CpG oligodeoxynucleotides attenuate ROR γ t-mediated Th17 response by restoring histone deacetylase-2 in cigarette smoke-exposure asthma

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Research

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Abstract

Background: Cigarette smoke (CS) exposure increases corticosteroid insensitive asthma related to increased Th17 phenotype, and new treatment strategies are needed for CS-associated asthma. Histone deacetylase 2 (HDAC2), found in airway epithelium, is critical in improving glucocorticoids insensitivity. We recently demonstrated the anti-inflammatory properties of CpG oligodeoxynucleotides (CpG-ODN) in CS-exposure asthma. However, CpG-ODN's effects on HDAC2 expression and enzyme activity remain unstudied. This study aimed to assess whether CpG-ODN protect against excessive Th17 immune responses in CS-induced asthma through HDAC2-dependent mechanisms, comparing their effects with corticosteroids'.

Methods: The effects of CpG-ODN alone and in combination with budesonide (BUD) on airway inflammation and Th2/Th17 related airway immune responses were determined using the *in vivo* model of CS-associated asthma and in cultured bronchial epithelial (HBE) cells administered ovalbumin (OVA) and/or cigarette smoke extract (CSE). HDAC2 and retinoid-related orphan nuclear receptor γ t (ROR γ t) were also assessed in mouse lung specimens and HBE cells.

Results: CpG-ODN and BUD *in vivo* synergistically attenuated CS-exposure asthmatic responses at various levels such as immune cell influx with mixed eosinophils and neutrophils, airway remodeling, Th2/Th17 associated cytokine and chemokine production, and airway hyperresponsiveness, along with blockade of ROR γ t-mediated Th17 inflammation through induced HDAC2 expression/activity. *In vitro*, CpG-ODN synergized with BUD to inhibit Th17 cytokine production in OVA- and CSE-challenged HBE cells while suppressing ROR γ t and increasing epithelial HDAC2 expression/activity.

Conclusions: CpG-ODN reversed CS-induced HDAC2 downregulation and enhances the sensitivity of CS-exposure asthma and CSE induced HBE cells to glucocorticoid treatment. This may be related to HDAC2 recovery via ROR γ t/IL-17 pathway regulation, suggesting CpG-ODN as a potential corticosteroid-sparing agent in CS-induced asthma with Th17-biased immune conditions.

Introduction

Asthma is an increasingly prevalent respiratory ailment. It affects at least 300 million individuals worldwide, with about 345000 deaths annually[1]. Approximately 25% of the adult population in developed nations smoke, and survey of asthma patients suggests a rate of smoking mirroring that of the general population[2]. Cigarette smoke (CS), directly and passively, increases asthma susceptibility, decreases the quality of life, and enhances symptom severity, as well as attack frequency and asthma exacerbations[3]. According to several clinical studies, asthmatic smokers show poor response to treatment with mainstay steroids compared with non-smokers[2]. Some asthma after exposure to CS progresses to uncontrolled asthma, also named severe asthma.

Although inhaled corticosteroids (ICS) are the standard therapeutic option for asthma, individuals show various responses, and most severe asthma patients may be insensitive to steroid-mediated

suppression[4]. Asthma is currently considered a heterogeneous ailment with many phenotypes, involving Th1, Th2 and Th17 cells[5]. In general, the polarized Th2 immune response featuring eosinophil influx substantially contributes to the development of allergic airway inflammation. Individuals with mild to moderate asthma are mostly of this type and could be treated with classic therapies, such as ICS. Meanwhile, severe asthma is hardly manageable, even with newest drugs. Severe asthma cases have a mixed Th1/Th2 phenotype comprising a Th17 component, with elevated neutrophil rates, or neutrophils co-existing with eosinophils, in the lung and sputum[6, 7]. We previously demonstrated that CS-exposure asthma shows Th17 differentiation, with budesonide (BUD) having limited effects on neutrophil infiltration in BALF, which suggested CS-exposed asthma may be relatively insensitive to corticosteroids (GCs)[8]. This could explain why asthma patients exposed to CS directly or passively may be less responsive to steroids. About 5–25% of severe asthma cases show poor symptom control even after administration of high-dose and/or systemic GCs, comprising nearly 50% of all asthma-associated treatment costs[9]. Accordingly, effective drugs are urgently required as mono or adjuvant therapies.

Corticosteroid insensitivity in mixed granulocytic asthma might be due to multiple factors. Classically, the molecular mechanisms of corticosteroid insensitivity mainly include the overexpression of proinflammatory transcription factors, phosphorylation of GC receptors (GR), and loss of histone deacetylase-2 (HDAC2) expression[10]. A recent study suggested that HDAC2 is required for corticosteroid-associated anti-inflammation[11]. To be specific, GCs decrease inflammatory reactions via HDAC2 recruitment to the promoters of proinflammatory genes, regulating their transcription[12]. According to several studies, HDAC2 activity is decreased in alveolar macrophages, peripheral blood mononuclear cells, and bronchial biopsies in asthma cases[13]. Moreover, studies have also demonstrated that HDAC2 protects airway inflammation in mouse and human epithelial cells. It is noteworthy that oxidative stress imposed by CS impairs HDAC2 function via ubiquitination-proteasome dependent degradation, leading to the amplification of the inflammatory response and GC insensitivity *in vitro* and *in vivo*[14–18]. Therefore, GC insensitivity is correlated with HDAC2, suggesting that drugs restoring HDAC2 activity and expression could improve GC insensitivity.

CpG oligodeoxynucleotides (CpG-ODN), unmethylated CpG dinucleotides, mimic the immunostimulatory activities of bacterial DNA to induce toll like receptor 9 (TLR9). CpG-ODN have demonstrated benefits in many rodent and primate models of asthma, with encouraging preliminary results in clinical studies[19]. The above reports suggested that CpG-ODN stimulate both Th1-inducing and Th2-limiting responses[20]. Our and other studies indicated that CpG-ODN have both potent preventive and therapeutic immune modulatory effects in allergic inflammatory diseases[21]. Treatment benefits of CpG-ODN in protecting against CS-induced airway inflammation are associated with reduced CS-induced excessive Th2/Th17 immune responses and increased Th1 responses based on our previous study[8], and further investigation of the underpinning mechanism is of great interest. Retinoid-related orphan nuclear receptor γ t (ROR γ t) represents a transcription factor regulating IL-17A[22]. Despite substantial efforts in understanding CpG-ODN-related anti-inflammation, it remains unclear whether and how CpG-ODN attenuate ROR γ t-mediated Th17 response by restoring HDAC2 activity and expression, thereby ameliorating GC insensitivity.

The current follow up study investigated the mechanism by which CpG-ODN regulate HDAC2 expression/activity, which modulates subsequent inflammatory responses in the mouse model of CS-related asthma and human bronchial epithelial (HBE) cells. We established a mouse model of ovalbumin (OVA)-related asthma after CS exposure, as well as *in vitro* cultures of HBE cells exposed to OVA and CSE, and administered CpG-ODN, BUD, to assess CpG-ODN's effects on airway inflammation and remodeling, as well as GC insensitivity via HDAC2 in mice co-exposed to chronic CS and OVA. We observed for the first time, to the best of our knowledge, that CpG-ODN could restore steroid sensitivity to block ROR γ t-induced upregulated IL-17 in CS-induced asthma *in vivo*, as well as in HBE cells induced by CSE, probably through the restoration of HDAC2 level and activity.

Materials And Methods

Mice and experimental design

Female specific pathogen free BALB/c mice (six- to seven-weeks old), provided by the Laboratory Animal Center of Southern Medical University, China (No.44002100019453), were housed under standard laboratory conditions such as 12h/12h light-dark cycle, and rodent chow and water for three days *ad libitum*.

Experimental animals were randomized to seven groups (12 animals/group), including the vehicle control, CS, OVA, OVA/CS, CpG-ODN, BUD and CpG-ODN/BUD groups. The latter 5 groups were sensitized and challenged with OVA. After each challenge, the mice of the last 4 groups were subjected to CS in ventilated whole-body smoking chambers as previously described[8]. CpG-ODN and/or BUD were administered intranasally to the last 3 groups half an hour post-OVA challenge as previously described[8]. The vehicle control and CS groups were not sensitized or challenged. Meanwhile, the vehicle control, CS and model groups were treated with NS as negative or positive controls. The chronic CS-exposure asthmatic murine model was established as described in a previous report, with minor modifications[7]. Details are described in the online supplementary material. A schematic diagram of CS-exposure asthmatic murine model and treatments is depicted in supplementary e-Fig. 1.

Laboratory Measurements in murine model

Additional details of bronchoalveolar lavage fluid (BALF) sampling, quantification of cytokines in BALF, tissue histology, immunohistochemistry, immunoblot, serum IL-17A and OVA-specific IgE level assessment, fluorescence microscopy, quantitative reverse transcription polymerase chain reaction (qRT-PCR), flow-cytometric analysis, and airway hyperresponsiveness (AHR) measurement are provided in the online supplemental material.

HDAC2 activity

HDAC2 activity in the nuclear extract was measured with HDAC2 IP & Activity Assay Kit (BioVision Mountainview, CA, USA) according to the manufacturer's instructions. We analyzed the fluorophore with

the excitation at 360nm and emission at 460nm by using a fluorescence plate reader.

CSE preparation

CSE preparation followed a previous report, with minor modifications[23]. Briefly, one cigarette (per cigarette: nicotine, 1.0 mg; tar oil, 10 mg; carbon monoxide, 13 mg; Tobacco Hunan Industrial Corporation, China) was combusted, and the smoke was passed through 10 mL of serum-free culture medium at 5 min/cigarette. The medium underwent pH adjustment to 7.4 and dilution as desired with culture medium. Freshly prepared CSE was employed within 30 min.

Cell culture

HBE cells, provided by the American type culture collection (ATCC® PCS-300-010™), underwent culture in RPMI 1640 containing 10% fetal bovine serum (FBS). Then, cells were administered 2.5, 5 and 10% CSE, respectively, for 6 h, 12h, 24h, 48h or 72h for detecting the dose/time effects of CSE on HBE cell proliferation.

Cytotoxicity Assay

The cell viability in response to the stimulation by different dose and time of CSE was performed using the standard 3-(4,5dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay[24]. Co-culture with CSE affected the proliferation and viability of HBE cells. A certain dose of CSE could stimulate the proliferation of HBE cells, while with increasing dose CSE showed cytotoxicity, not only inhibiting HBE cells but inactivating them, which suggested that a certain concentration CSE reduced the viability of HBE cells. Based on e-Fig.2 (see online supplementary e-Fig.2), HBE cells maintained with 2.5% CSE for 6 hours were more stable and close to natural cell proliferation; therefore, we chose 2.5% CSE to treat HBE cells for 6 hours in order to reduce experimental errors. Treatment dosages of CpG-ODN and BUD were determined in preliminary experiments.

Cell treatment

HBE cells were similarly divided into seven groups. (1) Vehicle control group: HBE cells were administered phosphate buffer saline (PBS), followed by PBS treatment. (2) CSE group: HBE cells were administered 2.5% CSE, followed by PBS treatment. (3) OVA group: HBE cells were administered 1 µg/ml OVA, followed by PBS treatment. (4) CSE/OVA group: HBE cells were simultaneously administered 1 µg/ml OVA and 2.5% CSE, followed by PBS treatment. (5) CpG-ODN group: HBE cells were simultaneously administered 1 µg/ml OVA and 2.5% CSE, followed by 5×10^{-6} M CpG-ODN treatment. (6) BUD group: HBE cells were simultaneously administered 1 µg/ml OVA and 2.5% CSE, followed by 10^{-8} M BUD treatment. (7) CpG-ODN/BUD group: HBE cells were simultaneously administered 1 µg/ml OVA and 2.5% CSE, followed by 10^{-8} M BUD and 5×10^{-6} M CpG-ODN treatment.

Laboratory Measurements in HBE cells

The amounts of IL-5, IL-13 (Th2 cytokines) and IL-17A (Th17 cytokine) were assessed by specific enzyme-linked immunosorbent assay (ELISA) kits (Bioss Inc., China) as directed by the manufacturer. The relative mRNA levels of cytokines in HBE cells were assessed by qRT-PCR. The protein expression levels of IL-17A (Invitrogen, USA), HDAC2 (Invitrogen, USA) and ROR γ t (Invitrogen, USA) were detected by Western blot and immunofluorescence. Flow cytometry antibodies detecting HDAC2, ROR γ t and IL-17A were provided by Abcam (US). Flow cytometry was carried out on a BD Calibur machine (BD, USA).

Statistical Analysis

Data are mean $\pm$ standard deviation (SD), and were assessed by one-way analysis of variance (ANOVA) for multiple groups, with post-hoc Tukey's multiple comparison test. GraphPad Prism 6.0 (GraphPad Software, USA) was employed for data analysis, with $P < 0.05$ indicating statistical significance.

Results

Suppression of combined granulocyte inflammation, airway structural remodeling, and AHR by CpG-ODN plus BUD in chronic CS-exposed asthmatic mice.

Histological data showed that lung specimens from OVA/CS mice had substantial peribronchial and perivascular connective tissues (e-Fig. 3A), multiple airway goblet cells containing mucus (e-Fig. 3B,D), and peribronchial collagen deposition (e-Fig. 3C,E). A combined granulocyte (neutrophil and eosinophil) inflammatory phenotype was confirmed as indicated by elevated Gr-1 (neutrophil-specific marker; Fig. 1B,E) and ECP (eosinophil-specific marker, Fig. 1A,D) immunohistochemical signals in the lungs, as well as marked expression of eotaxin 1 in BALF (Fig. 1C), which facilitates the recruitment of eosinophils and neutrophils[25]. Predominance of airway inflammation associated with neutrophils mixed with eosinophils was reduced; airway remodeling factors such as goblet hyperplasia and collagen accumulation were also diminished in the airway of CpG-ODN or BUD treated mice (e-Fig. 3) compared with CS-exposure asthmatic mice. Meanwhile, treatment with combined CpG-ODN and BUD caused almost no mucus hypersecretion alteration, negligible cell infiltration and airway wall thickness alteration, with suppression of AHR upon methacholine administration in animals with CS-exposure asthma (Fig. 1, e-Fig. 3).

Alteration of Th2/Th17 polarization and reduction of pro-inflammatory cytokines by CpG-ODN and BUD in CS-associated asthmatic mice.

Th2 markers (IL-5 and IL-13) were induced, while the Th1 marker IFN- γ was reduced after OVA + CS co-exposure in the mouse model ($p < 0.01$; e-Fig. 4C,D,E and $p < 0.01$; e-Fig. 4B). Pro-inflammatory cytokines (IL-8 and TNF- α) and TGF- β 1, and serum anti-OVA IgE increased also (all $p < 0.01$, e-Fig. 4A,F,G,H,I). The above-mentioned values changed substantially after treatment with CpG-ODN (e-Fig. 4). BUD also somewhat attenuated CS associated increase in pro-inflammatory cytokines and serum anti-OVA IgE. However, we also noted that CpG-ODN had additive beneficial effects with BUD treatment on Th1/Th2

homeostasis modulation, pro-inflammatory cytokines, TGF- β 1, and anti-OVA IgE, in the co-administration group (e-Fig. 4), which showed that CpG-ODN potentiated the effects of the corticosteroid.

Th17 cells exert their effects by producing multiple inflammatory cytokines such as IL-17A, which is known to enhance the chemotaxis of neutrophils in bronchial epithelial cells and airway smooth muscle cells[26]. More evidences have claimed Th17-associated neutrophilic airway inflammation in the mouse is GC insensitive[27]. As expected, Th17 cells in CS-exposure asthmatic mouse models were markedly elevated compared with the vehicle control group according to flow cytometry data (Fig. 2A,B). Moreover, significantly elevated serum, lung, and BALF IL-17A protein and mRNA amounts were found in the CS, OVA, and OVA/CS groups compared with the vehicle control group (Fig. 2C-G). Both CpG-ODN and BUD decreased the percentage of Th17 positive cells, and IL-17 mRNA and protein levels compared with untreated CS-related asthmatic mice (Fig. 2A-G). Meanwhile, joint treatment with CpG-ODN and BUD remarkably reduced Th17 cells, IL-17 mRNA and protein levels compared with the monotherapy groups (Fig. 2A-G).

Taken together, these data indicated that CS-exposure associated asthma induced a Th17/Th2-type response, and CpG-ODN and BUD synergistically decreased the exacerbated Th17- and Th2-associated cytokine amounts, enhancing the biosynthesis of IFN- γ , a Th1-associated cytokine.

HDAC2 activity and expression recovery upon treatment with CpG-ODN and BUD in CS-exposure asthmatic mice

CS reduces steroid responsiveness by modifying histone acetyltransferase, an essential epigenetic enzyme that mediates steroid anti-inflammatory action[28, 29]. Furthermore, HDAC2 activity and levels are substantially decreased by oxidative/nitrative stress, causing inflammation to be insensitive to the anti-inflammatory effects of GCs[30]. In this study, we assessed the levels of secreted HDAC2 in lung tissue samples by immunohistochemistry, ELISA and Western blotting. As shown in Fig. 3, OVA challenge and CS exposure both remarkably decreased HDAC2 mRNA and protein amounts (Fig. 3A-E). We also investigated the effects of CpG-ODN and BUD on CS-induced changes in HDAC2 mRNA and protein expression levels to verify whether CpG-ODN affect HDAC2 expression. Interestingly, it was found that after treatment with CpG-ODN or BUD only, HDAC2 gene expression levels were reversed compared with the untreated group, and this was more apparent after co-administration of CpG-ODN and BUD (Fig. 3A,B,C,E), although a statistically insignificant increase in HDAC2 protein expression levels was observed in mice administered CpG-ODN plus BUD ($P = 0.06$, Fig. 3D).

Moreover, based on studies reporting that patients with severe asthma have diminished GC sensitivity of peripheral blood monocytes (PBMCs) in comparison with non-severe asthma cases, in association with decreased HDAC2 activity that parallels the impaired GC sensitivity[31], we tested HDAC2 activity with a HDAC2 activity assay kit. As expected, similar to HDAC2 expression, HDAC2 activity in OVA + CS challenged mice was obviously suppressed and markedly recovered after administration of CpG-ODN or BUD, with significant differences between the OVA/CS and CpG-ODN/BUD groups, reflecting HDAC2 protein expression changes (Fig. 3F) .

These data suggested that HDAC2 was impaired, both at the expression and activity levels, in chronic asthmatic murine models. Meanwhile, CpG-ODN restored responsiveness to GC therapy by restoring HDAC2 expression and enhancing HDAC2 activity. When combined with BUD, CpG-ODN more substantially recovered HDAC2 activity and expression than either CpG-ODN or BUD alone.

Decreased RORyt expression and Th17 response under CS and OVA challenge after CpG-ODN and BUD treatment

HDAC2 is important in Th-17 cell differentiation from naive CD4⁺ T cells, and RORyt involvement attracts increasing attention[32, 33]. The catalytic activity of HDAC2 is important in inhibiting RORyt's transcriptional activity, and SUMOylated RORyt recruits HDAC2 to the IL-17 promoter for gene downregulation[34]. To explore the mechanism by which CpG-ODN treatment regulates the IL-17A cytokine due to HDAC2 up-regulation, we next examined the amounts of RORyt, an important biomarker of HDAC2-mediated Th17 response under CS-induced asthmatic conditions, by immunohistochemistry, ELISA and Western blotting. The results exhibited a distinct increasing trend in RORyt mRNA and protein expression levels in CS-exposed asthmatic mice in comparison with the vehicle control group (Fig. 4). Meanwhile, upon joint administration of CpG-ODN and BUD, the animals showed significantly decreased RORyt mRNA and protein amounts (Fig. 4), indicating that CpG-ODN in combination with BUD suppressed RORyt to a certain extent, thereby inhibiting IL-17A expression in Th17 cells.

CpG-ODN and BUD synergistically regulate interplay of HDAC2, RORyt and IL-17A, orchestrating inflammatory reactions in HBE cells.

Airway epithelial cells play a primordial role on body defense against allergens, viruses, and environmental pollutants, which are involved in asthma pathogenesis. Meanwhile, IL-17A is found in airway epithelial cells[35]. To further confirm whether CpG-ODN inhibit RORyt-mediated Th17 response via HDAC2, we next performed *in vitro* cultures of HBE cells exposed to OVA and/or CSE, and administered CpG-ODN and/or BUD. We performed ELISA, qRT-PCR, Western blotting, immunofluorescence and flow cytometry to assess the levels of cytokines, HDAC2 and RORyt in all groups.

Consistent with animal data, it is found that CSE-exposed or OVA-challenged HBE cells had elevated IL-5, IL-13 (Th2 cytokine) and IL-17A (Th17 cytokine) levels compared with the vehicle control group. The abovementioned cytokines were markedly increased in HBE cells after co-exposure to CSE and OVA (all $p < 0.01$, Fig. 5A-F). Moreover, OVA-stimulation only, CSE-exposure only, or both in HBE cells significantly reduced HDAC2 amounts, with remarkable RORyt and IL-17A amount increases at both the gene and protein levels, suggesting specific associations of HDAC2 and RORyt with the IL-17 promoter in HBE cells (all $p < 0.05$, Fig. 5G-H, Fig. 6). Since HDAC2 is the main HDAC contributing to GC effects, whether CpG-ODN influence the interplay of HDAC2, RORyt and IL-17A in HBE cells was examined. Thus, OVA and CSE-exposed HBE cells were treated with CpG-ODN and BUD. Interestingly, after administration of CpG-ODN or BUD, HDAC2 protein levels showed an increasing trend. Notably, the increasing trend of HDAC2

expression changes was more meaningfully after joint treatment with CpG-ODN and BUD. However, contrary to HDAC2 results, ROR γ t and IL-17A amounts were decreased in mice administered CpG-ODN and notably reduced after co-administration of BUD and CpG-ODN (all $p < 0.05$, Fig. 5G-H, Fig. 6).

Collectively, these data provided convincing evidence of an interplay between HDAC2 and ROR γ t in OVA-induced and CSE-exposed airway epithelial cells, substantially affecting allergic airway inflammation. Moreover, CpG-ODN could partly affect this interplay, specifically by improving HDAC2 expression and inhibiting ROR γ t expression simultaneously.

Discussion

Our previous and other studies revealed that CS-exposed asthma presents elevated inflammatory cell infiltration[8, 28], mucus production, airway remodeling, and Th2/Th17 polarization, which was further confirmed in the current study (Fig. 1,2, e-Fig. 3,4). As shown in Fig. 1, immunohistochemistry detecting Gr-1 (neutrophil-specific marker) and ECP (eosinophil-specific marker) confirmed substantially increased neutrophil and eosinophil influx into the lung, which suggested CS and OVA induced the infiltration of inflammatory cells into pulmonary tissues, including eosinophils and neutrophils. Neutrophilic inflammation is driven by IL-17, TNF- α , and IL-8[36]. Because of preponderant neutrophil inflammation in OVA/CS animals, whether the biosynthesis of cytokines/chemokines contributes to neutrophil recruitment was examined. This study showed that IL-17 and neutrophil chemokines, including IL-8 and TNF- α , were significantly elevated in the OVA/CS group, which may account for the enhanced infiltration of neutrophils into the pulmonary tissue. In addition, eotaxin 1 was increased in BALF of the CS/OVA group (Fig. 1). Eotaxin 1 promotes the recruitment of eosinophils and other immune cells such as neutrophils[37]. Mounting evidence suggests that decreased sensitivity to GCs is related to neutrophilic airway inflammation, and steroid-insensitive asthma is characterized by Th17 cytokines with neutrophilic inflammation[38, 39]. In this study, BUD alone markedly decreased Th2/Th17 cytokines in BALF, also reducing immune cells populating the pulmonary tissue (Fig. 1,2, e-Fig. 3,4), although to a lesser extent than shown in our previous study[8]. These findings indicate that the current model is not GC resistant but rather GC insensitive as far as lung cell inflammation is concerned. According to these findings, mice co-exposed to CS and OVA show exaggerated reactions to allergen inhalation, triggering inflammation that simultaneously involves eosinophils and neutrophils, elevated type 17-associated immune responses, and relative insensitivity to GCs.

Our previous study demonstrated that CpG-ODN alleviate mixed airway neutrophil and eosinophil inflammation in CS exposure OVA-induced asthma[8]. In this study, CS-exposure asthmatic mice treated with CpG-ODN also had decreased inflammatory cell infiltration, mucus accumulation and airway structural remodeling, and AHR(Fig. 1,e-Fig. 3). Several reports have revealed decreased HDAC2 activity in smokers and sputum cells in patients with respiratory diseases, as well as in CS-exposed asthma mice[13, 15, 22, 29]. Decreased HDAC2 amounts and activity are involved in steroid insensitivity and exacerbated disease in asthmatic individuals, indicating that insufficient transcriptional co-repressor amounts and activity might be critical for asthma pathogenesis[40]. Recently, emerging evidence has

suggested theophylline could downregulate the inflammatory response, locally and systemically, by increasing HDAC2 activity in patients with asthma[41, 42]. Since CpG-ODN reduce inflammatory response, we examined whether CpG-ODN modulate HDAC2 activity and expression, thereby enhancing the response to GCs in CS-exposure asthma mice. In the current CS exposure OVA-induced model, both HDAC2 mRNA and protein or activity were markedly suppressed, while as expected, they were increased after CpG-ODN administration. Jointly, these results provide CpG-ODN a novel avenue for developing specific HDAC2 regulators in restoring its activity and expression for enhancing steroid efficacy in asthma management. Furthermore, the combination of CpG-ODN and BUD more substantially recovered HDAC2 activity and expression, which may account for the improvement in corticosteroid insensitivity. Interestingly, in agreement with another study[43], BUD exerted great effects on the suppression of mediator release in relation to HDAC2 activity enhancement[42, 44]. CpG-ODN might act in an indirect manner with the corticosteroid to increase its effect on HDAC2 activity, suggesting that CpG-ODN exert corticosteroid-sparing effects.

There is increasing evidence that Th17 lymphocytes play a critical role in inducing neutrophilic airway inflammation. Th17 cells produce various inflammatory cytokines, including IL-17A, which regulates cellular immunity by upregulating downstream pro-inflammatory molecules in epithelial and mesenchymal cells, thereby mediating neutrophil infiltration and activation, and promoting neutrophil accumulation in pulmonary tissues. It is worth noting that IL-17A affects GC responsiveness via GR modulation. Specifically, increased expression of GR- β , a major suppressor of GR- α inducing and repressing target genes, is associated with severe asthma and upregulated IL-17A. These findings suggest a complex and potentially deleterious relationship association of IL-17A with GC insensitivity in severe asthma[45]. Of interest, in the present study, the opposite tendency between HDAC2 and IL-17A was found, with HDAC2 downregulated while IL-17A was upregulated in CS-exposure asthmatic mice. Several studies have pointed out that the striking interaction between HDAC2 and IL-17A forms a vicious circle, leading to the exacerbation of asthma[13]. According to a study reported by Tianwei Lai et al[13], HDAC2 impairment upregulates IL-17A, whose deficiency restores HDAC2 reduction, highlighting HDAC2 as a mediator that affects the secretion of IL-17A, thereby causing Th17-polarized response. As demonstrated in our previous research, CpG-ODN alleviate airway inflammation and remodeling by affecting DC-mediated Th17 differentiation in CS-exposure asthma. In the current study, CS-exposed mice administered CpG-ODN had elevated HDAC2 expression and attenuated IL-17A production. Collectively, CpG-ODN is likely involved in the interaction between HDAC2 and IL-17A. Therefore, the current results indicate that CpG-ODN may affect IL-17A secretion by modulating HDAC2 activity and expression, thereby inhibiting Th17 response.

On the other hand, ROR γ t represents an important transcription factor regulating IL-17A[46]. Several studies have revealed that ROR γ t-deficient T cells show suppressed differentiation of Th17 cells and downregulate IL-17A, while overexpression of ROR γ t induces IL-17A expression, conversely[47]. In addition, Amir Kumar Singh et al. elaborated a detailed mechanism that SUMOylation of ROR γ t promotes HDAC2 interaction with the IL-17 promoter and suppresses IL-17A transcription[33]. Furthermore, a research demonstrated ROR γ t acetylation in Th17 cells, with a significant enhancement by HDAC2

inhibitors[47], consistent with a recent study which revealed elevated RORyt acetylation in cultured CSE-induced HBE cells with HDAC2 silencing[22]. Taken together, these findings suggest that HDAC2 downregulation not only upregulates RORyt but also enhances its acetylation. Based on these findings, HDAC2 may inhibit RORyt-mediated IL-17A production, thereby attenuating Th17 response. Recent evidence suggests that the RORyt-associated transcriptional activation of IL-17A is induced by TCR stimulation and cytokines such as TGF- β , IL-6, and IL-23[48]. Therefore, whether CpG-ODN, which are capable of activating TCR 9, similarly affect RORyt was assessed. To investigate whether CpG-ODN are involved in specific interactions of HDAC2 and RORyt with the IL-17A promoter, RORyt expression levels were assessed in CS-exposure asthmatic murine models treated with CpG-ODN. Consistent with our hypothesis, the results indicated a negative correlation between HDAC2 and RORyt expressions in CS-exposure asthmatic mice. In addition, the expression of RORyt was recovered after treatment with CpG-ODN compared with mice with no treatments. Therefore, we hypothesized that CpG-ODN might mainly accelerate the binding capacity of RORyt to IL-17A via HDAC2, to inhibit IL-17A expression.

ICS represent the most effective anti-inflammatory anti-asthma drugs. According to previous findings, GCs can recover HDAC2 activity and inhibit RORyt expression[43]. This study indicated that both HDAC2 and RORyt expressions in co-exposed CS and OVA mice had more remarkable changes after sole administration of BUD or co-administration of BUD and CpG-ODN compared with the CpG-ODN alone group. This suggested that although GCs are still irreplaceable drugs for asthma, CpG-ODN, as a therapeutic immune modulator, have synergistic effects with GCs, dealing with severe asthma that may be less responsive to GCs.

Bronchial epithelial cells are increasingly considered to contribute to innate immunity. Recently, functional alterations in the airway epithelial barrier have been shown to contribute to asthma development and exacerbation[49]. We hypothesized that altered sensitivity to GCs in airway epithelial cells is substantially involved in GC insensitivity of lung's inflammatory responses[50]. Consistent with a recent study[7], we found that CSE exposure remarkably downregulated HDAC2 in HBE cells, and co-stimulation with OVA under CS-exposure asthmatic conditions further decreased HDAC2 expression (Fig. 5, 6). Contrary to the change of HDAC2 expression, elevated RORyt expression was observed in HBE cells cultured with CSE, with markedly increased expression in HBE cells co-administered CSE and OVA (Fig. 5, 6). The above results indicated an interplay between HDAC2 and RORyt in OVA and CSE-treated airway epithelial cells, thereby having an essential function in Th17 inflammation. Of interest, a report revealed that CpG-ODN promotes barrier integrity in healthy bronchial epithelial cells, thereby preventing inhaled molecules from entering into the submucosa and potentially causing allergen sensitization[49, 51]. Next, whether CpG-ODN regulate HDAC2 and RORyt in airway epithelial cells to change sensitivity to GCs since they enhance airway epithelial barrier integrity was investigated. We chose CpG-ODN and BUD to treat HBE cells cultured with OVA and CSE, and found that CpG-ODN regulated the interplay between HDAC2 and RORyt, synergistically with BUD to some extent in HBE cells, consistent with the above CS-exposure asthmatic murine model (Fig. 5, 6). It is known that IL-17A modulating the protective effects of HDAC2 on airway inflammation in asthma and HDAC2 activation and/or IL-17A downregulation could prevent allergic

airway inflammation[13]. Moreover, RORyt transcriptionally upregulates IL-17A, indicating that CpG-ODN may suppress RORyt-associated IL-17A expression via HDAC2 *in vitro* in line with the above evidences.

Conclusions

Overall, mouse and human epithelial cell assays demonstrated that CS-exposure asthma shows asthma phenotypes, characterized by enhanced infiltration of eosinophils and neutrophils, mucus production, airway remodeling, AHR and Th2/Th17-biased immune responses. CpG-ODN and BUD synergistically improved adverse CS-exposure asthma outcomes and inhibited, at least in part, RORyt-mediated Th17 response by restoring HDAC2, consequently ameliorating GC insensitivity. To the best of our knowledge, such a mechanism has not been previously described and may partly explain how CpG-ODN amplify the effects of steroid treatment. These data suggest that CpG-ODN may have a therapeutic value in reviving steroid effects in CS-exposure asthma, providing new insights into the mechanism by which CpG-ODN improve sensitivity to steroids.

Declarations

Ethics approval and consent to participate

All animal assays were approved by the ethics committee of the Third Affiliated Hospital of Sun-Yat-Sen University.

Consent for publication

All the co-authors consent to publish the work in Cell & Bioscience.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare that they have no competing interests.

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Author's contribution

Conceived and designed the experiments: LHT, YQM, ZTT. Performed the experiments: LHT, YQM, LYS, YXN, ZXL, YHL, WWB, MP. Analyzed the data: LHT, YQM, LYS, YXN, ZTT. Wrote the paper: LHT, YQM, LYS, YXN. All authors read and approved the final manuscript.

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Figures

Figure 1

CpG-ODN and BUD alleviate airway inflammation in the lung tissue in mice after OVA-challenge and CS-exposure. Lung tissue samples were histologically assessed 48h upon final OVA exposure.

Representative micrographs of ECP stained specimens ($\times 200$)(A). Representative micrographs of Gr-1 stained samples ($\times 200$)(B). Eotaxin 1 in BALF was assessed by ELISA(C). ECP-positivity rate was assessed as Gr-1-positive area/total bronchiole area(D). Gr-1-positivity rate was derived as Gr-1-positive area/total bronchiole area(E). Statistical significance denoted:*, # $P < 0.05$, **,## $P < 0.01$. * versus the vehicle control group; # versus the indicated group.

Figure 2

CpG-ODN and BUD synergistically alter Th17 responses in the lower airway. RORγt⁺CD4⁺ cells representing Th17 cells were assessed flow-cytometrically (A) and positive cells were quantitated (B). Th17-related cytokine amounts in serum (E) and BALF (F) were determined by ELISA, and relative mRNA expression was measured by qRT-PCR in lung tissues from mice (C). Immunoblot was carried out for assessing the protein amounts of Th17-associated cytokines in lung tissues, with GAPDH as a loading control (D); HDAC2/GAPDH ratios were assessed (E). Statistical significance denoted: *, #P<0.05, **, ##P<0.01. * versus the vehicle control group; # versus the indicated group.

Figure 3

CpG-ODN and BUD synergistically affect HDAC2 activity and expression in chronic CS-exposed asthmatic mice. Representative photomicrographs of immunohistochemical staining of lung sections for HDAC2 in various animal groups ($\times 200$)(A). HDAC2-positivity rate was determined as HDAC2-positive area/total bronchiole area(B). Immunoblot was carried out for assessing HDAC2 protein amounts in lung tissue specimens, with GAPDH as a loading control; HDAC2/GAPDH ratios were assessed(C,D). The mRNA expression levels of HDAC2 were evaluated by qRT-PCR in lung tissue samples from mice(E). HDAC2 activity was determined with the colorimetric EpiQuik HDAC2 Activity Assay Kit(F). Statistical significance denoted:*, # $P < 0.05$, **,## $P < 0.01$. * versus the vehicle control group; # versus the indicated group.

Figure 4

CpG-ODN and BUD synergistically affect RORγt expression in CS-exposed asthmatic mice. Representative photomicrographs of immunohistochemical staining of lung sections for RORγt from various animal groups ($\times 200$)(A). RORγt-positivity rate was determined as RORγt-positive area/total bronchiole area(B). The mRNA expression levels of RORγt were determined by qRT-PCR in lung tissue samples from mice(C). Immunoblot was carried out for assessing RORγt protein levels in lung tissue samples, with GAPDH as a loading control; RORγt/GAPDH ratios were determined (D,E). Statistical significance denoted:*, #P<0.05, **,## P<0.01. * versus the vehicle control group; # versus the indicated group.

Figure 5

CpG-ODN and BUD synergistically regulate the interplay of HDAC2, RORγt and IL-17A, orchestrating the inflammatory response in HBE cells. Th2-associated cytokines (A,C) examined by ELISA, and relative mRNA levels of Th2 cytokines in lung tissue specimens from mice (B,D), determined by qRT-PCR. Th17-associated cytokines (E) assessed by ELISA, and relative mRNA levels of Th2 cytokines in lung tissue samples from mice (F), evaluated by qRT-PCR. Immunoblot was carried out for assessing the protein amounts of HDAC2 (G) and RORγt in HBE cells, with GAPDH as a loading control; HDAC2/GAPDH (G) and RORγt/GAPDH (H) ratios were determined. Statistical significance denoted:*, #P<0.05, **,## P<0.01. * versus the vehicle control group; # versus the indicated group.

Figure 6

CpG-ODN and BUD synergistically affect HDAC2, RORγt and IL-17A in HBE cells. Representative micrographs of immunofluorescent staining of HBE cells for detecting tubulin GFP (green; HDAC2-positive) and H2BmCherry (Red; RORγt-positive) cells (×200) (A). HDAC2-positivity rate was determined as tubulin GFP (green)-positive area/total area (B). RORγt-positivity rate was evaluated as H2BmCherry (Red)-positive area/total area (C). Representative images depicting IL-17A+, HDAC2+, and RORγt+ cells in HBE cell populations (D). The ratios of specific protein-positive HBE cells to total HBE cells (E-G) were assessed by flow cytometry. Statistical significance denoted:*, #P<0.05, **,## P<0.01. * versus the vehicle control group; # versus the indicated group.

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