC, IL-23R, STAT3, MUC5AC, IL-17A, and IL-13 (Applied Biosystems, USA). The fold difference in expression of target cDNA was determined using the comparative CT method [36].

2.8. Western immunoblotting

Samples prepared from murine AECs/HBECs (30–40 µg protein/well) were separated on 10% SDS-PAGE as described earlier [3,10]. Proteins were transferred to nitrocellulose membranes and then probed either with rabbit HDAC2 monoclonal antibody (Abcam, USA) at a dilution of 1000, or GAPDH rabbit polyclonal antibody (Santa Cruz Biotechnology, USA) at a dilution of 1:5000. Bands were visualized using the enhanced chemiluminescence method (GE HealthCare, Mississauga, Canada) and quantified relative to GAPDH bands using the ImageJ image processing program (National Institutes of Health, Bethesda, USA). Western blot values are expressed as fold change after normalization to GAPDH levels.

2.9. Measurement of cytokines levels by ELISA

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

2.10. Measurement of myeloperoxidase activity

Myeloperoxidase (MPO) activity in the supernatants isolated from the lungs was taken as a measure of neutrophil inflammation as reported previously [25].

2.11. Lung histology

Lungs were removed from the thorax and fixed with formalin for histological analysis. Formalin-fixed lungs were sectioned at a thickness of 5 µm followed by staining with haematoxylin and eosin (H&E), and Periodic Acid Schiff (PAS)/alcian blue for inflammation related morphology and mucus production, respectively. Sections were examined by bright-field microscopy.

2.12. Harvesting of airway epithelial cells (AECs)

Airway epithelial cells from murine trachea were harvested by

scrapping the lumen of the trachea as described earlier [37]. For flow experiments, whole tracheas were cut open longitudinally and AECs were gently scraped using a mini plastic spatula in RPMI-1640. Scrapped epithelial cell clumps were passed through an 18G needle several times to prepare single cell suspension for flow cytometry experiments.

2.13. Normal human bronchial epithelial cell (HBEC) culture

Human primary bronchial epithelial cells (HBECs) of normal subjects (n = 3) were obtained from Lonza (Lonza, USA) and grown in commercially available BEGM™ BulletKit™ (Lonza, USA) as recommended by the manufacturer. HBECs were incubated at 37 °C in a humidified atmosphere with 5% CO₂ and grown until 70–80% confluence. All experiments were repeated twice and HBECs at 3–4 passage were used for all experiments. Prior to experiments, monolayers of HBECs were incubated in basal medium (supplement free) overnight. Cells were treated with BET inhibitor, (+)JQ-1 (Tocris, UK) or its inactive enantiomer (–)JQ-1 (Tocris, UK) at a final concentration of 100 nM or Dexamethasone at 20 µM, 1 h prior to stimulation with rhIL-17A (10 ng/ml; Biolegend). Cells were harvested for different analyses (flow cytometry/ western blot) 24 h post-IL-17A stimulation.

2.14. Reactive oxygen species (ROS) measurement

ROS generation was measured spectrofluorimetrically by oxidation of specific probe, 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) in BAL cells as described earlier [37]. Data is expressed as fold median fluorescence intensity (MFI) over control.

2.15. Chemicals

All chemicals were of the highest grade available unless stated otherwise and were purchased from Sigma Chemicals (USA).

2.16. Statistical analysis

The data were expressed as mean ± SEM (n = 10–12) and derived from two/three independent experiments. HBEC culture data were expressed as mean ± SEM (n = 3). Comparisons among different groups were analyzed by one-way ANOVA (analysis of variance) followed by Tukey's multiple comparison tests. A 'P' value of < 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 BET inhibitor, (+)JQ-1 on CE-induced mixed granulocytic airway inflammation and related parameters

Our modified cockroach allergen extract mouse model of asthma showed a robust mixed granulocytic phenotype (eosinophilic and neutrophilic inflammation) as well as mixed Th2/Th17 endotype in the lung (Figs. 2 and 3). In a preliminary experiment in this model, BET inhibitor (+)JQ-1 at 100 µg/mouse was found to be optimal as it led to > 90% reduction in CE-induced neutrophils/MPO activity with no further reduction at higher doses (Fig. 1A and B). Therefore, we chose this dose for further experiments. We then tested the efficacy of (+)JQ-1 on mixed granulocytic airway inflammation and other related parameters (Fig. 2A). Our data show that (+)JQ-1 abolishes neutrophilic inflammation/MPO activity (Fig. 2B and C) but has limited effect on eosinophilic inflammation (Fig. 2B). Reduction in neutrophilic inflammation was associated with decreases in MUC5AC expression/PAS staining (Fig. 2D–E) and overall inflammation (Fig. 2B and F). There was no significant difference in mixed airway inflammation and other inflammatory markers between (–)JQ-1 treated control and (+)JQ-1

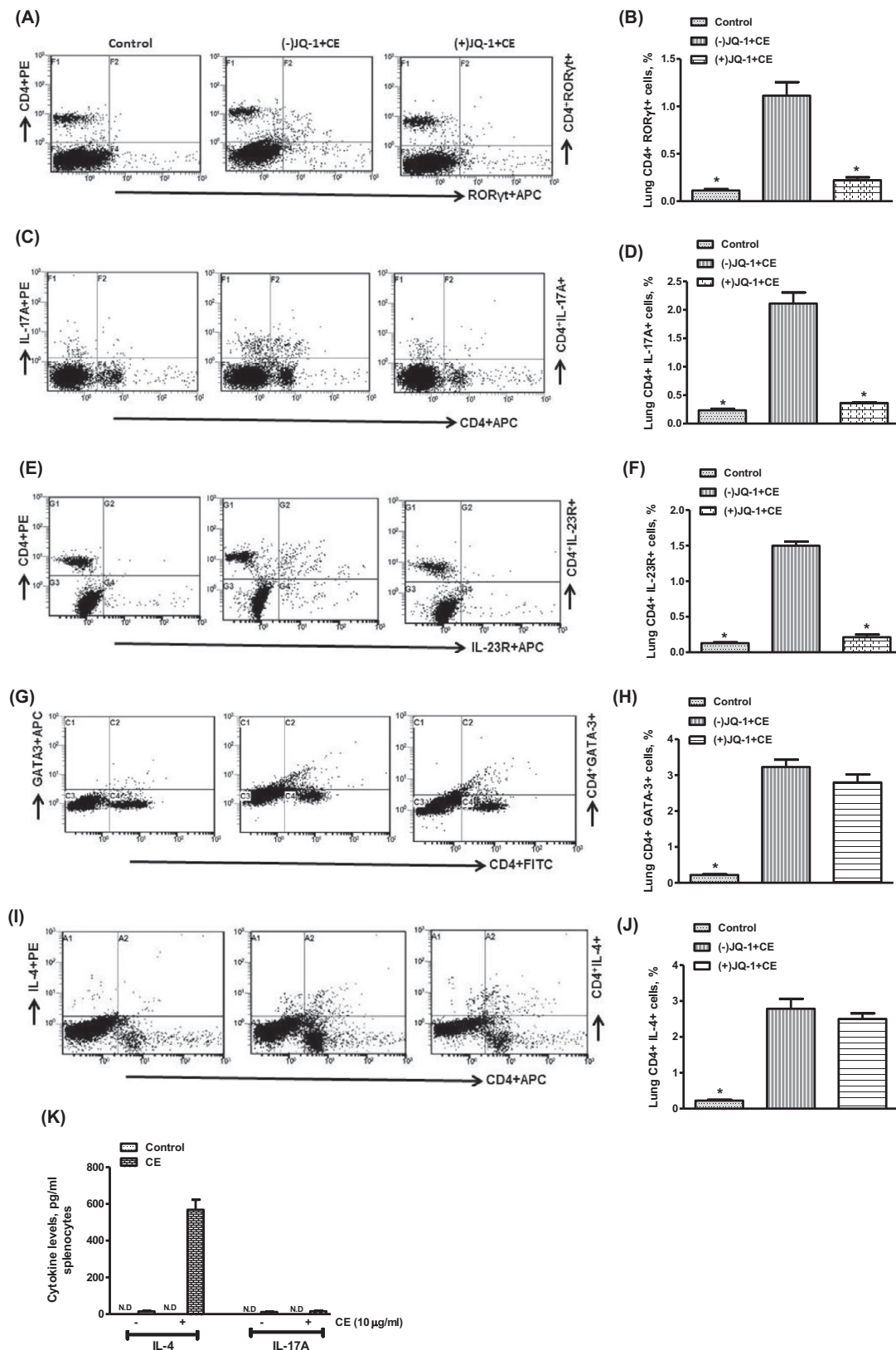


Fig. 3. Effect of BET inhibitor, (+)JQ-1 on CE-induced Th17/Th2 related markers in the lungs of mice. A) Representative dot plot for ROR γ t immunostaining in CD4⁺ T cells, B) % ROR γ t + CD4⁺ T cells; C) Representative dot plot for IL-17A immunostaining in CD4⁺ T cells, D) % IL-17A + CD4⁺ T cells; E) Representative dot plot for IL-23R immunostaining in CD4⁺ T cells, F) IL-23R + CD4⁺ T cells, G) Representative dot plot for GATA-3 immunostaining in CD4⁺ T cells; H) % GATA-3 + CD4⁺ T cells, I) Representative dot plot for IL-4 immunostaining in CD4⁺ T cells; and J) % IL-4 + CD4⁺ T cells, and K) IL-4 and IL-17A protein levels in splenocytes culture in the mice. Protein expression was measured by flow cytometry/ELISA. Dot plots represent events in lung cells taken from Control, (-)JQ-1 + CE and (+)JQ-1 + CE groups. Values are expressed as mean $\pm$ SEM, $n = 10$ –12/group from three independent experiments. * $P < 0.05$, vs. (-)JQ-1 + CE group.

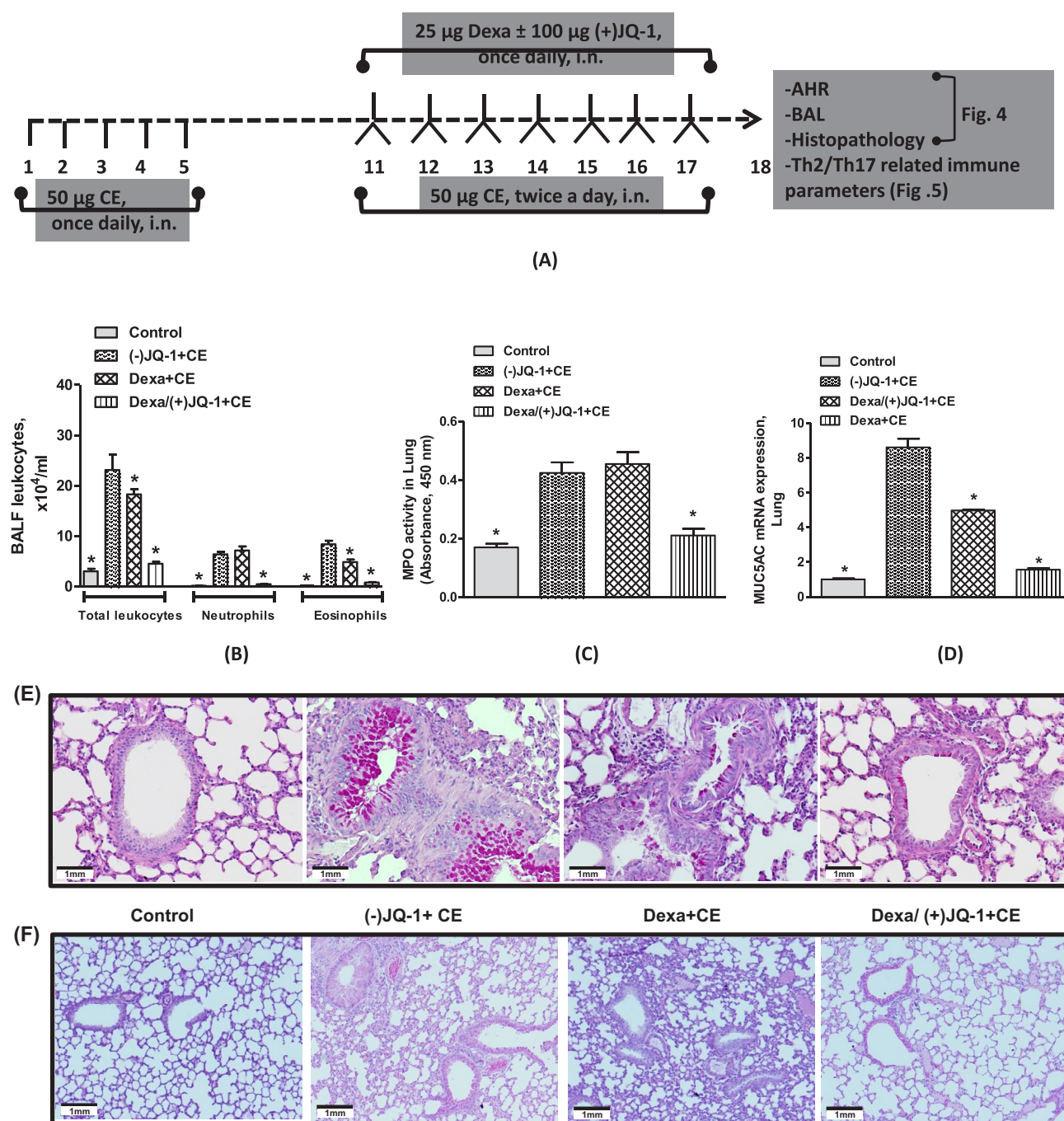


Fig. 4. Effect of dexamethasone (Dexa) alone and in combination with BET inhibitor, (+)JQ-1 on CE-induced mixed granulocytic airway inflammation and related parameters in the mice. A) Study design for the effect of Dexa and (+)JQ-1 in CE-induced mixed granulocyte asthma model, B) Total leukocytes, neutrophils, and eosinophils in BALF, C) Lung MPO activity, D) Lung MUC5AC, E) Lung sections stained with Alcian blue (upper horizontal panel; 200X), and F) H&E (lower horizontal panel; 100X). Each photomicrograph is a representative image from every group. BAL was performed 12 h after last CE challenge. Values are expressed as mean $\pm$ SEM, $n = 10$ –12/group from three independent experiments. * $P < 0.05$, vs. (-)JQ-1 + CE group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

treated control groups (Fig. 2); therefore (+)JQ-1 treated control group was not included in further experiments. Overall, these data suggest that (+)JQ-1 mainly suppresses neutrophils related airway inflammatory changes in CE-induced mixed granulocytic asthma model.

3.2. Effect of (+)JQ-1 on CE-induced changes in Th17 and Th2 markers

Since (+)JQ-1 mainly suppressed neutrophilic inflammation which was dependent on Th17 cells, we next asked the question whether BET inhibition would able to suppress Th17 related markers in the lung. Our

data show that (+)JQ-1 almost completely abolished CE-induced increase in Th17 markers in CD4+ T cells and lungs in general as shown in Fig. 3, and Table 1. (+)JQ-1 reversed CE-induced increases in RORC, IL-17A, and IL-23R expression as assessed by flow cytometry, and real-time PCR (Fig. 3A–F & Table 1). CE-induced increases in IL-17A protein levels in the lung (Table 2) were also greatly attenuated by the treatment of (+)JQ-1. However, there was no significant effect of (+)JQ-1 on STAT3 mRNA expression in CD4+ T cells (Table 1).

Since there was some inhibitory effect of (+)JQ-1 on eosinophils as well, we further assessed its effect on Th2 related markers in the lung.

Table 1

Fold difference in mRNA expression of Th2/Th17 related genes in pulmonary CD4+ T cells.

	Control	(-)JQ-1 + CE	(+)JQ + CE
RORC	1 ± 0.08*	26.7 ± 2.5	2.7 ± 0.15*
IL-17A	1 ± 0.09*	85.8 ± 5.9	4.1 ± 0.31*
IL-23R	1 ± 0.08*	56.6 ± 4.1	5.3 ± 0.46*
STAT-3	1 ± 0.06*	14.7 ± 1.1	18.4 ± 1.5
GATA-3	1 ± 0.06*	22.4 ± 1.8	21 ± 1.4
IL-4	1 ± 0.07*	71.5 ± 4.6	70.4 ± 3.5
IL-13	1 ± 0.08*	33.6 ± 2.1	31.9 ± 1.9

Values are expressed as Mean ± SEM, n = 10–12/group from three independent experiments. *P < 0.05, vs. (-)JQ-1 + CE group.

Table 2

Th17/Th2 cytokines levels in the lung (pg/mg protein).

	Control	(-)JQ-1 + CE	(+)JQ + CE
IL-17A	3.5 ± 0.19*	76.9 ± 6.1	9.2 ± 1.1*
IL-4	2.4 ± 0.10*	16.5 ± 1.3	15.8 ± 1.9
IL-5	2.8 ± 0.22*	25.5 ± 1.5	23.4 ± 1.6

Values are expressed as Mean ± SEM, n = 10–12/group from three independent experiments. *P < 0.05, vs. (-)JQ-1 + CE group.

Our data show that (+)JQ-1 failed to inhibit CE-induced increase in Th2 immune responses in the lungs of mice (Fig. 3G–J; Tables 1 and 2). Splenocytes isolated from CE-treated mice showed increase in only IL-4 but not IL-17A in response to *ex vivo* CE stimulation suggesting that Th17 differentiation conditions prevail only in the lung (Fig. 3K). Overall, these data show that (+)JQ-1 suppresses mixed granulocytic airway inflammation partially by abolishing Th17 driven immune responses in the lung.

3.3. Effect of dexamethasone alone and in combination with (+)JQ-1 on mixed granulocytic airway inflammation and related parameters

Th17 cells and neutrophils are associated with corticosteroid insensitivity; therefore we wanted to test whether corticosteroid therapy would be effective in mixed granulocytic asthma (Fig. 4A). Dexamethasone alone failed to inhibit both overall and neutrophilic inflammation (Fig. 4B,C and F), although it had significant effect on eosinophilic inflammation (Fig. 4B) and mucus secretion (PAS staining/ MUC5AC expression; Fig. 4D–E). Dexamethasone also had some degree of success in attenuation of CE-induced increase in Th2 immune responses in the lung, however it had no effect on Th17 driven immune responses (Fig. 5A–C). Based on the data obtained with (+)JQ-1 on mixed granulocytic inflammation, our next hypothesis was to test its efficacy in combination with corticosteroid therapy. Combination of (+)JQ-1 with Dexamethasone almost completely abolished both CE-driven mixed granulocytic inflammation and related inflammatory markers (Fig. 4B–F) as well as Th2/Th17 immune responses in the lung (Fig. 5A–C). These data show that elimination of Th17 driven immune response in the lungs of CE-treated mice leads to restoration of corticosteroid responsiveness.

3.4. Effect of (+)JQ-1 and dexamethasone on HDAC2 and nitrotyrosine in airway epithelial cells

AECs have been shown to be associated with corticosteroid insensitivity through reduction in HDAC2 expression, therefore we examined the effect of (+)JQ-1 and Dexamethasone on CE-induced changes in HDAC2 expression in AECs (Fig. 6). CE-treated mice had significant reductions in HDAC2 expression as assessed by western blot (Fig. 6A and B), and flow cytometry (Fig. 6C and D). Treatment of CE-administered mice with (+)JQ-1 almost completely restored HDAC2 expression whereas Dexamethasone treatment had a trend towards an increase,

however it was not significant (Fig. 6A–D). Since oxidative stress has been linked to a decrease in HDAC2 expression, we evaluated a well-known marker of oxidative stress, i.e. nitrotyrosine in AECs of CE-administered mice. CE administration led to a marked increase in immunostaining of nitrotyrosine in AECs, i.e. nitrotyrosine + EPCAM + cells (Fig. 6E and F). (+)JQ-1 almost completely inhibited CE-induced increase in nitrotyrosine + EPCAM + cells, whereas Dexamethasone had only partial inhibition (Fig. 6E and F). These data suggest that BET inhibition restores responsiveness of corticosteroid therapy through normalization of HDAC2 levels in AECs.

3.5. Effect of depletion of IL-17A and neutrophils on HDAC-2, oxidative stress and airway inflammation

Our next obvious question was to evaluate the direct effects of IL-17A and neutrophils on HDAC2 in CE-induced mixed granulocytic inflammation since (+)JQ-1 treatment inhibited both of them. For this purpose, we depleted IL-17A and neutrophils in CE administered mice by utilization of antibodies against IL-17A and neutrophils (Fig. 7). Our data showed that both IL-17A and neutrophil depletion in CE-administered mice led to an increase in HDAC2 expression (Fig. 7A and B). Depletion of neutrophils/IL-17A was associated with greatly attenuated neutrophilic infiltration into the lungs (Fig. 7C) and oxidative stress in BAL cells obtained from lungs (Fig. 7D) of CE-administered mice. Anti-IL17A antibody or anti-neutrophil antibody treated control groups did not have any differences in HDAC2 expression, BAL neutrophil numbers, and oxidative stress in BAL cells as compared to control group (data not shown). These data suggest that IL-17A driven neutrophilic infiltration and oxidative stress might play an important role in reduction of HDAC2 expression.

Next we wanted to evaluate the effects of BET inhibition, corticosteroids and IL-17A on HDAC2 expression in primary HBECs similar to murine AECs (Fig. 8). IL-17A treatment led to reduction in HDAC2 as shown by western blot and flow cytometry (Fig. 8A–C). Pretreatment with (+)JQ-1 reversed IL-17A-mediated reduction in HDAC2, whereas Dexamethasone did not have significant effect on HDAC2 expression (Fig. 8A–C). IL-17A treatment also led to increased nitrotyrosine expression as assessed by flow cytometry in HBECs which was attenuated by (+)JQ-1, whereas Dexamethasone had limited effect (Fig. 8D). There was no significant difference in HDAC2/nitrotyrosine expression between (-)JQ-1 treated and (+)JQ-1 treated control cells (data not shown); therefore (-)JQ-1 treated control group was used in further experiments. These data show that IL-17A also downregulates HDAC2 expression in HBECs which could be mediated through oxidative stress. Moreover, BET inhibitor, (+)JQ-1 was able to reverse IL-17A-mediated reduction in HDAC2 in HBECs. Overall, the data from the present study suggest that Th17 mediated inflammation plays a pivotal role in corticosteroid insensitivity in mixed granulocytic asthma. Further, our study shows that (+)JQ-1 restores corticosteroid responsiveness by abolishing Th17 driven immune responses in the lungs (please see graphical abstract).

4. Discussion

In this study we explored the effects of BET inhibitor (+)JQ-1 in a cockroach allergen-induced mouse model of asthma having both Th2/Th17 airway immune responses and mixed eosinophilic/neutrophilic inflammation, relevant to the pathogenesis of human asthma. There is a subset of asthma patients who have mixed Th2/Th17 endotype which is partially resistant/refractory to steroid therapy [11,16,18,38,39]. Our model recapitulated some of the features of this mixed granulocytic phenotype by showing partial resistance to steroids. A recent study also showed that Th2/Th17 adoptive cell transfer led to partial steroid resistant asthma in mice [38]. Our study shows that (+)JQ-1 leads to suppression of Th17 cells as well neutrophil inflammation without having any significant effect on Th2 related markers. Further, (+)JQ-1 was able to restore sensitivity of steroids in mixed granulocyte asthma

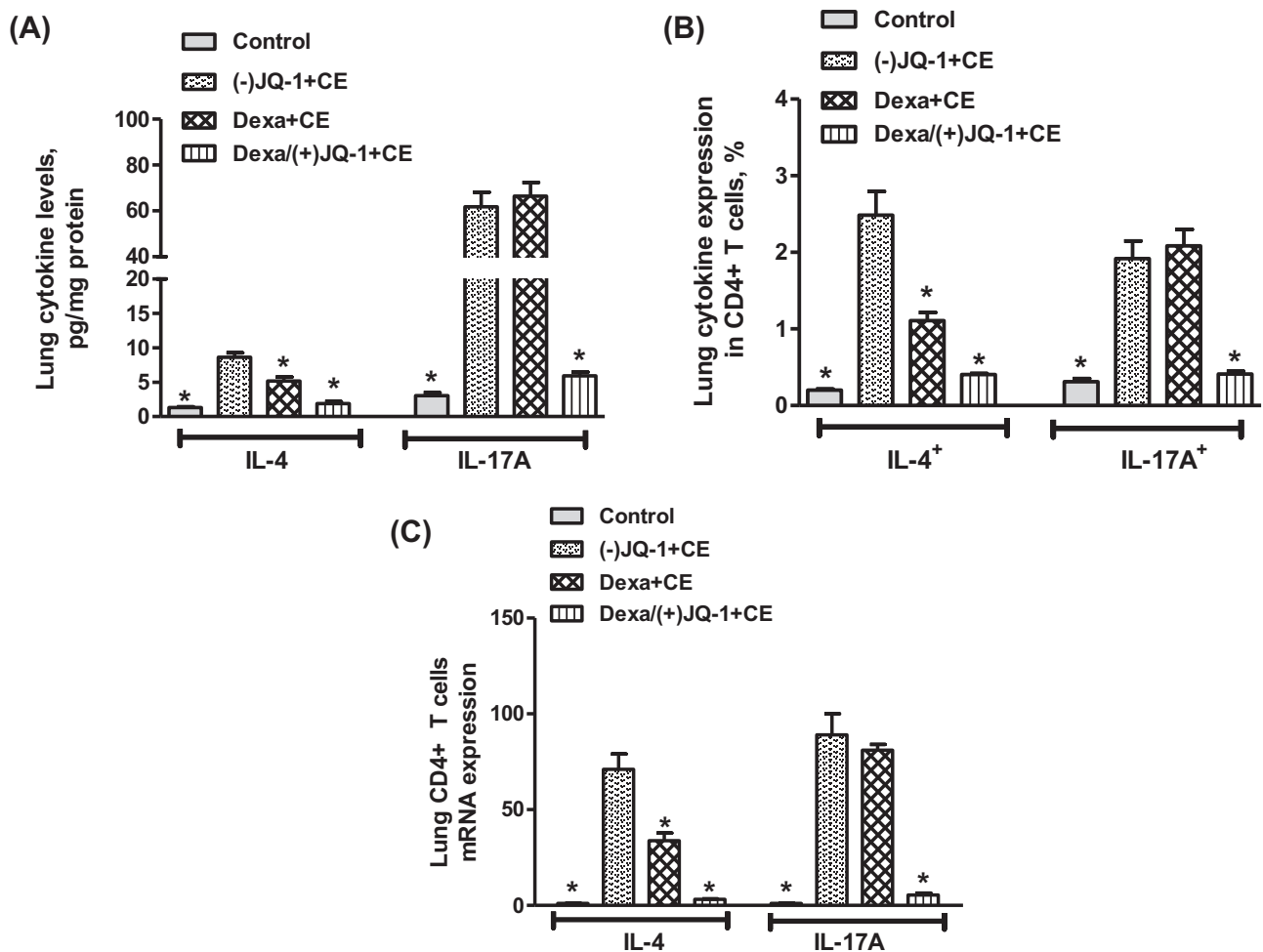


Fig. 5. Effect of dexamethasone (Dexa) alone and in combination with BET inhibitor, (+)JQ-1 on CE-induced Th2/Th17 related markers in the lungs of mice. A) IL-4 and IL-17A protein levels, B) % of IL-4 + CD4+ T cells and % of IL-17A + CD4+ T cells, and C) mRNA expression in CD4+ T cells. Protein expression was measured by flow cytometry/ELISA. mRNA expression level in lung CD4+ T cells of different groups was assessed by real time PCR. Values are expressed as mean $\pm$ SEM, $n = 10$ – 12 /group from three independent experiments. * $P < 0.05$, vs. (-)JQ-1 + CE group.

due to reductions in Th17 cells and associated neutrophilic inflammation.

Classically, asthma has been considered a Th2 driven airway disease. However numerous studies in the past decade have shown that it is a heterogeneous disease with different phenotypes which is likely dependent on the inflammatory microenvironment created by the aeroallergens [3,11,19]. Natural allergens originating from cockroach create such a microenvironment in the airways which allow differentiation of Th naïve cells into both Th2 and Th17 cells. Several studies in the past including ours have shown a mixed Th2/Th17 immune response in the lung of CE-induced asthmatic mice [8,19,31]. This may be due to increased IL-23/IL-6 released by epithelial and dendritic cells upon activation of PAR-2 and other innate immune receptors by natural allergens as shown previously [10,40,41]. This was further confirmed by lack of Th17 responses in the periphery whereas lung showed robust Th17 immune responses. These data show that CE-induced cytokines create a microenvironment specifically in the airways for the differentiation of Th17 cells.

Most of the steroid resistant models are based on adoptive transfer of antigen specific Th17 cells followed by antigen exposure or intranasal antigen exposure with a proinflammatory cytokine such as IL-17A/TNF- α [33,42,43]. These models don't wholly represent human mixed asthma phenotype which is a result of exposure to natural allergens such as cockroach allergens. Hence, use of BET inhibitor and corticosteroids in CE-induced mixed granulocytic phenotype allowed us

to dissect contribution of Th2 as well as Th17 cells in driving overall asthmatic inflammation.

The ability of the BET inhibitors to suppress inflammatory gene expression has now been demonstrated in a variety of inflammatory and autoimmune diseases [24,27]. BET inhibitors selectively inhibit expression of pro-inflammatory genes through interference in binding of BET proteins to histone acetylation sites [23,24]. BET proteins are usually involved in inflammatory signaling through association with transcription factors such as NF κ B, STAT3, and Nrf2 [26,27,44]. These studies suggest that BET bromodomains play a significant role in gene regulation epigenetically.

BET bromodomains are also known to associate with transcription factor, ROR γ t thereby playing an important role in Th17 differentiation. BET inhibitors have been specifically reported to inhibit Th17-mediated immune responses. For example, it has been shown that (+)JQ-1 inhibits disease progression in mouse model of multiple sclerosis through reduction Th17 immune responses [27]. BET inhibitors have recently been shown to attenuate pulmonary inflammation both *in vitro* and *in vivo* [34,45]. They have also shown efficacy in steroid resistant asthma models [32,33]. However, it not known whether BET inhibitors have the capacity to suppress the pathogenic mechanisms that underpin CE-induced mixed granulocytic airway inflammation.

One of the most important signature cytokines released by Th17 cells, i.e. IL-17A is increased in the lungs, sputum and BAL of asthmatic patients. IL-17A is further increased in severe asthmatics as compared

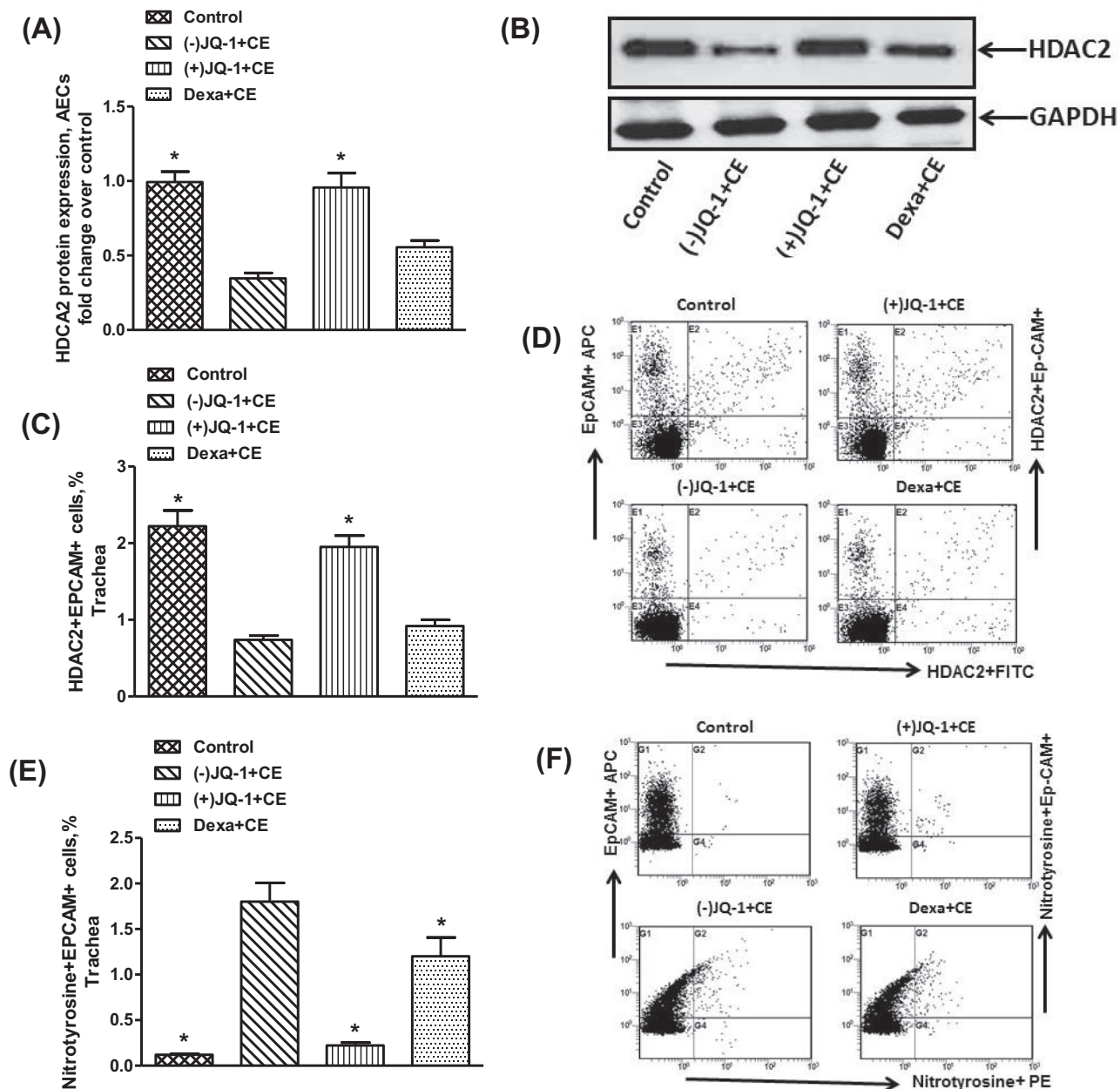


Fig. 6. Effect of BET inhibitor, (+)JQ-1 and corticosteroid therapy on HDAC2 and nitrotyrosine expression in the airway epithelial cells of mice. A) HDAC2 protein expression, B) representative immunoblot for HDAC2 protein expression, C) % of HDAC2 + EPCAM + cells, D) representative dot plot for HDAC2 immunostaining in EPCAM + cells, E) % of nitrotyrosine + EPCAM + cells, and F) representative dot plot for nitrotyrosine immunostaining in EPCAM + cells. Protein expression was measured by flow cytometry/western blot. Dot plots represent events in tracheal epithelial cells taken from Control, (-)JQ-1 + CE, (+)JQ-1 + CE, and Dexa + CE groups. Values are expressed as mean $\pm$ SEM, $n = 10$ –12/group from three independent experiments. * $P < 0.05$, vs. (-)JQ-1 + CE group.

to mild asthmatics [17,18–29]. The ability of Th17 cells/IL-17A to cause the infiltration of neutrophils suggests that their involvement in the pathogenesis of severe asthma [12,33]. In our study, only Th17 cells were markedly downregulated by (+)JQ-1 treatment. Th17 down-regulation led to abolishment of airway neutrophils without affecting Th2 responses. However, there was a slight but significant reduction in eosinophilic inflammation as well.

Therapies that target signaling mechanisms leading to differentiation of Th17 cells have been shown to cause reduction in both neutrophils and eosinophils in the airways. Anti-IL6R antibody has been shown to reduce not only neutrophils but also eosinophils along with IL-17A levels in the lung [41]. Lack of gp130, a signaling component required for IL-6R signaling also leads to decrease in airway neutrophils/eosinophils as well as IL-17A in a mouse model of asthma [42].

Furthermore, direct depletion of neutrophils in a mouse model of asthma has also shown reduction in airway eosinophils [9]. These observations along with our study suggest that therapeutic interruption of Th17 associated airway inflammation may also lead to decrease in airway eosinophils to some extent.

Th17-driven neutrophilic inflammation has emerging role in corticosteroids insensitivity. Th17 cell transfer results in corticosteroid-insensitive airway inflammation [46]. Corticosteroids are not able to attenuate Th17/neutrophilic airway inflammation while reducing Th2/eosinophilic inflammation [12,13,38,42]. ROR γ t overexpression leads to corticosteroid resistant asthma whereas GATA3 overexpression leads to corticosteroid sensitive asthma. Former is dependent on Th17/neutrophilic inflammation whereas the latter is dependent on Th2/eosinophilic inflammation [13]. On the other hand, BET inhibitors affect

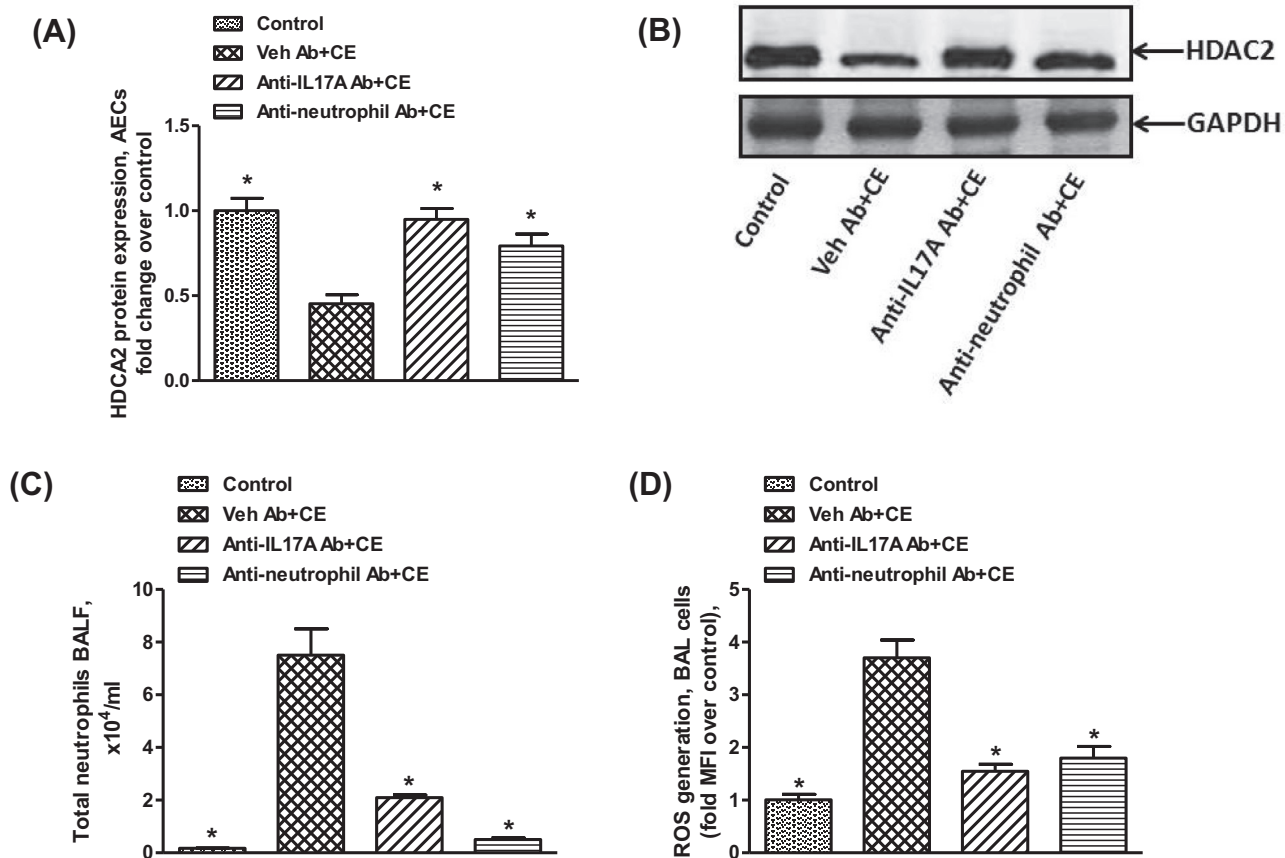


Fig. 7. Effect of IL-17A/neutrophil depletion on HDAC2 expression in AECs, neutrophilic airway inflammation, and oxidative stress in CE-driven asthma model in mice. A) HDAC2 protein expression in murine AECs, B) representative immunoblot for murine HDAC2 protein expression, C) Total neutrophils in BALF, and D) ROS generation in BAL. Protein expression was measured by western blot/ flow cytometry, and ROS generation was measured biochemically. Values are expressed as mean $\pm$ SEM, $n = 10$ –12/group for mice from two/three independent experiments. * $P < 0.05$, vs. Veh Ab + CE group.

mostly Th17 immune responses and neutrophilic inflammation as shown in the current study and previously [32,33]. Based on these observations, we hypothesized that BET inhibitor if combined with corticosteroids might be able to restore their efficacy due to abolishment of Th17 dependent neutrophilic inflammation. Our data showed that dexamethasone alone did not have much efficacy against mixed granulocytic airway inflammation which was due to Th17-induced airway neutrophilic infiltration. However, BET inhibitor, (+)JQ-1 in combination with dexamethasone totally inhibited both neutrophil and eosinophilic inflammation.

The basis for corticosteroid insensitivity in mixed granulocytic asthma may be multifactorial. However, one of the most important mechanisms for corticosteroid efficacy is recruitment of HDAC2, which shuts down inflammatory gene transcription by removal of acetylation marks from core histone proteins thereby leading to recoiling of DNA [47,48]. Overall HDAC activity and in particular HDAC2 expression has been reported to be low in peripheral blood mononuclear cells/alveolar macrophages and airway epithelium from patients with asthma [47,49,50]. It has also been shown in mouse model of steroid resistant asthma that a decrease in HDAC2 activity might be responsible for resistance to corticosteroid therapy [12]. Our study also showed diminished expression of HDAC2 in airway epithelial cells which was reversed by (+)JQ-1 treatment. This could be the basis by which (+)JQ-1 attenuates corticosteroid resistance in CE-induced mixed granulocytic asthma.

Our study shows that IL-17A may be responsible for decreased HDAC2 expression in AECs since its depletion in the lungs of CE-administered mice led to restoration of HDAC2 expression in AECs. Moreover, treatment of HBECS *in vitro* with IL-17A also led to reduction

in HDAC2 expression. IL-17A has been shown to induce corticosteroid insensitivity in AECs through reduction in HDAC2 [51]. Our study further shows that this reduction in HDAC2 could be either through IL-17A-induced signaling in AECs, or neutrophilic inflammation, both of which lead to oxidative stress. IL-17A signaling has been reported to cause oxidative stress through activation of different enzymes responsible for ROS generation such as inducible nitric oxide synthase and NADPH oxidase [52,53]. Further, oxidative stress has been shown to cause corticosteroid unresponsiveness in HBECS due to increased degradation of HDAC2 in previous studies [54,55]. Furthermore, neutrophilic airway inflammation in asthma has also been shown to be associated with reduced HDAC activity in blood monocytes [56]. All of these observations suggest a role of IL-17A/neutrophil mediated oxidative stress in reduction of HDAC2 expression and subsequent insensitivity of corticosteroid therapy. Our study further showed almost complete reversal of IL-17A-mediated reduction of HDAC2 in AECs by BET inhibition, but not by corticosteroid therapy. Therefore, the overall picture from this study suggests that BET inhibition may lead to restoration of corticosteroid responsiveness by blocking either IL-17A production in T cells or IL-17A-mediated signaling in AECs.

BET inhibition by reversing corticosteroid insensitivity enhances overall transcriptional blockade of inflammatory gene expression in the asthmatic lung. While BET inhibitor blocks acetylated lysines in histones thereby blocking transcriptional machinery required for inflammatory gene expression [23,24]. On the other hand, corticosteroids recruit HDAC2 which removes acetylation marks on histones to repress inflammatory gene expression [48]. Therefore, BET inhibitor by rescuing HDAC2 protein through suppression of Th17 mediated inflammation may be an alternative treatment strategy to reverse

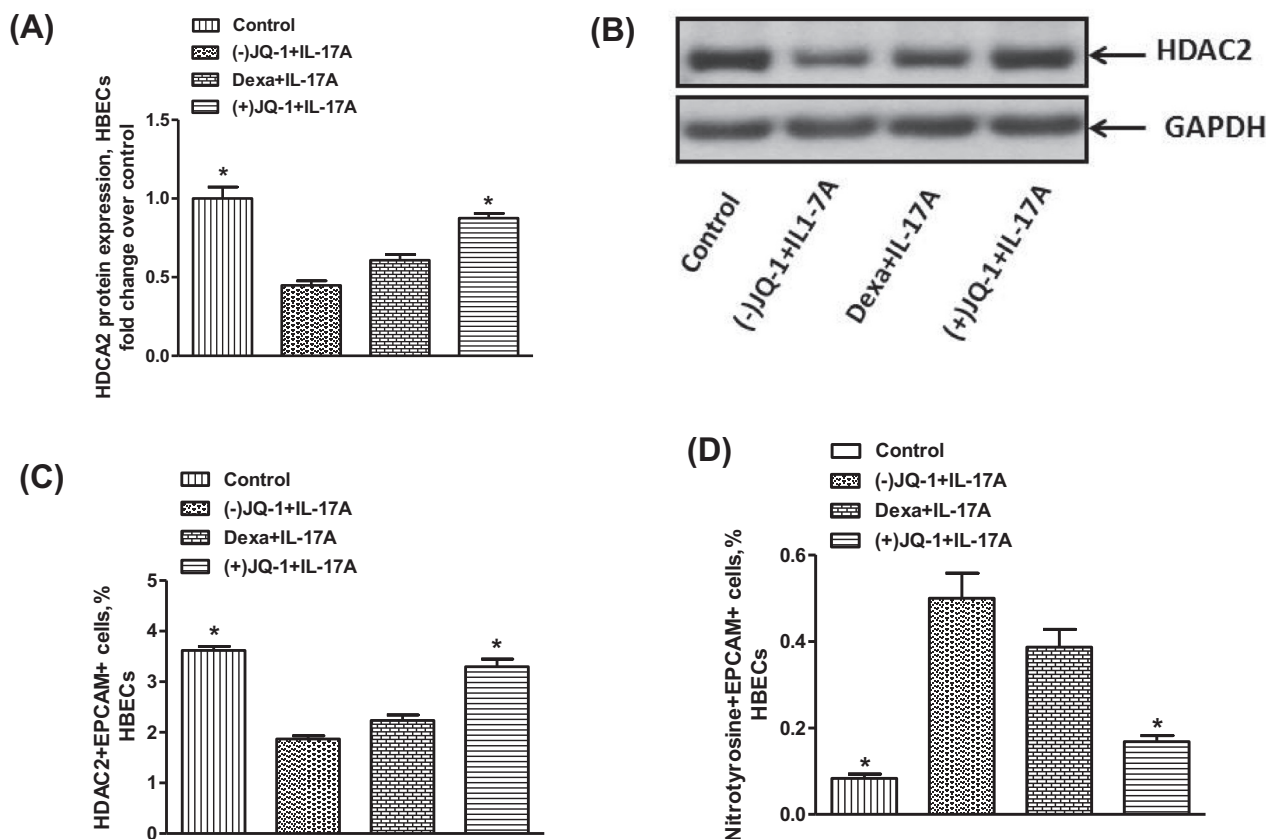


Fig. 8. Effect of IL-17A on HDAC2 expression in HBECs *in vitro*. A) HDAC2 protein expression, B) representative immunoblot for HDAC2 protein expression, C) % of HDAC2 + EPCAM+ cells; and D) % of nitrotyrosine + EPCAM+ cells. Protein expression was measured by western blot/ flow cytometry. Values are expressed as mean $\pm$ SEM, $n = 3$ /group. * $P < 0.05$, vs. (-)JQ-1 + IL-17A.

glucocorticoid resistance in a mixed granulocytic asthma phenotype.

IL-13 has been shown to be heavily involved in mucus hypersecretion and goblet cell metaplasia in allergic asthma [57]. Our study shows that MUC5A/mucus secretion is attenuated by BET inhibition. This could be due to BET inhibitor-induced reduction in Th17 cells/IL-17A as recent studies have shown that Th17 cells/IL-17A play an important role in upregulation MUC5AC and mucus secretion/goblet cells hyperplasia [58,59].

Dexamethasone has been used at a dose ranging from 0.2 mg/kg up to 5 mg/kg in different animal models of asthma, with former dose being equivalent to a clinically relevant human dose [60–62]. Importantly, dexamethasone at this dose, i.e. 0.2 mg/kg was sufficient to inhibit Th2 related allergic airway inflammation [60], therefore a dose of 1 mg/kg used in our study should be potent enough to suppress Th2 related airway inflammation. However, our results show only partial inhibition of Th2-related signaling at this dose which suggests that Th17/IL-17A/neutrophils play a significant role in corticosteroid resistance in this model.

In conclusion, combination therapy of BET inhibitor with corticosteroids may be pursued as a potential therapeutic strategy to combat Th2/Th17 associated mixed granulocytic airway inflammation in asthma.

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Author Contributions

AN conceived the study. AN, SFA, NS, KEI, and SMA performed and discussed the experiments. AN, SFA, NOA, NS, SMA, and KEI contributed to experimental design and data analysis/interpretation. AN, NS, and FAS wrote the manuscript with inputs from other authors. All authors read and approved the final manuscript.

Conflict of interest

All of the authors declare that there is no conflict of interest.

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